Fox Chase Cancer Center

Annual Progress Report: 2009 Formula Grant

Reporting Period

July 1, 2010 – June 30, 2011

Formula Grant Overview

The Fox Chase Cancer Center received $3,477,323 in formula funds for the grant award period January 1, 2010 through December 31, 2012. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Regulation of Genomic and Epigenomic Stability at CpG Sites - The purpose of this project is to understand how two base excision repair enzymes, thymine DNA glycosylase (TDG) and methyl-CpG-binding endonuclease 1(MED1)/ methyl binding domain 4 (MBD4), not only protect CpG sequences from transition mutations and ward off against endogenous deamination events, but also mediate DNA demethylation and modulate DNA methylation states, chromatin structure and gene transcription. This dual role in genomic and epigenomic stability of CpG sites is likely to be important for cancer formation, given the frequent occurrence of CpG to CpA (or TpG) point mutations and hypermethylation of tumor suppressor genes in cancer. Thus, knowledge accumulated in this project may lead to novel strategies for cancer prevention.

Anticipated Duration of Project

1/1/2010 - 12/31/2012

Project Overview

The conversion of cytosine into 5-methylcytosine (DNA methylation) is an important epigenetic modification of the mammalian genome that is potentially mutagenic. In fact, 5-methylcytosine has a tendency to spontaneously deaminate, generating thymine, which, if not removed from the G:T mismatch prior to replication, will lead to incorporation of adenine. Indeed, deamination at CpG sites is estimated to cause nearly one-third of all mutations in both cancer and human genetic diseases. Work conducted by us and others indicate that in order to maintain genomic stability at CpG sites, two base excision repair enzymes remove the offending thymine with their DNA *N*-glycosylase activity, MED1 (previously identified by us and also known as MBD4) and TDG.

DNA methyltransferases are known to initiate DNA methylation, whereas it is less clear how the removal of the methylation, or *demethylation*, takes place. Evidence in Arabidopsis and Zebrafish points to the role of the DNA repair machinery in affecting demethylation, either by

direct removal of 5-methylcytosine, or by its enzymatic conversion by deaminases into thymine, which is then removed from the resulting G:T mismatch. However, it is still largely unknown how mammalian cells demethylate the DNA and what functional consequences demethylation has in mammals. Given the ample remodeling of methylation patterns at CpG sites during development, and the hypermethylation of tumor suppressor genes and other CpG islands in human cancer, it is expected that a defect in demethylating activities may lead to a situation of *epigenomic instability* and contribute to developmental alterations and tumor formation.

The overall goal of this project is to test the hypothesis that the mammalian DNA repair enzymes TDG and MED1/MBD4 have dual functions in protection from tumorigenesis and developmental disorders: 1) Promote genomic stability at CpG sites by suppressing mutations; and 2) Maintain proper DNA methylation patterns by regulating CpG demethylation.

Additional funding was secured from NIH during the first year of this grant to support the work outlined below under aims 1 and 2. This project has been expanded to logically continue the research through the addition of aims 3, 4 and 5. Therefore, the focus of the remainder of this project will be to analyze the effect of a modification we have recently identified in MED1, sumoylation, on MED1 DNA damage response and repair capacity; to characterize the structural and functional properties of the AID-GADD45a-TDG demethylating complex, that we have recently described in mammalian cells; and to examine the effects of double inactivation of MED1 and TDG.

Thus, to test the general hypothesis outlined above, we now propose five Specific Aims:
Aim 1: Characterize the requirement of TDG during development and its role in DNA demethylation and chromatin modification, using knock-out and knock-in mice;
Aim 2: Evaluate the role of MED1 and TDG in tumorigenesis using animal models of intestinal cancer and analyzing human cancer specimens.
Aim 3: Analysis of MED1 sumoylation in DNA damage response and repair;
Aim 4: Characterization of the structure and function of the TDG demethylating complex;
Aim 5: Characterization of the phenotype of TDG-MED1-double mutant embryos.

Principal Investigators

Alfonso Bellacosa, MD, PhD
Associate Professor
The Fox Chase Cancer Center
333 Cottman Avenue
Philadelphia, PA 19111-2434

Other Participating Researchers

Salvatore Cortellino, PhD - employed by The Fox Chase Cancer Center

Expected Research Outcomes and Benefits

By evaluating the contribution of pathways of base excision repair (via MED1/MBD4 and TDG)

to the maintenance of CpG site integrity, we expect to obtain important information of relevance to several related issues. We anticipate that our studies will uncover the mechanisms by which endogenous mutagenesis by deamination is counteracted in mammalian cells, and will have immediate implications for cancer biology. Indeed, failure of these repair systems and acquisition of a mutator phenotype at CpG sites or loss of the epigenetic control afforded by CpG methylation may be critical for cancer formation. In the future, such knowledge might suggest innovative cancer prevention or therapeutic strategies.

Summary of Research Completed

In the timeframe 7/1/2010-6/30/2011, we made significant progress in the experiments related to Specific Aims 1 and 2. In fact, since Specific Aims 3-5 were added only in May 2011, upon discussion with Commonwealth grant officers, this report focuses on research completed for Aims 1 and 2.

Specific Aim 1 involves the characterization of the requirement of TDG during development, with an emphasis on determining whether TDG catalytic activity is required for development and DNA demethylation. This Aim is centered on the characterization of the knock-in TDG^{N151A} strain that we generated, bearing an inactivating point mutation in the glycosylase active site.

We had previously crossed $TDG^{N151A/+}$ mice with $TDG^{+/-}$, i.e. heterozygous mice bearing a null allele of TDG. No live pups with the $TDG^{N151A/-}$ genotype were obtained, suggesting that this genotype combination results in embryonic lethality. In the timeframe of the present report, we then conducted the definitive experiment by intercrossing $TDG^{N151A/+}$ heterozygotes. From these crosses, we could not detect livebirth pups of $TDG^{N151A/N151A}$ genotype and timed matings revealed that embryos with the $TDG^{N151A/N151A}$ genotype, similar to embryos with the $TDG^{-/-}$ genotype, die at midgestation. In fact, lethality occurs earlier in knock-in $TDG^{N151A/N151A}$ embryos (at approximately embryonic day E10.5) than in knock-out $TDG^{-/-}$ embryos (at approximately E11.5). These observations suggest that TDG catalytic activity is required for normal development, possibly through a direct role in DNA demethylation.

To further assess this possibility, we studied demethylation of the Tyrosine Amino Transferase (TAT) enhancer, which occurs at midgestation and is caused by the prenatal peak of glucocorticoids. We found that DNA demethylation of this enhancer was abrogated in *Tdg*$^{N151A/N151A}$ embryos. We conclude that the catalytic activity of TDG is essential for development and DNA demethylation. This conclusion confirms findings described in the previous report indicating a requirement of TDG for active demethylation of the Oct4 pluripotency gene promoter.

In order to clarify the mechanisms of active DNA demethylation, we characterized the complex of TDG-associated proteins. We found that in transfected HEK-293 cells TDG co-immunoprecipitate with the Activation-induced deaminase AID and the genome stability protein GADD45a, also involved in DNA demethylation. In addition, the TDG-AID and AID-GADD45a co-immunoprecipitations also take place at endogenous levels of expression of these proteins, in the developmentally relevant context of embryonic carcinoma P19 cells. In fact, shRNA-mediated down-regulation of TDG in P19 cells leads to reduction of AID expression,

which suggests that TDG may regulate the levels and/or the stability of AID. Thus, the TDG-AID-GADD45a interaction occurs *in vivo* and affects AID levels.

Recently, it has been proposed that 5-hydroxymethylcytosine, the hydroxylation product of 5-methylcytosine, is an intermediate in active demethylation. Specifically, it has been suggested that during active DNA demethylation in mammalian cells, AID/Apobec deaminases convert 5-hydroxymethylcytosine to 5-hydroxymethyluracil for subsequent processing by base excision repair. We found that TDG does not process 5-hydroxymethylcytosine, but has a robust glycosylase activity on a 5-hydroxymethyluracil:G mismatch in double-strand DNA, the expected product of deamination of 5-hydroxymethylcytosine.

Taken together, these findings suggest a two-step mechanism for DNA demethylation in mammals, whereby 5-methylcytosine and 5-hydroxymethylcytosine are first deaminated by AID and/or other deaminases to thymine and 5-hydroxymethyluracil, respectively, followed by TDG-mediated thymine and 5-hydroxymethyluracil excision repair.

These observations pertaining to Specific Aim 1 are described in our manuscript that has been recently published (Cortellino, S., Xu, J., Sannai, M., Moore, R., Caretti, E., Cigliano, A., Le Coz, M., Devarajan, K., Wessels, A., Soprano, D., Abramowitz, L. K., Bartolomei, M.S.; Rambow, F., Bassi, M.R., Bruno, T., Fanciulli, M., Renner, C., Klein-Szanto, A.J., Matsumoto, Y., Kobi, D., Davidson, I., Alberti, C., Larue, L. and Bellacosa, A. Thymine DNA Glycosylase Is Essential for Active DNA Demethylation by Linked Deamination-Base Excision Repair. *Cell*, 146, 67-79, 2011).

Specific Aim 2 involves a characterization of the role of MED1 and TDG in tumorigenesis. We previously reported that TDG protein levels are low to undetectable in human colorectal and melanoma cell lines. We therefore focused on the molecular mechanisms of reduced TDG expression. Our preliminary experiments suggest that the detected downregulation of TDG may be mediated by microRNAs; specifically, reduced levels of TDG in melanoma samples were associated with overexpression of a small microRNA cluster predicted to bind to the TDG mRNA. In preliminary experiments we found that exogenous expression of these microRNAs leads to TDG downregulation in cell lines that normally express normal levels of TDG.

Research Project 2: Project Title and Purpose

Estrogen Receptor-Mediated Gene Silencing in Tumorigenesis - The role of estrogen in contributing to women's cancers, especially those of the breast and uterus, is well established at the epidemiologic level. The molecular mechanism through which inappropriate timing and/or excessive levels of estrogen exposure initiate or promote human tumorigenesis remains largely unknown, however. The purpose of this study is to test the hypothesis that the activated estrogen receptor, which functions as a transcription factor, is also capable of silencing genes, through promoter hypermethylation, that normally function to suppress tumorigenesis in human cells subject to regulation by estrogen. In addition, we will explore the molecular mechanism of this methylation-dependent gene silencing.

Anticipated Duration of Project

1/1/2010 - 12/31/2011

Project Overview

The broad objective of this project is to test the hypothesis that the activated estrogen receptor silences the expression of critical tumor suppressor genes, and further, to test whether this methylation-mediated gene silencing involves the recruitment of DNA methyl-transferases (DNMTs) and/or histone deacetylase (HDAC) proteins to the promoter regions of target genes. A substantial body of preliminary data has been generated to support these hypotheses. These objectives will be tested through five specific aims. Aim one will be to confirm that treatment of estrogen receptor (ER)-positive human breast cancer cells with estrogen results in the down-regulation of expression of the genes encoding lipocalin 2 (LCN2) and corticotropin releasing hormone (CRH). Aim two will be to determine whether this down-regulation of expression is associated with hypermethylation of the promoter regions of these two genes, to map the specific methylation sites, and to determine the time course of methylation in relation to gene silencing. Aim three will be to test whether this phenomenon is associated with the physical association of activated ER with DNMT1 and/or DNMT3B, and the physical association of HDAC1 with this complex in the promoter regions of LCN2 and CRH. Further, we will test whether deacetylation and methylation occur at H3-K9 in conjunction with these biophysical events. In aim four, RNAi approaches will be used to test whether knockdown of DNMTs prevents the ER-activation associated methylation and silencing of LCN2 and CRH. Finally, aim five will be to determine whether exposure of human breast cancer cells to recombinant LCN2 or CRH has a growth inhibitory or apoptotic effect, confirming the physiologic relevance of this phenomenon. These aims will be accomplished using standard techniques of cell culture, qRT-PCR, western blotting, real-time MS-PCR, bisulfite sequencing, chromatin immunoprecipitation (ChIP)-PCR, stable transfection of shRNA expression vectors, and cell proliferation and apoptosis assays.

Principal Investigator

Jeff Boyd, PhD
Senior Vice President, Molecular Medicine
Executive Director, Institute for Personalized Medicine
The Fox Chase Cancer Center
333 Cottman Ave
Philadelphia, PA 19111-2434

Other Participating Researchers

Eric Ariazi, PhD - employed by The Fox Chase Cancer Center

Expected Research Outcomes and Benefits

Successful completion of the aims of this research project will provide additional insight into the mechanism through which estrogen contributes to human tumorigenesis, e.g., breast and

endometrial cancers. Such an understanding will allow for a more informed approach to the use of this hormone pharmacologically, in terms of risk-benefit analysis. Furthermore, a fundamental understanding of the molecular mechanism through which estrogen contributes to the development or progression of women's cancers is essential in order to develop more effective preventive strategies for these cancers, in light of the long-term exposure of females to this hormone over a reproductive lifetime. Finally, proof of the hypotheses proposed in this project will lead to a new model for the role of hormone receptor transcription factors in human carcinogenesis, which would provide greater insight into the process of tumorigenesis in a wide range of cancers not limited to those associated with estrogen exposure.

Summary of Research Completed

To investigate estrogen receptor alpha (ERα)-mediated changes in promoter methylation of tumorigenic genes, we have generated long-term estrogen-deprived T47D/LTED2 breast cancer cells. These cells were derived by selecting estrogen-dependent ERα-positive T47D cells in estrogen-free media (phenol red-free RPMI-1640 plus 10% dextran-coated charcoal-treated FBS) over a time period of 44 weeks.

We have previously identified LCN2 as a candidate target of ER-mediated methylation by gene expression microarray analysis of T47D cells treated with various combinations of the synthetic estrogen diethylstilbestrol (DES) and the methylation inhibitor 5-aza-cytidine. Hence, expression of ERα and lipocalin 2 (LCN2) at the protein and mRNA levels was characterized in the T47D cells throughout the time period of selection under estrogen-deprived conditions (Figure 1). ERα protein levels significantly decreased within the initial 72 hours (h) of estrogen deprivation, and continued to decrease until it was undetectable by immunoblotting by 5 weeks. Likewise, ERα mRNA levels progressively decreased by 30% within 24 h, by 79% within 1 week, and by 94% by 5 weeks of estrogen deprivation. At the end of 44 weeks of estrogen deprivation, ERα mRNA levels were only 0.3% of that before the selection began.

In contrast, LCN2 was undetectable by immunoblotting in T47D cells before estrogen deprivation was started, remained undetectable for 1 week, and only became detectable after 2-3 weeks of estrogen deprivation. LCN2 protein expression increased slowly with some variation from 2-24 weeks, and then showed a very large increase between 24 and 36 weeks. Similarly, LCN2 mRNA levels showed little change after 1 week of estrogen deprivation, and then increased by 7.5-fold and 19.4-fold after 2 and 3 weeks, respectively, of estrogen deprivation compared to time 0. After 24 weeks of estrogen deprivation, LCN2 mRNA levels had increased by 41-fold, and then showed a very large elevation between 24 and 36 weeks to ~200-fold over LCN2 levels at time 0. Noteworthy, the very high level of LCN2 expression remained so from 36 to 44 weeks, indicating that LCN2 expression had stabilized. Stabilized LCN2 expression also indicated that the T47D/LTED2 have fully adapted to an estrogen-free environment. Taking into account the overall changes in ERα and LCN2 levels, expression of these genes were inversely related. ERα levels dropped quickly after removal of estrogen from the media, and the T47D cells essentially became ERα-negative. Conversely, the T47D cells began as LCN2-negative, and only after ERα levels decreased significantly, did LCN2 levels slowly rise over 24 weeks to eventually become a very abundantly expressed gene after 36 weeks of estrogen deprivation. These data therefore indicate that ERα silenced LCN2 expression under estrogenized conditions,

and that loss of ERα due to estrogen deprivation allowed LCN2 expression to become re-activated.

The relatively slow yet progressive rise in LCN2 expression over 36 weeks of estrogen deprivation suggests that the mechanism may involve ERα-mediated promoter methylation. Methylation of DNA occurs on cytosine residues primarily in CpG dinucleotides. Methylation of CpG sites has been shown to be associated with gene inactivation. Therefore, we mapped potential CpG sites of methylation in the LCN2 promoter from 1 kb upstream to 100 bp downstream of the transcriptional start site (Figure 2). A map of this region of the LCN2 promoter is shown in Figure 2A. There are a total of 17 CpG sites and 1 estrogen response element (ERE).

Pyrosequencing is the gold standard to determine DNA methylation, but only short sequence reads of up to ~50 bases provide high quality data. Thus, we developed pyrosequencing assays for regions containing clusters of CpG sites that cover 11 of the 17 sites. To examine methylation status by pryosequencing, genomic DNA is first treated with bisulfite. Bisulfite converts unmethylated cytosine residues into uracil, whereas the methylated cytosine residue remain unchanged. Thus, bisulfite conversion results in different DNA sequences for methylated and unmethylated DNA. The first step in pyrosequencing analysis is to PCR amplify a DNA segment using a biotinylated reverse primer. Following capture of the biotinylated PCR product with streptavidin beads and denaturation, the biotinylated single-stranded PCR amplicon serves as a template for hybridization of a sequencing primer. The pyrosequencing reaction proceeds by stepwise incorporation of a single complementary dNTP for each cycle, and the stoichiometry of each incorporation event dictates the release of equimolar quantities of pyrophosphate (PPi). The released PPi is coupled to a luciferase reaction. This is achieved by converting PPi to ATP using ATP sulfurylase in the presence of APS. The ATP is a required co-factor in luciferase reactions that generates visible light proportional to the amount of ATP. The raw data output is seen as a peak (Pyrogram trace) and the height of the peak is quantitatively related to the amount of the specific nucleotide incorporated. Apyrase is included in these reactions, which degrades unincorporated nucleotides and ATP. This completes 1 cycle, and each subsequent cycle requires the addition of the next complementary nucleotide. This technology therefore allows quantification of sequence variation at a specific location, being either a methylated C or a T residue which corresponds to an unmethylated C before bisulfite conversion.

Pyrosequencing assays were developed to determine methylation status of 11 of the 17 CpG sites; 4 sites upstream of the ERE in the distal promoter, 5 sites downstream of the ERE in the proximal promoter, and 2 sites downstream of the transcriptional start (Fig. 2A). First, the methylation status of these CpG sites was determined at the onset (time 0), and after 24 and 36 weeks of estrogen deprivation (Fig. 2B). Before estrogen deprivation began, CpG sites 1 thru 5 and sites 10 and 11 were greater than 90% methylated, while sites 6 thru 9 in the proximal promoter were methylated from 18% - 61%. Thus the LCN2 promoter, under estrogenized conditions, was hypermethylated. After 36 weeks of estrogen deprivation, there were no changes in methylation of CpG sites 1 -4 upstream of the ERE in the distal promoter. However, CpG sites 5 thru 9 in the proximal promoter, and site 11 in transcribed region did show large decreases in methylation. It is interesting that all of the CpG sites that underwent demethylation were downstream of the ERE, and suggests importance to this topological arrangement. To examine

the kinetics of demethylation of CpG sites in the proximal promoter, the methylation status of CpG sites 7 thru 9 were determined every week during the first 8 weeks of estrogen deprivation (Fig. 2C). Methylation of CpG site 7 decreased from 20.8% to 0%, site 8 remained relatively constant (although this site showed demethylation at 24 and 36 weeks), and site 9 decreased from 54.5% to 6.5% over 8 weeks. Considering the LCN2 expression data, the rate of demethylation in the LCN2 proximal promoter was congruent with the rate of elevation in LCN2 expression.

Taken together, we hypothesize that under estrogenized conditions, ERα via the ERE recruits chromatin remodeling factors and DNA methyl-transferases (DNMTs) resulting in hypermethylation of the LCN2 promoter to silence its expression. Estrogen deprivation leads to loss of ERα expression, which results in demethylation of CpG sites in LCN2's proximal promoter causing reversal of silencing and abundant expression of LCN2.

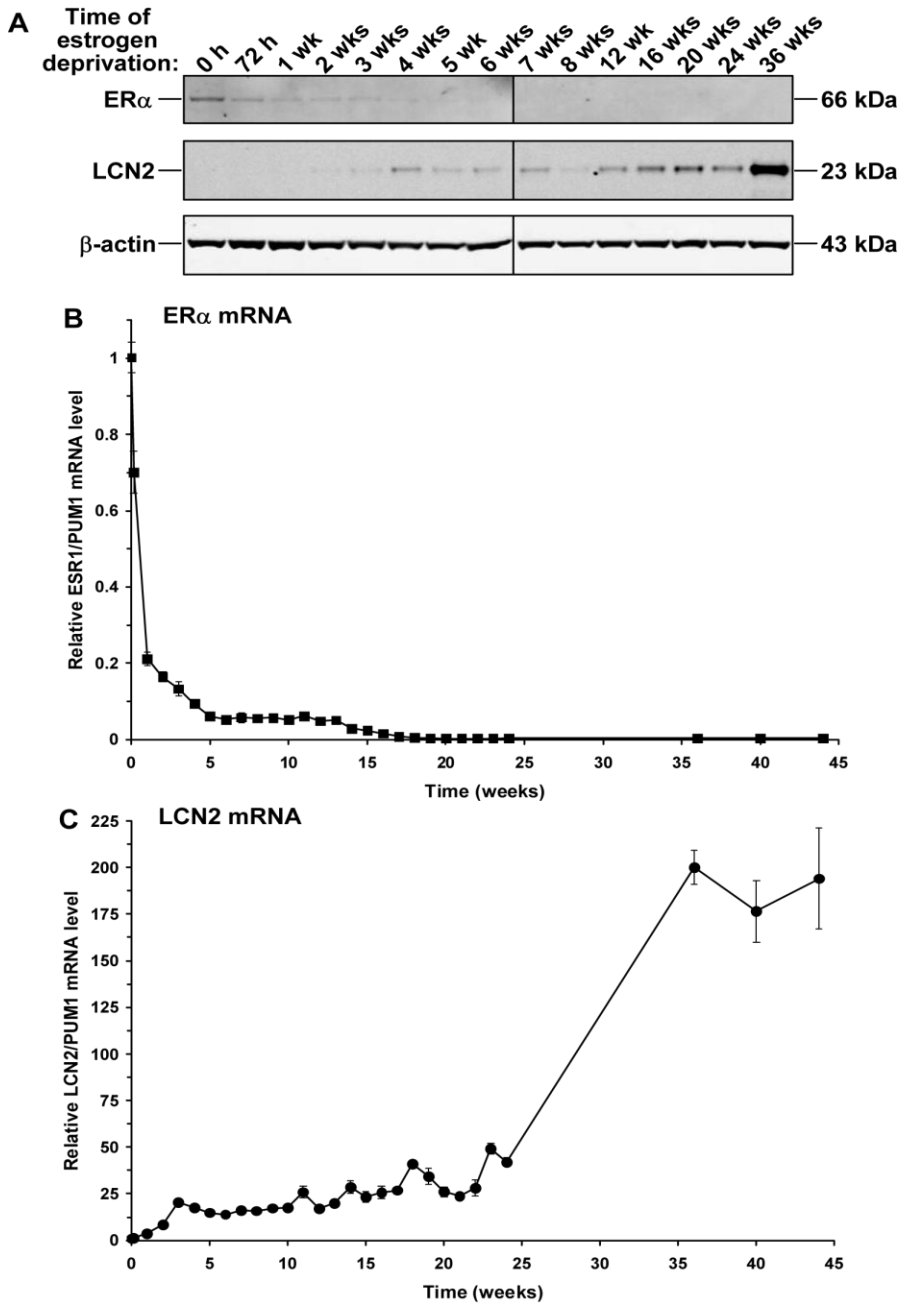

Figure 1. Long-term estrogen deprivation leads to loss of ERα and gain of LCN2 expression in T47Dcells. (A) Protein and (B-C) mRNA levels of ERα and LCN2 as a function of time of estrogen deprivation. Estrogen-dependent T47D cells were switched from fully estrogenized to estrogen-free media (phenol red-free RPMI-1640 + 10% dextran-coated charcoal-treated fetal bovine serum) for the indicated period of time. (A) Protein levels were measured by immunoblotting using 40 ug protein/lane and antibodies against ERα (clone AER611; Thermo Fisher Scientific), LCN2 (clone EPR5083; Epitomics) and β-actin (clone AC-15; Sigma-Aldrich). Blots were visualized using the Odyssey Infrared Imaging System (Li-Cor Biosciences). (B) RNA levels were measured by quantitative real-time PCR. Target mRNA levels were normalized to PUM1 [pumilio homolog 1 (Drosophila)] mRNA levels. Data were analyzed by the delta Ct method. Each data point represents the mean of 3-4 biological replicates and 3 technical replicates per biological replicate. Error bars = SD.

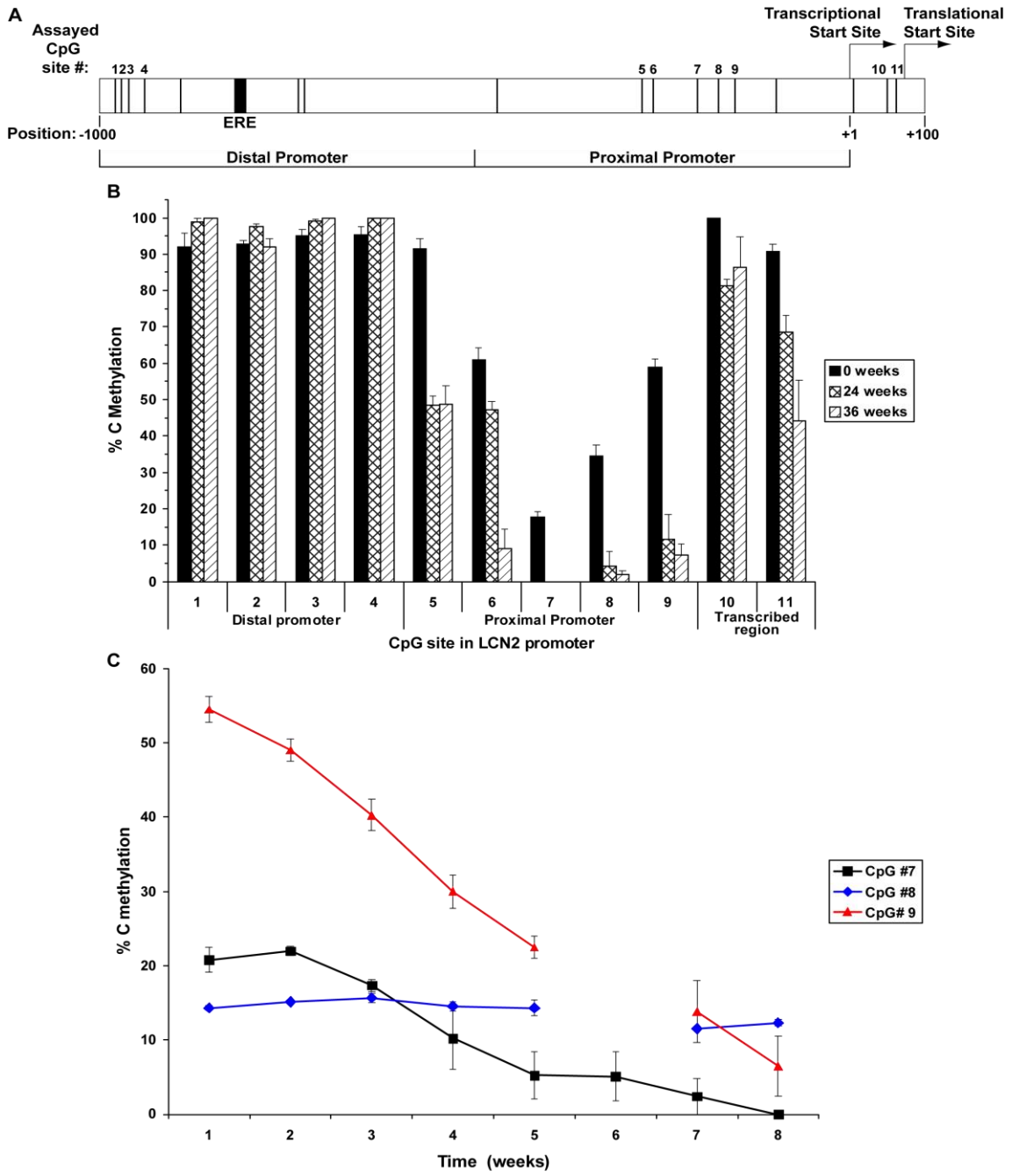

Figure 2. Methylation of CpG sites in LCN2's proximal promoter decreases during long-term estrogen deprivation in T47D cells. (A) Map of the LCN2 promoter spanning 1,000 bases upstream and 100 bases downstream of the transcriptional start site. Each line in the bar indicates a CpG site. The CpG sites assayed for methylation by pyrosequencing are numbered consecutively. (B) Methylation status of the LCN2 promoter at the start (0 weeks) and after 24 and 36 weeks of estrogen deprivation. (C) Time course of methylation changes at CpG sites 7 thru 9 in the proximal promoter. The 6 week data point for CpG sites 7 and 8 were due to failed reactions, but will be repeated. T47D cells were deprived of estrogen as in Figure 1. Genomic DNA was isolated and treated with bisulfite using the QIAamp DNA mini and EpiTect Bisulfite kits, respectively (Qiagen). Methylation of CpG sites was quantitatively measured by pyrosequencing using the PyroMark PCR kit and a PSQ 96MA instrument. Each data point represents the mean of 5 replicates and error bars the SEM.

Research Project 3: Project Title and Purpose

Markers of Adult Epithelial Stem Cells - The purpose of this project is to identify novel stem cell markers in adult epithelial tissues. Currently, no markers are available that consistently and robustly enable the *in vivo* identification and purification of adult stem cells from normal tissues or tumors. Moreover, the difficulty associated with *in vivo* identification of stem cells has hampered attempts to conduct functional analyses of genes thought to regulate stem cell function or contribute to the formation of tumor cells with self-renewing properties. We aim to determine the molecular identity and function of a new epithelial stem cell marker in the fly and assess its functional conservation in mammalian epithelial tissues. The information provided in this study will be a foundation for the development of agents that promote or target stem cell function for use in replacement therapies and anti-cancer treatment.

Anticipated Duration of Project

1/1/2010 – 6/30/2012

Project Overview

Substantial progress has been made in the identification of markers used to isolate and identify stem cells from adult tissues. In some cases, markers are relatively stem-cell specific (e.g. Oct4). In other cases, broadly expressed proteins exhibit differential levels of expression in stem cells vs. non-stem cell daughters. Despite these advancements, purification of adult tissue stem cells, identification of stem cells *in situ*, and the functional relevance of stem cell markers currently used in isolation protocols remain challenges for this field.

Our goal is to identify molecules that control stem cell identity and maintenance. Since identifying novel signals that regulate stem cell function is technically challenging in mammalian tissues, we are focusing on *Drosophila* ovarian Follicular epithelium Stem Cells (FSCs) as a model system for this analysis. In this system, epithelial stem cell formation can be visualized directly *in vivo*. Moreover, genetic mutational analysis allows us to pinpoint functional roles for specific genes in the cellular events required for stem cell commitment and self-renewal. The striking conservation of stem cell control signals in mammals and flies suggests that novel stem cell regulatory mechanisms identified in the fly system will generally be applicable for understanding epithelial stem cell regulation in mammalian tissues.

To identify novel stem cell regulators, we have initiated a screen for antibodies that specifically recognize FSCs *in situ*. Using this approach, we identified one antibody that recognizes a nuclear protein that is highly upregulated in FSCs and their differentiated progeny cells. *We propose that this follicle cell-nuclear antigen (FC-NA) is a novel epithelial stem cell marker that is important for promoting FSC commitment and function.* To test this hypothesis, we will 1) determine the molecular identity of FC-NA using biochemical approaches and molecular genetics, 2) define how FC-NA regulates FSCs *in vivo* through mutational analysis and imaging, and 3) define the protein expression patterns of FC-NA in mammalian epithelial tissues using immunostaining techniques. This project has the potential to uncover novel stem cell markers

with broad application in stem cell purification and identification as well as in functional analysis of normal and tumor stem cells.

Principal Investigator

Alana M. O'Reilly, PhD
Assistant Professor
The Fox Chase Cancer Center
333 Cottman Avenue
Philadelphia PA 19111-2434

Other Participating Researchers

None

Expected Research Outcomes and Benefits

The promise of stem cell research is two-fold. On the one hand, stem cell replacement therapies may provide cures for a diverse group of disorders, including cancer, diabetes, and traumatic injury. On the other hand, the identification of cancer cells with self-renewing properties that promote tumor growth and evade conventional anti-cancer therapies may lead to the development of potent new approaches to cancer treatment. Substantial progress has been made in the development of *in vitro* technologies that allow stem cells to be grown in culture and stimulated with factors that promote their differentiation into specific cell types. Far less progress has been made in identifying adult stem cells and their mechanisms of regulation in functioning adult tissues.

In this project, we aim to identify new markers of adult epithelial stem cells. Our preliminary results indicate that an unidentified nuclear protein is highly upregulated in epithelial stem cells in the *Drosophila* ovary, suggesting that this protein may be an important new stem cell marker. If the results of the experiments proposed support our hypothesis that this nuclear antigen is a novel regulator of epithelial stem cell function in flies and in mammals, a new tool for the identification, purification, and *in vivo* analysis of stem cells will be available. In addition, this study has clear implications for understanding the role of specific genes in promoting stem cell self-renewal and, importantly in the genesis of epithelial stem cells from uncommitted precursor cells. Conservation of the normal developmental mechanisms described in this study may have an important impact on the ability to recognize newly forming cancer cells with stem cell properties. Such a discovery would open new therapeutic avenues for treating cancer in its earliest stages. Finally, this tool may advance our understanding of the mechanisms regulating stem cells in their normal environments, information that is critical for the success of stem cell replacement therapies.

Summary of Research Completed

The goal of this work is to identify novel markers of epithelial stem cells. During the past year, we have characterized the roles of four novel genes in epithelial stem cell regulation in the

Drosophila (fruit fly) ovary. Our characterization of one of these genes was published in the Journal of Cell Biology in November 2010 (J Cell Biol. 2010 Nov 29;191(5):943-52). In the upcoming year, we expect to submit two manuscripts describing additional fly stem cell regulatory genes we have identified and a third manuscript that will be a continuation of the study we published in the Journal of Cell Biology. In addition to identifying novel stem cell control factors in the fly, we initiated studies to determine whether fly stem cell regulatory mechanisms are conserved in mammals. Specifically, we have focused on the role of the Hedgehog signaling pathway in the development of ovarian cancer in a mouse model. Our overall goal is to develop reagents that will enable the unambiguous identification of epithelial stem cells *in vivo* so that determining the mechanisms of action of specific genes and the tracking of developing cancer stem cells will become possible.

Epithelial stem cell proliferation is controlled by cells located outside of the stem cell niche

In our initial application, we described our efforts to generate antibodies that specifically mark epithelial stem cells. One of these, FC-NA was the main focus of our proposal, and work is still underway to determine the protein target of FC-NA. We also generated antibodies against two recently identified receptors for the growth factor Hedgehog (Hh). Hh controls the proliferation rates of several epithelial stem cells, including those in the cerebral cortex, skin, and prostate in mammals, and in *Drosophila* ovaries. In the fly, Follicle Stem Cells (FSCs) depend on Hh signals produced by cells lying far outside of the local FSC niche, but the mechanisms that control the levels of Hh in the niche and how Hh is delivered over a distance to promote FSC proliferation are not known. Since Hh is such a critical regulator of FSCs, we reasoned that Hh receptors might be specifically expressed in FSCs, making them potential stem cell markers.

Ihog and Boi were recently identified transmembrane proteins that function as Hh receptors. In several tissues, Ihog and Boi promote signaling in Hh receiving cells by binding to Hh and increasing the local Hh concentration. If Ihog and/or Boi were expressed on FSCs to enhance Hh signaling and FSC proliferation, they would be excellent candidate stem cell markers. To test this idea, we generated antibodies against each protein in rats and analyzed their expression patterns in the fly ovary. Whereas Ihog was not expressed at detectable levels in any cells in the ovary, Boi was robustly and specifically localized to the distant cells that produce Hh (Figure 1A [1]). This was surprising since we expected Boi to be expressed on FSCs, acting to promote Hh signaling within the FSCs themselves. Further work demonstrated that Boi functions to sequester Hh to the surface of the distant producing cells, limiting the levels of Hh available to the local FSC niche. In the absence of *boi*, Hh is released from the surface of the distant producing cells and accumulates in the extracellular matrix surrounding FSCs and their daughter cells (Figure 1B,C). As a result, FSCs are hyperproliferative and generate excess numbers of daughter follicle cells (Figure 1D,E). This function depends on the ability of Boi to bind directly to Hh, but is independent of the Boi-mediated pathway that promotes Hh signaling within receiving cells [1]. Taken together, these results suggest that expression of growth factor receptors on the surface of the cells that produce them is a critical mechanism for epithelial stem cell proliferation control in the fly ovary. If this mechanism is conserved in mammalian epithelial tissues, new avenues for manipulation of stem cells *in situ* and treatment of cancer cells with stem cell properties may be possible. Current work focuses on identifying the cues that

trigger Hh release from Boi in wild-type flies and how Hh is delivered to the FSC niche. We expect to submit a manuscript describing the cues that trigger Hh release in early fall, 2011.

Mechanistic conservation of Hh regulation in mammalian epithelia

The idea that growth factors can be sequestered and released in response to specific cues is exciting in terms of identifying a novel, general mechanism of stem cell regulation. Importantly, many epithelial cancers contain cells with stem cell characteristics that are associated with elevated levels of growth factor signaling pathways, suggesting that reducing the levels of secreted growth factors may reduce proliferation of stem-like cancer cells. Recent evidence suggests that the Hh pathway is inappropriately activated in serous type epithelial ovarian cancers, the most common and most deadly type of malignant ovarian cancer. In the past year, we initiated a project designed to address the potential role of mammalian homologs of Boi (Figure 2A) in the development and progression of epithelial ovarian cancer. To do this, we obtained mice that lack expression of the Boi homolog *Cell adhesion molecule-related/down-regulated by oncogenes* (*Cdon*, pronounced "kiddo"). Currently, we are analyzing expression of Hh pathway components in normal ovarian tissue and during tumorigenesis in wild-type and *Cdon* mutant mice. In addition, we are assessing the effects of Cdon mutation on the timecourse of ovarian tumor development using a model developed by Fox Chase Cancer Center investigators. If Cdon functions as a suppressor of stem-like cells, we expect to see elevated proliferation rates and a faster timecourse of tumor development in the mutant mice (Figure 2B). It is also possible that Cdon will function to promote Hh signaling, in which case we will see suppression of tumor growth and reduced rates of proliferation in the tumors (Figure 2C). This study will shed light on the role of Hh signaling in ovarian tumor development and progression, and possibly uncover a mechanism of regulation that may be a good target for treatment of ovarian cancers.

Other stem cell markers:

In addition to our completed study on the role of Boi in epithelial stem cell regulation, we have conducted genetic screens to identify novel markers of FSCs and the cells that regulate them. We previously described an antibody against FC-NA which is expressed at very high levels in FSCs and their progeny cells as well as in stem cells of the developing embryo. The purification of the FC-NA antigen is nearly complete. Once the molecular identify of FC-NA is known, extensive characterization of its role in stem cell regulation will be initiated. We also have identified new markers for FSCs, the distant growth factor producing cells, and the cells of the local FSC niche. Molecular identification and functional characterization of these genes is underway. We expect to uncover new mechanisms of stem cell regulation and genes that are important for stem cell function as a result of this characterization.

Publication

1. Hartman, T.R., Zinshteyn, D., Schofield, HK., Nicolas, E., Okada, A., O'Reilly, AM.., *Drosophila Boi limits Hedgehog levels to suppress follicle stem cell proliferation.* J Cell Biol, 2010. **191**(5): p. 943-52.

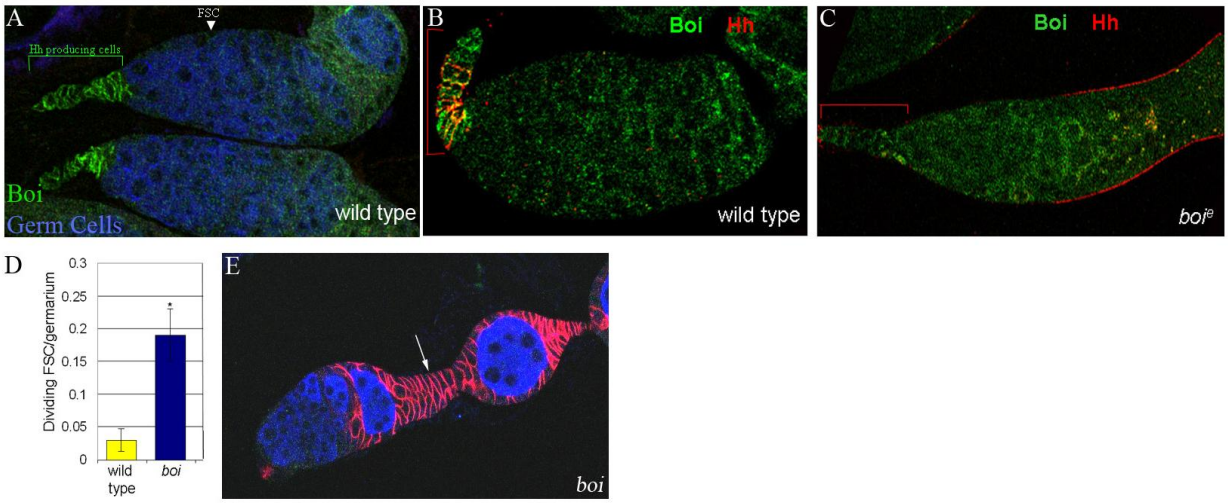

Figure 1: Boi acts in Hh producing cells to control FSC proliferation. A) Boi (green) localizes to Hh producing cells 6 cell diameters away from FSCs (white arrowhead). Germ cells are labeled (blue). B, C) Hh (red) and Boi (green) co-localize in Hh producing cells (red brackets) in wild-type ovaries (B). Hh is released to the extracellular space around FSCs and their daughters in boi mutants (C). D) boi mutant FSCs divide 10 times more frequently than wild-type FSCs. E). Excess FSC division leads to accumulation of daughter cells (red, arrow indicates excess follicle cells) between developing germline cysts (blue).

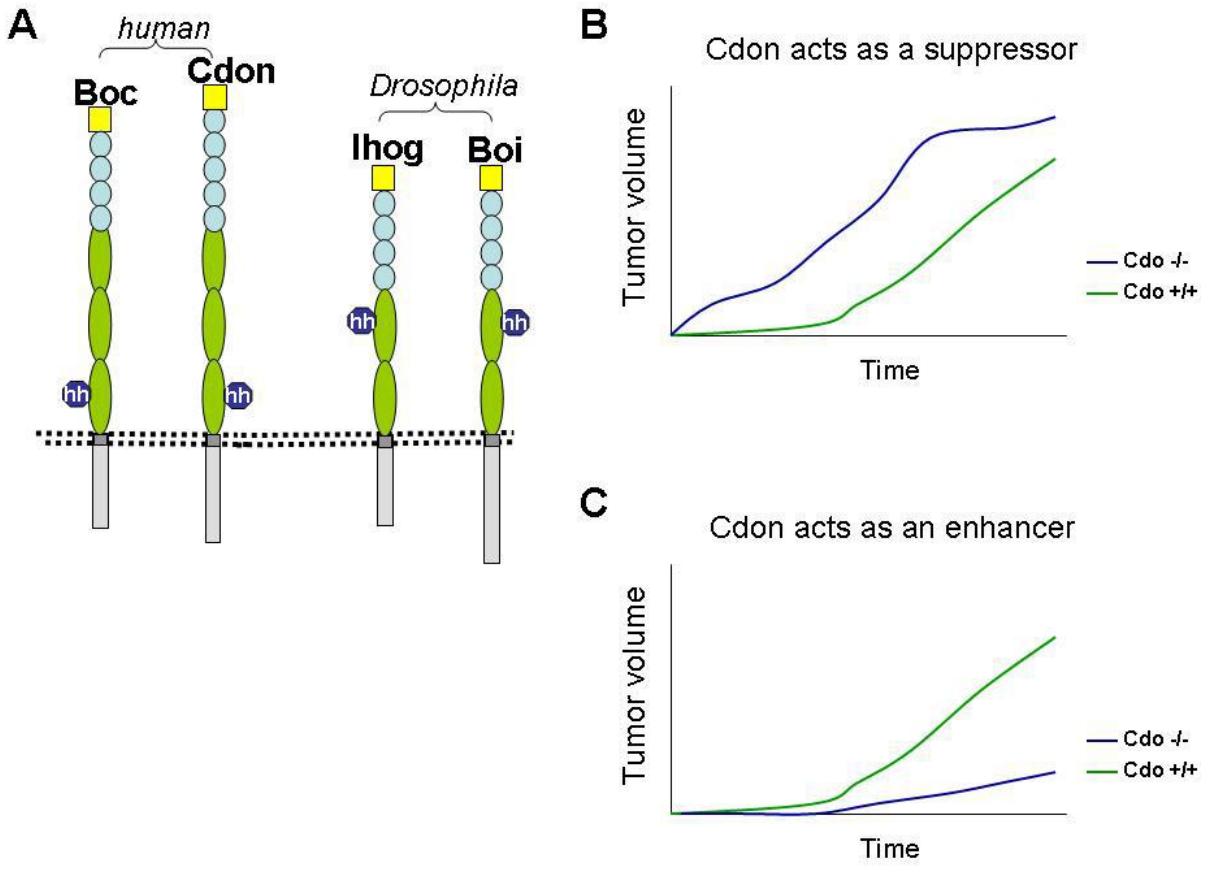

Figure 2: Role of mammalian Boi homologs in stem cell proliferation control. A) Boi has two mammalian homolgs, Boc and Cdon which bind to Hh and have a similar structure including Ig domains (blue circles), FNIII domains (green ovals) and a non-conserved cytoplasmic domain (gray). B,C) Predictions of tumor development experiment if Cdon acts as a suppressor of proliferation (B) or an enhancer (C). Loss of Cdon is predicted to increase tumor volume and development time if it acts as a suppressor (B). If it acts to enhance Hh signaling, Cdon loss will delay tumor development and growth (C).

Research Project 4: Project Title and Purpose

Role of a Novel Zinc Finger Factor in Hematopoietic Development - The purpose of this project is to understand the role of a novel erythroid factor (G4) in hematopoietic development using the zebrafish model system. Since the molecular basis for G4 function in controlling erythropoiesis is completely unknown, we will determine where *g4* fits in the hierarchy of transcription factors controlling hematopoietic development, identify its human ortholog, and perform structure/function analysis to identify the domains of *g4* that are essential for its function. Results from these studies will provide insights into how normal blood cell development is controlled and how those processes are perturbed in cancer.

Anticipated Duration of Project

1/1/2010 - 12/31/2011

Project Overview

A network of transcriptional regulators coordinates the cell fate choices and progressive differentiation of hematopoietic stem cells, a process that is incompletely understood. Hematopoiesis is conserved throughout vertebrate evolution, and we have employed the facile zebrafish experimental model system to identify a novel molecular effector, called *g4*, that is critical for erythroid development. *G4* is part of a cluster of highly homologous genes in zebrafish, and the corresponding human gene cluster encodes zinc finger factors of unknown function. The objective of this project is to investigate the role of *g4* in hematopoietic development using the zebrafish model system. We intend to do so according to the following aims:

Aim 1. Use epistasis analysis to determine where g4 function is positioned within the hierarchy of known regulators of erythroid development. This will be accomplished by performing morpholino knockdown of known molecular effectors of primitive hematopoiesis (*g4*, *scl*, *gata1*, *pu.1*), determining how this affects *g4* expression, and assessing whether forced expression of *g4* mRNA rescues the developmental blockade.

Aim 2. Utilize a complementation strategy to investigate the specificity of function among g4 family members and identify human paralogs. We will knock down *g4* expression and determine if forced expression of the other members of the zebrafish gene cluster or the human paralogs are able to restore development. Because the zebrafish family members are highly similar, this may inform the structure/function analysis if differences among family members are identified.

Aim 3. Perform structure/function analysis to identify domains or motifs of g4 that are essential for function. We will do so by determining if mutant *g4* constructs lacking conserved domains or motifs are able to rescue the developmental arrest caused by *g4* knockdown.

Together, these studies will provide insight into the function of g4 in normal development of erythroid precursors as well as to how its misregulation might contribute to their transformation.

Principal Investigator

Jennifer Rhodes, PhD
Assistant Professor
The Fox Chase Cancer Center
333 Cottman Avenue
Philadelphia, PA 19111-2434

Other Participating Researchers

Wittaya Pimtong, PhD – employed by the Fox Chase Cancer Center

Expected Research Outcomes and Benefits

Defects in the normal ontogeny of the hematopoietic system can contribute to the pathogenesis of these cells, leading to blood cell cancers or anemia. Furthermore, the continued advancement of molecularly targeted therapies to treat hematopoietic diseases is reliant on the identification of new, relevant factors and our understanding of the underlying biology. This study focuses on the *in vivo* function of a novel erythroid factor, and other highly homologous family members localized adjacent to one another in the genome. The potential importance of these genes in hematopoietic disease is evidenced by amplification of the locus encompassing this gene cluster in human hematologic cancers. Thus, results from this project will reveal insights into the function of a novel factor that regulates erythroid development and may also reveal a potential molecular target or generate animal models that could be exploited for diagnostic and therapeutic purposes.

Summary of Research Completed

The summary below describes the research completed between July 1, 2010 and June 30, 2011.

Aim 1. Use epistasis analysis to determine where g4 function is positioned within the hierarchy of known regulators of erythroid development.

Mammalian and zebrafish models have been used to establish essential roles for *pu.1* in white blood cell development and *gata1* in red cell development, defining a cross-antagonistic function for these factors in determining hematopoietic progenitor cell fate. This is evidenced by morpholino knockdown of zebrafish *gata1* which blocks hematopoietic progenitor cell differentiation to the erythroid lineage and instead causes the progenitor cells to transfate to the myeloid lineage. Similarly, *pu.1* morpholino knockdown causes a loss of myeloid cells and transfates the progenitor cells to the erythroid lineage. Since erythropoiesis occurs exclusively in the posterior hematopoietic region in the zebrafish embryo, while myelopoiesis occurs in both the posterior and anterior hematopoietic compartments, *pu.1* morpholino-injected (morphant) embryos display ectopic erythroid development in the anterior of the embryo. Our studies focus on the interplay of the novel gene *g4* with the *pu.1-gata1* axis regulating hematopoiesis.

To determine whether *g4* is essential for erythroid development, we examined the expression pattern of *gata1* in *g4* morphant embryos. We have found a decreased number of cells expressing *gata1* in 5-20% of the *g4* deficient embryos as determined by whole mount RNA in situ analysis (data not shown) at 24 hours post-fertilization (hpf). The incomplete penetrance was not due to the efficacy of *g4* knockdown, as affected and unaffected embryos had similar levels of wild-type *g4* message, which was depleted but not completely absent (data not shown). Surprisingly, in contrast to *gata1* morphants that show an expansion of *pu.1*-positive cells in the erythroid blood-forming region of the embryo, 24 hpf *g4* morphants have a normal *pu.1* expression pattern (data not shown). However, at 24 hpf, embryos deficient for *g4* have half the number of myeloid *l-plastin*-expressing cells compared with control-injected embryos (data not shown). These data indicate that *g4* is important for the development of both primitive erythroid and myeloid lineages.

The *g4* morphant does not simply mimic the *gata1* morphant, suggesting that G4 has additional functions beyond its partnership with Gata1. Possible explanations are that the stem/progenitor cells are impaired from further differentiation or/and the cells do not survive. The stem cell marker *scl* was present at comparable levels in control and *g4*-deficient embryos (data not shown), suggesting that the *g4*-deficiency does not ablate the stem cell population. We are currently testing whether knockdown of *g4* results in increased cell death, which we may be able to block by co-depletion of *p53* or forced expression of anti-apoptotic genes *bcl-2* or *bcl-xL*.

To establish if *pu.1* or *gata1* regulate the expression of *g4*, we performed morpholino knockdown of *gata1* and *pu.1* and examined whole mount *g4* RNA *in situ* hybridization (WISH) to examine the expression of *g4*. Our studies show that *gata1* knockdown resulted in a severe reduction in *g4* expression while *pu.1* knockdown caused an expansion of *g4* expression in the anterior myeloid compartment (data not shown). These data suggest reciprocal positive regulation between *gata1* and *g4* and indicate that *g4* is a new component of the *pu.1-gata1* network controlling hematopoiesis.

Recent publications, from Monteiro et al. and Belele et al., indicated that Gata1 activity is cell-type dependent, and in these examples Gata1 function differed in primitive versus definitive hematopoietic cells. The studies described above focused on the interactions of *pu.1*, *gata1* and *g4* during primitive hematopoiesis at 24 hpf. Thus, we examined the effects of *g4* knockdown on *gata1* expression during definitive hematopoiesis at 48 hpf. Surprisingly, at this age, *g4*-depleted embryos displayed a normal pattern of *gata1* expression but did have severely decreased numbers of *band3*-expressing and hemoglobin o-dianisidine stain positive cells in about half of the embryos in comparison to control embryos (data not shown). These data suggest that either a compensatory mechanisms rescues *gata1* expression but not cell maturation in this time frame or, alternately, that *g4* may have different roles in primitive versus definitive hematopoietic precursors. As *g4* function has never been examined and the *pu.1-gata1* regulatory network is highly conserved, the identification of *g4* as a new participant in this genetic pathway is significant and we are currently focused on completing these analyses for the purpose of publication.

Aim 2. Utilize a complementation strategy to investigate the specificity of function among g4 family members and identify human paralogs.

To test whether the *g4* family members are essential for normal zebrafish embryonic development, we designed morpholinos that simultaneously target all 4 *g4* family members, knockdown 3 out of the 4 genes, or a specifically deplete only the *g4* gene. We have found that the morpholinos induce the anticipated splicing defects as determined by RT-PCR analysis of individual and pools of morpholino-injected embryos (data not shown). The *g4*-specific morphant embryos have decreased erythropoiesis and the morpholinos that target multiple genes display widespread developmental defects (data not shown). Our experimental design was to use morpholino-injected embryos as a model to test the effect on expression of each *g4* family member and examine the ability of *in vitro* transcribed mRNA from each homolog to rescue the defects caused by gene knockdown.

We found that *g4* morphants display decreased levels of *g4* expression as determined by WISH. Since morpholinos do not inherently induce degradation of their target transcripts, our data suggests that either the abnormal splice variant is unstable or that the G4 protein promotes its own gene transcription. Similarly, published studies have shown that PU.1 and GATA1 drive transcription from their own promoters and WISH analysis of zebrafish *pu.1* and *gata1* morphant embryos showed a corresponding decrease in *pu.1* and *gata1* transcripts, respectively. Thus, we favor the hypothesis that G4 has autoregulatory function. Interestingly, knockdown of *g4* also resulted in decreased expression of other family members, most notably *g3*, suggesting a role for G4 in regulating the expression of this entire gene family.

We have isolated the full-length genes for each *g4* family member and two of the putative human paralogs and subcloned them into expression vectors. We have also generated in vitro transcribed mRNA of *g4* and the human paralogs to determine whether forced expression of these genes can rescue the defects observed in *g4*-deficient embryos. As it is still not clear whether *g4* functions upstream, downstream or in parallel to *gata1*, we are also using these reagents to test whether enforced expression of *g4* rescues defects due to *gata1* knockdown and vice versa. Preliminary experiments have shown that enforced expression of *g4* results in *g4*-knockdown gives rise to embryos with a normal *gata1* expression pattern at 24 hpf. Due to the incomplete penetrance of the *g4* knockdown-induced phenotype, Aims 1 and 2 studies demand a high number of embryos per analysis to achieve statistical significance. Currently, we are in the process of increasing the number of embryos analyzed for the studies described above.

Aim 3. Perform structure/function analysis to identify domains or motifs of g4 that are essential for function.

We have begun work to engineer the *g4* mutant isoforms for these studies and we are establishing a transcriptional reporter assay (see next paragraph) in cell lines to complement the in vivo analysis of *g4* wild-type and mutant isoforms in zebrafish embryos.

Our knockdown studies revealed similar responses of *gata1* and *g4* expression following the knockdown of *pu.1*, *gata1* or *g4* genes. We hypothesize that there is a regulatory domain that is conserved in the promoters of these two genes that accounts for the similarity in gene regulation. Supporting this hypothesis, we identified a roughly 100 base pair sequence with very high homology that is present in the promoters of both the *gata1* and *g4* genes (data not shown). The putative regulatory element contains a GATA consensus-binding site and is an intriguing candidate element for controlling the expression of *g4* and *gata1* genes and regulation by Gata1 and G4 proteins. We subcloned the *gata1* and *g4* consensus elements upstream of a promoter and luciferase reporter gene. We also generated zebrafish FLAG-G4 and HA-Gata1 expression constructs. In 293T cells we had difficulty expressing the zebrafish fusion proteins. A similar Gata1 fusion protein has been previously reported and the protein is expressed; thus, we do not think that the fusion protein is the underlying problem. Published studies examining the transcriptional activity of Gata1 have been performed in a variety of cell lines, including COS-7 cells which are readily available to our lab. We are currently testing our expression constructs in the COS-7 cell line.

Research Project 5: Project Title and Purpose

Reducing the Burden of Hepatocellular Carcinoma: Identification of New Targets against Hepatitis B virus (HBV) for Drug Development – A reduction in the burden of liver cancer depends on the development of effective therapies that prevent or arrest the growth of hepatocellular carcinomas. The purpose of the project is i) to develop rapid screening assays for primary cultures derived from hepatocellular carcinoma for drugs and drug combinations that induce cell death and to identify serine and tyrosine kinases that are required for the survival of tumor cells in the presence and absence of already available anticancer drugs and ii) to identify novel targets for antiviral therapy against hepatitis B virus.

Anticipated Duration of Project

1/1/2010 – 6/30/2012

Project Overview

The principal research objective of this project is to reduce the burden of hepatocellular carcinoma (HCC). HCC is the third leading cause of cancer mortality worldwide. So far, treatment options for patients with HCC are sparse and available medicines exhibit insufficient therapeutic efficacy to significantly prolong life. The overall goal of this project to develop a method for the rapid identification of new targets for the development of drugs that can prevent, arrest growth of HCC or even cure the disease. We will test the hypothesis that the survival of liver tumors depends on the expression of genes belonging to the cellular serine and tyrosine kinases and that inactivation of certain kinases will arrest tumor growth by increasing the rate of cell death of liver tumor cells. This hypothesis is supported by observations made with certain breast and lung cancers as well as by observations in our laboratory with an established cell line derived from a cervical carcinoma. Moreover, we will test the hypothesis that hepatitis B virus replication depends on cellular enzymes required for nucleotide excision repair of damaged DNA. If successful, our investigations will identify novel cellular targets for the development of antiviral therapies against hepatitis B virus, a major cause for liver cancer in the world.

Our goal to identify kinases required for survival of liver tumors will be achieved through two specific research aims: 1) to develop conditions for the screening of primary cell cultures derived from human liver cancers and 2) to use RNA interference technology to identify kinases that are required for survival of liver tumor cells and 3) to identify cellular enzymes required for the life cycle of hepatitis B virus.

Principal Investigator

Christoph Seeger, PhD
Professor
The Fox Chase Cancer Center
333 Cottman Avenue
Philadelphia, PA 19111-2497

Other Participating Researchers

Ronald Matteotti, MD – employed by The Fox Chase Cancer Center

Expected Research Outcomes and Benefits

The expected outcome of this project is the development of conditions to establish primary cell cultures from primary hepatocellular carcinomas and the identification of serine or tyrosine kinases that are essential for the development, growth and survival of these cancers. In addition, an expected outcome of this project is the identification of an endonuclease, polymerase and/or ligase required for hepatitis B virus replication. The benefits of the research project will be the identification of new targets for the development of drugs that in combination with already available medicines can prevent, arrest or cure liver cancers, and increase the survival rate of patients struck with this deadly disease.

Summary of Research Completed

Aim 3.1: Identification of the polymerase required for CCC DNA synthesis. The most widely held view in the field is that the conversion of RC DNA into CCC DNA occurs by a DNA repair mechanism normally used by cells during DNA replication or repair. For example, one could envision that the 5' ends of RC DNA signal the presence of two Okazaki fragments and recruit the lagging strand machinery for the maturation step leading to CCC DNA formation. In this model, the endonuclease Fen1 or DNA2 would cleave the RNA primer at the 5' end of plus strands and DNA polymerase δ or ε extend the 3' end and fill in the gap before the two ends are joined by a DNA ligase, i.e. DNA ligase I (Figure 1). The completion of minus strands would proceed in the same fashion except that an endonuclease would have to cleave the 5' end of minus strand distal to r5. An alternative model predicts that RC DNA is recognized in the nucleus as "damaged DNA" inducing a DNA repair reaction. Such a scenario could invoke excision of a larger segment from the 5' and/or 3' end of minus strand DNA, followed by DNA synthesis catalyzed by DNA polymerase β and other less characterized DNA repair enzymes.

To investigate this model, we developed an *in vitro* system based on frog oocyte extracts, which permits the conversion of RC into CCC DNA (Figure 2). In this system CCC DNA formation depends on ATP and a DNA polymerization step that is inhibited by aphidicolin, but not ddCTP, indicating that, in this system, CCC DNA formation depends on either polymerase δ or ε, but not β and on an ATP-dependent ligation reaction. However, we found that in tissue culture cells, formation of CCC DNA was not inhibited in the presence of aphidicolin, ddCTP or a combination of the two inhibitors (Figure 2). Thus, our results are inconsistent with a dependence on DNA polymerase δ, ε or β in infected cells. Hence, while these results demonstrated that RC DNA can be converted to CCC DNA by the cellular DNA repair machinery under certain *in vitro* conditions, they also demonstrated that hepatitis B viruses are likely to use a different mechanism for CCC DNA formation in a natural infection, such as the one used for excision of nucleotides that were damaged by UV or as a consequence of lipid peroxidation.

This so called nucleoside excision repair (NER) pathway requires enzymatic activities of

endonucleases to excise the damaged DNA, polymerases to fill in the gaps created by the excision and DNA ligase to seal the ends of DNA. Our experimental approach was based on cell culture systems that are permissive for DHBV and HBV replication. While the use of HepG2 cells for expression of hepatitis B viruses is well established and documented, 293T cells have more recently been identified as a cell line that supports DHBV replication including CCC DNA synthesis. The advantage of 293T cells over HepG2 cells is that they can be more efficiently transfected and that they can be very rapidly selected with puromycin following infection with lentiviruses that express shRNAs.

Using this approach, we have obtained very strong experimental evidence for a role of a Y-DNA polymerase in the formation of CCC DNA of both DHBV and HBV. Specifically, inhibition of expression of the polymerase led to a significant reduction in CCC DNA formation in 293T cells and HepG2 cells (Figure 3). While these results need to be confirmed, they are the first to demonstrate that HBV replication requires a cellular enzyme to repair its genomic RC DNA. These results are particularly exciting because the DNA polymerase is not required for cell viability. Therefore, the polymerase could serve as novel target for the development of drugs that inhibit HBV replication.

Aim 3.2: Identification of the exonuclease and DNA ligase required for CCC DNA synthesis. Using the same approach as described for the polymerase, we initiated experiments aimed at identification of the relevant endonuclease and DNA ligase required for the repair process. So far, we have obtained evidence for a role of an endonuclease (Figure 3). We are in the process of verifying these results.

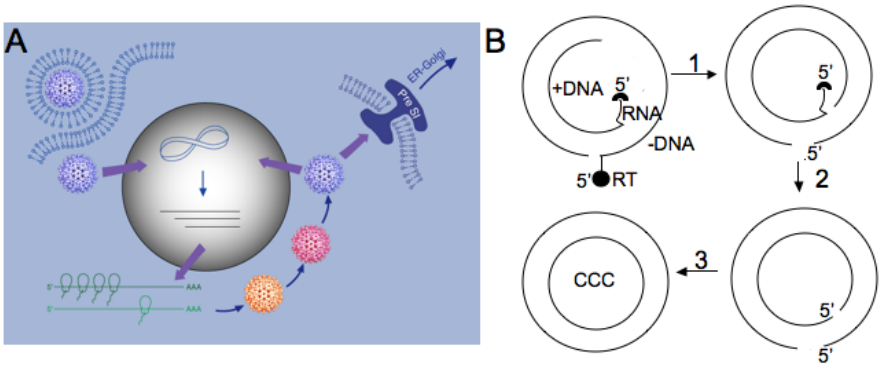

Figure 1. HBV Life cycle. A) Note, the dual fate of core particles. Retrograde transport leads to CCC DNA amplification. B) General model for conversion of RC to CCC DNA. 1; removal of reverse transcriptase (RT, filled circle) including at least 9 nts from 5' end of minus strand DNA, 2; removal of the RNA primer from plus strand DNA, 3; filling the gaps in RC DNA and ligation to form CCC DNA.

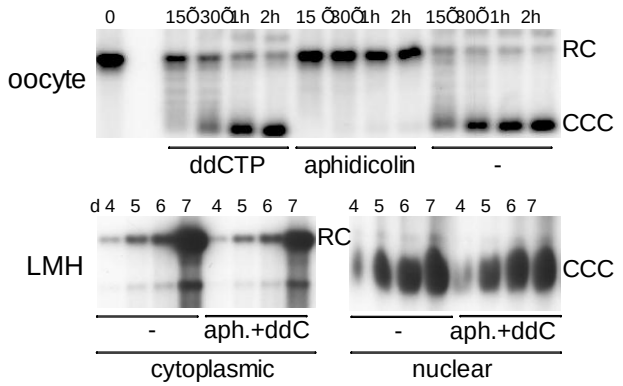

Figure 2. CCC DNA formation in oocyte extracts and LMH cells. Aph; aphidicolin. The difference in migration of CCC DNA isolated from oocyte extracts and LMH cells is caused by differences in the DNA extraction protocol.

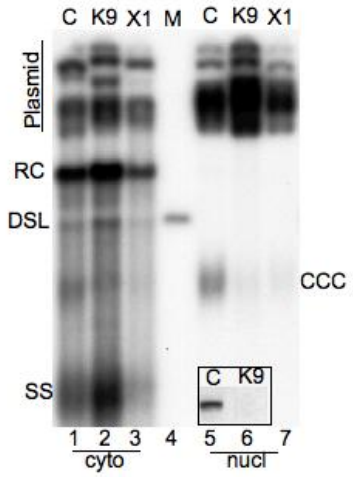

Figure 3. Inhibition of DHBV CCC DNA synthesis in 293T cells expressing shRNAs against the DNA polymerase (K9) and the endonuclease (X1). The left panel shows cytoplasmic core DNA, the right panel nuclear CCC DNA The inset shows a western blot developed with Ab to the DNA polymerase.