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TITLE: Early Diagnosis and Intervention Strategies for Post-Traumatic Heterotopic Ossification in Severely Injured Extremities

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13. SUPPLEMENTARY NOTES		
14. ABSTRACT This study will recruit wounded warriors with severe extremity trauma, which places them at high risk for heterotopic ossification (HO); bone formation at abnormal sites, which causes pain, limits motion and/or limits the use of a prosthetic device. There are three goals: 1) to understand the mechanisms involved in HO; 2) to define accurate and practical methods to predict where HO will develop; and 3) to define potential therapies for prevention or mitigation of HO.		
15. SUBJECT TERMS Wound healing		

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
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INTRODUCTION

The mechanism(s) involved in heterotopic ossification (HO) in our severely injured wounded warriors are unclear. Accurate, practical methods and assessment tools (macroscopic and cellular/molecular) need to be developed to characterize wounded tissues and predict where HO may or will develop. These tools need to provide insight into the biological wound environment and events that contribute to or elicit HO. These tools also need to provide effective methods for early diagnosis or risk assessment (prediction) so that therapies for prevention or mitigation of HO can be optimally targeted. This study seeks to contribute to advancement in each of these key areas.

The research teams at Cleveland Clinic Lerner Research Institute (CC), Walter Reed National Military Medical Center (WRNMMC) and Navy Medical Research Center,(NMRC) bring together robust and complimentary experiences. The research team at CC performs quantitative wound assessment using non-invasive imaging modalities (ultrasound), *in vitro* assay and characterization of tissue-resident connective tissue progenitors (CTPs) using image analysis of colony forming unit performance, and the teams at WRNMMC and NMRC perform Raman Spectroscopy and gene expression profiling in at-risk tissue from HO+ and HO- patients.

In Year 3 team members continued sample processing and data collection. Dr. Davis's lab (NMRC) shipped slides of patient 002's cultured wound site samples to the Muschler laboratory (CC). The Muschler lab performed colony analysis on these samples. Data tables with gene transcript analysis (NMRC) of biopsied wound site samples were viewed and discussed during bi-monthly team meetings. These two patient enrollments have provided three total extremity wounds for analysis.

A modification in our initial inclusion criteria now includes both below knee and upper extremity amputations and also includes severe military limb trauma with >75 cm^2 area of open tissue. In this population, these wounds are also at risk for HO, though less than that seen in the setting of transfemoral amputations and hip disarticulation. With these enrollment criteria changes in place, we remain ready to enroll at least a total of 5 subjects.

To extend our work in the most relevant and most practical setting, we proposed using a validated rat blast overpressure amputation model and requested a NCE. Our request for a NCE, and a revised SOW was approved October 9, 2015.

The Muschler lab (CC) continued work on Specific Aim 3, *to Identify one or more potential local or topical therapeutic agents that effectively inhibit colony formation, proliferation, differentiation and/or survival of CTP-Os in vitro that could be tested in a subsequent clinical trial.*

Year 3 Refinements include:

Rat Blast Overpressure amputation model (NMRC-CC)

Dr. Davis's lab (NMRC), has established a schedule to begin surgery for this animal amputation model starting Mid-November, 2015. Day-3 and Day-7 untreated groupings (5 rats/time point) and tissue samples from uninjured-naive rats will be collected and shipped to the Muschler lab (CC) by late November through December, 2015, and resume in January, 2016.

Colony Analysis-(NMRC-CC)

Dr Davis's laboratory (NMRC) processed biopsied wound site samples collected on Patient 002. The samples were set at 10,000, 15,000 and 20,000 cells /ml at oxygen tensions 0.3%, 3%, and 20%. These slides were harvested by fixation at day 6 and shipped to the Cleveland Clinic for colony analysis. For both patient 001 and 002 samples, all oxygen tensions at plating densities 10,000 and 15,000 cells/ml were stained and scanned for Colonyze™ processing. Colony prevalence data has been calculated and alkaline phosphatase analysis on these samples is in progress.

Cell and Colony Analysis (CC)

Using discard bone samples (CC) *in vitro* work continued on Specific Aim 3, to identify one or more potential local or topical therapeutic agents that effectively inhibit colony formation, proliferation, differentiation or survival. Trabecular surface cells, isolated from discard bone (n=11), were cultured in glucose concentrations elevated from normal 5mM glucose to 25mM glucose, **(Figure 1).** The culture protocol will now start cells in normal glucose media and 16% serum, shifting to low serum conditions for two days to obtain G0/G1, and then begin treatment conditions. Fixation and stain protocols have not been changed.

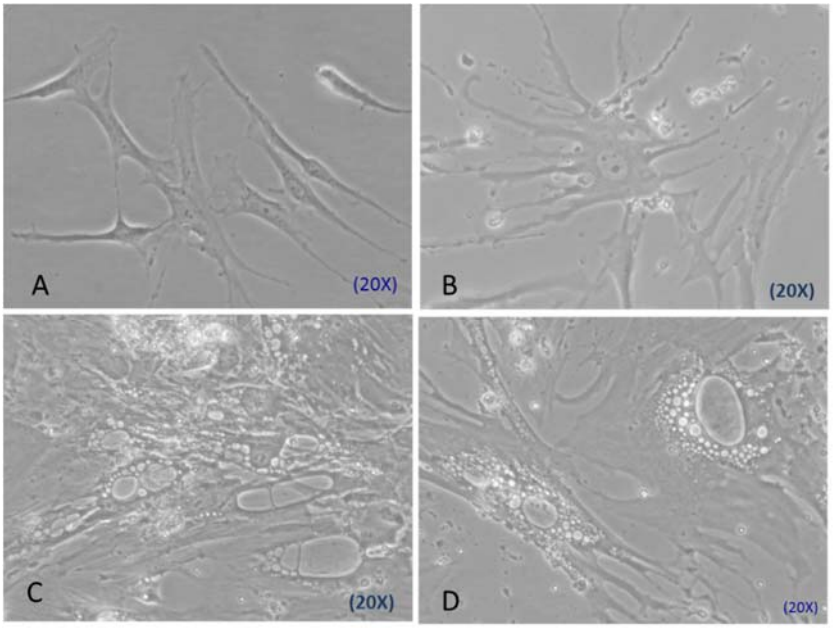


Figure 1. Phase contrast images of trabecular surface (TS) cells isolated from bone, cultured 14 days in 5mM glucose (A) and 25mM glucose, (B,C,D). Fat deposition present in high glucose (25mM) incubation conditions.

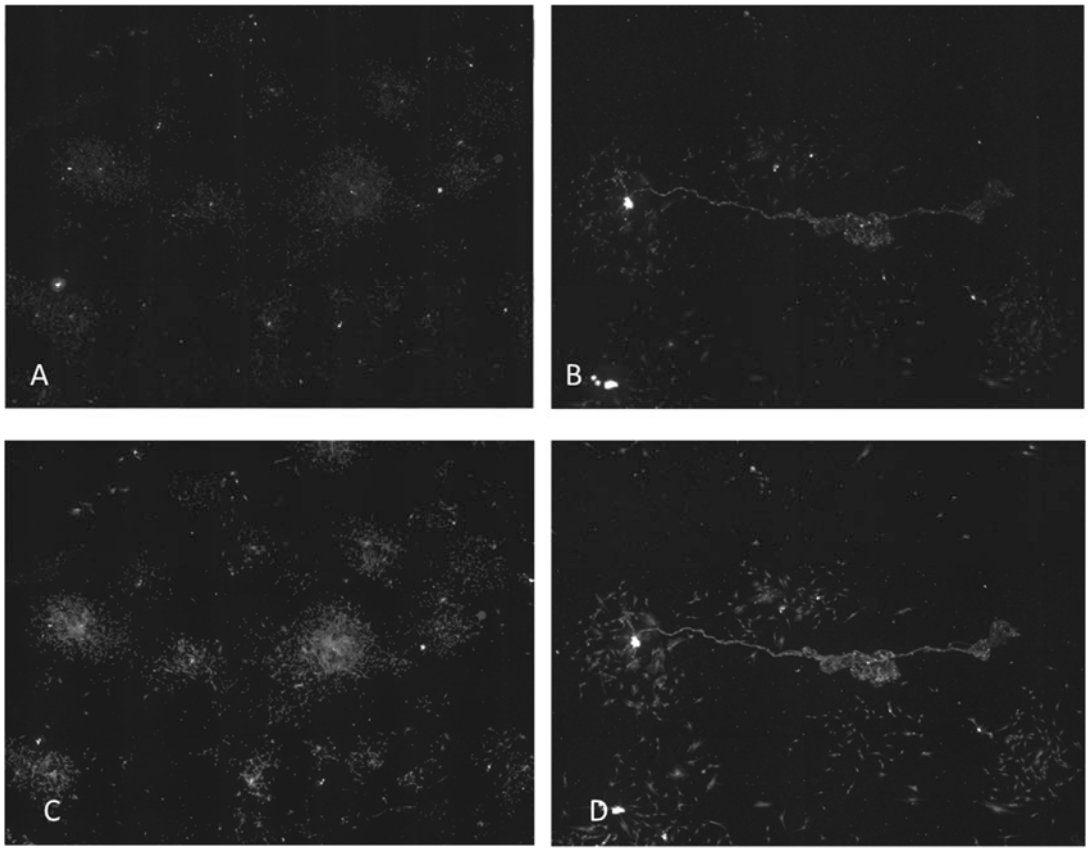


Figure 2. Trabecular surface bone cells cultured and harvested by fixation and stained with DAPI to identify cell nuclei and colonies. Cultured in 5mM glucose (A) and 25mM glucose (high glucose) (B).

Texas Red stained colonies of A and B, to identify cell alkaline phosphatase expression. Cultured in 5mM glucose (C) and 25mM glucose (high glucose) (D).

TS cultures were harvested by fixation and stained with DAPI, Nile Red, Alkaline phosphatase, and hyaluronan. Progress has been made toward optimizing image protocols for these cultures, capturing both osteogenic and adipocidic colony morphologies. On same patient samples, cultured TS cells are fixed and stained with DAPI and Nile Red stain (9-diethylamino-5H-bezo-αphenoxazine-5-one) to identify lipid droplets within the cell and on cell membranes. Imaging at (450-500nm) identifies neutral lipid droplets and imaging at (515-560 nm) identifies polarized lipid more closely associated with cell membranes and organelles, **(Figure 3).**

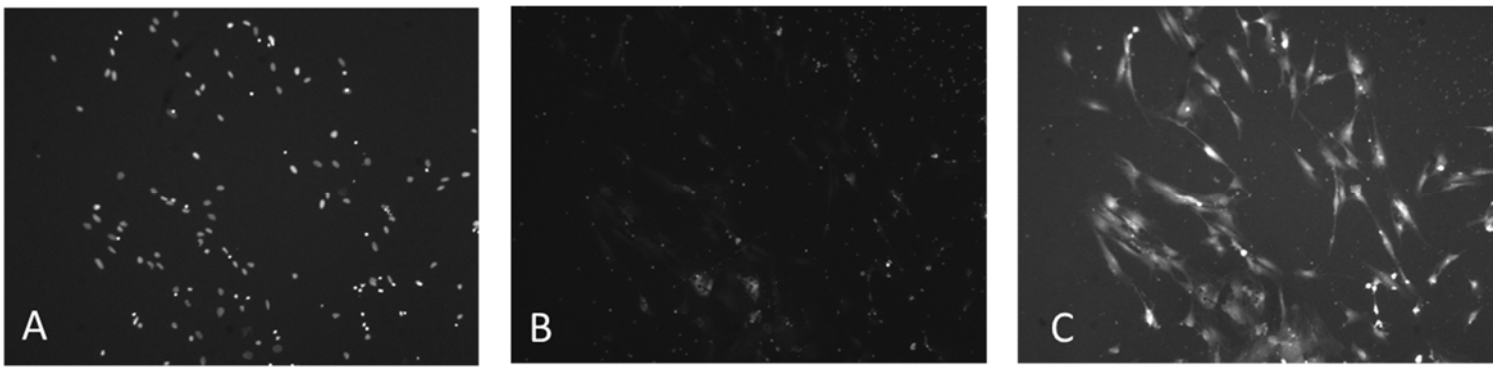


Figure 3. Trabecular surface bone cells cultured in 25mM glucose (high glucose) and stained with DAPI to identify cell nuclei,(A),and lipids stained with Nile Red , (B) and (C). (B) Imaged for neutral lipid visualization and (C) imaged for polarized lipids, same sample. All images, 10X.

A stain protocol for Hyaluronan (HA) now includes both permeabilized and non-permeabilized fixation conditions, to further characterize membrane and cellular HA. **Figure 4.** below shows the presence of HA in trabecular surface cells cultured in low (top panel) and high (bottom panel) Glucose. DAPI images show colony morphology difference in cells grown in high glucose, and image F shows increased HA expression in high glucose.

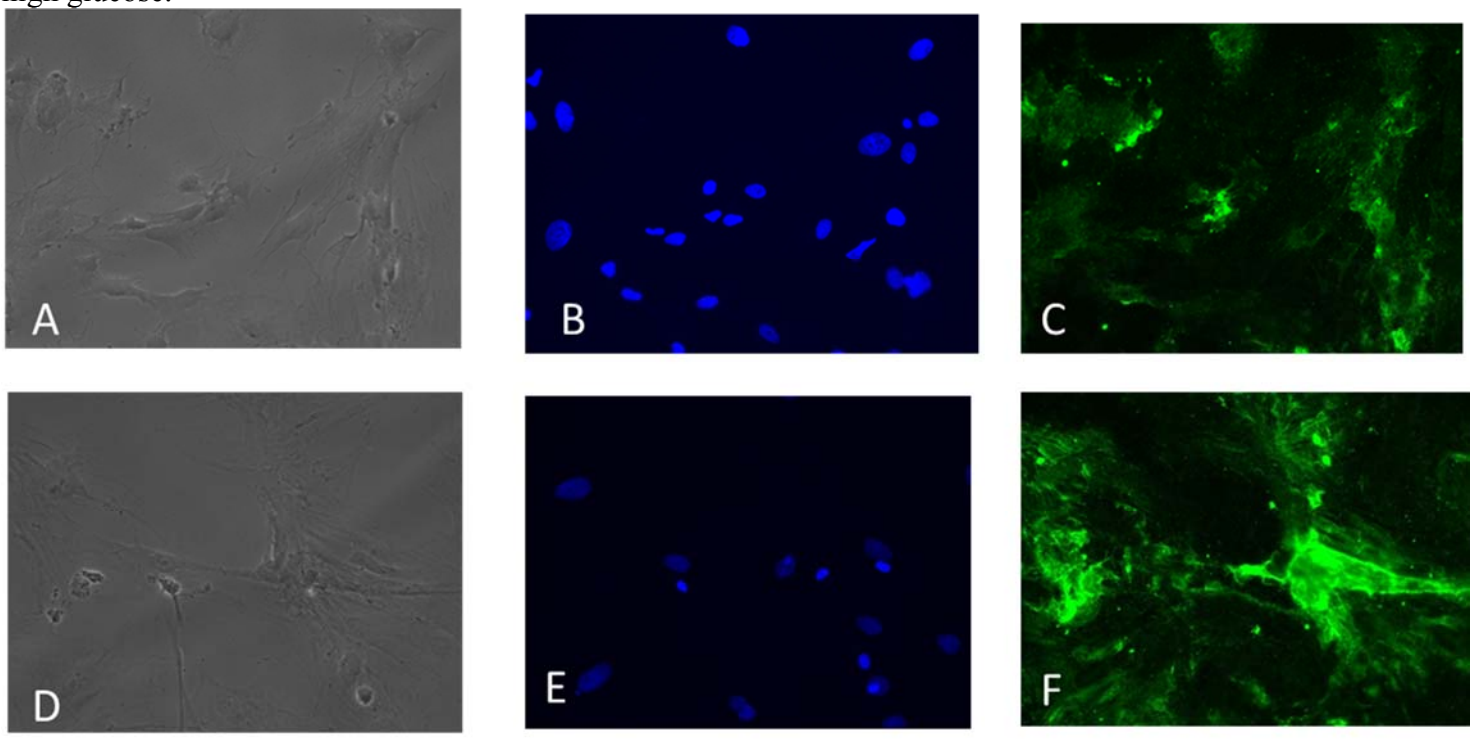


Figure 4. Trabecular surface cells cultured and harvested by fixation, non-permeabilized and imaged using phase contrast, (A,D), and stained with DAPI,(B,E) and HA, (C,F), all images 10X.

Gene Array (NMRC)
Using a custom gene array for assessing adipogenic, chondrogenic, osteogenic, angiogenic and wound healing mRNA transcripts, biopsied wound sites were analyzed based on either a 4 fold increase or 4 fold decrease, presented as genes over expressed or under expressed in injured muscle vs. control muscle. Gene analysis on Patients 001 and 002's biopsied tissue and control muscle tissue samples are complete. In samples collected from tissues sites that ultimately developed HO, 16 genes were identified with a 4-fold upregulation, and 5 genes were identified with a 4 fold decrease in gene expression. See Table in appendix .

Histology Analysis-(NMRC-CC)
Histoserv, Inc. received human samples from NMRC and processed this tissue to provide histology samples for H+E and Masson's trichrome stain, and immunostain for CD3, CD14 and MPO. Unstained sections were also provided for future shipment to CC for tissue HA analysis.

Ultrasound -(CC-WRNMMC)
Dr. Russell Fedewa completed the validation of the ultrasound data collection SOP, there has been no change in this protocol during this past funding period.

Raman Spectroscopy (WRNMMC)
An SOP for *in vivo* RS of injured muscle and pre-HO tissue is in place, there has been no change in this protocol during this past funding period.

Data Storage and Sharing-(WRNMMC-NMRC)
- Sharing includes only de-identified clinical data, wound descriptors, cell harvest and plating details, and CTP quantification and characterization.
- A SharePoint site hosted by WRNMMC remains as a dedicated site for use of this study under the Regenerative Medicine Department at NMRC and access from the WRNMMC and CC.
- SharePoint access is password protected per user and controlled by defined user roles.
- Data will be stored in Microsoft Access databases with separate data entry forms for each study site. Version control has been established via SharePoint utilities requiring databases to be "checked out" prior to editing.
- Data tables required to capture study data at each step - from patient enrollment through final sample analyses have been finalized. The interface between the individual, site-specific Access databases and the common, centralized database will require revision. Remaining tasks will not hinder the progress of the study at this time and will in no way affect patient care or data quality.

KEY RESEARCH ACCOMPLISHMENTS
- Methods and SOPs for the rat blast overpressure amputation model have been established **(NMRC).**
- Gene Expression data collection has been completed on biopsied wound samples from patients 001 and 002, **(NMRC)**
- Specific Aim 3 cell culture and stain SOPs for Fat and hyaluronan have been optimized, colony assay profiles to capture both osteogenic and adipocidic colony prevalence have been optimized, **(CC).**

REPORTABLE OUTCOMES
- In patient 001's four wound sampling sites, at 3 months from injury, only site 3 did not go on to form heterotopic ossification, (HO).
- In patient 001, when examining gene expression among a series of the 84 gene transcripts, it was found that site 3 was distinctly different than the other three sites.
- Blast injuries sustained by Patient 002 at 3 month from injury, showed sites 3, 5, 6, and 8 did not go on to form HO.

- Bone discard samples obtained from Dr. Muschler's surgery patients provide a suitable source of tissue sample that will continue to further elaborate cell characteristics to alter local colony performance.

CONCLUSION

The data from patient 001 offers substantial hope that a rational pattern or combination of gene expression might be used to predict HO occurrence. Of the four sites that were biopsied (1,2,3,4) only site 3 did not go on to develop HO. Gene expression among a series of the 84 gene transcripts, indicate site 3 was distinctly different than the other three sites. We are currently analyzing the gene expression wound site data for patient 002, to identify any similarities in sites that did not go on to develop heterotopic ossification, and to compare these sites to patient 001. Similar patterns of gene expression changes may provide important clinical information and assist in differentiating between future HO sites compared to non-HO sites. We continue to actively screen new patients presenting at WRNMMC.

APPENDICES

1. **Figure 1 Flow Chart**
2. **Table 1 Osteo MSCs**
3. **Quad Chart**
4. **Overexpressed Gene Table**

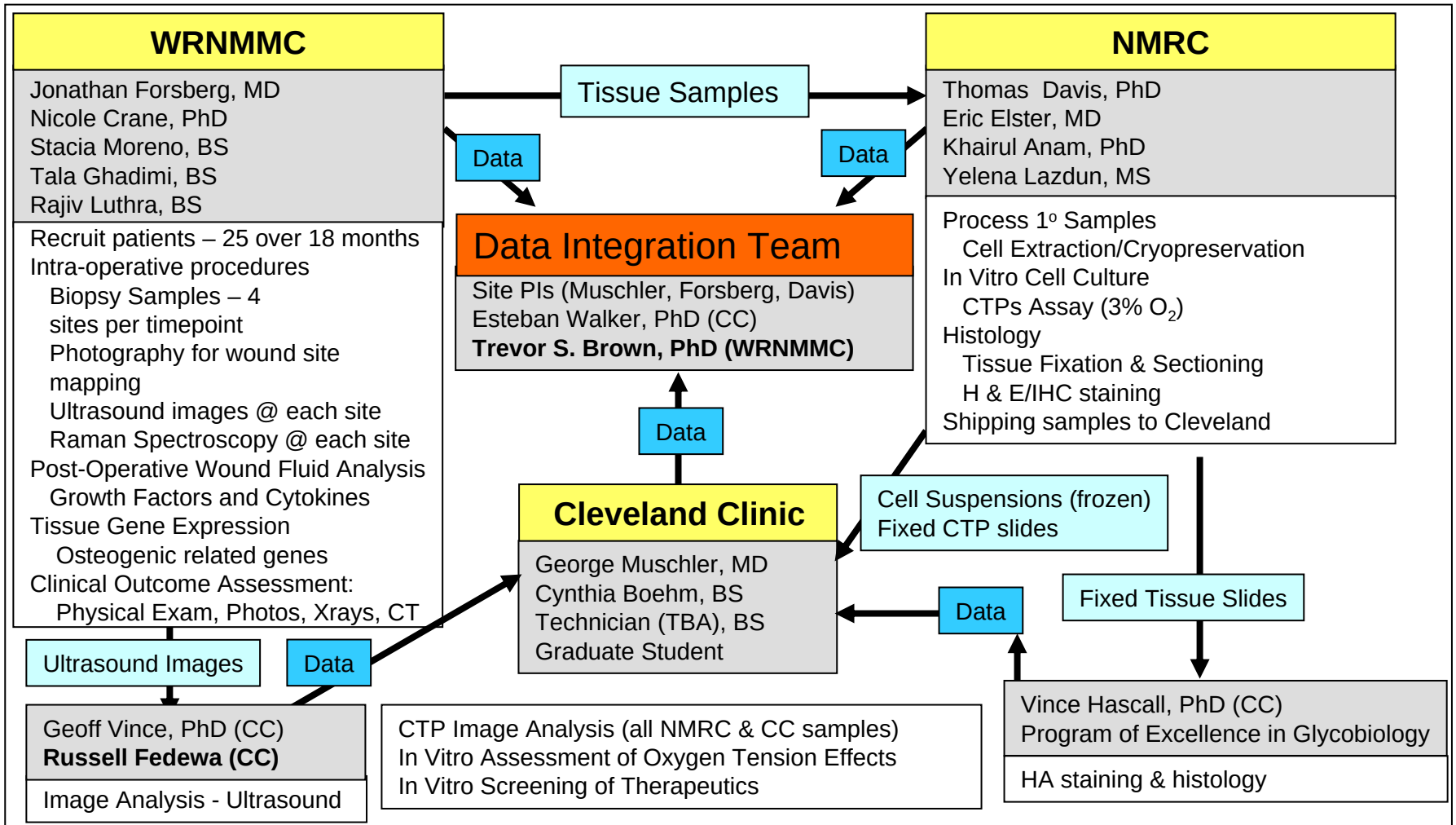


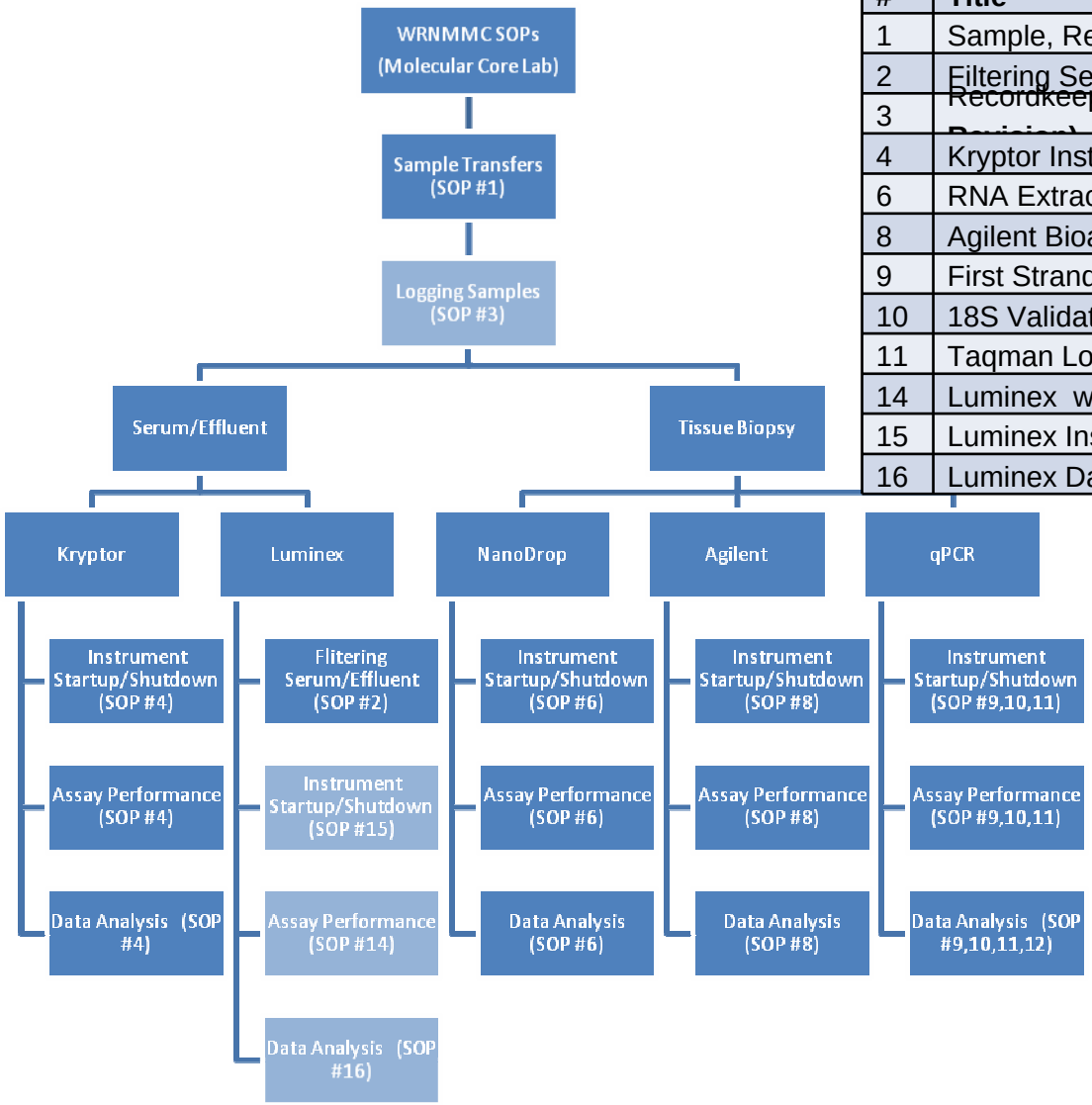
Figure 1. Lab Roles and Flow of Samples and Data

Figure 1

WRNMMC SOPs

Molecular Core Lab

Figure 2a



Molecular Core Lab Standard Operating Procedures	
#	**Title**
1	Sample, Receipt, Handling and Storage
2	Filtering Serum and Effluent Samples for Luminex
3	Recordkeeping for Laboratory Samples **(In Revision)**
4	Kryptor Instrument
6	RNA Extraction (Fibrous Tissue)
8	Agilent Bioanalyzer 2100
9	First Strand cDNA Synthesis
10	18S Validation Curve
11	Taqman Low Density Array
14	Luminex with Invitrogen Kit **(In Revision)**
15	Luminex Instrument **(In Revision)**
16	Luminex Data Analysis **(In Revision)**

Color Code
Complete
Under Revision
Not Available

NMRC SOPs
(DRAFT)

Figure 2b

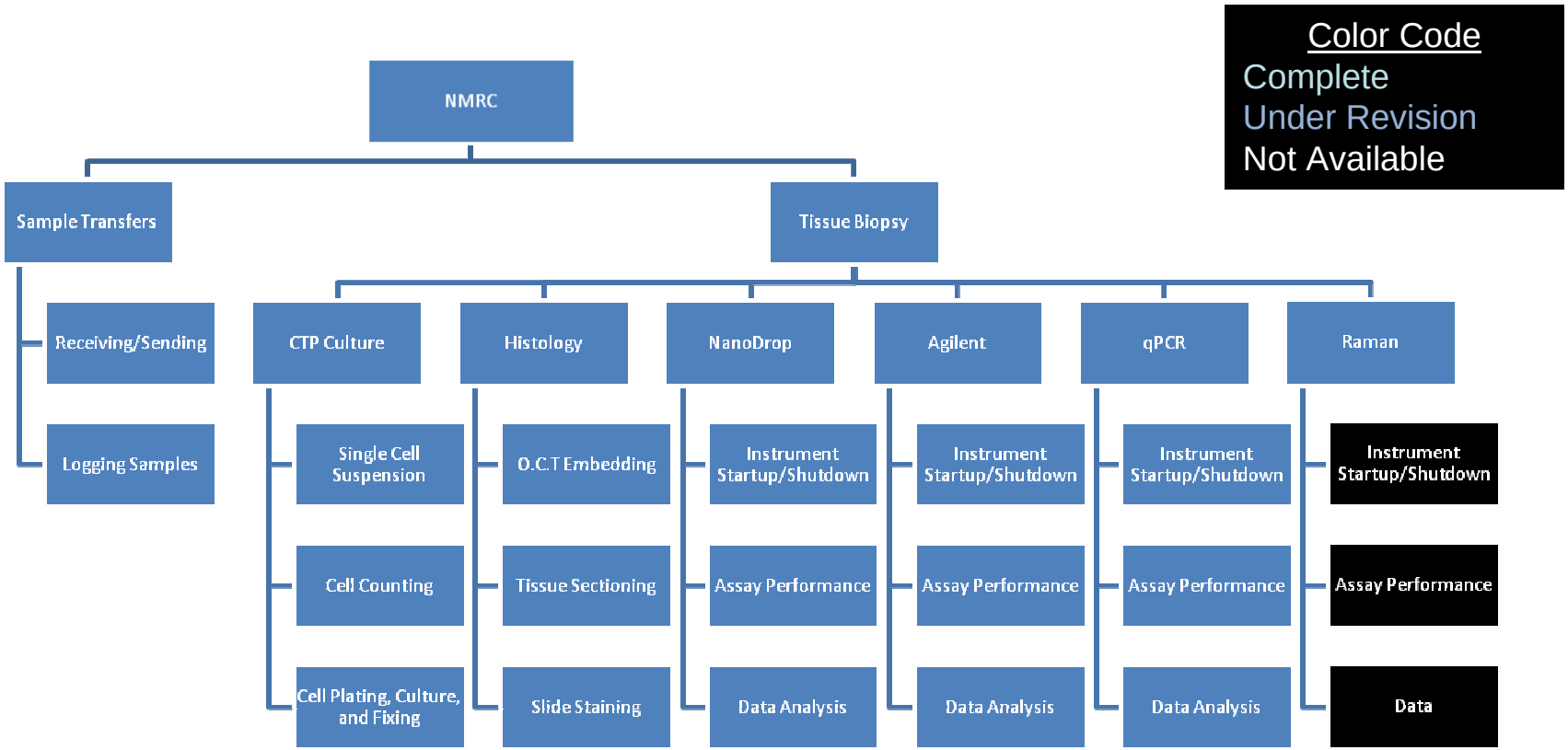
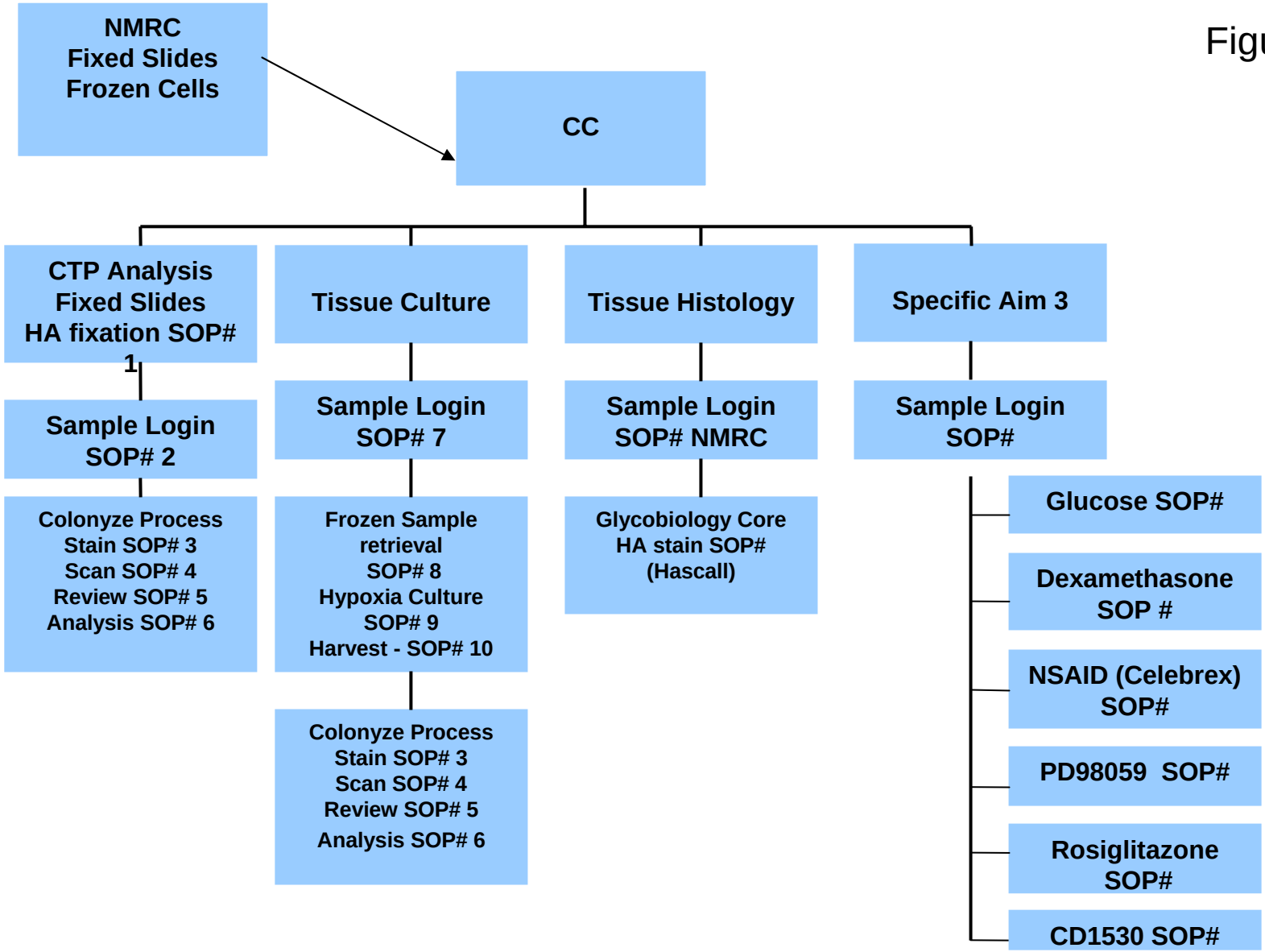


Figure 2c


NMRC
Fixed Slides
Frozen Cells
CC
CTP Analysis
Fixed Slides
HA fixation SOP# 1
Sample Login
SOP# 2
Colonyze Process
Stain SOP# 3
Scan SOP# 4
Review SOP# 5
Analysis SOP# 6
Tissue Culture
Sample Login
SOP# 7
Frozen Sample retrieval
SOP# 8
Hypoxia Culture
SOP# 9
Harvest - SOP# 10
Colonyze Process
Stain SOP# 3
Scan SOP# 4
Review SOP# 5
Analysis SOP# 6
Tissue Histology
Sample Login
SOP# NMRC
Glycobiology Core
HA stain SOP#
(Hascall)
Specific Aim 3
Sample Login
SOP#
Glucose SOP#
Dexamethasone
SOP #
NSAID (Celebrex)
SOP#
PD98059 SOP#
Rosiglitazone
SOP#
CD1530 SOP#

		Development/Cell Signaling Pathways							
Symbol	**Gene Name**	**Osteo**	**Angio**	**Adipo**	**Chondro**	**Myo**	**MSC**	**Inflam**	**Housekeeping**
ACAN	aggrecan								
ADIPOQ	adiponectin, C1Q and collagen domain containing								
ADIPOR1	adiponectin receptor 1								
ALPL	alkaline phosphatase, liver/bone/kidney								
ANGPT2	angiopoietin-2								
BMP2	bone morphogenetic protein 2								
BMP4	bone morphogenetic protein 4								
BMP6	bone morphogenetic protein 6								
BSP	bone sialoprotein								
CD44	*CD44* molecule (Indian blood group)								
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha								
COL10A1	collagen, type X, alpha 1								
COL11A1	collagen, type XI, alpha 1								
COL1A1	collagen, type I, alpha 1								
COL2A1	collagen, type II, alpha 1								
COL4A3	collagen, type IV, alpha 3								
COMP	cartilage oligomeric matrix protein								
CSF3	colony stimulating factor 3 (granulocyte)								
CXCL1 (GRO)	chemokine (C-X-C motif) ligand 1								
CXCL10 (IP-10)	chemokine (C-X-C motif) ligand *10*								
CXCL12 (SDF-1)	chemokine (C-X-C motif) ligand 12								
CXCXL5 (ENA-78	chemokine (C-X-C motif) ligand 5								
ENG	endoglin								
FADP4	fatty acid binding protein 4, adipocyte								
FGF1	fibroblast growth factor 1 (acidic)								
FGF10	fibroblast growth factor 10								
FGF2	fibroblast growth factor 2 (basic)								
FLT1	fms-related tyrosine kinase 1 (VEGFR)								
GLi2	GLI family zinc finger 2								
HAS1	hyaluronan synthase 1								
HAS2	hyaluronan synthase 2								
HAT1	histone acetyltransferase 1								
HDAC1	histone deacetylase 1								
HIF1a	hypoxia inducible factor *1 , alpha* subunit								
HNF1A	HNF1 homeobox A								
IGF2	insulin-like growth factor 2								
IL-10	interleukin 10								
IL-1B	interleukin 1, beta								
IL-6	interleukin 6 (interferon, beta 2)								
IL-8/CXCL8	interleukin 8								
ITGA1	integrin, alpha 1								
ITGA2	integrin, alpha 2								
ITGAM	integrin, alpha M								
ITGAV	integrin, alpha V (vitronectin receptor)								
ITGAX	integrin, alpha X								
JAG1	jagged 1								
KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)								
LEP	lLeptin								
LRP5	low density lipoprotein receptor-related protein 5								
MCP-1 (CCL2)	monocyte chemoattractant protein 1								
MIP-1a (CXCl3)	chemokine (C-C motif) ligand 3								
MMP9	matrix metallopeptidase 9								
MYOD1	myogenic differentiation 1								
NOTCH1	notch 1								
OCN	osteocalcin								
OCT4	octamer-binding transcription factor 4								
OMD	osteomodulin								
OPN	osteopontin								
PDGFA	platelet-derived growth factor alpha								
PHEX	phosphate regulating endopeptidase homolog, X-linked								
PPARG	peroxisome proliferator-activated receptor gamma								
PTCH1	patched 1								
PTK2	*PTK2* protein tyrosine kinase 2								
RHOA	ras homolog gene family, member A								
RUNX2 (Cbfa1)	runt-related transcription factor 2								
SCARB1	Scavenger receptor class B member 1								
SMO	smoothened, frizzled family receptor								
SMURF1	SMAD specific E3 ubiquitin protein ligase 1								
SMURF2	SMAD specific E3 ubiquitin protein ligase 2								
SOX2	SRY (sex determining region Y)-box 2								
SOX9	SRY (sex determining region Y)-box 9								
SP1	*Sp1* transcription factor								
Sp7 (OSX)	Sp7 transcription factor (Osterix)								
SPARC	secreted protein, acidic, cysteine-rich (*osteonectin*)								
TBX5	T-box 5								
TERT	telomerase reverse transcriptase								
TGFB1	transforming growth factor, beta 1								
TGFB3	transforming growth factor, beta 3								
TNF-a	tumor necrosis factor								
TWIST1	twist homolog 1								
VEGF-A	vascular endothelial growth factor A								
WNT5a	wingless-type MMTV integration site family, member 5A								
GUSB	glucuronidase, beta								
ACTB	Actin, beta								
B2M	beta-2-microglobulin								
GAPDH	glyceraldehyde-3-phosphate dehydrogenase								
HPRT1	hypoxanthine phosphoribosyltransferase 1								
RPL13A	ribosomal protein L13a								

Early Diagnosis & Intervention Strategies for Post-Traumatic Heterotopic Ossification in Severely Injured Extremities

OR110166

PI George F. Muschler, MD	**Org:** Cleveland Clinic	W81XWH-12-2-0118	Award Amount: $358,850
Co-PI Thomas Davis, PhD	Org: NMRC	W81XWH-12-2-0119	Award Amount: $276,172
Co-PI Jonathan Forsberg, MD	Org. WRNMMC	W81XWH-12-2-0120	Award Amount: $280,643

Study/Product Aims

Specific Aim 1: Characterize the heterogeneity and evolution of the biological environment in exposed tissue sites in wounded warriors under treatment for traumatic amputations of the lower extremity between week 1 and week 4 following injury using multiple modalities. (1-18 months)

Specific Aim 2: Develop a predictive model based on a minimum set of clinical variables collected in Aim 1, that will provide clinically useful prediction of where and in whom HO will form. (6-24 months)

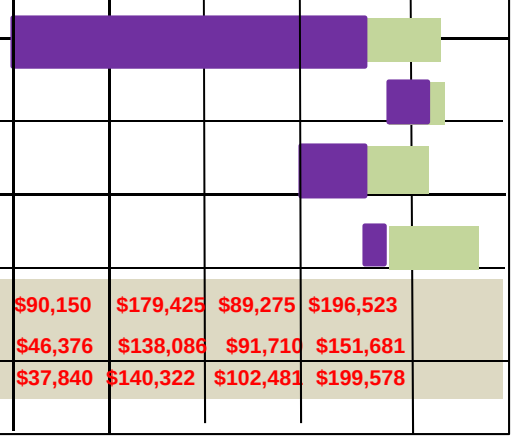
Specific Aim 3: Identify one or more potential local or topical therapeutic agents that effectively inhibit colony formation, proliferation, differentiation and/or survival of CTP-Os in vitro that could be tested in a subsequent clinical trial. (1-24 months)

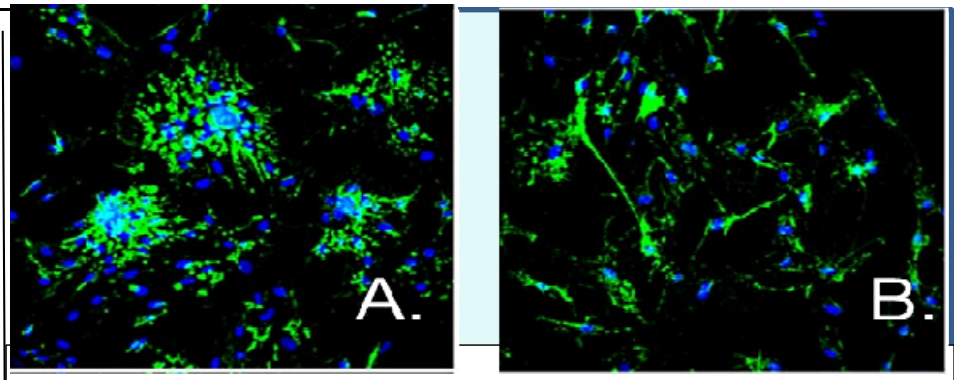
Specific Aim 4: Characterize the heterogeneity and evolution of the biological environment in tissue sites in traumatic amputations of the lower extremity in the Rat HO model between week 1 and week 3 following injury using multiple modalities.

Approach

The scope of this research effort is to perform quantitative wound assessment using noninvasive imaging modalities, such as ultrasound and Raman spectroscopy, as well as in vitro assay and characterization of tissue-resident connective tissue progenitors using image analysis of colony forming unit performance and gene expression profiling in at-risk tissue from HO+ and HO- patients.

Timeline and Cost





Human bone samples cultured in (A) 5mM glucose or (B) 25mM glucose. Cultures harvested by fixation at day 6 and hyaluronan imaged using Biotinylated Hyaluronan Binding protein (HABP) and streptavidin Alexa fluor 488. and DAPI for nuclei. **Accomplishments:** 1) ACURO approval obtained for new proposal "Evaluation of target therapies for inhibiting combat-related heterotopic ossification in a rat model of extremity injury", animal surgeries planned to start mid-November 2015 with surgery and culture complete mid-December, 2015. 2) Imaging protocols optimized for Aim 3 samples stained with Alkaline phosphatase, Nile Red and Hyaluronan (HA). 3) Request for one year NCE with revised budget and SOW approved October 9th, 2015.

Goals/Milestones

CY12 – Acquire clinical, local, cellular and molecular variables for completion of Aim 1
- ☑ Assay of CTP-Os, validation of US acquisition protocol.
- ☑ CTP-O assessment , ☑ serum & wound fluid analysis
- ☑ US virtual histology, ☐ Raman spectral imaging
- ☑ Osteogenic gene transcript analysis started

CY13 - Prototype HO diagnostic assessment tool.
- ☐ Correlate clinical, local, cellular and molecular variables
- ☐ Correlate US, Raman spectra and histopatholgy
- ☐ Develop Bayesian model for patient outcomes

CY14 – Assessment of Osteogenic Inhibitors. Identify candidate osteogenic inhibitors
- ☑ Qualitative assessment of inhibitors dose range
- ☐ Quantitate assessment dose and timing

Comments/Challenges/Issues/Concerns
- ☑ Patient recruitment below expectation. Expanding criteria to include all amputations.
- ☑ Burn rate slowed due to slow patient recruitment.
- ☑ Specific Aim 4 , rat blast amputation model, added to initial proposal.

Budget and Expenditures	CC	NMRC	WRNMMC	
W81XWH-	12-2-0118	12-2-0119	12-2-0120	Total
Projected Expenditure	$358,850	$276,172	$280,643	$915,665
Actual Expenditure	$162,327	$126,379	$81,066	$356,022

Genes over expressed in injured muscle vs. control muscle
(Gene data averaged for all wounds sites at each time period)

Gene Symbol	Fold Regulation (8 days post injury)
Spp1	172.4535
Col1a1	137.7958
Il1b	67.3332
Il10	49.9631
Has1	45.5771
Tac1	42.9908
Cxcl1	25.259
Mmp9	24.4814
Alpl	23.9021
Cxcl5	22.0299
Ifgax	19.3507
Ccl1	17.9644
Cxcl10	15.7957
Ccl3	12.0675
Col11a1	11.5082
Runx2	9.7222
Tgfb1	8.7068
Ifgam	8.4779
Il6	7.3628
Has2	6.8816
Hif1a	5.7466
Comp	5.7027
Cd44	5.5465
Twist1	4.3545

Gene Symbol	Fold Regulation (15 days post injury)
Spp1	1105.6776
Tac1	130.0625
Cxcl5	86.3414
Col1a1	84.2834
Il1b	74.488
Mmp9	69.8933
Il10	40.5062
Col11a1	38.3554
Cxcl1	32.1955
Il6	22.0141
Alpl	16.4488
Ccl2	16.058
Has1	14.6251
Ibsp	14.2341
Has2	13.6114
Cxcl10	12.9323
Runx2	11.7587
Ifgax	10.0565
Ccl3	8.5216
Hif1a	7.8294
Cd44	6.121
Twist1	5.8683
Ifgam	4.6578

Gene Symbol	Fold Regulation (8 days post injury)
Adipcq	-51.1911
Col4a3	-17.5839
Igf2	-15.1542
Myod1	-15.0561
Lep	-8.6769
Cebpa	-7.12
Fabp4	-5.4987
Hat1	-4.6948

Gene Symbol	Fold Regulation (15 days post injury)
Adipcq	-134.675
Col4a3	-40.6419
Myod1	-28.2165
Igf2	-22.7687
Bmp4	-11.6981
Lep	-8.5503
Bmp6	-5.3386
Cebpa	-4.6218

Genes under expressed in injured muscle vs. control muscle
(Gene data averaged for all wounds sites at each time period)