The Institute for Cancer Research

Annual Progress Report: 2011 Formula Grant

Reporting Period

July 1, 2013 – June 30, 2014

Formula Grant Overview

The Institute for Cancer Research (formerly Fox Chase Cancer Center) received $2,472,183 in formula funds for the grant award period January 1, 2012 through June 30, 2014. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Impacts of Inhibitory Receptors on Signaling Competence of NK Cells – Mature natural killer (NK) cells express inhibitory receptors that prevent autoimmune attack by recognizing MHC class I molecules on all normal cells of the body. During NK cell development, however, inhibitory receptor signaling paradoxically promotes maturation to functional competence. We have previously shown that NK cells from genetically-modified mice with reduced activation signaling are more mature and more functional. In this project, we will define how inhibitory receptors and their MHC class I ligands promote and suppress the signaling competence of NK cells. Our findings could lead to immunotherapies that enhance the signaling competence of NK cells to improve their attack of tumor cells in patients.

Duration of Project

1/1/2012 –12/31/2013

Project Overview

Natural killer (NK) cells can distinguish normal cells from tumor cells through signals derived from activating and inhibitory receptors. In mature NK cells, inhibitory receptors [Ly49 in mice and killer cell Ig-like receptors (KIR) in humans] are key regulators that prevent attack of normal cells through their detection of major histocompatibility complex class I (MHC-I) molecules on virtually every normal cell of the body. Tumor cells are often MHC-I-deficient and attract strong NK cell attack by lacking the inhibitory receptor ligands. Ly49, KIR, and their MHC-I ligands are encoded by highly polymorphic families of genes, and some inhibitory receptors are expressed in an individual that do not recognize the endogenous MHC-I, which is considered "self". Different inhibitory receptors are stochastically expressed during NK cell development, and the expression of a self-recognizing inhibitory receptor (SRIR) paradoxically promotes the maturation of a functionally competent NK cell that can elicit cytotoxicity and cytokine production. In contrast, NK cells lacking SRIR are non-functional, and it has been proposed that these cells are anergic due to chronic activation signaling in the absence of inhibitory signaling.

Supporting this hypothesis, we have previously reported that NK cells in mice lacking the activation signaling adaptor protein, B cell adaptor for phosphatidylinositol 3-kinase (BCAP), are more functional and more mature, even in a MHC-I deficient environment. A major gap in understanding, however, is how expression of SRIR impacts the competence of signal transduction through NK cell activating receptors and whether maturation plays a role in signaling competence. Our lab is well poised to make significant advances in understanding the intertwining roles of SRIR and maturation in promoting signaling and functional competence of NK cells in mice and humans. The results of these studies could help develop new therapies to improve NK cell competence to fight cancer. We will address the following specific aims:

1. How do inhibitory Ly49 impact the development of signaling competence in mouse NK cells?
2. How do inhibitory KIR impact the development of signaling competence in human NK cells?

Principal Investigator

Kerry S. Campbell, PhD
Associate Professor
The Institute for Cancer Research
333 Cottman Avenue
Philadelphia, PA 19111

Other Participating Researchers

Amanda K. Purdy, PhD; Alexander W. MacFarlane IV, PhD – employed by The Institute for Cancer Research

Expected Research Outcomes and Benefits

NK cells are important innate immune cells that can spontaneously attack malignant cells and virus infected cells to elicit cytotoxicity and cytokine release. Inhibitory receptors that recognize major histocompatibility complex class I (MHC-I) molecules as ligands are key regulators of NK cell function. NK cell inhibitory receptors play dual roles, however, since they tolerize NK cells from attacking normal MHC-I-expressing cells, but also provide important signals that promote the development of NK cells to achieve functional maturity. In this project, we propose to characterize the impacts of inhibitory receptors and their interactions with MHC-I ligands on the competence of activating receptor signaling by NK cells. By fully understanding the influences of inhibitory receptor signaling on activation signaling in NK cells, we will be better able to design therapeutic strategies to enhance NK cell function to treat cancer and viral infections. Therapeutic antibodies that block human inhibitory NK cell receptors from recognizing MHC-I are already entering clinical trials to potentiate NK cell function in cancer patients without full knowledge of how they may impact the functional competence of NK cells or for how long. Furthermore, hematopoietic stem cell transplantation has been shown to provide improved benefit in treating some blood cancers under defined conditions if allogenicity exists between donor NK cell inhibitory receptors and recipient MHC-I. Our studies will examine in detail how adoptive transfer of NK cells from MHC-I-deficient to MHC-I-sufficient mice (and vice versa) changes their activation signaling competence. Improved understanding of the kinetics of

signaling competence under these transplantation conditions should guide the design of better protocols for hematopoietic stem cell transplantation to improve patient outcomes.

Summary of Research Completed

In the most recent reporting period, we tested the functional impact of stimulating the different NK cell subsets by testing for interferon (IFN)-γ production by intracellular staining and flow cytometry to address Subaim 1c. In these studies, we found that the primary source of IFN-γ is from NK cells that express SRIR, but are also at a mid-level maturity stage designated by expression of CD11b and CD27 (R2 stage). We conclude from these data that, indeed, part of the "licensing" effect is due to the inherent advanced maturity state of the NK cells that express SRIR. In contrast, the less functional NK cells lacking SRIR were found to be enriched in less mature NK cells (CD11b-negative $CD27^+$, designated R1). We were, however, surprised that the most mature NK cells ($CD11b^+$ $CD27^-$, R3 stage) did not exhibit the strongest functional responses in these experiments.

Studies designed to address Subaim 1b were less productive than anticipated. Mouse splenocytes were stimulated as in Subaim 1a for various times, fixed, permeabilized and stained with various phosphoproteins and surface markers to distinguish NK cell subsets. While we were able to show some increased phosphorylation events in anti-NK1.1-stimulated NK cells, the intensity of signal was not adequate to reproducibly compare differential signals in the NK cell subsets, despite significant effort. In contrast, the calcium signaling strength was much higher, resulting in better resolution of differential signaling. Nonetheless, we acquired some preliminary data for future grant proposals through these phospho-specific antibody staining experiments.

For studies addressing Aim 2, we screened healthy donors for KIR and HLA-A,B,C ligand expression by flow cytometry and genotyping, in accordance with Subaim 2a. We used KIR SSP kits from Invitrogen and HLA-B and HLA-C SSP UniTray kits, also from Invitrogen, and we had these run in our genotyping facility at Fox Chase. In addition, we have begun to establish genomic PCR assays in our laboratory, using oligonucleotide primers that were previously described. We focused on KIR2DL1- and KIR3DL1-bearing individuals, since these are both common alleles found in humans, and specific antibodies are available for these KIR (most anti-KIR antibodies are cross reactive with other family members). To date, we have screened about 25 healthy donors in these assays, which provides a wealth of differential expression patterns of KIR2DL1 and/or KIR3DL1, either with or without their ligands. Importantly, most donors expressed both of these receptors, with about one third lacking ligand for each of the receptors.

To address Subaim 2b, we performed calcium mobilization experiments on peripheral blood lymphocytes from some of these donors using techniques established in mouse splenocytes in Subaim 1a. Different stimulating biotinylated antibodies were tested in combination with streptavidin crosslinker to find the best activating signal. Anti-CD16 antibody induced the most robust calcium signal. While anti-NKG2D or anti-NKp46 antibodies induced only barely detectable calcium signals, the combination of anti-NKG2D and anti-NKp46 induced a stronger response that was readily detectable, but less potent than anti-CD16 antibody. Therefore, we focused on the use of anti-CD16 antibody in subsequent studies, which limited our analysis to

the more mature CD56^{dim} NK cell subset that predominantly expresses this activating receptor. When we gated on NK cell subsets, based upon expression of SRIR or unligated IR (focusing on KIR2DL1 and KIR3DL1), we saw some enhanced signaling in NK cell subsets expressing SRIR, but the enhancement was not as pronounced as that found in murine NK cells. Similarly, the reduction of calcium signaling in NK cells expressing unligated IR was less pronounced than in NK cells from mice. While the results were less exciting than expected, the resolution may be hindered by lack of monoclonal antibodies specific for each of the individual inhibitory KIR, specifically KIR2DL2, KIR2DL3, and KIR3DL2. Available antibodies to these inhibitory KIR cross react with activating KIR, which limits their usefulness in fully resolving their impacts in our experiments. Based upon our data, we also hypothesize that calcium signaling in mouse NK cells may be more significantly influenced by SRIR, due to the cis interactions that occur between Ly49 and MHC-I on the surface of mouse NK cells themselves. In contrast, KIR appear to lack capacity to interact with MHC-I in cis on the surface of human NK cells, thereby allowing them to diffuse more freely in the plasma membrane on the surface and more readily and uniformly interact with and blunt early signaling events through NK cell activating receptors.

Research Project 2: Project Title and Purpose

Pre-BCR Function Selecting Novel B Cell Receptors in Chronic Lymphocytic Leukemia – B cell chronic lymphocytic leukemia (CLL) is the most common leukemia of adults in Western countries, characterized by usage of distinctive B cell receptor (BCR) heavy and light chain genes. We will test CLL heavy chains for capacity to associate with surrogate light chain into a pre-BCR. Our hypothesis is that the striking biases seen in CLL heavy chains arise because their precursors come from a distinctive B cell development process that selects for weak association with surrogate light chain. We will utilize procedures for measuring pre-BCR assembly and compare this with the induction of proliferation. Our work will establish the significance of BCR in development of CLL precursors, potentially revealing new avenues for treatment.

Duration of Project

1/1/2012 – 12/31/2013

Project Overview

B cell chronic lymphocytic leukemia (CLL) is the most common leukemia of adults in Western countries and is recognized by the accumulation of a clonal population of B cells with a surface phenotype distinguishing them from most mature B cells. Although the disorder has been known for over thirty years, surprisingly, its etiology remains unclear and standard treatments, established twenty years ago, entail significant side effects and do not effect a cure, but rather delay progression for a time. Analyses of B cell receptors (BCRs) from CLLs have revealed three distinctive features: 1) half match germline database sequences and half show extensive somatic hypermutation; 2) recurrent usage of certain heavy and light chains; 3) self-reactive binding. Thus CLL may arise from two distinct sets of B cells, some with highly mutated BCRs and the other lacking such mutations, but encoding autoreactivity. Furthermore, recent studies have described the presence of CLL-like monoclonal expansions in relatives of CLL patients.

As CLL is a disease whose incidence increases with age, rarely found in young individuals, it is tempting to speculate that clonal B cells often accumulate with age, but only infrequently progress to the CLL stage. Understanding the factors that generate these monoclonal B-cell lymphocytoses (MBLs) and determining how they progress to CLL could lead to breakthroughs in the treatment of CLL. Thus we will analyze BCRs from a mouse model of CLL, the TCL1 transgenic line (Aim 1), and also BCRs isolated from patients with unmutated CLL (Aim 2). In both cases, we will test whether the BCRs are enriched for heavy chains that show weak association with surrogate light chain, a feature we have found can serve as a marker for natural autoreactive B cells, potential precursors for these B cell leukemias. Thus we will ask whether generation of the striking biases seen in V genes of BCRs from both mouse and human $CD5^+$ B-cell leukemias involve an as-yet unappreciated mechanism that sculpts the repertoire of natural autoreactive B cells. Our work will establish the groundwork needed for further study of the role of BCR in development of CLL precursors, eventually providing insights that will lead to new therapeutic approaches for the treatment of CLL.

Principal Investigator

Richard R. Hardy, PhD
Professor
The Institute for Cancer Research
333 Cottman Avenue
Philadelphia, PA 19111

Other Participating Researchers

Srinivas Bandi, PhD – employed by The Institute for Cancer Research

Expected Research Outcomes and Benefits

Aim 1 (Mouse CLL): Determine whether late-developing $CD5^+$ B cell leukemias in mice are enriched for Ig heavy chains that mediate weak pre-BCR signaling. We have established a system for measuring the efficiency of pre-BCR assembly and signaling and will use it to evaluate Ig heavy chains isolated from clonal B cell expansions and leukemias that routinely appear in a group of aging (>1 year) mice. We will isolate cells from peripheral blood (progression series) or from the enlarged spleen (terminal animals) by electronic cell sorting. The VDJ regions obtained by PCR will be inserted into a mouse heavy chain expression vector and used for transduction of an Abelson line to test for pre-BCR assembly, and of $Rag1^-$ pro-B bone marrow cells to test for function in short-term stromal cell culture. This work will determine the incidence of weakly signaling VDJs in these $CD5^+$ B cell leukemias.

Aim 2 (Human CLL): Determine if human chronic lymphocytic leukemias are enriched for Ig heavy chain VDJs that assemble weakly with surrogate light chain. We will obtain heavy chain VDJ segments from the CLL Consortium. These VDJs will be inserted into a human heavy chain expression vector and evaluated for pre-BCR assembly and function, similar to the experiments of Aim 1 in mice.

Anticipated Outcomes: We have already carried out these assays with heavy chain VDJs prepared from normal B cells sorted from mouse spleen and human peripheral blood. We will determine if the representation of VDJs that show weak association with surrogate light chain is increased compared to this normal baseline value, which would support our hypothesis that natural autoreactive B cells constitute a precursor pool for B cell leukemia.

Summary of Research Completed

The overall aim of this project is to determine whether generation of the striking biases seen in immunoglobulin (Ig) variable genes of B cell receptors (BCRs) from both mouse and human $CD5^+$ B-cells and $CD5^+$ B-cell leukemias may arise, at least in part, by an as-yet unappreciated mechanism that regulates the repertoire of natural autoreactive B cells. In each developing B cell precursor, three DNA segments, a variable, a diversity, and a joining (V-D-J) are assembled to form the antigen recognition portion of the Ig heavy chain. Furthermore, at this "pre-B" stage of development, there is strong selection (Figure 1) based on assembly of the newly-formed Ig heavy chain with a "surrogate light chain" into a pre-B cell receptor (pre-BCR), and this "μ-selection checkpoint" may be important in understanding the development of distinctive heavy chains that encode BCRs with an autoreactive bias, often seen in leukemias. We have applied an assay with mouse cells that measures the capacity of Ig heavy chains to assemble into a pre-BCR and induce proliferation. This allows us to examine heavy chains isolated from a series of late-developing chronic lymphocytic leukemia (CLL)-like mouse leukemias (Aim 1). We have also established a comparable assay that enabled analysis of human cells, both normal (control) and leukemic (Aim 2). This work enabled us to assess pre-BCR assembly and signaling in both mouse leukemic models and in human B cell leukemias, comparing and contrasting them with normal B cells. Specifically, we asked whether the distinctive Ig heavy chain biases often seen in both mouse and human B cell leukemias may arise early in development (at the Pre-B stage), potentially providing important new insights into the growth and eventual transformation of such cells.

In carrying out the work of Aim 1, we performed an analysis of pre-BCR formation in a set of more than 40 Ig heavy chain VDJ segments cloned from a group of mouse B cell leukemias that developed in EmuTCL1 transgenic mice (that have B cell directed expression of the T cell leukemia 1 gene product). For each sample, VDJ segments are isolated by polymerase chain reaction (PCR) amplification using an Ig constant region specific primer together with a "consensus" primer that can bind to any of the V gene segments utilized in mouse. A secondary amplification utilizing similar primers, but containing unique restriction endonuclease sites, facilitates cloning the VDJ into a mu-heavy chain expression construct (Figure 2). The construct is inserted into a retrovirus vector that contains an IRES (internal ribosome entry sequence) followed by eGFP (enhanced green fluorescent protein) DNA segments, thereby coupling heavy chain expression with green fluorescence in cells transduced with the retrovirus. Retrovirus-containing helper cell supernatant is used to transduce (infect) a Pro-B cell line that lacks any endogenous heavy chain, but that does have surrogate light chain, allowing pre-BCR assembly to occur when an Ig heavy chain is provided. These cells are then tested for expression of mu-heavy chain and binding of a monoclonal antibody (SL156) that only recognizes surrogate light chain when complexed with Ig mu-heavy chain (i.e., it recognizes fully assembled pre-BCR). An example of this type of analysis is shown in Figure 3 that presents primary data from two

different VDJ heavy chains, one that strongly associates with surrogate light chain (marked "High") and one that does not (marked "Low"). Note that both heavy chains are expressed at similar levels (revealed by staining with antibody for IgH-mu) and that expression is correlated with green fluorescence (X-axis).

In the past six months of the project period we have continued to accumulate data from further analysis of TCL1 B cell leukemias, generating a data set of over 80. A number of these are repeats, where the same Ig heavy chain V-D-J was utilized, so these only are counted once. All told, we have approximately 50 unique sequences and analysis of these has shown that over 30% fail to assemble well with surrogate light chain. This is much higher than a prior analysis of normal B cells, where fewer than 10% show this property. The hypothesis we are testing in this work is that the TCL1 leukemias derive from a pool of autoreactive B cells that express CD5, termed B1a B cells. Importantly, a novel feature of these B cells is the usage of Ig heavy chains that show weak surrogate light chain association. We think that the reason for an increased number of such heavy chains is the production of these B cells from a fetal B cell developmental pathway that tends to favor low expression of the pre-BCR, thus enriching B cells with Ig heavy chains that show weak assembly with surrogate light chain.

In carrying out the work of Aim 2, we established a staining assay for examination of pre-BCR assembly with human immunoglobulin heavy chains, by staining transduced RS4;11 Pro-B cells (obtained from Dr. Tucker LeBien, University of Minnesota) with the anti-HSL2 monoclonal antibody that binds to human surrogate light chain (obtained from Dr. Hajime Karasuyama, Tokyo Medical and Dental University Graduate School), but only when assembled into a pre-BCR, exactly analogous to SL156 binding to mouse surrogate light chain. We have examined over 30 VDJs cloned from naïve B cells of healthy human peripheral blood. Most VDJs (>85%) associate strongly with surrogate light chain, as assessed by this staining assay. Simultaneously, we analyzed VDJ segments cloned from patient chronic lymphocytoic leukemia (CLL) cells. Some of these samples were obtained in house (Fox Chase Cancer Center) and some were obtained through the CLL Consortium with the help of Dr. Thomas Kipps (University of California San Diego Moores Cancer Center). VDJ clones are obtained by use of a Mu heavy chain primer and 5'-RACE (rapid amplification of cDNA ends). Clones obtained were sequenced to verify low-level or no somatic mutation (<3%), then inserted into the IgH expression vector, similar to the one we generated for mouse (Figure 2). Each VDJ-mu heavy chain was then inserted into a retrovirus, as described above and then used for production of retroviral supernatant. As in the mouse analysis, transduction of Pro-B cells with the Ig-heavy chain retrovirus generates cells, where GFP^+ (green fluorescent) cells are also Ig-heavy chain positive. These cells are permeabilized and then analyzed for expression of Mu heavy chain and HSL2-binding by flow cytometry.

Similar to our analysis comparing Ig heavy chains from TCL1 leukemias with normal follicular B cells in mouse, we also observed an enrichment for Ig heavy chains that only weakly associated with surrogate light chain when comparing CLL-origin with naïve B cell-origin samples. The results in both cases are not very extreme, in that we see 30-50% of the samples showing the novel characteristic, contrasting with an average of 10% or less showing this feature in control samples. We have now selected 5 "low" and 5 "high" CLLs and isolated their Ig light chains, allowing us to express the complete Ig molecule (IgH plus IgL), enabling assessment of

autoreactivity. We propose to carry this work forward as part of work proposed in an R01 application we recently submitted to the NIH.

Our analysis of mouse TCL1-promoted B cell leukemias has identified several VDJ segments recurrently utilized (and associated with specific light chains). This is key information that will enable further modeling of B cell leukemogenesis in immunoglobulin transgenic mice bearing these rearrangements. During the course of our work we developed a simple rapid procedure for making heavy chain "knock-in" mice, enabling precise physiologic expression levels compared with conventional transgenic mice. This zinc finger nuclease (ZFN) approach is much more rapid and much less labor intensive than the classic ES-cell knock-in procedure. One of these knockin models develops CLL-like leukemias, either promoted by TCL1, or with more time, on a wildtype background. Importantly, analyzing these leukemias, we have found repeated deletion of a DNA segment (by using array CGH) containing microRNA-15/16, which has been reported by Croce to be common in human CLLs. Thus we believe that this knockin line may be one of the closest models yet found for human CLL and plan to continue work with it in an R01 application submitted to the NIH.

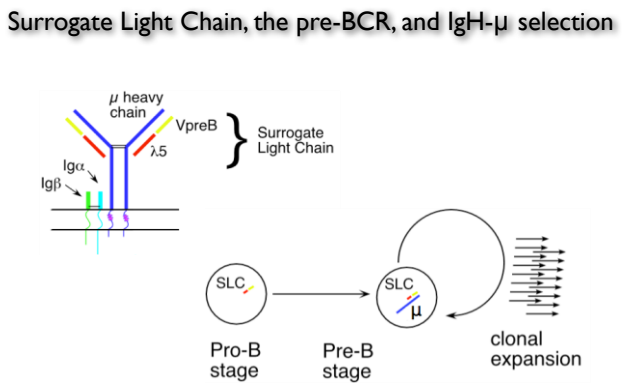

Figure 1. Pre-BCR mediated selection of mouse pre-B cells

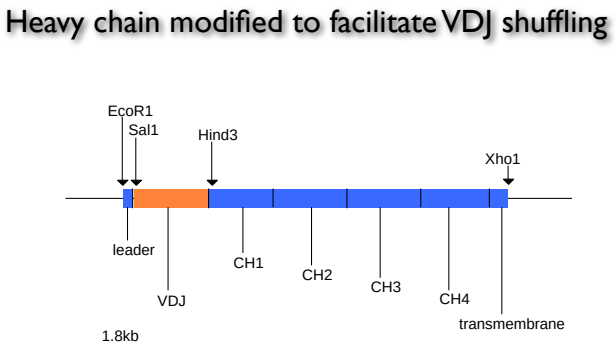

Figure 2. Ig heavy chain expression vector

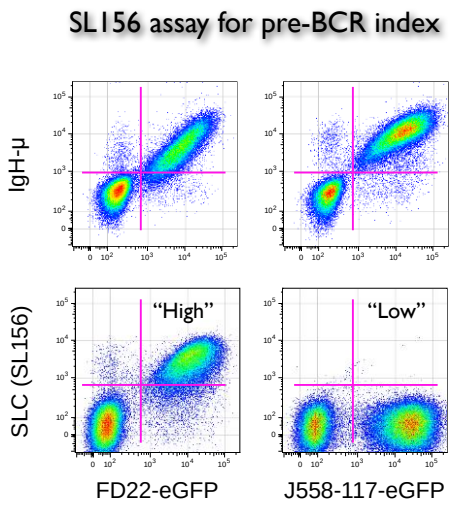

Figure 3. Example of two different IgH VDJs, one assembling well with surrogate light chain (FD22, "High") and the other other very weakly associating (J558-117, "Low")

Research Project 3: Project Title and Purpose

Dissecting Role of ThPOK in T Cell Development through TALEN-mediated Exon Swapping – ThPOK belongs to the POK family of transcription factors, which are known to play critical roles in development and cancer. We have shown that ThPOK has a non-redundant essential function in T cell development, and is a potent oncogene when overexpressed in T cells. Here we propose to elucidate how ThPOK carries out these functions using genetic approaches in mice. In particular, we will use site-specific TALE nucleases to generate knockin mice in which either the entire ThPOK coding region or individual functional domains are replaced by the corresponding region of related POK factors. This will establish whether the requirement for ThPOK in T lymphoid development and cancer reflects a unique function or expression pattern.

Duration of Project

1/1/2012 – 12/31/2013

Project Overview

We have identified the transcription factor ThPOK (T helper inducing POZ/Krueppel factor) as a key regulator of T lymphocyte development. ThPOK is essential for both directing commitment of αβ T cells to the CD4 lineage and promoting development of innate NKT γδ cells. In addition, it is a proto-oncogene, whose constitutive expression in thymocytes leads to aggressive thymic lymphoma. ThPOK belongs to the POK family of transcription factors that also includes Bcl6, Plzf and LRF/Pokemon. POK factors are characterized by a Zn finger DNA binding domain and a BTB or POZ domain, the latter implicated in interaction with components of the

histone deacetylase complex (HDAC) and transcriptional repression. It is unclear whether POK factors encode distinct or partly overlapping functions. To resolve this question, here we propose to develop new tools for targeting the ThPOK gene *in vivo*, and to use these tools for its functional dissection. In Aim 1, we propose to develop site-specific TALENS (transcription activator effector nucleases) to modify the ThPOK locus. TALENs will be introduced directly into mouse oocytes, where they will promote mutagenesis by introducing a site-specific double-stranded (ds) DNA break. Recent reports from other species suggest that effective site-specific TALENs are relatively easy to design and may greatly facilitate gene targeting, although this has yet to be directly demonstrated in mice. In Aim 2, we propose to use these site-specific TALENs to generate knockin mice in which either the entire ThPOK coding region or individual functional domains are replaced by the corresponding region of LRF or Plzf. This will establish whether all or part of the ThPOK protein is uniquely required for its role in T lymphoid development, or whether there is broad functional overlap with other POK family members. Functional dissection of ThPOK is critical to understanding its role in normal development and cancer.

Principal Investigator

Dietmar Kappes, PhD
Professor
The Fox Chase Cancer Center
333 Cottman Avenue
Philadelphia, PA 19111

Other Participating Researchers

Jayati Mookerjee Basu, PhD – employed by The Fox Chase Cancer Center

Expected Research Outcomes and Benefits

First, this project will advance our understanding of the role of POK family transcription factors in development and cancer. POK family factors are increasingly implicated in both processes, but it is unclear whether they employ the same mechanism. The current application will definitively resolve whether different POK factors perform unique or broadly overlapping functions. POK family members contain 2 major functional domains, the POZ domain implicated in transcriptional repression and/or activation, and the Zn finger domain, implicated in target recognition. Either or both domains may encode unique functions specific to particular POK factors. To examine this, we will generate unique new mouse models in which the endogenous ThPOK locus is modified by inserting coding exons from other related POK factors, while retaining normal transcriptional regulation under the control of ThPOK *cis* elements. Secondly, this project will lead to improvements in gene targeting technology in mice. Reverse genetics has become an important and widely used approach to dissect gene function *in vivo*, but remains laborious and expensive. The use of site-specific TALENs has the potential to considerably simplify and accelerate this approach. The major advantages are that mutations can be introduced directly into developing mouse oocytes, thus avoiding the need for ES cell culture,

and that target site selection is simpler and more predictable than for other existing methods. Hence, if successful in establishing the efficacy of TALEN-mediated gene targeting in mice, this project will represent an important technological advance.

Summary of Research Completed

Previously, we reported that we had designed and tested site-specific TALENs to target the coding exons of the ThPOK gene. While these TALENs proved effective at mediating the desired deletion event in cell lines, they proved inefficient at mediating homologous recombination when injected into mouse oocytes, and no correctly targeted mice were obtained by this approach. The current consensus in the gene targeting field is that the ZFN and more recently developed CRISPR methodologies are superior to the TALEN approach for gene targeting in mice. Given the proven efficiency of both ZFNs and CRISPRs in our hands for generating gene-targeted mice, and to accelerate the production of proposed ThPOK exon-swapped mice, we switched to a ZFN-mediated approach. Indeed, as previously reported, using site-specific ZFNs we were able to generate 9 founder lines in which all domains of ThPOK were replaced by those of the related LRF gene (LRF knockin, or LKI mice).

Since the previous reporting period ended, founder LKI mice were crossed to homozygosity to establish their phenotype with respect to T cell development and function. Note that the knockin construct precisely replaces the ThPOK coding exons with those of LRF, while surrounding sequences, including the small intron separating the 2 LRF coding exons, are all derived from the ThPOK gene. Note that 5' and 3' untranslated (UT) regions are also derived from the ThPOK gene, so that mRNA stability and translation should be similarly regulated. Hence, expression of the knockin allele should precisely replicate the levels and kinetics of endogenous ThPOK protein. RT-PCR analysis of sorted thymocyte subsets from heterozygous LKI/+ founder mice, demonstrates elevated LRF mRNA expression at the SP CD4 CD69+ stage, precisely when endogenous ThPOK is normally most highly induced. We have recently obtained our first homozygous LKI/LKI mouse, which demonstrates that these animals are viable.

Preliminary analysis of mature T cell composition in 4-week old homozygous LKI/LKI and heterozygous LKI/+mice reveals that 1) LKI/+ mice are phenotypically normal, indicating the knockin allele does not have a dominant effect, and 2) SP CD4 T cells are restored to about 50% of normal in LKI/LKI mice compared to ThPOK-/- mice. As a result, the relative proportions of mature SP CD4 and CD8 cells are markedly shifted towards the CD8 compartment. In addition, LKI/LKI mice exhibit unusual populations of mature CD4+8+ (DP) and SP CD4low cells. These effects presumably reflect altered development of MHC class II-restricted thymocytes, as the knockin allele should be expressed only in the former cells, like endogenous ThPOK.

We have previously reported that ThPOK is also required for development of CD4 Vα14+ iNKT cells, which are replaced by unusual CD8+ iNKT cells. In contrast, while LKI/LKI mice also fail to generate normal numbers of CD4 iNKT cells, they show elevated numbers of DN rather than SP CD8 iNKT cells. This suggests that LRF can compensate partly for ThPOK in iNKT development in preventing CD8 upmodulation, but not in promoting CD4 upmodulation.

We tentatively conclude that LRF cannot substitute fully for ThPOK in development of either conventional CD4 T cells or iNKT cells, and therefore that the 2 factors must possess distinct functions. We have not yet had the opportunity to analyze thymic development in these mice, since LKI/LKI homozygotes are presently too valuable for breeding to sacrifice.

Research Project 4: Project Title and Purpose

Characterization of Novel Epigenetic Factors – Epigenetics describes the process through which specific cell types are programmed from a single DNA blueprint. This is accomplished through chromatin modifications that direct activation or silencing of specific gene sets. Epigenetic factors that guide and place these modifications can thereby control cellular identities, e.g., differentiated, pluripotent, aged, and cancer. We used an unbiased functional screen to identify novel human factors that control epigenetic processes. The purpose of this research is to characterize these novel epigenetic factors and their networks. This work will contribute to our understanding of development, epigenetic diseases, and normal aging.

Duration of Project

1/1/2012 – 6/30/2014

Project Overview

The objective of this research is to characterize novel factors and interactions that control epigenetic gene silencing. The factors were revealed through a cell-based, unbiased genome-wide siRNA screen that has served as a hypothesis-generating tool. We thereby have identified novel epigenetic factors and networks that may contribute to development, human diseases and normal aging.

Aim 1. To characterize novel human proteins that were identified in a genome-wide functional screen for factors that control epigenetic gene silencing.

We will determine the detailed role of the previously uncharacterized human protein "Mammalian Genome Collection A" (MGCA). Our preliminary studies have shown that MGCA localizes to the nuclear lamina. Knockdown of MGCA interrupts epigenetic gene silencing, and produces nuclear distortions similar to that observed in human nuclear lamina diseases. We hypothesize that MGCA functions to bridge the nuclear lamina and silent chromatin. We will use a variety of standard molecular methods to determine the detailed localization of MGCA, identify functional domains and binding partners, and investigate its precise role in epigenetic gene silencing. Characterization of additional novel human proteins will be initiated.

Aim 2. To determine the role of sumoylation, and SUMO (small ubiquitin-like modifier) enzyme networks in global epigenetic gene silencing.

We will investigate a newly discovered and prominent role for the SUMO pathway in maintaining global epigenetic gene silencing in human cells. We will investigate sumoylation of

histones as a critical regulatory modification for gene silencing, and examine an unexpected role for a histone deacetylase in maintaining levels of a key SUMO pathway enzyme, UBC9.

Principal Investigator

Richard A. Katz, PhD
Research Professor
The Institute for Cancer Research
333 Cottman Avenue
Philadelphia, PA 19111

Other Participating Researchers

Andrey Poleshko, PhD – employed by The Institute for Cancer Research

Expected Research Outcomes and Benefits

Epigenetics describes the chromatin-based control of gene expression that programs development and establishes cellular identities. Errors in placement of epigenetic modifications on chromatin can lead to numerous diseases, including cancer. We are just beginning to understand how the epigenetic factors that place chromatin modifications can be manipulated to influence cellular identities, for both disease therapy and tissue engineering. It is therefore essential to develop a comprehensive view of cell-wide factor networks that control epigenetic processes. As nearly forty percent of human genes remain uncharacterized, we devised a functional screen with the aim of identifying novel human epigenetic factors.

Our screen identified over twenty candidate novel epigenetic factors, as well as known factors. We found that one novel factor, denoted here as MGCA, associates with the nuclear lamina, the meshwork of proteins that supports nuclear shape and anchors silent chromatin. Mutations in nuclear lamina and associated proteins are linked to muscular dystrophies, a premature aging syndrome, and cancer. Our preliminary results demonstrate that depletion of MGCA causes defects in epigenetic control, nuclear fragility, and disruption in nuclear positioning, as seen in nuclear lamina diseases.

This research is expected to reveal molecular details of how known and novel factors function in epigenetic control: their localization within the cell, their functional networks, and their potential disease associations. Epigenetic modifications are largely reversible, and can be manipulated for both disease therapy and tissue engineering. This research will improve health status by revealing the functions of novel and known factors that participate in epigenetic processes.

Summary of Research Completed

Our work has focused on Aim 1 of the proposal, to determine the detailed role of the previously uncharacterized human protein initially denoted "Mammalian Genome Collection A" (MGCA), now denoted by the gene name Proline Rich 14 (PRR14). Our preliminary studies had shown

that the PRR14 protein localizes to the nuclear lamina and that knockdown of PRR14 interrupts epigenetic gene silencing and produces nuclear distortions similar to that observed in human nuclear lamina diseases and cancer. We have now shown that PRR14 functions to attach heterochromatin to the nuclear lamina. Some of the work has been published:

Poleshko, A., Mansfield, K.M., Burlingame, C.C., Andrake, M.D., Shah, N.R. Katz, R.A. The human protein PRR14 tethers heterochromatin to the nuclear lamina during interphase and mitotic exit. *Cell Rep*. 5:292–301, 2013.

Poleshko, A., Katz, R.A. Specifying peripheral heterochromatin during nuclear lamina reassembly. *Nucleus* 5:32–39, 2014.

A conserved organizational feature of eukaryotic nuclei is the peripheral heterochromatin compartment, which provides a protected area for epigenetically silent genes and gene-poor DNA (Figure 1). In metazoan cells this compartment is associated with the nuclear lamina, the protein meshwork at the inner edge of the nucleus. Heterochromatin-nuclear lamina interactions promote epigenetic gene silencing. Important biological roles for the nuclear lamina as a docking site for peripheral heterochromatin continue to emerge within many normal and disease processes, including development, cancer, aging, and cell senescence. In particular, it will be important to explore nuclear lamina-heterochromatin "tethering" mechanisms in normal and diseased cells, as differential interactions may affect gene expression and thereby underlie human disease. In view of the broad biological impact of nuclear lamina-heterochromatin attachment, it is critical to obtain a comprehensive view of the attachment mechanisms. Below we provide a summary of work carried out within this reporting period that has addressed the mechanisms of PRR14-mediated heterochromatin tethering.

During this reporting period, we have developed quantitative imaging methods, implemented software for 3D-rendering of PRR14 during mitosis, and also carried out fine mapping studies relevant to PRR14-nuclear lamina interactions. We have now determined that a 1-272 fragment of PRR14 is sufficient to bind to the nuclear lamina. We also developed several stable cell lines expressing wt and mutant PRR14. Such cell lines allow time lapse imaging during mitosis. Among the lines is a stable RFP-PRR14 fusion cell line. This has allowed us to co-image two forms of PRR14 in the same cell. We have generally found that wild type and mutant forms of PRR14 do not co-localize. For example, wt RFP-PRR14 localizes to the nuclear lamina, while the GFP-1-225 fragment localizes to the nucleoplasm. These results suggest that PRR14 does not multimerize.

We also investigated a putative PRR14 Aurora B kinase site (T363). This site was of particular interest in that the binding partner of PRR14, HP1, is displaced from chromatin in early mitosis by Aurora B kinase phosphorylation of histone H3 serine 10. Also, a PRR14 phosphopeptide corresponding to this site was present in phosphopeptide databases. Substitution of PRR14 T363 with either alanine or phospho-mimic residues, had no overt effect on localization of PRR14. To determine whether there may be more subtle effects of these substitutions during mitotic disassembly and reassembly of PRR14 (Figure 2), we co-expressed substituted T363-substituted GFP-PRR14 along with wt RFP-PRR14. These experiments are still in progress.

During this reporting period we also began testing a new model in which PRR14 functions to guide reassembly of the nuclear lamina. We have reported that PRR14 knockdown results in nuclear lamina distortions, suggesting that PRR14 plays a role in the nuclear lamina structure. Our recent finding is that after PRR14 knockdown, specific defects in the assembly of Lamin A/C are observed in telophase. To monitor these defects we implemented the 3D-rendering methods mentioned above. The overall model will continue to be tested.

A major accomplishment during this reporting period was the publication of two papers. Some the work performed during this period was in response to journal reviewers' comments. In particular we added new experiments which featured image quantitation. Much of the work that contributed to the publications was performed during this reporting period. As such, we provide an overall summary of the published work:

The nuclear lamina is a meshwork composed of intermediate filament proteins, the A- and B-type lamins: Lamin A/C, Lamin B1, and Lamin B2. The PRR14 N-terminus binds to Heterochromatin Protein 1 (HP1), which is in turn bound to H3K9me3-modifed heterochromatin. The bivalent tethering function of PRR14 is facilitated by a second domain that binds to the Lamin A/C component of the nuclear lamina (Figures 1 and 2, and see below). Furthermore, we found that PRR14 disassembles and reassembles in a specific manner during mitosis, first binding to heterochromatin in anaphase, before reattaching to the assembling nuclear lamina (Figure 2). Our model predicts that PRR14 thereby provides a mechanism to specify heterochromatin for reattachment at the nuclear lamina. In order to study the dynamics of PRR14 during mitosis, we processed confocal images to produce 3D-renderings. This has allowed us to study specific defects in nuclear lamina-heterochromatin reassembly after PRR14 siRNA knockdown.

We obtained a detailed map of PRR14 functional domains. Using confocal imaging, we found that the PRR14 N-terminus binds to heterochromatin and central region is required for nuclear lamina association. The binding of the PRR14 to HP1 was demonstrated by mutating the HP1 consensus binding sequence at the PRR14 N-terminus. The V54E,V55E mutant in the context of the N-terminal 1-135 fragment fails to localize to heterochromatin. To further confirm that the 1-135 fragment was binding to HP1/heterochromatin, an HP1 siRNA knockdown experiment was carried out. Knockdown of HP1 resulted in the release of the N-terminal 1-135 fragment from heterochromatin. Fine mapping studies on the nuclear lamina binding site of PRR14 have also been carried out.

To confirm the binding of full length PRR14 to heterochromatin, we designed the following experiment. Full length PRR14 localizes to the nuclear lamina during interphase and attaches to neighboring peripheral heterochromatin through HP1 (Figures 1 and 2). The nuclear lamina is disassembled during early mitosis, releasing PRR14. PRR14 is also released from HP1/heterochromatin. HP1 is known to be displaced from heterochromatin early in mitosis through the activity of Aurora B kinase. It was therefore predicted that inhibition of Aurora B would result in heterochromatin retention of both HP1 and PRR14. Treatment with the Aurora B kinase inhibitor ZM447439 indeed resulted in retention of HP1 on chromatin in early mitosis, and PRR14 colocalized with HP1. To ask whether such localization of PRR14 was HP1-

dependent, siRNA knockdown of HP1 isoforms was carried out in conjunction with inhibitor treatment. Knockdown of all three HP1 isoforms resulted in loss of PRR14 heterochromatin binding.

To further address how PRR14 localizes to the nuclear lamina in interphase, siRNA knockdown of individual nuclear lamina components was carried out, and the effect on localization of GFP-tagged PRR14 was monitored by confocal microscopy. SiRNA knockdown of Lamin A/C promoted release of PRR14 from the nuclear periphery.

Having established that PRR14 attaches to both HP1-marked heterochromatin and the nuclear lamina, the proposed tethering function was tested in detail. We designed experiments that addressed the comparative roles of Lamin A/C, LBR (another proposed heterochromatin tether), and PRR14 in maintaining the peripheral heterochromatin compartment. These three proteins were knocked down using siRNA, and the effects on positioning of H3K9me3-marked peripheral heterochromatin were measured using image analysis. PRR14 knockdown uniquely promoted release of heterochromatin from the nuclear periphery. Only combined knockdown of LBR and LaminA/C produced a similar effect.

In summary, during the funding period we performed detailed mechanistic studies that have provided new and important insights into the organization of chromatin in human cells.

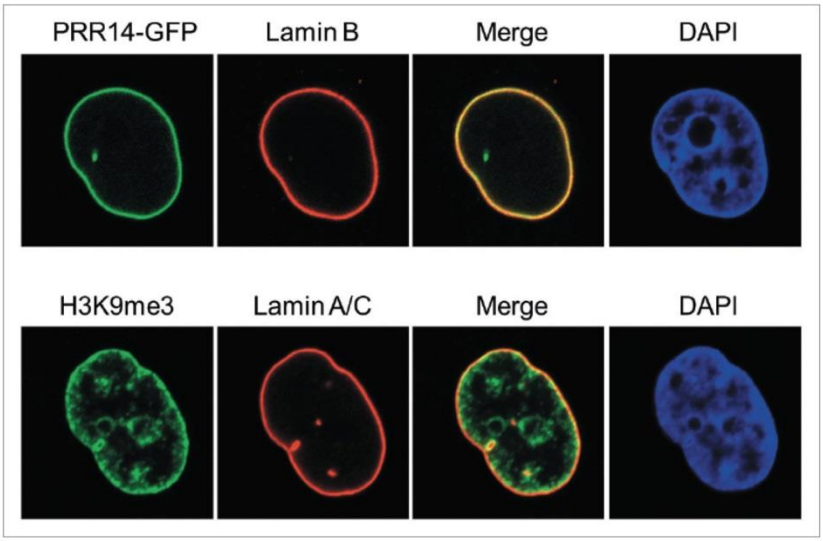

Figure 1. Localization of PRR14 in association with H3K9me3-marked heterochromatin at the nuclear periphery.

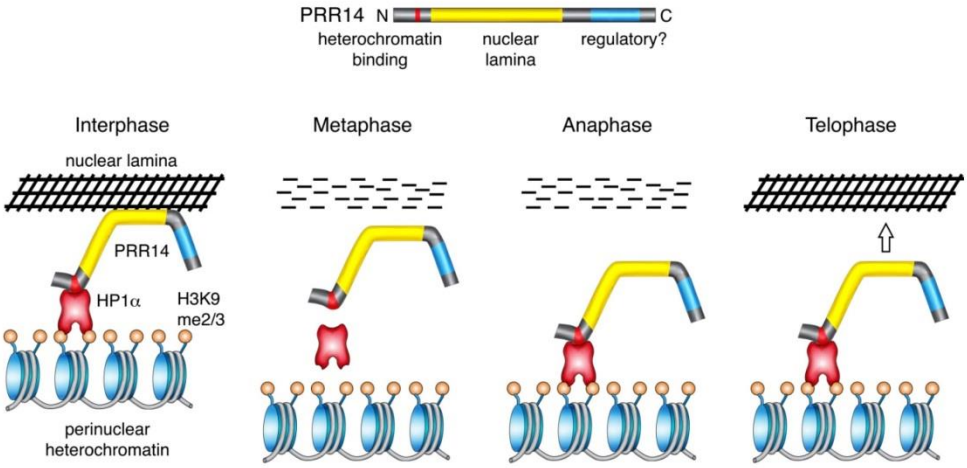

Figure 2. Model for role of PRR14 in the tethering of heterochromatin at the nuclear lamina. Depicted are PRR14-mediated attachment of HP1-H3k9me3-marked heterochromatin to the nuclear lamina, and the disassembly and reassembly steps that occur during mitosis.

Research Project 5: Project Title and Purpose

Role of Lin28B in the Enhanced Cancer Susceptibility Caused by Inactivation of Rpl22 – The purpose of this project is to investigate the basis for induction of the stemness factor, Lin28B, by inactivation of Rpl22 and to determine how Lin28B induction increases the susceptibility of Rpl22 mutant cells to malignant transformation.

Duration of Project

1/1/2012 – 06/30/2013

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at http://www.health.state.pa.us/cure.

Research Project 6: Project Title and Purpose

Chemosensitization of Cancer Cells by Inhibition of a Transcription Network – Tumor recurrence is a major obstacle to effective cancer therapies. This is of the utmost urgency for cancers that escape early detection because their response rate is usually poor by the time of diagnosis. We conducted a genome-wide siRNA screen of pancreatic cancer cells and identified a transcription network that was critical for survival to gemcitabine. We hypothesize that this transcription network was usurped during tumorogenesis to allow cancer cells to survive intrinsic and extrinsic stresses. This project will test: 1) if these transcription factors are also important for survival to other chemotherapeutics besides gemcitabine, and 2) if this pathway is used in other cancers such as ovary, lung and colorectal.

Duration of Project

1/1/2012 – 6/30/2014

Project Overview

The broad objectives of this research are to identify mechanisms that allow cancer cells to adapt and survive chemo and radiotherapies. This work is of significance because tumor recurrence after drug treatment remains a major obstacle to the long-term survival of cancer patients. Studying the mechanisms that cancer cells use to survive exposure to cytotoxic agents is critical to the development of strategies to enhance chemo-sensitization, and reduce the incidences of tumor recurrence. Our approach was to conduct a synthetic lethal screen using a genome-wide siRNA library. We chose pancreatic cancer for our studies because it is the 4th deadliest of all cancers in the US. Although gemcitabine is the drug of choice for pancreatic cancer patients, this drug only extends survival by a few months relative to other chemotherapies.

Our screen was conducted in the Panc 1 cell line, and candidate genes from our screen were validated in secondary screens using an independent set of si- or shRNA, as well as an additional cell line, BXPC3. Two of the genes encode transcription factors that are essential for the differentiation of specific tissues, but are not known to be involved in drug sensitization. Another two genes encode DNA damage response proteins, Chk1 (Checkpoint kinase 1) and 53BP1 (p53 tumor suppressor binding protein 1) that may be targets of these transcription factors. As the two transcription factors can form heterodimers, we hypothesize that they regulate a network of survival genes that cancer cells use to overcome the effects of DNA damage induced drugs such as gemcitabine. We propose the following aims to test our hypothesis:

1) Test drug sensitivities of a panel of cell lines derived from different origins of cancer to after inactivating this specific transcriptional network,
2) Test if sensitization is achieved with other genotoxic agents besides gemcitabine, and
3) Compare and identify critical downstream target genes to characterize the mechanism of drug sensitization.

Principal Investigator

Timothy J. Yen, PhD
Professor
The Fox Chase Cancer Center
333 Cottman Avenue
Philadelphia, PA 19111

Other Participating Researchers

Neil Beeharry, PhD – employed by The Fox Chase Cancer Center

Expected Research Outcomes and Benefits

Tumorogenesis may be seen as an evolutionary process whereby the cancer cell undergoes changes that uncouple proliferation from growth control. Because unrestrained growth exposes the cell to extrinsic and intrinsic stresses, the cancer cell must be able to rapidly adapt to many different adverse growth conditions. As part of the tumorogenesis process, the cell usurps existing pathways and re-tasks them to pro-survival and anti-apoptosis functions. The number of ways this occurs is not unlimited, and the ultimate targets that specify cell survival are likely to be conserved. We have identified a transcription network common in two pancreatic cancer cell lines that allows them to survive gemcitabine treatment. We predict that this transcriptional network and/or its target genes is not unique to pancreatic cancer and that it provides cytoprotective functions to many different types of cancer cells.

We have chosen to test if ovarian, lung and colorectal cancer cell lines rely on this transcriptional network for survival to chemotherapeutic agents. Although we expect that some will respond positively, we anticipate some non-responders as well. These non-responders will nevertheless provide valuable information when we compare their transcriptomes to that of the responders.

Differences in their patterns of gene expression will yield information about the molecular signatures that define responders and non-responders.

Our studies are translational in two ways. First, our studies provide immediate opportunities for clinical trials that combine existing inhibitors of these transcription factors with conventional chemotherapies. Second, results from our microarray analysis can be used to profile patient tumors to predict their response to the combination therapy.

Summary of Research Completed

Progress on Aim 1

We completed our studies of our screen to re-purpose clinically relevant kinase inhibitors as novel chemosensitizers. We determined that Bosutinib and its isomer (Bos-I) enhanced gemcitabine killing of pancreatic cancer cell lines in vitro and determined the concentration of drug that sensitized cells to killing at a comparable level as known inhibitors of Chk1, such as UCN-01. We submitted this as part of a manuscript, which required additional experiments as suggested by reviewers. These experiments included demonstrating that sensitization by Bos-I was also observed with other genotoxic drugs. Consistent with the observation for gemcitabine, Bos-I enhanced the cytotoxic effects of both cisplatin and doxorubicin. Sensitization was specific for DNA damaging drugs as no sensitization was observed with paclitaxel, which targets microtubules. We also tested the response of other cell lines such as MiaPaCa2 pancreatic adenocarcinoma cells and untransformed RPE cells. MiaPaCa2 cells treated with either gemcitabine or doxorubicin were more sensitive in the presence of Bos-I as compared to vehicle. Interestingly, normal RPE1 cells were not killed by these treatments suggesting that chemosensitization maybe preferentially achieved in tumor cells.

Progress on Aim 2

The characterization of the mechanism by which Bos-I enhances gemcitabine killing was completed. We showed by time-lapse, flow cytometry and immunofluorescence staining that Bosutinib isomer abrogated the DNA damage checkpoint and forced cells with incompletely replicated DNA into a catastrophic mitosis. In response to reviewers' comments, we next directly confirmed that Bosutinib and Bos-I dually inhibited Chk1 and Wee1, two kinases critical for the DNA damage checkpoint pathway. Furthermore, we showed by using phosphoH2AX as a pharmacodynamic marker that these drugs targeted these kinases in cells. When cells are treated with gemcitabine, phosphoH2AX staining increases. However, staining intensity further increased by 3-fold if the cells are also depleted of Wee1 (Fig.1). Treatment with Bos-I further increased staining. The Bos-I induced phosphoH2AX staining can be suppressed by overexpressing Wee1-wild type (Fig.1).

To understand the molecular basis for the differences in the specificity of bosutinib and Bos-I for Chk1 and Wee1, molecular models were constructed to visualize the interactions between the inhibitors and the ATP binding pocket. Structurally, the 2 compounds only differ in the positioning of the Cl and methoxy atoms on the aniline ring (Fig. 2), suggesting that the spatial position of these groups dictate their interaction within the ATP binding pocket. It is well established that the gatekeeper residue of kinases can dictate the ability for inhibitors to bind. Our molecular models showed that the methoxy group of authentic bosutinib (position 5) may

sterically clash with gatekeeper residue in Wee1 (Asparagine, N376) and possibly reduce its efficacy. By contrast, the methoxy group in Bos-I (position 3) is positioned away from the gatekeeper (Fig. 3). This suggests that the spatial relationship between the gatekeeper residue and the methoxy group in bosutinib and Bos-I may dictate the ability of the inhibitors to bind and thereby inhibit. These observations were supported by relative binding energy calculations derived from our models of bosutinib and Bos-I bound to Chk1 and Wee1. Consistent with cellular and biochemical data presented above, the calculations indicated that Bos-I had a stronger preference for both Wee1 and Chk1 compared with bosutinib.

To explore the importance of the gatekeeper residue in determining the sensitivity to bosutinib or Bos-I binding, we used the aforementioned kinase inhibitor database to identify kinases that are preferentially inhibited (>50%) by Bos-I or bosutinib and then compared the gatekeeper residues. For Bos-I, the gatekeeper residues were predominantly methionine, or valine, while similar analysis of bosutinib sensitive kinases identified predominantly methionine, threonine or phenylalanine as gatekeeper residues. This analysis showed that the gatekeeper residues of bosutinib-sensitive kinases were enriched for threonine or phenylalanine and provided us with an opportunity to test whether these gatekeeper residues might dictate selectivity for bosutinib over Bos-I. We performed in vitro kinase assays using recombinant Wee1 wild-type (WT) as well as gatekeeper mutants N376F and N376T and tested the inhibitory activity of bosutinib and Bos-I using phosphorylation of Cdc2, its physiological substrate, as a read out. We observed that phosphorylation of $Cdc2^{Y15}$ by Wee1 WT was potently inhibited by Bos-I, while approximately 4-fold more bosutinib was needed to achieve similar inhibition (IC_{50} values: Bosutinib 6.9 µM vs. Bos-I 1.8 µM). Importantly, both the N376T and the N376F mutant exhibited increased sensitivity to bosutinib as its IC_{50} was reduced to 1.9 µM and 0.9 µM, respectively. The sensitivity of the N376T mutant to Bos-I was unchanged (Bos-I Wee1 WT 1.8 µM vs. N376T 1.6 µM), while the N376F mutant was now less sensitive to Bos-I (IC_{50}: WT 1.8 µM vs. N376F 8.3 µM). These findings demonstrate that while the N376T mutant improves sensitivity to bosutinib, the sensitivity to the N376F mutant toward the inhibitors is flipped, compared with the Wee1 WT. We next generated molecular models of both inhibitors bound to the mutants. For the N376F mutant, the orientation of the benzyl group of the phenylalanine differed depending on which inhibitor was bound. When bosutinib was bound, the benzyl group was rotated away from the 5-methoxy group leading to reduced steric hindrance. In contrast, when Bos-I was bound, the benzyl group of N376F was orientated such that it was proximal to the 4-methoxy group. This orientation would generate a steric clash between the benzyl group and the 4-methoxy group that could explain the resultant decrease in ability to inhibit Wee1. In performing similar analysis of the 376T mutant (Fig. 3), we were unable to identify structural differences that may account for why Bos-I is slightly more effective compared with the WT Wee1, nor for the dramatic increase in effectiveness of bosutinib as compared with WT Wee1. Using a combination of in vitro kinase reactions and modeling studies, we provide evidence that both inhibitors can bind Wee1, albeit to slightly different abilities. Furthermore, the gatekeeper residue appears to be an important, but unlikely to be the sole, determinant for sensitivity.

Progress on Aim 3

We completed our xenograft studies that used patient derived pancreatic tumor cells. In response to reviewers' comments we had to repeat the xenograft experiment to verify that we could reproduce the response from the first set of experiments. In addition, we conducted

immunohistochemical studies to demonstrate efficacy of the drug combinations. As we demonstrated in our previous report, the new xenograft data showed that tumors responded to either gemcitabine or Bos-I alone for 17 days. However, tumors resumed growth after 17d and no longer responded to single drug treatment. By contrast, tumors treated with gemcitabine + Bos-I failed to grow up until the third week when the experiment was stopped.

To explore whether the mechanism of action for sensitization in vivo was similar to that in vitro, we used the phamacodynamic assay as employed above to measure γH2AX. In animals treated with gemcitabine alone there was an increase in γH2AX in the tumor cells compared with tumor cells from vehicle treated animals ($P < 0.01$). Moreover, γH2AX levels were highest in the tumor cells from animals treated with the combination of gemcitabine and Bos-I ($P < 0.0001$). We also observed that the mitotic index was reduced in tumors from animals treated with gemcitabine as compared with vehicle treated animals (vehicle 2.2 ± 0.7% of mitotic cells vs. gem-treated 0.4 ± 0.4% of mitotic cells; $P = 0.03$). However, in tumors from animals treated with the combination of gemcitabine and Bos-I, the mitotic index was increased compared with animals treated with gemcitabine alone (gem-treated 0.4 ± 0.4% of mitotic cells vs. gem+Bos-I treated 2.0 ± 0.6% of mitotic cells; $P = 0.05$), consistent with the in vitro data. Furthermore, the extent of DNA damage as assessed by γ-H2AX was approximately 2-fold higher in mitotic tumor cells in animals treated with gemcitabine and Bos-I compared with animals treated with vehicle alone. These findings show that Bos-I can enhance inhibition of tumor growth by gemcitabine in vivo.

Finally, we tested Bosutinib (at the same concentration as Bos-I) and found that it suppressed tumor growth as a single agent but tumor growth resumed after 17d. However, the combination of Bosutinib and gemcitabine did not suppress tumor growth as effectively as Bos-I+ gemcitabine, consistent with the weaker chemosensitization activity of Bosutinib.

Our work described in Aims 1-3 is now in press:
Beeharry et al. Re-purposing clinical kinase inhibitors to enhance chemosensitivity by overriding checkpoints. Cell Cycle. 2014 Jun 23;13(14). [Epub ahead of print] PMID: 24955955.

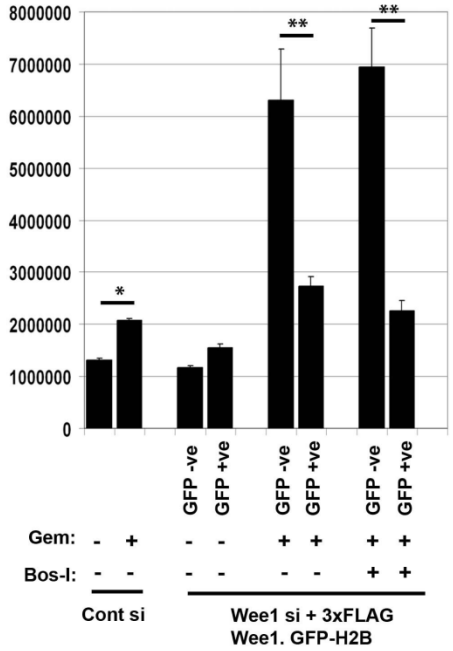

Fig.1. Wee1 is a target of Bos-I in cells. Panc1 cells were transfected with the indicated constructs, treated with Bos-I +/- gemcitabine and fixed and stained for pH2AX foci. The Wee1 expression construct also contained a gfpH2B that allowed identification of positively tranfected cells. Comparison of foci (Y axis) show that cells transfected with Wee1 (and thus gfpH2B positive) suppressed pH2AX foci that are induced by Gem +/- Bos-I.

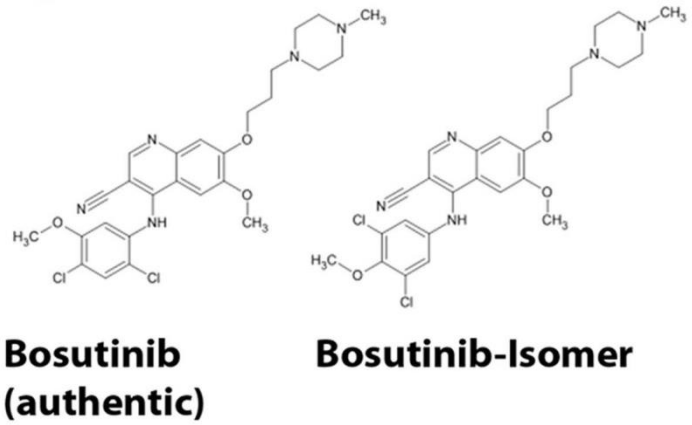

Fig.2. Comparison of the structures of Bosutinib and Bosutinib isomer. The aniline ring is at the bottom left of each structure. Note the differences in the positions of the –OCH3 methoxy and Cl between the structures.

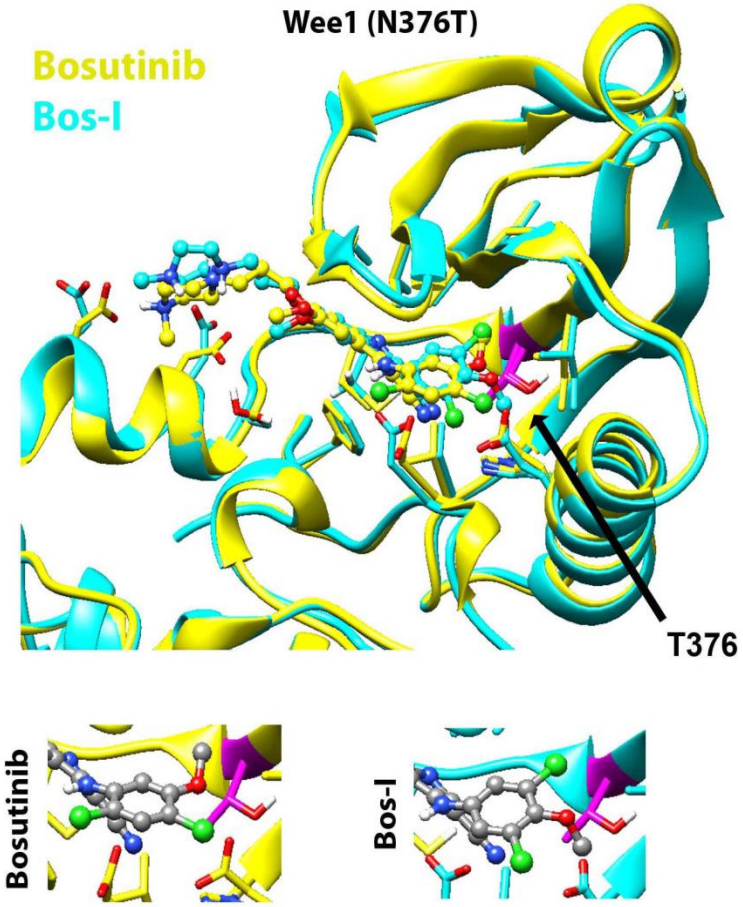

Fig.3. The gatekeeper residue in Wee1 contributes to inhibitor specificity. (**A**) Ribbon diagrams of published structures of Wee1 (PDB code 3BI6) bound with the inhibitors bosutinib or Bos-I which are superimposed (main). Insets show enlarged view of the pocket bound to their inhibitors (gray backbone). The gatekeeper residue T376 is shown in pink (black arrow). Note the steric clash of the Cl (Green) in Bosutinib with the gatekeeper.