Fox Chase Cancer Center

Annual Progress Report: 2009 Formula Grant

Reporting Period

July 1, 2013 – December 31, 2013

Formula Grant Overview

The Institute for Cancer Research (formerly Fox Chase Cancer Center) received $3,477,323 in formula funds for the grant award period January 1, 2010 through December 31, 2013. 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.

Duration of Project

1/1/2010 - 12/31/2013

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.

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Other Participating Researchers

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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 timeframe 7/1/2013-12/31/2013, we made significant progress in all the Specific Aims outlined above.

Specific Aim 1. We previously confirmed the essential role of TDG and TDG glycosylase activity in development. Work conducted by us and other groups identifies TDG as a critical effector of DNA demethylation pathways initiated by enzymes of the TET family. While the prominent role of the TET-TDG axis in DNA demethylation is established, the detailed mechanisms are poorly understood. One critical demethylation event occurring during development is the stripping of epigenetic marks, including imprinting marks, on primordial germ cells (PGCs) upon entering the gonadal ridge at mid-gestation. In an effort to study the role of TDG in PGC development, gametogenesis and imprinting erasure, we inactivated TDG in developing PGCs. Due to the lethality of TDG homozygous inactivation, we achieved PGC-specific inactivation by crossing our TDG conditional knock-out mice with BLIMP1::Cre transgenic mice. Timed matings were then conducted between sires with genotype TDG-/flox BLIMP1::Cre positive and wild type C57Bl/6 dams. The mothers were sacrificed at midgestation (E11.5 12.5). While some embryos appeared normal, other embryos exhibited reduced growth, with or without hemorrhagic features, malformation and resorption (embryonic lethality), i.e. variable phenotypes previously described for paternal TET1 knock-out offspring. These observations indicate that TDG is required for proper PGC development and strongly suggest a role of TDG in imprinting erasure downstream of TET1.

Specific Aim 2. In the past six months, we have continued the crosses between the TDG- and MED1-mutant mice with the APC-Min mice and FABP-Cre transgenic mice in order to generate the $TDG^{+/-}$ $MED1^{+/-}$ FABP-Cre mice that will be interbred with the $TDG^{+/flox}$ $MED1^{+/-}$ Apc-Min mice, in turn, to generate the experimental mice.

Specific Aim 3. We have been studying the functional significance of the novel modification of MED1, i.e. sumoylation, that we previously discovered. In the past six months, we confirmed our observation that the sumoylation pattern and extent is differentially changed in response to various DNA damaging agents, such as 5-fluorouracil, cisplatin, gamma-irradiation and N-methyl-N-nitrosourea. We also began writing a manuscript describing these observations.

Specific Aim 4. We had previously described, in addition to AID and GADD45a, other components of the TDG demethylating complex, including proteins previously known to play an important role in DNA repair. In the past period, we developed a robust in vitro assay to measure DNA demethylation. This is a fluorescence-based bi-enzymatic assay, amenable to

kinetic analysis. The assay employs a hairpin-shaped molecular beacon carrying a G:T or G:U mismatch (substrate) and makes use of highly active preparations of recombinant TDG and recombinant AP endonuclease (APE). In the folded hairpin substrate, the fluorescence of 6-FAM, used as fluorescent label at the 5' end, is quenched by a dabsyl "black hole" moiety at the 3' end. Upon removal of the mismatched T or U by TDG, and incision of the resulting apurinic/apyrimidinic (AP site) by APE, a short oligonucleotide containing 6-FAM is released. The resulting fluorescence is monitored by real-time qPCR over a 2-hour incubation period at 37ºC, providing a sensitive and quantitative measurement of repair activity. By using cell-free extracts instead of recombinant enzymes, this assay can be modified to accurately measure the base excision repair activity of extracts. We reasoned that this assay could be adapted to detect DNA demethylation, if the hairpin substrate was modified to contain a single G:5-methylcytosine (5mC) pair in a CpG context. In this case, demethylation would reflect oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) by TET activity, and of (a fraction of) 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), with removal of the two latter bases opposite G by TDG activity. Incision of the AP site by APE activity would trigger the fluorescence readout. We initially applied this assay to extracts from wild type and TDG knock-out mouse embryo fibroblasts (MEFs). Demethylating activity is present in wild type MEF extracts, but is largely absent in TDG knock-out MEFs .

Specific Aim 5. We previously interbred double heterozygous $TDG^{+/-}$ $MED1^{+/-}$ mice, in order to generate experimental TDG-MED1-double mutant embryos. TDG-MED1-double mutant embryos appear to have a more severe phenotype than TDG-single mutant embryos. In the past period, we began experiments to determine the requirement of TDG and MED1 for cellular reprogramming: single- and double-mutant mouse embryo fibroblasts were infected with polycistronic virus expressing the four reprogramming factors (Oct4, Sox2, cMyc, and Klf4). We are currently determining whether the cells generated have indeed properties of induced pluripotent stem cells (iPSCs).

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.

Duration of Project

1/1/2010 - 12/31/2012

Summary of Research Completed

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

Research Project 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.

Duration of Project

1/1/2010 – 12/31/2013

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

The goal of this work is to identify novel markers of epithelial stem cells. During this reporting period, we focused on completing studies aimed at utilizing a series of new tools for stem cell identification in determining the specific function of individual genes on stem cell function. This work has been submitted to the journal, Genetics. Going forward, we will use the tools developed throughout this project to identify novel genes that control stem cell function and their mechanisms of action.

Novel stem cell markers:

A major challenge in the stem cell field when this project started was the lack of definitive molecular markers that distinguish stem cells from neighboring cells. We knew from our previous studies that defects in integrin adhesion signaling had dramatic effects on the morphology, positioning, and proliferation rates of Follicle Stem Cells (FSCs) in the Drosophila ovary. However, in cases such as this where FSC morphology or positioning were altered due to genetic changes, it was difficult to identify a true mutant stem cell from a lineage labeled cell that was displaced from the normal niche and lost stem cell properties. Previously large scale genetic screens resulted in the generation of hundreds of transgenic fly lines bearing random insertions of the yeast transcription factor Gal4 in enhancer elements throughout the genome. The patterns of Gal4 expression throughout fly development depend on the normal expression of the enhancers adjacent to the insertion site. In most cases, expression patterns have been mapped in developing embryos and select developing tissues, but patterns of expression within FSCs and the cells that control their behavior in the ovary have not been clearly defined. We performed a genetic screen with these Gal4 lines and identified 21 lines with specific expression in either 1) FSCs, 2) somatic escort cells that directly contact FSCs and form part of its niche, 3) terminal filament and cap cells (collectively called apical cells) that produce the growth factors that control FSC proliferation, and 4) follicle cells, the daughters of FSC divisions.

In previous reports, we have described the use of newly identified Gal4 lines in the delineation of a novel cholesterol-dependent signal transduction pathway that controls FSC proliferation. In the final six months of the funding period, we focused our attention on FSC-specific Gal4 lines. Three lines were identified that exhibited expression in FSCs. Two lines exhibited highly similar patterns of expression in apical cells, escort cells and FSCs, and it turned out that they represented insertions into the same gene. The third line, 109-30 Gal4, exhibited expression in FSCs and their immediate daughter cells. Since our goal was to develop methods for genetically manipulating FSCs without affecting other cells that support their function, we were initially disappointed that we did not uncover a Gal4 line that was specific to FSCs alone. This may suggest that adult epithelial stem cells retain genetic characteristics of both the niche and the differentiating progeny cells, perhaps making them unique relative to all other cells in their environment.

In order to develop methods for specifically targeting FSCs, however, we took advantage of our ability to control FSC proliferation by diet. In starved flies, FSCs do not divide. Feeding stimulated proliferation within 3 hours, with peak levels of proliferation occurring at six hours. Using the Gal4 driver that is expressed in FSCs and their daughter cells, we marked FSCs

genetically, and then placed the flies under nutrient-restricted conditions to arrest FSC division. After three days, flies were fed to stimulate proliferation and the dynamics of FSC proliferation were assessed in wild-type versus integrin mutant FSCs. This method was impressively successful, enabling us to define 4 successive steps in FSC division after a period of starvation. Integrins were required very early in this process, indicating that adhesion is critical for all aspects of FSC division. This work has been submitted to the journal Genetics.

While the first method for assessing the effects of mutations on FSCs has significant merit for defining the roles of individual genes in FSC proliferation after a period of starvation, this technique is limited for use under steady state conditions. As the 109-30 Gal4 driver is also expressed in the differentiating daughter cells of FSCs, many cells are labeled in steady state conditions, as marked FSCs generate genetically identical daughters that also express the label. To address this issue, we utilized the second set of FSC Gal4 lines that are expressed in supporting escort cells and FSCs, but not in the daughters of FSC division. A lucky property of the system is that escort cells divide very infrequently relative to FSCs. Therefore, we took advantage of available tools for generating marked clones of cells using mitotic recombination. The use of a Gal4 line that is expressed only in non-dividing cells and dividing FSCs made it possible to specifically and uniquely label FSCs for analysis. This approach gave identical results in terms of defining the steps of FSC proliferation after a period of starvation, and also regarding the role of integrins in FSC proliferation control. Importantly, this approach will be useful for assessment of the roles of individual genes in FSC proliferation control in steady state conditions, as the FSCs will be easily distinguishable from their daughter cells which do not express the marker. Thus, in this reporting period, we have developed two new approaches for marking and genetically manipulating FSCs that will be useful to the broader fly oogenesis community and allow us to define novel signaling pathways that control epithelial stem cell function in vivo.

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.

Duration of Project

1/1/2010 - 12/31/2011

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's

Tobacco Settlement/Act 77 web page at http://www.health.state.pa.us/cure.

Research Project 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.

Duration of Project

1/1/2010 – 6/30/2012

Summary of Research Completed

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