AWARD NUMBER: W81XWH-20-1-0288

TITLE: Targeting Ligand-Dependent BMP Signaling in Melanoma

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

CONTRACTING ORGANIZATION: University of Massachusetts Medical School
Worcester, MA

REPORT DATE: July 2021

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2021	Annual Report	01Jul2020-30Jun2021

4. TITLE AND SUBTITLE
Targeting Ligand-Dependent BMP Signaling in Melanoma

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-20-1-0288

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Craig J. Ceol, Ph.D.

E-Mail: craig.ceol@umassmed.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Massachusetts Medical School

8. PERFORMING ORGANIZATION REPORT NUMBER
DUNS 603847393

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The studies in this project seek to understand the role of bone morphogenetic protein (BMP) signaling in melanoma and develop a treatment to target ligand-dependent BMP signaling. From previous studies we know that BMP signaling represses MITF expression and upregulates expression of neural crest genes, imparting a less-differentiated neural crest-like identity to melanoma cells. Inhibition of BMP signaling causes melanoma cells to express differentiation genes and die, preventing outgrowth of xenografted tumors. To target BMP signaling, we have created monoclonal antibodies that neutralize the BMP ligand GDF6. The GDF6 gene is copy number amplified in melanoma cells, and elevated expression of GDF6 is associated with a dependence of melanoma cells on BMP activity. Preliminary results suggest anti-GDF6 monoclonal antibodies effectively inhibit BMP signaling, blocking growth and causing death of melanoma cells. We will test if these antibodies shrink xenografted tumors and determine their effects on melanoma cells. We will also determine if the most effective of these antibodies complements existing melanoma therapies in shrinking melanoma xenografts. Additionally, the presence of BMP signaling in rare acral and mucosal melanoma subtypes will be determined, and, should BMP signaling be prevalent, the dependence of these subtypes on GDF6 will be tested in vitro and in xenograft studies. This combination of experiments will determine if the BMP activity that is evident in a majority of melanomas can be targeted as a monotherapy, in combination with existing therapies and as a treatment for rare acral and mucosal melanoma subtypes.

15. SUBJECT TERMS Acral melanoma, BMP, Bone Morphogenetic Protein, GDF6, Melanoma, Mucosal melanoma, Neural crest, Xenograft					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified	Unclassified	12	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

INTRODUCTION

The studies in this project seek to understand the role of bone morphogenetic protein (BMP) signaling in melanoma and develop a treatment to target ligand-dependent BMP signaling. From previous studies we know that BMP signaling represses MITF expression and upregulates expression of neural crest genes, imparting a less-differentiated neural crest-like identity to melanoma cells. Inhibition of BMP signaling causes melanoma cells to express differentiation genes and die, preventing outgrowth of xenografted tumors. To target BMP signaling, we have created monoclonal antibodies that neutralize the BMP ligand GDF6. The GDF6 gene is copy number amplified in melanoma cells, and elevated expression of GDF6 is associated with a dependence of melanoma cells on BMP activity. Preliminary results suggest anti-GDF6 monoclonal antibodies effectively inhibit BMP signaling, blocking growth and causing death of melanoma cells. We will test if these antibodies shrink xenografted tumors and determine their effects on melanoma cells. We will also determine if the most effective of these antibodies complements existing melanoma therapies in shrinking melanoma xenografts. Additionally, the presence of BMP signaling in rare acral and mucosal melanoma subtypes will be determined, and, should BMP signaling be prevalent, the dependence of these subtypes on GDF6 will be tested in vitro and in xenograft studies. This combination of experiments will determine if the BMP activity that is evident in a majority of melanomas can be targeted as a monotherapy, in combination with existing therapies and as a treatment for rare acral and mucosal melanoma subtypes.

KEYWORDS

Acral melanoma
BMP
Bone morphogenetic protein
GDF6
Melanoma
Mucosal melanoma
Neural crest
Xenograft

ACCOMPLISHMENTS

• Major goals of the project:

1) Test whether anti-GDF6 antibodies can cause melanoma regression in vivo
    a. ACURO review/approval of IACUC protocol (months 0-3; 100% complete)
    b. IRB approval, HRPO approval (months 0-3; 40% complete)
    c. Produce anti-GDF6 antibody for injection into xenografted mice (months 1-3; 100% complete)
    d. Inject anti-GDF6 antibody into xenografted mice (months 1-6; 50% complete)
    e. In harvested tumors quantify proliferation index, cell death, BMP activity (months 1-6; 50% complete)
2) Determine the ability of anti-GDF6 antibodies to complement existing melanoma therapies
    a. Produce anti-GDF6 antibody for injection into xenografted mice (months 1-6; 100% complete)
    b. Inject anti-GDF6 antibody into xenografted mice in combination with dabrafenib and trametinib (months 6-18; 0% complete)
    c. In harvested tumors quantify proliferation index, cell death, BMP activity (months 12-18; 0% complete)
    d. Inject anti-GDF6 antibody into xenografted mice in combination with anti-PD1 antibody (months 18-36; 0% complete)
    e. In harvested tumors quantify proliferation index, cell death, BMP activity (months 30-36; 0% complete)
3) Assess the extent of GDF6 expression and BMP activity in rare melanoma subtypes
    a. Staining of acral and mucosal melanoma samples with anti-GDF6 and anti-phospho-SMAD1/5/8 antibodies (months 1-18; 0% complete)
    b. Correlative analysis of GDF6 and phospho-SMAD1/5/8 staining with clinical outcome (months 12-18; 0% complete)

4) Determine the sensitivity of acral and mucosal melanomas to GDF6 inhibition
    a. In vitro knockdown of GFD6 in acral and mucosal melanoma cell lines and quantification of cell viability and cell death (months 12-24; 20% complete)
    b. Produce anti-GDF6 antibody for injection into xenografted mice (months 21-27; 50% complete)
    c. Knockdown of GDF6 in acral and mucosal melanomas and xenotransplant into immunocompromised mice (months 24-36; 0% complete)
    d. Inject anti-GDF6 antibody into mice xenografted with acral and mucosal melanomas (months 24-36; 0% complete)
    e. In harvested tumors quantify proliferation index, cell death, BMP activity (months 30-36; 0% complete)

• Accomplishments toward these goals:

1) Major activities

In this reporting period progress has been made on approvals to conduct experiments, producing reagents to conduct experiments, and initial experiments to determine efficacy of anti-GDF6 antibodies. With respect to approvals, revisions to our IACUC protocol have been made, and ACURO approval of our revised IACUC protocol has been obtained. For studies that require IRB approval, Dr Hornyak and Dr. Ceol have completed a protocol application and submitted internally for approval. Once that is obtained it will be submitted to HRPO for review. With respect to producing reagents to conduct experiments, substantial progress has been made in anti-GDF6 antibody production. Initial characterization of anti-GDF6 antibodies used antibody obtained from hybridoma supernatants. To produce large quantities of antibody, we cloned antibody genes and expressed them in CHO cells for more robust and larger-scale production. Using this strategy, sufficient quantities of antibody have been obtained for initial experiments and can readily be obtained for any subsequent experiments. Lastly, initial characterization of the efficacy of anti-GDF6 antibodies has been performed. Xenografted mice have been treated with anti-GDF6 antibodies, and results from these experiments are currently being analyzed. Together these activities are fulfilling the experimental plan for the project.

2) Specific objectives

The specific objectives during this reporting period were first to get procedures, approvals and reagents in place to conduct experiments in animals and on human tissues. The experiments within this reporting period conducted included anti-GDF6 treatment of mice that were xenografted with melanoma cells. In this experiment, immunocompromised mice were xenografted with SKMEL28 melanoma cells and tumors allowed to grow to 100mm3. Once tumors reached that volume, anti-GDF6 or control antibody treatment was begun. Tumor monitoring was performed twice per week to determine effects of anti-GDF6 treatment.

3) Significant results or key outcomes

To produce sufficient quantities of anti-GDF6 antibody, we cloned antibody genes and expressed them in CHO cells. Through this methodology we were able to obtain sufficient quantities of antibody for our initial experiments and can readily obtain enough antibody for all the experiments described in the proposal. The cloned antibody was tested in vitro to determine if it specifically recognized GDF6 protein (Figure 1). Immunofluorescence staining with anti-GDF6 #1 antibody was performed on control A375 cells and A375 cells that had been subjected to GDF6 knockdown. Staining was evident in control cells but was absent from GDF6 knockdown cells, demonstrating specificity of the anti-GDF6 #1 antibody. To determine if anti-GDF6 #1 could inhibit BMP activity, A375 cells were incubated with control media or media containing anti-GDF6 #1 antibody (Figure 2). Immunofluorescence staining of phospho-SMAD1/5/8, the canonical marker of BMP activity, was performed. Cells treated with anti-GDF6 #1 had substantially diminished phospho-SMAD1/5/8 staining to the same extent as cells treated with DMH1, a small molecule inhibitor of BMP signaling. Functional analyses were performed to determine if cloned anti-GDF6 #1 had the same impact on cell viability as did hybridoma-derived anti-GDF6 #1. As shown in Figure 3A, the cloned anti-GDF6 antibody performed similarly to hybridoma-derived anti-GDF6 antibody in that it could impair the viability of SKMEL28 cells upon incubation with them in vitro. Treatment with anti-GDF6 #1 significantly increased the death of A375 cells in culture (Figure 3B). This cloned anti-GFD6 antibody was used to treat mice with xenografted tumors to determine if treatment had an impact on tumor growth. As shown in Figure 3C, the

cloned anti-GDF6 antibody slowed growth of tumors. The tumors from this experiment are currently being analyzed to determine if anti-GDF6 treatment affected cell proliferation, cell death and BMP activity.

4) Other achievements
Nothing to Report.

• Opportunities for training and professional development:
The experiments performed to date have afforded professional development opportunities for Melissa Guerin, the technician in the Ceol laboratory. She worked closely with Dr. Ceol to familiarize herself with mouse husbandry and techniques and conducted some of the work on xenografted animals.

• Dissemination of results to communities of interest:
Nothing to Report.

• Plan for next reporting period to accomplish these goals:
In the next reporting period, we anticipate completion of xenograft studies that evaluate anti-GDF6 antibody treatment as a monotherapy. Additionally, we aim to begin testing the efficacy of anti-GDF6 antibody in combination with small molecule inhibitors of BRAF and MEK. For this work we have already produced sufficient quantities of anti-GDF6 antibody. In the next reporting period, we also anticipate obtaining IRB and HRPO approval so we may begin working with acral and mucosal melanoma patient samples. Once approvals are obtained, we will stain acral and mucosal melanoma samples to determine if BMP activity is present in these samples and whether this activity correlates with patient outcome. We anticipate beginning to characterize BMP activity, GDF6 expression and effects of GDF6 knockdown in acral and mucosal melanoma cell lines.

IMPACT
• Impact on the development of the principal disciplines of the project:
The principal discipline of this project is cancer biology and, more specifically, targeting bone morphogenetic protein (BMP) signaling as melanoma therapy. The project is the first to explore targeting BMP signaling in melanoma and thus has the potential to have a substantial impact on the discipline. The targeting of BMP signaling is to be accomplished by creating monoclonal antibodies that are directed against the BMP simulators ligand GDF6. Such antibodies have been generated and tested against melanoma cells in culture, and these antibodies can effectively cause the death of cultured melanoma cells. The primary goals of this project are to determine whether these antibodies are effective in shrinking melanoma tumors, are effective in combination with existing melanoma therapies, and are effective against rare acral and mucosal subtypes of melanoma. Thus far our results show that we can produce these antibodies at a large enough scale for our pre-clinical studies and that these antibodies can effectively shrink melanoma tumors in animal models. These studies and additional ones in this project are necessary to show efficacy of these antibodies and provide pre-clinical data prior to testing in humans. Because of the trajectory toward human studies, this project has potential to impact melanoma therapy and patient outcomes.

• Impact on other disciplines:
Nothing to Report.

• Impact on technology transfer:
Nothing to Report.

• Impact on society beyond science and technology:
Nothing to Report.

CHANGES/PROBLEMS
• Changes in approach and reasons for change:
Nothing to Report.

• Actual or anticipated problems or delays and actions or plans to resolve them:

There has been a delay in getting IRB approval for our planned studies of acral and mucosal melanoma samples. The IRB at Dr. Hornyak's institution was temporarily shut down due to COVID-19, and once their activities resumed there was a backlog of applications. The IRB application for our studies has been submitted and is currently under review. Once that is approved it will be submitted to HRPO for review and approval. We anticipate submission to HRPO to occur in the next 4 months.

• Changes that had a significant impact on expenditures:
The delay in IRB approval has meant that funds for our studies of acral and mucosal melanoma have not yet been transferred to BREF, our partner organization. Once IRB and HRPO approvals have been obtained, funds for these studies will be transferred to BREF to enable experiments to begin.

• Significant changes in use or care of human subjects, vertebrate animals, biohazards, or select agents:
Nothing to Report.

PRODUCTS

• Publications, conference papers, and presentations:

Publications:

• Gramann, A.K., Frantz, W.T., Dresser, K., Deng, A. and **Ceol, C.J.** (2021). BMP signaling promotes neural crest identity and accelerates melanoma onset. *Journal of Investigative Dermatology*, *141*, 2067-70.

• Frantz, W.T. and **Ceol, C.J.** (2020). From tank to treatment: modeling melanoma in zebrafish. *Cells*, *9*, 1289.

• Darp, R. and **Ceol, C.J. (**2020). Making a Melanoma: molecular and cellular changes underlying melanoma initiation. *Pigment Cell and Melanoma Research*, *34*, 280-7.

• Patton, E.E. … **Ceol, C.J.** … and Merlino, G. (2021) Melanoma models for the next generation of therapies. *Cancer Cell*, *39*, 610-31.

Presentations:

• Neural Crest Research Colloquia 2020
Rice University, UC Davis, Caltech

• International Pigment Cell Conference (Rising Star Award lecture) 2020
University of Yamagata, Yamagata, Japan
Cancelled due to COVID-19

• Melanoma Research Alliance Retreat 2020
Melanoma Research Alliance, Washington, DC

• Websites or Internet sites:
Nothing to Report.

• Technologies or techniques:
Nothing to report.

• Inventions, patent applications, or licenses:
Nothing to report.

• Other products:
Nothing to Report.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

• Individuals who have worked on this project:
Dr. Craig Ceol
Role: PI, University of Massachusetts Medical School
Researcher ORCID ID: 0000-0002-7188-7580

Months worked: 3
Contribution to project: Dr. Ceol has overseen all aspects of anti-GDF6 antibody testing.
Funding support: no change

Ms. Melissa Guerin
Role: Technician, University of Massachusetts Medical School
Researcher ID: n/a
Months worked: 10.2
Contribution to project: Ms. Guerin has conducted tests of antibody efficacy in vitro and assisted with in vivo testing of antibodies in xenografted mice.
Funding support: no change

Dr. Yang Wang
Role: co-PI, MassBiologics and University of Massachusetts Medical School
Researcher ID: n/a
Months worked: 0.24
Contribution to project: Dr. Wang has overseen production of anti-GDF6 antibodies.
Funding support: no change

Dr. Monir Ejemel
Role: Postdoctoral Fellow, MassBiologics and University of Massachusetts Medical School
Researcher ID: n/a
Months worked: 2.4
Contribution to project: Dr. Ejemel has conducted production and purification of anti-GDF6 antibodies.
Funding support: no change

• Change in active other support of the PD/PIs or senior/key personnel since last reporting period:
Nothing to Report.

• Other organizations involved as partners:
Nothing to Report. Once IRB and HRPO approvals have been obtained, our partner organization, Baltimore Research and Education Foundation, will receive funds to conduct planned studies on acral and mucosal melanomas.

SPECIAL REPORTING REQUIREMENTS
Nothing to Report.

APPENDICES
Please see Figures 1-3 appended in pages 6-8.

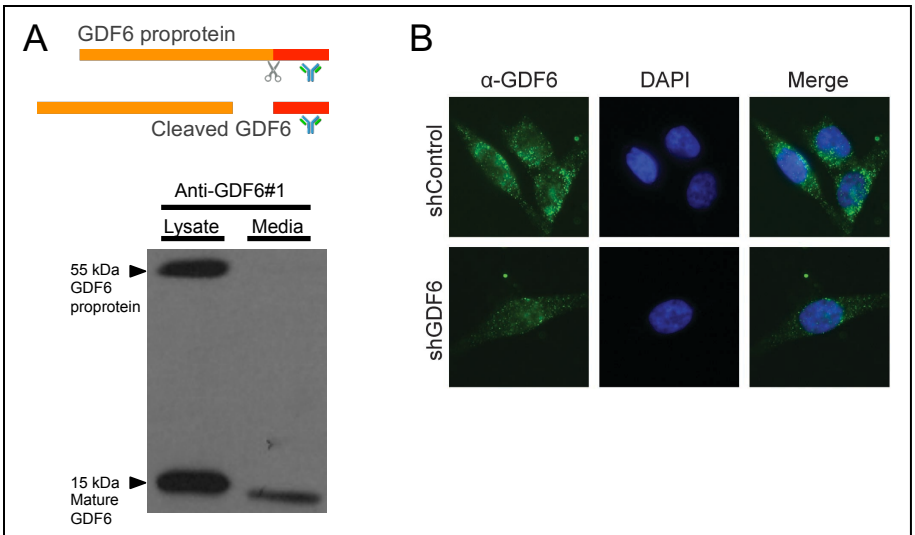

Figure 1: anti-GDF6 antibody design and specificity
A) Top, schematic of the GDF6 proprotein and ligand showing the anti-GDF6 antibody recognition site. Bottom, western blot of A375 melanoma cell lysate and conditioned media using anti-GDF6#1. B) anti-GDF6 antibody specificity. anti-GDF6 recognizes GDF6 protein in control A375 cells, and this staining is absent in A375 cells subjected to GDF6 knockdown.

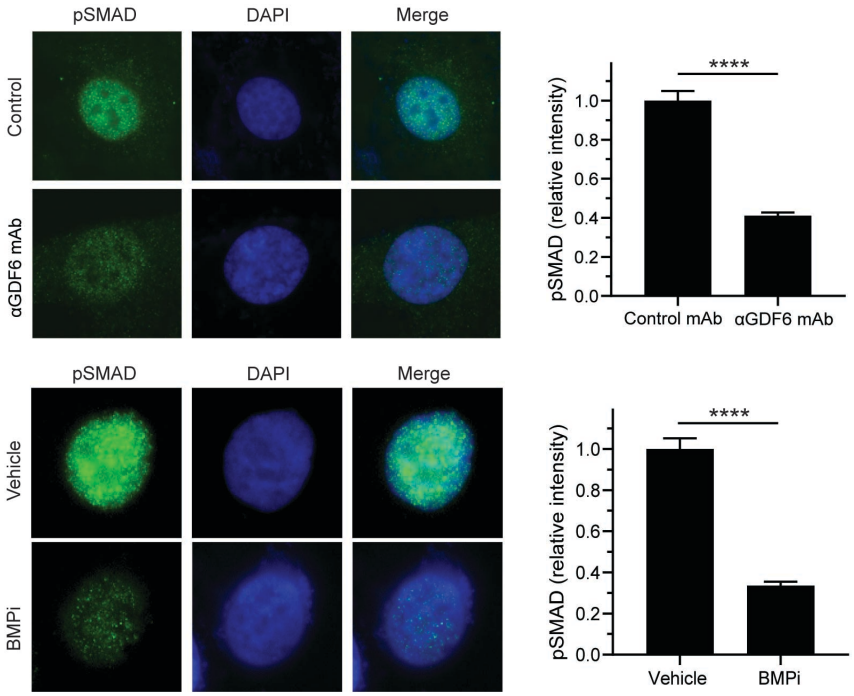

Figure 2: Effects of anti-GDF6 antibody treatment on BMP activity
Left, phospho-SMAD1/5/8 staining in nuclei of A375 cells treated with anti-GDF6#1 or DMH1. Right, quantification of phospho-SMAD1/5/8 signal. Treatment with anti-GDF6#1 inhibited BMP activity. **** $P < 0.0001$, Student's t test.

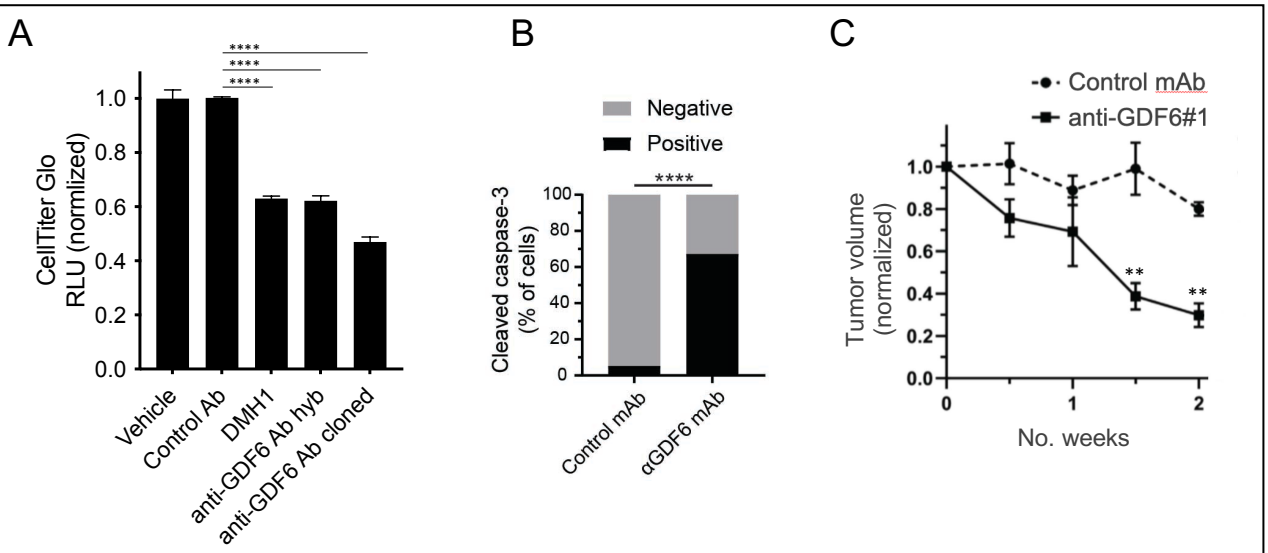

Figure 3: Effects of anti-GDF6 antibody treatment on cultured cells and xenografted tumors
A) Cell viability following treatment of melanoma cell cultures with anti-GDF6#1 antibody. Cultures were incubated with antibody or DMH1 for 24 hours and cell viability measured using the XTT assay. A375 cells, which are GDF6hi cells that are sensitive to GDF6 knockdown, were used. **** $P < 0.0001$, One-way ANOVA with Tukey's correction. B) Cleaved caspase 3 staining of control and anti-GDF6#1 treated cultures. **** $P < 0.0001$, Student's t test. C) Tumor volume measurement of nude mice xenografted with SKMEL28 cells and treated with anti-GDF6#1 or control antibody when tumors reached 100mm3. anti-GDF6#1 treatment caused rapid regression of xenografted tumors. ** $P < 0.01$, Student's t test.