November 17, 2017

James J. Foote
Foundation President, Trey Foote Foundation
jim_foote@treyfootefoundation.com

re: **pilot** research project, "Osteosarcoma Checkpoint Adaptation (OCA)"

Dear Jim,

Thank you for considering this proposal for $128,987 to support a pilot project exploring an Achilles heal of the bone cancer, osteosarcoma. **Our overall goal is to make osteosarcoma in children, teens & young adults more sensitive to frontline and secondary treatments, and thus more survivable**.

Background on our Organization: The Children's Cancer Therapy Development Institute (cc-TDI, www.cc-tdi.org) is a unique 501c3 non-profit organization focused on the 'preclinical gap' in childhood cancer research. Our mission is to bridge scientific discovery and the initiation of clinical trials. Through our efforts, **we will provide evidence-based testing for the selection of new drugs to be used in childhood, adolescent and young adult cancer clinical trials, thus seeding pediatric Phase I and II trials.** This concept was emphasized in the Institute of Medicine Report, *Making Better Drugs for Children with Cancer* in 2005. The goal of cc-TDI is to fill this needed role. Our longstanding work with mouse models of sarcomas is the cornerstone for basic science & target discovery, as well as preclinical studies, that fuel our mission.

Project-related Request: 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.

Project Timeline: The experiments above are expected to be completed in a one year period. The Trey Foote Foundation would be acknowledged in the resulting publication, and any efforts to move the findings to a clinical trial.

Thank you again for considering our pilot project!

Sincerely,

Charles Keller MD
Scientific Director, Children's Cancer Therapy Development Institute (cc-TDI.org)
Tel: 801-232-8038; Fax 970-237-6388; charles@cc-tdi.org

ps. because Charles Keller and James Foote are co-founders of First Ascent Biomedical, this potential conflict of interest will be declared and managed in accordance with the Boards of cc-TDI and TFF.

Osteosarcoma Checkpoint Adaptation (OCA) Pilot Project

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 G_2/M cell checkpoints [5,6]. In studies of osteosarcoma, this ability to evade G_2 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 G_2 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 G_2 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 G_1 [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.

Background

nuclear RTKs can drive chromatin remodeling, emergency transcription, checkpoint adaptation & radiation resistance

Our studies of checkpoint adaptation in the soft tissue sarcoma rhabdomyosarcoma led to the observation that nuclear RTK expression was induced in 4N cells by the same oncogenic transcription factor driving checkpoint adaptation [6]. This observation led us to explore nuclear RTK expression in cancer: recent data from our group suggests that receptor tyrosine kinases (RTKs) are curiously nuclear-localized [1,8] in tumors which have undergone checkpoint adaptation; a parallel set of studies infer that nuclear RTK's localize to gene loci bound to which phospho-Histone H3 (pHH3) protein also localizes, thereby activating transcription of these genes by RTK phosphorylation of Ser10 of pHH3. These M cell cycle phase genes marked by the M-phase marker pHH3 are perhaps not by coincidence the genes responsible for emergency transcription in times of cellular stress. Furthermore, nuclear RTKs have been shown to facilitate pHH3 marked stress response genes' transcription via chromatin remodeling [9-11].

Converging evidence suggests that nuclear-localized RTKs may have a broad role in cancers. Nuclear IGF1R, PDGFRA, FGFR1 and other RTKs have been shown to be present or to predominate over membrane-expressed RTKs in rhabdomyosarcoma (RMS), colon adenocarcinoma, breast cancer and renal cell carcinoma and to correlate with decreased survival (*e.g.*, in multivariate analysis for clear cell RCC the survival at 10 years worsened by 30% for intense nuclear IGF1R staining, $p < 0.05$; Figure 1) [1,2,8,12,13].

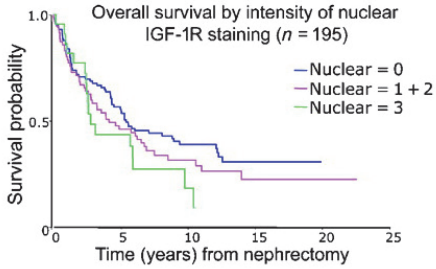

Figure 1. clear cell RCC survival is worsened by nuclear IGF1R expression [2].

A hint at the chemotherapy-resistance role of nuclear RTKs is that FGFR1 ligand appears to mediate substantial radiation protection [14]. These observations aside, kinase inhibitors given as monotherapy are largely ineffective and usually result in resistance; however, using inhibitors of FGF, PDGF and IGF receptors as front line therapy with timing that maximizes the effect of radiation, chemotherapies and G_2/M checkpoint inhibitors remains to be tested. Thus, **we hypothesize that RTK inhibitors targeting FGF, PDGF and IGF receptors for diseases with nuclear-predominant RTK expression will increase tumor cell kill and reduce tumor recurrence when added to chemotherapy or checkpoint inhibitor treatment regimens**. The concept of repurposing RTK inhibitors by capitalizing on the cell cycle dependent mechanisms if action for co-administered drugs is a relatively new clinical concept. What is needed now is supporting preclinical evidence for this novel approach to the use of RTK inhibitors in cancer chemotherapy that will address a significant unmet medical need by opening the door to new treatment paradigms for a variety of human malignancies.

Nuclear RTKs are found in common adult cancers

As stated earlier, nuclear IGF1R, PDGFRA, FGFR1 and other RTKs have been shown to be present in rhabdomyosarcoma (RMS), colon adenocarcinoma, breast cancer and renal cell carcinoma and to correlate with decreased survival [1,2,8,12,13]. Colon adenocarcinoma is an example of a pertinent adult cancer histology with nuclear RTK expression (Figure 2). In our unpublished preliminary data, the osteosarcoma cell line U2OS also has nuclear FGF receptor expression (Figure 2); furthermore, FGF receptor sensitivity is a characteristic of osteosarcoma cell lines recently described in a functional genomics screen [15].

The role of FGF signaling & nuclear RTKs for radioprotection were largely unappreciated until now

Studies more than 20 years ago, motivated by the quest for radiation protection, revealed that canonical FGFR1 ligand FGF2 administered to mice prior to whole body-irradiation afforded dose-dependent radioprotection [14,16]. Curiously, this radioprotection occurred with time zero treatment (during radiation), with pretreatment 24 hours before radiation, but not with pretreatment 4 hours before radiation – leaving the possibility open that Fgf2 signaling may have different effects during stress (such as that induced by radiation) than with G_1/S related growth factor signaling initiated under non-stress conditions that is interrupted by stress.

FGF ligand-induced FGF Receptor signaling and pHH3 binding is Rsk2-mediated and leads to chromatin remodeling

A series of studies elegantly demonstrate a ligand-dependent FGFR1 signaling pathway that can activate stress response & repair genes. Summarizing, FGF2-mediated FGFR1 nuclear localization leads to nuclear Ribosomal S6 Kinase 2 (RSK2) phosphorylation and activation [10], which in turn leads to phosphorylation of Histone H3 [9-11]. This phosphorylation then

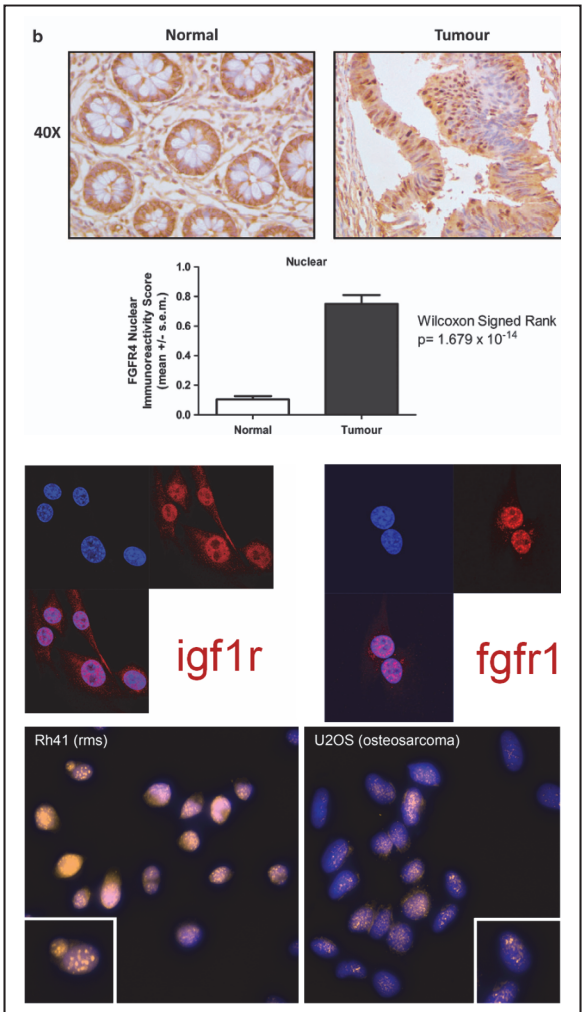

Figure 2. Nuclear FGF Receptors in colon adenocarcinoma[1], rhabdomyosarcoma (rms) & osteosarcoma (unpublished). top: in cell lines, FGFR4 inhibition reversed resistance to 5-fluorouracil (5-FU) and oxaliplatin. middle, confocal imaging of nuclear RTK expression in rms. bottom: side-by-side FGFR1 expression in osteosarcoma cell line U2OS and rhabdomyosarcoma cell line Rh41 via in-plane (apotome) imaging, demonstrate nuclear chromatin expression as well as trace cytoplasmic expression. dapi.

leads to chromatin remodeling and early-intermediate stress response gene expression [11]. A working model of the signaling mechanism is presented in Figure 3.

Overall, these results suggest that nuclear-localized RTKs may be targets for overcoming resistance to cell cycle checkpoint inducing therapies such as radiation, chemotherapy – and potentially even cell cycle checkpoint inhibitors of Wee1 or Chk1.

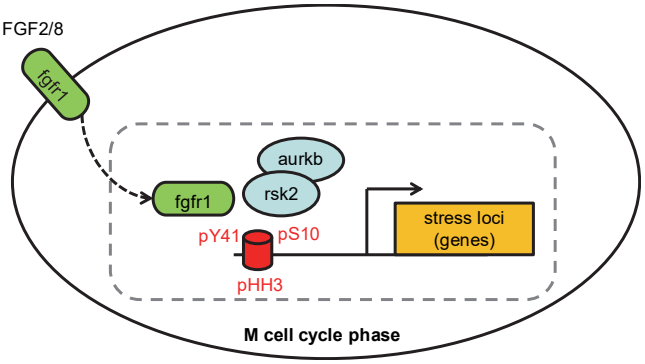

Figure 3. Working hypothesis for how RTKs facilitate M checkpoint adaptation and emergency response to stress.

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 G_2 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).

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 G_2/M cell cycle arrest – thus a candidate for checkpoint adaptation mediated chemotherapy resistance. Whereas radiation more heavily weights for a G_2 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.

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Budget (07/01/18-06/30/19)

Personnel					Amount
Name	Role on Project	% Effort	Salary	Fringe Benefits	
Charles Keller MD	Principal Investigator	2.5	4,080	1,224	5,304
Megan Cleary	Senior Research Associate	100	60,000	18,300	78,300
		Subtotals	64,080	19,524	83,604
Consultant Costs; Equipment					
none					
				Subtotal	0
Supplies (Itemize by Category)					
Disposables, reagents and media/solutions/drugs/antibodies for cell culture & drug screening & high content imaging, plus validation studies					
				Subtotal	23,976
Other Expenses (Itemize by Category)					
Travel to present results a national scientific conference $ 1,200 Every-4-month STR analysis of 7 cell cultures ($220 ea) = $4,620 Exome sequencing (100x; BGI) of 7 cell cultures (pre- and post-radiation; 480ea) = 6,720					
				Subtotal	12,540
				TOTAL DIRECT	$120,120
				RESEARCH SUPPORT COSTS (33.2%)	$39,880
				TOTAL COSTS	$160,000

Personnel:

Charles Keller, M.D. (0.3 FTE) is the Principal Investigator and will oversee all aspects of this project including experiments and analysis. Dr. Keller is Scientific Director and a Member of the Children's Cancer Therapy Development Institute (cc-TDI). Dr. Keller has extensive experience with mouse models of human cancer, including alveolar rhabdomyosarcoma (RMS), embryonal RMS, undifferentiated sarcoma and osteosarcoma.

Megan Cleary (1.0 FTE) is a Senior Research Scientist and will perform all experiments. She will participate in the process of culturing primary tumor cell cultures, performing STR fingerprinting, BGI DNA exome sequencing sendouts, chemical screens with and without irradiation and high content imaging.

SCIENTIFIC ABSTRACT

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). With respect to chromothripsis, Checkpoint Adaptation (CA) is a newly appreciated mechanism borrowed by osteosarcoma cells from yeast to ensure cell survival. 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. 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. Outside of cancer, CA is a known, evolutionarily-conserved (ancient) mechanism of survival for unicellular organisms experiencing stressful environmental conditions 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, with the potential to perform DNA repair later at G1. 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 – 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. The goal of our pilot study is to prevent checkpoint adaptation with small molecule inhibitors of causal epigenetic, kinase-related signaling pathways to improve the effectiveness of both radiation and chemotherapy.