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TITLE: Targeting the Six1/Eya Transcriptional Complex for Ovarian Cancer Therapy

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**ABSTRACT**

**Background:** The *Six1* homeobox gene encodes a transcription factor that is crucial for the development of many organs but is downregulated after organ development is complete. Its expression is low or undetectable in most normal adult tissues, but it is over-expressed in almost 70% of ovarian cancers, where its expression correlates with shortened survival. *Six1* is critical for proliferation and survival of ovarian cancer cells, which has been linked to both metastasis and resistance to chemotherapy. Since *Six1* does not have an intrinsic activation or repression domain, it requires the Eya coactivator proteins to activate transcription. Similar to *Six1*, Eya proteins are also not expressed in most normal adult tissues, but are re-expressed in cancers. Intriguingly, the Eya2 protein is amplified in 15% of epithelial ovarian cancers (EOCs), is overexpressed in as many as 94% of epithelial ovarian cancers, and like *Six1*, is associated with shortened survival in ovarian cancers.

**Hypothesis:** We hypothesized that the *Six1*/Eya interaction is critical for ovarian tumor growth and metastasis and that disrupting this interaction would significantly inhibit ovarian tumor growth and metastasis. Progress made is as follows:

**Aim 1.** Determine the role of the *Six1*/Eya interaction in tumor growth and peritoneal metastasis in EOC using a genetic approach. We have created stable ovarian cancer cell lines in which *Six1* or Eya are knocked down, and others in which Eya is overexpressed (WT or mutations that do not allow it to interact with *Six1* or to have Tyr Phosphatase activity). We have demonstrated that the interaction of *Six1* and Eya is critical for proliferation of ovarian cancer cells as well as for migration and alterations in adhesion.

**Aim 2.** Determine whether small molecules targeting the *Six1*/Eya interaction inhibit the proliferation and survival of EOC cells. We have tested our most promising *Six1*/Eya interaction inhibitors in ovarian cancer proliferation assays and found that they do inhibit proliferation. We are still optimizing our inhibitors for in vivo use (described in the progress report) but have been able to test them on a few ovarian cancer explants and have already found modest efficacy in growth inhibition in this assay.

**Subject Terms:** transcription, drug development
INTRODUCTION (taken from abstract of project):

Background: The Six1 homeobox gene encodes a transcription factor that is crucial for the development of many organs but is downregulated after organ development is complete. Its expression is low or undetectable in most normal adult tissues, but it is over-expressed in almost 70% of ovarian cancers, where its expression correlates with shortened survival. Six1 is critical for proliferation and survival of ovarian cancer cells, and it causes resistance to TRAIL-mediated apoptosis, which has been linked to both metastasis and resistance to chemotherapy. Since Six1 does not have an intrinsic activation or repression domain, it requires the Eya coactivator proteins (Eya1-4) to activate transcription. Similar to Six1, Eya proteins are also not expressed in most normal adult tissues, but are re-expressed in cancers. Intriguingly, the Eya2 protein is amplified in 15% of epithelial ovarian cancers (EOCs), is overexpressed in as many as 94% of epithelial ovarian cancers, and like Six1, is associated with shortened survival in ovarian cancers. These data suggest that the Six1/Eya complex may work together to stimulate ovarian tumor progression. Indeed, the Six1-Eya interaction is essential for proliferation and survival during development. Furthermore, we recently determined the crystal structure of the Six1/Eya2 complex and demonstrated that a single amino acid mutation (V17E) in Six1 disrupts the Six1/Eya2 interaction and inhibits Six1-mediated breast cancer metastasis in animal models, although the relevance of this interaction to ovarian cancer metastasis has not been examined. Thus, we hypothesized that the Six1/Eya interaction is critical for ovarian tumor growth and metastasis. We further hypothesized that disrupting this interaction will significantly inhibit ovarian tumor growth and metastasis. We have begun to test these hypotheses in the following specific aims using both genetic and pharmacological approaches. **Aim 1.** Determine the role of the Six1/Eya interaction in tumor growth and peritoneal metastasis in EOC using a genetic approach **in vitro and in vivo.** (Months 1-24).

1.1. **Determine the role of the Six1/Eya interaction in proliferation, survival, and TRAIL-resistance of ovarian tumor cell lines.** (Months 1-12)

For this subaim, we proposed to develop cell lines in which we would either express WT Six1, Six1 V17E (which cannot bind Eya), or CAT as a control to determine the role of the Six1/Eya interaction on tumor progression in EOC. In generating these cell lines, we determined that increased expression of Six1 in some of the lines did not impart the anticipated phenotypes, and subsequently found that this was likely because they already expressed relatively high levels of endogenous Six1 (not shown). However, we determined that while two particular cell lines, OV429 and OV432 contained high endogenous Six1, they did not express much Eya2 endogenously (not shown). Thus, we decided to approach the same question (is the interaction between Six1 and Eya required for EOC tumor growth and progression) from the angle of Eya2, rather than Six1. To be specific, we generated two new sets of cell lines, OV429 and OV432, which expressed high endogenous Six1 and to which we added Eya2. In this case, we added either WT Eya2, a mutant of Eya2 (A532R) that is unable to bind to Six1 (as previously shown by us in Patrick et al., *Nature Structural & Molecular Biology* 2013), an A532R mutant that also had an added NLS (as Eya2 will lose its ability to go to the nucleus when it cannot interact with Six1), and a mutant of Eya2 that lacks intrinsic tyrosine phosphatase activity (as this activity was
reported to be important for metastasis- Pandey et al., *Oncogene* 2010). The stable cell lines were examined for levels of Eya2 expression (to be sure all forms of Eya2 were expressed at approximately the same levels) (Fig. 1).

In addition, we took two ovarian cancer cell lines (SKOV3 and A2780) that express high endogenous Six1 and Eya2, and stably knocked down both proteins in those cell lines (Fig. 2). Once the stable cell lines were established, we tested them in a variety of assays to assess proliferative and migratory capacity. We found that Eya2 overexpression enhanced proliferation in both OV432 and OV429 cells, and that this was dependent on the interaction of Eya2 with Six1 but was not dependent on nuclear localization (in the absence of binding Six1) or on the tyrosine phosphatase activity of Eya2 (Fig. 3). We similarly found that KD of both Six1 and Eya2 led to decreased proliferation in SKOV3 and A2780 cell lines (Fig. 3). In addition, we saw substantial decreases in adhesion of the ovarian cancer cells to collagens and laminin that were also dependent on the interaction between Six1 and Eya2, but did not depend on the Tyrosine phosphatase activity of Eya nor its nuclear localization in the absence of binding to Six1 (Fig. 4). Finally, preliminary data also demonstrated that knockdown of Eya2 or Six1 in A2780 cells inhibited migration of these cells using Boyden chamber assays (data not shown).

Thus, these experiments have begun to provide the proof of principle genetic information that we wanted to validate the use of our Six1/Eya protein interaction inhibitors as a means to inhibit ovarian cancer growth and this aim was deemed to be about 80% complete (we still needed to perform more rigorous proliferation and apoptosis assays such as BrdU analysis and TUNEL staining etc, as outlined in the original proposal, as well as perform TRAIL related experiments).
However, we have recently had a significant setback as the main technician working on the project (who generated all these cell lines) very unexpectedly passed away. Unfortunately, we have been unable to retrieve these cell lines since his death, and thus have a new technician working to regenerate all the cell lines. Regeneration of these lines (as well as add-backs into the KD lines) is likely to take several months, and will set back the date and time in which we can continue to perform in vitro tumorigenesis/tumor progression related assays as well as in vivo assays using the genetically manipulated ovarian cancer cell lines.

1.2. Evaluate whether the interaction between Six1 and Eya is critical for tumor growth and metastasis in animal model (months 1-12 for animal approval, months 13-24 for animal experiments).

Submission of institution approved animal protocols and related material for DoD’s ACURO approval was performed and ACURO approval was received. Thus goal for months 0-12 achieved.

Fig. 3. The Six1-Eya2 interaction is critical for ovarian cancer proliferation. Cell titer-glo assays were used as a surrogate to measure growth rates of cells overexpressing WT Eya2, the Eya2-Six1 interaction mutant (A532R), NLS A532R (NLS), and a tyrosine phosphatase dead Eya2 (D274N). Similarly, these assays were used to assess proliferation in Eya2 and Six1 KD cells.

Fig. 4. The Six1-Eya2 interaction is critical for decreasing adhesion of ovarian cancer cells. Adhesion assays performed as outlined (Patrick et al., Nat Struct & Mol Bio 2013) by measuring adherence to the listed matrices. Mutants as outlined in Fig. 3 above.
Animal experiments are set to be performed in the upcoming months, however, as outlined above, cell lines need to be regenerated so this may not begin for another two or three months (thus more likely to start in month 16 rather than 13).

**Task 2.** Determine whether small molecules targeting the Six1/Eya interaction inhibit the proliferation and survival of EOC cells. (Months 1-24).

### 2.1. Confirm and characterize primary hits. (Months 1-12).

We have confirmed that all six classes of compounds inhibit the Six1/Eya interaction in ELISA assay (data not shown). We have established that the Chemotype 1 (the original class 4) and Chemotype 2 (the original class 5) compounds are the best classes of compounds to pursue moving forward due to their chemical characteristics (drug-like properties). We have tried to determine the mechanism of action of these compounds using a number of approaches including ITC, Surface Plasmon Resonance, and fluorescence. Unfortunately none of these approaches have unambiguously demonstrated which protein these compounds are binding, likely due to the low potency and weak binding of these primary hits. Using 2D NMR, we demonstrated that the chemotype 1 compound likely interacts with Eya2 leading to specific chemical shift changes (Fig. 5), although the spectrum quality is not sufficient to determine the exact binding site yet. We will try to improve the quality of the spectrum. At the same time, since the Six1/Eya interface is inaccessible in the current crystal form of Eya2, we are trying to generate new crystal forms of Eya2 to facilitate soaking experiments to determine compound binding site through crystallography. We are also trying to co-crystallize the compound with Eya2. As we continue optimizing these compounds in collaboration with our NIH colleagues, we will re-evaluate new analogs with higher potency through the above approaches, which will likely help us determine the mechanism of action of these compounds.

In collaboration with the NIH, we have been testing analogs of the original compounds to find inhibitors that a

**Fig. 5.** The addition of chemotype 1 compound to the Eya2 protein leads to specific chemical shift changes indicating that the compound is binding to Eya2. HSQC spectrum of Eya2 alone is shown in blue and Ey2+compound is in red.

**Fig. 6.** Analogs of original hits have IC50s in the low micromolar/high nanomolar range when tested on a MEF3 promoter luciferase construct (which has 6 tandem Six1 binding sites), and are also efficacious in the 3TP assay as a measure of TGF-beta signaling. In contrast, these analogs do no work on an unrelated promoter that does not contain Six1 binding sites (Dicer) even at the highest doses (50 mM).

more potent and that have better ADME characteristics. We have identified analogs of chemotype 2 with
IC50s in the low micromolar range, which is very promising for moving forward with this class (Fig. 6).

2.2. Examine the effect of promising small molecule hits in cell culture. (Months 13-18)

Because we had promising data in our genetic studies performed in aim 1, we began some experiments in cell culture with our promising hit compounds. Thus, we are ahead of schedule with this aim. First, we tested some additional analogs along with the 7571 analog shown above (4959 and 2877). We found that 4959 behaves similarly to 7571 in a second MEF3 reporter luciferase assay whereas the 2877 compound is inactive (Fig. 7). Interestingly, only the 7571 and 4959 compounds were able to inhibit the proliferation of A2780 cells (Fig. 7) as well as the SKOV3 cells (not shown), whereas the inactive compound was unable to inhibit proliferation. These data suggest that targeting the Six1/Eya2 interaction with our small molecules will be efficacious. Moving forward, we will test these compounds in additional assays (BrdU, TUNEL, migration, etc) to determine whether they can inhibit aggressive characteristics of ovarian cancer cells.

2.3. Determine whether small molecules targeting the Six1/Eya interaction inhibit survival of primary ovarian tumor explants. (Months 19-24)

Submission of institution approved IRB protocols and related material for DoD’s ACURO approval was performed. ACURO approval was received (Months 1-18)

We have begun the ovarian cancer explant experiments and have performed two such experiments to date. We have seen some efficacy of our inhibitors in this experiment, but given that the sample size is to date only two patients, we cannot yet report whether efficacy is consistent with Six1 and/or Eya2 expression until our sample size gets larger. Thus, this subaim is only just beginning.

IMPACT

Through this pilot project, we have begun to set the foundation toward the development of novel, tumor-specific agents that will target multiple aspects of ovarian tumorigenesis (proliferation, survival, tumor growth, and peritoneal metastasis), while sparing normal cells, by targeting the transcriptional complex Six1/Eya. Such specific, targeted agents are sorely needed in the treatment of EOC, where they can be combined with other therapies to increase overall therapeutic efficacy, while limiting side effects. Six1 and Eya are developmental genes that are over-expressed in a large percentage of ovarian cancers and their overexpression correlates with adverse outcomes in patients. In this pilot award, we have begun to test the hypothesis that disrupting the Six1/Eya interaction will significantly inhibit ovarian tumorigenesis and peritoneal metastasis, using both genetic and pharmacological approaches, and have preliminary evidence that disrupting this complex will
indeed be efficacious in inhibiting the growth (and potentially spread) of ovarian cancers. Since Six1 and Eya are regulators of embryonic development that are scarcely expressed in adult tissues, we believe that inhibition of the Six1/Eya interaction will lead to limited side effects in the adult. In addition, since a large percentage of patients with epithelial ovarian cancer overexpress Six1 (~70%) and Eya2 (~94%), therapies targeting this complex are likely to benefit a significant proportion of ovarian cancer patients. Thus, targeting the Six1/Eya transcriptional complex, which has never before been clinically targeted, could have tremendous impact on ovarian cancer treatment.

CHANGES/PROBLEMS

The largest change to the proposal (which is actual more of a technical change and is minor) is that we are assessing the role of the Six1/Eya interaction through creating mutations in Eya2 rather than Six1. This approach was taken because the cell lines expressed more endogenous Six1 on the protein level than we had realized.

The biggest problem we have had is that we have lost a number of the cell lines that we generated due to the unexpected and tragic death of one of the technicians on this project. Thus, we are in the processing of regenerating these lines and repeating the experiments he initially performed.

OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT

Nothing to report as this grant was not meant as a training grant but rather as a research grant.

HOW WERE THE RESULTS DISSEMINATED TO COMMUNITIES OF INTEREST?

Nothing to report as we have not yet published any of this work, but hope to within the next year.

PLANS FOR NEXT REPORTING PERIOD

We plan to continue with this work as outlined in the proposal, with the caveat that, as outlined above, we will need to regenerate all of our stable overexpression and knockdown lines which will set us back a few months. These cell lines will be regenerated as outlined in this progress report above, with a focus on examining the role of the Six1/Eya interaction through making mutations in Eya2 (rather than Six1). Once these cell lines are re-established, we will move into our in vivo experiments to examine the necessity of the Six1/Eya2 interaction in ovarian cancer progression using a mouse model. We will re-evaluate a number of chemotype 2 analogs in our biochemical and biophysical assays to identify mechanism of action. In addition, we will also now begin to more systematically test our small molecule compounds in a number of in vitro assays to measure the tumorigenicity and our metastatic characteristics of the ovarian cancer cell lines, and will test as many ovarian cancer explants as we can. These explants will be examined for Six1 and Eya2 expression as outlined in the original proposal, and will also be treated with lead compounds (as well as compounds from the same chemical class that are inactive) and MTS assays will be performed.

PRODUCTS

None to report yet. When we discover lead analogs that seem as if we can get them into in vivo experiments, we will likely patent these derivatives. We have not yet patented any of the analogs that we are using.
PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Rui Zhao, PhD, PI of proposal, 1.6 calendar months. Dr. Zhao coordinated and oversaw all biochemical and structural experiments in this proposal.

Lingdi Zhang, PhD, Research Instructor, 3.6 calendar months. Dr. Zhang was responsible for characterizing the Six1/Eya inhibitors using biochemical and structural approaches

Heide Ford, PhD, Collaborator, .54 calendar months. Dr. Ford coordinated and oversaw all cell culture experiments done to date.

Aaron Patrick, PhD, postdoctoral fellow, .93 calendar months. Dr. Patrick replaced Melanie Vincent on this grant and performed many cell culture experiments with the compounds. He left for a position in industry, and thus Joshua Cabrera (technician listed below) also worked on this proposal

Joshua Cabrera, MS, professional research assistant, 1.50 calendar months. Dr. Cabrera performed the genetic experiments (developing cell lines) as well as some of the compound experiments within the proposal.

Kian Behbakht, MD, Collaborator, .37 calendar months. Dr. Behbakht is a gynecologic oncology surgeon and ovarian cancer researcher. He oversaw experiments to test the effects of small molecules on primary ovarian cancer explants in Aim 2

Lubna Qamar, PhD, Senior Professional Research Assistant, 1.29 calendar months. Ms. Qamar was responsible for carrying out tissue explant experiments, and for helping in planning how to use these models.

Change in Active Support of PI or Key Personnel:

Rui Zhao (New support listed below that has come in at the same time or after the award of the DOD grant)

1. Cancer League of Colorado (Zhao/Ford) 7/1/14- 6/30/15 0 calendar
   $60,000 ($30,000 to Ford)
   Developing stapled peptides for breast cancer therapy
   Major goal: To develop novel stapled peptides that will disrupt the Six1/Eya interaction and inhibit breast cancer metastasis

2. Alex's Lemonade Stand Foundation (Ford) 7/1/2014-06/30/2016 1.2 calendar
   $125,000 ($62,500 to Ford)
   Targeting the Six1/Eya transcriptional complex to inhibit pediatric sarcoma
   Major Goal: This project addresses two overarching challenges in pediatric cancer therapy: 1) To identify means to eliminate the mortality associated with metastatic sarcoma, 2) To revolutionize treatment by replacing drugs that have life-threatening toxicities with safe and effective interventions, such as those that target the Six1/Eya transcriptional complex.

3. 1R21CA185752-01A1 (Zhao/Ford) 12/18/14-11/30/16 .96 calendar
   NIH/NCI $108,750
   Developing Cancer Therapies through Targeting the Six1/Eya Transcriptional Complex
The goal of this project is to identify and develop novel anti-cancer agents that target the Six1/Eya complex in breast cancer through characterizing and optimizing hit compounds identified through the use of a high throughput screen.

4. R01GM114178 (Zhao) 4/01/15 - 3/31/19 4.2 calendar months
NIH
$229,285 (including a subcontract to Dr. Hong Zhou at UCLA)
Title: Structure and function of U5 snRNP.
The major goal of this project is to understand the structure and function of U5 snRNP components using a combination of crystallography, electron microscopy, and yeast genetic approaches.

5. R21NS085514 (Zhao) 7/01/14 – 5/31/16 1.2 calendar months
NIH
$125,000 (including a subcontract to Dr. Chien-Ping Ko at USC)
Title: Spinal muscular atrophy therapy using recombinant SMN proteins.
The major goal of this project is to test the feasibility of using recombinant SMN proteins as a protein replacement therapy for Spinal Muscular Atrophy in cell culture and mouse models.

Heide Ford (New support listed below that has come in at the same time or after the award of the DOD grant)

1. Cancer League of Colorado (Zhao/Ford) 7/1/14 - 6/30/15 0 calendar months
$60,000 ($30,000 to Ford)
Developing stapled peptides for breast cancer therapy
Major goal: To develop novel stapled peptides that will disrupt the Six1/Eya interaction and inhibit breast cancer metastasis.

2. Alex’s Lemonade Stand Foundation (Ford) 7/1/2014-06/30/2016 1.2 calendar months
$125,000 ($62,500 to Ford)
Targeting the Six1/Eya transcriptional complex to inhibit pediatric sarcoma
Major Goal: This project addresses two overarching challenges in pediatric cancer therapy: 1) To identify means to eliminate the mortality associated with metastatic sarcoma, 2) To revolutionize treatment by replacing drugs that have life-threatening toxicities with safe and effective interventions, such as those that target the Six1/Eya transcriptional complex.

3. 1R21CA185752-01A1 (Zhao/Ford) 12/18/14-11/30/16 .96 calendar months
NIH/NCI $108,750
Developing Cancer Therapies through Targeting the Six1/Eya Transcriptional Complex
The goal of this project is to identify and develop novel anti-cancer agents that target the Six1/Eya complex in breast cancer through characterizing and optimizing hit compounds identified through the use of a high throughput screen.

Kian Behbakht: No new support
No overlap with DOD grant