OSTEOSARCOMA ALLIANCE

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Funding Meaningful Osteosarcoma Research Together

Guidelines: Proposal must benefit the osteosarcoma patient and be a new project or distinct portion of an ongoing larger project for which results can be expected in 12 months. At the completion of 12 months, results must be made available to share - regardless of succeed or fail outcome. The recipient must be available to present work underway and completed at the FACTOR conference in 2019. Fund may not be used for the formation of new organization or used for planning stages of research or other initiative. Presentation of check and tour of facility by MIB Agents is requested.

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Name: J. Andrew Livingston, MD and Chantale Bernatchez, PhD

Organization: University of Texas MD Anderson Cancer Center

Email: jalivingston@mdanderson.org Phone: (713) 792 - 3626

Initiative Name: Innovating Immunotherapy in Osteosarcoma through the use of Tumor-Infiltrating Lymphocytes (TIL) Amount Requested: $100,000

Desired Impact: To provide robust preclinical data for the development of a TIL therapy clinical trial in osteosarcoma

Projected Milestones: Sample collection (Q1-3), TIL/immune profiling via flow (Q2-3), TIL expansion and TCR sequencing (Q2-4), Final analysis and presentation of results (Q4)

I agree to Guidelines:

Immune therapies (aka immunotherapy) have been a major breakthrough in cancer treatment. Disappointingly, recent clinical trials with immune checkpoint inhibitors such as pembrolizumab only showed benefits in ≤ 5% of patients with recurrent/metastatic osteosarcoma. Given the poor results achieved with *in vivo* manipulation of the immune system (aka checkpoint blockade), we propose an *ex vivo* intervention: adoptive transfer of tumor-infiltrating lymphocytes (TIL).

We are uniquely positioned *to evaluate, develop, and implement TIL therapy in osteosarcoma* based upon long-standing expertise acquired in TIL therapy trials for metastatic melanoma lead by our dedicated team of scientists in the MD Anderson TIL Laboratory. We will translate this experience through pre-clinical studies in osteosarcoma. Our initial proposal will include tumor samples from 8 to 10 patients with osteosarcoma recruited over a 1 year to provide the basis for a potential clinical trial. For each patient, the sample will be split to be used for 3 purposes (extra tissue will be banked for future tumor assessment by histology):

1) Growth of osteosarcoma TILs for clinical proof of principle for feasibility.
2) Identification of suppression mechanism(s) or suboptimal T-cell (lymphocyte) activation tool within the tumor environment using multi-color flow cytometry.
3) T-cell receptor (TCR) sequencing to assess the diversity of the TILs residing in the fresh tumor in comparison to expanded TILs.

Growth of osteosarcoma TILs for clinical proof of principle for feasibility. We have previously shown in metastatic melanoma that TILs are like soldiers residing in the tumor trying to fight it. Because of different survival mechanisms deployed by the tumor, the TILs are unable to fight it alone, being outnumbered and missing proper training and weapons. Expanding these TILs *in vitro* (i.e. in the laboratory), in presence of high dose of interleukin-2 (training camp, see schematic, **Figure 1**) gives rise to billions of "ready to fight cancer" TILs. In TIL therapy, theses TILs are then infused back into the same patient to fight their specific cancer.

Post-infusion, the TILs will be returning to a hostile war. To prevent exhaustion of the troops, we propose to study the mechanisms used by the tumor to stop the TILs from attacking and use these mechanism against the tumor itself. This will be done through the **identification of suppression mechanism(s) or suboptimal T-cell activation tool within the tumor environment using multi-color flow cytometry.** Flow cytometry is a powerful tool that enables us to study every individual cell within the tumor. With our expertise, we can study the TILs ("soldiers"), the dendritic cells (DCs) and macrophages ("generals and captains") and identify by looking at their specific features if they are fighting for the troops or have been corrupted by the tumor. In depth analysis of these results obtained from the fresh tumors of the 8 to 10 osteosarcoma patients will allow us to select the appropriate "weapon" to add to our TIL therapy.

TCR sequencing to assess the diversity of the TILs residing in the fresh tumor in comparison to expanded TILs. In addition, we want to make sure that we keep our soldiers diverse in their killing capacity as each tumor cell requires a different level of "expertise" to be destroyed. The T-cell receptor (TCR, see schematic) of a T-cell makes each T-cell unique. We will assess the TIL identity in the fresh tumors by identifying all the TCRs within the fresh tumors and compare them with the expanded TILs to make sure we are not losing any important soldiers during the training camp.

Taken together, we believe that this pre-clinical and translational work on immune profiling and TIL expansion will greatly increase our knowledge of immunotherapy and immunoresistance in osteosarcoma and will lead to rapid breakthroughs in the treatment of patients with recurrent/metastatic disease by bringing TIL therapy into the clinic.

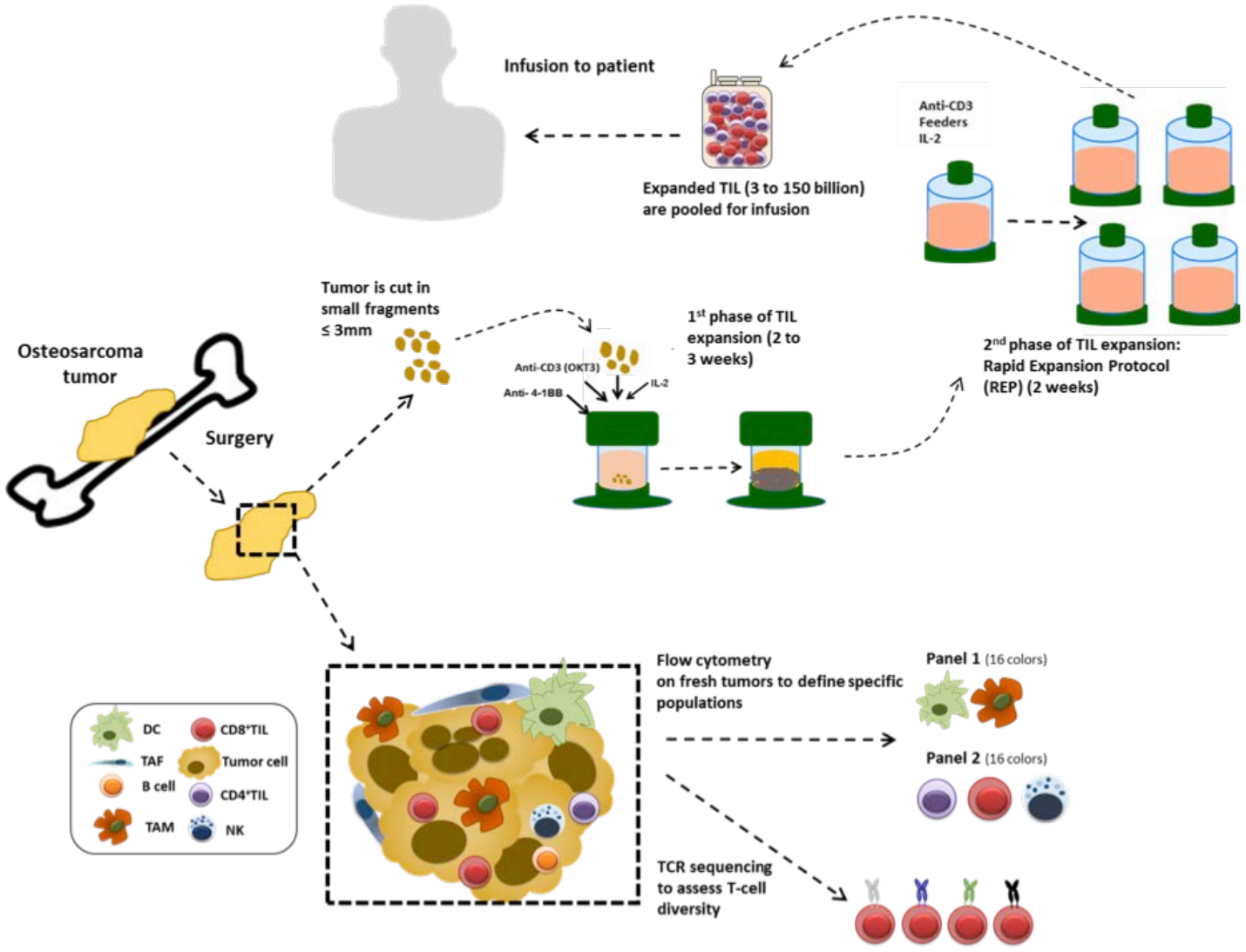

Figure 1. Schematic for the development of tumor-infiltrating lymphocyte (TIL) therapy in osteosarcoma.

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Name: Troy A. McEachron, PhD

Organization: Department of Translational Genomics, Keck School of Medicine, University of Southern California

Email: troy.mceachron@med.usc.edu Phone: 323-442-6049

Initiative Name: Combinatorial targeting of VEGFA in pre-clinical models of osteosarcoma Amount Requested: $100,000

Desired Impact: Our desired impact is to generate sufficient pre-clinical data to suggest that combination VEGFA inhibition is an effective therapeutic approach in a subset of molecularly defined osteosarcoma patients.

Projected Milestones: Milestone 1: Complete *in vitro* studies (0-4 months); Milestone 2: Complete in vivo studies (5-12 months); Milestone 3: Present data at the 2019 FACTOR conference

I agree to Guidelines: Troy McEachron

Osteosarcoma is an aggressive bone tumor typically observed in pediatric, adolescent, and young adult patients (AYA) and we are still treating patients the same today as we did in the mid-1980's. Over the last few decades the rate of overall survival has remained at roughly 65% for "localized" tumors and less than 30% for patients whose disease has spread to other organs. One reason behind this lack recent progress is that we have not been able to identify recurrent mutations in these tumors that can be utilized to design "targeted therapies". Recent technological advances have allowed us to understand that osteosarcomas do indeed have recurrent changes in their chromosomes and that these changes may be the key to unlocking new targeted therapies. As a young junior investigator, my goal of my laboratory is to use the genetic information contained within these complex tumors to guide rational therapies.

Approximately 20-25% of patients with osteosarcoma have chromosomal changes that lead to extra copies of the gene VEGFA, which encodes the vascular endothelial growth factor A. This gene is primarily responsible for growing new blood vessels that feed the tumor (angiogenesis) and instructing cancer cells to rapidly divide. These tumor blood vessels are not normal and prevent drugs and immune cells from penetrating the tumor while providing an escape route for tumor cells to spread to other organs (metastasis). Patients with osteosarcoma whose tumors produce high levels of VEGFA demonstrate shorter overall survival than those patients whose tumors do not have elevated VEGFA. Currently, there are multiple FDA-approved drugs that block angiogenesis and normalize tumor blood vessels, however, we know that individual drugs rarely produce long-term therapeutic effects. ***Together, these data lead us to hypothesize that the subset of patients whose tumors contain extra copies of VEGFA may be prime candidates for a combination therapy approach designed to inhibit VEGFA.*** We respectfully request $100,000 in funding to support our proposed study entitled "Combinatorial targeting of VEGFA in pre-clinical models of osteosarcoma." These funds will be used to investigate the efficacy of pharmacological inhibition of VEGFA using both in vitro and in vivo models of osteosarcoma. We anticipate that the data obtained from our investigation will serve as the foundation for further clinical investigations.

This project seeks to answer three specific questions:

1- Do osteosarcoma cell lines with extra copies of VEGFA display increased sensitivity to combination VEGFA inhibition in vitro?

2- Does the amount of VEGFA produced by the tumor cells dictate the therapeutic efficacy of combination VEGFA inhibition in vivo?

3- Does combination VEGFA inhibition provide durable therapeutic responses *in vivo?*

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Name: Giselle Sholler, MD MSc and Albert Cornelius, MD

Organization: Helen DeVos Children's Hospital / Beat Childhood Cancer

Email: giselle.sholler@helendevoschildrens.org Phone: 616-267-0334

Initiative Name: Personalized Medicine in Osteosarcoma Amount Requested: $100,000

Desired Impact: Identify novel pathways and drug combinations to target individual patient tumors

Projected Milestones: Analyze individual tumors for genetic changes activating tumors (AIM1: Months1-2). Use results to predict which drugs will be the most effective. Test drugs in tumor cells and mice models of matched patients. After treatment, the lab will study the tumor cells to see if the drug has targeted the genetic change effectively (AIM 2-3: Months 3-12).

I agree to Guidelines: 4/8/18

Background: Osteosarcoma (OS) is the most common bone tumor of children and young adults. Standard treatment with chemotherapy combined with aggressive surgical resection can cure 50-70% of patients, but survival among patients with metastatic or recurrent disease is poor. Treatment and outcomes have not improved significantly over the last 30 years, and more than a dozen clinical trials conducted over the past decade have failed to show any significant activity against relapsed OS. We must do better. As cancer treatments have advanced, it has been shown that the more targeted and personalized the treatment, the better the outcome. Personalized, precision medicine involves studying the genetic code of a child's tumor to identify what is driving the tumor to grow in order to find the best drugs to stop the patient's cancer. Our Beat Childhood Cancer Consortium has created a resource from 15 patients with relapsed osteosarcoma whose tumors have been sequenced to identify their genetic (DNA and RNA) changes, therefore identifying the mutations and pathways driving the tumors. Along with the genomic information, we have grown the tumor cells in our laboratory and have grown the tumors in mice models, creating mouse "avatars" from 11 of the 15 patients. This is a valuable resource to revolutionize the development of new and personalized therapies.

Hypothesis: Analyzing genetic changes that are driving tumors to grow can lead to identification of novel personalized drug combinations to treat children with osteosarcoma.

Aim 1: Analyze tumor genetic information to identify pathways driving individual osteosarcoma tumors using a systems biology approach. Patients enrolled on Molecular Guided Therapy NMTRC008/009 study "Feasibility Trial Using Molecular Guided Therapy for the Treatment of Patients with Relapse and Refractory Childhood Cancer" underwent a biopsy of their recurrent tumor. A complete genetic analysis was performed on their tumor in the form of whole-exome DNA sequencing and RNA sequencing (Figure 1 and 2). From this data, we will perform in depth biological analysis of the genetic changes and pathways driving each child's cancer to grow using additional advanced computational methods. This will identify drug targets to be tested in the following aims.

Figure 1. DNA Exome analysis of osteosarcoma tumors identified patients with mutations, amplification or deletions in each of TP53 (67%), NCOR1 (40%) or RB1 (33%). Copy number analysis identified MYC amplification in 40% of patients.

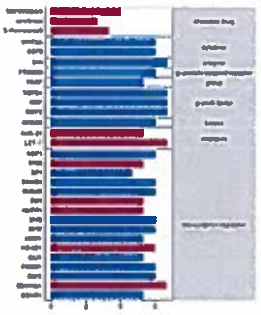

Figure 2 - Pathway Analysis of RNA expression. RNA pathways identified changes in the activity of upstream regulators for ERBB2, MYC, TGFB1, TP53, CDKN2A, HDAC1, VEGF, and HGF.

Aim 2: Identification and testing of drugs that are effective against patient derived osteosarcoma tumors in culture.
Cancer's often become resistant when treated with only one drug, and progress depends on finding multi-drug combinations to overcome tumor resistance to therapy. When fighting a fire one will be quickly engulfed in flames if targeting from only one hose, however the fire will be extinguished most effectively when several hoses are aimed at multiple angles. In order to find the multiple approaches needed to target each child's individual tumor, we will begin with our established resource of 11 patient osteosarcoma cell lines growing in culture. These will be tested using high throughput screening of 1400 drugs to evaluate which ones will be most successful for each patient. These drugs will then be tested in focused combination drug panels. In addition, correlation of these drug screening results with genomic analysis (from Aim 1) will help us to identify novel drug combinations to overcome the drug resistance which occurs in tumors when only using single drug therapy. These studies will help us to screen many agents to find out which combination is best for each individual child. These large studies can be done in osteosarcoma cell lines rapidly and then the best drug combination can then be confirmed in the animal models.

Aim 3: Focused testing of promising new drug combinations on patient Avatars.
Results of drug and pathway screening will identify the most promising combinations for each child with osteosarcoma. We will then test these predictions in animal models of each child's tumor (patient Avatar's). For each patient we anticipate the identification of four drugs that were effective against the cells in Aim 2. We will use these novel drug combinations to treat the patient Avatars to see which combination is most effective against each child's tumor.

Impact: This study will lead to the development of personalized treatment options identified through genetic analysis of tumors and high-throughput drug testing confirmed by Avatar mouse models. This will be translated to personalized treatment approaches to be used in real time for children with osteosarcoma.

Name : Subree Subramanian, MS, PhD

Organization: University of Minnesota

Email: Subree@umn.edu

Initiative name: **Combinatorial treatment of epigenetic regulation and immune response in Osteosarcoma**

Amount Requested: $100,000

Our goal in this project is to develop genomically informed combination therapies for osteosarcoma (OS). The molecular basis of osteosarcoma has received considerable attention during the last decade. However, clinical outcomes continue to be dismal and have landed this disease among the "most wanted" for development of new, effective therapies. The main barrier to successfully treat osteosarcoma is the lack of robust prognostic markers and the absence of effective therapies. The complex biology and tumor heterogeneity of osteosarcoma make it challenging to identify and evaluate new therapeutic targets and agents. Notably, we observed that **a unifying feature of highly aggressive osteosarcoma is the dysregulation of 14q32 microRNAs gene networks**. MicroRNAs are small non-protein coding RNAs that regulate gene expression and can function as tumor suppressors. One of the key functions of **14q32 microRNAs is to protect the expression of PTEN, a key tumor suppressor in OS.**

We and others have shown that microRNAs at the 14q32 locus are significantly downregulated in aggressive osteosarcoma and that this is directly correlated with chemoresistance and patient outcomes. We demonstrated that 14q32 miRNAs cooperatively regulate cMYC expression and function as tumor suppressors in OS. Further, deregulation of 14q32miRNAs network leads to overexpression of oncogenic miR-17-92 in OS. miR-17-92 is known to target a number of tumor suppressor genes including PTEN. **Downregulation of PTEN results in enhanced oncogenic PI3K activity. Activation of the PI3K pathway via PTEN loss promotes immune escape of cancers by driving immune checkpoint gene expression, including PD1/PD-L1.** Immune checkpoint blockade (ICB) using anti-PD1/PD-L1 is being investigated in OS.

We **hypothesize** that downregulation of 14q32 miRNAs in OS leads to the generation of a '*cold tumor*' and/or tumor microenvironment that support T cell exhaustion either by direct signaling using novel checkpoint inhibitors or secondarily by the redirection of metabolic intermediates into tumor cell division and away from immune cell replication. While this is likely an oversimplification of a complex situation it is essential that we learn whether activation of 14q32 microRNA by DNA and chromatin modifying drugs such as 5-AZA and SAHA along with immunotherapy anti-PD1 can lead to improved OS tumor control, representing a significant breakthrough in our ability to use combination immunotherapy in OS.

Aim. Evaluate the pre-clinical therapeutic efficacy of combination treatment of 5AZA/SAHA and anti-PD1 in osteosarcoma.

Dysregulation of 14q32miRNA/miR-17-92 networks is conserved and associated with aggressive osteosarcoma. Further, we showed that treatment with 5-AZA and SAHA restores expression of 14q32 miRNAs of aggressive osteosarcoma cells. Notably, this treatment also mimics RB expression

in osteosarcoma cells. Our working hypothesis is that modulation of 14q32miRNA/miR-17-92 in osteosarcoma will reduce tumor progression and sensitize to immunotherapy with anti-PD1. We will 1) evaluate the pre-clinical therapeutic efficacy of the 5-AZA and SAHA along with anti-PD1 in osteosarcoma and determine the biological endpoints (tumor regression and prevention of metastasis) of this combinatorial treatment in preclinical models, and 2) determine the mode-of action of combinatorial treatment.

Rationale. Our data showed that dysregulation of 14q32miRNA/miR-17-92 networks is conserved and associated with aggressive osteosarcoma. *14q32 miRNAs are epigenetically and epigenetic alterations downregulate their expression leading to loss of PTEN expression and immune suppression by PI3K activation.* Further, we showed that treatment of aggressive osteosarcoma cells with 5-AZA and SAHA restores expression of 14q32 miRNAs. This treatment also mimics RB expression in osteosarcoma cells. *These drug treated cells also showed increased expression of PTEN protein and significant levels of cell death through apoptosis in aggressive osteosarcoma cells. We propose to use various OS cell lines and orthotopic OS mouse model as platforms to investigate a combination of FDA approved DNA and chromatin-modifying drugs, specifically 5-Aza and SAHA in combination with anti-PD1 immunotherapy.*

Methods: We will evaluate the combination therapeutic effect in our established syngeneic immune competent model using mouse K7M2 osteosarcoma cells which more closely replicates clinical conditions of metastasis. To optimize drug dose *in vivo,* we will generate intratibial orthotopic osteosarcoma mouse model. We will use K7M2 cells stably expressing a luciferase gene construct that will be used to track *in vivo* metastasis using the Maestro imaging system**.** Upon establishment of orthotopic tumors 14-21 days, we will initiate treatment using the combine drugs 5AZA/SAHA/anti-PD1, and a vehicle control. 5-Aza (25 µg/kg); SAHA (50 mg/kg) will be delivered IP every day for five weeks. The anti-PD1, (BioXCell, i.p at 250 µg/mouse) will be IP administered once a weak and continued until these animals show either complete tumor regression i.e., tumor size reaches 1 cm^3 (sacrifice limit). Based on the power analysis that accounts for 80% success rate in establishing orthotopic tumors with K7M2 cells, 45 Balb/c mice will be randomized and divided into treatment (n=30) and control (n=15) groups.

We will plot the tumor regression or progression and measure the statistical significance of tumor volume (normalized to initial tumor in case of orthotopic models), metastatic response, and endpoints (tumor free survival, regression, or death). Resectable tumors will be surgically removed, weighed, and samples prepared for histological evaluation. The toxicity of these drug combinations will also be assessed by monitoring body weight, survival rate, and serum levels of enzymes, such as alkaline phosphatase.

Immune microenvironment and T cell infiltration/activation. Intra-tumoral immune cells will be analyzed by flow cytometry panels that will provide general information about the makeup of immune infiltrates as well as specific information about the state of T effector function.

Project mile stones. Upon the successful completion of these studies, we will have evaluated preclinical potential of modulation of microRNAs and immune regulation in osteosarcoma treatment. **These results will fundamentally advance our knowledge of how cancer cells modulate and suppress the immune response, provide novel targets, and form the basis for a new anticancer therapeutic strategy.**

Desired impact. These foundational and pre-clinical studies will help validate and prioritize this novel combinatorial treatment for potential osteosarcoma human clinical trials.

I agree to the Guidelines: 

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Name: Matteo Trucco, MD

Organization: University of Miami-Sylvester Comprehensive Cancer Center

Email: mxt722@med.miami.edu Phone: 305-243-4830

Initiative Name: Clinical trial of disulfiram to overcome chemotherapy resistance in sarcomas.

Amount Requested: $100,000 for purchase and compounding of disulfiram, and for ALDH testing.

Desired Impact: To prevent relapses by overcoming resistance to chemotherapy.

Projected Milestones: By early 2019, we anticipate having the trial approved and ready to enroll patients.

I agree to Guidelines: Matteo Trucco

Background: Adding chemotherapy to surgery greatly improved the survival of children and young adults with osteosarcoma. The treatments used, however, have significant short and long-term side effects, and the benefits are mostly for patients with localized disease. Even if the osteosarcoma seems to respond to therapy and whatever tumor seen is removed surgically, the tumors often come back. One explanation for this that there may be a small population of sarcoma cells that are resistant to chemotherapy but then grow recurrent (relapsed) tumors (Figure 1). These cells are often referred to as Cancer Stem Cells (CSCs). A way of identifying CSCs in several sarcomas including osteosarcoma is isolating the cells that have high levels of the enzyme Aldehyde Dehydrogenase (ALDH).[1-3] Recent data suggests ALDH is responsible for the resistance to chemotherapy seen in CSCs.[4-7] ALDH's job in cells is to break down aldehydes. Key chemotherapeutic drugs such as cyclophosphamide and ifosfamide are aldehydes. ALDH has also been associated with resistance to the many other chemotherapy drugs used to treat sarcomas, including cisplatin, doxorubicin, gemcitabine and docetaxel. This is thought to be because of ALDH's ability to inhibit reactive oxygen species (ROS) production, which is essential to how some chemotherapy drugs and radiation kill cancer cells (Figure 2).[5,8]

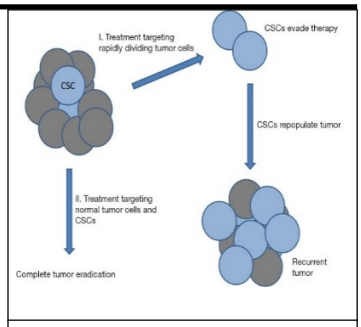

Figure 1. Depiction of CSC giving rise to recurrent tumor (Clark and Palle Annals of Translational Medicine 2016)

We recently completed a clinical trial of the mTOR inhibitor temsirolimus combined with liposomal doxorubicin for relapsed sarcomas showing that the combination prolonged survival more than either drug alone and that ALDH levels went down in the tumors of the patients that benefitted from the treatment (*manuscript submitted*). Blocking mTOR in cells has been shown to decreases ALDH levels, and subsequently increases osteosarcoma cells' sensitivity to chemotherapy-induced ROS and decreases their ability to form metastasis.[8] While mTOR inhibition appears to have some effect on ALDH-expressing sarcomas, ALDH can be blocked more directly and effectively with a medication called disulfiram. Disulfiram, originally developed as an anti-alcoholism drug over 60 years ago, specifically blocks ALDH, which is also necessary to breakdown alcohol. Several studies have shown disulfiram's ability to inhibit ALDH in cancer cells such as osteosarcomas, reversing their resistance to chemotherapy and killing them.[5,8-10] Disulfiram is safe and patients can take it for years.[11,12] Clinical trials testing disulfiram combined with chemotherapy are currently underway for breast, prostate, lung, and pancreatic cancer, among others. We propose a phase I clinical trial of disulfiram combined with gemcitabine/docetaxel for the treatment of children and young adults with relapsed osteosarcoma, Ewing sarcoma,

rhabdomyosarcoma and other sarcomas. We hypothesize that adding disulfiram to chemotherapy will make sarcoma CSCs sensitive to the chemotherapy leading to better and sustained responses to treatment.

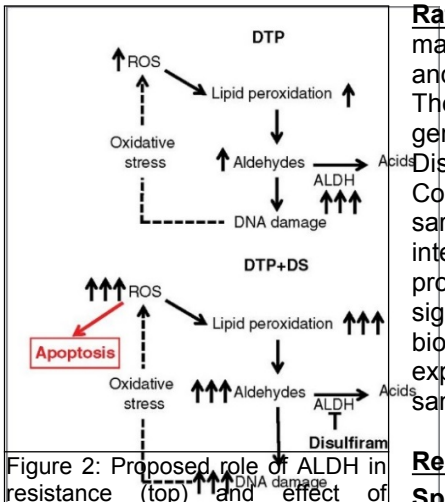

Figure 2: Proposed role of ALDH in resistance (top) and effect of disulfiram (bottom). DTP = drug-tolerant population; DS = disulfiram; ROS = reactive oxygen species; (Raha et al. Cancer Research 2014)

Rationale: Laboratory studies have shown disulfiram is toxic to sarcoma cells and make them sensitive to chemotherapy. Gemcitabine/docetaxel is a well-established and well-tolerated regimen for the treatment of several different relapsed sarcomas.[13,14] There is evidence that disulfiram makes cancer cells more sensitive to both gemcitabine and docetaxel, supporting a clinical trial of the proposed combination.[9,10] Disulfiram, however, has yet to be tested in patients with sarcomas. At the Sylvester Comprehensive Cancer Center (SCCC), we have a very active adult and pediatric sarcoma program, including dedicated oncologists, surgeons, pathologists and interventional radiologists, as well as very active adult and pediatric clinical trial programs. We have completed multiple clinical trials focusing on sarcomas. We have significant experience in clinical trials that include minimally invasive serial tumor biopsies to test response to our treatments. Furthermore, we have the reagents, experience, and resources to measure precisely ALDH levels in patient tumor samples.

Research Strategy:

Specific Aim1: Determine the safety and tolerability of disulfiram in combination with gemcitabine/docetaxel in pediatric and young adult patients with relapsed or resistant sarcomas. We will conduct a clinical (phase 1, 3+3, dose escalation) trial of disulfiram in combination with standard dose gemcitabine/docetaxel. For safety, the initial dose of disulfiram in this combination will be 80% of the standard dose (240mg/m²). If the initial dose level is determined to be safe, we will then give 100% standard dosing (300mg/m²). If 80% dosing is too toxic, a 50% dose (150mg/m^2) will be tried. The standard gemcitabine/docetaxel regimen to be used is gemcitabine (900 mg/m²) on Days 1 and 8 and docetaxel (75 mg/m²) on Day 8 of a 21-day cycle. Disulfiram will be administered once daily by mouth on Days 1 to 21. Inclusion criteria includes age 1-40 years old with a diagnosis of relapsed or refractory sarcoma who have adequate organ function. Adult patients (> 18 years old) must agree to minimally invasive tumor biopsy prior to treatment and after cycle 2, if deemed safe. Tumor biopsies will be optional for patients <18 years old. Patients must abstain from alcohol consumption and cannot take medication known to interact with disulfiram. An appropriate "washout" period from previous therapy must elapse prior to beginning treatment on this trial. Side effects, physical exam, and laboratory tests will be monitored to determine what dose of disulfiram is safe to give with gemcitabine/docetaxel using the National Cancer Institute - Common Terminology Criteria for Adverse Events (NCI-CTCAE), Version 4.0. Patients can continue treatment on the study for a maximum of 12 months as long as the patients appear to be benefiting from the treatment and they wish to continue on the study. Response to therapy will be assessed every 6 weeks with radiographic imaging and measured using Response Evaluation Criteria in Solid Tumors (RECIST) criteria.

Specific Aim 2: Test how effective Disulfiram is in eliminating Aldehyde Dehydrogenase (ALDH) expressing cancer stem cells and treating relapsed/refractory sarcomas. Minimally invasive core-needle biopsies of tumors will be performed by interventional radiology at two time points: 1) prior to beginning therapy and 2) after 2 cycles of treatment. Tumor tissue collected on patients enrolled on the study will be measured for ALDH levels. Tissue samples will be stored in liquid nitrogen until testing. On each patient, the two samples will be analyzed at the same time for more accurate comparison. Samples will be broken up into single cells and the ALDEFLUOR® reagent (Stem Cell Technologies, Vancouver, BC) will be used to label the ALDH. The cells will then be analyzed using a machine called a flow cytometer to measure the amount of ALDH in each cell and identify the CSCs population. We routinely performs this assay on healthy and cancer cells and have standardized protocols and controls for these tests. The technicians performing this testing will not be aware of whether the patients are responding to treatment to avoid bias. The ALDH measurements in pre- and post-treatment tumor samples will be sent to the team running the clinical trial who will see if ALDH levels match up with response to therapy. This will be helpful to determine what patients are likely to benefit from this therapy moving forward.

Innovation/Significance: While note exclusive to osteosarcoma, the proposed study is the first step in developing disulfiram as an addition to the treatment of sarcomas. This clinical trial proposes a novel strategy for targeting a source of resistance to chemotherapy thought to allow relapsed disease. It represents a major step toward more targeted, less toxic and more effective treatments for children and young adults suffering from sarcomas. By targeting CSCs with a well-studied drug, we can potentially eliminate the key cells responsible relapses and metastases, safely. The proposed studies measuring ALDH levels pre- and post-therapy are essential for confirming that disulfiram is really inhibiting ALDH and that ALDH levels predict which patients are likely to benefit from this type of therapy. The findings from this study will be used to secure additional funding from the NIH and other foundations to support expanded trials in specific sarcomas including osteosarcoma.

References:

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Name: ___Brian A. Van Tine M.D., Ph.D._____

Organization: _Washington University in Saint Louis _____

Email: __bvantine@dom.wustl.edu_____ **Phone:** _____314-747-3096_____

Initiative Name: **UNDERSTANDING OSTEOSARCOMA METABOLISM TO IMPROVE THERAPY**

Amount Requested: $100,000

Desired Impact: To improve the treatment of Osteosarcoma by obtaining the necessary preclinical justification to test PHGDH inhibitors in Osteosarcoma Patients. In addition, we wish to explore if we can use PHGDH expression as a biomarker to determine who should get high dose methotrexate.

Projected Milestones:

OS is a pediatric cancer that occurs in rapidly dividing bone. Current treatment for OS involves chemotherapy with high-dose methotrexate (HD-MTX), a therapeutic that inhibits an essential enzyme in the folate cycle, blocking proliferation and tumor growth. The broad objective of this project is to understand the role of serine biosynthesis in osteosarcoma (OS) so that it can be utilized not only as a therapeutic target for treatment, but also as a biomarker to determine who may not need to receive high dose methotrexate. Given that serine metabolism is a key source of single carbon units for the folate cycle, it is possible that targeting serine biosynthesis might be similarly effective at treating OS. We have identified that 3-phosphoglycerate dehydrogenase (PHGDH) [1] [2], the rate-limiting enzyme of serine biosynthesis, is overexpressed in OS that is associated with poor survival, suggesting its potential as a therapeutic target.

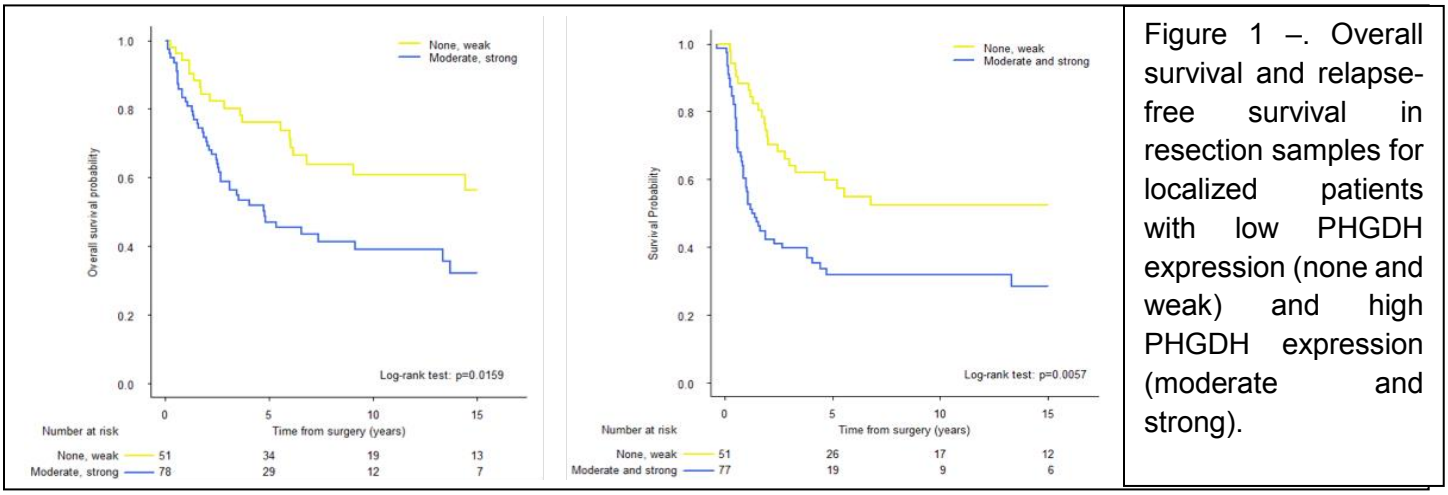

Figure 1 –. Overall survival and relapse-free survival in resection samples for localized patients with low PHGDH expression (none and weak) and high PHGDH expression (moderate and strong).

We have demonstrated that PHGHD over expression is associated with poor outcomes, as low expression of PHGDH is associated with an 18 year overall survival (unpublished results **Figure 1**). It is likely that low expressers of PHGDH may not benefit from high dose methotrexate therapy. There are now clinical PHGDH inhibitors NCT503 [3] and a compound from RAZE Pharmaceuticals (unpublished) that will need preclinical justification to justify testing them in osteosarcoma patients as opposed to breast cancer patients. These compounds kill osteosarcoma cell lines (Data not shown for space).

Long-term, we plan to identify PHGDH as a critical component of cellular metabolism and proliferation. This project will shed light on OS susceptibility to HD-MTX treatment due to upregulation of serine biosynthesis, and give rise to the possibility of more effective targeted therapeutics. **We hypothesize that PHGDH expression in osteosarcoma leads to dependence on serine biosynthesis only in patients that have a high expression of PHGDH.** If correct, this will provide the necessary preclinical justification for trials in osteosarcoma of the inhibitor from RAZE Pharmaceuticals. To test this hypothesis, we propose the following aims.

Aim 1. To determine if patients with a low expression of PHGDH benefit from high dose methotrexate
PHGDH has been reported to be overexpressed in OS. We will test a second pedigreed osteosarcoma micro-array set that has outcomes data and high dose methotrexate data to see if there is a correlation between tumor necrosis and high expression of PHGDH. **We hypothesize that osteosarcoma patients with low PHGDH expression will have a better overall survival independent of methotrexate exposure.**

Aim 2. To determine the utilization of extracellular versus intracellular serine in osteosarcoma.
It is important to elucidate the differential utilization of intracellular versus extracellular serine to determine the impact of the microenvironment on response to PHGDH inhibition. Preliminary data shows that growth rates of OS cell lines grown in serine-depleted media as compared to serine-rich media are identical. We have also shown that upon treatment with NCT-503, these cells die at similar rates irrespective of extracellular serine availability, suggesting that these cells are not reprogramming to utilize extracellular serine upon inhibition of *de novo* serine biosynthesis. **We therefore hypothesize that osteosarcoma cell lines do not rely upon extracellular serine, due to upregulated *de novo* serine biosynthesis.** We will test this by conducting [1,6-$^{13}C_2$]-glucose tracing using nuclear magnetic resonance spectroscopy (NMR) to measure the contribution of glucose carbons to the folate cycle. We will also conduct [3-^{13}C]-serine tracing to determine the metabolic fate of extracellular serine.

Aim 3. To determine how inhibition of serine biosynthesis induces cell death *in vitro* and *in vivo*.
Preliminary data obtained by fluorescence-activated cell sorting (FACS) shows that OS cell lines are susceptible to PHGDH inhibition. Based on this observation, it is important to determine the mechanism of cell death upon treatment. This will allow for potential dual targeting of OS, through simultaneous inhibition of serine biosynthesis and induction of the relevant cell death mechanisms. As PHGDH utilizes NAD to dehydrogenate 3PG, and the dysregulation of NAD-containing pathways has been shown to cause apoptosis, **we hypothesize that inhibition of PHGDH in osteosarcoma cell lines induces cell death by caspase 3-dependent apoptosis.** We will also generate cell-line derived xenografts and treat with NCT-503 to recapitulate the results *in vivo*. Tumor progression will be monitored, and tumor samples will be analyzed for caspase 3 cleavage and changes in other key proteins involved in cell death signaling pathways.

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I agree to Guidelines:

OSTEOSARCOMA ALLIANCE

MIB AGENTS OutSmarting OSTEOSARCOMA
Funding Meaningful Osteosarcoma Research Together

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Name: Charles Keller MD

Organization: Children's Cancer Therapy Development Institute

Email: charles@cc-tdi.org Phone: 801-232-8038

Initiative Name: Osteosarcoma Checkpoint Adaptation Amount Requested: $80,000

Desired Impact: new biology-based treatment options for osteosarcoma

Projected Milestones: Our overall goal is to make osteosarcoma in children, teens & young adults more sensitive to frontline and secondary treatments, and thus more survivable.

I agree to Guidelines:

THIS PROJECT WILL BE MATCHED 1:1 by The ERISTAND Charitable Fund and the Trey Foote Foundation (*i.e.,* for $80,000 from MIB Osteosarcoma Alliance, $80,000 more will be funded by EFC/TFF).

LAY SUMMARY: This project funds a childhood cancer research scientist to explore how resistance to chemotherapy and radiation can be reversed. We believe that cell surface receptors go to the tumor cell's nucleus in an unexpected way, and invoke genes that repair DNA damage. We believe that this can explain the clinical observation that radiation therapy is easily-resisted by osteosarcoma tumor cells, and in turn we believe we can reverse this resistance. Similarly, we believe we can improve the effectiveness of the second-line chemotherapy agent etoposide in a way that makes radiation and etoposide desirable front line therapies.

INTRODUCTION: The defining clinical and biological features of the bone cancer osteosarcoma are (i) the resilience of this bone cancer to chemotherapy and radiation, and (ii) the tumor cell tolerance of extreme chromosomal disorder (chromothripsis) (3). With respect to chromothripsis, Checkpoint Adaptation (CA) is a newly appreciated mechanism borrowed by osteosarcoma cells from yeast to ensure cell survival (4). This phenomenon is increasingly recognized as a mechanism by which sarcoma and other types of tumor cells evade cell death induced by chemotherapy & radiation at G2/M cell checkpoints (5, 6). In studies of osteosarcoma, this ability to evade G2 or M checkpoints is thought to be a process of stochastic clone selection where individual tumor cells evolve genetically during therapy (7). Outside of cancer, CA is a known, evolutionarily-conserved (ancient) mechanism of survival for unicellular organisms experiencing stressful environmental conditions (4) that increases the expression of G2 or M checkpoint-related proteins (or immediate early stress response genes transcribed from pHH3+ loci) to allow more time to repair DNA strand breaks or mitotic dysjunction. If repair is not complete at the end of a G2 or M checkpoint, elevated levels of IAP proteins (e.g.., Survivin) facilitate cell survival and checkpoint progression despite the incurred DNA damage or chromosomal

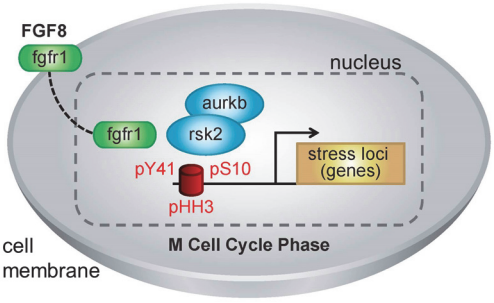
aberrations (6), with the potential to perform DNA repair later at G1 (4). Cells undergoing CA will frequently die in subsequent cell cycles if DNA damage goes unrepaired, yet some cells survive and proliferate in an aneuploid and hypermutated state (5) – expanding clonal evolution and fueling recurrences that are eventually resistant to all current forms of therapy. We hypothesize that checkpoint adaptation is a key vulnerability in osteosarcoma, and that reversing checkpoint adaption when cells are under treatment-related stress will decrease tumor recurrence.

Specific Aim and Approach

The goals of this pilot project are:

AIM 1. Define the classes of drugs that synergize with radiation in osteosarcoma.

Rationale: Radiation is an often-used palliative therapy for osteosarcoma, but in most instances the radiated tumor will re-grow. Radiation also induces G2 cell cycle checkpoint arrest and is associated with CA, making radiation a therapeutic modality that would potentially benefit from combination with a CA-reversing targeted therapy. CA-facilitating nuclear RTKs and nuclear kinases that modify histone H3 may be key targets (17). Classes of agents that may reverse CA are kinase inhibitors (e.g., RSK, AURK and FGF receptor inhibitors (9-11, 14, 16, 18)), epigenetic modifiers (19) and IAP inhibitors (*i.e.*, Survivin inhibitors).



Working hypothesis for how RTKs facilitate M checkpoint adaptation and emergency response to stress.

Approach: Using our SciCloneG3 robotic liquid handling system, we will print a custom 60-drug panel of promising agents that can abrogate CA, testing human primary osteosarcoma tumor cell cultures & cell lines across these drugs with or without radiation (up to 2Gy; using the 96hr IC25 dose for each culture). Radiation will be applied 24 hr after start of targeted therapy and will be given as a single dose delivered by our Faxitron instrument (in validation studies, hyperfractionated doses may be studied). The 96 hr drug IC50 for cell growth with & without radiation will be compared. Patient-derived primary tumors will include PDX explant cultures PCB151, PCB429 and PCB509 (co-developed by us with the Jackson Lab) and established human osteosarcoma cell lines will include U2OS (using which CA was originally described (5, 20)), MG63, SaOS2 and HOS-143B (21). As markers of drug-modified immediate early response and DNA damage (repair) response, 75 min post-radiation nuclear RTK expression and histone H3 Y41, S10 and S28 levels will be assayed. To measure CA abrogation, the 96 hr γ-H2AX, >4N ploidy count and Annexin (apoptosis) status will be measured (6) using our ArrayScan VTI high content imaging instrument. DNA exome sequencing will also be done pre/post radiation on steady-state cultures. Hits relative to radiation alone will be validated in 3 independent experiments in vitro, determining combination index (C.I.) as a measure of synergy. Validated hits will be a candidate for in vivo validation for follow-on grant applications. This mechanistically-oriented approach may add a new tool (effective radiation) to osteosarcoma treatment.

Alternative/Complementary Approach, if time allows: To define the classes of drugs that synergize with etoposide in osteosarcoma. Rationale: Etoposide has previously been a mainstay of multi-agent chemotherapy for osteosarcoma and an inducer of G2/M cell cycle arrest – thus a candidate for checkpoint adaptation mediated chemotherapy resistance. Whereas radiation more heavily weights for a G2 arrest/adaptation, etoposide weights for an M phase checkpoint arrest/adaptation. Approach: Taking the same approach as Aim 1, we will screen for targeted agents with synergy with etoposide. Etoposide will be added 24 hr after initiation of the targeted therapy. Validation will be performed as described in Aim 1.

Following completion of the above aims, we will have candidate drugs with which to pursue not only preclinical development of combined radiation/chemotherapy and targeted agents, but we will also have validated cell cultures and clinically-relevant drug treatment systems with which to interrogate the mechanism of checkpoint adaptation inhibition (prolonged cell cycle repair checkpoints, apoptosis resistance) in follow-on grant studies.

References 1. Cell Death Dis 5, e1046 (2014); 2. Cancer Res 70, 6412-6419 (2010); 3. Cell 144, 27-40 (2011); 4. DNA Repair (Amst) 8, 1101-1109 (2009); 5. Oncogene 26, 5833-5839 (2007); 6. PLoS genetics 10, e1004107 (2014); 7. Radiother Oncol 101, 24-27 (2011); 8. Mol Cancer Res 11, 1303-1313 (2013); 9. The Journal of biological chemistry 279, 29325-29335 (2004); 10. The Journal of biological chemistry 280, 25604-25610 (2005); 11. Mol Biol Cell 20, 2401-2412 (2009); 12. The Journal of biological chemistry 270, 30643-30650 (1995); 13. Cell 127, 185-197 (2006); 14. Acta Oncol 36, 337-340 (1997); 15. Cell Rep 14, 2490-2501 (2016); 16. Br J Cancer Suppl 27, S105-108 (1996); 17. Mol Cell 42, 274-284 (2011); 18. Oncogene, (2015); 19. Apoptosis 9, 583-589 (2004); 20. Cancer Res 66, 10253-10257 (2006); 21. Lab Invest 91, 1195-1205 (2011).

MIB AGENTS OSTEOSARCOMA ALLIANCE

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Funding Meaningful Osteosarcoma Research Together

Guidelines: Proposal must benefit the osteosarcoma patient and be a new project or distinct portion of an ongoing larger project for which results can be expected in 12 months. At the completion of 12 months, results must be made available to share - regardless of succeed or fail outcome. The recipient must be available to present work underway and completed at the FACTOR conference in 2019. Fund may not be used for the formation of new organization or used for planning stages of research or other initiative. Presentation of check and tour of facility by MIB Agents is requested.

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Name: Rosandra Kaplan, MD

Organization: National Cancer Institute, National Institutes of Health

Email: rosie.kaplan@nih.gov Phone: 240-383-6697

Initiative Name: Targeting tumor microenvironment in OS Amount Requested: 60,000

Desired Impact: Develop new therapies to limit metastatic progression in patients with OS

Projected Milestones: Generate preclinical studies to support clinical trial development for patients with OS

I agree to Guidelines: Yes

Osteosarcoma is a rare but too often deadly bone cancer that occurs in people of any age but mostly commonly in children and young adults. This cancer is the most common cancer to occur in bone. Further, Osteosarcoma is particularly challenging because it makes bone matrix. This matrix hardens and makes treatment to shrink once mineralized with calcium a particular challenge. Many patients with osteosarcoma have large bulky tumors by the time they are diagnosed and the main stay of treatment is to remove them and give chemotherapy before and after surgery to prevent/limit metastatic progression. When Osteosarcoma spreads it makes a milieu with bone matrix no matter the organ it grows in. This unique characteristic makes it critical to develop strategies that prevent metastatic tumor growth or treat these disseminated tumor cells before they develop a calcified osteoid matrix.

Our laboratory is interested in osteosarcoma metastasis and in particular the process of pre- and early metastatic niche formation which is a multi-step process that occurs first with a growing primary tumor secreting growth factors and exosomes (small subcellular microparticles) systemically that act on distant tissue sites and results in activation of hematopoietic stem and progenitor cells that proliferate in the bone marrow, enter circulation and home to distant metastatic sites or future metastatic sites and differentiate into immune suppressive myeloid cells. These monocytes can inhibit T cell proliferation and inhibit effective anti-tumor T cell mediated immunity. The hematopoietic cells home to particular sites of pre- and early metastatic microenvironments based on localized areas of up-regulated fibronectin production. Stromal cells and in particular vascular smooth muscle cells and pericytes normally reside adjacent to endothelium and provide survival and instructive cues to the endothelium. When activated by tumor secreted factors or inflammation or injury they can develop an altered phenotype marked by loss of usual perivascular cell markers, activation of specific markers such as PDGFRa, proliferation, motility and enhanced extracellular matrix production including increased fibronectin. This altered matrix promotes recruitment of the monocytes discussed above. Together this process creates a unique environment that supports disseminated tumor cells that are seeding in the lung. We have developed strategies to inhibit this process by targeting stromal cell plasticity or immune suppressive cells. One approach that we propose to develop is investigating ROCK1 inhibitor in treatment/prevent of metastatic disease. ROCK1 is a Rho GTPase that works to mediate cell shape, motility, extracellular matrix secretion and proliferation in vascular and perivascular cells as well as in certain tumors including Osteosarcoma. ROCK1 as a target was discovered after a network analysis performed by Theresa Beach after examining over 150 tumors from osteosarcoma patients. We also propose that not only will ROCK1 inhibition be potentially effective in targeting the tumor cells specifically but also the stromal cell plasticity that promotes the growth and survival of the disseminated tumor cells that lead to metastatic progression. We plan to investigate ROCK1 inhibitors in murine models with low and high metastatic potential and determine the impact and mechanism of this agent within the tumor cells and within the tumor microenvironment. We plan to examine tumor cells and alterations in both immune cells and stromal cells in tumor bearing treated and tumor bearing untreated controls. These preclinical studies if promising can inform development of a clinical trial in children and young adult patients with osteosarcoma.

We also plan to continue our investigations with immunotherapy in osteosarcoma. We currently completed a Phase I trial of PLX3397, a tyrosine kinase inhibitor that inhibitors CSF1R that is

expressed on immune suppressive myeloid cells that we have identified as a key component of a conducive microenvironment for disseminated metastatic tumor cells as well as a Phase I trial of GD2 CAR T cell therapy. We plan to preform combination studies targeting immune suppressive myeloid cells and macrophages with different immune therapy and stromal cell and tumor cell plasticity with ROCK1. We also plan to perform combination studies with other chemotherapies, targeted therapies and immunotherapies to determine the most effective strategy to move forward into a Phase II clinical trial. Currently we have some preliminary data to suggest that ROCK1 effectively targets a gene transcription factor protein KLF4, that is known to regulate stromal cell plasticity and metastatic progression. In vitro studies show some early efficacy against Osteosarcoma.

We plan to use the funds to support a post bac fellow interested in a career in pediatric oncology to dedicate a year before further graduate or medical school training to perform these pre-clinical studies under my direction in the laboratory.

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Name: Joshua Schiffman, MD

Organization: University of Utah (Huntsman Cancer Institute)

Email: Joshua.Schiffman@hci.utah.edu Phone: (801) 587-4745

Initiative Name: EP53 NPs and Pulmonary Metastases Amount Requested: $100,000

Desired Impact: Introduction of new indication of OS pulmonary lesions for treatment with EP53 NPs

Projected Milestones: (1) Determine best dose and (2) Demonstrate efficacy of EP53 NPs in pulmonary metastases

I agree to Guidelines: / Joshua Schiffman, MD

TESTING OF ELEPHANT P53 (EP53) NANOPARTICLES IN PULMONARY METASTASES

Osteosarcoma (OS) is the most common bone tumor in children and adolescents, and unfortunately, a very limited number of new drugs have been introduced for its treatment in over 40 years. Patients with OS, especially with metastatic tumors that have spread to other parts of the body, are still in desperate need of new therapeutic options. The biggest clinical impact in OS will be to discover and test new drugs for this group of patients. OS most commonly spreads to the lungs, and when this happens, treatment includes an aggressive combination of chemotherapy and surgery. Pulmonary metastases occurs in nearly half of OS patients and unfortunately these patients have extremely poor outcomes, with more than 70% dying from their lung disease.

Last year, our team was grateful to receive the MIB Agents Outsmarting Osteosarcoma Award that accelerated our elephant p53 therapeutic research. Nearly 100% of OS tumors will have disruption of the TP53 gene or its related pathway that interferes with DNA repair and cell death ("apoptosis") in tumor cells. Our research team discovered that elephants have extra elephant p53 (EP53) that naturally developed to protect their large and long-lived bodies from cancer. Using the MIB Agents Award, we began initial testing of an EP53-based medicine using nanoparticles (PEEL Therapeutics, Inc.) in the laboratory. We showed that EP53 nanoparticles (EP53 NPs) trigger OS cell death in tumors grown in a dish, *in vitro,* and also decrease tumor metabolism in mice transplanted with human and canine OS, *in vivo.* Dr. Schiffman reported on this progress during the Keynote Address at the FACTOR 2018 meeting. Even more recently, we have evidence in pathology slides of cell death and apoptosis in localized sarcoma tumors from mice treated with EP53 NPs. Based on this research, our team recently received new funding to continue *in vivo* testing of EP53 NPs in localized OS tumors in mice to document their safety, tolerability, and tumor response prior to the first human testing. ***Due to the very poor outcomes in OS patients with lung disease, the EP53 NPs need to be assessed for their ability to also target and destroy pulmonary metastases.***

TESTING OF ELEPHANT P53 (EP53) NANOPARTICLES IN PULMONARY METASTASES

For the current MIB Agents Award, we propose to expand our current work with EP53 NPs in localized OS to pulmonary metastases. For this study, we will work closely with Jason Yustein MD, PhD from Texas Children's Hospital at Baylor School of Medicine, who has great expertise in p53-related metastases and mouse models – and also was a speaker at FACTOR 2018. Dr. Yustein's lab has innovative and clinically relevant models derived from (1) novel p53-altered mouse models that spontaneously develop metastatic lung OS and (2) directly from pediatric metastatic lung lesions. Figure 1 shows the model directly derived from a patient's metastatic lung lesion, which can be monitored and tested with novel therapies. Besides the human models, Dr. Yustein has mouse models of metastatic disease that highly recapitulate the human disease. These one-of-a-kind models allow for additional therapeutic studies in mice that have normal immune systems, which add another layer of authenticity to testing our EP53 NPs.

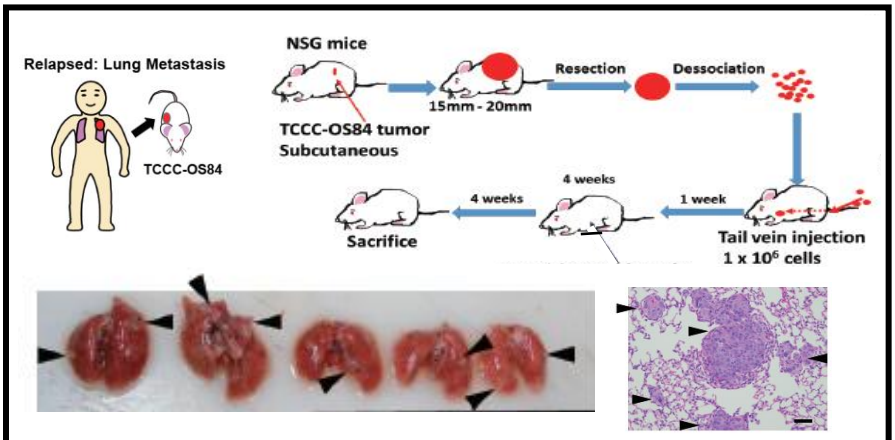

Figure 1. Patient-derived OS metastatic lung model for therapeutic testing. *TCCC-OS84 tumor was obtained from actual patient with metastatic lung lesion. Subsequently it was grown in a mouse and then used to further study the development of metastatic disease. Black arrows highlight lung OS lesions in images of the whole lungs (Left) and microscopic analysis of the lungs (Right).*

Based on our previous success with EP53 NPs and Dr. Yustein's success with OS pulmonary models of metastasis, we will work together to test the ability of EP53 NPs to being taken up and cause apoptosis in different sized pulmonary lesions. **AIM 1) MEASURE THE UPTAKE OF EP53 NPS IN PULMONARY LESIONS OF DIFFERENT SIZES.** We will use genomic barcodes and radiolabels within the NP carrier system to assess the ability of EP53 NPs to identify and deliver payload to OS pulmonary lesions of increasing size. This will allow us to determine the range of sizes that EP53 NPs can be used for OS pulmonary lesions, and importantly, the best dose schedule for maximum OS tumor saturation in the lungs. **AIM 2) TEST EFFICACY OF EP53 NPS IN OS PULMONARY METASTASES.** Using carefully controlled experiments, we will perform preclinical studies with the best dosage determined from our first study aim to assess OS tumor response in the lungs. We will perform these experiments using Dr. Yustein's models that recapitulate metastatic OS. These models include the one described in Figure 1, which takes human metastatic lung OS cells and injects them into a vein of the mouse that leads to the direct development of lung lesions. Additional studies using metastatic mouse OS cells will be used to confirm the effectiveness of the EP53 NPs in the setting of an intact immune system. Clinical assessment will be performed of OS pulmonary lesion through radiographic imaging (MRI/CT/PET), and gross anatomical and histological review under the microscope. Based on these results, we will be able to expand the indication of EP53 NPs for future preclinical trials in dogs and then children to include pulmonary metastases – in addition to just localized primary OS that is now being studied with EP53 NPs. Of note, the previous project funding does not support investigation into pulmonary metastases which is why this support is now needed. The current MIB Agent Award will be essential to expand the scope of EP53 NPs in OS treatment to include lung lesions and will foster a new collaboration between Dr. Schiffman and Dr. Yustein, two ongoing participants in MIB and FACTOR efforts. Working together, both Dr. Schiffman and Yustein are eager to join their complimentary clinical and scientific backgrounds to improve the lives of OS patients with pulmonary metastases.

Sujith K Joseph, PhD
Scientist, Pediatrics Hematology/Oncology
Baylor College of Medicine
832-824-6836

Texas Children's Hospital

BCM
Baylor College of Medicine
Department of Pediatrics
Section of Hematology/Oncology

April 20, 2018

MIB Agents Osteosarcoma Alliance
PO Box 858
Barnard VT 05031

Dear Members of the Scientific Advisory Board and Board of Directors:

Please accept our proposal for the MIB Agents Osteosarcoma Alliance research grant entitled **"Effective CAR T-cell Immunotherapy for Osteosarcoma"**; PI Sujith K. Joseph and Co-PI Nabil Ahmed. We have previously demonstrated promising responses for HER-2 targeted CAR T-cell therapy in children with metastatic and recurrent osteosarcoma in **The HEROS Trial** (NCT00902044; published in Ahmed *et al*., JCO 2015). In this study, we observed indicative responses for long term survival in a subset of children. Four children with treatment resistant OS are currently alive and well, 6 years after completing the trial. Additionally, in the currently ongoing trial, HEROS 2.0, we further established safety and achieved complete and durable remission in two sarcoma patients. We would like to build on these promising results by overcoming a major limitation to this approach: the scarcity of HER2 on OS tumor cells and the variability of its levels in tumors. In this proposal, we aim to use epigenetic agents to turn on the expression of tumor associated genes like HER-2 and improve the effectiveness of redirected CAR T-cells. The approach is readily translatable as part of our FDA and IRB approved clinical trials for HER-2 CAR T-cells and has future clinical impact to broaden their therapeutic reach.

We are requesting $100,000 for one year.

Thank you for your consideration for this award.

Sincerely,

Sujith K. Joseph, PhD
Staff Scientist

Nabil Ahmed, MD, MPH
Associate Professor

Department of Pediatrics
Section of Hematology/Oncology
Center for Cell and Gene Therapy
Baylor College of Medicine, Houston, Texas

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Name: Sujith K. Joseph, PhD

Organization: Baylor College of Medicine

Email: Sujithj@bcm.edu Phone: 832-824-6836

Initiative Name: Effective CAR T-cells for Osteosarcoma Amount Requested: $100,000

Desired Impact: Improve therapeutic reach and efficacy of HER2 CAR T-cells for rapid clinical translation.

Projected Milestones: Study OS target changes and redirected CAR T-cell efficacy with Epigenetic Agents.

I agree to Guidelines: Sujith Joseph

Title: Effective CAR T-cell Immunotherapy for Osteosarcoma.

Background: Osteosarcoma (OS) is an aggressive cancer of the bone that affects children and young adults. Escalation of treatment measures does not improve outcomes for children with metastatic and recurrent disease. Additionally, the treatment related toxicities are unacceptable. The 5-year survival rate still remains less than 20%. Novel treatments that are tumor-targeted can improve outcomes and lower treatment related side effects. Hence, we engineered the patient's immune cells to seek and kill OS cancer cells by attaching to a cancer protein, called HER-2. To achieve this, immune cells (T-cells) are grafted with an artificial molecule called CAR (Chimeric Antigen Receptor), which recognizes HER-2 molecules on OS with high accuracy. Naturally, this approach completely spares normal body tissues. We tested the safety of HER2 CAR T-cells in a phase 1 clinical trial for advanced sarcoma (NCT00902044; published in Ahmed *et al*., JCO 2015). In this study, we saw indicators of long term survival. Four children with treatment resistant OS are currently alive and well, 6 years after completing the trial. We would like to build on these promising results, but have identified a major limitation to this approach: the scarcity of HER2 on OS tumor cells.

Hypothesis: We therefore reason that an agent that increases HER2 on OS cancer cells will make them more likely to be killed by CAR T-cells and improve our promising results (Figure 1).

Epigenetics is the area of science that pertains to changing gene activity without changing the genes themselves. An Epigenetic Agent (EA) is a medication that causes such changes and as an example, turn on the HER2 gene resulting in more HER2 expression. We are therefore proposing to use epigenetic agents to turn the HER2 gene on so CAR T-cells can see them and kill the tumor more efficiently. We expect that this will significantly improve our clinical results.

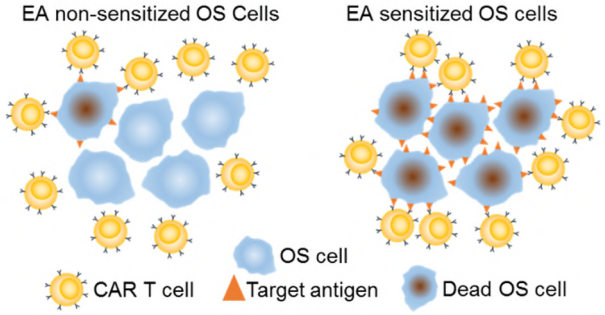

***Figure 1**: Increased target expression after EA sensitization improves CAR directed T-cell killing*

Scientific Rationale: Epigenetic mechanisms affect expression of genes without changing the DNA template. In OS, these changes drive tumor development by silencing tumor suppressor pathways and involve mechanisms like DNA methylation and histone modification. Epigenetic agents (EAs) inhibit these mechanisms to treat cancer (Morrow & Khanna, 2015). They belong to two classes of medications namely DNA methyltransferase inhibitors (DNMTi) and histone deacetylase inhibitors (HDACi). Treatment with EAs also induce expression of aberrant tumor associated genes and fetal self-antigens in multiple cancers. For example, decitabine, a DNMTi, increases expression of MAGE-A family and NY-ESO-1 antigens in OS (Li *et al*., 2014). Entinostat, an HDACi, induces specific production of NKG2D ligand on sarcoma cells (Zhu *et al*., 2015). Similarly, Zoledronate treatment sensitizes OS cells to T-cell killing in presence of trastuzumab, a HER2 targeting monoclonal antibody (Liu *et al*., 2015). These expression changes are effectively targetable with immune therapies like CAR T-cells. However, the influence of EA medications on expression patterns for HER-2 and other target antigens in OS, a HER-2 non-amplified cancer, remains relatively unexplored. Defining such changes associated with EA medications can help redirect CAR T-cells to these antigens and increase susceptibility of OS cells to CAR T-cell therapy.

Approach: We will investigate the effect of epigenetic agents on surface expression of HER-2 and other targets in OS lines to redirect CAR T-cells (Aim 1). To achieve this aim, we will expose multiple OS cancer lines (U2OS, HOS, LM7, MG-63 and SaOS) with selected EAs that have proven to be successful in the clinical setting (DNMTi - Azacytidine and Decitabine and HDACi - Zoledronate, Valproic acid; vorinostat, Panobinostat and entinostat). We will probe the sensitized OS lines to study expression changes (duration and intensity) of validated tumor targets like HER-2 with techniques like mass cytometry or flow cytometry.

Following this, we will evaluate the efficacy of epigenetic agents to sensitize OS lines towards CAR T-cells redirected against HER-2 and/or other targets (Aim2). For this aim, we will treat OS lines with selected EAs and CAR T-cells redirected against HER-2 and/or other antigens to decipher functional efficacy. We will use long-term and short-term tumor killing assays on *in vitro* cancer models and validate their efficacy and safety in animal models of OS. We will use immune deficient mouse models that allow human tumor engraftment and spontaneous metastasis for this purpose. We will also optimize development of a novel CAR construct for targetable antigens where none exists.

Projected Milestones: Four months into the study, we expect to understand the effect of EAs on OS target expression. At the end of twelve months, we anticipate to complete assessment of EA's ability to improve CAR T-cell activity directed against HER-2 and/or other OS targets.

Anticipated Challenges: A subset of patients can develop resistance to treatment with single EA medication. To overcome this, we will use combination of agents to describe target expression. Resetting epigenetic patterns can also favor tumor resistance. We will use multivalent CAR T-cells that target multiple antigens to overcome this challenge (Bielamowicz et al., 2018). EA medications may likely have direct effects on immune cells, such as CAR T-cells. To date, such effects described are beneficial and includes decrease in checkpoint inhibitors, increase in T-cell attractants and inhibition of immune suppressive cells. We will investigate such effects of EA on CAR T-cells, as required.

Impact: Several epigenetic agents are currently available on the market. These have been established for other medical indications. Enhancement of HER-2 CAR T-cell activity observed in combination with EA is therefore immediately translatable to the clinic. This will be incorporated into our HER2 CAR T-cell trials that are FDA and IRB approved. The combination approach can also expand eligibility of patients for HER2 directed T-cell therapy. Other OS molecules validated during this study can serve as additional targets for multivalent CAR approach extending the therapeutic reach of CAR T-cells. The synergy with EA may also improve T-cell function and thus enhance anti-tumor responses. Effectively, these improvements in OS-targeted therapy can translate to a cancer free life without debilitating treatment related toxicities for children with metastatic and recurrent disease.

Conclusion: Epigenetic agents can increase the expression of OS tumor specific antigens that are targetable with CAR redirected T cells. This combination approach can lead to effective CAR T-cell based therapy for OS and improve outcomes for this hard to treat high-risk malignancy.

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Name: Robert M. Hoffman, PhD

Organization: AntiCancer Inc.

Email: all@anticancer.com Phone: 858-654-2555

Initiative Name: Transformational therapy of osteosarcoma Amount Requested: $100,000

Desired Impact: See below

Projected Milestones: See below

I agree to Guidelines:

Transformative therapy for osteosarcoma identified in patient-derived orthotopic xenograft (PDOX) mouse models

Background and significance: Osteosarcoma (OS) is the most common pediatric cancer. First line chemotherapy is usually administered before and after surgery. Commonly used chemotherapy, such as cisplatinum, doxorubicin and ifosfamide, has not bettered the poor outcome for patients with metastatic disease or non-resectable tumors and has not improved in 30 years [1]. To achieve better outcome, our laboratory has developed the patient-derived orthotopic xenograft (PDOX) mouse model for osteosarcoma [2,3]. In this model, primary osteosaroma or metastatic osteosaroma from the patient is implanted in the femur (leg bone) ("orthotopic" [correct place]) of immunodeficient mice, for example, athymic "nude" mice which lack T cells to reject the human tumors. Metastatic tumors can also be implanted orthotopically on the corresponding organ in the nude mice such as the lung, in the PDOX models. In the PDOX models, the patient osteosarcoma replicates the behavior it had in the patient. The PDOX model of osteosarcoma can be used for individualizing therapy for each patient as well as for developing novel transformative therapy which can be used for all osteosarcoma patients.

We previously established a PDOX from a metastatic osteosarcoma of a patient who failed cisplatinum (CDDP) therapy. Osteosarcoma resected from the patient was implanted orthotopically in the femur of mice to establish the PDOX model. We tested and identified effective standard drugs administered singly on this PDOX model, while accurately maintaining the CDDP resistance of PDOX tumor, thereby mimicking the patient [4]. Subsequently, we identified combination chemotherapy with standard drugs, that significantly regressed the osteosarcoma PDOX tumor [5].

Our laboratory is developing the transformative therapeutic tumor-targeting bacteria, *Salmonella typhimurium* A1-R (*S. typhimurium* A1-R). *S. typhimurium* A1-R has been engineered to selectively target tumors and not infect normal tissue [6]. The other transformative therapeutic being developed in our laboratory is recombinant methioninase (rMETase) that targets the elevated requirement for common the amino acid methionine and that occurs in all tested cancer types [7]. rMETase can arrest tumor growth with no or minimal effect on normal cells or tissues.

In a pilot study to test the potential of these transformative therapeutics on osteosarcoma, the CDDP-resistant, lung-metastatic osteosarcoma PDOX was implanted in the lung ofnude mice and treated with *S. typhimurium* A1-R and rMETase [6,7]. rMETase combined with both *S. typhimurium* A1-R and CDDP was the most effective of all therapies and eradicated the metastatic osteosarcoma PDOX [8].

The overall aim of the present application is to demonstrate that the combination of *S. typhimurium* A1-R and rMETase, *S. typhimurium* A1-R and a standard chemotherapy drug can eradicate osteosarcoma in a series of 5 PDOX models of this disease in order to prepare for future clinical application of this transformative therapeutic strategy. We have recently made very promising developments showing that both rMETase [9] and *S. typhimurium* A1-R [10] can be administered orally, making such treatment both safe and easily administered.

Specific Aims and Milestones:

1. Determine the efficacy or oral rMETase (o-rMETase) alone on 5 PDOX models of metastatic osteosarcoma.
2. Determine efficacy of tumor-targeting *S. typhimurium* A1-R alone or on 5 PDOX models of metastatic osteosarcoma.
3. Determine efficacy of oral rMETase combined with *S. typhimurium* A1-R and various chemotherapy drugs on 5 PDOX models of metastatic osteosarcoma with the goal of eradicating the tumor in each case.

Methodology: Osteosarcoma from the primary tumor is minced into small fragments (3–4 mm) and after nude mice are anesthetized, a 10 mm skin incision is made on the right thigh and the biceps femoris muscle is split to reach the distal femur. An incision is made sparing the knee joint and then the a single 3 to 4 mm tumor fragment is implanted orthotopically into this space to establish a PDOX model that represents the primary tumor of the patient [3].

The lung-metastatic osteosarcoma PDOX will also be established in the lung of nude mice. After anesthesia, mice are put under anesthesia. A 1.5 cm transverse incision of the skin is made in the left chest wall. Chest muscles are separated by sharp dissection and costal and intercostals (chest) muscles are exposed. The chest wall is opened. The left lung is taken up with a forceps, and tumor fragments are sewn promptly into the lower lung with one suture. The lung is then returned into the chest cavity and the incision in the chest wall is closed [6].

Impact: The results of the present proposal will deliver a curative therapeutic strategy for metastatic osteosarcoma.

Investigators: Robert M. Hoffman, Ph.D., Professor of Surgery, UCSD, President, AntiCancer Inc., has been doing cancer research for 53 years. Hoffman received his PhD from Harvard University, did post-doctoral research at Massachusetts General Hospital, Harvard Medical School and has been on the faculty of the UCSD School of Medicine since 1979. Hoffman has published almost 1,000 scientific papers and is a world-leader in cancer research. Hoffman's team developed the PDOX model and pioneered the development of rMETase and *S. typhimurium* A1-R for cancer treatment.

References:

1. Blattmann, C., et al. Establishment of a patient-derived orthotopic osteosarcoma mouse model. J Trans Med 13, 136, 2015.
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Name: Alex Y. Huang, MD, PhD

Organization: Case Western Reserve University / Angie Fowler AYA Cancer Institute / UH Rainbow Babies

Email: alex.y.huang@case.edu Phone: 216-368-1271

Initiative Name: Anti-integrin Therapy for Lung OS Amount Requested: $100,000

Desired Impact: Paradigm-shifting immunotherapy for lung OS using FDA-approved medicine

Projected Milestones: Submission of IND-enabling pre-clinical data to FDA for an OS-specific clinical trial

I agree to Guidelines: Looking forward to share results at the 2019 FACTOR conference!

Osteosarcoma (OS) is an aggressive malignant primary bone cancer with a high propensity for lung metastasis. OS frequently originates from primitive mesenchymal bone-forming cells in the long bones during periods of rapid bone growth. Consequently, OS represents the most prevalent bone cancers affecting children and adolescent and young adults (AYA), with ~400-600 cases a year and accounts roughly half of all new cases of OS diagnosed in the United States. Despite aggressive combination chemotherapy and surgery, the outcome for metastatic OS remains dismal, and the overall survival in children and AYA patients with metastatic OS has not improved significantly over the past 3 decades. A high proportion of OS patients develop metastatic disease at distant sites either at the time of diagnosis (one in five patients; 20%) or after initiation of multimodal therapy including combination chemotherapy and surgery (in ~30% of patients). The lung accounts for >80% of all OS metastatic sites. Unfortunately, almost all of the patients who develop surgically unresectable pulmonary metastatic OS (pOS) invariably succumb to this devastating disease. Therefore, pOS represent a disease with urgent unmet needs. As OS contains extremely complex and heterogeneous chromosomal and genetic alterations, recent advances in molecular precision medicine therapy approaches to target OS-specific mutations will likely be challenging in the immediate future. Immunotherapy is the new kid on the block as a potential new addition in the armamentarium to treat pOS. Immunotherapy targets existing common immune pathways through which different cancers thrive by interacting with the host immune system. Such immune-mediated factors influencing tumor immunogenicity may or may not be directly related to known characteristic genetic alterations intrinsic to any particular cancer histology. Our multi-disciplinary investigator team at the Angie Fowler AYA Cancer Institute / UH Rainbow Babies & Children's Hospital in Cleveland, Ohio is determined to bring novel immune-based therapeutic options to pediatric and AYA patients suffering from this devastating disease by **providing novel "outside-of-the-box" and paradigm-shifting immunotherapeutic approaches.** As described below, we are now on the verge of brining one such readily available therapy to the clinic for patients with pOS, and we are extremely optimistic to make this happen within the next 12 months.

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Accumulating data in cancer immunology make it abundantly clear that immune cells within the tumor microenvironment (TME) play pivotal roles in cancer pathogenesis. Ongoing pre-clinical research in **our laboratory has discovered that one of the critical "enablers" of pOS survival and maintenance in the lung tissue is pulmonary macrophages (MACs).** We identified tumor-expressed Vascular Cell Adhesion Molecule-1 (VCAM-1) as playing a pivotal role in OS metastasis through its interaction with $\alpha4\beta1$ integrin on MACs. Compared to non-metastatic, localized OS tumor (K7), metastatic OS cells (K7M2) express high surface level of VCAM-1. This is a major finding that was also independently corroborated in mouse model of triple-negative breast cancer (4T1) that metastasizes to the lung. When we genetically delete VCAM-1 expression on K7M2, tumor cells behave similar to K7 with a loss of lung metastatic potential. Furthermore, depletion of pulmonary MACs both prevents pOS formation and also ameliorates already-established pOS disease in more than 60% of the time.

The presence of MACs in the lungs is important for infectious control; therefore we sought to functionally interfere the communication between pOS and MACs rather than depleting MACs all together. *Our novel concept was that pOS thrives due to the "enablers" which are the MACs, and that disrupting their communication will be highly effective in not only causing pOS disappearance but also preserve immune function of MACs for infectious control.* Indeed, we tested the efficacy of using an antibody against the $\alpha4$ portion of the $\alpha4\beta1$ integrin on MACs, so that communication with VCAM-1 on pOS can be disrupted. Indeed, we are able to **CURE** 60% (!) of mice with established pOS by giving the anti-$\alpha4$ antibody via intranasal / inhaled routes. Similarly, we were able to develop a peptide mimicking a portion of VCAM-1 that can interfere with VCAM-1 / $\alpha4\beta1$ interaction. Administration of the anti-VCAM-1 peptide was equally effective.

These ongoing data are extremely exciting, because for the first time we have been able to identify a set of molecular targets (VCAM-1/$\alpha4\beta1$) that are responsible for lung metastasis mediated by the immune "enablers" in the lung microenvironment. What's more exciting about this finding is that ***antibody against human $\alpha4$ has already been FDA-approved*** for the treatment of multiple sclerosis and inflammatory bowel disease (Natalizumab or Tysabri™, Biogen), where $\alpha4\beta1$ is critical for allowing disease-causing lymphocytes to migrate to the CNS or the gut. **Major aims of this MIB Agent grant application** are to secure funding to: **1)** perform additional *in vivo* treatment efficacy validation of anti-$\alpha4$ antibody in 3 other pOS mouse models to establish generalizability of this approach; **2)** complete IND-enabling safety and toxicity studies to apply Natalizumab intravenously or intratracheally for refractory / unresectable pOS. A clinical protocol is near completion and will be going through institutional clinical trial review during the next 4 months; and **3)** characterizing changes in MAC function and characteristics in pOS lung tissues and peripheral blood undergoing treatment with anti-$\alpha4$ antibody, with a goal to examine whether soluble VCAM-1 molecule, which are detectable in the serum, can serve as a bio-marker of disease or therapy response.

Significance / innovation / expected outcome: Our exciting ongoing project provides a strong scientific rationale and efficacy data for proposing the use of FDA-approved biologic, Natalizumab (Tysabri™) for the immunotherapeutic treatment of refractory or unresectable pOS, a disease that lacks any alternative therapeutic options currently. The re-purposing application of a FDA-approved drug for a different disease indication where no efficacious standard of therapy has been established is likely to enhance its chances of being approved for a rapid translational clinical trial. The clinical protocol approval process will be completed in the next 4 months, followed by IND filing with the FDA. We are highly enthusiastic that ***an immunotherapy clinical trial for pOS will be a reality within the next 12 months.*** Our chances of success in this highly impactful venture is further sustained by a focused institutional commitment to build a sarcoma immunotherapy translational and research program in AYA oncology at the Case Comprehensive Cancer Center and the Angie Fowler AYA Cancer Institute in Cleveland, Ohio, recognized leaders in AYA Oncology Innovation. We will also leverage our extensive collaborations to offer this new therapeutic approach to other local and national institutions (including patients treated at the Cleveland Clinic). Dr. Huang will leverage his leadership in Cancer Immunotherapy initiative and co-leadership in Hematopoiesis & Immune Cancer Biology Program in the Cancer Center to collaboratively learn new biology about how such therapy works on a cellular and molecular level, thereby creating additional opportunities for future exploration of improved novel or combination approaches. Furthermore, Dr. Huang will continue to champion the cause of AYA sarcoma research and therapy needs through his involvement as a member of the National Moonshot Initiative Blue Ribbon Panel Working Group on Cancer Immunotherapy & Prevention.

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Name: Ching C. Lau, M.D., Ph.D.

Organization: The Jackson Laboratory for Genomic Medicine; Connecticut Children's Medical Center

Email: ching.lau@jax.org; clau@connecticutchildrens.org Phone: 860-837-2374; 860-545-9174

Initiative Name: Single Cell Genomics of Osteosarcoma Amount Requested: $100,000

Desired Impact: Establish the rational basis and identify targets for developing immunotherapy in osteosarcoma

Projected Milestones: In 12 months, analyze 8 cases of OS by single cell genomics and long read RNA sequencing.

I agree to Guidelines:

Background and Significance

This is a new initiative that I developed shortly after my arrival at The Jackson Laboratory for Genomic Medicine (JAX-GM) and Connecticut Children's Medical Center (CCMC), where I am currently the Chief of Hematology-Oncology. As a co-Principal Investigator on the NCI-funded TARGET (Therapeutically Applicable Research to Generate Effective Targets) project, which began while I was at Texas Children's Hospital/Baylor College of Medicine, I have been co-leading a multi-national team of investigators using multiple genomic platforms to analyze the largest cohort of osteosarcoma cases ever assembled. The preliminary conclusion of the TARGET project is that osteosarcoma has a very high level of genomic instability and very few recurrent therapeutically targetable mutations. However, in a subset of cases, there is a definite immune signature which is associated with poorer prognosis. This has led to **our current hypothesis that immune cells could have been recruited by the tumor cells to facilitate their own growth, therefore suggesting immunotherapy as a viable therapeutic strategy.** However, based on the RNAseq data from the TARGET cases, we cannot be certain about the functional status of tumor-infiltrating immune cells. Specifically, we could not accurately determine the proportion of each type of immune cells in the tumor and whether the cytotoxic T-cells present were active and capable of eliminating tumor cells. This is due to the fact that all the RNAs used for the TARGET project have been extracted from bulk tumor tissue, making it difficult to confirm the status of individual immune cells. However, this limitation can be overcome by applying single-cell transcriptomic (SCT) analysis in which gene expression profiles of thousands of individual cells from the same tumor tissue are generated. Such analyses would allow us to pinpoint the various cell types in the tumor, including subtypes of tumor and infiltrating immune cells as well as their potential interactions with one another. With this approach, we could directly test various hypotheses such as whether tumor-infiltrating immune cells are inactivated through tumor cell-mediated immune checkpoint activation, which could be overcome by currently available immune checkpoint inhibitors.

In addition, we will further explore the potential of using immunotherapy to treat osteosarcoma by testing **the hypothesis that the genomic instability of osteosarcoma might be associated with expression of novel fusion transcripts arising from chromosomal breakage and recombination or increased production of rare transcript isoforms from perturbed alternative splicing. Such transcript aberrations could give rise to antigenic proteins**

recognizable by antibodies or T-cell receptors, depending on whether these proteins ultimately reside on the cell surface or remain intracellular. This hypothesis is based on our observations in the TARGET study of more than 200 breakpoints in each of the osteosarcoma genomes analyzed so far and a few abnormal transcripts as a result of alternative splicing. However, we are confident that we have missed a large percentage of the aberrant transcripts because of the intrinsic limitation of the short-read sequencing-based Illumina platform that was used to generate the RNAseq data for the TARGET project. At JAX-GM, our team has built a robust pipeline to overcome this limitation by utilizing long-read RNA sequencing (LRseq) based on the PacBio platform that can sequence full-length transcripts with high sensitivity and accuracy. This pipeline has been validated in a triple-negative breast cancer pilot study that has just been completed recently. In that study, hundreds of alternatively spliced transcripts were discovered and validated, many of which have prognostic significance.

Because of the cost of doing single-cell transcriptomic analysis as well as LRseq, we propose to carry out the following specific aims in only 8 cases of newly diagnosed osteosarcoma from CCMC and collaborating institutions (see below):

Aim 1: Generate transcriptome profiles for approximately 12,000 single cells per case. Cells from each case sample will be presorted for viability and CD45+ versus CD45- cells, each of which will be analyzed separately **using droplet-based technology (10x Genomics)**. Cell types will be identified by comparing each cell's individual expression profile to established reference databases for normal cell types and osteosarcomas as controls. RNAseq data of the TARGET project (89 cases) will be used for subsequent validation followed by immunophenotyping using Imaging Mass Cytometry (ICM by Fluidigm) and immunohistochemistry (IHC) on paraffin sections in matched cases.

Aim 2: Generate LRseq transcript profiles in parallel to conventional RNAseq. For each of the new case samples, we will analyze LRseq data generated with the PacBio RS II/Sequel platform to identify full-length isoforms and candidate fusion transcripts in comparison to conventional RNAseq data. These transcripts will be validated at the RNA level using RT-PCR and Nanostring, as well as at the protein level by mass spectrometry whenever fresh frozen tissues from the same cases are available or by IMC/IHC on paraffin sections. The final step of large-scale validation will be carried out using the TARGET datasets that contain RNAseq, WES and WGS data.

Future directions: Annotation of the isoforms and fusion transcripts will include localization of the expressed proteins which would be useful in prioritizing further exploitation of these potential targets for immunotherapy. For example, cell surface proteins could be targeted by monoclonal antibodies or genetically engineered CAR-T cells, while intracellular neo-antigens could be targeted by tumor vaccines.

Personnel

At JAX/CCMC, we have assembled a team of seasoned investigators with expertise in various disciplines to address the clinical challenges of osteosarcoma. These include:

- Ching Lau, MD PhD – Team leader who has extensive experience in cancer genomics and drug development, co-leader of the TARGET Ostoeosarcoma Project
- Paul Robson, PhD – Pioneer in single cell genomics and Director of SCG Program at JAX
- Jacques Banchereau PhD – Pioneer in cancer immunology and leader of the LRseq Program at JAX
- Karolina Palucka, MD – Vast experience in cancer immunotherapy
- Michael Isakoff, MD – Experienced Phase I/II trial investigator at COG and other consortia
- Adam Lindsey, MD – Experienced orthopedic surgeon specializing in treating bone tumors

Together we will collaborate with investigators from other institutions including:

- Paul Meltzer, MD PhD – Chief of Cancer Genetics Branch at NCI and co-leader of TARGET OS Project
- Richard Gorlick, MD – Chief of Pediatric Oncology at MDACC and Chair of Bone Committee of COG
- Chand Khanna, DVM PhD – Senior investigator in osteosarcoma research
- David Geller, MD – Co-Director of Orthopaedic Oncology Service, Montefiore Medical Center

Budget

Single-cell transcriptome profiling	$3,436 x 2/case x 8 cases = $54,976
Imaging mass cytometry (for Aim 1*)	$503/case x 8 cases = $4,024
Long read RNA sequencing**	$5,125/case x 8 cases = $41,000
TOTAL	**$100,000**

*Cost of ICM and IHC for Aim 2 including testing of new antibodies for aberrant proteins will be covered by other research funds. **Cost of conventional RNAseq for 8 cases will be covered by my other research grants. *Salary support will be covered by other research funds.*

Deliverables

At the end of 12 months, we will have completed SCT profiling and LRseq of 8 newly diagnosed osteosarcoma cases and validated the findings with the TARGET dataset. The final results will form the objective criteria and foundation based on which the decision can be made whether immunotherapy is a viable therapeutic strategy for osteosarcoma.

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Name: Monika A. Davare, Ph.D. & Lara E. Davis, M.D.

Organization: Oregon Health and Sciences University

Email: davarem@ohsu.edu Phone: 503-706-8422

Initiative Name: ROS1: Achilles Heel in a subset of Osteosarcoma? Amount Requested: $100,000

Desired Impact: Test and establish utility of targeting ROS1 with FDA approved ROS1 inhibitors in subset of osteosarcoma

Projected Milestones: Complete evaluation of the functional role of ROS1 in osteosarcoma using targeted gene knockdown and test efficacy of ROS1 inhibitors, alone or in combination with chemotherapeutic agents in a panel of 15 osteosarcoma cell lines

I agree to Guidelines: Monika A. Davare & Lara E. Davis

Hypothesis: We propose that a subset of osteosarcoma patient's tumors are driven by a cancer causing gene called ROS1 that promotes the growth and spread of these cancer cells.

Why look into ROS1: We and others have previously shown that when ROS1 is present at high levels in cells, its biological activity aggressively drives the growth and the metastatic spread of cancer. Importantly, *ROS1 is actionable* - meaning that there are several FDA approved drugs that very effectively block its activity and shrink tumors. In fact, lung cancer patients whose tumors are identified as having high ROS1 activity are now treated orally with a ROS1 blocking drug - a pill taken at home. And notably, these patients have seen impressive benefits in terms of tumor shrinkage, improvement of symptoms, and prolonging survival. Very little is known about the role of ROS1 in osteosarcoma. Osteosarcoma patients whose tumors have higher levels of ROS1 may benefit from these oral ROS1 - targeted drugs, at least one of which is already FDA approved. We propose to test this concept here.

Rationale and some data: What is the evidence that prompted us to consider ROS1 as a potential gene driving osteosarcoma? Recently, investigators at St. Jude's Children's Hospital analyzed hundreds of cancer samples from children with sixteen different types of cancer, and have made all these data available for public use (reference 1, back page). We mined these data, and discovered that ROS1 is most highly expressed in osteosarcoma tumors, compared to fifteen other cancers in children and young adults; see graph A on second page. The second hint comes from examining another public dataset where investigators from The Institute for Cancer Research (London, England) eliminated 117 genes (reference 2, back page), some of which are involved in driving cancer, and measured if cancer cells stayed alive or died. Their focus was not specifically on ROS1. But with our hypothesis in mind, we examined these data, and noted that of the top 15 cancer cell lines where ROS1 controls cell growth, almost half (7 out of 15) were osteosarcoma cell lines. See graph B on second page.

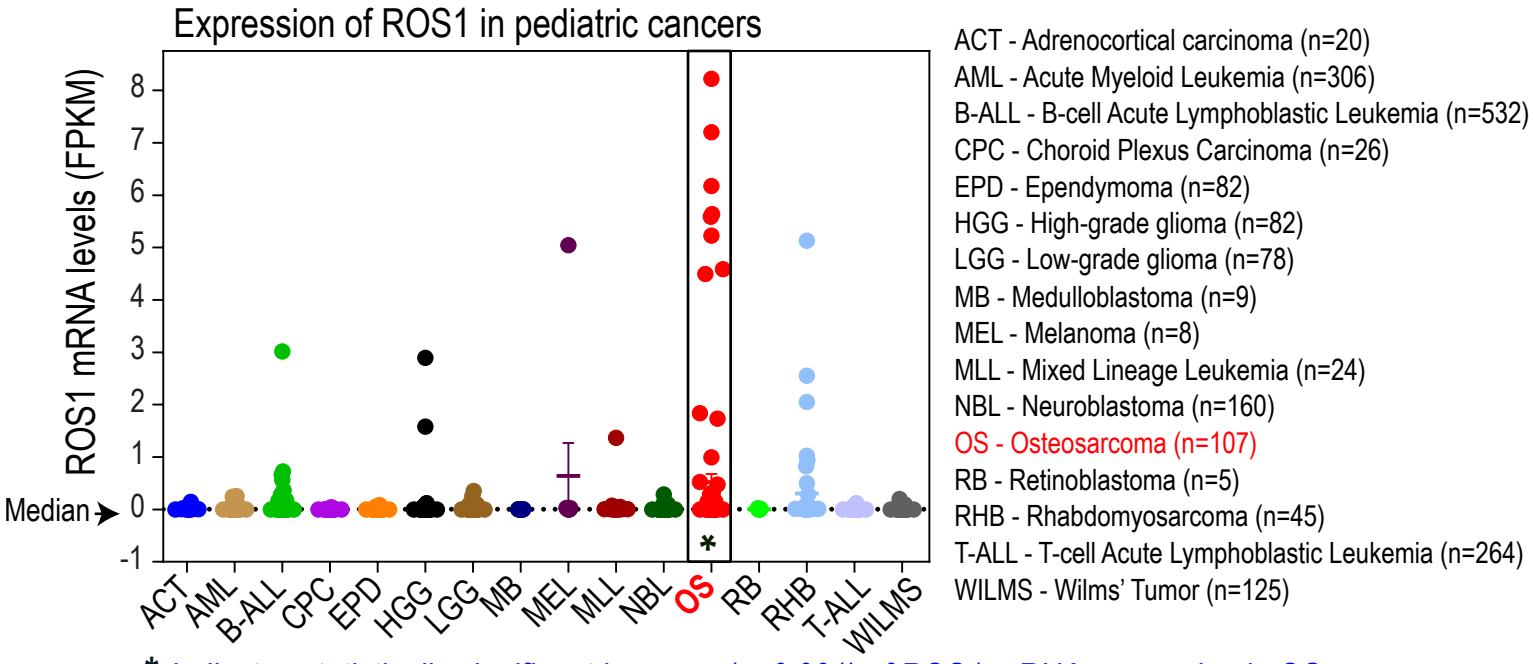
What we propose to do: We want to systematically examine whether: (a) ROS1 is an Achilles Heel in the subset of osteosarcoma tumors where it is also highly expressed, and (b) examine the cell-killing effects of ROS1 targeted drugs on these ROS1-positive as compared to ROS1-negative osteosarcoma cells as well as combination treatment with existing chemotherapeutic drugs with the goal of reducing the amount of chemotherapy used.

Innovation and potential clinical impact for osteosarcoma patients: There has been no focused study on the role of ROS1 in osteosarcoma to date. Given that ROS1 is an excellent drug target, and adult lung cancer patients with tumors that are driven by ROS1 are experiencing dramatic improvements and outcomes, there is impetus to test the proposed hypothesis in a systematic and rigorous fashion. The outcomes of this study may guide specific testing for ROS1 in osteosarcoma and potentially propel new clinical trials and development of novel, highly effective, targeted therapies.

APPLICANTS: Monika A. Davare, PhD & Lara E. Davis, MD
Instituion: OHSU

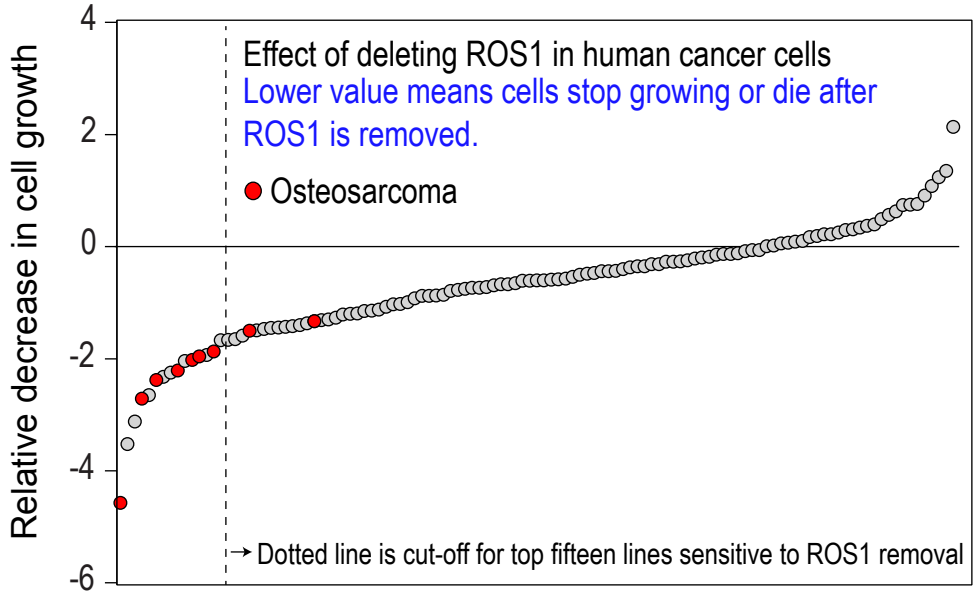
"back page"

A. ROS1 is highly expressed in some osteosarcoma tumors



* *indicates statistically significant increase ($p<0.001$) of ROS1 mRNA expression in OS as as compared to all but MEL and RHB cancer types*

B. Almost half of the top fifteen cancer cell lines that are killed by ROS1 elimination are osteosarcoma cell lines (7 of 15)



Each gray circle represents one of 117 human cancer cell lines.
Types of cancers tested: breast, ovary, lung, endometrium, brain, cervical, head & neck, pancreas, esophagus, and osteosarcoma.
Red circles are osteosarcoma cell lines.

Reference 1: https://www.stjude.org/research/pediatric-cancer-genome-project.html
Reference 2: Campbell et al. Large-Scale Profiling of Kinase Dependencies in Cancer Cell Lines. Cell Rep. 2016 Mar 15;14(10):2490-501.

Guidelines: Proposal must benefit the osteosarcoma patient and be a new project or distinct portion of an ongoing larger project for which results can be expected in 12 months. At the completion of 12 months, results must be made available to share - regardless of succeed or fail outcome. The recipient must be available to present work underway and completed at the FACTOR conference in 2019. Fund may not be used for the formation of new organization or used for planning stages of research or other initiative. Presentation of check and tour of facility by MIB Agents is requested.

Please fill out the form below, proposals will be submitted as a layman's summary and are limited to front and back of this page. Completed RFP will be available for the public to view on MIB Website and social media so the public can vote. Deadline for submission is April 20, 2018. Email to info@MIBagents.org

Name: John H. Healey, MD

Organization: Memorial Sloan Kettering Cancer Center

Email: healeyj@mskcc.org Phone: 212-639-7611

Initiative Name: Preclinical Development of Novel Therapies for Metastatic Osteosarcoma Amount Requested: $100,000

Desired Impact: Unique characteristics of lung metastases and individual patient tumors will be identified and analyzed. These unique characteristics of lung metastases of osteosarcoma can then be targeted in a patient-specific fashion with appropriate chemotherapeutic agents.

Projected Milestones: Isolation of exosomes & establishing PDX models (months 1-6); characterization of exosomes, premetastatic niche, and tumor initiating cells (months 6-9); genetic analysis and target identification (months 6-12)

I agree to Guidelines: Yes

Preclinical Development of Novel Therapies for Metastatic Osteosarcoma

Investigators: John H. Healey[1] [principal investigator], Vinagolu K. Rajasekhar[1], David C. Lyden[2]
Affiliations: (1) Orthopaedic Service, Dept of Surgery, Memorial Sloan Kettering Cancer Center, (2) Dept of Pediatrics, Memorial Sloan Kettering Cancer Center

BACKGROUND:

Patients with osteosarcoma (OGS) have an urgent need for new therapies. Metastases are most common in the lung. Although about 15% of patients are cured by surgical removal of the primary tumor alone, the microscopic metastases in the remainder are addressed by chemotherapy with failure in about 30% of patients with localized disease. Those presenting with overt lung metastases have poor survival (32%) [Chou A, *Cancer* (2009)115:5339-48]. Surgical resection of limited lung lesions can salvage the minority of patients. Metastases that develop during chemotherapy have a particularly bleak prognosis. There are no effective drug treatments for them.

Treatment for the last several decades for all patients has used three drug regimens of standard drugs (doxorubicin, platinum, methotrexate and sometimes ifosfamide). New strategies should address the unique biologic characteristics of OGS. (1) We hypothesize that metastases arise preferentially from tumor initiating cells (TICs): a distinct but a minor population in primary tumors and enriched within metastatic tumors. TICs have considerable plasticity, remaining dormant or altering their metabolism as needed to adapt to new and distant local environments. Characterizing and targeting these cells has not been investigated as a therapeutic strategy in osteosarcoma and may have potential to improve the present day patients' outcome. (2) The metastatic process is influenced by the action of cancer exosomes. Cancer exosomes, the lipid bilayer-enveloped vesicles that contain tissue-specific proteins and genetic material, are shed into the circulation in body fluids in the early stages of tumorigenesis, promoting tumor growth and establishing premetastatic niche in select target organs, which are in turn appeared to be determined by integrin types expressed on exosome surface. We have preliminarily confirmed this in many cancers, including metastatic osteosarcoma [*Nature* (2015)527:329].

RESEARCH PLAN:

Aim 1: Rapid characterization of osteosarcoma metastases and the premetastatic niche. We will identify unique characteristics of metastatic TICs and the metastatic tissue microenvironment *in vivo*. The first part of this aim leverages our expertise with PDX modeling of freshly harvested specimen of a patient's treatment-refractory osteosarcoma primary tumor in bone. We then propagate the specimen as patient-derived xenograft (PDX) in immunocompromised/humanized mice to increase the tumor volume in a humanized tumor microenvironment and thus the levels of TICs as we did with other cancer models [*Nature Commun* (2011)2:162; *Nature Commun* (2016)7:10442]. PDXs can be used to conduct precise co-clinical trials with a battery of potential therapeutic agents (the "mouse hospital" concept). The development of specifically humanized (patient-personalized) PDXs would further enhance the value of this approach. The second part of this aim focuses on the role of cancer exosomes in osteosarcoma metastasis. By isolating and characterizing exosomes from patients with potential to end up with metastatic osteosarcoma, we will investigate the potential for these exosomes as to where in the body establishes pre-metastatic niches in PDX models. If successful, we will have an exceptional opportunity to characterize a wealth of *in vivo* molecular and proteomic characteristics of the microenvironment before and after tumor formation. These *in vivo* biologic characteristics will be compared with that of patients to identify clinically relevant therapeutic targets.

Aim 2: Delineate the functional interactions between metastatic tumor cells and their microenvironment, and any exosome-associated factors mediating these interactions. We have previously identified horizontal genetic transfer and molecular signaling crosstalk between cancer-associated fibroblasts (CAFs) in the tumor stroma and breast TICs to foster therapy resistance [*Cancer Res* (2017)77:5438]. However, the specific pathways of genetic transfer and signaling crosstalk, as well as the role of exosomes in these interactions, have not yet been fully characterized. We will use our in-house expertise and ongoing collaborations in whole-genome single-cell copy number and gene expression profiling assays, chromosome instability characterizations, and adoptive immunotherapy approaches [*Mol Cancer Ther* (2017)16:2701; *Nature* (2018)553:467] to identify and verify any novel, functionally associated cell surface biomarkers in the treatment-refractory osteosarcoma TICs from primary and metastatic tumors.

Aim 3: Development of novel therapeutic approaches for metastatic osteosarcoma. In addition to our integrated systems biology related approaches [*Clin Orthop Relat Res* (2016) 474:178; *Cell Rep* (2017)20:1623], our center also performs integrated mutation profiling of actionable cancer targets (MSK-IMPACT) testing (approved by the FDA) to characterize mutations in about 458 cancer-related genes in clinically resected tumors. As a result of tumor heterogeneity in humans, a single primary tumor may give rise to metastatic clones with different genomic profiles; each clone may respond differently with respect to the uptake of the same candidate drugs, a problem often encountered in solid tumors. We plan to compare IMPACT data from the primary osteosarcoma patients to those of metastatic patients to prospectively identify possible and functionally conserved therapeutic targets. Our metastatic PDX model derived spheroids and organoids will allow us to perform *in vitro* miniscreens for identifying novel investigational therapeutics so that they can be loaded in patients own exosomes to deliver only at the metastasizing locations with any systemic toxicities often encountered in systemic chemotherapies. If the above approaches fail, we will still have the epitopes of the exosomes and novel surface proteins present only in the metastasis TICs determined and they could be employed in targeted/adoptive immunotherapy approaches pioneered by ongoing collaborators in our center.

Patients will benefit by identifying and targeting the unique tumor initiating cells and exosomes that characterize the metastatic lung tumor. By comparing the actionable (IMPACT) genetic changes in the primary and metastatic tumors, the unique characteristics of the individual patient tumors can then be targeted in a patient-specific fashion with appropriate chemotherapeutic agents.

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Funding Meaningful Osteosarcoma Research Together

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Name: Nancy Gordon M.D. and John Andrew Livingston M.D.

Organization: The Children Cancer Hospital University of Texas MD Anderson Cancer Center

Email: ngordon@mdanderson.org Phone: 713-5635418

Initiative Name: Autophagy Inhibition to enhance chemotherapy sensitivity Amount Requested: $100,000

Desired Impact: Improve treatment responses and clinical benefits of standard treatments for Pediatric and Adolescents with relapsed/recurrent Osteosarcoma

Projected Milestones: Complete the initial Phase 1 patient enrollment, maximum tolerated dose determination and preliminary biomarker studies.

I agree to Guidelines: - Nancy Gordon M.D./ John Andrew Livingston M.D.

Prognosis for Pediatric and Adolescent (P&A) patients with relapsed/recurrent osteosarcoma (OS) is dismal with no new agents identified for > 30 years. Chemotherapy continues to be the main treatment for new and relapsed patients. Given the lack of alternative therapies and until new options emerge, identifying approved agents that can augment chemotherapy activity is a novel strategy for rapid translation into clinical trials for P&A OS patients.

Autophagy is a survival pathway that helps tumor cells recover after chemotherapy and has been shown to protect OS cells from chemotherapy-induced cell death. We showed that blocking autophagy enhanced the activity of gemcitabine (G), a drug used with docetaxel (D), to treat recurrent OS with limited efficacy. Although efficacy data is limited, given the lack of other therapeutic options, G alone or in combination with D remains the National Comprehensive Cancer Network guideline recommendation for second-line therapy in OS.

Our goal is to increase the therapeutic activity of G+D by adding an agent that blocks autophagy. Chloroquine (CQ) and its derivative hydroxychloroquine (HCQ), (used for malaria prophylaxis), inhibit autophagy. We demonstrated in vitro and in vivo that G combined with HCQ resulted in increased sensitivity to G and increased OS tumor cell death. In addition, we demonstrated that the protective effect of autophagy correlated with the expression of phosphorylated heat shock protein 27 (pHSP27) after chemotherapy treatment. In other words, when OS cells were treated with G, not only autophagy was detected but also increase expression of pHSP27. Further inhibition of autophagy with HCQ made the tumor cells more sensitive to the drug, causing cell death.

Furthermore, we recently published an autophagy biomarker study where we demonstrated that in 260 OS patient samples, the autophagy marker LC3B punctate was significantly higher in OS lung metastasis suggesting that chemotherapy may induce autophagy in OS and is a potential contributor to metastatic disease recurrence. Moreover, the expression of HSP27 at diagnosis and after chemotherapy was predictive of an inferior overall survival.

Based on our extensive pre-clinical and biomarker data, we *hypothesize* that autophagy inhibition will enhance the efficacy of G+D in OS patients and that pHSP27, a biomarker of the protective effect of autophagy against chemotherapy, will predict

whether adding an autophagy inhibitor to the regimen will provide clinical benefit. We predict that patients with increased expression of pHSP27 will benefit from the use of HCQ in combination with chemotherapy.

We therefore propose a clinical trial in P&A relapsed/recurrent OS patients to investigate the safety of this combination, and whether combining HCQ with G+D increases therapeutic response and as a consequence tumor cell death. We will obtained tumor samples before and after treatment to confirm that autophagy was blocked by HCQ and to look for biomarkers that correlate with therapy response. **While there are numerous trials in adult cancer patients demonstrating the therapeutic benefit of combining HCQ with chemotherapy, to date there are no data in P&A patients on the safety of combining HCQ with chemotherapy, and no trials investigating therapeutic activity in relapsed OS. These studies can identify a new therapeutic strategy for treating P&A OS patients.**

Our hypothesis is that combining HCQ with G+D will enhance therapeutic response in patients with relapsed disease. The clinical efficacy of G+D in relapsed/metastatic OS in retrospective studies show conflicting results and limited efficacy; objective response rates vary widely from 5-30%. Our preclinical data demonstrated that combining HCQ with G enhanced chemosensitivity and led to OS cell death. In addition, we observed increased expression of autophagy markers in OS patient metastases further supporting the rationale for combining G+D with HCQ in OS patients.

Objective responses (measurable responses) in OS are rare, even with known active drugs such as high-dose methotrexate, doxorubicin, and cisplatin (MAP). Therefore, the Bone Tumor Committee of the Children's Oncology Group (COG) has recently advocated for using disease control rate (percent of patients with advance or metastatic cancer who have achieved complete response, partial response or stable disease to a treatment in clinical trials) as the primary endpoint for evaluating efficacy in OS trials. The proposed phase I/II study has been submitted to the IRB and will evaluate safety and efficacy of G+D+HCQ.

The **primary objectives** are **1)** To determine the maximum tolerated dose (MTD) and preliminary safety of G+D+HCQ in children and adolescents with recurrent/metastatic OS, and **2)** to determine whether G+D+HCQ increases the disease control rate at 4 months as compared to historic COG controls. **Secondary objectives** are to determine the objective response rate , estimate the event free survival, progression free survival and overall survival, to estimate the rates of toxicity of G+D+HCQ, and to investigate pharmacokinetics (drug levels in the blood) of HCQ in patients with recurrent OS. **Alternative objectives** will be to evaluate the effect of the combination treatment on autophagy and the utility of pHSP27 as a predictive biomarker of HCQ benefit using samples collected from patients.

Based on the exceptional volume of P&A OS patients at our center, our proposal will be a single institution study. The proposed clinical trial will be conducted within both the Department of Pediatrics and the Department of Sarcoma Medical Oncology (adult oncology) at MD Anderson Cancer Center. We have seen >230 relapsed OS patients over the past 5 years. We expect to average enrolling 2 patients per month, with the target accrual of up to 31 patients. We would anticipate completing patient accrual within 18 months with planned additional follow up of 12 months for each patient, making the total duration of the study 30 months or 2.5 years for all data to mature. Funds provided will help jump start the study. The clinical trial protocol has been fully developed and is ready to start enrolling patients upon receiving funding. By 12 months, we should be able to complete the initial phase 1 patient enrollment, MTD determination and preliminary biomarker studies.

Our research plan has the potential to immediately impact the care of children and adolescents with recurrent/metastatic OS. Currently, there are limited treatment options for patients who relapse after standard treatment and few clinical trials available nationally for these patients. We will conduct a clinical trial specifically for pediatric and adolescent patients with recurrent/metastatic OS which seeks to improve upon the standard of care by adding a 3rd drug (hydroxychloroquine) to a standard chemotherapy regimen in order to overcome treatment resistance. This addition has significantly improved treatment responses and clinical benefits of standard treatments in other cancer types but has not been tested in pediatric or adolescent cancer patients or patients with OS. If successful, this research has the potential to improve survival for pediatric and adolescent patients with OS.

In addition to the patients who may benefit from participating in the clinical trial, the lessons learned from the clinical trial and the translational research conducted on patient samples in the laboratory will improve our understanding of why certain patients may or may not benefit from the treatment. If we can identify which patients are most likely to benefit (by developing biomarkers), this could be used to select patients for this treatment or related clinical trials in the future.

If the results are promising, because all 3 drugs used in this protocol are approved by the Food and Drug Administration and commercially available, this treatment could be made readily available to all patients with recurrent/ metastatic OS in the short to medium term.