

Armed Forces Institute of Regenerative Medicine

Annual Report 2010



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**Armed Forces Institute of Regenerative Medicine
Annual Report 2010**

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Executive Summary



The use of improvised explosive devices in Iraq and Afghanistan has caused a marked increase in severe blast trauma. Due to advances in body armor, **quicker evacuation from the battlefield, and advanced medical care**, many of the injured survive to face the challenge of overcoming severe limb, head, face, and burn injuries that can take years to treat **and usually result in significant lifelong impairment.**

The **burgeoning field of regenerative medicine** provides hope for restoring the structure and function of damaged tissues and organs and curing previously untreatable injuries and diseases. The concept of regenerative medicine—in its simplest form—is to replace or regenerate human cells, tissues, or organs to restore or establish normal function. Advanced technologies such as tissue regeneration, bone scaffolding, and stem cell-enabled

treatments are needed to revolutionize the clinical rehabilitation of severely injured service members.

The Department of Defense established the Armed Forces Institute of Regenerative Medicine (AFIRM) in 2008 with the mission of developing new products and therapies to treat severe injuries suffered by U.S. service members. This multi-institutional, interdisciplinary network of scientists has been designed to accelerate the delivery of regenerative medicine therapies for severely injured U.S. service members. Centered around well-established, proven research investigators, the AFIRM has been able to expand the rehabilitative medicine knowledge base, develop models of injury, and test advanced technology products.

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Executive Summary

Creating Partnerships and Collaborations

The AFIRM's success to date can be ascribed at least in part to the program's emphasis on establishing partnerships and collaborations. The AFIRM is a six-way partnership among the U.S. Army, Navy, and Air Force, the Veterans Health Administration, the Defense Health Program, and the National Institutes of Health. The AFIRM is composed of two independent research consortia working with the U.S. Army Institute of Surgical Research and the Major Extremity Trauma Research Consortium (METRC). One research consortium is led by Wake Forest University Baptist Medical Center and the McGowan Institute for Regenerative Medicine in Pittsburgh (WFPC [Wake Forest-Pittsburgh Consortium]) while the other is led by Rutgers, the State University of New Jersey, and the Cleveland Clinic (RCCC [Rutgers-Cleveland Clinic Consortium]). Each research consortium contains approximately 15 member organizations, which are mostly academic institutions. Notably, AFIRM member organizations have established collaborations/partnerships with over 50 academic or industrial institutions, located both within and outside of the United States (including Australia, China, Finland, Germany, and the Netherlands).

Research activities are organized into five program areas: Limb and Digit Salvage, Craniofacial Reconstruction, Scarless Wound Healing, Burn Repair, and Compartment Syndrome. Over 80 projects have been funded by the AFIRM to date. A Program Synergy Group has been established to identify collaborative opportunities and build bridges between the programs and projects. One example of a successful collaborative effort among scientists working at different institutions is found in the Craniofacial Reconstruction Program. Researchers at three leading academic institutions—Dr. David Kaplan and colleagues at Tufts University; Drs. Peter Rubin and Kacey Marra and colleagues at the University of Pittsburgh; and Drs. James Yoo and Sang Jin Lee and colleagues at Wake Forest University—formed a partnership to develop and deliver a clinically useful engineered soft tissue replacement that can be either used as a stand-alone therapy or integrated with composite tissue

Limb and Digit Salvage

The Limb and Digit Salvage Program seeks to develop novel solutions using regenerative medicine that will allow victims of severe military or civilian trauma to recover more efficiently and reliably from their injuries and retain their limbs as they return to productive life.

A total of 19 projects were funded in Year 2. Projects span the following clinical challenge areas: Bone, Soft Tissue, and Nerve Repair/Regeneration; Composite Tissue Injury Repair; Transplantation; and Epimorphic Regeneration.

regenerative medicine therapy of burns, craniofacial injuries, and extremity injuries (Projects 4.1.4 and 4.1.5). The main scientific approach involves the use of the patient's own adipose-derived stem cells and fibroblasts, combined with carrier biomaterials, to achieve soft tissues with functional networks of blood vessels. The researchers will determine the optimal combination of their successful biomaterials and cellular elements and finalize a clinical therapy model, which will involve integrating engineered adipose tissue (Drs. Rubin, Marra, and Kaplan) with the connective tissue system (Drs. Yoo and Lee).

Second Year Research Highlights

Although the program has only recently completed its second year of funding, research efforts have already yielded a substantial number of noteworthy accomplishments. For example, Drs. Cathryn Sundback and Joseph Vacanti at Massachusetts General Hospital/Harvard University (Craniofacial Reconstruction Program, Project 4.1.2) have made significant progress in defining a "living ear prosthesis" using the patient's own cartilage cells to develop an engineered structure that would be more patient-friendly than any of the artificial prostheses used today. The Vacanti laboratory has also achieved proof-of-concept for the de novo engineering of functional

human muscle tissue, the first application being the restoration of movement in a damaged eyelid. This project addresses the significant problems encountered by warriors who have lost control of their eyelids and are unable to maintain the hydration of the cornea through regular blinking.

Dr. Sang Jin Lee and colleagues at Wake Forest University (Compartment Syndrome Program, Project 4.3.5) have been developing an approach to recruit a patient's own stem/progenitor cells to the site of compartment syndrome injury to increase the regenerative response. They are using biomaterials containing muscle-inducing factors that can be implanted within the injured muscle compartment. This group has now shown proof-of-principle in vitro and validated their method in a small animal model of tissue injury.

Another Compartment Syndrome Project (4.3.6), led by Drs. Thomas J. Walters, Robert Christy, and Christopher Rathbone at the U.S. Army Institute of Surgical Research (USAISR), is focused on developing cell-based regenerative medical approaches to reduce the magnitude of injury, hasten healing, and improve the outcomes of wounded soldiers suffering from ischemic-reperfusion



Closeup of a fabricated ear scaffold created by AFIRM researchers.

(I/R) muscle injuries (can be caused by tourniquet application, vascular trauma, or acute compartment syndrome). They have demonstrated improved muscle function in the short term following I/R through the early injection of skeletal muscle progenitor/stem cells.

Drs. Carmine Iovine and Niraj Ramachandran and colleagues at the New Jersey Center for Biomaterials (Burn Repair Program, Project 4.6.4) developed a novel antibacterial dressing containing complexed iodine for the treatment of burn skin and soft tissue wounds. They achieved promising results in initial porcine-infected burn trials with the new wound dressing. The polymer exhibited good antimicrobial activity, little biological reactivity, and dressing changes incurred no trauma to the skin-generating wound bed.

Dr. Patricia Hebda and colleagues at the University of Pittsburgh's McGowan Institute for Regenerative Medicine (Scarless Wound Healing Program, Project 4.5.4) are developing novel anti-inflammatory therapies aimed at improving the quality of healing following burn injury. The research team has demonstrated that early, short-term topical treatment with the anti-inflammatory agents nimesulide and prostaglandin E2 (PGE2) attenuates the wound inflammatory response, which leads to the promotion of healing.

Craniofacial Reconstruction

The Craniofacial Reconstruction Program aims to generate both soft and hard tissues through novel regenerative medicine approaches to reduce the impact of devastating, disfiguring facial injuries on wounded warriors.

A total of 13 projects were funded in Year 2. Projects span the following clinical challenge areas: Bone and Soft Tissue Regeneration, Cartilage Regeneration (with a focus on the ear), and Virtual Modeling.



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Dr. George Muschler and colleagues at the Cleveland Clinic (Limb and Digit Salvage Program, Project 4.2.1) completed a detailed competitive evaluation of new biomaterials for the fabrication and characterization of three polymer-based bone regeneration scaffolds. They down-selected scaffold materials in the canine femoral multidefect (CFMD) model. Among the materials tested to date, the porogen-leached tyrosine-derived polycarbonate (Tyr-PC) with beta tri-calcium phosphate (TCP) performed best. The researchers also established a **defined track record of historical performance standards** that can be used to rapidly benchmark the performance of new or competing scaffold materials using the CFMD model.

The projects highlighted in the preceding paragraphs are just a few examples of a long list of research developments and successes resulting from AFIRM-funded laboratories over the past year.

Second Year Program Highlights

The AFIRM involves the efforts of more than 450 individuals, including faculty members, postdoctoral fellows, graduate students, scientific and technical staff, and undergraduates. AFIRM faculty members are highly accomplished scientists—over the second year of the program, 46 awards/honors were conferred upon AFIRM faculty, including selection to membership or leadership

Scarless Wound Healing

The Scarless Wound Healing Program encompasses a continuum of technologies aimed at the various stages of wound healing to find new treatment options to prevent and manage scars.

A total of 10 projects were funded in Year 2. Projects span the following clinical challenge areas: Control of Wound Environment and Mechanics, Therapeutic Delivery to Wounds, Attenuation of Wound Inflammatory Response, and Scar Mitigation.

positions in professional societies, honorary degrees from research/academic institutions, awards from private foundations, and recognition of exemplary AFIRM meeting presentations. Their AFIRM-related research efforts have substantially contributed to the scientific literature—over the second year of the program, they published 73 articles in peer-reviewed journals and produced 106 presentations and non-peer-reviewed publications. AFIRM scientists have also been making **novel patentable discoveries in the field of regenerative medicine**—over the second year of the program, they filed 10 invention disclosures, of which 2 have resulted in government patent applications.

The Technology Readiness Levels (TRLs) of products generated by AFIRM-funded researchers have been steadily rising. At the start of the program, 61 products (93%) were nearly evenly distributed across TRLs 1, 2, and 3; and the other 5 products were at TRL 4 or 5. By the end of the second year of the program, no products remained at TRL 1, and only 9 products were at TRL 2. The vast majority of products (50) were at TRLs 3 and 4, and the other 10 products were distributed between TRLs 5, 6, and 7. Of note, approximately twice as many projects increased by one TRL during the second year of the program compared to the first year.

The AFIRM has recruited a substantial amount of young talent into the field of regenerative medicine since its inception. During the second year of the program, more than 110 students (undergraduate and graduate) received practical scientific training through AFIRM-sponsored research projects. The AFIRM is developing fellowship programs to provide unique educational opportunities for aspiring scientists. For example, RCCC, in concert with the Henry M. Jackson Foundation, established the Henry M. Jackson-AFIRM Regenerative Medicine Traveling Fellowship to foster knowledge exchange and collaborative relationships among members of the military, civilian scientists, and clinicians. In addition, the RCCC designed and applied for NIH-funding for a Translational Research in Regenerative Medicine: Stem Cells on Scaffolds Fellowship to provide young scientists or clinicians with opportunities to learn regenerative medicine approaches within the AFIRM consortia.

Burn Repair

The Burn Repair Program seeks to design innovative regenerative medicine therapies for victims of severe military or civilian trauma so they can recover from their injuries with improved function and aesthetics.

A total of 17 projects were funded in Year 2. Projects span the following clinical challenge areas: Intravenous Treatment of Burn Injury, Topical Treatment of Burn Injury, Wound Healing and Scar Prevention, and Skin Products/Substitutes.

From the Laboratory to the Battlefield

AFIRM-funded researchers share a strong commitment to developing commercial products and bringing therapies to wounded warriors and the civilian sector as quickly as possible. The ultimate goal of AFIRM-funded projects is the conduct of clinical trials; in fact, some AFIRM researchers have initiated clinical trials while many others anticipate the commencement of clinical trials within the next few years.

For example, WFPC researchers Drs. Geoffrey C. Gurtner and Michael T. Longaker at Stanford University (Scarless Wound Healing Program, Project 4.5.1) developed a region-specific device capable of stress-shielding mechanical forces to minimize scar formation following burns. A novel pressure-sensitive adhesion dressing has been developed that is capable of offloading wound forces. This material has been shown to manipulate wound forces to either increase or decrease fibrosis and scar formation in a pig model. Preliminary human trials using the researchers' stress-shielding polymer device have begun in collaboration with Dr. Bill Beasley at Neodyne Biosciences, Inc. (Scarless Wound Healing Program, Project 4.5.9), and the researchers have already observed significant improvement in scarring compared to control within-subject wounds. The

researchers are in the process of completing a Phase 2 study with a broader surgical patient population encompassing a wider variety of wounds. They plan to further refine the device to custom design treatments for various size wounds and tension states, which will allow for body-specific regional stress-shielding to address a wide variety of surgical wounds.

Two parallel clinical studies are under way in Dr. Maria Siemionow's laboratory at the Cleveland Clinic (Craniofacial Reconstruction Program, Project 4.3.1). The researchers hope to transform standards for clinical immunomodulation, making transplantation of composite tissue allografts (CTAs; large segments of complex, vascularized tissue) safer and more widely available to victims of disease and traumatic injury. They are using a therapeutic antibody, TOL101, as a conditioning agent prior to transplantation to enhance allograft tolerance. A clinical trial protocol for immunomodulation with TOL101 has been established for patients undergoing kidney transplantation to confirm the safety and efficacy of TOL101. The antibody has undergone various regulatory tests and has passed a pre-Investigational New Drug screening with the U.S. Food and Drug Administration (FDA). Patient enrollment is expected to start within 12 months. The researchers are also screening potential recipients for face transplantation to be performed at the Cleveland Clinic, in conjunction with USAISR. This pro-

Compartment Syndrome

The Compartment Syndrome (CS) Program seeks to prevent or reverse secondary damages resulting from trauma so that repair and regeneration of wounded tissue are enhanced, and healing and return to functionality are improved.

A total of 6 projects were funded in Year 2. Projects span the following clinical challenge areas: Cellular Therapy of CS and Biological Scaffold-Based Treatment of CS.



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cess is defining consensus within and across services regarding rigorous standards for patient screening and education related to CTA procedures. The expectation is that at least one, and perhaps two, transplants will be performed during Year 3. Dr. James Holmes and colleagues at the Wake Forest University Baptist Medical Center (Burn Repair Program, Project 4.2.7) are conducting a multicenter FDA approval trial for ReCell[®]. ReCell is a technique whereby healthy skin cells are harvested from patients in the operating room and are placed in a suspension so the cells can be sprayed onto the burn. From ~4 cm² of healthy skin, ~320 cm² of burn can be “grafted” using the patient’s own cells without the need for any culture techniques. The cells multiply and create new skin tissue. Participants are being enrolled from among 10 U.S burn centers including USAISR.

Another Burn Repair project that has been progressing toward clinical trials is led by Dr. Steven Boyce at the University of Cincinnati (Project 4.7.2). In collaboration with Dr. Richard Clark at Stony Brook University, the

researchers are designing and testing new prototypes of engineered skin substitutes that restore skin color and develop vascular networks thereby resulting in improved outcomes in recovery from life-threatening burns. They have restored skin color in an animal model. Their technology has been licensed to Lonza Walkersville, Inc., which has initiated technology transfer and has received funding from the AFIRM to perform an initial clinical study.

Notably, the RCCC has established a clinical trials core at Case Western University and has invited the WFPC to participate in efforts to standardize the approach to clinical trials at military sites and to ensure electronic data collection for all clinical trials in the AFIRM using the commercial OnCore[®] software. A clinical trials text, authored by Stanton Gerson, MD, director of the RCCC clinical trials core, has been provided to USAISR to facilitate generation of a USAISR clinical trials manual.



I: Introduction



BACKGROUND

The wars in Iraq and Afghanistan have resulted in more than 5,700 U.S. military fatalities and more than 40,000 injuries.¹ Treatment of combat-related injury and trauma is particularly complex. Advances in body armor have substantially improved protection of the torso, which contains the vital organs. **In addition, evacuations from the battlefield have become faster, and medical care has advanced.** Due to all of these factors, survivability has increased. However, those who survive often have seriously

debilitating injuries. Conventional weapons and the destructive force of improvised explosive devices ravage the face, neck, head, and limbs, causing massive trauma and tissue loss. According to the *Journal of Orthopaedic Trauma*, the use of improvised explosive devices in Operation Iraqi Freedom/ Operation Enduring Freedom has led to a substantial increase in severe blast trauma, which is now responsible for approximately 75% of all combat-related injuries.

¹ September 27, 2010 <http://www.defense.gov/news/casualty.pdf>.



I: Introduction

The emerging field of regenerative medicine focuses on restoring the structure and function of tissues and organs that have been damaged and finding methods of curing previously untreatable injuries and diseases. Regenerative medicine holds great potential for healing military personnel with debilitating, disfiguring, and disabling injuries of the extremities. Scientists working in the area of regenerative medicine use tissue-engineering techniques to prompt the body to regenerate cells and tissues, often using the patient's own cells combined with degradable biomaterials. Use of a patient's own cells eliminates the possibility of tissue rejection. Technologies for engineering tissues are developing rapidly. The ultimate goal is to deliver advanced therapies, such as whole organs and engineered fingers and limbs, to injured members of the military as well as civilians.

Research Goals

The AFIRM is a multi-institutional, interdisciplinary network focused on developing advanced treatment options for severely wounded warfighters. The AFIRM is designed to speed the delivery of regenerative medicine therapies to treat the most severely injured U.S. service members from around the world. It is anticipated that the AFIRM will be able to translate many of its technologies to patients within the next 3 to 4 years.

The AFIRM Has Five Major Research Programs:

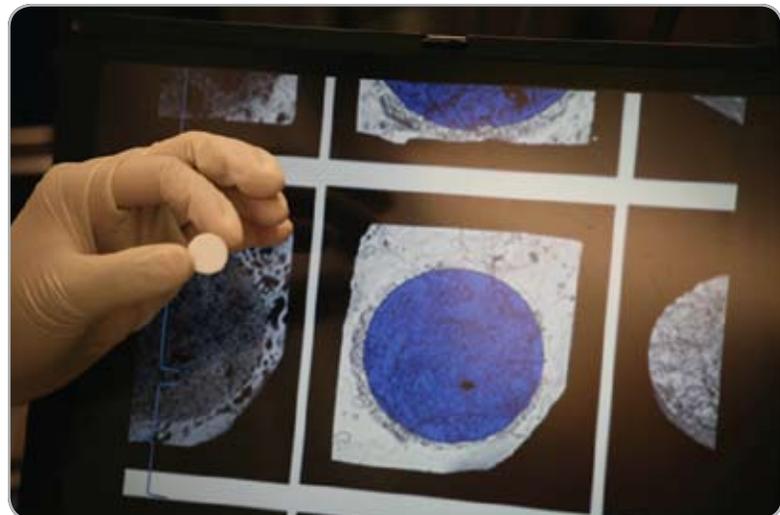
Limb and Digit Salvage

Saving the limb, also referred to as "limb salvage," at a minimum requires (1) bridging large bony defects to re-establish a strong connection and mobility along the entire limb; (2) bridging soft tissues, such as muscle, nerves, tendons, and ligaments, to lend stability and enable movement; and (3) growing healthy skin to cover the injured area to provide a durable barrier to infection. This AFIRM program is dedicated to developing regenerative medicine therapies to help

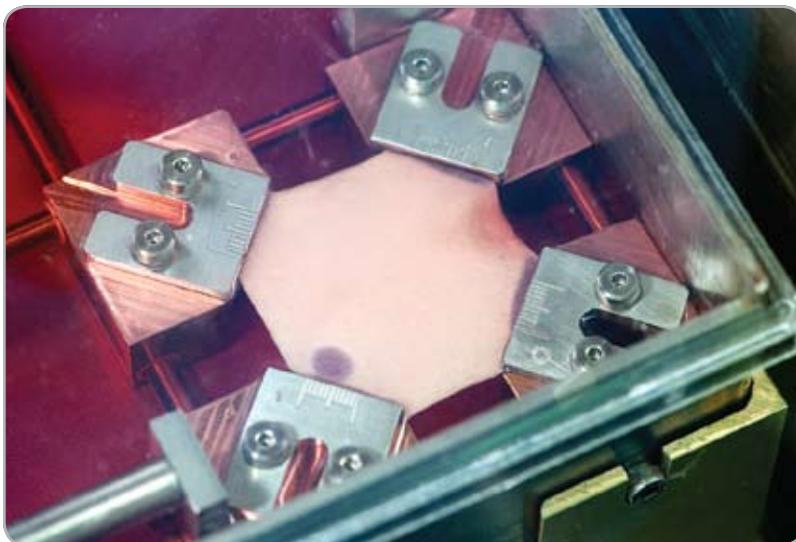
save and rebuild injured limbs. The program focuses on using new technologies in regenerative medicine and tissue engineering to provide health care providers with advanced tools and new options for repair and regeneration of these critical tissues. The goal is to allow victims of severe military or civilian trauma to be able to recover from their injuries more rapidly, more reliably, and also regain the function of injured limbs as they return to productive life.

Craniofacial Reconstruction

Massive bone and tissue loss to the face and head is a large problem of blast injuries for our warfighters. This complex area involves multiple levels and tissue types, which require different strategies for repair. The AFIRM Craniofacial Reconstruction Program is designing and developing therapies that health care providers can use to treat the wounded warfighter. These therapies will (1) regenerate functional bone and cartilage to calvarial-, upper- and mid-facial anatomies and the mandible; (2) restore sensate and motor competencies through muscle and nerve regeneration; (3) mitigate scar formation; (4) prevent infection; and (5) eliminate skin coverage deficits through tissue engineering. The creation and delivery of new polymers and tissues will preserve and



Tyrosine-derived polycarbonate and calcium phosphate composite scaffolds developed by AFIRM-funded researchers. The bone regenerating capacity of these scaffolds is being evaluated using a critical size defect in the rabbit skull.



AFIRM-funded researchers have developed a bioreactor system that can incrementally expand skin, which provides a new option for generating additional skin for grafting.

regenerate bone and soft tissue capable of administering stem cells, growth factors, bone derivatives, and drugs.

Scarless Wound Healing

Military tissue trauma and burns create not only large wounds but also large scars. These scars are often very visible and can draw unwanted attention to the wounded warrior. In some instances, the scars become so thick that they can limit movement of joints and greatly restrict the patient's ability to move. Scars are the result of the body's complex series of wound-healing processes that begin at the onset of injury and can continue for months. This AFIRM program is investigating all phases of wound healing and scar formation to find new treatment options to prevent and mitigate scars.

Burn Repair

Although recent advances in critical care and resuscitation have helped, there is still high long-term morbidity and mortality associated with burns. Current treatment options include the administration of antibiotics and tissue excision for deeper burns, which are then replaced with skin grafts. The AFIRM Burn Repair Program is tackling the issues associated with these methods and using regenerative medicine to (1) prevent wound infec-

tion, (2) prevent burn inflammation and injury extension, (3) speed generation of a viable wound bed and reduce reharvest time of autograft donor sites, (4) improve skin substitutes for burn wound grafting when autografts are not immediately available, and (5) prevent and manage scars. The overall goal of the program is to allow victims of severe military or civilian trauma to be able to recover from their injuries more rapidly, more reliably, and with improved function and aesthetics.

Compartment Syndrome

Compartment syndrome is often a secondary sequelae resultant from blast injuries, severe blunt or penetrating trauma, fractures, and vascular injuries. Muscles are encased in com-

partments of nonyielding tissue called fascia. Bleeding or tissue swelling within a muscle compartment raises the pressure in the compartment that, if unchecked, can become high enough that blood flow into the compartment is reduced or completely stopped, which can destroy the nerves and muscles within the compartment. The only current treatment for compartment syndrome is a surgical procedure called a fasciotomy, which leaves an open wound that is susceptible to infection and added complications. To be effective, the fasciotomy must be performed within hours of onset; however, the detection of compartment syndrome is limited by the need to treat extensive primary traumatic injuries and the involvement of multiple tissue types. This AFIRM program focuses on attacking the problem of compartment syndrome through regenerative medicine therapies to prevent the syndrome, stabilize tissues, and reduce the onset of late effects of nerve and muscle damage. The goal is to prevent or reverse the secondary damages resulting from trauma so that repair and regeneration of wounded tissue are enhanced, and healing and return to functionality are improved.



I: Introduction

History

In 2005, Dr. Anthony Atala presented some of the latest advances in the field of regenerative medicine at the Advanced Technology Applications in Combat Casualty Care Conference. This talk alerted the combat casualty care research community to the near-term potential for regenerative medicine products that could make a substantial difference in the care of our wounded warriors. The following year, the Army's Director of the Combat Casualty Care Research Program, COL Bob Vandre, developed the idea of a regenerative medicine institute similar to the Department of Defense's (DoD's) Multi-disciplinary University Research Initiatives but aimed at near-term, translational research. COL Vandre received U.S. Army Medical Research and Materiel Command (USAMRMC) approval in 2006 to pursue funding for the project. He subsequently briefed the DoD Technology Area Review and Analysis panel, which reviews medical research and development for the DoD. The concept received high approval from the panel.

In 2007, USAMRMC, the Office of Naval Research, the U.S. Air Force Office of the Surgeon General, the National Institutes of Health (NIH), and the Veterans Health Administration of the Department of Veterans Affairs (VA) agreed to co-fund the new institute. Taking their funds and adding in \$10 million (M) from the 2007 War Supplemental bill provided \$8.5M per year in funding for the AFIRM, which was deemed sufficient to proceed.

A Program Announcement was released in August 2007, and seven proposals were received in October 2007. In December 2007, two finalists were selected for oral presentations. Both received scores of "excellent" and one was selected for funding. White House staffers heard about the AFIRM and invited representatives from USAMRMC to come and discuss the new institute. After two meetings and upon hearing that there was funding for only one AFIRM finalist, the DoD was tasked

to provide funding for the second AFIRM finalist. Within 1 week, an additional \$8.5M per year was transferred to USAMRMC's budget lines. Both AFIRM finalists signed USAMRMC cooperative agreements in March 2008.

Funding – A Six-Way Partnership

The AFIRM is financed with basic research through exploratory development funds and is expected to make major advances in the ability to understand and control cellular responses in wound repair and organ/tissue regeneration. The program is managed and funded through USAMRMC with funding from the following organizations:

- U.S. Army
- U.S. Navy, Office of Naval Research
- U.S. Air Force, Office of the Surgeon General
- Veterans Health Administration
- Defense Health Program
- National Institutes of Health

Total funding for the first 5 years of the AFIRM amounts to close to \$300M:



The Wake Forest Institute for Regenerative Medicine.

- \$100M from U.S. Government funding (Army, Navy, Air Force, VA, and NIH).
- \$80M from matching funds received from state governments and participating universities.
- \$109M from pre-existing research projects directly related to deliverables of the AFIRM from the NIH, Defense Advanced Research Projects Agency, congressional special programs, the National Science Foundation, and philanthropy.

Structure

The AFIRM is composed of two independent civilian research consortia working with the U.S. Army Institute of Surgical Research (USAISR) at Fort Sam Houston, Texas, and the Major Extremity Trauma Research Consortium (METRC). USAISR, which includes the Brooke Army Medical Center (newly renamed the San Antonio Military Medical Center – North), serves as the AFIRM’s primary government component and is home to the DoD’s only burn unit. The partnership with METRC will enable the timely identification of AFIRM-developed advanced treatment options for clinical evaluation by METRC clinical trial investigators. The two AFIRM research consortia are responsible for executing the management of overall therapeutic programs and individual projects within their consortia. One consortium is led by the Wake Forest Institute for Regenerative Medicine and the McGowan Institute for Regenerative Medicine in Pittsburgh, and the other is led by Rutgers, the State University of New Jersey, and the Cleveland Clinic. Each of these civilian consortia is itself a multi-institutional network, as shown in the following paragraphs.

Wake Forest-Pittsburgh Consortium (WFPC)

The WFPC is directed by Anthony Atala, MD, Director of the Wake Forest Institute for Regenerative Medicine and Professor and Chair of the Department of Urology at Wake Forest University, and co-directed by Rocky Tuan, PhD, Director of the Center for Cellular and Molecular Engineering at the University of Pittsburgh.

The WFPC consists of the following member institutions:

- The Wake Forest Institute for Regenerative Medicine/ Wake Forest University

- The McGowan Institute for Regenerative Medicine/ University of Pittsburgh
- Allegheny-Singer Research Institute
- Carnegie Mellon University
- Georgia Institute of Technology
- Institute for Collaborative Biotechnologies (includes University of California, Santa Barbara; Massachusetts Institute of Technology; and California Institute of Technology)
- Oregon Medical Laser Center
- Stanford University
- Rice University
- Tufts University
- University of Texas Health Science Center at Houston
- Vanderbilt University
- Sanford-Burnham Medical Research Institute/University of California, Santa Barbara
- University of California, Berkeley
- University of Wisconsin
- The Johns Hopkins University School of Medicine

Rutgers-Cleveland Clinic Consortium (RCCC)

The RCCC is directed by Joachim Kohn, PhD, Director of the New Jersey Center for Biomaterials and Board of Governors Professor of Chemistry at Rutgers University, and co-directed by George Muschler, MD, Director of the Orthopaedic Research Center and the Clinical Tissue Engineering Center at the Cleveland Clinic.

The RCCC consists of the following member institutions:

- Rutgers/New Jersey Center for Biomaterials
- Cleveland Clinic Foundation
- Carnegie Mellon University
- Case Western Reserve University
- Dartmouth Hitchcock Medical Center/Thayer School of Engineering
- Massachusetts General Hospital/Harvard Medical School



I: Introduction

- Massachusetts Institute of Technology
- Mayo Clinic College of Medicine
- Northwestern University
- Stony Brook University
- University of Cincinnati
- University of Medicine and Dentistry of New Jersey
- University of Virginia
- Vanderbilt University

Additional Collaborators to AFIRM

AFIRM researchers have established a wide variety of both national and international partnerships with academia and industry, which has contributed to the success of the program to date. These include collaborations with:

- Avita Medical, LLC
- Biologics Consulting Group
- Biosafe-America
- BioStat International, Inc.
- Bonwrx, Inc.
- Brooke Army Medical Center
- CV-Path Institute Inc.
- Cynvenio Biosystems
- Emory University
- Fidia Advanced Biopolymers (Italy)
- Glycosan BioSystems, Inc.
- Healthpoint, Ltd./DFB Bioscience
- Integra Spine/Integra LifeSciences
- Johann Wolfgang University (Germany)
- Kensey Nash Corporation
- KeraNetics, LLC
- Lexmark, Inc.
- LifeNet Health
- Loyola University Medical Center
- Maricopa Integrated Health Systems
- Massachusetts Eye and Ear Infirmary
- MedDRA Assistance, Inc.
- Morgridge Institute for Research
- Neodyne Biosciences
- Nitinol Development Corporation
- Organogenesis, Inc.
- Osteotech, Inc.
- PeriTec Biosciences
- Philadelphia University
- Porex Corporation
- Proxy Biomedical
- Queensland University of Technology
- Radboud University of Nijmegen Medical Centre (The Netherlands)
- Royal Perth Hospital (Australia)
- Shanghai 9th People's Hospital (China)
- SimQuest, LLC
- Special Operations Medical Command-Fort Bragg
- The University of Texas at Arlington
- Tolera Therapeutics
- University of Alabama – Birmingham
- University of Florida
- University of Indiana
- University of Massachusetts, Lowell
- University of North Carolina – Chapel Hill
- University of Tampere (Finland)
- University of Tennessee Health Science Center
- University of Texas, Austin
- University of Utah
- University of Washington
- University of Wisconsin, Madison
- Washington Hospital Center (Washington, DC)

Programs and Projects

Within each consortium, research activities are organized into programs (Limb and Digit Salvage, Craniofacial Reconstruction, Scarless Wound Healing, Burn Repair, and Compartment Syndrome). Scientists or clinicians responsible for coordinating the research activities of an entire program are called program leaders. Each program consists of numerous projects, and the scientist or clinician responsible for a specific project is called a team leader.



Dr. Joachim Kohn, Director of the RCCC, in the laboratory.

Consortium members evaluate all levels of the consortium annually to monitor progress and guide the consortium's activities. In 2009, the RCCC established a clinical trials core to emphasize standardized approaches to clinical trials at military sites and authored documents to facilitate the generation of a USAISR clinical trials manual. Active project management by each consortium has reshaped the programs, leading to the termination or reduced funding of some projects and the addition of projects that are more promising for accelerated development. Additionally, information for the public including clinical trial opportunities has been made available through web sites developed and maintained by the consortia.

In addition to the three core groups (RCCC, WFPC, and USAISR), intramural researchers from the NIH and/or the Veterans Health Administration can participate in the AFIRM although none have chosen to do so as of yet. With the approval of a program leader, the intramural researchers can lead projects.

Management and Oversight

Day-to-day execution of the AFIRM's Science and Technology and Advanced Development portfolio is managed by the newly established AFIRM Project Management Office (PMO), located within the U.S. Army

Medical Materiel Development Activity (USAMMDA) at Fort Detrick, Maryland. The AFIRM PMO is chartered to enhance product management as a core business process and competency to execute strategic business investments under the AFIRM. The AFIRM PMO is working as part of an integrated project management team, across the AFIRM consortia, to incorporate the strategic, developmental, and tactical aspects of product management. The integrated product management philosophy of the AFIRM PMO will serve as an execution system to improve strategic business investment, enhance product deliverables and serve as an accountability model to ensure "added value" in the execution of the AFIRM portfolio. Moreover, the AFIRM PMO will ensure limited financial resources are working on the most important initiatives that will successfully execute the strategic mission and move the most advanced AFIRM products towards FDA licensure.

The AFIRM is guided by a Board of Directors (BOD) and an Integrated Project Team (IPT), which contains a Steering Group. A Program Synergy Group is responsible for research coordination and communication between the three components of the AFIRM. The roles and membership of each of these entities are described as follows.



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Board of Directors

The AFIRM's BOD is chaired by the Commanding General of USAMRMC and contains flag-level representatives from the Army, Navy, Air Force, NIH, VA, Office of the Assistant Secretary of Defense for Health Affairs, TRICARE Management Activity and the Uniformed Services University of the Health Sciences. The Principal Assistant for Research and Technology of USAMRMC serves as the Deputy Chair of the BOD. The main purpose of the BOD is to provide high-level guidance for the AFIRM by presiding over the IPT and the Program Synergy Group.

Integrated Project Team

The AFIRM's IPT is chaired by the Director of the Clinical and Rehabilitative Medicine Research Program (CRM-RP). IPT membership consists of a group of experts who represent the interests of the funding agencies, experts in military needs, external scientists knowledgeable in regenerative medicine, and specialists in contracting and product development. The overall function of the IPT is to ensure that the AFIRM meets military needs, funds superior science, and is well managed.

The specific responsibilities of the IPT are to:

- Approve the annual report and program plans that are presented to the BOD.
- Ensure that all AFIRM research projects are aligned with military requirements.
- Monitor and evaluate the activities and progress of the AFIRM programs and management.
- Facilitate the military's evaluation and purchasing of products developed by the AFIRM.
- Assist consortia Directors and Management Teams in internal communication within the DoD and in understanding and meeting DoD regulation and reporting requirements relative to AFIRM performance.
- Facilitate the leveraging of AFIRM resources by coordinating with other funding agencies that support closely related research.

The IPT's Steering Group has day-to-day decision-making authority over the AFIRM and recommends major changes in research direction or funding to the voting members of the IPT. This group is chaired by the AFIRM Project Director and also includes the USAISR Commander, the Combat Casualty Care Senior Scientist, the Contracting Officer, and the Directors and Co-Directors of the RCCC and the WFPC. Among other activities, the Steering Group ensures that all AFIRM research projects are aligned with military requirements, reviews AFIRM research allocation, establishes decision points and continuation criteria, assesses project and program achievements in relation to milestones and time lines, and recommends continuation or termination of programs and individual projects to the IPT.

The IPT contains additional members from the Army, Navy, Air Force, and VA (one representative from each of these organizations), three representatives from the NIH (sharing one vote), and four external scientists. The IPT also contains ex officio advisors from the Judge Advocate General, the DoD Human Use office, a commercialization expert, and a regulatory expert appointed by the CRM-RP.

The Steering Group and the additional IPT members are voting members of the IPT. They are assisted by the ex officio members of the IPT and the Program Synergy Group to ensure that the AFIRM is progressing toward solutions for militarily relevant injuries.

Program Synergy Group

The Program Synergy Group includes representatives from each of the major programs in each of the consortia, members of the NIH or VA intramural research programs (as deemed appropriate), and USAISR. The Program Synergy Group is chaired by one of the consortia Co-Directors. It serves as a conduit for information exchange among the cores and seeks to build bridges between the programs and projects. It identifies and promotes opportunities to share or combine best practices and to accelerate existing projects or initiate new projects to bring therapies to our wounded service members. The Program Synergy Group reports its findings and recommendations twice a year to the Steering Group.



II: Limb and Digit Salvage



BACKGROUND

Injuries to arms and legs following severe trauma often result in the loss of large regions of tissue in the middle portion of the limb, disrupting the healing and use of the hand or foot. Despite many advances in reconstructive surgery, current methods to reconstruct these tissues are inadequate in many settings. The AFIRM Limb and Digit Salvage Program seeks to give wounded warriors innovative solutions to the most severe, devastating limb injuries through regenerative medicine. The goals of this program are to preserve and restore damaged or missing tissue following injury, reduce the need for amputation, reduce the time and risk involved in recovery, and enable the warrior's return to a fully independent, fully functional life, and ideally, a return to duty.

Rutgers-Cleveland Clinic Consortium (RCCC) researchers are working on a series of integrated projects focused on developing tissue-engineering solutions for bone, nerve, vessels, fascia, menisci, and skeletal muscle. They are collaborating with researchers funded by AFIRM's Craniofacial Reconstruction Program in advancing methods of immunomodulation to enable limb and face transplantation with minimal immunosuppression, using composite tissue allografts when salvage is not possible. RCCC investigators are also aligned with ongoing work in AFIRM's Burn Repair program, developing therapies for restoration of massive skin loss, including in the limb.

our science for their healing



II: Limb and Digit Salvage

Wake Forest-Pittsburgh Consortium (WFPC) researchers are pursuing an interdisciplinary, multipronged approach to the reconstruction/replacement of functional limb and digit tissue. Approaches they are pursuing include transplantation (composite tissue allografts), epimorphic regeneration, tissue regeneration by traditional tissue-engineering approaches, and enabling technologies. These diverse approaches represent the highly interdisciplinary background and experience of the program team leaders and the level of expertise required to address the challenging problem of limb and digit reconstruction.

U.S. Army Institute of Surgical Research (USAISR) researchers are using various scaffolds that will allow the focal and time-dependent release of antibiotics along with growth factors that encourage the influx of blood vessels and bone-producing cells to the wound site for enhanced fracture healing and prevention of infection. They anticipate that this scaffold will result in a better clinical outcome than the current staged treatment.

Projects in the AFIRM Limb and Digit Salvage Program have established numerous industry partnerships in anticipation of commercialization, a critical step in delivering products to wounded warriors.



Graduate student Catherine Ward preparing to analyze how a novel oxygen-generating material affects skeletal muscle (WFPC).

Unmet Needs

The unconventional weapons used in current conflicts in Iraq and Afghanistan are resulting in unconventional wounds that demand better solutions. Modern battlefield medicine is saving wounded warfighters who, in previous wars, would not have survived. These injuries often involve a massive loss of tissue, including large defects in continuity of bone (up to 20 cm), nerve, vessels, muscle, tendon, ligament, and skin. Musculoskeletal/extremity injuries are present in more than 80% of all combat injuries, fractures are present in 26%, and 82% of these fractures are open and complicated by extensive soft tissue loss.

Wounded warriors frequently sustain polytrauma (i.e., injury to several body areas and/or systems). In fact, there is a mean of 4.2 wounds reported for each wounded warrior. Wounds almost always involve injury to more than one tissue (e.g., bone, nerve, muscle, tendon, or vessel). As a result, combined approaches are envisioned. However, most often, the injury to one particular tissue becomes the limiting factor in salvage or functional restoration, most often bone and nerve, but vascular injury and muscle/tendon discontinuity are also common. Projects in the Limb and Digit Salvage Program are therefore targeted to make specific advances in these important areas of critical unmet needs: bone, nerve, artery, and soft tissue, as well as composite tissue injury repair and transplantation.

Limb salvage is currently possible in a large fraction of injured warriors but many still face amputation. Most of these are lower extremity amputations for which well-designed and well-tolerated prostheses are available. However, 20% of amputations involve the upper extremity and although prosthesis technology is advancing rapidly, fully functional, well-tolerated upper limb prostheses are still not available. The technology to salvage these limbs is progressing, as evidenced by projects in this program. However, when salvage fails, the capability to provide an identically functional replacement through engineering or transplant is crucial for wounded warriors. The main obstacle to successful transplantation of composite tissue to repair segmental defects in a limb, or to replace a whole limb, is control of the immune response.

A critical need therefore exists to improve immunomodulation techniques to reduce the obstacles to composite tissue transplantation.

Areas of Emphasis

AFIRM researchers are pursuing a complementary mix of research projects focused on various aspects of limb and digit salvage. Projects can be grouped into six “clinical

challenge” topic areas: Bone Repair and Regeneration, Soft Tissue Repair and Regeneration (excluding nerve), Nerve Repair and Regeneration, Composite Tissue Injury Repair, Transplantation, and Epimorphic Regeneration (and associated methods). Additional details on projects in each of these topic areas can be found in **Table II-1** and subsequent sections of this chapter.

Table II-1. Projects funded by RCCC, WFPC, and USAISR per clinical challenge topic area.

Clinical Challenge	Consortium/Institution	Project Number	Project Title
Bone Repair and Regeneration	RCCC	4.2.1	Advanced 3D Scaffolds for Large Segmental Bone Defects
		4.2.2	Optimizing Cell Sources for the Repair of Bone Defects
		4.2.3	Advancing Bone Repair Using Molecular Surface Design (MSD)
		4.2.4	Systematic Review of Innovative Combined Therapies for Repair of Bone Defects
	USAISR	4.4.9	Bone Regeneration in a Contaminated Defect
Soft Tissue Repair and Regeneration (excluding nerve)	WFPC	4.4.6	Oxygen-Generating Biomaterials for Large Tissue Salvage
		4.5.8	Isolation and Expansion of Native Vascular Networks for Organ Level Tissue Engineering
	RCCC	4.3.2	Development of Bioabsorbable Tissue-Lined Stent for Vessel Trauma
		4.4.3a	Functional Scaffold for Musculoskeletal Repair and Delivery of Therapeutic Agents
		4.4.3b	Functional Scaffolds for Soft Tissue Repair and Joint Preservation
Nerve Repair and Regeneration	WFPC	4.4.4	Peripheral Nerve Repair for Limb and Digit Salvage
		4.4.5	Modular, Switchable, Synthetic, Extracellular Matrices for Regenerative Medicine
	RCCC	4.4.1/4.4.2	Repair Segmental Nerve Defects
		4.4.2a	Cell and Bioactive Molecule Delivery to Enhance the Repair of Segmental Nerve Defects
Composite Tissue Injury Repair	WFPC	4.4.3	Engineered Delivery of Spatial and Temporal Cues for Composite Tissue Injury Repair
Transplantation	WFPC	4.4.2	Hand Transplantation for Reconstruction of Disabling Upper Limb Battlefield Trauma – Translational and Clinical Trials
Epimorphic Regeneration (and associated methods)	WFPC	4.4.1	Blastemal Approach to Digit Reconstruction
		4.4.7	High Throughput Approaches Applied to Tissue Regeneration
		4.4.8	Magnetophoretic Cell Sorting for Transplant Therapies



II: Limb and Digit Salvage

Bone Repair and Regeneration

Studies at RCCC

RCCC's bone program contains four integrated projects designed to address critical gaps that currently limit the medical therapy option and outcomes for warriors with injured limbs, whose challenges include the need to regenerate a segmental defect in an extremity. The researchers are defining optimal methods for bone regeneration, focusing on synthetic scaffold development in combination with a patient's own bone-forming osteogenic connective tissue progenitors (CTP-Os) to regenerate bone in large post-traumatic segmental defects more rapidly, effectively, and reliably. This mission involves three core research project areas: defining an optimal osteoconductive scaffold (Project 4.2.1), selecting the preferred cell-sourcing methods (Project 4.2.2), and optimizing the osteoconductive and osteoinductive milieu (Project 4.2.3). Oversight of the three core research projects is provided by Project 4.2.4.

The **Muschler group** (Project 4.2.1) at Cleveland Clinic, Mayo Clinic, Massachusetts Institute of Technology (MIT), and Rutgers seeks to identify an optimized degradable osteoconductive scaffold that will function better than the existing standard of allograft bone matrix as a building block upon which to build other key biological elements. Candidate materials include, but are not limited to, tyrosine-derived polycarbonate (Tyr-PC), poly(L-lactide:ε-caprolactone) (PLCL), and poly(propylene fumarate) (PPF). The researchers are utilizing a standardized defect (the canine femoral multi-defect model) to competitively assess biological and preclinical differences in scaffold performance. Among the eight materials tested to date, porogen-leached Tyr-PC scaffolds with a calcium-containing coating were the most promising. The researchers plan to optimize the



Researcher Anthony Radice at the lab bench (RCCC).

calcium-containing coatings applied to porogen-leached Tyr-PC scaffolds during Year 3. Only one additional scaffold technology (slow-degrading PPF) may be advanced for further development in Year 3. The research team is actively working to define the next appropriate step toward clinical trials of these materials.

The bone-forming cells are severely depleted in the region of a large bone defect. The **Muschler/Zborowski group** (Project 4.2.2) at Cleveland Clinic is investigating methods to optimize the clinical harvest, intraoperative processing, and transplantation of bone-forming CTP-Os to enhance bone regeneration in large defects. The researchers intend to provide surgeons treating injured warriors with effective and practical clinical methods to concentrate, select, and transplant CTP-Os from a patient's own bone marrow (BM) and thereby enable and accelerate bone regeneration with minimal risk. Three basic methods for cell processing are being explored: density separation, selective retention (SR), and magnetic separation. These methods will be competitively compared in vivo during Year 3 using the canine femoral multidefect model. The most effective method for cell sourcing and processing will be further assessed in a rigorous, clinically relevant, long bone defect model. The researchers' methods for cell sourcing and processing will be combined with top-performing scaffolds from

other AFIRM projects and advanced into clinical trials, most likely in collaboration with the Major Extremity Trauma Research Consortium (METRC).

Placing a scaffold and optimal cell source in a bone defect has been shown to improve bone formation. However, under current conditions most transplanted cells do not survive. Rather, they die as a result of the profound drop in oxygen within the transplant site through a process of apoptosis (programmed cell death). This challenge becomes progressively greater as the distance from a transplanted cell to the nearest blood vessel increases from 1-3 mm (in simple fractures or small animal models) to 3-10 mm in larger defects. The **Muschler/Griffith/Kohn/Clark group** (Project 4.2.3) at Cleveland Clinic, MIT, Rutgers, and Stony Brook University is developing methods designed to enhance the survival and performance of transplanted cells:

- **MSD is a biomaterials strategy that involves tethering** a growth factor or signaling molecule on the surface of an implant to change the way that cells interact with the material. This can change cell attachment, migration, proliferation, differentiation, or survival in a controlled manner. The researchers of Project 4.2.3 have explored tethering of growth factors and proteins using both purely chemical strategies and a binding strategy based on a biological system. Based on data obtained to date, the research team selected epidermal growth factor (EGF) as the most promising agent for tethering. During the upcoming year, they will optimize the fabrication of EGF-tethered scaffolds and validate their bioactivity. The researchers hope to translate at least one advanced bioactive MSD surface into an appropriate human trial by Year 5 of the project.
- **Oxygen Delivery (O_2D)** within an implanted scaffold for bone repair is being developed using polymer methods that have previously been used for controlled drug delivery. Rather than using a drug, polymers are being developed that will release oxygen at very low levels over a few days following implantation. Data generated to date in this project have demonstrated that even a small amount of local O_2D may have profound effects on the ability of transplanted cells to survive a rapid

drop in oxygen levels. The researchers plan to advance the O_2D technology platform to in vitro toxicity testing. Beginning in 2011, they plan to assess in vivo efficacy in the canine femoral multidefect model.

Project 4.2.4: Systematic Review of Innovative Combined Therapies for Repair of Bone Defects. This project is not a research activity and has no specific cost or deliverables associated with it (therefore, no progress report is included in this chapter for this project). George Muschler, MD and Michael Yaszemski, MD, PhD use personal and collaborative interactions to reach out and communicate with the military treatment community, the musculoskeletal trauma community (orthopedic and emergency medicine clinicians), and the tissue-engineering and research community. The purpose of these activities is to ensure that the work within the RCCC bone-related projects of AFIRM remains well targeted to address evolving needs and to keep abreast of new information and opportunities that may influence or advance the AFIRM work. Noteworthy is that (1) Dr. Muschler has taken a role on the Steering Committee of the METRC, and (2) Drs. Yaszemski and Muschler met with Mark Melkerson of the U.S. Food and Drug Administration (FDA) to explore the most appropriate mechanisms for communication among AFIRM, METRC, and FDA.

Studies at USAISR

The **Wenke group** (Project 4.4.9) at USAISR is using various scaffolds that will allow the focal and time-dependent release of antibiotics along with growth factors that encourage the influx of blood vessels and bone-producing cells to the wound site for enhanced fracture healing and prevention of infection. They anticipate that this scaffold will result in a better clinical outcome than the current staged treatment. The researchers determined that early debridement (removal of a patient's dead, damaged, or infected tissue) and antibiotic administration decrease the rate of infections. Delivering both bone morphogenetic protein (BMP) and the antibiotic vancomycin to a contaminated critical size rat femur defect, they found that it is possible to both reduce infection and regenerate bone in a contaminated defect.



II: Limb and Digit Salvage

Soft Tissue Repair and Regeneration (excluding nerve)

Studies at WFPC

The **Harrison group** (Project 4.4.6) at Wake Forest University is developing an injectable material capable of generating oxygen. Preparing an injectable oxygen-generating material would allow the delivery of oxygen in controlled amounts to engineered tissue scaffolds or pre-existing tissue. The researchers isolated a leg muscle from the rat and injected tiny particulate oxygen-generating particles (POGs) into it. They found that the POGs dispersed throughout the tissue. They also determined that mechanical injection into a functioning muscle does not hinder the active force of the muscle. Structurally, the researchers observed that muscles injected with POGs were able to maintain the characteristics of healthy muscle. The researchers plan to test the oxygen-generating material in both muscle ischemia (restricted blood supply) and wound graft models to determine the extent of the ability of the POGs to provide enough oxygen for large three-dimensional tissue and skin. Because composite tissues are composed of multiple cell types, the research team will analyze several tissue systems including skeletal muscle, bone, nerve, and skin. Optimization of the most promising tissue systems benefiting from the technology will continue in later years along with identifying the best possible clinical application.

Tissue engineering holds the promise of creating replacement limbs and organs outside of the human body. However, two major obstacles have hindered the development of techniques to fabricate limbs and organs: (1) the inability to adequately grow vascular networks into tissue constructs outside the body and (2) the inability to reintegrate these tissues into the systemic circulation. The **Gurtner/Longaker/Langer group** (Project 4.5.8) at Stanford University and MIT has developed novel strategies that use preformed native circulatory networks that can be supported outside the body during organ fabrication, expanded using progenitor cell-based techniques, and then readily integrated into the systemic circulation. Their preliminary work has demonstrated the feasibility of sustaining explanted microvascular beds (EMBs) outside the body for up to 7 days. They have

been able to transduce EMBs with vectors that deliver therapeutic proteins and demonstrate expression of the proteins after reimplantation. In the upcoming years, the research team plans to isolate and expand native vascular beds using a multifaceted approach to tissue engineering. They will employ bioreactor systems, angiogenic hydrogels (stimulate blood vessel growth into an area), and progenitor cells to fabricate organ-level vascular networks.

Studies at RCCC

Current metal stents are designed for the treatment of late-stage peripheral vascular disease and leave young patients at risk for graft failure due to fracture or the recurrence of stenosis (narrowing of a blood vessel), and are not designed to last the lifetime of the individual. The **Sarac group** (Project 4.3.2) at Cleveland Clinic is developing bioabsorbable and/or fracture-resistant, tissue-lined stent grafts for minimally invasive treatment of arterial and venous trauma in young patients. During the past year, the researchers established the design of the stent and the attachment pattern of the tissue lining. They developed both a fatigue-resistant Nitinol stent and a bioabsorbable stent. They are conducting a variety of mechanical tests on these stents, and initial results show that the stents are meeting specifications. In Year 3, the researchers will evaluate and compare their stent prototypes in the iliac arteries of the pig. In Years 4 and 5, a 6-month animal trial and sterilization and delivery system modifications will be completed in preparation for design freeze and a shift to clinical trials.

Fascia is a thin, fibrous, resilient connective tissue that surrounds, protects, and supports the body's muscles. Fascia lata, the deep fascia of the thigh, provides a natural, strong, and mechanically robust scaffold for rotator cuff tendon repair, for the repair of abdominal wall fascia, or for bridging large tendinous deficits between muscle and bone. However, the suture retention properties of fascia lata limit the early mechanical strength of associated repairs.

The **Derwin group** (Project 4.4.3a) at Cleveland Clinic seeks to provide injured warriors with biological materials for reconstruction of tendon and muscle-tendon defects,



Dr. Ophir Ortiz analyzes results from the Quartz Crystal Microbalance with Dissipation (QCM-D) system (RCCC).

using human fascia lata allograft tissue, reinforced with polymer fibers. They believe that these materials will have sufficient strength and suture retention properties to enable robust soft tissue repairs and early mobilization, as well as biological properties that accelerate tissue incorporation and functional remodeling. In Year 2, the researchers completed design and testing methods for reinforced fascia patches. Three critical feasibility studies for rotator cuff repair are ongoing and are expected to be complete by May 2011. These studies are being funded by the Musculoskeletal Transplant Foundation (MTF). The funding of the rotator cuff studies by MTF will allow the researchers to shift their focus from tendon regeneration to abdominal wall reconstruction, which has been identified as a critical gap in current therapies available to wounded warriors.

Due to limited healing capabilities, injuries to the meniscus (internal cartilage) of the knee are often treated with resection. This treatment generally leads to early symptom relief but frequently leads to the development of degenerative arthritis of the knee. The **Gatt/Dunn group** (Project 4.4.3b) at the UMDNJ is developing an "off-the-shelf" tissue-engineered meniscus scaffold that can be used to replace a severely damaged meniscus in

an injured service member. The scaffold is composed of an anatomically designed fiber-reinforced scaffold and a collagen-based ECM similar to the native meniscus. The therapeutic goal is to return service members to duty as quickly as possible and to avoid the costly consequence of osteoarthritis of the knee in later years. Year 2 studies focused on the design, fabrication, and evaluation of "second generation" scaffolds with improved mechanical and biological properties. The researchers improved the scaffold by modifying the fiber reinforcement pattern, increasing the collagen content, and adding glycosaminoglycans (GAGs). Further optimization of the scaffold and surgical protocol during the upcoming year is expected

to improve the in vivo performance in a sheep model. Particular emphasis is being placed on examination for evidence of degenerative changes of the articulating cartilage surfaces beyond 16 weeks of implantation.

Nerve Repair and Regeneration

Studies at WFPC

Following trauma, incomplete nerve regeneration and permanent demyelination (damage to the protective sheath surrounding nerves) may result, leading to life-long disability. The **Marra/Kaplan/Smith group** (Project 4.4.4) at the University of Pittsburgh, Tufts University, and Wake Forest University is developing a proactive biodegradable nerve guide system that delivers chemical cues (e.g., growth factor incorporation and protein coatings) and biophysical cues (e.g., surface patterning) to regenerating peripheral nerves. The researchers identified a drug delivery strategy to deliver neurotrophic factors for approximately 60 days in vivo in a rat nerve defect model. They are now examining the use of keratin-filled bovine collagen nerve guides (NeuraGen®) to treat a 1 cm nerve defect in the median nerve of non-human primates. The animals were doing well at



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13 weeks post surgery. Results of this project, if successful, will lead to a human pilot study.

Functional limb and digit tissue restoration involves a hierarchically defined process that often requires precise spatial and temporal coordination among multiple biological systems and processes. The **Tirrell group** (Project 4.4.5) at the University of California, Berkeley is pursuing an approach to induce peripheral nerve growth following traumatic amputation by modulating components of the naturally occurring extracellular matrix (ECM) (e.g., fibronectin and laminin). The researchers are using synthetic chemistry to construct peptide amphiphiles (PAs) with controlled physicochemical and bioactive properties and testing their ability to promote cell adhesion, migration, and nerve regrowth. They have developed several biologically active PA gels with well-defined three-dimensional structures. They plan to correlate rheological and structural features of the PAs with biological activity to refine and define injectable three-dimensional matrices for in vivo applications (e.g., nerve gap injury model). They will conduct animal studies using biologically responsive PA gels in collaboration with the Marra and Guldborg groups. They will also design the multicomponent synthetic ECM system to regenerate nerve and/or bone at the wound site over multiple time domains.

Studies at RCCC

RCCC's Nerve portfolio is composed of four parallel, synergistic, and interdependent projects (three are incorporated under Project 4.4.1/4.4.2 and the fourth is Project 4.4.2a). In aggregate, these projects seek to provide wounded warriors with injuries to large sensory and mixed motor nerves with an opportunity for recovery that does not currently exist. Novel materials are being developed and their mechanical properties are being tested to identify those best suited to nerve repair. Biocompatibility is being tested in vitro using standardized tissue culture model systems and in vivo using rodent models.

Like the work in RCCC's Bone portfolio, collaboration between laboratories involved in the Nerve portfolio is designed to enable competitive assessment in a tournament design. Each lab brings forward its best material(s) for testing. Successful outcomes in the 1 cm rat sciatic

nerve defect justify further assessment in an intermediate-gap (rabbit 10 cm) and then in a large-gap (sheep 20 cm) animal model. In parallel, essential steps for clinical transition including sterilization techniques and scale-up under current Good Manufacturing Practice (cGMP) guidelines are being implemented.

The **Windebank/Yaszemski group** at Mayo Clinic, the **Kohn/Schachner group** at Rutgers, and the **Anderson/Langer group** at MIT (Project 4.4.1/4.4.2) have developed a model for the early assessment of promising scaffolds in a human setting that involves the opportunity to repair defects of the sural nerve (a small sensory nerve on the back of the leg), which is routinely biopsied and left unrepaired during investigation of possible nerve diseases. The researchers established a standardized nerve defect and tube implantation model system in the rat that enables the competitive evaluation



Dr. Basak Clements making a biodegradable, porous nerve conduit (RCCC).

of polymer scaffolds across the consortia. Screening of over eight scaffold materials was completed resulting in the selection of a poly(caprolactone-fumarate) (PCLF) tube as the candidate scaffold to move into clinical trials. The researchers developed a method for incorporating aligned electrospun fibers into the nerve scaffold in a uniform manner with no fiber aggregation. They incorporated anti-inflammatory agents in electrospun fibers to decrease the inflammatory response to synthetic materials. The research team also developed a rat hindlimb model including a soft tissue defect and fibrosis. This constitutes an important advance for studying nerve injury and repair strategies in the setting of extensive soft tissue injury that often accompany nerve injuries in war casualties. In Year 3, the researchers will initiate a clinical trial "Repair of 6 cm Peripheral Nerve Gaps" in sural nerve biopsy patients using PCLF tubes. Subsequent Phase 2 clinical trials in the setting of traumatic injury are envisioned, involving both upper extremity and lower extremity cohorts, potentially in collaboration with METRC.

Bone marrow stem cells (BMSCs) comprise a heterogeneous population of cells that contribute to the regeneration of multiple body tissues. The **Siemionow group** (Project 4.4.2a) at Cleveland Clinic seeks to improve nerve regeneration by enhancing the performance of allograft nerve sheath conduits using culture-expanded BMSCs. The researchers have determined that an allogenic epineural conduit without immunosuppression is a feasible method of peripheral nerve gap repair. The addition of BMSCs resulted in better functional outcomes than saline-filled (control) conduits. BMSCs also increased the amount of myelin surrounding the regenerated nerve fibers when compared to controls. Overall, allogenic epineural conduits supported with BMSCs in 2 cm rat sciatic nerve defects provided recovery comparable to that achieved by an autograft. In Year 3, the researchers will continue development of the conduit in the rat model. By the beginning of Year 4, it is anticipated that further testing will be performed in the sheep 6-8 cm median nerve defect using the best performing constructs from Year 3. Advancement into clinical trials is projected in Year 5, assuming favorable performance is achieved in the sheep model.

Composite Tissue Injury Repair

Studies at WFPC

The **Guldberg/Boyan group** (Project 4.4.3) at the Georgia Institute of Technology seeks to develop and test technologies that will enable the restoration of limb function following composite tissue trauma. The researchers have established promising regenerative strategies for bone and nerve using nanofiber mesh spatial guidance and sustained delivery of a clinically approved inductive protein, bone morphogenetic protein-2 (BMP-2). They have developed composite multi-tissue injury models to simulate complex combat injuries and test spatial and temporal guidance strategies that take advantage of synergistic interactions among the tissues observed during development and repair. Notably, they have established a rat model of composite tissue loss that is now used by all project leaders within the AFIRM Limb and Digit Salvage Program. A large animal model is currently being planned as the next step toward clinical translation. Once proof of concept has been demonstrated in the large animal model, the goal is to initiate a human clinical trial pilot study in Year 5.

Transplantation

Studies at WFPC

While composite tissue allografts (e.g., hand transplants) are now a clinical reality and have been performed in multiple centers worldwide, the procedure has not reached widespread clinical use because recipients require lifelong high-dose multidrug immunosuppression to prevent graft rejection. The **Lee group** (Project 4.4.2) at the University of Pittsburgh is developing a protocol for hand transplantation using donor BMSCs in combination with novel fusion proteins (the "Pittsburgh protocol") that will minimize maintenance immunosuppressive therapy. The researchers have successfully achieved their clinical translation milestone. Three patients have received hand transplants. The first patient was treated in March 2009 using the newly developed Pittsburgh protocol (developed in part under AFIRM funding). The patient remains on minimal immunosuppressant therapy and has excellent use of the transplanted hand. The second patient was a bilateral transplant and the third patient was a unilateral transplant. These patients are 6 and 3 months



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Hand transplant recipient, Corporal Maloney, visited by General James N. Mattis, Commander of the U.S. Central Command (WFPC).

post transplantation, respectively, and their progress is excellent. The researchers have also established a pre-clinical hindlimb transplant model in Yucatan miniature swine. They are optimizing the number of cells required for bone marrow transplantation and optimizing the immunosuppressive therapy protocol in the swine model. In Year 3, the research group aims to prolong limb allograft survival by using targeted skin immunotherapy with inhibitors of white blood cell migration in combination with optimal protocols from Year 2.

Epimorphic Regeneration (and associated methods)

Studies at WFPC

The **Badylak group** (Project 4.4.1) at the University of Pittsburgh is investigating mechanisms for recruiting large populations of stem cells to the site of limb and digit injury and then developing strategies to induce the formation of functional limb and digit tissue to replace the damaged or missing structures. They have established a model of digit amputation in an adult mouse where the second joint of a digit is amputated. In their mouse model, the researchers have shown that treatment with

peptides derived from ECM leads to the recruitment of a population of cells that express markers that are universally recognized as markers of primitive stem cells (i.e., Sox2, Rex1, and Sca1). This is an important finding because the essential first step to promoting epimorphic regeneration is the endogenous recruitment of a population of stem cells that are capable of forming all of the tissues in the missing limb or digit. Future work will focus on further refining strategies to increase the number of primitive stem cells that can be recruited to a site of amputation, as well as developing a device that will allow one to control the stem cells. Clinically, the researchers used a biologic scaffold composed of porcine-derived ECM to treat a soldier with massive loss of quadriceps muscle. Results showed the new regrowth of 10%-15% of the missing muscle. This clinical application is remarkable for the potential to provide a viable option for patients suffering from traumatic muscle tissue loss.

The **Stewart/Thomson group** (Project 4.4.7) at the Morgridge Institute for Research and the University of Wisconsin, Madison is studying tissue regeneration using high-throughput technologies (e.g., microarrays and next-generation sequencing). In collaboration with the

Badylak group, the researchers have analyzed amputated mouse digit tips both from untreated mouse digits and those treated with ECM factors that are designed to enhance regenerative capabilities. The short-term purpose of this collaboration is to identify genes and gene networks that are activated (or deactivated) in the treatment case. The longer term purpose is to harness this knowledge in conjunction with methods from the research team's prior work on reprogramming cells to activate or deactivate appropriate genes and gene networks to foster regeneration of tissues. The researchers have provided whole transcriptome data and analysis of the mouse digit tip amputation time course for both treated and untreated time courses. Their analysis revealed the upregulation of ECM components, structural components, and cell adhesion molecules. In addition, the analysis identified several upregulated genes involved in bone/cartilage formation, angiogenesis, and syndactyly (fusion of digits). These preliminary studies lay the groundwork for providing transcriptome analysis for the multipotent cell cluster in the coming year. Also in the coming years, the research team would like to conduct whole transcriptome analysis of the axolotl limb blastema and perform bioinformatics analyses comparing the data to the mouse digit system.

The **Soh group** (Project 4.4.8) at the University of California, Santa Barbara is developing ways to sort and isolate cells from complex mixtures of cells. The researchers have generated a family of devices that solve severe shortcomings inherent in current methods of magnetic cell sorting. In particular, they have developed the **Multi-Target Magnetic Activated Cell Sorter (MT-MACS) platform to achieve, for the first time, simultaneous sorting of multiple targets at high levels of purity, recovery, and throughput.** In addition, they have developed the **Continuous-Trapping Magnetic Activated Cell Sorter (CT-MACS) device, which allows purification of extremely rare cells from complex mixtures with unprecedented cell recovery.** To fully exploit the utility of these microfluidic devices, the researchers have closely interacted with the Badylak and Muschler laboratories to isolate pluripotent progenitor cells from tissues of model animals. They are also in the process of commercializing the technology with an industrial partner. In Year 3, the research team plans to shift the focus to utilizing its microfluidic purification systems to efficiently generate affinity reagents for stem cell markers. Overall, this unique cell-sorting capability may provide a critical technical solution to isolate target stem cells for clinical therapeutics.



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Progress Reports: Bone Repair and Regeneration

Advanced 3D Scaffolds for Large Segmental Bone Defects

Project 4.2.1, RCCC

Team Leaders: George F. Muschler, MD (Cleveland Clinic)

Project Team: Viviane Luangphakdy, MS, Hui Pan, MD, PhD, Kentaro Shinohara, MD, PhD, Brian Lampe, BS (Cleveland Clinic); Joachim Kohn, PhD, Aniq Darr, PhD (NJCBM); Linda Griffith, PhD, Linda Stockdale, MAT (MIT); Michael Yaszemski, MD, PhD, Suzanne Segovis, BS, and Marokh Dadsetan, PhD (Mayo Clinic)

Collaborators: Sunil Saini, PhD (IntegraTM, Plainsboro, NJ)

Therapy: Advanced Regeneration of Segmental Bone Defect

Deliverable: Advanced 3D Scaffolds for Large Segmental Bone Defect

TRL Progress: *Start of Year 1, TRL 3; End of Year 1, TRL 3; End of Year 2, TRL 3*

Key Accomplishments: The researchers completed fabrication and characterization of three polymer-based scaffold platforms. They completed competitive assessment and down-selection of scaffold materials in the canine femoral multidefect (CFMD)

model. Among the materials tested to date, the porogen-leached Tyr-PC with beta tri-calcium phosphate (TCP) performed best. The researchers also established a defined track record of historical performance standards that can be used to rapidly benchmark the performance of new or competing scaffold materials using the CFMD model.

Key Words: Bone repair, bone defect, bone graft, scaffold, osteogenesis, osteoinduction, osteoconduction, connective tissue progenitors (CTP-O), canine femoral multidefect (CFMD)

Introduction

Treating military fractures represents an ongoing challenge because high-energy blast injuries from improvised explosive devices are increasingly common. Military extremity injuries traditionally comprise the majority (58%-88%) of traumatic injuries in the U.S. armed conflicts. A large proportion (23%-39%) of extremity wounds are fractures, of which most (82%) are open injuries. These defects are most frequent in the tibia; however, the femur, humerus, radius, and ulna are also common anatomic sites. These fractures are frequently comminuted, complicated by extensive soft-tissue loss, and have segmental bone loss of 5-20 cm. As a result, the biological challenge of treating these defects is not just the challenge of assembling a combination of scaffold, cells, and growth factors to fill the defect, as it is frequently described. The challenge here also demands that the solution(s) offered will function in an environment that is defined by the tissue envelope that exists surrounding these defects and that these solutions fit into the clinical-care environment in which these defects are managed and treated. These treatment environments include (a) the management of bone loss in the acute

stage, in which mechanical stability and prevention and treatment of infection are the primary goals, and subsequently (b) reconstruction of the defect in the chronic stage, after the limb has been preserved, but when the environment around the defect is often characterized by dense scarring and compromised vascularity.

This biological environment of a large bone gap in a compromised tissue envelope represents one of the greatest challenges for military surgeons. Methods to ensure rapid, safe, reliable bone regeneration in a bone defect with a compromised tissue bed are greatly needed. The researchers of this project seek to identify an optimized degradable osteoconductive scaffold that will function better than the existing standard of allograft bone matrix as a building block upon which to build other key biological elements.

To accomplish this charge, highly promising biomaterials and scaffold fabrication methods in laboratories at Rutgers, MIT, and Mayo Clinic have been identified, all of which have effective transition opportunities. The scaffolds' substrate materials evaluated by the researchers include: Tyr-PC; poly(L-lactide-co-glycolide acid) (PLGA);

PLCL; poly(propylene fumarate) (PPF); and TCP ceramic granules. Several clinically practical methods for fabricating three-dimensional scaffolds are used, including three-dimensional printing, laser stereolithography (SLA), and the porogen-leached method. These scaffolds and fabrication methods have been systematically evaluated and compared using an established canine model of bone defect repair, the CFMD model. Between March 2008 and March 2010, a series of eight experiments was executed to directly compare scaffold options. In each comparison, the superior scaffolds moved forward in subsequent comparisons.

Summary of Research Completed in Year 1

During the first year of the project, the researchers identified, designed, and fabricated four distinct families of copolymer-based osteoconductive scaffolds for in vivo testing. They synthesized new, second-generation polyester and tyrosine-based copolymers and their composites with osteogenic inorganic particles. They fabricated complex three-dimensional printed scaffolds with controlled porosities from several of the copolymers and found them to be easily sterilizable. They completed all animal surgeries planned for Year 1 along with micro-CT (mCT) scanning for statistical analysis of bone volumes and preparation of histology samples for the initial evaluation of copolymer and composite scaffolds. They achieved good in vivo performance using tyrosine-based copolymer and poly(alkyl ester) composites. Using SLA, they prepared complex composite scaffold architectures and assessed sterilization methods for the scaffolds including gamma irradiation and ethylene oxide.

Research Progress – Year 2

The CFMD model is an effective and sensitive tool to rapidly screen and compare scaffold materials, cell transplantation strategies, and effects of soluble factors (e.g., BMPs) in a bone defect of clinically relevant size. The CFMD model provides four scaffold-ready cylindrical defects in the lateral canine femur measuring 10 mm in diameter x 15 mm in height. Each scaffold construct is manufactured as a cylinder 9.9 mm in diameter x 15 mm

in height. A description of the surgical and mCT analysis protocol for the femoral defect surgery has been detailed in Takigami's osteogenic protein-1 paper and in two recent reviews on the design and use of animal models for bone regeneration to advance clinically valuable bone regeneration methods and materials.

Overall, the research team completed eight in vivo scaffold assessment experiments. The mCT and histology results demonstrated that the best-performing degradable scaffolds—Tyr-PC with TCP, PPF slow degrading, and PLCL with TCP—did not compare to the bone allograft matrix in either region (pericortical [PC] and intramedullary [IM]) (Figure II-1). Among the three AFIRM polymers, there was no single, definitive scaffold that outperformed the others. However, there were specific formulations in each platform that were clearly preferred over others. The addition of TCP to any scaffold seemed to significantly improve bone ingrowth. The relative performance of individual scaffolds appeared to be systematically different in the PC and IM regions. The best synthetic scaffolds in the PC region were Tyr-PC with TCP and PLCL with TCP. The best synthetic materials in the IM region were Tyr-PC with TCP, PPF slow and fast, and PLCL-TCP.

The scaffold-testing tournament confirmed that the allograft matrix remained the preferred choice, with far greater bone formation especially in the IM region. The use of platelet-rich plasma (PRP) to generate the clot environment rather than BM appears to increase bone formation, particularly in the deeper/central (last vascularized) areas of the defect.

The researchers initiated a rigorous plan for assessing strategies for rapidly deployable calcium-containing scaffold coatings to improve scaffold performance. They completed a leveraged (externally funded) assessment of allograft in the CFMD model. Finally, they established an expanded team to develop the goat chronic tibia defect (GCTD) model to provide a more appropriate tissue envelope for assessing bone defect regeneration strategies within AFIRM.



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Key Research Accomplishments

- Completed fabrication protocols and characterization of each scaffold platform at the three participating bio-materials laboratories (Rutgers, MIT, and Mayo Clinic).
- Established a defined track record of historical performance standards that can be used to rapidly benchmark the performance of new or competing scaffold materials in the CFMD model.
- Made the decision to move forward and integrate Project 4.2.2 to optimize cell delivery using a bone allograft matrix.
- Down-selected among initial scaffold options to just two AFIRM-funded platforms: Tyr-PC (Rutgers), which stood out as most promising, and PPF (Mayo Clinic).

Conclusions

The participating laboratories in this project have operated with an exceptional level of collaboration and col-

legality and have advanced the work at a rate that has exceeded the expectations of the initial AFIRM proposal.

The CFMD model has provided a sensitive, robust, standardized tool that is effective in defining biologically and clinically relevant differences in performance characteristics between scaffold preparations in a competitive comparison environment. It has revealed significant differences between scaffolds and also deficiencies in performance of graft preparations that have previously performed exceptionally well in smaller defects in smaller animals.

Among the materials tested to date, the porogen-leached Tyr-PC with TCP performed best. Three-dimensional printed PLCL with TCP and stereolithography-fabricated slow-degrading PPF also demonstrated potential utility and may undergo further evaluation. However, these results fail to demonstrate mCT or histological performance that meets or exceeds that of the current "gold standard" for osteoconductive scaffold, i.e., the allograft bone matrix.

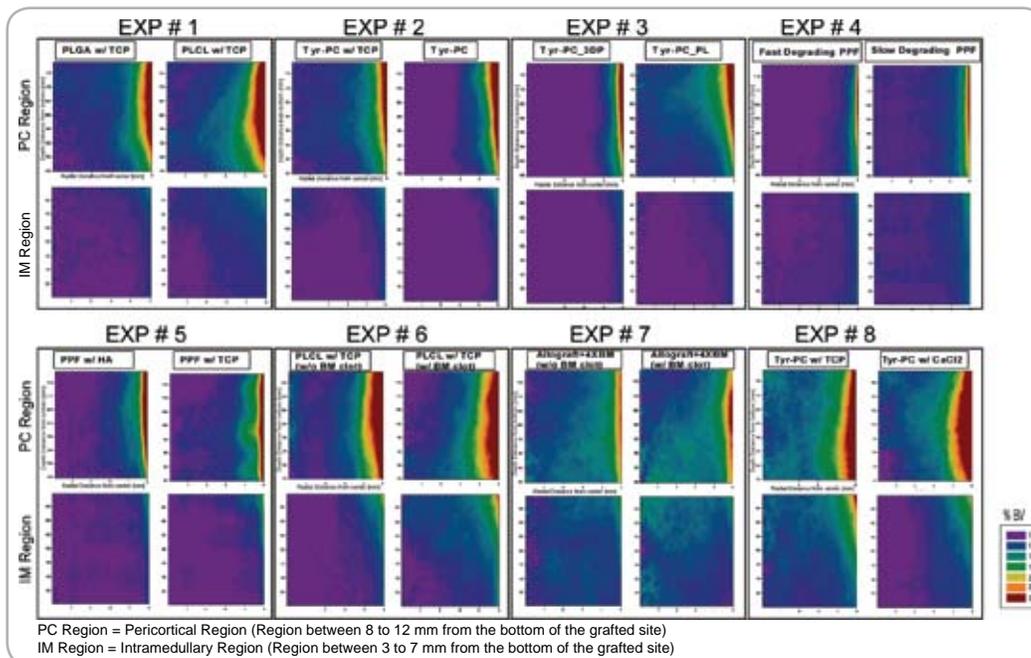


Figure II-1. Mean percentage of bone volume (% BV) formed by the osteoconductive scaffolds plotting by radial position (x-axis) and depth position (y-axis).

Research Plans for the Next 3 Years

The test results from this scaffold-testing tournament have demonstrated that the addition of calcium-containing coatings or materials can enhance the performance of three leading scaffolds (i.e., Tyr-PC, PLCL, and PPF). Based on this finding, the Limb and Digit Salvage Program has decided to extend the planned optimizing and down-selection of available scaffolds in Project 4.2.1 beyond the initial 2 years of AFIRM funding into Year 3 to determine if adding calcium-containing coatings or materials may also advance the performance of Tyr-PC and/or PPF scaffolds into or beyond the range of allograft bone matrix. To facilitate this exploration, the researchers established a consulting/collaboration relationship with Dr. Racquel LeGeros (of New York University), an expert in calcium-containing biomaterials and the holder of potentially relevant patents in this domain. The potential for advancement in this area is also expected to positively affect the downstream capacity of these AFIRM scaffolds to be integrated into cell-sourcing and delivery strategies developed in Project 4.2.2 and MSD strategies developed in Project 4.2.3.

The research team established active outreach to enable competitive assessment of these platforms with existing options that may be relevant to the injured warrior. For example, proposals have been set in place with industrial partners to evaluate other clinical standard materials already commercially available, including BMP-2 Infuse™ (Medtronic Spinal and Biologics Division, Memphis, TN) and Trinity™ Evolution (Musculoskeletal Transplant Foundation, Edison, NJ).

The research team is actively working to define the next appropriate step toward clinical trials of these materials. An inventory of available animal models has been completed with the finding that *none* of the current animal models of segmental bone defects provides a tissue envelope environment that is satisfactory in mimicking the wound environment of the bone defects being treated in our wounded warriors. In response to this finding, a team has been established to develop a more rigorous segmental defect model. This team includes members

of the current AFIRM team and other leading experts. This team has defined a chronic bone defect model in the goat tibia, which (a) includes a defined soft-tissue defect and (b) establishes a scarred soft-tissue envelope around a 5 cm bone defect, as a pregrafting state in which new materials and strategies can be tested. The team proposes work to integrate surgery at three separate surgical centers, using a carefully standardized protocol to both speed the work and to demonstrate reproducibility and translatability of the model protocol across centers. A Peer Reviewed Orthopaedic Research Program proposal was submitted but not funded in 2009. Active work is ongoing to identify an appropriate funding source to advance this critical element of the project.

MIT, Rutgers, and Mayo Clinic will continue ongoing contact with industrial partners (Integra™, Trident, BonWrx) and the FDA to identify pathways to commercialization (510(k) or Investigational Device Exemption [IDE] depending on the scaffold selected) for their biodegradable scaffolds in Year 3. In addition, the integrated bone team has recognized the need for collaborative work related to standardize and optimize a means for scaffold sterilization. A subgroup has been meeting monthly via teleconference and webinar to discuss and standardize a sterilization procedure.

Overall, the project is considered to be on track for delivering a well-characterized and competitively vetted scaffold into transition and clinical trials by 2013.

Planned Clinical Transitions

Clinical trials in Year 5 will require the collaboration of a large clinical trial network of civilian and military trauma centers. The integrated bone team is proactively developing resources, models, and venues for clinical trials with the METRC clinical trials group. The METRC consists of a network of clinical centers, plus one data-coordinating center, that will work together with USAISR to conduct multicenter clinical research related to AFIRM regenerative strategies in limb salvage. Clinical trials are most likely to be funded by one or more industrial partners, depending on the scaffold, cell source, or molecularly designed surface that is involved.



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Corrections/Changes Planned for Year 3 and Rationale for Changes

The three scaffold platforms were offered the opportunity to progress in further assessment. However, Integra, which controls the three-dimensional printing fabrication methods, has elected not to continue development of this strategy within AFIRM. This leaves the Tyr-PC and PPF scaffold platforms as the two remaining scaffolds that are currently being explored in the project.

Calcium-containing coatings may allow advancement of the performance of degradable scaffolds to a level that approaches or exceeds allograft performance. Therefore, work under this project will extend in Year 3 with more investigation of scaffolds with calcium phosphate coatings.

Until evaluation of optimal calcium coatings on degradable polymer scaffolds is completed, allograft matrix remains (for now) the preferred scaffold upon which to advance the cell transplantation method in Project 4.2.2.

As described previously, a critical assessment of the established canine segmental defect model system (canine bilateral ulnar defect model [2.5 cm defect] and the canine femoral segmental defect [5 cm]) suggests that these models are not rigorous enough to model the current clinical challenge. Thus, the GCTD model is now planned as the next step beyond the CFMD model in Years 3-4. This goat model will provide the standardized platform for assessing the optimized scaffolds (Project 4.2.1) and cell preparations (Project 4.2.2) during 2010-2012, in advance of clinical studies, which are projected for a 2013 start date.

Optimizing Cell Sources for the Repair of Bone Defects

Project 4.2.2, RCCC

Team Leaders: George Muschler, MD and Maciej Zborowski, PhD (Cleveland Clinic)

Project Team: Cynthia Boehm, BS, Tonya Caralla, MS and Pownima Joshi, PhD (Cleveland Clinic)

Collaborators: Vince Hascall, PhD (Cleveland Clinic)

Therapy: Advanced regeneration of segmental bone defects

Deliverable: Preferred clinical method for progenitor cell concentration, selection, and delivery

TRL Progress: *Start of Year 1, TRL 3; End of Year 1, TRL 4; End of Year 2, TRL 4*

Key Accomplishments: The researchers evaluated two density-separation devices for concentration of bone marrow cells. They tested one of the preferred degradable scaffolds from Project 4.2.1 and found that it showed modest performance as a substrate for selective retention (SR). They down-selected the option of CD45 depletion as a means of enhancing the prevalence of osteogenic connective tissue progenitors (CTP-Os) in favor of hyaluronic acid (HA)-positive

selection. They designed, fabricated, and validated a new magnetic separator system for selection of CTP-Os. The system is simple to use, easily adaptable for intraoperative single-step procedures, capable of processing 2×10^9 cells (required for scale-up to the CFMD model) and provides improved separation over the small-scale commercial magnet used previously.

Key Words: Connective tissue progenitors, magnetic separation, cell sourcing, bone graft, hyaluronan, density separation, selective retention

Introduction

The AFIRM bone-related projects collectively seek to improve medical therapy and outcomes for today's injured warriors, whose challenges include the need to regenerate a segmental defect in an extremity. This project addresses the need to select a clinically available and optimized method for harvesting, processing, and transplanting CTP-Os that are severely depleted or missing in these bone defects and surrounding tissues. Three clinically feasible methods for rapid intraoperative processing of marrow-derived cells to concentrate CTP-Os are being investigated: (1) density gradient separation, (2) SR, and (3) magnetic separation (MS).

Density separation (DS) devices are already used to process blood to prepare platelet-rich plasma (PRP) gels. DS can also be used to increase the concentration of CTP-Os. DS systems use programmable intraoperative processing equipment and can be standardized. However, the value of DS processing of bone marrow in improving the efficacy of bone-grafting procedures has not been established. While DS can be used to con-

centrate CTP-Os in a small volume, methods have not been established that optimize the use of DS-processed cells so that they can be transplanted and retained effectively in a graft site. Such methods could include combining cell populations, isolated using DS methods, with effective biomaterials. DS methods also have the disadvantage of requiring specialized equipment and a technician to operate. Samples need to be passed off of the sterile operative field to allow processing. This project will clarify the likely value of DS processing to benefit wounded warriors and to help physicians who wish to optimize treatment for bone defects.

SR is another strategy that provides the opportunity to increase the concentration of local CTP-Os and to decrease or limit the concentration of competing or undesirable cells. SR uses the intrinsic properties of many CTP-Os that enable them to rapidly attach to some surfaces, including some implantable scaffolds. This property enables some scaffolds to be used as an affinity column to rapidly concentrate and select CTP-Os, even when such cells are present as a minor population in a dilute mixture of cells. SR has already been vali-



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dated in preclinical animal testing and clinically in the DePuy Collect™ device. Work in this project is advancing methods and materials to optimize the opportunity to use SR methods in the operating room, to process a patient's own cells, and to enhance bone regeneration in complex bone defects.

MS is a method for processing cells that involves labeling or tagging cells that can be selectively collected or eliminated. This project utilizes an MS system developed in Dr. Zborowski's laboratory that takes advantage of the important observation that CTP-Os derived from BM can be selectively labeled, based on the presence of HA in the ECM that is retained on the surface of the CTP-Os after harvest from BM by aspiration.

Each of these methods, alone or together, offers significant potential value to the wounded warrior, by providing methods to effectively repopulate or "reseed" the region of a bone defect with an effective population of cells that are capable of regenerating the missing bone tissue.

Summary of Research Completed in Year 1

During the first year of the study, the researchers defined methods to increase the surgical yield of BM harvest procedures without an increase in morbidity. Three practical methods were characterized to enable the processing of human or canine BM: DS, SR, and MS.

Research Progress – Year 2

Density Separation

The researchers performed a screening assessment on several commercially available systems that conduct DS processing of blood to prepare plasma or PRP. These assessments found that DS processing could prepare a suspension of BM-derived cells that include CTP-Os at a concentration 3-5-fold greater than native marrow. However, when compared to manual buffy-coat methods commonly used in laboratory protocols, the yield of cells and CTP-Os was substantially less. This result suggests that many CTP-Os were lost in the course of processing—perhaps sticking to the walls of the bags and tubing used in the processing procedure. This result

may reduce the effectiveness of DS devices. The effectiveness of DS processing as a means of enhancing the in vivo performance of a graft material will be tested between 2010-2012.

Selective Retention

Allograft materials and several scaffold preparations have been evaluated as substrates for affinity columns used in SR processing. Individual substrate materials demonstrated significant differences in surface area, pore size, connectivity, surface chemistry, and texture. Each of these variables may have a profound effect on the capacity for a given scaffold to function as a substrate for SR. The researchers are actively evaluating and optimizing allograft bone matrix, ceramic granules, and three-dimensional scaffolds fabricated from Tyr-PC and from polypropylene fumarates (PPF). Other AFIRM scaffolds may also be evaluated for SR performance.

Magnetic Separation

Down-selection of the MS system used for scale-up occurred in Year 2. The system selected is simple, user-friendly, and capable of handling the cell loads required for scale-up of magnetic separation into the in vivo CFMD model. An example of the separation system, used on a starting sample of 0.8×10^9 cells, shows robust enrichment of CTP-Os in the positive fraction, 18-fold higher than the unselected marrow (Figure II-2).

Key Research Accomplishments

- Evaluated several DS devices for concentrating bone marrow cells.
- Demonstrated SR, using several promising, clinically available, degradable scaffold materials.
- Established and scaled a magnetic cell separation system, based on HA-labeling and the use of a fixed magnet, to enable both in vivo large animal studies and clinical studies.

Conclusions

DS, SR, and magnetic cell separation each have the potential to offer the wounded warrior substantial new capabilities for therapy strategies that can repopulate

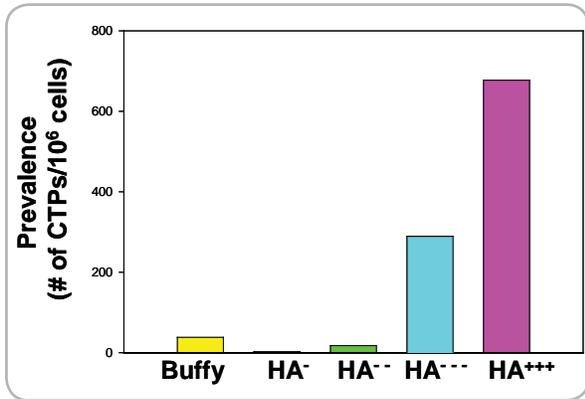


Figure II-2. Prevalence of CTP-Os in different fractions before (buffy) and after magnetic separation. Note the effect of 18-fold enrichment in the HA+++ fraction. The apparent CTP-O partitioning to the HA--- fraction is undesirable and will be minimized by further optimization of scale-up operation in Year 3.

missing stem cells and progenitor cells in the setting of a large bone defect using the patient's own cells. These methods can be used rapidly and effectively in the operating room and could be used separately or combined to optimize the cellular environment to accelerate bone regeneration.

Research Plans for the Next 3 Years

In Year 3, a series of in vivo experiments will be performed, using the established CFMD model to competitively assess the capacity of DS, SR, and/or MS to

enhance the magnitude and rate of new bone formation in the setting of grafting into a bone defect site. Outcomes will be assessed using both mCT and histology. The most effective method for cell sourcing and processing will be advanced into further assessment, in a rigorous long bone defect model that includes tissue loss and scarring around a PMMA spacer to reproduce the common clinical setting in which grafting is performed in the setting of bone defects following military extremity injuries.

Planned Clinical Transitions

These methods for cell sourcing and processing will be combined with top-performing scaffolds and advanced into clinical trials, most likely in collaboration with the METRC. Depending on the preferred method, clinical trial methods involving "minimal manipulation" of autogenous cells could begin as early as 2011. The METRC consists of a network of clinical centers and one data-coordinating center that work together with the USAISR to conduct multicenter clinical research related to AFIRM regenerative strategies in limb salvage.

Corrections/Changes Planned for Year 3

The researchers have modified their original work plan to make allograft, rather than a degradable scaffold, the first substrate in which alternative cell-sourcing strategies are competitively assessed.



II: Limb and Digit Salvage

Progress Reports: Bone Repair and Regeneration

Advancing Bone Repair Using MSD

Project 4.2.3, RCCC

Team Leaders: George F. Muschler, MD (Cleveland Clinic); Linda Griffith, PhD (MIT); Joachim Kohn, PhD (Rutgers/State University of New Jersey, New Jersey Center for Biomaterials [NJCBM]); and Richard Clark, MD (Stony Brook University [SBU])

Project Team: Luis Alvarez, PhD, Jaime Rivera, BS, James Serdy, MTh, Linda Stockdale, MAT (MIT); Vivek Raut, MS, and Chris Heylman, BS (Cleveland Clinic)

Collaborators: Sunil Saini – Integra™ Life Sciences (formerly Therics)

Therapy: Advanced regeneration of segmental bone defects

Deliverable: 1: To develop an advanced bone scaffold that presents tethered epidermal growth factor (EGF) to improve the survival and performance of local and transplanted progenitors.

2: To design an oxygen delivery system to increase survival and performance of local and transplanted progenitors in a hypoxic wound environment.

TRL Progress: *Start of Year 1*, EGF-based MSD TRL 3, Peptide P12-based MSD TRL 2, Oxygen Delivery Strategies TRL 2; *End of Year 1*, EGF-based MSD TRL 3, Peptide P12-based MSD TRL 2, Oxygen Delivery Strategies TRL 2; *End of Year 2*, EGF-based MSD TRL 3, Peptide P12-based MSD TRL 3, Oxygen Delivery Strategies TRL 3

Key Accomplishments: The researchers found that tEGF increased proliferation of mesenchymal stem cells (MSCs) while preserving differentiation, while soluble EGF does not preserve differentiation of MSCs. They employed β -tri-calcium phosphate binding peptide (β -TCPBP) technology to design new proteins for the survival and protection

of CTP-Os. They demonstrated that tethered P12 (tP12) increased CTP-O colony-forming efficiency (CFE) and migration, as well as the proliferation of hTERT cells. The research team established a system for local oxygen delivery using polymeric microparticles based on drug delivery methods. They discovered that slowing the rate of decline of oxygen is sufficient to increase CTP-O CFE and increase proliferation of cells and colonies.

Key Words: Tethered EGF, connective tissue progenitors, large bone defect, scaffolds, β -tri-calcium phosphate binding peptide, tethered P12, oxygen tension, hypoxia, tethered heregulin, serum deprivation

Introduction

The AFIRM bone-related projects have been designed to address critical gaps that currently limit the medical therapy option and outcomes that are available to injured warriors whose challenges include the need to regenerate a segmental defect in an extremity. This project focuses on highly promising methods to enhance the survival and performance of CTP-Os, which may be transplanted on a scaffold. Transplanted cells face a very harsh wound environment, in which oxygen levels plummet, and chemical signals tend to drive cells to die via a path of necrosis or a path of programmed cell death called apoptosis.

The researchers of this project are developing two practical methods aimed at improving cell survival: (1) MSD to ensure that cells transplanted onto a scaffold receive signals from that scaffold that will inhibit early cell death and even stimulate early phases of regeneration and (2) local O_2 D into the area where cells are most threatened.

MSD involves linking bioactive molecules to the surface of a biomaterial as a means of improving control over the cell and tissue response to the implant material. Bioactive molecules that have been considered in this project include: EGF, P12 (a fibronectin fragment), and soluble Type I Neuregulin-1, also called heregulin (HRG). Each molecule can be linked ("tethered") to the surface of an implanted scaffold. Tethering ensures that the signaling molecule does not diffuse away from the cells and enables control over the concentration and presentation of the signal so that the effect can be optimized. Two methods of tethering have been explored: (1) linking covalently using "comb polymer" technology, and (2) tethering molecules to the surface of scaffolds that contain calcium-phosphate coatings (e.g., β -tri-calcium phosphate [β -TCP]) using high-affinity noncovalent bonding of a novel β -TCPBP.

O_2 D involves the use of biologically compatible salts that, when combined with water (H_2O), will recombine to

release elemental oxygen (O₂). In this project, salts that have this property are incorporated into a degradable polymer in a form that can be strategically placed into the area of a large bone defect. By controlling the rate of degradation of the polymer, oxygen can be delivered over the course of a few hours to over a week.

Competitive Technologies

There are no current products designed to deliver local oxygen or to enhance the performance of transplanted cells using MSD. The Infuse Bone Graft with recombinant human bone morphogenetic protein-2, which is delivered by adsorption on an implanted scaffold and is rapidly released in a soluble form, is thought to act on both local and transplanted cells but is not considered to be a pro-survival factor. As a result, MSD methods as well as O₂D methods are projected to not only enable more effective cell transplantation, but also to be synergistic with the local delivery of BMP-2 and other scaffolds and signaling technologies in the highly compromised bone defect environment.

Leveraged Funding

Work on the basic cell biology of tEGF for cell migration and survival is supported by NIH R01 DE019523-10 (\$250K direct costs per year), which provides partial support of student salaries for production of growth factor ligands and for analyzing behavior of CTPs in the Griffith lab. It is also supported by an anonymous foundation grant (\$425K per year), which provides equipment for protein synthesis and characterization.

Summary of Research Completed in Year 1

During the first year of the project, the researchers fabricated two-dimensional scaffolds presenting tEGF and a fibronectin-derived peptide P12 that enhance new bone tissue formation. They developed and implemented a protocol for spin coating two-dimen-

sional scaffold surfaces with and without comb coating to which hTERT MSCs attached and proliferated. They were able to detect β-TCP on scaffold surfaces. They also established methods for the quantitative measurement of in vitro cell response (attachment, survival, proliferation, and migration), and methods/protocols to characterize two-dimensional scaffold design using radiolabeling and analytic chemistry techniques. Additionally, they developed an optimized β-TCPBP that incorporates multiple β-TCP binding sites in fusion with EGF (Figure II-3). They began in vitro MSD studies using CTP-O cells. They determined that tEGF can exert a strong proliferative response in CTP-O cells seeded on three-dimensional β-TCP scaffolds even while cultured under osteogenic conditions. They also determined that tEGF does not reduce the early differentiation potential of CTP-O cells as evidenced by sustained alkaline phosphate levels at Day 7 versus controls. They determined that oxygen deprivation (as opposed to glucose deprivation) is the cause of CTP-O cell death in a hypoxic cell environment. Finally, they successfully fabricated oxygen-generating microbeads and confirmed oxygen delivery from the microbeads.

Research Progress – Year 2

MSD: Based on data collected in the first 2 years of this project, EGF was selected as the most promising agent for tethering and is being developed in a targeted effort to make these capabilities available for the care of injured warriors. The researchers have shown that EGF increases proliferation of MSCs, culture expanded on β-TCP scaffolds and PLGA/β-TCP two-dimensional surfaces, without compromising the ability of MSCs to differentiate into osteoblasts. They have also shown that

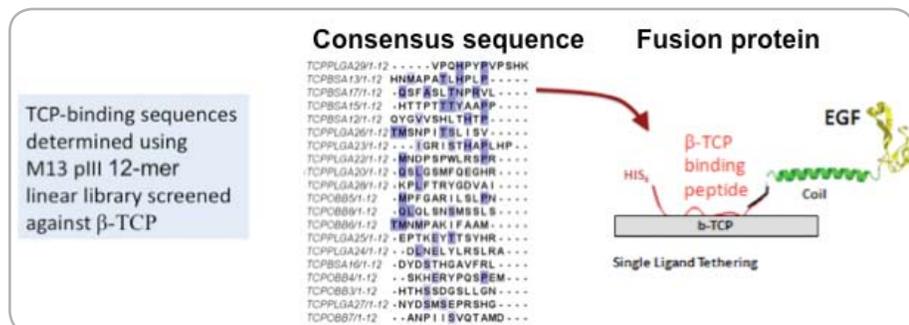


Figure II-3. High-affinity TCP-binding proteins identified by phase display.



II: Limb and Digit Salvage

Progress Reports: Bone Repair and Regeneration

tP12 improves the proliferation of hTERT cells and the CFE of freshly isolated human marrow-derived CTP-Os. However, due to the fact that the effect size appears to be larger for EGF and because the delivery methods for EGF are more technically advanced, AFIRM investments in MSD are now focusing exclusively on the rapid advancement of EGF MSD.

O₂D: The researchers established a system for local O₂D using polymeric microparticles based on drug delivery methods arising from the laboratory of Vinod Labhasewar, PhD, in the Department of Biomedical Engineering at the Cleveland Clinic. Data generated to date have demonstrated that even a small amount of local O₂D may have profound effects on the ability of transplanted cells to survive a rapid drop in oxygen levels. The feasibility of O₂D methods has been clearly established. An O₂D formulation targeting a 96-hour delivery profile will be ready for testing in the CFMD model before June 2011. The researchers have demonstrated that CTP-O CFE and proliferation can be augmented by slowing the rate of decline of oxygen in vitro over a period as short as 48 hours. This justifies further work to discover methods that may blunt the development of local hypoxia in the wound environment using O₂D. The researchers have also found that red blood cells (RBCs) are detrimental to CTP-O CFE and proliferation (cells per colony) in vitro. This effect is unrelated to steric interference with attachment and is mediated by soluble factors, justifying future work to limit RBCs in the wound-healing environment.

Key Research Accomplishments

- Demonstrated that MSCs cultured on β -TCP scaffolds and PLGA/ β -TCP two-dimensional surfaces tethered with EGF proliferate at a greater rate than those cultured on mock scaffolds.
- Demonstrated that tEGF but not soluble EGF conserves the multipotency of MSCs cultured on β -TCP scaffolds.
- Designed new proteins (β -TCPBP-HRG and β -TCPBP-HABP) for MSD using β -TCPBP technology.
- Produced recombinant β -TCPBP-HRG protein in BL-21 *E. coli* cells, with subsequent ongoing work to optimize the affinity-chromatography procedures to isolate the recombinant protein.
- Demonstrated that the P12 peptide when tethered to two-dimensional surfaces using comb polymers, increases proliferation of hTERT cells at Day 6.
- Demonstrated that the P12 peptide when tethered to two-dimensional surfaces using comb polymers, increases CFE and migration (in the form of reduced colony density) in CTP-O-derived colonies at Day 6.
- Established a system for local O₂D using polymeric microparticles based on drug delivery methods.
- Developed a mathematical model that will enable rational design and interpretation of O₂D, based on the interaction of cell-consumption rate and O₂D rates.
- Demonstrated that CTP-O CFE and proliferation can be augmented by slowing the rate of decline of oxygen in vitro over a period as short as 48 hours.
- Demonstrated that RBCs are detrimental to CTP-O CFE and proliferation (cells per colony) in vitro.

Conclusions

MSD and O₂D represent new and highly promising clinically relevant strategies that have a high probability of enhancing the performance of the degradable bone scaffolds being developed in Project 4.2.1 and also enhancing the clinical performance of cell harvest, processing, and transplantation methods being developed in Project 4.2.2. While each of these technologies is projected to make substantial contributions as new clinical therapy options in their own right, the coordinated and highly interactive development of these technologies within the AFIRM expanded and open Bone Network creates an unprecedented opportunity to design and optimize an integrated approach to large bone defects. This new paradigm of collaboration, in which synergies between technologies can be explored and the most promising approaches rapidly developed, presents a new and unprecedented opportunity to serve the injured warrior.

Research Plans for the Next 3 Years

Product 1 (MSD Scaffolds Presenting Tethered EGF): In Year 3, researchers at MIT will optimize the fabrication of bioactive β -TCPBP-EGF-tethered scaffolds, with consideration of manufacturing, storage, and regulatory constraints. They will also complete the validation of the scaffold bioactivity using third passage MSCs. Cleveland Clinic Foundation will characterize the performance of GMP-certified β -TCPBP-EGF-tethered scaffolds in vitro (using human and canine progenitor cells). If successful, the researchers will evaluate the performance of GMP-certified β -TCPBP-EGF-tethered TheriLok crosses in vivo in the CFMD model and subsequently a large segmental-defect goat model (subject to additional funding). In Year 5, the researchers will translate at least one advanced bioactive MSD surface into an appropriate human trial, most likely with an optimized cell source.

Product 2 (O₂D System): Cleveland Clinic Foundation will advance the O₂D through in vitro toxicity testing to meet ISO 10993 standards of biocompatibility. Efficacy in vivo will be assessed in the CFMD model. In vivo study work will begin in 2011. Cleveland Clinic Foundation will actively pursue a corporate partner to supplement funding for clinical trials in Years 4 and 5.

Planned Clinical Transitions

Product 1 (MSD Scaffolds Presenting Tethered EGF): The technology transfer office at MIT is actively engaged in commercialization efforts around MSD strategies. Researchers at MIT have filed an invention disclosure for patent protection related to the presentation of tethered biomolecules and the β -TCPBP. Researchers at MIT and Cleveland Clinic Foundation recognize that proof of efficacy in an in vivo setting is considered to be a critical step in establishing a commercial value proposition for a potential partner and have made plans to advance into in vivo studies. A regulatory pathway that includes a pharmaceutical manufacturing engineering process must be expected in this product class. Therefore, MIT and

Cleveland Clinic Foundation have scheduled a pre-Investigational New Drug (IND) meeting with a representative of the FDA.

Product 2 (O₂D System): The technology transfer arm of the Cleveland Clinic, Cleveland Clinic Innovations, is actively involved in developing the intellectual property position and business model for the O₂D technology platform. Cleveland Clinic Foundation is developing the understanding of the in vitro toxicity testing that is needed to meet ISO 10993 standards of biocompatibility. Researchers at Cleveland Clinic Foundation have also made plans to advance into in vivo studies. They have scheduled a pre-IND meeting with a FDA representative.

Corrections/Changes Planned for Year 3

This project has closely followed the initial plan, in rapidly screening (a) potential ligands and tethering strategies related to MSD and (b) substrates and delivery strategies for O₂D. In each case, systems of metrics for in vitro performance have been established and met to demonstrate biological feasibility and bioactivity.

Based on the in vitro efficacy data and analysis of the regulatory pathway for advancing MSD as a therapy, the researchers decided to focus this project exclusively on the delivery of tEGF using β -TCPBP. Comb polymer strategies and other ligands will be developed secondarily and with non-AFIRM resources unless additional funding becomes available.

The development of the β -TCPBP and the opportunity to deliver tEGF using this versatile platform were not part of the initial proposal, which focused on the use of comb-polymer tethering methods. However, β -TCPBP-EGF offers a robust method of MSD of β -TCP-containing scaffolds with characteristics that afford cellular protection, enhanced proliferation, and preservation of osteogenic potential. Achieving this effect may have clinical implications, such as increasing patient-native progenitor populations to improve bone wound healing.



II: Limb and Digit Salvage

Progress Reports: Bone Repair and Regeneration

Bone Regeneration in a Contaminated Defect

Project 4.4.9, USAISR

Team Leaders: Joseph C. Wenke, PhD, USAISR

Project Team: Kate V. Brown, MD and Tega Guda, PhD

Collaborators: Scott A. Guelcher, PhD (Vanderbilt University)

Therapy: Bone regeneration

Deliverable: Agents to improve bone regeneration

TRL Progress: *Start of Year 1, TRL 3; End of Year 1, TRL 3; End of Year 2, TRL 4*

Key Accomplishments: Delivering both BMP and the antibiotic vancomycin to a contaminated critical size rat femur defect, the researchers found that it is possible to both reduce infection and regenerate bone in

a contaminated defect. They also determined that earlier debridement and antibiotic administration decrease infections. In addition, they found that sustained release of vancomycin from polyurethane (PUR) scaffolds inhibits infection of bone wounds in a rat femoral segmental defect model.

Key Words: Bone repair, infection, growth factors, antibiotics

Introduction

Bone fractures are common and although most heal without complication, 10%-15% of the approximately 6.5 million bone fractures that occur annually in the United States are complicated by disrupted patterns of bone healing. The type, severity, and complications of the fracture are, however, different in a military setting. Due to the nature of injuries that occur during combat, open fractures (fractures that have direct communication with the environment) are the most common fracture (constituting 19% of all injuries and 67% of fractures in combat). Most of the combat-related open fractures are caused by missile injury, have a large segment of missing bone, and often have concomitant periosteal, vascular, and neural damage. Malunion or delayed union complicates many of these open fractures (e.g., delayed or nonunion complicated 3 of 5 open tibial fractures sustained in Somalia). This disrupted fracture healing causes morbidity and lengthy hospitalization; thus, delayed or nonunions prevent soldiers from returning to duty. The fact that open fractures are contaminated with bacteria makes the clinical challenge that orthopedic surgeons face even more daunting. To prevent infection, the wound is debrided, irrigated frequently, and routinely packed with antibiotic-impregnated (PMMA) beads, which may remain in the wound for up to 6 weeks. This results in a delay in definitive bone grafting and recovery

of limb function. An alarming statistic came from this work: approximately 50% of all patients who suffered leg-threatening injuries, whether the limb was amputated or salvaged, had not returned to work for a single day by 2 years. This statistic illustrates that the current standard of care for these patients is insufficient.

When definitive bone grafting occurs, a foreign material, with osteogenic properties, is placed in an avascular, contaminated wound. This may serve as the nidus for infection. It is well known that vascularized, highly perfused tissue is less likely to become infected. It is also well known that vascularization is initiated immediately and occurs within 4-6 weeks. Therefore, it stands to reason that an osteogenic bone graft that releases antibiotics for a period of time will protect the graft until it is vascularized and will help reduce infection.

We propose to use various scaffolds that will allow a focal release of growth factors and antibiotics in an effort to exploit a biological template for enhanced fracture healing and prevention of infection. The biological template includes angiogenesis, chemoattraction of osteoblast lineage cells, and their mitogenic amplification in situ. The antibiotic or antimicrobial used will be broad spectrum and will be released for at least 8 weeks in an effort to prevent infection. Therefore, we propose that dual delivery of a growth factor and an antibiotic in

a scaffold that allows focal and temporally tuned release kinetics will result in a better clinical outcome than the current staged treatment.

Research Progress

(Funded in 2009)

At the initiation of this project, platelet-derived growth factor (PDGF) appeared to be a good candidate for a growth factor. Being a chemoattractant and a mitogen, we believed it would have an additive effect to BMP. Moreover, Biomimetics Pharmaceuticals appeared to be bringing it to market for orthopedics. Recently, reports suggest that PDGF is not very effective in nonaged or nonosteoporotic models. Biomimetics has had difficulty bringing this product to market. BMP is the most powerful growth factor for bone. The more ideal release kinetics of this material has improved its performance. Therefore, we do not believe using PDGF would be the best use of time and money at this point.

This project is focused on (1) developing animal models that are appropriate for evaluating dual delivery implants, (2) evaluating promising technologies and approaches in proof-of-concept studies, and (3) translating successful technologies/products toward the clinic. The researchers modified an established contaminated segmental critical size defect model to have the desired characteristics (i.e., established infection is reduced but still persists with antibiotics). They used this contaminated model to evaluate PUR scaffolds (Figure II-4) to control the release of antibiotics and BMP to prevent infection and regenerate bone, respectively.

The PUR scaffold was shown to release vancomycin for more than 8 weeks at a level of 20 microns for *Staphylococcus aureus*. This approach was as effective as the currently used PMMA beads with antibiotics in the contaminated defect. The PUR scaffold with different release kinetics was then compared to collagen sponge with rhBMP (a currently used therapy) (Figure II-5). The PUR scaffold with a burst and

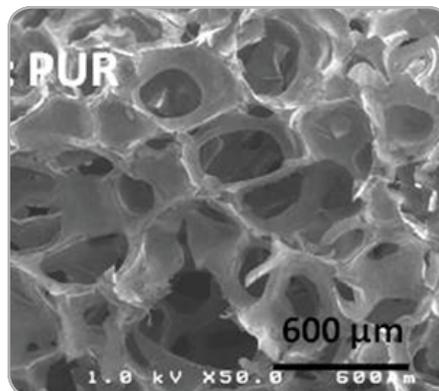


Figure II-4. Morphology of PUR scaffold.

sustained release regenerated more bone than the collagen sponge (capable of burst release only) (Figures II-6 and II-7). The sustained release promoted very little bone regeneration. The more effective release kinetics for BMP design was used in conjunction with vancomycin. Preliminary results indicate that it was effective in reducing infection in regenerating bone. This may be the first project to use tissue-engineering approaches to develop a controlled-release dual-purpose implant.

During the extended vivarium closure in fall 2009, the researchers screened the effect of 20 different antibiotics on osteoblast viability and osteogenic potential. The local delivery of antibiotics is currently being explored as a means to circumvent systemic toxicity while minimizing infection. In addition, dual-delivery devices are being developed to deliver antibiotics while simultaneously improving bone regeneration.

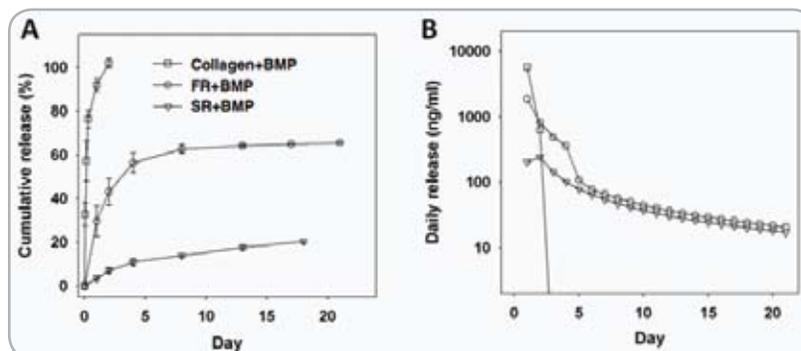


Figure II-5. In vitro release kinetics for rhBMP-2 released from FR+BMP and SR+BMP scaffolds and the collagen sponge: (A) Cumulative release. (B) Daily release.

II: Limb and Digit Salvage

Progress Reports: Bone Repair and Regeneration

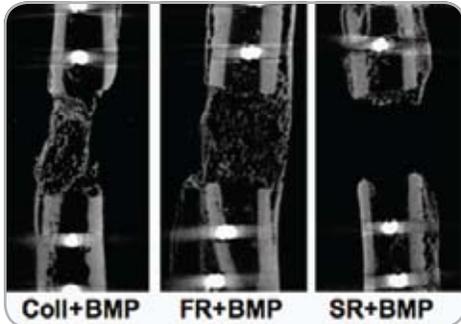


Figure II-6. Representative mCT images of new bone formation.

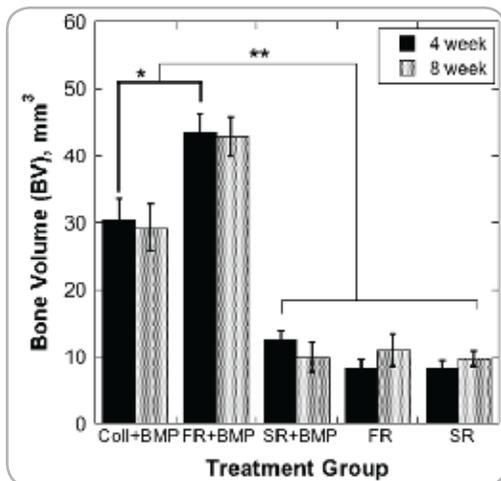


Figure II-7. Analysis of new bone formation as measured by mCT. Collagen+BMP and FR+BMP were significantly higher than the other at 4 and 8 weeks. FR+BMP had significantly more bone than Collagen+BMP at both time periods.

When choosing an antibiotic for a local delivery system, it is important to consider the significant diversity among antibiotics with regard to which pathogen they target, their mechanism of action, and the levels at which they become effective. An often overlooked factor that must be considered is the effect that they have on osteogenic cell viability. Although it is obvious that cell toxicity will affect bone regeneration, it may also be important to consider the osteogenic potential of surviving cells as well.

Treatment with $\geq 200 \mu\text{g}/\text{mL}$ reduced cell number and alkaline phosphatase (AP) by $\geq 25\%$ in 9 and 15 of the

antibiotics, respectively. In general, decreases in AP were measured at lower doses than for cell number. Antibiotics within a class generally had similar effects in terms of their effect on cell number and osteogenic potential, with a few exceptions. Vancomycin caused the least decrement in cell viability and AP activity.

This study is the first comprehensive evaluation of several antibiotics' effects on osteoblast viability and osteogenic potential for extended periods of treatment. This reference will help clinicians and researchers choose the optimal antibiotic for the treatment of infection and the maintenance of healthy host tissue.

Key Research Accomplishments

- Developed a long bone defect animal model that was appropriate for evaluating dual-purpose implants.
- Determined that delay in debridement and antibiotics increases infection.
- Evaluated the ability of implants to deliver a known growth factor (BMP).
- Initiated the evaluation of a biodegradable porous PUR scaffold as the delivery vehicle for antibiotics.
- Evaluated implants in a contaminated defect.

Conclusions

The majority of battlefield wounds occur to the extremities, and the most common fractures are open fractures. Common complications of open fractures include infection and delayed union or nonunion. This disrupted fracture healing causes morbidity and lengthy hospitalization; thus, delayed unions or nonunions prevent soldiers from returning to duty. Data from the rat calvarial critical size defect model suggests that the earliest introduction of antibiotics and debridement may reduce the infection rate. Further, the PUR scaffolds support tunable, sustained release of vancomycin and significantly reduced the infection compared to the clinical standard (PMMA). A dual-delivery device that delivers (1) antibiotics/antimicrobials to prevent or treat infection and (2) growth factor and/or pluripotent stem cells may further improve the standard of care of open fractures. Therefore, the development of a bone graft that can reduce infection

and promote bone regeneration are advantageous over the current staged treatment.

A contaminated critical size rat femur defect was utilized to demonstrate that both early and late debridement and local antibiotic administration reduce infection. And the results suggest that the earliest introduction of antibiotics and debridement may reduce the infection rate. A biodegradable porous PUR scaffold was evaluated as a delivery vehicle for vancomycin. The decreased solubility of hydrophobic vancomycin-free base (V-FB) resulted in extended vancomycin release profile and translated

to improved infection control in vivo in a contaminated critical size fat femoral segmental defect. Compared to PMMA, PUR is a biodegradable system that does not require the extra surgical removal step in clinical use and suggests that PUR scaffolds incorporating V-FB could be a potential clinical therapy for treatments of infected bone defects. The physical characteristics of the scaffold and the burst with sustained release of BMP regenerated more bone than the current standard of care. Delivering both BMP and vancomycin, the research team demonstrated that it is possible to both reduce infection and regenerate bone in a contaminated defect.



II: Limb and Digit Salvage

Progress Reports: Soft Tissue Repair and Regeneration (Excluding Nerve)

Oxygen-Generating Biomaterials for Large Tissue Salvage

Project 4.4.6, WFPC

Team Leader(s): Benjamin Harrison, PhD (Wake Forest University)

Project Team Members: Catherine Ward, BS and Sirinrath Sirvinsoot, PhD (Wake Forest University)

Collaborator(s): Robert Guldberg, PhD (Georgia Tech); George Christ, PhD (Wake Forest University); Kacey Marra, PhD (University of Pittsburgh);

and James Yoo, MD, PhD (Wake Forest University)

Therapy: Supply temporary oxygen to hypoxic tissue

Deliverable(s): Injectable oxygen-generating materials for tissue salvage

TRL Progress: *Start of Program, TRL 2; End Year 1, TRL 2; End Year 2, TRL 3*

Key Accomplishments: The researchers have created a controllable, injectable, oxygen-generating biomaterial. They have tested the material for sustained release of oxygen in vitro and feasibility of injection in vivo.

Keywords: Oxygen, tissue engineering, tissue salvage, hypoxia, ischemia

Introduction

Replacement or restoration of tissue loss caused by traumatic injury, congenital defects, tumor removal or severe burns is a challenge. For example, current treatment for reconstruction of volumetric muscle loss is associated with donor-site morbidity and limited functional restoration. Therefore, tissue engineering and regenerative medicine offer the possibility of generating functioning tissue for patients.

However, there are some significant challenges for generating large volumetric tissues. Metabolically active cells can only survive up to a few hundred micrometers away from a blood supply because of oxygen diffusion limitations. In newly formed engineered tissue, this supply is not present in the early stages and the avascular engineered tissue quickly becomes hypoxic internally. Investigators have used biological approaches to promote angiogenesis, and while such approaches are able to stimulate host tissue responses associated with neovascularization, the extended time needed to establish the vascular network may be inadequate. The Harrison group hypothesizes that oxygen-generating biomaterials can provide a source of oxygen to counter the effects of hypoxia to maintain the viability of engineered tissue.

In this project, this laboratory has been developing an injectable material capable of generating oxygen. Prepar-

ing an injectable oxygen-generating material would allow delivery of oxygen in controlled amounts to engineered tissue scaffolds or pre-existing tissue. The ability to control the amount of oxygen delivered is important because different cell types can have different biological oxygen demands, and different oxygen tension can trigger different biological effects in cells.

Summary of Research Completed in Year 1

During the first year of the study, the researchers produced an injectable solution capable of in situ oxygen generation. They demonstrated that oxygen production can be sustained for up to 3 days. They also determined that the injectable oxygen-generating material is nontoxic to cells.

Research Progress – Year 2

During the past year, the researchers started evaluating their novel oxygen-generating material in a biological system. Muscle tissue was initially chosen to evaluate the material. This included determining the conditions needed to inject the test material into a muscle as well as determining how well the material can be distributed in tissue. Then, the aim was to assess its effect. To do this, POGs were fabricated in sizes less than 25 μm in diameter. These particles were dispersed into polyethylene glycol and methyl green dye and then injected into rat

tibialis anterior to determine distribution throughout the muscle. The formulations were optimized to allow distribution evenly throughout the muscle after injection, which was an improvement from the previous generation. Histological sections of the muscle showed that up to 6 hours after the muscles had been removed from the body, POG-treated muscle maintains better morphology than the untreated group (Figure II-8).

Enabling Technologies

The deliverable for this project is a system for providing localized delivery of oxygen. Therefore this project is best described as an enabling technology for tissue regeneration. For composite tissues, such as a limb or digit, it became necessary to evaluate the feasibility of this technology in specific tissue types including bone, muscle, and nerve. After preparing the oxygen-generating material, the researchers have begun testing it in relevant tissue systems to investigate the feasibility of using the material.

Collaborative Skeletal Muscle Functional Studies

The real litmus test of a new technology is evaluating its functional impact on tissue. A burgeoning collaboration with the Christ lab (AFIRM project leader at Wake Forest University) was established to evaluate the effect of POGs on skeletal muscle. An AFIRM graduate student worked in Dr. Christ's lab through an NIH stimulus R01 award to gain relevant physiology experience to evaluate the effect of POGs on muscle function.

For in vitro whole muscle function studies, the rat extensor digitorum longus muscle was isolated, sutured to a mounting post and force transducer, and evaluated in an organ bath at 37°C using elec-

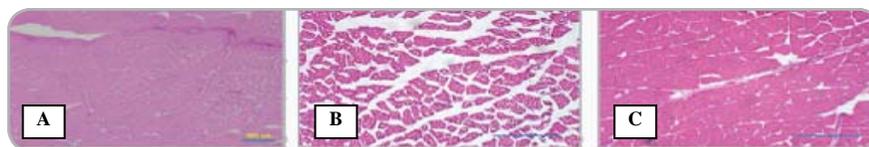


Figure II-8. Histological sections of muscle tissue removed from a rat, stained with hematoxylin and eosin. A: Native tissue upon removal; B: Tissue 6 hours after removal at 37°C, injected with a vehicle alone; C: Tissue 6 hours after removal, injected with POG material and an antioxidant. Muscles injected with POG were able to maintain histological characteristics of native muscle where control muscles began to exhibit signs of necrosis, including spacing between fibers and apoptosis. (Scale represents 200 μm .)

trical field stimulation (22V, 0.2 ms pulse) (Figure II-9). Muscles were injected with POGs dissolved in vehicle or with vehicle alone prior to different contractile protocols. Preliminary results show that mechanical injection into a functioning muscle does not hinder the active force, which suggests that the treatment method proposed (direct muscle injection) may not be detrimental to the tissue. This was observed in functional data where active force at 200 Hz for POG injections is not significantly different from all other controls. Structurally, it was observed that muscles injected with POGs were able to maintain histological characteristics of healthy muscle in the organ bath, whereas controls began to show signs of a necrotic core as evidenced by compact fibers on the outside perimeter and the spaced fibers in the center of the tissue. Future work will include optimizing the dosage to gain better functional recovery.

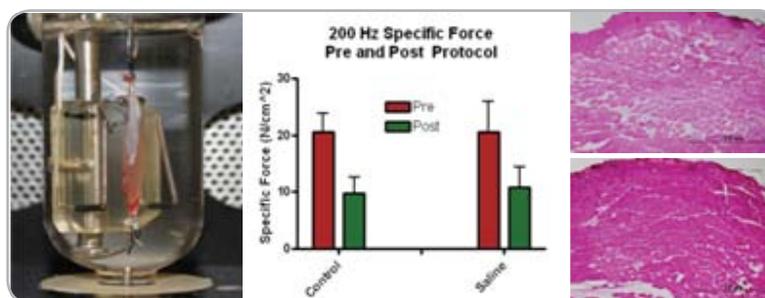


Figure II-9. In vitro skeletal muscle assays. Skeletal muscle is removed from a rat and receives no treatment, a needle injection, saline, or POG. It was observed that the injections produced the same forces suggesting that the method of injection was not detrimental to the tissue (middle). Also, structurally, muscle injected with POGs showed no signs of a hypoxic core (right, bottom), which was obvious in control muscle (right, top).



II: Limb and Digit Salvage

Progress Reports: Soft Tissue Repair and Regeneration (Excluding Nerve)

Bone Regeneration Studies

To evaluate the potential utility of POGs in bone regeneration, porous biodegradable scaffolds capable of producing oxygen were prepared (Figure II-10). The porous scaffolds were then placed into a 6 mm femur defect model in rats. This was followed by analysis at 4 and 8 weeks post operation. Quantitative mCT bone volume results showed a trend toward increased bone volume in the POG scaffolds. However, no statistical significance was observed. The failure to achieve statistical significance may be due to the relatively low number of animals tested in this pilot study and the variability in the data.

Collaborative efforts with the Guldberg lab at Georgia Tech, which has considerable expertise in this animal

model, were established to better test the feasibility of using oxygen-generating materials in remodeling bone defects.

Key Research Accomplishments

- Optimization of an injectable oxygen-generating solution.
- Demonstrated that oxygen-generating material can be injected and dispersed throughout tissue.
- Demonstrated that the oxygen-generating material can maintain histomorphological characteristics of skeletal muscle in a nonvascularized environment.
- Demonstrated that the oxygen-generating material may have potentially useful properties in oxygenating hypoxic environments within skeletal muscle tissue.

Conclusions

Limb and digit regeneration is arguably the most ambitious program within AFIRM. As such, there is a need for developing novel technologies that can aid the regenerative process. This project has been focused on developing a chemically based oxygen delivery system. As this technology matured during Year 2, the research team began to explore the feasibility of delivering oxygen in in vivo models. In addition, the researchers initiated collaborative efforts with other AFIRM investigators to leverage their expertise as well their tissue-specific project application. The results suggest that POGs may aid in engineering large tissue constructs that have had limited success thus far because of oxygen diffusion limitations. The most traction in animal models thus far has been in skeletal muscle systems. Future work will include testing the material in both a muscle ischemia and a wound graft model to determine the extent of the ability of the POGs to provide enough oxygen for both large three-dimensional tissue and skin.

Research Plans for the Next 3 Years

Preclinical studies will be initiated to evaluate the efficacy of the oxygen-generating material. The technology will be tested in systems where hypoxia may cause detrimental effects and POGs may be beneficial. During Year

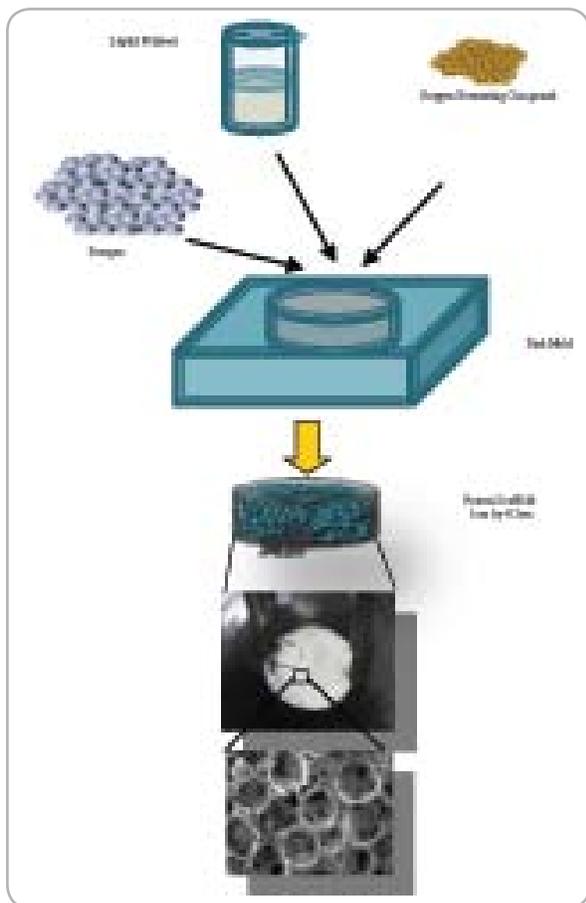


Figure II-10. Schematic of bone scaffolds incorporating POGs.

3, POG technology will be tested in different systems to determine areas that would benefit the most from this approach. Because composite tissues are composed of multiple cell types, several tissue systems will be analyzed including skeletal muscle, bone, nerve, and skin. Optimization of the most promising tissue systems benefiting from the technology will continue in later years along with identifying the best possible clinical application.

Planned Clinical Transitions

While no immediate human clinical trials are currently slated under this AFIRM project, options are being

explored including multiple pathways to incorporate into other AFIRM research as well as to leverage other funds to accelerate the time to clinic.

Corrections/Changes Planned for Year 3

For next year, identification and testing of the technology in systems where hypoxia may cause detrimental effects and POGs may be beneficial will occur. Since composite tissue is made of multiple tissue types, we intend to explore four areas including skeletal muscle, bone regeneration, nerve regeneration, and skin regeneration.



II: Limb and Digit Salvage

Progress Reports: Soft Tissue Repair and Regeneration (Excluding Nerve)

Isolation and Expansion of Native Vascular Networks for Organ Level Tissue Engineering

Project 4.5.8, WFPC

Team Leader(s): Geoffrey C. Gurtner, MD, Michael T. Longaker, MD, MBA (Stanford University); and Robert Langer (MIT)

Project Team Members: Michael Sorkin (Stanford University)

Collaborator(s): None

Therapy: Vascularized tissue engineering

Deliverable(s): Hydrogel-encased vascularized networks for organ-level engineering

TRL Progress: *Start of Program*, TRL 1; *End Year 1*, TRL 1

Key Accomplishments: This research group has initiated experiments to isolate the vascular network of rat superficial inferior epigastric artery

flaps using detergents and enzymes. Experiments to define the native matrix surrounding the explanted vessels have begun, and the group plans to test various decellularization strategies.

Keywords: Vascular engineering, hydrogel, bioreactor

Introduction

Injured or missing extremities, failing organs, and significant burn injuries continue to place a huge burden on wounded soldiers and society. Tissue engineering holds the promise of creating replacement limbs and organs outside of the human body. However, two major obstacles have hindered the development of techniques to fabricate limbs and organs: (1) the inability to adequately vascularize tissue constructs *in vitro* and (2) the inability to reintegrate these tissues into the systemic circulation. Many tissue-engineering strategies start with cells implanted onto matrices and then attempt to induce the formation of a *de novo* vascular system. This approach has proven difficult because the complexities of *in vivo* neovascularization are difficult to recapitulate *ex vivo*. In contrast, the Stanford group has developed novel strategies that utilize preformed native circulatory networks that can be supported *ex vivo* during organ fabrication, differentiated using progenitor cell-based techniques, and then readily integrated into the systemic circulation.

The researchers hypothesize that a native circulatory bed can be isolated and expanded *ex vivo* using progenitor cell- and small molecule-based modalities to create a fundamental vascular unit from which to bioengineer limbs and organs. Their preliminary work has demonstrated the feasibility of sustaining EMBs *ex vivo* for up

to 7 days. They have successfully transduced EMBs with nonviral vectors and demonstrated post-reimplantation expression of therapeutic peptides. In addition, they have seeded EMBs with progenitor cells and induced sustained differentiation *ex vivo* following reimplantation. The technology has advanced beyond “proof of principle” toward a flexible regenerative environment based on a bioreactor system. This innovative approach has allowed utilization of the pre-existing vascular system as a scaffold that can be manipulated *ex vivo* and subsequently reconnected to the circulatory system *in vivo* using standard microsurgical techniques.

Research Progress

(Note: This project was just funded during the past year.)

The Stanford group has begun studies investigating the optimal methods to isolate and expand native vascular beds (Figure II-11). The MIT group is continuing to refine the angiogenic encasing hydrogels for use with the bioreactor system. Ongoing experiments will build upon this starting point to add more complex biochemical signals and progenitor cell populations to build an organ from the “inside-out.”

Key Research Accomplishments

- Initiated experiments to isolate vascular beds using the rat as an in situ bioreactor and are testing various microsurgical, physical, and enzymatic debridement techniques.

Research Plan for the Next 3 Years

In the upcoming years, the research team plans to continue to pursue the original aims to isolate and expand native vascular beds using a multifaceted approach to tissue engineering. The team will employ bioreactor systems, angiogenic hydrogels, and progenitor cells to fabricate organ-level vascular networks.

Planned Clinical Transitions

The researchers' approach to engineering vascularized organs using pre-existing vascular beds could be widely applicable to humans. Tissues such as omentum or free flaps are often used for reconstructive surgery and the experiments planned in this project could be readily applied to decellularize and seed autologous progenitor cells onto autologous human tissues.



Figure II-11. Zoom photograph of elevated superficial inferior epigastric artery flap being perfused in situ while various debridement techniques are being performed. These preliminary experiments allow us to determine the extent of safe processing that can be performed without adversely affecting microcirculatory patency.



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Development of Bioabsorbable Tissue-Lined Stent for Vessel Trauma

Project 4.3.2, RCCC

Team Leaders: Timur P. Sarac, MD (Cleveland Clinic)

Project Team: M. Bastan, MD (Cleveland Clinic)

Collaborators: M. Wiggins, PhD, M. Sattiraju (PeriTec Biosciences); P. Gingras, P. Mulrone (Proxy Biomedical); C. Bonsignore, and J. Wang (Nitinol Development Corporation)

Therapy: Treatment of arterial and venous trauma

Deliverable: Bioabsorbable tissue-lined polymeric stent graft

TRL Progress: *Start of Year 1, TRL 1; End of Year 1, TRL 3; End of Year 2, TRL 3*

Key Accomplishments: The researchers developed two tissue-

lined stent grafts for the treatment of traumatic blood vessel injuries. They also developed a novel anhydrous solution for the storage of bioabsorbable materials.

Key Words: Stent, peritoneum (tissue), polydioxanone, mineral oil, blood vessel trauma

Introduction

The purpose of this project is to develop a bioabsorbable and/or fracture-resistant, tissue-lined stent graft for minimally invasive treatment of arterial and venous trauma. The stent graft has the requirement of being a durable, bioabsorbable material or fatigue-resistant structure with enough stent strength for the short term to cover the injury and prevent exsanguination. Absorbable stent designs are advantageous, for as the stent absorbs, the tissue heals into the arterial wall, overcoming long-term fatigue issues. The fracture-resistant stent may supplant the bioabsorbable stent if structural design modifications can resolve fatigue issues. The tissue lining used in this project (bovine peritoneum) is known to be resistant to thrombosis and intimal hyperplasia. It also is less susceptible to infection than Dacron or ePTFE-lined stent grafts. The combination of bovine peritoneum and bioabsorbable and/or fracture-resistant stent in this hybrid technology is designed to allow the body to incorporate the tissue into the vessel wall and seal the injury within 3 to 6 months.

Summary of Research Completed in Year 1

The researchers evaluated the tensile strength of several polymers in the first year of the project. They chose polydioxanone (PDO) as an appropriate polymer for fabrica-

tion of a bioabsorbable tissue-lined stent to treat traumatic arterial and venous injuries, with poliglecaprone as a second choice, and polyglactin as a third choice. They identified a 3-6-month window for the degradation time line of the polymers. They also discovered that mineral oil will serve as an effective storage emollient for the stent graft. Finally, they completed a first-generation prototype and design of a bioabsorbable stent.

Research Progress – Year 2

In Year 2 of the project, four major tasks were undertaken and accomplished:

1. Refine and manufacture a PDO bioabsorbable stent (Figure II-12) and a fracture-resistant nitinol stent (Figure II-13).
2. Mechanically test tissue following storage in anhydrous fluid (Figure II-14).
3. Conduct fatigue tests on fracture-resistant stents (Figure II-15).
4. Mechanically evaluate the compressive strength of both stent designs (Figure II-16).

Each of the stated goals was met, yielding unique tissue-lined stent grafts that are the first of their kind. With the mechanical criteria met, the technology is ready to progress to preclinical animal studies.

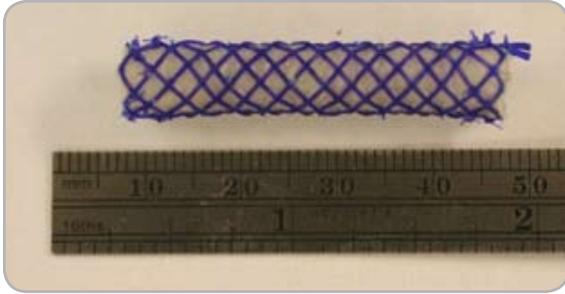


Figure II-12. PDO bioabsorbable tissue stent graft.

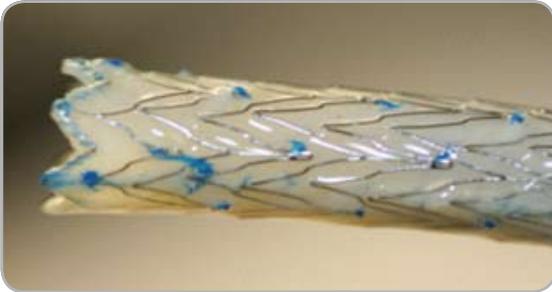


Figure II-13. Fracture-resistant nitinol stent graft.

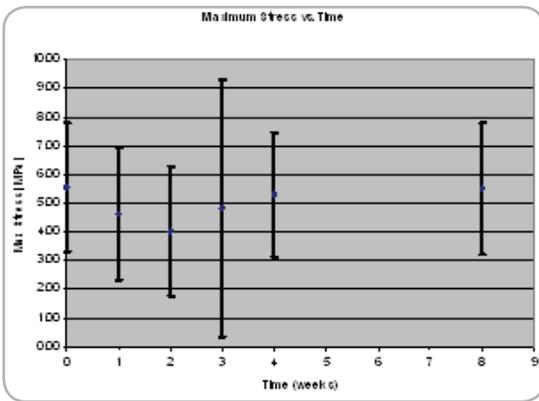


Figure II-14. Maximum stress/time of tissue in anhydrous fluid.

Key Research Accomplishments

- Manufactured two novel tissue-lined stent grafts.
- Both stent graft designs passed fatigue and compression mechanical tests.
- Developed an anhydrous storage method for a bioabsorbable tissue-lined stent, which passed fatigue testing.

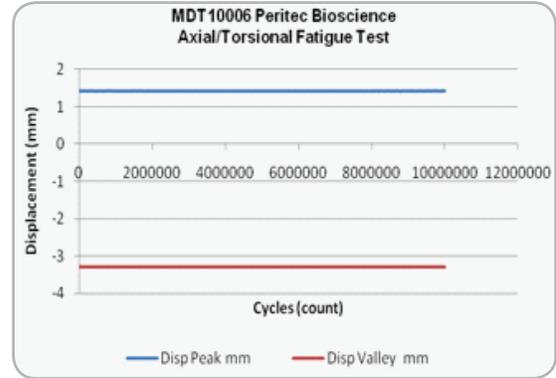


Figure II-15. Fatigue-resistant stent graft 10-year simulation.

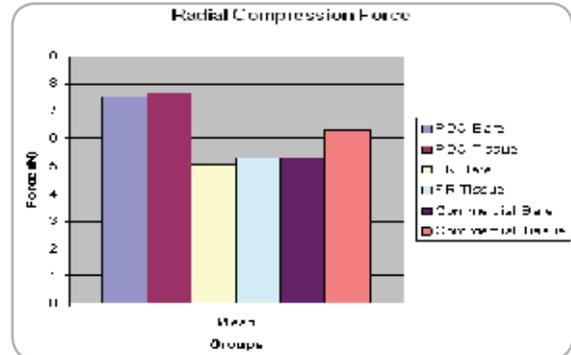


Figure II-16. Compression force of bioabsorbable and fracture-resistant stents.

Conclusions

The development of a tissue-lined bioabsorbable stent graft and a fracture-resistant stent graft is an important advancement for the treatment of arterial and venous injuries. To date, the following objectives have been accomplished: identified a polymer and storage solution for the development of a bioabsorbable stent graft, developed a PDO bioabsorbable tissue-lined stent, developed a fracture-resistant tissue-lined nitinol stent, and mechanically tested both stents. Both designs performed exceptionally well in mechanical testing. These two designs are ready for performance evaluation in preclinical animal models.



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Research Plans for the Next 3 Years

The majority of activities in Year 3 will focus on evaluation of stent prototypes in porcine iliac arteries. The goal will be to test the bioabsorbable, tissue-lined stent and compare it to a fracture-resistant nitinol self-expanding, tissue-lined stent. This will involve explant, post-processing, and histological analyses beginning at 30 days. The best stent will be chosen from this study based on development of intimal hyperplasia and/or thrombosis. The preferred stent will be compared to the nested cell design (if manufacturing is feasible), and the best stent will be selected from these to progress to long-term animal studies.

Years 4 and 5 will concentrate on design control validation history. At that time, a 6-month animal trial as well as sterilization and delivery system modifications will be completed in preparation for design freeze and a shift to clinical trials. While predicate devices may be available at that time, it is anticipated that the regulatory pathway will continue to be through premarket approval. Industry support will be required for the clinical trials. However, recent FDA decisions allowing historical controls will make these trials less burdensome than in the past.

Planned Clinical Transitions

The FDA Center for Devices and Radiologic Health published on January 13, 2005 a "Guidance Document" that establishes guidelines (including ISO standards) for the preclinical evaluation of devices. The Sarac group has followed these to the letter. To date, there is one FDA-approved stent graft (Viabahn-Gore, Inc.) and one

FDA-approved bare metal stent (LifeStent, Edwards Lifesciences/Bard) for use in blood vessels. However, the FDA has since announced that they will allow historical controls, which decreases the number of patients required by 30%-50%. There is precedent for the tissue processing in a 510(k), but preliminary discussions with the FDA indicate this device design will likely require an IDE. This evaluation considers that no bioabsorbable stent is on the market today. Additionally, pre-IDE meetings will be held. While approval is garnered for the use of the stent graft for blockages, a parallel application for a Humanitarian Device Exemption for trauma will allow quicker approval. Precedent has been set for covered stents for use in coronary arteries.

Corrections/Changes Planned for Year 3 and Rationale for Changes

When the initially planned industry partnership did not materialize, the researchers identified an exceptional partner in Proxy Biomedical. In this partnership, a bioabsorbable stent has been defined and advanced as far or further than initial targets set in discussions with the initial potential partner. The researchers continue to work closely with PeriTec Bioscience on the tissue lining and have designed a nested self-expanding stent structure to accommodate the tissue-lined constructs in conjunction with PeriTec and Nitinol Development Corporation.

In addition, the planned collaboration on a canine model of injury met unforeseen and insurmountable obstacles. A porcine model will be used for large animal studies, requiring minimal alterations of the stent design.

Functional Scaffold for Musculoskeletal Repair and Delivery of Therapeutic Agents

Project 4.4.3a, RCCC

Team Leaders: Kathleen Derwin, PhD (Cleveland Clinic)

Project Team: Amit Aurora and Ryan Milks (Cleveland Clinic)

Collaborators: A commercial partner is collaborating in the development of this device.

Therapy: Repair of large tendon and muscle defects with good suture retention and enhanced wound healing

Deliverable: A fiber-reinforced fascia lata device for effective musculoskeletal repair

TRL Progress: *Start of Year 1*, TRL 3; *End of Year 1*, TRL 5; *End of Year 2*, TRL 5

Key Accomplishments: The researchers have developed the design and testing methods for the reinforced fascia patches. They finalized the rat model for dorsal subcutaneous pouch

implantation. They also finalized the human cadaver model for rotator cuff tendon repair, with and without scaffold augmentation. Three critical feasibility studies for rotator cuff repair are ongoing. The research team also developed and validated a fluoroscopic method for measuring tendon repair gap formation.

Key Words: ECM, scaffold, tendon repair, fascia lata, rotator cuff

Introduction

No tissue repair technology currently exists for large tendon and muscle defects that is natural, strong, large, has good suture retention, and provides enhanced wound healing. Engineered human fascia lata potentially offers all of these advantages. However, despite the favorable mechanical properties of fascia and its established track record in orthopedics, its suture retention properties are poor and currently limit the clinical utility of this biomaterial for load-bearing applications. Hence, the objective of this project is to engineer a fascia lata device for effective musculoskeletal repair by stitching polymer fibers into the scaffold in a unique, controlled manner to impart targeted mechanical strength while maintaining biocompatibility.

Military Relevance

The large volume of military musculoskeletal trauma underscores the critical need for developing regenerative technologies that target large soft tissue defects. In particular, scaffolds aimed at bridging massive tendon and muscle defects over to bone would restore some degree of limb function in salvage procedures. An engineered fascia scaffold that can be crafted as a patch large enough to bridge a defect spanning several inches will be useful for these applications. Future work could

explore ways to promote neo-muscle formation onto the fascia bridge.

The initial design criteria for the engineered fascia scaffold address the clinical need for better repair strategies for rotator cuff injury. Rotator cuff injuries affect approximately 30%-40% of the population over age 60. Surgical repair of chronic tears is indicated when conservative treatment fails to improve the patient's symptoms. Despite improvements in the understanding of rotator cuff pathology and advances in surgical treatment options, repairs of large, chronic rotator cuff tears fail to heal in 20%-95% of cases. Hence, scaffolds are being investigated as a means to augment rotator cuff repairs and reduce the rate of re-tear. While rotator cuff studies are recognized to be of secondary military importance, efficacy studies for this indication have the greatest likelihood of first reaching clinical trials and commercialization with industry partners because of the large civilian patient population that is available. Furthermore, improved outcomes for rotator cuff repair would have a positive impact on the combat readiness of soldiers who experience these injuries in the course of training or active duty.

An engineered fascia patch may have an additional indication for closure of abdominal wall hernias, espe-



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cially at the midline (linea alba) where high mechanical forces tend to make these repairs difficult. Soldiers are deliberately overhydrated during transport to the treating hospital and may require abdominal wall release to relieve compartment syndrome that occurs secondary to the overhydration. These patients would benefit from a scaffold that would improve outcomes of their abdominal wall repair. Furthermore, an engineered fascia patch has potential application in the repair of fascia that has been released in extremity compartment syndromes.

Summary of Research Completed in Year 1

During the first year of the study, the researchers developed two new, clinically relevant bench tests for suture retention properties of scaffolds: the modified-ball-burst test and the tension-with-side-constraint test. They developed a rat subcutaneous model for evaluating the in vivo host response, degradation, and loss of suture retention following implantation of large (4x4 cm) scaffolds, and used the model in pilot studies. They established a magnetic displacement tracking system and a multiactuated mechanical test system for human cadaver studies. Finally, they developed clinically relevant surgical methods for performing rotator cuff repair with scaffolds on cadaver shoulders, including a human cadaver model for testing the extent to which augmentation with scaffolds improves the biomechanical outcomes of rotator cuff repairs.

Research Progress – Year 2

Subtask A: Identify and survey new fibers.

A.1 Identification of New Fibers

In the first half of Year 2, several polymer braids were investigated. Two braids were chosen for subsequent feasibility studies based on a battery of mechanical tests.

A.2 Stitch Pattern Configuration

The reinforcing pattern and size of the fascia patches for rotator cuff tendon repair applications were optimized according to design criteria for human rotator cuff repair.

Subtask B: Perform time zero and in vivo degradation testing of devices stitched with the preferred fiber in the rat subcutaneous model.

B.1 Time Zero Patch Suture Retention Test

Time zero failure testing of fascia reinforced with the two chosen braids is ongoing. Fatigue testing of fascia reinforced with both braids will be completed.

B.2 Rat Subcutaneous Implantation Feasibility Study

Fascia patches (5x5 cm) reinforced with the polymer braids were manufactured and implanted into a rat dorsal subcutaneous pouch model (n=22 rats per braid). Reinforced patches (1x1 cm) were implanted into an abdominal wall defect of the same rats. Rats from this ongoing study will be euthanized at 4 and 12 weeks (11 rats per braid per time point). The 5x5 cm patches will be tested for mechanical properties using the tension-with-side-constraint test used for time zero evaluation. The loss of mechanical properties due to graft remodeling will be evaluated for each braid at both 4 and 12 weeks. The 1x1 cm patches will be evaluated histologically for the presence of neutrophils, lymphocytes, macrophages, and giant cells. A preferred polymer braid from the two tested will be evaluated in the human cadaver model and a preclinical canine model.

Subtask C: Test the efficacy of the reinforced fascia device in a human cadaver rotator cuff injury and repair model.

C.1 Human Cadaver Rotator Cuff Injury and Repair Model Development

A surgical protocol was developed for rotator cuff tendon repair on a human cadaver model, with and without scaffold augmentation of the repair (Figure II-17). All of the surgical methods, sutures, anchors, and tools are consistent with the manner in which repairs and augmentation might occur in a human patient intraoperatively.

C.2 Human Cadaver Biomechanical Testing Method Development

For the first 7 months of Year 2, the researchers worked to develop a custom magnetic system for tracking tendon repair gap formation during testing. Such an approach

is necessary when the tendon repair is covered by an augmentation patch and cannot be tracked optically. However, in Month 8 a decision was made to abandon this magnetic system for a fluoroscopic approach, for the requisite accuracy and reproducibility could not be obtained using the magnetic system. The fluoroscopic method was successfully validated against a gold standard optical method.

To date, eight nonaugmented repaired shoulders have been tested using this fluoroscopy method for tracking repair gap formation. Shoulders were cycled between 5-180N at 0.25 Hz until failure or 5,000 cycles. Following selection of the preferred polymer braid, reinforced fascia patches will be manufactured and augmented repairs will be tested. Ten pairs of nonaugmented and augmented repaired cadaver shoulders will be tested. The extent to which augmentation with reinforced fascia improves the biomechanical outcomes (e.g., repair gapping, stiffness, and failure load) of rotator cuff repairs in human cadaver shoulders will be evaluated.

Key Research Accomplishments

- Identified two fibers as appropriate for feasibility studies.
- Finalized the rat model for dorsal subcutaneous pouch implantation.
- Finalized the human cadaver model for rotator cuff tendon repair, with and without scaffold augmentation.
- Developed and validated a fluoroscopic method for measuring tendon repair gap formation.

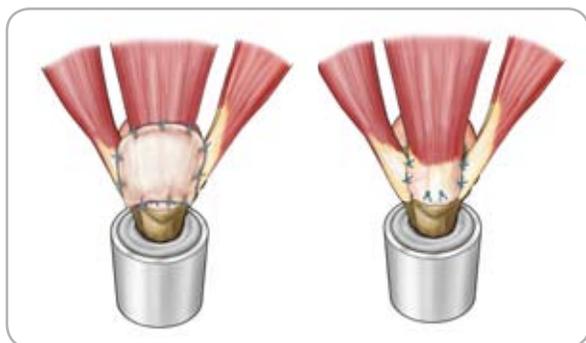


Figure II-17. Human rotator cuff tendon repair augmented with reinforced tissue graft.

Conclusions

All of the design and testing methods for the reinforced fascia patches have been completed. Three critical feasibility studies for rotator cuff repair are ongoing.

Research Plans for the Next 3 Years

Three critical feasibility studies for rotator are ongoing and are expected to be complete by the end of Year 3. (An in vivo canine rotator cuff injury and repair study is ongoing with outside funding recently secured.) Feasibility studies with reinforced fascia patches will be undertaken following the selection of a polymer braid. Data from animal studies and manufacturing tests will be collected and submitted for FDA approval through the 510(k) mechanism. The scope of work described in this progress report will move the technology through TRL 6 by the end of Year 3. By Years 4-5, it is expected that the researchers' device will be advanced into clinical trials for rotator cuff repair.

Planned Clinical Transitions

As cadaver and animal studies are completed during Year 3, the commercial partner will take the lead in addressing the necessary production and regulatory steps required for FDA clearance of the researchers' device (identify packaging components, prepare a design history file, perform shelf-life/stability study, etc). The device is expected to be ready to advance to clinical trials for rotator cuff repair by Years 4-5.

Corrections/Changes Planned for Year 3 and Rationale for Changes

The principal change in this project is a shift in focus of AFIRM funding from tendon regeneration to abdominal wall reconstruction, which has been identified as a critical gap in current therapies available to wounded warriors. This has been made possible by the agreement of the MTF to fund the rotator cuff feasibility studies and to transfer the technology for manufacturing, toxicity testing, packaging, and shelf-life studies internally.



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Functional Scaffolds for Soft Tissue Repair and Joint Preservation

Project 4.4.3b, RCCC

Team Leaders: Charles J. Gatt, Jr., MD and Michael G. Dunn, PhD (University of Medicine and Dentistry of NJ [UMDNJ])

Project Team: Aaron Merriam, BS (UMDNJ and Rutgers – the State University of New Jersey) and Asa Vaughan, PhD (Rutgers – the State University of New Jersey, New Jersey Center for Biomaterials [NJCBM])

Collaborators: Joachim Kohn, PhD and Sanjeeva Murthy, PhD (NJCBM)

Therapy: Regeneration of fibrocartilaginous tissue such as the meniscus of the knee

Deliverable: An implantable scaffold composed of biodegradable polymer fiber-reinforced collagen sponge for repair of knee meniscus

TRL Progress: *Start of Year 1, TRL 3; End of Year 1, TRL 3; End of Year 2, TRL 4*

Key Accomplishments: The researchers designed, fabricated, and tested a

second-generation scaffold. They began a nonfunctional small animal study with the second-generation scaffold. The Kohn laboratory obtained mechanical properties and degradation profiles for five polycarbonates. Significant enhancements using automated synthesis accelerated the evaluation of wide banks of polycarbonates for ACL/meniscus scaffolds.

Key Words: Meniscus, scaffold, degradable polymeric fibers, collagen, regeneration

Introduction

This project addresses a large burden to the military and its personnel: injuries to the musculoskeletal system, specifically, the meniscus and cartilage of the knee joint. Musculoskeletal injuries are the most common types of injuries seen in the military and occur six times more than any other injury type. Severe damage to the knee meniscus, the focus of this project, can significantly impair the normal activity of service members. Due to limited healing capabilities, meniscal injuries are often treated with resection. This treatment leads to symptom relief, but in many cases, results in the development of degenerative arthritis of the knee. The pain associated with damaged meniscal tissue as well as osteoarthritis in the knee can have a debilitating effect on the ability of service men and women to perform, inhibiting their operational capability in both the short and long term.

The focus of this project is development of a tissue-engineered meniscus scaffold that will offer a solution to service members who have moderate to severe meniscal damage. The overall goal is to develop an off-the-shelf clinical device that can be implanted at the site of a meniscal resection and result in knee joint preservation. The clinical role of the device is twofold: (1) to provide

symptom relief and rapid return to function for active military personnel and (2) to prevent the progression to degenerative knee arthritis that commonly requires costly total knee replacement surgery later in life.

The meniscus scaffold (see **Figure II-18**) is composed of a cross-linked collagen sponge reinforced with resorbable polymer fibers, which provide (1) mechanical function, immediately bearing loads in the knee joint and (2) allow for ingrowth and remodeling of neo-meniscal tissue during degradation and resorption of the device.

Summary of Research Completed in Year 1

During the first year of the study, the researchers developed a novel meniscus scaffold consisting of high-strength resorbable tyrosine-derived polymeric fibers arranged within a collagen matrix, which promotes the synthesis of new, organized tissue when implanted as a total meniscal replacement in sheep. They completed full meniscal scaffold implantation surgeries in sheep. Half of the implants failed due to improper positioning of the posterior anchor attachment of the scaffold; the researchers developed a new surgical procedure to address this issue. They demonstrated a new synthesis



Figure II-18. Sheep meniscus (left) and the meniscus scaffold (right) under development in the Gatt-Dunn lab.

of collagen at 8 weeks after meniscal scaffold implantation, which tends to organize along the longitudinal axis of polymer fibers. Immune staining demonstrated the presence of types I and III collagen, which are major constituents of fibrocartilage.

Research Progress – Year 2

A second-generation scaffold was designed and evaluated for future implantation in a long-term, functional, in vivo model. Sponges with higher collagen content and GAGs—polysaccharides important to the integrity and resilience of connective tissue—had improved stiffness and viscoelastic properties making them more comparable to native meniscus and more appropriate for the sheep model. Alterations in the structure of the device and the operative procedure aimed to improve long-term survivability of the implant and to avert common operative complications.

The meniscal device must achieve a balance between maintaining structural integrity long enough to allow ingrowth of native tissue and degradation within 1 year to prevent interference of the device with new native tissue. Parallel studies ongoing at NJCBM are evaluating polycarbonate copolymers as potential replacements for the poly(desaminotyrosyl-tyrosine dodecyl dodecanedioate) (poly[DTD-DD]) fibers in the meniscus scaffold. Polycarbonate copolymers would allow for a more rapid degradation rate if required for long-term function. It is important to note that, the development of a polycarbonate for the meniscus scaffold is unique to the NJCBM.

Key Research Accomplishments

- Designed and tested a second-generation scaffold.
 - Modified the fiber reinforcement pattern for radial stability.
 - Increased collagen content of sponge to improve mechanical properties.
 - Added GAGs to improve compressive properties and influence tissue ingrowth.
- Obtained mechanical properties and degradation profiles for five polycarbonates as potential replacements for poly(DTD-DD).
 - This work has been accomplished by the Kohn laboratory at the NJCBM.

Conclusions

The focus of this project is development of a tissue-engineered meniscus scaffold that will offer a solution to service members who have moderate to severe meniscal damage. The overall goal is to develop an off-the-shelf clinical device that can be implanted at the site of a meniscal resection and result in knee joint preservation. The goal is to return service members to duty as quickly as possible and to avoid the costly consequence of osteoarthritis of the knee in later years.

Overall, the researchers have shown that this novel hybrid meniscus scaffold promotes the synthesis of organized, new tissue when implanted as a meniscal replacement. In Year 3, second-generation, optimized scaffolds will undergo further in vivo evaluation over longer time frames, using modified surgical techniques in the sheep meniscus replacement model.

The NJCBM team has found that polycarbonates resorb faster than polyarylates and that tuning composite formulations can control the degradation rate. Studies are under way to identify an ideal polycarbonate formulation to balance mechanical properties with optimal degradation and resorption rates. In vitro screening assays and in vivo animal models have been developed and validated; therefore, as soon as a suitable polycarbonate is discovered, further evaluation will move forward.



II: Limb and Digit Salvage

Progress Reports: Soft Tissue Repair and Regeneration (Excluding Nerve)

Research Plans for the Next 3 Years

For the Gatt-Dunn laboratory:

- Second-generation meniscus scaffolds will undergo in vivo evaluation in sheep using the “meniscus plug” (partial meniscectomy) model, with sacrifices at 8, 16, and 32 weeks post operation to determine the amount and type of tissue ingrowth.
- Second-generation meniscus scaffolds will undergo in vivo evaluation in sheep using the total meniscectomy model at 16 and 32 weeks post operation. At sacrifice, the meniscus scaffolds will be retrieved and analyzed using mechanical testing, histological analyses, and immunohistochemical analyses to determine types I, II, and III collagen deposition. In addition, the articular cartilage will be examined grossly and histologically.
- Second-generation meniscus scaffolds will undergo in vivo evaluation in sheep using the total meniscectomy model at 52 weeks post operation.

The research plan for NJCBM is to determine if there is a suitable polycarbonate replacement for poly(DTD-DD) fibers. This will consist of the following:

- Vary compositional parameters until a suitable polycarbonate is discovered for the meniscus scaffold.
- Once this is completed, in vivo testing will be conducted in a rabbit model and sheep models.

Planned Clinical Transitions

The meniscus scaffold likely will require an IDE, with preclinical data from the large animal studies and clinical trials. In Year 3 of the project, we will seek guidance from AFIRM staff concerning our preclinical safety and efficacy data and will arrange a pre-IDE meeting with the FDA.

An international patent is pending; and licensing discussions are under way with two orthopedic companies (nondisclosure agreements preclude identifying the companies). The team will meet separately with both parties to begin discussions regarding licensing the technology and providing additional funding to accelerate preclinical research studies. In addition, \$100,000 in matching funding has been received from the NJ Commission on Science and Technology.

Corrections/Changes Planned for Year 3

In the original proposal, large animal studies with second-generation scaffolds were scheduled to begin in Year 2, but they were delayed until the beginning of Year 3 because of extensive optimization and mechanical and biological testing of the second-generation scaffolds in Year 2.

Peripheral Nerve Repair for Limb and Digit Salvage

Project 4.4.4, WFPC

Team Leader(s): Kacey Marra, PhD (University of Pittsburgh); David Kaplan, PhD (Tufts University); and Tom Smith, PhD (Wake Forest University)

Project Team Members: Mostafa Ramadan, MD, Lauren Kokai, PhD, Ryan Nolan, Samantha Beckowski, Danielle Minter, Wesley Sivak, MD, PhD, Jedidiah McAtee, BS (University of Pittsburgh); Marie Tupaj, Alexander Nectow (Tufts University); Jonathan Barnwell, MD, Zhongyu John Li, MD, PhD, Mark Van Dyke, PhD, and Lauren Pace (Wake Forest University)

Collaborator(s): Tirrell Laboratory (University of California, Santa Barbara);

Harrison Laboratory (Wake Forest Institute for Regenerative Medicine); and University of Virginia Department of Orthopaedic Surgery (Hand Surgery)

Therapy: Combined strategy for regeneration over long (>3 cm in human) peripheral nerve gaps

Deliverable(s): Proactive biodegradable nerve guide system for peripheral nerve regeneration

TRL Progress: *Begin Year 1*, TRL 1; *Begin Year 2*, TRL 2; *End Year 2*, TRL 3

Key Accomplishments: The examination of polymer/growth factor guides in a critical size rat defect model

demonstrating enhanced nerve repair resulted in the identification of a drug delivery strategy to deliver bioactive neurotrophic factors for ~60 days in vivo. The researchers have initiated nonhuman primate studies utilizing keratin-filled nerve guides. Preclinical testing of keratin biomaterials has been completed, and a pre-IDE package has been prepared for the FDA.

Keywords: Limb regeneration, silk fibroin, nerve guides, drug delivery, keratin, functional electrical stimulation, clinical trial

Introduction

Approximately 1.9 million people are living with limb loss in the United States as a result of trauma, cancer, vascular problems, or congenital defects. It is well known that in some amphibians, the presence of a copious nerve supply is a key factor for their regenerative ability following amputation. Peripheral nerve regeneration is a critical issue in humans as well, as 2.8% of trauma patients present with this type of injury. Following trauma, incomplete nerve regeneration and permanent demyelination may result, leading to lifelong disability. Several nerve regeneration strategies are currently being employed in this project, including biophysical guidance, biochemical applications, and protein modification.

The researchers of this project seek to (1) create a biodegradable nerve guidance system that delivers nerve growth factors (NGF, glial cell line-derived neurotrophic factor [GDNF], NT-3) and biophysical guidance to regenerating peripheral nerves, (2) move this nerve guidance system in vivo, and (3) utilize expertise from all three laboratories (biomaterial expertise and small and

large animal facilities) at Tufts University, University of Pittsburgh, and Wake Forest University.

Summary of Research Completed in Year 1

During the first year of the project, the researchers developed a novel biodegradable conduit for nerve repair (Figure II-19) and conducted several in vitro and in vivo studies. Specifically, they examined nerve guides consisting of FDA-approved materials (collagen, silk, and polycaprolactone [PCL]) in a rat sciatic nerve defect. Neurotrophic growth factors (e.g., GDNF) were encapsulated in double-walled polymer microspheres, resulting in an extended, long-term release. These microspheres were embedded in the walls of both the PCL and silk nerve guides. The researchers began to conduct sophisticated functional analyses on rats (Figure II-20). Histological analysis of the guides included axon counting and inflammatory response evaluation. Preliminary results from a rabbit study indicated that keratin-filled collagen nerve guides could bridge a 2 cm rat tibial defect in >50% of the animals tested.



II: Limb and Digit Salvage

Progress Reports: Nerve Repair and Regeneration

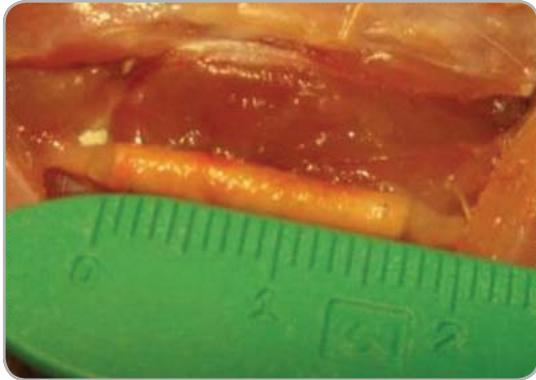


Figure II-19. Nerve guide in rat sciatic nerve defect.

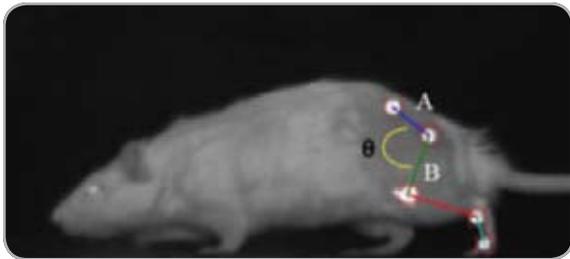


Figure II-20. Examination of functional improvement using video gait kinematics.

Research Progress – Year 2

During the past year, all three laboratories involved in this project made significant progress.

The Kaplan group at Tufts University used silk biomaterial protocols and integrating regenerative approaches toward a biodegradable nerve guidance system. Regenerative approaches include incorporating biophysical cues (e.g., surface patterning and electrophysiology applications) and chemical cues (e.g., protein coatings and growth factor incorporation) into silk nerve guides. Three different approaches were studied for nerve growth factor incorporation into silk films: (1) mixing NGF with the silk solution, (2) layering NGF on top of flat methanol-treated silk films, and (3) forming multiple layers of NGF between methanol- and nonmethanol-treated silk films. NGF layered between nonmethanol-treated silk fibroin films provided the best option for a 2-week linear release response.

The Smith group at Wake Forest University placed four nonhuman primates (*macaca fascicularis*) on a study examining the use of keratin-filled bovine collagen nerve guides (NeuraGen®) to treat a 1 cm nerve defect in the median nerve. These animals are 13 weeks post surgery and are doing very well. A spinoff company, KeraNetics LLC, has established a GLP-compliant manufacturing facility that will produce keratin biomaterials for the first clinical trial of this product. A grant to fund this trial has been awarded by the Congressionally Directed Medical Research Programs (CDMRP). KeraNetics will also sponsor an IDE application to the FDA. Electrophysiological and functional studies are following the recovery process.

The Marra group at the University of Pittsburgh has identified an optimal formulation for an off-the-shelf nerve guide involving PCL and 60-day delivery of GDNF. PCL/GDNF guides were implanted in a 1.5 cm rat sciatic nerve defect model, and results were assessed after 6 weeks and 16 weeks. This model has shown promise (Figure II-21), and nonhuman primate studies are being initiated. The Pitt lab received more keratin gels from the Wake Forest lab and implanted the gels within the PCL/GDNF conduits in the rat sciatic nerve defect model in December 2009. Conduits were harvested and histological assessment is under way.

In addition to the work described previously, a device was developed to increase axon outgrowth by means of electrical stimulation. Results revealed statistical differences within the first week following neuron differentiation between neuron axons exposed and unexposed ($n=30$, $p < 0.05$).

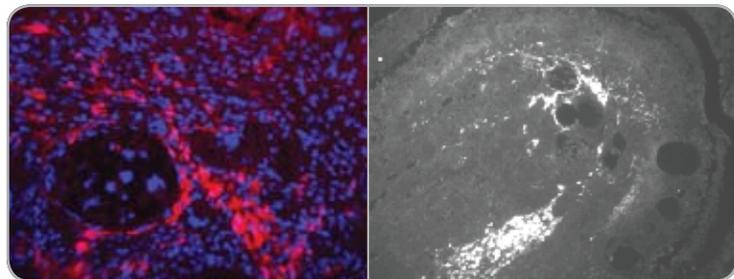


Figure II-21. Cross-section of nerve guide with Schwann cell invasion surrounding GDNF-containing microspheres.

Key Research Accomplishments

- Identified a drug delivery strategy to deliver bioactive neurotrophic factors for approximately 60 days in vivo (based on examination of polymer/growth factor guides in a critical size rat defect model that demonstrated enhanced nerve repair).
- Initiated nonhuman primate studies utilizing keratin-filled nerve guides.
- Initiated GLP-compliant manufacturing of keratin biomaterials through a spinoff company.
- Completed preclinical testing of keratin biomaterials and prepared a pre-IDE package for the FDA.
- Obtained a grant for a clinical trial to study nerve repair using the keratin biomaterial from the CDMRP.

Conclusions

The researchers are making substantial progress toward the development of a proactive biodegradable nerve guide system for peripheral nerve regeneration.

Research Plans for the Next 3 Years

The Pittsburgh-Wake Forest-Tufts labs will begin integrating electrode, drug delivery, and chemical coupling

techniques with silk nerve guides in vitro. Functionalization studies will be carried out with silk nerve guides in vitro. In an in vivo rat model, implantations will continue with the next generation silk nerve guides (e.g., porosity, multiple growth factors, and biophysical stimulation).

Planned Clinical Transitions

All three teams have begun planning a pathway to clinical studies. At the University of Pittsburgh, Dr. Marra has met with members of the Clinical Translation Science Institute twice to begin the process of transitioning from preclinical to clinical studies. After completion of the nonhuman primate model, Dr. Marra will be able to conduct a Phase 1 clinical trial.

At Wake Forest, a pre-IDE meeting with FDA is planned for the second quarter of 2010 with a formal IDE application to follow. A Phase 1 clinical trial is expected to begin enrollment by the fourth quarter of 2010.

Corrections/Changes Planned for Year 3

One important change has been the removal of the rabbit tibial nerve defect model and the rapid progression into the nonhuman primate median nerve defect model. This will permit earlier clinical translation.



II: Limb and Digit Salvage

Progress Reports: Nerve Repair and Regeneration

Modular, Switchable, Synthetic, Extracellular Matrices for Regenerative Medicine

Project 4.4.5, WFPC

Team Leader(s): Matthew Tirrell, PhD (University of California, Berkeley)

Project Team Members: Won H. Suh, MS/PhD and Katie Megley, BS (University of California, Berkeley)

Collaborator(s): Kacey Marra (University of Pittsburgh); Bob Guldberg (Georgia Institute of Technology); and Stephen Badylak (University of Pittsburgh)

Therapy: Injectable synthetic extracellular matrices for regenerative medicine

Deliverable(s): Task 1 (Year 1): Model peptide amphiphile (PA) synthesis and characterization; Task 2 (Years 1-2): Employ fibrous networks composed of worm-like micelles; Task 3 (Year 2): Neural stem cell attachment, proliferation, and differentiation; Task 4 (Year 2): Shear responsive peptide amphiphile development; Task 5 (Year 3): Additional peptide amphiphiles construction; Task 6 (Years 4-5): Test injectable synthetic matrices in animal models.

TRL Progress: *Start of Program*, TRL 1; *End Year 1*, TRL 2; *End Year 2*, TRL 2

Key Accomplishments: The researchers have developed several biologically active PA gels with well-defined three-dimensional structures.

Keywords: Synthetic ECM, peptide amphiphile, nerve regeneration, tissue engineering, micelles, vesicles

Introduction

Functional limb and digit tissue restoration involves a hierarchically defined process that often requires precise spatial and temporal coordination among multiple biological systems and processes. Even in amphibians, the regeneration process occurring after a traumatic limb wound is often limited by peripheral nerve damage. As part of a team effort with other research groups in AFIRM's Limb and Digit Salvage Program, the Tirrell group is pursuing an approach to induce peripheral nerve growth following traumatic amputation by modulating components of the naturally occurring ECM (i.e., fibronectin and laminin). Specifically, synthetic chemistry was utilized to construct PAs (lipopeptides) with controlled physicochemical and bioactive properties (i.e., cell adhesion promoted by RGD [arginine-glycine-aspartic acid]) and their *in vitro* efficiencies in adhesion, proliferation, and differentiation of neural stem cells on engineered surfaces were tested. The PA gel system will be further tested in animal injury models in the form of gel-like materials.

Summary of Research Completed in Year 1

During the first year of the project, the researchers synthesized double-tailed PAs with controlled bioactive components and found that double-tailed PAs that incorporate RGD units can promote attachment, proliferation, and differentiation of human neural stem cells (hNSCs). Also, the research team developed an alanine-rich peptide head group containing PAs (C16-WAAAA-KAAAAKAAAAKA = C16-W3K, **Figure II-22**) that transform from spherical micelles into three-dimensional structured worm-like micelles (a fibrous gel) based on the modulation of physicochemical properties (i.e., shear, temperature, and concentration). Based on these Year 1 accomplishments, biologically responsive fibrous PA gels were further developed and tested against hNSCs.

Research Progress – Year 2

- Shear responsive bioactive PAs C16-W3K and C16-W3K-RGD were mass-produced and characterized physicochemically and biologically. Both PAs formed gels, displayed three-dimensional structures with

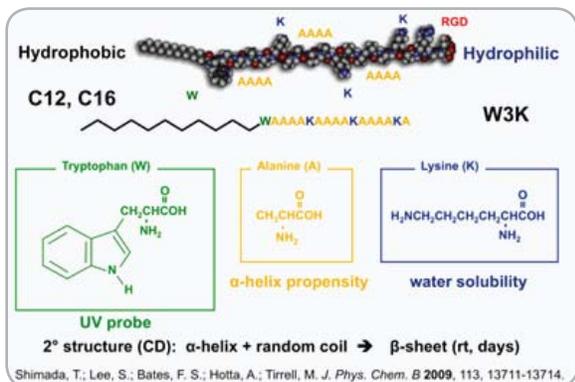


Figure II-22. Shear-responsive PA C16-W(AAAAK)3A (C16-W3K). W3K appended with a single hydrocarbon tail will transform from spherical micelles into worm-like micelles based on modulation of physicochemical properties such as shear, monomer concentration, and temperature.

- nanometer features and, in the case of cross-linked C16-W3K-RGD, promoted mammalian cell adhesion.
- Additional PAs were synthesized to further expand on the synthetic ECM series. C16-W(A4E)3A (C16-W3E), C16-W3E-RGDS, and C16-W3K-RGDS PAs were synthesized in addition to C16-W3K and C16-W3K-RGD. The switching out of lysine (K) units with glutamic acid (E) units allowed charge reversal of individual PAs from positive to negative. The aim was to modulate the charge densities within the three-dimensional synthetic ECM that may play a critical role in mammalian cell adhesion and/or nerve regeneration. In the case of C16-W3E, they acted as gel inducer forming beta-sheet structures in an accelerated fashion.
 - Cytotoxicity, cell adhesion, and long-term cell growth of hNSCs were modulated based on the mixing ratio of C16-W3E and C16-W3K-RGD. The ideal ratio allowing the best cell growth was between 15%-30% of C16-W3K-RGD mixed with 70%-95% C16-W3E.
 - The researchers have established an active collaboration with Marra's group at the University of Pittsburgh to further test the efficacy of PA gels on nerve regeneration. These laboratories have exchanged ideas about how best to proceed with animal testing and plan to conduct active research involving rat injury models during 2010 and 2011. Additional collabora-

tions will come from Guldberg's group at Georgia Institute of Technology to conduct composite injury model studies using Tirrell's group PA systems and from Badylak's group at the University of Pittsburgh to diversify biologically responsive peptide head group moieties in the PAs.

Key Accomplishments

- Determined that single-tailed C16-W(A4K)3A and C16-W(A4K)3ARGD(S) PAs form worm-like micelles (gels) and are responsive to shear.
- Demonstrated that bioactive C16-W(A4K)3ARGD gel matrices can encapsulate biological fluids and have worm-like structures.
- Observed that surfaces engineered with C16-W(A4K)3ARGD promote mammalian cell adhesion.
- Determined that C16-W(A4E)3A, C16-W(A4E)3ARGDS, and C16-W(A4K)3ARGDS PAs can be readily prepared via standard solid-peptide chemistry synthesis methods.
- Found that C16-W(A4K)3A and C16-W(A4E)3A PAs have distinct gelation profiles and exhibit unique secondary structures.
- Demonstrated that C16-W(A4E)3A PAs are noncytotoxic, but C16-W(A4K)3A PAs are cytotoxic (sub-10 micromolar LD₅₀) to human NSCs.
- C16-W(A4E)3A PAs mixed with C16-W(A4K)3A-RGD PAs promote hNSC growth in vitro at an optimal ratio (i.e., 15%-30% of C16-W3K-RGD mixed with 70%-85% C16-W3E).

Conclusions

The researchers have successfully developed several biologically active PA gels with well-defined three-dimensional structures.

Research Plan for the Next 3 Years

The researchers plan to correlate rheological and structural features with biological activity of prepared PAs to refine and define injectable three-dimensional matrices for in vivo applications. They will correlate in vitro biologi-



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Progress Reports: Nerve Repair and Regeneration

cal activity of different PA gel formulations with in vivo regenerative applications (i.e., nerve gap injury model). The bioactive PA gel remodeling profiles by proliferating cells will be assessed. The researchers will conduct animal studies using biologically responsive PA gels in collaboration with the Marra and Guldberg groups. They will expand on biologically responsive amphiphilic construct platforms via the multicomponent approach (i.e., include nucleic acids and/or proteins) based on experimental results. The multicomponent synthetic ECM system will be designed to positively regenerate nerve and/or bone at the wound site over multiple time domains. Finally, the researchers will lipidate bioactive peptide oligomers that will modulate biological function at the site of injury in collaboration with the Badylak group and incorporate such new amphiphilic constructs into the three-dimensional PA gel system and test their in vivo efficacy.

Corrections/Changes Planned for Year 3

- Tasks 3 and 4: Extend into Years 3-4; the hNSC experiments will be critical for the continued development of the PA gel system.
- Task 5: Extends into Years 3-5; the researchers have found that mixing different PAs with different biological and physicochemical properties can create unique synthetic ECMs.
- Task 6: The start time line has been changed from Years 4-5 to Years 2-5.
- Increase collaborations with the Marra, Guldberg, and Badylak groups.
- Incorporate animal skin testing to conduct at University of California, Berkeley (Years 3-5).

Repair Segmental Nerve Defects

Project 4.4.1/4.4.2, RCCC

Team Leaders: Anthony Windebank, MD, Michael Yaszemski, MD, PhD (Mayo); Joachim Kohn, PhD, Melitta Schachner, PhD (Rutgers); Daniel G. Anderson, PhD and Robert Langer, ScD (MIT)

Project Team: Robert Spinner, MD, Huan Wang, MD, PhD, Bingkun Chen, MD, PhD, Mahrokh Dadsetan, PhD, Andrew Knight, PhD, Jing Rui, MD, Suzanne Segovis (Mayo Clinic); Melitta Schachner, PhD, Kathryn Uhrich, PhD, David Shreiber, PhD, Jian Chen, PhD, Shirley Masand, Mindy Ezra, Jeremy Griffin, Joachim Kohn, PhD (Rutgers, the State University of NJ); Sally Meiners, MD (UMDNJ/Robert Wood Johnson Medical School); Daniel G. Anderson, PhD, Robert Langer, ScD, Hao Cheng, PhD, Paulina S. Hill, PhD, and Nathaniel Vacanti (MIT)

Collaborators: Bonwrx™; Sanjeeva Murthy, PhD (Rutgers, the State

University of NJ) and Christopher Pastore, PhD (Philadelphia University)

Therapy: Treatment of peripheral nerve injuries

Deliverable: Polymer conduits suitable to repair large motor nerve defects

TRL Progress: Biodegradable polymeric nerve conduits, *Start of Year 2, TRL 3; End of Year 2, TRL 4*

Nerve conduits with bioactive growth factors, *Start of Year 2, TRL 3; End of Year 2, TRL 4*

Adult stem cell therapy, *Start of Year 2, TRL 5; End of Year 2, TRL 6*

“Biorubber” nerve conduits, *Start of Year 2, TRL 3; End of Year 2, TRL 4*

Biodegradable nerve conduits with growth factors, *Start of Year 2, TRL 3; End of Year 2, TRL 3*

Key Accomplishments: The researchers found that candidate

polymer PCLF and single-channel poly(xylitol-co-sebacate) (PXS) conduits promoted better functional regeneration than the FDA-approved collagen conduit. They synthesized a novel library of polyesters to further optimize the nerve guide material. They developed a method for incorporating aligned electrospun fibers into the nerve conduit lumen in a uniform manner with no fiber aggregation. They encapsulated anti-inflammatory agents in electrospun fibers to decrease the inflammatory response to synthetic materials. The researchers also established a complex soft tissue injury model and obtained approval for a clinical trial for “repair of 6 cm peripheral nerve gaps in sural nerve biopsy using PCLF tube.”

Key Words: Peripheral nerve, conduit, scaffold, biodegradable polymer, stem cells, growth factors, tyrosine-based polycarbonates, electrospinning, topographical cue

Introduction

Due to limitations of autologous nerve grafting, the current clinical gold standard to repair nerve defects, alternative nerve repair materials are needed. Synthetic nerve conduits have been approved for clinical use, but current commercially available conduits fail to regenerate nerve across critical size gaps (>3 cm in humans). The researchers of this project aim to reconstruct severed nerves resulting in critically large gaps by fabricating biodegradable, polymeric, porous conduits that provide physical and biologic guidance to regenerating nerves. The synthetic polymer nerve guidance strategies are complementary to the cadaver-derived, epineurial sheath peripheral nerve regeneration strategy being pursued by the Siemionow lab at the Cleveland Clinic.

Military Relevance

During wartime, 50% of injuries resulting from gunshots and grenades cause a complete transection of peripheral nerves. Once severed, these nerves can regenerate; however, they often result in poor functional recovery due to their tendency to extend randomly across the gap created. There is an immediate need to help guiding regenerating nerves to increase functional recovery following peripheral nerve injury in the battlefield.

Summary of Research Completed in Year 1

During the first year of the project, the researchers effectively encapsulated and released neuronal growth factors from fumarate-derived polymer scaffolds and polymer microspheres. The growth factors were released in a biologically active form and with a time course suit-



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able to enhance peripheral nerve regeneration. The research team also showed that MSCs provide a potential cell-based delivery platform for growth factor delivery.

Research Progress – Year 2

At the individual sites, *in vivo* studies were performed at Rutgers to investigate the use of a nonporous, fast-degrading Tyr-PC as well as a porous, fast-degrading Tyr-PC conduit made from the same polymer composition in the mouse femoral nerve model. Functional studies showed that Tyr-PC is significantly superior to polyethylene as an entubulation material to treat peripheral nerve injuries, with some groups of mice receiving Tyr-PC conduits regaining locomotive functions equivalent to pre-injury capacity. Retrograde tracing demonstrated the correct motoneurons projecting through nerve conduit to the femoral nerve motor branch. These studies provided insights for the team to identify several design parameters as being crucial for the desired performance of the next-generation conduit: polymer composition, degradation, porosity, and filler.

The Rutgers team is fabricating nerve guidance conduits specifically for the newly adopted rat sciatic nerve injury model. Several fabrication methods such as extrusion, electrospinning, dip-coating, or braiding are being explored to impart the desired physical and mechanical properties to the conduits. An *in vivo* implantation study, currently under way, will characterize the degradation rates and changes in mechanical properties of various conduits over time in a rat subcutaneous implantation model.

In collaboration with the Mayo group, single channel polyester conduits developed at MIT were tested in a 1 cm long rat sciatic nerve defect model (Figure II-23). This first-generation conduit showed better functional nerve regeneration than the commercially available collagen conduit as assessed by electrophysiological response and muscle weight. With this exciting data, the MIT team has been working on improving the mechanical properties of the conduits and fabricating intraluminal fillers to provide better guidance for regenerating nerves.

Although the first-generation polyester conduits showed good efficacy in the 1 cm rat sciatic nerve defect model, the elasticity of these materials was not optimal under hydrated conditions. Furthermore, the polyester conduit was found to degrade too quickly for long nerve gaps. To help conduits maintain excellent elasticity in the hydrated state and decrease the degradation rate, the MIT team synthesized a library of polyesters with different physical and biochemical properties. One newly developed polymer proved to have excellent elasticity in both the hydrated and dehydrated states. Histological analysis showed that this elastomer had improved biocompatibility and a slower degradation rate. Systematic screening of this polyester library led to the fabrication of a second-generation conduit with optimized material properties.

To generate a three-dimensional intraluminal scaffold, the MIT team developed a method for incorporating aligned electrospun fibers inside the nerve conduit lumen. Uniform alignment within the conduit was achieved by changing the surface properties of the fibers to prevent aggregation. The MIT team showed that bioactive agents such as anti-inflammatory drugs can be encapsulated into electrospun fibers and released in a controlled manner over time. It was found that the release kinetics of the drug could be controlled depending on the type of synthetic material used. For example, certain synthetic fibers showed a burst release of encapsulated drug while

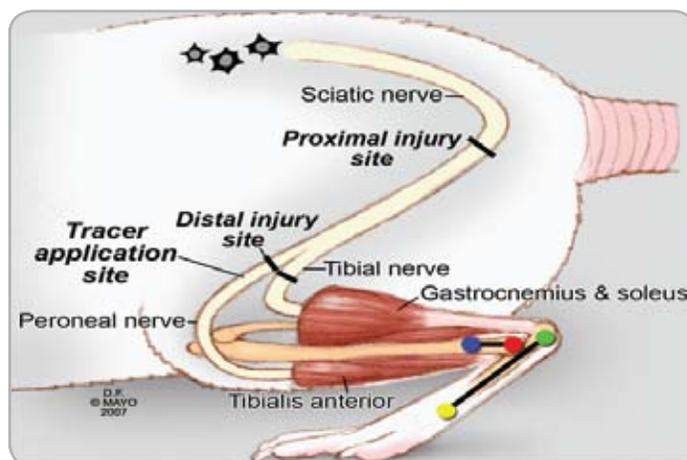


Figure II-23. Nerve injury repair with a biodegradable nerve conduit (shown in blue) implanted on the sciatic nerve in an *in vivo* model.

others showed controlled release for prolonged periods of time. Implantation of electrospun constructs in vivo showed that fibers with encapsulated anti-inflammatory agents had significantly less scar tissue formation around the implant than those without drug.

The Mayo group has completed screening of their candidate polymers by comparing the effectiveness of nerve regeneration after implanting synthetic polymer conduits in a rat 1 cm nerve gap. The polymers being compared are PLGA, PCLF, two types of hydrogel, i.e., positively charged oligomer of poly(ethylene-glycol) fumarate (OPF+) and a noncharged oligomer of poly(ethylene-glycol) fumarate (OPF). Nerve conduction study, isometric force measurement and muscle mass results showed that PCLF performs better than other polymers.

The Mayo group also completed an in vivo study of microsphere delivery of NGF and showed that the PLGA microsphere delivery system does not inhibit or obstruct outgrowing axons when placed in the lumen of the nerve conduit. This is the first requirement for the design and use of a delivery system. These microspheres are easy to fabricate and use, and the release kinetics can be regulated by changing the ratio of lactic to glycolic acid. This makes it possible to tailor the release profiles of various growth factors to suit their biological effects.

The Mayo group has successfully created an ischemic/fibrosis model for nerve injury and repair. The results showed that ligation of vessels and caustic injury to the muscles caused fibrosis and scarring of the nerve bed and hence poor nerve regeneration. Blast injuries occurring in the battle field very rarely cause a simple, clean-cut nerve injury. The surrounding soft tissue is always traumatized, leaving a fibrotic and/or ischemic tissue bed for the surgeon performing nerve reconstruction. This new model mimics real clinical scenarios and will lead to studies of complex nerve injury, and repair and further development of strategies to enhance nerve regeneration in a complex wound often seen in the war injured.

Comparison of scaffolds between groups

Each group has developed synthetic, biodegradable scaffolds from Tyr-PC (Rutgers), PXS – MIT, PCLF –

Mayo, OPF – Mayo with a positively charged or neutral surface. These have been compared to the “gold standard” for repair (autologous graft), the best commercially available collagen scaffold, and against each other in the same animal model. Using standardized comparisons, a best candidate was selected for advancement to a new (human) model system.

Clinical trial proposal

Six centimeters of the sural nerve (behind the ankle) is routinely removed for diagnostic purposes. The resulting nerve defect, which is not routinely repaired, provides an ideal model for testing novel nerve tubes. In the proposed study, a 6 cm gap in the sural nerve will be repaired using a biodegradable conduit.

The clinical trial proposal was approved for funding through the AFIRM clinical trials request for applications. This will support upscaling of manufacture under GMP-compliant conditions and submission for approval to conduct the trial to the FDA and Mayo Institutional Review Board (IRB). Thirty patients will be randomized to repair or no repair and followed for 1 year with clinical and physiological measures of nerve repair.

Key Research Accomplishments

- Determined that Tyr-PC conduits filled with saline significantly enhanced functional recovery over a polyethylene conduit filled with saline.
- Characterized the influence of conduit filler material and incorporated peptide mimics on functional recovery after nerve injury.
- Synthesized a library of novel polyester elastomers to optimize the biocompatibility, mechanical properties, degradation rate, and suturability of nerve conduits.
- Developed a method for incorporating aligned electrospun fibers into the nerve conduit lumen in a uniform manner with no fiber aggregation and for encapsulating anti-inflammatory drugs into electrospun fibers to decrease the immune response to implants.
- Designed microspheres with regulatable release kinetics for various growth factors.



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- Created a rat hindlimb soft tissue defect and fibrosis animal model that constitutes an important advancement in studying nerve injury and repair strategies. This model includes aspects of extensive soft tissue injury that often accompany nerve injuries in war casualties.
- Established a standardized nerve defect and tube implantation and evaluation model. This enables the standardized evaluation of multiple polymer scaffolds that enter the downselect process across the consortia.
- Completed the screening of in-house polymer scaffolds for downselect and decided on PCLF tube as candidate scaffold to move forward to clinical trial.
- Obtained approval to proceed with a clinical trial for “repair of 6 cm peripheral nerve gaps in sural nerve biopsy using PCLF tube.”
- Developed a complex wound model involving a rat hindlimb soft tissue defect and fibrosis.
- Completed in vivo studies of growth-factor-releasing microspheres and adipose-tissue-derived stem cells and their effect on nerve regeneration.

Conclusions

The nerve teams at Cleveland Clinic, Mayo, MIT, and Rutgers have formed a highly successful and integrated approach to bringing forward novel scaffolds for nerve repair. The collaboration involves on-site visits to compare and standardize techniques, monthly teleconferences, and face-to-face meetings at AFIRM’s “All Hands” conference and at one of the partner institutions during the year. As a result of this collaboration, new materials will be brought to clinical trial by the end of the third year of the AFIRM consortium funding. This represents a major step forward in a field that has been making little progress for more than 40 years.

Research Plans for the Next 3 Years

The researchers at Rutgers plan to translate their success with the femoral nerve model by optimizing nerve conduits for the rat sciatic nerve injury model during

Year 3 by using the strategies outlined previously. After completing animal cohorts to evaluate the efficacy of the Tyr-PC in this model, they are planning to conduct studies in the nonhuman primate model as the next step to transitioning into clinical trials. In this large animal model, researchers will be evaluating regeneration of nerve gaps that correspond to 5-20 cm gaps that are encountered in humans after massive trauma, such as those incurred in war. The team is planning to commence nonhuman primate studies in the beginning of Year 4. The best conduit design will be carried forward to clinical studies in Year 5 (TRL 4).

The MIT group will complete evaluation of single channel conduits made of newly screened polyesters in rat sciatic nerve defect model; complete development of aligned electrospun intraluminal matrix fillers; conduct in vivo nerve regeneration testing of conduits with electrospun intraluminal axon guidance scaffolds that promote axon growth and decrease inflammation; conduct safety and toxicity testing; obtain 510(k) FDA approval; and initiate human pilot study by the end of Year 5 (TRL 4).

The Mayo group will work with their industrial partner, Bonwrx, to complete the upscaling for GMP-compliant manufacture of 6 cm PCLF tubes and validation of sterilization procedures to reach TRL 5 at the end of Year 3. They will initiate the clinical trial “Repair of 6 cm Peripheral Nerve Gaps” in sural nerve biopsy patients using PCLF tube in Year 3 (TRL 5). The Mayo group will also carry out studies to further optimize candidate polymer scaffold by designing various delivery systems for vascular endothelial growth factor release to enhance nerve regeneration. They will test optimized/enhanced candidate polymer conduits in a complex injury environment in Year 4 (TRL 3) and continue PCLF clinical trial (TRL 5). In Year 5, 50% of a Phase 1 clinical trial of reconstructing sural nerve biopsy defect with PCLF nerve conduit will be completed (TRL 6).

Planned Clinical Transitions

The Rutgers team is in the early phases of animal studies. The researchers’ goal is to achieve clinical transitions by the end of Year 5. To this end, they will design animal studies that demonstrate the safety and

efficacy of the tyrosine-derived nerve guidance conduit as compared to the FDA-approved and marketed nerve guidance conduits.

The MIT group has significant experience in translating medical technologies from their laboratory into the clinic, with more than 40 products that are approved for human use or currently in clinical trials. Their strategy to translate this technology would focus on (1) the development of the technology and appropriate patent coverage and (2) human proof of utility as soon as possible. To this end, MIT is collaborating with Professors Yaszemski and Windabank at the Mayo Clinic to better understand the clinical utility of these constructs and prepare for human trials. The laboratories of Langer and Anderson are planning to prepare a next-generation conduit to further improve the efficacy of their synthetic scaffold on a level close to autograft. Depending on their performance in vivo, the MIT team anticipates testing these in a physician-sponsored IDE with their clinical partners at the Mayo Clinic. Assuming appropriate in vivo performance, MIT team anticipates connecting with both venture capital companies (e.g., Polaris ventures, Flagship ventures, Atlas, and Healthcare Ventures) and medical device companies with which they have current relationships (e.g., Medtronic and Genzyme).

The Mayo group has proposed a new balanced risk-benefit parallel process in developing and translating into practice novel materials for nerve repair, using a carefully defined clinical model: sural nerve biopsy and reconstruction with 6 cm PCLF tube. This clinical trial was approved (AFIRM clinical trials funding). The Mayo group has already submitted this clinical trial to the IRB web site last September. They will complete the upscaling for GMP manufacture of 6 cm PCLF tubes and validation of sterilization procedures with their industrial partner Bonwrx and file an IDE with FDA for the 6 cm PCLF polymer scaffold. Their industrial partner has a complete business plan to commercialize this polymer nerve tube.

Corrections/Changes Planned for Year 3 and Rationale for Changes

The Rutgers group is going to initiate additional in vitro study to characterize and optimize mechanical properties of Tyr-PC. They will also implant these tubes subcutaneously to investigate in vivo degradation. These studies will be done outside the AFIRM funding. An optimized polycarbonate tube will be tested in animal nerve models when available.



II: Limb and Digit Salvage

Progress Reports: Nerve Repair and Regeneration

Cell and Bioactive Molecule Delivery to Enhance the Repair of Segmental Nerve Defects

Project 4.4.2a, RCCC

Team Leaders: Maria Siemionow, MD, PhD (Cleveland Clinic)

Project Team: Grzegorz Brzezicki, MD, Amanda Mendiola, MD, Maria Madajka, PhD, and Joanna Cwykiel, MSc (Cleveland Clinic)

Therapy: Nerve gap repair

Deliverable: An epineural tube that can be supplemented with cell therapies for large peripheral nerve gaps

TRL Progress: *Start of Year 1, TRL 3; End of Year 1, TRL 4; End of Year 2, TRL 4*

Key Accomplishments: The researchers completed a study on allogenic epineural tube repair of a 2 cm rat sciatic nerve defect. They demonstrated that allogenic epineural tube repair without immunosuppression is a feasible method of peripheral nerve gap repair. The researchers' results

were comparable to autograft repair when an epineural tube was used in conjunction with local BMSC therapy.

Key Words: Nerve repair, natural nerve conduits, epineural tubes, autogenic nerve conduits, allogenic nerve conduits, autogenic mesenchymal stem cells, allogenic mesenchymal stem cells

Introduction

Peripheral nerve injury can result in a significant burden. The resultant morbidities can be both permanent and significant. Effective nerve gap repair remains an elusive enemy to even the most accomplished surgeon. The standard of care for the surgical treatment of peripheral nerve lacerations or segmental defects is primary repair or nerve grafting. Direct coaptation provides the optimum chance for effective nerve regeneration. If direct repair is not possible, then an autologous nerve graft is used but it has limitations due to donor nerve availability and the potential for donor site morbidity.

Alternatives to autografts have been explored including vein segments and nerve sheath segments. Synthetic scaffold conduits have also been investigated in repairing small gaps (2 cm or less) but have shown little to no efficacy for the repair of longer defects. The Siemionow group has previously shown superb nerve regeneration when transected nerves were repaired with the use of epineural sleeve technique work. The sleeve of epineurium over the coaptation site created chamber-like structure providing neuroregenerative environment for axon growth. Therefore, it is hypothesized that the epineural tube may be an optimal tool for bridging nerve defects.

BMSCs have been studied in recent years for tissue regeneration. They comprise a multipotential heterogeneous population of cells that contribute to the regeneration of multiple body tissues. In the epineural tube, the multipotency of the cells is augmented by the presence of neurotrophic factors that the tubes naturally contain. The Siemionow group proposes to introduce a novel method of cellular therapeutics by local administration of BMSCs into transplanted epineural tubes (Figure II-24).

Summary of Research Completed in Year 1

During the first year of the study, the researchers successfully performed the transplantation of the isogenic epineural tube and bone marrow-derived MSC delivery into transplanted tube. They began initial harvesting of transplanted tubes with positive measures of functional outcomes. Preliminary electrophysiological measurements using somatosensory-evoked potential technology showed successful regeneration over the gap; however, no significant differences between groups were noted. Preliminary immunohistochemical data supported nerve regeneration in the presence of transplanted isogenic bone marrow-derived MSC therapy. Histo-morphometric analyses indicated a positive effect of isogenic bone transplantation on nerve regeneration.

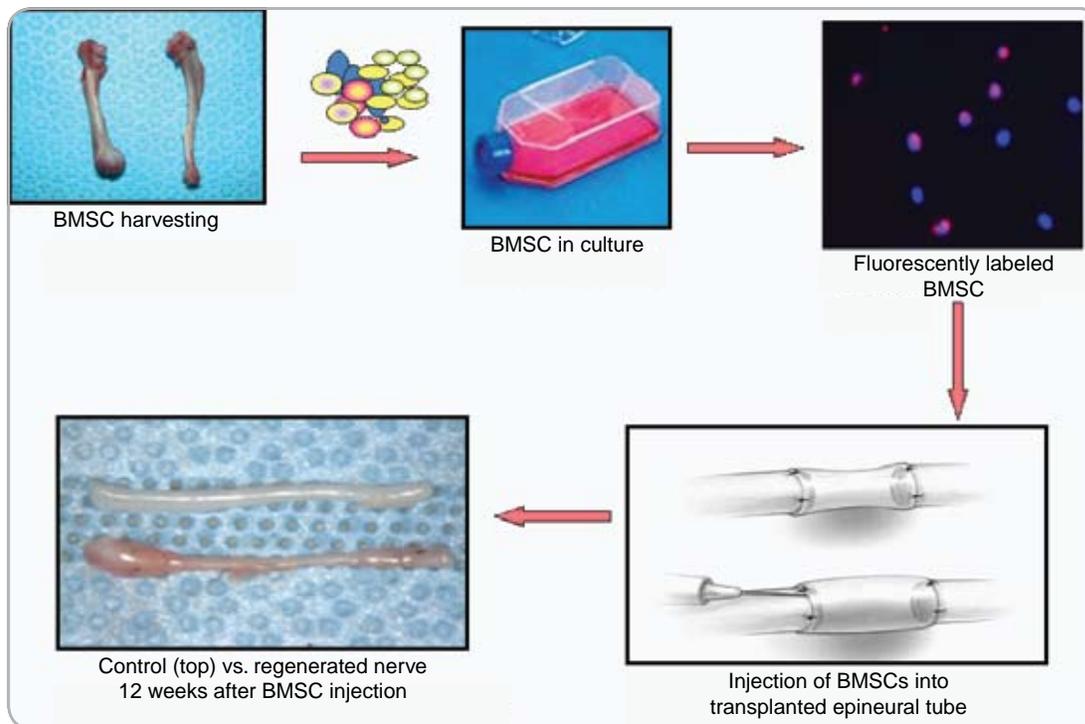


Figure II-24. Procedure for BMSC therapy via transplanted epineural tubes. Isogenic and allogenic bone marrow stromal cells (BMSCs) were harvested to support peripheral nerve regeneration. The bone marrow was placed in culture for 10-14 days to isolate the stromal cells, which were then stained with PKH. Next, a 2 cm segment of an allogenic rat sciatic nerve was harvested, fascicles were removed and the empty epineural sheath was used to bridge a 2 cm gap in a Lewis rat. Approximately 3×10^6 BMSCs were then injected into the empty epineural sheath.

Research Progress – Year 2

Macroscopic observation

In all animals, the transplanted tube appeared to be intact and bridged the nerve gap. Minimal amounts of fibrous tissue surrounding allogenic conduits were observed while neuroma formation or axon growth outside the tube was not observed.

Pinprick Test

Experimental groups had comparable sensory recovery as assessed by pinprick (response to painful stimuli graded 0–no response, 1–response at ankle level, 2–response at metatarsal level, 3–response at toe level) at all time points. All animals from each group received the maximal score of 3 at 24 weeks post nerve repair, indicating full return of sensation.

Toe-Spread Test

The toe-spread test was used for evaluation of motor recovery—reinnervation of hindlimb musculature. In the uninjured hind limb, the rat extends and abducts the toes when suspended by the tail. The toe spread was graded from 0 to 3 in the following manner: no toe movement = grade 0, any sign of toe movement = grade 1, toe abduction = grade 2, and toe abduction with extension = grade 3.

After 6 weeks of recovery, epineural conduits filled with saline (the saline group) had significantly lower scores when compared to other groups ($p = 0.0149$). At 12 and 24 weeks, no differences were noted; however, the saline group tended to show the poorest motor recovery while epineural conduits supported with BMSCs improved motor recovery to a level equivalent to that achieved by an autograft.



II: Limb and Digit Salvage

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Electrophysiology (Somatosensory-Evoked Potentials)

Somatosensory-evoked potentials (SSEP) were recorded by two intracranial electrodes placed over the somatosensory cortex. Achilles tendon and dorsum of the foot were stimulated bilaterally to assess sensory nerve regeneration. In these SSEP measurements, the waveform morphology consisted of a series of negative and positive potentials, with P1 and N2 being most robust and consistent. The latencies at this point were used to compare sensory recovery between different treatment groups. Wave amplitudes were also measured as a means of assessing the degree of axonal regeneration—number of axons available for electrical current conduction.

With regard to comparisons between P1%, N2%, and Amp% values, no significant differences were observed at the 6-week time point although the ACI BMSC group trended toward the most elongated P1 and N2 latencies. After 12 weeks of recovery, the latencies and amplitudes were comparable among all groups. The ACI BMSC group had a tendency toward lower amplitudes compared to other epineural conduits and the autograft. There were no significant differences in all measured parameters between different epineural constructs and the autograft positive control at 24 weeks.

Histomorphometric Analysis

The distal epineural tube transversal sections stained with toluidine blue, just proximal to the distal coaptation site was used for analysis. Six representative fields from each section were chosen by an investigator blinded to the treatment group. Each image was captured via a computer and evaluated using Image Pro Plus (Media Cybernetics, Silver Spring, MD) software for the following parameters: myelin thickness, axonal density, fiber diameter, axon diameter, and percentage of myelinated nerve fibers. These parameters allow for an assessment of the quantity (axonal density) and quality (level of myelination) of nerve regeneration through the epineural tubes and enables a comparison with the autograft repair.

The autograft group and the Lewis BMSC group had significantly thicker myelin compared to the ACI BMSC

group at 12 weeks. At 24 weeks, the autograft group had thicker myelin than the saline group while no differences were observed when compared to BMSC-supported constructs. The saline group at 12 weeks presented with thicker axons than the ACI BMSC group. The Lewis BMSC group had thicker nerve fibers as compared to the ACI BMSC group at 12 weeks. At 24 weeks, the autograft group had larger diameter nerve fibers than saline and Lewis BMSC constructs. Autograft and BMSC-supported conduits had equal percentage of myelinated nerve fibers at 6 weeks. At the same time, the saline group had significantly less myelinated nerve fibers compared to all other groups. Similar findings were observed at 12 weeks; however, the ACI BMSC group was not significantly better than the saline group. No differences were observed in axonal density up to 24 weeks; however, the saline group trended toward the highest densities at 24 weeks.

Gastrocnemius Muscle Index (GMI)

The grade of gastrocnemius muscle reinnervation by motor component of the sciatic nerve was assessed by GMI. Wet weight of the ipsilateral muscle was compared to the contralateral muscle weight and the muscle weight index was calculated. The index percentage produced represented the recovery in denervation atrophy of the gastrocnemius muscle on the operated side, with a 100% GMI indicating full recovery of the operated side.

BMSC-supported constructs had significantly better muscle reinnervation compared to autograft and saline group at 12 weeks. At 24 weeks, the saline group had significantly lower levels of muscle reinnervation when compared to the autograft and Lewis BMSC groups. The ACI BMSC group failed to show muscle weight recovery from 12 to 24 weeks as observed in other experimental groups. Lewis BMSC-supported constructs showed equal muscle reinnervation as autograft at 24 weeks.

Immunostaining

Laminin B2 is constitutively expressed in neuronal tissue and constitutes a major component of the Schwann cell basal lamina, and its expression is upregulated in peripheral nerve regeneration. Masaki et al. reported that laminin B2 in the presence of dystroglycan progressively

increased in remyelinating Schwann cells during axonal regeneration. Artificial conduits, which lack laminin B2 expression, perform significantly worse when compared to decellularized allografts or autografts, which are rich in laminin B2. Laminin B2 expression was observed throughout the transplanted epineural constructs up to 24 weeks post-nerve repair indicating the presence of scaffolding required for nerve regeneration and proper environment for nerve function.

BMSCs were prelabeled with red membrane dye PKH-26 (Sigma-Aldrich, UK) to assess their survival, migration, and transdifferentiation after transplantation into the epineural tubes.

PKH-26 stained, transplanted BMSCs were observed inside the tube up to 24 weeks after nerve repair. Additionally, BMSCs expressed NGF as demonstrated by double-positive staining for PKH and NGF.

Presented results are superior to outcomes presented by Whitlock et al. who studied collagen conduits and decellularized allografts in a 14 mm sciatic nerve gap model. Processed allografts, technology directly competing with epineural tubes, are under investigation as an alternative for allografts as no immunosuppression is required in the recipient. Decellularized allografts also tend to provide significant improvement over currently marketed artificial conduits. At 12 weeks post repair the Whitlock group reports a GMI of 0.34 for the processed allograft while Siemionow's group reports a GMI of -0.57 for a defect longer by 6 mm. Similarly, at 6 weeks post implantation the nerves reconstructed with a 2 cm epineural tube/BMSC construct had thicker nerve fibers compared to nerves reconstructed with 1.4 cm acellular allogenic nerve grafts.

Key Research Accomplishments

- Completed a study on allogenic epineural tube repair of 2 cm rat sciatic nerve defect.
- Demonstrated that allogenic epineural tube repair without immunosuppression is a feasible method of peripheral nerve gap repair.
- Determined that the results obtained were comparable to autograft repair when an epineural tube was used in conjunction with local BMSC therapy.

Conclusions

The researchers have proven that an allogenic epineural conduit without immunosuppression is a feasible method of peripheral nerve gap repair. The addition of BMSCs resulted in better functional outcomes than saline-filled conduits and also increased myelination of the regenerated nerve fibers when compared to saline-filled conduit. In summary, allogenic epineural conduits combined with BMSC local therapy in 2 cm rat sciatic nerve defects provide recovery comparable to that achieved by an autograft. However, further studies are warranted to prove this efficacy in longer gaps and in large animal models before introduction of this technique into clinical trials.

Research Plans for the Next 3 Years

Year 3 Q1-2:

Continuation of conduit development in rat model.

- 2 cm sciatic nerve defect repair (6- and 12-week time points, same evaluation methods as in Year 1 and Year 2 experiments) with "enhanced" constructs:
 - Epineural tube supported with BMSCs suspended in fibrin matrix filler
 - Ex vivo precultured epineural tube/BMSC constructs
- 4 cm sciatic nerve defect repair (6-, 12-, and 24-week time points, same evaluation methods as in Year 1 and Year 2 experiments).
 - Epineural tube supported with BMSCs
 - Best performing "enhanced" conduits—either fibrin matrix constructs or precultured constructs

Year 3 Q3-4:

Large animal study: sheep 6-8 cm median nerve defect repair with:

- "Standard" epineural tube repair supported with BMSCs derived from Year 1 and Year 2 (autograft as control)
- Best performing "enhanced" constructs: Either fibrin matrix constructs or precultured constructs



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Year 5:

Clinical trial of best performing construct derived from sheep study (TRL 5).

Planned Clinical Transitions

The researchers plan to initiate a clinical trial in Year 5 and anticipate submitting an IRB application during the second quarter of Year 4—upon availability of first data from the sheep study. Preliminary discussions have been held with the MTF for collaboration on research involving human cadaveric nerve for use in allograft transplantation.

Corrections/Changes Planned for Year 3

Based on favorable findings from Year 2 experiments, the researchers' main goal for Year 3 is to advance allogenic epineural constructs supported with BMSC local therapy into a large animal trial. They will use an established model of 6-8 cm median nerve defect in sheep.

Simultaneously, the Microsurgery Laboratory plans to perform additional studies in the rat sciatic nerve model concentrating on further enhancement of the construct's neuroregenerative potential. The preliminary in vitro study showed increased secretion of NGF from BMSCs when an empty epineural tube was added to the culture. **To further evaluate this interesting finding, the research team plans to pre-culture in vitro epineural/BMSC constructs before in vivo implantation into rat sciatic nerve defect.** The results will be compared to "standard" epineural/BMSC conduits and autografts. Additionally, **the researchers will test constructs with fibrin matrix filler,** which should act to keep the lumen of the conduit open and support the survival of the transplanted BMSCs—an important consideration for repair of longer defects. The best performing construct from Year 2, as well as any promising construct newly developed in Year 3, will be also tested in a previously tested rat model of long (4 cm) sciatic nerve defect, as an intermediate step toward a large animal model.

Engineered Delivery of Spatial and Temporal Cues for Composite Tissue Injury Repair

Project 4.4.3, WFPC

Team Leader(s): Robert Guldberg, PhD (Georgia Institute of Technology) and Barbara Boyan, PhD (Georgia Institute of Technology)

Project Team Members: Ravi Bellamkonda, PhD (Georgia Institute of Technology); Robert Taylor, MD, PhD (Emory University); Yash Kolambkar, PhD (Georgia Institute of Technology); Natalia Landazura, PhD (Emory University); Nick Willett, PhD, Brent Uhrig, Isaac Clements, and Angela Lin (Georgia Institute of Technology)

Collaborator(s): Dietmar Hutmacher (Queensland University of Technology); Andres Garcia (Georgia Institute of Technology); David Kaplan (Tufts University); Benjamin Harrison (Wake Forest Institute for Regenerative Medicine); Shawn Gilbert (University of Alabama-Birmingham); Thomas

Clemens (Johns Hopkins University); George Muschler (Cleveland Clinic); and Josh Wenke (USAISR)

Therapy: Functional limb regeneration following severe combined bone, nerve, and vascular injuries

Deliverable(s): Develop composite injury animal models that simulate complex military wounds. Establish and test spatiotemporal delivery strategies for regeneration of bone, nerve, and vascularity.

TRL Progress: *Start of Program*, TRL 1; *End Year 1*, TRL 2; *End Year 2*, TRL 3

Key Accomplishments: The researchers have established composite injury models in the rat that simulate bone/nerve and bone/vascular injuries. Results thus far have demonstrated that functional recovery from composite

injuries is significantly more challenging than recovery from injuries to a single tissue. Using quantitative outcome measures, the researchers have also shown promising results for highly translatable nanofiber biomaterials delivery systems that provide spatial and temporal cues to guide improved bone and nerve regeneration. They have demonstrated that oriented nanofiber meshes can guide functional axon growth across critically sized peripheral nerve defects and that a novel nanofiber mesh/hydrogel protein delivery system fully restores the mechanical function of massive long bone injuries at lower doses than the current clinical standard.

Keywords: Bone, nerve, vascularization, composite injury, animal model

Introduction

Traumatic injury to the extremities in combat is a significant problem for reconstruction and restoration of function. Complicated fractures and fragmented bone can cause loss of limb function even if the limb is restored esthetically. One reason for this is traumatic injury to the nerve, with resulting loss of the musculature or bone tissue. Another reason is the lack of adequate vasculature needed to supply nutrients and connective tissue progenitor cells. There is a clear need for regenerative technologies that enable the restoration of limb function following composite tissue trauma. However, current preclinical testing models generally involve injury to only a single tissue type.

To address this limitation, a goal of this project is to establish animal models of composite tissue trauma that combine a massive segmental bone defect in the rat with peripheral nerve resection and/or femoral artery ligation.

The models are being used to quantitatively evaluate spatial and temporal delivery of biological cues that direct nerve, vascular, and bone growth in a synchronized manner. The rationale for this approach is based on recent observations that vascular and neural development occur in tandem and perhaps synergistically during fetal bone formation and growth.

The aims of this project are to:

- Develop composite bone/nerve and bone/vascular injury rodent models
- Quantitatively evaluate strategies for delivering spatial and temporal information to direct segmental bone regeneration, peripheral nerve repair, and vascular regrowth

Dr. Guldberg has provided the design and protocol for his rat segmental bone defect model to investigators at Wake Forest University (Drs. Mark Van Dyke and Tom



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Smith). The model has been successfully implemented at Wake Forest and used to test their bone regenerative technologies.

An exciting collaboration has been initiated with Dr. David Kaplan at Tufts using their silk hydrogel technology to deliver both BMP and a small molecule (DFO) that activates the hypoxia inducible factor (HIF) pathway. The silk hydrogel can be integrated directly into the nanofiber mesh/hydrogel system developed in Dr. Goldberg's laboratory. It provides a longer period of release than the current hydrogel being used (RGD modified alginate) and is more degradable long term. In vitro studies have been completed to determine release kinetics from the silk hydrogel and verify that the released DFO is functional, as assessed by increased VEGF expression. The HIF pathway has been shown to link angiogenesis and osteogenesis and therefore activators of this pathway may be a potent therapeutic for treating composite tissue injuries.

Summary of Research Completed in Year 1

During the first year of the project, the researchers established a novel spatial (nanofiber mesh) and temporal (alginate hydrogel) delivery strategy of a clinically approved osteoinductive factor (BMP-2) that can fully restore function to massive (8 mm) rat bone defects (Figure II-25). After just 12 weeks, the fixation plates could be removed and the animals could ambulate normally on their regenerated limbs. It is important to note that the 8 mm femoral defect is 60% larger than the standard critical size (5 mm) for rat long bone defects and thus represents a highly challenging model. It was shown that a perforated nanofiber mesh design accelerates early bone repair at 4 weeks by enhancing the ingrowth of vascularity and/or osteoprogenitors from the surrounding soft tissues (data not shown). This observation supports the hypothesis that composite tissue injuries will require a spatially and temporally coordinated treatment approach.

Research Progress – Year 2

In the past year, the researchers extended their first year work by performing a dose-response study indicating that an 8 mm bone defect could be fully bridged using only 1 μ g of BMP. In addition, a direct comparison to collagen sponge delivery of BMP showed that the nanofiber mesh/hydrogel delivery system is superior to the current clinical standard delivery system. A patent was submitted in November 2009 on this technology. The next step is to test the nanofiber mesh/alginate delivery system in a large animal study.

For peripheral nerve repair, the researchers demonstrated that nanofiber-based guidance channels promote robust levels of axonal regeneration, even across critical-length nerve gaps. Significantly, this regeneration can occur in the absence of any exogenous ECM or trophic factors. The aligned topography of the interposed nanofiber thin films stimulates endogenous repair processes, promoting a sequence of regenerative events that normally fail to occur over critical-length nerve gaps. The most recent studies indicate that a single nanofiber membrane produces superior axonal growth and organization compared to multiple membranes within guidance channels.

Over the past year, the researchers have established two composite injury models that simulate complex military injuries. In the first, a bone/vascular composite injury model was created by combining the 8 mm segmental bone defect model with a femoral artery ligation model. The animals tolerate the procedure well. Vascular mCT analysis was used to evaluate the vascular growth response in the limb following ischemic injury. The next step in Year 3 will be to quantify the effects of the ischemic injury on segmental bone repair.

Finally, the research team has established a bone/nerve composite injury model by combining the 8 mm segmental bone defect model with a peripheral nerve injury model. A full study is ongoing to evaluate the effects of dual injury to bone or nerve single injuries treated using the delivery systems described previously. Longitudinal

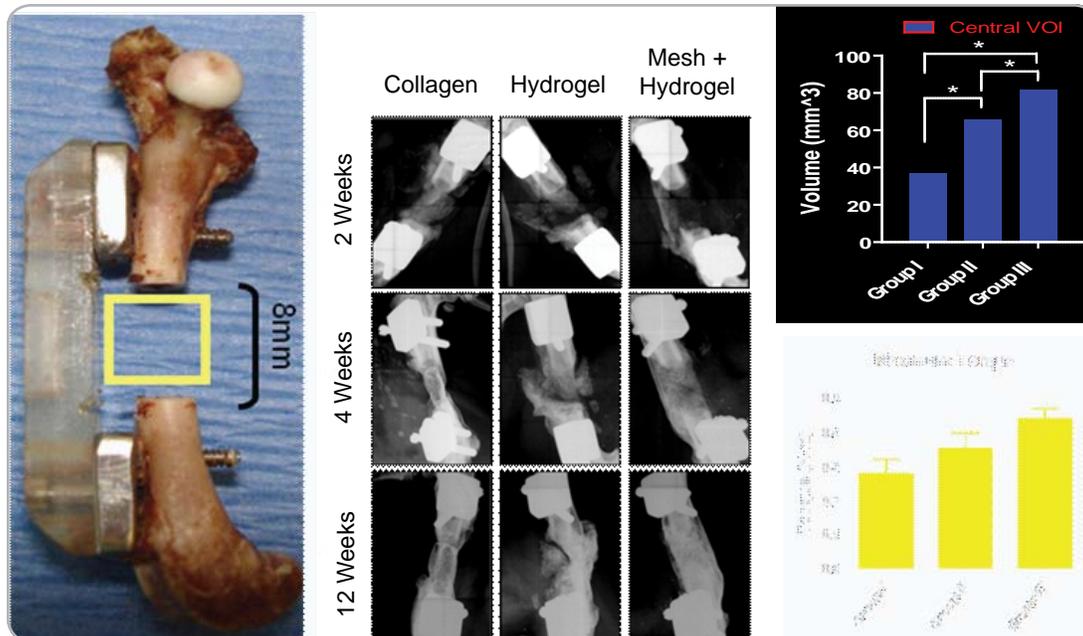


Figure II-25. Segmental bone defect model developed at Georgia Tech for evaluation of bone regenerative technologies (left). The model allows in vivo quantification of bone ingrowth volume via mCT imaging and biomechanical testing of functional restoration. A study comparing delivery of 5 μ g BMP-2 within collagen sponge (Group I), hydrogel (Group II), and nanofiber mesh + hydrogel (Group III) showed superiority of the new spatiotemporal delivery strategy in terms of both bone ingrowth volume and biomechanical strength (middle and right).

gait analysis has demonstrated that composite bone and nerve injury has a greater than additive effect on functional deficit compared to the effects of bone and nerve injury alone.

Key Research Accomplishments

- Established a composite bone/nerve injury model, including quantitative analysis of effects of injury severity on limb function.
- Established a composite bone/vascular injury model.
- Developed a contact nerve guidance scaffold and filed a patent for it.
- Developed a nanofiber mesh/hydrogel spatiotemporal growth factor delivery system and filed a patent for it.
- Identified a partner and leveraged funds for a large animal study.
- Initiated a composite bone/muscle study with leveraged funds.

Conclusions

The researchers have established promising regenerative strategies for bone and nerve using nanofiber mesh spatial guidance and sustained delivery of a clinically approved inductive protein (BMP-2). They have developed composite multi-tissue injury models to simulate complex combat injuries and test spatial and temporal guidance strategies that take advantage of synergistic interactions among the tissues observed during development and repair. They chose the rat model since it provides the opportunity for larger in vivo studies and is amenable to highly quantitative assessment methods (e.g., mCT assessment of vascularization and bone formation). Variations of the composite injury model include bone/nerve injuries and bone/vascular injuries. The researchers are making their models available for testing regenerative strategies developed by other AFIRM investigators.



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Research Plans for the Next 3 Years/Planned Clinical Transitions

As results from the Year 3 rat studies are completed, the group will begin planning large animal studies of the most promising technologies for Year 4. Discussions have been initiated and additional funds identified for sheep defect studies. Dr. Guldberg will also coordinate with Dr. Muschler on the development of a standardized goat bone defect model within AFIRM. Intellectual property will be marketed in Year 3 to members of the Georgia Tech industry partners program. The group

previously had success licensing patent rights to industry partners following successful large animal studies. Once proof of concept has been demonstrated in the large animal model, the goal is to initiate a human clinical trial pilot study in Year 5.

Corrections/Changes Planned for Year 3

Preclinical studies in a more clinically relevant large animal model have been added to the project plan to accelerate progression toward clinical translation of the spatiotemporal delivery systems.

Hand Transplantation for Reconstruction of Disabling Upper Limb Battlefield Trauma – Translational and Clinical Trials

Project 4.4.2, WFPC

Team Leader(s): W.P. Andrew Lee, MD (University of Pittsburgh)

Project Team Members: Gerald Brandacher, MD, Vijay S. Gorantla, MD, PhD, Stefan Schneeberger, MD, Xin Xiao Zheng, MD, and Galen S. Wachtman, MD (University of Pittsburgh)

Collaborator(s): None

Therapy: Reconstructive transplantation of upper extremity under a novel bone marrow/stem cell-based immunomodulatory protocol

Deliverable(s): Phase 1 – Translational/Preclinical Trials: Novel

immunosuppressive protocol that combines systemic stem cell-based therapy with local immunomodulation in a swine heterotopic hindlimb model of composite tissue allotransplantation

Phase 2 – Clinical Trial: Reconstructive transplantation as treatment for hand or forearm loss under a novel cell-based immunomodulatory protocol

TRL Progress: *Start of Program*, TRL 4; *End Year 1*, TRL 5; *End Year 2*, TRL 5

Key Accomplishments: In Phase 1, the researchers employed a swine model to determine that a bone marrow cell infusion of 60 million

cells/kg achieves greater microchimerism at early time points after transfusion. They have also shown prolongation of allograft survival using CTLA4/Ig fusion protein, though experiments have not reached their designated end points. In Phase 2, a unilateral hand transplant recipient has demonstrated highly encouraging functional, immunological, and graft survival outcomes on a single immunosuppressive medication (FK506).

Keywords: Hand transplantation, immunosuppression, immunomodulation, swine

Introduction

Composite tissue allografts (e.g., hand transplants) are now a clinical reality and have been performed in multiple centers worldwide. To date, approximately 57 hands have been transplanted globally. Apart from excellent and highly encouraging functional results, composite tissue allotransplantation has not reached widespread clinical use because recipients require lifelong high-dose multidrug immunosuppression to prevent graft rejection. These regimens carry a high risk for serious side effects. In light of these challenges, a protocol for solid organ transplantation at the University of Pittsburgh has utilized a minimization strategy consisting of recipient conditioning (induction therapy), donor bone marrow infusion, and monotherapy maintenance immunosuppression.

This project has two arms: (1) a preclinical model of heterotopic hindlimb transplantation in Yucatan miniature swine and (2) clinical trials of human hand transplantation. Research trials are parallel and complementary, and work in each arm is being reported separately.

Summary of Research Completed in Year 1

In Phase 1 Year 1 in a preclinical swine model, the researchers found that a bone marrow cell infusion of 60 million cells/kg provided a significantly higher amount of microchimerism at time points early after infusion. Building on this information, the goal for Phase 1 Year 2 is to use that optimal bone marrow infusion dose and add custom CTLA4/Ig fusion protein to augment induction and possibly reduce the amount of chronic immunosuppression necessary to promote long-term allograft survival.

In the human clinical trial (Phase 2), the overall goal is to establish hand transplantation as a treatment strategy for reconstruction of disabling combat injuries involving hand or forearm loss using a novel bone marrow/stem-cell based protocol (Pittsburgh Protocol). One patient received a unilateral hand transplant in March 2009 and has since been maintained on one immunosuppressive medication.



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Research Progress – Year 2

As described earlier, Phase 1 Year 2 contains large animal experiments designed to test the efficacy of the CTLA4/Ig fusion protein.

Phase 1: Establish a protocol that combines systemic stem cell-based therapy with local immunomodulation enabling graft survival minimizing systemic immunosuppressive treatment in a pre-clinical swine model for composite tissue allotransplantation.

Research progress was delayed over 6 months in this set of experiments due to the lack of availability of appropriate research animals. However, after successfully learning and performing the SLA typing assay, SLA-mismatched swine suitable for use in transplantation studies could be obtained. Preliminary results show that the control group, which received a donor bone marrow infusion, rejected the skin portion of the allograft at approximately 50 days post transplantation. Remaining portions of the allograft have survived indefinitely. This result is consistent with our earlier preliminary data. In the experimental group, which included treatment for 30 days of FK506 and 4 doses of CTLA4/Ig fusion protein, prolonged survival of the entire allografts (skin and all underlying tissues) has been observed. Even after 70 days post transplantation, no visible signs of acute rejection were observed.

Phase 2: Establish hand transplantation as a treatment strategy for reconstruction of disabling combat injuries involving hand or forearm loss using a novel bone marrow/stem-cell based protocol (Pittsburgh Protocol) to minimize immunosuppressive therapy.

The group continued to screen and accrue candidates for hand transplantation via an approved IRB protocol. Three patients have now been treated. In March 2009, the group successfully performed a hand transplantation on a former Marine who lost his hand in a training accident while on active duty. The patient experienced one very minor episode of rejection at the 6-month time point, which was successfully treated. He is now more than 14 months post transplant, remains on minimal

immunosuppressant therapy, and continues to regain motor and sensory function of his transplanted hand. He undergoes occupational hand therapy two times per week and has returned to work as an electrician's apprentice. The second patient was a bilateral transplant and the third patient was unilateral. These patients are 6 and 3 months post transplantation, respectively, and their progress is excellent.

Key Research Accomplishments

- Demonstrated a statistically significant increase in microchimerism for bone marrow cell infusion of 60 million cells/kg versus 15 and 30 million cells/kg groups.
 - Bone marrow cell infusion is a critical component of the Pittsburgh Protocol for immune modulation, and an optimal dose of bone marrow infusion has never before been documented in composite tissue allotransplantation.
- Produced stable long-term microchimerism (1×10^6 %) at all doses.
- Successfully performed swine SLA typing; one of only five laboratories worldwide able to perform this assay.
- Initiated heterotopic hindlimb transplantation utilizing a custom chimeric CTLA4/Ig fusion protein with the intent to reduce the need for chronic calcineurin inhibitor-dependent immunosuppression.
 - Demonstrated increased allograft survival in animals transplanted using CTLA4/Ig fusion protein; animals have not rejected the allograft and are awaiting experimental end points.
- Performed a unilateral hand transplant on a retired Marine in March 2009, who continues to regain motor and sensory function in his transplanted hand and has had no adverse effects related to the transplantation.
- Performed a unilateral hand transplant on one patient and a bilateral hand transplant on another patient.
 - The progress of both patients is excellent to date.

Conclusions

This group has developed a preclinical heterotopic hindlimb transplant model for composite tissue allotrans-

plantation using a novel immunomodulatory protocol. It has been determined that the optimal dose of bone marrow cell infusion in this model is 60 million cells/kg, and the group has adopted this optimized dose in current animal studies. The group has also achieved stable levels of microchimerism in swine in all groups after bone marrow cell infusion. The use of chimeric CTLA4/Ig fusion protein in the hindlimb transplant model to reduce the amount of chronic immunosuppression and augment induction is currently under way, showing promising early results, and will be finished within the next 4 months.

The group has applied these results from Phase 1 studies to human hand transplantation using the Pittsburgh Protocol, a novel immunomodulatory strategy that aims to reduce maintenance immunosuppression necessary for successful composite tissue allotransplantation. One patient has received a unilateral hand transplant and is over 14 months post operation. He has been maintained on a single immunosuppressive drug at low levels and continues to have increased motor and sensory function of his transplanted hand. It is hoped that success of this experimental protocol will allow for greater clinical application of hand transplantation for the reconstruction of upper extremity amputations.

Research Plans for the Following Years

Based on the data obtained in Year 1, in Year 2 the group will supplement recipient conditioning with total

body irradiation using fusion protein combinations active against CTLA4Ig. In Year 3, the group aims to prolong limb allograft survival by using targeted skin immunotherapy with leukocyte migration inhibitors in combination with optimal protocols from Year 2. Such targeted immunomodulatory protocols together with cell-based strategies could establish tolerance and ultimately eliminate the need for prolonged immunosuppression to maintain graft survival. Subsequently in Year 4, spaced dosing of tacrolimus monotherapy followed by weaning will be attempted under the cover of local immunotherapy.

Planned Clinical Transitions

In Phase 2 of this project, the group has successfully treated one patient using the Pittsburgh Protocol. The goal is to promote long-term hand transplant acceptance while minimizing the need for immunosuppressive drug therapy. By Year 5, the group plans to translate findings from Phase 1 to Phase 2. In addition, the group plans to implement an optimized strategy combining targeted immunomodulation, bone marrow stem cell/fusion protein induction and topical migratory inhibitors to further reduce maintenance immunosuppression and allow weaning of systemic drug therapy. This regimen should reduce side effects related to high-dose immunosuppressions and will hopefully enable widespread clinical application of hand transplantation for the reconstruction of upper extremity amputations.



II: Limb and Digit Salvage

Progress Reports: Epimorphic Regeneration (and associated methods)

Blastemal Approach to Digit Reconstruction

Project 4.4.1, WFPC

Team Leader(s): Stephen F. Badylak, DVM, PhD, MD (University of Pittsburgh)

Project Team Members: Vineet Agrawal, Scott Johnson, Neill Turner, Alex Huber, Li Zhang, Janet Reing, and Stephen Totley (University of Pittsburgh)

Collaborator(s): Ron Stewart, James Thomson (University of Wisconsin); Susan Braunhut (University of Massachusetts, Lowell); David Kaplan (Tufts University); Eileen Moss, Muthu Wijesundara (The University of Texas at Arlington)

Therapy: Treat digit loss with epimorphic regeneration strategies

Deliverable(s): A biologic scaffold-based strategy for inducing epimorphic regeneration in limb and digit soft tissues. A biomaterial that can facilitate epimorphic regeneration in soft tissues (multiple forms, solid sheet, gel, powder, etc.).

TRL Progress: *Start of Program*, TRL 3; *End Year 1*, TRL 4; *End Year 2*, TRL 5

Key Accomplishments: This group has used a mouse model of mid second phalanx digit amputation to show that treatment with bioactive molecules derived from ECM can recruit endogenous multipotential stem cells

to the site of injury. This “endogenous stem cell therapy” has used the concept of bioactive signals from the ECM as a “homing signal.” These multipotential cells have the ability to differentiate into tissues from all three germ layers (i.e., ectoderm, mesoderm, and endoderm) and cause a significant shift in the wound-healing potential of mammals.

Keywords: Limb regeneration, extracellular matrix (ECM), epimorphosis, multipotential cell cluster (MCC)

Introduction

The present work investigates mechanisms for stimulating non-blastemal epimorphic regeneration in tissues beyond the very few that presently exist in adult mammals. The liver, skin, bone marrow, and intestinal lining of epithelial cells are examples of tissues that exhibit non-blastemal epimorphic regeneration in adult mammals. However, virtually every other tissue does not have this capacity as a component of the default mechanism for wound healing. A large part of the present work is based upon resurrecting this non-blastemal regenerative capacity in alternative tissues. The signals to facilitate this resurrection of non-blastemal regeneration reside within the ECM. Developing therapeutic strategies that can take advantage of this matrix-based approach is the fundamental objective of the present work.

Summary of Research Completed in Year 1

Using the C57Bl/6 mouse digit amputation model, the researchers established a method during the first year of the project for recruiting multipotential stem cells to the site of amputation by regional injection. They identified a partial genetic signature of the MCC in vivo. They also

isolated a more potent fraction of ECM degradation that shows in vitro chemoattractant results for perivascular stem cells.

Research Progress – Year 2

In a mouse model of digit amputation where the second joint of a digit is amputated, the researchers have shown that treatment with peptides derived from ECM leads to the recruitment of a population of cells that express markers that are universally recognized as markers of primitive stem cells (Sox2, Rex1, Sca1). This is a very important finding because the essential first step to promoting epimorphic regeneration is the endogenous recruitment of a population of stem cells that are capable of forming all of the tissues in the missing limb or digit. Since digit tissue consists of nerve, vessels, bone, cartilage, skin, fat, and connective tissue, primitive stem cells are the ideal source of “endogenous stem cells” for regeneration because they have the ability to differentiate in all the aforementioned types of tissue. Research has shown that the primitive stem cells are indeed capable of forming nerve, bone (Figure II-26), cartilage, fat, and even other cell types such as islet cells. Future work will focus on further refining strategies to increase

the number of primitive stem cells we can recruit to a site of amputation, as well as developing a device that will allow one to control the stem cells.

The researchers used a biologic scaffold composed of porcine-derived ECM to treat a soldier with massive loss of quadriceps muscle. Results showed de novo restitution of between 10% and 15% of the missing muscle. This clinical application is remarkable for the potential to provide a viable option for patients suffering from traumatic muscle tissue loss. With the exception of tissue transposition procedures that require harvesting tissue from another part of the body of the same patient, there is no treatment for these patients. A study for 15 additional patients with this regenerative medicine approach is currently planned and awaiting IRB approval at Brooke Army Medical Center in San Antonio, Texas.

Key Research Accomplishments

- Established a consistent model of digit amputation in an adult mouse.
- Established a method for recruiting multipotential stem cells to the site of amputation by regional injection.

- Isolated peptides from ECM that are capable of recruiting primitive stem cells in the laboratory and in animals.
- Used a biological scaffold composed of porcine-derived ECM to treat a soldier with massive loss of quadriceps muscle.
 - Results showed de novo restitution of 10%-15% of the missing muscle.

Conclusions

The research group has demonstrated the ability to recruit endogenous multipotential cells to the site of injury in a nonregenerating mammalian system (i.e., a step toward non-blastemal epimorphic regeneration). Work continues to further define and ultimately refine the proteins and peptides of the ECM that are involved in the recruitment of the MCC to the site of injury. The group is continuing to further define the population of cells involved in the formation of the MCC and to examine the ability of those cells to differentiate into different functional tissues. The ability to specifically direct the differentiation of the MCC into functional tissue is one of the next major hurdles. It is believed that control of the

“microenvironmental niche” will be required; and to that end, this group has developed a conceptual prototype for a BIODOME device. The device will eventually be used to control microenvironmental conditions including hydration state, pH, oxygen tension, electrical potential, and other factors known to affect stem cell fate. Development and testing of the BIODOME device are ongoing through small amounts of leveraged funding.

Research Plans for the Next 3 Years

The researchers will continue in the upcoming years to fully characterize the composition of the MCC through co-localization of various stem cell and differentiated cell markers. They

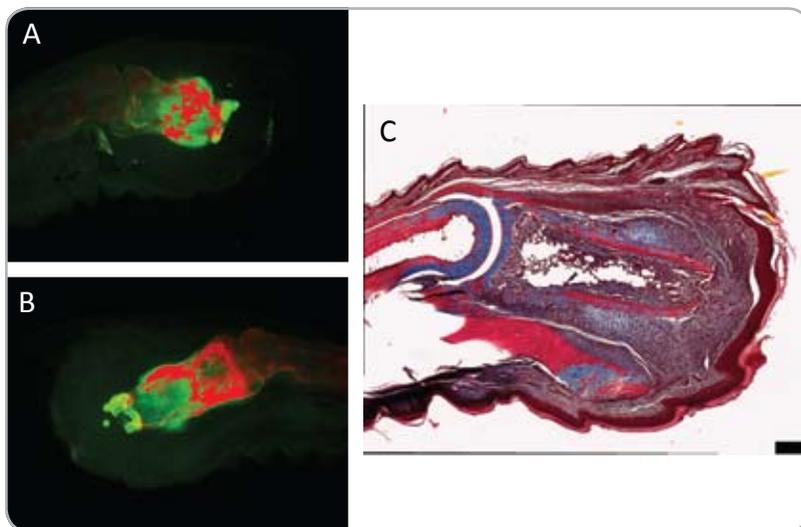


Figure II-26. A: An isolated matricryptic peptide promotes localized bone formation (red) following amputation of a digit in a mouse model. B: Matricryptic peptides promote localized bone formation (red) following amputation of a digit in a mouse model. C: An isolated matricryptic peptide promotes localized bone formation following amputation of a digit in a mouse model.



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will also continue to fractionate the ECM into constituent peptides that will be screened to test their ability to induce or differentiate the MCC. Within the next 3 years, this research group will seek industry partners for the mass production, clinical translation, and further development of bioactive peptides for treatment of limb/digit loss. Additionally, further development of the concept of the BIODOME as a functional device, with an objective of eventual use in human trials, will continue.

Planned Clinical Transitions

The group is working with Dr. Peter Rubin at the University of Pittsburgh to evaluate the clinical efficacy of treating distal digit amputations in patients with a commercially available, FDA-approved form of powdered ECM. This process will help further understanding of the types of patients who would be candidates for a future study with ECM fractions as outlined in this project. Additionally, IRB approval for a pilot clinical study to investigate the efficacy of ECM scaffolds upon musculotendinous regeneration following traumatic injury and loss is pending. The proposed work involves the treat-

ment of 15 patients, all of which have suffered from loss of large amounts of functional musculotendinous tissue as a result of trauma. Ten of the patients will be at least 6 months post trauma and five of the patients will have sustained injury within the previous 6 months. The injury in each of these patients will be that either heroic tissue transplantation procedures would be required or amputation would be necessary under the current standard of care. These patients will be implanted with a custom-designed biologic scaffold composed of SIS-ECM (i.e., the RESTORE™ device, manufactured by DePuy, Inc.). The use of this device will be consistent with the labeling approved by the FDA. The measured end points will include both structure/morphology (via biopsy) and function (via strength testing and electromyographic measurements) of the remodeled tissue. A minimum of 6 months follow-up will be collected for each of the 15 patients. All patients will be treated and follow-up completed within an 18-month period of time. The surgeries will be conducted by Dr. Steve Wolf at the USAISR at Fort Sam Houston, San Antonio, Texas.

High-Throughput Approaches Applied to Tissue Regeneration

Project 4.4.7, WFPC

Team Leader(s): Ron Stewart, PhD (Morgridge Institute for Research) and James Thomson, VMD, PhD, Diplomate ACVP (Morgridge Institute for Research and University of Wisconsin, Madison)

Project Team Members: Srikumar Sengupta, PhD and Mitch Probasco, BS (Morgridge Institute for Research)

Collaborator(s): Steve Badylak, MD, PhD (University of Pittsburgh) and H. Tom Soh, (University of California, Santa Barbara)

Therapy: The long-term therapy for this work will be improved limb and tissue

regenerative outcomes in mammals (including humans)

Deliverable(s): Whole transcriptome measures on ECM-treated and untreated P2 amputated mouse digits

TRL Progress: *Start of Program*, TRL 2; *End Year 1*, TRL 2; *End Year 2*, TRL 2

Key Accomplishments: The research team successfully produced information on all the genes expressed (the “transcriptome”) on ECM-treated and untreated P2 amputated mouse digits over a 14-day time course. The team exceeded the deliverable in providing

detailed information about the most upregulated and downregulated genes including enriched Gene Ontologies (GO) that identified key groups of genes that are up- and downregulated on treatment. This information is useful in determining gene networks that are activated after ECM treatment and will guide further studies aimed at dissecting out the genes expressed in MCCs at the amputation site.

Keywords: Mouse, transcriptome, regeneration, axolotl

Introduction

The research team has been investigating tissue regeneration using high-throughput technologies (microarrays and next-generation sequencing). In collaboration with Steve Badylak’s group, the researchers have analyzed amputated mouse digit tips both from untreated mouse digits and those treated with ECM factors that are designed to enhance regenerative capabilities. The short-term purpose of this collaboration is to identify genes and gene networks that are activated (or deactivated) in the treatment case. The longer term purpose is to harness this knowledge in conjunction with methods from our prior work on reprogramming of cells to activate or deactivate appropriate genes and gene networks to foster regeneration of tissues.

Dr. Badylak’s group has sent RNA samples for microarray analysis, which represent two time courses. The first time course includes amputation at P1 followed by treatment of the mouse digit tips with ECM at Days 0, 1, 4, 7, 10 and 14 after amputation. The second time course consists of mice with amputated digits that do not receive treatment post amputation and are analyzed at the same time points as the ECM-treated animals.

The team is using the NimbleGen whole genome mouse gene expression chip (NimbleGen 60 mer chip, design MM8). The team has produced a whole transcriptome analysis of all the genes expressed in the genome for the ECM-treated and untreated P2-amputated mouse digit time courses.

Summary of Research Completed in Year 1

During the first year of the project, the researchers completed a pilot microarray study of mouse digit tips, which showed that treatment with ECM factors designed to enhance regenerative capabilities led to the expression of ECM remodeling genes and genes indicative of stem cell activity. In addition, they developed methods for sample preparation and RNA amplification from small quantity RNA samples for microarray and next-generation sequencing analysis. They established data analysis pipelines for microarray and RNA sequencing analysis. They developed lentiviral-based methods for reprogramming cells based on altering transcriptional networks. They also established methods for predicting gene networks based on co-regulation analysis.



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Research Progress – Year 2

The team performed quality control on all samples for microarray analysis. Quality control shows all samples are of high quality. The transcriptome time course data for both time courses has been completed. The genomic DNA signal has a small dynamic range, as expected. The RNA channel has a wide dynamic range, as expected. This is an indication that the sample quality is very good.

The team analyzed the data and identified GO terms that are enriched, representing groups of genes that are enriched upon treatment (see **Figure II-27**). These include ECM components, structural molecules, and molecules involved in cell adhesion. Adhesion is also enriched at Day 14. In addition to the GO analysis, the analyses identified genes specifically upregulated late in the ECM-treated time course that are not upregulated in the untreated time course. These include genes expressed in the developing limb bud (DDAH1) as well as angiogenesis (MEST). It also includes a gene involved in syndactyly (a condition where two digits are fused together), FBN2. In addition to these genes, transcription factors involved in bone and cartilage formation (i.e.,

SP7, RUNX3, RUNX1, and SOX4), and genes expressed in the developing limb bud (i.e., RUNX1 and MEF2A) are identified.

To complement the work on mouse digit regeneration, next-generation sequencing using the Illumina GAI1 is being performed on RNA from the axolotl (*Ambystoma mexicanum*) as a model system for limb regeneration. The ability to fully regenerate an adult amputated limb is found in certain species of newts and salamanders including the axolotl. This ability is unique within the vertebrate phylum and thus makes these animals important models for limb regeneration. An axolotl colony has been established in the Thomson lab, and the team has performed RNA-seq analyses of various portions of the axolotl limb. The team has identified genes expressed in the axolotl limb, showing the feasibility of our protocols and technology.

Key Research Accomplishments

- Performed quality control on mouse digit time course samples.
- Performed microarray analysis of mouse digit time course samples.
- Performed detailed data analysis (included gene enrichment and GO enrichment) of the mouse digit time course samples.
- Identified genes and GO categories enriched specifically in treated time course samples.
- Performed preliminary analysis of axolotl limb transcriptome showing feasibility.

Conclusions

The research team has provided whole transcriptome data and analysis of the mouse digit tip amputation time course for both treated and untreated time courses. This analysis shows the upregulation of ECM components, structural components, and cell adhesion molecules. In addition, the analysis identifies several upregulated genes involved in bone/cartilage formation, angiogenesis, and syndactyly. These preliminary studies lay the groundwork for providing transcriptome analysis for the multipotent cell cluster in the coming year, funding

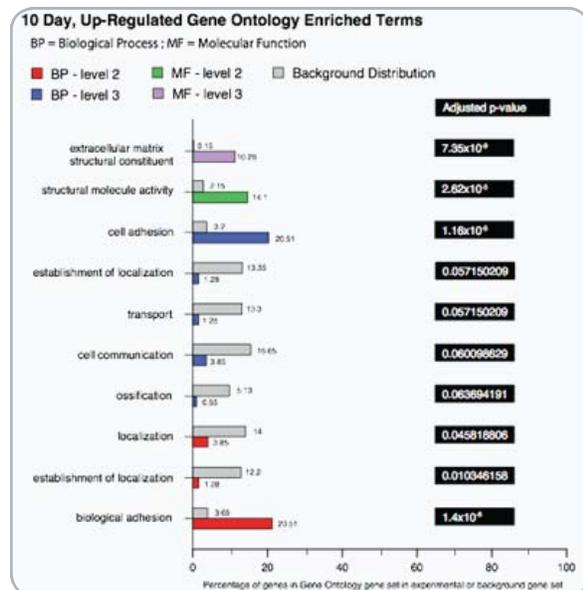


Figure II-27. Gene Ontology terms enriched in the upregulated treated Day 10 sample.

permitting. In addition, in the coming years, the team would like to produce whole transcriptome analysis of the axolotl limb blastema and perform bioinformatics analyses comparing this to the mouse digit system, funding permitting.

The potential implications of this work are substantial. Identification of similarities and differences in the active gene networks in the mouse digit model system and the axolotl blastema will provide information on the factors needed to modulate gene expression to enhance limb/digit regeneration. The ongoing collaboration with Dr. Soh on affinity reagent development and sorting technologies will allow us to dissect cell types within the regenerating axolotl blastema and MCC. This, combined with the acquired gene network similarities and differences, will inform the team on how to manipulate the mouse model system to enhance digit regeneration by activating regenerative or “multipotent cell cluster” activities within the mouse digit. Even a partial activation of these activities would represent a substantial advance in regenerative medicine.

Research Plans for the Next 3 Years

Funds permitting, in the next 3 years, the team, in collaboration with Dr. Steve Badylak, will provide a detailed transcriptome analysis of the axolotl blastema and the MCC. The microarray analysis and subsequent bioinformatics work are very expensive. The team cur-

rently does not have sufficient funding to perform these analyses. As part of this plan, if funding permits, the team will compare the axolotl and mouse transcriptome results, identifying genes and gene networks specifically involved in regeneration. To do this, it is necessary to produce a time course of the limb blastema over the course of regeneration to identify genes and gene networks activated or deactivated during regeneration, funds permitting. The blastema is likely to be composed of heterogeneous cell types. The team would also collaborate with another member of this consortium, Dr. Hyongsok Soh (University of California, Santa Barbara), in developing methods to manufacture affinity reagents for various rare precursor cell types present in the blastema. The team would use these reagents in conjunction with Dr. Soh's dielectrophoretic cell sorter to sort out cell types within the blastema. The sorted cells would then be tested for expression differences via RNA-seq. The team would then compare this information with nonregenerating model systems such as the mouse digit. From this information, the team would identify gene networks that are specifically activated in regenerating axolotl blastemas and the pattern of activation of these networks within the blastema. This knowledge lays the groundwork for activating regenerative activities in nonregenerating species such as the mouse digit. The Morgridge Institute for Research/University of Wisconsin group would plan on coordinating activities on the axolotl with functional testing strategies in the mouse to be performed in collaboration with Dr. Steve Badylak.



II: Limb and Digit Salvage

Progress Reports: Epimorphic Regeneration (and associated methods)

Magnetophoretic Cell Sorting for Transplant Therapies

Project 4.4.8, WFPC

Team Leader(s): Hyongsok (Tom) Soh, PhD (University of California, Santa Barbara)

Project Team Members: D.P. Bothman, J.D. Adams, and B.S. Ferguson (University of California, Santa Barbara)

Collaborator(s): Cynvenio Biosystems (Westlake Village, CA); Stephen Badylak, (University of Pittsburgh); and Muschler laboratory (Cleveland Clinic); and James Thomson, (University of Wisconsin)

Therapy: Therapeutic applications of magnetophoretic cell sorting developed

under this contract will be improved transplant outcomes using the purified cells collected by the devices.

Deliverable(s): Development of microfluidic magnetophoretic cell sorting devices and systems for isolation of pluripotent, rare stem cells. Development of magnetophoretic devices for identification of new markers for nuclear reprogramming.

TRL Progress: *Start of Program*, TRL 2; *End Year 1*, TRL 2; *End Year 2*, TRL 2

Key Accomplishments: The researchers have developed the first high purity, multitarget magnetic

separation device and demonstrated extremely high recovery of rare target cells from whole blood (92% recovery of 10 cells/mL). In addition, in situ culturing capability has been successfully integrated within the separation device for contamination-free expansion of stem cell products. Finally, using the microfluidic separation devices, the group has obtained key results toward rapid isolation of aptamer reagents for stem cell surface markers.

Keywords: Magnetic cell sorting, microfluidics, aptamers, stem cell transplant

Introduction

Cell sorting is a critical technology for cell-based therapeutics, wherein the rare target cells must be isolated from complex mixtures such as blood and homogenized tissue. The performance of cell sorting is benchmarked by three key metrics: purity (the fraction of target cells among collected cells), recovery (the fraction of input target cells successfully collected after sorting), and throughput (number of cells sorted per unit time). Currently, the two most widely used methods of cell sorting are MACS and fluorescence-activated cell sorting.

Toward this goal, this group has been focusing on developing a novel magnetic separation termed Ultrahigh-Gradient Magnetic Activated Cell Sorter (UHG-MACS). The central advantage of UHG-MACS technology is that, through the unique fluidic and magnetophoretic physics that occur only at the microscale, separation forces can be controlled with exquisite precision and reproducibility. This enables exceptional purity and ultrahigh recovery at high throughputs. Furthermore, due to the fact that the researchers' cell-sorting process is performed in a dis-

posable chip format, it allows the integration of multiple assay steps into a single, closed system. These features are highly advantageous for stem cell transplant therapies because it obviates the need for system cleaning and maintenance and eliminates sample contamination.

Summary of Research Completed in Year 1

During the first year of the project, the researchers developed a unique device that can achieve, for the first time, the simultaneous sorting of multiple targets at high levels of purity, recovery, and throughput. They also developed a device that allows purification of extremely rare cells from complex mixtures with unprecedented cell recovery. Additional details on both of these devices follow.

Research Progress – Year 2

Background

Magnetic cell sorting allows high-throughput sorting of target cells based on surface markers. The technique is extensively used in biotechnology for a wide range

of applications ranging from in vitro diagnostics to cell-based therapies. Existing methods (e.g., magnetic columns) suffer from two main disadvantages. First, separation is only based on a single parameter (i.e., the presence or absence of magnetization). Therefore, the simultaneous sorting of multiple targets at high levels of purity, recovery, and throughput is not possible. Second, current methods are not suitable for the isolation of small numbers of rare cells from complex mixtures due to low rates of recovery. To address these critical problems, this laboratory, in collaboration with Cynvenio Biosystems, has developed two revolutionary methods of magnetic

separation, and they are now utilizing these systems to isolate pluripotent, rare stem cells from blood and tissues (in collaboration with the Badylak laboratory). First, the lab developed the MT-MACS using UHG technology, which makes use of microfluidics technology to achieve the simultaneous, spatially addressable sorting of multiple target cell types in a continuous flow manner for the first time (Figure II-28). Second, to handle extremely rare cells from small samples, the researchers have developed the CT-MACS also using UHG technology and have shown integration of separation and culturing in a single device.

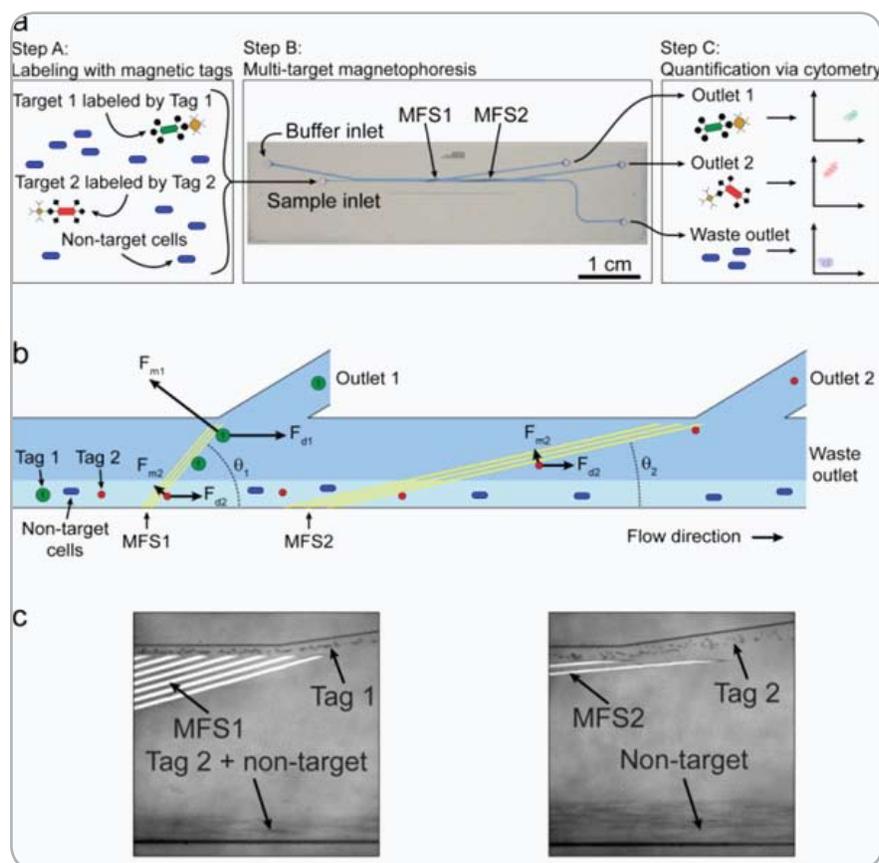


Figure II-28. MT-MACS separation architecture. (Step A) The sample contains an excess of nontarget cells and two different target cells (target 1 and target 2) that are labeled with two different magnetic tags (tag 1 and tag 2) via specific surface markers. (Step B) The sample is continuously pumped into the device where the two target cell types are sorted into spatially segregated, independent outlets. Separation occurs in two regions of high magnetic field gradient generated by the microfabricated ferromagnetic strips (MFS 1 and MFS 2). (Step C) After sorting, the eluted fractions from each outlet are analyzed via flow cytometry.

Development of the MT-MACS

The MT-MACS makes use of microfluidics technology to achieve simultaneous, spatially addressable sorting of multiple target cell types in a continuous-flow manner. The laboratory used the MT-MACS device to purify two types of target cells that had been labeled via target-specific affinity reagents with two different magnetic tags with distinct saturation magnetization and size. The device was engineered so that the combined effects of the hydrodynamic force produced from the laminar flow and the magnetophoretic force produced from patterned ferromagnetic structures within the microchannel result in the selective purification of the differentially labeled target cells into multiple, independent outlets. For the first time, the capability to simultaneously sort multiple magnetic tags was demonstrated with >90% purity and >5,000-fold enrichment

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and multiple bacterial cell types with >90% purity and >500-fold enrichment at a throughput of 10^9 cells per hour. Upon further development, this innovative capability will have a significant impact for research as well as clinical cell-sorting applications.

Development of CT-MACS Device for Rare Cell Isolation

In many applications in clinical stem cell therapies, the target cells occur at low concentrations. Thus, to isolate extremely rare cells from complex background matrices, the CT-MACS device was developed (Figure II-29).

This device offers significant advantages over the conventional magnetic separation apparatus (e.g., magnetic columns) because it offers the capability to precisely control the hydrodynamic and magnetophoretic forces within the microchannel, enabling highly efficient manipulation of a small number of cells without any loss and imposing washing conditions that are stringent and reproducible. In collaboration with Cynvenio Biosystems,

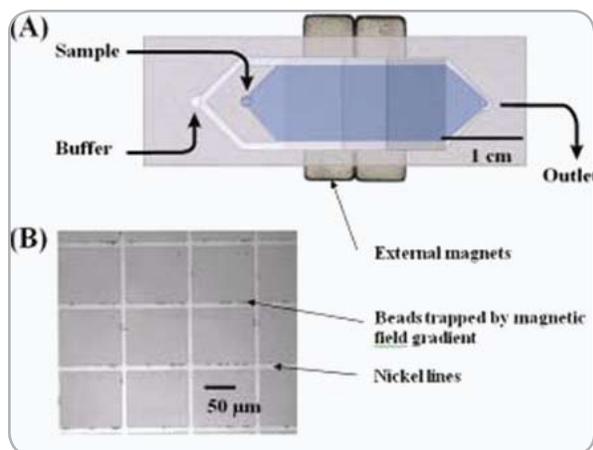


Figure II-29. CT-MACS device architecture. (A) Photograph of the microdevice with the external rare earth magnet. The device is 64 mm × 15.7 mm × 1 mm (L × W × H) and contains two inlets (sample and buffer) and one outlet. The microchannels inside the device are 30 μm in height and 12 mm in width. The microchannel contains microfabricated nickel strips with widths of 10 μm. During operation, the sample stream is flanked by two buffer streams. (B) Optical micrograph of the trapped magnetic beads. The beads are captured at the edge of nickel patterns where the magnetic field gradient is the strongest.

the most recent generation of CT-MACS system has been used to successfully capture extremely rare cells (50, 30, and 10 cells/mL) from an excess of background cells ($\sim 10^9$ cells/mL) with exceptional recovery (>90%).

The chip is optically transparent, which allows for direct optical analysis after the separation. This is a unique feature that is not available in commercial systems. In addition, the small chamber volume (300 microliters) naturally offers low reagent consumption and reduced reaction time.

Furthermore, using the microfluidics technology, the group has demonstrated the capability to integrate the cell-sorting step with in situ culturing within the chip so that stem cell products can be expanded without contamination. In collaboration with Cynvenio Biosystems, the group purified CD 34+ hematopoietic stem cells (HPCs) from cord blood samples and expanded them on a chip. Briefly, mononuclear cells from cord blood (AllCells, Emeryville, CA) were labeled with CD34+ magnetic particles (Miltenyi Biotech) and FcR blocker. After purification in the chip, serum-free expansion medium with a cytokine cocktail (Stem Cell Technologies, Vancouver, Canada) containing Flt-3 ligand, SCF, IL-3, and IL-6 was used to expand the cells in the chip for 7 days, which showed significant growth of the HPCs (Figure II-30). Staining with anti-CD34 antibodies conjugated to streptavidin-PE on the expanded cells confirmed that the expanded cells indeed express the CD34 antigen (Figure II-30 inset).

Key Research Accomplishments

- Developed the first MT-MACS chip, which can simultaneously sort multiple target cells with high purity, recovery, and throughput.
- Developed a custom CT-MACS device, which allows high performance magnetic sorting of extremely rare target cells even in complex backgrounds containing a tremendous excess of background cells.
- Demonstrated the capability to integrate the cell-sorting step with in situ culturing within the chip so that stem cell products can be expanded without contamination.

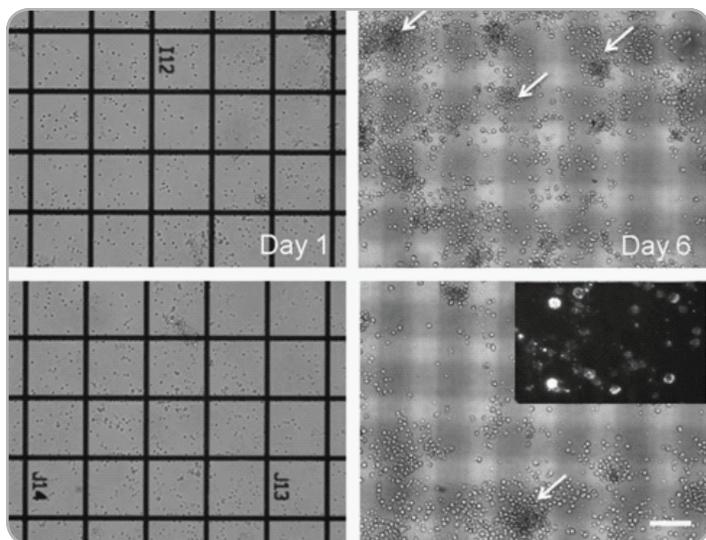


Figure II-30. Magnetically labeled CD34+ cells in cord blood MNCs captured and cultured in the chip. The images show before (Day 0) and after (Day 6) from two different locations on the chip. Note the cells have detached from the grid and are expanding in clusters on the bottom of the chip (arrows). The scale bar is 100 μm . (Inset bottom right) Staining with anti-CD34 antibodies conjugated to streptavidin-PE on the expanded cells confirm that the expanded cells express the CD34 antigen.

Conclusions

In this second year of AFIRM support, the researchers have developed a family of devices that solve severe shortcomings inherent in current methods of magnetic cell sorting. In particular, they have developed the MT-MACS platform to achieve, for the first time, simultaneous sorting of multiple targets at high levels of purity, recovery, and throughput. In addition, they have developed the CT-MACS device, which allows purification of extremely rare cells from complex mixtures with unprecedented cell recovery. To fully exploit the utility of the microfluidic devices, they have closely interacted with the Badylak and Muschler laboratories to isolate pluri-

potent progenitor cells from tissues of model animals. The technology is now in the process of commercialization through an industrial partner (Cynvenio Biosystems, Westlake Village, CA) – commercial availability will benefit many researchers within AFIRM who have a need for rare cell isolation. This unique cell-sorting capability may provide a critical technical solution to isolate the target stem cells for clinical therapeutics.

Research Plans for the Next 3 Years

The development of magnetophoretic cell sorting devices has progressed rapidly in Years 1-2 and is now being transitioned to industry for commercialization. The research team plans to facilitate this transition so that a commercial device will be available to AFIRM investigators who are interested in using this system. Beyond the device development, the team found that the key bottlenecks in stem cell purification are the lack of adequate surface markers to identify the

target cells, as well as affinity reagents that specifically bind to them with high affinity. To address these critical issues, in the next 3 years, the team plans to perform *in vitro* directed evolution to generate affinity reagents for stem cell markers utilizing the novel microfluidic separation system.

Corrections/Changes Planned for Year 3

The cell-sorting system development has progressed rapidly and is now currently being commercialized. In Year 3, the research team plans to shift the focus to utilizing the microfluidic purification systems to efficiently generate affinity reagents for stem cell markers.



III: Craniofacial Reconstruction



BACKGROUND

A total of 26% of wounded warriors treated in U.S. military facilities during the current conflicts have sustained maxillofacial injuries.¹ These injuries range from simple fractures to extensive bone defects, severe burns, and soft tissue avulsion. A warrior with a significant craniofacial injury has lost his or her interface with the world; when one's face is gone, so is one's identity. A warrior with severe facial injury may lose the ability to communicate—speech may be difficult to understand and facial expressions nonexistent—even the ability to eat. Significant disfigurement excludes one from society—a mangled, contracted, or absent face does not invite interaction. These soft tissue and bony injuries to the face and cranium are excruciating physical injuries with devastating psychological impact. Restoring form and function to these traumatized warriors is critical to rehabilitation.

AFIRM's Craniofacial Reconstruction (CFR) Program seeks to restore wounded warriors with devastating, disfiguring facial injuries to fully functional lives, including integration with society through the application of regenerative medicine technology. The CFR Program comprises a multidisciplinary, multi-institutional collaborative research team conducting projects ranging from single tissue regeneration to complete face transplants, from emerging products in the early stages of bench research to products entering clinical trials. Drawing on the strengths of each investigator on the team, an optimal set of complementary technologies have been identified to achieve hard and soft tissue regeneration. The CFR Program will give wounded warriors a new face to present to the world.

¹ Hale, Robert. Slide Presentation "The Regenerated Face for Maxillofacial Battle Injuries – Joint Theatre Trauma Registry, Oct. 19, 2001 to Dec. 12, 2007," presented May 19, 2010.



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Unmet Needs

Current treatments in cranio-mandibulo-maxillofacial (CMF) reconstruction practices are inadequate to treat the unique and massive craniofacial deficits resulting from blast injuries. Presently, massive bone loss to the craniofacial complex incurred in combat is reconstructed with nonresorbable synthetic materials, bone implants, or metallic devices that restore anatomical form to some degree and limited function. The currently available synthetic bone materials, for example, do not remodel or integrate with host tissue and can become infected and require extensive, multiple revision surgeries. A biodynamic, biocompatible substitute for complex bone defects is a critical need in facial injury.

Current techniques and therapies neither mitigate scar contracture nor achieve complex soft tissue coverage esthetics of structures such as the ears and nose. Allogenic and autogenous grafts are options with limited availability for repair of injuries in which a prosthesis is unacceptable, such as those to the ear and nose. Current standard treatments are hindered by high donor site morbidity and poor long-term results. A readily available, or readily generated, replacement tissue for complex soft tissue structures is an unmet need in facial injury.

In the most severe facial injuries, the loss of tissue is massive. In such cases, there is no satisfactory surgical approach for facial replacement other than transplant. However, composite tissue allografts (CTAs) that constitute the face are highly antigenic and require aggressive immunosuppression regimens to prevent rejection of the transplanted tissue. This puts the host at substantial risk, both from the immunosuppressive agents and from opportunistic infections, and has led to a significantly shortened life expectancy for all transplant recipients. Development of a technique or therapy providing modulation of the immune response to allografts without indiscriminate immunosuppression is necessary to provide adequate treatment to severe facial injuries in wounded warriors.

High order explosive devices cause severe blast injuries to unprotected craniofacial tissue, including the small muscles of facial expression and function. A severely

damaged orbicularis oculi prevents eyelid closure, resulting in dry, painful, irritated eyes and potential blindness. In the catastrophically injured soldier, autologous donor sites for soft tissue transfers may be missing or compromised. Allogeneic donor muscle transplantation represents the sole near-term therapy option but requires lifelong immunosuppression with significant attendant risks. Engineered skeletal muscle restores form and function of critical tissues using a patient's own cells and fills an unmet need in facial reconstruction.

Soft tissue deficits may be treated with pedicled muscle and skin flaps and allogenic skin substitutes. But these treatments do not restore myogenic and neurogenic competence; the warrior's face may no longer have large defects, but it does not move and does not feel. The implications for independence, communication, and integration are substantial. Restoring facial nerve and muscle competence is a critical need to significantly improve the outcome for wounded warriors with severe facial injuries.

Finally, planning the complex reconstruction and regeneration needs of wounded warriors with severe facial injuries is a critical need. AFIRM researchers are developing a visualization tool for patient-specific wounds and injuries. This will help integrate specific tissue regeneration strategies both within the CFR Program and throughout AFIRM. The approach will be staged, proceeding from the least biomechanically challenging to the most challenging zones in the CMF complex.

Areas of Emphasis

The Rutgers/Cleveland Clinic Consortium (RCCC), The Wake Forest/Pittsburgh Consortium (WFPC), and the U.S. Army Institute of Surgical Research (USAISR) are pursuing a complementary mix of research projects focused on various aspects of CFR. Projects can be grouped into four "clinical challenge" topic areas: Bone Regeneration, Soft Tissue Regeneration, Cartilage Regeneration (with a focus on the ear), and Virtual Modeling for CFR. Additional details on projects in each of these topic areas can be found in the following table and subsequent sections of this chapter.

Bone Regeneration

Studies at WFPC

The **Mikos/Wong group** (Project 4.1.2) at Rice University and the University of Texas Health Science Center (UTHSC) is developing porous space maintainers that will maintain the proper anatomical relationship of the tissue adjacent to a craniofacial defect. They are also developing an “in vivo bioreactor” technology that involves the production of a bone/soft tissue flap with a blood supply at a site in the body away from the wound and then transplanting it into the skeletal defect once the wound has been optimized for reconstruction. The researchers completed a preclinical study to evaluate the ability of several space maintainer formulations to prevent soft tissue collapse in a mandibular critical size bone defect in a rabbit model. They also initiated a study to evaluate the release of antibiotics from porous space

maintainers in an infected rabbit mandibular defect model. They are collaborating with investigators in China to determine the efficacy of their “in vivo bioreactor” approach. The team has also developed a protocol for Institutional Review Board (IRB) submission to obtain approval to initiate clinical studies of their space-maintaining devices.

The **Sfeir group** (Project 4.1.3) at the University of Pittsburgh is developing nanostructured bioactive bone cements that contain the essential components to mimic bone architecture, composition, and mechanical strength while providing the bone-generating characteristics required for bone tissue regeneration. They have conducted in vivo screening experiments to rapidly determine if nano-calcium phosphate-containing materials will be optimal in the proposed bone regeneration strategy either with or without the incorporation of the

Table III-1. Projects funded by RCCC, WFPC, and USAISR per clinical challenge topic area.

Clinical Challenge	Consortium/Institution	Project Number	Project Title
Bone Regeneration	WFPC	4.1.2	Space Maintenance, Wound Optimization, Osseous Regeneration, and Reconstruction for Craniomaxillofacial Defects
		4.1.3	Novel Synthetic Bone for Craniofacial Application
	RCCC	4.5.1a	Regeneration of Bone in the Cranio-Mandibulo-Maxillofacial Complex Using Allograft Bone/Polymer Composites
		4.5.1b	Regeneration of Bone in the Cranio-Mandibulo-Maxillofacial Complex
	USAISR	4.1.7	Improving Cell Engraftment for Bone Repair
Soft Tissue Regeneration	WFPC	4.1.4 / 4.1.5	Soft Tissue Regeneration
		4.1.6	Bioreactors and Biomaterials for Tissue Engineering of Skeletal Muscle
	RCCC	4.1.2	Develop Innervated, Vascularized Skeletal Muscle
		4.3.1	Composite Tissue Allograft Transplantation Without Life-Long Immunosuppression
Cartilage Regeneration (Focus: Ear)	WFPC	4.1.1	Engineered Cartilage Covered Ear Implants for Auricular Reconstruction
	RCCC	4.5.4a	Regeneration of Ear
		4.5.4b	Regeneration of Ear – Optimization of Scaffold
Virtual Modeling for CFR	RCCC	4.5.5	Visualization of Patient-Specific Wounds and Injuries



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bone-inductive growth factors. Their data show that their nano-calcium phosphate-containing bone cements are indeed very good candidates for further assessment as a therapy for bone regeneration. The research team has recently developed injectable forms of their bone cements. Continued effort on the project is focused on further developing the bone cements to gather more data and proceed further for U.S. Food and Drug Administration (FDA) approval and eventual clinical trials.

Studies at RCCC

The **Guelcher group** (Project 4.5.1a) at Vanderbilt University is developing an injectable allograft/polymer bone-void filler, Plexur LV[®], with and without recombinant human bone morphogenetic protein-2 (rhBMP-2) for the treatment of skull defects. They have identified the final formulation for Plexur LV. Preclinical studies in rabbit skull and jaw models of bone regeneration show that the material supports rapid cellular infiltration and new bone formation and that the material is a useful delivery system for rhBMP-2. Moreover, the product degrades, thus limiting the amount of inorganic material at the site of injury. The researchers are pursuing FDA approval for the bone-void filler with corporate support from Osteotech, Inc. Pending the successful completion of the rabbit skull defect study, the technology will progress to ISO 10993 studies (a series of standards for evaluating the biological compatibility of a medical device prior to a clinical study) in 2011. Once Plexur LV has been approved for long bones, the research team anticipates filing a 510(k) device application for CMF applications, which likely will require a clinical trial.

The **Hollinger group** (Project 4.5.1b) at Carnegie Mellon University (CMU), Rutgers University, and the Mayo Clinic is developing biodegradable scaffolds containing tyrosine-derived polycarbonate (Tyr-PC) or poly(ϵ -caprolactone fumarate [PCLF]), which may provide compelling therapeutic solutions for the regeneration of craniofacial bone. The first preclinical studies with these scaffolds have been completed. The researchers found that their Tyr-PC scaffolds, with the addition of rhBMP-2, could regenerate bone in the rabbit skull defect model in 6 weeks. They also found that the Tyr-PC scaffolds were gradually resorbed and replaced with new bone during

the 6-week period. While the research team emphasized the skull and upper face in Year 2, they will gradually transition to the jaw in Years 3 and 4. The researchers will proceed with standardized models, first in the rabbit and later, the goat. Based on the results, further testing of the most promising materials will proceed to nonhuman primates. In collaboration with industry partners, the researchers may deliver a bone regeneration therapy for a small Phase 1 clinical trial as early as Year 4.

Studies at USAISR

The **Rathbone/Wenke group** (Project 4.1.7) at USAISR is developing a strategy for preconditioning stem cells for the treatment of muscle and bone injuries. The researchers are first determining the best type of stem cell to use in their experimental paradigm. They have successfully isolated mesenchymal stem cells (MSCs), bone marrow-derived stem cells (BMSCs), and adipose-derived stem cells (ASCs) from mice using standard isolation procedures. They completed preliminary experiments to begin to optimize dosages and will follow up these experi-



Graduate student Hanshella Magno is assessing the bone-regenerating capacity of scaffolds using a critical size defect in the rabbit calvaria (RCCC).



Hung-wok Park fabricating an ear scaffold (WFPC).

ments using freshly isolated and cultured stem cells. The researchers plan to use transgenic mice and the skull defect model to obtain *in vivo* measurements of cell survival in a bone defect and to determine the potential for the stem cells to stimulate bone regeneration. They recently attained regulatory approval for animal studies.

Soft Tissue Regeneration

Studies at WFPC

The **Rubin group** (Projects 4.1.4 and 4.1.5) at the University of Pittsburgh, Tufts University, and Wake Forest University is seeking to develop and deliver a clinically useful engineered soft tissue replacement that can be used as a stand-alone therapy or integrated with composite tissue regenerative medicine therapy of burns, craniofacial injuries, and extremity injuries. The researchers are generating silk-based scaffolds that contain autologous (the patient's own) ASCs and fibroblasts, combined with carrier biomaterials, to achieve vascularized soft tissues. Recent preclinical studies in small animal models have demonstrated the effectiveness of several variants of their general approach in generating vascularized soft tissue constructs that maintain their size and shape for more than 6 months.

The investigators are aggressively pursuing translation of the ASC technology into clinical trials for the production of vascularized fat pads.

The **Christ group** (Project 4.1.6) at the Wake Forest Institute for Regenerative Medicine (WFIRM) seeks to develop a technological tool that preconditions and accelerates muscle tissue maturation and function. The researchers have developed and implemented a rodent model of volumetric muscle loss (VML). Their model has been designed to evaluate tissue-engineered skeletal muscle constructs generated using an innovative bioreactor system that mechanically stimulates the constructs during development. The tissue-engineered skeletal muscle (TE-SKM) constructs in the preclinical study demonstrated clinically relevant contractile responses within 2 months of implantation, and the submaximal contractile force generated by the constructs was indistinguishable from the native muscle tissue. The validation provided through the preclinical proof-of-concept (POC) studies will facilitate the clinical translation of the bioreactor technology for skeletal muscle development to address the critical clinical need for muscle constructs capable of generating clinically relevant forces in the craniofacial complex.

Studies at RCCC

The **Sundback/Vacanti group** (Project 4.1.2) at Massachusetts General Hospital (MGH) is engineering skeletal muscle with physiological connections to the host's neurovascular (nerve and blood vessel) network using biodegradable polymer scaffolds. During the past year, the researchers engineered three-dimensional skeletal muscle similar to immature skeletal muscle and developed protocols for the development of functional blood vessels and nerves in the tissue. To create vascularized skeletal muscle, they co-seeded stem cells with muscle cells. They found that this network effectively integrated with the host vasculature *in vivo*. The researchers transferred a branch of a functional nerve to their engineered muscle that had been implanted in the jaw of a rat. They were able to re-establish neuromuscular connections in nonfunctional native muscle using this surgical protocol. Another AFIRM laboratory, led by Dr. Joachim Kohn, has developed a prototype scaffold to support the scale-up



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Researchers Jake McFadden and Erik Bassett in the laboratory (RCCC).

of these engineered muscles over the next 3 years to a muscle the size of the human orbicularis oculi muscle (closes the eyelid). During Year 5, the MGH team will develop a surgical model to implant the engineered muscle construct in the orbicularis oculi site. A POC study will demonstrate functionality of the construct, and preclinical trials in a large animal model will be initiated.

The varying nature of CTAs, which differ in their immunological responses, raises new challenges for transplant immunologists. The Siemionow group (Project 4.3.1) at Cleveland Clinic hopes to transform standards for clinical immunomodulation, making transplantation of CTAs (large segments of complex, vascularized tissue) safer and more widely available to victims of disease and traumatic injury. They are investigating therapies that incorporate bone marrow transplantation (BMT) to improve the outcome of CTAs. A therapeutic antibody, TOL101, is being used as a conditioning agent prior to transplantation to enhance allograft tolerance. The antibody has undergone various regulatory tests and has passed a pre-Investigational New Drug (IND) screening with the FDA. The researchers expect to complete safety and efficacy studies for TOL101 in approximately 30 kidney transplant recipients by the end of the third quarter of 2010. They are also exploring the use of donor-recipient “chimeric” cells as potential immunomodulators. They anticipate the

first human therapeutic use of human chimeric cells as early as the fourth quarter of 2011. The research team is also enrolling potential patients for face transplants at the Cleveland Clinic. They expect to complete at least one face transplant in Year 3 of the project pending suitable recipient identification and a suitably matched donor.

Cartilage Regeneration (Focus: Ear)

Studies at WFPC

The Yoo group (Project 4.1.1) at Wake Forest University seeks to accelerate the delivery of reconstructive applications to injured armed forces personnel through the development of an engineered cartilage covered ear implant. The researchers are continuing to (1) develop a system that requires a minimum tissue biopsy for cell isolation and expansion using different cell sources, (2) develop standard operating procedures (SOPs) for autologous cell sourcing and an associated expansion system, and (3) refine the cell delivery system to facilitate clinical translation of the cartilage-coated auricular implants. Studies initiated in a rabbit model have demonstrated the feasibility of autologous cell harvest and transfer in the approach and are guiding the development of SOPs for the technology. Results from preclinical studies in a rodent model and the ongoing studies in

the rabbit model demonstrate the biological compatibility and structural stability of the cartilage-coated implants and point to the viability of the approach for eventual clinical translation.

Studies at RCCC

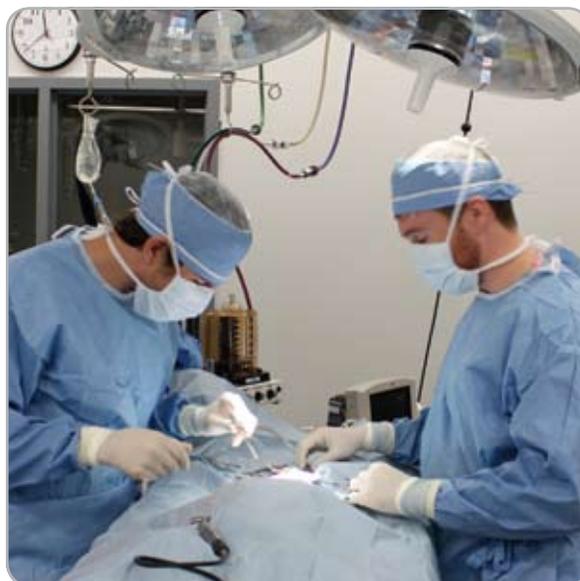
The **Sundback/Vacanti group** (Project 4.5.4a) at MGH and the **Anderson/Langer group** (Project 4.5.4b) at the Massachusetts Institute of Technology (MIT) seek to expedite the development of a permanent, implantable, living external ear for the injured warfighter and to achieve cosmetic outcomes that meet patient expectations. They made significant progress during the past year in preclinical testing. Testing progressed from a small animal, immunocompromised model (nude mouse) to a large animal, immunocompetent model (sheep). Autologous neocartilage formation on porous collagen scaffolds was demonstrated in the sheep model, with minimal inflammatory response noted. The team's dynamic (versus static) culture method generated a robust increase in autologous cartilage formation in vivo and reduced the inflammatory response. Use of an internal, titanium wire support for maintenance of ear size and shape was proven effective, completely eliminating shrinkage of the construct. Wired constructs retained all gross characteristics and flexibility of cartilage and showed no visible evidence of wire extrusion. In the next 3 years, the researchers will enhance cartilage formation from nasal-septal and auricular sources by optimizing cell seeding density and bioreactor culture. They will continue to pursue enhanced culture techniques to reduce inflammation and improve construct integration. Overall, they are on track for performing a pilot clinical trial in humans by the beginning of Year 5.

Virtual Modeling for CFR

Studies at RCCC

The **Kelliher/Champion group** (Project 4.5.5) at SimQuest LLC is developing a virtual reality visualization tool for patient-specific wounds and injuries. During the past year, the researchers have developed the platform for

translating AFIRM results from laboratory prototypes into clinical practice. Specifically, the technology comprises injury data analysis tools, preparation of patient-specific wound and injury models, and physics-based surgical simulation. The injury analysis tools create a statistical basis for determining high-impact areas of focus and for tracking effectiveness of surgical and regenerative techniques. They identify successful or unsuccessful outcomes and serve to identify the common elements of treatment that may be the causative agents. The researchers have obtained IRB approval to begin patient data collection. The tool is first being used at the Walter Reed Army Medical Center (WRAMC), where the initial focus is on capturing data for warfighters who have received craniofacial implants. Over the next 3 years, the researchers plan to delve into the segmentation and modeling of targeted soft-tissue structures such as specific muscles, nerves, vasculature, and scar tissue. They also plan to develop new algorithms to segment these structures from computed tomography data.



Patrick Spicer and Jim Kretlow (both MD/PhD students at Rice University) performing a surgery to create a non-healing 10 mm mandibular defect in a rabbit model (WFPC).



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Progress Reports: Bone Regeneration

Space Maintenance, Wound Optimization, Osseous Regeneration, and Reconstruction for Craniomaxillofacial Defects

Project 4.1.2, WFPC

Team Leader(s): Antonios G. Mikos, PhD (Rice University) and Mark E. Wong, DDS (UTHSCH)

Project Team Members: F. Kurtis Kasper, PhD, Lucas Kinard, BS, James D. Kretlow, PhD, Meng Shi, PhD, Patrick Spicer, BS (Rice University); Nagi Demian, DDS, MD, and Simon Young, DDS, PhD (UTHSC)

Collaborator(s): Shanghai 9th People's Hospital, Shanghai, China and Radboud University of Nijmegen Medical Centre, Nijmegen, The Netherlands

Therapy: Staged reconstruction of large osseous defects in the craniofacial region restoring function and esthetics

Deliverable(s): (1) Biocompatible, antibiotic-releasing implants to maintain bony wound spaces; (2) "in vivo bioreactor" that will allow for the generation of vascularized bone; (3) injectable system for delivery of growth factors necessary for bone regeneration and wound healing

TRL Progress: *Start of Program*, TRL 2; *End Year 1*, TRL 3; *End Year 2*, TRL 4

Key Accomplishments: The researchers have fabricated and characterized in vitro a variety of porous implant formulations presenting a range of porosities and mechanical properties and have evaluated them for efficacy in maintaining a bony defect space while supporting soft tissue healing in

a rabbit mandibular defect model in vivo. Additional in vitro studies have demonstrated that antibiotics can be incorporated into space-maintaining implants and released in a controlled fashion. In addition, the release kinetics can be altered by tunable construct fabrication parameters. Initial results from an ongoing in vivo study in a sheep model suggest the feasibility of an "in vivo bioreactor" approach for the generation of vascularized autologous bone flaps and the transfer of the flaps to fill mandibular defects.

Keywords: Craniofacial bone reconstruction, space maintenance, bone flap, controlled drug delivery, in vivo bioreactor

Introduction

Ballistic injuries resulting in significant soft and hard tissue loss and devitalization are commonly encountered clinical scenarios in current U.S. military combat theaters. This project seeks to develop a method to facilitate effective staged reconstruction of large osseous defects in the craniofacial region and extremities of injured military personnel, thus restoring function and esthetics in these individuals. The purpose of this research is to decrease the complications and infections associated with large bony reconstructions in this particular patient population through three mechanisms: (1) the initial implantation of a biocompatible, antibiotic-releasing space maintainer within a large osseous defect during the early phases of treatment, (2) implantation of an "in vivo bioreactor" construct away from the site of injury that will allow for the generation of a vascularized bone flap to be used as donor tissue for second-stage reconstruc-

tive surgeries, and (3) augmentation of the implanted vascularized bone flap within the recipient defect site by using an injectable system tailored for both the delivery of growth factors needed to promote bone regeneration and wound healing until sufficient integration of the bone flap has occurred.

Summary of Research Completed in Year 1

During the first year of the project, the researchers fabricated and characterized a variety of carboxymethylcellulose poly(methyl methacrylate) (CMC/PMMA) porous space maintainer formulations presenting a range of porosities and mechanical properties. They also initiated the identification of antibiotics to be released from the space maintainers as a prophylaxis against infection of the craniofacial wound site. They explored and characterized in vitro the release of the antibiotic(s) of interest from poly (L,D-lactic-co-glycolic acid) (PLGA) micro-

spheres to be incorporated into the space maintainers. They also identified a clinically viable antibiotic (colistin) to treat a battlefield-relevant target bacterial species (*Acinetobacter baumannii*). Finally, they initiated a pilot study to evaluate several CMC/PMMA space maintainer formulations for their ability to prevent soft tissue collapse in a large osseous defect in a rabbit model.

Research Progress – Year 2

A number of studies were completed or initiated in the past year investigating and optimizing the technologies envisioned for application of the researchers' approach for craniofacial bone regeneration. First, an in vivo study was performed to investigate the effects of PMMA space maintainer porosity on the healing of surrounding soft tissues. PMMA was selected due to its regulatory status, ability to be molded, and because the envisioned modifications (i.e., porosity and drug release) could be accomplished using combinations of FDA-regulated materials. The study was performed using a rabbit mandibular defect that was left open to the oral cavity to allow bacterial seeding, such as might be encountered in a clinical setting. The study identified porosity values that facilitated or allowed soft tissue closure with a minimal inflammatory response. Examples of healed and nonhealed wounds are shown in Figure III-1.

Second, an ongoing study is investigating whether in situ PMMA polymerization has an effect on the tissue response to space maintainers. Third, in vitro studies investigated drug release from these porous space maintainers using degradable microparticles incorporated into

the PMMA matrix. Colistin, an antibiotic effective against *A. baumannii*, was released in a controlled fashion over 28 days. It is envisioned that these formulations and others under investigation will allow for controlled, local delivery of antibiotics and/or growth factors to optimize the tissue microenvironment for the later transfer of the vascularized bone flap.

Fourth, in vivo studies investigating the effect of antibiotic release in a wound infected with *A. baumannii* have been initiated. Fifth, ongoing work is also being performed investigating the de novo formation of vascularized bone flaps. In collaboration with colleagues at the 9th People's Hospital in Shanghai, China leveraging non-AFIRM funds, the current study is designed to determine how the filler material affects bone growth during flap generation and following flap transfer. Bone is being generated within chambers placed adjacent to the cambial side of the periosteum covering a sheep's rib, and the generated bone is then transferred to a bone defect created in the sheep's mandible. The effect on bone growth of having a flap transferred with a pedicle is being compared to transferring the generated bone as a nonvascularized graft. Examples of the vascularized bone flap and the flap following transfer and microsurgical anastomosis are shown in Figure III-2. Results from this study are expected in late 2010. Current issues remaining in this area include the determination of an ideal anatomical site that contains periosteum of a large enough size to generate a large volume bone flap as well as an available vascular supply that can be easily harvested with the generated bone.



Figure III-1. Representative images of rabbit mucosal defects 12 weeks after implantation of PMMA space maintainers. In 3 of 6 animals with solid PMMA space maintainers, the soft tissue failed to close over the implant (A, implant marked by arrows). In 5 of 6 animals implanted with porous space maintainers, wound closure was complete after 12 weeks (B,C).



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Progress Reports: Bone Regeneration

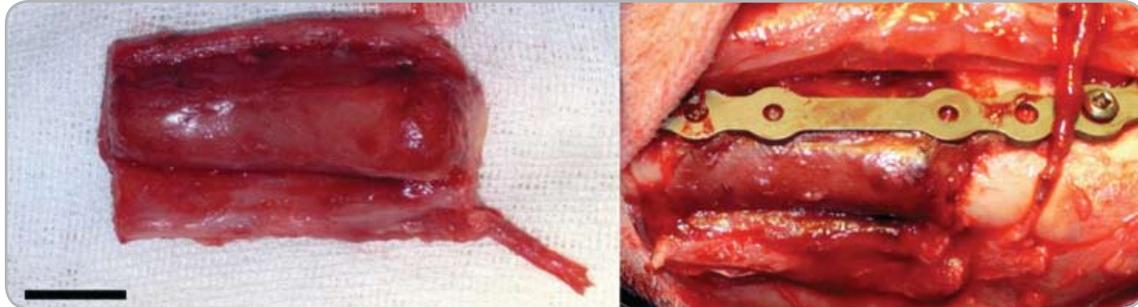


Figure III-2. De novo generated vascularized bone flap following explanation with the adjacent intercostal vessel (left, scale bar = 1 cm). After being generated for 10 weeks, the bone flap was transferred to a mandibular bone defect, and the vessel was anastomosed to a branch of the facial artery (right). The study is being conducted in collaboration with investigators at the 9th People's Hospital in Shanghai, China, leveraging non-AFIRM funds.

Key Research Accomplishments

- Completed a pilot study evaluating several CMC/PMMA space maintainer formulations presenting a range of porosities for their ability to support soft tissue healing in a rabbit mandibular defect model.
- Initiated a study investigating whether in situ PMMA polymerization has an effect on the tissue response to space maintainers in a nonhealing mandibular defect in a rabbit model.
- Completed in vitro studies characterizing the release of the antibiotic colistin from PMMA-based space maintainers incorporating PLGA microspheres and demonstrated that antibiotic release can be controlled through tunable parameters of space maintainer fabrication.
- Initiated an in vivo study to evaluate the efficacy of colistin-releasing CMC/PMMA/PLGA space maintainers toward eradicating *A. baumannii* in an infected nonhealing mandibular defect in a rabbit model.
- Initiated ongoing in vitro studies characterizing the release of antibiotics from PMMA space maintainers incorporating gelatin microspheres, which may allow for intraoperative antibiotic loading and potentially facilitate regulatory approval.
- Initiated an ongoing pilot study in collaboration with colleagues at the 9th People's Hospital in Shanghai, China leveraging non-AFIRM funds, investigating the

“in vivo bioreactor” approach for the generation of vascularized bone flaps, and the durability of these flaps when transferred to a mandibular defect in a sheep model.

Conclusions

Considerable progress has been made over the course of the past year toward the development of antibiotic-releasing implants for bony space maintenance and an “in vivo bioreactor” approach for the generation of vascularized autologous bone flaps, which collectively are envisioned to facilitate the staged reconstruction of large bone defects in the craniofacial region. Specifically, the introduction of porosity into PMMA-based space maintainers was shown to positively influence the tissue response to the implants in a nonhealing rabbit mandibular defect model. Further, in vitro studies demonstrated that the antibiotic colistin can be released from PMMA-based space maintainers incorporating PLGA microspheres in a controlled fashion and that the release kinetics can be modulated through manipulation of tunable parameters of the construct fabrication. Initial results from an ongoing in vivo pilot study employing a sheep model demonstrate that PMMA chambers filled with osteoinductive materials can be effectively employed in an in vivo bioreactor strategy to generate vascularized autologous bone flaps and that these flaps can be transferred to fill mandibular defects.

Research Plans for the Next 3 Years

Future research plans involve continued exploration of the release of antibiotics from PMMA-based space maintainers, with a particular emphasis on the characterization of the efficacy of antibiotic release in eradicating target species in infected nonhealing rabbit mandibular defects. Ongoing studies investigating the in vivo bioreactor approach for vascularized bone flap generation will continue to be explored in a sheep model to optimize the parameters for bone flap generation and to evaluate the durability of repair of mandibular defects upon transfer of the bone flaps. Finally, future studies will involve the synthesis of degradable materials for controlled growth factor delivery to support bone regeneration and aesthetic contouring.

Planned Clinical Transitions

Productive interactions have been established between the investigators on this project and personnel from the UTHSC Center for Clinical and Translational Sciences and Center for Translational Injury Research regarding plans for translation of the space maintainer technology into clinical trials. A working plan has been developed in cooperation with these centers for the preparation and submission of the associated clinical trial protocols for studies investigating the space-maintaining devices under development in this project. It is expected that the first clinical trial will be initiated by December 2010.



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Progress Reports: Bone Regeneration

Novel Synthetic Bone for Craniofacial Application

Project 4.1.3, WFPC

Team Leader(s): Charles Sfeir, DDS, PhD, Elia Beniash, PhD, and Prashant Kumta, PhD (University of Pittsburgh)

Project Team Members: Abhijit Roy, PhD, Shinsuke Onishi, DDS, PhD, and Sabrina Noorani, MS (University of Pittsburgh)

Collaborator(s): None

Therapy: Bone tissue engineering

Deliverable(s): Novel bioactive bone cements incorporating nanostructured calcium phosphate (NanoCaPs)

TRL Progress: *Start of Program, TRL 2; End Year 1, TRL 2; End Year 2, TRL 4*

Key Accomplishments: Developed and characterized novel bioactive bone cements and scaffolds based on the use of natural polymers that incorporate NanoCaPs. Initiated in vitro assessments and found that the synthesized forms of cement materials are biocompatible. Performed initial in vivo screening experiments using rabbits to determine if the material will provide the best bone regeneration strategy. The data show that the newly

synthesized resorbable cements containing NanoCaPs with and without BMP-2 are very good candidates for further assessment of the system as a bone regeneration therapy.

Keywords: Bone regeneration, craniofacial defects, synthetic bone, NanoCaPs, bioresorbable

Introduction

This project focuses on developing novel bone regeneration strategies for CFR by exploiting the combined attributes of nano-scale inorganic bioactive cements and naturally derived polymer hybrid materials that possess excellent bioreactivity, biocompatibility, safety, and regenerative capability. This combination of materials would result in development of structurally and functionally normal bone that is free of infection for injured military personnel. The proposed technologies will also be used for regenerating large osseous defects in the extremities where bone fracture is a major clinical problem contributing to nearly 50% of all Armed Forces personnel injuries.

This project aims to develop a synthetic bone-like environment that involves bioactive nanostructured amorphous and/or NanoCaPs, nano-structured CaP-based bioactive bone cements, and extracellular matrix (ECM)-derived materials such as urinary bladder membrane (UBM). These nanostructured systems contain essential components to mimic the bone architecture, composition, and mechanical strength while providing the osteoinductive and osteoconductive characteristics required for bone tissue regeneration. The combined

nanoscale hybrid system will incorporate BMP-2 or BMP-7, which are known for their bone regeneration ability. This strategy will create an organic/inorganic scaffold system that would simulate the unique composition and architecture of bone.

There are three specific aims for this project:

- **Specific Aim 1:** Synthesize and characterize nano-structured apatitic bioactive bone cements and scaffolds based on natural ECM-derived polymers that incorporate NanoCaPs (amorphous and/or nanocrystalline). These hybrid scaffold systems will incorporate BMP-2 or BMP-7 and ECM from the porcine UBM.
- **Specific Aim 2:** Preclinical assessment of the regenerative capacity of the bone cements and ECM-derived polymers in a well-established rabbit calvarial critical size defect (CSD) model.
- **Specific Aim 3:** Initiation of clinical testing of synthetic bone for craniofacial defects.

Summary of Work Completed in Year 1

During the first year of the project, the researchers developed and characterized novel nanostructured apatitic

bioactive bone cements and scaffolds based on the use of natural ECM-derived polymers that incorporate NanoCaPs. They initiated the in vitro assessment of the cements and scaffolds and found that the synthesized forms of the cement materials are biocompatible. A preliminary experiment in a rabbit ulna defect model with bone cements containing BMP-2 showed some bone regeneration (bridging) at 8 weeks post surgery.

Research Progress – Year 2

Since previous compositions did not perform well in in vivo rabbit screening experiments, the researchers synthesized a novel composition of the cement with UBM. They are currently assessing this new composition in a rabbit calvarial CSD model. In addition to the new cement/UBM design, they developed injectable cements as they found it difficult to fit the preformed scaffolds in the defect site. They therefore directed research at identifying a suitable plasticizer and stabilizer. They achieved injectability by the addition of 2%-3% of a biocompatible cohesion promoter. They found that addition of the cohesion promoter to the cements (cement and porogen cement) improved the injectability of the cement (>95%). They noted that the injected formulations did not disintegrate when added into the buffer solutions or when mixed in with the rabbit blood. Thus, these cements can be used as a paste on site during surgeries into the rabbit calvarial defects of ~15 mm in diameter.

The researchers also studied the initial and final cement setting times for their injectable cement formulations. The initial setting time increased slightly due to the use of more liquids to generate a useable injectable formulation. The initial x-ray diffraction results of these injectable cements did not show any noticeable changes in the phase evaluations compared to the noninjectable formulations. The researchers found the handling characteristic of this injectable cement to be very similar to the injectable form comprising porogen cement. Detailed handling characteristics and structural and morphological evolutions of these modified cements (porogen cement and porogen cement UBM) are currently being assessed and will be reported in subsequent reports.

Since injectable cements have been developed, implants of these cements in critical size defects in rabbit calvariae are currently being assessed by the research team. They have tested four different conditions. During the procedure, it was determined that there were no complications observed with regard to the formation and application of the cement in the defect site. In addition, it was observed that there was sufficient time for the initial setting to become effective. The researchers performed regular skull x-rays at three different time points after surgery to observe new bone formation or any changes in the defects over time. At 8 weeks after surgery, all rabbits from the first batch were euthanized and dissected, and the samples were fixed in 10% formalin. Microcomputed tomography (mCT) scans were obtained, and the data are currently being analyzed. The second batch of rabbit samples were fixed, and they will receive the mCT scan to analyze bone regeneration.

Key Research Accomplishments

- Developed and characterized several novel nanostructured apatitic bioactive bone cements and scaffolds containing NanoCaP carriers of growth factors based on the use of natural ECM-derived polymers.
 - Results from the in vitro assessment of the cements show that these materials are biocompatible.
- Initiated an in vivo screening experiment to rapidly determine if a given material will provide the best bone regeneration strategy.
 - The data show that the newly synthesized bioresorbable cement containing NanoCaPs with and without BMP-2 are very good candidates for further assessment as a therapy for bone regeneration.
 - The AFIRM funding will be focused on further developing these cements to gather more data and for obtaining FDA approval.
- Performed in vivo experiments in the rabbit calvarial model using four different treatment groups.
 - The calvarial samples were retrieved and mCT scans were obtained.
 - The data are currently being analyzed for analysis of new bone formation.



III: Craniofacial Reconstruction

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Conclusions

In conclusion, novel nanostructured apatitic bioactive bone cements and scaffolds based on the use of natural ECM-derived polymers containing NanoCaPs have been successfully developed and characterized. The inorganic cements with or without BMP-2 appear to perform very well in vitro as well as in vivo.

Research Plans for the Next 3 Years

Future efforts will be focused on expanding the preclinical in vivo experiments to further develop these cements and gather more data to proceed further for FDA 510(k) submission and approval. In vivo experiments using a rabbit calvarial model are currently being executed, and further analysis for bone regeneration will be conducted.

Planned Clinical Transitions

Clinical transition plans will initially focus on the cement alone to proceed further toward FDA approval.

This project was selected by the Department of Defense (DoD) to accelerate transition from the laboratory to clinical use. This funding will enable the researchers to perform all the necessary aspects needed for manufacturing the cement for human use, biocompatibility, and preclinical experiments under Good Laboratory Practice (GLP) conditions required for submitting a 510(k) FDA application.

The AFIRM Program will be critical to continue the developmental research and to gather preclinical data for the UBM-cement and cement BMP-2 systems. The initial in vivo screening results have shown excellent bone regeneration, and these data will be critical for the successful completion of the project and later FDA submission.

Regeneration of Bone in the Cranio-Mandibulo-Maxillofacial Complex Using Allograft Bone/Polymer Composites

Project 4.5.1a, RCCC

Team Leaders: Scott A. Guelcher, PhD (Vanderbilt University)

Project Team: Pamela Brown-Baer, DDS, Joseph C. Wenke, PhD (USAISR); Kasia Zienkiewicz, MS, and Jerald E. Dumas, BS (Vanderbilt University)

Collaborators: Subha Bhattacharyya, PhD (Osteotech, Inc.) and COL Robert Hale, MD (Brooke Army Medical Center)

Therapy: Injectable biomaterial for regeneration of craniofacial bone

Deliverable: Injectable allograft bone/polymer composite

TRL Progress: *Start of Year 1, TRL 3; End of Year 1, TRL 3; End of Year 2, TRL 4*

Key Accomplishments: The researchers identified the final formulation for Plexur LV® injectable

bone-void filler. They also identified lead candidate weight-bearing composites for testing in the mandible. They showed that Plexur LV allograft/polyurethane (PUR) bone void filler incorporating rhBMP-2 regenerated bone in the rabbit calvarial model.

Key Words: Composite, allograft, bone, polymer, polyurethane, injectable, mandible

Introduction

Injuries to the face and neck are common and have high morbidity. Recent studies have examined the battle-injury patterns and resource impacts of injuries for the two current conflicts, Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF). These studies have highlighted the significance of the injuries to the head and neck. Owens et al. reported that the frequency of head and neck injuries has increased in the current conflicts in Iraq and Afghanistan in comparison to previous wars.¹ In addition, this research showed that head and neck injuries account for 29.4% of all battle injuries sustained in OIF and OEF. Looking at the same cohort, Masini et al. found that head and neck injuries required 20% of all treatment resources, had the highest mean disability rating (52%), and will command 27% of all total projected benefit costs.²

Further research has studied the CMF battle injuries of OIF and OEF. Lew et al. characterized the facial

fractures in current conflicts and found that the highest percentage, 36%, were to the mandible. They also reported that 76% of the CMF battle injuries were classified as open fractures.³ The high incidence of open fractures and the associated high complication rate result in a large military patient population that requires improved treatments. This project focuses on evaluating these improved materials for treating traumatic bone defects. Considering the frequency and characteristics of these injuries, with the resultant mean disability rating, we hypothesize that the current surgical techniques are not sufficient to treat these injuries.

Currently available treatment options are not optimal. The current methods of CMF bone repair incorporate the use of autografts or allografts. New surgical techniques, such as distraction osteogenesis, have been successful in restoring smaller portions of the mandible. However, due to the size and the geometry of battle injuries, such surgery has limited application. Autografts have been the

1 Owens BD, Kragh JF, Jr., Wenke JC, Macaitis J, Wade CE, Holcomb JB. Combat wounds in Operation Iraqi Freedom and Operation Enduring Freedom. *J Trauma* 2008;64(2):295-9.
 2 Masini BD, Waterman SM, Wenke JC, Owens BD, Hsu JR, Ficke JR. Resource utilization and disability outcome assessment of combat casualties from Operation Iraqi Freedom and Operation Enduring Freedom. *J Orthop Trauma* 2009;23(4):261-6.
 3 Lew TA, Walker JA, Wenke JC, Hale RG. Characterization of craniomaxillofacial battle injuries sustained by U.S. service members in the current conflicts of Iraq and Afghanistan. *J Oral Maxillofac Surg* (in press).



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standard of care but have the downside of causing morbidity of the donor site, which is typically the iliac crest for smaller defects or CTAs from the fibula for larger defects. In addition, the autologous implants are subject to resorption and at times fail to integrate, especially without sufficient site vascularization.

Summary of Research Completed in Year 1

During the first year of the project, the research team fabricated injectable porous bone particle/polymer composites with tunable porosities, mechanical properties, and working times. They showed that these materials could remodel in a rabbit distal femur model. The researchers achieved sustained controlled release of osteogenic agent from the bone particle/polymer composites for up to 21 days. They fabricated low-porosity injectable bone/PUR composite cements with high bone content that produced wet compressive strengths of up to 60 MPa, more than 5 times stronger than the first-generation high-porosity material. The researchers demonstrated that their bone particle/PUR composites passed the ISO 10993 systemic toxicity test. In a non-survival test, they injected the bone particle/PUR bone void filler into a rabbit calvarial defect and found the working and tack-free times to be comparable to those observed in vitro.

Research Progress – Year 2

Remodeling of allograft/PUR composites in a rabbit calvarial model. The rabbit study has been completed at USAISR in collaboration with Pam Brown-Baer and Josh Wenke. In the study design, there were three treatment groups: (1) empty defect, (2) Norian injectable calcium phosphate bone cement, and (3) injectable allograft/PUR bone-void filler ($n = 10$ /treatment group). Two time points, 6 and 12 weeks, were investigated. **Figure III-3** shows a photograph of the material after injection and an x-ray image taken at sacrifice at 6 weeks. The low-magnification image (top) of the composite shows extensive cellular infiltration into the interior of the composite, and new bone formation near the host-bone interface. Higher magnification of regions near the host-bone interface (bottom) shows active remodeling,

including allograft resorption, osteoid formation, and new bone formation. Unremodeled allograft particles embedded in polymer matrix are also evident. Histological sections taken at 12 weeks showed that $>80\%$ of the polymer had resorbed (data not shown).

These observations show that resorption of the allograft creates pores into which cells subsequently migrate, thereby presenting an alternative pathway (in addition to migration through open pores) by which cells can infiltrate the composite. At USAISR, histological analysis of the brains showed no signs of inflammation on the dura for the allograft/PUR treatment group.

In a similar study, an allograft/PUR composite comprising 34 vol% allograft bone particles and 40% porosity was injected into bilateral femoral condyle plug defects in athymic rats. Histological sections showed regions of allograft resorption, cellular infiltration, osteoid formation, and active regions of bone remodeling (funded by Osteotech).

Remodeling of allograft/PUR + rhBMP composites in a rabbit calvarial model. To investigate the effects of rhBMP-2 on bone remodeling, allograft/PUR composites incorporating rhBMP-2 were injected into rabbit calvarial defects. rhBMP-2 loaded on a collagen sponge (Infuse) was used as the clinical control. Allograft/PUR composites incorporating 420 $\mu\text{g/mL}$ rhBMP-2 were injected into 15 mm rabbit calvarial defects. Histological sections at 6 weeks show extensive new bone formation along the

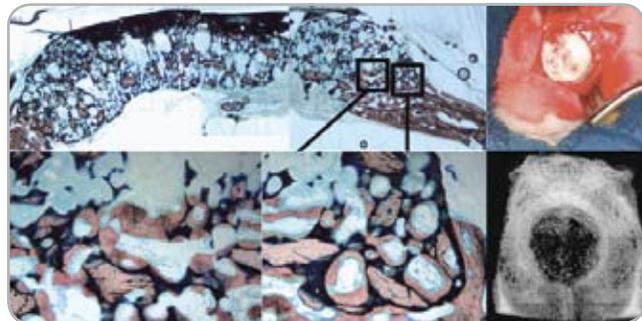


Figure III-3. Plexur LV allograft/PUR bone void filler injected into 15 mm calvarial defects in rabbits supports cellular infiltration and new bone formation. Higher magnification images of the regions shown in boxes in the top left figure are presented in the two bottom left images.

upper surface of the composites and near the host bone interface (Figure III-4). In many of the animals, new bone had completely bridged the upper surface of the defect. Higher magnification images (20X and 40X) show active bone remodeling by OBs and OCs, as well as the formation of new BVs. Interestingly, the rate of polymer degradation was higher compared to the samples without rhBMP-2, as evidenced by the absence of a significant amount of polymer at 6 weeks. In contrast, the collagen + rhBMP-2 samples exhibited no significant new bone formation and were comparable to the negative control.

Key Research Accomplishments

- Multiple batches of lysine triisocyanate poly(ethylene glycol) (LTI-PEG) prepolymer and polyester triol have been manufactured by Ricerca for FDA-required preclinical testing and ISO 10993 testing.
- Identified the final formulation for Plexur LV injectable bone-void filler.
- Injected Plexur LV allograft/PUR bone void filler into 15 mm critical size rabbit calvarial defects and found that it supported cellular infiltration and new bone formation and did not induce a severe inflammatory response.

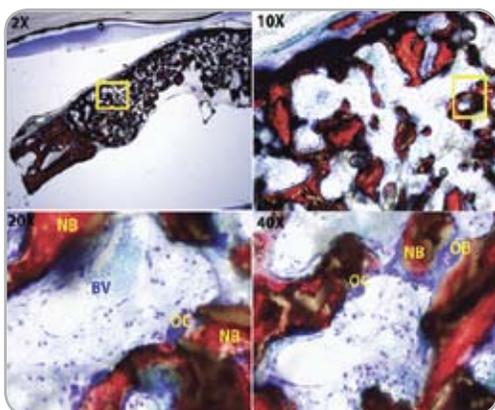


Figure III-4. Allograft/PUR (420 µg/mL rhBMP-2) injected into 15 mm calvarial defects in rabbits supported new bone formation. NB: new bone, OBs: osteoblasts, OCs: osteoclasts, BVs: blood vessels. In vitro release kinetics shows ~20% release of BMP-2 at 25 days.

- Injected Plexur LV allograft/PUR bone void filler incorporating rhBMP-2 into 15 mm critical rabbit calvarial defects and found that it promoted enhanced new bone formation relative to a collagen sponge + rhBMP-2.

Conclusions

The final formulation for the injectable bone void filler Plexur LV has been identified. The product is undergoing commercial development by Osteotech, Inc. Preclinical studies in rat and rabbit models of bone regeneration show that the material supports rapid cellular infiltration and new bone formation and that the material is a useful delivery system for rhBMP-2.

Research Plans for the Next 3 Years

The allograft/PUR composite technology has been licensed to Osteotech. Plexur LV is undergoing commercial development as an injectable bone-void filler for orthopaedic applications. A preclinical study in a rabbit femoral condyle plug defect is planned for summer 2010. Osteotech will complete the current Good Manufacturing Practice-compliant manufacturing development in 2010, followed by ISO 10993 testing and filing of a 510(k) device application. In addition to the injectable bone-void filler, an injectable weight-bearing bone cement is also under development for the reconstruction of mandibular defects, which require a biomaterial with superior mechanical strength. The performance of the weight-bearing cement will be validated in rabbit and sheep models of mandibular bone regeneration with and without rhBMP-2.

Planned Clinical Transitions

Plexur LV for orthopaedic applications will be commercialized via the 510(k) pathway without a clinical trial. Pending the successful approval of Plexur LV for long bone, a 510(k) for craniofacial applications will be filed. However, the craniofacial application will require a clinical trial for regulatory approval. Osteotech is commercializing the allograft/PUR technology, which it has licensed from Vanderbilt.



III: Craniofacial Reconstruction

Progress Reports: Bone Regeneration

Regeneration of Bone in the Cranio-Mandibulo-Maxillofacial Complex

Project 4.5.1b, RCCC

Team Leaders: Jeffrey Hollinger, DDS, PhD (CMU), Joachim Kohn, PhD (Rutgers University); and Michael Yaszemski, MD, PhD (Mayo Clinic)

Project Team: Jinku Kim, PhD, Aditi Sharma, Sean McBride, Pedro Alvarez, Abiraman Srinivasan, PhD (CMU); Aniq Darr, PhD, Hanshella Magno, Das Bolikal, PhD (Rutgers); Brett Runge, PhD, and Mahrokh Dadsetan, PhD (Mayo)

Collaborators: Bryan Lovas, Sunil Saini, PhD (Integra Spine, was Therics); and Amit Vasani, PhD (Cleveland Clinic)

Therapy: Bone regeneration in the CMF complex

Deliverable: Bioactive Tyr-PC or poly(ϵ -caprolactone fumarate) (PCLF) scaffolds for bone regeneration fabricated by salt leaching or solid free-form fabrication methods

TRL Progress: *Start of Year 1, TRL 3; End of Year 1, TRL 3; End of Year 2, TRL 4*

Key Accomplishments: The researchers have shown in vitro and in vivo biocompatibility of Tyr-PC and PCLF scaffolds and efficacy of the scaffolds containing rhBMP-2 for bone regeneration using the rabbit CSD calvarial model.

Key Words: Bone, regeneration, cranio-mandibulo-maxillofacial, tyrosine-derived polycarbonates, poly(ϵ -caprolactone fumarate) (PCLF), BMP, PDGF

Introduction

In a review of approximately 28,000 patients injured in Afghanistan and Iraq over a period of roughly 2 years, from late 2001 to early 2004, head and neck injuries ranked second to extremity injuries in incidence. Although the head and neck only comprise 12% of the body surface area, 64% of combat deaths are due to these injuries. Historically, head and neck injuries have accounted for 16% to 21% of all injuries in major U.S. conflicts (WWII, Korea, Vietnam) and a recent survey of OIF/OEF from 2001 to 2007 showed over 25% of the trauma to military service members occurred in the CMF anatomy. CMF injury is a serious problem not only in numbers but also in intensity because wounded warriors who suffer traumatic brain injury or blindness are more severely handicapped and have more difficulty returning to active duty and productive life than those with extremity injuries.

Massive bone loss of the CMF complex incurred in combat is typically reconstructed with either (1) nonresorbable synthetic materials, such as PMMA; (2) other synthetic polymers (HTR [hard tissue replacement]: poly(ethyl methyl methacrylate)); or (3) metallic devices (e.g., titanium) that may restore anatomical form and limited function. When available, (4) allogeneic and autogenous

grafts are options, and soft-tissue deficits may be treated with pedicled muscle, skin flaps, and allogeneic skin substitutes. Contemporary surgical solutions are profoundly inadequate to regenerate massive osseous avulsion in the CMF complex.

Restoring the CMF anatomy's form and function with a regenerative composition will be a significant improvement over contemporary options. However, available synthetic materials do not remodel and integrate with host tissue, become infected, and require extensive, multiple revision surgeries. Moreover, PMMA, HTR, and metallic devices lack controlled-delivery capability for biological factors (antibiotics, growth factors). Consequently, current treatment options do not regenerate tissues, and thus produce a less than satisfactory esthetic and functional outcome. Therefore, the wounded warfighter with CMF injuries often suffers low self-esteem, with uncertainty and discomfort about going out in public, on top of his or her significant functional tissue deficits.

The opportunities for Tyr-PCs and PCLF in such therapies promise to deliver regenerative therapeutics, and the biomaterials should degrade to innocuous, metabolizable products. Consequently, the expected outcome will be tissue regeneration and the restoration of form and function.

Summary of Research Completed in Year 1

During the first year of the project, the researchers prefabricated tyrosine-based copolymer scaffolds to fit into the CSD of rabbit skull. Their initial results showed that tyrosine-based three-dimensional scaffolds induced significant osteogenic differentiation and mineralization of pre-osteoblasts, compared to two-dimensional tissue culture surfaces. In vitro cyto-compatibility and cell attachment data with tyrosine-based scaffolds showed no cytotoxicity and robust cell attachment. The researchers initiated a study on the effect of ethylene oxide (EtO) sterilization on molecular weight loss and began nuclear magnetic resonance analysis of EtO-sterilized scaffolds. Gel permeation chromatography analyses of scaffolds that have been subjected to EtO sterilization and degassed showed approximately 35% loss in molecular weight. Finally, both injectable and implantable compositions based on two types of polyester copolymers were identified for craniofacial applications.

Research Progress – Year 2

In Year 2, the research team developed and fabricated Tyr-PC or PCLF-based scaffolds for bone regeneration

in a rabbit calvarial CSD model. They demonstrated both in vitro and in vivo performance of the Tyr-PC and PCLF scaffolds. Cell viability, proliferation, and osteogenic differentiation of hMSCs and MC3T3-E1 cells were determined. A rabbit calvarial CSD model was used to assess new bone formation with rhBMP-2-containing Tyr-PC and PCLF scaffolds.

Tyr-PC and PCLF formulations were synthesized and fabricated into three-dimensional porous scaffolds. Cell attachment on all tested scaffolds was robust, regardless of polymer compositions. The in vitro and in vivo assays revealed that Tyr-PC and PCLF scaffolds appeared to be biocompatible. Moreover, the efficacy of rhBMP-2-supplemented scaffolds was validated in the stringent 15 mm diameter rabbit CSD calvarial model. It was noteworthy that rhBMP-2 incorporation into the scaffolds significantly increased new bone formation, as determined by mCT and histology (**Figures III-5–7**).

At 6 weeks, in the rabbit CSD model (Figure III-7), the following outcomes were determined:

- A. The scaffolds containing rhBMP-2 had more trabecular bone volume than did scaffolds without rhBMP-2.

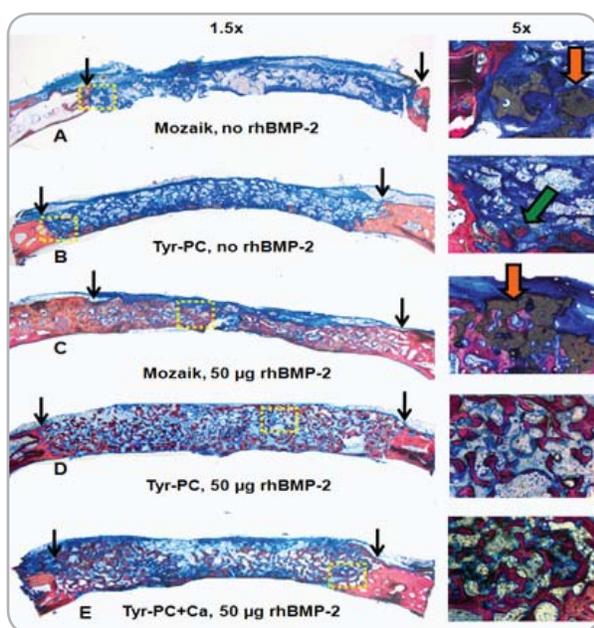


Figure III-5. Bone healing in rabbit calvaria with different scaffolds at 6 weeks post implantation. New bone formation was assessed by histology. The coronal plane of the histological section was stained with Sanderson's Rapid Bone Stain and counterstained with van Gieson's picrofuchsin: the image indicates soft tissue staining in blue, and bone staining in pink/red (panels on the left at 1.5x magnification; insert (yellow dotted lines) at 5x magnification on the right). Black arrows outline the defect site. Little bone formation was found in Mozaik scaffolds without rhBMP-2 (A) while the marginal bone formation can be seen along the dural surface and the host bone in the Tyr-PC scaffold (B, C, D, and E). All of the rhBMP-2 treated scaffolds revealed robust trabecular bone formation throughout the implanted scaffolds, which is consistent with mCT data. No macroscopic difference in new bone formation among the groups was found. In addition, a large amount of remaining Mozaik (orange arrows) scaffolds was still present in the defect site, regardless of rhBMP-2 treatment at 6 weeks post implantation. Conversely, Tyr-PC-based scaffolds were almost completely degraded over the same time period.



III: Craniofacial Reconstruction

Progress Reports: Bone Regeneration

- B. Tyr-PC+Ca scaffolds with 50 μ g of rhBMP-2 had significantly more trabecular bone volume than did other groups.
- C. The Mozaik scaffold with 200 μ g of rhBMP-2 had significantly more bone volume than Tyr-PC and PCLF+HA, but was not different than Tyr-PC+Ca with 200 μ g of rhBMP-2.
- D. The Mozaik scaffold with 200 μ g of rhBMP-2 had significantly more bone volume than the Mozaik scaffold with 50 μ g of rhBMP-2. For the other scaffolds, there was no significant difference between the use of 50 or 200 μ g of rhBMP-2.

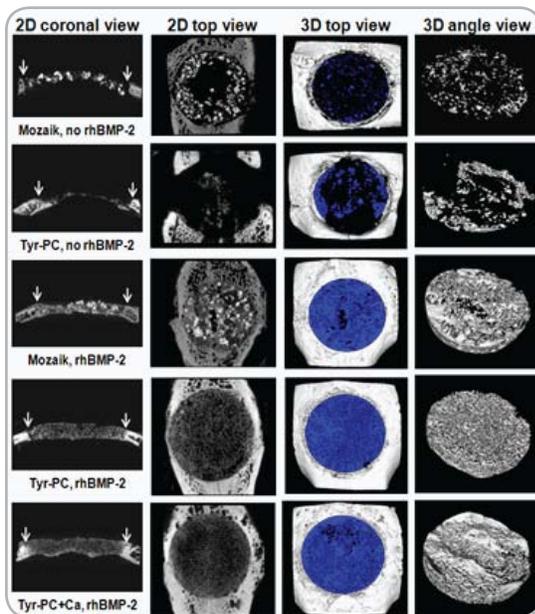


Figure III-6. mCT images of rabbit calvarial bone regeneration. Two-dimensional sections (coronal and transverse) of mCT calvarial specimens of each treatment showed that without rhBMP-2, Mozaik and Tyr-PC scaffolds barely induced new bone formation, whereas rhBMP-2-treated scaffolds (50 μ g/scaffold) appeared to induce substantial bone regeneration at 6 weeks. White arrows in the first column identify the defect site. Remaining β -TCP fragments can be seen in the two-dimensional transverse images of Mozaik scaffolds. three-dimensional reconstructed images confirmed that new bone formation in the defect site almost completely occupied the defect site when the scaffolds were treated with rhBMP-2.

Key Research Accomplishments

- Produced in vitro and in vivo performance data of scaffolds made from Tyr-PC and PCLF.
 - Also showed efficacy of Tyr-PC and PCLF scaffolds containing rhBMP-2 for bone regeneration, using the rabbit CSD calvarial model.
- Completed the second round of rabbit surgeries to determine the efficacy of Tyr-PC scaffolds (including Tyr-PC+CaP) containing rhPDGF-BB in a CSD rabbit calvaria model.

Conclusions

Scaffolds made from Tyr-PC or PCLF and rhBMP-2 were shown to regenerate bone in the stringent rabbit calvarial CSD model in 6 weeks. The porous three-dimensional scaffolds were fabricated using a combination of salt-leaching and phase separation techniques. In the rabbit CSD model, Tyr-PC scaffolds containing rhBMP-2 were gradually resorbed and replaced with new bone over the 6-week study period; whereas PCLF scaffolds degraded slowly, thus having less new bone formation in a rabbit calvarium when compared to Tyr-PC scaffolds.

Research Plans for the Next 3 Years

This project is on target. The focus of the project in the next 3 years will be on CMF anatomical zones.

In Year 3, the researchers will assess the efficacy of Tyr-PC scaffolds containing rhBMP-2 and/or rhPDGF-BB using rabbit and goat calvaria and mandible models. A total of 80 goats will be used to determine the efficacy of rhBMP-2-containing Tyr-PC scaffolds using goat CSD calvarial and mandibular models. A total of 35 rabbits will be used to determine the efficacy of dual delivery of rhBMP-2 and rhPDGF-BB using the rabbit CSD calvarial model (TRL 5).

If the researchers are successful with the goat model in Year 3, they will determine if a nonhuman primate model may be needed for subsequent animal testing prior to a Phase 1 human clinical trial. If the team decides on the need for such a model, they will implement it in Year 4

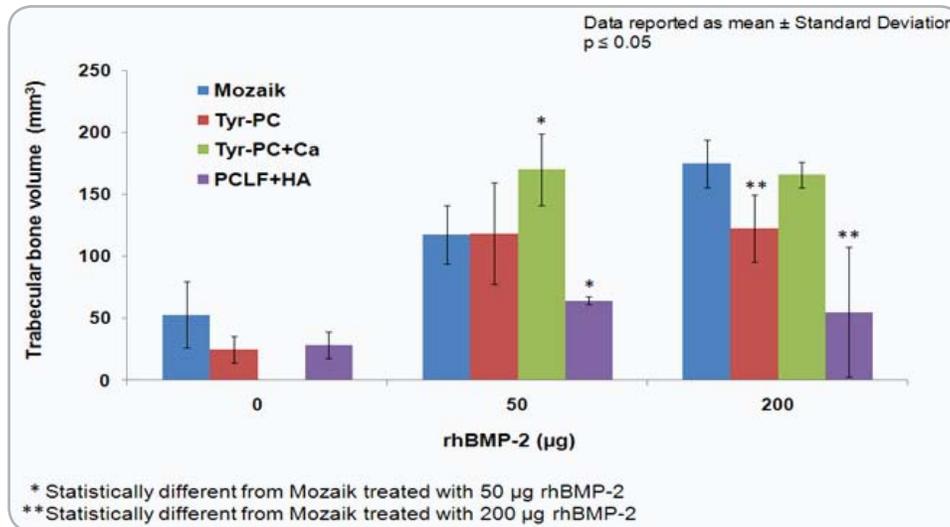


Figure III-7. Trabecular bone formation in CSD rabbit calvaria at 6 weeks post implantation with experimental scaffolds, assessed by mCT image analysis. Data are reported as a mean ± standard deviation for n=3-5.

and develop a CMF product for a Phase 1 clinical trial in Year 5. If the goat model is sufficient to verify safety and efficacy, the team will prepare a Phase 1 clinical study protocol in Year 4 and will finish the study in Year 5 (TRL 6).

Planned Clinical Transitions

The researchers may deliver a bone regeneration therapy for a small Phase 1 clinical trial as early as Year 4 and will prepare a regulatory strategy with both the industry partner Trident and the Rutgers University-retained regulatory consultant. The regulatory strategy may include both an Humanitarian Device Exemption and a combination of IND/Investigational Device Exemption (IDE).

Corrections/Changes Planned for Year 3 and Rationale for Changes

In Year 2, CMU and Rutgers have shown the potential of Tyr-PC as a bone regeneration therapy. The researchers plan to determine efficacy of rhBMP-2-containing Tyr-PC scaffolds using goat CSD calvarial and mandibular models in Year 3. They will also determine the efficacy of dual delivery of rhBMP-2 and rhPDGF-BB using the rabbit CSD calvarial model in Year 3.



III: Craniofacial Reconstruction

Progress Reports: Bone Regeneration

Improving Cell Engraftment for Bone Repair

Project 4.1.7, USAISR

Team Leaders: Chris Rathbone, PhD and Joseph C. Wenke, PhD (USAISR)

Therapy: MSC transplantation

Deliverable: A strategy for using environmental and/or pharmacological preconditioning of MSCs for the treatment of muscle and bone injuries

TRL Progress: *Start of Year 1, TRL 3; End of Year 1, TRL 3; End of Year 2, N/A*

Key Accomplishments: Due to the vivarium closure and short period of funding, accomplishments are limited. However, the researchers successfully isolated BMSCs and ASCs using standard isolation procedures when tissues were available. In addition,

they isolated BMSCs using Magnetic Activated Cell Sorting (MACS) from transgenic animals and confirmed their feasibility for use in imaging. They also attained regulatory approval for animal studies.

Key Words: Stem cells muscle, bone, injury

Introduction

The majority of combat wounds experienced by soldiers during the current conflicts are to the extremities, and a significant portion of these extremity injuries are fractures. Delayed union or nonunion is a well-known complication of fractured bone, which is a problem that greatly diminishes the functionality of the affected limb. Severe lower extremity injuries, which are common to soldiers on the battlefield, are often nonunion.

Currently, the most common treatment for fractures and delayed or nonunion is the use of an autogenous bone graft. Undesirable effects of this treatment include the need for an additional surgical procedure and donor-site morbidity. To overcome these insufficiencies, several types of scaffolds are being developed, and the use of growth factors to promote healing is being explored. However, the successful repair and regeneration of damaged tissue, especially traumatic injuries where a frank loss of tissue occurs, will likely involve the use of MSC delivery, either alone or in conjunction with scaffolds and/or growth factors since stem cells offer several benefits that cannot be delivered with only a drug or scaffold.

To maximize the use of MSCs for bone repair, it is important to address several questions related to their delivery. First, a pertinent question is whether ASCs or BMSCs are better for bone repair. There are point-of-care devices available for both stem cell types that give

clinicians the option of choosing. However, each stem cell type has its logistical and biological advantages and disadvantages. Regardless of the clinician's preference, it would be optimal to maximize their use by choosing the best culture strategy and potentially preconditioning treatment to obtain the maximum benefit out of the stem cells. A second relevant question is to address whether freshly isolated or cultured stem cells are superior for improving bone repair. Although culturing and expanding cells in culture have the advantage of increasing the number of cells available for transplantation, it is important to determine if this culture expansion is necessary or detrimental for bone repair. Finally, it has been reported that MSCs have significant paracrine effects when implanted as they have the ability to secrete a vast array of growth factors and recruit other cells to the site of injury.

The experiments in this project will attempt to determine preconditioning strategies that can augment the paracrine effects of MSCs while improving their survivability. Importantly, the preconditioning strategies tested will be those that are currently FDA approved or those for which FDA approval is feasible.

The overall experimental approach can be subdivided into two studies:

- The first study will be performed in vitro to determine the optimal cell type, culture condition, and preconditioning treatment based on markers of angiogenesis,

resistance to apoptosis, and homing and retention. The markers chosen for each of these processes involved in regeneration will have been well described in the literature, associated with, and sometimes at least in part responsible for cell engraftment.

- Based on the results from study 1, study 2 will be completed to test the effect of the chosen cell type, culture condition, and preconditioning treatment on cell engraftment and bone regeneration in vivo using a mouse calvarial defect model. Transgenic animals will be used as donor animals and cell survival will be monitored in vivo using an Xenogen imaging system.

Research Progress – Year 1

Note: This study was just funded during the past year.

Due to the closure of the vivarium, it was not possible to perform complete experiments on primary ASCs and BMSCs. By extension, it was not possible to use freshly isolated cells. However, preliminary experiments were completed using immortalized stem cells to begin to optimize dosages. These experiments will be followed up using freshly isolated and cultured ASCs and BMSCs. The drug doses will again be optimized in primary cells. When possible, tissue from other investigators was obtained to optimize mouse stem cell isolation procedures.

ASCs and BMSCs were isolated from inguinal fat pads and long bones of mice, respectively, and were plastic-adherent and their morphology consistent with MSCs derived from mice. These techniques used for isolation are sufficient to answer the questions in the proposal; however, BMSCs were also isolated from transgenic

mice (FVB-Tg[CAG-luc,-GFP]) using MACS, and their ability to be used for cell survival and tracking was confirmed using a muscle injury model that is well established at USAISR. This is the method of isolation to be used for future studies on bone regeneration. The use of these transgenic mice and the calvarial defect model will allow for in vivo measurements of cell survival in a bone defect and will also allow for the determination of bone regeneration. Regulatory approval for animal studies was attained and will commence following the results of study 1.

Key Research Accomplishments

Due to the vivarium closure and the short period of funding, accomplishments are limited.

- Isolated BMSCs and ASCs using standard isolation procedures.
- Isolated BMSCs using MACS from transgenic animals and confirmed their feasibility for use in imaging.
- Attained regulatory approval for animal studies.

Conclusions

The majority of the in vitro work has been limited to immortalized cells; however, stem cell isolation procedures have been worked out to allow for the transition to primary cells. The results of the in vitro analyses will be used in in vivo models. In this regard, the ability to isolate BMSCs from transgenic mice (FVB-Tg[CAG-luc,-GFP]) and track them in vivo was confirmed using a muscle injury model that is well established at USAISR, which supports the feasibility of using this experimental model as described in the proposal.



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Progress Reports: Soft Tissue Regeneration

Soft Tissue Regeneration

Projects 4.1.4 and 4.1.5, WFPC

Team Leader(s): Peter Rubin, MD, Kacey Marra, PhD (University of Pittsburgh); David Kaplan, PhD (Tufts University); James Yoo, MD, PhD, and Sang Jin Lee, PhD (Wake Forest University)

Project Team Members: Evangelia Bellas, BS, Bruce Paniliatis, PhD, Natasa Miljkovic, MD, PhD (Tufts University); Mostafa Ramadan, MD, Han Li, MD, Rachel Hoyer, Donna Ward, PhD (University of Pittsburgh); Chang Mo Hwang, PhD, Weijie Xu, PhD, and Tom Shiner, BS (Wake Forest University)

Collaborator(s): Jeff Gimble (Louisiana State University) and Steve Badylak (University of Pittsburgh)

Therapy: Long-term soft tissue restoration of traumatic defects with cell based-degradable injectable and implantable scaffolds, resulting in sustained shape and volume over time

Deliverable(s): Vascularized connective tissue and fat pad (Years 1 and 2). Development of injectable/implantable vascularized soft tissue composed of connective tissue and fat (Years 2 and 3). Demonstration of the applicability of using injectable/implantable soft tissue composites for limb, burn, and craniofacial applications in a large animal model (Years 3 and 4). Initiation of clinical testing of small defects soft tissue replacement (Years 4 and 5).

TRL Progress: *Start of Program, TRL 2; End Year 1, TRL 3; End Year 2, TRL 4*

Key Accomplishments: The researchers have conducted numerous preclinical studies using biomaterials, ASCs, and fibroblasts. They demonstrated the feasibility of using cell-seeded silk scaffolds for long-term soft tissue restoration. Vascularized

adipose constructs maintained shape and volume, and functional adipose outcomes, in vitro over a 6-month period. The researchers have initiated clinical studies using lipoaspirate as a fat graft. They have also developed and refined an injectable hydrogel system based on hyaluronic acid (HA) and fibrin as scaffold materials. When combined with cellular elements, these hydrogels form stable soft tissue constructs. Importantly, both mechanical properties of the hydrogels and their ability to release angiogenic factors have been tested.

Keywords: Adipose tissue, connective tissue, adipose-derived stem cells, fibroblasts, lipoaspirate, regeneration, silk scaffold, injectable hydrogel

Introduction

The current standard of care for fat depot regeneration has relied on three approaches: (1) surgical flaps that move adipose tissue from one site to another while maintaining an intact blood supply, which is associated with medical risks, high costs, scarring, and functional loss; (2) artificial fillers, such as Teflon paste, silicone implants, and bovine collagen, that lack any metabolic activity; and (3) free fat transplants that involve the implantation of autologous adipose tissue fragments without an intact blood supply. Often, the free fat transplants lose volume over time, which is attributed to traumatic rupture, avascular necrosis, apoptosis of the adipocytes, inflammation secondary to cell death, fibrosis and contraction of the graft, and/or delipidation of the adipocytes with subsequent volume loss.

The restoration of traumatic soft tissue defects must start with a strategy that will restore tissue size and shape to near normal dimensions. Furthermore, this goal must be addressed with a strategy that will provide sustained retention of such improvements for at least 1 year while the body gradually remodels and regenerates the site into seminormal or normal soft tissue structure and function. Nondegradable scaffold systems have been used to provide rapid restoration of morphological features, but these fail to integrate and regenerate native tissue, thus remaining a barrier to tissue function. The use of degradable scaffold systems based on collagen or PLGA offer benefits, but these tend to fail within about 3 months due to premature degradation and subsequent loss of transport, leading to necrosis and collapse of the soft tissue.

The main scientific approach of this project involves the use of autologous ASCs and fibroblasts, combined with

carrier biomaterials, to achieve vascularized soft tissues. The specific aims are: (1) engineering of vascularized connective tissue and fat pad, incorporating cellular elements and custom-designed biomaterial scaffolds; (2) development of implantable and injectable composite vascularized soft tissue; (3) demonstration of the applicability of using implantable and injectable soft tissue composites for limb, burn, and craniofacial applications in a large animal model; and (4) initiation of clinical testing of soft tissue replacement for small defects.

Summary of Research Completed in Year 1

During the first year of the project, the researchers developed four types of silk fibroin scaffolds and demonstrated that each scaffold could support the growth of soft tissue. They developed a collagen gel delivery system that releases vascular endothelial growth factor with the goal of enhancing vasculogenesis *in vivo*. They implanted cell-hydrogel constructs in athymic mice and began to examine the dimensional changes in the mice at various time points post implantation. They also developed polymeric microspheres containing proteins known to promote angiogenesis; these microspheres are expected to enhance tissue formation by the cell-hydrogel constructs.

Research Progress – Year 2

In the past year, the research team has conducted numerous preclinical studies using injectable and implantable biomaterials, ASCs, and dermal fibroblasts. They had previously shown that unseeded silk scaffolds could maintain their size and shape for longer than 1 year in a subcutaneous rat model. The researchers have now determined that silk particles mixed with lipoaspirate have the potential for adipose tissue repair. When the optimal number of silk particles was used, there was minimal fibrous tissue reaction. The particles appeared to serve as a “silk skeleton,” giving structure and mechanical strength to the lipoaspirate. Notably, silk particles promoted vascularization in the lipoaspirate.

The research team found that silk gel mixed with lipoaspirate also has the potential for adipose tissue repair. When the optimal amount of silk gel was used,

a minimal fibrous tissue reaction was observed. Silk gel promoted vascularization in lipoaspirate scaffolds. After 6 weeks, composite scaffolds composed of silk gel and lipoaspirate retained their weights more than with lipoaspirate alone.

The researchers also demonstrated the long-term (6-month) feasibility of vascularized connective tissue and adipose constructs with measurable adipogenic and vascular outcomes. Preliminary experiments showed that dynamic *in vitro* co-cultures on silk scaffolds maintained morphology and tissue development. Pre-culturing endothelial cells under low oxygen (5%) conditions led to better cell survival than normal oxygen (21%) levels. Preliminary studies on endothelial cell sources showed differences in cell proliferation.

Additionally, the researchers have developed and refined an injectable hydrogel system based on HA and fibrin as scaffold materials. When combined with cellular elements (dermal fibroblasts), these hydrogels have also formed stable soft tissue constructs. Importantly, both the mechanical properties of the hydrogels and their ability to release angiogenic factors have been tested. Preliminary studies showed that addition of angiogenic factors promoted neovascularization and improved dimensional stability of the cell-hydrogel constructs. Preliminary studies also showed that gel porosity affected new tissue formation as well as improved integration with the host tissue.

Overall, these accomplishments are on track with the milestones outlined for this project, and the project is well on the way to developing clinically useful injectable and implantable soft tissue therapies that can improve facial deformities with greater precision.

Key Research Accomplishments

- Developed injectable hydrogels with different formulations.
 - When combined with dermal fibroblasts, these hydrogels form stable soft tissue constructs.
- Demonstrated the feasibility of using cell-seeded silk scaffolds (injectable and implantable) for long-term soft tissue restoration.



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- Determined that vascularized connective tissue and adipose constructs maintained their shape and volume, and functional adipose outcomes, in vitro over a 6-month period.
- Initiated clinical studies using lipoaspirate as a fat graft.

Conclusions

Silk-based scaffolds are able to maintain cellularity and sustained morphology for at least 6 months in vitro. In vitro co-culture/silk constructs maintained functional outcomes for adipose tissue. In vivo studies with co-cultured constructs as well as lipoaspirate infused silk scaffolds are progressing well and demonstrate the utility of a silk scaffold mechanical skeleton within the soft tissue. Injectable hydrogels (fibrin/HA-based) with cells are able to maintain structural integrity and tissue volume. This hydrogel system can present sustained release of growth factors that resulted in the formation of neovascularization and improved dimensional stability of the engineered tissue.

Research Plans for the Next 3 Years

Continued optimization of the co-culture/silk scaffold constructs both in vitro and in vivo is planned. In addition, continued optimization of the injectable cell-hydrogel system, both in vitro and in vivo is planned. Ongoing work is continuing in dynamic culture systems to improve cellular ingrowth and vascularity of the construct. In vivo studies of co-culture constructs and lipoaspirate-infused silk scaffolds are in progress and will be conducted for up to 2 years in a mouse model. The researchers will determine the optimal combination of their successful biomaterials and cellular elements and finalize the clinical therapy model. This will involve integrating engineered adipose tissue (Rubin, Marra, and Kaplan) with the connective tissue system (Yoo and Lee).

Planned Clinical Transitions

The ultimate goal of this project is to produce long-term soft tissue restoration of traumatic defects with cell based-degradable injectable and implantable scaffolds, resulting in sustained shape and volume over time. With this project currently at TRL 4, it is anticipated that a clinical trial will be ready soon. In fact, proposals for clinical translation will be pursued again this coming year to move the current technology ahead. Based on recent success with new silk-based medical devices in industry, a path forward for this clinical step is feasible and achievable once resources are identified.

Bioreactors and Biomaterials for Tissue Engineering of Skeletal Muscle

Project 4.1.6, WFPC

Team Leader(s): George J. Christ, PhD (WFIRM)

Project Team Members: James Yoo MD, PhD, Sang Jin Lee, PhD, Benjamin T. Corona, PhD, and Masood A. Machingal (WFIRM)

Collaborator(s): Tom Walters, PhD (USAISR) and David Kaplan, PhD (Tufts University)

Therapy: Skeletal muscle reconstructive procedures required to repair complex facial injuries

Deliverable(s): A skeletal muscle tissue implant capable of generating clinically relevant force/tension.

TRL Progress: *Start of Program*, TRL 2; *End Year 1*, TRL 3; *End Year 2*, TRL 3

Key Accomplishments: The researchers established a rodent model of surgically created trauma (i.e., VML) in which 50% of the latissimus dorsi (LD) muscle is removed. In this model, the missing tissue was replaced with TE-SKM or with only a scaffold (i.e., no cells). Alternatively, no muscle repair was performed. Within 2 months of implantation of TE-SKM, the retrieved LD muscle containing the tissue engineered construct was capable of generating nearly 75% of the maximal contractile response observed in native

LD muscle from the same animal. This observation stands in stark contrast to findings with retrieved LD muscle that contained only the scaffold or was unrepaired. Overall, the research team found that the first-generation TE-SKM was capable in their model of physiologically significant, and therefore, clinically relevant contractile responses within 2 months of implantation.

Keywords: Tissue engineering, skeletal muscle, bioreactors, muscle precursor cells, biomaterials

Introduction

Current management of tissue coverage and augmentation involves the use of existing host tissue to construct muscular flaps or grafts. In many instances, this approach is not feasible, delaying the rehabilitation process as well as the restoration of tissue function. In fact, the inability to engineer clinically relevant functional muscle tissue remains a major hurdle to the successful skeletal muscle reconstructive procedures required to repair the complex facial injuries suffered by warfighters. The long-term goal of this project is the creation of an autologous skeletal muscle tissue implant capable of generating clinically relevant force/tension. This proposal will continue the development of a technology to further probe the feasibility and applicability of creating contractile skeletal muscle tissues through use of a bioreactor system in conjunction with novel biomaterials/scaffolds and optimized bioreactor protocols. The overall goal is to use this technology in injured soldiers to assist with rehabilitation and restoration of skeletal muscle function. The initial clinical application will be repair and restoration of craniofacial battlefield wounds.

The Specific Aims of this project are:

Specific Aim 1: Demonstrate “proof of concept” for engineering functional (i.e., contractile) skeletal muscle tissue for craniofacial defects (Years 1-2).

Specific Aim 2: Conduct feasibility study – implantation of engineered skeletal muscle in a rat skeletal muscle replacement model (Years 2-3).

Specific Aim 3: Conduct feasibility study – implantation of engineered skeletal muscle in a large animal model of craniofacial defects (Years 4-5).

Specific Aim 4: Determine the feasibility of using biopsies from human patients for the engineering of functional skeletal muscle (Years 3-5).

Summary of Research Completed in Year 1

During the first year of the project, the researchers developed an SOP for the use of rat muscle precursor cells (MPCs) in their experiments. They generated an organized muscle tissue from human MPCs in vitro on (1)



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a decellularized scaffold and (2) an aligned polycaprolactone (PCL)/collagen scaffold. They demonstrated myotube formation both in culture and on the surface of the aligned PCL/collagen scaffold. They introduced the LD defect model and completed retrieval and physiological analysis of the first control defect. The researchers also established an assay for the neuromuscular junction. They created a custom-designed seeding chamber for muscle scaffolds using an FDA-approved pharmaceutical-grade silicone rubber. Finally, they completed initial characterization of engineered tissue function in vivo, which indicated the necessity to further modify/optimize the PCL/collagen-based scaffold system.

Research Progress

The two major goals/tasks for this past year were to (1) complete development of the mouse LD VML model for functional replacement and (2) implant TE-SKM constructs generated using the SOPs established in the first year of this grant (Figure III-8) to the site of injury on the LD and begin a rigorous characterization of the retrieved tissue, as well as the native LD muscle from the same animal.

The primary research focus over the past year has been to characterize the ability of first-generation TE-SKM constructs to restore tissue function in the rodent LD model of VML. To characterize muscle tissue engineered in vivo, development of a mouse LD VML model for implantation of TE-SKM constructs was finalized. Briefly, in this model the medial 50% of the native LD is excised, leaving only half of the endogenous LD muscle. A TE-SKM construct is then sutured in place of the excised tissue. Of note, following surgery, mice exhibit normal ambulation with the use of the surgery-side arm. Following either 1 month or 2 months after implantation, the surgically defected LD muscles with TE-SKM construct implantation are explanted. At these times, visual inspection of injured LD muscle gross morphology with TE-SKM constructs implanted illustrates construct

fusion with the host tissue as well as degradation of the bladder acellular matrix (BAM) scaffold.

However, the driving question of this body of research is: *Does the implantation of a TE-SKM construct enhance functional restoration in the rodent LD VML model?*

Figure III-9 demonstrates the force produced by isolated LD muscle with and without TE-SKM constructs in an organ bath. Maximal tetanic force generated by the TE-SKM repaired group after 1 month of implantation (14.7 ± 7.3 g) was not statistically greater than the nonrepaired group at 1 month (10.5 ± 4.5 g, $p=0.22$). However, at 2 months, the maximal tetanic force generated by the TE-SKM repaired group (22.71 ± 5.0 g), which is ~75% of that observed in native LD muscle (30.6 ± 4.1 g) was significantly greater than the nonrepaired group at 2 months (14.2 ± 5.8 g; $p < 0.05$). Additionally at 2 months, the TE-SKM repaired group generated greater force than the group repaired with nonseeded scaffold (12.2 ± 3.2 g; $p=0.022$). Therefore, implantation of TE-SKM constructs results in a significantly greater force recovery 2 months after implantation than would occur without replacement at all or with replacement of BAM

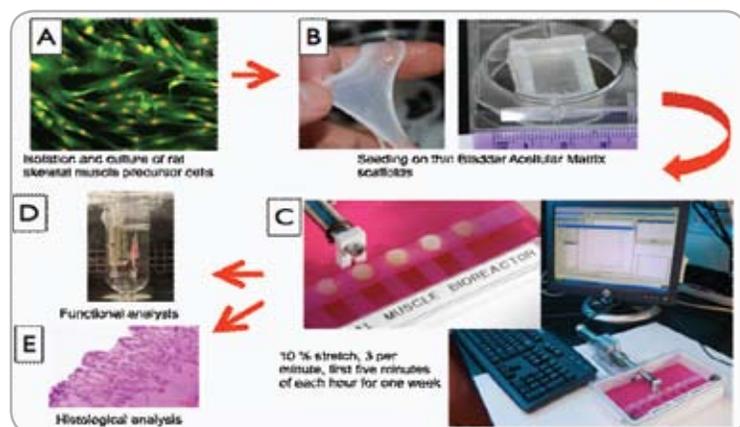


Figure III-8. SOP for creation of TE-SKM. Rat muscle progenitor cells are isolated (A) and expanded until passage two when they are seeded onto scaffolds (B). Following 10 days of static culture, the seeded scaffolds are then preconditioned in a skeletal muscle bioreactor for 1 week (C). At this stage, the scaffolds are then implanted in vivo into a rodent model. Following a given time interval, tissue-engineered skeletal muscles are explanted and analyzed for function in an in vitro organ bath (D) as well as tissue morphology via histological and immunohistochemistry analyses (E).

scaffold without seeded cells or bioreactor preconditioning. The researchers also found that sarcoplasmic reticulum (SR) calcium release appeared to be intact in retrieved tissue with a bioengineered construct.

Key Research Accomplishments

- Optimized the LD VML defect model.
- Characterized the histology and innervation of the native mouse LD muscle.
- Recovered approximately 75% of maximal native LD tetanic muscle contractility (isometric) within 2 months of implantation of TE-SKM in a 50% VML rodent model.
- Observed little or no difference in contractility to electrical field stimulation in what may be considered the majority of the physiologically relevant levels of submaximal stimulations.
 - Similar results were not observed for scaffold alone or no repair.
- Demonstrated that SR calcium release was intact in retrieved tissue with a bioengineered construct.

Conclusions

Significant progress has been made during the first 2 years of funding, and completion of all milestones/deliverables remains on target. All assays and SOPs are now in place for rapid completion of the POC mouse

studies and quick translation in the first quarter of the coming year to a more clinically relevant rat model. The most important POC accomplishment this past year is the demonstration that the developed TE-SKM technology is capable of producing physiologically significant, and therefore, clinically relevant, contractile responses within 2 months of implantation in a mouse LD VML model.

Research Plan for the Next 3 Years

In Year 3, the researchers plan to complete their work with the mouse LD VML model. They will also begin a POC study, in collaboration with Dr. Walters at USAISR, with the larger defect rat tibialis anterior (TA) muscle VML model, which is a thicker muscle than mouse LD muscle and approximates facial muscles such as the zygomaticus. At the beginning of Year 4 through Year 5, a large animal feasibility study for functional recovery of skeletal muscle surgical defects in dog facial muscle is planned. Additionally, research efforts will continue for the identification of a suitable replacement for the originally proposed PCL/collagen scaffold that remodels too slowly for the proposed use in TE-SKM (4+ months required for significant remodeling). In the interim period, the BAM scaffold will continue to be used for POC work. A recent collaboration with Dr. David Kaplan at Tufts will provide additional options with respect to the source of biomaterials available for this purpose (e.g., silk-based

scaffolds). In addition, the possibility remains for the continued use of a modified PCL/collagen-based electrospun scaffold with Drs. Yoo and Lee, who are co-investigators on this project. Moreover, in the third year of this work the researchers will begin to document the applicability of human muscle progenitor cells for restoration of skeletal muscle function in the rodent VML model.

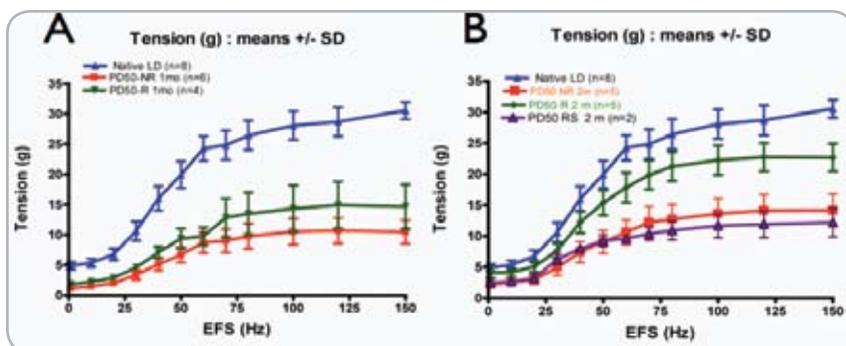


Figure III-9. Contractile response of paired skeletal muscle. Functional response of isolated LD muscles 1 month (A) and 2 months (B) after implantation. Muscles belonged to the following groups: TE-SKM repaired defect (PD50-R; green), non-repaired defect only controls (PD50-NR; red), native contralateral LD (blue), and defect repaired with non-seeded scaffolds only (PD50 RS; purple).



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Planned Clinical Transitions

Development of this technology to the TRL 4 level will occur by completion of the original 5-year plan of study. The BAM scaffold will be studied for potential clinical development of this technology. This seems a reasonable approach given that several porcine-derived ECM products/scaffolds have already received FDA approval (e.g., Medeor Matrix, Strattice, and SIS). However, FDA activity on this matter will be monitored in consultation with the WFIRM Regulatory Affairs Director. As described elsewhere in this report, over the next 12-15 months parallel development of other FDA-approved biomaterials (i.e., silk fibers and PCL/collagen) will be pursued. The rationale is that it will provide a potential alternative biomaterial in the event that unexpected regulatory hurdles associated with the use of porcine-

derived products are encountered. A firm commitment to the most suitable biomaterial by the second quarter of Year 4 is required to ensure completion of the proposed/required large animal feasibility studies.

Corrections/Changes Planned for Year 3

The proposed corrections/changes to this project are as follows:

- Conduct POC studies in a larger defect rat TA VML model.
- Evaluate novel scaffold systems for bioengineered muscle, i.e., electrospun silk-based scaffolds and modified electrospun PCL/collagen scaffolds.

Develop Innervated, Vascularized Skeletal Muscle

Project 4.1.2, RCCC

Team Leaders: Cathryn Sundback, ScD and Joseph Vacanti, MD (MGH)

Project Team: Craig Neville, PhD, Mei Li, MS, Eric Finkelstein, PhD, Caitlyn Dickinson, BS, Kenneth Rask, MBA (MGH); Douglas Henstrom, MD, Tessa Hadlock, MD (Massachusetts Eye and Ear Infirmary, [MEEI]); Sanjeeva Murthy, PhD, and Joachim Kohn, PhD (Rutgers)

Therapy: Replacement of severely injured facial skeletal muscles

Deliverable: Innervated, vascularized skeletal muscle

TRL Progress: *Start of Year 1, TRL 2; End of Year 1, TRL 2; End of Year 2, TRL 2*

Key Accomplishments: The researchers engineered vascularized skeletal muscle in vitro by co-seeding endothelial cells and MSCs along with muscle cells in the three-dimensional muscle fabrication process. They developed an innervation model

in immunocompromised rats and demonstrated effective innervation in control studies in which neuromuscular junctions were re-established in denervated native muscle. Finally, they developed prototype scaffolding to support scale-up of the engineered muscles from the size scale of human muscle fascicles to an orbicularis oculi.

Key Words: Tissue engineered skeletal muscle, vascularization, innervation, fibrin

Introduction

Blast injuries of the eye and eyelid significantly impact the quality of life of the injured warfighter. A severely damaged orbicularis oculi prevents eyelid closure, ultimately resulting in blindness. No autologous donor sites for soft tissue transfers exist for this muscle so only tissue engineering holds the promise of fabricating a replacement muscle from a patient's own cells.

The researchers initially engineered a physiologic immature muscle of a size that approximates a muscle fascicle (subunit). During the past year, they evaluated this immature muscle for vascularization and innervation as key steps toward engineering functional replacement facial muscle.

Specifically, the deliverables for the Year 2 of the project were to:

- Establish a prevascular network within the immature muscle and demonstrate effective vascularization in vivo.
- Characterize innervation of the immature muscle upon in vivo implantation.

- Develop scaffolding to support muscle implantation and scale-up as well as allow neurovascular invasion from the host. These scaffolding studies are being funded through a leveraged Congressionally Directed Medical Research Programs-funded program.

Summary of Research Completed in Year 1

During the first year of the project, the researchers tested biodegradable polymer scaffolds that improved the ability to handle engineered myoids for skeletal muscle regeneration. They produced vascular-like networks and immature muscle in vitro using biopolymer gels. They employed MSCs to support the formation of the prevascular network. They also implanted engineered immature muscles into adipose tissue of mice and found explanted muscle constructs to continue to express muscle contractile proteins.

Research Progress – Year 2

Vascularization. Immature muscles were produced in vitro by seeding and proliferating a co-culture of myoblasts and fibroblasts (mouse embryonic fibroblasts [MEFs]) on bovine fibrin and then differentiating the myoblasts to myotubes. Contraction of the fibrin gel induced



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the myotube sheet to align and roll, ultimately forming an immature muscle. Fibrin supported cellular proliferation and differentiation and readily degraded during muscle construct self-assembly; inhibition of fibrin degradation was found to prevent cell sheet rolling and ultimately myotube maturation.

To support rapid perfusion of the muscle construct upon implantation, a prevascular network was established in vitro within the engineered immature muscle. A co-culture of human endothelial cells (human umbilical vein endothelial cells [HUVECs]) and MSCs was co-seeded with the muscle cells (myoblasts and MEFs) to form an endothelial-lined network within the muscle construct; MSCs support and maintain the endothelial-lined network. MSCs were found to inhibit bovine fibrin degradation, but they were competent to degrade human fibrin. A permanent protocol change was made.

Vascularized muscle constructs have been engineered in vitro (**Figure III-10**). The morphology of the self-assembled vascularized muscle was similar to self-assembled muscle, with the addition of an extensive vessel network lining the muscle construct periphery (**Figure III-11**). A more highly branched vessel network was observed when the endothelial cells and MSCs were seeded into the fibrin instead of on top of the fibrin with the myoblasts and MEFs (**Figure III-12**).

Human Fibroblast Cell Source. To support the shift toward a human-based muscle system, the MGH team qualified potential human fibroblast cell sources. To as-

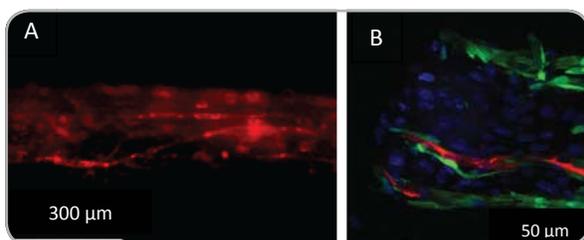


Figure III-10. Longitudinal section of engineered vascularized muscle construct, 6 days after in vitro differentiation. HUVECs (td Tomato-labeled) and MSCs (eGFP-labeled) were co-seeded with myoblasts and fibroblasts on fibrin scaffold. (A) Endothelial-lined vessel networks (red) aligned along construct length. (B) MSCs (green) closely associated with endothelial-lined vessels (red).

sess the impact on myoblast differentiation and myotube viability, human fibroblast sources were screened in two-dimensional myoblast/fibroblast co-cultures, with and without MSCs. Skeletal muscle fibroblasts and foreskin fibroblasts supported superior myotube formation in comparison with MEFs and dermal fibroblasts (data not shown). However, MSCs slightly decreased myotube formation in each myoblast/fibroblast combination.

Innervation. An innervation implantation model was developed in which engineered muscle was implanted into a vascular-rich muscle bed in the submandibular space of an immunocompromised rat. To simulate orbicularis oculi innervation, a branch of the facial nerve (marginal mandibular) or the hypoglossal nerve was transferred to the implanted engineered muscle. Neural signals from the transferred nerve will direct maturation of the implanted engineered muscle. To validate the approach, surgically created nerve stumps from the hypoglossal or marginal mandibular nerves were transferred to native denervated digastric muscles. Under all conditions, neurite outgrowth occurred and neuromuscular junctions were re-established within 4 weeks (**Figure III-13**).

Muscle Scale-Up. A muscle equivalent in size to the human orbicularis oculi will be engineered by bundling MGH immature muscles, which are on the scale of muscle fascicles. A highly porous sleeve was designed and fabricated by the Kohn laboratory to enclose the engineered muscles prior to implantation. The diameter of the sleeve can be expanded to allow for easy insertion of three to five self-assembled muscles. When the scaffold is stretched, the sleeve tightens around the muscle bundle, promoting effective muscle-muscle contact. During in vivo implantation, the scaffold containing the muscle bundle will be anchored in the animal in a stretched position. The material composition of the sleeve is being fine-tuned to degrade over several months.

Key Research Accomplishments

- Vascularized skeletal muscle has been engineered in vitro using a vasculogenesis approach in which endothelial progenitor cells and MSCs are co-seeded with muscle cells. This vascularized muscle has an extensive endothelial-lined network that encircles the periphery of the engineered muscle.

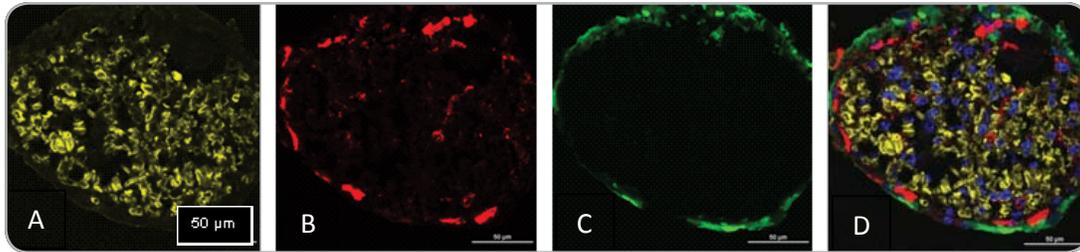


Figure III-11. Cross sections of engineered vascularized muscle construct, 6 days after in vitro differentiation. HUVECs (td Tomato-labeled) and MSCs (eGFP-labeled) were co-seeded with myoblasts and MEFs on the fibrin scaffold. (A) Dense, aligned myotubes were observed throughout the cross section (fast myosin heavy chain [My32], yellow). (B) The endothelial-lined vessel network was largely concentrated along the muscle periphery (red, HUVECs) (C) in close association with the MSCs (green); the MSCs supported the formation and maturation of the vessel network. (D) The merged image depicted a dense muscle structure encapsulated with an endothelial-lined network of vessels. Nuclei stained with DAPI (blue).

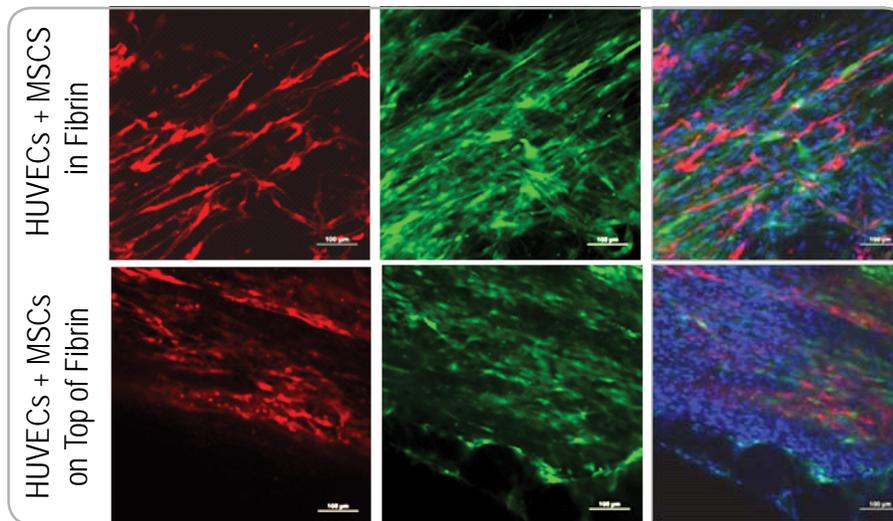


Figure III-12. Engineered vascularized muscle construct, 6 days after in vitro differentiation. HUVECs (td Tomato-labeled) and MSCs (eGFP-labeled) were seeded either in or on top of the fibrin scaffold while myoblasts and fibroblasts were seeded on top of the fibrin scaffold. A more highly branched vessel network was observed when vascular cells were seeded in the fibrin. Nuclei stained with DAPI (blue).

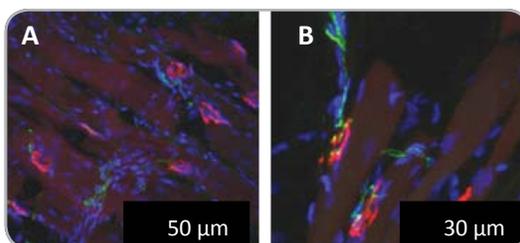


Figure III-13. Denervated posterior digastric muscle in nude rats re-innervated with either (A) marginal mandibular nerve or (B) hypoglossal nerve, 4 weeks after denervation and nerve transfer. Nerve stumps were attached with human fibrin glue. Neurite outgrowth (green, neurofilament) and NMJs (red, α -bungarotoxin) are observed, indicating re-innervation has occurred. Nuclei lining the muscle fibers is stained with DAPI (blue).



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- To simulate orbicularis oculi innervation, a branch of the facial nerve or the hypoglossal nerve was transferred to the engineered muscle that had been implanted in the submandibular space of an immunocompromised rat. Neuromuscular junctions were re-established in denervated native muscle using this surgical protocol.
- The Kohn laboratory has developed a prototype scaffold to support the scale-up of the engineered muscles to a muscle the size of the human orbicularis oculi muscle.

Conclusions

Toward the goal of engineering replacement orbicularis oculi muscles, the researchers have engineered three-dimensional skeletal muscle with morphology and maturation metrics similar to immature skeletal muscle. Upon implantation, these muscle constructs must be vascularized and innervated to be functional; the research team has made significant progress toward accomplishing both objectives. In addition, the Kohn lab has developed prototype scaffolding to support scale-up of the MGH engineered muscle to the size scale of the human orbicularis oculi muscle.

Research Plans for the Next 3 Years

In Year 3 (starting and ending with TRL 2), the researchers will continue developing protocols to vascularize and innervate the engineered muscle in immunocompromised rodents. Human cell sources will replace rodent cell sources, and protocols will be tailored to optimally process human cells. In collaboration with the Kohn team, the researchers will complete scaffold development to scale-up the engineered muscle to a muscle the size of orbicularis oculi; the degradation profile and mechanical properties of the scaffold material will be optimized.

In Year 4 (ending with TRL 3), the researchers will conduct a POC study in immunocompromised rodents to demonstrate contractile human muscle on the scale of the orbicularis oculi. A Request for Designation will

be submitted to the FDA to determine the regulatory path for the engineered eyelid muscle. In preparation for Year 5, a large animal model will be selected, and protocols will be developed to transition from human cell sources to autologous large animal cell sources. The supporting scaffold will be tailored, as necessary.

In Year 5 (ending with TRL 4), a surgical model will be developed to entopically implant an engineered autologous orbicularis oculi muscle, and a POC study will be conducted to demonstrate orbicularis oculi function. The research team will be ready to initiate a GLP preclinical trial in the selected large animal model by the end of Year 5.

Planned Clinical Transitions

An agreement will be signed in Year 4 with an industrial partner to fabricate the scaffold used to bundle the engineered muscles for scale-up. Trident Biomedical LLC will be the likely partner if the final material is a Tyr-PC.

Engineered muscle is a combination product. In Year 4, the researchers will submit a Request for Designation to the FDA Office of Combination Products to determine the regulatory path. Subsequently, the research team will hold a pre-IND or IDE meeting with the appropriate FDA agency to review existing data and receive guidance on documentation preparation.

Corrections/Changes Planned for Year 3 and Rationale for Changes

The initial scaffold concept evolved during Year 2 from a fibrous network that internally supports each engineered muscle in a porous sleeve that supports a bundle of engineered muscles. The scaffold concept was modified because the mechanical forces generated during the muscle self-assembly process were insufficient to tightly roll the three-dimensional construct containing supporting fibers. Development of this revised scaffold concept continues into Year 3.

Composite Tissue Allograft Transplantation Without Lifelong Immunosuppression

Project 4.3.1, RCCC

Team Leaders: Maria Siemionow, MD, PhD (Cleveland Clinic)

Project Team: Aleksandra Klimczak, PhD, Joanna Cwykiel, MSc, Arkadiusz Jundzill, MD, Agata Matejuk, PhD, Selman Altuntas, MD, Bahar Bassiri Gharb, MD, and Antonio Rampazzo, MD (Cleveland Clinic)

Collaborators: Jim Herrman, PhD, CEO (Tolera Therapeutics, Kalamazoo, MI)

Therapy: Chimeric cell therapy for CTA transplantation without lifelong immunosuppression

Deliverable: Method to induce tolerance in allograft recipient(s) by administering donor-recipient chimeric-cell therapy created ex vivo, allowing graft recipients to avoid the negative effects of lifelong immunosuppression

TRL Progress: *Start of Year 1*, TRL 3; *End of Year 1*, TRL 3; *End of Year 2*, TRL 4

Key Accomplishments: The researchers confirmed the importance of chimerism in tolerance induction and graft survival. They conducted donor-recipient cell fusion both in vivo

and ex vivo leading to chimera creation and confirmed the therapeutic effect of chimeric cells by demonstrating the prolonged survival of skin allografts. In addition, they brought a new, selectively blocking induction antibody (TOL101) for solid organ and CTA transplants through GMP manufacturing, assurance testing, and pre-IND screening processes with the FDA.

Key Words: In vivo created chimeric cells, ex vivo created chimeric cells, vascularized skin allograft survival, cell fusion, tolerance induction, prevention of transplant rejection, cellular therapy

Introduction

In the current military conflicts, U.S. soldiers are experiencing unprecedented traumatic injuries from improvised explosive devices. Such massive trauma often results in catastrophic injury or “polytrauma” to the victim, who may end up missing several limbs, being blind, and missing part or all of the face. While prosthetics and plastic surgery can help restore the injured warfighter to partial function, the potential ability to transplant large segments of vascularized tissue, allowing partial restoration of faces and limbs, will provide help and hope to victims of disease and traumatic injury. Having one’s face restored to a more human appearance, especially, makes an amazing impact on an individual’s morale—which can vastly aid their physical healing and rehabilitation and enhance their quality of life.

The Cleveland Clinic has performed successful CTAs during surgeries of the hand, larynx, abdominal wall, and a partial face transplant. The immunological character of CTAs, which contain skin, lymph nodes, and bone marrow (BM), may generate a high immunological response; this raises challenges for transplant immunologists. To reduce or prevent immune reactions and potential trans-

plant rejection, recipients must take lifelong immunosuppressant drugs, which can have severe side effects, including making the recipient vulnerable to diseases. To improve the outcome of CTA, a supportive therapy with donor BMT is used in clinical practice. Currently, scientists are still investigating the clinical, technical, and biological criteria for using BMT in cell therapy protocols; frequent complications include long hematopoietic recovery and graft failure.

An alternative approach to BMT cellular therapy that may or may not create chimera cells in vivo, is direct chimera application. The terms *chimera*, *chimeric*, *chimerism* refer to one body containing cells from two genetically distinct individuals—in this case, donor and recipient. We produced chimeric cells in our laboratory by fusion of BM cells derived from donor and recipient. Fused cells, which have characteristics of both cell types, were further transferred to a transplant recipient. Chimeric cells as a new cellular therapy can be used alone or together with “conventional” BM therapy. We will test both applications to optimize silencing the recipient’s immune reaction and thus lessening the patient’s need for immunosuppressant drugs and their negative side effects.



III: Craniofacial Reconstruction

Progress Reports: Soft Tissue Regeneration

The researchers aim to use cell fusion to create ex vivo donor-recipient chimeric cells. Their aim is to apply chimerism to human transplants and to develop donor(s)-specific tolerance. Chimerism can offer transplant patients a best-case scenario: the opportunity to achieve a restored body, an improved self-image, and freedom from worry about the immunosuppressive side effects of current transplantation regimens. For this reason, supportive therapy with donor-recipient chimeric cells represents a potentially groundbreaking modality in solid organ and CTA transplants. The researchers hypothesize that creation of chimeric cells ex vivo will constitute a more successful application of tolerance induction in CTA compared to BMT.

The ultimate goal of this project is to change the human body's way of modulating its immune system reaction to make allograft transplant of composite tissues (i.e., skin, lymph, and bone) safer and more widely available to victims of disease and traumatic injury. These methods

will provide the injured warrior with the opportunity to receive large blocks of tissue and even whole limbs from a donor through the current tissue-donor systems while reducing or eliminating the need for long-term immunosuppression and associated costs and risks. These same strategies are also relevant to advancement in solid organ transplantation.

Summary of Research Completed in Year 1

During the first year of the study, the researchers confirmed the presence of donor-origin cells in the peripheral blood and BM compartment of recipients at different time points. This confirmed the efficacy of the chosen immunodepletive protocol for chimerism induction. The research team also accomplished in vivo cell fusion in the first group of animals and created ex vivo donor-recipient cells by cell fusion (Figure III-14). The first clinical observations confirmed the supportive role

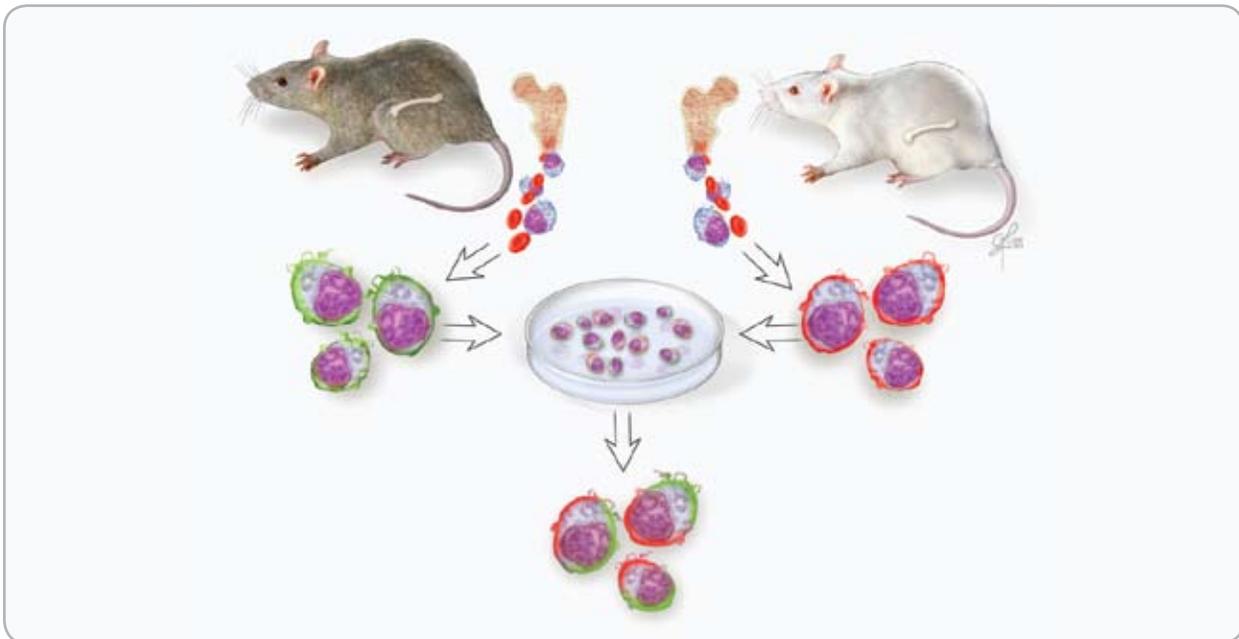


Figure III-14. Ex vivo creation of fusion cells. This novel experimental approach toward tolerance induction is currently tested on animal model and utilizes both donor and recipient bone marrow-derived cells. In the first step, hemiface transplantation is performed across MHC barrier between ACI (RT1a) rat as a donor and LEW (RT1l) rat as a recipient. Hemiface recipient rat undergoes short immunosuppressive protocol with the use of $\alpha\beta$ - T cell receptor mAb and CsA. Isolated bone marrow-derived cells from donor and recipient are fluorescently labeled and fused ex vivo with the use of PEG. Based on fluorescent staining, double-stained cells (fused cells) are separated and collected for the therapeutic purpose.

of donor-recipient chimeric cells created ex vivo by cell fusion for prolonged skin allograft survival.

Research Progress – Year 2

To support the aim of combining donor and recipient cells, to induce chimerism and reduce the need for immunosuppressants, the researchers conducted investigations of BMT using vascularized allograft transplants (e.g., limb and bone) in a rat model. The results of the therapy were that myeloid and lymphoid cells developed both (a) stable, multi-lineage chimerism and (b) long-term immunological tolerance (up to 750 days).

To confirm the role that donor BMCs may play in inducing chimerism, the lab developed a surgical model of a vascularized BM transplant that contains intact vascularized bone with a BM microenvironment in rats. The researchers confirmed that donor BMCs showed migratory potential and engraftment and that led to maintenance of chimerism (i.e., cells from both donor and recipient lived on in the recipient animal's body). These results suggest that the presence and successful engraftment of hematopoietic cells (i.e., donor BMCs) and creation of chimera are key events in tolerance induction. This study led to the idea to create chimeric cells directly in a laboratory setting and make them available for cellular therapy in rats.

Based on these results, the research group hypothesized that chimeric cells might induce immune tolerance as part of a supportive therapy for allograft transplants. To assess the application of chimeric cells in multiorgan transplants from two unrelated donors, the lab team used an in vivo rat model to create a second generation of multi-chimeric cells, based on the lab's established method of intraosseous BMT. The researchers verified that multi-chimerism had occurred when the vascularized skin allograft transplants survived for up to 540 days. These results in a rat model of chimerism are promising and suggest that the researchers have the ability to apply the chimerism approach to human CTAs and/or solid organ transplantation (Aim 2 of the project).

Finally, the researchers have taken a new, selectively blocking induction antibody (TOL101) for solid organ and

CTA transplants through GMP-compliant manufacturing, assurance testing, and pre-IND screening processes with the FDA.

Key Research Accomplishments

- Confirmed in vivo spontaneous fusion of donor-recipient cells in BM, by detecting the presence of donor-origin DNA in the cell population using polymerase chain reaction (PCR), flow cytometry analysis, and immunostaining.
- Confirmed the presence of donor-origin cells in the peripheral blood of LEW primary chimera at different time points.
- Confirmed the presence of ex vivo created chimeric cells in the peripheral blood at different time points in all 8 assessed experimental groups.
- Confirmed a supportive role for donor-recipient chimeric cells, created ex vivo by cell fusion, in prolonged skin allograft survival for up to 86 days.

Conclusions

The research team found that chimerism was induced soon after transplantation. They confirmed the presence of donor-origin cells in the peripheral blood and BM compartment of LEW recipients using flow cytometry, immunostaining, and the PCR technique at different time points. These results confirmed that spontaneous fusion of donor-recipient cells had occurred, along with successful engraftment and re-population of donor-origin cells. Also, the researchers successfully created ex vivo donor-recipient chimeric cells by cell fusion and confirmed their beneficial effect in skin allograft survival (up to 86 days). They also detected long-term chimerism in the peripheral blood, in a rat model, after application of ex vivo created donor-recipient chimeric cells.

The creation in laboratory settings of chimeric cells with potential to induce immunologic tolerance when introduced to a recipient body is an innovative method to prevent rejection in face transplants. This cellular/chimeric therapy may create the basis for future clinical applications. This work has advanced the original goal, i.e., to develop cells that combine characteristics of both



III: Craniofacial Reconstruction

Progress Reports: Soft Tissue Regeneration

donor and recipient, and transplant those with the allograft in the hope of reducing or eliminating the immune response, thus making it possible for transplant recipients to lead more normal and safe lives without the need for immunosuppression. Aiming for clinical application, the researchers initiated pre-IND screening processes with the FDA for a new, selectively blocking induction antibody (TOL101) for solid-organ CTA transplants.

Research Plans for the Next 3 Years

Year 3, Q1-2:

The researchers plan to develop optimal conditions required for the preservation and banking of ex vivo fused cells. They will also establish a fusion and cell culture procedure for the ex vivo creation of fused human cells, including selection of the best cell line (e.g., from umbilical cord blood cells) available from cell banks. (TRL 3)

Year 3, Q3-4:

The research group plans to evaluate the viability and competence of human chimeric (fused) cells. They will perform human cell fusion ex vivo and establish methodology, cell preservation, and banking. They will also evaluate different preservation and banking conditions. Finally, the plan to establish a human protocol for supportive therapy with chimeric cells (safety and efficacy). (TRL 4)

Year 4–Year 5:

The researchers plan to initiate the first clinical trial using human BM for CTA transplants such as limb and face during these years.

Planned Clinical Transitions

The research team is planning the start of a clinical trial in Year 4. They anticipate the submission of an FDA application in the second quarter of Year 4 when data from human blood or BMC experiments are expected to be available.

Engineered Cartilage Covered Ear Implants for Auricular Reconstruction

Project 4.1.1, WFPC

Team Leader(s): James J. Yoo, MD, PhD (Wake Forest University)

Project Team Members: Sang Jin Lee, PhD, Chang Mo Hwang, PhD, Young Min Ju, PhD, Jae Hyun Kim, PhD, Bukyu Lee, DDS, PhD, and Denethia Green, BS (Wake Forest University)

Collaborator(s): Greg Sword (Porex Corporation)

Therapy: Reconstruction of the external ear

Deliverable(s): Engineered cartilage tissue covering the commercially available alloplastic implant

TRL Progress: *Start of Program*, TRL 3; *End Year 1*, TRL 3; *End Year 2*, TRL 4

Key Accomplishments: The researchers have demonstrated in vivo structural stability of their engineered cartilage covered ear implants for clinical application. The engineered cartilage homogenously covered

alloplastic ear implants by a cell spraying method. Their research to date indicates that cartilage tissue-covered ear implants are able to maintain device contour and placement without causing skin necrosis when implanted in vivo.

Keywords: Auricular cartilage, alloplastic implant, tissue engineering

Introduction

Traumatic injuries constitute a major cause of morbidity and mortality for the Armed Forces. The incidence of craniofacial injuries has been rapidly increasing due to the frequent ballistic and explosive injuries on the battlefield. Protruding tissues such as ear and nose are frequently affected in these injuries. Although the loss of ear tissues does not pose life-threatening danger, it is functionally and cosmetically debilitating and hinders injured soldiers from returning to society.

The standard treatment method for auricular reconstruction uses autologous costal cartilage as a graft material. However, autologous costal cartilage is limited in supply, provides inadequate dimensions, and is progressively absorbed after implantation. Currently, alternative approaches use alloplastic ear implant devices composed of silicone or polyethylene (PE). These implants are approved by the FDA, and they are nontoxic, cause minimal foreign body reactions, and possess adequate mechanical properties for use in non-load-bearing tissues of the craniofacial region.

Although alloplastic ear implants are able to effectively eliminate the morbidity associated with the costal cartilage graft, the use of these implants is often associated

with complications that include inflammation, infection, erosion, and dislodgement. As a result, implant extrusion occurs frequently due to the limited vascularization and constant abrasion against the surrounding tissues. A common practice to overcome these complications includes the use of a temporo-parietal tissue flap from the side of the head to cover the implant, which provides vascularized tissue cushion against the abrasive implant. In this project, the researchers have developed an engineered cartilage that entirely covers the abrasive ear implant, and this could prevent implant exposure and extrusion while maintaining appropriate mechanical properties. Creation of cartilage tissue using a soldier's own cells would bring benefits and minimize the morbidity associated with implant dislodgement.

The strategy for developing an engineered cartilage tissue covering the alloplastic ear implant consists of two steps: (1) surface modification of the device's innate hydrophobic characteristics to achieve hydrophilic environment for cells and (2) coverage of chondrocyte-hydrogel conjugation to a MedPor® implant to achieve cartilage tissue cushion against the device. In this project, the processing system will be refined and optimized for a smooth translation into soldiers who require auricular reconstruction.



III: Craniofacial Reconstruction

Progress Reports: Cartilage Regeneration (Focus: Ear)

This group has previously demonstrated that cartilage tissues could be engineered to serve as a biological cover for a commercially available ear implant. The chondrocyte-fibrin constructs successfully formed neocartilage tissue with adequate mechanical strength and the long-term stability required for successful clinical application. This system may improve the structural and functional interactions between the implant and recipient tissue, and this in turn may enhance the outcome of auricular cartilage reconstruction by eliminating many of the problems associated with the use of the current ear implant alone.

Summary of Research Completed in Year 1

During the first year of the project, the researchers prepared fibrin hydrogels with various concentrations of fibrinogen and thrombin, mixed cultured chondrocytes with the hydrogels, and implanted the constructs subcutaneously into athymic mice. They harvested the mice at various times post implantation. While nontreated ear implants resulted in severe skin necrosis at 2 weeks post implantation, cartilage-covered ear implants were able to maintain device contour and placement without causing skin necrosis.

Research Progress – Year 2

During Year 2, the researchers continued to focus on in vivo preclinical demonstrations of their engineered cartilage-covered ear implants in a rodent model. To demonstrate clinical feasibility, they performed in vivo studies demonstrating total auricular cartilage reconstruction using an ear-shaped implant. Their ear-shaped implants were homogeneously covered by chondrocyte-fibrin constructs using a cell spraying system (Figure III-15). The preparation contains fibrinogen and thrombin solutions with chondrocytes. The spraying device uses two liquid phases that can be either extruded through a dual chamber applicator or sprayed through the applicator with an inert gas carrier. The cell-fibrin suspension was homogeneously

applied to the surface of ear implants. The engineered cartilage-covered ear implants that were implanted in athymic mice showed no evidence of skin necrosis, implant exposure, or extrusion. The histomorphological evaluations consistently showed neocartilage formation on the ear implants and revealed the presence of evenly dispersed triangular and ovoid-shaped chondrocytes that inhabited normal-appearing lacunae, and these were surrounded by perichondrium.

Key Research Accomplishments

- Optimized the cell delivery system for creating cartilage-covered auricular implants.
- Evaluated the biocompatibility and structural stability of engineered cartilage ear implants in a rodent model.
- Demonstrated the structural and functional integrity of engineered cartilage ear implants in a rodent model.

Conclusions

This study demonstrates that cartilage tissues can be engineered to serve as a biological cover for a commercially available ear implant. The chondrocyte-fibrin constructs successfully formed neocartilage tissue with adequate mechanical strength and the long-term stability required for successful clinical application. This system improves the structural and functional interactions between the implant and recipient tissue and this in turn may enhance the outcome of auricular cartilage reconstruction by eliminating many of the problems associated with the use of the current ear implant alone.

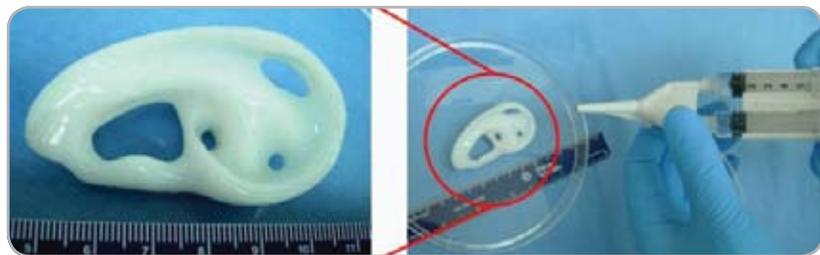


Figure III-15. Cell spraying system. The ear-shaped implants were homogeneously covered by cell-fibrin constructs using the gas-assisted spray system.

Research Plans for the Next 3 Years

The researchers will continue to evaluate the biocompatibility and structural stability of engineered cartilage ear implants in a large preclinical model. They will also continue to demonstrate the structural and functional integrity of engineered cartilage ear implants in a large preclinical model. Finally, they will continue to analyze isolation efficiency, as well as cell growth, maintenance of phenotypic and functional expression, and ECM production in the preclinical model.

Planned Clinical Transitions

The researchers plan to continue to develop a system that requires a minimum tissue biopsy for cell isolation and expansion using different cell sources, including

ear, nose, and rib cartilage tissues. They will continue to develop SOPs for autologous cell sourcing, an associated expansion system, and surgery. They will refine the cell delivery system to facilitate clinical translation of the engineered cartilage-covered ear implants. They will prepare materials for FDA discussions, will initiate a clinical trial of engineered cartilage ear implants, and will monitor the clinical trial as needed.

Corrections/Changes Planned for Year 3

A larger preclinical model involving implantation of an engineered cartilage-covered ear implant in the auricular region using autologous cells has been added to the project. The objective is to further confirm the clinical applicability and to develop SOPs for surgical methods.



III: Craniofacial Reconstruction

Progress Reports: Cartilage Regeneration (Focus: Ear)

Regeneration of Ear

Project 4.5.4a, RCCC

Team Leaders: Cathryn Sundback, ScD and Joseph P. Vacanti, MD (MGH)

Project Team: Mack Cheney, MD, Tessa Hadlock, MD, Douglas Henstrom, MD (MEEI, Facial Plastic Surgery); Irina Pomerantseva, MD, PhD, Ken Rask, Erik Bassett, Katherine Kulig, Libin Zhou, MD (MGH, Tissue Engineering); Mark A. Randolph, David A. Bichara, MD, Xing Zhao, MD, and Matt Johnson (MGH, Plastic Surgery Research Laboratory)

Collaborators: Nathaniel Hwang, PhD, Daniel Anderson, PhD, Robert Langer, ScD (MIT); and Chris Bowley (Kensey Nash Corp.)

Therapy: Tissue-engineered cartilage constructs for ear replacement

Deliverable: A permanent, implantable, engineered, living external-ear replacement for the wounded warfighter

TRL Progress: *Start of Year 1*, TRL 3; *End of Year 1*, TRL 3; *End of Year 2*, TRL 3

Key Accomplishments: The researchers demonstrated robust autologous cartilage formation on porous fibrillar collagen scaffolds in an immunocompetent sheep model without a significant inflammatory or foreign-body response. In a POC study in nude mice, they demonstrated the effectiveness of internal support in maintaining the size and shape of human-ear-shaped neocartilage constructs.

Key Words: Tissue-engineered ear, cartilage, porous collagen, chondrocytes

Introduction

Limited reliable options exist for reconstruction of blast injuries to the head and neck. Current clinical approaches for reconstructing the external ear include implanting either an autologous hand-carved costal-cartilage framework or porous PE (Medpor). Both options are prone to complications, require multiple surgeries, and have unpredictable and often suboptimal aesthetic outcomes. The MGH team has shown initial success in engineering ear-shaped cartilage using biodegradable scaffolds and chondrocytes in immunocompromised animals.

Tissue-engineered autologous ear replacements would combine the best of both current clinical approaches: the precisely defined architecture of Medpor implants and the autologous properties of carved cartilage. For this new design, the number of surgeries could also be reduced from four to two. A critical, remaining design goal is to maintain the complex three-dimensional structure of this largely unsupported cartilage when it is subjected to the mechanical forces of the surrounding tissues during cartilage maturation and wound healing after implantation. These forces are much greater in a human or a large animal than in a rodent model.

The goal of this project is to expedite the development of a permanent, implantable, living external ear for the injured service member and to achieve cosmetic outcomes that meet patient expectations.

Summary of Research Completed in Year 1

During the first year of the project, the researchers developed an ovine model for subcutaneous implantation of ear-shaped constructs. They engineered cartilage from both ovine and human cartilage cells and MSCs in immunocompromised mice. They also designed ear-shaped scaffolds using computer-aided design (CAD) principles and tested the scaffolds with numerous materials.

Research Progress – Year 2

Scaffold Material and Dynamic Culture Screening in Immunocompromised Mice. The research team evaluated four formulations of porous fibrillar collagen produced by Kensey-Nash Corp., and nine synthetic blends of copolymers provided by the MIT collaborators, comparing (1) their ability to form cartilage in nude mice, after being seeded with sheep auricular chondrocytes and (2) the effects of static and dynamic preculture conditions

over different time periods. Based on the results of this screening study, two scaffold materials (low density collagen and poly (DL-lactide-co-caprolactone) 40:60 (PLA/PCL)) and two dynamic culture conditions (rotational oxygen-permeable bioreactor system and RotoMix) were selected for future studies (Figure III-16).

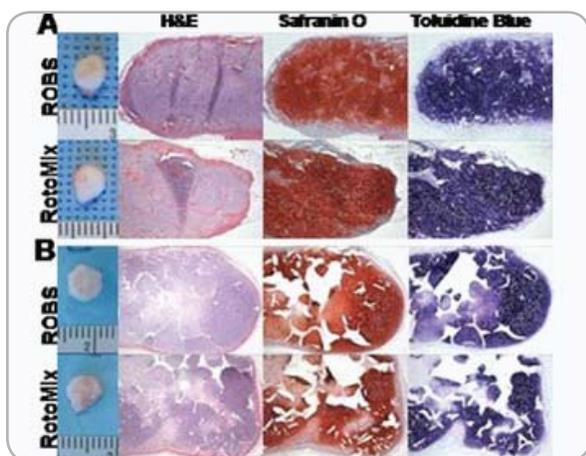


Figure III-16. Cartilage formation (A) in low density collagen and (B) in PLA/PCL, after 14 days of dynamic in vitro culture and 6 weeks in vivo. Scale bar, 100 μ m.

Tissue Reaction to Scaffold Materials and Components of Culture Medium in Immunocompetent Mice. Pre-incubation of samples in serum-free medium caused the least inflammatory response while incubation in phosphate-buffered saline (PBS) and complete medium (with fetal bovine serum, FBS) caused a severe reaction. A severe reaction was observed for PLA/PCL samples preincubated in PBS, with multiple giant-cell formation and neovascularization of the scaffold material at 2 weeks. Among low density collagen samples, materials preincubated in complete medium had the greatest severe inflammatory response at 2 weeks. The team plans in future studies to substitute FBS with autologous serum for several days before implantation; this is expected to help reduce inflammation.

Autologous Cartilage Formation in Sheep. The study design is presented in **Figure III-17**; two 3-month-old animals and two 11-month-old animals were used for the study. In both older sheep, neocartilage formation was seen throughout the scaffolds and was interrupted by

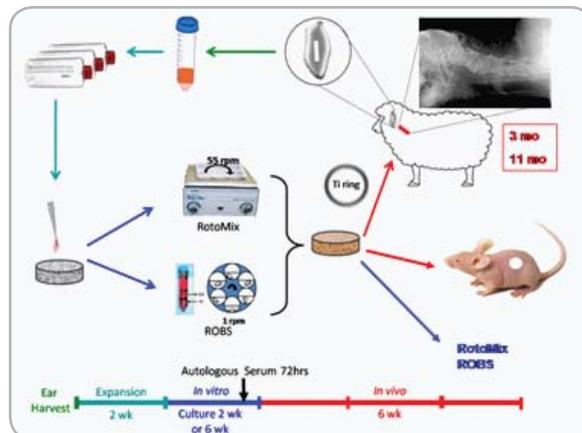


Figure III-17. Experimental sheep study design. Sheep auricular chondrocytes were expanded and seeded on collagen scaffolds. After in vitro culture for 2 or 6 weeks, seeded scaffolds were implanted in sheep.

residual scaffold fibers; in both younger sheep, cartilage formation was noncontiguous (Figure III-18). Minimal inflammatory reaction and no foreign-body response was seen in the older sheep, and the histological picture resembled that seen in the corresponding control mice. Severe inflammatory and foreign-body reactions were observed in response to constructs made with PLA/PCL. The younger animals were the Polypay breed, and the older animals were the Finn breed; the immune response in sheep may be dependent on age, sex, genetic factors, and breed. The research team is currently performing a study to determine if the difference in neocartilage formation and associated immune status of the animals was due to sheep age or breed.

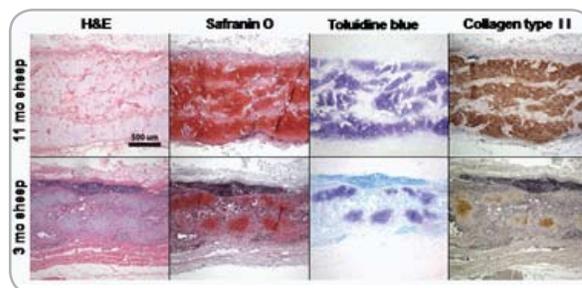


Figure III-18. Cartilage formation in sheep. In older sheep, neocartilage formation was seen throughout the scaffolds and was interrupted by residual scaffold fibers (stained red, H&E); in younger sheep, cartilage formation was noncontiguous.

III: Craniofacial Reconstruction

Progress Reports: Cartilage Regeneration (Focus: Ear)

Human Ear-Shaped Cartilage Formation in Mice. Half-size adult human ear scaffolds with and without internal support were fabricated from low density collagen for implantation on the backs of nude mice. After 2 weeks in vitro culture, visible shrinkage occurred in the constructs without support (Figures III-19 and 20). Total re-

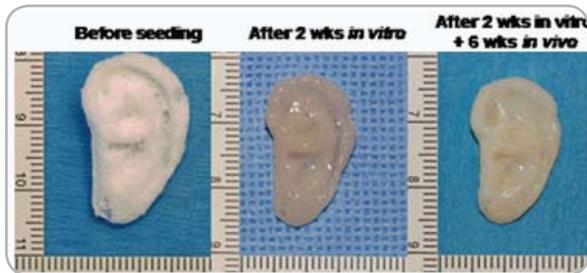


Figure III-19. Ear-shaped constructs without internal support shrunk after 2 weeks in vitro culture, without further significant reduction in size after 6 weeks in vivo. Ear shape was retained.

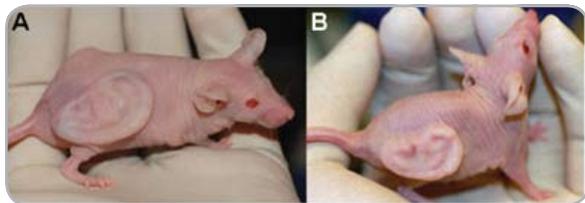


Figure III-20. Ear-shaped construct without internal support (B) is smaller than the one with support (A) after 6 weeks in vivo; both types of constructs retained a characteristic ear shape.

duction in size for the ear-shaped constructs without wire support after 6 weeks in vivo culture averaged 14.8% in length and 16.8% in width while reduction in size for constructs with wire support averaged 3.7% in length and 5.6% in width. Both construct types retained a characteristic human ear shape (Figure III-20). Grossly, the tissue resembled cartilage, and all ear-shaped constructs were flexible (Figure III-21). Histologically, neocartilage formation was observed throughout the constructs, except for a small area in the mid-construct that was likely a scaffold-production artifact (Figure III-22).

Development of a Scaffold Shaped and Sized Like the Adult Human Ear. A CAD file of the ear scaffold (Fig-

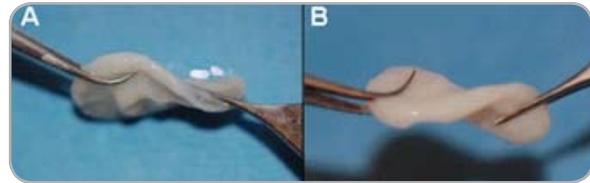


Figure III-21. Flexibility was similar of (A) ear-shaped cartilage constructs with internal support and (B) constructs without support, after 6 weeks in vivo.



Figure III-22. Ear-shaped construct was cut along the black line and sectioned. The composite image on the right demonstrates shape retention and cartilage formation throughout the construct. Safranin O staining.

ure III-23A) was created and used to rapidly prototype a full-size model (Figure III-23B). Using molds made from the model, Kensey Nash Corp. will manufacture the scaffolds. The thickness of the final scaffold will vary from 1.2 to 2.4 mm, and the total volume will be 2 mL; those parameters approximate the ones of native adult human ear cartilage.

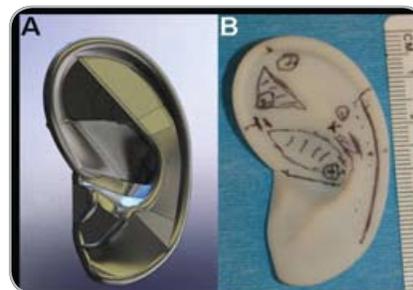


Figure III-23. (A) A computer-aided simulation of the ear scaffold and (B) a full-size rapid-prototype model.

Key Research Accomplishments

- Completed studies on both material screening and in vitro culture optimization and identified a biocompatible scaffold material and the dynamic in vitro culture conditions that result in consistent and robust neocartilage formation in immunocompromised mice.

- Engineered autologous cartilage in an immunocompetent sheep model, using auricular chondrocytes, porous fibrillar low density collagen, and dynamic in vitro culture conditions prior to implantation.
- In a POC study in nude mice, demonstrated maintenance of the size of human ear-shaped neocartilage constructs with internal support. Control constructs without internal support failed to maintain their original size.

Conclusions

The research team selected porous, fibrillar, low density collagen as a material for fabricating ear-shaped scaffolds, based on the results of in vitro and in vivo studies in immunocompromised animals and the finding that this material consistently supported neocartilage formation. Dynamic culture of the seeded constructs prior to implantation resulted in more robust cartilage formation in vivo. Formation of autologous neocartilage from auricular chondrocytes on porous fibrillar collagen scaffolds was demonstrated in an immunocompetent sheep model. Significant progress has been made in POC studies of human ear-shaped constructs, which retained their size and shape after implantation in nude mice. Significant shrinkage occurred in the control constructs.

Research Plans for the Next 3 Years

In Year 3 (starting at TRL 3), the research team will continue developing the cell sources for an engineered ear. Primary chondrocytes will be expanded in culture; up to 100 million cells will be required for one human-size ear scaffold. Several strategies to prevent de-differentiation and/or to induce redifferentiation of culture-expanded chondrocytes will be evaluated in vitro; cartilage formation from redifferentiated chondrocytes will be confirmed in nude mice. The research team will submit a Request for Designation to the FDA to determine the regulatory path for the engineered ear. The surgical implantation model in sheep will be further modified to more closely approximate auricular reconstruction techniques in humans.

The next generation of full-size ear-shaped constructs will be manufactured for the POC studies in sheep. POC studies in sheep will be completed in Year 4 to assess size and shape retention of the ear-shaped construct in an immunocompetent sheep model. The successful completion of this study will bring the ear program to TRL 4. In preparation for clinical trials, researchers will perform a GLP preclinical trial in sheep to demonstrate safety and efficacy of the engineered ear. The team will develop a protocol for a pilot clinical trial. The research team will be ready to start a pilot clinical trial by the beginning of Year 5 (see the following for additional information).

Planned Clinical Transitions

The engineered ear is a combination product (defined by the FDA as being composed of two or more regulated components that are physically, chemically, or otherwise combined or mixed and produced as a single entity). Therefore the research team will submit a Request for Designation to the Office of Combination Products of the FDA to determine the regulatory path. Consequently, the researchers will hold a pre-IND or IDE meeting with the appropriate agency of the FDA and will prepare and file the required documentation. Team representatives will audit Kensey Nash Corp., the manufacturer of the scaffolds, and the Harvard Medical School GMP facility, where cells and seeded scaffolds will be cultured. The team will also develop a clinical study protocol, which will include preclinical data from small and large animal studies, and submit it to the local IRB. After the approval from the local IRB is obtained, the team will submit the clinical protocol to the USAMRMC for review. Once approved, a clinical trial will commence. The trial and its 6-month follow-up will be completed during Year 5; the primary endpoints will be safety and implant integrity. Successful conclusion of the clinical trial will bring the ear program to TRL 5.



III: Craniofacial Reconstruction

Progress Reports: Cartilage Regeneration (Focus: Ear)

Corrections/Changes Planned for Year 3 and Rationale for Changes

The researchers need to further develop the source of cells for the engineered ear. Originally it was proposed that supplementing primary chondrocytes with MSCs would yield a sufficient cell population for seeding scaffolds the size and shape of the adult human ear. This approach is being pursued by collaborators at MIT. The results are encouraging; however, it remains

unclear whether it will be feasible to use MSCs for auricular cartilage repair in a clinical trial in 2 years. The research team is refocusing attention on the expansion of primary chondrocytes in vitro and addressing the de-differentiation/redifferentiation issues. Such studies will be conducted in vitro and in nude mice. In addition, the team will refine the sheep implantation model to more closely approximate auricular reconstruction in humans. In particular, a staged approach will be developed with the engineered ear elevated off of the neck's surface.

Regeneration of Ear – Optimization of Scaffold

Project 4.5.4b, RCCC

Team Leaders: Daniel G. Anderson, PhD and Robert S. Langer, ScD (MIT)

Project Team: Nathaniel Hwang, PhD (MIT)

Collaborators: Joseph Vacanti, MD, PhD, Cathryn Sundback, ScD, Irina Pomerantseva, MD, PhD, Ken Rask, Gwen Owens (MGH); Mark Randolph, and David Bichara (MGH Plastic Surgery Research)

Therapy: A permanent, implantable, tissue-engineered ear for the wounded soldiers

Deliverable: A technology platform for tissue engineering of cartilage based on stem cells for auricular, nasal, and articular applications

TRL Progress: *Start of Year 1, TRL 2; End of Year 1, TRL 3; End of Year 2, TRL 3*

Key Accomplishments: The researchers optimized both in vitro and

in vivo systems for efficient cartilage formation in using MSCs, chondrocytes, and resorbable materials. They identified an optimal biomaterial composition (poly L-lactic acid, poly(L-lactide-co-caprolactone-co-glycolide). They also demonstrated cartilage formation in an animal model.

Key Words: Ear, auricular cartilage, scaffolds chondrocytes, mesenchymal stem cells, tissue engineering

Introduction

Current standard treatments for total external ear reconstruction involve harvesting of autologous costal cartilage and carving it into the shape of external ear cartilage. However, this method is clearly associated with donor-site morbidity and demands precise techniques. An alternative method involves the transplantation of the Medpor biomaterial ear framework, composed of porous high-density PE. This product is formed by sintering small particles of high-density PE to make firm materials that can be molded using hot water. The Medpor ear implant is fabricated with pore sizes from 100-250 μm to encourage tissue ingrowth and cell engraftment; however, this implant is nondegradable and may impose long-term complications including skin necrosis (reported via Dr. Vacanti's group). The MIT group believes that tissue engineering may provide an alternate source and better solution for engineering a replacement ear.

There are two major barriers to the development of a fully resorbable, tissue engineered ear: (1) the development of a biomaterial with appropriate mechanical and biological properties and (2) the isolation and/or generation of an adequate number of cells with appropriate activity. The researchers have been working with the

MGH (Sundback/Vacanti) team to address both of these issues.

A suitable scaffold should have the following properties: (1) biocompatibility; (2) biodegradability, at a rate desirable for tissue formation; (3) a composition that promotes appropriate cell proliferation and matrix accumulation; (4) the proper size and shape for human clinical application, i.e., the size and shape of an adult human ear; (5) appropriate internal mechanical support; (6) a design that provides a satisfactorily low-inflammatory response to the native tissues; and (7) a design that enables integration with native tissues.

The researchers have been evaluating a library of different FDA-approved materials and composites using the aforementioned criteria, both in vitro and in vivo, and have developed lead scaffolds using FDA-approved materials that may address these issues. The ultimate potential of these scaffolds must be determined in large animal studies at an appropriate scale, and the scaffolds need to be subject to appropriate stress levels during healing. Large animal studies of these materials are currently being planned in Year 3 in collaboration with the MGH team.



III: Craniofacial Reconstruction

Progress Reports: Cartilage Regeneration (Focus: Ear)

The researchers have also dedicated a portion of their studies toward the generation of a supplementary source of chondrocytes. In particular, they have investigated the potential of different combinations of chondrocytes, BM-derived MSCs, and differentiation conditions. These studies are ongoing, with the hope of identifying a means of increasing the starting cell population.

In summary, the MIT group is pursuing the following specific aims to achieve the overall deliverable of an engineered ear:

- To utilize morphogenetic factors from chondrocytes.
- To co-culture auricular chondrocytes with MSCs.
- To fabricate and evaluate biodegradable scaffolds.

Summary of Research Completed In Year 1

In Year 1, this project was part of Project 4.5.4 (Regeneration of Ear). The research team, which included scientists at MGH and MIT, developed an ovine model for subcutaneous implantation of ear-shaped constructs. They engineered cartilage from both ovine and human cartilage cells and MSCs in immunocompromised mice. They also designed ear-shaped scaffolds using CAD principles and tested the scaffolds with numerous materials.

Research Progress – Year 2

The researchers have demonstrated that chondrocyte-secreted morphogenetic factors can be used to direct and generate *in vivo* cartilaginous tissue from MSCs. Co-culture of chondrocytes and MSCs showed promising methods to augment the total cell number for ear-tissue engi-

neering. Histological analysis indicated that a 1:1 ratio of chondrocytes to MSCs could be used to induce chondrogenic commitment of MSCs (Figure III-24). Constructs supplemented with MSCs alone resulted in minimal collagen secretion and lower cellularity compared to the constructs supplemented with chondrocytes alone. The researchers have successfully demonstrated that the chondrocyte-MSC co-culture methodology provides sufficient cells for making a human ear replacement.

The research team's research indicates that the PLCL based scaffold is optimal for engineering ear tissues. From an array of different scaffold compositions examined *in vivo* for 6 weeks. Three different compositions of materials demonstrated the least deformation and best tissue maintenance. The research team performed autologous chondrocyte experiments with poly L-lactide acid: poly (L-lactide-co-caprolactone-co-glycolide) compositions (referred to as "C2 compositions") in a sheep

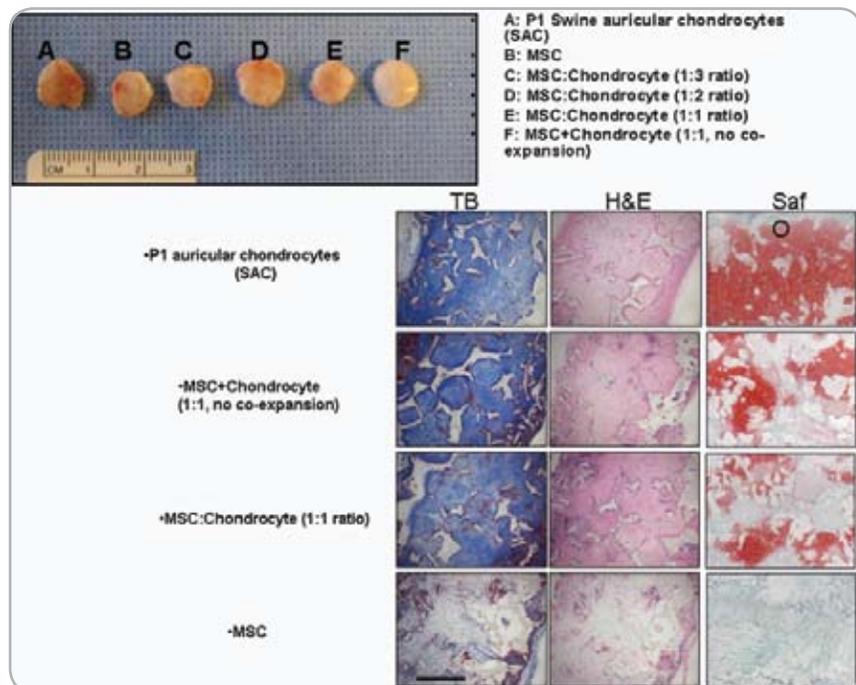


Figure III-24. Chondrocyte isolation and co-culture: Isolated chondrocytes were co-cultured with adipose-derived MSCs in various densities for 3 days in DMEM supplemented with 10% FBS, 5000 U/mL penicillin, 5000 U/mL streptomycin, and 1 mM L-glutamine on PLLA/PLCL scaffolds. Cell-scaffold constructs (MSC alone, chondrocytes alone, MSC:chondrocyte 1:1 ratio) were transplanted subcutaneously into the dorsal region of 6- to 8-week-old athymic mice for 6 weeks.

model. Histological analysis indicated that the constructs in immunocompromised animal models showed significantly enhanced cartilaginous tissues as compared to the constructs used in the autologous sheep transplantation model. The reduced cartilaginous tissue is probably due to an inflammatory reaction stemming from the degraded products in sheep. Inflammation and foreign body response to the current FDA-approved materials may be problematic for tissue engineering of scaffolds, as demonstrated with the C2 composition. Therefore, in addition to reviewing and testing a library of polymeric scaffolds for auricular tissue engineering, the research team is also aiming to improve the biological function of current FDA-approved materials. Specifically, they wish to modify the materials' surface with biologically active ECM components, to minimize the inflammatory response, reduce the foreign-body reactions, and promote vascular ingrowth.

Key Research Accomplishments

- Optimized a cell population for auricular tissue engineering.
 - Determined that MSCs expanded with a chondrocyte-conditioned medium displayed a basophilic ECM deposition characteristic of neocartilage *in vivo*.
 - Observed that the morphogenetic factors from chondrocytes may provide a microenvironment for priming MSCs for auricular tissue engineering.
 - In view of the limited cell sources for regenerating ear tissue, the co-culture of MSCs and chondrocytes shows a promising mechanism that may help overcome the cell-number limitation in the clinical setting.
- Synthesized and optimized fully biodegradable synthetic biomaterials.
 - Investigated an array of FDA-approved materials to support the formation of cartilage tissue in a mouse model and identified PLLA/PLCL as an optimal biomaterial for tissue engineering of the ear.
 - Discovered that scaffold composition can influence *in vivo* cartilage formation in sheep models.

- Developed a method to modify the surface of scaffolds for bioactivity.

Conclusions

To provide an adequate number of cells for tissue engineering of the ear, the research team has been investigating how to use MSCs with (a) optimal soluble factors and (b) morphogenetic factors from chondrocytes to maximize both the number of cells and the formation of cartilage tissue. In Year 2, the researchers evaluated the *in vivo* and *in vitro* mechanical properties of a library of degradable polymers, after seeding scaffolds made from such polymers with chondrocytes, MSCs, or a mixture of chondrocytes and mesenchymal cells. In particular, they identified a subset of polymeric scaffolds that enhanced cartilaginous tissue formation. Currently, these scaffolds are under the investigation in large animal models.

Research Plans for the Next 3 Years

In Year 3, cells in various stages of expansion and differentiation will be tested in large animals in collaboration with the MGH team. The goal will be to take one of the MGH team's scaffolds into the clinic within 2 years.

The deliverables for Year 3 are to:

- Develop a strategy to utilize autologous stem cell-based approaches for stem cell-based ear tissue engineering (to provide a long-term solution to obtain a sufficient quantity of cells).
- Fabricate and optimize a fully synthetic and biodegradable scaffold—one that possesses controllable mechanical and degradation properties.
- Improve the chemical and mechanical properties of engineered ear cartilage by optimizing *in vitro* culture conditions in bioreactor systems.
- Conduct large animal studies (as detailed in the following).

In Years 3-5, the researchers will perform large animal studies in sheep using autologous stem cells and optimized scaffolds in collaboration with the MGH group. Long-term stability of the engineered cartilage



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is a prerequisite for success of engineered tissues; therefore, the research team is planning to monitor human-ear-shaped constructs for extended periods (up to 24 months). They are supplementing the chondrocytes with autologous MSCs as well. For the stem cell-based engineered tissue to be successful, the researchers must monitor the stability and maintenance of the tissue phenotype. Therefore, they will also monitor calcification or any tissue resorption seen in the sheep model. They will further modify the C2 composition as well, to try to achieve a low inflammatory response in immunocompetent animals.

Planned Clinical Transitions

The research team is currently utilizing FDA-approved materials and autologous cell therapy. In addition, they will collaborate with the MGH team to prepare an IDE or IND for clinical application (upon successful completion of large-animal studies). Furthermore, once the optimal

composition of biomaterials is identified in large-animal studies, the researchers plan to work with Concordia Biomedical, a company with clinically approved biomaterials, which will provide a GMP-compliant facility for fabricating scaffolds for clinical trials.

Corrections/Changes Planned for Year 3 and Rationale for Changes

In the research team's prior annual report, they had proposed to complete animal studies in 2010. However, it is now hypothesized that long-term, large animal studies are needed to demonstrate the efficacy of stem cell-based cartilaginous tissue formation on fully biodegradable scaffolds. Therefore, the research team has changed the time frame for initial clinical trials accordingly.

Visualization of Patient-Specific Wounds and Injuries

Project 4.5.5, RCCC

Team Leaders: Tim Kelliher and Howard Champion, MD (SimQuest LLC)

Deliverable: Craniofacial injury-mapping software that can be used to create a visualization and simulation of patient-specific craniofacial wounds and injuries

TRL Progress: *Start of Year 1, TRL 3; End of Year 1, TRL 3; End of Year 2, TRL 4*

Key Accomplishments: The research team selected the definition and data-collection system for wounds and injuries. They developed the first prototype of the craniofacial injury-

mapping (CFIM) software and delivered it to the WRAMC. They also began defining tissue-material properties to prepare a facial model for simulation.

Key Words: Simulation, craniofacial injury, treatment strategy, surgical planning

Introduction

The SimQuest team is collaborating with other members of AFIRM-funded projects to develop a virtual-reality application that will (1) visually model and simulate patient-specific wounds and injuries and (2) integrate specific treatment strategies, including wound healing, repair, flaps, hard-tissue implants, and specific tissue-regeneration strategies for skin, soft tissues, muscles, nerves, and vessels, with or without absorbable platforms.

Military Relevance

Due to the severity of combat-related craniofacial injuries, especially those caused by explosive devices, there is a critical need to develop modern technologies, tactics, and techniques to treat such injuries. In Year 2, the SimQuest team leveraged a related project at WRAMC, CFIM. This project addresses the first major step in improving care, i.e., providing an analyzable database, complete with injury-coding tools, as a prelude to planning and optimizing any reconstructive intervention. In collaboration with the existing Center for Excellence for management of complex craniofacial injuries at WRAMC, SimQuest expects to enhance surgical techniques and improve patient outcomes by (1) compiling a database of past craniofacial surgeries on combat casualties; (2) developing tools to encode the case information, to help understand the effects of injury mechanisms and treatments on wounding and restorative care; and (3) creating individualized, detailed facial-structure

models for surgical planning that are tailored to individual patients. This optimized approach promises an improved result in a shorter time, thus improving the quality of life for warfighters with craniofacial injury, helping them to more quickly return to work and productive life, and also having direct applicability to civilian craniofacial injury management.

The SimQuest team will build on the CFIM project, in which a database is being developed that extends the basic surface-wound mapping data to include enhanced detail about craniofacial injury and the treatments that are used. In the CFIM project, the database focus is limited to tracking implants and documenting injuries while the project team's needs include a wider scope including treatment parameters. The researchers selected the database elements in consultation with surgeons at WRAMC in the departments of Neurosurgery; Plastic Surgery; Ear, Nose, Throat; and three-dimensional prototyping. This wide range of input from surgeons ensures a high degree of overlap among the elements of the database and items of interest to the project team. The database schema will be extended to include these elements chosen by surgeons and suitable graphic user interface (GUI) components added to the injury-mapping tool to collect and maintain them. The SimQuest team will begin preparations to integrate reengineered or transplanted tissues into their physics-based simulator in Year 3. The simulator uses tissue properties to drive the physics of the tissue motion, which will be used to



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develop realistic parameters for the reengineered or transplanted tissues that the CFIM team prioritized as focus areas. For engineered tissues, the team will design mechanical tests in consultation with the AFIRM partners who are developing tissue regeneration products.

Craniofacial Injury Mapping

The facial injury database contains a searchable body of knowledge on facial reconstruction. The facial-norm component provides a model of normal facial anatomy as a fixed frame of reference for cross-case analysis of results. The database and the norm together form the foundations for developing tools to capture detailed injury and treatment data. Injury aspects incorporated into the database taxonomy will define the future scope of inquiry. That is, the database taxonomy gives the definition of what aspects of the injury will be captured and become searchable elements of the database. Researchers looking to use the database will only be able to query elements that are in the taxonomy; questions that require further information will remain unanswerable using the tools. Thus, it is important that a diligent taxonomy be established.

Summary of Research Completed In Year 1

During the first year of the project, the researchers developed a virtual reality application that enables visualization of patient-specific wounds and injuries and integrates specific treatments. They determined the granularity of tissue rendition necessary to capture iconic injuries for application in repair/regeneration integration and treatment. The SimQuest team employed a twofold approach to design the injury database. First, they identified which data currently existed in disparate databases and determined what additional data elements were required. Second, they developed an injury taxonomy schema suitable for encoding the data. Assembly of data and data needs from historical and current surgical cases at WRAMC provided the basis for the taxonomy. From this initial gathering of data, the group designed an injury database schema to encode data and, in parallel, developed a three-dimensional model of normal facial anatomy suitable for use in capturing and spatially organizing the data. Finally, they developed a means to

store the markup information in the database, using a space-efficient encoding method that allows the user to search across cases.

Research Progress – Year 2

The SimQuest developers met several times with medical collaborators at WRAMC to discuss and refine the data to ensure maximum applicability to their clinical work. From these discussions, the team compiled a list of more than 100 candidate data elements for potential inclusion in the data taxonomy, subsequently refining and reducing them from the original compilation into a prioritized set for implementation. The team further refined and reorganized these elements into conditions and measurements. Conditions are the elemental medical abnormalities for which the patient is being treated. Measurements are the results of any test or diagnostic. These together provide the initial population of the schema. The refined definition is encoded in the XML format, which plugs into SimQuest's database-generation engine.

Because of the expansive nature of treatments and conditions that might be entered into the database, the team designed and completed the definition for a schema that can expand to accommodate information that was not known at the time the schema was developed. This flexibility will make the resulting software both more robust to change and easier to maintain. A second benefit of this design decision is that the team was able to proceed with the schema definition prior to IRB approval to access patient records. This approach effectively split data definition into an earlier and a later phase, with the later phase being distributed over the course of the data-entry task. As the data are entered, the schema will be expanded as needed. SimQuest extended its data generation engine to accommodate the evolutionary data schema approach.

Methods

1. Data Elements

SimQuest conducted a series of interviews with clinicians at WRAMC and other facilities to elicit the clinical needs for data collection and tracking. The SimQuest team is finishing the alpha version of a CFIM database,

which they will deploy at WRAMC. To accommodate the anticipated growing needs of the CFIM team for data capture, the SimQuest team modified the injury-mapping tool to handle expansion of the data elements without requiring a change to the data schema. This approach will greatly reduce the maintenance needs over the lifespan of the tool. The initial version of the tool will be delivered without the graphical input turned on. The graphical-input portion of the tool, in which users identify precisely where injuries have occurred, is scheduled to be completed later in the year. The three-dimensional surface models that were developed as part of the Year 1 effort will be included as part of the graphical-input version of the tool. Prior to beginning the final development phase for the injury-mapping tool, the SimQuest team plans to coordinate with other CFIM team members to discuss the means of data capture (GUI) and the grouping of inputs into categories that will facilitate researchers' access to the data.

2. Injury Taxonomy

To address the second task, SimQuest is beginning by comparing the tissue properties currently being used for a suture simulation, in which the soft tissue is located in the forearm, with published reports for soft tissue in the facial area. Being a nonhomogeneous area, the face requires different regions to have differing values for material properties. The comparison against literature serves to establish a baseline for the range of values that the simulator will need to support.

At the conclusion of Year 2, the initial results of the project are entering clinical trials. A sample data-entry screen that reflects the updated schema was developed as part of the AFIRM work. This tool is going into first use at WRAMC where the initial focus will be on capturing the data for warfighters who have received craniofacial implants.

The schema will expand over the course of Year 3 as cases are entered and unanticipated conditions are encountered. A further driver for expansion will be the coordination of data collection with the Cleveland Clinic's face transplant team. The SimQuest and Cleveland Clinic teams have conducted initial discussions to highlight

areas in which the parameters that are currently used in selecting transplant candidates could be captured in the injury-mapping tool. As the injury-mapping tool enters clinical use for wounded warfighters, with the intention to expand the system to capture data on all implant recipients, a natural extension of this work will be to capture these parameters of interest for CTA—an extension that fits directly into the AFIRM goal of transitioning research efforts into clinical practice. This coordination with the transplant team will also serve to guide SimQuest's simulation work, ensuring that the focus remains on development of clinically useful technology.

Key Research Accomplishments

- Compiled a list of more than 100 candidate data elements for potential inclusion in the data taxonomy.
 - Refined and reduced the list to a prioritized set of data elements for implementation.
- Developed and delivered a prototype injury-mapping tool.
- Obtained IRB approval to begin patient-data collection.

Conclusions

The complete program will serve the needs of casualties with craniofacial injury. Having direct access to a database of craniofacial injuries and outcomes will facilitate understanding among clinicians and researchers about the long-term impacts of treatment decisions and will pave the way for integrating regenerative surgical techniques into clinical practice.

During work on the data-definition tasks, it became clear that the current injury-coding mechanisms (Abbreviated Injury Scale, Injury Severity Score, and International Classification of Diseases) fail to adequately describe injuries to the facial complex. Although these mechanisms are sufficient for gross anatomical comparison, they do not distinguish among a wide range of injuries, instead grouping injuries within a broad category. The injury taxonomy developed by SimQuest may provide a base from which a more detailed input-coding system can evolve. The three-dimensional markup tools, while not yet completed, provide the basic data-entry and coding structures required for the user to input and



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compare injuries across a population of individuals with craniofacial injuries, thus leading to a platform in which reconstructive and regenerative therapy can be planned and optimized.

The completed project will provide, for the first time, a means to code and track facial injury at a level of detail that provides researchers with tools to study the effectiveness of various reconstructive methods, using a broad-based, systematic approach. Further, it will give a means to track and follow up with individual patients, to help ensure that they continue to receive appropriate medical care as they transition from post-injury care to lifetime sustainment within the Department of Veterans Affairs (VA) system.

Research Plans for the Next 3 Years

Building the three-dimensional aspects of a patient-specific model is a more involved process than extending the relational database of craniofacial injury. The relational database is flat, limited representation of textual data. The three-dimensional representation relates all of that data spatially across and among anatomical boundaries. The CFIM project provides a start in this area, but as a pilot is limited in scope to a focus on hard structures, with a minimum of attention given to soft tissues, beyond a representation of the air-skin boundary. CFR and CTA require significant knowledge of the internal state of soft tissue. Segmentation, registration, and modeling will be employed on specific patients of interest to the CTA team. The patient-specific modeling for AFIRM will delve into the segmentation and modeling of targeted soft-tissue structures such as specific muscles, nerves, vasculature, and scar tissue. New algorithms will be developed to segment these structures from CT data.

After the patient-specific models are sufficiently robust from a geometric standpoint, the next phase will be to use SimQuest's surgical-simulation technology to develop a surgical-simulation tool. Leveraging this technology requires the developers to enhance the geometric model with information about the mechanical properties of the constituent tissues and tissue replacements. Further, an application must be produced that bundles the simulation technology into a clinically relevant package. The simulation tool will be able to show how moved or transplanted tissue will appear, given the geometry and physics of the tissue. This will allow clinicians to take measurements of the way tissues are predicted to interact and indicate the volume of transplant tissue that they will need to achieve satisfactory coverage. This work is currently at TRL 3, with plans to move to TRL 4/5 over the next 3 years.

Planned Clinical Transitions

SimQuest and WRAMC have received IRB approval to begin data entry into the prototype CFIM software. Data entry will begin as soon as a WRAMC craniofacial data coordinator is hired; interviews are currently being held for this position. This software will mature from the current TRL 4 to TRL 5 over the course of the next year. SimQuest and WRAMC have met with the VA representatives to plan coordination of the data-mapping efforts between the DoD medical service and the VA.

Corrections/Changes Planned for Year 3

The connection to the VA will add additional focus to the data collection effort to ensure that there is compatibility between the injury-mapping tools and the VA system. This will not substantially alter the project.



IV: Scarless Wound Healing



BACKGROUND

Scar formation following injury is a major biomedical burden for the U.S. health care system. Both soldiers and civilians suffer from the consequences of dysregulated wound repair, which can lead to severe functional disability and disfigurement. The costs associated with treatment of fibrosis¹ in the United States are estimated to be over \$4 billion per year. Current treatment regimens involving surgery, silicone sheeting, anti-inflammatory medications, and laser/radiation have been disappointing. This is largely due to a lack of understanding of the fibrotic process. The pathophysiology of scar formation suggests the need to regulate numerous aspects of the wound environment, including cells, extracellular matrix (ECM), mechanics, and biochemical signaling.

Wound healing proceeds through overlapping and well-defined phases of repair. This process continues for months and often results in irreversible scar formation with resultant contractures and disfigurement. For any therapeutic approach to be truly successful, it must be comprehensive and encompass the myriad inputs regulating wound healing. Studies of tissue regeneration have implicated the inflammatory environment, matrix components, mechanical context, and cellular players in producing a “scarless” wound profile. The approach taken by AFIRM researchers encompasses a broad continuum of technologies aimed at modulating the tissue response to injury. Collectively, the AFIRM projects represent a collaborative effort to address every aspect and stage of wound repair in a single research program, with the overarching aim of developing a more effective wound management paradigm.

¹ The formation of excess fibrous connective tissue in an organ or tissue as a reparative or reactive process.

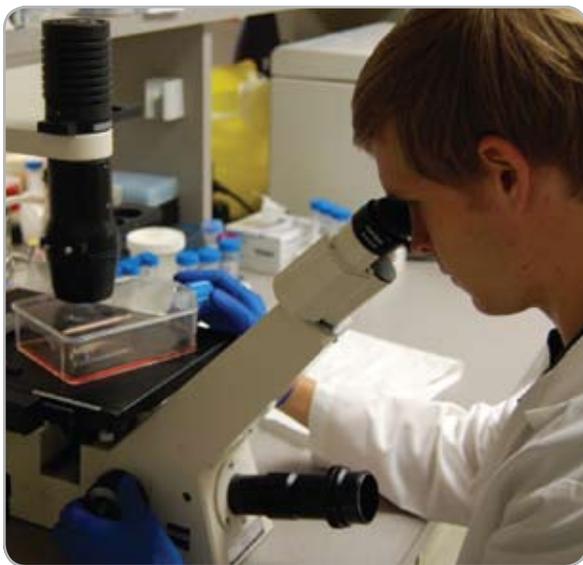


IV: Scarless Wound Healing

Unmet Needs

Effective strategies to promote wound regeneration and prevent scar formation are needed, especially given the increasing survival of injured soldiers returning from the battlefield. The burden of scarring that follows the 230 million surgical procedures performed worldwide each year is enormous. Although the exact incidence of pathologic scarring is unknown, soldiers and civilians continue to suffer from functional disabilities caused by wound contracture and severe disfigurement from hypertrophic scarring. In some instances, the scars become so thick that they can limit movement of joints and greatly restrict the patient's ability to move.

Multiple factors are known to influence wound repair (such as inflammation, oxygen tension, and ischemia) but therapeutic modalities aimed at these targets have been largely unsuccessful. Although antifibrotic biomolecules have demonstrated effectiveness *in vitro*, a major hurdle for clinical translation has been the ability to maintain drug release and bioactivity in a complex wound environment. There is also a lack of effective animal models to study scar formation. Therefore, the development of more appropriate and clinically relevant animal models of hypertrophic scarring remains another unmet need.



Jedidiah McAtee isolates human ACSs, a process which involves enzymatic digestion (WFPC).

Areas of Emphasis

The AFIRM Scarless Wound Healing Program consists of a synergistic combination of nine leading research groups focusing on every aspect of scarless wound healing. Industrial partners have contributed to the initiation of two clinical trials. This program uses complementary approaches (e.g., device, pharma, and biotechnology) to balance short- and long-term objectives. Projects can be grouped into four "clinical challenge" topic areas: Control of Wound Environment and Mechanics, Therapeutic Delivery to Wounds, Attenuation of Wound Inflammatory Response, and Scar Mitigation. Additional details on projects in each of these topic areas can be found in **Table IV-1** and subsequent sections of this chapter.

Control of Wound Environment and Mechanics

Studies at Wake Forest-Pittsburgh Consortium (WFPC)

Using a mouse model of hypertrophic scarring based on increasing the skin stress of healing wounds, the **Gurtner/Longaker team** (Project 4.5.1) at Stanford University found that the skin's biomechanical properties correlated with the amount of scarring following wounding. They have validated the red Duroc pig as an ideal choice for biomechanical skin studies. They have developed computer-modeled wound simulations that allow for the precise characterization of region-specific mechanics. They have also developed a pressure-sensitive, "stress-shielding" device that can modify mechanical forces to control scar formation in the red Duroc pig model. In collaboration with the **Beasley team** (Project 4.5.9) at Neodyne Biosciences, Inc., the Stanford researchers have completed a Phase 1 first-in-man study demonstrating safe and dramatic reduction in hypertrophic scar formation using the stress-shielding devices. The study outcomes demonstrated a significant and dramatic reduction in scar formation in treated wounds compared to untreated within patient controls.

The Gurtner/Longaker team plans to investigate the underlying molecular mechanisms and pathways responsible for this effect to produce next-generation devices more appropriate for burn injuries. They will complete

studies in transgenic mice to elucidate novel pathways in fibrosis that can potentially be used to develop targeted biologic therapies. In collaboration with the Beasley team at Neodyne Biosciences, Inc., they plan to complete a Phase 2 study with a broader surgical patient population encompassing a wider variety of wounds.

Therapeutic Delivery to Wounds

A novel approach is needed to alter the trajectory of wound healing in the initial days following injury to promote the regeneration of tissue. Such an approach could involve the delivery of cells, molecules, proteins, or genes to the wound surface.

Studies at Rutgers-Cleveland Clinic Consortium (RCCC)

The Mustoe group (Project 4.6.3) at Northwestern University is investigating the different wound-healing capabilities and the potential for scar-free healing of curcumin, fibronectin peptide P12, and human bone marrow mesenchymal stem cells (bMSCs) in various rabbit ear wound-healing models. In Year 2, the researchers found that intravenous curcumin promoted wound healing and

scar reduction in a nonischemic (i.e., no deficiency in blood flow) rabbit ear model. In addition, investigations were initiated on curcumin treatment of wounds under conditions of ischemia (deficient blood flow) and ischemia-reperfusion (deficient blood flow followed by return of blood to the affected tissue) in the rabbit ear model. Preliminary results indicate that curcumin improves healing under ischemic conditions. Preliminary results regarding topical application of human bone marrow MSCs in the rabbit ear wound-healing model demonstrated lack of effectiveness. In the upcoming year, the researchers will continue to apply curcumin intravenously in the rabbit ear wound-healing models to confirm and extend the results obtained to date. In addition, they will evaluate topical delivery of curcumin via tiny nanospheres from the Kohn laboratory.

The Katz group (Project 4.7.1) at the University of Virginia is developing regenerative therapies using adipose stem cells (ASCs). The researchers are developing a “wound paste” platform for dermal (skin) repair and replacement that involves the combination of ASCs (and other cells) and a cell-free dermal scaffold. In the

Table IV-1. AFIRM-funded projects per clinical challenge topic area.

Clinical Challenge	Consortium/Institution	Project Number	Project Title
Control of Wound Environment and Mechanics	WFPC	4.5.1	A Device to Actively Control the Mechanobiology During Wound Healing and Prevent Scar Formation
		4.5.9	Neodyne’s Device to Actively Control the Mechanobiology During Wound Healing and Prevent Scar Formation – Clinical Trial
Therapeutic Delivery to Wounds	RCCC	4.6.3	Therapy to Limit Injury (TLI) and Promote Non-Scar Healing After Burns and Severe Battle Trauma
		4.7.1	Adipose-Derived Therapies for Wound Healing, Tissue Repair, and Scar Management
	WFPC	4.5.2	Regenerative Bandage for Battlefield Wounds
		4.5.5	Scarless Wound Healing Through Nanoparticle-Mediated Molecular Therapies
Attenuation of Wound Inflammatory Response	WFPC	4.5.3	Multifunctional Bioscaffolds for Promoting Scarless Wound Healing
		4.5.4	Regulation of Inflammation, Fibroblast Recruitment, and Activity for Regeneration
Scar Mitigation	WFPC	4.5.6	Delivery of Therapeutic Compounds into Injured Tissues
		4.5.7	Scar Mitigation via Matrix Metalloproteinase-1 Therapy



IV: Scarless Wound Healing

coming year, the researchers plan to complete studies that support the filing of an Investigational New Drug (IND) application for the wound paste technology. Over the next 3 years, the research team plans to move the wound paste technology through Phase 1 clinical trials. In addition to these efforts, the Katz group will pursue collaborative studies with other AFIRM members. One such interaction will involve exploring the feasibility of adding ASCs (cell suspensions and/or prefabricated spheroids) to engineered skin substitutes prepared by the Boyce lab, to determine the impact on blood vessel growth within the constructs and/or the development of a subcutaneous adipose (fat) layer. Similar studies are planned with the Christy lab at the U.S. Army Institute for Surgical Research (USAISR), in which ASC spheroids will be evaluated within their established system of blood vessel development and remodeling.

Studies at WFPC

The **Gurtner/Longaker team** (Project 4.5.2) at Stanford University is capitalizing on the ability of wounded fetal tissue to regenerate with minimal scarring by developing a regenerative bandage that contains a fetal-like matrix and wound progenitor (stem) cells. The goal of this bandage is to maintain an acute wound in a pro-regenerative state and prevent the onset of scarring, fibrosis, and infection. The researchers have developed a modifiable hydrogel scaffold that can deliver matrix components, cells, and/or wound-healing drugs. They have seeded these matrices with progenitor cell populations and demonstrated improved cell viability. These matrices integrated well with the host tissue in a mouse excisional wound model and improved early wound healing. Over the next 3 years, the researchers plan to optimize the hydrogel delivery of stem cells into wounds in their mouse model. They will also investigate drug delivery systems using the hydrogel biomatrix to deliver potent antifibrotics into the wound. The research team anticipates the start of a clinical trial of the cell-free matrix by Year 4.

The **Kathju team** (Project 4.5.5) at the Allegheny Singer Research Institution continues to progress in the use of tiny nanoparticles as a nonviral means of delivering molecules into wounds. The researchers have identified a gene (CCT-eta) that is normally decreased in healing

fetal wounds but elevated in adult wounds. They have developed nonviral nanoparticle-mediated delivery systems to selectively decrease the expression of CCT-eta in complex adult wounds. Over the next 3 years, the investigators will continue to investigate the best means of embedding small inhibitory RNAs in plasmid vectors. They will also continue to investigate the possibility of other means to augment delivery into the complex wound environment, including ultrasound enhancement of gene transfer. This research project will not transition to the clinic until after Year 5.

Attenuation of Wound Inflammatory Response

Following injury, an intense inflammatory response ensues and is necessary for normal wound healing. However, aberrations in this process result in chronic wounds and have been strongly implicated in fibrotic scar formation. Redirecting this process toward a regenerative outcome requires controlling the inflammatory response and is the focus of two AFIRM projects.

Studies at WFPC

The **Washburn group** (Project 4.5.3) at Carnegie Mellon University is developing hyaluronic acid biogels that contain monoclonal antibodies or peptides (short versions of proteins) with specific affinities for cytokines and other mediators of inflammation to absorb proinflammatory cytokines and decrease inflammation. The researchers have identified formulations of cytokine-neutralizing gels that are able to control inflammation in wound-healing models. These gels have been shown to rescue viable tissue from inflammation-driven necrosis in a rat burn model. The researchers have licensed their technology to a startup company to aid with manufacturing and commercialization. In the upcoming year, they will conduct follow-up experiments in a burn model in pigs. This will provide a real indication of whether the gels lead to improvements in healing. The team will also perform preclinical tests necessary for regulatory approval. They hope to begin Phase 1 clinical trials to test the cytokine-neutralizing gels in burn patients in 2011 or 2012.



Richard Koepsel, PhD, prepares an enzyme assay for MMP-1 0044 (WFPC).

The **Hebda group** (Project 4.5.4) at the University of Pittsburgh's McGowan Institute for Regenerative Medicine is working to clarify fibroblast and inflammatory mediator interactions with the goal of developing novel anti-inflammatory therapies to improve the quality of healing. The research team has demonstrated that early, short-term topical treatment with the anti-inflammatory agents nimesulide and prostaglandin E2 (PGE2) attenuated the wound inflammatory response, which led to the promotion of healing. In the upcoming year, the group will characterize the contribution of different fibroblast phenotypes on inflammation and collagen production, with the hypothesis that there are unappreciated differences in fibroblast biology that lead to prolonged inflammation in the wound. The researchers will continue preclinical testing in animal models with plans to identify a systemic treatment that can be advanced to a clinical trial after Year 5 of the project.

Scar Mitigation

AFIRM researchers are studying transforming growth factor- β 1 (TGF- β 1) and matrix metalloproteinase-1 (MMP-1) in scar mitigation. TGF- β 1 has been shown to be a major factor in wound repair and skin fibrosis while

MMP-1 has been implicated in the repair of muscle scars and has been shown to improve muscle regeneration when directly injected into fibrotic muscle.

Studies at WFPC

The **Ruoslahti group** (Project 4.5.6) at the Sanford-Burnham Medical Research Institute has identified peptides that home to wounds and deliver therapeutic payloads. They have targeted these molecules to early wound and scar tissue and have shown effective penetration deep into the wound tissue in a mouse model.

They are using the antifibrotic protein decorin to home therapeutics to injured tissue, resulting in the inhibition

of excessive TGF- β 1 activity. Over the next 3 years, the researchers plan to further refine delivery and homing of decorin to demonstrate preclinical efficacy. They also plan to initiate Phase 1 clinical trials and are seeking external funding from both industry and public sources to advance this process as soon as possible.

The **Russell/Koepsel group** (Project 4.5.7) at the University of Pittsburgh is examining MMP-1 collagen matrix interactions in vitro and in preclinical rat and dog studies. The researchers have developed a method for the manufacture of active human MMP-1 that results in a single homogeneous product without degradation products, something not previously attainable. Because of disappointing results with a chemically modified enzyme (loss of activity), the researchers decided to proceed with the native enzyme for future studies. Over the next 3 years, they will continue their preclinical studies in rat and will initiate canine studies to determine optimal timing and dosing and ultimately assess the efficacy of MMP-1 therapy in preventing fibrosis. The researchers aim to transition their product into a clinical trial by the end of Year 5.



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Recently Added Clinical Trial

Autologous Fat Transfer for Scar Prevention and Remediation (AFT-SPAR) – Clinical Trial

This project is a Phase 1/2 study designed to test the safety and efficacy of using Autologous Fat Transfer (AFT) to favorably impact the formation and remodeling of scar tissue that forms in association with burn or other open wounds that heal either by secondary intention or with the use of split-thickness skin grafts.

During the past year, a University of VA (UVA) research nurse, Dr. Catherine Ratliff, was integrated into the AFT-SPAR team. Dr. Ratliff is an accomplished clinical research nurse with many years of experience managing clinical trials at UVA. The

UVA team and the AFIRM Clinical Core reviewed several iterations of the protocol. The protocol was finalized within 6 months and submitted to UVA Institutional Review Board (IRB). In November 2009, the team received final approval from the UVA IRB, achieving a major milestone. The DoD Human Research Protection Office approved the protocol in May 2010.

Patient screening and enrollment are under way at UVA. UVA is the lead site and is expected to enroll between 12-20 patients within 12-18 months. After appropriate approval, USAISR will function as a second site and is expected to enroll between 6-10 patients within 12-18 months. These relative proportions may change depending on subject enrollment rates at each site. The first procedure will be performed in early July 2010.



Dr. Catherine Ratliff, Research Nurse, using a colorimeter for measuring the color intensity of the scar for the AFT-SPAR clinical trial (RCCC).

A Device to Actively Control the Mechanobiology During Wound Healing and Prevent Scar Formation

Project 4.5.1, WFPC

Team Leader(s): Geoffrey C. Gurtner, MD (Stanford University) and Michael T. Longaker MD, MBA (Stanford University)

Project Team Members: Reinhold Dauskardt, PhD (Stanford University)

Collaborator(s): Neodyne Biosciences, Inc.

Therapy: Control of wound environment to minimize scarring

Deliverable(s): Battlefield-ready, region-specific devices capable of

stress-shielding mechanical forces to minimize scar formation

TRL Progress: *Start of Program*, TRL 4; *End Year 1*, TRL 5; *End Year 2*, TRL 6

Key Accomplishments: The Stanford group has validated the red Duroc swine as a viable large animal model to study the mechanical wound environment in the context of skin fibrosis. They designed a novel effective polymer device capable of stress-shielding wounds and off-loading pathologic

mechanical forces to prevent fibrosis in preclinical studies. They also developed incisional and excisional models for hypertrophic scarring in the red Duroc pig based on the manipulation of mechanical wound forces.

Keywords: Hypertrophic scarring, mechanobiology, wound device

Introduction

Scar formation following injury causes significant functional and cosmetic problems. Fibrotic healing causes contractures that prevent joint and other body part movements, and poor skin healing results in considerable permanent disfigurement. Current treatments for scarring are largely ineffective, in part due to our poor understanding of the disease process. One factor thought to affect scar formation and being increasingly studied is mechanical force. The laboratories at Stanford have been highly interested in investigating the mechanisms linking mechanical force with fibrosis and have developed a novel external device capable of controlling the mechanical forces experienced by healing wounds. The goals of this project can be divided into three aims.

Aim 1: To understand the fundamental mechanical properties of unwounded and wounded swine skin in various mechanical stress environments.

Aim 2: To minimize scarring by creating a regenerative stress state in healing swine wounds.

Aim 3: To bring the device to clinical trials and field use.

In the previous year, the Stanford group characterized the mechanical properties of swine skin to develop surgical models to investigate mechanical activation of scar formation. They developed a novel polymer device that was able to control the mechanical forces applied to healing wounds and this dramatically affected scar formation. In collaboration with a commercial company, Neodyne Biosciences, Inc., they completed a highly promising Phase 1 first-in-man trial demonstrating the enormous potential of this device to reduce scar formation.

Summary of Research Completed in Year 1

During the first year of the project, the researchers determined that the red Duroc pig is an ideal choice for biomechanical skin studies. They developed finite element methods to model wound stresses, which allow for precise characterization of region-specific skin mechanics. They also developed the first generation of safe, durable, and modifiable pressure-sensitive adhesive (PSA) devices that could modify mechanical forces and subsequently alter scarring and fibrosis after injury.



IV: Scarless Wound Healing

Progress Reports: Control of Wound Environment and Mechanics

Research Progress – Year 2

The Stanford group has made significant progress in translating preclinical large animal studies into human clinical trials. They have performed extensive biomechanical testing of region-specific skin in both the red Duroc pig and human volunteers. They have developed a finite element model-based computer program to predict mechanical wound forces. Their material science team has developed a novel stress-shielding polymer capable of controlling mechanical wound forces to direct either fibrosis or regeneration (**Figure IV-1**). Their surgical team has developed both incisional and excisional wound models in the red Duroc that allow for mechanobiology studies of hypertrophic scarring. When these devices were configured to increase mechanical forces, there was a significant increase in scar formation based on gross examination and with histological analyses, including collagen production, vascularity, and fibroblast quantities (markers of hypertrophic scarring). When the devices were used in a stress-shielding configuration, there was minimal to absent scar formation on gross and histologic examination with preservation of unwounded epithelial architecture. The research group's collaboration with Neodyne Biosciences, Inc. has resulted in a completed Phase 1 first-in-man clinical trial

with a dramatic reduction in hypertrophic scar formation (**Figure IV-2**).

Key Research Accomplishments

- Characterized fundamental skin properties of *swine* skin in different regions.
- Examined basic biomechanical skin properties of *human* skin in various regions.
- Determined that intrinsic stress levels between swine and humans were similar, thus validating the choice of animal model.
- Developed computer modeling based on finite element analysis to predict wound stress based on injury type, size, and region.
- Developed first-generation polymeric PSA devices for experimental use and determined that they are safe, durable, and modifiable.
- Determined that PSA used on *incisional swine* wounds could effectively and reliably regulate wound stress.
- Observed that elevation of wound stress using PSAs adjacent to incisional wounds led to increased fibrosis, demonstrating that PSAs can be used to modify mechanical force.

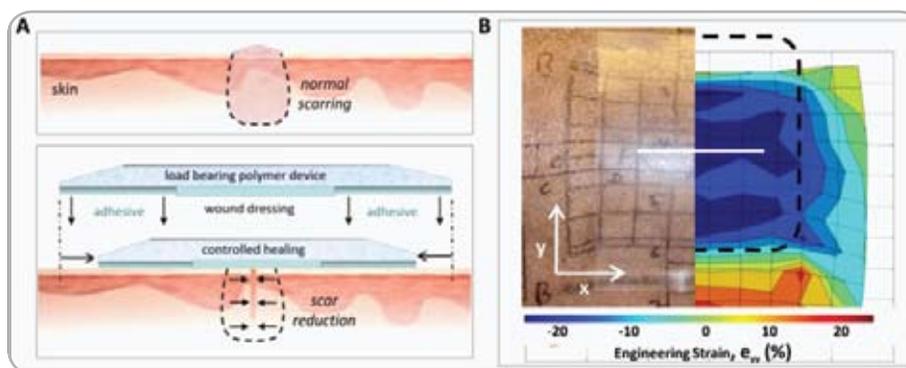


Figure IV-1. A pressure-sensitive polymer device was constructed that could be applied to skin and precisely control the degree of stress modulation based on its material properties and prestrained condition (A). The team validated that this material could provide sustained control of skin forces and that the mechanical skin environment could be tightly regulated with this device (B). These data suggested that this device could potentially be used to increase mechanical forces to create a profibrotic wound environment and conversely, that mechanical wound forces could be mitigated and potentially decrease scar formation. This served as the fundamental hypothesis, namely, that mechanical forces could be manipulated to control scar formation.



Figure IV-2. First-in-man study. Bilateral abdominoplasty incisions were treated on one side with the new stress-shielding device while the other side underwent standard of care without stress shielding. After 6-9 months, a lay panel review was completed using the VAS Scar Scale. Pair-wise assessment showed a statistically significant difference between treated and untreated incisions of 14.1 based on the non-parametric Wilcoxon signed-rank test ($p = 0.004$). A formal panel review by 3 independent plastic surgeons showed a statistically significant improvement in VAS score of 32 points, favoring the treated side ($p = 0.0039$). These dramatic results demonstrate proof-of-principle and device safety in humans, confirming the relevance of the team's large animal studies and establishing the groundwork to begin larger clinical trials.

- Determined that application of PSAs overlying incisional wounds in a “stress-shielding” configuration led to attenuation of wound stress and minimal subsequent fibrosis.
- Obtained strong evidence supporting the fundamental hypothesis, namely, that this novel PSA technology can modify wound mechanical forces and subsequently alter fibrosis following injury.
- Collaborated with Neodyne Biosciences, Inc. to complete a first-in-man study focused on modifying the wound mechanical environment following elective abdominoplasty. The study outcomes demonstrated a significant and dramatic reduction in scar formation in treated wounds compared to untreated within patient controls.

Conclusions

The Stanford group has successfully extended pre-clinical studies to Phase 1 clinical trials over the past 2 years. Proof-of-concept research in the red Duroc pig has yielded significant insight into the ability of polymeric devices to externally manipulate the mechanical wound environment. The researchers have developed models to study the role of mechanotransduction in fibrosis in a large animal known to scar in a similar fashion to human hypertrophic scarring, and these models will highly benefit the scientific research community. The polymeric device has proven extremely safe and effective in human patients, and the research team has achieved a dramatic and significant reduction in scar formation in a Phase 1 trial.

Research Plans for the Next 3 Years

In the next 3 years, the Stanford group has several goals related to the basic science of skin mechanobiology and to the advancement of clinical trials using advanced versions of the polymeric device. They plan to investigate the molecular mechanisms underlying skin mechanofibrosis using biomolecular techniques to study gene transcription and protein secretion. Preliminary targets implicated in transcriptional analysis of porcine wounds suggest that cellular focal adhesion components play a



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key role in communicating and regulating mechanical signaling to induce fibrosis. Mouse knockout models are highly suited to elucidate important mechanisms in this process and will help guide the development of targeted biologic therapies to prevent scarring. These findings would be of significant interest to other researchers examining molecular targets for fibrosis, and the Stanford group itself could apply a wide array of these biologic therapies to their large animal fibrosis model.

Planned Clinical Transitions

Neodyne Biosciences, Inc. has licensed the PSA technology from Stanford University and is in the process of starting Phase 2 trials that will recruit a larger patient population. In conjunction with the Materials Science and Engineering Department at Stanford University, they will further refine the polymeric device to custom design treatments for various size wounds and tension states. This will allow for body-specific regional stress-shielding to address a wide variety of surgical wounds.

Corrections/Changes Planned for Year 3

As explained previously, the Stanford group plans to add murine studies in knockout mice to study the molecular mechanisms of scar mechanotransduction. These studies cannot be performed in large animal models given the inherent difficulties in generating and maintaining transgenic animals. However, insights into the scarring process observed in the red Durocs can be thoroughly studied in a murine model and will potentially reveal novel targets in hypertrophic scar formation that can be used to develop effective antifibrotics. These data will be highly important to other AFIRM researchers studying small molecule and biologically targeted therapies to prevent scarring.

In addition, the red Duroc models of mechanofibrosis are well suited to test any biologic strategies identified in the proposed murine research. Preliminary targets include mice lacking key mechanotransduction components such as focal adhesion kinase, integrin subsets, or other focal adhesion complex elements. These studies would be performed in conjunction with continuing pig and human studies and would add significantly to the progress already achieved in this project by elucidating important underlying molecular mechanisms.

Neodyne's Device to Actively Control the Mechanobiology During Wound Healing and Prevent Scar Formation – Clinical Trial

Project 4.5.9, WFPC

Team Leader(s): Bill Beasley (Neodyne Biosciences, Inc.) and Geoffrey C. Gurtner, MD (Stanford University)

Project Team Members: John Zepeda, Jasper Jackson, and Christy Cowley (Neodyne Biosciences, Inc.)

Collaborator(s): Michael T. Longaker, MD, MBA, Reinhold Dauskardt, PhD, and Paul Yock, MD (Stanford University); Clinical Trial Investigators: Josh Korman, MD, David Kaufman, MD, Regina Rosenthal, MD, Jane Weston, MD, Howard Rosenberg, MD, Daryl

Hoffman, MD, Barry Press, MD, and Laurence Berkowitz, MD

Therapy: Control of wound environment to minimize scarring

Deliverable(s): Commercially available devices capable of stress-shielding mechanical forces to minimize scar formation

TRL Progress: *Start of Program*, TRL 5; *End Year 1*, TRL 6

Key Accomplishments: Neodyne Biosciences, Inc. completed follow-up

and statistical analysis on the Phase 1 first-in-man clinical trial with their device capable of stress-shielding wounds and off-loading pathologic mechanical forces to prevent fibrosis, which showed a dramatic reduction in hypertrophic scar formation in treated wounds compared to untreated within patient controls. The research team has begun a second human clinical trial with a newly designed stress-shielding device.

Keywords: Hypertrophic scarring, mechanobiology, wound device

Introduction

Scar formation following trauma and burn injury leads to severe functional disability and disfigurement. Multiple factors are known to influence wound repair (e.g., inflammation, oxygen tension, and ischemia), but therapeutic modalities aimed at these targets have been largely unsuccessful. Mechanical force has long been recognized to influence cellular behavior in vitro and clinical observations based on Langer's lines and hypertrophic scarring corroborate this phenomenon in vivo.

Recently, the Gurtner laboratory at Stanford University published the first murine model of hypertrophic scarring based on increasing the skin stress of healing wounds. The researchers found that intrinsic skin mechanics correlated with scarring phenotype following wounding, as low mechanical stress fetal wounds exhibited minimal fibrosis and stiffer human skin displayed robust scarring. These findings prompted the initial studies to examine the role of mechanical stress in scar formation and to develop a novel device to actively control wound environment mechanics to mitigate fibrosis.

Today, there are no commercially available wound care products that specifically address the mechanical stress state of healing wounds to reduce scarring. Elastic bandages and pressure dressings provide a widely variable range of compressive forces and are generally used for hemostatic purposes not directly for scar attenuation. Negative pressure wound sponge devices (WoundVac) are used on large, open exudative wounds but require elaborate components and an electrical energy source. In contrast to existing wound care options, Neodyne's technology enables the precision stress shielding of area-specific wound forces through a portable, simple pressure adhesive dressing (**Figure IV-3**). Neodyne is creating battlefield-ready, region-specific devices for different wounded areas of the body, capable of precision stress shielding of mechanical forces to minimize scar formation. Neodyne hopes to commercially distribute these devices for treating wounds of civilians and soldiers post surgery or injury.



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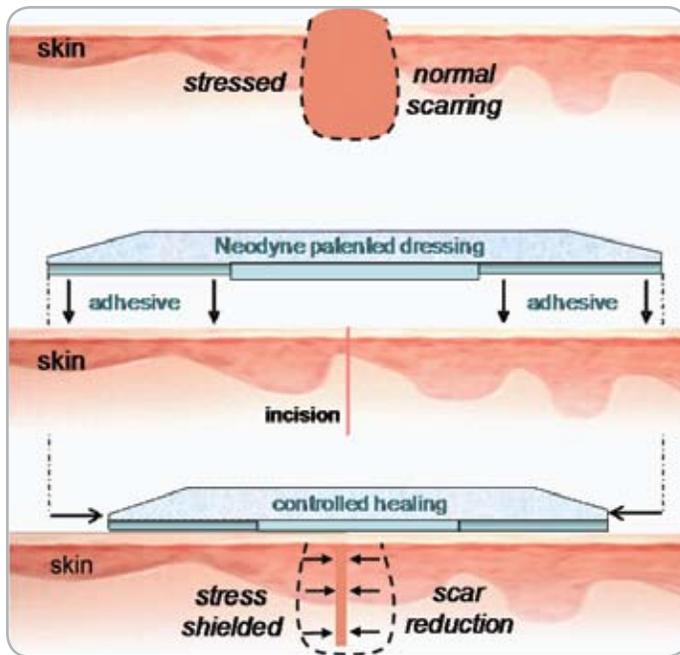


Figure IV-3. Normal cutaneous scarring in humans is dramatically reduced using Neodyne's technology to actively control the wound-healing environment (applicator not shown).

Summary of Research Completed in Year 1

During the first year of the project, Neodyne Biosciences, Inc., in collaboration with the Stanford group (Project 4.5.1), determined that the application of PSA devices overlying incisional wounds in a "stress-shielding" configuration resulted in attenuation of wound stress and minimal subsequent fibrosis. They also obtained strong evidence supporting the hypothesis that their novel PSA technology could modify wound mechanical forces and subsequently alter fibrosis following injury. Neodyne licensed the technology from Stanford University and completed a first-in-man study to modify the wound mechanical environment following elective abdominoplasty (see the following).

Research Progress – Year 2

Neodyne Biosciences, Inc. completed follow-up and statistical analysis on the Phase 1 first-in-man clinical trial, which showed a dramatic reduction in hypertrophic scar formation. Bilateral abdominoplasty incisions were

treated on one side with our stress-shielding device while the other side underwent standard of care without stress shielding. After 6-9 months, a lay panel review was completed using the VAS Scar Scale. Pair-wise assessment showed a statistically significant improvement in treated compared to untreated incisions of 14.1 points (range 0-100). A formal panel review by three independent plastic surgeons showed a statistically significant improvement in VAS score of 32 points, favoring the treated side. These dramatic results demonstrate proof of principle and device safety in humans, confirming the relevance of the large animal studies performed at Stanford in the red Duroc pig and establishing the groundwork to begin larger clinical trials.

Neodyne has also started a second clinical trial called "The Louvre Clinical Protocol," which was approved by the Western Institutional Review Board on June 17, 2009. The study objective was to evaluate the performance of the Neodyne device when used for post-surgical incision care.

The study was designed as a prospective, open label, multicenter study with the goal of enrolling up to 45 subjects. Qualified subjects included those who had an abdominoplasty, abdominoplasty revision, breast lift, and/or breast reduction procedures within 1 week of study enrollment. The researchers have almost completed this study of 61 patients and are in the process of analyzing the data. Further, second-generation devices of varying strain levels have also been released.

Key Research Accomplishments

- Conducted study initiation and training visits with the investigators and study personnel at the 8 clinical investigation sites.
- Screened and enrolled 61 subjects (i.e., 37 abdominoplasty, 3 abdominoplasty revision, and 21 breast reduction/lift).
- Performed 18 monitoring visits to the clinical sites to review and collect study data.
- Released devices with 3 new strain levels (i.e., 50%, 40%, and 30%).

Conclusions

Neodyne has extended the Stanford animal work and its first-in-man clinical study to a second-generation clinical trial over the past year. Proof-of-concept research in the red Duroc pig has yielded significant insight into the ability of polymeric devices to externally manipulate the mechanical wound environment. Neodyne has advanced the work completed at Stanford to demonstrate that the work completed on red Duroc pigs also applies in a human model. The Neodyne device has proven extremely safe and effective in human patients, and their research team has achieved a dramatic and significant reduction in scar formation in a Phase 1 trial.

Research Plans for the Next 3 Years

In the next 3 years, Neodyne has several goals related to the commercialization of its technology using advanced versions of the polymeric device. The researchers plan

to continue with clinical studies in both military and civilian populations to validate the full range of potential uses for the product and to test hypotheses for use on incisional wounds as well as scar revision procedures. Neodyne will continue to collaborate with Stanford University to conduct human trials with advanced versions of the technology that are developed in the Gurtner lab.

Planned Clinical Transitions

Neodyne Biosciences, Inc. is in the process of completing Phase 2 trials in a larger patient population. In conjunction with the Materials Science and Engineering Department at Stanford University, Neodyne will further refine the polymeric device to custom design treatments for various size wounds and tension states. This will allow for body-specific regional stress shielding to address a wide variety of surgical wounds. Neodyne has also been awarded a grant from AFIRM to conduct a clinical trial with military personnel as well as a civilian trial(s) to test a commercial-ready device and applicator.



IV: Scarless Wound Healing

Progress Reports: Therapeutic Delivery to Wounds

Therapy to Limit Injury (TLI) and Promote Non-Scar Healing After Burns and Severe Battle Trauma

Project 4.6.3, RCCC

Team Leaders: Thomas Mustoe, MD (Northwestern University)

Project Team: Seok Jong Hong, MD, Sheng-Xian Jia, MD, PhD, Marina Vracar-Grabar, MS, Yanan Zhao, MD (Northwestern University)

Collaborators: Richard Clark, MD (Stony Brook University)

Therapy: Enhance healing and attenuate scarring

Deliverable: Intravenous treatment with curcumin, P12, and MSCs

TRL Progress: *Start of Year 1*, Curcumin TRL 2, MSCs TRL n/a, P12 TRL 2; *End of Year 1*, Curcumin TRL 3, MSCs TRL 2, P12 TRL 2; *End of Year 2*, Curcumin TRL 4, MSCs TRL 3, P12 TRL 3

Key Accomplishments: The researchers demonstrated the wound-healing potential of systemic pure curcumin and the significant reduction of scarring in the nonischemic rabbit ear model. They developed an ischemia-reperfusion (I/R) injury rabbit ear model to study the therapeutic effect on wound healing in compromised vascular

conditions. They found that xenogeneic cells (i.e., cells derived from donor or other species) in the rabbit ear model induced inflammation and scarring. Finally, systemic delivery of fibronectin peptide P12 in the rabbit ear model had little or no effect on wound healing, suggesting the predominant mechanism is limiting injury progression rather than accelerating wound healing.

Key Words: Burn, wound-healing, hypertrophic scarring, rabbit ear wound model, curcumin, adipose-derived stem cells, fibronectin

Introduction

I/R injury is defined as injury to tissues upon restoration of blood flow, occurring under such conditions as a blast injury, chronic wounds, or myocardial infarction and stroke. During the second year of the project, the Mustoe lab developed an I/R rabbit ear model that replicates some important elements of the I/R injury. Agents such as curcumin and P12 with proven anti-inflammatory, anti-apoptotic, antiproliferative properties were tested in the rabbit ear hypertrophic scar (excess scar tissue) model, ischemic (restriction in blood supply), and for their wound-healing capabilities. The main objective of this research is to identify novel treatment modalities to promote wound healing and reduce scar formation that can be translated into clinical practice.

Summary of Research Completed in Year 1

During the first year of the project, the Northwestern group's initial experiments with crude curcumin demonstrated a modest, but significant, reduction of scarring

in the rabbit ear model at a calculated tissue dose. In addition, the researchers started to evaluate the effects of human bMSCs and rabbit ASCs on rabbit ear wound healing and scar prevention.

Research Progress – Year 2

Aim 1: *Promotion of wound healing and prevention of hypertrophic scar using topical and systemic administration of curcumin and P12 within the rabbit ear hypertrophic scar model.*

1. Curcumin

Study of the curcumin effect on scar formation in the nonischemic rabbit ear model has been completed. Histological results showed a significant reduction in early scar formation with systemically administered low and medium doses of a purer form of curcumin (0.2 and 1 μM) but an adverse effect of the higher dose (2 μM). This work advanced the systemic curcumin treatment therapy to TRL 4 during second year of the program. Encouraged by the positive results of systemic curcumin in the nonischemic rabbit ear model, further experimen-

tation was initiated to evaluate curcumin's effects in the I/R model. Initial results indicate that curcumin had a significant beneficial effect on wound healing and the ability to sustain healing in the I/R rabbit ear model. Curcumin given intravenously at 1 and 2 mM tissue concentrations significantly promoted wound epithelialization and sustained healing when compared to the wounds receiving no drug treatment (Figure IV-4). Systemic curcumin in the I/R study will continue in Year 3.

2. Fibronectin peptide P12

The effect of P12 on nonischemic wound re-epithelialization (re-growth of epithelial tissue over a denuded surface) and granulation (new) tissue formation was tested using three doses of systemically administered P12: 1, 3, and 10 mg/kg. All three P12 doses showed no effect on nonischemic wound re-epithelialization when compared to untreated wound. The wound-healing response in compromised blood supply conditions was also not supported by P12 treatment. The highly encouraging results with P12 in the rat and swine burn comb models suggest the predominant mechanism is to limit injury progression rather than accelerate wound healing. Further experiments with P12 will be considered in the I/R rabbit model and also in collaboration with Kohn's group possibly using different formulations of P12 in the form of topical nanosphere gel therapy.

Aim 2: Promotion of wound healing and prevention of hypertrophic scar using topically administered human bMSCs and rabbit ASCs.

Histological evaluation indicates that human bMSCs and rabbit ASCs failed to promote wound healing measured by re-epithelialization in the rabbit ear model compared to untreated wounds. However, ASC treatment significantly increased the epidermal area of the wounds when compared to the effect of human bMSCs. In addition, rabbit ASC treatment markedly stimulated granulation tissue formation compared to saline (no drug) or human bMSCs. Further, human bMSCs showed increased scar formation while rabbit ASCs

demonstrated a trend toward reduced scar formation. In addition, histologically human bMSCs showed excess inflammation and increased scarring compared to controls, suggesting that xenogeneic cells in the rabbit ear model are challenging. It could be suggested that with immunosuppression treatment this outcome would be different.

In summary, the comparison of human bMSC and rabbit ASC treatment groups indicates that rabbit ASCs have some beneficial effects on wound healing and scar reduction and provide a rationale for stem cell therapy.

Key Research Accomplishments

- Determined that curcumin, given intravenously as a single micromolar dose, promotes wound healing and scar reduction in the nonischemic rabbit ear model and also improves wound healing under limited blood supply conditions.
- Developed two additional wound systems—the ischemic and I/R rabbit ear models—and began to examine the effect of curcumin treatment in these models.

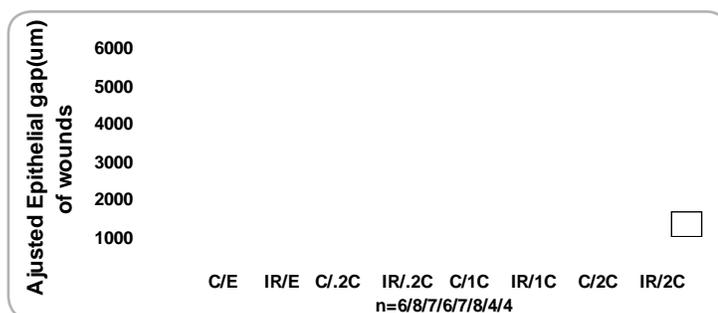


Figure IV-4. Wound epithelialization in the I/R injury rabbit ear model upon curcumin treatment. One and 2 μM concentrations of curcumin have a statistically significant beneficial effect on wound healing in I/R model; t-test, p value: I/R/E vs. I/R/0.2C = 0.364; I/R/E vs. I/R/1C = 0.015; I/R/E vs. I/R/2C = 0.016. There are multiple control groups in this experimental setting. Control non I/R (C/E) refers to the group that has not received I/R cycling nor curcumin treatment (wounding and Et-OH/PBS vehicle injection only); (I/R/E) refers to the group that had received I/R but no curcumin. C/0.2C – Non I/R control that has received 0.2 μM curcumin; I/R/0.2C – I/R cycling and 0.2 μM curcumin; C/1C - Non I/R control that has received 1 μM curcumin; I/R/1C - I/R cycling and 1 μM curcumin; C/2C - Non I/R control that has received 2 μM curcumin; I/R/1C - I/R cycling and 2 μM curcumin; n-number of wounds



IV: Scarless Wound Healing

Progress Reports: Therapeutic Delivery to Wounds

- Obtained preliminary results regarding the efficacy of the topical application of human bMSCs in the rabbit ear wound-healing model.
 - Determined that hMSC treatment induced inflammation and failed to promote wound healing compared to treatment with autologous rabbit ASCs.
- Determined that systemic administration of a single dose of fibronectin peptide P12 had little or no effect on wound healing in both the nonischemic and ischemic rabbit ear models suggesting the predominant mechanism is to limit injury progression rather than accelerate healing.

Conclusions

The effect of curcumin on wound healing and scar reduction in the nonischemic rabbit ear wound model has been confirmed. Study of curcumin on wound healing in two additional wound models, ischemic and I/R, have been initiated and will continue in Year 3. Rabbit ASCs showed a positive trend on wound healing and scar reduction across several of the parameters measured. Human bMSC treatment induced inflammation and failed to promote wound healing in the rabbit ear model, suggesting that xenogeneic cells in this model are challenging. Different cell delivery systems will be considered in future human xenogeneic cell therapy runs in the rabbit ear model. Systemic administration of a single dose of fibronectin peptide P12 had little or no effect on wound healing in both the nonischemic and the ischemic rabbit ear models, suggesting the predominant mechanism is to limit injury progression rather than to accelerate healing.

Research Plans for the Next 3 Years

The evaluation of additional curcumin formulations for topical delivery in the rabbit ear model will continue in Year 3 in collaboration with the SheiHet (New Jersey

Center for Biomaterials – Rutgers University) and Clark (Stony Brook University) laboratories. Proof of concept for improved wound healing and reduced scarring in the nonischemic rabbit ear wound model has been obtained for the intravenous administration of curcumin. For Year 3, studies will be extended to the rabbit ear I/R model, and the delivery of curcumin via nanospheres (therapy developed in The New Jersey Center for Biomaterials – Rutgers University) will be tested. Thus, it is estimated that in Year 3 systemic curcumin therapy will reach TRL 4/5 and topical therapy with curcumin-loaded nanospheres will be advanced to TRL 3. In Year 4, systemic curcumin treatment will be at TRL 5, and assessment of the effectiveness of topical curcumin formulations in all three rabbit ear models will continue, focusing on the refinement of dosing and delivery.

Planned Clinical Transitions

In Year 4, the definitive animal studies will be performed for both therapies (systemic and topical). Pending the results of discussions with the U.S. Food and Drug Administration (FDA) and the Center for Devices and Radiological Health, pre-IND review will be initiated. In Year 5, safety and toxicity studies (TRL 5) will be conducted according to Good Laboratory Practice guidance. Finally, in Year 5, the IND will be submitted and the design of a clinical trial initiated. The pathways to commercialization of both products, systemic and topical treatments, are being pursued in collaboration with the Clark laboratory (Stony Brook University) and industry partners—Neomatrix Formulations, Inc. for systemic delivery and Trident Biomedicals, Inc. for topical delivery.

Adipose-Derived Therapies for Wound Healing, Tissue Repair, and Scar Management

Project 4.7.1, RCCC

Team Leaders: Adam J. Katz, MD (University of Virginia)

Project Team: Ning Yang, PhD, Hulan Shang, MS, and Anna Parker, MD (University of Virginia)

Collaborators: LifeNet Health, Virginia Beach, VA and Glycosan BioSystems, Inc., Salt Lake City, UT

Therapy: Wound healing, scar prevention, and management

Deliverable: Autologous adipose cell-based therapies

TRL Progress: *Start of Year 1, TRL 2; End of Year 1, TRL 3; End of Year 2, TRL 3-4*

Key Accomplishments: The researchers determined that multicell spheroids could undergo spontaneous, self-directed migration into a bilaminar-like orientation when placed onto dermal scaffold. They also demonstrated the ability to seed and subsequently proliferate suspensions of ASCs and/or spheroids onto intact and/or microperforated acellular dermal scaffolds (ADSs), and the ability of such

constructs to assist in wound healing in vivo while also decreasing wound contraction. Finally, they developed a novel product formulation, wound paste, composed minimally of ASCs and particulated ADSs, and demonstrated the feasibility and proof of concept of its delivery to open wounds and its ability to incorporate into and close wounds while minimizing wound contraction.

Key Words: Scar, burn injury, fat grafting, adipose stromal cells, autologous cell therapies

Introduction

Essentially all battlefield wounds involve some component of skin and/or soft-tissue injury and in many cases the tissue loss/damage can be quite extensive. The definitive and often ideal closure of many burn and traumatic wounds involves the use of a skin graft. Most often, split thickness skin grafts are used even though full-thickness skin grafts provide a better aesthetic and functional result. Interestingly, it is the amount (% thickness) of the dermal component of a graft that primarily determines its susceptibility for secondary contraction. Secondary contraction can lead to scar contracture, causing deformity and functional deficit. As a general rule, the thicker the graft (e.g., full-thickness), the less contraction and the better the functional and aesthetic outcome. Currently, there is no full thickness dermal equivalent (with or without an epidermal layer) that contains a patient's own cells (autologous cells) except that excised from the patient.

The main objective of research conducted by the Katz group is to develop a dermal equivalent/replacement

that provides clinical benefit akin to a full-thickness skin graft and uses readily available adipose tissue as a cell source. By minimizing the need for dermal harvest, one can minimize donor morbidity and/or lessen the time needed for cell expansion and product manufacture, thereby enhancing therapeutic availability. Adipose tissue (fat) is an excellent source of autologous cells that are abundant, expendable, replenishable, safe, and easy to harvest. In addition, these cells are known to produce a wide variety of soluble and insoluble factors that favorably impact wound healing and the repair of damaged tissues through the modulation of angiogenesis, inflammation, apoptosis, cell homing, cell proliferation, and cell migration.

At present, engineered constructs for the repair and replacement of skin and subcutaneous tissue differ in their composition: some contain cells while others do not (acellular scaffolds). The cell-based products include allogeneic cells—usually dermal fibroblasts and/or keratinocytes from fetal foreskin samples. None of these products utilizes autologous cells and therefore none of them engrafts permanently. Thus these products would have



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suboptimal properties for definitive closure and healing of traumatic and thermal wounds. There are several potential ways in which adipose-derived cell therapies could offer alternatives, advantages, and/or enhancements to existing and emerging technology platforms:

- The use of ASCs as the cellular foundation of the dermal component of skin constructs may provide expedited manufacturing time frames for autologous tissues and/or reduce the donor site deformity associated with the initial tissue/cell harvest.
- ASCs added to existing constructs may enhance/ expedite the revascularization and “take” of such constructs, given their documented angiogenic properties.
- Acellular dermal scaffolds seeded with autologous ASCs may provide an expeditious and effective autologous dermal substitute.

Strategic Rationale:

The general approach of the Katz group toward the repair and/or regeneration of soft tissue defects involves the combination of autologous cells with some type of scaffold. In the case of dermal defects, the scaffold is ideally intended to provide an immediate “fill” so as to prevent significant contraction of the wound edges while also facilitating the ingrowth and incorporation of cells. Although several scaffold options exist, both synthetic and natural, the Katz lab has focused its research efforts to date on the use of commercially available, de-cellularized human dermal products (e.g., Alloderm[®], DermMatrix[®], FlexHD[®], and DermAcell[®]). In the context of dermal defects, these products offer the advantages of FDA clearance and clinical use, as well as satisfying the reconstructive dictum of replace “like with like.”

From an autologous cell-sourcing perspective, the researchers have focused efforts on the application of adipose-derived cells to its strategic vision. Although dermal fibroblasts represent the logical foundational cell type to repair dermal defects, the harvest and isolation of such cells, especially in the context of immediate, point-of-care therapy, are associated with either significant donor-site morbidity or inadequate cell dose. Subcutaneous adipose tissue, on the other hand, lies right below

the dermis and represents an abundant, expendable, and easily accessible tissue source loaded with high numbers of progenitor/regenerative type cells that are readily implicated in the tissue repair and wound-healing process.

Summary of Research Completed in Year 1

Studies from Year 1 yielded two important findings: (1) ASCs formulated into self-assembling three-dimensional spheres show significantly different biological and therapeutic characteristics as compared to cells grown in “traditional” monolayer culture, and (2) ASCs can readily attach to ADSs, proliferate, and migrate thereafter; however, it is extremely difficult to get rapid, thorough, and uniform incorporation of the cells into the scaffold. This is especially true (and critical) if one is attempting to develop a point-of-care therapy whereby ASCs would be isolated, seeded onto/combined with a dermal scaffold, and applied to an open wound all within the context of a single operative session.

Research Progress – Year 2

During the second year of the project, the research team continued to follow the hypothesis that a dermal replacement scaffold that is thoroughly and uniformly pre-seeded with biologically active progenitor cells would enhance and expedite its incorporation into the recipient wound bed, and thereby enhance favorable wound closure and tissue repair.

The primary technical challenges associated with this study relate to the development of strategies for the (1) efficient and effective seeding/incorporation of cells into a given scaffold/matrix and (2) efficient and effective incorporation and vascularization of a given construct into the host bed/recipient site. During Year 2, the researchers explored several variables/questions in the context of these challenges including:

- **Scaffold type and format:** Is it preferred to use ADS formulated as sheets or as particulates? Are certain brands of ADSs more efficient than others?

- **Cell types and formulation methods:** Can adipose-derived stem/stromal cells serve/function as a cell substitute for dermal fibroblasts? Are single cell suspensions more effective than prefabricated multicellular spheroids?
- **Strategies for seeding cells onto scaffolds:** Are there ways to achieve uniform and patterned seeding of cells onto a scaffold? Is it possible to achieve such in a point-of-care setting?

The research team demonstrated the feasibility of forming self-assembling cell spheroids composed of multiple (up to 3) cell types. They also demonstrated the ability of multicell spheroids (e.g., ASCs and keratinocytes) to undergo spontaneous, self-directed migration into a bilaminar-like orientation when placed onto dermal scaffold (Figure IV-5). They gathered new/additional data to support the hypothesis that ASCs can serve as a cell substitute (in part or in total) for dermal-derived fibroblasts, the harvest of which is associated with lower yield and/or greater donor-site morbidity. The researchers also demonstrated the ability to seed, and subsequently proliferate, ASC suspensions and/or spheroids onto intact and/or microperforated ADSs, and the ability of such constructs to assist in wound healing in vivo while also decreasing wound contraction.

The research team developed a novel product formulation, “wound paste,” composed minimally of ASCs and particulated ADSs (Figure IV-6). The wound paste formulation strategy embodies a strategic balance between the desire/advantages of using of human dermal ECM (or other) as a cell scaffold material and delivery vehicle while also maximizing expedient, efficient, and uniform seeding of therapeutic cells into the scaffold. Furthermore, wound paste represents a cell-scaffold construct formulation that is clinically flexible and amenable to point-of-care therapeutic paradigms. The researchers demonstrated the feasibility and proof of concept of formulating wound paste, its delivery to open wounds, and its ability to incorporate into and close wounds while minimizing wound contraction.

Key Research Accomplishments

- Determined that multicell spheroids could undergo spontaneous, self-directed migration into a bilaminar-like orientation when placed onto dermal scaffold.
- Demonstrated the ability to seed, and subsequently proliferate, ASC suspensions and/or spheroids onto intact and/or microperforated ADSs, and the ability of such constructs to assist in wound healing in vivo while also decreasing wound contraction.

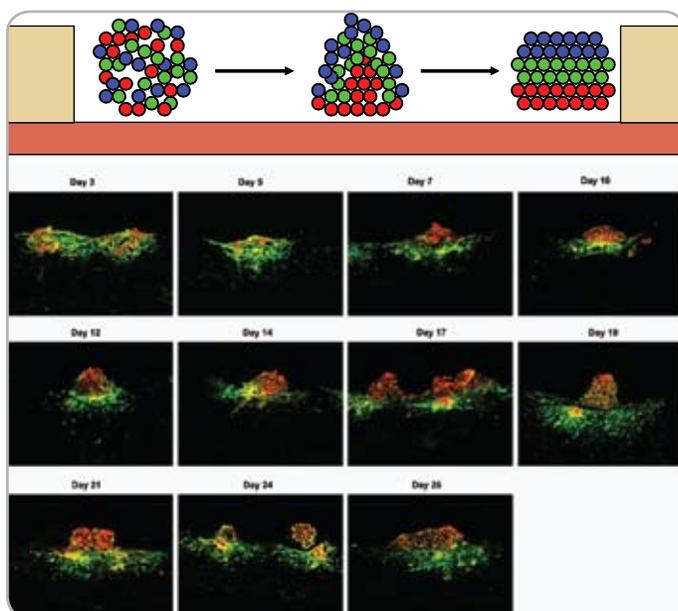


Figure IV-5. *Top:* Schematic diagram of theorized multicellular aggregate spheroid action in wound-site environment. Each color represents a different cell type. This diagram depicts how the cells may migrate from their originally mixed orientation and self-assemble to form the native skin layers and thereby regenerate/repair healthy skin tissue. *Bottom:* Pre-stained ASCs and keratinocytes cocultured as spheroids and seeded onto a dermal template/scaffold. ASCs are labeled with DiO (green) and keratinocytes are labeled with Dil (red). Over 4 weeks, and as early as 1 week, the two cell types localize (i.e., self-assemble) to discrete regions relative to each other with keratinocytes migrating to the top and ASCs migrating and localizing toward/within the dermal scaffold.



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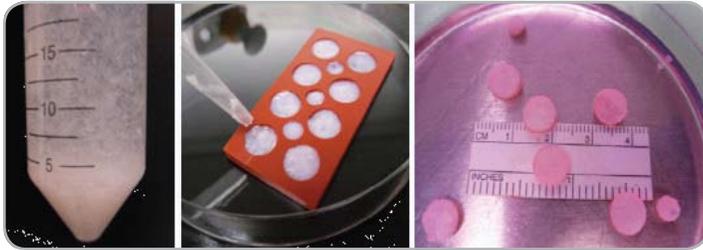


Figure IV-6. Feasibility of wound paste: Acellular dermal “pulp” can readily be mixed with autologous cells and/or other components (e.g., soluble factors and matrix proteins) (left), and delivered directly to wounds (templates) of various size and depth/thickness (middle), followed by subsequent gellation (right).

- Developed a novel product formulation, wound paste, composed minimally of ASCs and particulated ADSs, and demonstrated the feasibility and proof of concept of its delivery to open wounds and its ability to incorporate into and close wounds while minimizing wound contraction.

Conclusions

The Katz group has met its projected major milestone for the end of Year 2: the down selection of several potential strategies involving adipose-derived cell therapies for wound healing and soft tissue repair to a single, lead therapeutic approach/formulation. Given the results of Year 1 studies and continued awareness and evaluation of similar technologies/products and unmet therapeutic needs, as well as manufacturing and regulatory considerations, the research team has decided to pursue the

further development of wound paste as an autologous dermal replacement for the treatment of open wounds. The wound paste approach offers novelty, flexibility, and several other advantages compared to the other strategic approaches evaluated by the team and represents a platform that is realistically amenable to achieving a TRL 5 by the end of Year 3.

Research Plans for the Next 3 Years/Planned Clinical Transitions

Over the next 3 years, the Katz group plans to move the wound paste technology through Phase 1 Clinical Trials (TRL 6). In Year 3, the team will carry out a strategic analysis of potential regulatory paths, including a meeting with RCCC Clinical Core advisors, and a possible pre-IND meeting with FDA. A request for designation or a submission to the Tissue Reference Group of FDA may be indicated. Preclinical studies that address chemistry, manufacturing, and control and quality control parameters will be completed during Year 3. In Years 4 and 5, a clinical trial will be implemented and completed. In preparation and support of these objectives, the research team has identified and initiated discussions with potential partners/companies that can provide anticipated and necessary infrastructure, materials, and expertise.

Regenerative Bandage for Battlefield Wounds

Project 4.5.2, WFPC

Team Leader(s): Geoffrey C. Gurtner, MD and Michael T. Longaker MD, MBA (Stanford University)

Project Team Members: Anthony Oro, MD, PhD (Stanford University)

Collaborator(s): None

Therapy: Improved wound healing and reduced scarring

Deliverable(s): Regenerative bandage that promotes fetal-like wound healing instead of scarring

TRL Progress: *Start of Program*, TRL 1; *End Year 1*, TRL 2; *End Year 2*, TRL 3

Key Accomplishments: The researchers have developed a novel biomaterial scaffold with modifiable open porosity and matrix components. This composite matrix is highly biocompatible with numerous cell types important for wound repair. They have initiated characterization of the dermal architecture of fetal murine skin and unwounded murine skin using advanced

microscopic techniques. They have also demonstrated predictable degradation properties in vivo and shown that the biomaterial scaffold improved early wound healing in a humanized excisional wound model in mice.

Keywords: Dermal matrix, wound healing, fetal skin

Introduction

Wounded soldiers returning from Iraq have sustained significant trauma to the head, neck, face, and limbs. Timing is critical to optimize salvage of traumatic wounds; once wounds are surgically debrided, coverage is important to reduce a prolonged inflammatory state, infection with subsequent contraction, and disability. A novel approach is needed to minimize this inflammatory and fibrotic cascade in the initial days following injury while promoting tissue regeneration. The research team's technical approach begins immediately post injury with a regenerative bandage consisting of a fetal biomimetic matrix and human progenitor cells to maintain an acute wound in a proregenerative state of "suspended animation" and prevent the onset of scarring, fibrosis, and infection. Using their knowledge of fetal skin development, scarless repair, and burn therapy, the researchers hope to preserve wounds in a "fresh state" by recreating a fetal-like wound-healing milieu to promote regeneration and optimize the results of definitive therapy provided back in the United States.

There are several commercial products used for skin engineering based on human or pig skin. These decellularized matrices are effectively used in a variety of

surgical and wound settings, and clinical results are improved in many cases compared to no treatment at all. However, natural skin sources are limited by availability, cost, and risk of disease transmission. Further, clinical results using skin substitutes remain suboptimal due to poor cosmetic and functional outcomes. Synthetic skin substitutes offer the promise of a widely available, disease-free, cheaply produced replacement skin that can potentially improve current clinical outcomes. Fetal wound healing is known to be "scarless" up to the third trimester in mammals, and fetal skin architecture is known to evolve during embryogenesis.

The Stanford group hypothesizes that specific aspects of fetal skin microarchitecture are responsible for the regenerative profile observed with fetal wounding and that changes during adult skin morphogenesis predispose to scar formation. The goals of this project can be divided into three aims.

- **Aim 1:** To design hygroscopic dressings mimicking fetal micropatterning.
- **Aim 2:** To determine the ability of progenitor cells to maintain wounds in suspended animation.



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- **Aim 3:** To utilize progenitor cells to determine their regenerative capacity in wounds delivered via micro-patterned dressings.

The researchers have begun to characterize fetal skin microarchitecture and have developed a novel fabrication technique to create modifiable porous scaffolds amenable to skin and wound applications. Their preliminary experiments demonstrate high biocompatibility both in vitro and in vivo, and early wound-healing results demonstrate a marked improvement in early wound repair processes.

Summary of Research Completed in Year 1

During the first year of the study, the researchers developed porous, modifiable pullulan-collagen hydrogel scaffolds. They determined that microdomain pore sizes can be modulated to mimic those found in fetal collagen patterning. They used microprinting technology to pattern fibronectin on the pullulan-collagen hydrogel scaffolds. The researchers also modified pullulan hydrogels to deliver small molecules (deferoxamine) into murine wounds. They observed that the molecules maintain their efficacy and are effectively released by the dressing biomaterial. They seeded MSCs and fibroblasts into the bioscaffolds and demonstrated excellent cellular biocompatibility. In addition, they determined through preliminary in vivo studies in mice that the pullulan-collagen hydrogels are biocompatible, maintain their architecture, do not incite a robust inflammatory response, and allow cellular incorporation.

Research Progress – Year 2

During the second year of the project, the Gurtner/Longaker group began to characterize fetal murine skin architecture. They have modified their synthetic bioscaffold to mimic the structure of fetal and unwounded murine skin. The porous biomaterial is fabricated using salt-induced phase inversion techniques, and it

displays predictable swelling, degradation, and rheologic behavior. In vitro studies demonstrate high biocompatibility with endothelial cells, fibroblasts, and MSCs. In vivo studies show a predictable degradation profile and no impairments in wound healing when used in a subcutaneous position. When used in a humanized excisional wound model in mice, the researchers noted a significant improvement in early wound healing, possibly related to the induction of granulation tissue formation. This is illustrated in **Figure IV-7** and **Figure IV-8**.

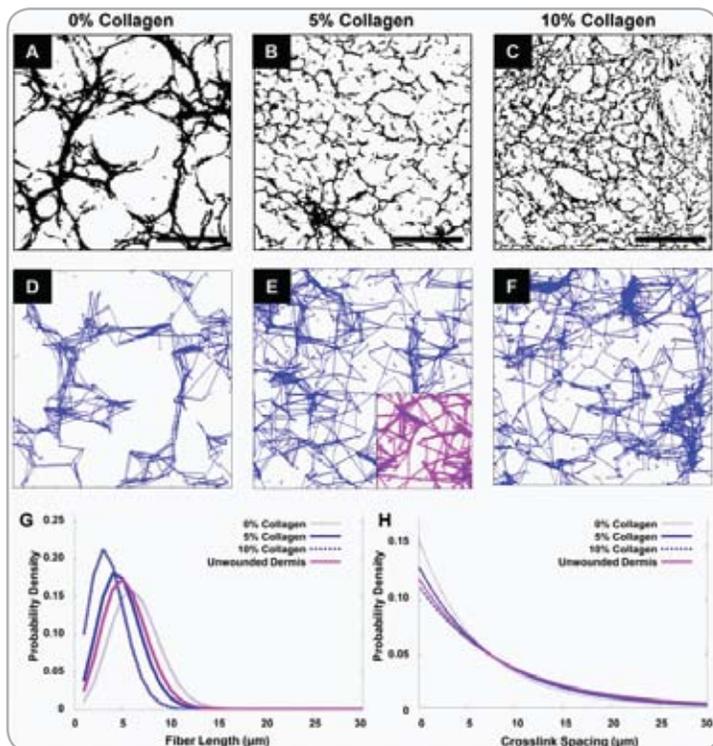


Figure IV-7. Network extraction analysis. A network extraction algorithm was used to analyze the microstructure of the hydrogels. Topographical data were extracted from representative SEM images for 0%, 5%, and 10% collagen-pullulan hydrogels (A-F). The same algorithm was applied to acellular human dermal matrix (E, inset). Based on quantitative analysis of fiber length (G) and crosslink spacing (H) distribution, the team determined that the 5% collagen hydrogel scaffolds (solid blue line) best approximated the microarchitecture of unwounded human dermis (purple line). Scale bar is 100 μm. These data validate the matrix properties of this hydrogel dressing and allow precise control to hydrogel properties to mimic natural skin architecture. This technique can be used to validate the fetal-like hydrogel matrices currently being developed.

Key Research Accomplishments

- Developed a porous biocompatible hydrogel that is highly modifiable and based on the FDA-approved carbohydrate pullulan (widely used in the food industry).
 - Characterized the biomechanical, degradation, swelling, and drug-binding capabilities of the bioscaffold.
- Defined fetal matrix elements that may be critical in scarless healing (e.g., collagen I, collagen III, and fibronectin).
- Refined biomaterial fabrication techniques to allow modification of scaffold pore size and permit micropatterning of matrix elements.
- Demonstrated biocompatibility with murine MSCs, fibroblasts, endothelial cells, and human fibroblasts *in vitro*.
- Demonstrated *in vivo* biocompatibility in a murine subcutaneous model and characterized its degradation and morphology.
- Utilized the bioscaffold in a humanized excisional wound model and demonstrated improved early wound healing.

Conclusions

The research team has successfully fabricated a biocompatible dermal scaffold based on the architecture of fetal murine skin. This material is highly modifiable with predictable swelling, degradation, and rheologic properties. Preliminary *in vitro* studies have demonstrated significant biocompatibility with several wound repair cell types, including endothelial cells, fibroblasts, and MSCs. This wound dressing does not impair wound healing in a subcutaneous model and predictably degrades

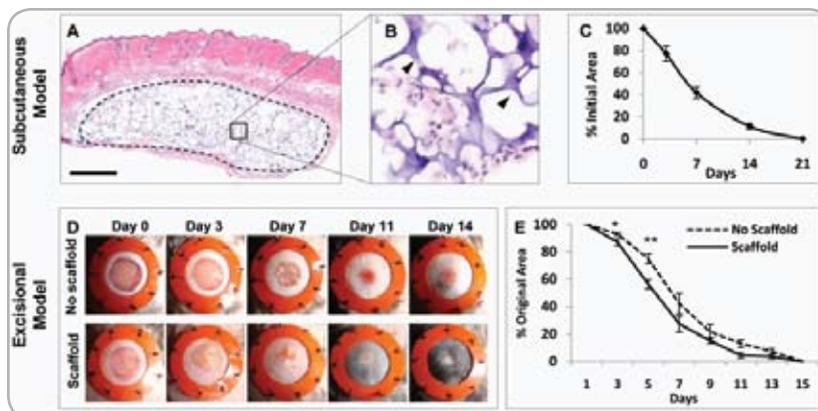


Figure IV-8. *In vivo* studies. Five percent collagen-pullulan hydrogels retained their reticular architecture following subcutaneous implantation in wild-type mice at Day 7 post implantation (A). Zoomed image shows cellular infiltration within the porous scaffold and porous dermal-like domains (B, arrowheads). Quantification of remaining hydrogel area after implantation indicates gradual degradation profile of hydrogels *in vivo* within the subcutaneous pocket (C). Gross photographs of excisional wounds treated with no scaffold (top row) or 5% collagen-pullulan scaffolds (bottom row) from Days 0 to 14 post-injury (D). Five percent collagen-pullulan hydrogel scaffold-treated wounds demonstrated significantly improved wound closure at Days 3 and 5 post injury (E). Scale bar is 0.5 mm. * $p < 0.05$, ** $p < 0.005$. These data demonstrate the *in vivo* applicability and potential of these dermal hydrogel constructs. Their reticular architecture is maintained *in vivo* and allows for cellular incorporation. They exhibit a predictable degradation pattern that permits incorporation into the wound during skin repair. More significantly, hydrogel-treated wounds exhibit improved early wound healing in an excisional wound model. Future experiments will characterize the mechanisms for improved wound closure, and the research team has already begun using the material for progenitor cell delivery into wounds.

over a period of 3 weeks. In a humanized excisional wound model, the pullulan-collagen composite hydrogel significantly improved early wound healing, potentially by stimulating granulation tissue formation.

Research Plans for the Next 3 Years

In the next 3 years, the Gurtner/Longaker group has several goals related to the use of this biomatrix as a regenerative engineered skin application. First, they will use the hydrogel scaffold as a stem cell delivery vehicle to augment wound regeneration. They will need to determine the optimal seeding density and conditions to sustain progenitor cells and fully use their regenerative capacity for wound repair. They plan to assess wound healing using these cell-scaffold constructs in



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healthy wild-type mice and potentially in diseased mice including aged and diabetic animals. In addition, the researchers plan to modify the scaffold to permit the release of growth factors and cytokines that may aid in wound regeneration. Various control release strategies will be employed to recapitulate a "fetal-like" scar-free environment.

Planned Clinical Transitions

The research team plans to continue in vivo small animal studies to further characterize the observed improvement in early wound healing. They plan to file a 510(k) for a cell-free matrix if continued positive results are obtained. This could set the path for clinical trials using this dressing on open wounds to enhance granulation tissue formation.

Corrections/Changes Planned for Year 3

The Gurtner/Longaker group is expanding its use of progenitor cells to include adipose- and bone marrow-derived stem cells to seed into these biomatrices. The use of these cells has greater clinical applicability compared to embryonic stem cells (as initially proposed) and their use bypasses ethical concerns regarding the use of embryonic tissues. Autologous cells can potentially be harvested from injured patients and used to seed biomatrices in vitro for subsequent use as a regenerative wound bandage.

Scarless Wound Healing Through Nanoparticle-Mediated Molecular Therapies

Project 4.5.5, WFPC

Team Leader(s): Sandeep Kathju, MD, PhD (Allegheny-Singer Research Institute)

Project Team Members: Latha Satish, MSc, MPhil, PhD (Allegheny-Singer Research Institute)

Collaborator(s): None

Therapy: 1. Formulation containing small interfering RNA (siRNA) that can be applied to wounds to mitigate scar formation. 2. Probiotic therapy to burn wounds to inhibit pathogenic infection and reduce scarring.

Deliverable(s): To arrive at a formulation of molecular agents that can be applied to healing wounds so that they repair with diminished or absent scar formation using nanoparticulate technology. Also, probiotic therapy to reduce scarring in a burn wound scenario.

TRL Progress: *Start of Program*, TRL 1; *End Year 1*, TRL 2; *End Year 2*, TRL 3

Key Accomplishments: The researchers are using siRNA in novel nanoparticulate formulations to mitigate scar formation in healing

wounds. siRNA versus the eta subunit of the CCT-eta applied to incisional wounds demonstrated no apparent toxicity and successfully decreased the accumulation of two important markers of fibrosis, α -smooth muscle actin and collagen, in the wound bed. In addition, they found that a novel probiotic therapy for infected burn wounds decreased both the load of pathogenic bacteria in the wound and the expression of markers for subsequent scar formation.

Keywords: Scarless wound healing, nanoparticles, siRNA, probiotics, burns

Introduction

The purpose of this project is to arrive at technologies that will enable the reduction of scar formation after injury. Scar, while useful in sealing an injured area, is also the source of significant morbidity, including restriction of movement (e.g., in tendons and muscle), narrowing of viscera, and entrapment of nerves as well as the psychosocial damage associated with severe disfigurement. Burn injuries are particularly prone to extensive and crippling hypertrophic scarring.

This laboratory has investigated mammalian fetal wound healing as a model of scarless healing after integumentary injury. Mammalian fetuses (until the beginning of the third trimester) heal their injuries regeneratively, without attendant scar deposition. The research team has previously used differential display, PCR (polymerase chain reaction) suppression subtraction hybridization, and microarray analysis to identify multiple candidate genes that are differentially expressed in healing fetal wounds. The ultimate goal is to use these gene products to modu-

late the adult wound environment to abolish or mitigate scar formation in healing adult wounds.

To this end, the research team has chosen CCT-eta (found to be specifically reduced in healing fetal wounds) as the initial candidate test gene and has sought to evaluate technologies that would allow for efficient transfection of this candidate molecular agent into healing skin wounds. In particular, the team has evaluated nanoparticle-complexed formulations as a nonviral means of molecular delivery in animal wound models.

Summary of Research Completed in Year 1

During the first year of the project, the research team established in situ hybridization and immunohistochemical protocols for determining the levels of CCT-eta genes and proteins, respectively, in adult wounds. They determined that CCT-eta is increased in healing adult integumentary wounds, which is in contrast to levels of CCT-eta in healing fetal wounds. They identified multiple cell types that upregulate CCT-eta in response to wound-



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ing in an adult organism. They also determined that multiple nanoparticulate carriers of molecular constructs, including atelocollagen and agarose, can be used to modulate gene expression in a wound milieu. Finally, they designed and validated an siRNA-expressing plasmid vector that can be used to manipulate CCT-eta in wounds.

Research Progress – Year 2

Over the past year, the team began the process of evaluating which formulation for an siRNA would yield the most efficient modulation of target gene expression in vivo, and they compared the standard liposomal transfection technique with innovative nanoparticulate formulations using atelocollagen and agarose. They have made significant progress on in vivo administration of siRNA into incisional/excisional wounds using agarose and have clarified the likely mechanism by which CCT-eta suppression can inhibit fibroblast and tissue contraction.

The researchers observed that inhibition of CCT-eta by siRNA led to a marked decrease in alpha-smooth muscle actin (α -SMA). α -SMA is a principal molecular factor in the ability of fibroblast cells to generate force for contraction, and it is postulated that such a decrease can lessen fibroblast contractility and its ability to deform tissue into scar. With their improved ability to deliver an siRNA into a healing wound in an animal model, the Kathju group found that repeated administration of siRNA formulations successfully increased the magnitude and persistence of gene inhibition. A protocol of repeated administration of CCT-eta siRNA appears to decrease multiple molecular markers of scar formation, suggesting this may be an effective therapeutic strategy. The researchers determined that use of this modified approach has no apparent toxicity to the animal and has no apparent negative effect on the rate of wound closure.

The researchers have also made significant progress combating scarring in burn injury. Burn wounds are

frequently superinfected with pathogenic bacteria, particularly *Pseudomonas aeruginosa*. This infection has long been felt to exacerbate the fibrotic inflammatory response to burn injury, and the team's hypothesis is that by suppressing this infectious phenomenon, it will be possible to simultaneously suppress the resultant hypertrophic scarring that can ensue. To accomplish this, the team has established a rabbit model of burn injury that includes the ability to examine the behavior of full-thickness wounds infected with *P. aeruginosa*. The team has used probiotics (living microorganisms that confer a health benefit to the host) as a novel means of managing infected burn wounds. By applying nonpathogenic bacteria (*Lactobacillus plantarum*) to a burn wound, they found that they were able to suppress the ability of *P. aeruginosa* to establish an infection, and this can mitigate the scarring that results as measured by total collagen deposition in the wound (Figure IV-9).

Key Research Accomplishments

- Determined the likely mechanism by which inhibition of CCT-eta can influence adult fibroblast physiology so that it becomes more “fetal-like.”
- Demonstrated that inhibition CCT-eta can reduce the amount of collagen deposited into a healing wound, which is tantamount to reducing the scar burden of the wound.
- Determined that a schema of three administrations of siRNA in agarose can lead to effective suppression of the target molecule (CCT-eta) in a healing wound at 4-5 weeks.

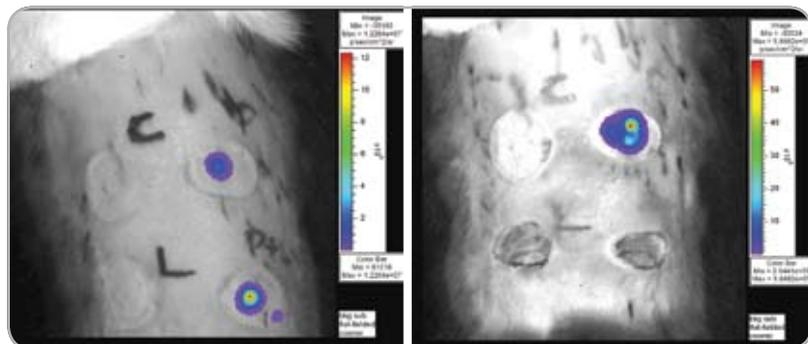


Figure IV-9. Probiotic therapy abrogates a pseudomonas burn infection. Note the absence of light-producing bacteria in a wound in the right panel compared to the left.

- Demonstrated that use of this modified approach has no apparent toxicity to the animal and has no apparent negative effect on the rate of wound closure.
- Established a rabbit burn wound model, including an arm that explores the behavior of infected burn wounds.
 - Demonstrated that probiotic therapy of burn wounds can (1) greatly diminish the ability of the pathogen *P. aeruginosa* to establish an infection and (2) reduce collagen deposition as a marker for scar formation on challenge with *P. aeruginosa*.

Conclusions

The research team concludes that both of the approaches investigated here, nanoparticle-mediated siRNA therapy in incisional wounds and probiotic therapy of burn wounds, demonstrate little to no toxicity, have strong in vivo evidence of efficacy, and warrant further development and refinement.

Research Plans for the Next 3 Years

The research team is presently gathering additional data points for the animal cohorts discussed previously; the intention is to continue to refine the approach so as to maximize in vivo effects. To improve the length of

efficacy of siRNA administration, the group will continue to investigate the best means of embedding siRNAs of interest in plasmid vectors. Their goal is to generate siRNAs in situ for longer periods of time. Although agarose has proven to be an adequate medium for siRNA administration, the group will also continue to investigate the possibility of other means to augment delivery into the complex wound environment, including ultrasound enhancement of gene transfer. Ultimately, the group plans to recapitulate this system in a porcine model (which more closely mimics human skin) as a prelude to potential clinical tests in humans.

With regard to burn injury and probiotics, the group will confirm the results presented herein and expand the metrics of measurement of scar formation. They will also test the ability of probiotics to reduce the local and systemic inflammation elicited by an infected burn wound. The group will also test the ability of probiotics to treat already infected burn wounds and determine how to counteract other burn wound pathogens with probiotics.

Planned Clinical Transitions

Both strategies considered here, nanoparticles and probiotics, are easily applied and are clear trajectories to clinical use although neither is at the stage where this is imminent.



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Progress Reports: Attenuation of Wound Inflammatory Response

Multifunctional Bioscaffolds for Promoting Scarless Wound Healing

Project 4.5.3, WFPC

Team Leader(s): Newell R. Washburn, PhD (Carnegie Mellon University)

Project Team Members: Liang Tso Sun, BS (Carnegie Mellon University)

Collaborator(s): Stephen Badylak, DVM, PhD, MD, Robert Christy, PhD (USAISR); and Michael Lotze, MD (University of Pittsburgh)

Therapy: Cytokine-neutralizing gels

Deliverable(s): Gels that locally control inflammation and promote scarless wound healing

TRL Progress: *Start of Program*, TRL 1; *End Year 1*, TRL 2; *End Year 2*, TRL 3

Key Accomplishments: The researchers have identified formulations

of cytokine-neutralizing gels that control inflammation in wound-healing models. These gels have been shown to rescue viable tissue from inflammation-driven necrosis in a rat burn model.

Keywords: Antibody, cytokine, gel, hyaluronic acid, inflammation, wound healing

Introduction

Inflammation is a critical component of the healing trajectory, but exuberant inflammatory responses can be deleterious for healing. Local control of inflammation could provide a safe method for improving outcomes in a broad range of injuries. Wound healing is an integrated process in which the response to injury follows a trajectory starting with the formation of a blood clot, followed by inflammation, proliferation of connective tissue and epithelial cells, and ending in a remodeling phase. Each phase in wound healing may be characterized by a triad of cells, ECM, and soluble signaling proteins (**Figure IV-10**).

Burns are a significant source of injury and morbidity in combat conditions. Serious thermal injuries result in a cascade of signaling events that include continued tissue necrosis and the formation of hypertrophic scars instead of regenerated tissue. A critical challenge in casualty care is developing advanced therapies for improving burn outcomes.

The trajectory of burn wound healing is a complex process starting with necrosis due to the thermal injury, followed by a two-stage inflammatory process, delayed cell death, formation of granulation tissue, and remodeling. The complications from partial- or full-thickness burns are broad ranging, including compromised protection by the epidermis and loss of resident leukocytes and lymphocytes, edema, reduced host defenses to bacterial colonization, multiple organ failure, and loss of connective tissue cells that would normally contribute to the repair response (**Figure IV-11**). Burned tissue has been modeled as having three concentric zones: (1) irreversibly damaged tissue in the zone of coagulation, (2) hypoperfused tissue in a zone of stasis, and (3) edematous tissue in a zone of hyperemia. The central necrotic zone often progresses into surrounding zones, which increases the likelihood of hypertrophic scarring and patient morbidity.

The extensive tissue necrosis and hyperinflammation associated with burns make them physiologically distinct from other types of acute injuries to soft tissue, but the general framework of wound healing still provides a basis for understanding tissue responses.

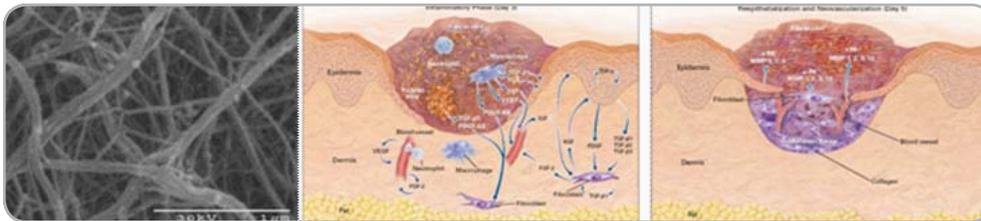


Figure IV-10. Progression from fibrin clot (left) to inflammation (center) to proliferation (right) in cutaneous wound healing.

The goals of treating burns are to promote healing while minimizing scarring and wound contracture. Standard practices of treating partial-thickness burns include cooling with chilled water, topical application of antibiotics, debridement using enzymatic or mechanical methods, and covering with a protective dressing. Current theories of burn progression suggest that the fundamental mechanism of continued tissue damage is driven by inflammatory responses due to the initial injury. The approach used by the Washburn group is to deliver gel therapeutics that locally modulate inflammation by selectively neutralizing cytokines and growth factors that mediate the deleterious physiological responses to thermal injuries.

While there is clearly an immunological basis to the physiological responses to burns, systemic immunotherapies cannot be considered because the patient's immune system is often already compromised, or other medical conditions associated with the injury (e.g., blast damage) preclude this treatment option.

The research team hypothesized that cytokine-neutralizing antibodies immobilized in a gel matrix could locally modulate inflammatory responses at sites of tissue injury. The following specific aims summarize the strategy for developing this new class of therapeutic:

- Specific Aim 1: Identify formulations of cytokine-neutralizing gels that are capable of controlling inflammation *in vivo*.
- Specific Aim 2: Validate cytokine-neutralizing gels in a scar-forming animal model.
- Specific Aim 3: Optimize formulation of cytokine-neutralizing gels in preclinical models and prepare for clinical trials.

In the past year, the team has worked closely with the group of Dr. Robert Christy at USAISR in validating the lead formulation of cytokine-neutralizing gels in a rat burn model.

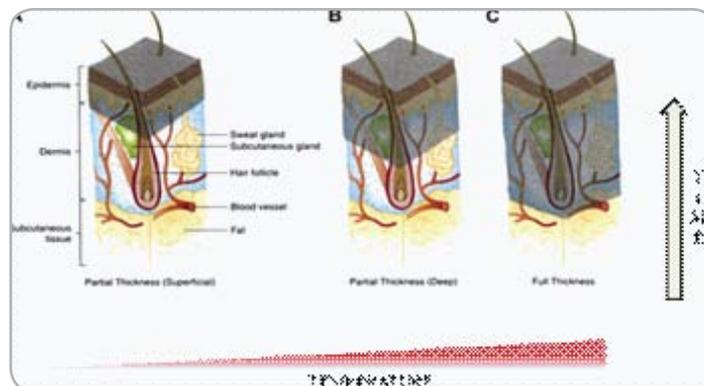


Figure IV-11. The effects of time and temperature on the extent of tissue necrosis at the site of thermal injury.

Summary of Research Completed in Year 1

During the first year of the project, the researchers created uncrosslinked hyaluronic acid (HA) gels by coupling monoclonal antibodies (mAbs) to HA. They also created crosslinked HA gels by covalently attaching the RGD peptide to HA-mAb gels. They determined that HA gels could modulate macrophage phenotype without coupled antibodies. They demonstrated that neutralization of tumor necrosis factor- α (TNF- α) alone may provide significant reductions in inflammatory signaling. They also determined that maximum reduction of inflammatory responses occurs when both TNF- α and interleukin-1 β (IL-1 β) have been neutralized. Finally, they identified material design parameters that optimize the activities of covalently attached monoclonal antibodies.

Research Progress – Year 2

The research team has recently completed the first round of testing in burns on a rodent using novel HA gels functionalized with antibodies against TNF- α and IL-1 β in collaboration with the USAISR. Sites treated with HA gels functionalized with antibodies against TNF- α and IL-1 β also showed evidence for the formation of granulation tissue 2 days following thermal injury, compared with 4 days for sites treated with saline or HA.

The researchers also measured the rate of wound closure as a function of time, which showed that cytokine-neutralizing gels also enhanced the rate of closure at



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later times. Interestingly, the concentrations of IL-1 β were highest at the sites treated with HA-[anti-IL-1 β /anti-TNF- α], suggesting that the gels are indeed sequestering a significant fraction of IL-1 β . However, IL-6, which is produced by cells stimulated by IL-1 β and TNF- α and is an important downstream mediator of burn pathology, was significantly lower at sites treated with HA-[anti-IL-1 β /anti-TNF- α], which suggests that the cytokines inhibited by the gel are inactive.

Key Research Accomplishments

- Identified formulations of cytokine-neutralizing gels based on antibodies against TNF- α and IL-1 β conjugated to HA that are effective at modulating acute inflammation.
- Demonstrated that cytokine-neutralizing gels preserve viable tissue in a rat burn model.

Conclusions

Cytokine-neutralizing gels appear to be a promising therapy for treatment of partial-thickness burns. In Year 3, the researchers will develop a mechanistic understanding of their mode of action, optimize the formulation, and test in a pig burn model to see whether they improve healing outcomes as well.

Research Plans for the Next 3 Years

The research team is currently analyzing samples from a large study in a rat burn model treated with cytokine-neutralizing gels. They expect to publish this in 2010. Also in 2010, the new company will be initiating a pilot study in treating psoriasis patients with cytokine-neutralizing gels (see following paragraphs). As part of this work, skin irritation tests with the gels will be conducted that will be applicable to the approval process for a burn study in humans.

In early 2011, the team plans on performing follow-up experiments in a burn model in pigs. This would constitute a definitive test of the effects of cytokine-neutralizing gels and would provide a real indication of whether the gels rescue viable tissue from necrosis and whether this leads to improvements in healing outcomes. Some-

time in 2011, the researchers plan to perform relevant preclinical tests necessary for regulatory approval, such as measuring toxicity and systemic exposure of the gels. In 2011 or 2012, the researchers plan to initiate Phase 1 clinical trials (as follows).

Planned Clinical Transitions

Team members are working actively toward taking cytokine-neutralizing gels into clinical trials.

Currently, the team is initiating discussions with Dr. Larry Jones, a leading trauma surgeon at Western Pennsylvania Hospital, who has run several clinical trials for other burn therapeutics, on how to run a burn clinical trial. One arm of a clinical trial of cytokine-neutralizing gels could be run in Pittsburgh by Dr. Jones.

The team is also working to establish a partnership with the manufacturer of a commercial anti-TNF- α therapy who would supply therapeutic antibodies for studies in humans. If such an agreement could be reached, it would significantly streamline the regulatory approval process under a 505(b)(2) mechanism.

Finally, the team is planning in late 2010 on enrolling 15 patients in a double-blind, placebo-controlled study in treating psoriasis. Patients will undergo daily treatment on plaques ranging from 10-20 cm² for 2 weeks and outcomes will be assessed by analyzing photographs of treated and control sites. As part of the pilot study in psoriasis, a partnership for performing GMP-compliant production of cytokine-neutralizing gels will be established. This study will also provide a good benchmark to assess the effects of cytokine-neutralizing gels in treating a condition already treated with traditional anti-TNF- α therapy.

In 2011 or 2012, Phase 1 clinical trials to test cytokine-neutralizing gels in burn patients will be initiated. The timing will depend on the outcome of the type B meeting with representatives from the Dermatological and Dental Products Division in Center for Drug Evaluation and Research/FDA that was scheduled for August 18, 2010 to finalize the preclinical requirements for using cytokine-neutralizing gels in treating partial-thickness burns.

Regulation of Inflammation, Fibroblast Recruitment, and Activity for Regeneration

Project 4.5.4, WFPC

Team Leader(s): Patricia A. Hebda, PhD (University of Pittsburgh, McGowan Institute for Regenerative Medicine)

Project Team Members: Joseph E. Dohar, MD and Tianbing Yang, PhD (University of Pittsburgh, McGowan Institute for Regenerative Medicine)

Collaborator(s): None

Therapy: Attenuate local inflammatory responses to reduce scarring and promote healing.

Deliverable(s): Combinatorial anti-inflammatory topical therapy to reduce scar formation

TRL Progress: *Start of Program*, TRL 3; *End Year 1*, TRL 3; *End Year 2*, TRL 4

Key Accomplishments: The research group has demonstrated that early, short-term topical treatment with the anti-inflammatory agents nimesulide and PGE2 can attenuate the wound inflammatory response following skin incisional wounds in the rat, leading

to reduced scarring and increased healing. In addition, the group has characterized the effects of therapeutic treatment on collagen production and organization in the healing wounds. The researchers also established donor cell strains of different phenotypes, including cell strains with regenerative healing capability, for continuing work with cell therapy.

Keywords: Scarless healing, inflammation, fibrosis, cell therapy

Introduction

The Hebda laboratory is focusing on two related processes highly relevant to scar formation: inflammation and fibroblast activity. The overriding hypothesis is that the development of fibrosis can be prevented by blunting early wound healing processes leading to fibroblast recruitment and activation of synthetic properties. To achieve regeneration, it is first essential to regulate the inflammatory response and the influx of host fibroblasts. Control of these two fibrogenic processes will serve to establish an optimal foundation for therapies and interventions leading to regenerative healing. The early inflammatory phase of tissue repair has been shown to be important for the long-term outcome of wound healing.

This project has three Specific Aims:

- To determine the potential of combinatorial anti-inflammatory therapy in decreasing subsequent fibroblast activity in the wound bed.
- To precisely characterize the contribution of the fibroblast phenotype to the overall degree of tissue fibrosis.
- To design interventions, based on the results of the first two aims, that provide a wound environment for rapid, regenerative healing.

The researchers in Hebda's laboratory propose to use a combinatorial yet specific anti-inflammatory therapy to significantly reduce or eliminate scarring associated with dermal wound healing. They hypothesize that a combined treatment of inflammation will result in a synergistic effect not achievable with single-agent therapies. The proposed mechanism of action is a reduction in the chemoattractant gradients normally used by invading fibroblasts along with a diminished profibrotic cue.

The second goal of this study is to precisely characterize the role of fibroblasts in the development of fibrosis. While this issue has been addressed extensively by other researchers, it remains unclear how much of the fibroblast response to injury is an intrinsic property or a response to soluble wound factors. The researchers propose to use a novel method—transplantation of fetal fibroblasts into an adult dermal wound bed—to precisely characterize the impact of inflammatory and other soluble mediators on the fibroblast phenotype. This approach will allow them to determine if the fibroblast phenotype is a dynamic one, largely influenced by the wound environment. Should this be the case, then prevention of fibrosis/scarring could be primarily a matter of reducing profibrotic signals in the wound bed.



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Summary of Research Completed in Year 1

During the first year of the project, the research team demonstrated that early, short-term topical treatment with the anti-inflammatory agents nimesulide and PGE2 can attenuate the wound inflammatory response following skin incisional wounds in the rat, leading to a reduced amount of scarring and the promotion of healing. This determination was based on: clinical assessment of healing, wound histology, tissue levels of ECM components, tissue biomechanics, and collagen organization.

Research Progress – Year 2

The investigators are continuing their targeted anti-inflammatory agent studies. They are also testing the use of cell transplantation together with anti-inflammation for improving healing, using cells that have a capacity for regenerative healing without scar formation, such as fetal fibroblasts and bone marrow stem cells.

The researchers' combination anti-inflammatory treatment revealed:

1. Improved regain of tensile strength in the early to mid-stages of healing.
2. No net increases of total collagen, but a shift in the collagen composition toward a more regenerative (scarless fetal healing) profile.
3. Better organized collagen matrix that presumably contributed to greater tensile strength. (**Figure IV-12**).
4. Slightly increased level of hyaluronan, which needs further verification. Hyaluronan is also believed to be a major contributor to scarless fetal healing.
5. No significant change in the cellular infiltration of total inflammatory cells or fibroblasts. However, this needs to be examined more closely in future experiments to look at specific inflammatory cell subtypes.

The researchers also established donor cell strains carrying the

green fluorescent protein (GFP) marker from GFP rats and initiated cell therapy experiments. This work is ongoing and will continue into Year 3 of the project.

Key Research Accomplishments

- Demonstrated that early, short-term topical treatment with the anti-inflammatory agents nimesulide and PGE2 can attenuate the wound inflammatory response in the rat following skin incisional wounds, which leads to reduced scarring and increased healing. This determination was based on:
 - Clinical assessment of healing
 - Wound histology
 - Tissue levels of ECM components
 - Tissue biomechanics—tensiometry
 - Collagen organization—tissue morphometrics
- Established donor cell strains carrying the GFP marker from GFP rats and began cell therapy experiments.

Conclusions

The researchers have demonstrated that early, short-term treatment with anti-inflammatory agents can attenuate the wound inflammatory response with downstream effects on healing. The combination of nimesulide and PGE2 has specific and potentially useful effects on the healing process. The finding that nimesulide and PGE2-treated wounds have a better collagen organization with similar collagen deposition, yet exhibit higher regain of tensile strength supports the premise that controlled modulation of inflammation can decrease fibrosis without impeding the healing process. These results are very encouraging, and they suggest that the experimental

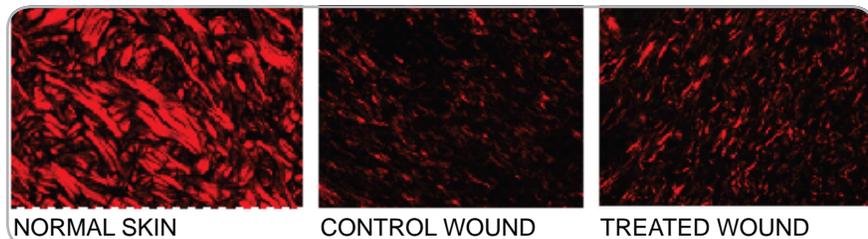


Figure IV-12. The early topical anti-inflammatory treatment caused the collagen fibers (shown in red) to be better organized and more similar to normal skin in wounds shown after 2 weeks of healing has occurred.

plan is feasible and the milestones are achievable. The work will continue with additional studies to verify and optimize results to date. Meanwhile, the project has progressed to the second aim, which includes introducing donor cells into the wounds alone and combined with the anti-inflammatory therapy.

Research Plans for the Next 3 Years

The researchers are in the process of preparing strains of donor fibroblasts and will characterize, expand, and store these cell strains for planned experiments under Aim 2.

The original goals for Year 2 have been accomplished. Hence, the researchers will be able to move ahead to the next objective, which is to precisely characterize the contribution of the fibroblast phenotype to the overall degree of tissue fibrosis. For this work, they are preparing rat fibroblasts of several distinct phenotypes (with respect to their healing properties), as well as adult rat stem cells derived from bone marrow. Each cell strain is labeled with a stable and benign fluorescent tag—GFP. Each cell phenotype will be tested as donor cells in animal wound-healing models and identified in the healing wounds because of the GFP marker. The researchers will be able to tell how well each type of cell strain survives and distributes itself in the wound bed and how it contributes to the healing process. They will use markers for inflammation and collagen production, as well as wound histology and tensiometry, to determine the qualitative and quantitative effects on healing. The researchers anticipate that this part of the project should be done

in Year 3, during which time they plan to complete the analysis of fibroblast contribution to healing outcome.

In Years 4 and 5, the researchers will address Aim 3 (the design of interventions) based on results of the first two aims to provide a wound environment for rapid, regenerative healing. They will combine anti-inflammatory treatments with donor fibroblast delivery to see whether donor fibroblasts (of favorable phenotypes) have a greater impact on healing outcomes. The focus of these studies will be the basis for developing a wound treatment regimen for future clinical trials.

Planned Clinical Transitions

The researchers have selected therapeutic agents that have the advantage of already being approved for use in humans. This should facilitate the pathway toward a Phase 1 clinical trial. The optimal treatment will first be tested in an animal model analogous to the clinical target (still to be determined but possibly a burn injury). The results will determine whether the treatment is ready for clinical testing or whether additional refinement in the animal model is necessary. These clinical studies, however, will likely commence after Year 5 of this project.

Corrections/Changes Planned for Year 3

This project has not had substantial changes made to it. However, the researchers intend to expand upon the original plan by including the testing of systemic treatment (in addition to topical delivery) to maximize the clinical applications for which this approach can be used.



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Delivery of Therapeutic Compounds into Injured Tissue

Project 4.5.6, WFPC

Team Leader(s): Erkki Ruoslahti, MD, PhD (Sanford-Burnham Medical Research Institute at University of California, Santa Barbara [UCSB])

Project Team Members: Tero Järvinen, MD, PhD (Sanford-Burnham Medical Research Institute at UCSB and University of Tampere, Finland); Sajid Hussain, PhD, and Chris Brunquell (Sanford-Burnham Medical Research Institute at UCSB and Institute for Collaborative Biotechnologies)

Collaborator(s): None

Therapy: Drug targeting to injured tissues/preventing scarring.

Deliverable(s): Systemic and local wound targeting with peptides that penetrate into wound and scar tissue.

TRL Progress: *Start of Program*, TRL 1 (Decorin); *End Year 1*, TRL 2 (Decorin); *End Year 2*, TRL 3 (Decorin), TRL 5 (CendR)

Key Accomplishments: The researchers have accomplished three major goals: (1) identification of peptides that home to wounds and can deliver a therapeutic payload to wounds and other injured tissues, (2) development of wound-targeting peptides that penetrate into wound and early scar tissue, and (3) design of a wound-targeted biological anti-scarring agent.

Keywords: Wound angiogenesis, homing peptides, anti-scarring, TGF- β

Introduction

Scar formation takes place in tissue injuries caused by trauma, surgery, inflammation, and ischemia. Current options in reducing scar formation are limited to local intervention. Numerous growth factors and other agents that could potentially enhance tissue regeneration have been identified, but their therapeutic application has been limited in clinical medicine for several reasons. It is difficult to maintain bioactivity of locally applied therapeutic agents in regenerating tissue because of lack of retention of the agent, poor tissue penetration, and instability of protein therapeutics in the protease-rich environment of a tissue injury. Moreover, injuries to organs beneath the skin and multiple sites of injury further limit the usefulness of local treatments.

Clearly, systemic approaches to tissue repair would be valuable. The research team uses wound-homing peptides to achieve effective systemic delivery of therapeutic agents into injured tissues. They have assembled a panel of peptides for this purpose and have demonstrated the effectiveness of the approach by targeting the physiological proteoglycan inhibitor TGF- β into wounds with a resulting reduction in scarring. The latest advance relates to peptides that appear to be capable of penetrating into wound and scar tissue outside the blood vessels.

Aim 1. Peptide-mediated delivery. The research group previously identified two wound-homing peptides that recognize wound blood vessels at different stages of healing. One of these peptides recognizes a wound-specific form of heparan sulfate; the target molecule for the other is not known. The group has established an effective methodology for peptide-mediated delivery of therapeutic compounds into injured tissues (see Aim 2). More recent work has uncovered a revolutionary delivery system based on a novel cell and tissue-penetration system that can be activated by so-called CendR-peptides in a tissue-specific manner. The CendR-peptides provide an avenue of targeting any pharmaceutical agent into a desired location without physically coupling the agent into peptides. Current efforts are focused on establishing the CendR-based targeted delivery of therapeutics for tissue injuries during the healing period.

Aim 2. Wound-targeted decorin. The group has used the wound-homing peptides to target the anti-scarring protein decorin to skin wounds in mice. As reported earlier, the wound-targeted decorin was effective in enhancing the closure of skin wounds and significantly reducing several indicators of scarring. The wound treatment with targeted decorin has been submitted for publication, and a revised version of the manuscript is being prepared.

The results were described in preliminary form in the Annual Report for Year 1; but since the decorin project has been the main focus during the past year, the group has generated additional and revised data, which are described here. The group plans to advance the targeted decorin toward clinical trials and is taking the steps to acquire funding for this venture.

Summary of Research Completed in Year 1

During the first year of the project, the researchers produced and purified a target-seeking antifibrotic agent (recombinant decorin fusion protein) in mammalian expression vectors and baculovirus. They established the *in vitro* biological activity of the decorin fusion protein. They achieved targeted delivery of the decorin fusion protein into regenerating tissue following intravenous injection in mice. They also demonstrated that the decorin fusion protein could inhibit TGF- β -dependent scar-associated processes. Finally, they determined that the decorin fusion protein could inhibit scar formation in mice during wound healing.

Research Progress – Year 2

The researchers previously identified two peptides (small pieces of protein) that recognize the blood vessels in wounds. These so-called “homing” peptides can be used to deliver therapeutic compounds into injured tissues so that the injured tissue receives a higher concentration of the therapeutic than normal tissue (Figure IV-13). The advantages of this approach include increased efficacy of the therapeutic agent at the site of injury and reduced side effects in normal tissues.

The research team has recently uncovered a novel delivery principle based on homing peptides that penetrate into the target (injured) tissue and into cells in that tissue (Figure IV-13). They activate a transport system that sweeps along co-injected drugs into the target tissue. This discovery greatly broadens

the utility of the targeting technology; the peptide no longer has to be chemically coupled to the drug to be delivered.

The earlier coupling-based technology has been successfully used to deliver an anti-scarring protein decorin to skin wounds in mice. The wound-targeted decorin improved the closure of skin wounds and reduced scarring in them under conditions where nontargeted decorin was ineffective. An effort is under way to raise funds to advance the wound-targeting decorin to clinical trials.

Key Research Accomplishments

- Identified peptides that home to wounds and can deliver a therapeutic payload to wounds and other injured tissues.
- Developed wound-targeting peptides that penetrate into wound and early scar tissue.
- Designed a wound-targeted biological anti-scarring agent.

Conclusions

Good progress is being made on the development of systemic delivery of therapeutic agents to injured tissues. The tissue-penetration technology, the development of which has started in the past year, offers particular promise. Scar tissue is dense and particularly impermeable to drugs. Preliminary data from this project suggest that it may be possible to enhance drug delivery to already es-

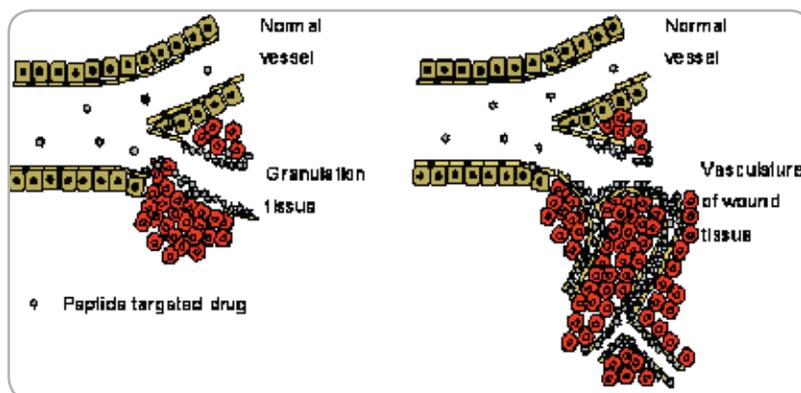


Figure IV-13. Schematic presentation of peptide-targeted drug accumulation in the target.



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established scar tissue. The data also show that a known anti-scarring agent, decorin, can be made much more active by homing peptide delivery.

Research Plans for the Next 3 Years

The researchers plan to develop a technology that is generally applicable to systemic targeting of injured tissues. This technology is intended for applications that cannot be dealt with by local delivery to injured areas. The focus will be on injury-specific targeting, wound and scar tissue penetration, and demonstrating the efficacy of the technology with decorin, an anti-scarring agent that had time and again been proven to be effective in experimental systems.

Planned Clinical Transitions

The researchers hope that the improved efficacy of the targeted decorin will encourage clinical trials. The advantages are that less of the recombinant protein needs to be manufactured and that patent coverage will extend into the late 2020s. Discussions with potential commercial partners and public funding agencies are under way to advance the project.

Corrections/Changes Planned for Year 3

The main new element that was not foreseen when the original application was submitted is that it is possible to use tissue-penetrating homing peptides to deliver drugs to a target tissue without coupling the drug to the peptide. The peptide activates a transport system in the specific target tissue that sweeps along any compound in the blood. Major improvements in the delivery of drugs to injured tissues may ensue.

Scar Mitigation via Matrix Metalloproteinase-1 Therapy

Project 4.5.7, WFPC

Team Leader(s): Alan Russell, PhD and Richard Koepsel, PhD (University of Pittsburgh)

Project Team Members: Yong Li, MD, PhD, Johnny Huard, PhD, and Harry Blair, MD (University of Pittsburgh)

Collaborator(s): None

Therapy: Treatment of muscle scars

Deliverable(s): A formulation, method, and treatment regimen for remediation of pre-formed muscle scars using MMP-1.

TRL Progress: *Start of Program, TRL 2; End Year 1, TRL 2; End Year 2, TRL 3*

Key Accomplishments: In the course of the research for this project, a method for the manufacture of active human MMP-1 that results in a single homogeneous product without the degradation products seen in previous batches of the enzyme was developed. The enzyme was modified with polyethylene glycol, which enhanced stability of the enzyme, and a collagen-binding peptide that demonstrated enhanced binding to collagen. A disconnect between results obtained with the most commonly used assay for MMP-1 and the ability of the enzyme to cleave type 1 collagen, its natural

substrate, was shown, and results from assays of the modified enzymes with collagen showed that the modifications significantly reduced activity toward collagen and diminished their potential as therapeutic agents. Protocols for in vivo analysis were approved by Institutional Animal Care and Use Committee (IACUC) and Animal Care and Use Review Office (ACURO).

Keywords: Muscle scars, remediation, matrix metalloproteinase I

Introduction

Previous pilot studies have demonstrated a possible connection between MMP-1 and the healing of muscle scars. Scarring often occurs during the healing of skeletal muscle injuries. This scarring inhibits complete healing of the muscle and can result in significant loss of muscle function. MMP-1 is an enzyme normally involved in remodeling of ECM and works by hydrolyzing type I collagen. It has been shown injection of MMP-1 into a muscle scar can improve muscle regeneration by breaking down the collagen fibrils within the scar tissue.

The first phase of this project was designed to investigate the interaction between MMP-1 and collagen in vitro with the aim of gaining a better understanding of relevant kinetic parameters. The native active enzyme was compared to chemically modified variants designed to enhance stability or binding to determine the best form of the enzyme for therapeutic purposes.

Summary of Research Completed in Year 1

During the first year of the project, the researchers developed a method to produce pure homogeneous MMP-1 enzyme for use in preclinical trials, modified MMP-1 with a collagen binding peptide, developed the Dual Polarization Interferometer as a platform for determining real-time MMP activity, and demonstrated differences in activity of MMP-1, PEG-MMP-1, and peptide-MMP-1.

Research Progress – Year 2

The researchers have been characterizing the kinetic properties of MMP-1 and modified versions of MMP-1. It was shown that the unmodified MMP-1 has significantly higher activity against collagen than either of the modified versions. The enzyme modified with a collagen binding peptide did demonstrate enhanced binding to collagen. Several methods were developed to assess the diffusion rate of MMP-1 through collagen matrices. None of these methods resulted in acceptable answers. Experiments that incubated MMP-1 with an adherent col-



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lagen layer showed dramatically different activities as the temperature was increased suggesting that the in vivo conditions encountered by MMP-1 might be significantly different from those of in vitro experiments.

A protocol was developed to study the effect of MMP-1 on muscle scars in rats. The protocol uses an excision model of a muscle scar and a dosing regimen calculated from previous pilot studies in mice. The protocol has received IACUC and ACURO approval. Assumptions from the in vitro experiments, namely that MMP-1 binding to collagen does not significantly change its diffusion and that the temperature and physical form of collagen have very large effects on MMP-1 activity led to the development of a mathematical model for predicting the range of effective doses for a given scar. The model will be solved and the solution used to inform the selection of doses in the animal experiments.

Key Research Accomplishments

- Developed a method to produce pure homogeneous enzyme for use in preclinical trials.
- Modified MMP-1 with a collagen-binding peptide.
- Demonstrated the differences in activity of MMP-1, PEG-MMP-1, and peptide-MMP-1.
- Developed and obtained approval of an animal protocol for testing MMP-1 in rats.
- Developed a mathematical model for calculating therapeutic dosing.

Conclusions

The research team has shown that modification of MMP-1 to improve stability or binding characteristics of the enzyme results in significant reduction of the activity of the enzyme against collagen. Further, while modification with PEG did enhance stability of the enzyme, the enhancement was negligible under physiological conditions. The collagen-binding enhancement obtained by modifying MMP-1 with a collagen-binding peptide was offset by the reduction in the activity of the modified enzyme. The group has thus down-selected unmodified MMP-1 for animal trials.

Using the unmodified MMP-1 as the therapeutic agent reduces the number of animals that will be needed. The effective dose of MMP-1 can be determined by a dose ranging study using the dose from a pilot study in mice as the basis for dosing decisions. An animal protocol based on these conclusions has been approved by the IACUC. The development of a model for behavior of MMP-1 at the site of a scar will be used to recalculate the therapeutic doses (if necessary) for the rat studies.

Research Plans for the Next 3 Years

The group is still on track to proceed with Aims 2 and 3 of the project with minor alterations that remove an animal trial that was intended to select the form of the enzyme for therapy. The amended future aims of the project are thus:

Aim 2: Determine the efficacy of MMP-1 against muscle scars in rats. The tasks in this aim will provide the parameters of a treatment protocol that will be translated to a large animal (canine) model. Specifically, the researchers aim to complete: (1) a trial that will determine an effective dose of the active enzyme using the best conditions from Aim 1 as a guide, (2) a trial giving multiple doses of enzyme, and (3) a single dose trial to determine optimum time after injury for delivery of the MMP-1.

Aim 3: Translate the findings of the rat studies to a canine model. This task will be a pilot study to determine whether the technology can be scaled up and be used to treatment of large injuries like those that may be encountered in the field.

Planned Clinical Transitions

Successful completion of the aims of this project will lead directly to clinical trials.

Corrections/Changes Planned for Year 3

As reported previously, an animal trial that down-selects the enzyme form to be used in further studies will be eliminated since the modified enzymes are significantly less active.



V: Burn Repair



BACKGROUND

Changes in weaponry have resulted in a higher frequency of burns, often in combination with other severe injuries. These injuries are costly in both human and economic terms and carry risks to survival, independence, and function, both in the short term and over time as the injuries mature with scarring and movement-limiting contractures. The current standard of care for burn injuries includes early excision of the damaged tissue and autografting (i.e., transplanting tissue from one part of the body to another), and these procedures have not fundamentally changed in more than 30 years. Finding

novel solutions to the challenge of burn injuries will improve outcomes for wounded warfighters suffering not only from burns but from other debilitating and disfiguring injuries where vasculature, wound coverage, or scarring are problematic. It is anticipated that the therapies developed by AFIRM researchers will serve the military population as well as later translate to the civilian population, where there are more than 1 million burns in the United States each year, resulting in 900,000 hospital days, 4,500 deaths, and more than \$1 billion annually in treatment costs and lost productivity.



V: Burn Repair

Unmet Needs

Although respiratory distress is often the critical issue immediately after burn injury, skin loss becomes the major problem within the next 24 hours. Disruption of the skin barrier results in fluid and heat loss, and a predisposition to infection. Progressive inflammation and extension of burns during the first few days after injury compounds these problems. Acutely, deep second-degree burns often extend to become full-thickness, third-degree burns with resultant increased tissue loss, longer healing times, and excess morbidity and mortality. Over the long term, burn progression results in increased scarring, wound contractures, and poor quality of life. Therapies such as nonsteroidal anti-inflammatory drugs (NSAIDs) and antioxidants have not shown substantial benefit in preventing injury extension. Hence, a critical unmet need is prevention of burn inflammation and injury progression.

Nonviable tissue within the burn favors bacteria colonization and infection. Despite a reduced incidence of invasive infection, this complication remains the most common cause of morbidity and mortality in patients with extensive burns. Although Sulfamylon[®], Silvadene[®], and silver nitrate are frequently used to prevent burn infections, each has disadvantages or adverse side effects. Sulfamylon[®], a suspension of mafenide acetate in a hydrophilic cream, is bacteriostatic for both gram-positive and -negative organisms, and penetrates the eschar (scab that forms over a burn injury) extremely well. However, it is painful when applied to partial-thickness burns, and it inhibits carbonic anhydrase, which can lead to metabolic acidosis if applied over an extensive surface. Silvadene, a suspension of silver sulfadiazine in a hydrophilic base, does not induce pain or disturb acid-base balance but fails to penetrate the eschar well and often does not protect against *enterobacter* and *pseudomonas*. Furthermore, it may induce neutropenia (a disorder characterized by an abnormally low number of neutrophils, a type of white blood cell that fights infection) or even pancytopenia (a medical condition characterized by reduced levels of white and red blood cells and platelets). Silver nitrate 0.5% solution is active against a broad spectrum of bacteria but cannot penetrate the eschar and is caustic, damaging otherwise viable tissue. Clearly

new topical antibiotics are needed for prevention of infection in burn patients.

After burn wound excision, cutaneous autografts (skin grafts taken from an unburned part of the same patient) are optimal for closure; however, this mandates a viable wound bed and available donor sites. In patients with extensive burns, the area in need of grafting may outsize available donor sites. Thus, donor site reharvesting would be necessary as soon as possible, and therefore optimal, ongoing care of the site(s) becomes critical. Maximum rate of re-epithelialization and minimum trauma to the donor site are key goals of good care. A critical need therefore exists to speed re-epithelialization of autograft donor sites to reduce reharvest time.

Insufficient normal skin availability can limit the burn area covered, even with meshed autografts and donor site reharvesting. If a temporary covering is indicated for the excised wound site, fresh cadaver skin allograft is currently favored although silver dressings, composite hydrogels, and cultured epithelial autografts can be useful. Frozen cutaneous allografts (tissue grafted from one individual to a genetically nonidentical member of the same species) and porcine cutaneous xenografts (tissue grafted from one species to an unlike species) are the two most readily available skin substitutes, but they are (a) less adherent to the wound bed than fresh autografts, (b) less able to control the bacterial population of the underlying wound, and (c) usually do not become well-vascularized (infiltrated with blood vessels) from the underlying wound bed. Another alternative is cultured autologous keratinocyte sheets, but these are limited by a 3-4 week preparation time, sheet fragility, and susceptibility to infection. Synthetic skin substitutes have also been used with limited success. An effective synthetic skin substitute should be compatible with the patient's own tissue, have no antigenicity or toxicity, have water vapor permeability similar to that of skin, be impermeable to microorganisms, adhere to the wound, be readily vascularized, and have an indefinite shelf life. The available skin substitutes need to be modified to increase their clinical usefulness by enhancing both their resistance to infection and their ability to accelerate the formation of either neodermis or granulation tissue (the fibrous connective tissue that replaces a clot in healing wounds).

The therapies and innovations proposed by AFIRM researchers and described in this chapter should reduce wound scarring and contractures, as well as prevent burn injury progression, reduce inflammation, and induce healing following burn injury.

Areas of Emphasis

Rutgers-Cleveland Clinic Consortium (RCCC), Wake Forest-Pittsburgh Consortium (WFPC), and U.S. Army

Institute of Surgical Research (USAISR) researchers are pursuing a complementary mix of research projects focused on various aspects of burn injury. Projects can be grouped into four “clinical challenge” topic areas: Intravenous Treatment of Burn Injury, Topical Treatment of Burn Injury, Wound Healing and Scar Prevention, and Skin Products/Substitutes. Additional details on projects in each of these topic areas can be found in **Table V-1** and subsequent sections of this chapter.

Table V-1. Projects funded by RCCC, WFPC, and USAISR per clinical challenge topic area.

Clinical Challenge	Consortium/Institution	Project Number	Project Title
Intravenous Treatment of Burn Injury	RCCC	4.6.1	Therapy to Limit Injury Progression, Attenuate Inflammation, Prevent Infection, and Promote Non-Scar Healing After Burns and Battle Trauma
		4.6.2	Mesenchymal Stem Cells for Burn and Wound Healing
Topical Treatment of Burn Injury	WFPC	4.2.3	Novel Biomaterials That Support the Survival of Damaged Cells and Tissues
	RCCC	4.6.4	Polymeric Iodophor Absorbent Antimicrobial Wound Dressing
		4.6.5	Topical P12 Delivery via Biodegradable Fibro-Porous Mats for Burn Treatment
		4.6.6	Topical Curcumin-Containing Therapies to Promote Scarless Healing
Wound Healing and Scar Prevention	WFPC	4.2.2	Delivery of Stem Cells to a Burn Wound via a Clinically Tested Spray Device--Exploring Human Fetal Skin Progenitor Cells for Regenerative Medicine Cell Based Therapy Using Cell Spray Deposition
		4.2.4	Artificial Extracellular Matrix Proteins for Regenerative Medicine
		4.2.5	In Situ Bioprinting of Skin for Battlefield Burn Injuries
		4.2.7	A Multicenter Comparative Study of the ReCell® Device and Autologous Split-Thickness Meshed Skin Graft in the Treatment of Acute Burn Injuries
	USAISR	4.6.7	The Impact of Trauma on the Potency of Adult Stem Cells
Skin Products/Substitutes	WFPC	4.2.1	Tissue Engineered Skin Products – ICX-SKN
		4.2.1a	Tissue-Engineered Skin Substitute for Burns
		4.2.6	Amniotic Fluid Stem Cells for Burn
		4.2.8	In vitro Expanded Living Skin for Reparative Procedures
	RCCC	4.7.2	Engineered Skin Substitutes
	USAISR	4.6.8	Adipose-Derived Stem Cells for Tissue Engineered Dermal Equivalent



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Intravenous Treatment of Burn Injury

Studies at RCCC

The **Lin group** (Project 4.6.1) at Stony Brook University conducted a focused screening investigation for agents that might inhibit the progression of burn injury. Agents were selected for their reported ability to inhibit oxidative stress (the nutraceutical curcumin, which is found in the spice turmeric), cytokine stress (pentoxifylline, approved by the U.S. Food and Drug Administration [FDA] in oral form), or apoptosis (fibronectin peptide P12, recently discovered in the Clark laboratory, two patents pending). During the past year, the research team further characterized the actions of each of these three agents. They are conducting experiments aimed at determining the optimal doses of curcumin and P12 in a porcine hot comb burn model. As soon as proof-of-principle is obtained from experiments with either of these agents, the researchers will file for orphan drug designation with the FDA. Phase 1 clinical trials could commence as early as Year 4.



Dr. Thomas Morrow working on fabrication of curcumin therapy (TyroMat™) to promote healing and reduce scarring (RCCC).

The **Caplan/Sorrell group** (Project 4.6.2) at Case Western Reserve University is developing an off-the-shelf, cell-based therapeutic product (adult human mesenchymal stem cells [MSCs]) that will be applied to the surfaces of extensive human skin wounds and/or burns to enhance wound repair and reduce scar formation. During the past year, the researchers demonstrated that human adult bone marrow-derived MSCs that were expanded in cell culture and fluorescently labeled for cell tracking could be successfully introduced into large, full-thickness skin wounds in mice. Initial experiments in the rat hot comb model performed at Stony Brook University found that MSCs provided by the Caplan laboratory, but cultured in the Clark laboratory, demonstrated minimal activity in the hot comb model. Several issues, however, arose about the protocol; thus, repeat experiments are pending. The researchers also plan to develop a porcine wound model to obtain necessary preclinical data regarding efficacy and safety of the product in a situation that more closely mimics human skin. Using data from the porcine studies, they will develop a plan for Phase 1 clinical trials.

Topical Treatment of Burn Injury

Studies at WFPC

Keratins are tough, fibrous structural proteins found in structures that grow from the skin (e.g., hair and nails). The **Van Dyke group** (Project 4.2.3) at Wake Forest University School of Medicine (WFUSM) is exploiting the thermoprotective properties of keratins to try and ameliorate the progression of injury immediately following a burn. They are aiming to deliver a topical therapy that would limit the progression of the “zone of coagulation” following a burn, thus limiting the overall size and severity of the injury. The researchers have developed a cell culture heat shock model using mouse skin cells. They have used this model to identify a keratin subtype, gamma-keratose, that promotes cell survival following thermal injury. Initial gene expression experiments suggest that gamma-keratose causes a decrease in the expression of inflammatory genes in the surviving cell population and induces the expression of a “repairing” fibroblast genotype. The researchers will conduct a large, definitive, preclinical burn study in swine in Year 3.

They also plan to determine whether other types of skin cells (e.g., keratinocytes) are affected by the keratin biomaterials following thermal injury. They have prepared a pre-Investigational New Drug (IND) package for submission to the FDA to seek regulatory approval for clinical studies, and anticipate commencing a clinical trial during Year 4.

Studies at RCCC

In three related projects, researchers at RCCC are testing the topical application of three therapeutic agents for the treatment of burns: iodine, peptide P12, and curcumin.

The **Iovine/Ramachandran group** (Project 4.6.4) at the New Jersey Center for Biomaterials (NJCBM) initially identified a polymer system that could release molecular iodine from a wound dressing, potentially preventing infection. However, various technical and toxicity-related issues limited the usefulness and applicability of this system. The research team abandoned research efforts on the initial polymer system and instead developed a novel antibacterial dressing containing complexed iodine for the treatment of burn skin and soft tissue wounds. They have achieved promising results in initial porcine-infected burn trials with the new wound dressing. The polymer exhibited good antimicrobial activity, little biological reactivity, and dressing changes incurred no trauma to the skin-generating wound bed. The research team plans to continue monitoring different process variables and working toward development of a prototype. By Year 5, they hope to attain 510(k) clearance by the FDA to enable the production of commercial material for use in a clinical study.

The **Macri group** (Project 4.6.5) at Stony Brook University is engineering a drug delivery scaffold for the topical therapy of large, acute burn injuries that are unable to close. The researchers are exploring the effects of sustained release of the fibronectin-derived peptide P12 in this scaffold model. They produced fibroporous mats containing varying amounts of P12 and determined that the mats could release P12 in a rate-controlled manner. They demonstrated the biocompatibility of the fibroporous mats in vivo in a porcine model. At 28 days, treatment



RCCC researcher, Lauren Macri, PhD candidate at Stony Brook University, prepares sterile P12 fiber mats for topical application to burn injury progression.

sites demonstrated decreased wound contraction and no evidence of inflammation. In a larger study, macroscopic evaluation suggested that granulation tissue accumulated in the wounds so that most wounds were completely filled in with new tissue on Day 7. The researchers plan to evaluate P12 fibromats in two additional models: split thickness wounds to determine whether topical P12 speeds re-epithelialization, and excised burns to determine whether topical P12 limits burn injury progression. The researchers plan to collect the necessary P12 stability and safety data during the upcoming year to be able to prepare an IND application. They hope to initiate a clinical trial in Year 4 or 5.

The **Sheihet group** (Project 4.6.6) at the NJCBM is testing the topical delivery of curcumin using nanospheres in a gel and fibroporous mats. The researchers demonstrated that both nanospheres and fibroporous mats successfully incorporate high quantities of curcumin and provide substantial enhancement to its stability. They also showed that nanospheres and fibroporous mats



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Larisa Sheihet, PhD, Rutgers Research Professor working on dynamic light scattering to determine the size distribution profile of curcumin-loaded nanospheres.

release curcumin in a rate-controlled manner, which can provide the ability to maintain curcumin levels within the therapeutic window, preventing both toxic and non-therapeutic doses. The researchers will evaluate the efficacy and safety of both topical curcumin therapies in porcine and rabbit models during Year 3. They will design human clinical trials based on the outcome of their preclinical evaluations. They plan to initiate human clinical trials in Year 5.

Wound Healing and Scar Prevention

Studies at WFPC

The **Gerlach group** (Project 4.2.2) at the University of Pittsburgh is finding that skin progenitor cells derived from human fetal skin tissue may provide an interesting new cell source for regenerative cell-based therapy for acute and chronic skin disease and burn patient treatment. The researchers are expanding on successful work conducted previously in Germany in which fetal skin cells are sprayed onto excised burn wounds using a precision device (as known as the "SkinGun"). They have established methods for isolating and culturing fetal skin cells from human skin. They expect to have established working cell banks by the completion of Year 3. The researchers have initiated plans for obtaining FDA

approval of their skin cell delivery device, with approval anticipated by the completion of Year 3. They plan to conduct a clinical feasibility study in Year 5.

The **Tirrell group** (Project 4.2.4) at the California Institute of Technology aims to develop and produce artificial extracellular matrix (aECM) proteins that can be utilized in tissue repair. The researchers have prepared several variants of aECM constructs and observed accelerated rates of wound healing in vitro. They developed a mechanistic model to explain the origin of the increase in wound-healing rates. They are now ready to begin animal trials, which will be conducted in collaboration with the Van Dyke laboratory (Project 4.2.3). The researchers note that numerous opportunities exist to couple this technology with existing burn therapies or with evolving technologies in the AFIRM Burn Repair program. Additionally, they feel that this technology has the potential to be used as the platform/scaffold for a future skin substitute.



WFPC researcher Dr. Jörg Gerlach with the skin cell spray device.



WFPC researchers have developed a prototype system that uses ink jet technology to print skin cells directly on burns.

The **Yoo group** (Project 4.2.5) at Wake Forest University is using ink-jet technology to achieve the “printing” of skin onto an excised burn wound. Since bio-printing has been successfully used to fabricate other tissues, skin should be similarly amenable to this approach especially given its minimally three-dimensional, linear spatial orientation. The researchers have developed a portable skin printing device for direct in situ applications for a large animal study. This process involved design, construction, laser scanner integration, software development and validation. The researchers achieved delivery of skin cells directly onto skin defects in a mouse model using the device. They anticipate progressing to porcine burn injury models within the next 1-2 years.

The **Holmes group** (Project 4.2.7) at Wake Forest University is conducting a multicenter FDA approval trial for ReCell®. ReCell is a technique whereby a small (~4 cm²) split-thickness skin graft/biopsy is harvested from a burn patient and prepared in the operating room so that cells from the dermo-epidermal junction are harvested and immediately applied to an excised burn wound via a syringe at an expansion ratio of 80:1. From ~4 cm² of skin, ~320 cm² of burn can be “grafted” using the patient’s own cells without the need for any culture techniques. This technology has the potential to radically alter modern burn surgery. Patient enrollment began in May 2010, and study completion is anticipated to occur during Year 5.

Studies at USAISR

Severe burn leads to a long-term hypermetabolic response, which is associated with increased incidence of infection, delayed wound healing, impaired immunity, loss of lean body mass and muscle strength, loss of bone mineral content and bone strength, and growth delay in children. The **Wu/Walters group** (Project 4.6.7) hypothesizes that reducing the hypermetabolic response would help to keep a patient’s stem cells functioning properly and would thus improve tissue regeneration after injury. The researchers established an animal model that induces a clinically relevant systemic response to burn and found that a 40% total body surface area burn in rats resulted in a reduction of body weight, lean body mass, fat mass, and bone mineral content, and an increase of serum proinflammatory cytokines. They believe that a sustained inflammatory response plays an important role in determining the functionality of harvested adult stem cells, and the efficacy of adult stem cell transplantation after severe burn. Over the next 3 years, the researchers will investigate the ability of systemic therapeutic approaches to modulate the regenerative activity of a patient’s own adult stem cells, as well as improve survival, engraftment, and differentiation efficacy of transplanted adult stem cells.

Skin Products/Substitutes

Studies at WFPC

The need for an skin replacement that is instantly available and alleviates the need to take a split- or full-thickness skin graft has long been sought. Two industry partners, **DFB/Healthpoint, Ltd.** (Project 4.2.1), and **Organogenesis** (Project 4.2.1a) are rapidly developing separate and distinct products that will be tested in a clinical trial scheduled to begin by the second quarter of 2012. The Healthpoint team is developing a permanent dermal skin graft replacement (ICX-SKN), which can be integrated and remodeled by the host. Over the past year, the researchers characterized the ICX-SKN matrix during maturation including: composition, density and pore size, cell distribution, and overall thickness. They also established an approved Institutional Animal Care and Use Committee (IACUC) protocol for performing burn studies in pigs. The Organogenesis team has made



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considerable progress toward the development of an advanced human cell-based therapy for the treatment of deep and extensive burns. The researchers have completed the groundwork for a porcine preclinical model and have developed three burn product constructs leveraging their volumetric computed tomography (VCT) platform technology and advanced biomaterials. They plan to begin a large animal efficacy study and start safety and biocompatibility studies in the first quarter of 2011.

The **Furth group** (Project 4.2.6) at the Wake Forest Institute of Regenerative Medicine is developing an improved “off-the-shelf” bioengineered skin product for the treatment of extensive burns that uses broadly multipotent stem cells from amniotic fluid (amniotic fluid-derived stem [AFS] cells). The researchers are capitalizing on the capability of AFS cells to differentiate into skin progenitor (stem) cells without forming tumors, thus facilitating the healing of a burn wound following the introduction of cells. AFS cells were only recently identified, and as such, this project is truly in the early stages. Nonetheless, significant progress has been made in cell isolation and culture techniques. The researchers have determined that AFS cells grown under certain conditions express transcription factors that are shared with other stem cells, including embryonic stem cells. When grown in appropriate inductive media, AFS cells can be induced to express markers of basal stratified epithelia (skin layer containing progenitor cells), suggesting usefulness in the generation of living skin equivalents. In Year 3, the researchers will further characterize the expression of stem cell-related markers by the AFS cells at the RNA and protein levels, before and after induction.

The **Lee/Yoo/Holmes group** (Project 4.2.8) at Wake Forest University has developed an in vitro tissue expander system that permits a rapid increase in surface dimensions of donor skin while maintaining tissue viability for subsequent skin transplantation. The researchers are conducting an initial Phase 1 trial that will analyze the safety of harvesting, expanding, and then grafting a piece of skin from a burn patient. An ~40 cm² split-thickness piece of skin will be harvested at the initial operation from a burn patient who is anticipated to require



In WFPC's ReCell[®] clinical trial, cells are applied to an excised burn wound via a syringe at an expansion rate of 80 to 1.

multiple acute operations. The skin will be expanded in a bioreactor to ~100 cm² over 2 weeks. The skin will then be grafted back onto the patient in the standard manner, with or without meshing, at the next operation. This technology is noteworthy in that it will produce an alternative for generating “more skin” for grafting when faced with limited donor sites. Patient enrollment will commence during Year 3.

Studies at RCCC

Engineered skin substitutes (ESSs) have been developed and tested clinically as an adjunctive treatment for burn repair. Although ESSs reduce the requirements for harvesting skin autografts (i.e., skin grafted from one part of the body to another), there are two major deficiencies: (1) incomplete pigmentation, which does not resolve with time and (2) the absence of a network of blood vessels, which limits the thickness and rate of engraftment of ESSs. The **Boyce/Clark group** (Project 4.7.2) of the University of Cincinnati and Stony Brook University is designing and testing new prototypes of ESSs that restore skin color and develop vascular networks thereby resulting in improved outcomes in recovery from life-threatening burns. Skin color has been

restored in an animal model of ESS, and the researchers will use flow cytometry to calibrate melanocyte density in Year 3. Research related to the formation of vascular networks has progressed to animal studies. The researchers' technology has been licensed to Lonza Walkersville, Inc., which has initiated technology transfer and has received funding from AFIRM to perform an initial clinical study (see below).

Studies at USAISR

Adipose-derived stem cells (ASCs) have the potential to grow into endothelial or epithelial cell lineages. The **Christy/Baer group** (Project 4.6.8) hypothesized that a person's own ASCs could be used to produce a clinically relevant tissue engineered skin equivalent. The researchers used ASCs and a polyethylene glycol (PEG)-based biomaterial that mimicked ECM as a strategy to regrow blood vessels into traumatized tissue. They found that cells began to form vascular tube-like networks in the biomatrix in the absence of additional soluble cytokines. ASCs inside matrices were not only viable but proliferated and formed microvessels. Further analysis using specific markers identified ASCs that had differentiated into vascular cells. This ability to form capillaries is central to developing new therapies for wound healing and tissue engineering. The researchers characterized the ASC phenotype from various patients and initiated preclinical animal wound-healing studies.

Recently Added Clinical Trials

WFPC

An Open-label, Multicenter, Proof of Concept Study of the Safety & Efficacy of Stratagraft® Skin Substitute as an Alternative to Autografting in Promoting the Healing of Excised, Deep Partial-thickness Burns

Principal Investigators (PIs) – Booker King, MD, and James H. Holmes IV, MD (WFPC)

This multicenter, Phase II trial will assess the safety and efficacy of Stratagraft® Skin Substitute when utilized as an autograft substitute in excised, deep-partial thickness burn injuries. At 4 clinical sites, the enrollment of 30 patients will likely be completed during the upcoming year, with planned 24-month follow-up.

RCCC

Translation of ESS (Project 4.7.2) to clinical trials. With an initial prototype of ESS studied clinically for several years, the regulatory and logistical requirements for clinical trials of this therapy are well understood. A commercial developer, Lonza Walkersville, Inc., currently holds a license to the ESS technology. During the past year, the PI of Project 4.7.2 collaborated in an application for support of initial clinical studies through the AFIRM. Preparations progressed including:

- Completion of protocols for cGMP-compliant manufacturing and characterization of the ESS device,
- Preparation of regulatory submissions for FDA and study protocols for the IRB at the USAISR and Human Research Protection Office (HRPO). These protocols will be designed for electronic data collection by the Clinical Trials Office of the AFIRM,
- Recruitment of a Clinical Research Organization to staff and operate the clinical trial, and,
- Orientation and training of USAISR surgeons and staff for enrollment and treatment of 10 burn patients at the USAISR Burn Center for the Phase 1 clinical study.

Additional funding from the DoD Office of Technology Transition is pending for this work.



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Progress Reports: Intravenous Treatment of Burn Injury

Therapy to Limit Injury Progression, Attenuate Inflammation, Prevent Infection, and Promote Non-Scar Healing After Burns and Battle Trauma

Project 4.6.1, RCCC

Team Leaders: Fubao Lin, PhD (Project Leader), and Richard Clark, MD (Program Director), Stony Brook University

Project Team: Richard Clark, MD and Adam Singer, MD, Stony Brook University

Collaborators: Molly Frame, PhD and Marcia Tonnesen, MD, Stony Brook University

Therapy: Therapy for burn injury progression

Deliverable: Intravenous (IV) P12 and curcumin

TRL Progress: *Start of Year 1*, TRL 1; *End of Year 1*, TRL 4; *End of Year 2*, TRL 5

Key Accomplishments: The researchers confirmed the proof-of-principle that P12 infusion could limit burn injury progression in a large animal model.

Key Words: burn, injury progression, P12, curcumin

Introduction

Battlefield polytrauma secondary to blasts and explosions is increasingly common, affects multiple sites, and is complex. Many of these injuries, but particularly burns, are subject to progressive tissue damage over subsequent days that is likely secondary to repetitive reperfusion injury. Progressive extension of burns and other battlefield injuries can have a devastating effect. Acutely, deep second-degree burns often become full-thickness third-degree burns leading to increased tissue loss, longer healing time, excess morbidity, and mortality. Chronically, increased scarring, wound contractures, and poor quality of life are seen. Therapies, such as NSAIDs and anticoagulants (heparin) have not shown substantial benefit in preventing burn injury progression to date.

Summary of Research Completed in Year 1

During the first year of this project, the researchers established a rat hot comb model for burn injury progression. They tested five therapeutic agents, with low risk profiles, that target three of the major sequelae of reperfusion injury on the rat hot comb model: (1) cytokine

release, (2) generation of reactive oxygen species, and (3) markedly increased programmed cell death (apoptosis). The five therapeutics agents were: (1) human bone marrow-derived MSCs (BM-MSCs), (2) pentoxifylline, which inhibits the production of tumor necrosis factor-alpha (TNF- α), (3) curcumin, a potent antioxidant, (4) desferrioxamine (DFO), a potent iron chelator that blocks free radical chain reactions, and (5) a 14 mer peptide (P12) from fibronectin with remarkable antiapoptotic properties (Lin and Clark, unpublished data). The studies demonstrated that only P12 and curcumin showed significant inhibition on burn injury progression in a rat hot comb model. In contrast, BM-MSCs, pentoxifylline, and DFO showed little effects.

Research Progress – Year 2

The studies during the second year of the project focused on P12 and curcumin using a swine hot comb model to develop novel therapies to prevent burn injury progression.

1. P12 inhibits burn injury progression in a swine hot comb model. IV infusion of P12 limited burn injury progression in the rat hot comb model. To further confirm

this finding, P12 was evaluated on a large animal using the swine hot comb model. When 25 kg swine were used, the necrotic interspaces for control swine was more than 90% (Figures V-1b and V-1d). Although 10 mg/kg P12 treatment showed little effect, 1 mg/kg and 3 mg/kg P12 infusion at 1 hour and 24 hours after the burn significantly inhibited burn injury progression (Figures V-1c and V-1d). The viable interspaces increased to about 60% after 1 mg/kg P12 infusion from 3% of control swine (Figure V-1d). Further studies showed that one dose P12 infusion 1 hour after burn was enough to inhibit burn injury progression in the swine hot comb model (Figure V-2).

2. IV administration of curcumin enhanced burn injury progression in a swine hot comb model. In contrast to the effect in the rat hot comb model, curcumin enhanced burn injury progression significantly in the swine hot comb model. As shown in Figure V-3, for control swine, necrotic interspaces were only 24% when 45 kg swine were used. After 0.4, 1.33, and 4 μ g/kg curcumin treatment, necrotic interspaces increased to 84%, 85%, and 42%, respectively. These results are consistent with the in vitro studies in which curcumin

enhances oxidative stress-mediated fibroblast killing. It indicated the differential response of rat and swine to curcumin treatment in the hot comb model.

3. Apoptosis and necrosis in the zone of ischemia surrounding burns in pig hot comb model. Necrosis progressed laterally from the burn edge over time, with most cells displaying evidence of necrosis by 24 hours postburn. Though no apoptosis was seen at 1 and 4 hours post burn, by 24 hours a gradient of apoptosis was observed in the dermis with no apoptosis at the burn edge but increased apoptosis moving laterally from the burn injury. Apoptosis rates were zero in controls. Progression of cell necrosis surrounding a burn was assessed with staining for HMGB1 (high mobility group box-1 protein), allowing for discernment of early, intermediate, and late-stage necrosis. Though apoptosis at 24 hours was observed concomitant with early and intermediate signs of necrosis, cell necrosis appears to be the most prominent mechanism of cell death in burn injury progression (data not shown).

4. Microvascular responses to P12. P12 was found to limit burn injury progression in both rat and swine hot

comb models. To elucidate the mechanism of P12 on limiting burn injury progression, the vasoactive response to locally applied P12 was determined using cheek pouch mode. As shown in Figure V-4, P12 dilated terminal arterioles at nanomolar concentrations and constricted terminal arterioles at micromolar concentrations. The figure shows that 1 nM provides the peak dilation to P12, yet higher concentrations preferentially constricted. When compared to micropipette applied full-length fibronectin, there was a clear difference in response. Full-length fibronectin only dilated these terminal arterioles.

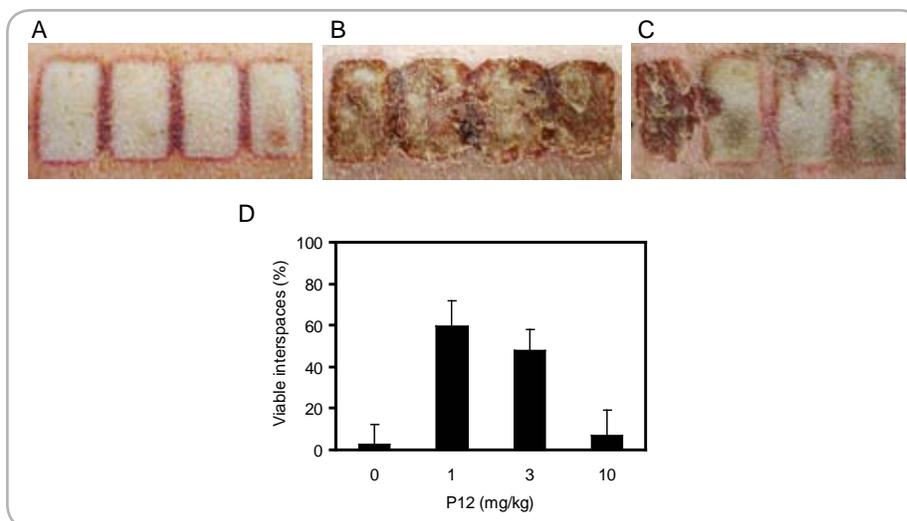


Figure V-1. P12 treatment inhibited burn injury progression in a swine hot comb model. Buffer or P12 was IV administrated from ear vein at 1 hour and 24 hours after burn. On Day 7, the number of interspaces that had progressed to full-thickness necrosis was assessed by an observer blinded to the protocol. a. Control burn, 24 hours. b. Control burn 7 days after burn. c. Burn treated with 3 mg/kg P12 7 days after burn. d. Macroevaluation of burn injury progression (n = 42).

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Progress Reports: Intravenous Treatment of Burn Injury

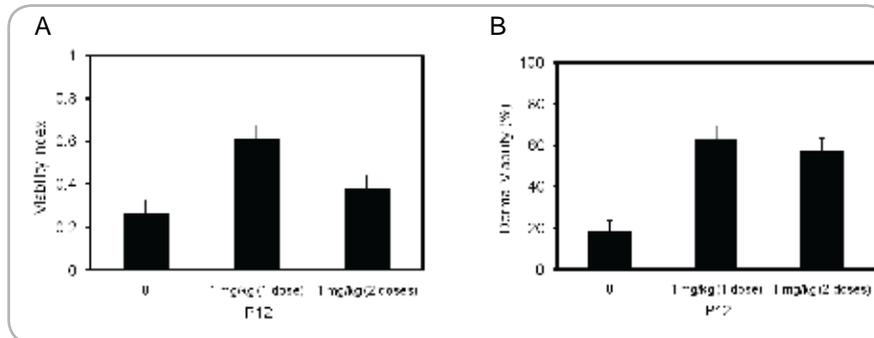


Figure V-2. One dose of P12 infusion inhibited burn injury progression in a swine hot comb model. Buffer or P12 (1 mg/kg) was IV administered from ear vein at 1 hour (one dose) or 1 hour and 24 hours (two doses) after burn. At Day 7, burn injury progression was assessed by observers blinded to the protocol. a. Macro-evaluation. b. Histological analysis.

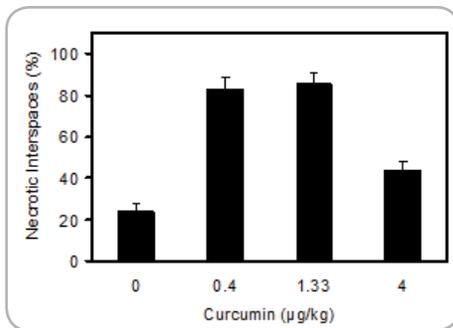


Figure V-3. IV administration of curcumin enhances burn injury progression in a swine hot comb model. Buffer or curcumin was IV administered from the ear vein at 1 hour and 24 hours after burn. Full-thickness biopsies from the interspaces 7 days after injury were evaluated for evidence of necrosis after hematoxylin and eosin staining by a board-certified dermatopathologist (histologic analysis) (n = 72).

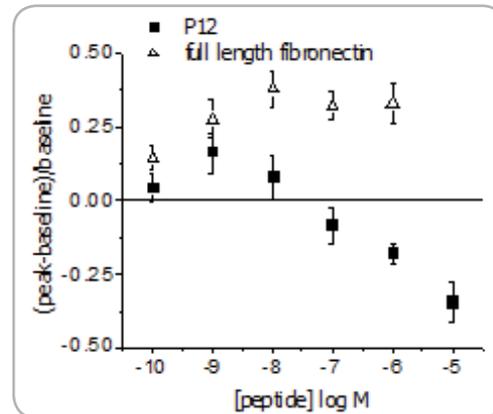


Figure V-4. Responses of terminal arterioles to P12 and fibronectin. Hamster was anesthetized, and P12 or fibronectin at different concentration was applied to the adventitial surface of small arterioles via pneumatic ejection under intravital microscopy. The change of arteriole diameter was recorded (fractional change from baseline) (n=12).

Higher concentrations of fibronectin were problematic due to precipitation. These effects may contribute to the inhibition of P12 on burn injury progression.

5. Microvascular responses to curcumin. Curcumin showed an opposite effect on burn injury progression in rat and swine hot comb models. To better understand the mechanism of curcumin on burn injury progression, the vasoactive response to locally applied curcumin was determined using cheek pouch mode. The results

showed that curcumin dilated terminal arterioles from picomolar to micromolar concentrations and peaked at 100 nM for early response (data not shown). Further studies demonstrated that curcumin is acting via the adrenergic receptor system (data not shown).

Key Research Accomplishments

- Confirmed that P12 was effective in limiting burn injury progression in a large animal model.

- Determined that one dose of P12 infusion limited burn injury progression in the swine hot comb model.
- Defined the mechanisms of cell death in the tissue surrounding the burn: necrosis and apoptosis.
- Demonstrated that P12 dilated terminal arterioles at nanomolar concentrations and constricted terminal arterioles at micromolar concentrations.
- Demonstrated that curcumin dilated terminal arterioles at picomolar to micromolar concentrations.

Conclusions

Both necrosis and apoptosis were involved in the cell death surrounding the burn. IV infusion of P12 was found to be effective in limiting burn injury progression in both rat and swine hot comb models. Importantly, one dose of P12 infusion 1 hour after burn was found to be enough to limit burn injury progression. The effects of P12 to limit burn injury progression were consistent with the multi-functions of P12 to dilate terminal arterioles, protect cells from oxidative stress-mediated death, inhibit apoptosis, and enhance cell survival. These findings may lead to the development of a novel therapy to limit burn injury progression by one dose of P12 infusion. Alternatively, curcumin was found to enhance burn injury progression at nanomolar concentration in the swine hot comb model although it inhibited burn injury progression in the rat hot comb model. These results indicated a differential response of large and small animals.

Research Plan for the Next 3 Years

In Years 3-5, the optimal dose and time of P12 and curcumin infusion to limit burn injury progression will be evaluated in the swine hot comb model. Based on the

results of these studies, P12 stability and pharmacokinetics studies will be carried out. To detect nanomolar concentration of P12, mass spectrum analysis, enzyme-linked immunosorbent assay (ELISA) or a similar assay using P12 antibody or labeled P12 will be established. The tissue distribution of P12 will be studied using mass spectrum, ELISA, or fluorescence-labeled P12. Orphan drug and IND applications using P12 infusion to limit burn injury progression will be filed. Phase 1 and Phase 2 clinical trials will be completed.

Planned Clinical Transitions

Jules Mitchel, PhD, President of Target Health, will submit a request for Orphan Drug Designation of P12 by the end of Year 2. Microconstants, Inc. has been selected to initiate protein stability studies by the end of Year 2. American Peptide, Inc. has been instructed to move P12 peptide synthesis to their Good Manufacturing Practice (GMP)-compliant facility. They will use the same manufacturing procedure as currently in use and no further scale-up will be needed at this time. Hyaluron, Inc has been selected to produce the final product, i.e., “finish and fill.” NeoMatrix Formulations, Inc., founded by Richard Clark, has been activated with selection of a Board of Directors and initiation of a CEO search. The company will negotiate a license from Stony Brook University for all relevant intellectual property and then submit a pre-clinical grant application to AFIRM as well as a fast-track Phase 1/2 Small Business Innovation Research to the National Institutes of Health. Jules Mitchel, in collaboration with Stan Gerson, Richard Clark, Adam Singer, and a designated burn surgeon, will be responsible for writing Phase 1 and Phase 2 clinical trial protocols beginning in Year 3 and then submitting an IND to the FDA in Year 4.



V: Burn Repair

Progress Reports: Intravenous Treatment of Burn Injury

Mesenchymal Stem Cells for Burn and Wound Healing

Project 4.6.2, RCCC

Team Leaders: Arnold Caplan, PhD and Michael Sorrell, PhD (Case Western Reserve University)

Project Team: Marilyn Baber, BA and Randall Young, VMD (Case Western Reserve University)

Collaborators: Glenn Prestwich, University of Utah, Fidia Advanced Biopolymers, Italy

Therapy: Topical delivery of adult human bone marrow-derived MSCs

Deliverable: Adult human MSCs

TRL Progress: *Start of Year 1, TRL 1; End of Year 1, TRL 3; End of Year 2, TRL 3/4*

Key Accomplishments: The researchers demonstrated proof-of-

principle for therapeutic cell delivery to full-thickness skin wounds in mice. **Effective refinements in the procedures** have been noted and are being implemented.

Key Words: MSC, skin, wound repair, mouse

Introduction

Battlefield polytrauma produces complex, multiple wounds of the skin and internal organs that present extreme difficulties in healing. Using standard technologies, each wound requires individual intervention. Therefore, novel technologies are required to promote rapid, systemic healing of these multiple wounds. One such mechanism is the introduction of therapeutic cells into injured victims that either home systemically to wound sites or are applied directly to wound sites. Therapeutic cells are defined as cellular populations that have the capability to home to sites of injury and upon arrival release multiple bioactive factors that promote healing with minimal scar formation. Examples of therapeutic cells are adult bone marrow-derived cells and adult ASCs. Both types of cells are obtained from adult donors and do not present the ethical problems that arise from using human fetal cells. These cells have been identified as stem cells owing to their abilities to differentiate into multiple types of MSCs upon proper induction. In addition, these cells have now been shown to exert therapeutic potentials without cellular differentiation.

The mechanisms of this therapeutic mechanism are still under investigation. Nonetheless, there is growing evidence that cellular interactions between therapeutic cells and wound cells are critical. One type of interaction occurs between therapeutic cells and vascular endothe-

lial cells. Therapeutic cells promote neovascularization, stabilize newly formed vessels, and potentially release potent bioactive factors into adjacent blood vessels. In addition, the therapeutic cells possess immunomodulatory functions that attenuate scar formation. Thus, the application of these cells has the potential of hastening wound repair and of reducing fibrosis and scar formation at wound site.

The mechanisms of therapeutic cell application poses problems. An important issue is how to optimally deliver these cells to target sites in a timely and effective manner. There are essentially two approaches for cellular delivery: systemic delivery and direct topical delivery. A problem with systemic delivery is that cells may become entrapped in nonrelevant sites. This entrapment may either delay homing of cells to wound sites or prevent delivery entirely. The direct delivery method avoids these issues. However, problems arise with regard to the direct delivery of therapeutic cells to internal organs. The direct delivery of therapeutic cells to internal organs requires an invasive approach through surgery or injection of cells. The issues related to invasiveness do not arise when the cells are directly applied to skin wounds. In the delivery of therapeutic cells to skin wounds, it is essential that concentrated populations of cells be presented to the wounds. Furthermore, these cells must have demonstrated therapeutic effectiveness.

The solution proposed in this project is to delivery concentrated populations of therapeutic cells in an ECM carrier. The carrier cannot be toxic or rejected, and ideally, the carrier would positively influence wound repair on its own. This combination magnifies the therapeutic potential.

Summary of Research Completed in Year 1

During the first year of the project, the researchers developed a mouse excisional skin defect wound model that allowed quantitative fluorescent imaging of the localization of infused adult MSCs. They received approval on all animal protocols and documentation. They completed pilot experiments in June 2009.

Research Progress – Year 2

Year 2 studies demonstrated that human adult BM-MSCs that were culture expanded and labeled for cell tracking could be successfully introduced into large, full-thickness skin wounds in mice (**Figure V-5**). These cells migrated into the wounds principally at the boundaries between the wound bed and surrounding non-wound tissue. This migration appears to be attracted by blood vessels located at this site. At later times, the delivered therapeutic cells aligned along a newly developed vascular plexus at the base of the dermis. This vascular plexus provides blood to the newly forming skin at the wound site, affording the opportunity for therapeutic cells to provide bioactive factors to the blood supply. Wounds treated with therapeutic cells healed with a more mature

dermis than wounds that were not treated. These studies provide the basis for future studies to better understand how therapeutic cells function. In addition, these studies provide the basis for establishing a means for the effective delivery of cells in a clinical setting.

Key Research Accomplishments

- Developed an effective delivery vehicle using hyaluronan matrices.
- Successfully introduced therapeutic cells to murine wounds.
- Successfully tracked cells in wounds.

Conclusions

During Year 2, the researchers developed a proof-of-principle study for the delivery of therapeutic cells to full-thickness skin wounds in mice. These cells were concentrated in hyaluronan carriers and applied directly to wounds at the time of surgery. By Day 6 post surgery, the transplanted cells migrated from the carriers into the wound beds following a defined pathway. This pathway led to the wound margin and terminated in the vascular plexus at the base of the developing dermis. Here, the delivered cells co-aligned with mouse blood vessels. These studies indicate the feasibility of delivery of human therapeutic cells to animal wounds and provide the basis for extending the studies into a large animal model. Preliminary results indicate an efficacious outcome for wounds that received therapeutic cells compared with wounds that did not receive these cells.

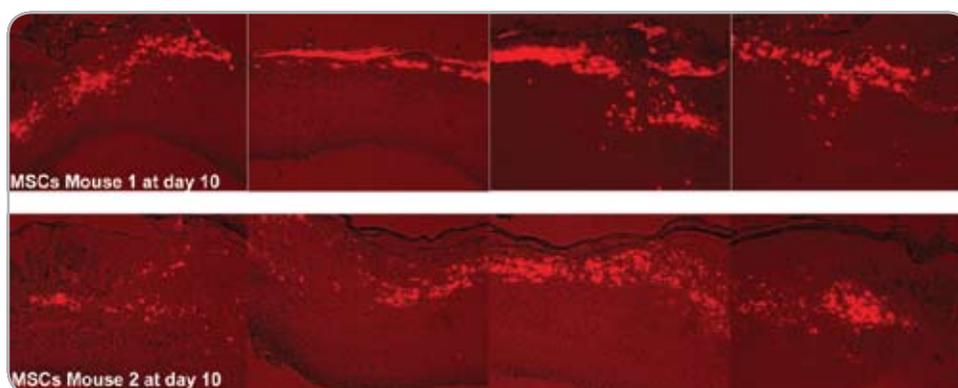


Figure V-5. CM-Dil labeled hMSCs in wound beds of two mice at Day 10. The entire wound bed is shown for both mice. hMSCs have entered the wound beds.



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Research Plans for the Next 3 Years

The primary research goal for this project is the development of an off-the-shelf, cell-based therapeutic (adult hMSCs) product that will be applied to the surfaces of extensive human skin wounds and/or burns to enhance wound repair and to reduce scar formation. The basic proof-of-principle for this study has been completed in a mouse model (TRL 3). The next 3 years will be devoted to the modification of the delivery of the product to wounds that are in the early granulation tissue phase to make the product more practical for clinical application. The development of a product that can be stored long term will be performed concurrent with other studies. These studies will be combined with the application of selection technology that has been developed for these studies (TRL 4). A porcine wound model will be developed and used to obtain necessary preclinical data regarding efficacy and safety of the product in a situation that more closely mimics human skin (TRL 5). With these data, it will be possible to develop a plan for Phase 1 clinical trials using an off-the-shelf product that delivers allogeneic therapeutic cells to human wounds (TRL 6).

Planned Clinical Transitions

The plans for the translation of preclinical studies to Phase 1 clinical studies requires the acquisition of sufficient data from a porcine model to demonstrate the

effectiveness of the approach and to assess the safety of the approach. The porcine model is critical in that the skin of pigs has an anatomical structure nearly identical to that of human skin. This contrasts with a rodent skin architecture that is substantially different from human skin. Skin wound repair in pigs is a close match to that of humans, in contrast to that of rodent skin repair. The larger size of the animal and wounds will require the development of larger implants. This is feasible, but it will require modifications of the current procedures. As soon as sufficient efficacy and safety data have been obtained from the pig model, plans will be developed for regulatory submission for a Phase 1 clinical trial. This is expected to occur during Year 4. Discussions are in progress with commercial partners regarding the progress of the study. Plans to commercialize the product will also depend upon results of the large animal model.

Corrections/Changes Planned for Year 3

There are no major changes planned for the proposed third year studies. One addition has been made to the plan. In past studies, hMSCs were loaded onto hyaluronan-carriers immediately before implantation onto mouse skin wounds. A modification of this has begun to develop methodologies to freeze these carriers with loaded cells to maintain cellular viability and function. This will be necessary for a commercial product.

Novel Biomaterials That Support the Survival of Damaged Cells and Tissues

Project 4.2.3, WFPC

Team Leader(s): Mark Van Dyke, PhD (WFUSM)

Project Team Members: Deepika Poranki, MS (Graduate Student); Carmen Gaines, PhD (Postdoctoral Fellow); and Olga Roberts, PhD (Research Associate Professor)

Collaborator(s): James H. Holmes IV, MD, Mark Lively, PhD (WFUSM); David Tirrell, PhD (CalTech); Luke Burnett, PhD (KeraNetics LLC); and Joseph Molnar, MD, PhD

Therapy: Burn repair

Deliverable(s): Keratin biomaterial-based burn treatment, including preclinical testing and a clinical trial.

TRL Progress: *Start of Program*, TRL 3; *End Year 1*, TRL 3; *End Year 2*, TRL 4

Key Accomplishments: The researchers have investigated a fundamental mechanism for keratin biomaterial burn treatment. They have identified a key component, gamma-

keratose, and have developed new formulations using this material that they are currently testing in a pivotal animal study. They have established manufacturing capabilities at a spinoff company and are submitting an investigational device exemption (IDE) application to FDA to obtain approval for a clinical study.

Keywords: keratin, biomaterial, burn, hydrogel, cell survival, TBSA, wound, skin

Introduction

Studies in mice and pigs have shown that keratin hydrogel treatment can mitigate damage after thermal injury and prevent tissue in the so-called “zone of stasis” from becoming necrotic. In one chemical burn study, wounds treated with keratin hydrogel healed significantly faster than wounds treated with a conventional biomaterial hydrogel. In a thermal injury model in pigs, re-epithelialization was more rapid in keratin-treated wounds compared to conventional treatment (Van Dyke et al., unpublished data). These pilot study data suggest a role for keratins in the salvage of cells and tissue after burn injury. Any technology that serves to protect cells from thermal injury or promotes cell survival would be of tremendous value as a post-burn treatment. Tissue preservation in the zone of stasis will prevent a burn wound from growing larger during the nascent stages of recovery when cell death normally contributes to an overall increase in total burned surface area (TBSA). This is especially important as every 1% increase in TBSA correlates with a 6% increase in mortality. Keratin biomaterials may be able to provide stability to the wound while promoting normal healing and be deployable directly to the field so that it can be used immediately after burn injury. However, the mechanism of cell and tissue salvage is not

known and an optimal formulation and treatment protocol have yet to be determined. The working hypothesis for this project is that keratin biomaterials act on the membrane receptors expressed on thermally injured cells in the zone of stasis. Further, certain keratin subtypes may have greater efficacy than others.

The goal of this project is to investigate the specific structure-activity relationships between keratin and skin component cells, as well as the signaling mechanisms by which these keratins promote cell survival, with the goal of producing an optimal biomaterial for thermal injury treatment that could be tested in a pivotal animal trial. The specific aims of the project are as follows:

Specific Aim 1. To investigate the thermoprotective characteristics of keratin biomaterials in vitro. (Years 1 and 2)

Specific Aim 2. To test the thermoprotective characteristics of keratin biomaterials in a pig burn injury model. (Year 3)

Specific Aim 3. To conduct the first clinical investigation of a keratin biomaterial treatment for burn injury. (Years 4 and 5; not currently funded)



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Summary of Research Completed in Year 1

During the first year of the project, the researchers developed a porcine heat shock model and a standard method for the production of keratin biomaterials. They established a primary mouse skin cell isolation and culture system. They initiated ISO 10993 safety testing on keratin biomaterial. They began testing keratin materials in the pig model to determine the basis of their thermoprotective mechanism and ability to accelerate wound healing. They determined that re-epithelialization occurred more rapidly when keratin hydrogel treatment was applied in the pig thermal burn model.

Research Progress – Year 2

As reported in February 2010, a cell culture heat shock model has been developed using mouse dermal fibroblasts. This model has been used to show that a particular keratin subtype, gamma-keratose, is primarily responsible for the tissue-sparing phenomenon, producing a 300% increase in the number of surviving cells. The cell culture model system is also being used to determine dosing ranges for animal studies, as well as to study the cellular and molecular basis of cell survival following thermal injury. In initial experiments, it was determined that dermal fibroblasts experience an initial necrosis phase wherein the heat treatment causes rapid cell death, followed by a second apoptotic phase. It is during this second apoptotic phase where gamma-keratose treatment facilitates almost 100% cell survival. Initial gene expression experiments suggest that gamma-keratose causes a decrease in expression of inflammatory genes in the surviving cell population and induces the expression of a “repairing” fibroblast genotype. A dosing study has also been conducted to optimize the concentration of gamma-keratose used in the burn treatment hydrogel formulation. A swine burn study protocol also received IACUC and Animal Care and Use Review Office (ACURO) approval.

Keratin biomaterials have undergone extensive safety testing and passed a series of ISO 10993 standardized assays including cytotoxicity, systemic toxicity, mutagenic-

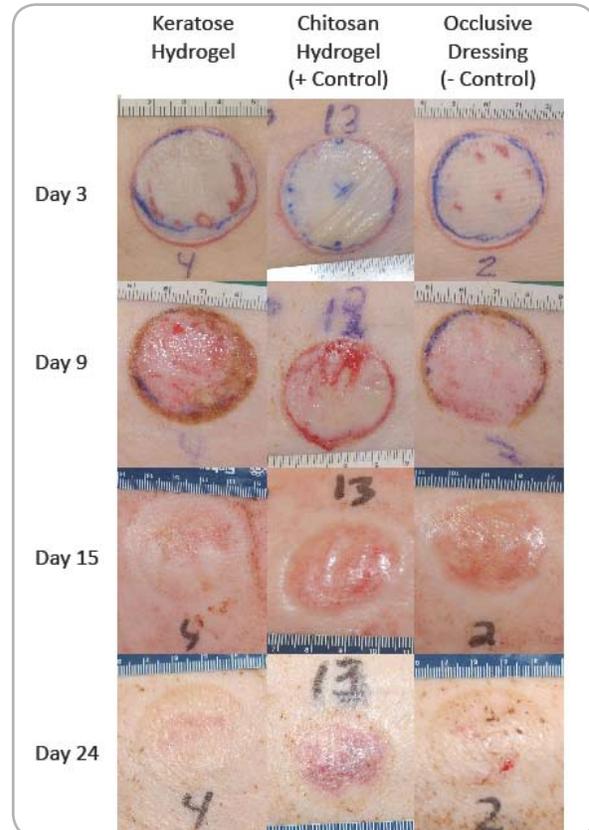


Figure V-6. Digital photographs of thermal burns in swine. Burns in the keratose treatment group showed no growth of the initial wound area, faster re-epithelialization, and complete healing by Day 15.

ity, sensitization, irritation, pyrogenicity, and sterilization validation. These studies were conducted at a contract research organization (CRO) under Good Laboratory Practice (GLP) standards. These data have been summarized in a pre-IDE package to be submitted to FDA so that the process of regulatory approval for clinical studies can begin. A thermal injury study in swine has also been initiated with training of study personnel and experimental parameter determinations completed.

Key Research Accomplishments

- Developed a heat shock cell culture model.
- Completed testing of keratin subtypes.
- Completed initial gene expression experiments.
- Completed gamma-keratose dosing experiments.

- Obtained IACUC and ACURO approval for swine burn study.
- Completed ISO 10993 safety assays.
- Completed GLP keratin production lab set-up (startup to conclude in August 2010).
- Drafted an IDE application package to the FDA.
- Initiated pilot experiments to define parameters for definitive preclinical study.

Conclusions

A fundamental mechanism for keratin biomaterial burn treatment has been investigated. A key component, gamma-keratose, has been identified, and new formulations using this material have been developed and are being tested in a pivotal animal study. In addition, manufacturing capabilities at a spinoff company have been established, and an IDE application to FDA is being submitted to obtain approval for a clinical study.

Research Plans for the Next 3 Years

The mechanistic study continues with gene expression experiments currently being conducted. In addition, other skin cell types (e.g., keratinocytes and vascular cells) are also being tested to determine if keratin biomaterials affect these cells after thermal injury. These ongoing studies are essential to defining the mode of action to meet regulatory requirements and demonstrate the safe use of keratin biomaterials in humans. A large, definitive, preclinical burn study in swine will be completed in Year 3. This study will support an application to the FDA for product marketing approval. KeraNetics' regulatory consultant has recommended a request for 510(k) approval contingent upon an equivalency trial. This is a

human study designed to demonstrate that the results obtained in swine are predictive of use in humans and is planned to be conducted in Years 4 and 5, contingent on obtaining funding.

Planned Clinical Transitions

A pre-IDE package is ready to be submitted to FDA at the time of this report. A meeting with FDA will be requested to solicit their feedback on the preclinical data package and proposed clinical study design. Once agreement is reached, a final IDE package will be submitted. If approved, local Institutional Review Board (IRB) and Department of Defense (DoD) approval will be obtained. KeraNetics is currently working to validate their keratin biomaterials production facility so that product can be manufactured for this study. The earliest anticipated enrollment for the Phase 1 portion of the study is the fourth quarter of 2010. It is expected that the study will require 18 to 24 months to complete.

Corrections/Changes Planned for Year 3

Based on feedback from review of the first annual report, major changes to the project aims were made, including the removal of a planned rodent study and acceleration of the swine experiments. Through the partnership with KeraNetics, much of the preclinical work required for transition to clinical trials has been completed. This also represents an aggressive acceleration of the originally proposed time line. An expeditious regulatory pathway has also been adopted with the use of a 510(k) application that will be contingent on an equivalency study in humans. This approach is justified based on the encouraging outcomes in the preclinical testing and very favorable safety testing results (i.e., GLP toxicity testing).



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Polymeric Iodophor Absorbent Antimicrobial Wound Dressing

Project 4.6.4, RCCC

Team Leaders: Carmine Iovine, Niraj Ramachandran, PhD (Project Leaders) (NJCBM); and Richard Clark, PhD (Program Director) (Stony Brook University)

Project Team: Adam Singer, MD (Stony Brook University) and Joachim Kohn, PhD, (NJCBM)

Therapy: Treatment of infected and heavily exuding traumatic or thermal wounds of war soldiers

Deliverable: Polymeric iodophor absorbent antimicrobial wound dressing (PIP-BU [Buruli ulcer])

TRL Progress: *Start of Year 1, TRL 2; End of Year 1, TRL 3; End of Year 2, TRL 4*

Key Accomplishments: The researchers made significant progress in the design and fabrication of a novel polymeric wound dressing, PIP-BU. They also completed proof-of-concept of testing of the PIP-BU wound dressing in pigs.

Key Words: iodine, polymeric iodophor, disinfection, wound dressing, antimicrobial, sustained release

Introduction

A major barrier to wound healing is the presence of infection. Thus, the development of novel therapies aimed at preventing or reducing wound infections is crucial in both military and civilian settings. A number of commercially available antibacterial dressings are currently marketed, each with its own advantages and limitations. The goal of this project is to develop an absorbent antimicrobial wound dressing that releases iodine on demand and keeps infection rates under control. The researchers have designed and fabricated a polymer-based matrix capable of efficiently complexing iodine as a foamed polymer network (Figure V-7). This technology has proven to (1) address the requirements of effective wound dressing properties, (2) be applicable to a variety of medical applications, and (3) be cost effective. The objective of this study was to evaluate the antibacterial and wound-healing properties of this novel iodine-based dressing and compare its performance to commercially available dressings in two animal wound models.

Unlike most injuries encountered in hospitals, battle-field wounds are polytraumatic in nature, involving multiple mechanisms of injury to multiple anatomical sites. Essentially all of these wounds involve some component of skin and/or soft-tissue injury. Some of the key factors in the treatment of traumatic injuries are

bleeding control (to prevent critical blood loss), ischemia control (to ensure adequate perfusion of nondamaged tissue), and contamination control (for the management of microbial and chemical contaminants). The PIP-BU wound dressing is intended to aid in the management of microbial wound contamination and infection. Since infected wounds produce exudates from the necrosis of tissue, wounds will also benefit from the absorption of the exudate by a wound dressing. The PIP-BU absorbent antimicrobial wound dressing is a nonsterile, nonadherent, moist, polymeric dressing that contains complexed and elemental iodine that is released into the wound as exudate is absorbed. Iodine has continually proven its antimicrobial effectiveness since its introduction almost two centuries ago. Not only is topically applied iodine effective against all deleterious microbes, it does not produce resistance in bacteria nor does it produce allergies, unlike antibiotic drugs, which makes iodine ideal treatment for traumatic wounds requiring immediate and effective treatment.

Initial proof-of-concept was demonstrated and a prototype of this novel dressing has been developed under the AFIRM Program at the NJCBM in Rutgers University. In conjunction with Dr. Richard Clark at Stony Brook University, scientists at NJCBM are spearheading this effort to establish the product specifications, test methods, and



Figure V-7. Niraj Ramachandran, PhD, Postdoctoral Associate, in the Kohn laboratory conducting a complexing procedure for a wound dressing

biocompatibility as well as compile and file a Premarket Notification Application (510(k)) with the FDA. Following FDA clearance, the PIP-BU dressing will be included in clinical trials as a nonsignificant risk device. It has been well established that infection control is a key determinant in successful management of both acute and chronic wounds. As a result, this novel dressing will be also useful for nonmilitary applications, such as infected wounds, pressure, venous, and diabetic ulcers.

Summary of Research Completed in Year 1

The research team identified a polymer system that could release molecular iodine from a wound dressing, potentially preventing infection. The system, called the I-Plex Absorbent Antimicrobial Wound Dressing, is a nonadherent, moist, formalin-treated polyvinyl alcohol (PVA) sponge that releases molecular iodine into wounds as exudates are absorbed by the polymer. The researchers completed feasibility studies for the preliminary evaluation of the device, including fabrication of the PVA sponge and complexing iodine with the sponge. They estimated the iodine loading and release rates

from the PVA sponge for both starch- and air-foamed materials and established a quality control procedure in which samples made from each batch were tested for the weight of iodine loaded.

Research Progress – Year 2

The formalized PVA-based system failed 1.5 years into the program due to toxicity concerns. But the project was brought back on track by the development of the novel polymeric system (PIP-BU) for infection control.

After the choice of appropriate polymer chemistry, two prototypes for the absorbent antimicrobial wound dressing were developed and tested in a clinically relevant infected porcine model. Based on the analytical work performed to date, the weight percentage of iodine loaded into the matrix is higher than any commercially available iodine-based wound dressing. It is hypothesized that the PIP-BU dressing strongly binds iodine and a specific and significantly stronger force is required to pull it out. This was confirmed by no iodine release measured when dressing was incubated in the following liquids: tap water, deionized water, phosphate-buffered saline (PBS) with or without calcium and magnesium ions. However, the PIP-BU dressing released therapeutically relevant doses of iodine in the presence of wound exudates and bacteria making it an ideal on-demand release system for the treatment of infected wounds.

The histological analysis of the in vivo evaluation of PIP-BU therapy is not available yet and hence only gross observations that were made during the animal study (April 2010) are reported here. At every dressing change, the complexed iodine dressings had turned from brown/black to a lighter shade of yellow/white. This change in color indicates the release of some iodine although based on visual inspection of these dressing approximately 50% of the initial iodine was still present in the dressings. Upon removal of the PIP-BU dressings, the wounds appeared noninflamed and clean from any exudates or biofilms. In addition, removal of the PIP-BU dressings was easy—they did not stick to the wound bed as compared to the Silverlon® dressing that is currently the standard of care in the U.S. Army.



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Key Research Accomplishments

- Developed a novel and versatile class of polymeric iodophors that release iodine on demand and can be used in the treatment of variety of medical conditions.
- Determined that PIP-BU dressings bind significantly higher amounts of iodine compared to currently used therapies.
- Determined that PIP-BU therapy is easy to administer, has superior handling properties, and can serve as an effective treatment for prolonged periods of time.
 - PIP-BU releases iodine slowly on-demand for more than 5 days so would not require frequent dressing changes.
 - Simple visual indication of iodine depletion by change in color from brown/black to light yellow or white indicates the need for a dressing change.

Conclusions

In the past 9 months, the team at Rutgers has developed a novel antibacterial dressing (PIP-BU) that contains complexed iodine for the treatment of burn skin and soft tissue wounds. This novel technology utilizes completely novel chemistry, and it has been tested in vitro for its effectiveness in binding and releasing iodine. The PIP-BU dressings release iodine slowly on-demand for more than 5 days and hence would not require frequent dressing changes. Simple visual indication of iodine depletion by change in color from brown/black to light yellow/white will indicate the need for the dressing change. In addition, the dressing has good handling properties and can potentially incorporate significantly higher amounts of iodine than any other previously used iodophor. Further, all the raw materials used to make this product are commercially available and based on the preliminary cost

evaluation estimates this infection control dressing will be cheaper than any currently commercially available wound dressing.

Research Plans for the Next 3 Years/Planned Clinical Transitions

Product development will be broken into three main phases: Research and Development, Pre-Commercial Development, and Commercial. The primary objectives and activities of each phase are described below:

Research and Development Phase: The team will continue monitoring different process variables and working toward developing a prototype. Since proof-of-concept has already been established, drafting of the product design matrix (design input, output, verification, etc.) will be initiated and completed during Year 3 of the project.

Pre-Commercial Phase: The product testing procedures will be finalized and adapted, and the manufacturing will be transferred to a GMP-compliant facility (i.e., a contract facility). Development of the quality system and standard operating procedures will be initiated. Production and testing (release, shelf-life) of GMP-prototype product will be completed during this phase and are likely to happen during Year 4 of the project. This product will be used in the GLP-certified biocompatibility testing required for FDA clearance.

Commercial Phase: In Year 5, following 510(k) clearance by the FDA, commercial material will be produced for use in a clinical study. Since the dressing will be cleared prior to human use, the clinical study will only require IRB and DoD review. In addition, as a cleared device, it could also be deployed to the troops.

Topical P12 Delivery via Biodegradable Fibro-Porous Mats for Burn Treatment

Project 4.6.5, RCCC

Team Leaders: Lauren Macri, PhD Candidate (Project Leader) and Richard Clark, MD (Program Director) (Stony Brook University)

Project Team: Adam Singer, MD (Stony Brook University); Larisa Sheihet, PhD, and Joachim Kohn, PhD (NJCBM)

Collaborators: None

Therapy: Topical therapy to limit burn injury progression and accelerate cutaneous wound healing

Deliverable: Peptide-eluting tyrosine-based copolymer device

TRL Progress: *Start of Year 1*, TRL 2; *End of Year 1*, TRL 3; *End of Year 2*, TRL 3

Key Accomplishments: The researchers produced P12-loaded fibroporous mats with desired fiber morphology and high peptide loading and determined that the mats could release P12 in a rate-controlled manner.

They demonstrated the biocompatibility of the fibroporous mats in vivo in a porcine model.

Key Words: Polymers, drug delivery, electrospinning, peptide, wound healing, burn

Introduction

Burn injuries claim the lives of 4,000 U.S. civilians each year and send another 500,000 to seek medical attention. Approximately 8% of those 500,000 burn victims require hospitalization, of which 25,000 are admitted to the 125 specialized U.S. burn centers. This accounts for more than 900,000 hospital days per year and more than \$1 billion per year in associated costs, including loss of productivity. In addition, 25% of people with burns greater than 75% of total body surface area result in death. Although burn wound care has advanced over the years (e.g., resuscitation, emergency care, and transportation), there is still a critical need to develop treatments that prevent burn injury progression and promote robust burn wound healing.

P12, a peptide derived from fibronectin (ECM glycoprotein), has recently been elucidated by the Clark laboratory at Stony Brook University and shows significant promise in the treatment of burns. This peptide enhanced the survival of adult human dermal fibroblasts, which plays a critical role in wound healing, when cells were exposed to stressful conditions. In addition, P12 reduced burn injury progression in a rat hot comb burn model. Therefore, the goal of this project is to produce a biocompatible, degradable device that can bind and elute P12 to decrease the time required to treat and recover

from burns and cutaneous wounds. The competitive advantage of this product over those commercially available and in development is the limitation of burn injury progression, which is currently not addressed by any FDA-approved therapy. In addition, this is the first topical therapy proposed to attenuate burn injury progression and accelerate healing with a single device.

Summary of Research Completed in Year 1

During the first year of the study, the researchers identified polymer compositions that can degrade in the appropriate time frame relevant to the most crucial stages of cutaneous wound repair after third-degree burns. They achieved fabrication and characterization of the engineered P12 delivery scaffold: P12-containing fibroporous mats were successfully produced using the electrospinning technique. The evaluation of P12 release kinetics from electrospun fibroporous mats is ongoing.

Research Progress – Year 2

1. Production and characterization of P12-loaded tyrosine-derived electrospun fibroporous mats

Although it has been previously shown that injection of P12 attenuated burn injury progression in the rat model, the topical route of its delivery would be more favor-



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able for the following reasons: ease and convenience of application, suitability for self-medication, avoidance of first pass metabolism, and localized delivery of the drug to the wound site with the possibility to achieve higher efficacy with a lower therapeutic dose. Therefore, the researchers hypothesized that the incorporation of P12 into a biocompatible, degradable, polymer matrix would produce controlled topical delivery of P12 to the wound site over a therapeutically relevant period of time.

Tyrosine-derived polycarbonates are a class of novel and proprietary polymers invented in the laboratory of Joachim Kohn at the NJCBM at Rutgers University. They are created from naturally occurring metabolites thus ensuring the nontoxic properties of both polymer and its degradation products. The rationale in choosing these polymers is based on their ability to (1) bind and elute drug(s), (2) promote sustained (gradual) drug release, and (3) resorb benignly in vivo at a predetermined period of time. In addition, tyrosine-based copolymers have been employed in FDA-approved implantable medical devices, PIVITM (2005), PIVITM AB (2006), and AIGISRX™ (2008) (TyRx Pharma, Inc.).

In this project, two polymer compositions were used to produce electrospun fibroporous mats, abbreviated TyroMat-Fast or TyroMat-Slow, which deliver P12 to the injured site either fast (within 1 day) or slow (within 7 days), respectively. The morphology of the fibers was affected by the input concentration of P12 (Figure V-8). Electrospinning polymer without P12 produced TyroMats with a “bead-on-a-string” morphology. However, increasing the input concentration of P12 produced smooth fibers with minimal beading, no webbing, and smaller diameter as shown in Figure V-8. Similar results were observed with the TyroMat-Slow formulation. The binding efficiency of P12 to both TyroMat-Fast and TyroMat-Slow was 70%-80% and independent of: (1) polymer composition, (2) P12 input, and (3) choice of electrospinning solvent.

Shelf-life is defined as the duration of time that TyroMats can be stored at specified conditions (i.e., temperature and humidity) without deviating from their initial specifications. The shelf-life of TyroMat-Fast with and without P12 was evaluated at -20°C, 4°C, and 25°C. At

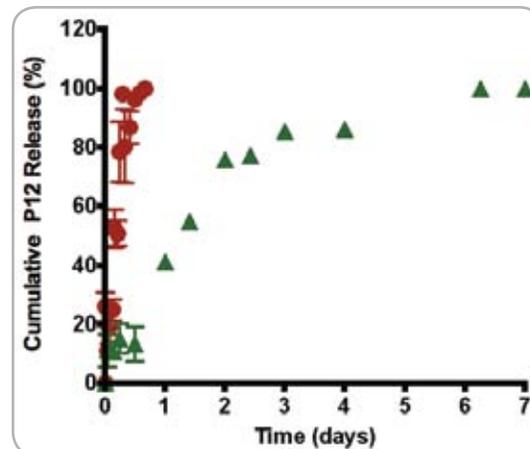


Figure V-8. Scanning electron micrographs of TyroMat-Fast with (0.06, 0.6, and 6% by weight) and without P12 (0%).

select time points, each sample was evaluated for fiber morphology, polymer molecular weight, and P12 content. No significant change in fiber morphology and diameter was measured during 21 weeks at all storage conditions. In addition, P12 content was retained for 21 weeks independent of storage temperature. However, TyroMat-Fast lost 5%, 18%, and 30% of its molecular weight when stored at -20°C, 4°C, and 25°C, respectively (Figure V-9). Degradation of the polymer can be attributed to the residual water (5 wt%) measured in the TyroMat-Fast post-packaging.

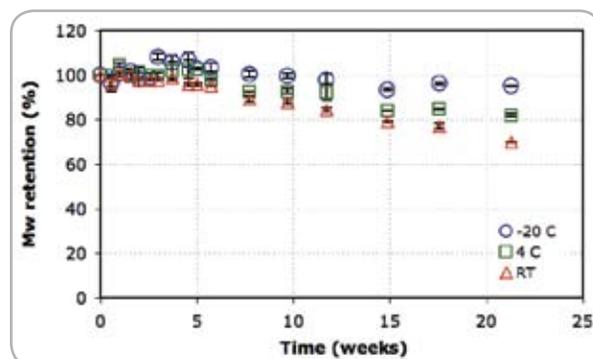


Figure V-9. Stability of electrospun TyroMat-Fast stored at -20°C, 4°C, and 25°C for 21 weeks. P12 content was retained for 21 weeks independent of storage temperature; however, polymer molecular weight was affected by storage at 4°C and 25°C.

2. *In vitro* polymer degradation and peptide release from TyroMats

The optimal timing to deliver P12 to the injured site remains unclear at the present time. Therefore, the release of P12 was profiled *in vitro* from fast and slow dissolving/degrading TyroMats. TyroMat-Fast and TyroMat-Slow formulations dissolve and release P12 within 16 hours and 7 days (Figure V-10). The TyroMat-Fast formulation dissolved significantly faster, thus most of the peptide was rapidly released to the injured site. In the case of the TyroMat-Slow formulation, a more sustained (gradual) therapeutic treatment is expected. In addition, the release of P12 from TyroMat-Slow fibers paralleled the degradation of TyroMat-Slow, whereas the release of P12 from TyroMat-Fast paralleled the dissolution of TyroMat-Fast.

3. *In vivo* biocompatibility of TyroMats with and without P12

A pilot study for the treatment of full-thickness excisional wounds with P12-eluting TyroMats in the porcine model was completed in early 2010. In the study, TyroMat-Fast and TyroMat-Slow with and without P12 were evaluated for their biocompatibility *in vivo*. In addition, these TyroMats contained varying amounts of P12 to evaluate the therapeutic dose of P12. Twenty full-thickness wounds (2.5x2.5 cm) were created on each animal's backs

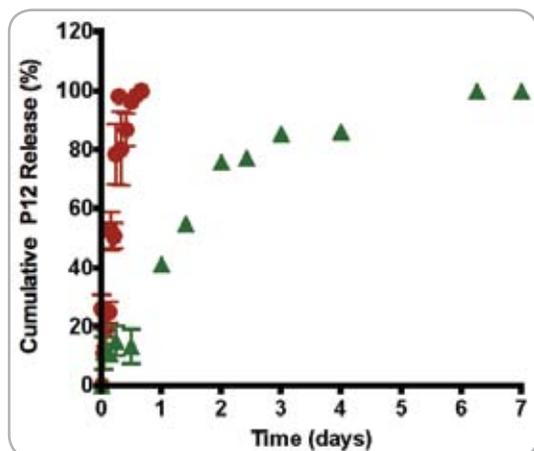


Figure V-10. Control of the P12 dose delivered to the injured site can be achieved using different polymer compositions of TyroMats: (●) TyroMat-Fast and (▲) TyroMat-Slow

and flanks. Each wound received weekly applications of 2.5x2.5 cm of all types of TyroMats. Full-thickness biopsies were taken and histopathologic studies were performed to assess inflammation and healing.

Macroscopic evaluation of wounds 4 days post injury suggested that new tissue (granulation tissue-GT) formation was present in the majority of the wounds. The accumulation of GT progressed throughout the next 3 days so that most wounds were completely filled in with new tissue on Day 7. Microscopic inspection of wounds on Days 4 and 7 showed fibroplasia (creation of new tissue) and neovascularization (new blood vessels) with minimal to no inflammation within the wound space. These observations were in agreement with the macroscopic analysis. No wound contraction was evident at Days 4 and 7, and minimal contraction was observed at Day 14. However, marked contraction was evident at Day 28, where the size of the wound surface was smaller and appeared hourglass-shaped. Together, these data provide insight into P12 doses to evaluate in upcoming pig studies.

Key Research Accomplishments

- TyroMats with and without P12 were fabricated with uniform fiber morphology and high peptide loading.
- Control of the peptide dose delivered to the injured site can be achieved using different polymer compositions of TyroMats: fast (within 1 day) and slow (4 to 7 days) release.
- *In vivo* biocompatibility of fast- and slow-release formulations of TyroMats was obtained in a full-thickness excisional wound porcine model.
- Preclinical evaluation of P12-containing TyroMats in excisional wound porcine models began in Year 2 and will continue throughout Year 3.

Conclusions

Peptide-loaded fibroporous mats (TyroMats) were fabricated with uniform and consistent fiber morphology and high peptide loading. Control of the peptide dose delivered to the injured site can be achieved using different polymer compositions of fibroporous mats.



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TyroMat-Fast resorbed significantly faster, thus most of the peptide was rapidly released to the injured site. Alteration of polymer composition resulted in both slower polymer resorption and peptide release, thus providing a sustained treatment with P12. In vivo biocompatibility of TyroMat formulations was obtained in the full-thickness excisional wound porcine model. TyroMats containing varying doses of P12 are currently being evaluated for their effects on limitation of burn injury progression and enhancement of scarless healing.

Research Plans for the Next 3 Years/Planned Clinical Transitions

In Year 3, the efficacy and safety of topical P12 therapy will be evaluated in porcine models, advancing the technology to TRL 4. The outcome of these studies will provide feedback for further optimization of these technologies to achieve the desired therapeutic effect. In Year 4, the definitive animal studies will be performed, and pending the results, discussions with the FDA and the Center for Devices and Radiological Health will be initiated for pre-IND review. In Year 5, safety and efficacy studies (TRL 5) will be conducted in GLP guidance. Finally, in Year 5, IND will be submitted and design of clinical trial initiated.

Topical Curcumin-Containing Therapies to Promote Scarless Healing

Project 4.6.6, RCCC

Team Leaders: Larisa Sheihet, PhD (Project Leader) (NJCBM) and Richard Clark, MD (Program Director) (Stony Brook University)

Project Team: Lauren Macri, Adam Singer, MD (Stony Brook University); and Joachim Kohn, PhD (NJCBM)

Therapy: Topical therapy for cutaneous wound healing

Deliverable: A controlled release formulation of curcumin

TRL Progress: *Start of Year 1*, TRL 1; *End of Year 1*, TRL 2; *End of Year 2*, TRL 3

Key Accomplishments: The researchers demonstrated that both nanospheres and fibroporous mats successfully incorporate high quantities of curcumin and provide substantial

enhancement to its stability. They also showed that nanospheres and fibroporous mats release curcumin in a rate-controlled manner, which can provide the ability to maintain curcumin levels within the therapeutic window, preventing both toxic and nontherapeutic doses.

Key Words: Wound healing, scarring, curcumin, nanospheres, fibroporous mats, topical delivery

Introduction

There are more than 1 million burns each year in all age groups in the United States, resulting in 900,000 hospital days and 4,500 deaths. Mortality and morbidity from burns, trauma, and other skin loss injuries remain significant medical and socioeconomic problems estimated to cost more than \$1 billion annually in treatment costs and lost productivity. From 2003-2007, the burn unit at Fort Sam Houston (USAISR) had 1,497 hospitalizations, including 656 military, of which 540 were related to the conflict in Iraq. Although recent advances in resuscitation and critical care have led to a significant reduction in the morbidity and mortality of burn victims, improved burn wound management might further minimize acute complications and chronic functional impairment. Improved wound care and prevention of burn injury progression are highly significant to the mission of AFIRM as the treatments being explored in this program are applicable to all burn patients: minor to medium burns may heal faster and allow the expedited return of the wounded warrior to active duty. In the case of severe burns, prevention of burn injury progression may save lives and may significantly reduce the cost of subsequent treatment and rehabilitation.

Progressive inflammation and extension of burns during the first few days after injury can have a devastating effect both acutely with deep second-degree burns often becoming full-thickness third-degree burns leading to increased tissue loss, longer healing time, excess morbidity and mortality, and chronically with increased scarring, wound contractures and poor quality of life. Currently clinically used therapies, such as NSAIDs and antioxidants have not shown substantial benefit to date. The therapy proposed in this project could potentially address one or more of these issues resulting in reduced wound scarring and contractures and burn injury progression.

Curcumin, a yellow extract from the rhizome of turmeric, has been shown to possess many biological actions including anti-inflammatory, anti-cancer, antioxidant, wound healing, and antimicrobial. Curcumin has been used to enhance wound healing for hundreds of years in Asia and recently has been shown to enhance wound healing in several animal models. Despite this considerable promise, its use is still suggested only as a dietary supplement mainly due to its poor bioavailability, rapid metabolism, and rapid systemic elimination. In addition, curcumin is almost insoluble in water, thus creating an additional challenge to its administration and perhaps later bioavailability.

The earlier-discussed limitations of curcumin use require development of an appropriate solubilization and delivery method to increase its *in vivo* stability and bioavailability. Within the framework of this project, tyrosine-derived polymers developed in the Kohn lab (Rutgers) have been chosen to fabricate delivery systems due to their biocompatibility and biodegradability (Figure V-11). Two delivery approaches, nanospheres and fibroporous dressings, have been developed using these polymers that will allow topical controlled delivery of curcumin to the injured site.

Summary of Research Completed in Year 1

In the first year of the project, the researchers formulated curcumin-loaded nanospheres to contain stable high amounts of curcumin compared to curcumin dispersion in PBS. They confirmed the nonconcentration dependent and sustained-release profile of curcumin from curcumin-loaded nanospheres. They determined that nanospheres significantly enhanced permeation of lipophilic Nile red and curcumin to deeper human cadaver skin layers, compared to a nonparticulate vehicle, PEG. They showed that nanospheres formulated as gel or aqueous suspension delivered significantly higher amounts of Nile red to the porcine skin compared to the PEG formulation. The gel formulation delivered greater amounts of Nile red than the aqueous solution.

Research Progress – Year 2

Curcumin-loaded tyrosine-derived nanospheres

Tyrosine-derived nanospheres (here referred to as nanospheres) substantially enhance (6-fold) the stability and aqueous solubility (180 times) of curcumin compared to PBS over the course of 24 hours. This may potentially result in its improved bioavailability and thus higher efficacy to improve wound healing and reduce scarring (Figure V-12). Nanospheres release curcumin in a rate-controlled manner, which could provide the ability to maintain curcumin levels within the therapeutic window, preventing both toxic and nontherapeutic doses (Figure V-13-blue). To improve applicability of this treatment, curcumin-loaded nanospheres were dispersed in gel that provided better contact with the wound site compared to

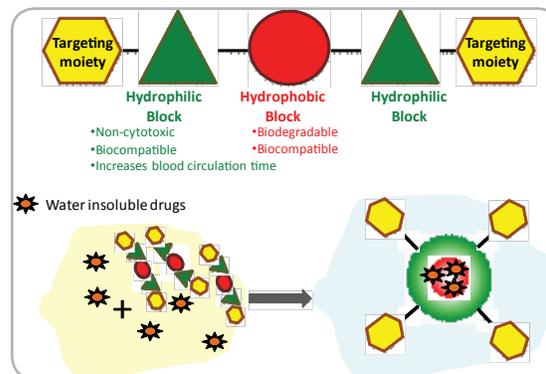


Figure V-11. Targeted drug-loaded nanospheres are obtained by spontaneous self-assembly of the polymer solution in the presence of drug.

aqueous dispersion of nanospheres that have previously shown a run-off effect. As shown in Figure V-13 (blue vs. red), presence of gel does not affect the release kinetics of curcumin with both formulations releasing approximately 60% and 80% of the curcumin by Days 7 and 10, respectively. In addition, the effect of curcumin concentration in the nanospheres on its release kinetics was investigated. While linear release was measured regardless of the amount of curcumin in the nanospheres, the daily release (dose) was strongly dependent on how

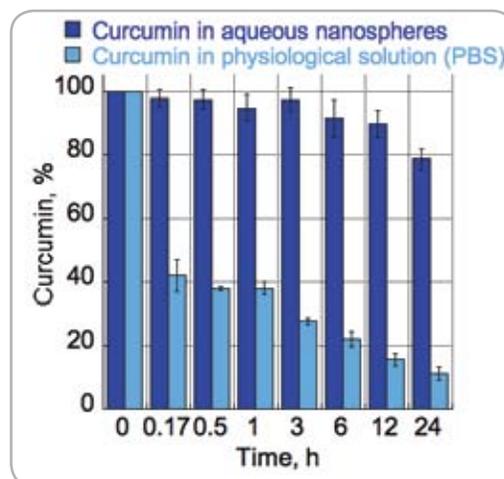


Figure V-12. Curcumin stability over a period of 24 hours as a function of the dispersion media: physiological solution, PBS (dark blue bars) vs. nanospheric dispersion in PBS, pH 7.4 (light blue bars).

much curcumin is present in nanospheres. For instance, 1 mL formulations containing 1.8, 0.36, or 0.12 mg of curcumin released 0.6, 0.1, or 0.45 mg of curcumin on Day 3, respectively. Thus, control of curcumin dose delivered to the site of injury can be achieved using different initial input of curcumin into nanospheres.

The results described previously and reported in Year 1 confirm the potential of curcumin-loaded nanospheres formulation as a topical therapy for cutaneous wound healing. Preclinical evaluation of nanospheres containing varying doses of curcumin for treatment of cutaneous tissue repair in rabbit ear model began in April 2010.

Curcumin-loaded tyrosine-derived fibroporous mats

Based on the extensive knowledge of tyrosine-derived polymers established in Dr. Kohn's laboratory, two polymer compositions were chosen as fast-degrading (FD) and slow-degrading (SD) matrices to produce fibroporous mats (referred herein as fibers). It is yet to be confirmed if curcumin should be delivered in a bolus (high quantity) or gradually (sustained release) to result in the desired therapeutical outcome for cutaneous wound healing. It was anticipated that due to the rapid degradation of the FD polymer matrix it would provide a bolus-type release, and a sustained release would be achieved using fiber fabricated from SD polymer.

Numerous experimental conditions such as polymer composition, curcumin input and various fabrication parameters were explored to produce optimal curcumin-containing fibroporous mats. Figure V-14 depicts images of both types of fibers containing curcumin: A-FD, B-SD. No significant difference was observed in the size, morphology, and incorporation of curcumin as a function of polymer composition used.

As expected, FD-fibers released approximately 85% of its payload with fibers completely dissolving within 8 hours of incubation time (Figure V-14-A, red circles). After 12 hours, the polymer had lost more than 60% of its original molecular weight (Figure V-14-A, blue squares, left Y axis). These results confirmed the validity of the chosen FD fibers as a matrix for the bolus administration of curcumin.

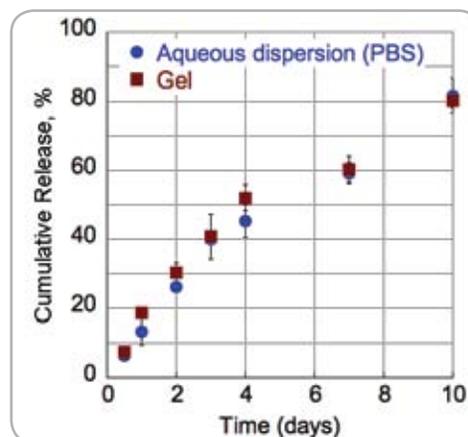


Figure V-13. In vitro release of curcumin from aqueous dispersion of nanospheres (●) and nanospheres dispersed in gel (■).

A very different release profile of curcumin was obtained using SD fibers. No significant release was observed during the first 2 days and only 32% of curcumin was released by Day 7. As anticipated by the design of SD fibers, their degradation (loss of molecular weight) occurred significantly slower than in FD fibers: by 12 hours and Day 7 only 20% and 80%, respectively, of molecular weight was lost (Figure V-14-B, blue squares, left Y axis). However, unexpectedly no significant release of curcumin was measured even by Day 7: only 30% of curcumin was released (Figure V-14-B, red circles, right Y axis). These results suggest that currently composition of SD fibers should be further modified to address the requirement of a gradual 7-day release of curcumin. This work will be performed during the Year 3 of the program.

The results obtained so far for curcumin-loaded FD fibers confirm the potential of this technology in topical therapy for cutaneous wound healing. Preclinical evaluation of this formulation as a treatment for cutaneous tissue repair in full thickness excision wound model in pigs began in May 2010.

Key Research Accomplishments

- Demonstrated that both nanospheres and fibroporous mats successfully incorporate high quantities of curcumin and provide substantial enhancement to its stability.

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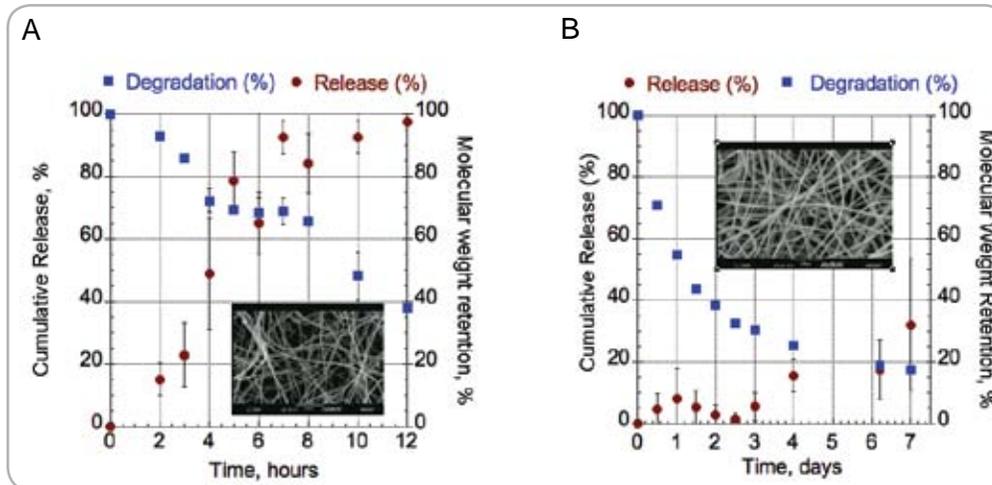


Figure V-14. In vitro curcumin release (●) and biodegradation of the fibers (■): (A) FD and (B) SD.

- This may result in improved bioavailability of curcumin delivered via proposed matrices.
- Demonstrated that both polymer matrices release curcumin in a rate-controlled manner, thus control of the curcumin dose delivered to the injured site can be achieved using different delivery matrices.
- Obtained proof-of-concept for both delivery matrices (not containing curcumin) in a number of laboratory and porcine models.
- Began preclinical evaluation of curcumin-containing nanospheres and curcumin-containing fibroporous mats in April and May 2010, respectively.

Conclusions

During Year 2, it was clearly demonstrated that water insoluble curcumin could be efficiently incorporated into different types of tyrosine-derived matrices, thus providing the ability to deliver desired concentrations of curcumin to an injury site. Both nanospheres and fibroporous mats release curcumin in a rate-controlled

manner, which can provide the ability to maintain curcumin levels within the therapeutic window, preventing both toxic and nontherapeutic doses. Curcumin therapy delivered via tyrosine-derived polymeric matrices offers the potential to address the key military requirements for wound management to reduce burn wound progression.

Research Plans for the Next 3 Years/Planned Clinical Transitions

In Year 3, the efficacy and safety of both topical curcumin therapies will be evaluated in porcine and rabbit models advancing the technology to TRL 4. The outcome of these studies will provide an optimized technology to evaluate for therapeutic effect. In Year 4, the definitive animal studies will be performed and pending the results discussions with the FDA and the Center for Devices and Radiological Health will be initiated for pre-IDE review. In Year 5, safety and efficacy studies (TRL 5) will be conducted in GLP guidance. Finally, in Year 5, an IDE will be submitted and design of a clinical trial will be initiated.

Delivery of Stem Cells to a Burn Wound via a Clinically Tested Spray Device—Exploring Human Fetal Skin Progenitor Cells for Regenerative Medicine Cell-Based Therapy Using Cell Spray Deposition

Project 4.2.2, WFPC

Team Leader(s): Jörg C. Gerlach, MD, PhD (University of Pittsburgh)

Project Team Members: Patrick Over, Matthew Young, and Christa Johnen

Collaborator(s): James Holmes, MD (Wake Forest) and Steven Wolf, MD (USAISR) (Years 4-5)

Therapy: Skin stem cell delivery for cell-based treatment of burn wounds

Deliverable(s): (1) Optimized cell isolation and spraying methodologies and (2) FDA-approved spray device that

can deposit fetal skin stem cells onto wound surfaces

TRL Progress: *Start of Program*, TRL 1; *End Year 1*, TRL 1; *End Year 2*, TRL 2

Key Accomplishments: The research group has established cell isolation methods for fetal epidermal basal progenitor cells. Additionally, fetal dermal MSC isolation and culture have been enabled. Characterization of both cell types is ongoing. An in vitro cell spray model and an in vitro wound capillary membrane model have been

designed for testing of an active wound dressing for the support of skin cells, which works with epidermal progenitors and dermal stem cells. Including the mesenchymal dermal stem cells allows the project to extend from development of second-degree burn therapy to third-degree burn therapy.

Keywords: Skin stem cells, burn wounds, human fetal tissue, progenitor cells, cell spray method

Introduction

The surgical treatments available to burn patients have had a dramatic effect on survivability, yet the outcome for a severely burned patient is still poor. The human body has an innate tendency to respond to injury by producing scarring and fibrosis, which often results in functional limitations and esthetically nonsatisfactory results.

Currently, the gold standard for treatment of severe burns is autografting. However, in cases where there is insufficient healthy skin for use in grafting, other materials have been used. A host of graft materials are available to treat full-thickness burns. Although these therapies have improved patient outcomes, they all result in severe scarring and are poor means by which to induce wound-healing responses. Regenerative medicine research may provide novel opportunities for burn treatment.

This field seeks to accelerate regenerative processes that essentially recapitulate fetal-like wound healing in the adult. In a landmark study by Hohlfield et al. (*Lancet* 366:840-842), fetal-derived skin fibroblasts were banked and then used as a cell source to culture living skin equivalents that were grafted onto pediatric patients. Although these fetal-derived constructs produced a more fetal-like healing response (i.e., rapid wound closure and no hypertrophic granulation tissue), the approach still delivered the cells in a simple and uncontrollable manner. Interestingly, however, Hohlfield and colleagues demonstrated that fetal fibroblasts were not detectable after several weeks; therefore, the cells appear to have established an appropriate environment for autologous healing in the wound bed. Thus, skin progenitor cells derived from human fetal skin tissue have the potential to serve as a regenerative cell-based therapy for acute and chronic skin disease and burn injuries. The focus of this project is to develop methods of isolating and character-



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izing fetal skin cell populations, particularly the epidermal basal keratinocyte progenitor cells and dermal MSCs.

In addition, a method to deliver the fetal cells to a burn wound is required. The Gerlach group has developed a spray device that can “seed” the burn site with any given cell type. The first clinical tests in Berlin, Germany, used expanded keratinocyte cultures that were cultured *ex vivo* and then delivered to the wound site of burned patients. In Berlin and now in Pittsburgh, the Gerlach group transitioned to the use of noncultured cells that are isolated and sprayed in a single session. The Gerlach group proposed to combine the efforts of their clinical work and the work of Hohlfeld by exploring the use of the skin spray device with banked fetal-derived skin progenitor cells. A summary of the skin cell spray concept is shown in Figure V-15.

Summary of Research Completed in Year 1

During the first year of the project, the researchers established an *in vitro* cell spray model and an *in vitro* wound capillary membrane model of an active wound dressing for the support of progenitor cells, which work with fetal skin fibroblasts and keratinocytes. They also established laboratory methods for the isolation and cell culture of fetal skin stem cells.

Research Progress – Year 2

The research team’s long-term clinical goal is to refine and control the deposition of fetal skin progenitor cells in a burn wound with the potential to control their differentiation into basal keratinocytes and other terminally differentiated skin cells. Their clinical results so far indicate that a spray delivery of highly proliferative skin progenitor cells improves patient outcomes. The group hypothesized that a fetal-like healing response initiated by these progenitor cells prior to their elimination from the body will establish a significant improvement in wound healing. Moreover, *in vitro* expansion of skin progenitor cells may be able to form the basis of an “off-the-shelf/ out-of-the-freezer” skin cell-based therapy product that can be made readily available for immediate therapy and does not require complex patient-specific *in vitro* cell culture methods.

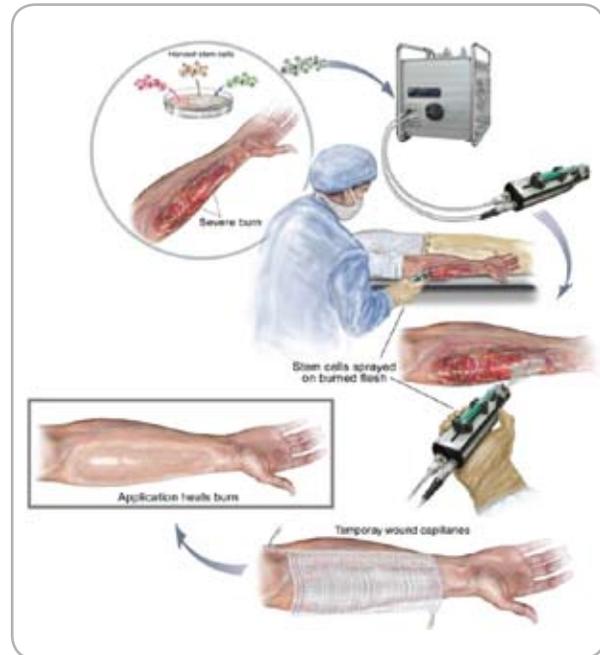


Figure V-15. Perspective of skin spray application.

During the last 2 years, the Gerlach group established fetal tissue donation procedures and obtained IRB approval and oversight for obtaining and using these tissues and the cells derived from them. In addition, cell-sourcing logistics and collaborations have been established. The group also developed isolation and cell culture methods for fetal skin progenitor cells. Methods to isolate fetal skin progenitor cells include a mechanical “dry swab off” method using the surface of collagen-coated dry polystyrol cell culture dishes followed by immediate covering with cell culture media, as well as methods based on collagenase tissue digestion, mechanical scraping with a scalpel, trypsin tissue digestion, and capturing outgrowth from tissue pieces placed on a collagen-coated dish surface. Additionally, after an extensive literature search, experimental *ex vivo* immunofluorescence staining, and discussions with colleagues, the group decided to focus on fetal skin tissues from before gestation Week 9. The rationale for this was based on the confirmation that fetal skin consists of only one cell layer at this stage. In addition, hair structures with subsequent well-known progenitor specialization have not yet developed prior to Week 9.

The immunological markers for use in designing experiments to characterize the skin, as well as a functional assay, were also determined after a thorough literature review. The results show that the raw dermal cell fraction that was isolated contains at least 60% MSCs, which appears sufficient as a starting point for cell-clone generation and cell-banking work. The MSCs could be taken into culture and expanded. Initial clonal growth of dermal MSCs and epidermal progenitors has been completed and compared to adult skin cells; cell stability was tested up to seven passages.

Earlier experimental in vitro cell spray deposition method results were reevaluated. This is of importance for the cell deposition aspects of the project. Those experiments were performed on test paper, using the same amount of trypan blue dye on comparable surface areas. A test person who had 10 minutes to be trained on each of the different methods performed the spraying procedure.

The group finalized in vitro evaluations of cell spraying with the SkinGun using the developed experimental “test wound” in Petri dishes; the results show that there is no significant difference between cells sprayed or dripped or deposited by a syringe (Figure V-16). These positive results confirm the group’s earlier work on adult keratinocytes and the finding that the SkinGun does not expose the cells to greater injury than conventional and routine laboratory pipetting.

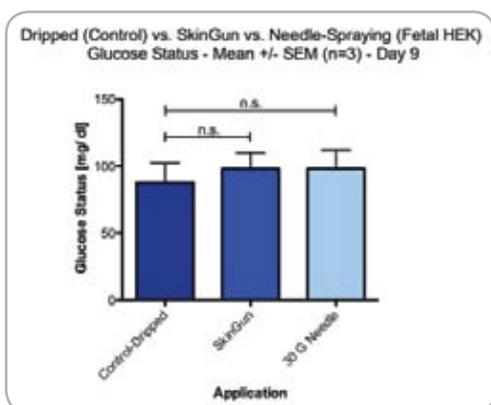


Figure V-16. Comparison of skin gun application, needle spraying, and controls with dripping from pipette into dish.

Overall, development of the isolation techniques for the key cells of this project plan has been accomplished. In addition an in vitro cell spray model and an in vitro wound capillary membrane model of an active wound dressing for the support of sprayed cells have been established. The system can deliver progenitor cells, which work with fetal skin epidermal keratinocyte progenitors to treat second-degree burns, as well as dermal MSCs for the treatment of third-degree burns. Further cell characterization work and investigations into the best methods for freezing and cell banking are ongoing.

Key Research Accomplishments

- Established methods for isolating fetal epidermal basal progenitor cells and fetal dermal MSCs from human skin.
 - Obtained Ethics Committee and IRB approval for obtaining and using these tissues and the cells derived from them.
- Designed an in vitro cell spray model and an in vitro wound capillary membrane model for testing of an active wound dressing for the support of skin cells, which works with epidermal progenitors and dermal stem cells.
 - Performed spray parameter tests to achieve maximal viability and attachment after spraying.

Conclusions

These results demonstrate that skin progenitor cells derived from human fetal skin tissue may provide an interesting new cell source for regenerative cell-based therapy for acute and chronic skin disease and burn patient treatment. The studies reported here describe preliminary isolation, characterization, and application methods for both fetal dermal fibroblasts and epidermal keratinocyte progenitor cells. Ongoing work will determine whether cryopreservation and cell-banking methods can be established to provide a reliable and convenient cell source for skin progenitor cell-based burn therapy. The knowledge gained will contribute to scientific knowledge in the field of skin stem cell research. As a potential medical product, the group proposes cryopreserved, cell-banked cells for an immediately available “out-of-freezer” therapy.



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Research Plans for the Next 3 Years

Optimization of isolation and banking techniques for cells is well under way with completion expected during Year 3 of the project. Under an Ethics Committee and IRB-approved protocol, the Gerlach group will isolate fetal skin stem cells in Pittsburgh. They will use a collagenase-based cell isolation procedure to separate the skin stem cells from the underlying connective tissue layers and use FACS (fluorescence activated cell sorting) and immunohistochemistry to demonstrate that a pure source of cells expressing appropriate markers has been isolated. Functional in vitro assays will be developed to track the differentiation of these fetal skin stem cells into basal keratinocytes. Using this assay, markers that Hohlfeld has reported for her clinically applied cell population will

be confirmed. The researchers will compare and contrast the ability of clonally derived and mixed populations of their isolated skin stem cells to synthesize de novo skin equivalents on matrices similar to those used by Hohlfeld. They will also compare and contrast the stability of mixed cell populations and clonally derived cell populations to standard cryopreservation techniques. By taking cells that exhibited potential in prior experiments, they will then determine the adhesion efficacy of these cells after deposition into an artificial wound.

Planned Clinical Transitions

Preparation of documentation needed to obtain FDA approval for a planned clinical feasibility study in project Year 5 is ongoing.

Artificial Extracellular Matrix Proteins for Regenerative Medicine

Project 4.2.4, WFPC

Team Leader(s): David A. Tirrell, PhD (California Institute of Technology)

Project Team Members: Phoebe Tzou, PhD (Postdoctoral Fellow) and Eileen Fong, BS (Graduate Student) (California Institute of Technology)

Collaborator(s): Mark Van Dyke (WFUSM)

Therapy: Burn therapy

Deliverable(s): Optimized, aECM proteins for clinical burn repair

TRL Progress: *Start of Program*, TRL 1; *End Year 1*, TRL 1; *End Year 2*, TRL 2

Key Accomplishments: The researchers have shown that rates of wound healing in vitro can be accelerated fivefold by increasing the density of cell-adhesion ligands in ECM proteins. They developed a mechanistic model to explain the origin of the increase in wound-healing rates. They found that rates of wound healing on aECM proteins can be greater than

on fibronectin, in part due to increased rates of proliferation. Recent success in improving expression yields of epitope-free aECM proteins has positioned the laboratory to begin animal trials, which will be conducted in collaboration with the Van Dyke laboratory, ahead of schedule.

Keywords: Burn repair, tissue scaffolds, wound healing, extracellular matrix proteins, protein engineering

Introduction

Artificial ECM proteins are designed by combining elements drawn from natural ECM proteins such as fibronectin, collagen, laminin, keratin, and elastin. The needed elements are encoded into artificial genes and the corresponding proteins are expressed in bacterial cells. The modularity of the gene design allows rapid and systematic variation in mechanical and biological properties and in the rate of protein degradation by proteolytic or hydrolytic processes. Matrices can therefore be optimized individually for regenerative therapies with distinct performance requirements. Under the auspices of the AFIRM, the Tirrell laboratory is exploring variations in ECM protein design to optimize matrices for burn repair, and ultimately, for other regenerative therapies. Specific aims for the first 5 years of the project are:

- **Specific Aim 1.** Design and expression of optimized aECM proteins. Optimization parameters include density and identity of cell-adhesion ligands and crosslink density.
- **Specific Aim 2.** Preparation of matrix constructs for wound-healing studies in vitro. Constructs include adsorbed films, spin-coated films, and porous matrices.

- **Specific Aim 3.** Determination of rates of wound healing on aECM matrices.
- **Specific Aim 4.** Evaluation of aECM proteins in animal models of burn repair.

Studies conducted during the initial 5-year period will position the laboratory for evaluation of artificial ECM proteins for use in clinical burn repair.

Summary of Research Completed in Year 1

During the first year of the study, the researchers completed the design and construction of two new artificial ECM proteins with high-affinity fibronectin-derived cell-binding elements combined with elastin-like domains. They also prepared the first generation of thin film matrix constructs to be used for aECM protein evaluations.

Research Progress – Year 2

Increased rates of wound healing on aECM proteins containing full-length fibronectin domains. In Year 1, the researchers introduced a new class of aECM proteins containing full-length fibronectin domain 10 (FN10) and fibronectin domains 9 and 10 (FN910). In Year 2, Caltech investigators compared the rates of healing of



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wounded fibroblast cell sheets on these constructs, on a previous generation of aECM proteins (which contain shorter fibronectin-derived arginine-glycine-aspartic acid [RGD] domains), and on fibronectin. The results show enhanced healing on the aECM proteins containing FN10 and FN910. Remarkably, healing on these substrates is faster than that observed on fibronectin itself.

Full-length cell-binding domains promote higher cell migration rates. In the experiments described in the previous paragraph, cells at the migrating wound edge were tracked for 10 hours from the start of wounding. Individual cells migrated with similar rates on FN910 ($9.2 \pm 0.8 \mu\text{m/hr}$), FN10 ($8.3 \pm 0.6 \mu\text{m/hr}$), and FN ($9.4 \pm 0.8 \mu\text{m/hr}$). In contrast, cells migrated significantly more slowly on the aECM protein containing the shorter RGD domain ($5.9 \pm 0.4 \mu\text{m/hr}$; $P < 0.05$).

Increased proliferation observed on aECM proteins. The mitotic activity of the migrating cell sheet was probed by BrdU incorporation. BrdU-positive cells were found predominantly at the front of the sheet. The percentage of BrdU-labeled cells was also found to be similar on all aECM protein surfaces. However, this percentage was significantly lower for FN, suggesting that proliferation plays a reduced role in wound healing on FN.

Inhibition of proliferation reduces the rate of wound closure on aECM proteins but not on fibronectin. To test the hypothesis that proliferation plays a substantial role in the wound-healing process on aECM proteins, Caltech investigators inhibited fibroblast mitosis using mitomycin-C (MMC). Treatment with $10 \mu\text{M}$ MMC for 24 hours had no observable effect on cell morphologies and cell migration rates (data not shown). When the cell sheets were treated with MMC, there was significant reduction in the distance traveled by the cell sheet for all aECM proteins. There was however no effect on the rate of wound closure for FN (or glass), suggesting that proliferation did not contribute significantly to wound closure on FN.

Increasing RGD density increases the rate of wound healing fivefold. In Year 2, the researchers completed

an in vitro wound-healing study of epithelial cell sheets cultured on ECM protein matrices containing increasing amounts of the RGD adhesion sequence derived from fibronectin. Cell sheets advance fivefold more rapidly on highly adhesive surfaces than on surfaces characterized by lower cell adhesion. Surprisingly, faster wound closure is not a result of faster cell migration. The Caltech team proposed that this apparent contradiction might be explained by an increased probability of boundary crossing from fibronectin to highly adhesive ECM proteins. On this basis, they developed a dynamic Monte Carlo simulation that reproduces nicely the dependence of the wound-healing rate on the concentration of cell-adhesion ligands at the artificial protein surface. This is a qualitatively new perspective on the determinants of the rate of wound closure.

Gram-scale expression of epitope-free aECM constructs. Early generations of aECM proteins expressed in the Tirrell laboratory contained known epitopes derived from the expression vectors used to drive synthesis of the proteins in bacterial cells. New constructs were generated in Year 2 to allow gram-scale synthesis of aECM proteins without these epitope tags. The Caltech team has now succeeded in preparing both elastin- and keratin-based aECM proteins in epitope-free form. These successes will provide the basis for in vivo evaluation of aECM proteins in the pig model in collaboration with the Van Dyke laboratory, either later in Year 2 or in Year 3 of this project.

Key Research Accomplishments

- Achieved increased rates of wound healing on aECM proteins containing full-length fibronectin domains.
- Observed increased proliferation on aECM proteins.
- Elucidated the role of proliferation in wound closure on aECM proteins and on fibronectin.
- Demonstrated that increasing RGD density in aECM proteins increases the rate of wound healing fivefold.
- Achieved gram-scale expression of epitope-free aECM constructs.

Conclusions

The artificial ECM proteins project is ahead of schedule. Several variants of matrix constructs have been prepared, accelerated wound healing has been observed in vitro, and a new model of the wound healing process explains the observed dependence of wound-healing rate on the adhesive properties of the matrix. The Caltech team has solved the problem of high-yield expression of aECM proteins that do not carry the T7 and histidine-tag epitopes and has expressed both elastin- and keratin-based aECM proteins in gram quantities. Evaluation of aECM proteins in the pig burn model will begin in the next several weeks in collaboration with the laboratory of Mark Van Dyke.

Research Plans for the Next 3 Years

In the next 3 years, the project team will focus on scale-up of expression of generation 1 (G1) variants for evalu-

ation in the pig burn model, and on preparation of G2 (keratin) variants in collaboration with Mark Van Dyke. All aspects of the G2 work have been accelerated with respect to the original proposal.

Planned Clinical Transitions

Clinical transitions must await completion of evaluation of aECM protein constructs in animal models. Clinical transitions will probably be accelerated with respect to the original proposal, but the timing of such transitions remains uncertain.

Changes Planned for the Following Years

Evaluation of G1 constructs in animal models and preparation of G2 (keratin) constructs have been accelerated with respect to the original proposal.



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In Situ Bioprinting of Skin for Battlefield Burn Injuries

Project 4.2.5, WFPC

Team Leader(s): James J. Yoo, (Wake Forest University)

Project Team Members: Kyle W. Binder, Weixin Zhao, Dennis Dice, Josh Tan, Hyun-Wook Kang, and Shengli Zhang

Collaborator(s): Lexmark, Inc. and Organogenesis, Inc.

Therapy: Burn repair

Deliverable(s): Biologically functional skin grafts that can be applied directly onto burn wounds using a portable bioprinter/skin generator

TRL Progress: *Start of Program*, TRL 1; *End Year 1*, TRL 2; *End Year 2*, TRL 3

Key Accomplishments: The researchers developed a portable skin-printing device system for direct in situ applications for a large animal

study. This process involved design, construction, laser scanner integration, software development, and validation. The researchers achieved bioprinting of full-thickness skin using human skin cells and dermal matrices in a murine wound model.

Keywords: Burn, skin, bioprinting, allogeneic transplantation

Introduction

Burn injuries typically account for 5%-20% of combat casualties. However, the changing nature of war, especially the rapid increase in the use of improvised explosive devices in combat zones, has drastically affected the number and types of burn injuries. Most battlefield burns are massive injuries and require grafts for coverage and repair since any loss of full-thickness skin of more than 4 cm in diameter will not heal by itself. Current treatment options such as autografts and commercially available skin products are limited in size and some require a lengthy preparation time, making them unusable in severe cases that require prompt and aggressive measures to maintain the lives of wounded soldiers.

Therefore, a new approach that permits immediate burn wound stabilization with functional recovery is necessary. To address the present limitations, the Yoo laboratory seeks to develop a novel delivery system that would allow on-site in situ repair of battlefield burn injuries using tissue-engineered skin grafts produced with a portable skin-printing system.

The unique advantages of the proposed in situ skin-bioprinting system include:

(1) The ability to treat massive burns immediately after stabilization of the wound in the battlefield as skin cells

and matrices can be accurately delivered onto the injured sites. Moreover, bioprinting using inkjet technology involves a rapid printing process with a firing speed of greater than 250,000 drops of cells per second in terms of digital fabrication. This would result in a skin fabrication procedure ranging from minutes to a few hours, depending on the size and type of burn.

(2) The ability to deliver several dermal cell types and matrices simultaneously onto target sites to generate anatomically and functionally adequate dermal tissues. The amount and ratio of cells and matrices as well as the thickness of the skin layers can be precisely controlled by means of the drop-on-demand mechanism of inkjet bioprinters. The delivery of major skin tissue elements onto the injured site would allow for a rapid restoration of the skin and may minimize scarring and enhance cosmetic recovery.

Summary of Research Completed in Year 1

During the first year of the project, the researchers designed a portable skin-printing system and associated control software. They demonstrated the feasibility of using the printer as a delivery system by printing two different skin cells directly onto a wound bed in an athymic mouse model. The skin cells delivered through the

printer nozzles remain viable in vitro and survive after in vivo implantation.

Research Progress – Year 2

The tasks for Year 2 include the following activities:

- Develop a portable skin-printing device system for direct in situ applications. (Years 1-2)
- Bioprint partial and full-thickness skin using human skin cells and dermal matrices. (Years 2-3)

Development of a Portable Skin-Printing Device System

The researchers established the following criteria for the novel system to account for the difficulties of treating patients with large wounds:

- The system must be portable and capable of being quickly transported to patients with extensive burn wounds.
- The system must accommodate a wide range of body types.
- The system should be capable of tailoring cell therapy to an individual patient's specific needs.

This design accomplishes these goals by using a cartridge-based delivery system with a laser scanning system, both mounted on a portable XYZ plotting system (Figure V-17). The cartridge system is similar to that used in traditional inkjet printing so that each cell type is loaded into an individual cartridge in the same way different color inks would be contained in different cartridges. These printheads use pressure-based nozzles instead of the thermal or piezoelectric microfluidic delivery devices used in traditional inkjet printers. A pressure-based delivery system allows the printer to remain a safe distance above the patient to accommodate a variety of body types.

The dimensions of the system are large enough to cover the torso of an average patient but small enough to easily pass through most doorframes. Design goal 3 is accomplished by combining a wound-scanning system with a cartridge-based delivery system. To print a skin

construct exactly matching a patient's wound, the current prototype incorporates a three-dimensional laser scanner mounted above the patient. The cartridge-based delivery system is similar to that used in traditional inkjet printing so that each cell type is loaded into an individual cartridge in the same way different color inks would be contained in different cartridges.

Bioprinting of Full-Thickness Skin Using Human Skin Cells and Dermal Matrices

To demonstrate the feasibility of in situ skin printing, human fibroblasts and keratinocytes were isolated from foreskin. A computer-controlled XYZ plotter and cell deposition system were used to print skin cells directly in a nude mouse wound model. A layer of fibroblasts was printed followed by a layer of keratinocytes. A full-thickness excisional wound was made on the dorsa of female Nu/nu mice (L x W, 3x2.5 cm). Three experimental groups (cell printed, collagen and fibrin, and untreated) were examined every week for 6 weeks to determine the size of the wound and the amount of scarring. Histology was performed at 1, 3, and 6 weeks post surgery.

Wounds repaired using cell printing demonstrated faster wound closure compared to untreated and gel-treated controls. Overall, printed skin cells were able to close the entire wound at 3 weeks post surgery compared to 5 weeks for both negative controls. These results demonstrate the feasibility of in situ bioprinting for rapid care of skin wounds by demonstrating that two different skin cell types could be directly delivered onto a wound. This experiment proves that full-thickness skin defects can be repaired with an inkjet delivery system.

Key Research Accomplishments

- Developed a portable skin-printing device system for direct in situ applications, which included:
 - a. Design of noncontact valve delivery system
 - b. Development of software for bioprinter
 - c. Optimization of a more reliable pressure system for delivery
 - d. Integration of scanning system
 - e. Successful scale-up of skin printer for large animal study

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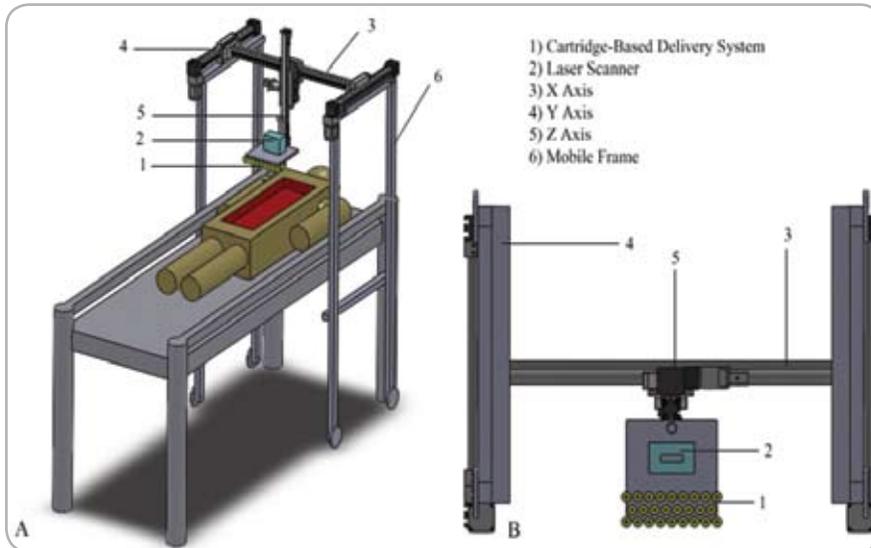


Figure V-17. Concept design of the skin delivery system. (A) Trimetric view of system demonstrating in situ skin printing. A cartridge-based delivery system (1) and laser scanner (2) are mounted on an independent Z axis (5) that is mounted on a fixed XY platform (3,4). The XY platform is mounted on a mobile frame (6) that fits over the patient bed. (B) Patient perspective of the skin delivery system demonstrating multiple rows of nozzles (1) for high-throughput coverage of large wounds.

- f. Development of multiple interchangeable print-heads for various applications
- g. Preliminary design of clinical skin printer
- Achieved bioprinting of full-thickness skin using human skin cells and dermal matrices.
 - a. Successful delivery of two different skin cell types onto a wound in a murine model
 - b. Skin cells delivered through the printer nozzles remain viable
 - c. Demonstration of skin tissue formation and full-thickness wound repair

Conclusions

During the past year, this group has successfully designed and constructed a working bioprinter. The cartridge-based delivery system can be easily transported from location to location. The scanning system

integrated into the bioprinter allows for tailored cell delivery. The group has also successfully built a scale-up bioprinter for large animal studies. Using the bioprinter, the group demonstrated the feasibility of in situ bioprinting for rapid care of skin wounds by delivering two different skin cell types onto a full-thickness wound in a murine model. The group has also demonstrated that these skin cells remain viable and survive after printing. In addition, printed skin cells form normal skin tissue and integrate with the surrounding skin. Therefore, this experiment proves that full-thickness skin defects can be repaired with an inkjet delivery system. Research into in situ skin

printing will continue by expanding the current study into a porcine model using autologous cells.

Research Plans for the Next 3 Years

The researchers plan to validate the second-generation prototype (scale-up unit), refine the large animal bioprinter, and deliver cells to a critical size burn defect in a porcine model.

Planned Clinical Transitions

This basic research project is not slated for clinical trials during the next 5 years. The goal is to demonstrate the feasibility of developing a skin delivery system and test its applicability clinically.

A Multicenter Comparative Study of the ReCell[®] Device and Autologous Split-Thickness Meshed Skin Graft in the Treatment of Acute Burn Injuries

Project 4.2.7, WFPC

Team Leader(s): James Holmes, MD (Director) (WFUBMC Burn Center, Assistant Professor of Surgery, Wake Forest University Winston-Salem, NC)

Project Team Members: Joseph Molnar, MD (Wake Forest University); Rajiv Sood, MD (University of Indiana); William Hickerson, MD (The University of Tennessee Health Science Center); David Mazingom, MD (University of Florida); Bruce Cairns, MD (University of North Carolina at Chapel Hill); Booker T. King, MD (USAISR); Kevin Foster, MD (Maricopa Integrated Health Systems); Marion Jordan, MD (Washington Hospital Center, DC); David Heimbach (University of Washington); and Richard L. Gamelli, MD (Loyola University Medical Center)

Collaborator(s): Fiona Wood, MD (Royal Perth Hospital, Perth, Australia);

William Dolphin (Avita Medical LLC, Woburn, MA); John Geisel (Avita Medical LLC, Woburn, MA); Annette Fagnant (MedDRA Assistance Inc. Foster, RI); Susanne Panzera (BioStat International, Inc. Tampa, FL); and Maureen Lyden (BioStat International, Inc. Tampa, FL)

Therapy: Transplantation of autologous epidermal cells for treatment of second-degree burn injuries. The autologous epidermal cells are separated from a small split-thickness skin biopsy using the ReCell Autologous Cell Harvesting (ACH) System (Avita Medical LLC, Woburn, MA)

Deliverable(s): FDA marketing approval for the ReCell ACH System for the treatment of second-degree burn wounds, which is to be achieved through

a focused clinical study (106 patients, 4-month follow-up).

TRL Progress: *Start of Program*, TRL N/A; *End Year 1*, TRL 7; *End Year 2*, TRL 7

Key Accomplishments: The researchers obtained both FDA and USAMRMC Office of Research Protections (ORP)/Human Research Protection Office (HRPO) approval of the study protocol, executed contracts with key vendors for support of the clinical trial (regulatory affairs, clinical management, biostatistics, data management, and the independent reading facility for primary endpoint adjudication) and initiated subject enrollment.

Keywords: ReCell system, cell spray, skin grafting, burns

Introduction

The skin, as the largest organ in the body, performs a range of vital protective, immunologic, neurosensory, thermoregulatory, and homeostatic functions. Therefore, any wound involving thermal, electrical, or chemical burn, trauma, abrasion, or laceration may seriously compromise the participation, performance, health, and ultimately, the life of the patient. In addition to the acute, short-term effects of inadequate wound management, the long-term effects of wounds and wound scars include pain, restriction of movement, occupational limitations, disfigurement and potential psychological impairment leading to lifelong disabilities, under-employment, and failure to fully reintegrate into society. The

rapid and effective management of wounds of an injured warfighter is, therefore, a critical factor in the determination of wound outcome and consequential morbidity and mortality.

The ReCell Device is based on previous work of Wood & Stoner and the recognition that autologous transplantation of epidermal cells could offer long-term wound closure in a clinically advantageous time frame while optimizing the patient's outcome. The device is designed to provide a simple, safe technique for the harvesting of epidermal cells for enhancement of epidermal repair. The initial step involves harvesting a split-thickness skin biopsy, followed by separation of the dermis from the epidermis to harvest the cells of the epidermal-dermal junc-



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tion. The separated cells are combined in suspension consisting of a mixed population of live keratinocytes, melanocytes and papillary fibroblasts. The suspension is then sprayed onto the prepared wound bed. The cells migrate over the surface providing epidermal reconstruction with site-matched characteristics of color and texture. The applied cells are incorporated into the developing epidermis. The speed of re-epithelialization is very important as the “sealing” of the skin surface limits the inflammation that has been implicated as the pivotal factor in hypertrophic scar formation. By providing a source of viable and metabolically responsive epithelial cells on the wound surface, the ReCell Device technology may facilitate rapid wound healing while minimizing donor site morbidity and potentially eliminating or minimizing scar formation.

The main goal of this project is to collect clinical data to demonstrate the safety and effectiveness of the ReCell Device compared with the standard of care, STSGs, for the treatment of second-degree burn wounds. The results from this study will be used to support a premarket application to the FDA for the ReCell Device. For the regulatory application, the hypotheses to be supported are: (1) noninferiority with the primary efficacy end point defined as recipient site wound closure at the Week 4 follow-up visit of the ReCell-treated area as compared to that of the STMG-treated area and (2) superiority in the healing of the ReCell donor site as compared to the STMG donor site at Week 1. However, in accordance with the AFIRM grant, subjects will be followed for up to 52 weeks following randomization to collect additional data pertaining to wound-healing appearance/scar formation. The target enrollment in this study for evaluation of the regulatory hypotheses is 106 subjects (adjusted upward by 15% to account for potential withdrawals or nonevaluable subjects). This number of accrued subjects is also sufficient to assess the longer term outcomes of scar formation consistent with the AFIRM grant objectives.

Since the ReCell Device is currently an unapproved technology in the United States, initiation of the clinical trial was contingent on obtaining approval from FDA/

Center for Biologics Evaluation and Research (CBER) for an IDE application. Avita Medical submitted the AFIRM study protocol to FDA in April, 2009, and through successful negotiations with the Agency obtained final approval for the IDE in December 2009. In parallel with the IDE process, the program collaborators identified and engaged with potential investigational sites and set up the infrastructure (clinical monitoring, clinical management, data management, and central reading facility) necessary to execute the trial.

Research Progress

(Funded in 2009)

The main competitive technology for ReCell for treating burn patients is the split-thickness skin graft. This has been the standard of care for more than 80 years and is used as either a full sheet or meshed graft depending on the size of the area to be covered and the availability of donor sites. The Cultured Epithelial Autograft (CEA) also is a technology that has been used to treat burn patients since the early 1990s. This is a lab-based procedure that takes skin cells from the patient, isolates the keratinocytes, and uses them to grow sheets of new skin. The process requires special equipment and personnel and takes up to 14-21 days to develop the autograft sheets. They are typically used for large burns that are greater than 30% total body surface area and where there are limited donor sites. There are still questions about final patient outcome and scar quality associated with CEAs.

Following submission of the AFIRM/ReCell clinical study protocol in April 2009, FDA/CBER initially responded with a conditional approval letter; however, many of the conditions cited required re-evaluation of the clinical study's primary endpoint. A meeting was held between the study collaborators and FDA in June 2009 to obtain better clarification/input for FDA's concerns. Based on the initial conditional approval letter and subsequent input received from FDA, the following major program/protocol modifications were incorporated into the clinical study design:

- Primary end point revised from comparison of percentage of epithelialization at 8 weeks between

- ReCell- and STMG-treated sites to comparison of proportion of healed wounds at 4 weeks (noninferiority hypothesis)
- A coprimary end point was included within the protocol to establish superiority in the healing of the ReCell donor site as compared to the STMG donor site at Week 1.
- Evaluation of the coprimary end points is to be based both on local investigator assessment and also via review by an independent clinical expert panel (via review of photographs taken of the wounds and planimetric data).
- The study sample size was increased from 54 to 106 subjects to have adequate power to evaluate the coprimary end point hypotheses.
- The study duration for the regulatory endpoint was increased from 6 weeks to 16 weeks to ensure FDA that any potential safety concerns would be captured.
- Due to the increased sample size, the number of participating sites was increased from 8 sites up to 12 sites.

The revised clinical protocol was reviewed favorably by the Agency, and a final IDE approval letter was issued on December 4, 2009.

Key Research Accomplishments

- Contracted with key vendors for support of clinical study execution including:
 - a. Clinical Research Organization (BioStat International, Inc.) for biostatistical support and clinical and data management
 - b. Photographic Central Reading Facility (Canfield Scientific) for management of study photographic records and primary end point adjudication
 - c. Regulatory Affairs Consult/FDA Liaison (MedDRA Assistance Inc.) serving as the primary correspondent with the FDA's CBER on all matters pertaining to the study protocol and IDE application
- Obtained FDA approval for the study IDE
- Obtained Final USAMRMC ORP/HRPO approval for study protocol

- Obtained local site IRB approval for 6 participating investigational sites
 - a. 4 of 10 sites have received or are pending receipt of ORP/HRPO approval
 - b. 1 site has undergone a study initiation visit/study protocol training and may initiate enrollment
- Initiated subject enrollment on 27 May 2010 at WFHUS

Conclusions

Considerable progress has been made to date—the researchers have obtained both FDA and USAMRMC ORP/HRPO approval of the study protocol, executed contracts with key vendors for support of the clinical trial (regulatory affairs, clinical management, biostatistics, data management and the independent reading facility for primary endpoint adjudication), and initiated subject enrollment.

Research Plans for the Next 3 Years

The research plans for the next 3 years include completing trial enrollment goals (106 subjects) and initiate progress to program status level TRL 8.

Planned Clinical Transitions

The clinical program will be transitioning from a planning phase to an execution phase with subject accrual anticipated over the course of the next year. IRB approval has either been obtained or is currently pending for investigational sites participating in this program. Avita Medical Inc. continues to be an industry collaborator on this program.

Corrections/Changes Planned for Year 3

The program time line has been extended by approximately 1 year due to program delays as a result of the lengthy regulatory approval cycle required to obtain IDE approval from FDA/CBER.



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The Impact of Trauma on the Potency of Adult Stem Cells

Project 4.6.7, USAISR

Team Leaders: Xiaowu Wu, MD and Thomas Walters, PhD

Project Team: Associate Investigators: Robert Christy, PhD, Christopher Rathbone, PhD, and Thomas Walters PhD

Technical: Nicole Wrice and Melissa Sanchez

Therapy: Cell-based trauma treatment (extremity, burn)

Deliverable: Determination of the optimal time for harvesting and delivery of adult stem cells after burn

TRL Progress: *Start of Year 1*, TRL 3; *End of Year 1*, TRL 3; *End of Year 2*, TRL 4

Key Accomplishments: The researchers established an animal model that induces a simulated systemic response to burned patients. They found that a 40% TBSA burn in rats

induced a systemic response, which was characterized by an increase of serum proinflammatory cytokines and a reduction of body weight, lean body mass, fat mass, and bone mineral content.

Key Words: Bone marrow-derived stem cell, muscle-derived stem cell, adipose derived stem cell, potency, burn

Introduction

Soft tissue injuries caused by burn, gunshot wound, and blast injuries comprise the majority of the wounds received by U.S. military personnel in war time activities and make up the largest percentage of surgical procedures performed on battlefield injuries. Using autologous stem cells to repair, replace, and accelerate the healing process holds great promise for tissue engineering and regenerative medicine, which can be applied for combat casualty care. These approaches will eventually involve harvesting stem cells from the injured subjects and then delivering stem cells to the site of tissue damages and injuries such as bone fractures, burn wound, muscle injury, and volumetric muscle loss. While adult stem cells have been identified from different organs and tissues, efforts have been focused primarily on adipose tissue, bone marrow, and skeletal muscle as adult stem cell sources. It should be apparent that the patient from whom the stem cells are extracted will have experienced a severe traumatic injury. However, consideration has not yet been given to the fact that trauma itself may have systemic and/or local effects on the ability of stem cells to proliferate and differentiate.

Severe burn is one of the most physiologically challenging insults to the human body. Severe burn not only

causes skin damage, but also induces damage of multiple body systems and alteration of normal physiologic responses, which therefore causes a high incidence of shock, sepsis, multiple organ failure, and sustained muscle atrophy. There are numerous studies suggesting that cellular functionality is altered in response to trauma. For example, it has been shown that severe burn increases cell apoptosis in multiple tissues such as skeletal muscle, fat, gut mucosal, liver, and cardiac muscle. Besides the primary tissue damage or loss, severe burn induces a series of systemic responses, such as stress and acute phase response, inflammatory response, immune depression, and hypermetabolic response. These pathophysiologic changes have been found in all trauma conditions but are more serious and persistent in severe burn. It is therefore reasonable to postulate that burn may also exert systemic influences on stem cells' functionalities in both injured and remote locations within the body. The results of this study will demonstrate the impact of traumatic tissue injury on the functionality of adult stem cells and therefore be able to determine the optimal time of stem cell harvesting, as well as the delivery of stem cells to sites of injury.

Research Progress

(Funded in 2009)

A sustained and prolonged hypermetabolic response is one of the major hallmarks of severe burn, which is present throughout hospitalization into convalescence. This long-term hypermetabolic response is associated with increased incidence of infection, delayed wound healing, impaired immunity, loss of lean body mass and muscle strength, loss of bone mineral content and bone strength, and growth delay in children. The researchers found that the rat with a 40% TBSA induced a similar acute hypermetabolic response to burned human subjects characterized by a decrease in skeletal muscle weight in the hindlimb, total body lean body mass, fat content, bone mineral content (Figure V-18). They hypothesize that the normal homeostasis of cellular turnover in the tissue is broken due to the severe burn, and reduction of the hypermetabolic response is beneficial for maintaining autologous stem cell functionality and thus improving tissue regeneration after injury. The researchers will isolate those adult stem cells in this animal model and determine their functionality (proliferation, migration, differentiation, and paracrine capability) in vitro. They will further investigate systemic therapeutic approaches

to improve the efficacy and outcome of adult stem cell therapy in severe burn.

There is still controversy about the optimal time when the adult stem cells are applied with maximum efficiency and best biologic benefit after tissue damage and injury. Some studies have shown that stem cells are actively arrested within inflamed tissue, and proinflammatory mediators released either locally or systemically exert certain detrimental effects on stem cells and progenitor cells. However, other studies have shown that stem cells may help restore the immune system and reduce the local inflammatory response. The acute inflammatory response was induced in rats with 40% TBSA, and a sustained inflammatory response is associated with protein catabolism, cellular apoptosis, delayed wound healing, and immune depression and thus interference in outcome. The researchers found that serum levels of the pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α were significantly increased at Day 3 and Day 7 after burn. They hypothesize that sustained inflammatory response plays an important role in determining the functionality of harvested adult stem cells and the efficacy of adult stem cell transplantation after severe burn.

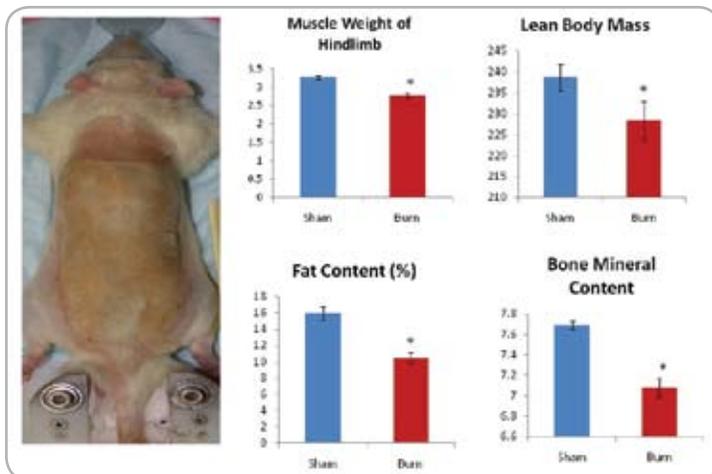


Figure V-18. The rat received 40% TBSA of scald burn (the burn wound is made at back and belly of the trunk). The muscle weight of the hindlimb is significantly decreased at Day 14 after burn compared to sham control. By using DEXA scanning the total body lean body mass, fat content, and bone mineral content significantly decreased in burn compared to sham control.

Restoration of normal function is an important outcome after severe burn. Retrospective studies indicate that although many burned patients eventually return to normal functioning, up to 25% cannot return to previous levels of productivity. In the researchers' rat model, similar to the burned subjects, they found that muscle cross-section area, muscle protein weight, and muscle strength were significantly decreased after burn compared to the sham control, suggesting burn-induced skeletal muscle atrophy. Satellite cells (SCs) are the adult stem cell in skeletal muscle (also named muscle progenitor cells), and the primary cell type involved in skeletal muscle regeneration during physiological conditions. Application of exogenous SC transplantation will enhance muscle regeneration under various conditions of muscle injury. The researchers found that gene expression of myogenic differentiation



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1 (MyoD1), insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF) was significantly decreased in the skeletal muscle at Day 1 after burn. The reduction of IGF-1 and VEGF was attenuated, as well as MyoD1 gene expression, at Days 3 and 7 after burn. This may suggest that SC activity is acutely inhibited, and this is associated with decreased expression of IGF-1 and VEGF in response to burn.

The researchers will isolate SCs in this model and determine the functionality of SCs in vitro. Further study will focus on improving autologous SC functionality by using systemic therapeutic approaches (e.g., using IGF-1 or VEGF) or delivering preconditioned SCs to diminish muscle atrophy and improve muscle regeneration after severe burn.

Key Research Accomplishments

- Established an animal model that induces a clinically relevant systemic response to burn.
- Determined that a 40% TBSA in rats induced a systemic response, which was characterized by an increase of serum proinflammatory cytokines and a reduction of body weight, lean body mass, fat mass, and bone mineral content.
- Obtained IACUC approval for animal protocol.

Conclusions

A 40% TBSA in rats induced a systemic response, which was characterized by an increase of serum proinflammatory cytokines and a reduction of body weight, lean body mass, fat mass, and bone mineral content. This model demonstrates that the burn-induced systemic response causes a pathophysiologic alteration at remote sites, e.g., burn wounds on the trunk resulted in skeletal muscle atrophy. It has been further shown that muscle atrophy is associated with decreased functionality of muscle SCs, which might be in part regulated by the local paracrine capability of growth factors. Understanding the temporal changes of functionality of adult stem cells in response to severe trauma (burn) is beneficial for clinical decision making by determining the optimal time of harvesting and delivering adult stem cells for patients with traumatic injury.

Research Plans for the Next 3 Years

Future studies will focus on investigation of the ability of systemic therapeutic approaches to modulate the regenerative activity of endogenous adult stem cells (in vivo), as well as improve survival, engraftment, and differentiation efficacy of transplanted adult stem cells.

Tissue-Engineered Skin Products – ICX-SKN

Project 4.2.1, WFPC

Team Leader(s): Eric Rolland, PhD and Paul Kemp, PhD (Healthpoint, Ltd)

Project Team Members: Dennis L. Carson, PhD, DABT, Duncan Aust, PhD (Healthpoint, Ltd.); and Kathi Mujynya Ludunge (DFB Bioscience)

Collaborator(s): Pat Hebda, PhD (University of Pittsburgh)

Therapy: A permanent dermal skin graft replacement (ICX-SKN), which can be integrated and remodeled by the host

Deliverable(s): Initiation of human clinical evaluation of ICX-SKN

TRL Progress: *Start of Program*, TRL 1; *End Year 1*, TRL 2; *End Year 2*, TRL 3

Key Accomplishments: The researchers characterized the ICX-SKN matrix during maturation including: composition (fibrin/collagen), density and pore size, cell distribution, and overall thickness. Asset transfer from Intercytex to Healthpoint, Ltd. was

completed. Healthpoint's in vivo pig burn model was approved by IACUC and model evaluation has been completed.

Keywords: Burn, collagen, matrix, skin graft replacement, human dermal fibroblasts

Introduction

The need exists for an “off-the-shelf” skin replacement that is instantly available and alleviates the need to take a full-thickness skin graft. Several so-called “living skin equivalents” (LSEs) and “living dermal equivalents” (LDEs) approved by the FDA are currently available. Although these materials, such as Apligraf, Dermagraft, and OrCel, work as artificial skin grafts, in reality no current living product meets the rigorous requirements necessary to accomplish this function.

The failure of these artificial skin grafts results from lack of a constant structural element due to the quantitative and qualitative nature of the ECM into which the cellular elements were deposited. The intended aim in these first generational constructs was of gradual implant remodeling while maintaining a structure sufficiently robust to resist degradation in the wound. With these first-generation materials, the implant rapidly degraded producing a failed element within a wound that then slowly healed by secondary intent.

The intent of this project therefore is to extend the findings of Neidert et al. (*Biomaterials* 23:3717–3731) who had shown that cells grown within a fibrin scaffold gradually remodeled into a cell-synthesized matrix.

Summary of Research Completed in Year 1

During the first year of the project, the researchers initiated the development of a casting dish for preclinical production of the ICX-SKN biological dermis. They also identified a suitable freeze-drying process for the constructs. They developed a burn pig protocol and characterized maturation of the ICX-SKN constructs by scanning electron microscopy and immunostaining. Finally, they identified ultrasound stimulation protocols that greatly increase the mechanical and biological handling characteristics of the constructs.

Research Progress – Year 2

Development of Skin Casting Dish

During the ICX-SKN product development at Intercytex (ICX), several types of casting dishes were tested and/or developed as research prototypes to allow casting and maturation of the ICX-SKN matrix. The design proved to be successful with regard to casting and maturation of the ICX-SKN matrix, which allowed for easier feeding and reduced the risk of contamination by reducing the number of manipulations required (one feed manipulation per two constructs each time). Additionally there was a relative reduction in the volume of feeding medium required. Dishes had good handling properties. The base



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was skirted to allow easy lifting and carriage. Higher external walls reduced chance of medium spillage and contamination. A handle was incorporated into the lid for ease of lifting when feeding. Plates were stackable and self-contained, saving on space within the incubator. The design of the double 100x100 mm dish seemed to be successful.

Number of Cells Within SKN Constructs During Manufacture

The number of live, apoptotic, and dead HDF cells in the SKN constructs during manufacturing was assessed. The average total number of cells in the construct 3 hours after casting was 1.35×10^6 with 4% apoptotic cells and 8% dead cells. On Day 7 the average total number of cells had significantly increased to 6.72×10^6 cells ($p = 0.005$). Between Days 7 and 42, the average total number of cells fluctuated between 6.72×10^6 and 7.93×10^6 . However, the number of live cells remained relatively constant at an average of 5.41×10^6 . The percentage of apoptotic cells was 13.5% and dead cells was 9% between Day 7 and Day 35 but increased to 17% apoptotic and 18.5% dead cells after Day 35. On Day 49 the average total number of cells significantly decreased to 6.08×10^6 cells ($p = 0.016$), and the average number of live cells decreased by 26% compared to Day 42.

The Effect of Cross-Linking Reagents on ICX-SKN Matrix

A study was performed to establish whether chemical cross-linking of ICX-SKN collagenous matrix would increase desirable characteristics of the matrix with regard to its strength and resistance to collagenase digestion but still remain cell friendly. Two cross-linkers of particular interest were: 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC or EDAC) and 1,1-carbonyldiimidazole (CDI). Both are chemically synthesized and therefore available at high purity, both are easily dissolved in aqueous solution, and after reaction with free amino acid groups both produce inert reaction byproducts that can be removed easily from final formulation of the product.

The results from this study have shown that the best outcome was obtained when sSKN matrix was cross-linked with 5-10 mM EDC between 2-4 hours at RT. Thus,

EDC cross-linking of sSKN has been recommended as an adjunct treatment to sSKN to increase the desirable characteristics of SKN constructs.

ICX-SKN in Pig Burn Model

A pig burn study under the direction of Dr. Pat Hebda at the University of Pittsburgh completed the in-life phase during the current reporting year. The objective of this pilot study was to assess the effect of several iterations of ICX-SKN prototype living skin equivalent grafts on excised third-degree burn injuries in pigs in the short-term after application and the effect of the wound environment on each of the LSE prototypes.

Study Setting and Animal Subjects

The pig has been favored as a burn model for human skin for several reasons. Epidermal turnover occurs over a similar time in both species and is similar in composition. This is not true for phylogenetically lower mammalian species, such as rats or mice. In addition, the pig presents a model of sufficient size to create multiple injuries, which facilitates comparison of several applications with controls in the same animal. This study was performed using approved protocols in compliance with IACUC regulations. Two young female Yorkshire pigs were used and housed in separate cages, and three prototype LSE iterations were tested (i.e., SKN-A, SKN-B, and SKN-C).

Results

Summary of clinical observations: All of the dressings remained in place and were hydrated, with the exception of a few in the caudal region that were rubbed off by animal movement in the pen. Otherwise, there was no indication of any inflammatory or other adverse effects of the LSE treatments.

Wound size tracings: The wounds were traced over the first 3 weeks of the study or until full closure. This is only an approximation of the degree of wound contraction to be verified by histological measurements. The summary of the effect of treatment on wound size (tracings) is shown in Figure V-19. Based on these observations, it would appear that the double layers of LSEs in Groups B, D, and F achieved some inhibition of wound con-

traction, notably on Days 6 and 8, compared with the untreated control Group G.

The histological analysis is in progress. These results will assess the quality of healing and look for any residual LSE material in the wounds.

Technology Transfer from Intercytex to Healthpoint

Various master and working fibroblasts cell banks have been transferred from Intercytex to DFB BioScience. The first step in the technology transfer was to verify cell integrity after shipment and whether the cell culture process was under control at DFB BioScience. Selected cells from ICX banks have been cultured in the conditions previously established by ICX. Control cultures have been grown in DFB culture medium. A fibroblast cell bank established at DFB has been used as a control as well. Cell viability at thawing and cell yield during 3 consecutive passages have been evaluated and compared to predefined specifications. The results demonstrated that the cells transferred from ICX meet the requirements and that the cell culture process is under control at DFB BioScience. Moreover, it was observed that DFB culture medium allowed for better cell yield than the medium previously used at ICX.

Pig Burn Model Development at Healthpoint

Healthpoint has established an approved IACUC protocol for performing burn studies in pigs. The methodology is similar to the pig burn studies performed at the University of Pittsburgh. Under the Healthpoint protocol each of six pigs will receive 20 burn wounds 2 cm in diameter. A recent burn study completed by Healthpoint personnel demonstrated that the model functioned appropriately. Finalization of the paperwork is ongoing and the protocol will be sent shortly to the DoD USAMRMC ACURO for review and approval.

Key Research Accomplishments

- Determined that during the maturation period of the pSKN (product of casting HDFs in human fibrinogen), an increased amount of collagen is produced giving a more porous, structured, and denser matrix with increasing mechanical strength.
- Demonstrated that freeze-drying of the matured collagenous pSKN (producing dSKN followed by sterilization to produce sSKN) serves as an intermediate product during the manufacturing process to allow a longer shelf-life without compromising the structure and function of the final product.
- Determined that the development of a 10x10 cm casting dish will allow scale-up of production.
- Initial studies using ultrasound stimulation are indicating increased collagen production by HDFs further increase the mechanical strength of the construct.
- Completed asset transfer from Intercytex to Healthpoint, Ltd.
- Completed confirmation and validation of the master cell banks.
- Completed the in-life phase of a pig burn study with three iterations of the intermediate matrix.

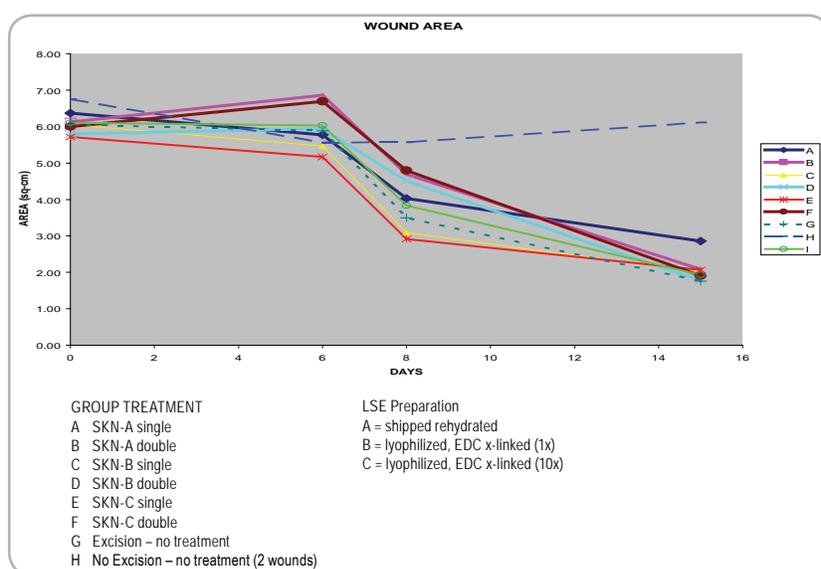


Figure V-19. Wound-tracing measurements.



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- Obtained IACUC approval of the Healthpoint pig burn study and conducted a model evaluation study.

Conclusions

The researchers continue to make substantial progress in the development of a permanent dermal skin graft replacement (ICX-SKN), which can be integrated and remodeled by the host.

Research Plans for the Following Years

Moving forward, Healthpoint will continue to produce ICX-SKN. The immediate need is to determine how to ensure that the collagen matrix is robust in a burn environment. In vitro assays are being developed to determine the relative strength of the matrix in comparison to

various proprietary manipulations in the manufacturing process. Once lead candidates are selected they will be evaluated in the pig burn model. The lead candidate from these studies will be selected for testing in humans. As this project moves forward, interactions with the FDA will begin as plans are finalized for an IND.

Planned Clinical Transitions

The project plans include evaluating the ICX-SKN in humans. An IND will be assembled and filed with FDA. Thus once the lead candidate is selected, the development of the IND will begin. This will require the completion of an appropriate preclinical package along with a clinical protocol. In addition, the protocol will have to be approved through the appropriate IRB for the clinical site(s).

Tissue-Engineered Skin Substitute for Burns

Project 4.2.1a, WFPC

Team Leader(s): Eric Rolland, PhD and Paul Kemp, PhD (Organogenesis)

Project Team Members: David Hurley, MD, Vincent Ronfard, PhD, Michael Segal, and Xianyan Wang, PhD, (Organogenesis Inc.)

Collaborator(s): Lan Cao, PhD, Matthew Wong, MS, Katie Faria, Mark Tuden, Cecile Rousseau, PhD, Parid Sava, Esin Yesilalan, MS, Lorraine Laham, PhD, Gabriel Muraca, Jon Leman (Organogenesis Inc.); James

Holmes, MD (Wake Forest); Jean-Michel Rives, MD (Plastic Surgeon); and Yann Barrandon, MD, PhD (Ecole Polytechnique Fédérale de Lausanne Centre Hospitalier Universitaire Vaudois)

Therapy: Burn therapy

Deliverable(s): An advanced human cell-based therapy for the treatment of deep and extensive burns

TRL Progress: *Start of Program*, TRL 1; *End Year 1*, TRL 1; *End Year 2*, TRL 2

Key Accomplishments: The research group completed the groundwork for the porcine preclinical model and developed three burn product embodiments leveraging Organogenesis' VCT platform technology and advanced biomaterials.

Keywords: Burns, wound healing, fibroblast, tissue engineering, animal model, silk scaffold

Introduction

The researchers have made substantial progress in both the development of a porcine model for preclinical testing and the development of biological constructs as well as in the actual production of potential products. An assigned Project Manager is responsible for the project time line, team meetings, meeting minutes, monthly four panel reports to senior management, budget tracking, tracking and trending of short- and long-term deliverables, project presentations, and independent review of design control phases. The team meets on a regular monthly basis and the Project Manager reports directly to Executive Management. Organogenesis is using a professional project management approach to facilitate product development.

Summary of Research Completed During Year 1

During the first year of the project, the research team established a project management structure including the initiation of Design Control. They also established porcine fibroblast and keratinocyte cell banks. They refined the porcine self-assembly dermal matrix. Finally, they began development of a porcine wound model and, in parallel, a human dermal matrix.

Research Progress – Year 2

Porcine fibroblast and keratinocyte cell banks were developed from 4 pig breeds and yielded over 1,200 frozen vials. Over 2,500 constructs have been produced from both pig and human cells. Porcine and human versions of potential products were developed over several iterations and initial preclinical testing has been performed in both murine (170 mice) and porcine models (3 pigs) of full-thickness wounds. Three potential burn product embodiments have been developed and further investigation is under way to finalize the product.

Project Management continues as a formal activity. Development of an appropriate Target Product Profile (TPP) was accomplished during this grant period. This TPP was developed by consulting with both internal company experts and external experts, specifically in the field of surgical burn care. The AFIRM burn product and pig model development time lines have also been generated. The research team has contributed to another AFIRM project, providing multiple pig cell lines to Dr. James Yoo's research team.

Key Research Accomplishments

Porcine Preclinical Model Development

- Completed porcine fibroblast and keratinocyte master cell banking and working cell banking from domestic

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(Yorkshire) and multiple miniature porcine breeds (Figure V-20).

- Characterized the growth characteristics of the cells.
- Optimized the porcine fibroblast-derived ECM cell culture process and achieved more than double the thickness of the matrix developed during Year 2 (Figure V-21).
- Developed a robust cell culture system to resolve the contraction issues during cell culture.
- Prototyped apparatus for the assembly of new cell culture inserts for potential scaling-up.

Burn Product Development

- Created two strategies for developing burn products: (A) incorporated Organogenesis' platform technology with biodegradable natural biomaterial; (B) leveraged Organogenesis' VCT platform technology.
- Developed an in vitro angiogenesis assay that allows assessing different embodiments effectively.
- Designed and prototyped a new cell culture system for scaling up.
- Developed an in vitro angiogenesis assay that allows assessing different embodiments effectively.
- Completed the first small animal screening study using a subcutaneous nude mouse implantation model on 3 burn product embodiments; obtained promising preliminary results.
- Performing an ongoing assessment on the four embodiments of the product comparing them with commercial burn products using a subcutaneous standard mouse implantation model.
- Scheduled the next preclinical study using full-thickness wound mouse and pig models.

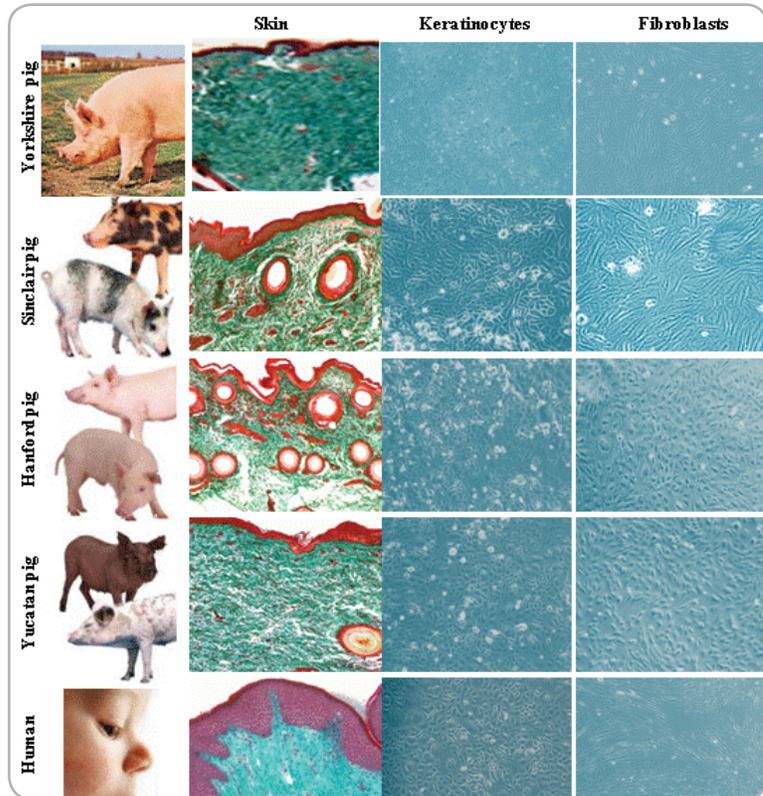


Figure V-20. Comparison of pig and human skins. Similarities and differences are observed between pig and human skins and among breeds.

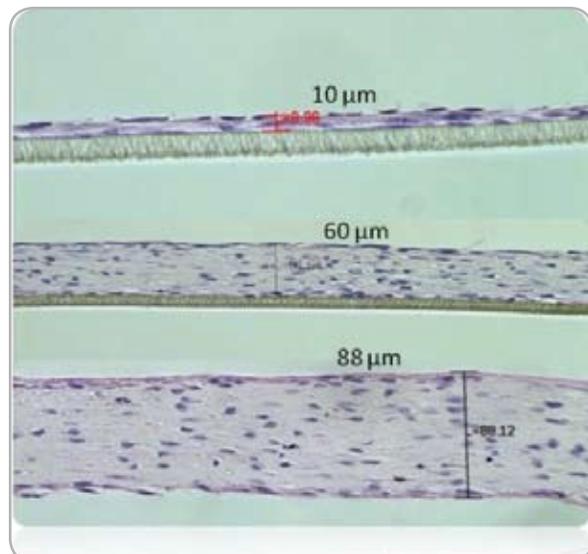


Figure V-21. Progress in increasing the thickness of porcine fibroblasts-derived ECM.

Conclusions

The researchers have made substantial progress in both the development of a porcine model for preclinical testing and the development of biological constructs as well as in the actual production of potential products.

Research Plans for the Next 3 Years

The research team will start the final mouse feasibility study and a large animal feasibility study for the burn product in the third quarter of 2010. They will also start the final feasibility in vitro characterization, final feasibility safety biocompatibility study, VCT01™ porcine pig con-

struct model study, and Apligraf® re-application porcine pig construct model study during the third quarter of 2010. In the fourth quarter of 2010, the researchers will start the porcine VCT03 and construct model study and the porcine version of the burn product model study. They will begin a large animal efficacy study and start the safety/biocompatibility studies for the burn product in the first quarter of 2011.

Planned Clinical Transitions

A pre-IND meeting with the FDA is scheduled for the fourth quarter of 2010. The research team anticipates beginning preparations for a clinical trial in the first quarter of 2011.



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Amniotic Fluid Stem Cells for Burn

Project 4.2.6, WFPC

Team Leader(s): Mark E. Furth, PhD (WFIRM)

Project Team Members: Chad D. Markert, PhD (WFIRM)

Collaborator(s): Mark Van Dyke, Colin Bishop, Emily Moorefield, Shantaram Bharadwaj, Martin Childers, Peter Antinozzi (Wake Forest University); and Luis Solchaga (Case Western)

Therapy: AFS cells to treat severe burns. In particular, LSEs prepared

using undifferentiated AFS cells and differentiated cells obtained from them

Deliverable(s): An improved off-the-shelf bioengineered skin product for the treatment of extensive burns that utilizes broadly multipotent stem cells from amniotic fluid

TRL Progress: *Start of Program*, TRL 1; *End Year 1*, TRL 1; *End Year 2*, TRL 2

Key Accomplishments: The researchers demonstrated the immunomodulatory activity of AFS cells against human T-lymphocytes. They optimized the expression of stem cell markers Oct4 and SOX2 by AFS cells under new culture conditions. They also induced cytokeratin 14 (CK14), a marker of epithelial differentiation, in AFS cells cultured in inductive media.

Keywords: Stem cells, regenerative medicine, living skin equivalents

Introduction

The project seeks to meet the need for an off-the-shelf LSE for military personnel and noncombatants who suffer extensive burns using AFS cells. One of the potential and important advantages of these cells would be to improve immunological matching of graft to recipient without the time and expense that are required to harvest and expand autologous cells for each recipient. Several bioengineered skin products utilizing allogeneic cells have proven safe and effective in human testing for wound healing, and some have shown value in treating burns. However, there is evidence that allogeneic epidermal cells do not engraft permanently, but rather that they are eventually rejected and must be replaced by the recipient's own cells. In the case of extensive burns, the limited amount of remaining epidermis makes this problematic.

Although only autologous cells provide a perfect genetic match, a careful analysis of donor pools indicated that as few as 10 carefully selected self-renewing stem cell lines potentially could offer "the maximum practical benefit" for matching of human HLA (major histocompatibility) antigens. This number appears small enough to enable manufacturing and maintenance of inventory for rapid delivery in treatment settings. This group has begun to map out a detailed plan for the banking and scale-up

manufacture of AFS cells, in compliance with regulatory requirements of current Good Tissue Practice (cGTP) and current Good Manufacturing Practice (cGMP).

Summary of Research Completed During Year 1

During the first year of the project, the researchers demonstrated that human AFS cells express CD146, a cell surface marker protein recognized as a marker of perivascular cells. They also demonstrated enhanced skin wound healing by AFS cells in immune-deficient mice. They also found initial evidence for the expression of stratified epithelial lineage markers p63 and CK14 by some AFS cell lines in response to in vitro differentiation conditions.

Research Progress – Year 2

Immunomodulatory Activity of AFS Cells

AFS cells were primed with IL-1 β , added to PHA stimulated T-cells, and T-cell proliferation and activation were assayed by EliSpot assay for IFN- γ . The results show that AFS cells can inhibit T-cell proliferation to about the same extent as MSCs (Figures V-22 and V-23; collaborations with Luis Solchaga, Emily Moorefield, Colin Bishop). The initial assay was carried out with lines h-AFS-A1 and h-AFS-H1. Both showed immunomodulatory activity,

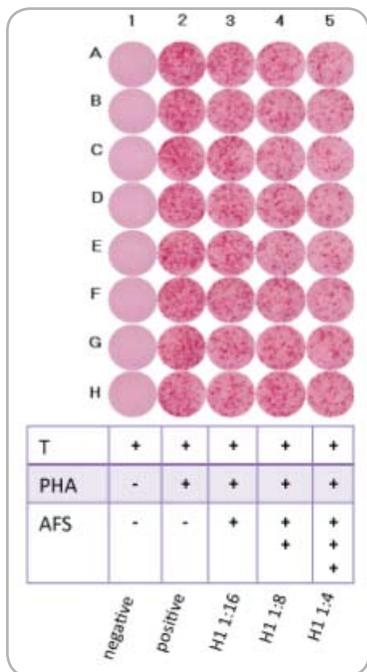


Figure V-22. Immunomodulation by AFS cells – EliSpot assay for T-cell proliferation.

with H1 being stronger. Another line, hAFS-CB3, which has somewhat different morphology and other properties, was negative. Very recent data indicate that several newly isolated h-AFS cell lines show strong immunomodulatory activity in the same assay used in Figures V-22 and V-23.

Differentiation to Keratinocyte Lineage

By combining the hanging drop technique with culture in O₂ levels that more closely mimic the in vivo environment, recent research results from this group now demonstrate more clearly that a newly isolated AFS cell line expresses two nuclear markers that have been associated with the stemness of embryonic stem (ES)

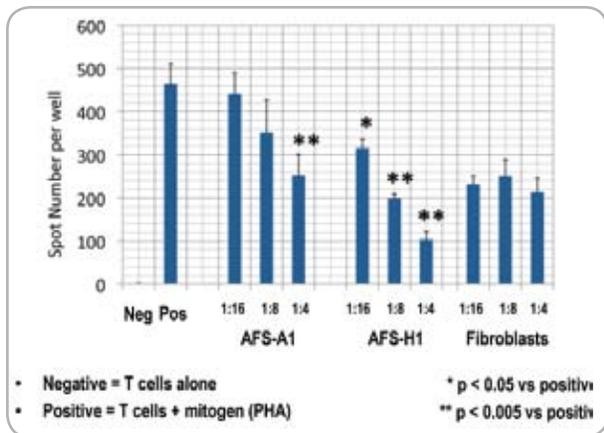


Figure V-23. AFS cells inhibit T-cell proliferation, compared to fibroblast controls.

cells; namely, Oct4 and SOX2 (Markert et al., *Journal of Immunological Methods*, submitted April 2010). This suggests that the manipulations of culture techniques may significantly enhance the differentiation potential of AFS cells. The variability in previous results might be explained by the fact that AFS cells had been grown routinely in conditions that poorly mimic the in vivo environment.

With the new findings, the original hypothesis that AFS cells can serve as a cell source in the generation of LSEs gains its strongest support to date.

AFS cells were primed with IL-1 β , added to PHA-stimulated T-cells, and T-cell proliferation and activation were assayed by EliSpot assay for IFN- γ . The results show that AFS cells can inhibit T-cell proliferation to about the same extent as MSCs (collaborations with Luis Solchaga, Emily Moorefield, and Colin Bishop). Very recent data indicate that several newly isolated h-AFS cell lines show strong immunomodulatory activity in the same assay.

Differentiation of AFS Cells

In the previous year, this group documented the ability to induce expression of early-stage markers of the keratinocyte lineage by AFS cells; namely, p63 and CK14. In the current year, the group found that the conditions used for this induction, which was based on published reports for MSCs, stress the cells because of overgrowth. Viability is marginal, and recovery of RNA is poor. Therefore, they developed new conditions under which to assay for differentiation. Under these conditions, by adjusting cell density and timing more carefully, excellent recovery of RNA is achieved from highly viable cell populations. This allowed the group to search for keratinocyte differentiation under more robust conditions.

The group developed the use of a bioimager (Becton Dickinson Pathway 855) to carry out efficient screening (moderate throughput in pharmaceutical industry terms). As shown in Figure V-24, the group first optimized immunofluorescent staining for the nuclear marker p63 to have an assay sufficiently reliable for screening purposes. In addition, the assays have been adapted for use on a bioimager.

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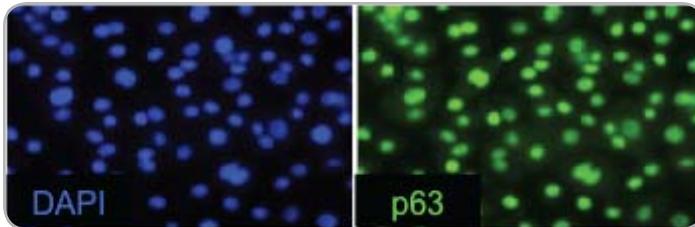


Figure V-24. Optimization of p63 immunofluorescent staining on human keratinocytes in culture. DAPI nuclear staining (left) and p63 localization in nuclei (right).

New AFS Culture Conditions for Enhanced Stem Cell Phenotype and Differentiation

Several lines of evidence from this group's work and that of others led to the conclusion that both aggregate formation and culture in reduced O_2 might be beneficial in achieving the desired differentiation of AFS cells to the keratinocyte lineage, a derivative of embryonic ectoderm.

Accordingly, pilot experiments were initiated to attempt to induce expression of p63 and CK14 in recently isolated lines of human AFS cells. The strategy was to grow AFS cells in low O_2 , place them under aggregation conditions for 4 days, confirm nuclear expression of Oct4 and SOX2 by immunofluorescent staining, and grow the cell aggregates in potentially inductive media formulations. While starting with keratinocyte-conditioned medium, the group plans to rapidly attempt to switch to defined components to enhance translational potential. The immediate readout is expression of markers of candidate keratinocyte progenitors, CK14 and p63.

Preliminary results are presented in **Figures V-25 and V-26**. AFS cells were grown in hanging drops in low O_2 for 4 days. As shown in Figure V-25, two recently isolated AFS cell lines showed positive nuclear staining for Oct4 and SOX2, similar to that seen in teratocarcinoma cells (positive control). Normal keratinocytes were negative in these transcription factors. The aggregates were then transferred to a low-adherence plate for 7 days in a commercially available keratinocyte serum-free medium (KSFM), "conditioned" for 24 hours by human keratinocytes. Strong CK14 expression was observed by immunofluorescence (Figure V-26A), with staining intensity comparable to that seen in authentic human keratinocytes (Figure V-26B). The group plans to move

forward by comparing other media formulations with conditioned KSFM and by staining for both CK14 and p63. They will then seek to expand the cells that express candidate basal cell markers and test their ability to actually yield differentiated keratinocytes in vitro and in vivo. Previously, the principal rate-limiting target was induction of differentiation of AFS cells to the epidermal lineage. The Key Research Accomplishments listed suggest that this challenge can be overcome.

Finally, this research group plans to study the feasibility of cell spraying or in situ bioprinting of induced and non-induced AFS cells in animal models of burn injury and develop in vitro induction conditions to promote the dif-

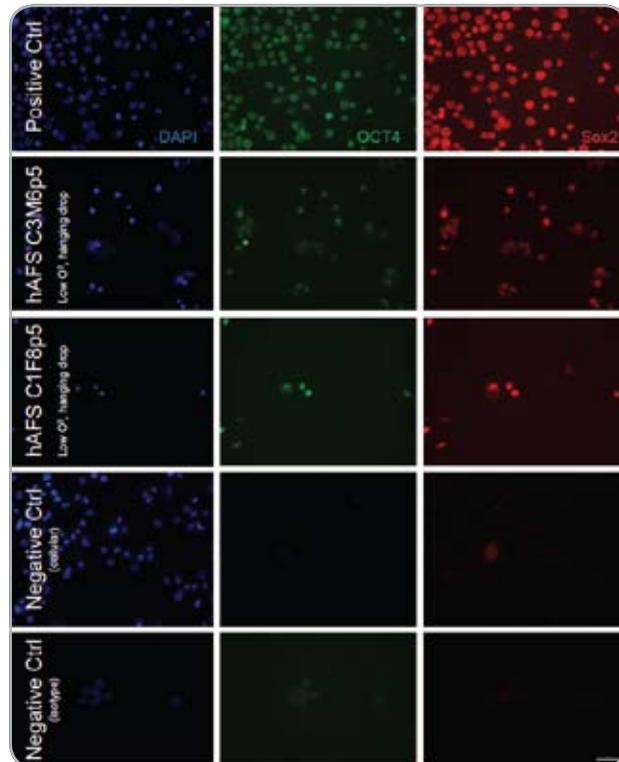


Figure V-25. Oct4 and SOX2 expression in aggregates formed in hanging drops at low O_2 . Immunofluorescence in two independent, recently isolated human AFS clonal cell lines for Oct4 (green) and SOX2 (red) with appropriate monoclonal antibodies. DAPI staining (blue) shows nuclei. Positive control is human teratocarcinoma (NCCIT). Negative cell control is human keratinocytes. Negative isotype control is mouse IgG. Scale bar = 50 microns.

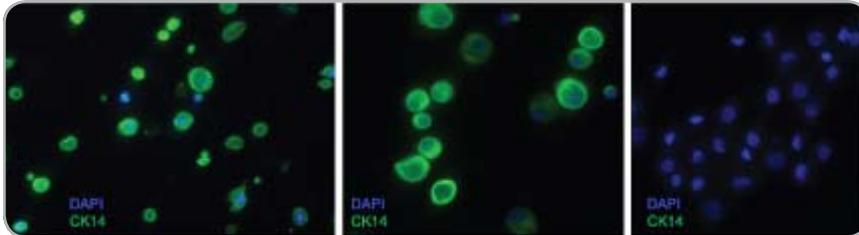


Figure V-26. CK14 Expression in Induced AFS Cells Under New Culture Conditions. Human AFS cells were maintained for 4 days in hanging drop culture (low O_2), then transferred to culture for 7 days in KSFM conditioned by keratinocytes. Staining of CK14 (green) was assessed by indirect immunofluorescence using a mouse monoclonal antibody and FITC-goat-anti mouse IgG. Nuclei were stained with DAPI. Photomicrographs represent matched exposures. (A) Induced AFS cells. (B) Normal human keratinocytes (positive control). (C) Induced AFS cells with isotype control.

ferentiation of AFS cells toward the melanocyte lineage. They will also incorporate AFS cell-derived melanocytes into LSE or use for bioprinting/cell spraying *in vivo*.

Key Research Accomplishments

- Demonstrated immunomodulatory activity of AFS cells against human T-lymphocytes.
- Observed stem cell markers Oct4 and SOX2 in AFS cells under improved culture conditions.
- Observed CK14, a marker of epithelial differentiation, in AFS cells cultured in inductive media.

Conclusions

AFS cells grown under certain conditions express certain transcription factors (e.g., Oct4 and SOX2) shared with other stem cells, including ES cells, and when grown in appropriate inductive media, may then be induced to express markers of basal-stratified epithelia (CK14 and p63), suggesting usefulness in the generation of LSEs. Furthermore, the cells may secrete several growth factors important for creating a trophic, permissive microenvironment. In particular, they can produce significant levels of VEGF, which would likely prove beneficial in supporting angiogenesis for regenerating skin.

Research Plans for the Next 3 Years

A top priority of the research team is to further characterize the expression of markers of pluripotent stem cells, various adult stem cells, skin progenitor cells, and more differentiated keratinocyte lineage cells, by AFS cell lines, at both the RNA and protein levels, before and after induction. Initially, the group will compare expression in the undifferentiated stem cells grown as monolayers (in low or

standard O_2), aggregated cells grown as hanging drops in low O_2 , and cells induced in conditioned KSFM. (The group also must establish whether unconditioned KSFM will suffice, and/or whether addition of additional defined factors to KSFM can substitute for keratinocyte conditioning). Markers of interest will include an array of stem cell markers, epithelial lineage markers, and appropriate controls.

Planned Clinical Transitions

Although it is too early to plan translation to clinical trials at this time, the *in vitro* work described here presents many opportunities for hypothesis-testing experiments in animal models of wound healing. These models are in place at WFIRM and are readily available. As previously noted, transition of this 5-year project to clinical studies is anticipated to occur 2 years after the project ends. Success in bioprinting with undifferentiated stem cells and AFS-derived epidermal lineage cells could potentially accelerate clinical translation.



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In Vitro Expanded Living Skin for Reparative Procedures

Project 4.2.8, WFPC

Team Leader(s): Sang Jin Lee, PhD, James J. Yoo, MD, PhD, and James H. Holmes (Wake Forest University)

Project Team Members: Jae Hyun Bae, MD, PhD, Hyun-Wook Kang, PhD, Mitchell Ladd, BS, Oscar Gonzalez, BS, Kevin Johnson, PhD, and Paul Scarpinato, BS (Wake Forest University)

Collaborator(s): Steven E. Wolf, MD (USAISR)

Therapy: Treatment of burn injuries

Deliverable(s): Autologous skin grafts

TRL Progress: *Start of Program*, TRL 4; *End Year 1*, TRL 4; *End Year 2*, TRL 5

Key Accomplishments: This group has developed an in vitro tissue expander system that permits a rapid increase in surface dimensions of donor skin while maintaining tissue viability for subsequent skin transplantation. The expander system utilizes a computer-controlled bioreactor capable of providing an accurate expansion rate for yielding target skin dimensions over a defined time period. This system was successfully tested and validated

on human skin samples. The group has been able to consistently double the surface area of donor skin within 2 weeks while maintaining cell viability. The expanded skin, grafted in small (mice) and large (pig) animal models, showed viable graft take when implanted on a recipient dermal bed. The researchers recently designed and built a clinically applicable bioreactor system.

Keywords: Autologous skin grafts, in vitro skin expander, bioreactor, burn injury

Introduction

Many reparative procedures due to battlefield trauma and burn may require additional skin for coverage. The standard of care for skin defect replacement is the use of autologous skin grafts. However, donor-site tissue availability is a major obstacle to the successful replacement of skin defects. Because of this limitation, other approaches are commonly employed to cover skin defects. These include commercially available skin products based on biomaterials and tissue engineering, allografts, and xenografts. However, these approaches also have limitations, such as the need for concomitant autograft, insufficient mechanical properties, high cost, lack of permanence, potential for infectious disease transmission, and inadequate biocompatibility. Nevertheless, many commercial skin products are being used as acceptable skin substitutes when autologous donor tissue is unavailable.

Alternatively, subcutaneous tissue expanders or STSGs are used clinically to generate larger segments of autologous skin when donor-site tissue is limited. Subcutaneous tissue expanders are balloon implants that are sequentially filled with incremental volumes of saline to increase the amount of overlying skin. The

physico-mechanical stress of the tissue expander results in biologic creep, greater mitotic activity of cells, and increased vascularity, which ultimately leads to expanded skin. Subsequently, the expanded skin can be used as a tissue flap or harvested for use as a skin graft. However, the use of a subcutaneous tissue expander is associated with an additional surgical procedure(s), which increases donor site and overall morbidity. In addition, this technique requires a lengthy wait time (on the order of months) to obtain sufficient tissue for intervention. Moreover, the discomfort associated with the increasing expander volume and the frequent tissue fibrosis remains as major limitations. Alternatively, meshed STSGs use a graft mesher that cuts the skin into a mesh pattern, which results in greater surface dimensions before application on the wound bed. However, meshed STSGs are not considered ideal for many applications because they leave large gaps of the open wound, which requires a longer healing time and results in a cross-hatched or cobblestone pattern of healed skin as scar tissue fills the gaps.

The researchers of this project are conducting an initial "Phase 1" trial that will analyze the safety of harvesting, expanding, and then grafting a piece of skin from a burn

patient. An ~40 cm² split-thickness piece of skin will be harvested at the initial operation from a burn patient who is anticipated to require multiple acute operations. The skin will be expanded in a bioreactor to ~100 cm² over 2 weeks. The skin will then be grafted back onto the patient in the standard manner, with or without meshing, at the next operation.

The overall goal of the project is to provide wounded soldiers with large dimensions of autologous skin for reparative procedures. The project consists of the following three specific aims:

- Optimize expansion parameters for maximizing surface dimensions of human skin.
- Establish SOPs for skin expansion parameters and delivery.
- Determine the applicability in wounded soldiers through a clinical trial.

Research Progress

(Funded in 2009)

In the past year, the researchers have developed a clinically applicable bioreactor system composed of a closed stand-alone system and a new gripping system (Figure V-27). The gripping points are maximized to generate uniformly distributed expansion capability on the skin resulting in maximized surface dimension. The force sensor is incorporated for real-time monitoring of the tension of skin matrix during the expansion procedure. And, automatic systems such as a temperature controller, an automatic media exchange system, and an air/CO₂ controller are incorporated into the system for realizing the closed stand-alone system.

This system was successfully tested and validated on human skin samples. The group has been able to consistently double the surface area of donor skin within 2 weeks while maintaining cell viability. The expanded skin, grafted in small (mice) and large (pig) animal models, showed viable graft take when implanted on a recipient dermal bed (Figure V-27).

The group is currently defining parameters that would maximize the surface dimensions of autologous skin grafts for the treatment of battlefield burns—experiments to discover relationships between mechanical expansion stimuli, tissue culture conditions, and the tissue expansion protocol are ongoing. The researchers have come up with a strategy to perform a clinical trial, which requires processes that include toxicity testing under GLP, establishment of SOPs, initiation of communication with the FDA and IRB and FDA application submission.

Key Research Accomplishments

- Built an in vitro tissue expander system that uses a computer-controlled bioreactor capable of providing an accurate expansion rate for yielding target skin dimensions over a defined time period.
- Tested and validated the bioreactor system on human skin samples.

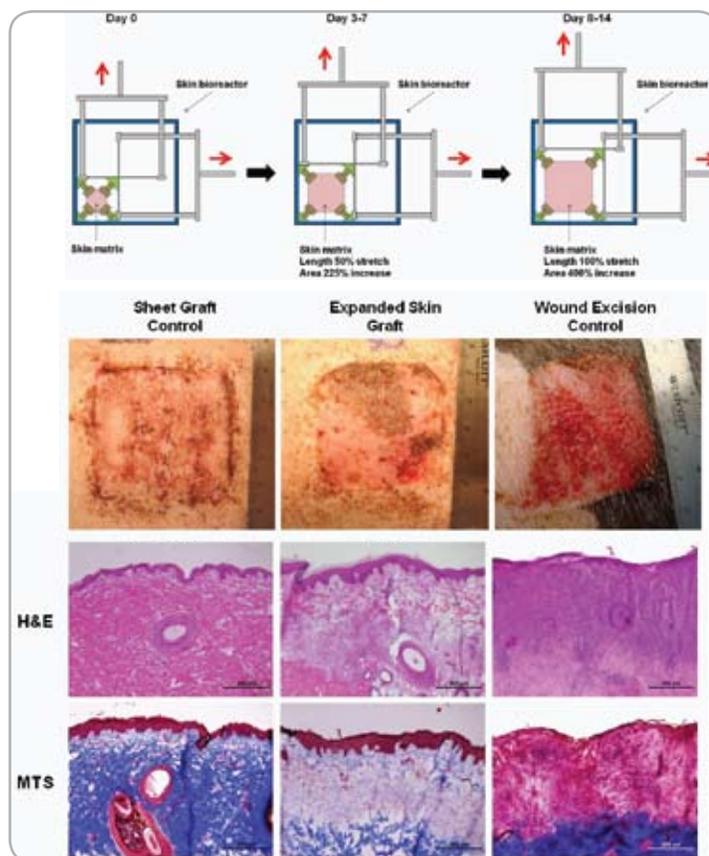


Figure V-27. In vitro expanded living skin for reparative procedures.



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- Consistently doubled the surface area of donor skin within 2 weeks while maintaining cell viability.
- Designed a clinically applicable bioreactor system for a clinical trial, with construction under way of a prototype.
- Prepared documents for pre-IDE/IND submission.

Conclusions

These results show that skin can be incrementally expanded in vitro using a computer-controlled biaxial bioreactor system. Cell viability and proliferative potential of the tissue were maintained, dermal structural integrity was preserved, and pore size was increased. Thus, this technology provides an opportunity to generate large amounts of living skin for burn repairs and may overcome current limitations. These studies indicate that this technology would allow a surgeon to take a small, split-thickness skin biopsy using a dermatome and expand it in vitro using the expander system to create a large segment of skin for subsequent repair procedures in wounded soldiers.

Research Plans for the Next 3 Years

The research team plans to conduct materials characterization and biocompatibility testing of the device and its components. They will perform a pilot study of human skin expansion in the prototype device. They will assemble study reports and information for submission to the Wake Forest IRB. They will hold a pre-IDE/IND meeting with the FDA and prepare an IDE/IND submission. They will construct a clinically applicable skin expander system. Finally, they will seek IRB and FDA approval for clinical trial and begin a Phase 1 clinical trial.

Planned Clinical Transitions

Because the skin expansion uses equipment without any cellular components, it is defined as a device and will be under regulation of the devices section of the FDA. The research team is working toward obtaining FDA approval for a prospective, multicenter, nonrandomized, uncontrolled pilot study (Feasibility/Phase 1). The researchers are currently seeking IRB approval. They intend to initiate the clinical study soon.

Engineered Skin Substitutes

Project 4.7.2, RCCC

Team Leaders: Steven Boyce, PhD (University of Cincinnati) and Richard Clark, MD (State University of New York, Stony Brook)

Project Team: Dorothy Supp, PhD (University of Cincinnati)

Therapy: Advanced therapy for extensive, deep burns

Deliverable: Autologous Engineered Skin Substitutes (ESSs)

TRL Progress: *Start of Year 1*, TRLs 3-4; *End of Year 1*, TRLs 3-4; *End of Year 2*, TRL 4

Key Accomplishments: The researchers calibrated human melanocytes (HMs) densities in co-cultures of HMs and human keratinocytes (HKs) for ESS using flow cytometry. They completed animal studies of HM transplantation with restoration of skin color. They developed

an ELISA for CD31 to track human dermal microvascular endothelial cells (HDMECs) in ESSs. They transplanted human fibroblasts (HFs)-HDMEC co-cultures to athymic mice. Finally, they used CAD-CAM engineering of microperforations in biopolymer substrates to develop vascular channels in ESSs.

Key Words: Burns, engineered skin, pigmentation, blood vessels

Introduction

Prompt and effective wound closure remains a rate-limiting factor in recovery from extensive, deep burn injuries. To address this limitation, ESSs have been developed and tested clinically as an adjunctive treatment to conventional skin grafting. Completed clinical studies show a reduction in the requirement for harvesting of autologous skin to complete wound closure. Technology for ESSs has been licensed to Lonza Walkersville, Inc. (LWI), which has initiated technology transfer and product development under cGMP standards. Successful completion of product development may allow reductions in morbidity and mortality for soldiers who are casualties of combat-related burn injuries.

To date, ESSs remain the only autologous skin device with dermal and epidermal substitutes that has been tested quantitatively in clinical trials for permanent closure of extensive, full-thickness burns. Several models of allogeneic skin substitutes (Apligraf[®], StrataGraft[®], OrCel[™], Transcyte[™]) are available commercially, but they are not permanent, and they function as biologic dressings. Acellular materials (Integra Dermal Regeneration Template, AlloDerm[®]) promote the ingrowth of autologous fibrovascular tissue but do not provide permanent wound closure with autologous epidermis. The only commercially available autologous skin cell product is

EpiCel[®] that consists of epidermal keratinocytes without a dermal substitute and is known to blister and ulcerate repeatedly that leads to chronic morbidity from open wounds, secondary healing, and long-term scar.

ESSs consist currently of cultured keratinocytes and fibroblasts attached biologically to a collagen-based matrix. ESSs have been shown to be efficacious in treatment of excised, full-thickness burns of greater than 50% of the total body surface area. This composite of cells and biopolymers is designated as a medical device by FDA. Although ESSs reduce requirements for harvesting of skin autograft, they have deficiencies, including but not limited to:

- Incomplete pigmentation that does not resolve with time
- Absence of a vascular plexus that limits the thickness of ESSs that will engraft predictably

This research report describes studies to address these biological deficiencies. The researchers are pursuing two aims focused on modifying ESSs for the treatment of extensive, deep burn injuries. Aim 1 evaluates restoration of pigmentation by addition of cultured HMs to the epidermal component of the device, and Aim 2 pursues the development of a vascular network in ESS by population of the dermal substitute with cultured HDMECs.



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Summary of Research Completed During Year 1

Advanced models of ESSs with pigmentation and vascular analogs were established during Year 1, and feasibility was demonstrated for advancement to animal studies in Year 2.

Research Progress – Year 2

Overall, progress with the preclinical studies has been steady and productive. Both prototypes of ESSs with either melanocytes (Figures V-28 and V-29) or endothelial cells (Figures V-30 and V-31) have been tested in animals with promising results. Particularly, the transplantation of melanocytes may be feasible for an initial clinical study by Year 5 of the first cycle of the AFIRM program. In addition, the current model of ESSs is proceeding to a clinical study in partnership with the licensee of the ESS technology, Cutanogen Corporation, a subsidiary of LWI.

Key Research Accomplishments

- Calibrated HM densities in co-cultures of HMs and HKs for ESSs using flow cytometry.

- Completed animal studies of HM transplantation with restoration of skin color.
- Developed an ELISA for CD31 to track HDMECs in ESSs.
- Transplanted HF-HDMEC co-cultures to athymic mice and qualitatively assessed perfusion by labeling with intravascular TITRC-lectin.
- Used CAD-CAM engineering of microperforations in biopolymer substrates to develop vascular channels in ESSs.

Conclusions

The investigators consider the progress with this project to be consistent with eventual translation to clinical trials of advanced models of ESSs with melanocytes and/or with endothelial cells. Skin color has been restored in an animal model of ESSs, and flow cytometry will be used to calibrate melanocyte density in project Year 3. Formation of vascular channels has progressed to animal studies, and new analytical methods have been established to track HDMEC densities in ESSs and to verify with labeled lectins the perfusion of human

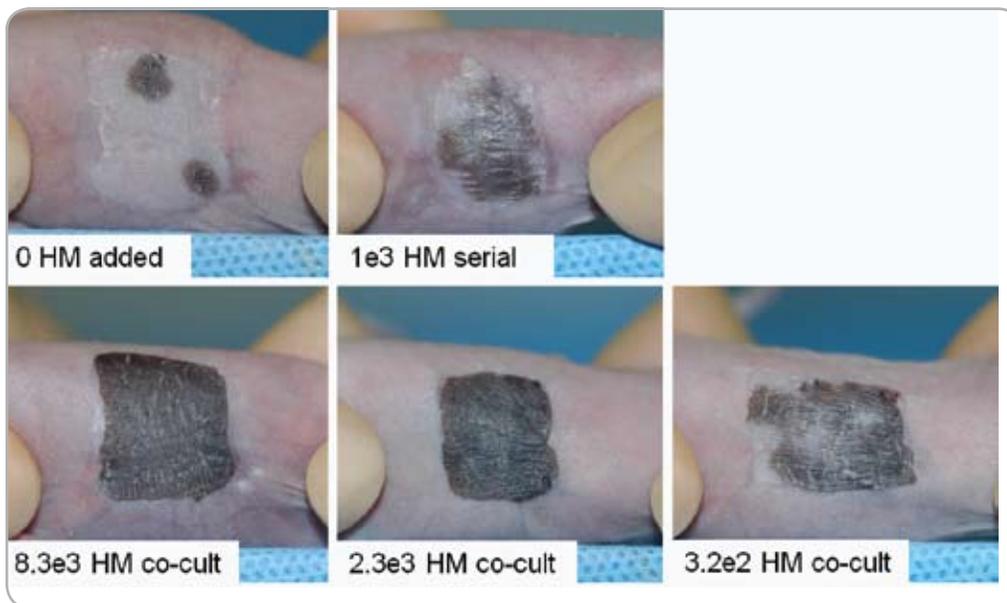


Figure V-28. Restoration of pigmentation in ESSs. Upper left, irregular pigmentation results if no HMs are added. Upper right, addition of HMs before HKs, and without co-culture results in partial pigmentation at a density of $1e3/cm^2$. Lower row, co-culture of HMs and HKs prior to inoculation increases pigmentation at 8 weeks after grafting and restores uniform skin color.

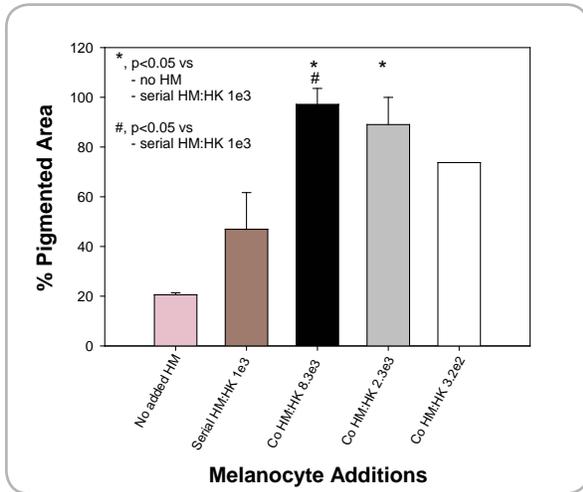


Figure V-29. Percentage pigmentation (mean ± SEM) in ESSs at 8 weeks after grafting to full-thickness wounds in athymic mice. Values are presented left to right. If no human melanocytes (HM) were added to ESSs, pigmentation was irregular and covered 20.6 ± 0.8% of the grafted area. Serial addition of 1e3 HM/cm² covered 46.9 ± 14.8%. Co-culture of HM with human epidermal keratinocytes and subsequent inoculation of 8.3e3, 2.3e3, or 3.2e2 HM/cm² onto ESS generated pigmented areas of 97.2 ± 6.4%, 89.0 ± 10.9%, and 73.7 ± 0.0% respectively. These results suggest that complete pigmentation of ESSs can be accomplished at a density of 1e3 HM/cm² that makes consistent repigmentation of healed ESSs a practical consideration.

lumens in grafted ESS in mice. Microperforation of biopolymer scaffolds has proven feasible to form vascular channels in ESS after population with fibroblasts, endothelial cells, and other cells as needed. Together, these results show promise of continued progress toward clinical studies with advanced models of ESSs. Concurrently, the licensee of the ESS technology is collaborating with the RCCC-AFIRM to initiate a limited clinical trial of autologous ESSs for treatment of soldiers with massive burns. This cycle of technology development and transfer enables delivery of an advanced therapy and new medical benefits to both military and civilian populations.

Research Plans for the Next 3 Years

The research team proposes to study novel prototypes of ESSs after transplantation in Year 3. Future plans remain focused on delivery of advanced therapies for life-threatening burns to the military and civilian populations. An initial model of engineered skin has been tested successfully in pediatric burn patients. This model has received support to perform a limited clinical study in burned military personnel admitted to the Burn Unit of USAISR. If successful, this model would be followed with advanced models of engineered skin that could match color and/or become vascularized more rapidly. If successful, later models could incorporate hair, glands, and nerve.

Planned Clinical Transitions

With an initial prototype of ESSs studied clinically for several years, the regulatory and logistical requirements for clinical trials of this therapy are well understood. A commercial developer, LWI, currently holds a license to the ESS technology. During project Year 2, the investigator collaborated in an application for support of initial clinical studies through the AFIRM. Preparations progressed including:

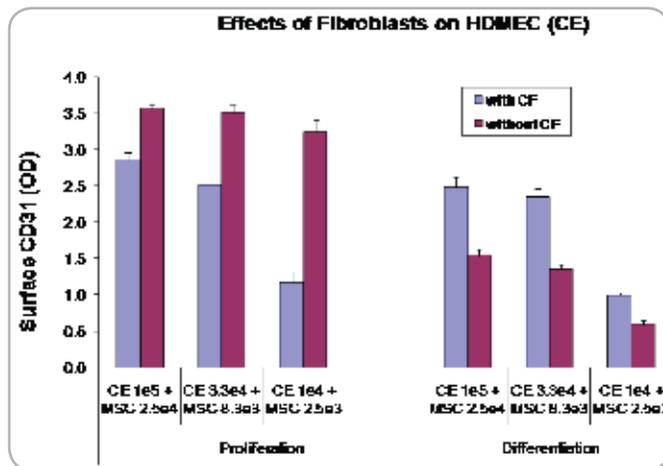


Figure V-30. Responses of HDMECs to proliferation or differentiation media, with or without human fibroblasts. HDMEC cultures with fibroblasts maintain CD31 expression (viability) independently of growth media.



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- Completion of protocols for cGMP-compliant manufacturing and characterization of the ESS device
- Preparation of regulatory submissions for FDA and study protocols for the IRB at USAISR and U.S. Army HRPO. These protocols will be designed for electronic data collection by the Clinical Trials Office of the AFIRM
- Recruitment of a CRO to staff and operate the clinical trial
- Orientation and training of USAISR surgeons and staff for enrollment and treatment of 10 burn patients at the USAISR Burn Center for the Phase 1 clinical study.

Additional funding from the DoD Office of Technology Transition is pending.

Corrections/Changes Planned for Year 3

A third aim has been added to explore addition of adipocyte stem cells to ESSs for possible development of a layer of subcutaneous fat. This study will be performed in collaboration with Adam Katz, MD, the principal investigator of AFIRM Project 4.7.1.

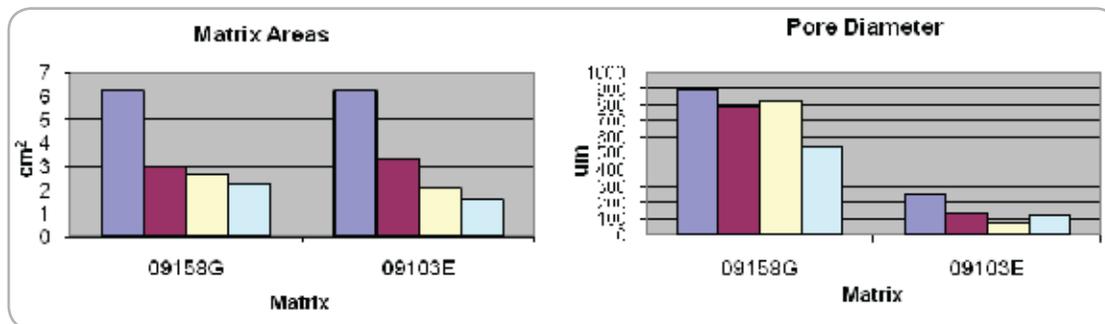


Figure V-31. Changes in matrix areas and pore diameters during fabrication of ESSs. Blue, before hydration; Red, after hydration; Yellow, 3 days after HF inoculation; Cyan, 4 days after HDMEC inoculation. (Left) Overall areas of matrices decreased by more than 50% during incubations. (Right) Pore diameters decreased from 900 μm to an average of $\sim 540 \mu\text{m}$, and from 250 μm to an average of $\sim 120 \mu\text{m}$. The last value begins to approach the diameters of arterioles and venuoles.

Adipose-Derived Stem Cells for Tissue-Engineered Dermal Equivalent

Project 4.6.8, USAISR

Team Leaders: Robert J. Christy, PhD and David G. Baer, PhD

Project Team: Postdoctoral: Shanmugasundaram Natesan, PhD

Technical: Nicole Wrice and Sharanda Hardy

Collaborators: Laura Suggs, PhD (University of Texas, Austin)

Therapy: Burn injury

Deliverable: Dermal equivalent using autologous stem cells

TRL Progress: *Start of Year 1, TRL 3; End of Year 1, TRL 3; End of Year 2, TRL 4*

Key Accomplishments: The researchers used a PEGylated-fibrin biomaterial and ASCs for use as

a strategy for revascularization of traumatized tissue. They characterized the ASC phenotype from various patients and initiated preclinical animal wound-healing studies.

Key Words: Human adipose-derived stem cells, burn therapy, revascularization

Introduction

Thermal injury accounts for approximately 5% of combat casualties, involving large TBSA and continues to be a significant source of morbidity. While a wide variety of dermal matrices has been developed, the lack of a host cell source and the time involved for cell expansion has limited their clinical application. The overall objective of this project is to develop a laminar skin substitute for military personnel using a dermal equivalent containing hydrogel-based biomatrices and ASCs eventually differentiating into both epidermal and vascularized dermal layers. The biomatrices will be engineered to control cell differentiation in the absence of growth factor supplementation of culture media. An epidermal cell sheet derived from ASCs will be layered over the dermal equivalent to get a laminar skin substitute.

The discovery of multipotent MSCs within the stromal fraction of adipose tissue prompted their use for the healing and reconstruction of many tissues. MSCs derived from adipose tissues differentiate into multiple phenotypes including adipose, muscle, bone, neuronal, endothelial, hepatocyte, and epithelial-like cells. ASCs are easily isolated from the stromal vasculature of subcutaneous adipose tissue by liposuction with a minimally invasive procedure and the excised adipose contains 100 to 1,000 times more pluripotent cells per

cubic centimeter than bone marrow. The wound-healing effects of ASCs were also verified with an *in vivo* animal study, demonstrating that ASCs significantly reduced wound size and accelerated re-epithelialization. This makes adipose tissue an attractive *in vivo* cellular source of autologous stem cells for regenerative therapies. The Christy lab has hypothesized that autologous ASCs can be used to produce a clinically relevant tissue-engineered skin equivalent. ASCs possess a heterogeneous cell population with the potential to differentiate into endothelial and epithelial cell lineages.

Research Progress

(Funded in 2009)

Vascular Differentiation of ASCs Directed by a PEGylated Fibrin Biomatrix

ASC have received attention because of their ability to differentiate toward connective tissue cell types. There is mounting evidence that ASCs have an unexplored plasticity toward vascular cell types, and it may be that this plasticity can only be fully unlocked by a structural ECM mimic. Previously, strategies for neovascularization of damaged tissue have focused on the delivery, either directly or through gene therapy of angiogenic agents to induce formation of vasculature from existing vessels. Unfortunately, these strategies have met with



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limited clinical success. We hypothesized that the correct three-dimensional matrix could direct ASC differentiation toward specific cell types. Using a PEG-based fibrin ECM mimic, we have developed a method to differentiate ASCs toward vascular cell types. We found ASCs seeded in PEGylated fibrin after 7 days showed distinct morphologies. Cells began to form vascular tube-like networks in the biomatrix in the absence of additional soluble cytokines (Figure V-32).

Cell viability and proliferation of ASCs that were embedded in the three-dimensional matrix were measured using assay (data not shown). The amount of vascularization was related to the initial cell number plating density (data not shown). The ASCs inside the matrices were not only viable but proliferated and formed microvessels. Expression levels of endothelial specific genes, von Willebrand factor (vWF) and CD31, and perivascular specific genes, NG2 and PDGFR β , significantly increased through the course of differentiation (Figure V-33).

Immunohistochemical analysis identified ASCs that had differentiated into vascular cells using endothelial and perivascular cell-specific markers (Figure V-34). This neovascularization to form capillaries is central to developing new therapies for wound healing and tissue engineering.

Layered Matrix and Cell Migration and Differentiation

We have combined ASCs loaded onto chitosan microspheres and sandwiched between the PEGylated fibrin gel and a type I collagen gel, to approximate both the dermal connective tissue as well as the vascular bed to nourish it. Cells that had been seeded onto chitosan microspheres were able to migrate through either PEGylated fibrin (Figure V-35 A-C) or collagen (Figure V-35 D-F). Migration was seen in both gels as early as Day 2 after seeding. Migration and/or proliferation continued throughout the times monitored (Day 8 for PEGylated fibrin and Day 12 for collagen). ASCs migrating into the PEGylated fibrin demonstrated the characteristic tubular morphology as seen in the gel matrix alone while ASCs migrating through

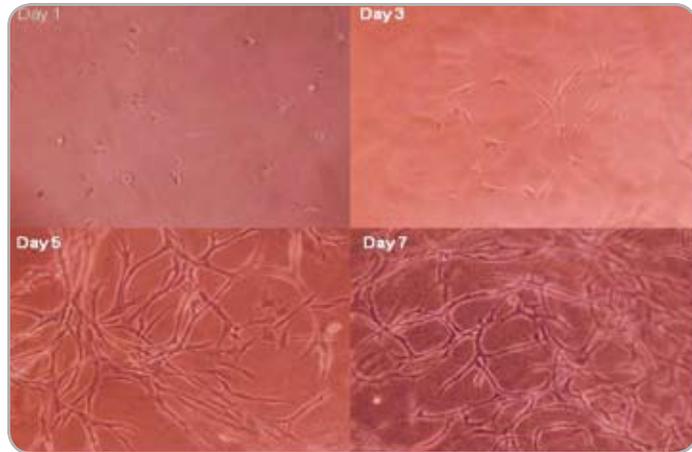


Figure V-32. Light microscopic images of differentiation time course of ASCs into vascular-like structures. Cells began to form vascular tube-like networks in the PEGylated fibrin gel in the absence of additional soluble cytokines.

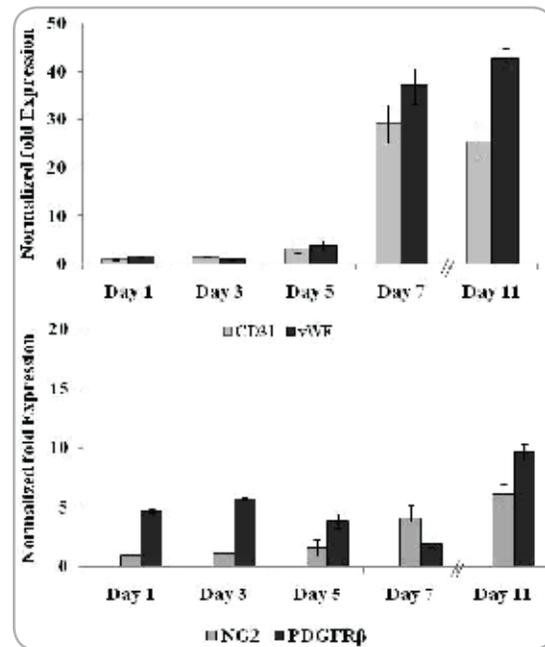


Figure V-33. Endothelial- and pericyte-specific markers expressed by the differentiated ASCs in PEGylated fibrin gels. Expression levels of endothelial cell-specific markers (CD31 and vWF) and pericyte-specific markers (NG2 and PDGFR β) were analyzed using real-time polymerase chain reaction (RT-PCR). There was significant increase in endothelial cell-specific markers; CD31 (25-fold) and vWF (42 fold), in comparison to pericyte markers, NG2 (6-fold) and PDGFR β (9-fold) by Day 11.

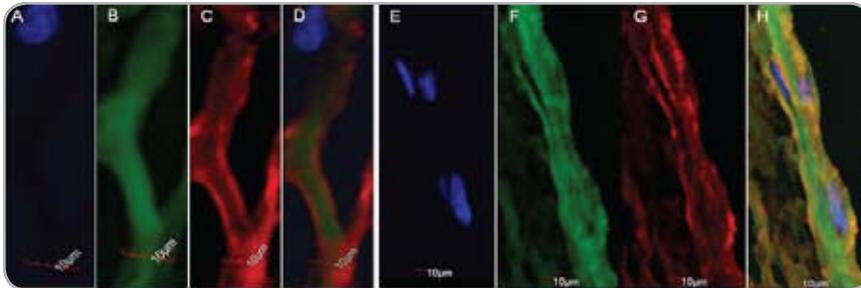


Figure V-34. Confocal Z-stacked images of tube-like structures formed by ASCs in PEGylated fibrin gel. ASCs when seeded in PEGylated fibrin exhibit an endothelial phenotype expressing both vWF (B) and CD31 (C). Figure V34-D shows the merged image of B and C stained with Hoeschst (A) for nuclei. The formed tubes were positive for both pericyte specific markers NG2 (G) and the endothelial cell-specific marker (F). Figure V34-H shows vWF, NG2 and Hoeschst (E) overlay.

Key Research Accomplishments

- Evaluated viability and confirmed multilineage differentiation potential of ASCs.
- Characterized the differentiation of ASCs in PEGylated fibrin hydrogels for viability and endothelial cell phenotype.
- Designed a bilayered dermal equivalent using ASCs loaded CSMs in collagen and PEGylated fibrin matrices.

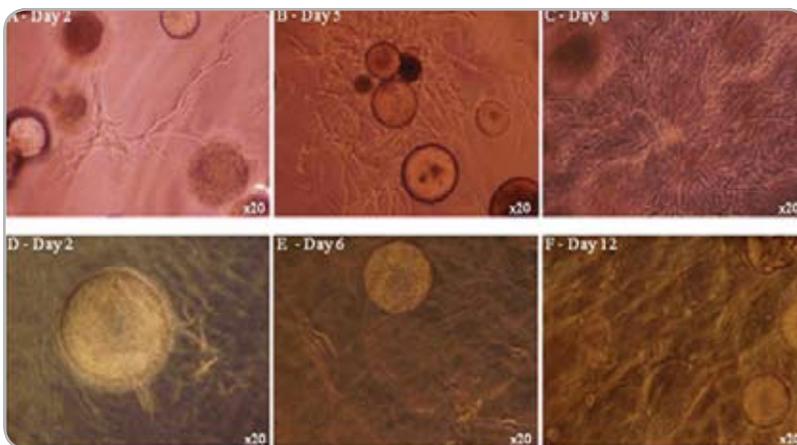


Figure V-35. ASCs released from chitosan microspheres (CSMs) *in vitro* in PEGylated fibrin and collagen gels. Phase contrast images of ASCs migrated from chitosan microspheres into collagen (A, B, and C) and PEGylated fibrin (D, E, and F). ASCs that have migrated from CSMs attached to the PEGylated fibrin show classical sprouting (A, Day 2) followed by differentiating into tube-like structures (B, Day 5). Over the time course of differentiation, they migrate into the gel forming a dense multicellular network (Day 8, C). ASCs released from the CSMs into collagen were more spindle in appearance (Day 2, D) which developed filopodias (Day 6 E). Over time they formed more elongated morphological structures stretching along fibril assemblies resembling cells that are associated with stromal tissues.

the collagen matrix had a spindle-shaped morphology. This experiment underscores the importance of the ECM in controlling the lineage specification and is not driven by soluble factors. Utilizing this insoluble bilayer matrix to direct ASC differentiation will allow for the development of both vasculature as well as dermal connective tissue from a single population of ASCs.

Conclusions

Advances in tissue engineering and stem cell research promise future treatments that may re-grow skin from patients themselves. Tissue engineering for a number of tissues is now entering clinical practice including skin. Tissue-engineering strategies for skin that are currently marketed in the United States suffer from very high costs, logistic difficulties in delivering a living product to patients, limitations in the timely application of equivalents, the potential for immunogenic response from non-self-donor cells and the potential for graft failure due to lack of robust blood vessel in growth. The proposed strategy relies on same-self stem cells that can be isolated at the point-of-care in a rapid fashion following injury. Furthermore, the use of a cell/matrix construct that can be assembled in the surgical suite at the time of injury provides a unique avenue for limiting infection risk. The high costs, logistic difficulties, and lack of timely application are eliminated by the use of this proposed dermal equivalent.



VI: Compartment Syndrome



BACKGROUND

In the arms and legs, thick layers of connective tissue called fascia surround groups of muscles, holding them in place and protecting them. Within these layers of tissue that do not readily expand are **confined spaces called compartments, which contain muscles, nerves, and blood vessels.** Compartment syndrome (CS) is a potentially serious medical condition in which **increased pressure or swelling within a compartment compromises the blood supply to the muscles located within that space.** CS can result from fractures, blunt and penetrating trauma, blast trauma, injury to blood vessels, and **the return of blood flow to a muscle after surgical intervention.** CS can also result from the use of a **combat tourniquet in the field.** The treatment of CS requires the surgical release of the fascia that

encloses the muscle compartment as soon as CS is diagnosed. **The fascia should be cut open (known as a fasciotomy) within the first 3 to 6 hours to prevent irreversible injury to the muscles, nerves, and blood vessels (vasculature).**

Soldiers that develop CS have prolonged recovery times and rarely recover complete muscle function, and they usually do not return to active duty at the same level of performance. Most CS injuries of the extremities result in permanent disability. A **safe and effective new therapy to replace and regenerate cells and tissues damaged by CS is urgently needed** as there are no effective treatments available for military surgeons to combat this important problem.

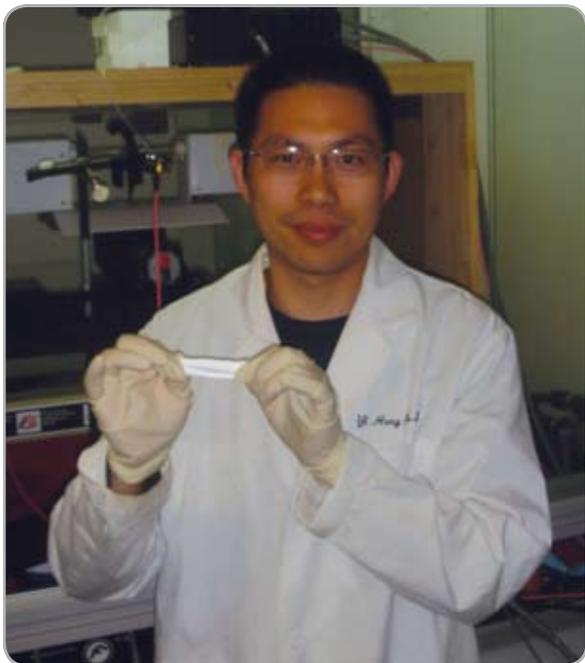


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The overall goal of the AFIRM CS Program is to reduce the impact of CS on wounded warriors and improve their functional recovery through the application of regenerative medicine. The projects seek to increase the salvage of injured limbs that have been affected by CS with an interdisciplinary approach based upon a combination of stem cells and inductive biodegradable scaffolds for the reconstruction of functional compartment tissues. The regenerative medicine technologies described herein have been used by AFIRM investigators and others for civilian tissue injuries safely and effectively and thus substantiate the rationale for using this approach to solve an important unmet need in the treatment of battlefield injuries.

Unmet Needs

While CS is a well-recognized cause of severe extremity injury that frequently results in chronic disability due to irreversible muscle and nerve damage, there has been almost no improvement in the treatment of this problem in over 100 years since the advent of surgical fasciotomy. One reason for the lack of progress is the fact that there have been no satisfactory surrogate animal models of



Yi Hong, PhD, with a synthesized material for treating CS (WFPC).

the syndrome in which to study new prospective treatments, despite decades of research attempts. AFIRM is supporting the successful creation of platform animal models of small and large wounds complicated by CS. These animal models have laid the foundation to test hypotheses that can advance regenerative medicine technologies designed to treat this important battlefield injury. Additionally, instrumentation has been developed within the program to quantitatively assess muscle and nerve regeneration in a precise, reproducible manner in these models.

Untreated CS can lead to Volkmann's contracture, which is a permanent shortening of musculature of the hand at the wrist and results in a claw-like deformity of the hand and fingers. Advanced CS can lead to permanent paralysis due to the failure of muscles and nerves in an affected compartment to recover. At that stage, amputation of the affected limb may be the patient's only remaining treatment option. Therefore, partial replacement of the dysfunctional tissue by living engineered muscle tissue is an attractive concept and an unmet need. AFIRM researchers are generating technologies that will provide an improved functional recovery for injured soldiers through the regeneration of muscle, nerve, and blood vessels lost to CS and other battlefield wounds.

Areas of Emphasis

AFIRM researchers are pursuing a complementary mix of research projects focused on various aspects of treatment of CS. Projects can be grouped into two "clinical challenge" topic areas: Cellular Therapy of CS and Biological Scaffold-Based Treatment of CS. Additional details on projects in each of these topic areas can be found in **Table VI-1** and subsequent sections of this chapter.

Cellular Therapy of Compartment Syndrome

Studies at Wake Forest-Pittsburgh Consortium (WFPC)

WFPC researchers are using human muscle-derived and bone marrow-derived stem and progenitor cells to reconstruct functional compartment tissues following the development of CS.

Table VI-1. Projects funded by WFPC and USAISR per clinical challenge topic area.

Clinical Challenge	Consortium/ Institution	Project Number	Project Title
Cellular Therapy of CS	WFPC	4.3.1	Cellular Therapy for the Treatment of the Consequences of Compartment Syndrome
		4.3.2	Use of Bone Marrow Derived Stem Cells for Compartment Syndrome
	USAISR	4.3.6	Regenerative Medicine Approaches to the Treatment of Ischemia/Reperfusion Injury in Skeletal Muscle
Biological Scaffold-Based Treatment of CS	WFPC	4.3.3	Biodegradable Elastomeric Scaffolds Microintegrated with Muscle-Derived Stem Cells for Fascial Reconstruction Following Fasciotomy
		4.3.4	Use of Autologous Inductive Biologic Scaffold Materials for Treatment of Compartment Syndrome
		4.3.5	Material-Induced Host Cell Recruitment for Muscle Regeneration

The **Huard/Soker group** (Project 4.3.1) at the McGowan Institute for Regenerative Medicine (MIRM) and the Wake Forest Institute of Regenerative Medicine (WFIRM) is developing a two-pronged effort to enhance regeneration through the use of autologous (a person's own) muscle-derived stem cells (MDSCs) in combination with inhibitors of the fibrotic process. The first therapeutic cell treatments are now under way after development of a small animal model of CS. The researchers found that MDSCs can be implanted and tracked in the damaged muscle area and can lead to a significant reduction in fibrosis in the injured area. In Year 3, the researchers will evaluate safety and efficacy of the MDSCs. The Huard/Soker group also determined that the U.S. Food and Drug Administration (FDA)-approved drug losartan (used for hypertension and to prevent pathologic scarring after a heart attack) reduces fibrosis in damaged muscle in humans. They plan to recruit muscle injury patients into a preclinical study using losartan (orally) to gather data that would be used to obtain funding for the use of the drug in a double-blinded clinical trial.

The **Gregory group** (Project 4.3.2) at the Oregon Medical Laser Center (OMLC) has developed a large animal CS model in Sinclair mini-swine that can be used to test whether the application of bone marrow progenitor or stem cells can enhance the healing and function of an injured limb. These cells are identical to the cells that have been used successfully to regenerate heart



Denethia Green preparing a sample for histological analysis on a compartment syndrome project with Dr. Sang-jin Lee (WFPC).

muscle after a heart attack. The Gregory group has also worked to adapt an automated bone marrow stem cell processing system for use as an inexpensive deployment method for stem cell treatments within a military hospital environment. After completion of their first large animal trial, the Gregory group will begin preparing for a human clinical trial.

Studies at the U.S. Army Institute for Surgical Research (USAISR)

Ischemic/reperfusion injury (I/R) can be caused by tourniquet application, vascular trauma, or acute CS. USAISR researchers are delivering autologous cells in an effort to improve muscle function following I/R.



VI: Compartment Syndrome

The **Walters/Christie/Rathbone group** (Project 4.3.6) at USAISR is developing cell-based regenerative medical approaches aimed at reducing the magnitude of injury, hastening healing, and improving the outcomes of wounded soldiers suffering from I/R-related muscle injuries. It is focusing on autologous cell sources and methods of delivery that it expects will lead to the most rapid transition into clinical practice. The researchers have generated dose/response curves for functional recovery following I/R. They also demonstrated improvements in muscle function following I/R through the administration of skeletal muscle progenitor cells (MPCs). They plan to conduct long-term experiments and monitor the time course of the recovery of muscle function *in vivo*. They will also attempt to deliver MPCs to humans via intravenous injection in future studies.

Biological Scaffold-Based Treatment of Compartment Syndrome

Studies at WFPC

WFPC researchers are developing animal models of CS and implantable scaffolds that can be used to treat this potentially devastating condition.

The **Wagner group** (Project 4.3.3) at the MIRM has been making substantial progress in developing biodegradable scaffolds with elastic properties that can be integrated with autologous MDSCs for the reconstruction of fascia (thick, fibrous tissue that encloses and protects the organs) after abdominal and other CS injuries. The Wagner group has now demonstrated the safety and efficacy of these materials in a small animal model of wound defects. It will be evaluating this promising technology in large animal, battlefield-relevant models in Year 3.

The **Badylak group** (Project 4.3.4) at the University of Pittsburgh has been developing strategies to improve the therapeutic potential of endogenous (i.e., originates from within the organism) biomaterial scaffolds. The use of endogenous materials reduces the adverse inflammatory reaction and enhances the therapeutic regenerative potential of the biomaterial, which may be used to replace and regenerate muscle and nerve tissue



Nicholas Amoroso, BSE, working on synthesizing material for treating CS (WFPC).

following CS injuries. The **Badylak group** has developed large animal models of CS injury, and it is evaluating several therapeutic strategies *in vivo*. Initial results have indicated improved muscle regeneration and function. Year 3 will be focused on preclinical trials of refined scaffold preparations.

The **Lee group** (Project 4.3.5) at Wake Forest University has been developing an approach to enhance the recruitment of endogenous stem and progenitor cells to the site of CS injury to increase the regenerative response. This group is using biomaterials containing myogenic (muscle cell)-inducing factors that can be implanted within the injured muscle compartment. It is now shown proof-of-principle *in vitro* and demonstrated that cells expressing muscle satellite/precursor cell markers were mobilized into implanted biomaterials containing myogenic-inducing factors. In Year 3, the researchers will see the development of refined myogenic-inducing factor evaluation and long-term effects on muscle regeneration in a small animal model of muscle injury.

Cellular Therapy for Treatment and Consequences of Compartment Syndrome

Project 4.3.1, WFPC

Team Leader(s): Johnny Huard, PhD MIRM, (University of Pittsburgh) and Shay Soker, PhD (WFIRM)

Project Team Members: Burhan Gharaibeh, PhD, Nick Oyster, Minakshi Poddar, Giovanna DiStefano, Michelle Witt (MIRM); Tracy Criswell, PhD, and Zhan Wang, PhD (WFIRM)

Collaborator(s): Dr. William Wagner and Dr. Stephen Badylak (MIRM)

Therapy: Cellular therapies for the treatment of CS

Deliverable(s): Method of muscle tissue regeneration through the delivery of muscle stem and progenitor cells together with angiogenic and antifibrosis factors

TRL Progress: *Start of Project*, TRL 1 (CS Model), TRL 2 (Co-cultured cells); *End of Year 1*, TRLs 3, 2; *End of Year 2*, TRLs 4, 2

Key Accomplishments: The researchers have developed a rat model of CS where muscle and other tissue injuries imitate the injuries observed in human CS biopsies. They found that

MDSCs can be implanted and tracked in the damaged muscle area and lead to a significant reduction in fibrosis in the injured area. In addition, the researchers have shown that endothelial cells co-cultured with myoblasts enhance muscle fiber formation and tissue growth on collagen-based scaffolds in vivo. Finally, they have shown that the angiotensin receptor II blocker losartan reduces fibrosis in damaged muscle in humans.

Keywords: compartment syndrome, muscle, rat model, losartan, endothelial cells, stem cells

Introduction

Musculoskeletal injuries strongly impact the Army in terms of human suffering, direct and indirect monetary costs, loss of time for work or training, and, perhaps most importantly, military readiness. These injuries alone account for a large number of disabled soldiers. According to the Medical Surveillance Monthly Report issued by the Department of Defense, musculoskeletal injuries were the leading cause of hospitalization of active duty members in 1993. In 1997 and 2001, these injuries again were highlighted on the list of frequent causes of service member hospitalization, ranking second and fifth overall in these years, respectively.

Among the musculoskeletal injuries that result from battlefield trauma, CS requires the most clinical intervention. CS occurs when the circulation within tissues in a closed space is compromised. As the pressure within an osseofascial compartment rises to a level that decreases the perfusion gradient across tissue capillary beds, cellular anoxia and muscle ischemia occur and these, ultimately, cause tissue necrosis. Necrosis leads to contracture, muscle weakness, sensory deficits, muscle

degeneration through rhabdomyolysis, renal failure, and even death. In fact, acute CS is a potentially devastating condition for soldiers. A variety of combat-related injuries including fractures, contusions, burns, trauma, post-ischemic swelling, gunshot wounds has been found to be the initiating factors for CS. Diagnosis is primarily made by clinical observations, including severe pain and lack of pulse in the limb, which are supplemented by compartment pressure measurements.

The specific aims of this project are to: (1) determine and compare the regenerative capacities of human myoendothelial and pericyte cells with that of human myoblasts after implantation in injured skeletal muscle after CS, (2) investigate the effect of angiogenesis on the regenerative capacity of human muscle-derived stem cells (hMDSCs) injected into the injured skeletal muscle after CS, and (3) develop biological approaches to prevent and eliminate scar tissue and improve muscle healing after CS injury.

Specifically, the researchers seek to: (1) create a CS model in rats that will mimic the human pathology and deliver muscle stem and progenitor cells, (2) deliver an-



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giogenic growth factors and endothelial progenitor cells combined with muscle stem and/or progenitor cells, and (3) deliver the nonpeptide angiotensin receptor antagonist losartan to CS patients.

Summary of Research Completed in Year 1

During the first year of the project, the researchers created a model of CS in a rat leg muscle using a combination of a tourniquet and an external compression device. They performed a microscopic evaluation of the tourniquet model using a variety of stains. They isolated, characterized, and banked human and rodent muscle-derived cell populations that will be used in stem cell repair strategies. They also examined muscle biopsies obtained from human CS patients using a variety of stains and a procedure that reveals differences in genes and found that the marker profile of several genes in human CS biopsies was appreciably different from that of human control biopsies.

Research Progress – Year 2

To better understand CS, the researchers have created two similar animal models of CS in rats. The first model (created during the first year of the study, as noted previously) involves the use of a tourniquet and an external compression device while the second model employs a modified blood pressure cuff on the hindlimb (details follow).

The histology of muscle biopsies obtained from human CS patients was examined to emulate the conditions in the animal models and better understand the histology and molecular profile of this syndrome in humans. The time at which the muscle is collected is important—later stages show severe muscle damage. The results observed in the two human tissue samples (less than 8 hours of CS and 10 days after injury) show the extent of the muscle damage that can occur in this time period. Molecular analysis revealed a great increase in different growth factors and cytokines. It is of interest to note that CD4, CD31, and CD34 all increased, showing that there may be an increase of vascularity in the injured area in an attempt to enhance healing. In addition, transforming

growth factor (TGF)- β 2 and myostatin (GDF8) were analyzed by western blot and found to increase dramatically in injured limb biopsies.

Tissue obtained from later time points would probably show an increase in TGF- β 1, an important growth factor in the stimulation of fibrosis. These results are very important, not only to establish an accurate animal model but also to determine the efficiency of biological approaches to improve tissue healing after CS occurs. Based on the data that the researchers have reported on murine models, they examined the effect of an orally active, nonpeptide angiotensin receptor antagonist (losartan) on two patients with injured hamstring muscles, and dramatic improvement in their muscle injury was observed. It is likely that with more patients and more data obtained from randomized and double-blinded experiments, it will be possible to gain additional insight into the mechanism of repair mediated by losartan and its effects on muscle injury. Data from these case studies will be used in therapy for CS patients.

The Soker laboratory has shown that fluorescently labeled co-cultured myoblasts, human umbilical vascular endothelial cells (HUVECs), and pericytes allow for visualization of tissue growth and differentiation. Co-culture of endothelial cells with myoblasts enhances myofiber formation and tissue growth on collagen-based scaffolds. Combination of endothelial cells and vascular endothelial growth factor together with muscle progenitors improves tissue viability and size. The researchers have created an animal model of CS in rats using a modified blood pressure cuff on the hindlimb that allows precise control of the amount of pressure received by each animal. An initial experiment showed that 120-140 mmHg of pressure for 3 hours allowed for the greatest representation of CS in the tibialis anterior (TA) within the minimal amount of time as determined by histological analysis. The damage induced in the TA has been correlated to quantitative data obtained from organ bath studies performed on extensor digitorum longus (EDL) muscles that were harvested simultaneously with the damaged TA muscles.

Finally, the ability to grow and visualize fluorescently labeled HUVECs, pericytes, and myoblasts in a co-culture system has been demonstrated. Additionally, the presence of HUVECs enhances the growth and differentiation of myoblasts grown on an acellular scaffold in vitro and in vivo. These experiments demonstrated that the use of fluorescently labeled cells is a viable method of following cell fate during neovascularization and muscle tissue regeneration in vitro, with the future purpose of using this method to follow regenerating cells in the CS model in vivo.

Key Research Accomplishments

- Developed a new animal model of CS in the rat using modified neonatal blood pressure cuffs that could maintain constant pressure at 120-140 mm Hg in the TA muscle.
- Determined that damage induced in the TA using the new CS rat model can be correlated to damage that can be quantitated in the EDL muscle using organ bath studies.
- Observed that direct injection of fluorescently labeled myoblasts into the TA can be detected upon gross examination of the muscle.
- Determined that endothelial cells co-cultured with myoblasts enhance muscle fiber formation and tissue growth on collagen-based scaffolds in vivo.
- Demonstrated that the angiotensin receptor II blocker losartan, which is approved for clinical use as antihypertensive drug, reduces fibrosis in damaged muscle in humans.

Conclusions

The researchers are making steady progress in developing a reliable model for understanding CS as well as cellular therapies to overcome its effects.

Research Plans for the Next 3 Years

Direct muscle physiological testing via contractile testing using in situ force testing by stabilizing the hindlimb will be performed. The optimum length for the development of maximum isometric tetanic force and a frequency

force curve will be developed for the muscle from records of the force exerted during periods of stimulation at increasing frequencies.

Indirect testing by computerized gait analysis using high-resolution video capturing (DigiGait™ system) of the animals' movements, including paw use and placement, is an integral aspect of the proposed analysis of the animals' motor function on the treadmill, and these studies will begin. All data will be analyzed within the Rodent Behavior Analysis Core (Director Floh Thiel, PhD) at the University of Pittsburgh.

Specifically, the researchers propose to:

- Optimize the injection conditions of murine MDSCs and test hMDSCs in athymic (nude) rats
- Analyze the beneficial effect of using losartan as an antifibrotic agent to reduce fibrosis in the animal model of CS
- Test the effect of stem cell therapy on improving angiogenesis and the recovery of CS-injured muscle
- Recruit more subjects for the case study of losartan
- Determine the progression of CS symptoms in the rat model of CS
- Deliver muscle progenitors cells to injured muscles of rats with CS symptoms
- Perform combined delivery of muscle and endothelial progenitors and angiogenic growth factors to injured muscles of rats with CS symptoms

Planned Clinical Transitions

The Huard/Soker group will seek more CS patients, study the molecular profile, and detail the pathology of their injury. Muscle injury patients will be recruited into a preclinical study using losartan (orally) to accumulate data that would be used to obtain funding for the use of the drug in a double-blinded clinical trial. Use of losartan could preclude a long and expensive drug development path and, because it is off-patent, it would be exceedingly inexpensive to deploy. Most general practitioners in the United States have extensive experience using this important medication. Losartan has been safely used for



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more than 20 years in millions of patients worldwide for other nontrauma conditions and could easily be adapted to CS trauma patients in a military hospital/rehabilitation environment. This drug can now potentially be used in AFIRM clinical trials of battlefield CS to prevent patho-

logic scarring, thus moving the Technology Readiness Level (TRL) from 2 to 5 if clinical funding can be identified. The group intends to seek an industry partner for a clinical trial on the use of losartan once all the data from Years 2 and 3 have been evaluated.

Use of Bone Marrow-Derived Cells for Compartment Syndrome

Project 4.3.2, WFPC

Team Leader(s): Kenton Gregory, MD (OMLC)

Project Team Members: Bo Zheng, MD, Michael Rutten, PhD, Jeff Teach, RN, Hua Xie, MD, PhD, Ping-Chen Wu, MS, and Rose Merten, BS (OMLC)

Collaborator(s): OMLC, CV-Path Institute Inc., USAISR, Special Operations Medical Command-Fort Bragg, Biosafe-America, Biologics Consulting Group, and Torston Tonn, MD (Johann Wolfgang University, Frankfurt, Germany)

Therapy: Autologous bone marrow stem cell treatments for CS

Deliverable(s): A large animal CS model to evaluate the ability of stem cell treatments to regenerate muscle and nerve in extremity wounds complicated by CS.

TRL Progress: *Start of Project*, TRL 2; *End of Year 1*, TRL 2; *End of Year 2*, TRL 3

Key Accomplishments: The researchers determined that Sinclair mini-swine can be used in a large animal model of CS to test whether the application of bone marrow progenitor cells can enhance the healing and function of an injured limb. They also developed a protocol for harvesting and

isolating porcine bone marrow cells using an automatic cell processing machine with a sterile, closed flow path. Additionally, they created and modified cell invasion assays as well as flow cytometry protocols to help assess the function, viability, and identification of cells used in the bone marrow treatment.

Keywords: Extremity CS, autologous bone marrow-derived stem cells, porcine model, TA, automated bone marrow stem cell separator, gait analysis

Introduction

Tissue wounds to the extremities are among the most common battlefield injuries sustained by soldiers during Operation Iraqi Freedom and Operation Enduring Freedom. A particularly common trauma caused by improvised explosive devices is a blast injury to the extremities resulting in CS. Unless adequately treated, the related swelling and increased pressure within tissue compartments that occurs in CS can quickly lead to permanent muscle, nerve, and vascular cell death. Soldiers developing CS also have prolonged recovery times and rarely recover complete function. A central question for complete CS injury recovery is how to regenerate lost muscle mass.

Clinical trials of autologous bone marrow-derived stem cell therapy to treat extremity injury, as well as acute myocardial infarction, continue to show safety and efficacy. This research program is uniquely suited to using bone marrow as a safe and cost-effective regenerative medicine approach for extremity injury. This group

continues to refine procedures and protocols using the SEPAX automated sterile processing device to isolate bone marrow progenitor cells. In combination with the development of a large animal model to study CS, the researchers are now determining parameters that are critical to a successful regenerative strategy, including the time of bone marrow cell harvest, administration, and the number of needed treatments. Research in earlier phases of the preclinical program had shown that 1 week following CS injury there is significant amplification of stem cell niches within the bone marrow. There is also a local increase of stem cell homing signals within the injured tissue. Based on these results, the researchers hypothesize that the harvesting of bone marrow stem cells 1 week post-injury will result in a therapeutically efficacious stem and progenitor cell product for the treatment of CS.

The goal of this project is to improve the endogenous cellular regeneration response through the use of autologous bone marrow stem cell therapy. The bone marrow treatment should shift the balance from cellular atrophy



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and formation of fibrosis to the generation of physiologically active cells resulting in improved muscle function. This approach to accelerating healing and regenerating tissue lost to battlefield blast and other extremity trauma offers a unique, safe, practical, and significant opportunity to improve functional recovery for the injured soldier.

The specific aims of the project are to (1) optimize cell separation flow paths and protocols for an automated bone marrow processing device and (2) develop an animal model of CS.

Summary of Research Completed in Year 1

During the first year of the project, the researchers developed a large animal (porcine) model of CS. They also developed an automated bone marrow stem cell harvesting and isolation system with associated protocols. They developed a cell invasion assay protocol for adult porcine bone marrow in response to the homing factor stromal cell-derived factor-1. They developed a cell colony forming unit assay protocol for adult porcine bone marrow to examine the effects of stem cell colony formation in response to bone marrow cells loaded with and without the cells trackers Dil or quantum dots. They completed a comparative flow cytometry analysis of bone marrow stem cells in uninjured and CS-injured pigs. Their preliminary results suggested that certain identifiable cell phenotypes (i.e., CD90, CD56, CD144, CD105, and VEGFR2) were upregulated in response to trauma at different time points.

Research Progress – Year 2

Progress Related to Aim 1

The researchers' previous methods of purifying bone marrow relied on manual density gradient procedures that can sometimes produce variable outcomes as well as cell preparations that can be contaminated with red blood cells (RBCs), platelets, and granulocytes. Further, the manual density gradient procedure is highly labor intensive and is limited to processing small volumes of bone marrow. An important advancement in this group's bone marrow processing method was the procurement of a SEPAX device (Biosafe-America, Houston, Texas)—

an automatic cell-processing machine that has a sterile, closed flow path. The SEPAX device uses a new computerized density gradient protocol for the separation of bone marrow mononuclear cells from contaminating RBCs and granulocytes. The ease of deploying a small footprint, wall plug compatible, and inexpensive device to process autologous bone marrow at a tertiary military hospital is relevant to the application of our treatment strategy in a military medical environment.

Over the past year, the Gregory group developed a close relationship with the engineers at Biosafe-America to refine the device's flow path and processing software to adapt the system for porcine bone marrow cell isolation. They also developed modifications to a three-color fluorescent probe assay for measuring apoptotic, necrotic, and live cells. This assay provides a convenient method for quantifying apoptotic cells (green), necrotic cells (red), and healthy cells (blue) within the same cell population by confocal microscopy. As shown in **Figure VI-1**, under the same experimental conditions, the researchers found the three-color apoptosis assay to be more sensitive at detecting injured (i.e., apoptotic or necrotic) bone marrow cells compared to the fluorescence Live-Dead assay.

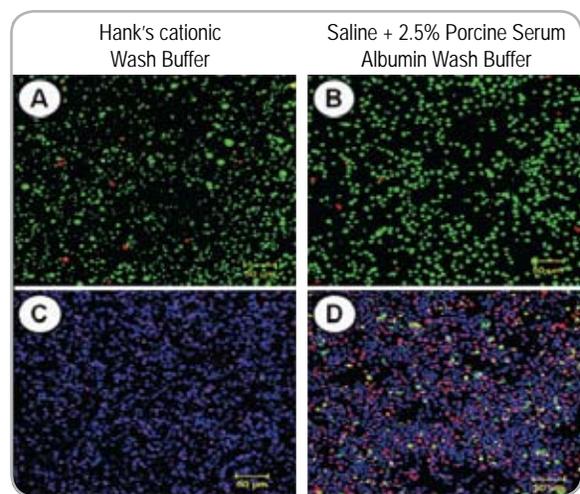


Figure VI-1. Representative confocal photographs of SEPAX processed porcine bone marrow comparing two different wash buffers and analyzed for viability using the Live-Dead (A,B) or apoptosis (C,D) assays. In (D), note the greater sensitivity of the apoptosis assay for apoptotic (green), necrotic (red), and live (blue cells) detecting dead and necrotic cells.

The level to which the SEPAX device is able to purify bone marrow cells may be a critical factor in the treatment's success. It was found that the SEPAX machine could eliminate ~99.5% of the RBCs from processed bone marrow. Monitoring the SEPAX processing results for RBC depletion will continue to be important in the use of processed bone marrow cells for the treatment of CS. This will be useful to determine if any positive or negative outcome from bone marrow treatment of the injury is due to the treatment itself and not due to RBC contamination.

Progress Related to Aim 2

To replicate a common battlefield skeletal muscle injury that could serve as a model for treatment with autologous stem cells, the researchers initially developed a porcine extremity-injury CS model using domestic juvenile swine. The domestic swine model showed promising preliminary results in being able to track treatment-related cell injections. However, it also unveiled potential limitations as a long-term, injury-healing model. The juvenile age of domestic swine and their exponential growth rate posed issues both for their housing as well as for drawing a realistic comparison of muscle regeneration to that in injured adult soldiers.

As an alternative to juvenile domestic swine, the researchers examined the possibility of using sheep for the model of CS. There are multiple advantages to using sheep including their slow growth rate that in turn allows studies using adult stem cells in adult recipients. Also, sheep have a thicker, multilayered fascia overlaying their TA muscle, which compared to the swine's thinner, single-layered fascia, facilitates its ability to maintain fluid within the compartment. Great effort was expended over the last year to switch to a sheep model, which included training staff in proper handling and surgical procedures. However, it was determined that the sheep model was not adequate, a major factor being the tendency of the sheep to die unexpectedly during routine procedures.

The researchers are now focusing on the use of Sinclair miniature swine for their CS model. Sinclair mini-swine were originally developed for research purposes at the University of Minnesota in 1949. Sinclair mini-swine has the advantage over other mini-swine (such as Yucatan)

in that their leg structure is similar to that of a domestic swine (i.e., it is not shortened in length). Also, the Sinclair does not develop a potbelly to the degree that the Yucatan does, thus avoiding complications to gait assessment due to the animal's abdomen dragging on the ground. The Sinclair has an overall slower growth rate compared to domestic swine, an advantageous characteristic that allows the performance of long-term studies within the weight restrictions of the researchers' animal care facility.

The researchers conducted a feasibility study to test their new functional systems, which evaluate muscle damage and regeneration. They were able to also develop and audit the protocols needed before beginning the main studies. Animal gait was measured with the use of a pressure mat system that captures multiple sequential foot strikes of the animal for analysis of foot function and gait. This system records force, pressure, timing, center of force, and area of contact. The system is also capable of capturing video and synchronizing it with the pressure mat data to quantify the degree of foot drop. Muscle function was measured through the use of a biomechanical muscle tester specially designed to accommodate large animals to measure the biomechanical behavior of the animal's hindlimbs. This biomechanical tester measures isometric forces. Motor nerve conduction and dorsiflexion force measurements for the control animal demonstrate a decrease in muscle and nerve functionality as shown in **Figure VI-2**. Gait analysis was completed prior to injury and showed symmetry between right and left sides. Analysis of gait symmetry for the control animal post injury showed that the uninjured right rear leg was favored over the injured left rear leg. The injury did not affect the symmetry of the front limbs.

The researchers are currently conducting feasibility studies with two Sinclair mini-swine to determine protocols for new function testing equipment including the Torque Muscle Function Tester and Gait Analysis System, as well as characterization of Sinclair Bone Marrow Cell Profiles.

Key Research Accomplishments

- Determined the species (i.e., Sinclair mini-swine) to use in a large animal model of CS.



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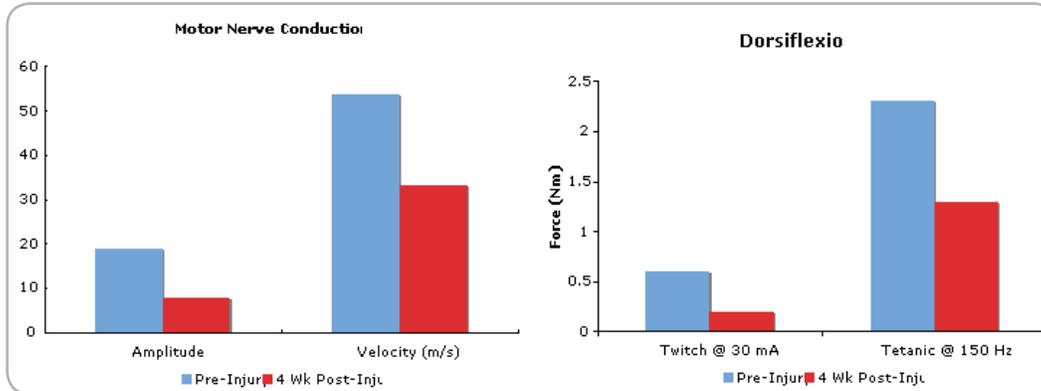


Figure VI-2. Muscle function of control animal's TA pre-injury and 4 weeks post-injury.

- Refined the SEPAX device's flow path and processing software to adapt the system for the isolation of porcine bone marrow stem cells.
 - Determined that the SEPAX machine could eliminate ~99.5% of the RBCs from processed bone marrow.
- Modified a three-color fluorescent probe assay for measuring apoptotic, necrotic, and live cells to serve as a convenient method of quantifying these types of cells within the same cell population by confocal microscopy.

Conclusions

The Gregory group continues to make substantial progress toward the development of a large animal model of CS that can be used to test whether the application of bone marrow progenitor cells can enhance the healing and function of an injured limb. This approach to accelerating the healing and regeneration of tissue lost to battlefield blast and other extremity trauma offers a unique, safe, and practical opportunity to improve functional recovery for the injured soldier.

Research Plans for the Next 3 Years

In their large animal model of CS, the Gregory group will begin to evaluate multiple treatment time points up to 28 days post injury and will compare these data to untreated controls at time points up to 3 months post-extremity CS injury. A minimum threshold of 50%

improvement in functional recovery (muscle strength, gait improvement, nerve conduction velocity) without adverse clinical events is required for the preclinical milestone deliverable and movement forward toward clinical trials. After completion of the first large animal trial, the Gregory group will begin preparing for a human clinical trial as described in the following.

Planned Clinical Transitions

Upon the completion and successful results of the Multi-Dose Treatment Study, the Gregory group will prepare an Investigational New Drug (IND) application for the FDA and begin a Phase 1 human clinical trial during Year 5. They will perform a cytotoxicity study in rodents aimed at evaluating the toxicology, biodistribution, tumorigenicity, and microbiological effects of their treatment. This will be done simultaneously with the IND preparation. The rodent study will be paid for by non-AFIRM funds. The Gregory group will also begin to work closely with their FDA consultant to make a smooth transition from preclinical animal trials into a Phase 1 human trial. The trial will include 20 patients and will be performed in conjunction with Brooke Army Medical Center.

Corrections/Changes Planned for Year 3

Preclinical trials have been moved from Year 2 to Years 3 and 4. Years 4 and 5 will now focus on preparing and starting the Phase 1 human clinical trial.

Regenerative Medicine Approaches to the Treatment of Ischemia/Reperfusion Injury in Skeletal Muscle

Project 4.3.6, USAISR

Team Leaders: Thomas J. Walters, PhD, Robert Christy, PhD, and Christopher Rathbone

Project Team: X. Kelly Chen, PhD, Janet Roe, BS, LTG, and Melissa Sanchez, BS

Collaborators: Roger Farrar, PhD (University of Texas, Austin)

Therapy: Cell-based therapy for CS

Deliverable: Improved functioning of skeletal muscle following ischemia/reperfusion injury

TRL Progress: *Start of Project*, TRL 3; *End of Year 1*, TRL 3; *End of Year 2*, TRL 4

Key Accomplishments: The researchers developed an in vivo system for assessing muscle function in rats. They determined dose/response

curves for functional recovery (ischemic time/muscle function) following I/R. In addition, they demonstrated improvements in muscle function following I/R through the administration of skeletal MPCs.

Key Words: Adipose-derived stem cells, bone marrow-derived stem cells, muscle precursor cells, skeletal muscle, ischemia reperfusion injury

Introduction

Extremity trauma historically constitutes more than 70% of battlefield injuries. As with previous wars, extremity trauma constitutes the majority of all injuries in Iraq and Afghanistan. As of July 19, 2010, more than 17,700 soldiers had suffered combat-related injuries that were too severe to allow them to be returned to active duty. Each of these soldiers averaged 2.3 extremity injuries. Extremity trauma is the source of the greatest health care burden for the current wars, consuming 65% of the health care dollars spent on wounded warriors. By definition, all extremity injuries involve muscle trauma. Muscle trauma can take a number of forms including blunt trauma, crush, penetrating, and I/R injury. I/R can be caused by vascular trauma, tourniquet application, or acute CS. The rate of extremity vascular injury is now three to five times that reported from previous wars, dramatically impacting the rate of I/R. CS has been specifically acknowledged as a major source of morbidity in the current war.

Recently, there has been encouraging research aimed at accelerating and improving muscle healing following sports-related types of injuries using therapies based on the use of mesenchymal stem cells (MSCs). MSC-based therapies offer an attractive treatment possibility

for skeletal muscle I/R. Another potential therapeutic cell source is skeletal MPCs. These cells can be obtained from muscle biopsies and have recently been shown to be present in debrided muscle tissue in war-traumatized muscle. Transplantation of MPCs has been shown to improve muscle function in animal models of muscular diseases, denervation, toxins, cryo injuries, and volumetric muscle loss and have been used to treat Duchenne muscular dystrophy and cardiovascular diseases in clinical trials. Advantages of MPCs as an autologous cell source for transplantation include their abundance in skeletal muscles, high proliferative potential under culture conditions, commitment to myogenic lineage (eliminating the risk of tumorigenicity), and high resistance to ischemia. In addition, recent observations suggest that MPCs may be beneficial for muscle repair based on their ability to support angiogenesis and neurogenesis. Despite the promising beneficial effects of MPCs in muscle repair in other animal models and in in vitro studies, their ability to restore muscle function following I/R is untested.

The researchers of this project seek to develop cell-based regenerative medical approaches to reduce the magnitude of the injury, hasten healing, and improve outcomes of wounded soldiers suffering from I/R-related muscle injuries. They are focusing on autologous cell



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sources and methods of delivery that they expect will lead to the most rapid transition into clinical practice.

Research Progress

(Funded in 2009)

The researchers previously established a rat model of tourniquet-induced I/R and tested early treatments for I/R using their model. However, these studies focused primarily on early time points (days versus months). Testing any cell-based therapy designed to hasten and improve restoration of muscle function requires (1) actual measurement of muscle function (versus histological or immunohistological correlates); (2) long-term studies with multiple time points; and (3) an injury that is too severe to recover spontaneously, yet not so severe that it is beyond treatment.

Long-term studies are labor intensive and require large numbers of animals. The researchers developed an in vivo method for determining muscle function that allows repeated measurements in the same animal, which dramatically reduced animal numbers and labor. After initial development, they used their method to examine the relationship between ischemic time and muscle function (**Figure VI-3**). The results of this study demonstrated that 2 hours of ischemia does not result in a permanent

loss of function and is therefore not severe enough to satisfy the established standards. In contrast, 3 hours of ischemia resulted in an injury severity too great to heal completely in the 8-week period of the study. Based on these results, at least 3 hours of ischemia is required for screening of cell-based therapies designed to improve functional outcomes.

Stem cell-based therapy using MPCs was investigated by transplanting approximately 10^6 cells into the TA muscle 2 days following 3 hours of ischemia. Muscle function was then determined in situ 14 days later (**Figure VI-4**). At this time point, MPCs significantly improved specific force (N/cm²); indicating that MPC transplantation improved the quality of the muscle (**Figure VI-4E**). This provides exciting evidence that MPCs may provide a beneficial therapy although confirmation of whether this translates to a better final outcome remains to be confirmed by ongoing long-term studies.

Key Research Accomplishments

- Developed an in vivo system for assessing muscle function in rats.
- Determined dose/response curves for functional recovery (ischemic time/muscle function) following I/R.

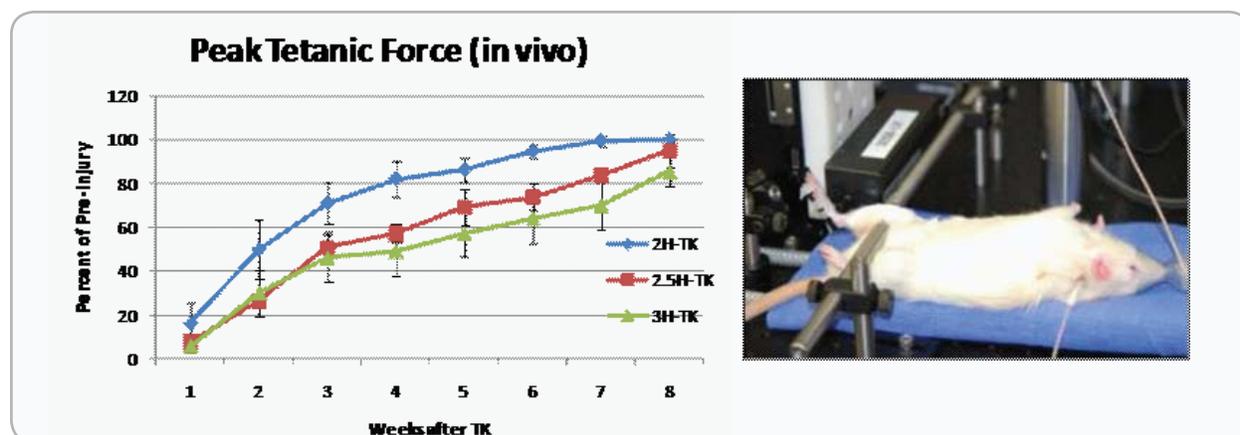


Figure VI-3. A graph of the relationship between ischemic time and muscle function (left) The values are expressed relative to pre-injury values. The setup for the experiment is shown (right). Animals first underwent surgical implantation of a nerve-cuff electrode. The leads were externalized with a plug between the shoulders. Animals were allowed 1 month to recover before baseline measurements were made. Muscle function was assessed by fixing the leg in a custom jig and fixing the paw of the affected limb to a footplate secured to dual-mode galvanometer (force transducer). The muscle was activated with the implanted electrode. Control and data acquisition were performed using custom LabView™ developed in our lab. Animals were anesthetized with isoflurane during the procedure.

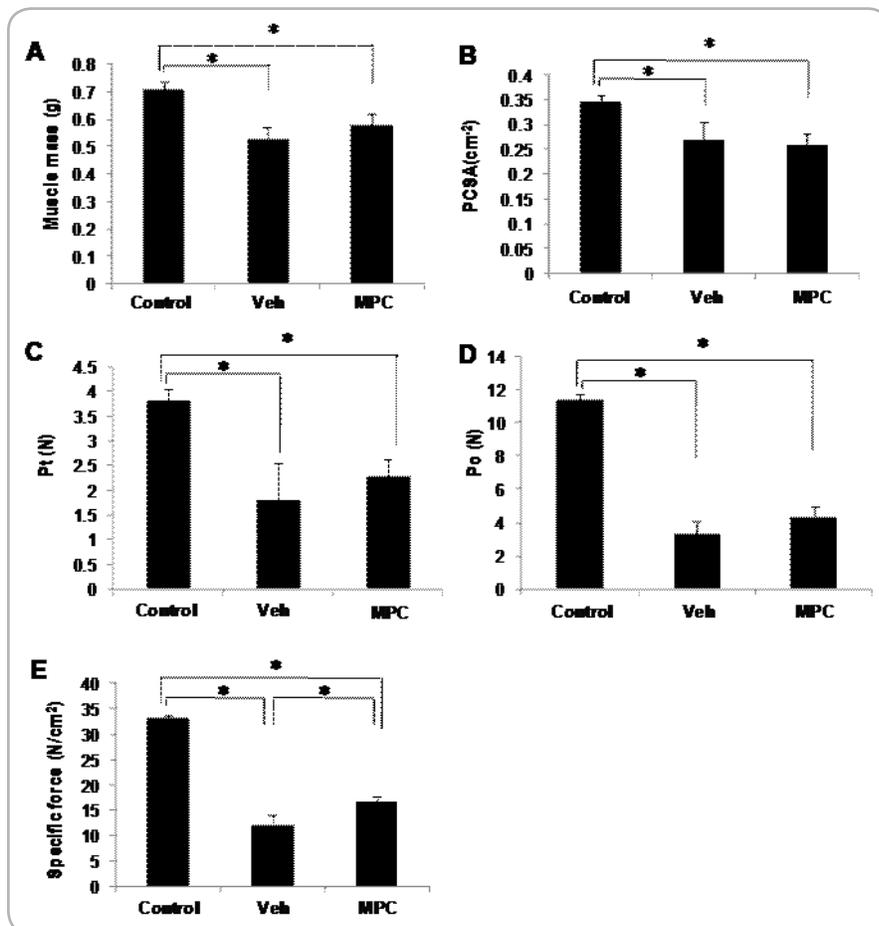


Figure VI-4. Muscle mass (A), physiological cross-sectional area (B), maximum twitch force (Pt) (C), maximum tetanic force (Po) (D), and muscle specific force (E) at 2 weeks after tourniquet application. Values are expressed as mean \pm SEM. *denotes significance at $p < 0.05$.

- Demonstrated that MPCs improve muscle function following I/R in the short-term.

Conclusions

The results of the completed experiments are the first to demonstrate the benefits of cell-based therapy following I/R. In these studies, cells were transplanted via direct injection. The preliminary findings show that the early

treatment of I/R with direct injection of MPCs can improve muscle function in the short term. While this has been the standard experimental method for treating muscle injury and diseases, the practicality of scaling up this method to soldiers suffering from trauma involving a large muscle mass is questionable. A more practical approach is cell delivery via intravenous injection.

Research Plans for the Next 3 Years

To demonstrate that the treatments with MPCs also improve the magnitude of muscle healing, long-term experiments must be performed. The researchers will conduct long-term experiments and monitor the time course of the recovery of muscle function in vivo.

The mechanism for the ability of MPCs to hasten the short-term return of muscle function is unknown. The researchers will investigate whether these cells undergo engraftment and contribute to the cell population of the host muscle or whether their benefit is via paracrine influences.

Finally, the researchers will attempt to deliver MPCs to humans via intravenous injection in future studies.



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Progress Reports: Biological Scaffold-Based Treatment of CS

Biodegradable Elastomeric Scaffolds Microintegrated with Muscle-Derived Stem Cells for Fascial Reconstruction Following Fasciotomy

Project 4.3.3, WFPC

Team Leader(s): William R. Wagner, PhD (MIRM)

Project Team Members: Ryotaro Hashizume, MD, Kazuro L. Fujimoto, MD, PhD, Yi Hong, PhD, and Nicholas J. Amoroso, BSE (MIRM)

Collaborator(s): Stephen Badylak, DVM, MD, PhD and Johnny Huard, PhD (MIRM)

Therapy: Treatment of abdominal compartment syndrome (ACS); development of fascial repair technology

Deliverable(s): An engineered tissue based on an elastic biodegradable

synthetic material, electrospun poly(ester urethane) urea (ePEUU) that aids in reconstruction of the abdominal wall in CS

TRL Progress: *Start of Project*, TRL 2; *End of Year 1*, TRL 2; *End of Year 2*, TRL 3

Key Accomplishments: The researchers developed a new biomaterials technology that may be applicable to fascial reconstruction in a variety of settings. Their in vivo testing compared: (1) expanded polytetrafluoroethylene (ePTFE) as a control, (2) dry ePEUU, (3) a new wet

electrospinning technique where serum-based medium was electrospayed concurrently with electrospinning of PEUU (**wet ePEUU**), and (4) ePEUU blended with digested porcine dermal extracellular matrix (ECM), in a full thickness abdominal defect rat model. Results from this in vivo study showed **wet ePEUU** has suitable characteristics for further investigation given the mechanical properties observed, which mimic native abdominal wall tissue.

Keywords: Abdominal compartment syndrome, biodegradable elastomer, abdominal defect, extracellular matrix, mechanical property, rat model

Introduction

ACS represents the pathophysiologic consequence of a raised intra-abdominal pressure, which causes progressive hypoperfusion and ischemia of the intestines and other peritoneal and retroperitoneal structures. Damage control laparotomy is performed for repair of the primary injury and hemorrhage control. In many cases, massive edema of the bowel, caused by primary injury and/or operative procedures, precludes the primary closure of the abdominal wall fascia, which might lead to secondary ACS. Open abdomen management has been adopted in the initial stage of treatment. After successful avoidance of ACS, however, the large abdominal wall defect needs to be reconstructed. In that circumstance, when autogenous tissue reconstruction is planned, mobilization of the components of the abdominal wall is difficult, leading to repairs under tension and an increased incidence of hernia formation over time.

Many techniques are advocated for the repair of these defects. Some of the most commonly applied approaches utilize prosthetic materials. The most obvious advantages with the use of prosthetic materials are their ready availabilities and fairly simple techniques of implantation. Disadvantages with prosthetic materials include the risks of intestinal fistula formation, prosthetic infection, adhesions, and recurrent hernias. Excellent clinical and animal results with biodegradable material derived from animal ECM have been obtained with these materials in a variety of placements. The disadvantage of these materials is that the mechanical properties, particularly elasticity, and sometimes tensile strength, are limited.

Given these observations, the Wagner group hypothesized that application of an engineered tissue based upon an elastic biodegradable synthetic material, ePEUU, would result in improved outcomes in the reconstruction of the abdominal wall and other sites of fascia reconstruction. The elastic and biodegradable

properties of this material may facilitate the generation of a mechanically appropriate tissue both in the early and late stages of healing for an extensive abdominal wall defect. The group is investigating approaches where scaffolds are implanted that will encourage cell migration and remodeling of the wall tissue, as well as approaches where cell-seeded scaffolds are implanted for wall regeneration. The former have been the focus of the past year of research.

Summary of Research Completed in Year 1

During the first year of the project, the Wagner group created biodegradable, elastic scaffolds for reconstruction of the abdominal wall after the development of ACS. They integrated their scaffolds with ECM from the dermal layer of the skin as well as MDSCs to aid in abdominal wall regeneration. They produced abdominal wall patch materials, a series of novel dermal ECM digests and ePEUU blends, tissue constructs combining MDSCs and ePEUU, and an abdominal wall defect model in the rat for the in vivo assessment of the biodegradable scaffolds.

Research Progress – Year 2

Methods

PEUU was synthesized from polycaprolactone diol ($M_n=2000$), 1,4-diisocyanatobutane, and putrescine according to previously described methods. For the current study, a wet ePEUU was fabricated by a combination of electrospinning and electrospaying. Cell culture medium (DMEM with 10% fetal bovine serum, 10% horse serum, and 1% penicillin/ streptomycin) was fed by a syringe pump at 0.2 mL/min into a sterilized capillary (1.2 mm inner diameter) charged at 7 kV and suspended 4 cm above the target mandrel (6 mm diameter). Concurrently, PEUU in hexafluoroisopropanol solution (12%, w/v) was fed at 1.5 mL/h from a capillary, charged at 12 kV and perpendicularly located 20 cm from the target mandrel. The mandrel was charged at -4 kV and rotated at 250 rpm while translating 8 cm along the x-axis at 0.15 cm/s. As a control, a dry ePEUU sheet was prepared using only electrospinning (without media electrospaying) using the same parameters described previously.

A defect (1x2.5 cm) involving all of the layers of the abdomen was created in the abdominal wall of Lewis rats. Subsequently, the created defect was repaired by one of three types of patches, each 400 μ m thick. For each group, the implanted samples were retrieved at 4 and 8 weeks post-operation ($n=7$ per group per time point).

Many native tissues of the body possess a high degree of mechanical anisotropy in their response to loads encountered throughout their everyday function. Ideally, constructs designed for the repair and replacement of such tissues should possess similar mechanical properties, not only to better replicate native tissue, but also to avoid a marked mismatch in behavior at the anastomoses. For this reason, the functional efficacy of the candidate abdominal wall reconstructive patches were evaluated using a biaxial mechanical testing device designed to apply simultaneous load in two axes. Biaxial mechanical property measurements were performed for retrieved samples at each time point (4 and 8 weeks).

Results

A full thickness rat abdominal wall defect was implanted with (1) ePTFE, (2) dry ePEUU, (3) a scaffold created using a wet electrospinning technique where serum-based medium was electrospayed concurrently with electrospinning of PEUU (wet ePEUU), and (4) dermal ECM-blended ePEUU. Histological assessment showed markedly improved infiltration and remodeling for wet ePEUU scaffolds (**Figure VI-5**). Biaxial mechanical assessment at 8 weeks revealed ePTFE-patched walls to be isotropic with low compliance while wet ePEUU-patched walls were anisotropic with compliance curves similar to native tissue (low tension at small stretch, rapidly increasing tension at high stretch). Dermal ECM blending with ePEUU improved ECM mechanical properties and resulted in thicker constructs in vivo although cell infiltration was not markedly different from ePEUU. Very little cell ingrowth was observed in ePTFE while wet ePEUU degradation was accompanied by extensive alpha-smooth muscle actin positive ingrowth and collagenous fibers.

Key Research Accomplishments

- Completed an in vivo study of abdominal wall replacement using a small animal (rat) model with 3 groups: animals receiving an ePTFE patch, a dry ePEUU patch, or a wet ePEUU patch.
- Measured biaxial mechanical properties for pre-implant ePTFE, dry ePEUU, and wet ePEUU as well as 4- and 8-week explanted patches of ePTFE, dry ePEUU, and wet ePEUU from the rat model.
- Data showed similarities to the native abdominal wall tissue for wet ePEUU, unlike the stiffer ePTFE control material.

Conclusions

The use of a microfibrinous, wet-processed ePEUU material for abdominal wall reconstruction resulted in more physiologic mechanical behavior with cellular remodeling, possibly leading toward a functional autologous replacement. This result is encouraging not just for ACS, but also for other fascial wall reconstruction efforts. In addition, this new biomaterial approach may find use

in other applications (e.g., blood vessel generation and other soft tissue wall repair).

Research Plans for the Next 3 Years

In the coming year, the Wagner group will continue to broaden these in vivo studies in terms of the materials implanted (e.g., PEUU processed in alternative manners with ECM gel) and implant periods (4, 8, and 12 weeks) and assessments, including the histological biocompatibility, adverse events, intestinal fistula, prosthetic infection, and recurrent hernias for each of the implanted constructs. The group will also further evaluate the cell microintegrated scaffolds both in terms of in vitro characterization and in the small animal model. They plan to use green fluorescent protein transgenic MDSCs to allow cell tracking in the in vivo studies with the microintegrated scaffolds.

With optimization of scaffold design ongoing in Years 2 and 3, the Wagner group plans to initiate large animal trials with the porcine model later in Year 3 using

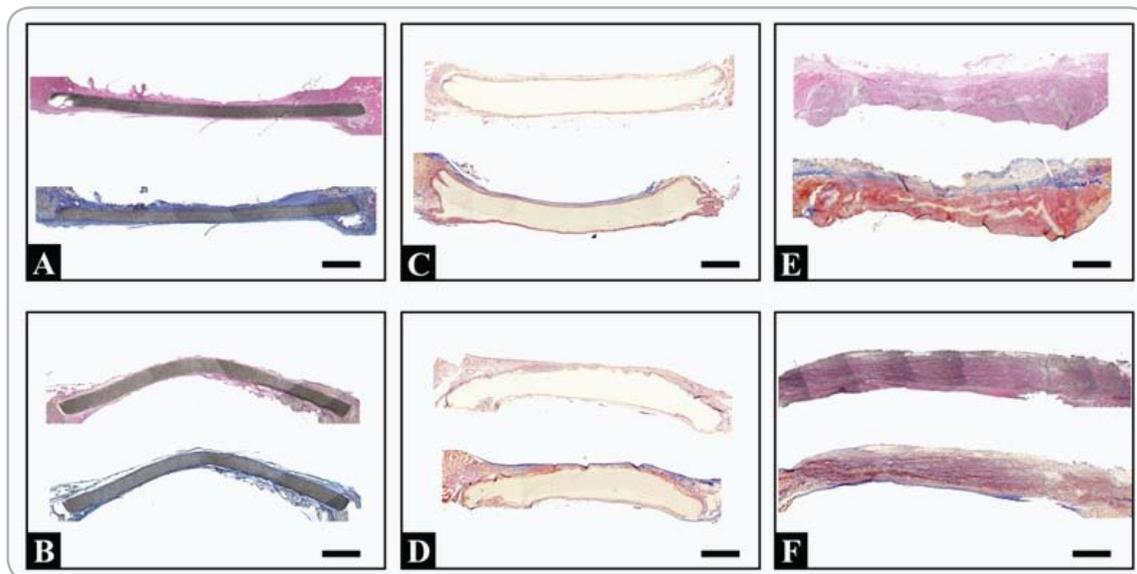


Figure VI-5. Representative cross-sections of implanted ePTFE (A and B), dry ePEUU (C and D), and wet ePEUU (E and F). The upper row is from 4-week explants (A, C, and E) and the lower row from 8-week explants. In each box, staining for the upper image is hematoxylin and eosin (H&E), and Masson-trichrome for lower image (scale bar: 1 mm).

acellular constructs. Complexity will be built into the porcine model (in terms of construct design, evaluation methods, and implant time) in Years 4 and 5. In Year 5, the researchers anticipate being in communication with the FDA regarding clinical trials. The developed materials will be considered for other applications as the technology matures. Potential applications include skin, craniofacial, and soft tissue reconstruction. When the large animal model is achieved, it is anticipated that this technology may potentially be evaluated in concert with approaches developed in other AFIRM CS projects.

Planned Clinical Transitions

The Wagner group expects to be exploring partnering opportunities with industry for the use of the developed materials in the coming years. Upon successful completion of Aim 2, the FDA will be engaged in discussions to determine the preclinical data that would be required to justify filing for an investigational device exemption. This work would occur in Years 6 and 7. Once this milestone is met, clinical trials can commence, potentially in Year 7.



VI: Compartment Syndrome

Progress Reports: Biological Scaffold-Based Treatment of CS

Use of Autologous Inductive Biologic Scaffold Materials for Treatment of Compartment Syndrome

Project 4.3.4, WFPC

Team Leader(s): Stephen F. Badylak, DVM, PhD, MD (University of Pittsburgh)

Project Team Members: Kerry Daly, BScV, BVSc, PhD, Matt Wolf, BS, and Scott Johnson, BS, MS (University of Pittsburgh)

Collaborator(s): None

Therapy: Treatment for peripheral CS

Deliverable(s): (1) Optimization of skeletal muscle ECM and characterization of its properties.
(2) Reconstruction of functional

compartmental muscular tissue in animal models utilizing the inductive properties of biologic scaffolds and stem cells

TRL Progress: *Start of Project*, TRL 2; *End of Year 1*, TRL 2; *End of Year 2*, TRL 3

Key Accomplishments: Using techniques developed during the first year of the project, the researchers optimized the standard operating procedure for preparing skeletal

muscle ECM. They also characterized the skeletal muscle ECM properties considered to be important for biocompatibility in vitro and regeneration of functional skeletal muscle tissue in vivo. They are conducting preclinical studies to evaluate the suitability of currently available biologic scaffolds and stem cells for treatment in animal models.

Keywords: Compartment syndrome, biologic scaffolds, extracellular matrix

Introduction

Peripheral CS represents a serious complication of traumatic extremity injury, especially the type of trauma sustained by soldiers in combat. The fundamental problem is thought to be the severe swelling that occurs within a confined space (compartment), typically in the lower limb. The swelling and associated increased intracompartmental pressure severely compromise blood flow resulting in ischemic necrosis of all tissues within the compartment (e.g., muscle, nerves, and associated structures). The loss of functional tissue is frequently severe enough to require amputation of the affected limb. The standard of care for peripheral CS is fasciotomy with an attempt to salvage the viability of as much functional tissue as possible. Morbidity is high and includes severe aesthetic abnormalities (because of lost compartmental space).

The Badylak group is investigating a method for using the inductive properties of ECM as a scaffold for the recruitment of endogenous stem cells and the attachment, proliferation, and spatial organization of these cells into functional tissue. Previous work has shown that

manufactured forms of ECM (e.g., porcine small intestinal submucosa [SIS], porcine urinary bladder, porcine and bovine dermis and pericardium) have the potential to promote constructive remodeling of damaged or missing body parts in place of inflammation and scarring. The present work extends this concept by investigating methods for use of ECM scaffolds (with and without the presence of stem cells) in conjunction with traditional treatment methods to better facilitate regeneration of affected skeletal muscle compartments. In addition, they are developing methods of in situ decellularization of the necrotic tissue while retaining the native ECM (autologous ECM). Stated differently, the ECM within the compartment would be isolated from its original cell population (which has now become necrotic), and this matrix would then be used as a template for tissue reconstruction.

The specific aims of this project are to (1) optimize skeletal muscle compartment ECM and characterize its properties in vitro and (2) reconstruct functional compartmental tissue in animal models using the inductive properties of biologic scaffolds and stem cells (either derived from bone marrow or human muscle).

Summary of Research Completed in Year 1

During the first year of the project, the Badylak group established a reproducible model of peripheral CS in two species (rabbit and dog). This model has now become the basis for current preclinical animal studies evaluating the ability of ECM scaffolds to facilitate functional muscle tissue regeneration when compared to traditional treatment modalities. The researchers established the biocompatibility of exogenous ECM for supporting the growth of human microvascular endothelial cells (HMECs), 3T3 fibroblasts, and perivascular stem cells. Previous work also included the development of methods for the complete decellularization of the anterior tibial compartment in situ.

Research Progress – Year 2

Four methods of decellularization of skeletal muscle were developed in the laboratory, which differ in detergents, proteases, and concentrations used. All four methods resulted in scaffolds that contained no visible nuclear material histologically and only small amounts of low molecular weight DNA (**Figure VI-6a**), which may be important in facilitating constructive remodeling. Multiple cell types proliferated on this skeletal muscle ECM in vitro, suggesting its biocompatibility (Figure VI-6b) while preliminary studies using a rodent partial abdominal wall defect have demonstrated that skeletal muscle ECM scaffolds induced a mononuclear cell infiltrate and an angiogenic response after 14 days post implantation (Figure VI-6c).

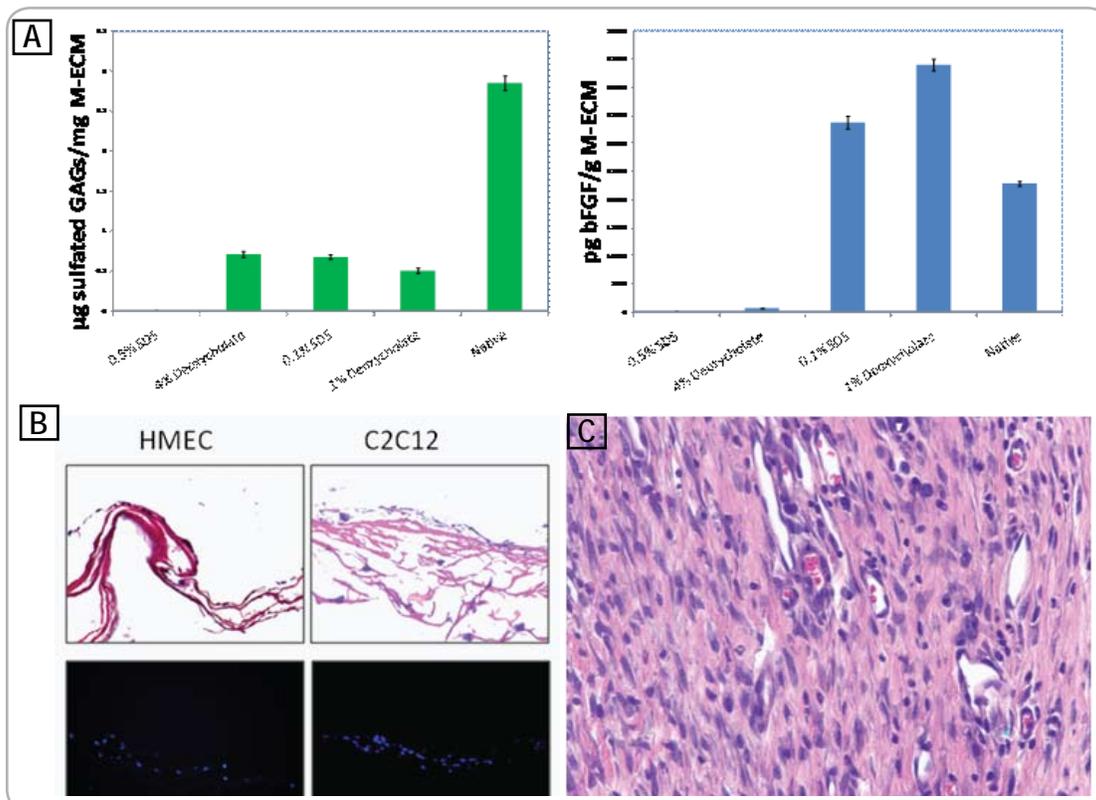


Figure VI-6. a. GAGs and the basic fibroblast growth factor (bFGF) are retained in muscle ECM scaffolds. b. H&E (top) and DAPI (bottom) of HMEC endothelial cell and C2C12 myoblast cell seeded scaffolds. c. H&E image of muscle ECM scaffold using 1% deoxycholate after 14 days of implantation, 400X. There is a mononuclear cell infiltrate and blood vessels (arrows) in the implant area.

VI: Compartment Syndrome

Progress Reports: Biological Scaffold-Based Treatment of CS

The Badylak group also prepared a model of peripheral CS in the anterior tibial compartment in the rabbit. Saline infusion with intermittent crushing increased mean intra-compartmental pressures and induced rhabdomyolysis, significant hypocalcemia, and increased serum CPK levels immediately post induction, which is consistent with the clinical syndrome. The ability of SIS ECM to facilitate constructive remodeling of anterior tibial compartment is being investigated in this model. Powdered and sheet SIS was used to replace musculoskeletal defects created by a fasciotomy in the rabbit model. Rabbits treated with SIS implants returned to normal gait by 3 weeks, but this was 4 weeks in fasciotomy controls.

By 1-month post treatment, rabbits receiving only a fasciotomy had deposition of fibrous tissue within the defect (**Figure VI-7**). In comparison, the SIS implant was still visible and infiltrated with inflammatory cells in rabbits treated with implants (**Figure VI-7**). By 3 months, there was adipose and fibrous tissue in place of muscle in fasciotomy rabbits, but mononuclear cell pressure and angiogenesis were present in animals treated with SIS implants (**Figure VI-7**). Longer time points are required to fully evaluate the potential of SIS to remodel function musculotendinous tissue in this peripheral CS model.

Key Research Accomplishments

- Optimized decellularization protocols for preparing skeletal muscle ECM.
- Evaluated the bioactive properties of skeletal muscle ECM with regard to in vitro cell viability, growth factors, and GAGs.
- Further characterized a peripheral CS model with respect to the clinical parameters identified in human patients.
- Using their model of CS in the rabbit, established clear differences in outcome between “fasciotomy only” animals and those treated with SIS at both 1 and 3 months after treatment.

Conclusions

Skeletal muscle ECM manufacturing methods have been developed and the resulting ECM demonstrates biocompatibility in vitro and contains growth factors and GAGs that suggest in vivo suitability. The new model of peripheral CS shares many clinical features with that seen in human patients. Fasciotomy treatment in conjunction with an exogenous ECM (SIS) implant shows promise for regeneration of functional skeletal muscle.

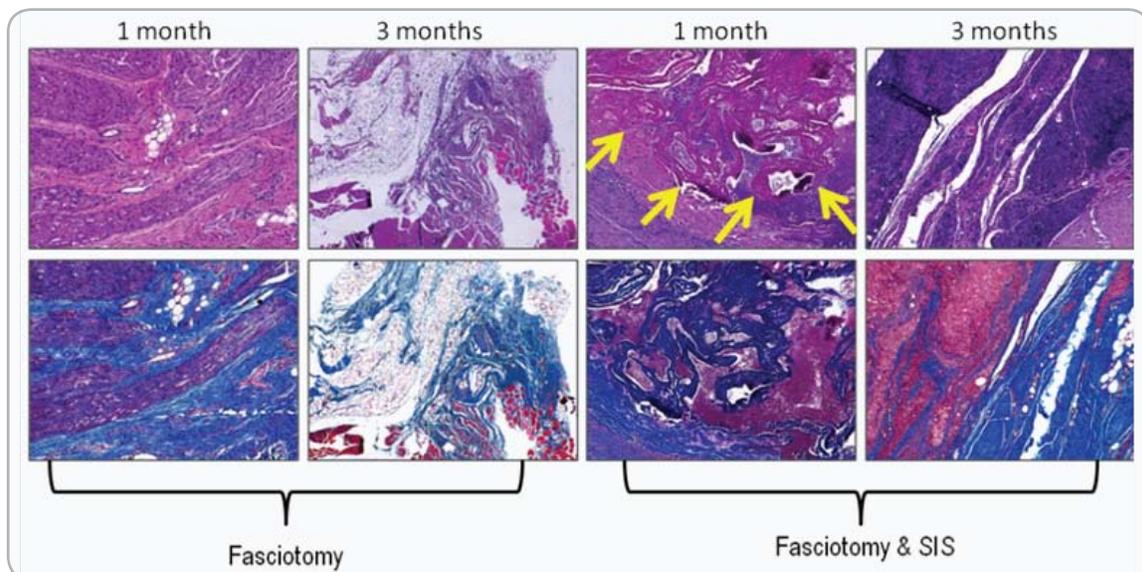


Figure VI-7. H&E (top) and Masson's trichrome (bottom) images of anterior tibial compartment after 1 and 3 months. Fibrosis is seen at 1 month, and fibrosis and adipose tissue at 3 months in fasciotomy-treated animals. The SIS implant is still visible (arrows) at 1 month in the implant group, and active inflammation and remodeling are still undergoing at 3 months.

Research Plan for the Next 3 Years

Year 3 work will continue to evaluate the exogenous ECM (SIS) and begin investigation of the suitability of autologous ECM and skeletal muscle ECM in animal models. The study time will be extended to 6 months in rabbits so that the host functional and morphologic healing response can be fully examined. In addition, characterization of the cellular infiltration of the debrided area and structures associated with healing and regeneration (macrophages, myocytes, blood vessels, and nerves) will begin.

Planned Clinical Transitions

There are few options for the treatment of advanced peripheral CS. Complete loss of compartmental functional

musculature results in the need for prosthetic devices in most cases. Alternative approaches to reconstructing individual components of the compartment such as muscle, blood vessels, and nerves are in progress, but to current knowledge, there is no work currently being done to reconstruct the complex architecture of the innervated, vascularized skeletal muscle architecture of the compartment. Many approaches are investigating the use of stem cells, deposited at the site of damaged tissue, for their ability to reconstitute functional tissue, but this approach would combine such cells with the ECM bioscaffold to optimize outcome. If our large animal preclinical studies in Year 3 show positive results, a human clinical trial will be prepared for Year 4.



VI: Compartment Syndrome

Progress Reports: Biological Scaffold-Based Treatment of CS

Material-Induced Host Cell Recruitment for Muscle Regeneration

Project 4.3.5, WFPC

Team Leader(s): Sang Jin Lee, PhD (Wake Forest University)

Project Team Members: James J. Yoo, PhD, MD, Benjamin Harrison, PhD, Young Min Ju, PhD, Chang Mo Hwang, PhD, and In Kap Ko, PhD (Wake Forest University)

Collaborator(s): Shay Soker, PhD (Wake Forest University)

Therapy: Treatment of CS through in situ muscle tissue regeneration

Deliverable(s): Demonstration of in situ muscle tissue regeneration using a target-specific scaffolding system

TRL Progress: *Start of Project*, TRL 2; *End of Year 1*, TRL 2; *End of Year 2*, TRL 3

Key Accomplishments: In the past year, the Lee laboratory has demonstrated that host muscle satellite/progenitor cells can be mobilized into implanted biomaterials

in situ and that these cells can be differentiated into myogenic lineage using myogenic-inducing factors in vitro. In addition, scaffolds that incorporate myogenic-inducing factors for in vivo demonstration of host muscle satellite/progenitor cell differentiation have been developed.

Keywords: Muscle satellite/progenitor cells, cell recruitment, biomaterial, myogenic-inducing factor, compartment syndrome, muscle regeneration

Introduction

CS is a common traumatic injury that results in muscle, nerve, and vessel damage due to increased pressure within a confined space in the body. Although CS can affect any limb or muscle compartment, it frequently occurs after trauma to the lower leg such as fracture. The standard treatment is fasciotomy, which is considered as the definitive and only treatment for acute CS. Although this procedure is able to relieve immediate concerns, muscle weakness and atrophy are a continued sequel. Various management approaches have been introduced, including physical therapy, muscle transplantation, and myoblast cell therapy. However, none has entirely addressed the problems associated with the long-term consequences of the CS in wounded soldiers.

In this project, the researchers aim to use stem or progenitor cells residing in the host to regenerate muscle tissue through the use of a target-specific scaffolding system. This approach is based on the demonstration that almost every tissue in the body contains some type of stem or progenitor cell. The specific aims of this project are to (1) investigate this possibility using an animal model to initiate stem/progenitor cell mobilization, recruitment, and differentiation in vivo and (2) demonstrate the

in situ muscle tissue regeneration using a target-specific scaffolding system.

Summary of Research Completed in Year 1

During the first year of the project, the Lee group demonstrated that host stem/progenitor cells can be mobilized and recruited into the implanted biomaterials. They also demonstrated that these stem/progenitor cells were capable of differentiating into multi-lineage cells (e.g., osteogenic, adipogenic, myogenic, and endothelial cells). When these biomaterials were implanted in the muscle region, host muscle stem/progenitor cells that express PAX3, PAX7, and myoD were localized within the scaffolds.

Research Progress – Year 2

During the past year, the researchers focused on developing myogenic inducing factor-incorporated biomaterials that would promote host muscle satellite/progenitor cell migration, proliferation, and differentiation. To evaluate the effectiveness of myogenic-inducing factors such as hepatocyte growth factor, SDF-1 α , bFGF, fibroblast growth factor-6, epidermal growth factor, insulin-like growth factor-I, and insulin-like growth factor-II, two

types of skeletal muscle cells, consisting of (1) MPCs isolated from human skeletal muscle tissue and (2) C2C12 muscle satellite cells, were tested for in vitro cell proliferation, migration, and differentiation assays and in vivo host muscle satellite/progenitor cell recruitment.

The researchers' results indicate that the introduction of myogenic-inducing factors into scaffolds enhances host muscle satellite/progenitor cell migration into the implanted biomaterial (**Figure VI-8**). Ongoing investigations include the use of functional biomaterials containing several myogenic-inducing factors for in situ muscle tissue regeneration. Further investigations into the levels of host stem cell recruitment, proliferation, and differentiation will be conducted.

Key Research Accomplishments

- Demonstrated host muscle satellite/progenitor cell mobilization into biomaterials.
- Characterized infiltrating host cells within biomaterials.
- Completed an in vitro evaluation of muscle satellite/progenitor cell migration, proliferation, and differentiation.
- Fabricated a reliable biomaterial system (prototype).
- Incorporated myogenic-inducing factors within the biomaterial system (ongoing).
- Conducted an in vivo demonstration of host muscle satellite/progenitor cell infiltration into myogenic-inducing factor-incorporated scaffolds (ongoing).

Conclusions

The Lee group demonstrated that myogenic-inducing factors effectively promoted myogenic cell migration, proliferation, and differentiation in vitro. In addition, they showed that cells expressing muscle satellite/precursor cell markers were mobilized into implanted biomaterials containing myogenic-inducing factors. They believe that these cells would be capable of differentiating into muscle cells for in situ muscle tissue regeneration. Therefore, it may be possible to enrich the infiltrate with specific

cell types and control their fate, provided the proper substrate-mediated signaling can be imparted into the scaffold for in situ regeneration of functional muscle tissue through host cell recruitment.

Research Plans for the Next 3 Years

The researchers plan to continue optimization and evaluation of the myogenic inducing factor-incorporated biomaterials in vitro and in vivo. They also plan to demonstrate the applicability of the myogenic-inducing factor-incorporated biomaterials for in situ muscle tissue regeneration. In addition, they will conduct long-term in vivo studies investigating in situ muscle tissue regeneration.

Planned Clinical Transitions

This basic research project is not slated for clinical trials during the first 5 years of the award.

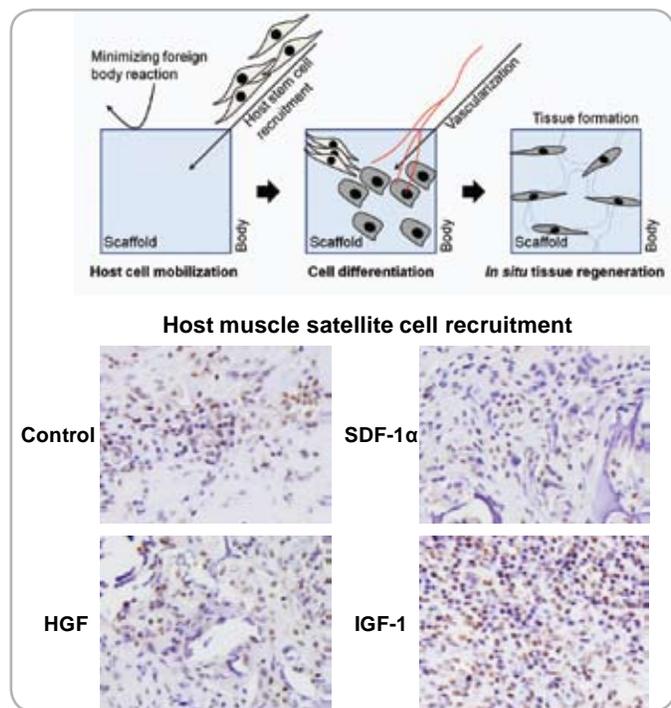


Figure VI-8. Material-induced host cell recruitment for muscle regeneration.



VII: AFIRM Statistics



INTRODUCTION

As indicated throughout this annual report, the AFIRM is composed of a host of researchers across many research institutions. While the previous chapters demonstrate the depth of their research projects, the breadth of the program also can be seen from a global perspective, viewing the research consortium as a whole, rather than as many individual components. This chapter demonstrates **the extent and quality of scientific and technical expertise** being applied to the problems of regenerative medicine. This chapter also demonstrates **tangible, scientific outcomes attributable to AFIRM**

supported research: inventions disclosed, patent applications filed, research or review articles published or accepted, conference and meeting presentations and posters presented or accepted, and the advancement of products through research and development stages. The AFIRM program data shown in this chapter are based on the first and second program years (PY1 and PY2). For the purpose of this annual report, PY1 is defined as the period from the initiation of research projects in May 2008 through the end of May 2009, and PY2 is defined as the period from June 1, 2009 to May 31, 2010.



VII: AFIRM Statistics

Personnel

A substantial workforce has been funded through the AFIRM program to conduct research on regenerative biology and medicine, from faculty members to undergraduate students (Figure VII-1). Notably, nearly 120 research faculty members were funded through the AFIRM in PY2. Another 81 postdoctoral associates and fellows and 77 scientific and technical staff were funded through the AFIRM in PY2.¹ Finally, with more than 40 graduate students and nearly 20 undergraduate students funded through AFIRM projects to conduct research, the program is substantially contributing to the training of the next generation of scientists to advance regenerative medicine research and development into the future.

In addition to the many scientists directly supported by the AFIRM, numerous others conducting research for the program were not directly supported with AFIRM funds (shown as the orange or light green bar extensions in Figure VII-2). These scientists are providing the complementary technical expertise needed to achieve the goals of the program. For example, 23 faculty contributed to Wake Forest-Pittsburgh Consortium (WFPC) and Rutgers-Cleveland Clinic Consortium (RCCC) research projects without being funded by the AFIRM. An additional 23 postdoctoral fellows, 18 graduate students, 33 undergraduate students, and 17 staff scientists and technicians contributed to AFIRM research projects in the second program year without being funded by the program (Figure VII-2).

Another highlight of the AFIRM is the substantial recruitment of young talent into the field of regenerative medicine. More than 110 students (62 graduate students and 50 undergraduate students) received practical scientific training through AFIRM-sponsored research projects in PY2 (shown in Figure VII-2). The number of AFIRM-supported graduate students who completed their degree requirements is shown in Figure VII-3. From PY1 to PY2, the number of students who completed master's degrees increased from 1 to 3, and the number of students who completed PhDs increased from 2 to 9.

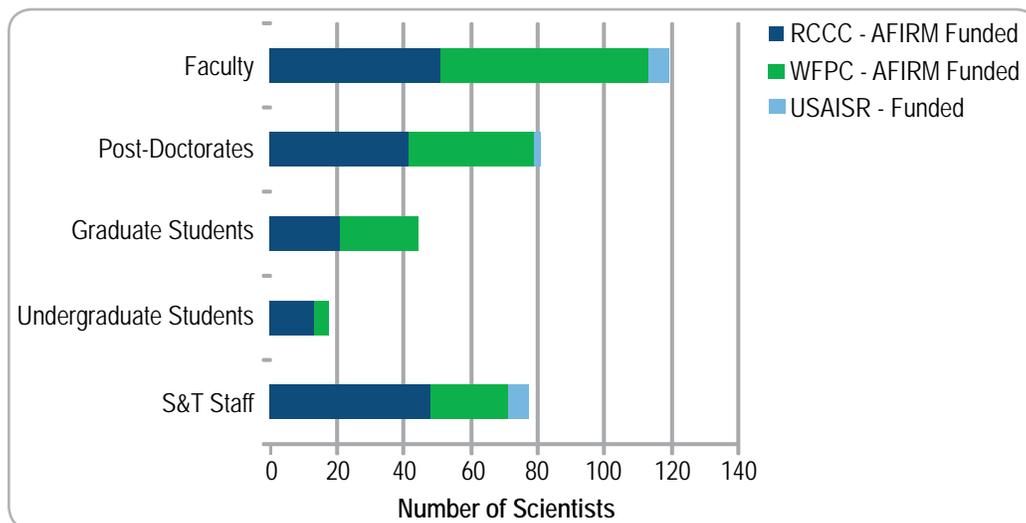


Figure VII-1. Numbers of scientists and students supported through the AFIRM program during PY2.²

¹ The numbers may be slight overestimates due to some individuals working on multiple projects. Any scientist or student who contributed to more than one project was counted only once. This was verified by tracking the names of all scientists and students by project. However, not all individuals who worked on AFIRM projects were named; thus it is possible that some individuals working on two or more projects could have been counted in each project.

² This is defined as the number of unique individuals supported by the AFIRM program during any part of the second program year. See footnote 1 for caveat regarding these data.

The AFIRM is developing fellowship programs to provide unique educational opportunities for aspiring scientists. For example, RCCC, in concert with the Henry M. Jackson Foundation, established the Henry M. Jackson-AFIRM Regenerative Medicine Traveling Fellowship to foster knowledge exchange and collaborative relationships among members of the military, civilian scientists, and clinicians. Military personnel selected for the fellowship will visit sites at both AFIRM consortia to learn of advances in regenerative medicine applicable to wounded warriors. Likewise, civilian scientists and clinicians will visit military facilities to understand the capabilities and mission of these institutions, and the needs of the wounded warriors they serve. In addition, RCCC designed and applied for NIH funding for a Translational Research in Regenerative Medicine: Stem Cells on Scaffolds Fellowship to provide young scientists or clinicians with opportunities to learn regenerative medicine approaches within the AFIRM consortia.

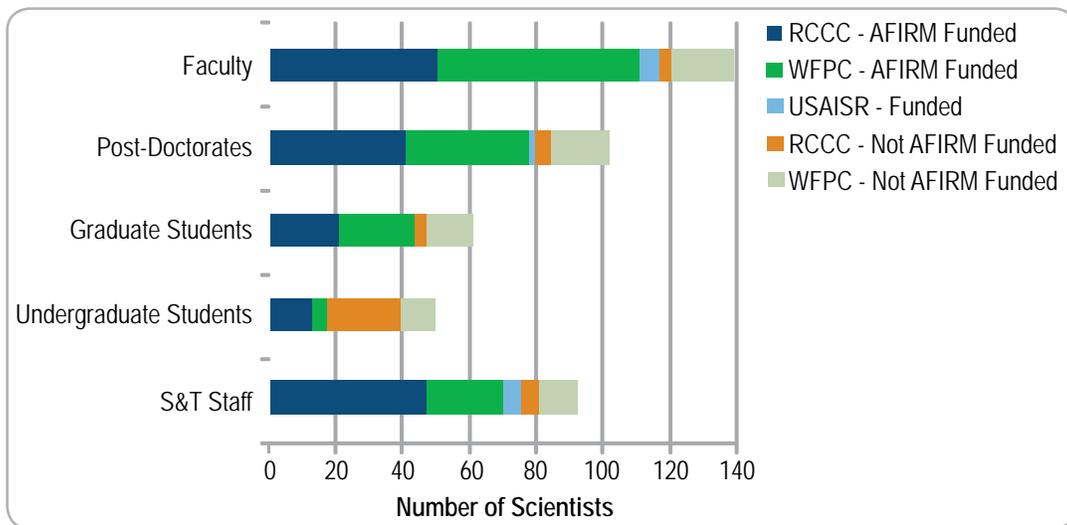


Figure VII-2. Numbers of scientists and students who conducted research on AFIRM projects during PY2.

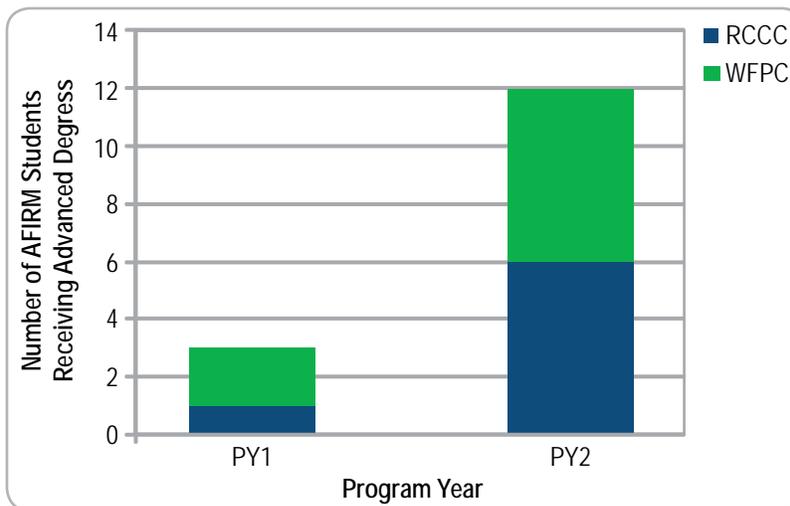


Figure VII-3. Number of graduate degrees awarded to AFIRM contributing students in PY1 and PY2.



VII: AFIRM Statistics

The AFIRM is composed of five overarching research program areas: Limb and Digit Salvage, Craniofacial Reconstruction, Burn Repair, Scarless Wound Healing, and Compartment Syndrome. Figure VII-4 depicts the approximate proportion of all personnel, funded and unfunded, who worked on the different program areas in PY2.³

Honors and Achievements

The AFIRM's faculty are highly accomplished in their respective scientific fields. From June 2009 through May 2010, 46 honors and awards were conferred upon AFIRM faculty, as self-reported by the researchers. These honors include selection to membership or leadership positions in professional societies, honorary degrees from research/academic institutions, awards from private foundations, and recognition of exemplary AFIRM meeting presentations. The distribution of the honors received is displayed according to the type of conferring organization in Figure VII-5.⁴ The complete lists of honors and awards received by AFIRM faculty during PY2 are shown in Appendix A.

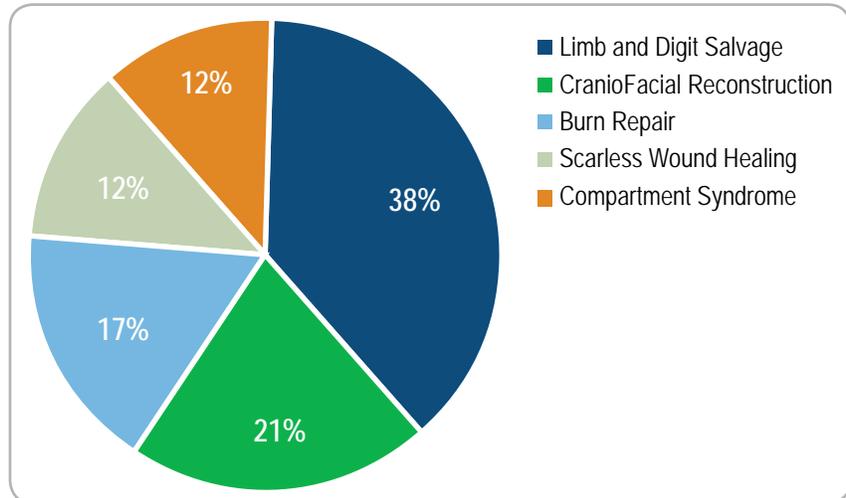


Figure VII-4. Percentage of personnel conducting research in the AFIRM program across the five program areas.

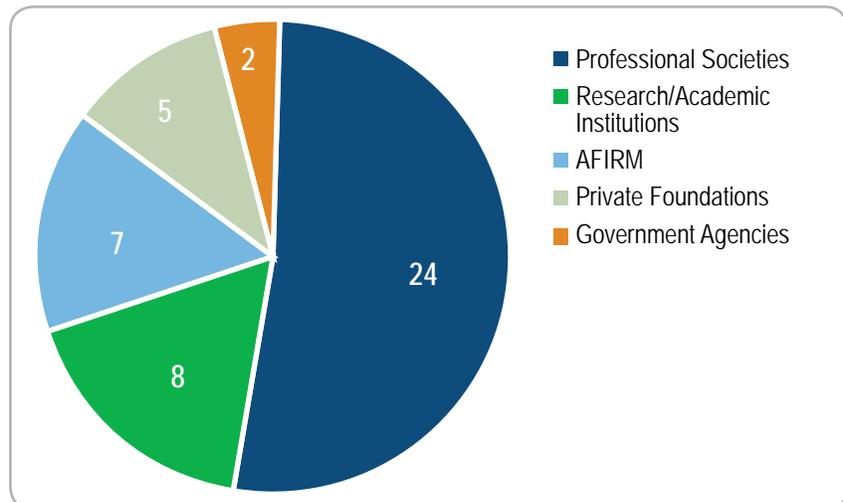


Figure VII-5. Distribution of honors and awards to AFIRM faculty by type of conferring organization.

In addition to awards and honors received by AFIRM researchers, many AFIRM investigators have successfully competed for new research funds. AFIRM investigators submitted 29 proposals in PY2; and 38 newly competed grants, contracts, or subcontracts began in PY2.⁵

³ Figure VII-4 counts each person once only; however, a small number of researchers worked on projects in two or more program areas. These researchers are only represented once in the chart according to their principal research area.

⁴ Awards to faculty exclude awards to postdoctorate fellows and students and also excludes the awarding of competed grants and contracts.

⁵ The number of proposals includes those with reported submission dates falling within PY2 as well as proposals that were reported without submission dates. The number of new grants or contracts includes those reported with funding start dates within PY2 and those that did not report funding dates.

Publications and Presentations

The presentation and publication of research findings are the most immediate output accomplishments of AFIRM-supported researchers.

For the purposes of this report, the following definitions have been applied for consistency:

Non-Peer-Reviewed Publications and Presentations

Meeting symposia, invited talks, oral presentations, and posters delivered or accepted between May 2008 and May 2009 or from June 2009 through May 2010 are included in the PY1 and PY 2 numbers, respectively, regardless of the review process for accepting a presentation or the eventual publication of an abstract in a scientific journal. Additionally, editorial comments, letters, non-peer-reviewed book chapters, and other types of non-peer-reviewed published works are included.

Peer-Reviewed Publications

Research or review articles accepted to, in press, or published in peer-reviewed journals or peer-reviewed edited books in PY1 and PY2 are included. Research or review manuscripts submitted to a journal or in preparation are not included in this annual report.

The number of non-peer-reviewed publications resulting from AFIRM-sponsored research decreased from 118 in PY1 to 106 in PY2 (Figure VII-6).⁶ Meanwhile, the number of peer-reviewed publications resulting from the investigators' research increased from 59 in PY1 to 73 in PY2. The complete lists of AFIRM researchers' publication and presentation citations counted in PY2 are shown in Appendix B.

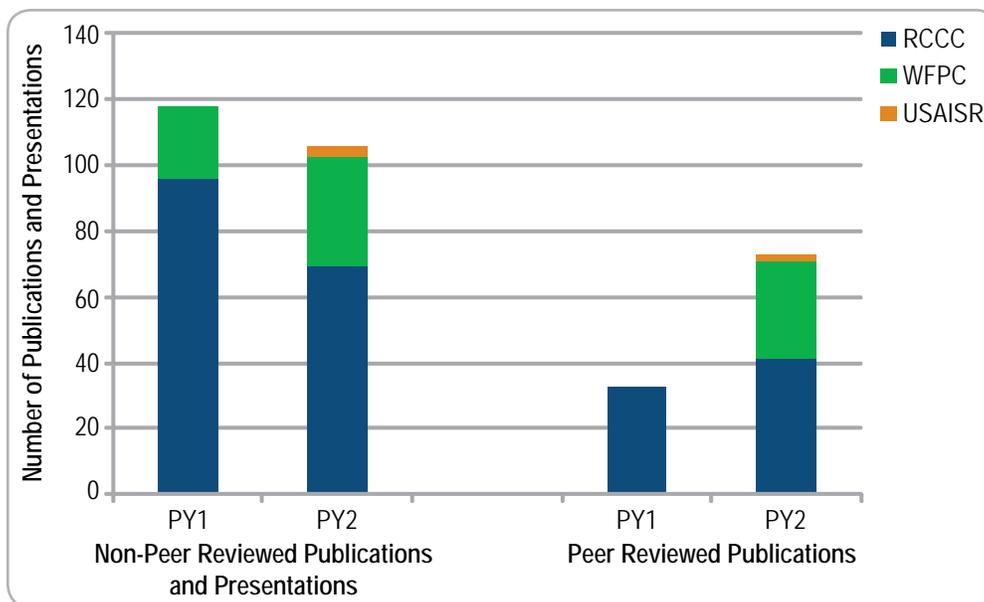


Figure VII-6. Dissemination of AFIRM-sponsored research findings to the scientific community in PY1 and PY2.

⁶ Articles published or accepted and presentations and posters delivered or accepted during the years covered by either PY were included if the self-reported citations did not list the month of publication. Publications and presentations counted in PY1 were excluded from the number in PY2.



VII: AFIRM Statistics

Inventions, Patent Applications, and Patents

The successful development of tangible products or inventions can be tracked across three milestone phases: (1) an invention disclosure is filed by a researcher with his/her institutional technology licensing office, (2) a patent application is submitted to the government patent office (e.g., U.S. Patent and Trademark Office or USPTO), and (3) a patent is awarded by the government patent office for the intellectual property.

Many of the AFIRM's principal investigators were already developing regenerative medicine-related research products at the time the program was initiated. Products developed entirely before the AFIRM program existed are not recognized as AFIRM outcome accomplishments.⁷ However, products initially developed prior to AFIRM support but refined during the AFIRM program period are considered AFIRM outcome accomplishments as are all newly disclosed intellectual property.

In the first year of the program, AFIRM investigators made a combined 26 invention disclosures to their institutional technology licensing offices. Of those disclosed inventions, 12 government patent applications have been filed (6 in each of the first 2 program years). In PY2, AFIRM investigators filed 10 new invention disclosures. Two of those invention disclosures led to patent application submissions in PY2, resulting in a total of 8 patent application submissions in PY2 (Figure VII-7). No patents attributable to AFIRM support were awarded to AFIRM researchers during the first 2 years of funding, which is expected considering the length of time for a patent to be issued.⁸ The complete lists of inventions disclosed and patent applications filed in PY2 that are attributable to AFIRM-sponsored research are shown in Appendix C.

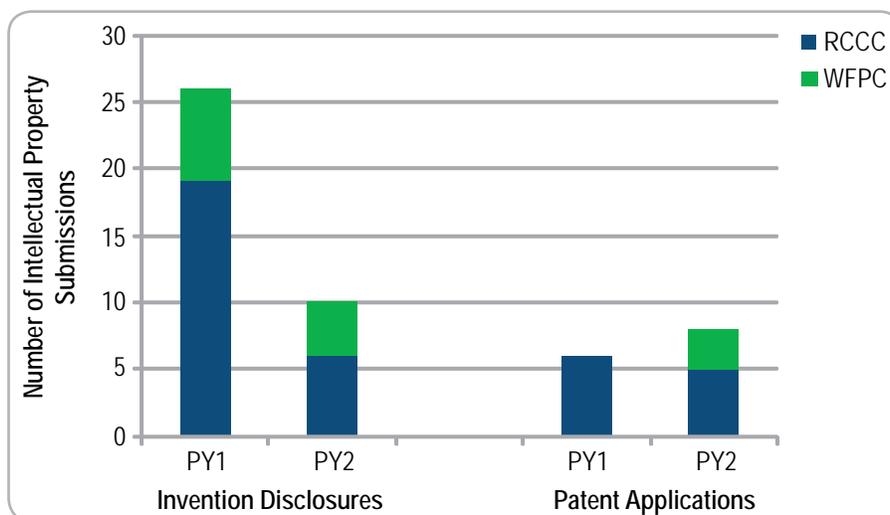


Figure VII-7. AFIRM-attributable invention disclosures and government patent applications filed in PY1 and PY2.

⁷ Definitions of AFIRM-attributable inventions, patent applications, and patents were developed to standardize the self-reported program data and are described in Appendix C.

⁸ The average time for the USPTO to render the final disposition on Biotechnology and Organic Chemistry applications is 35 months according to the USPTO's Performance and Accountability Report Fiscal Year 2008; however, accelerated examination status for patent application reviews can reduce the time an application is in pendency at the USPTO to 12 months or less.

Developmental Accomplishments and Milestones

Technology Readiness Levels of Products

Research progress can be measured in terms of the transitional progress of products.⁹ Biomedical research and development activities funded through USAMRMC are categorized by Technology Readiness Levels (TRLs). TRLs identify a given product's research and development stage along a 9-point scale, which for therapeutic products extends from basic research at TRLs 1 and 2 to proof-of-concept studies (TRLs 3-4), preclinical (TRL 5) and clinical (TRLs 6-8) technology development stages, and post-marketing surveillance (TRL 9).

At the start of the program, 61 products (93%) were nearly evenly distributed across TRLs 1, 2, and 3; and the other 5 products were at TRL 4 or 5. By the end of PY1, 5 products remained at TRL 1, and 56 products were at TRL 2 or 3. Another 12 products were distributed between TRLs 4, 5, and 6. By the end of PY2, no products remained at TRL 1, and only 9 products were at TRL 2. The vast majority of products (50 of 69) were at TRLs 3 and 4, and the other 10 products were distributed between TRLs 5, 6, and 7. **Figure VII-8** shows the AFIRM's overall research and development progression from predominantly basic research to proof-of-concept and technology development stages.

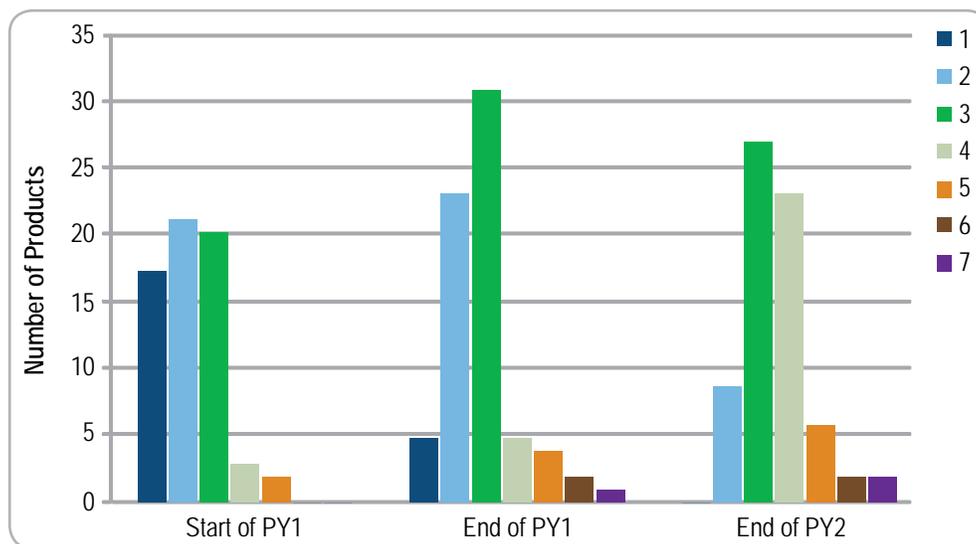


Figure VII-8. AFIRM research products' progression through research and development stages.

⁹ A given research project can advance one or more products through development. Each developed product is separately assigned a TRL; therefore, a given project may have 2 or more products and thus 2 or more TRLs. In PY2, 6 AFIRM projects were developing more than one product: four projects were each developing 2 products, and 2 other projects were developing 3 products. For simplicity, the term product will be used to describe either the project (if no specific product has resulted from basic research) or product (if a specific product is being further developed).



VII: AFIRM Statistics

While Figure VII-8 displays the status of all products' TRL stages, it does not track how each product transitioned from one year to the next. **Figure VII-9** summarizes the transition of each product by showing the number of R&D products that remained at the same TRL or that advanced by one or more TRL in one program year. Figure VII-9 shows that approximately twice as many projects increased by one TRL in PY2 compared to PY1. Of 63 products having self-reported TRLs at the beginning of the program and the end of PY2, 34 products (54%) advanced one TRL, another 22 products (35%) advanced two or more TRLs, and only 7 products (11%) did not advance in TRL during the first 2 years of the program (data not shown).

Preclinical Models and Clinical Studies

Products at TRL 4 or 5 are being tested in in vivo animal models, some of which had to be newly developed or validated for the purpose of testing the products in an adequate injury model. In PY2, AFIRM researchers completed the development and/or validation of 12 experimental models for studying injury mechanisms, developing therapeutic approaches, and conducting preclinical studies to demonstrate the potential of therapeutic products.

Products at TRL 6 and above are being evaluated in human clinical studies that require federal regulatory approval and approval through institutional review boards (IRBs). In PY2, AFIRM investigators advanced products through clinical study planning, approval, and execution stages. Two Phase 1 clinical trials were completed, four clinical trials opened to enroll patients (3 Phase 1 and 1 Phase 2 trials), and five additional planned clinical studies' protocols (four Phase 1 and one Phase 2) had been submitted to IRBs. **Figure VII-10** shows the number of unique clinical protocols by the most advanced stage of development. Of note, one project not only completed the Phase 1 clinical trial but also progressed to open enrollment for the subsequent Phase 2 study in PY2, and this project is only included in the bar for Phase 2 Trial Open to Enrollment in Figure VII-10.

Commercialization Plans

Commercial partnerships are important to the final development and fielding of medical materiel products. The collaboration of AFIRM investigators with commercial partners will enable clinical trials to be conducted, as commercial and venture capital is leveraged with government funds. The formal agreement between an investigator and an industry partner is also a surrogate measure of the demonstrated potential utility of the product being developed. In PY2, 13 commercial partnerships with AFIRM project investigators were in place to advance the development of products,¹⁰ and for six more products, investigators have identified potential commercial partners or have initiated talks with potential partners.

¹⁰ Some of the commercial partnerships preceded the start of the program, including some investigations of off-the-shelf products or products licensed by the partnering company.

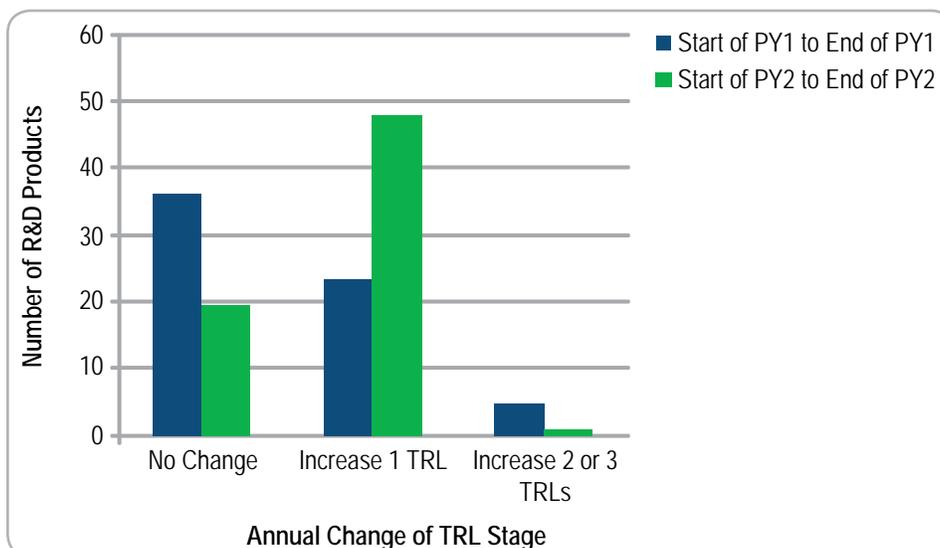


Figure VII-9. Advancement of TRLs for AFIRM products from the start of PY1 to the end of PY1 and from the start of PY2 to the end of PY2.

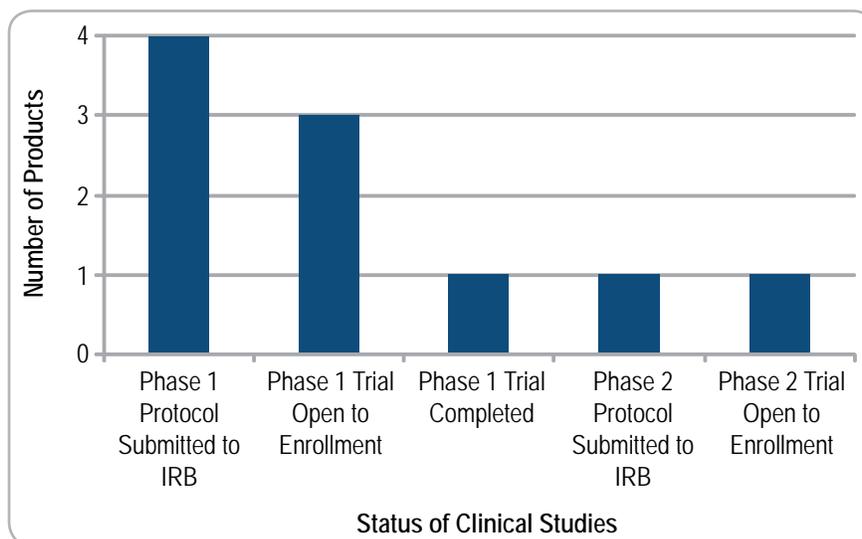


Figure VII-10. Most advanced stage of clinical product development for unique AFIRM projects in PY2.

Appendix A: Honors and Awards to AFIRM Faculty

During the reporting period from June 2009 through May 2010, 46 honors or awards were received by AFIRM faculty, as self-reported.

Rutgers – Cleveland Clinic Consortium

Alvarez, L (Massachusetts Institute of Technology): Top Poster Award, Second International Conference on Stem Cell Engineering, American Institute of Chemical Engineers and Society for Biological Engineering, 2010.

Boyce, S (University of Cincinnati): Board of Trustees, American Burn Association, 2010.

Dewar, A (Stony Brook University): Best Poster Award, AFIRM All Hands Meeting, AFIRM, 2010.

Griffith, L (Massachusetts Institute of Technology): Radcliffe Fellow, Radcliffe Institute for Advanced Study, 2009.

Langer, R (Massachusetts Institute of Technology): Founding POLY Fellow, Division of Polymer Chemistry, American Chemical Society, 2010.

Langer, R (Massachusetts Institute of Technology): Biomedical Research Leaders Award, Massachusetts Society for Medical Research, 2009.

Langer, R (Massachusetts Institute of Technology): Honorary Degree, Rensselaer Polytechnic Institute, 2010.

Langer, R (Massachusetts Institute of Technology): Honorary Degree, Willamette University, 2010.

Macri, L (Stony Brook University): 1st Place People's Choice Award, AFIRM All Hands Meeting, AFIRM, 2010.

Macri, L (Stony Brook University): Oral Presentation Selection, Society for Biomaterials, 2010.

Sarac, T (Cleveland Clinic Foundation): Charles C. Guthrie Award for Outstanding Basic Science Research, Midwestern Vascular Surgical Society, 2009.

Siemionow, M (Cleveland Clinic Foundation): 1st Place Oral Presentation Award (Limb and Digit), AFIRM All Hands Meeting, AFIRM, 2010.

Siemionow, M (Cleveland Clinic Foundation): Clinical Researcher of the Year Award, American Association of Plastic Surgeons, 2010.

Siemionow, M (Cleveland Clinic Foundation): Proclamation from the mayor of Cleveland, in recognition of professional accomplishments, City of Cleveland, 2009.

Siemionow, M (Cleveland Clinic Foundation): Portraits of Polish Medicine - Physician, Health Market (Rynek Zdrowia), 2009.

Siemionow, M (Cleveland Clinic Foundation): Award for Excellence, Lerner Research Institute, 2010.

Siemionow, M (Cleveland Clinic Foundation): 2nd Place, Oral Presentation, Ohio Valley Society of Plastic Surgeons, 2010.

Siemionow, M (Cleveland Clinic Foundation): Medal of Recognition Award for Pioneering Plastic Surgeon, The Board of Trustees of The Kosciuszko Foundation, 2009.



Appendix A: Honors and Awards to AFIRM Faculty

Siemionow, M (Cleveland Clinic Foundation): Orrefors Kosta Boda Crystal Inspiration Award, When U Dream A Dream Foundation, 2009.

Siemionow, M (Cleveland Clinic Foundation): President, International Society of Hand and Composite Tissue Allografts, 2009.

Wang, H (Mayo Clinic): 2nd Place Poster Award, AFIRM All Hands Meeting, AFIRM, 2010.

Windebank, AJ (Mayo Clinic): Councilor Nominee, American Neurological Association, 2010.

Windebank, AJ (Mayo Clinic): Honorary Doctorate, University of Salzburg – Paracelsus Medical University, Austria, 2010.

Yaszemski, Michael (Mayo Clinic): Achievement Award, American Academy of Orthopaedic Surgeons, 2010.

Yaszemski, Michael (Mayo Clinic): Clemson Award for Applied Research, Society for Biomaterials, 2010.

Wake Forest-Pittsburgh Consortium

Boyan, B (Georgia Institute of Technology): Manny Horowitz Award, ASTM (originally American Society for Testing and Materials), 2009.

Guldberg, R (Georgia Institute of Technology): Director of the Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, 2009.

Gurtner, G (Stanford University): James Barrett Brown Award (Best Plastic Surgery Paper), American Association of Plastic Surgeons, 2009.

Gurtner, G (Stanford University): Associate Chair, Department of Surgery, Stanford University, 2010.

Huard, J (McGowan Institute): 1st Place Oral Presentation Award (Compartment Syndrome), AFIRM All Hands Meeting, AFIRM, 2010.

Longaker, M (Stanford University): 1st Place Oral Presentation Award (Scarless Wound Healing), AFIRM All Hands Meeting, AFIRM, 2010.

Longaker, M (Stanford University): Basic Science/Translational Researcher of the Year Award, American Association of Plastic Surgeons, 2010.

Marra, K (McGowan Institute): Emerging Female Scientist Honorable Mention, Carnegie Science Center, 2010.

Mikos, AG (Rice University/University of Texas Health Science Center): Greater Houston Section Award, American Chemical Society, 2009.

Mikos, AG (Rice University/University of Texas Health Science Center): Food, Pharmaceutical and Bioengineering Award in Chemical Engineering, American Institute of Chemical Engineers, 2010.

Mikos, AG (Rice University/University of Texas Health Science Center): Meriam/Wiley Distinguished Author Award, American Society for Engineering Education, 2010.

Mikos, AG (Rice University/University of Texas Health Science Center): Fellow, Biomedical Engineering Society, 2009.

Mikos, AG (Rice University/University of Texas Health Science Center): Distinguished Scientist Award, Isaac Schour Memorial Award, International Association for Dental Research, 2010.

Tirrell, D (California Institute of Technology): Dickson Prize for Science, Carnegie Mellon University, 2010.

Tirrell, D (California Institute of Technology): Founding POLY Fellow, Division of Polymer Chemistry, American Chemical Society, 2010.

Wachtman, GS (McGowan Institute): Best Poster Award, AFIRM All Hands Meeting, AFIRM, 2010.

Wachtman, GS (McGowan Institute): Best Paper, Basic Science, 55th Annual Scientific Meeting, Ohio Valley Society of Plastic Surgeons, 2010.

Wachtman, GS (McGowan Institute): Plastic Surgery Education Foundation Research Fellowship, Plastic Surgery Education Foundation, 2009.

Wagner, WR (McGowan Institute): Executive Board Selection, International Federation for Artificial Organs, 2010.

Wagner, WR (McGowan Institute): NIH College of Reviewers, National Institutes of Health, 2010.

Wagner, WR (McGowan Institute): Stevenson Biomaterials Lectureship, Syracuse University, 2009.



Appendix B: Publications and Presentations

Peer-reviewed journal articles are defined as research articles and review articles “accepted” to, “in press,” or published in scientific and technical journals from June 2009 through May 2010. Additionally, book chapters are included as peer-reviewed publications. The articles shown in Tables B-1a, B-1b, and B-1c were self-reported by the AFIRM investigators.

Table B-1a. Peer-Reviewed Publications: Rutgers - Cleveland Clinic Consortium

Amos PJ, Kapur SK, Stapor BS, Shang H, Bekiranov S, Khurgel M, Rodeheaver GT, Peirce S, and Katz AJ. Human Adipose-Derived Stromal Cells Accelerate Diabetic Wound Healing: Impact of Cell Formulation and Delivery. <i>Tissue Engineering: Part A</i> , v16, No.5, 2010, pp 1595-1606.
Bailey AM, Lawrence MB, Shang H, Katz AJ, Peirce SM. Agent-Based Model of Therapeutic Adipose-Derived Stromal Cell Trafficking During Ischemia Predicts Ability to Roll on P-Selectin. <i>PLoS Computational Biology</i> , Vol. 5, No.2, 2009.
Bettinger C, Bruggeman J, Borenstein J and Langer R. In Vitro and In Vivo Degradation of Poly (1,3-diamino-2-Hydroxypropane-Co-Polyol Sebacate) Elastomers. <i>Journal of Biomedical Materials Research</i> , 91:1077-1088, 2009, PMID: 19107786.
Bettinger C, Bruggeman J, Misra A, Borenstein J, and Langer R. Biocompatibility of Biodegradable Semiconducting Melanin Films for Nerve Tissue Engineering. <i>Biomaterials</i> , 30:3050-3057, 2009, PMID: 19286252.
Bettinger C, Kulig K, Vacanti J, Borenstein J, and Langer R. Nanofabricated Collagen-Inspired Synthetic Elastomers for Primary Rat Hepatocyte Culture. <i>Tissue Engineering</i> , 15:1321-1329, 2009, PMID: 18847357, PMCID: PMC2789736.
Bettinger C, Langer R, and Borenstein J. Engineering Substrate Topography at the Micro- and Nanoscale to Control Cell Function. <i>Angewandte Chemie</i> , 48:5406-5415, 2009, PMID: 19492373, NIHMSID: NIHMS148783.
Bhang S, Cho S, Lim J, Kang J, Lee T, Yang H, Song Y, Park M, Kim H, Langer R, Anderson D, and Kim B. Locally Delivered Growth Factor Enhances the Angiogenic Efficacy of Adipose Derived Stromal Cells Transplanted to Ischemic Limbs. <i>Stem Cells</i> , 27:1976-1986, 2009, PMID: 19544425.
Chan J, Zhang L, Tong R, Ghosh D, Gao W, Liao G, Yuet K, Gray D, Rhee J, Cheng J, Golomb G, Libby P, Langer R, and Farokhzad O. Spatiotemporal Controlled Delivery of Nanoparticles to Injured Vasculature. <i>Proceedings of the National Academy of Sciences</i> , 107:2213-2218, 2010, PMID: 20133865.
Cho S, Goldberg M, Son S, Xu Q, Yang F, Mei Y, Bogatyrev S, Langer R, and Anderson D. Lipid-Like Nanoparticles for small Interfering RNA Delivery to Endothelial Cells. <i>Advanced Functional Materials</i> , 19:3112-3118, 2010.
Costache D, Sheihet L, Zaveri K, Knight DD, and Kohn J. Polymer-Drug Interactions in Tyrosine-Derived Triblock Copolymer Nanospheres: A Computational Modeling Approach. <i>Mol Pharm.</i> 2009 Sep-Oct;6(5):1620-7.
Dang T, Xu Q, Bratlie K, O'Sullivan E, Chen X, Langer R, and Anderson D. Microfabrication of Homogenous, Asymmetric Cell-Laden Hydrogel Capsules. <i>Biomaterials</i> , 30:6896-6902, 2009, PMID: 19800116, NIHMSID147749.
Dumas JE, Zienkiewicz K, Tanner SA, Prieto EM, Bhattacharyya S, and Guelcher SA. Synthesis and Characterization of Injectable Allograft Bone/Polymer Composite Scaffolds. <i>Tissue Engineering</i> , In Press.
Fisher O, Khademhosseini A, Langer R, and Peppas N. Bioinspired Materials for Controlling Stem Cell Fate. <i>Accounts of Chemical Research</i> , in press (advance web pub: 10.1021/ar900226q), PMID: 20043634, NIHMSID: NIHMS168065.
George P, Saigal R, Lawlor M, Moore M, LaVan D, Marini R, Selig M, Makhni M, Burdick J, Langer R, and Kohane D. Three-Dimensional Conductive Constructs for Nerve Regeneration. <i>Journal of Biomedical Materials Research</i> , 91:519-527, 2009, PMID: 18985787.
Gerecht S, Ferreira LS, and Langer R. Vascular Differentiation of Human Embryonic Stem Cells in Bioactive Hydrogel-Based Scaffolds. <i>Methods in Molecular Biology</i> , 584:333-354, 2010, PMID: 19907986.
Harris T, Green J, Fung P, Langer R, Anderson D, and Bhatia S. Tissue-Specific Gene Delivery Via Nanoparticle Coating. <i>Biomaterials</i> , 30: 3926-3933, 2009, PMID: 19850333, PMCID: PMC2796451.
Hook A, Williams P, Anderson D, and Langer R. High Throughput Methods Applied in Biomaterial Development and Discovery. <i>Biomaterials</i> , 31:187-198, 2010, PMID: 19815273.



Appendix B: Publications and Presentations

Table B-1a. Peer-Reviewed Publications: Rutgers - Cleveland Clinic Consortium (cont.)

Jun Y, Li J, Runge MB, Dadsetan M, Chen Q, Lu L, and Yaszemski MJ. Crosslinking Characteristics and Mechanical Properties of an Injectable Biomaterial Composed of Polypropylene Fumarate and Polycaprolactone Copolymer. <i>Journal of Biomaterials Science: Polymer Edition</i> , in press.
Khademhosseini A and Langer R. Nanotechnologies: Emerging Applications in Biomedicine. <i>BBA General Subjects</i> , in press.
Khademhosseini A, Rajalingam B, Jinno S, and Langer R. Nanoengineered Systems for Tissue Engineering and Regeneration, in <i>Nanotechnology</i> , 2009, 361-384. Ed., Vogel, V., Wiley-Vch Verlag GmbH and Co., KGaA, Weinheim, Germany.
Kim J, Dadsetan M, Ameenuddin S, Windebank AJ, and Yaszemski MJ. In Vivo Biodegradation and Biocompatibility of PEG/Sebacic Acid-Based Hydrogels using a Cage Implant System. <i>Journal of Biomedical Material Research, Part A</i> . (2010-in press)
Kraehenbuehl T, Ferreira L, Zammaretti P, Hubbell J, and Langer R. Cell-Responsive Hydrogel for Encapsulation of Vascular Cells. <i>Biomaterials</i> , 30:4318-4324, 2009, PMID: 19500842.
Lahann J and Langer R. Nanobiomaterials: The Interface of Nanotechnology and Biomaterials. <i>IQT Quarterly</i> , 1:11-15, 2010.
Lee KW, Wang S, Dadsetan M, Yaszemski MJ, and Lu L. Enhanced Cell Ingrowth and Proliferation Through Three-Dimensional Nanocomposite Scaffolds with Controlled Pore Structures. <i>Biomacromolecules</i> . 2010 Mar 8;11(3):682-9.
Levenberg S, Ferreira L, Chen-Konak L, Kraehenbuehl T, and Langer R. Isolation, Differentiation and Characterization of Vascular Cells Derived from Human Embryonic Stem Cells. <i>Nature Protocols</i> , in press.
Li B, Yoshii T, Hafeman AE, Nyman JS, Wenke JC, Guelcher SA. The Effects of rhBMP-2 Released from Biodegradable Polyurethane/Microsphere Composite Scaffolds on New Bone Formation in Rat Femora. <i>Biomaterials</i> . 2009 Dec;30(35):6768-79. Epub 2009 Sep 17.
Magno MH, Kim J, Srinivasan A, McBride S, Darr A, Bolikal D, Darr A, Hollinger JO, and Kohn J. Synthesis, Degradation and Biocompatibility of Tyrosine-Derived Polycarbonate Scaffolds for Use in Bone Tissue Engineering. Accepted. <i>Journal of Materials Chemistry</i> , 2010.
Muschler GF, Raut VP, Patterson TE, Wenke JC, and Hollinger JO. The Design and Use of Animal Models for Translational Research in Bone Tissue Engineering and Regenerative Medicine. <i>Tissue Eng Part B Rev</i> . 2010 Feb;16(1):123-45.
Nguyen D, Green J, Chan J, Anderson D, and Langer, R. Polymeric Materials for Gene Delivery and DNA Vaccination. <i>Advanced Materials</i> , 21:847-867, 2009.
Park H, Karajanagi S, Wolak K, Aanestad J, Deheron L, Kobler J, Lopez-Guerra G, Heaton J, Langer R, and Zeitels S. 3D Hydrogel Model Using Adipose-Derived Stem Cells for Vocal Fold Augmentation. <i>Tissue Engineering Part A</i> , 16:535-543, 2010, PMID: 19728785.
Park H, Yip M, Kost J, Kobler J, Langer R, Zeitels S, and Chertok B. Indirect Low-Intensity Ultrasonic Stimulation for Tissue Engineering. <i>Journal of Tissue Engineering</i> , in press.
Raut VP, Patterson TE, Wenke JC, Hollinger JO, Muschler GF. Assessment of Biomaterials: Standardized In Vivo Testing. <i>An Introduction to Biomaterials</i> , S.A. Guelcher, J.O. Hollinger (eds), CRC Press, 2010 (in print).
Runge MB, Dadsetan M, Baltrusaitis J, Knight AM, Ruesink T, Lazcano E, Lu L, Windebank AJ, and Yaszemski MJ. The Development of Electrically Conductive Polycaprolactone Fumarate-Polypyrrole Composite Materials for Nerve Regeneration. <i>Biomaterials</i> , in press.
Singer AJ, Taira BR, Lin F, Lim T, Anderson R, and Clark RAF. Curcumin Reduces Injury Progression in a Rat Comb Burn Model. <i>J Burn Care Res</i> , in press, 2010
Sorrell JM, Baber MA, and Caplan AI. Influence of Adult Mesenchymal Stem Cells on In Vitro Vascular Formation. <i>Tissue Engr A</i> , 2009 15:1751-1761.

Table B-1a. Peer-Reviewed Publications: Rutgers - Cleveland Clinic Consortium (cont.)

Taylor M, Urquhart A, Anderson D, Langer R, Davies M, and Alexander M. Partial Least Squares Regression as a Powerful Tool for Investigating Large Combinatorial Polymer Libraries. <i>Surface and Interface Analysis (Spec. Iss.)</i> , 41:127-135, 2009.
Tholpady SS, Ogle RC, and Katz AJ. Adipose Stem Cells and Solid Organ Transplantation. <i>Current Opinion in Organ Transplantation</i> , 14:51-55, 2009.
Yang F, Cho S, Son S, Bogatyrev S, Singh D, Green J, Mei Y, Park S, Bhang S, Kim B, Langer R, and Anderson D. Genetic Engineering of Human Stem Cells for Enhanced Angiogenesis Using Biodegradable Polymeric Nanoparticles. <i>Proceedings of the National Academy of Sciences</i> , 107:3317-3322, 2010.
Yang F, Green J, Dinio T, Keung L, Cho S, Park H, Langer R. and Anderson D. Gene Delivery to Human Adult and Embryonic Cell-Derived Stem Cells Using Biodegradable Nanoparticulate Polymeric Vectors. <i>Gene Therapy</i> , 16:533-546, 2009, PMID: 19129861, PMCID: PMC2688702.
Yang F, Mei Y, Langer R, and Anderson D. High-Throughput Optimization of Stem Cell Microenvironments. <i>Combinatorial Chemistry and High Throughput Screening</i> , 12:554-561, 2009, PMID: 19601753, PMCID: PMC2748120.
Yao L, de Ruitter GC, Wang H, Knight AM, Spinner RJ, Yaszemski MJ, Windebank AJ, and Pandit A. Controlling Dispersion of Axonal Regeneration Using a Multichannel Collagen Nerve Conduit. <i>Biomaterials</i> . 2010 Apr 27. [Epub ahead of print]

Table B-1b. Peer-Reviewed Publications: Wake Forest – Pittsburgh Consortium

Agrawal V, et al. Epimorphic regeneration approach to tissue replacement in adult mammals. <i>Proc Natl Acad Sci U S A</i> , 2009. 107(8):3351-5.
Boerckel JD, Dupont KM, Kolambkar YM, Lin ASP, and Guldberg RE. In Vivo Model for Evaluating the Effects of Mechanical Stimulation on Tissue-Engineered Bone Repair. <i>Journal of Biomechanical Engineering</i> , 131(8):084502, 2009.
Brayfield CA, Marra KG, and Rubin JP. Adipose Stem Cells for Soft Tissue Regeneration. <i>Handchirurgie, Mikrochirurgie, Plastische Chirurgie</i> , 2010, 42(2):124-128.
Choi JH, Bellas E, Glettig DL, and Kaplan DL. Adipogenic Differentiation of Human Adipose-Derived Stem Cells on 3D Silk Fibroin Scaffolds. <i>Methods in Molecular Biology</i> . Ed. Jeffrey M. Gimble & Bruce Bunnell. Humana Press, In press.
Choi JH, Gimble JM, Lee K, Marra K, Rubin PJ, Yoo JJ, Vunjak-Novakovic G, and Kaplan DL. Adipose Tissue Engineering for Soft Tissue Regeneration. <i>Tissue Engineering Part B: Reviews</i> . 2010 March 24.
Clements IP, Kim YT, English AW, Lu X, Chung A, and Bellamkonda, RV. Thin-Film Enhanced Nerve Guidance Channels for Peripheral Nerve Repair. <i>Biomaterials</i> , 30(23-24):3834-46, 2009.
Dupont KM, Sharma K, Stevens HY, Boerckel JD, Garcia AJ, and Guldberg RE. Human Stem Cell Delivery for Large Segmental Bone Defect Repair. <i>Proceedings of the National Academy of Sciences USA</i> , 107(8):3305-10, 2010.
Ghaznavi M, Kokai LE, Tuffaha SH, Lovett ML, Kaplan DL, and Marra KG. Silk Fibroin Conduits in a Rat Model: A Cellular and Functional Assessment of Peripheral Nerve Repair. <i>Annals of Plastic Surgery</i> , 2010, In Press.
Golub J, Kim YT, Duvall CL, Bellamkonda RV, Gupta D, Lin ASP, Weiss D, Taylor WR, and Guldberg RE. Sustained VEGF Delivery Via PLGA Nanoparticles Promotes Vascular Growth. <i>American Journal of Physiology. Heart and Circulatory Physiology</i> , 298(6):H1959-65, 2010.
Guldberg R.E. Spatiotemporal Delivery Strategies for Promoting Musculoskeletal Tissue Regeneration. <i>Journal of Bone and Mineral Research</i> , 24(9):1507-11, 2009.
Hashizume R, Fujimoto KL, Hong Y, Amoroso NJ, Tobita K, Miki T, Keller BB, Sacks MS, and Wagner WR. Morphological and Mechanical Characteristics of the Reconstructed Rat Abdominal Wall Following Use of a Wet Electrospun Biodegradable Polyurethane Elastomer Scaffold. <i>Biomaterials</i> . 2010;31:3253-3265.



Appendix B: Publications and Presentations

Table B-1b. Peer-Reviewed Publications: Wake Forest – Pittsburgh Consortium (cont.)

Hoffman-Kim D, Mitchel JA, and Bellamkonda RV. Topography, Cell Response, and Nerve Regeneration. <i>Annu Rev Biomed Eng</i> , In Press.
Johnson MR, Lee HJ, Bellamkonda RV, and Guldberg RE. Sustained Release of BMP-2 in a Lipid-Based Micro-tube Vehicle. <i>Acta Biomaterialia</i> , 5(1):23-28, 2009.
Kang JH, Gimble JM, and Kaplan DL. In Vitro 3D Model for Human Vascularized Adipose Tissue. <i>Tissue Eng Part A</i> . 2009, 15:2227-2236.
Kim U and Soh HT. Simultaneous Sorting of Multiple Bacterial Targets Using Integrated Dielectrophoretic Magnetic Activated Cell Sorter. <i>Lab on a Chip</i> (9) 2313-2318 (2009).
Kokai LE, Ghaznavi AM, and Marra KG. Incorporation of Double-Walled Microspheres into Polymer Nerve Guides for the Sustained Delivery of Glial Cell Line-Derived Neurotrophic Factor. <i>Biomaterials</i> , 2010, 31(8):2313-2322.
Kokai LE, Tan H, Jhunjhunwala S, Little SR, Frank J, and Marra KG. Protein Bioactivity and Polymer Orientation is Affected by Stabilizer Incorporation in Double-Walled Microspheres. <i>Journal of Controlled Release</i> , 2010, 141:168-176.
Kolambkar Y and Guldberg RE. Colonization and Osteogenic Differentiation of Different Stem Cell Sources on Electrospun Nanofiber Meshes. <i>Tissue Engineering</i> , (In Press).
Kretlow JD, Shi M, Young S, Spicer PP, Demien N, Jansen JA, Wong ME, Kasper FK, and Mikos AG. Porous Polymethylmethacrylate Space Maintainers Promote Soft Tissue Coverage of Clean/Contaminated Alveolar Bone Defects. <i>Tissue Eng Part C Methods</i> . 2010 in press (DOI: 10.1089/ten.tec.2010.0046).
Lee H, McKeon RJ, and Bellamkonda RV. Sustained Delivery of Thermostabilized chABC Enhances Axonal Sprouting and Functional Recovery After Spinal Cord Injury. <i>Proceedings of National Academies of Sciences USA</i> , 107(8):3340-5, 2010.
Lou XH, Qian J, Xiao Y, Viel L, Gerdon AE, Lagally ET, Atzberger P, Tarasow TM, Heeger AJ, and Soh HT. Micro-magnetic Selection of Aptamers in Microfluidic Channels. <i>Proceedings of the National Academy of Sciences, USA</i> , 106 (9) 2989-2994 (2009).
Markert CD, et al. Immunofluorescence Microscopy for Imaging of Nuclear p63 in Human Primary Keratinocytes: A Comparison of Antibodies and Fixation Methods. <i>Journal of Immunological Methods</i> , 352 (2010), 174-177.
Santiago LY, Clavijo-Alvarez J, Brayfield C, Rubin JP, and Marra KG. Delivery of Adipose-Derived Precursor Cells for Peripheral Nerve Repair. <i>Cell Transplantation</i> , 2009, 18(2):145-158.
Satish L, Johnson S, Abdulally A, Post JC, Ehrlich GD, and Kathju S. Cloning and Expression of Rabbit CCT Subunits eta and beta in Healing Cutaneous Wounds. <i>Cell Stress Chaperones</i> . 2010 Apr 15. [Epub ahead of print]
Satish L, Johnson S, Wang JH, Post JC, Ehrlich GD, and Kathju S. Chaperonin Containing T-Complex Polypeptide Subunit eta (CCT-eta) is a Specific Regulator of Fibroblast Motility and Contractility. <i>PLoS One</i> . 2010 Apr 30;5(4):e10063.
Shi M, Kretlow JD, Nguyen A, Young S, Baggett LS, Wong ME, Kasper FK, and Mikos AG. Antibiotic-Releasing Porous Polymethylmethacrylate Constructs for Osseous Space Maintenance and Infection Control. <i>Biomaterials</i> . 2010 May;31(14):4146-56.
Suh WH and Tirrell M. Surface Engineering Using Peptide Amphiphiles. <i>Comprehensive Biomaterials</i> , 2010, in press.
Tan H, Ramirez CM, Miljkovic ND, Li H, Rubin JP, and Marra KG. Thermosensitive Injectable Hyaluronic acid Hydrogel for Adipose Tissue Engineering. <i>Biomaterials</i> , 2009, 30(36):6844-6853.
Tan H, Rubin JP, and Marra KG. Injectable In Situ Forming Biodegradable Chitosan-Hyaluronic Acid Based Hydrogels for Adipose Tissue Regeneration. <i>Organogenesis</i> , In Press.
Wojtowicz AM, Oest ME, Dupont KM, Templeman KL, Hutmacher DW, Guldberg RE, and Garcia AJ. Collagen-Mimetic Peptide Coating of Biomaterial Scaffolds for Bone Defect Repair. <i>Biomaterials</i> , In Press.

Table B-1c. Peer-Reviewed Publications: U.S. Army Institute of Surgical Research

Brown KV, Walker J, Cortez D, and Wenke J. Delay in Debridement and Antibiotics Increases Infection. <i>J Surg Orth Adv</i> 2010 19(1):18-22.
Li B, Brown KV, Wenke JC, and Guelcher SA. Sustained Release Of Vancomycin from Polyurethane Scaffolds Inhibits Infection of Bone Wounds in a Rat Femoral Segmental Defect Model. <i>Journal of Controlled Release</i> . 2010 Aug 3;145(3):221-30.

Tables B-2a, B-2b, and B-2c display non-peer-reviewed publications and all presentations. These publications and presentations were self-reported by AFIRM investigators. The non-peer-reviewed publications are defined as editorials, letters, or opinion writings that have been “accepted” to, “in press,” or published in scientific and technical journals from June 2009 through May 2010. Presentations include all invited talks, symposia, oral presentations, and posters presented at scientific research conferences and meetings regardless of the peer review process. All such presentations made and all presentations “accepted” from June 2009 through May 2010 are included in the following tables. Presentations not specifically labeled as “accepted” in the researchers’ progress reports were not assumed to be accepted and were not included in the following tables.

Table B-2a. Presentations and Non-Peer-Reviewed Publications: Rutgers – Cleveland Clinic Consortium

Alvarez L, Sanchez Palacios E, Stockdale L, Muschler G, and Griffith L. Manipulation of Mesenchymal Stem Cell Migration Through the Use of EGF-Family Growth Factors. <i>Proceedings of American Institute of Chemical Engineers/Society for Biological Engineering Second International Conference on Stem Cell Engineering</i> , (2010).
Alvarez L, Stockdale L, Saini S, Muschler G, and Griffith L. Novel Molecular Surface Design of Bone Regeneration Scaffolds. <i>Proceedings of Armed Forces Institute of Regenerative Medicine All Hand Meeting</i> , (2010).
Arnold PB and Katz AJ. Tissue Processing Considerations for Autologous Fat Grafting. In <i>Autologous Fat Transfer: Art, Science, and Practice</i> . Melvin A. Shiffman, Editor, Springer (Berlin), (2009).
Boyce ST, Pruszka J, Lockhart M, Estes M, Rust M, Supp DM, and Kagan RJ. 2010. Formation of Vascular Channels in Engineered Skin Substitutes. Proc Armed Forces Institute for Regenerative Medicine, All Hands Meeting; St. Pete’s Beach, FL.
Boyce ST, Zimmerman R, Swope VB, Supp DM and Kagan RJ. 2010. Regulation of Pigmentation in Engineered Skin Substitutes. Proc Armed Forces Institute of Regenerative Medicine, All Hands Meeting; St. Pete’s Beach, FL.
Boyce ST. Stem Cell Isolation, Propagation, Differentiation and Transplantation; Moderator, Correlative Session 2; Armed Forces Institute of Regenerative Medicine; St. Pete’s Beach, FL; Jan, 2010.
Boyce ST. Translational Research with Engineered Human Skin; Correlative Session 3; Armed Forces Institute of Regenerative Medicine; St. Pete’s Beach, FL; Jan, 2010.
Clark RAF. Fibronectin in Wound Healing: Lessons from Normal Healing Processes Applied to Therapies for Progressive Burn Injury, Society for Investigative Dermatology National Meeting, Atlanta, GE, May 6, 2010.
Clark RAF, Lin F, Macri L, Lanier S, Tonnesen MG, and Singer AJ. Novel Fibronectin (FN) Peptide Protects Adult Human Dermal Fibroblasts (AHDF) from Oxidative- and Cytokine-Induced Death and Inhibits Burn Injury Progression, European Society for Investigative Dermatology, Budapest, Hungary, September 12, 2009.
Dadsetan M and Yaszemski MJ. Doxorubicin Release from Microspheres Encapsulate within Oligo (Polyethylene Glycol) Fumarate Hydrogel. American Chemical Society National Meeting, Washington DC, August 16-20, 2009.
Dadsetan M and Yaszemski MJ. Incorporation of Electrical Charge into Oligo (Polyethylene Glycol) Fumarate Hydrogel for Cartilage Regeneration. American Chemical Society National Meeting, Washington DC, August 16-20, 2009.
Devore D, Peddada L, and Roth C. Graft Copolymer–Liposome Complexes Enhance Extracellular Delivery of Antisense Oligonucleotides. 83rd Colloid and Surface Science Symposium, June 19, 2009.



Appendix B: Publications and Presentations

Table B-2a. Presentations and Non-Peer-Reviewed Publications: Rutgers – Cleveland Clinic Consortium (cont.)

Dewar A, Clark RAF, and Frame MD. Vasoactive Effects of Curcumin in Arterioles Is Mediated by Adrenergic Receptors. American Heart Association annual meeting, San Diego, CA, January 7-10, 2010.
Dewar A, Clark RAF, Singer AJ, and Frame MD. Curcumin at Nanomolar Doses Vasodilates the Terminal Arteriole of the Capillary Network. AFIRM All Hands Meeting, St Pete's Beach, FL, Jan. 15, 2010.
Dewar A, Taira B, Singer AJ, Clark RAF, and Frame MD. Curcumin at nM Concentrations Limits Burn Injury Progression and Causes B-Adrenergic Receptor-Mediated Vasodilation. Society for Investigative Dermatology National Meeting, Atlanta, GE, May 6, 2010.
Heylman CM and Muschler GF. The Effect of Oxygen Kinetics on Colony Formation by Osteogenic CTPs In Vitro. <i>Proceedings of Armed Forces Institute of Regenerative Medicine All Hands Meeting</i> , (2010).
Heylman CM, Patterson TE, Rozic R, Boehm CA, Nakamoto C, and Muschler GF. Distribution of Human Connective Tissue Progenitors in Trabecular Bone and Bone Marrow. <i>Proceedings of Advanced Technology Applications for Combat Casualty Care</i> , (2009).
Heylman CM, Patterson TE, Rozic R, Boehm CA, Nakamoto C, and Muschler GF. Distribution of Human Connective Tissue Progenitors in Trabecular Bone and Bone Marrow. <i>Proceedings of Bones and Teeth Gordon Research Conference</i> , (2009).
Hwang NS, Anderson D, and Langer R. Guided Differentiation of Mesenchymal Stem Cells on Cell-Patterned Substrates. Abstract submitted to Experimental Biology Meeting 2010.
Hwang N, Anderson D, and Langer R. Use of Morphogenetic Factors from Chondrocytes for Stem Cell-Based Articular Tissue Engineering. Abstract submitted to Orthopaedics Research Society 2010.
Shengxian J, Yanan Z, Singer A, Clark RAF, and Mustoe TA. Systemically Administrated Curcumin Can Significantly Promote Wound Healing and Improve Hypertrophic Scarring.
Khademhosseini J, Vacanti J, and Langer R. Progress in Tissue Engineering. <i>Scientific American</i> , 300:64-71, 2009.
Kim D, Sperandeo M, Palat S, Clark RAF. The Impact of Pyruvate and Cell Density on Oxidative Stress Assays. AFIRM All Hands Meeting, St Pete's Beach, FL, Jan. 15, 2010.
Kim J, Magno MHR, Alvarez P, Darr A, Kohn J, and Hollinger JO. rhBMP-2 Treated Tyrosine-Derived Polycarbonates for Craniofacial Bone Regeneration: In vitro. AFIRM all hands on meeting 2010 in Tampa, FL.
Kim J, Magno MHR, Doll B, Waters H, Sharma A, McBride S, Darr A, Kohn J, and Hollinger JO. Bone Regeneration In Rabbit Calvaria Using rhBMP-2 Treated Tyrosine-Derived Polycarbonates. AFIRM all hands meeting 2010 in Tampa, FL.
Kim J, Magno MHR, Srinivasan A, Darr A, Kohn J, and Hollinger JO. Development of Tyrosine-Derived Polycarbonates as Bone Tissue Engineering Scaffolds, SFB annual meeting 2010 in Seattle, WA.
Kim J, Magno MHR, Srinivasan A, Darr A, Kohn J, and Hollinger JO. Pre-Osteoblast Cell Response on Three Dimensional Porous Tyrosine-Derived Polycarbonate Scaffolds. BMES annual meeting 2009 in Pittsburgh, PA.
Kim J, Magno MHR, Srinivasan A, Kohn J, and Hollinger JO. Potential of Tyrosine-Derived Polycarbonates as Tissue Engineering Scaffolds for Treatment of Craniofacial Bone Defects. 2nd TERMIS International Congress 2009 in Seoul, Korea.
Kohn J, et al. Poster presentations at the AFIRM conference "All Hands Meeting," St. Pete Beach, Florida, USA, January 2010.
Langer R. Perspectives and Challenges in Tissue Engineering and Regenerative Medicine. <i>Advanced Materials</i> , 21:3235-3236, 2009 (Editorial).
Lanier AT, McClain SA, Lin F, Tonnesen MG, Singer AJ, and Clark RAF. Spatiotemporal Progression of Tissue Death Surrounding Burns. Society for Investigative Dermatology National Meeting, Atlanta, GA, May 6, 2010.
Lanier ST, McClain SA, Lin F, Singer AJ, and Clark RAF. Apoptosis in the Zone of Ischemia Surrounding Burns AFIRM All Hands Meeting, St Pete's Beach, FL, Jan. 15, 2010.
Li B and Guelcher SA. Dual Delivery of Growth Factors and Antibiotics from Polyurethane Scaffold Improves Tissue Regeneration in Infected Bone Wounds Abstract submitted to the TERMIS 2nd World Congress 02WC, August 31 – Sept 3, 2009, Seoul, KOR.

Table B-2a. Presentations and Non-Peer-Reviewed Publications: Rutgers – Cleveland Clinic Consortium (cont.)

Li B, T Yoshii, and SA Guelcher. Controlled Delivery of BMP-2 from Polyurethane Scaffolds Promotes New Bone Formation in Rat Femoral Defect. Abstract submitted to the Society for Biomaterials Annual Meeting, 2009.
Lim T, Lin F, Taira BR, Singer AJ, McClain SA, and Clark RAF. Effect of IV deferoxamine on Burn Wound Progression. American College of Emergency Physicians Annual Meeting, Boston, MA, October 5-6, 2009.
Lin F, Tonnesen MG, and Clark RAF. Novel Fibronectin-Derived Peptide Protects Human Fibroblast from Oxidative/Cytokine Stress-Mediated Cell Death. AFIRM All Hands Meeting, St Pete's Beach, FL, Jan. 15, 2010.
Luangphakdy V, Shinohara K, Pan H, Griffith LM, Kohn J, Yaszemski MJ, and Muschler GF. Systematic Evaluation of Advanced Osteoconductive Scaffolds for Bone Repair in the Canine Femoral Multi-Defect Model (poster). Armed Forces Institute of Regenerative Medicine (AFIRM) All Hands Meeting, Saint Petersburg, FL, January 13-15, 2010.
Macri LK, Sheihet L, Singer AJ, Kohn J, and Clark RAF. Peptide-Deliverig Fibro-Porous Mats to Accelerate Skin Regeneration. AFIRM All Hands Meeting, St Pete's Beach, FL, Jan. 15, 2010.
Magno MHR, Kim J, Srinivasan A, Darr A, Hollinger JO, and Kohn J. Tyrosine-Derived Polycarbonate Scaffolds for Bone Regeneration in the Craniofacial Complex. The ATACCC Conference 2009 in Tampa, FL.
Magno MHR, Kim J, Srinivasan A, Darr A, Hollinger JO, and Kohn J. Tyrosine-Derived Polycarbonate Scaffolds for Craniofacial Bone Regeneration. AFIRM All Hands on Meeting 2010 in Tampa, FL.
Niraj Ramachandran, Carmine Iovine, Richard Clark, Adam Singer, Masood Saeed and Joachim Kohn. Polymeric Iodine Based, Absorbent, Antimicrobial Wound Dressing. St. Pete Beach, Florida, USA, January 2010. Poster presentations at the AFIRM conference "All Hands Meeting."
Ramachandran N, Iovine C, Diamond M, Rosenblatt S, Clark R and Kohn J. Polymeric Iodophor Based Antimicrobial Wound Dressing. St. Pete Beach, Florida, USA, August, 2009. Poster presentation at the ATACC conference.
Raut VP, Stockdale LA, Boehm CA, Patterson TE, Clark RAF, Griffith LG, Muschler GF. Molecular Surface Design with Tethered Bioactive Molecules for Enhancement of In-Vitro Performance of Connective Tissue.
Raut VP, Stockdale LA, Boehm CA, Patterson TE, Clark RAF, Griffith LG, Muschler GF. Advancing Bone Repair Using Molecular Surface Design with Tethered Fibronectin-Derived Peptide (P12) for Enhancement of Connective Tissue Progenitors. <i>Proceedings of Advanced Technology Applications for Combat Casualty Care</i> , (2009).
Runge MB, Dadsetan AK, Windebank A, Yaszemski MJ. Development of Electrically Conductive Polymeric Scaffolds for Nerve Regeneration (poster). Armed Forces Institute of Regenerative Medicine (AFIRM) All Hands Meeting, Saint Petersburg, FL, January 13-15, 2010.
Runge MB, Dadsetan M, Baltrusaitis J, Ruesink T, and Yaszemski MJ. Biocompatibility of Polycaprolactone Fumarate-Polypyrrole Composite Materials: Effect of Anionic Dopant on Cell Viability. American Chemical Society National Meeting, Washington DC, August 16-20, 2009.
Runge MB, Dadsetan M, Baltrusaitis J. Ruesink T, and Yaszemski MJ. Evaluation of Electrically Conductive and Non-Conductive Porous Three-Dimensional Scaffolds. American Chemical Society National Meeting, Washington DC, August 16-20, 2009.
Runge MB, Dadsetan M, Ruesink T, and Yaszemski MJ. Evaluation of Electrically Conductive and Non-Conductive Porous 3-Dimensional Scaffolds (poster). Armed Forces Institute of Regenerative Medicine (AFIRM) All Hands Meeting, Saint Petersburg, FL, January 13-15, 2010.
Runge MB, Dadsetan M, and Yaszemski MJ. Development of Conducting Polymer Composites for Peripheral Nerve Regeneration. Armed Forces Institute of Regenerative Medicine (AFIRM) All Hands Meeting, Saint Petersburg, FL, January 13-15, 2010.
Salomon S, Sheihet L, Batheja P, Singer A, Michniak-Kohn B, Clark R and Kohn J. Topical Curcumin-Containing Therapies to Limit Burn Injury Progression. St. Pete Beach, Florida, USA, 2010.
Sharma A, Kim J, Runge B, Alvarez P, Dadsetan M, Yaszemski MJ, and Hollinger JO. Potential of Poly (ε-caprolactone fumarate) at Bone Tissue Engineering Scaffolds SFB annual meeting 2010 in Seattle, WA.
Sharma A, Kim J, Runge MB, Alvarez P, Dadsetan M, Yaszemski MJ, and Hollinger JO. The Potential of Poly(ε-Caprolactone Fumarate) as Bone Tissue Engineering Scaffolds. AFIRM all hands on meeting 2010 in Tampa, FL.



Appendix B: Publications and Presentations

Table B-2a. Presentations and Non-Peer-Reviewed Publications: Rutgers – Cleveland Clinic Consortium (cont.)

<p>Sheihet L, Salomon S, Batheja P, Singer A, Michniak B, Clark R and Kohn J. Curcumin-Loaded Nanospheres as a Therapy to Limit Burn Injury Progression and to Promote Non-Scar Healing. St. Pete Beach, Florida, USA, August 2009. Poster presentations at the ATACCC All Hands Meeting.</p>
<p>Shinohara K, Luangphakdy V, Pan H, Griffith LM, Kohn J, Yaszemski MJ, and Muschler GF. Systematic Evaluation of Advanced Osteoconductive Scaffolds for Bone Repair in The Canine Femoral Multi-Defect Model (poster). 56th Annual Meeting of the Orthopedic Research Society, New Orleans (LA), March 6-9, 2010.</p>
<p>Singer AJ, Taira BR, Lin F, McClain SA, Lim T, Andersen R, and Clark RAF. A Novel Fibronectin-Derived Peptide Reduces Injury Progression in a Rat Comb Burn Model. AFIRM All Hands Meeting, St Pete's Beach, FL, Jan. 15, 2010.</p>
<p>Singer AJ, Taira BR, Lin F, McClain SA, Lim T, Andersen R, and Clark RAF. Reduction of Burn Injury Progression After Intravenous Purified Curcumin in Rat Comb Burns. AFIRM All Hands Meeting, St Pete's Beach, FL, Jan 15, 2010.</p>
<p>Sorrell JM and Caplan AI. Fibroblasts—A Diverse Population at the Center of It All. <i>Int Rev Cell Mol Biol</i>, 276, 161-214, 2009.</p>
<p>Sorrell JM, Baber MA, and Caplan A I. Influence of Adult Mesenchymal Stem Cells on In Vitro Vascular Formation. In: <i>Advances in Tissue Engineering</i>, Volume 1, Angiogenesis. Johnson PC and Mikos AG eds. Mary Ann Liebert, New Rochelle, NY, pp. 230-240, 2010.</p>
<p>Tanner SA and Guelcher SA. Injectable Allograft Bone/Polymer Composites for the Treatment of Craniofacial Bone Defects. Abstract submitted to the TERMIS 2nd World Congress 02WC, August 31 – Sept 3, 2009, Seoul, KOR.</p>
<p>Tholpady ST, Arnold PB, Morgan RF, Ogle RC, and Katz AJ. Fat Grafting and Stem Cell Biology. In <i>Fat Injection: From Filling to Regeneration</i>. Sydney R. Coleman and Riccardo F. Mazzola, Editors; Quality Medical Publishing, Inc., St. Louis, Missouri, 2009.</p>
<p>Vaughan AD, Gatt C, Dunn M, and Kohn J. Degradable/Resorbable Polymer Fibers for Anterior Cruciate Ligament (ACL) and Meniscus Repair and Replacement, <i>2010 AFIRM Conference</i>, January 11-15, 2010.</p>
<p>Vaughan AD, Gatt C, Dunn M, and Kohn J. Development of Resorbable Polymer Fibers for Tissue Engineered Scaffolds for Anterior Cruciate Ligament (ACL) and Meniscus Repair and Replacement, <i>2009 Advanced Technology Applications for Combat Casualty Care (ATACCC) Conference</i>, August 10-12, 2009.</p>
<p>Wang H, Hébert-Blouin MN, Spinner RJ, Yaszemski MJ, and Windebank AJ. Creation of an Ischemia/Fibrosis Limb Model and Its Impact on Nerve Regeneration. AFIRM All-Hands Meeting, St. Petersburg, FL, January 11-15, 2010.</p>
<p>Wang H, Hébert-Blouin MN, Windebank AJ, Spinner RJ, and Yaszemski MJ. Nerve Regeneration in the Scarred and Ischemic Limb. Annual Meeting of American Society for Peripheral Nerve, January 8-10, 2010, Boca Raton, FL.</p>
<p>Wang H, Hébert-Blouin MN, Windebank AJ, Yaszemski MJ, and Spinner RJ. Nerve Regeneration in the Scarred and Ischemic Limb. The 18th Meeting of the Sunderland Society, October 31 –November 3, 2009, Shanghai, China.</p>
<p>Windebank A, Spinner R, Dyck PJ, Bishop A, Razonable R, Wang H, Wettstein P, and Yaszemski M. A Clinical Trial to Assess the Safety of a Novel Scaffold Biomaterial. AFIRM All-Hands Meeting, St. Petersburg, FL, January 11-15, 2010.</p>
<p>Yaszemski MJ, Windebank AJ, and Wang H. Tissue Engineering: Application in Bone, Cartilage and Nerve Regeneration. 3rd World Congress of Gene, December 1-7, 2009, Foshan, China.</p>
<p>Yaszemski MJ, Windebank AJ, and Wang H. AFIRM Integrated Nerve Regeneration Program: Program Status and Research Projects. AAOS Extremity War Injuries Symposium V: Barriers to Return of Function and Duty. January 27-29, 2010, Washington DC.</p>
<p>Yanan Z, Shengxian J, Nunez J, Hong SJ, Vracar-Grabar M, and Mustoe TA. Laboratory for Wound Repair and Regenerative Medicine, Department of Surgery, Northwestern University, Chicago, IL 60611, USA. Differential Effects of Topically Applied Rabbit Adipose- and Human Bone Marrow-Derived Stem Cells on Wound Healing in a Rabbit Ear Model.</p>

Table B-2b. Non-Peer-Reviewed Publications and Presentations: Wake Forest – Pittsburgh Consortium

Bae JH, Kang H-W, Ladd M, Atala A, Yoo JJ, and Lee SJ, Development of In Vitro Skin Expansion Bioreactor System for Burn Injuries. 2010 AFIRM All-Hands Meeting, January 11-15, St. Pete Beach, FL, USA.
Christ GJ. Rodent Latissimus Dorsi as a Model System for Evaluating Tissue Engineered Skeletal Muscle. TERMIS Meeting, Seoul, South Korea, August 31st, 2009.
Chun Y, Ingham SJM, Irrgang J, Hagen T, Fu Freddie, Gharaibeh B, Wright V, and Huard J. Improving Recovery Following Recurrent Hamstring Injury Using an Angiotensin II Receptor Blocker: Two Case Studies. Orthopaedic Research Society. New Orleans. March 2010.
Criswell TL, Wang Z, Corona BT, and Soker S. Imaging Vasculogenesis in Regenerating Muscle Tissue In Vitro. TERMIS-EU. Galway, Ireland. June 2010.
Davissou N, McCann P, Ingham S, Sicari B, DiStefano G, Lavasani M, Gharaibeh B, and Huard J. Local Tissue Damage in a Rat Model of Hindlimb Compartment Syndrome. Children's Hospital Summer Student Poster Session, Lawrenceville Campus, Rangos Research Center, Pittsburgh, PA, July 22, 2009.
Galvez MG, Wong VW, Chang EI, Major M, Carre L, Kandimalla R, Bhatt KA, Rajadas J, Longaker MT, and Gurtner GC. Pullulan-Collagen Hydrogel Scaffold as a Dermal Substitute. American College of Surgeons 95th Annual Meeting, Chicago, IL. Oct. 11-15, 2009. Abstract/podium presentation.
Guldberg RE. Engineered Delivery of Spatial and Temporal Cues for Composite Tissue Injury Repair, Armed Forces Institute of Regenerative Medicine (AFIRM) All-Hands Meeting, January 12, 2010, Tampa, Florida.
Guldberg RE. Methods for Evaluating Vascularization Within Regenerating Bone. Invited speaker, Evaluation and Manipulation of Angiogenesis in Bone Regeneration Workshop, Orthopaedic Research Society Meeting, March 7, 2010, New Orleans, Louisiana.
Guldberg RE. Significance of Vascularity for Bone Defect Healing. Invited speaker for the Biomechanics and Biology of Bone Regeneration Conference, November 19, 2009, Berlin, Germany.
Ingham SJM, DiStefano G, Sicari B, Fu F, Gharaibeh B, and Huard J. A New Reliable Murine Model for Compartment Syndrome. Orthopaedic Research Society. New Orleans. March 2010.
Ingham SJM, Sun B, DiStefano G, Sicari B, Kragh JF, Gharaibeh B, and Huard J. Characterization of Human Muscle Degeneration After a Compartment Syndrome to the Lower Limb. Orthopaedic Research Society. New Orleans. March 2010.
Ju YM, Atala A, Yoo JJ, and Lee SJ. Biomaterial Induces Host Stem Cell Recruitment for In Situ Muscle Tissue, North Carolina Tissue Engineering & Regenerative Medicine Conference, November 13, 2009, Salem College, Winston-Salem, NC, USA.
Ju YM, Hwang CM, Atala A, Yoo JJ, Atala A, and Lee SJ. Biomaterial induces Host Stem Cell Recruitment for In Situ Muscle Regeneration, the Society for Biomaterials 2010 Annual Meeting and Exposition: Giving LIFE to a World of Materials, April 21-24, 2010, Seattle, WA, USA.
Ju YM, Hwang CM, Atala A, Yoo JJ, and Lee SJ. Biomaterial Induces Host Stem Cell Recruitment for In Situ Muscle Tissue Regeneration, 2010 All-hands Meeting, January 11-15, St. Pete Beach, FL, USA.
Kathju S and Satish L. The Chaperonin Containing T-Complex Polypeptide: Do Monomeric Subunits Have Discrete Individual Functions? In: <i>Handbook of Molecular Chaperones</i> , Durante P and Colusci L, eds. Nova Science Publishers, 2009.
Kolambkar YM, Boerckel JD, Dupont KM, Bajin M, Huebsch ND, Mooney DJ, Huttmacher DW, and Guldberg RE. Spatiotemporal BMP Delivery Enhances Functional Repair of Segmental Bone Defects. The 56th Annual Meeting of the Orthopaedic Research Society, New Orleans, Louisiana, March, 2010.
Ladd M, Romero V, Lee SJ, Atala A, and Yoo JJ, In Vitro Expanded Living Skin for Burn Injuries, Tissue Engineering and Regenerative Medicine International Society 2nd World Congress, August 31-September 3, 2009, Lotte Hotel World, Seoul, Korea.
Ladd M, Romero V, Lee SJ, Atala A, and Yoo JJ, In Vitro Expanded Living Skin for Burn Injuries, North Carolina Tissue Engineering & Regenerative Medicine Conference, November 13, 2009, Salem College, Winston-Salem, NC, USA.



Appendix B: Publications and Presentations

Table B-2b. Non-Peer-Reviewed Publications and Presentations: Wake Forest – Pittsburgh Consortium (cont.)

Ladd M, Romero V, Lee SJ, Bae JH, Kang HW, Atala A, Yoo JJ. In Vitro Expanded Skin for Burn Injuries, the Society for Biomaterials 2010 Annual Meeting and Exposition: Giving LIFE to a World of Materials, April 21-24, 2010, Seattle, WA, USA.
Lee SJ, Green D, Broda C, Atala A, and Yoo JJ, Cartilage Covered Alloplastic Medical Device Improves Implant Stability, 2010 AFIRM All-Hands Meeting, January 11-15, St. Pete Beach, FL, USA.
Lee SJ, Lee DJ, Broda C, Atala A, and Yoo JJ, Engineered Cartilage Covered Ear Implants for Auricular Reconstruction, Tissue Engineering and Regenerative Medicine International Society 2nd World Congress, August 31-September 3, 2009, Lotte Hotel World, Seoul, Korea.
Lee WPA. Bone Marrow Cell Utilization in Composite Tissue Allotransplantation: Translational and Clinical Trials. American Society of Reconstructive Transplantation Section, Annual Meeting of the American Society of Reconstructive Microsurgery, Boca Raton, FL, January 2010.
Lee WPA, Brandacher G, Schneeberger S, Shores JT, Wachtman GS, Keith JD, and Gorantla VS. The Pittsburgh Hand Transplant Program - Early Experience with a Novel Immunomodulatory Protocol in Two Patients. 89th Annual Meeting of the American Association of Plastic Surgeons, March 23, 2010.
Machingal MA, Corona BT, Kesireddy V, Andersson K, Herco M, Vishwajit S, Bishwokarma B, Zhao W, Yoo JJ and Christ GJ. Wake Bioengineered Skeletal Muscle for Defect Replacement in a Rodent Model. <i>FASEB J</i> . 2010;24:842.843.
Machingal M. Bioengineered Skeletal Muscle for Functional Defect Replacement in Rodent Muscle Injury Model. Armed Forces Institute of Regenerative Medicine All Hands Meeting, 2010.
Markert CD, Bharadwaj S, and Antinozzi P, and Furth ME. Screening of Potential Stem Cell Sources for Skin Equivalents.
Roy A, Hong W, Fatima SP, Costello BJ, Sfeir C, Mooney M, and Kumta PN. Novel Synthetic Porous and Biodegradable Bone Cement for Orthopaedic and Craniofacial Regeneration. Poster presented at the 2009 Biotech conference, Pennsylvania Convention Center, Philadelphia, November 17, 2009.
Roy A, Hong W, Fatima SP, Jinhua Li, Sfeir C, and Kumta PN. Novel Synthetic Bone for Orthopaedic and Craniofacial Regeneration. Biomedical Engineering Society Annual Meeting, October 7-10, 2009, Pittsburgh, PA, USA, Oral presentation(OP-9-1-3D).
Rustad KC, Wong VW, Galvez MG, Major MR, Nehama D, Sorkin M, Januszyk M, Rajadas J, Longaker MT, and Gurtner GC. Pullulan-Collagen Hydrogel Scaffold Based on Fetal Dermal Microarchitecture for Regenerative Wound Healing. Armed Forces Institute of Regenerative Medicine All Hands Meeting, St. Pete, FL. January 13, 2010. Poster presentation.
Wachtman GS, Jindal R, Unadkat JV, Gorantla VS, Brandacher G, Schneeberger S, and Lee WPA. Titration of Bone Marrow Cell Infusion in a Preclinical Model of Composite Tissue Allotransplantation. AFIRM All Hands Meeting 2010, St. Petersburg, FL, January 2010.
Wachtman GS, Jindal R, Unadkat JV, Gorantla VS, Brandacher G, Schneeberger S, and Lee WPA. Titration of Bone Marrow Cell Infusion in a Preclinical Model of Composite Tissue Allotransplantation. 56th Annual Scientific Meeting of the Robert H. Ivy Society of Plastic and Reconstructive Surgeons, Hershey, PA, March 6, 2010.
Wachtman GS, Jindal R, Unadkat JV, Gorantla VS, Brandacher G, Schneeberger S, and Lee WPA. Titration of Bone Marrow Cell Infusion in a Preclinical Model of Composite Tissue Allotransplantation. 55th Annual Scientific Meeting of the Plastic Surgery Research Council, San Francisco, CA, May 22, 2010.
Wong VW, Bhatt KA, Vial IN, Dauskardt RH, Longaker MT, and Gurtner GC. Mechanomodulation of the Wound Environment to Decrease Scar Formation in a Porcine Model. Armed Forces Institute of Regenerative Medicine All Hands Meeting, St. Pete, FL. Abstract/podium presentation.
Yang TB, Dohar JE, Hebda PA. Combinatorial Anti-Inflammatory Therapy in Decreasing Subsequent Fibrotic Fibroblast Activity in the Wound Bed. (Selected for Podium Presentation) Symposium for Advanced Wound Care-Wound Healing Society Joint Meeting. Orlando, FL. April 17-20, 2010. <i>Wound Repair Regen</i> 18:A16 (2010).

Table B-2c. Presentations and Non-Peer-Reviewed Publications: U.S. Army Institute of Surgical Research

Brown KV, Li B, Guda T, Perrien D, Guelcher S, and Wenke JC. Decreasing Complications in Open Fractures Using a Novel Bone Graft. British Trauma Society 2010.
Brown KV, Li B, Guda T, Perrien D, Guelcher S, and Wenke JC. Decreasing Complications in Open Fractures with a Novel Bone Graft. Orthopaedic Research Society, New Orleans 2010.
Brown KV, Murray CK, and Wenke JC. Earlier Debridement and Antibiotic Administration Decrease Infections. Combined Services Orthopaedic Society 2010.



Appendix C: Patent Applications and Invention Disclosures

The attribution of inventions and patent applications to specific research support is subject to varying interpretations in the absence of a standard definition. Optimally, only those patents and patent applications displaying the AFIRM contract number in the government Interest field in the U.S. Patent and Trademark Office (USPTO) patent application record should be included as directly attributable to the AFIRM program; however, this strict definition would exclude provisional patent applications left undisclosed to the public and recently filed applications not yet included in government databases. Rather than using this rigid definition for the analysis, the following nonvalidated definitions were applied to self-reported intellectual property milestones:

- A self-reported invention disclosure filed with the inventor's institutional technology licensing office during either PY1 or PY2 is attributed to the AFIRM program in that program year.
- A self-reported patent application filed with a government patent office between October 2008 and May 2009 is attributed to the AFIRM program in PY1, and a self-reported patent application filed with a government patent office from June 2009 through May 2010 is attributed to the AFIRM program in PY2.
- No self-reported patent issued in 2008 or 2009 is attributable to the AFIRM program. (According to the USPTO Performance and Accountability Report Fiscal Year 2008, the average patent pendency period for Biotechnology & Organic Chemistry is approximately 35 months. Even applications filed under accelerated examination status can take up to 12 months before final disposition.) In subsequent years, a patent award will be attributable to the AFIRM based on the original patent application filing date meeting the minimal criteria for patent applications above.

All self-reported patent application numbers and inventors (i.e., principal investigators) were queried against the World Intellectual Property Organization (WIPO) patent application database <http://www.wipo.int/pctdb/en/> and the USPTO Ap-pFT patent application database <http://patft.uspto.gov/>. The database queries were used to (1) identify patent applications filed for self-reported inventions and (2) identify and validate filing dates for patent applications.

Fourteen government-filed patent applications were self-reported by the AFIRM researchers. Six of these applications did not indicate a filing date nor were they identified on USPTO or WIPO databases and are not included in the count of second year patent applications attributed to the AFIRM program. The remaining 8 patent applications are shown below and are included in the PY2 count.

Patent Applications: Rutgers - Cleveland Clinic Consortium

Low Dose IV and Topical Treatment of Curcumin for Blood Vessel Injury. Filed on October 16, 2009. Clark, R (Stony Brook University).

Tissue Engineered Fibrocartilage Replacement (PCT/US2009/045985). Filed on June 2, 2009. Gatt C, Balint EA, and Dunn MG (University of Medicine and Dentistry of New Jersey).

Compositions and Methods for Regulating Extracellular Matrix Production in Adipose Derived Cells (Alternate Title: "Katz-ECM": Generation of ECM and Clinical Applications of Such from Human SCs Cultured in 3-D Suspension") (Serial Number 12/580417). Filed on October 16, 2009. Katz AJ (University of Virginia).

Compositions and Methods for Modular Soft Tissue Repair (Alternate Title: "Katz-Free": Serum-Free Adipogenesis of Human ASCs Formulated as 3-D Aggregates) (Serial Number 12/580419). Filed on October 16, 2009. Katz AJ and Shang H (University of Virginia).

Bone/Polyurethane Composites and Methods Thereof (Alternate Title: Synthesis and Characterization of Injectable Allograft Bone/polymer Composite Scaffolds) (PCT/US2009/062621). Filed on October 29, 2009. Dumas JE, Zienkiewicz K, Tanner SA, Prieto EM, Bhattacharyya S, and Guelcher SA (Vanderbilt University).



Appendix C: Patent Applications and Invention Disclosures

Patent Applications: Wake Forest - Pittsburgh Consortium

Systems and Methods to Affect Anatomical Structures (PCT/US2009/065754). Filed on November 24, 2009. Gulberg R, Kolambkar Y, and Hutmacher DW (Georgia Institute of Technology).

The Multi-Target Magnetic Activated Cell Sorter. Filed in 2009. Soh H-T, Bothman DP, Adams JD, and Ferguson BS (University of California, Santa Barbara).

Novel Nanostructured Smart Injectable Bone Cements for Bone Regeneration. Filed on September 17, 2009. Sfeir C, et al. (University of Pittsburgh).

Invention disclosures are not publicly reposed in standard databases; therefore, the AFIRM consortium reports are the only information source for these records. The provided information did not always indicate a date when the inventions were filed with the institutional technology licensing office, and most records did not indicate a case reference number assigned to the invention. Due to these limitations, all invention disclosures without a date or reference number were assumed, but not validated, to have been filed from June 2009 through May 2010. Also, self-reported patent applications that only listed an invention disclosure number, but not a patent application filing number or serial number, were considered invention disclosures and not patent applications.

In total, 8 invention disclosures were made by AFIRM faculty during this period (see Chapter VII, Figure VII-7). In addition, 2 patent applications listed above were not listed as invention disclosures in PY1 but are included in the sum of invention disclosures, based on the assumption that the inventions must have been disclosed to the institutional technology licensing office prior to the submission of the patent application.

Invention Disclosures: Rutgers - Cleveland Clinic Consortium

Storage Solution. Sarac T (Cleveland Clinic Foundation).

A Method for Engineering of A Replacement Autologous Outer Ear. Sundback C (Massachusetts General Hospital).

β -TCP Binding Peptide. Griffith L (Massachusetts Institute of Technology).

Mechanism of Action of IV Curcumin. Filed on June 10, 2009. Clark, R (Stony Brook University).

Invention Disclosures: Wake Forest- Pittsburgh Consortium

Compositions and Methods for Reduced Scarring in Healing Wounds and for Treatment and Prevention of Fibrosis. Kathju S and Satish L (Allegheny-Singer Research Institute).

A Wet-Electrospun Biodegradable Scaffold and Uses Thereof. Wagner WR, et al. (McGowan Institute for Regenerative Medicine).

A Novel Biohybrid Composite Scaffold of Extracellular Matrix Gel and Biodegradable Elastomeric Fibers. Wagner WR, et al. (McGowan Institute for Regenerative Medicine).

Combined Space Maintenance and Bone Regeneration System for the Reconstruction of Large Osseous Defects. Mikos AG, Wong ME, Young S, Kretlow JD, Shi M, and Kasper FK (Rice University).

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