Fox Chase Cancer Center

Annual Progress Report: 2009 Formula Grant

Reporting Period

January 1, 2010 – June 30, 2010

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, 2011. 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 - 12/31/2011

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 indicates 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 a dual function 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. To test this hypothesis we propose two 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.

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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 first six months of this project, in agreement with the anticipated milestones presented in the original strategic plan, we conducted an initial characterization of the TDG N140A knock-in

mutant mice (Specific Aim 1) and started the analysis of TDG alterations in human cancer (Specific Aim 2).

For Specific Aim 1, we crossed $TDG^{N140A/+}$ mice with $TDG^{+/-}$, i.e. heterozygous mice bearing a null allele of TDG. No live pups with the $TDG^{N140A/-}$ genotype were obtained, suggesting that this genotype combination results in embryonic lethality. We then conducted timed matings and found that embryos with the $TDG^{N140A/-}$ genotype, similar to embryos with the $TDG^{-/-}$ genotype, die at midgestation. This observation suggests that TDG catalytic activity is required for normal development, possibly through a direct role in DNA demethylation.

In order to determine whether TDG catalytic activity is required for development, we will next cross heterozygous $TDG^{N140A/-}$ mice and analyze the resulting offspring (mice/embryos) with the $TDG^{N140A/N140A}$ genotype.

In order to determine conclusively whether TDG is involved in active DNA demethylation, we turned to a cellular model, reactivation of a methylated Oct4 pluripotency gene in embryonic carcinoma P19 cells, using an Oct4 promoter::EGFP reporter. The unmethylated reporter was expressed at equal levels in parental P19 cells and a P19 derivative line with a knock-down of TDG. However, the SssI in vitro-methylated reporter was efficiently demethylated only in parental P19 cells within 12 hours of transfection, whereas the extent of demethylation was severely impaired in the derivative line. The short time frame of the demethylation and the fact that the reporter plasmid used lacks an origin of replication rules out any potential effect of passive demethylation and confirms that TDG is involved in an active demethylation process.

For Specific Aim 2, while we just started the crosses to generate mice with double inactivation of MED1 and TDG in the intestine, in the context of the APC^{Min} background, we have obtained interesting data on the regulation of TDG expression levels in human cancer specimens.

We reported that TDG protein levels are low undetectable in human colorectal and melanoma cell lines. We therefore focused on the molecular mechanisms of reduced TDG expression. In a pilot experiment on a small number of human colorectal cancer samples (<10), we failed to detect evidence of promoter hypermethylation. In addition, TDG is not present in the list of genes mutated in colorectal cancer.

We were able to obtain new data suggesting that the detected downregulation of TDG may be mediated by microRNAs. In fact, reduced levels of TDG in melanoma samples were associated with overexpression of a small microRNA cluster predicted to bind to the TDG mRNA.

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.

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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

We have generated model systems to investigate estrogen receptor alpha (ERα)-mediated changes in DNA methylation of promoters of genes involved in breast cancer. The models we generated are derived from ERα-positive MCF-7 and ERα-positive T47D human breast cancer cells. We chose these cells based on prior studies aimed at investigating breast cancer resistance to aromatase inhibitors, which block extragonadal conversion of androgens to estrogens. In such reported studies, MCF-7 and T47D cells were deprived of estrogen for greater than a year and thereby adapted to grow under estrogen-free conditions to reasonably mimic aromatase inhibitor resistance. These reports showed in two clones of long-term estrogen deprived (LTED) MCF-7 cells termed MCF-7:2A and MCF-7:5C that ERα expression levels were significantly increased 2-4-fold compared to the parental cells. In contrast, in other studies employing T47D cells, selection under long-term estrogen deprivation conditions resulted in the clone T47D:C42 cells that had become permanently ERα-negative. We hypothesized that in these reports, the persistent alterations in ERα expression were due to changes in ERα promoter methylation. Further, since ERα expression was increased in one cell type (MCF-7:2A and MCF-7:5C), but silenced in the other cell type (T47D:C42), producing similar models for our studies may prove particularly useful.

Therefore, during the current reporting period, we have generated polyclonal MCF-7/LTED and polyclonal T47D/LTED cells from parental estrogen-dependent MCF-7 and T47D cells. The parental, or wild-type cells, are routinely maintained under fully estrogenized condition, i.e. in RPMI-1640 media supplemented with 10% whole fetal bovine serum (which contains endogenous estrogens). Hence, wild-type cells were switched to estrogen-free conditions by culturing them in phenol red-free RPMI-1640 media supplemented with 10% dextran-coated charcoal-treated fetal bovine serum. After 6 months of estrogen deprivation, MCF-7 and T47D cells had adapted to estrogen-free conditions and were termed MCF-7/LTED and T47D/LTED cells, respectively. These cells were characterized relative to their parental cells for changes in mRNA levels of ERα, LCN2, and CRH by real-time PCR. Also, growth responses to 17β-estradiol (E_2) and to the synthetic estrogen diethylstilbestrol (DES) were assessed using a DNA-specific fluorescent dye (Hoechst 33258) to measure DNA mass per well after 6 days of compound exposure.

Assessment of growth responses of MCF-7/LTED cells showed they were not stimulated by E_2 or DES, however the complete antiestrogen fulvestrant yet inhibited basal growth by ~50%. Thus unliganded ERα provided a growth advantage to the MCF-7/LTEDcells, but E_2 did not provide any additional growth support. Examination of MCF-7/LTED cells showed a substantial 10.9-fold increase in ERα mRNA, and an 85.1% decrease in LCN2 mRNA expression. This indicates that LCN2 was inversely regulated by ERα. We hypothesize that the substantially increased ERα expression led to hypermethylation of the LCN2 promoter to silence its expression. CRH was below the limit of detection by real-time PCR in both MCF-7/LTED cells and parental cells, indicating that ERα may not regulate this gene.

Characterization of growth responses of T47D/LTED cells showed they had become completely insensitive to estrogenic stimulation: neither E_2 or DES stimulated their growth, nor did fulvestrant affect growth. Evaluation of ERα expression indicated that T47D/LTED cells had also become ERα negative at the protein level by immunoblotting, and real-time PCR showed ERα mRNA expression substantially decreased to only 0.25% of its level in parental cells. Thus, long-term estrogen deprivation caused T47D cells to silence ERα, whereas this selective pressure caused MCF-7 cells to overexpress ERα. Further examination of alterations in gene expression by real-time PCR indicated that LCN2 increased by 8.6-fold and CRH decreased by 95.7%. These data provide further evidence in a second cell line that LCN2 is inversely regulated by ERα expression, and again we hypothesize that as T47D/LTED cells became increasingly ERα negative, that LCN2's promoter region correspondingly became demethylated. However, since CRH expression decreased as did ERα expression, it is unlikely that CRH's promoter methylation state is regulated by ERα, and therefore, we will no longer continue to evaluate CRH expression and its promoter methylation in future studies. However, we will search for other candidate genes whose promoter's methylation status is regulated via ERα.

To track changes in ERα and LCN2 expression and promoter methylation status as a function of time, another experiment to derive a second estrogen-deprived T47D polyclonal cell line termed T47D/LTED2 is currently being conducted, only this time RNA and genomic DNA samples are being collected every week. At the end of the current reporting period, the T47D cells have been deprived of estrogen for 8 weeks. By this time, the T47D cells already show no estrogenic responses in terms of proliferation. Additionally, over the 8 weeks of estrogen deprivation, ERα mRNA expression has steadily decreased such that it is only 11.7 % that of the parental cells, while LCN2 has steadily increased by 28.2-fold. Therefore, the results regarding ERα decreases and LCN2 increases as a result of estrogen deprivation are reproducible. Determination of the methylation status of ERα and LCN2 promoters is underway.

To further establish that changes in ERα expression regulates LCN2 expression, and in a larger context, regulates methylation of other target promoters to reprogram cells, we are again selecting the original T47D/LTED cells using DES to derive T47D/LTED/DES cells. Derivation of T47D/LTED/DES cells has been ongoing concurrently with derivation of T47D/LTED2 cells. After 8 weeks of DES exposure, DES once again stimulates growth of these T47D/LTED/DES cells. Importantly, the T47D/LTED/DES cells have re-expressed ERα (which increased by 143-fold relative to T47D/LTED cells and corresponds to 39% the level of wild-type T47D cells). Further, in the T47D/LTED/DES cells, the re-expression of ERα was accompanied by a 72.4% decrease in LCN2. Thus, an inverse relationship was again observed between ERα and LCN2

expression, consistent with our hypothesis that ERα mediates methylation of the LCN2 promoter to silence its expression.

In summary, we have used long-term estrogen deprivation to drive selection of MCF-7 and T47D cells, which exhibited persistent and substantially increased ERα expression (MCF-7/LTED) or silenced ERα expression (T47D/LTED) cells. Silencing of ERα expression was reproduced in a second experiment deriving T47D/LTED2 cells. Finally, endogenous ERα became re-expressed by long-term DES exposure of T47D/LTED cells to derive T47D/LTED/DES cells. In all cases, ERα and LCN2 expression were inversely related. Future experiments will focus on characterizing the methylation status of ERα and LCN2 as a function of time, and the mechanism of ERα-mediated methylation involving recruitment of DNA methyl-transferases (DNMTs) and/or histone deacetylase (HDAC) proteins to the promoter regions of target genes.

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 - 12/31/2011

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.

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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

Our goal is to identify markers of epithelial stem cells that will enable functional analysis of genes involved in stem cell regulation and label stem cells unambiguously *in vivo*. In our initial application, we reported the isolation of an antibody that recognizes a nuclear antigen (FC-NA) which is expressed at high levels in Follicle Stem Cells (FSCs) and their progeny in the fly ovary. In our first six months of funding, we found that FC-NA also is expressed in all cells of the fly embryo, suggesting that it may also be an important marker of fly embryonic stem cells. Moreover, we have established conditions for biochemical purification of FC-NA and are now poised to identify the purified protein by mass spectrometry analysis. A new postdoctoral fellow with extensive experience in biochemistry and mouse genetics will arrive in the lab September 1, 2010 to apply his expertise to the biochemical analysis of FC-NA and evaluation of its role in mammalian epithelial stem cells.

<u>Stem cell expression of FC-NA</u>

Although FC-NA is expressed at low levels in most cells of the fly ovary, its expression is dramatically upregulated in FSCs and is maintained in the differentiating follicle cells produced by asymmetric FSC divisions. The contrast in expression of FC-NA between FSCs and neighboring cells of the transient stem cell niche makes it possible to unambiguously identify FSCs for the first time. Moreover, this expression pattern suggests that upregulation of FC-NA in epithelial stem cells may be important for their function.

In order to molecularly identify FC-NA, we have taken a biochemical approach. Ovaries can be used as a protein source for biochemical purification, but the proximity of the gut and its resident proteases coupled with labor intensive dissection required for ovary isolation makes the system less than an ideal protein source. In flies, the developing embryo can be considered a bag of epithelial stem cells. All tissues are derived from a synchronized population of epithelial cells that differentiate to form specific organs. Since our hypothesis is that FC-NA is a general marker of epithelial stem cells, we reasoned that it might be expressed at high levels in embryonic progenitor cells, which are epithelial in origin. To test this, we analyzed patterns of FC-NA expression in fixed embryos at various stages of development. Specifically, embryos developing from eggs laid by wild-type female flies over a 24 hour time period were fixed and stained with antibodies against FC-NA. Co-labeling with antibodies against the Hedgehog receptor Smoothened revealed the segmentation patterning of mid-stage embryos and enabled determination of the developmental stage. This experiment clearly demonstrated high levels of expression of FC-NA in all cells of early embryos and a more restricted pattern later in

development (Figure 1). Highest levels of expression in late embryos were observed in the developing epithelial tissues, with highest levels observed in the gut.

These preliminary results support our idea that FC-NA may be an epithelial stem cell marker. Further, these results suggest that FC-NA marks embryonic stem cells, at least in the fly. Although our primary goals for this project are to molecularly identify FC-NA and determine its function in the well-characterized FSCs of the fly ovary, we plan to analyze its role in embryonic development in the future.

Biochemical purification of FC-NA

In initial experiments, we established conditions for isolating FC-NA from the fly ovary by immunoprecipitation using anti-FC-NA antibodies. Bands of 27kD, 40kD, 64kD, 72kD, 100kD, 108kD, and 135kD were observed, suggesting that the antibody was capable of immunoprecipitating proteins from tissue lysates (Figure 3A). The robust expression of FC-NA in early stages of embryonic development described above indicates that embryos also may be rich source of FC-NA protein for purification. Embryo collection techniques are straightforward and efficient and large volumes of protein (on the order of grams) can be collected with very little time or effort. To verify that the protein recognized by FC-NA antibodies in ovaries was the same in embryos, we compared the patterns of bands immunoprecipitated by the same antibody from the two different tissues. Proteins of approximately 27kD, 40kD, and 72kD were detected by Western blotting from both tissues (Figure 2). Additional proteins of 52kD and 56kD were observed in embryos but not in ovaries. Based on this analysis, our attention is focused on the three bands which are apparently the same in both tissues.

Our next step was to perform a preparative immunoprecipitation with FC-NA antibodies. Two grams of protein were isolated from 24 hour embryo collections. Anti-FC-NA immunoprecipitation was performed in the presence or absence of a peptide antigen that competes for binding of the antibody. Silver staining revealed a single protein band of approximately 40kD in the immunoprecipitated material that was lost in the presence of the competing peptide. These results suggest that the 40kD band observed in both embryos and ovaries is the FC-NA protein. We are now prepared to generate samples for analysis by mass spectrometry for definitive identification of FC-NA.

Screening for additional stem cell markers

FC-NA was isolated as part of a screen for new markers of epithelial stem cells. Further antibody and genetic screening in the lab has uncovered one additional marker of FSCs and two markers of the FSC support cells that produce FSC proliferation control factors. The FSC marker is in a fly line that contains a P-element insertion that encodes the Gal4 protein. Expression of Gal4 under control of the promoter into which the P-element is inserted drives expression of UAS-containing reporter genes FSCs and their progeny cells. Similarly, a Gal4-containing P-element inserted in a different locus drives reporter gene expression in FSC support cells, suggesting that it may be an important non-autonomous regulator of FSCs. Finally, we identified an antibody that recognizes a plasma membrane bound antigen in FSC support cells.

Current work is directed at molecularly identifying these three proteins using plasmid rescue techniques and determination of expression patterns in genetic mutants.

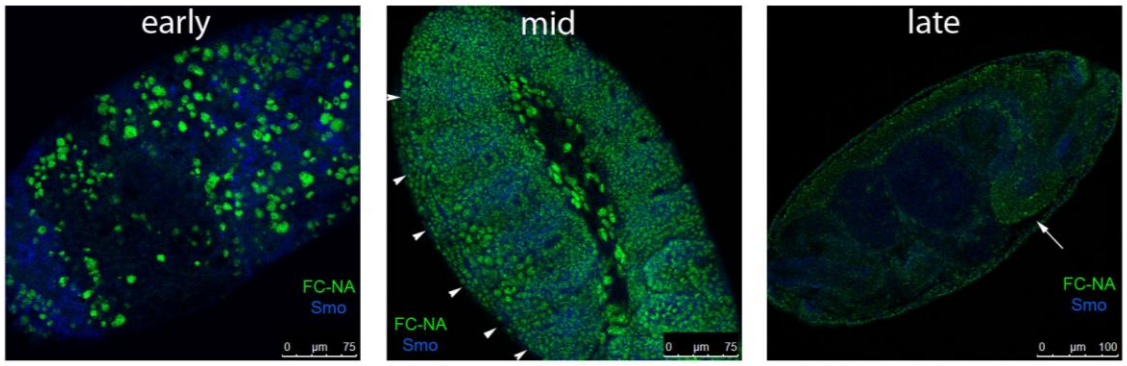

Figure 1: FC-NA is expressed in early fly embryos. Embryos fixed in 4% paraformaldehyde were stained with antibodies against FC-NA (green) and Smoothened (Smo, blue). In early embryos (left) nuclear expression of FC-NA is observed in most cells. In mid-stages of embryogenesis (center), when Smo marks stripes of cells that respond to Hedgehog signaling (arrowheads), FC-NA is still expressed in most cells. By late stage of embryogenesis (right), high levels of FC-NA are restricted to epithelial tissues including the developing gut (arrow).

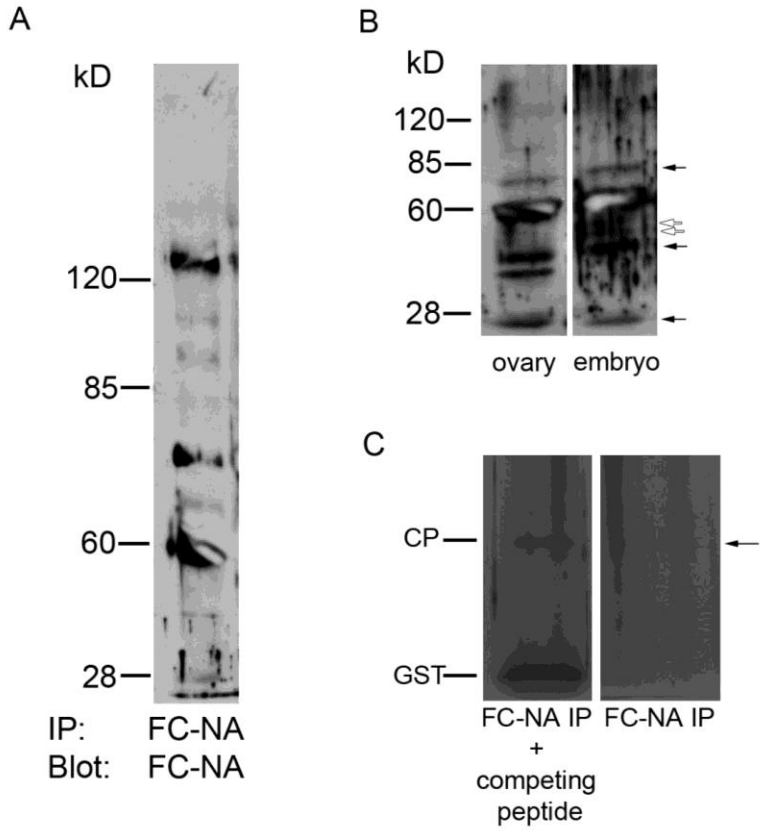

Figure 2: Biochemical characterization of FC-NA. A) Ovary lysates were subjected to anti-FC-NA immunoprecipitation followed by immunoblotting with FC-NA antibodies. Bands of 27kD, 40kD, 64kD, 72kD, 100kD, 108kD, and 135kD were observed. Molecular weight markers are indicated in Kilodaltons (kD), B) Ovary (right) or embryo (left) lysates were subjected to anti-FC-NA immunoprecipitation followed by immunoblotting with FC-NA antibodies. Proteins of approximately 27kD, 40kD and 72kD were detected in both tissues (black arrows). Additional proteins of 52kD and 56kD (white arrows) were observed in embryos but not in ovaries. C) Silver stain of FC-NA immunoprecipitated in the presence or absence of competing peptide (CP). The competing peptide is a GST-fusion protein. Both the 26kD GST alone and the competing peptide fusion protein (CP) are indicated. A 40kD band present in the IP only lane (arrow) was competed by the presence of the competing peptide.

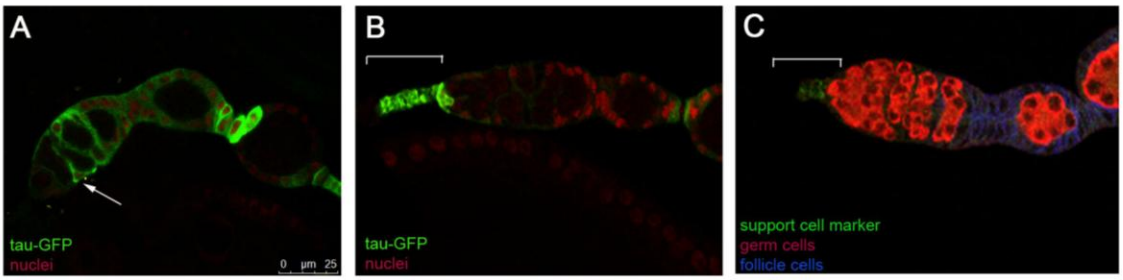

Figure 3: Markers of FSCs and their support cells identified in screens. A) Germaria isolated from flies bearing a Gal4-containing P-element insertion in an unidentified locus and UAS-tauGFP, which labels microtubules were stained with anti-GFP antibodies (green) and nuclear marker (red). Expression is seen in FSCs (arrow) and their progeny for the first three stages of oogenesis. B) Germaria isolated from flies bearing a Gal4-containing P-element insertion in a locus that drives UAS-tauGFP expression (green) in the terminal filament and cap cells (bracket) that support FSC proliferation from a distance. Nuclei are labeled (red). C) An antibody isolated in our screen recognizes a membrane-bound antigen (green) in terminal filament and cap cells (bracket). Germ cells (red) and follicle cells (blue) are also labeled.

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.

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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

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. To establish if *pu.1* or *gata1* regulated the expression of *g4*, we performed antisense morpholino knockdown of *gata1* and *pu.1* and examined whole mount *g4* RNA *in situ* hybridization (WISH) to examine the expression of *g4*. Our preliminary 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 indicate that *g4* is likely to be a new component of the network controlling hematopoietic progenitor cell differentiation. However, due to the high homology between *g4* family members, we question whether the *g4* WISH probe only recognizes *g4* RNA or may cross-react with other closely related transcripts. Thus, we are corroborating our WISH analysis of the morphant embryos with semi-quantitative or quantitative RT-PCR analysis using primers that are specific for each of the *g4*-related genes.

To test if *g4* is essential for hematopoietic development, we examined the expression pattern of *pu.1* and *gata1* in *g4* morphant embryos. Interestingly, at 24 hours post-fertilization, *g4* morphant embryos displayed decreased *gata1* expression (data not shown), indicating that *g4* is essential for normal *gata1* expression and that these factors have a reciprocal positive-regulatory relationship. Surprisingly, in contrast to *gata1* morphants that show an expansion of *pu.1*-positive cells in the erythroid blood-forming region of the embryo, *g4* morphants have a normal *pu.1* expression pattern (data not shown). Thus, the *g4* morphant does not simply mimic the *gata1* morphant, suggesting that G4 has additional functions beyond its partnership with Gata1. We are currently exploring possible mechanisms underlying this additional function.

Our studies highlight the similarities between zebrafish *gata1* and *g4* gene regulation. We hypothesize that there is a regulatory domain that is conserved in the promoters of these two genes. 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 domain 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.

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 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 morphant embryos have decreased erythropoiesis and the morpholinos that target multiple genes also cause morphological defects (data not shown). Morpholino-injected embryos will be used to test the effect on expression of each *g4* family member and as models to examine the ability of *in vitro* transcribed mRNA from each homolog to rescue the defects caused by gene knockdown. Interestingly, we have 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 suggest 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.

As a first step in testing if forced expression of individual *g4* family members and human homologs can rescue the defects in the morphant embryos, we have isolated the full-length genes for each family member and subcloned them into expression vectors. This effort proved to be difficult, but is now complete. Furthermore, by analyzing the synteny between the human and zebrafish genomes and the homology of zebrafish and human proteins (data not shown), we have identified two human factors that are putative homologs of the *g4* gene. We have obtained full-length clones of these candidates. These efforts will now allow us to investigate the effects of forced expression of *g4*, family members and the human genes during normal development and in the hematopoietic defects found in *g4* morphant embryos.

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

Working with the Molecular Modeling Facility at our Center, we have identified distinct regions of the G4 protein that are NOT conserved between family members as well as a stretch of 12 zinc-finger motifs that are HIGHLY conserved between family members (data not shown). The strategy that we originally proposed has been refined by this molecular modeling analysis, although the fundamental constructs that we are engineering remain the same as originally proposed. Due to the recent cloning of the full-length *g4* gene, we are currently engineering deletion constructs and a domain swap mutant form in which the 12 zinc-finger motifs of *g4* will be exchanged for those of a closely related family member. We plan to inject *in vitro* transcribed mRNA encoded by these constructs to test which forms can rescue the *g4* or *g4*-family morphants.

Research Project 5: Project Title and Purpose

Reducing the Burden of Hepatocellular Carcinoma: Identification of New Targets 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 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.

Anticipated Duration of Project

1/1/2010 - 12/31/2011

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 is to develop a method for the rapid identification of new targets for the development of drugs that can 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.

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.

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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. 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 arrest or cure liver cancers, and increase the survival rate of patients struck with this deadly disease.

Summary of Research Completed

Specific Aim 1. To develop conditions for the screening of primary cell cultures derived from human liver tumors

Efforts have been initiated to establish primary cultures from hepatoma tissue. We have obtained two tissue samples derived from hepatomas. In each case, following the resection, the tissue was transferred to a tube containing F-12 serum free media, put on ice and transported from the operating room to the laboratory. The tissue was then minced between two scalpels until finely chopped and then transferred to a flask containing collagenase-dispase liver digestion medium (InVitrogen) and incubated for 2 hours at 37°C. After incubation the cells were pelleted by centrifugation and washed four times, resuspended in HepatoZYME-SFM medium (InVitrogen) containing Cipro. A major problem with the cultures has been the contamination with erythrocytes, which reduced the plating efficiency. Efforts are under way to remove red blood cells with the help of Ficoll gradients.

Specific aim 2. To identify kinases required for survival of primary hepatocellular carcinomas

To enhance our chances to identify kinase(s) required for survival of hepatoma cells, we proposed to perform our screen in the presence of an already available therapeutic agent. To identify the appropriate drug, we have performed several screens with HeLa and hepatoma-derived Huh7 cells. Drugs that were tested included, the Smac mimic, LBW242, sorafenib and velcade. The drugs were also analyzed in combination with interferon alpha, interferon gamma and tumor necrosis factor alpha. The results showed that; i) Huh7 cells were generally much more resistant to drug induced cell death compared with HeLa cells; ii) sorafenib was effective in killing HeLa and Huh7 cells; velcade had no effect on either cell line; and, iii) LBW242 differentially induced cell death in HeLa, but not in Huh7 cells.

The observation that Huh7 cells were resistant to the smac mimic LBW242 was particularly interesting and raised the question about differences between Huh7 and HeLa cells in terms of their response to the drug. Since LBW242 induced apoptosis depends on TNFα, we investigated the effect of the cytokine in combination with LBW242 on the activation of caspase 3 and its downstream target PARP. The results showed that Huh7 cells poorly respond to TNFα compared with HeLa cells and that as a result, the combination of TNFα and LBW242 only weakly induces caspase 3. We interpret theses results to mean that a survival pathway is activated in Huh7 preventing TNF-induced apoptosis in the presence of LBW242. It is anticipated that we will be

able to identify the relevant pathways through the planned kinase screen in the presence of LBW242.