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TITLE: Uncovering the Role of BMP Signaling in Melanocyte Development and Melanoma Tumorigenesis

PRINCIPAL INVESTIGATOR: Craig J. Ceol, Ph.D.

CONTRACTING ORGANIZATION: University of Massachusetts Medical School
Worcester, MA 01655

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Fort Detrick, Maryland 21702-5012

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**6. AUTHOR(S)**
Dr. Craig Ceol, PhD

E-Mail: Craig.Ceol@umassmed.edu

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Massachusetts Medical School
55 Lake Ave N.
Worcester, MA 01655-0002

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
Melanoma is the most aggressive and lethal form of skin cancer. In 2013 over 75,000 Americans were diagnosed with melanoma, and nearly 10,000 died from this disease. It has been known for over a decade that mutations that overactivate the BRAF and NRAS genes promote melanoma formation. At the same time it has also become clear that these mutations are not sufficient for melanoma formation and other genes are involved. Using genomic studies and cross-species comparisons, we identified the BMP factor GDF6 as a gene that may cooperate with mutant BRAF to promote melanoma. The aims of this grant are to determine if GDF6 does in fact cooperate with mutant BRAF and uncover the mechanisms by which GDF6 acts in melanomas and normal melanocytes. Toward these aims, we have used our zebrafish model to demonstrate cooperativity between GDF6 and mutant BRAF in accelerating melanoma onset. Furthermore, we have knocked down GDF6 in human melanoma cells, finding that loss of GDF6 causes cells to cease proliferating. These and other data suggest that GDF6 promotes melanoma progression and its withdrawal is detrimental to melanoma cell growth. We are currently investigating whether blocking GDF6 function is a viable therapeutic strategy.

**15. SUBJECT TERMS**
Nothing listed

**16. SECURITY CLASSIFICATION OF:**

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**18. NUMBER OF PAGES**
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USAMRMC

**19b. TELEPHONE NUMBER (include area code)**

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INTRODUCTION:
Melanoma is the most aggressive skin cancer, and every year it kills nearly 10,000 Americans and roughly 60,000 people worldwide. A greater understanding of the genetic basis for melanoma is essential for designing new ways to diagnose and treat this disease. Nearly a decade ago, it was discovered that mutations that inappropriately activate the \textit{BRAF} gene are present in over half of all human melanomas. Activated \textit{BRAF} mutations are necessary for formation of these melanomas, but numerous studies have shown that they are not sufficient. To find other genes that cooperate with \textit{BRAF} in creating melanomas, we have used genomic studies and cross-species comparisons to identify several candidates. One of these candidates, \textit{GDF6}, is a BMP factor that is recurrently amplified and upregulated in human and zebrafish melanomas. The purpose of this study is to functionally analyze the role of \textit{GDF6} in melanoma progression. In addition, this study aims to use gain and loss of function studies to determine how \textit{GDF6} acts in melanomas and normal melanocytes. A major goal of this research is to determine if \textit{GDF6} can be used as a diagnostic or prognostic marker in melanoma and is a potential therapeutic target.
As requested in the Technical Reporting Requirements, this section describes research progress in reference to each task outlined in the Statement of Work. Below, I restate each task and briefly describe its components. With each task an update on progress made is included.

**Task 1: Perform gain and loss of function studies in zebrafish embryos and mammalian cultured cells to determine if GDF6 antagonizes melanocyte development.**

In this task, studies in zebrafish and mammalian cultured cells were proposed to determine the effects of *gdf6b* overexpression and *gdf6b* loss on melanocyte development. Zebrafish expressing *gdf6b* in melanocyte progenitors fail to develop melanocytes, suggesting that *gdf6b* inhibits terminal differentiation of melanocytes. We have created a zebrafish strain with a targeted mutation in *gdf6b*. *gdf6b* mutant animals have excess melanocytes, consistent with a role for *gdf6b* in inhibiting melanocyte development. These animals are being further characterized to determine the stage of melanocyte development during which *gdf6b* acts. We have also performed knockdown and overexpression experiments in human cultured melanoma cells. As described below, alteration of *GDF6* levels has profound effects on cell viability and tumorigenic potential.

**Task 2: Use established screening procedures in zebrafish to determine if GDF6 overexpression accelerates melanoma onset or exacerbates other properties of melanomas. In addition, use human melanoma cells to determine if GDF6 knockdown in GDF6-positive cells or overexpression in GDF6-minus cells affects tumorigenicity.**

To address this task, a zebrafish screening scheme, termed the ‘MiniCoopR’ assay\(^1\),\(^2\), was used to determine if *gdf6b* has an effect on melanoma progression. In this assay, melanocyte-deficient animals are injected with DNA that can both rescue melanocytes and overexpress a gene of interest. Zebrafish with rescued melanocytes are monitored weekly for tumors to determine if the gene of interest affects tumor onset as compared to a control gene. When *gdf6b* was overexpressed using MiniCoopR, melanomas arose more quickly as compared to *EGFP* controls (Fig. 1A). *GDF6* was also expressed in cultured human A375 melanoma cells. *GDF6* overexpressing cells were xenotransplanted into nude mice and tumor progression monitored as compared to control A375 cells. *GDF6* overexpression caused tumors to grow much more quickly than controls (Fig. 1B,C). A375 and other human melanoma cell lines express endogenous *GDF6*, so we determined the effects of *GDF6* knockdown in these cell lines. When *GDF6* was knocked down using multiple, independent shRNAs A375 and other melanoma cells underwent programmed cell death (Fig. 2). When knockdown cells were xenotransplanted prior to death, melanoma progression was markedly decreased (Fig. 3). Taken together, these results indicate that *GDF6* is an oncogene and the cell death resulting from its knockdown makes it an excellent target for anti-melanoma therapy.

Knockdown and overexpression cells are being used to determine how *GDF6* acts. The GDF6 protein is initially made as a proprotein, which is cleaved in cells to generate mature, secreted GDF6\(^3\). To determine if mature GDF6 acts as a pro-survival factor, we added recombinant, mature GDF6 to media of *GDF6* knockdown cells. Recombinant GDF6 rescued the effects of *GDF6* knockdown (Fig. 4), indicating that GDF6 can act as a secreted protein to promote melanoma cell survival. These data suggest that targeting soluble, extracellular GDF6 is a therapeutic strategy for melanoma and possibly other types of tumors. Currently we are characterizing the transcriptomes of *GDF6* to further address the effects of *GDF6* knockdown and overexpression. Analyses thus far are consistent with a role for *GDF6* in promoting cell survival.

**Task 3: Use BMP pathway reporters to determine the dynamics of BMP activity in normal melanocytes and melanoma cells. Examine GDF6 expression and mutation status in human melanomas, benign melanocytic lesions and normal melanocytes to determine if modulation of GDF6 activity is consistent with a role in melanoma formation.**

A major goal of this task is to assess the effects of *GDF6* on BMP signaling activity. In zebrafish we initially proposed to use a fluorescent reporter to monitor transcriptional output of BMP activity – however, technical difficulties have made this approach untenable. Instead, we have used antibodies that recognized phosphorylated SMAD1/5/8 to measure BMP signaling activity. In zebrafish, melanomas have high levels of GDF6 protein as well as robust phospho-SMAD expression (Fig. 5). In cultured melanoma cells we similarly detect GDF6 and phospho-SMAD1/5/8 expression. When *GDF6* is knocked down, phospho-SMAD1/5/8 levels go down (Fig 6), consistent with the notion that GDF6 signals through SMAD1/5/8 and the BMP signaling pathway.
Additional experiments were performed to determine if GDF6 acts via the BMP signaling pathway. Knockdown of SMAD1 resulted in the same cell death phenotype as GDF6 knockdown, suggesting that both genes act in the same pathway. To directly assess whether GDF6 acts via the BMP signaling pathway we performed genetic epistasis analyses (Fig. 7). In these epistasis experiments an activated variant of SMAD1 was used. This variant, SMAD1DVD, contains amino acid substitutions in key catalytic residues, resulting in a constitutively active protein. When GDF6 knockdown was performed in A375 cells expressing SMAD1DVD, cell death was suppressed, indicating that GDF6 acts upstream of or in parallel to SMAD1. When such cells were xenotransplanted into immunocompromised mice, they grew much more quickly than GDF6 knockdown cells, again indicating that GDF6 acts upstream of SMAD1.

Stainings of zebrafish and human tissue samples were used to further investigate the role of GDF6 and SMAD1 in melanoma (Fig. 8). In zebrafish melanomas we discovered robust expression of GDF6 and phospho-SMAD1. Similar, robust expression of GDF6 and phospho-SMAD1 was observed in human melanoma sections. To determine if there is a correlation between GDF6 or phospho-SMAD1 expression and clinical outcome, we have recently stained a tissue microarray of human melanoma tissue cores, each of which has associated clinical data. We are currently analyzing these stainings to determine if increased GDF6 or phospho-SMAD1 expression correlates with a poor clinical outcome.
KEY RESEARCH ACCOMPLISHMENTS:

- Overexpression of GDF6 accelerates melanoma onset in zebrafish.
- Overexpression of GDF6 accelerates melanoma onset of A375 cells in xenotransplanted mice.
- Knockdown of GDF6 causes programmed cell death.
- Recombinant GDF6 protein rescues effects of GDF6 knockdown.
- Knockdown of SMAD1 causes programmed cell death.
- Expression of an activated SMAD1 variant, SMAD1DVD, suppresses the effects of GDF6 knockdown.
- GDF6 and phospho-SMAD1 are robustly expressed in zebrafish melanomas.
- GDF6 and phospho-SMAD1 are robustly expressed in human melanomas.
- Zebrafish with mutant GDF6 have supernumerary melanocytes.
REPORTABLE OUTCOMES:

Presentations during this reporting period include:
• 7th Zebrafish Disease Models Conference, selected talk (abstract appended)
  Title: Mechanistic insights into oncogenic glutamate receptor signaling in melanocytes and melanoma
• 22nd International Pigment Cell Conference, selected talk (abstract appended)
  Title: Dual mechanisms combine to mediate regeneration of zebrafish melanocytes following injury
• 52nd Annual Meeting of The American Society of Dermatopathology, selected talk (abstract appended)
  Title: The novel oncogene \textit{GDF6} promotes melanoma cell survival
• PanAmerican Society for Pigment Cell Research, selected talk (abstract appended)
  Title: Identifying GDF6 as a novel pro-survival melanoma oncogene
• Tufts University, American Cancer Society seminar
  Title: Understanding melanoma initiation using the zebrafish
• University of Massachusetts Medical School Hematology/Oncology seminar
  Title: The BMP factor \textit{GDF6} is a novel pro-survival melanoma oncogene
• MassBiologics research seminar
  Title: The novel melanoma oncogene \textit{GDF6} as a therapeutic target

Cell lines created during this reporting period include:
• GDF6-overexpressing melanoma cell lines
• GDF6-knockdown melanoma cell lines
• SMAD1-overexpressing melanoma cell lines
• SMAD1-knockdown melanoma cell lines
• SMAD1DVD-overexpressing cell lines

Zebrafish strains created during this reporting period include:
• Strains with loss-of-function mutations in \textit{GDF6}
• Strains with \textit{GDF6} overexpression in melanocytes

Publications include:

Personnel paid by research effort:
Craig Ceol, Ph.D.
Fang Liu, Ph.D.
Arvind Venkatesan
CONCLUSION:

In this reporting period we have obtained data that show GDF6 is a new oncogene in melanoma. These data include gain-of-function data that show GDF6 can promote tumor progression. Conversely, loss-of-function data indicate that GDF6 is required for melanoma cell survival and, its loss abrogates tumor progression. Genetic epistasis and other data show that GDF6 acts via the BMP signaling pathway to promote melanoma progression and melanoma cell survival. Our current hypothesis is that GDF6 normally prevents terminal differentiation of melanocytes, thereby keeping cells in a progenitor-like state. Less differentiated progenitor-like cells are more apt to proliferate and support tumor progression.

These findings are important because GDF6 represents a prime target for anti-melanoma therapy. GDF6 encodes a secreted protein that is required for melanoma cell survival. Inhibition of GDF6 has the potential to cause melanoma cell death and reduce tumor mass. Targeting GDF6 protein could potentially be accomplished by inhibitors, such as monoclonal antibodies, that do not need to cross cell membranes. We are currently beginning to test this possibility by generating anti-GDF6 antibodies.
REFERENCES:


APPENDICES:

Please see appended *curriculum vitae* for Dr. Ceol.
Please see appended meeting abstracts.
CRAIG JOSEPH CEOL  
Assistant Professor  
Program in Molecular Medicine and Department of Molecular, Cell and Cancer Biology, University of Massachusetts Medical School  
Albert Sherman Center, AS6.1041, 368 Plantation Street, Worcester, MA 01605  
Telephone: (508) 856-5509  
Email: Craig.Ceol@umassmed.edu  
Date prepared: August 1, 2015

EDUCATION

Yale University, New Haven, CT  1989-1993  
B.S./M.S. combined degree in Molecular Biophysics and Biochemistry  
Research Advisor: Dr. Lynne Regan

Massachusetts Institute of Technology, Cambridge, MA  1995-2003  
Ph.D. degree in Biology  
Research Advisor: Dr. H. Robert Horvitz

Massachusetts Institute of Technology, Cambridge, MA  2003-2004  
Postdoctoral Fellow, Department of Biology, HHMI  
Research Advisor: Dr. H. Robert Horvitz

Harvard Medical School, Children’s Hospital Boston, Boston, MA  2004-2008  
Postdoctoral Fellow, Division of Hematology/Oncology, HHMI  
Research Advisor: Dr. Leonard I. Zon

PROFESSIONAL EXPERIENCE

Research Associate, Eli Lilly and Company  1993-1995  
Division of Bioprocess Development

Instructor, Harvard Medical School, Children’s Hospital Boston, 2008-2009  
Division of Hematology/Oncology

Assistant Professor, University of Massachusetts Medical School  2010-  
Program in Molecular Medicine and Program in Cell Dynamics  
Department of Cancer Biology

HONORS AND AWARDS

Yale University:  
B.S./M.S. four-year degree, Molecular Biophysics and Biochemistry  1991-1993  
Yale University Summer Study Grant  1991  
Distinction in Molecular Biophysics and Biochemistry  1993

Massachusetts Institute of Technology:  
Koch Predoctoral Research Fellow  1999-2000

Children’s Hospital Boston and Harvard Medical School:  
Damon Runyon Cancer Research Foundation Postdoctoral Fellowship  2005-2007  
American Cancer Society Postdoctoral Fellowship (declined)  2005  
Winner, poster prize, Keystone Symposium, Santa Fe, NM  2006  
Advances in the Understanding and Treatment of Melanoma  
Winner, Presentation Prize, Harvard Stem Cell Institute Symposium  2008

Charles A. King Trust of The Medical Foundation Postdoctoral Fellowship  2008-2009

NIH Pathway to Independence Award (K99/R00), NIAMS  2009-2013

University of Massachusetts Medical School:  
Worcester Foundation for Biomedical Research Award  2011-2012  
American Cancer Society Research Scholar Award  2012-2016  
Kimmel Scholar Award  2013-2015
FUNDING

Active:
RSG-12-150-01-DDC Research Scholar Award, American Cancer Society, Ceol (PI) Epigenetic determinants of melanoma initiation and maintenance.
R01AR063850-01 NIH/NIAMS, Ceol (PI)
Use of comparative oncogenomics to identify novel regulators of melanoma progression.
CA120099 Dept of Defense Peer Reviewed Cancer Career Development Award, Ceol (PI)
Uncovering the role of BMP signaling in melanocyte development and melanoma tumorigenesis.
SKF-13-123 Kimmel Scholar Award, Ceol (PI)
Mechanisms underlying melanoma initiation and maintenance.
UL1TR000161 UMMS NHMPP Award, Ceol & Yang (PIs)
GDF-6 blocking antibodies as cancer therapeutics.

Concluded:
R00AR056899-04 Pathway to Independence Award, NIH/NIAMS, Ceol (PI)
Identifying events and genetic regulators of melanoma progression.
P60016170000122 Worcester Foundation for Biomedical Research, Ceol (PI)
Use of comparative genomics to identify oncogenes.
Scientific Meeting Grant, The Company of Biologists, Ceol (PI)
Zebrafish Disease Models 7 conference, Madison, WI, June 28-July 1, 2014

TEACHING AND MENTORING

Teaching:
M.D./Ph.D. Research Tutorial, one discussion group (3hr). 2010
Ph.D. Summer RAPS (Reading, Analysis, Problem Solving paper review), one discussion group (2hr). 2010
Cancer Biology, one lecture (2hr), one discussion group (2hr). 2010-
Molecular Biology of the Cell Cycle, one lecture (0.5hr), one discussion group (2hr) 2011, 2015
Stem Cell and Regenerative Biology. Co-coordinator, two lectures and discussion groups (4hr) plus organizational responsibilities. 2011-2012
RAPS, Block II (2hr). 2011-
Topics in Molecular Medicine, one lecture and discussion group (2hr). 2012
MDP740 Developing solutions to research problems, lecture and discussion (2hr) 2014

Advisory and supervisory responsibilities:

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<th>Name</th>
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<th>Year(s)</th>
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<tr>
<td>Rajesh Vyas</td>
<td>Postdoctoral Fellow</td>
<td>2014-</td>
</tr>
<tr>
<td>Ana Neto</td>
<td>Postdoctoral Fellow</td>
<td>2011- NRSA Fellow, NCI</td>
</tr>
<tr>
<td>Fang Liu</td>
<td>Postdoctoral Fellow</td>
<td>2013</td>
</tr>
<tr>
<td>Corrie Painter</td>
<td>Postdoctoral Fellow</td>
<td>2012-4 CRI Irvington Inst. Fellow</td>
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<tr>
<td>Sharanya Iyengar</td>
<td>Graduate Student</td>
<td>2010-</td>
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<tr>
<td>James Neiswender</td>
<td>Graduate Student</td>
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<td>Arvind Venkatesan</td>
<td>Graduate Student</td>
<td>2011-</td>
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<tr>
<td>Revati Darp</td>
<td>Graduate Student</td>
<td>2014-</td>
</tr>
<tr>
<td>Alec Gramann</td>
<td>MD/PhD Student</td>
<td>2015-</td>
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<tr>
<td>Tyler Frantz</td>
<td>MD/PhD Student</td>
<td>2015-</td>
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<tr>
<td>Eli Freiman</td>
<td>Medical Student</td>
<td>2012</td>
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<tr>
<td>Alysia Bryll</td>
<td>Rotating MD/PhD Student</td>
<td>2015</td>
</tr>
<tr>
<td>Cierra Smith</td>
<td>Rotating Graduate Student</td>
<td>2014</td>
</tr>
<tr>
<td>Heather Kolpa</td>
<td>Rotating Graduate Student</td>
<td>2010</td>
</tr>
</tbody>
</table>
Jennifer Maurer Rotating Graduate Student 2010
James Ritch Rotating Graduate Student 2010
Lin Lin Rotating Graduate Student 2010
Justin Peter Hess Undergrad. Student (WPI) 2012
Sukanya Murali Undergrad. Student 2013
(Anna University – Chennai)
Brittney Logan Undergrad. student 2013
(W. New England University)

Dissertation committees:
Shawna Guillemette, UMass Medical School, Cancer Biology Program
Tomoko Tabuchi, UMass Medical School, Interdisciplinary Graduate Program
David Driscoll, UMass Medical School, Cancer Biology Program
Anna Malinkevich, UMass Medical School, Interdisciplinary Graduate Program
Cheng Chang, UMass Medical School, Cancer Biology Program (Chair)
Nomeda Girnius, UMass Medical School, Interdisciplinary Graduate Program
Lin Lin, UMass Medical School, Cancer Biology Program (Chair)
James Ritch, UMass Medical School, Interdisciplinary Graduate Program
Nicola Kearns, UMass Medical School, Interdisciplinary Graduate Program

Qualifying examination committees:
Christopher Clark, UMass Medical School, Neuroscience Program
Caitlin Fogarty, UMass Medical School, MD/PhD Program
Nomeda Girnius, UMass Medical School, Interdisciplinary Graduate Program
Chien-Min Hung, UMass Medical School, Interdisciplinary Graduate Program
James Ritch, UMass Medical School, Interdisciplinary Graduate Program
Lin Lin, UMass Medical School, Cancer Biology Program (Chair)
Ly-She Ee, UMass Medical School, Interdisciplinary Graduate Program
Shubham Dutta, UMass Medical School, Interdisciplinary Graduate Program
Nicola Kearns, UMass Medical School, Interdisciplinary Graduate Program
Hsi-Ju Chen, UMass Medical School, Interdisciplinary Graduate Program
Nicholas Panzarino, UMass Medical School, Cancer Biology Program (Chair)

SERVICE

University of Massachusetts Medical School and local:
Sherman Center Labs NI/GTC/CVC/Diabetes Focus Group 2010
Diabetes and Endocrinology Research Center (grant reviewer, ad hoc) 2011
AP Biology High School Outreach Program (host) 2011-
University of Massachusetts Medical School Convocation (Dinner and Dialogue event speaker and panelist) 2011
University of Massachusetts Medical School visit of Young President’s Organization & World President’s Organization (speaker) 2011
University of Massachusetts Medical School Development Council meeting (speaker) 2012
University of Massachusetts Medical School BARG Organization (speaker) 2012
LCME accreditation of University of Massachusetts Medical School (Junior Faculty cohort) 2012
University of Massachusetts Chancellor’s Review (Faculty Review Committee) 2012
Wachusett High School Science Seminar 2012
University of Massachusetts Medical School Science to Trades Seminar 2013
WSRS interview w/ Greg Byrne in support of UMMS Cancer Walk 2013
UMMS Development Office – lab tours with donor groups 2013-
MassAHEC Network Frontiers in Science Seminar 2014
NIH BEST Award Focus Group 2014
Hudson Hoagland Society annual meeting (speaker) 2014
WSRS interview W/ Jordan Levy in support of UMMS Cancer Walk 2014
Original reports:

   ‡ This paper is highlighted by the Faculty of 1000.


   ‡ This paper is highlighted by the Faculty of 1000.


‡ This paper is highlighted by the Faculty of 1000.


Reviews and commentary:


Cover art:


ORAL PRESENTATIONS
Meeting presentations:
East Coast C. elegans Meeting, Boston, MA 1998
International C. elegans Meeting, Madison, WI 1999
East Coast C. elegans Meeting, Durham, NH 2002
Keystone Symposium, Advances in the Understanding and Treatment of Melanoma, Santa Fe, NM 2006
Gordon Conference, Cancer Models and Mechanisms, Les Diablerets, Switzerland 2008
8th International Conference on Zebrafish Development and Genetics, Madison, WI 2008
Harvard Stem Cell Institute Research Symposium, Boston, MA 2008
9th International Conference on Zebrafish Development and Genetics, Madison, WI 2009
3rd Zebrafish Disease Models Conference, Boston, MA 2010
Connecticut Valley Zebrafish Meeting, Middletown, CT 2010
Gordon Conference, Cancer Genetics and Epigenetics, Ventura, CA 2011
Biotechcellence 2012 National Technical Symposium Anna University, Chennai, India (via videoconference) 2012
10th International Conference on Zebrafish Development and Genetics, Madison, WI (workshop co-coordinator) 2012
International Federation of Pigment Cell Societies, Pigment Cell Development Workshop, Edinburgh, UK 2013
5th European Melanoma Conference, Basic and clinical research join forces to defeat melanoma, Marseille, France 2013
6th Zebrafish Disease Models Conference, Murcia, Spain 2013
7th Zebrafish Disease Models Conference, Madison, Wisconsin 2014 (in place of maternity leave postdoc Ana Neto)
22nd International Pigment Cell Conference, Bringing colours to life, Singapore 2014
52nd Annual Meeting of The American Society of Dermatopathology, San Francisco, CA 2015
PanAmerican Society for Pigment Cell Research Conference, Irvine, CA 2015

Invited seminar presentations:
Hubrecht Institute, Utrecht, Netherlands 2008
Cancer Genomics and Developmental Biology Programme Seminar
Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA 2008
Whitehead Seminar Series for High School Teachers: Controlling Genes
Providence College, Providence, RI 2011
Biology Department Seminar
UMass Medical School, Worcester, MA 2011
Cutaneous Tumor Board, Pathology Department
University of Rochester Medical Center, Rochester, NY 2011
Biomedical Genetics Department Seminar
Quinsigamond Dermatological Society, Worcester, MA 2011
Grand Rounds
Carnegie Institution, Baltimore, MD 2012
Department of Embryology Seminar
National Institutes of Health, Bethesda, MD 2012
NIH Comparative Biomedical Scientist Program Symposium
University of Massachusetts Medical School, Worcester, MA 2012
Cancer Biology Retreat
Assumption College, Worcester, MA 2012
Seminar in Life Sciences
University of Massachusetts Medical School, Worcester, MA 2013
Microbiology and Physiological Systems Department Seminar
Tufts University School of Medicine, Boston, MA 2013
Molecular Physiology and Pharmacology Retreat (Keynote)
Centro Andaluz de Biología del Desarrollo, Seville, Spain 2013
CABD Institute Seminar
University of Michigan, Ann Arbor, MI 2014
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<td>Molecular, Cellular and Developmental Biology Seminar</td>
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<td>Biology and Bioengineering Seminar</td>
<td>Tufts University, Medford, MA</td>
<td>2015</td>
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<td>American Cancer Society Relay for Life Seminar</td>
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<td>University of Massachusetts Medical School, Worcester, MA</td>
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<tr>
<td>Division of Hematology/Oncology Grand Rounds</td>
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Mechanistic insights into oncogenic glutamate receptor signaling in melanocytes and melanoma

Ana Neto and Craig Ceol

Program in Molecular Medicine and Department of Cancer Biology, University of Massachusetts Medical School, Worcester, Massachusetts, USA

Glutamate signaling, which is important in the central nervous system and in glial cell function, has recently been shown to have a role in melanoma progression. Human melanoma exome sequencing studies have identified activating mutations in metabotropic glutamate receptor 3 (GRM3). We hypothesize that altered glutamate signaling affects the development and function of melanocytes, endowing these cells with properties important for melanoma progression. Accordingly, understanding the role of glutamate signaling in melanocytes may inform how dysregulation of glutamate signaling is involved in melanoma progression. To test our hypothesis we use the miniCoopR assay, in which transgene-bearing melanocytes are derived in a $mitfa^{lf}$ background. Using this assay, we have determined how oncogenic GRM3 variants affect developing melanocytes and impact tumor formation. In embryonic melanocytes oncogenic GRM3 mutants disrupt trafficking of melanosomes, the pigment-producing organelles, whereas wild-type GRM3 does not. These and other data indicate that oncogenic GRM3 variants dysregulates cyclic AMP (cAMP) signaling, a heretofore unknown role for these oncogenes. Extending our analyses to tumors, we have found that expression of oncogenic GRM3 affects melanoma onset. These and additional data suggest that altered cAMP signaling can impact melanoma progression. Recent data have implicated defective cAMP signaling in the melanoma susceptibility of red-haired, fair-skinned individuals. Our data support the notion that disrupted cAMP signaling is a more pervasive contributor to melanoma, including in individuals that have incurred GRM3 mutations.
Dual mechanisms combine to mediate regeneration of zebrafish melanocytes following injury

Sharanya Iyengar, Craig Ceol

Program in Molecular Medicine and Department of Cancer Biology, University of Massachusetts Medical School, Worcester, MA, USA

Melanocytes, which can be lost during hair graying, injury and disease-related depigmentation, are replenished in mammals by resident stem cells. To gain insight into melanocyte regeneration we set out to identify whether melanocyte stem cells are present in adult zebrafish and how such cells might reconstitute the pigment pattern following injury. Using a targeted cell ablation approach we determined that mitfa is expressed not only in differentiated melanocytes but also in the cells that mediate melanocyte regeneration. When mitfa-positive cells are selectively ablated no melanocyte regeneration occurs. However, when ablation is performed in a p53-deficient background, melanocyte regeneration occurs, suggesting that death of the cells that mediate regeneration is dependent on p53. We then used mitfa-positivity to perform lineage-tracing experiments and assay whether unpigmented mitfa-expressing cells have stem cell properties. During regeneration, mitfa-positive cells can divide asymmetrically with one daughter cell differentiating and the other daughter remaining uncommitted; these are melanocyte stem cell divisions. In addition, some mitfa-positive cells directly differentiate during regeneration. Taken together, these data indicate that multiple mechanisms are used to re-establish pigmentation following injury and enable regeneration following subsequent rounds of ablation. We have used reporter assays and drug studies to assess whether pathways important for melanocyte development are also involved in regeneration. We found that Wnt signaling gets turned on during melanocyte regeneration and that Wnt inhibition after ablation of differentiated melanocytes delays regeneration. These studies have established a system by which regeneration can be traced with single-cell resolution and perturbations to regeneration analyzed in exquisite detail.
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The novel oncogene GDF6 promotes melanoma cell survival

Arvind M. Venkatesan, Rajesh Vyas, Sanchita Bhatnagar, Karen Dresser, Yvonne Edwards, Michael Green, April Deng, Craig Ceol

1 Program in Molecular Medicine, UMass Medical School, Worcester, MA, USA
2 Department of Molecular, Cell and Cancer Biology, Worcester, MA, USA
3 Dept. of Pathology UMass Medical School, Worcester, MA, USA

The prevalence of BRAFV600E in nevi indicates that this mutation is not sufficient to cause melanoma. To identify new melanoma genes that could cooperate with BRAFV600E, we searched for abnormalities shared in both human melanomas and in a zebrafish BRAFV600E-driven melanoma model. We hypothesized that these conserved abnormalities would be enriched for genes that affect melanoma progression. In these analyses, we identified the GDF6 gene, which encodes a member of the bone morphogenetic protein (BMP) family. GDF6 genes in humans and zebrafish were recurrently copy number amplified in melanoma, and expression of the GDF6 gene was observed in human and zebrafish melanomas but absent from normal melanocytes in both species. In functional analyses, overexpression of GDF6 accelerated melanoma progression, whereas knockdown of GDF6 in cultured A375 cells compromised melanoma formation in xenotransplantation assays. Knockdown of GDF6 caused programmed cell death, which was rescued by an activated variant of the SMAD1 transcription factor, indicating that GDF6 acts through the canonical BMP signaling pathway. Strikingly in tissue sections, GDF6 protein was readily detectable in more than 90% of melanomas, but was absent from melanocytes in normal adjacent skin. BMP pathway activity was likewise apparent in melanomas in a pattern that overlapped with GDF6 staining. Taken together, these data indicate that GDF6 is a new melanoma oncogene that promotes melanoma cell survival and can be therapeutically targeted to induce melanoma cell death.
Identifying GDF6 as a novel pro-survival melanoma oncogene

Arvind M Venkatesan\textsuperscript{1,2}, Rajesh Vyas, Ph.D.\textsuperscript{1,2}, Sanchita Bhatnagar, Ph.D.\textsuperscript{2}, Karen Dresser\textsuperscript{3}, Feng Qi\textsuperscript{4}, Jian-Liang Li, Ph.D.\textsuperscript{4}, April Deng, M.D., Ph.D.\textsuperscript{3}, Michael Green, M.D., Ph.D.\textsuperscript{2}, Craig Ceol, Ph.D.\textsuperscript{1,2}

\textsuperscript{1} Program in Molecular Medicine, UMass Medical School, Worcester, MA, USA
\textsuperscript{2} Department of Molecular, Cell and Cancer Biology, Worcester, MA, USA
\textsuperscript{3} Dept. of Dermatopathology UMass Medical School, Worcester, MA, USA
\textsuperscript{4} Sanford Burnham Medical Research Institute, Orlando, FL, USA

To identify genes involved in tumor progression we defined regions of recurrent copy number variation in zebrafish melanomas and compared these regions to ones recurrently altered in human melanomas. In the set of genes that were recurrently amplified in both species we found the BMP factor GDF6. In analyses of both zebrafish and humans, GDF6 mRNA and protein were upregulated in melanomas as compared to normal melanocytes. In functional assessments, we found that overexpression of GDF6 accelerated melanoma onset in zebrafish and mouse xenotransplantation assays. Furthermore, knockdown of GDF6 in melanoma cell lines led to apoptotic cell death in culture \textit{in vitro} and in GDF6-deficient tumors \textit{in vivo}. Addition of recombinant GDF6 protein to the media prevented melanoma cells from undergoing GDF6 shRNA-induced apoptosis, suggesting that GDF6 acts as a secretory factor in aiding melanoma cell survival. GDF6, like other BMP factors, is predicted to signal through SMAD1/5/8 transcription factors, and similar defects were observed when SMAD1 was knocked down. To further define the relationship between GDF6 and SMAD1 in melanoma, GDF6 knockdown was performed in cells expressing a constitutively active SMAD1 variant. This variant rescued the death caused by GDF6 knockdown, suggesting that, at least in part, GDF6 acts through SMAD1 to promote melanoma cell survival. These data establish a role for BMP signaling in melanoma and identify a novel secretory factor, GDF6 that mediates this role. Discovery of this novel secretory factor that is present in a majority of human melanomas provides an excellent therapeutic target.
Figure 1. GDF6 expression accelerates melanoma onset. A) Expression of gdf6b accelerates melanoma onset. In this assay melanocytes expressing the zebrafish GDF6 ortholog gdf6b or EGFP were reconstituted in a BRAFV600E-positive and p53-negative background. B) GDF6 expression in control and GDF6 overexpression cell lines. C) Cells that overexpress GDF6 have accelerated melanoma onset in xenotransplantation.
Figure 2. GDF6 knockdown causes programmed cell death. A) Western blot of GDF6 knockdown cells. B) Clonogenic assay of cells subjected to GDF6 knockdown. C) Annexin V positivity of GDF6 knockdown cells. D) Cleaved caspase 3 positivity of GDF6 knockdown cells.
Figure 3. GDF6 knockdown abrogates tumor progression.
Figure 4. Recombinant GDF6 rescues the proliferation defect caused by GDF6 knockdown. Experiments were performed with MeWo melanoma cells, and recombinant GDF6 protein was added to culture media for rescue experiments.
Figure 5. High levels of GDF6 and phospho-SMAD1/5/8 in zebrafish. A) H&E staining of zebrafish melanoma. B) phospho-SMAD staining. C) GDF6 staining.
Figure 6. *GDF6* knockdown reduces phospho-SMAD1/5/8
Figure 7. Genetic epistasis of GDF6 and SMAD1. Rescue of GDF6 knockdown in shown in A) clonogenic assays, B) Annexin V positivity, C) cleaved caspase 3, D) xenotransplantation.
Figure 8. GDF6 and SMAD1 staining in human melanomas. A) H&E stains are shown (left). Robust GDF6 (middle) and phospho-SMAD1/5/8 expression are typically found in human melanomas but not normal melanocytes. B) Quantification of staining.