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

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Craig J. Ceol, Ph.D.

E-Mail: craig.ceol@umassmed.edu

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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					
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1. 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 (Figure 1). 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.

2. KEYWORDS:

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

3. ACCOMPLISHMENTS:

What were the major goals of the project?

• 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; 100% 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; 50% 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; 50% complete)
   b. Produce anti-GDF6 antibody for injection into xenografted mice (months 21-27; 80% 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)

What was accomplished under these goals?

Major activities, specific objectives and significant results:

1) Test whether anti-GDF6 antibodies cause melanoma regression in vivo
   • In this reporting period we have confirmed the effectiveness of anti-GDF6 antibodies in nude mice engrafted with SK-MEL28 tumors. SK-MEL28 cells were injected into the flanks of nude mice and allowed to establish tumors. When tumors reached ~100mm3, animals were treated with anti-GDF6 antibody. In these studies, we used two doses of anti-GDF6 antibody (50mg/kg and 10mg/kg) and found both doses to have some effect (Figure 2A). This enables future studies to be conducted with lower dosing, thus consuming less antibody. We are currently assessing treated and control tumors to determine if anti-GDF6 antibody activity was on target. Namely, we are measuring the BMP marker phospho-SMAD1/5/8 using immunohistochemistry and quantifying whether it is reduced in anti-GDF6-treated tumors as compared to control tumors.

2) Determine the ability of anti-GDF6 antibodies to complement existing melanoma therapies
   • In preparation for these studies we have assessed the effect of targeting GDF6 in melanoma cells. Using bulk RNA sequencing we have obtained transcriptional profiles of melanoma cells subjected to GDF6 knockdown and assessed its effect on proteins that are relevant to immune checkpoint inhibitor therapy. Interestingly, we found that class I MHC expression was elevated in cells subjected to GDF6 knockdown (Figure 2B). MHC class I is critical to presentation of tumor neoantigens that drive responses to immune checkpoint inhibitor (ICI) therapy. Furthermore, reduced MHC class I expression is a common cause of ICI resistance.

Thus, in addition to cooperating with ICIs due to their own, ICI-independent anti-tumor activity, anti-GDF6 antibodies may also enable ICI-resistant cells to regain sensitivity to ICI therapies. In the next reporting period we will combine anti-GDF6 treatment with ICI therapy to determine if there is cooperativity.

3) Assess the extent of GDF6 expression and BMP activity in rare melanoma subtypes
   • Using immunohistochemistry we have stained a subset of acral melanomas for GDF6 and the BMP activity marker, phospho-SMAD1/5/8. GDF6 was expressed at high levels in the majority of these tumors, and phospho-SMAD1/5/8 was also expressed in these tumors (Figure 3A,B). 3 of the 6 invasive primary acral melanomas had a prominent eccrine pattern of immunohistochemical signal (Figure 3A), with expression of GDF6 along with the melanocyte and melanoma markers Melan-a and SOX10 prominent in eccrine structures. These primary melanomas may fit the profile of syringotropic melanomas previously described (Zembowicz & Kafanas, 2012). We are staining additional samples for sufficient power to determine if there is an association between the presence of GDF6 and/or phospho-SMAD1/5/8 and patient clinical outcomes.

4) Determine the sensitivity of acral and mucosal melanomas to GDF6 inhibition
   • We obtained acral melanoma cell lines and one mucosal cell line and have grown these lines in culture. The growth characteristics of the mucosal cell line are not conducive to further study, e.g. xenograft. The acral melanoma cell lines are being injected into mice to determine the number of cells that can be used to generate xenografts. In the next reporting period we will inhibit GDF6 in acral melanoma cells in vitro and determine effects on cell growth and survival. Additionally, assuming suitable cell numbers for xenograft can be achieved, we will determine if GDF6 inhibition affects acral melanoma growth in vivo.

Other achievements:

The findings from this study have been used to obtain additional funding through the University of Massachusetts Chan Medical School BRIDGE fund. This fund is designed to promote translational opportunities for research being conducted at UMass Chan. The funding secured will allow my laboratory to develop human anti-GDF6 antibodies that can be used to obtain preliminary data for and support an IND filing.

What opportunities for training and professional development has the project provided?

Dr. Revati Darp was funded by the award and was a trainee in my laboratory. In this reporting period she received one-on-one mentorship, in particular about scientific writing, preparing scientific figures, preparing a manuscript for submission, and navigating the manuscript review process.

During this reporting period, Dr. Ceol participated in conferences, including the UMass Chan Medical School Clinical and Translational Sciences Symposium and Boston Area Zebrafish Research Conference.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

Plans for the next reporting period:

1) Test whether anti-GDF6 antibodies cause melanoma regression in vivo
   This task in nearly complete. In the next reporting period we will quantify proliferation index, cell death, BMP activity in tumors harvested from xenografted mice treated with anti-GDF6 and control antibodies.

2) Determine the ability of anti-GDF6 antibodies to complement existing melanoma therapies
   In the next reporting period we will treat melanoma xenografted mice with combination therapies. These combinations will be anti-GDF6+anti-PD1 and anti-GDF6+dabrafenib+trametinib and tumor growth in these animals will be compared to that of appropriate control-treated animals. Harvested tumors will be assessed for proliferation index, cell death and BMP activity.

3) Assess the extent of GDF6 expression and BMP activity in rare melanoma subtypes
   Additional immunohistochemistry of acral melanomas will be performed, and levels of GDF6 and phospho-SMAD1/5/8 will be quantified in these samples. The quantified levels of these markers will be compared to patient outcomes to determine if there is an association between the levels of these markers and patient survival, metastatic spread, response to therapy and other features.

4) Determine the sensitivity of acral and mucosal melanomas to GDF6 inhibition
   In vitro knockdown of GDF6 and antibody treatment will be performed in vitro on acral melanoma cell lines. The growth rate and survival of treated cells will be quantified. Xenografts of acral melanoma cell lines will be performed, and xenograted mice will be treated with anti-GDF6 antibodies to determine their sensitivities to treatment.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

New therapies for melanoma patients are a means to address the current shortcomings of immune checkpoint inhibitor (ICI) treatment. The technology that is the subject of this proposal – anti-GDF6 antibodies – does this.

Several studies have evaluated mechanisms by which melanomas, both cutaneous and acral, evade ICI therapy. One thread of studies has shown that resistance is associated with de-differentiation of melanoma cells. In a second thread of studies, ICI resistance was associated with reduced expression of MHC class I antigen presentation on melanoma cells. Loss of MHCI presentation on melanoma cells makes them invisible to ICI-stimulated T cells. Recently, a tight linkage between these two mechanisims, de-differentiation and MHCI downregulation, was shown.

Previous studies in the Ceol laboratory have shown that BMP signaling in melanomas, which is stimulated by the BMP ligand GDF-6, acts to promote de-differentiation and enable melanoma cell survival. As part of the current study, we have shown that anti-GDF6 antibodies on their own inhibit melanoma growth in vitro and in vivo. Additionally, we have also shown that GDF6-driven BMP signaling represses expression of MHCI genes. Thus, targeting BMP signaling

by the use of anti-GDF6 antibodies has the potential to upregulate MHCI expression and counteract an important mechanism by which melanomas evade ICI therapy. In ongoing work we are testing whether anti-GDF6 can cooperate with ICI therapies to further inhibit melanoma growth in vivo.

The approach of targeting BMP signaling in melanoma is unique since no drugs currently do this. Indeed, the concept of differentiating melanoma cells to enable ICI sensitivity is novel. Such a drug would potentially benefit the tens of thousands of Americans and hundreds of thousands of patients worldwide who are diagnosed with stage 2 through 4 melanoma and would develop resistance to ICI therapy. Furthermore, nearly 75% of melanomas exhibit BMP activity, indicating that a large fraction of patients could benefit from anti-GDF6/BMP13 therapy. Targeting of BMP signaling is through monoclonal antibodies directed against GDF6/BMP13. The use of monoclonals is beneficial because of their favorable tolerance, and since GDF6/BMP13 is specifically expressed by melanoma cells and not adult tissues, the risk for on-target toxicity is low.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

There has been one minor change in the approach during the reporting period. As mentioned above, the mucosal cells which we had planned to evaluate for our study do not have proper growth characteristics for in vitro or in vivo analysis. To our knowledge, this is the only mucosal melanoma cell line that is available. We therefore will focus our studies of rare melanomas on acral melanomas exclusively.

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

There have been few delays in this reporting period, although the project experienced significant delays in previous reporting periods related to the COVID-19 pandemic. These include the inability to perform research during a period of laboratory closure, restrictions on the use of animal facilities during the early part of the pandemic, and difficulties in hiring and retaining individuals due to pandemic-related issues.

Changes that had a significant impact on expenditures

We have experienced delays in hiring staff during this reporting period. In the wake of the COVID-19 pandemic it has been more difficult to find qualified individuals for research studies. For this reason and the reasons listed above, we have requested a no-cost extension for 12 months. During this extended period we anticipate hiring an individual who can complete the proposed studies.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

Publications, conference papers, and presentations

Darp, R., Vittoria, M.A., Ganem, N.J. and Ceol, C.J. (2022). Oncogenic BRAF induces whole-genome doubling through suppression of cytokinesis. *Nature Communications*, *13*, 4109.

Journal publications

Wilcock, D.J., Badrock, A.P., Owen, R., Guerin, M., Southam, A., Johnston, H., Ogden, S., Fullwood, P., Watson, J., Ferguson, H., Haworth, J., Richardson, D., Dunn, W., Wellbrock, C., Lorigan, P., Ceol, C., Francavilla, C., Smith, M.P., Hurlstone, A. (2022). Oxidative stress from DGAT1 oncoprotein inhibition in melanoma suppresses tumor growth when ROS defenses are breached. *Cell Reports*, *39*, 110995.

Vittoria, M.A., Kingston, N., Xia, E., Hong, R., Huang, L., McDonald, S., Tilston-Lunel, A., Darp, R., Campbell, J., Lang, D., Xu, X., Ceol, C., Varelas, X. and Ganem, N.J. (2022).

Inactivation of the Hippo tumor suppressor pathway promotes melanoma. *Nature Communications*, *13*, 3732.

Frantz, W.T., Iyengar, S., Neiswender, J., Cousineau, A., Maehr, R. and Ceol, C.J. (2023). Stem cell heterogeneity and reiteration of developmental signaling underlie melanocyte regeneration in zebrafish. *eLife*, in press.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Invited talks:

Local:
Clinical and Translational Science Research Symposium 2022
University of Massachusetts Chan Medical School, Worcester, MA

Regional
Boston Area Zebrafish Meeting (BAZAR) 2023
Boston College, Newton, MA

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Patent:
Title: Targeting GDF6 and BMP signaling for anti-melanoma therapy, European Patent EP3268025, US Provisional Application No. 62/130,749.

Invention disclosure:
University of Massachusetts Chan Medical School invention disclosure UMMS23-06: Anti-GDF6 antibodies for treatment of BMP-driven diseases

Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the 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: this award

Ms. Revati Darp
Role: Graduate Student, University of Massachusetts Medical School
Researcher ID: n/a
Months worked: 3
Contribution to project: Ms. Darp has conducted tests of antibody efficacy in vitro and assisted with in vivo testing of antibodies in xenografted mice.
Funding support: this award

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: this award

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: this award

Dr. Thomas Hornyak
Role: co-PI, Baltimore Research and Education Foundation
Researcher ID: n/a
Months worked: 0.6 (no salary requested)
Contribution to project: Dr. Hornyak has overseen collection and immunohistochemistry of acral melanomas.
Funding support: n/a

Mr. Emmanual Kalapurakal
Role: Researcher, Baltimore Research and Education Foundation
Researcher ID: n/a
Months worked: 1.8
Contribution to project: Mr. Kalapurakal has performed immunohistochemistry on acral melanomas.
Funding support: n/a

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The key personnel on the project are Drs. Craig Ceol (UMass Chan Medical School), Yang Wang (UMass Chan Medical School and MassBiologics) and Thomas Hornyak (BREF and University of Maryland). In the last reporting period, Dr. Ceol activated an NIH R01 award, AR081355-01A1, which replaces an NIH R56 award which was reported in the previous annual report. Dr. Ceol's effort with respect to his NIH awards R56 to R01 did not change, and his effort level on this Department of Defense award has not changed. There were no changes in active support for Drs. Wang and Hornyak since the last reporting period.

What other organizations were involved as partners?
Organization Name: Baltimore Research and Education Foundation
Location of Organization: Baltimore, Maryland USA
Partner's contribution to the project:

Subaward (e.g., partner's staff work with project staff on the project):
A subaward that is part of this grant goes to collaborator Dr. Thomas Hornyak and his laboratory for research on acral melanomas. Dr. Hornyak is part of the Baltimore Research and Education Foundation and a dermatologist at the University of Maryland and the US Veteran's Administration in Maryland. Dr. Hornyak's laboratory is performing immunohistochemistry for GDF6 and phospho-SMAD proteins in human acral melanomas.

8. SPECIAL REPORTING REQUIREMENTS

Dr. Hornyak and his laboratory operate under a subaward. The activities undertaken by his laboratory are specified above, and his laboratory's work on immunohistochemistry of acral melanomas is described.

9. APPENDICES:

Figures 1 and 2 are attached as an appendix.

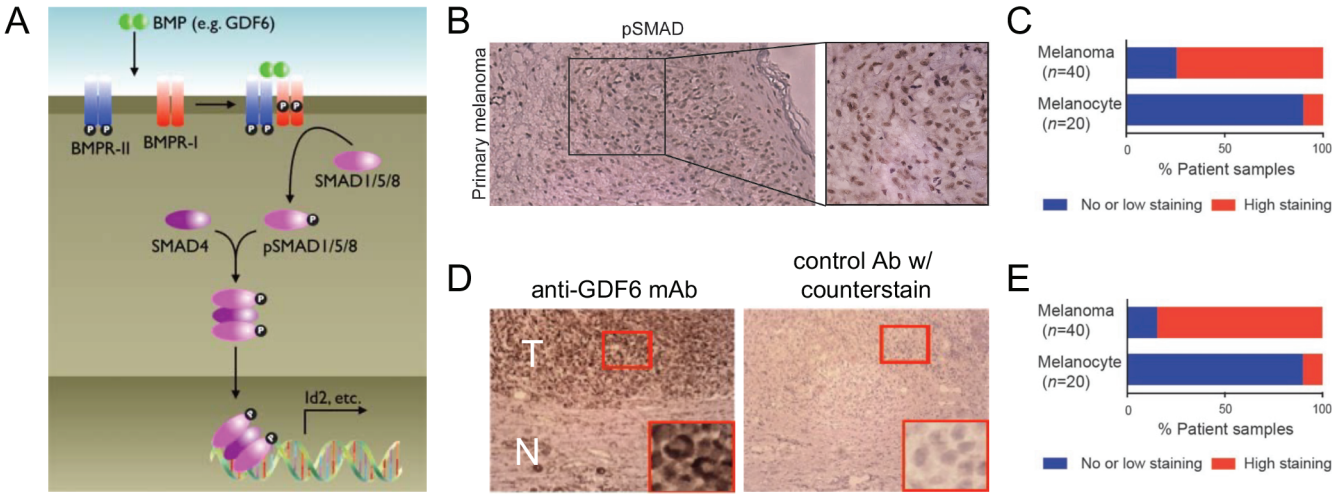

Figure 1: BMP signaling in melanomas.
A) Canonical BMP signaling: ligand binding induces receptor heterotetrameric assembly, leading to phosphorylation of type I receptors and subsequent phosphorylation of downstream SMAD1/5/8 effectors. Phosphorylated SMAD1/5/8 proteins enter and are retained in the nucleus. B) Staining of a primary cutaneous melanoma with an antibody against phospho-SMAD1/5/8. phospho-SMAD1/5/8 is predominantly localized to nuclei. C) Percentages of cutaneous melanomas and normal melanocytes that stain for high and low levels of phospho-SMAD1/5/8. Normal melanocytes have little to no BMP activity. D) Staining of a cutaneous melanoma with an antibody against GDF6. GDF6 is localized to the cytosol. T, tumor. N, normal tissue. E) Percentages of cutaneous melanomas and normal melanocytes that stain for high and low levels of GDF6.

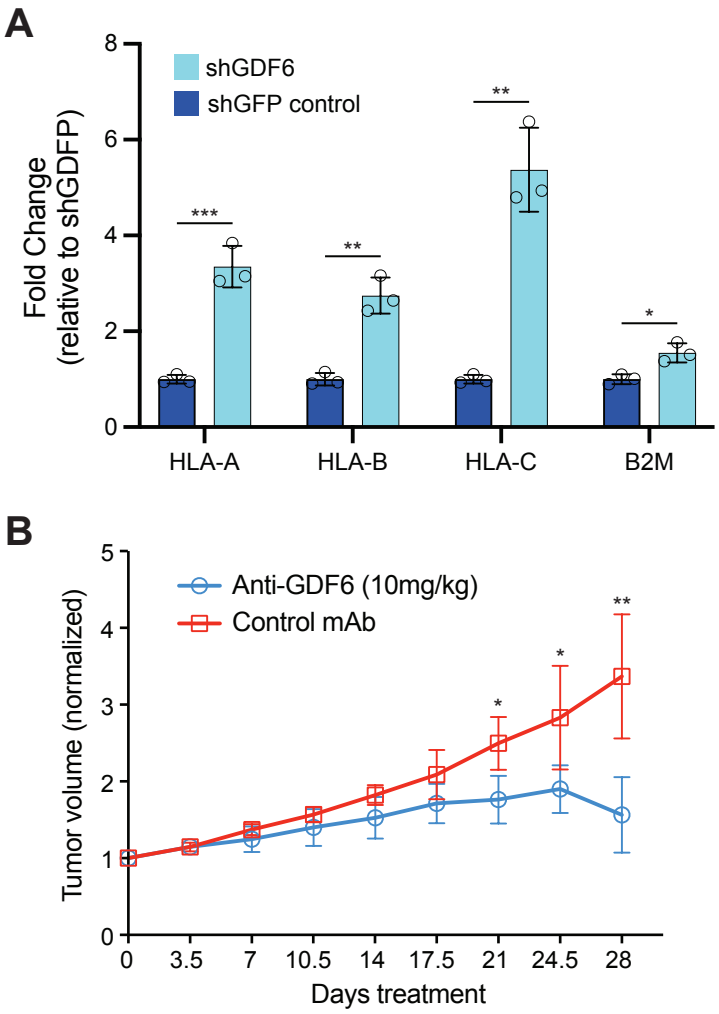

Figure 2: Effect of GDF6 inhibition on MHCI expression and tumor growth
A) GDF6 negatively regulates expression of MHC class I genes HLA-A, HLA-B, HLA-C adn B2-macroglobulin. Quantitative PCR showing increased expression of MHC I genes following knockdown of GDF6-dependent BMP signaling. B) Tumor volume measurement of nude mice xenografted with SKMEL28 cells and treated with anti-GDF6 or control antibody (each at 10mg/kg) when tumors reached 100mm3. anti-GDF6 treatment caused regression of xenografted tumors. *P < 0.05, **P < 0.01, and ***P < 0.001, by 2-tailed Student's t test.

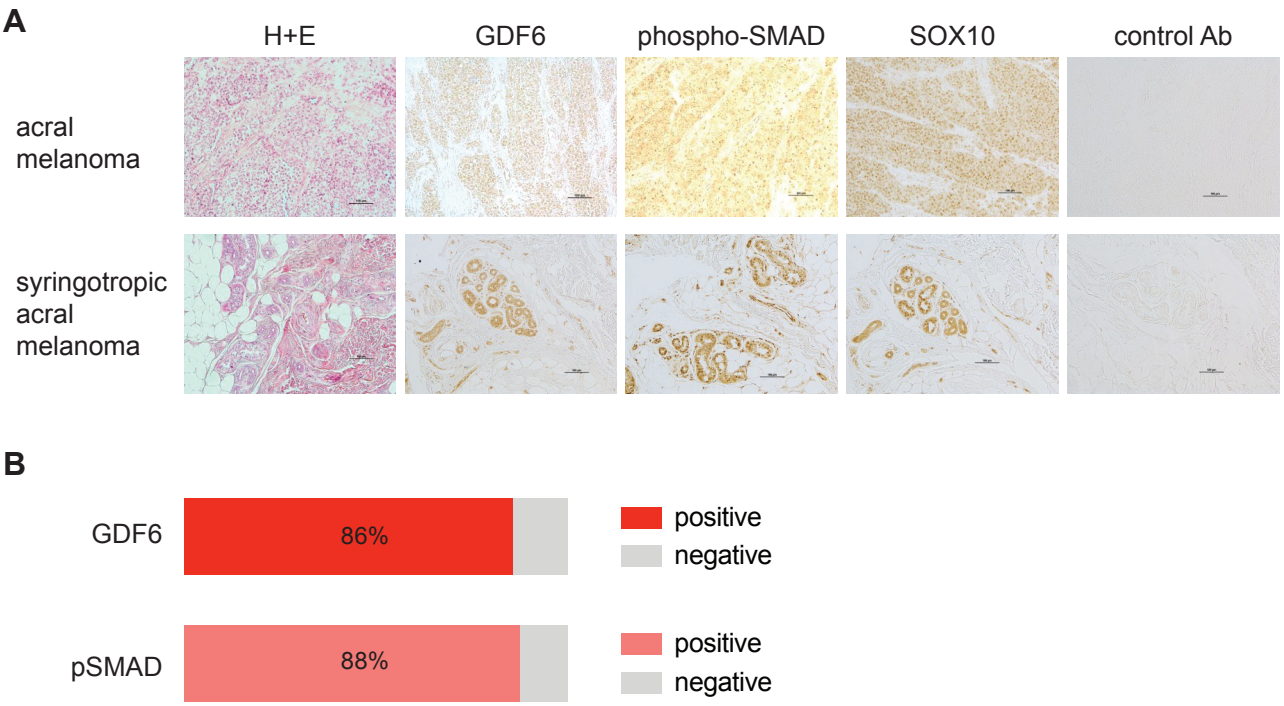

Figure 3: GDF6 and phospho-SMAD1/5/8 expression in acral melanomas
A) Immunohistochemistry of GDF6 and phospho-SMAD1/5/8 in acral melanomas. Top, an invasive acral melanoma. Bottom, an invasive syringotropic acral melanoma with an eccrine staining pattern. B) Percentages of acral melanomas that are positive for GDF6 (top; n=7) and phosphoSMAD1/5/8 (bottom; n=8).