Pennsylvania State University

Annual Progress Report: 2009 Formula Grant

Reporting Period

January 1, 2010 – June 30, 2010

Formula Grant Overview

The Pennsylvania State University received $8,412,824 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

Modifications of Histone H3 in Diabetic Retinopathy - The overall purposes of this project are twofold. First we plan to define some of the changes in chromatin structure that occur in the retinas of an animal model of Type I diabetes. This will give us fundamental information about the range of retinal changes seen in this disease and will offer new insights into the types of change that need to be treated. The second objective is to monitor how treatment with insulin can reverse these changes and restore the retina to its normal state. This set of experiments will test the hypothesis that some of the retinal changes in diabetes become irreversible with time.

Anticipated Duration of Project

1/1/2010 – 12/31/2010

Project Overview

The overall goal of this research project is to define a specific set of molecular changes that occur in a mouse model of Type I diabetes and to test the hypothesis that some of these changes become irreversible with longer periods of hyperglycemia. To achieve this goal the project has two specific aims.

Aim 1: We will test the hypothesis that two histone modifications, H3K9Ac and H3K4me2, will be detected at different sites in the genomes of normal and diabetic mouse retina. To test this hypothesis we will carry out three sets of experiments. First, to better define the appropriate ages for more detailed molecular experiments we will carry out an immunocytochemical analysis using antibodies that recognize the two modifications. We will test tissue from animals of 1 to 20 weeks of age. Second, we will take animals of defined ages and carry out a ChIP-seq analysis. The approximately 10 million short DNA sequences resulting from each experiment will be aligned to the mouse genome to give us a quantitative map of the frequency of occurrence of each modified histone at each site across the genome. We will then use custom algorithms to search for differences between control and diabetic samples. We will identify

genes associated with these modified histone changes by identifying coding sequences -2.5kb to +1kb from the modified histone binding sites. We will cluster genes associated with histone modification changes using bioinformatic approaches to identify any specific categories.

Aim 2: We will test the hypothesis that restoration of normal glucose levels by implantation of insulin pellets will cause some, but not all, epigenetic changes to revert to normal. The phenomenon of "metabolic memory" in which extended periods of poor glycemic control can lead to progressive retinopathy even after insulin therapy is an important issue as it affects the efficacy of any treatment. We will implant insulin pellets in mice either at the onset of hyperglycemia (4 weeks) or after retinopathy and vascular changes are well established (10 weeks). At 16 weeks, animals will be examined for reversion of changes in H3 modifications using the same ChIP-seq methods. In this way we will identify genes whose expression may be subject to metabolic memory. Such information is key to developing therapies to be used in conjunction with insulin to prevent retinopathy even after glycemic control.

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Expected Research Outcomes and Benefits

The anticipated outcomes of this project are 1) a description of two specific epigenetic changes across the whole genome that will define the retinal changes seen in a mouse model of Type I diabetes; 2) an understanding of how these changes might alter expression of genes in the retina; 3) a description of the effectiveness of insulin in restoring the epigenome and gene expression to a normal condition. The benefits from this project are twofold. First, the result will give us new information about the ways in which lack of insulin and hyperglycemia affect the structure of the retinal genome. From these results we will develop a fuller understanding of the ways in which diabetes can affect the expression of many genes in the retina. Second, we will have quantitative information about the ways in which insulin can reverse the retinal effects of diabetes at the early and late stages of the disease.

The health benefits of this project are that, first, we will define new therapeutic targets; second, we will define a set of biomarkers by which we can define the disease progression and the effectiveness of any therapy; and third, we will provide an accurate assessment of the ability of insulin therapy to reverse the damage to the retina at early and late stages of the disease.

Together these outcomes will also speed the development and characterization of new therapies to treat diabetic retinopathy.

Summary of Research Completed

The overall goal of this research project is to define a specific set of molecular changes in epigenetic posttranslational modification of core histones H3 and H4 that occur in a mouse model of Type I diabetes and to test the hypothesis that some of these changes become irreversible with longer periods of hyperglycemia in the retina. During the first half year of the project we generated a colony of our mouse model for Type I diabetes (Ins2 –Akita/+ mice), developed methods for ChIP-Seq on retina tissue and carried out ChIP-Seq for several modifications of histones H3 and H4.

1.Animal model of type I diabetes.

All mice were maintained by the Penn State University College of Medicine Animal Facility in accordance with IACUC guidelines under specific pathogen-free conditions and monitored by quarterly sentinel testing. We use C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) as wild type animals. C57BL/6J-Ins2 Akita/+ mice (The Jackson Laboratory, Bar Harbor, ME) are continuously maintained by crossing with C57BL/6j stock at each generation. Due to the asynchronous replication and resulting inconsistency of the diabetes in Ins2 Akita/+ female mice, only male mice were used for the experiments. Akita designates a point mutation in Ins2 gene which leads to accumulation of misfolded Ins2 protein and pancreatic beta cells death. Ins2 Akita mice are typically hyperglycemic by 5 weeks of age and were phenotyped in our experiments by blood glucose level at this age. At 16 weeks of age Akita mice and wild type littermates were phenotyped once more for verification and retinas were isolated from the animals.

2. *ChIP preparation.*

Retinas were cross-linked with 1% formaldehyde and digested into mononucleosomes. For micrococcal nuclease digestion, an aliquot of nuclear preparation containing 0.5mg/ml DNA was resuspended in 1ml RSB buffer and 0.5mM PMSF (phenylmethylsulfonyl fluoride, a protease inhibitor). Calcium chloride was added to a final concentration of 1mM, and micrococcal nuclease (Nuclease S7, Roche) was added at 2.5 units/ml. The reaction was carried out at 37ºC for 1hour and terminated by adding EDTA to a final concentration of 5mM. After centrifugation for 7 min at 5,000xg, the pellet containing almost all of the chromatin was resuspended in ChIP lysis buffer, sonicated briefly (2 times for 10 sec) and used directly for chromatin immunoprecipitation (ChIP) with several types of antibodies against histones H3 and H4 modification. Using wild type mouse retinas, we have tested the purities of mono-nucleosomes after micrococcal nuclease digestion at different time points as shown in Fig. 1.

After reversing the crosslinks, mononucleosome DNA was purified for SOLiD fragment library generation by ligating adaptors through end repair. After quality control, libraries were sequenced on the SOLiD™ 3 system. All sequencing of ChIP libraries was performed in the Genome Core Facility, Huck Institutes for Life Sciences at the University Park Campus of the

Pennsylvania State University**.** After sequencing ChIP DNA on SOLiD sequencer the reads were mapped to mouse genome with NextGEne software.

3. *Preliminary results.*

To date we have sequenced 4 ChIP samples from Akita mice and wild type littermates. Table 1 summarizes the numbers of matched and unmatched reads for each experiment. These samples include the H3K4me2 proposed in the first specific aim. We have prepared samples for H3K9Ac, the other modification listed in Aim 1, and these have been submitted for SOLiD sequencing.

The matched reads were then aligned to the mouse genome using NextGene software. We are developing custom algorithms to exclude artificially high signals due to repetitive elements in the genome. There may be important information in these data but we currently have no good way to analyze it. We are focusing on coding regions and adjacent promoter regions to determine chromatin changes that might be associated with diabetes. This analysis is ongoing but below we have included two examples in which we see increased histone modification at specific genes.

A complete list of genes with differing histone modification patterns is being assembled. As with our previous developmental ChIP-Seq experiments, we will verify significant changes by standard ChIP analysis.

Though preliminary, our analysis is showing that there are chromatin changes between diabetic and normal mice.

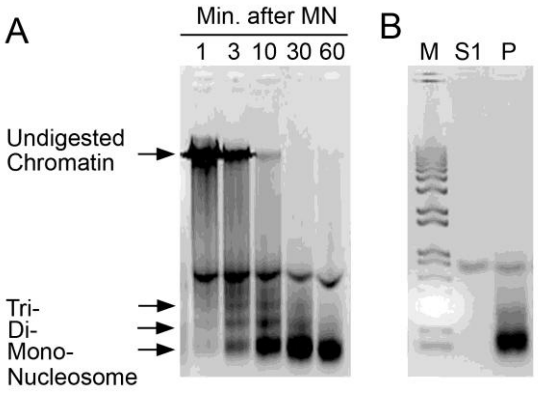


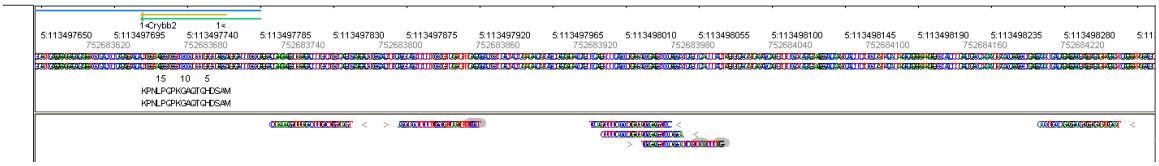
Fig.1: Verification of nuclease digestion. A**.** Time series of micrococcal nuclease digestion. B. Small 20ul aliquots of supernatant S1 (treated with Proteinase K and SDS as in test MN digest above) and pellet P (resuspended in ChIP lysis buffer) were checked on 1% agarose electrophoresis in Tris-Acetate-EDTA buffer. Pellet P contained chromatin consisting of mononucleosomes and was subjected to ChIP.

TABLE 1

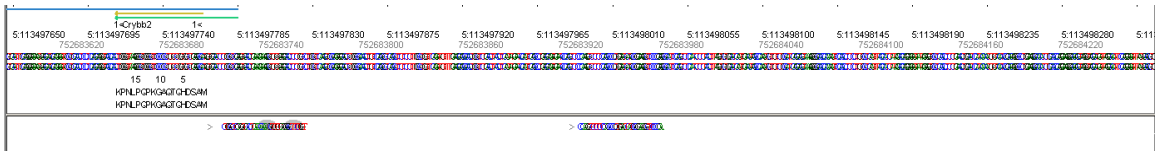
Probe #	Genotype	Antibody	Butyrate	perfect	match	unmatch	covered bases
1	wt	H4K12ac	No	2102646	4712444	4687192	239271758
2	akita	H4K12ac	No	3999849	8707115	8225320	401264216
3	wt	H4K12ac	Yes	4303379	9331330	8419037	420104416
4	akita	H4K12ac	Yes	3631525	7994367	7162542	371570825
5	wt	H3K4me2	No	3935320	8768099	8697627	401890426
6	akita	H3K4me2	No	5641485	11990867	10950423	519161745
7	wt	H3K27me3	Yes	4103642	8935444	8842843	404894920
8	akita	H3K27me3	Yes	2768904	6036316	5984453	294932431

Fig.2. An example for comparison distribution of reads for Akita and wild type littermates for H3K4me2 ChIP-Seq.

A. Beginning of gene crystalline betab2 in sample from Ins2 Akita/+ mice retina.

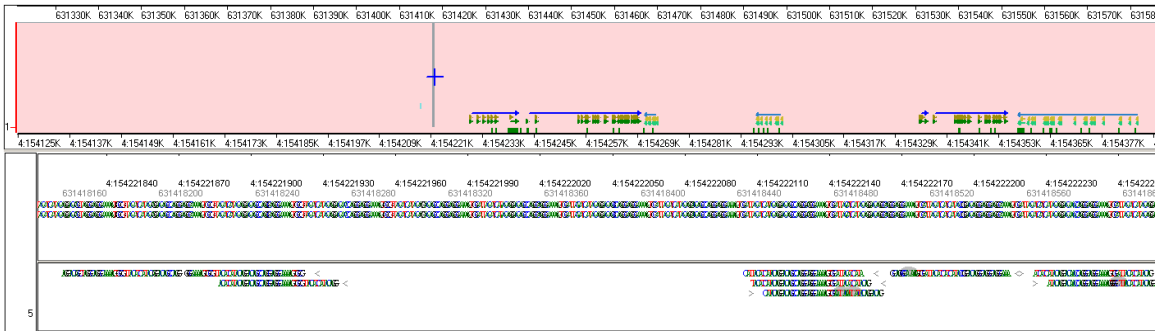


B. Beginning of gene crystalline betab2 in sample from wt mice retina.

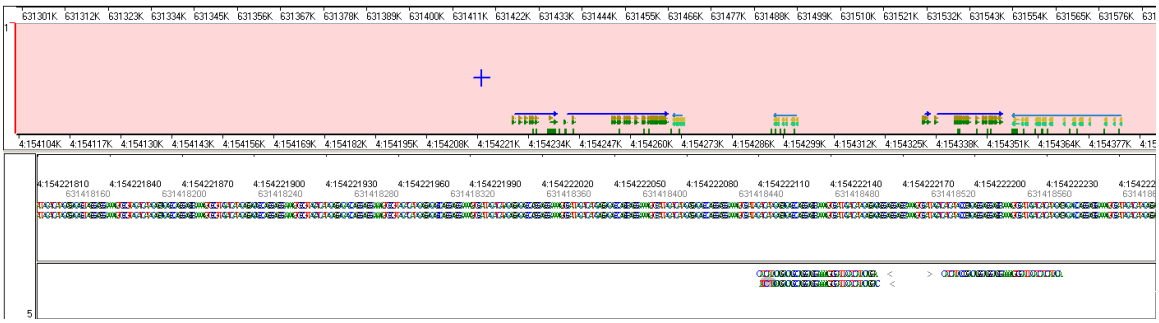


More reads in crystalline promoter area in the Akita sample could suggest more open chromatin structure and upregulation of this gene in the diabetic animals.

C. Region of chromosome 4 with matched reads in sample from Ins2 Akita/+ mice retina.



D. Same region in sample from wt mice retina.



More reads in this position could suggest more open chromatin structure in diabetes.

Research Project 2: Project Title and Purpose

Brain and Behavior in Early Iron Deficiency - Aggressive iron repletion following iron deficiency in infancy will result in excessive accumulation of this potential neurotoxin in the brain and is associated with alteration in brain genomic level and myelin composition. The purpose of this project is to determine if aggressive dietary iron intervention to replenish brain iron concentrations in formerly iron deficient animals is neurotoxic.

Anticipated Duration of Project

1/1/2010 – 12/31/2010

Project Overview

Our *long-term objective* is to determine *when* and *how much iron to give* to prevent the long-term neurobehavioral sequelae of early iron deficiency (ID). There is the potential for neurotoxicity if excessive iron is given to treat early ID. Thus, the *specific aim* of this study is to determine if larger amounts of iron taken up by brain tissue during aggressive dietary iron treatment result in changes in brain genomic and structural sequelae. We *hypothesize* that aggressive iron intervention during lactation is associated with neurotoxicity.

Strategies: We will assess the early ID in rat pups by cross-fostering of rat pup to mothers given <5 ppm Fe (ID group) or 50 ppm Fe diet (control group). Pups with early ID will receive the 50 ppm (control) or 1000 ppm iron diet (high iron treatment) between P8 and P21. We chose P8 to represent the rodent neuronal equivalency to the intervention being done in the human study at 6 weeks post-partum. The dietary levels of iron to achieve "iron loading" of the brain through this route was estimated from published data where lactating dams were provided from 400-20,000 ppm Fe.

To determine the consequences of aggressive iron repletion, we will measure *brain iron concentration, targeted gene expression profiles for oxidative stress and myelin-associated protein expression* at P21, P90 and 6 months of age. We chose P21 to document the model's acute effects, P90 to document the persistence of the genomic and myelination and 6 months of age to document long-term effects of aggressive iron repletion. Brain iron concentration will be measured with atomic absorption spectrophotometry. The gene expression profile of the brain will target the oxidative stress gene and be determined with gene array. We will use RT-PCR to verify the changes from the gene array experiments. Myelin analyses will be focused on the expression of myelin-associated proteins (Myelin Base protein, CNPase and Proteolipid protein) in white matter regions. Measurements of brain iron concentration, gene expression and myelin composition in two iron treatment groups (50 and 1000 ppm Fe) will be compared to the control groups (never ID) and H67D mice. We will include H67D mice because the H63D HFE or hemochromatosis gene variant is associated with iron overload and increased oxidative stress and it has been proposed as a genetic modifier for risk of neurodegenerative disorders. Therefore, comparing gene expression and myelin-analyses of the P8 treated group given severe iron intervention (1000 ppm) to H67D mice will further elucidate the potential for neurotoxicity if excessive iron is given to treat early ID.

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Expected Research Outcomes and Benefits

We anticipate that similar to H67D mice, which have significant increases in brain and liver iron concentration (preliminary data), P8 treated group given the severe degree of iron treatment (1000 ppm) will have increased brain iron concentration due to enhanced iron transport upregulated by the early ID. The cause of the increased transport will be a future direction for our analyses. P8 treated rats given 1000 ppm iron are also expected to have alterations in gene expression in the brain and myelin composition compared to the control groups (50 ppm Fe treatment and never ID groups). The interpretation is that aggressive iron repletion is associated with potential neurotoxicity if P8 treated rats given severe iron intervention display increased iron concentration and have similar pattern in alteration in expression of genes and myelin-associated proteins as H67D mice, an *in vivo* model for human H63D mutation, which is proposed to be a genetic modifier for risk of neurodegenerative diseases.

Therefore, this project will reveal if aggressive repletion of iron is associated with neurotoxicity. From the gene array analysis, we will learn the molecular footprint of brain iron overload resulting from aggressive iron intervention and the potential use of an antioxidant therapy with the iron supplementation. These data will help inform the future analyses being performed at the protein level suggesting mechanisms underlying these higher order analyses, including behavior, and the level at which intervention strategies must be targeted. The project may narrow down the optimal dosing and timing of iron treatment that is sufficient but not neurotoxic. Therefore, findings from this study will inform the non-human primate and human projects with respect to the efficacy and safety of iron treatment in young mammals and it may also have important public health implications.

Summary of Research Completed

We have made progress in two areas of the planned study. First, in the rat studies, it was not technically possible to get feed for the rats at 1000ppm levels and we had to revise protocol to feed 400ppm which is more physiologically realistic. Because of this change in protocol, we have had a slower than expected start. Rat samples are currently being collected but no analyses have been performed to date. We anticipate these animals will be available by October of 2010.

The second portion of the study involved using the new mouse line we created that carries the H67D mutation of the HFE gene (the mouse analogue of the human H63D gene) by first knocking out the HFE wt gene and then knocking in the H67D mutation. The mice are viable and grow into adulthood. The first set of analyses was to determine if the mutation was functional and altered brain iron status. We determined the total iron level in the brains of 6-month-old wild type (+/+), heterozygous (+/H67D) and homozygous (H67D/H67D) H67D mice using atomic absorption spectrophotometry (AA).

Total brain iron level was 22% higher in H67D/H67D and 18.6% higher in +/H67D mice compared to +/+ mice. Total liver iron level was also 40% higher in H67D/H67D mice than in +/+ mice. These results provide evidence that the allelic variant is functional (Figure 1).

We demonstrated that there were changes in iron management protein expressions in brains of 6-month-old H67D/H67D mice compared to the wild type. Total H-ferritin expression was significantly increased while the expressions of divalent metal transporter (DMT-1) and transferrin receptor (TfR) tended to decrease in H67D/H67D mice compared to the wild type (Figure 2). DMT-1 immunoreactivity was decreased in the motor cortex, striatum and cerebellum while TfR immunoreactivity was decreased in the motor cortex of H67D/H67D mice (data not shown). These findings are consistent with the increased brain iron level in H67D knock-in mice.

We previously reported abnormal glutamatergic secretion and release in the H63D cells. Because glutamate toxicity is considered part of the pathogenic mechanism in a number of neurodegenerative diseases, we wanted to interrogate the potential for glutamate toxicity in association with the in vivo model. We, therefore, determined the expression of the cystine glutamate antiporter, xCT, which is associated with glutamate release and oxidative stress. Increased xCT antiporter level was observed in the brains of H67D/H67D compared to +/+ mice (Figure 2D) similar to that found in the cell lines.

These studies demonstrate that the H67D knock-in mutation is functional and challenges the long held paradigm that the brain is protected from HFE mutations. Collectively, these data support our hypothesis that the presence of this HFE gene variant creates a supportive and enabling milieu for neuropathogenic processes and justify our planned studies that exposure to a high iron diet in the presence of this mutation could significantly and negatively impact neuronal function. The iron exposure studies will begin in the second half of this funding period.

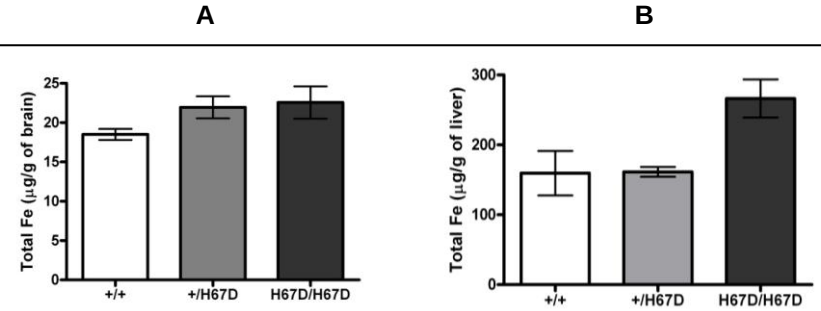

Figure 1. Total iron concentration was 22% increased in the brain (A) and 40% increased in the liver (B) of H67D/H67D mice compared to the wild-type (+/+) mice. Bars represent mean ± standard error. (* = p < 0.05; n = 4-6 per genotype).

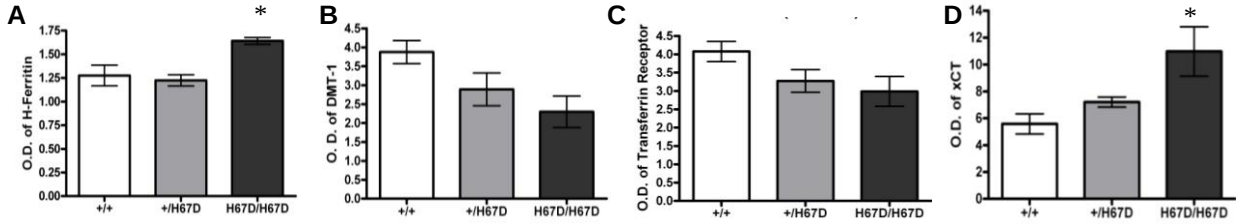

Figure 2. *A*: H-ferritin expression was 22% increased in H67D/H67D compared to the wild type mice. *B, C*: DMT-1 and TfR expression tended to decrease in H67D/H67D compared to the wild type mice. D: xCT expression was 49% increased in H67D/H67D compared to the wild type mice. Bars represent mean ± standard error. (* = p < 0.05; n = 4-6 per genotype).

Research Project 3: Project Title and Purpose

Cell Signaling Mechanisms Necessary for High-Fidelity Replication of Repetitive Sequences - The integrity of an organism depends on faithful replication of the genome with every cell division. The human genome contains six billion bases, many of which can be present in structures that make it difficult for replicative enzymes to work efficiently and accurately. Human cells have 15 different DNA polymerases that function to copy the genome. Mutations caused by polymerase errors within repetitive microsatellite sequences pose a significant threat to genome integrity, as is evident in colorectal cancers. The goal of this project is to elucidate the polymerases and cell signaling mechanisms that are necessary for high-fidelity replication of microsatellites. Understanding the dynamics of microsatellite replication will lead to new approaches for detecting and treating colorectal cancer, one of the most common cancers in the United States and Pennsylvania.

Anticipated Duration of Project

1/1/2010 - 12/31/2010

Project Overview

Broad objective: To elucidate the polymerases and cell signaling mechanisms that are necessary for high-fidelity replication of microsatellites.

Specific Aim : To test the hypothesis that Y-family polymerases, Pol κ and Pol η, are required for accurate microsatellite replication, using a human cell culture model system.
Subaim 1: To determine the effects of altered Y-family polymerase levels on microsatellite mutagenesis.
Subaim 2: To determine the effect of mutations in the ubiquitin binding zinc-finger (UBZ) domain of Pol κ on microsatellite mutagenesis.

Rationale: Replicative polymerases are inhibited by microsatellite sequences, and other DNA sequences that form non-B structures require Pol κ and/or Pol η for effective replication. Our working model is that the faithful replication of microsatellite sequences necessitates coordination of several polymerases, including Y family polymerases. Switching among cellular polymerases has been proposed to be regulated by ubiquitination.

Experimental approach: To examine the roles of Pol κ and Pol η in microsatellite replication (Subaim 1), we will engineer human colorectal carcinoma cell lines to increase and decrease levels of the two polymerases Pol κ and Pol η, both individually and in combination. To determine the role of monoubiquitination of PCNA in signaling polymerase switching (Subaim 2), colorectal carcinoma cell lines will be created in which the conserved aspartic acid (D644) of the Pol κ UBZ domain is mutated to alanine. To measure mutagenesis in both aims, we will use a shuttle vector assay developed in our laboratory for determining mutation frequencies of both microsatellite and non-repetitive coding sequences. Mutation rates at the microsatellite sequences will be compared with those in the coding region of the HSV-tk gene in at least three independent determinations for each parental cell line and derivative.

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Expected Research Outcomes and Benefits

The immediate scientific outcome of this project is to define the specific roles of Pol κ and Pol η in human genome stability. The importance of Y-family DNA polymerases to genome stability is illustrated in disorders resulting from their absence. For example, DNA Pol η is mutated in the

hereditary cancer syndrome, xeroderma pigmentosum-variant; these patients are very susceptible to sunlight-induced skin cancers. Previous research on Y-family polymerases has focused almost exclusively on DNA damage tolerance. Our working hypothesis states that microsatellite sequences are recognized by a cell in a similar manner as damaged DNA, and that Y family polymerases are activated to assist in replication of microsatellites. Our work will determine whether altered DNA polymerase levels are tolerated in tumor cells, and what mutational effects can be expected by alterations in their regulation.

After this model system is established, it can be used to explore the role of other polymerases in microsatellite replication as well as the role of the cell cycle regulators, p53 and p21. Several potential health benefits of our research can be expected in the long-term. First, by determining the mechanisms of DNA polymerase alterations in tumors, new clinical tests may be developed to aid diagnosis and prognosis, especially of colorectal cancers that display microsatellite instability. Second, since altered DNA polymerase levels are implicated in resistance to cancer therapy, our research could be used to individualize patient treatment options, with the goal of optimizing an anti-tumor response.

Summary of Research Completed

A. Manipulation of Y family polymerase levels in human colon cancer cell lines (Subaim 1)

1. *Choice and characterization of cell lines for use in the model*.

Two well described human colorectal cancer (CRC)-derived cell lines were chosen for this study. HCT-116 cells do not express the mismatch repair (MMR) protein MLH1, and consequently have a greatly elevated mutation frequency and a microsatellite instability high phenotype. DLD-1 cells harbor a mutation in the MSH6 MMR gene, and display an elevated mutation frequency. DLD-1 cells display microsatellite instability at mononucleotide repeats, but are relatively stable at dinucleotide repeats.

We are characterizing the gene and protein expression of endogenous Pol κ and Pol η in these cell lines. To date, we have focused on Pol κ. We have examined pol κ protein levels via Western analyses, and found that the polymerase is expressed in the nuclear fraction of both HCT-116 and DLD-1 cells. However, because these are cancer cell lines, the possibility exists that they harbor a mutation within the gene. To test for the presence of genomic mutations, we created PCR primers to amplify each of the 15 POLK gene exons. We have successfully sequenced ~60% of the exons from both HCT116 and DLD1 genomic DNA, and have found no mutations. Some of the DNA sequences from the PCR reactions were ambiguous. We are in the process of cloning the PCR fragments of the remaining exons into pDrive, using the Qiagen PCR Cloning kit, and sequencing independent clones from each exon to test whether there are mutations present in a heterozygous state.

2. *Establishing culture conditions for genetic manipulation of CRC cell lines*.

The molecular genetic experiments used in this study utilize both transient and stable DNA transfection techniques. We performed initial experiments using a GFP expression plasmid (pEGFP-N1) and the Fugene lipofectin reagent to determine the optimal conditions needed to achieve very high (>80%) transfection efficiencies for each cell line. Optimal Fugene:DNA ratios for HCT-116 and DLD-1 cells were determined to be 3:2 and 4:2, respectively. Next, we

determined cell survival in the presence of varying concentrations of the antibiotics G418 and hygromycin. Effective drug concentrations for selection of stable transformants of HCT-116 and DLD-1 were determined to be, respectively: 100 and 300 μg/ml for hygromycin and 0.3 and 1.0 mg/ml for G418.

3. *Establishing the tet-on system for regulating Y family polymerase expression.*
Overexpression of Y family polymerases may be deleterious to cell survival. Therefore, we are using a system in which polymerase expression can be experimentally induced. We have chosen to use the pTetOnAdvanced system from Clontech.
3.1. Isolation of DpT, HpT clones: DLD1 and HCT116 cells were transfected with the pTetOn regulation vector. Selection of stable transfectants was performed using 1.0mg/ml G418, and independent clones were isolated by limiting dilution. Clones of each cell line (29 for HCT116, named HpT, and 8 for DLD1, named DpT) were isolated from 1 cell/well or 2 cell/well dilutions, and maintained in the presence of 0.5 mg/ml G418.
3.2. Characterization of clones using a GFP vector: To screen the clones for doxycycline inducibility, we created a Tet-On GFP expression vector. The pEGFP-N1 and pTRE tight plasmids were digested with NotI and EcoRI, and the 0.7kB fragment from pEGFP-N1 was ligated into pTRE-tight. HpT clones were transfected with pTRE-GFP in the absence of serum, and 16 hours post transfection, the media was replaced with 10% fetal bovine serum (FBS, Tet-free –Clontech) DMEM-F12, with or without 1.0 ug/ml Doxycycline. As shown in Figure 1, *GFP expression in several HpT clones is inducible by doxycycline, to varying levels.*
3.3. Construction of polymerase expression vectors: We purchased a cDNA expression vector for Pol η from Open Biosystems (pOTB7). We are in the process of subcloning an EcoRI to SmaI fragment into the MCS of the pTRE-tight regulated vector. We received wild-type and catalytically inactive forms of the human Pol κ cDNA in the pcDNA3.1 vector from the laboratory of Dr. Jean Sebastian Hoffman, and are subcloning the NheI to PmeI fragments into pTRE-tight.

B. Development of microsatellite mutagenesis assays (Subaims 1 and 2)
1. *Origin-dependent HSV-tk shuttle vector assay.*
1.1. Attempts to use the published oriP-tk assay in CRC cell lines.
Our initial approach to studying mutagenesis in CRC cell lines was to use our published oriP-tk shuttle vector assay. For this, we cloned the EBV-derived EBNA-1 gene into the pCDNA3 expression vector. Stable, EBNA-1 expressing clones were isolated from HCT116 and DLD1 cells (HE2 and DE1, respectively). The oriP-tk shuttle vector (pJY1) was transiently transfected into HE2 cells, and plasmid DNA was isolated by Hirt extraction at various times after transfection. The DNA was digested with DpnI to remove DNA molecules that were not replicated in human cells, and the digested DNA was used to transform bacterial strain FT334. As shown in Figure 2A, we detected extremely low levels of replicated oriP-tk plasmids in this cell line. Also, several attempts to isolate stable transfectants of the oriP-tk vector in either cell line were unsuccessful. *We conclude that oriP-derived plasmids are not efficiently replicated in either HCT-116 or DLD-1 cells.*

1.2. Comparison of viral origins for efficiency of DNA replication in tumor cell lines.
We next determined whether the number of replicated plasmid molecules recovered from human tumor cell lines is influenced by the virally-derived DNA replication origin sequence. We

compared the EBV-derived oriP sequence to the SV40 ori sequence using HEK-293 cells expressing the cognate origin-binding proteins. For this experiment, HEK-293TN cells (expressing the SV40 large T antigen) or HEK-293EBNA cells (expressing the EBV EBNA 1 antigen) were transfected with SV40ori-GFP (pEGFP-N1) or EBVoriP-GFP (pNC1) vectors, respectively. Visual inspection for green fluorescence confirmed that >70% of the cells were transfected. After 4 and 6 days, plasmid DNA was extracted, digested with DpnI, and used to transform bacteria. The number of *E. coli*, drug-resistant colonies recovered is shown in Figure 2. Clearly, the SV40 origin (panel C) is superior to the EBV origin (panel B) in this experimental protocol.

1.3. Determination of SV40ori plasmid replication in CRC cells.
Finally, we determined whether the SV40 ori plasmid would be efficiently replicated in the CRC cell lines. For this experiment, the pEGFP-N1 plasmid was co-transfected with or without a plasmid expressing the SV40 large T-Ag. Plasmid DNA were harvested 48 hours later, digested with DpnI, and used to electroporate *E. coli*. The number of kanamycin-resistant colonies observed is shown in Figure 2D. *From these results, we conclude that an SV40 origin-containing plasmid replication is robust in the CRC cell lines, providing an appropriate base for our mutational assay.*

1.4. SV40-TK vector construction. To construct a new SV40ori-tk shuttle vector, we digested the HSV-tk expression plasmid pGTK4 with XbaI. Separately, we amplified the SV40 origin from the pEGFP-N1 plasmid using PCR. The primers included the XbaI recognition sequence (TCTAGA) and 20 nucleotides of the pEGPF-N1 plasmid upstream and downstream of the SV-40 origin. The PCR product was digested with XbaI and ligated into pGTK4.

2. *HSV-tk gap-filling assay*.
We have adapted a published mutagenesis assay utilizing gapped heteroduplex plasmids for use in our laboratory. The assay is shown schematically in Figure 3.
2.1. Assessment of DNA recovery
Our published, HSV-tk containing pSStuI/RStuI gapped plasmid (500 ng) was used to transfect HCT116 and DLD-1 cells in the presence of 5 μg pEGFP plasmid. The purpose of pEGFP is to determine the human cell transfection efficiency (GFP) and the efficiency of plasmid recovery (kanamycin resistance). Plasmid DNA was harvested from the human cells 24 hours later, using an alkaline lysis method. In this protocol, any unfilled gap DNA (input DNA that was not replicated in the human cells) should be degraded in the presence of the alkaline lysis buffer used during DNA extraction. To test the efficiency of gapped plasmid degradation, human cells are harvested and "spiked" with gapped plasmid immediately before alkaline lysis. Fewer than 600 Kan^R colonies were observed for this control, whereas 10^4 - 10^5 Kan^R colonies were observed after allowing replication of the gapped plasmid in human cells.

2.3. Preliminary results of the mutagenesis assay in HCT-116 cells
The HSV-tk mutant frequency measured after transfection of a gapped plasmid containing the HSV-tk gene only was 9.3×10^{-2}. The mutant frequency measured after transfection of a gapped plasmid containing a GT_{13} microsatellite and the HSV-tk gene was 41×10^{-2}. These high mutant frequencies are consistent with the reported MMR-deficient, mutator phenotype of HCT-116 cells.

To determine the mutational pathways that are monitored using the gap assay, independent mutants derived from both gapped plasmids were characterized (Table 1). We recovered molecules in which a mutation was observed within the HSV-tk gene MluI-StuI target sequence (the ssDNA portion of the gapped plasmid), corresponding to a mutation frequency of ~1.9×10^{-2} for both plasmids. These events likely result from errors made by DNA polymerases during gap-filling DNA synthesis, and were the expected products. However, the majority (70-80%) of plasmids recovered have undergone major deletion events within the human cells (Table 1). These rearrangements resulted in the complete loss of the MluI-StuI target sequence along with additional sequences within the plasmid. The frequency of such deletions is ~5-fold higher after replication of the GT_{13}-containing gap in human cells (34×10^{-2}), relative to the HSV-tk only gap (6.3×10^{-2}). Unexpectedly, we observed a highly unusual class of mutations after replication of the HSV-tk gene only gap in human cells, in which the MluI-StuI target sequence is replaced with sequences either from elsewhere in the plasmid or from the human chromosomal DNA. Both the large deletions and the insertion events are analogous to chromosomal instability changes that have been documented to occur in tumor cells. *These preliminary results demonstrate that the gap-filling assay is a robust approach for the analyses of several mechanisms of genome instability in human cells.*

Figures and Tables

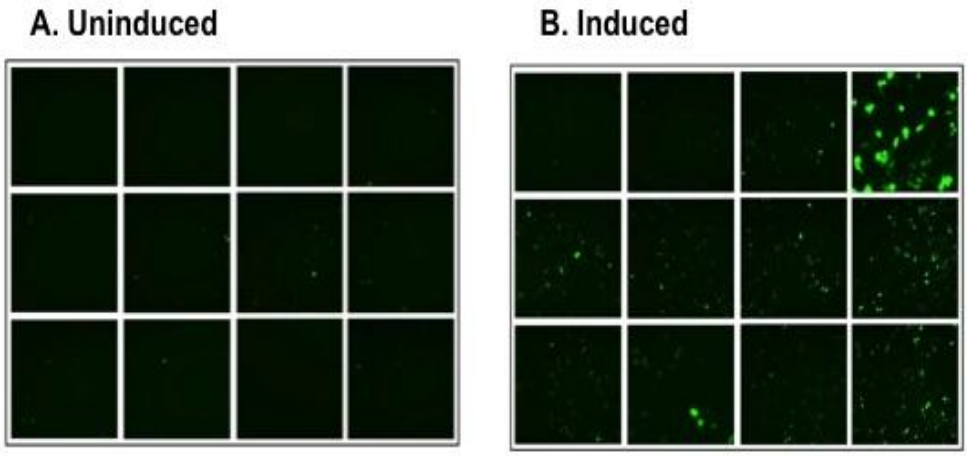

Figure 1. Experimental test of inducibility of HCT116/pTetOn clones. Independent clones were transfected with pTRE-GFP vector DNA, grown for 24 hours in the absence (panel A) or presence (panel B) of 1 μg/ml doxycycline, and examined microscopically for GFP expression. Positive clones are those that display green fluorescence in panel B but not in panel A.

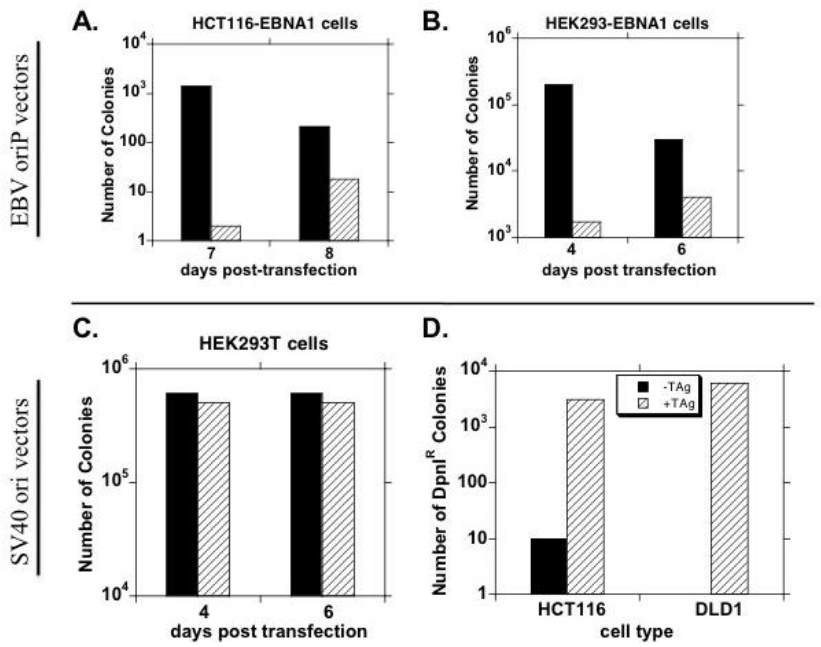

Figure 2. Replication and recovery of viral origin-containing plasmids in human tumor cell lines. Replication was assessed by harvesting plasmids on the indicated days after transfection, and determining the number of antibiotic-resistant bacteria resulting from E. coli transformation. Solid bars, uncut plasmid; hatched bars, DpnI-digested plasmid. (A-B). EBV-oriP vector replication in EBNA-1 expressing tumor cell lines. (C). SV40 ori vector replication in large T-antigen expressing cell line. (D). Co-transfection of SV40ori plasmid with or without large T-antigen expressing (Tag) plasmid.

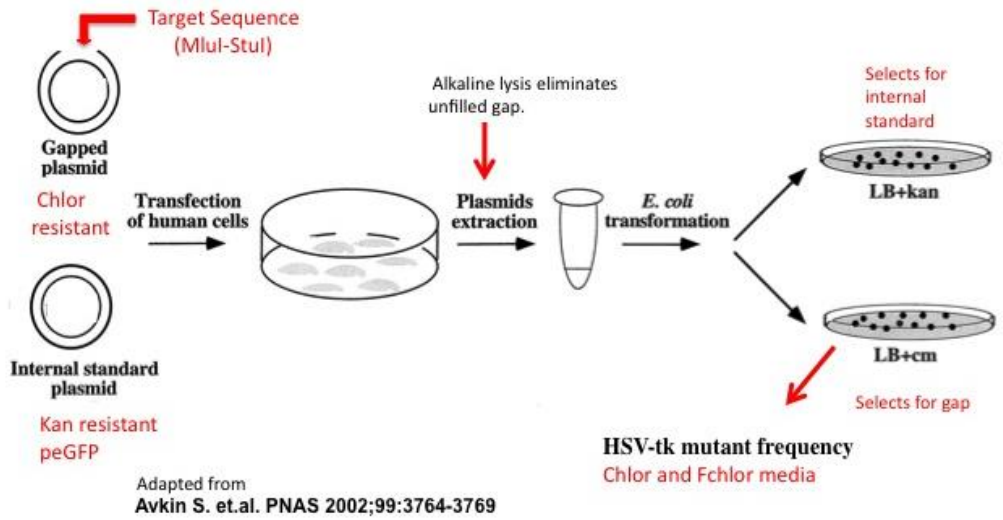

Figure 3. Schematic of the gapped plasmid mutagenesis assay in human cells**.** Adaptations made from the published procedure for use in our laboratory's HSV-tk mutagenesis assay are shown in red.

	Number of Events (Proportion of total)	
Mutational Event	HSV-tk gene only	GT13
Intact Target Sequence	4 (0.20)	4 (0.18)
no mutation	0	3
mutation	4	1
Deleted Target Sequence	13 (0.68)	18 (0.82)
Target Sequence Replacement	2 (0.10)	0
plasmid	1	0
chromosomal	1	0
Total Events	19	22
Observed HSV-tk mutant frequency	9.3×10^{-2}	41×10^{-2}

Table 1. Mutational events involving the MluI-StuI target sequence (HCT116 cells)

Research Project 4: Project Title and Purpose

Impact of the Penn State Diabetes Patient Registry - Disease registries (or electronic patient databases) are being implemented extensively by health care systems with the goals of improved records management, individualized patient care, and population-based disease management. They are a central feature of improving quality care for chronic illnesses and feature prominently in the national effort towards redesigning primary care as the Patient Centered Medical Home

(PCMH). Although used extensively, little data are available on the overall impact of registries on care. The Penn State Diabetes Registry (PSDR) was first implemented in the Penn State Hershey system in 2002 to achieve the aforementioned goals and to simultaneously provide a research network for patient recruitment for diabetes research. The purpose of this project is to identify the value of this chronic illness registry for quality improvement of clinical outcomes, to examine its effectiveness in promoting high quality clinical research and to identify opportunities for PSDR refinements through assessments of the impact of the PSDR.

Anticipated Duration of Project

5/1/2010 - 6/30/2011

Project Overview

Despite widespread use of diabetes registries for a variety of functions, little is known regarding the value of these registries to improve clinical care, foster research recruitment, and determine what factors facilitate adoption and implementation. The current project has the following specific aims:

1. Evaluate the impact of introductions of a chronic disease registry (Penn State Diabetes Registry - PSDR) to improve clinical care. As the PSDR is introduced into new clinics, pre-post comparisons of clinical parameters (Hemoglobin A1c (A1c), Blood Pressure (BP) and LDL-cholesterol (LDLc) and process measures (annual testing and screening) for the patient populations will demonstrate the impact of this implementation on diabetes care.

2. Evaluate the impact of the PSDR for subject recruitment for research trials. A process approved by the Penn State Human Subjects Institutional Review Board allows researchers to make patients aware of opportunities to participate as human subjects in new research through letters sent by care providers using the PSDR. Consort diagrams constructed for each research project will enable comparisons of response, enrollment and completion rates, while using trial participation requirements such as time, risks and discomfort as co-variables.

3. Evaluate facilitating factors and identify barriers for the adoption and implementation of the PSDR within individual clinics. Focus groups, surveys and related qualitative data on PSDR usage will be analyzed to identify factors that determine the success of PSDR implementations. As the PSDR and similar electronic medical record systems become standard in health care settings, this knowledge is important for new improvements.

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Expected Research Outcomes and Benefits

The expected *outcomes* of this research are:
1. Assessment of the impact of a chronic disease registry (PSDR) on patient care;
2. Identification of barriers to acceptance and use of a chronic disease registry for diabetes (PSDR) by health care providers;
3. Identification of specific elements most crucial for both clinical and research applications of the PSDR;
4. Summary of the effectiveness and identification of opportunities for process improvement in the use of registries as a research recruitment tool; and
5. Modification of the PSDR as appropriate to improve and enhance its usefulness for both clinical and research applications.

Based on these outcomes, the benefits expected are:
1. Dissemination of key features required for chronic disease registries as part of ongoing PCMH efforts throughout the commonwealth and nationally;
2. Modifications of the PSDR to improve usage compliance and enhance the PSDR design to more readily integrate it into the clinical setting;
3. Modifications of the PSDR that will enhance its usefulness to researchers;
4. Publication and dissemination of these results to support improvements to other registries;
5. Dissemination of these findings to the Pennsylvania Chronic Care Commission to improve the diabetes registry implementation process that is part of the Commonwealth's healthcare reform initiative;
6. Identification of the most successful process for using chronic disease registries to inform providers and patients about research opportunities while minimizing the burdensome aspects of clinician involvement; and
7. Identification of the data fields most crucial for both clinical and research processes to encourage clinician compliance with their use.

Summary of Research Completed

Support staff were hired to develop an electronic survey for data collection, set up a focus group and collect historic data from the registry. The project is in its early stages. There is nothing substantive to report yet.

<u>Research Project 5: Project Title and Purpose</u>

Epigenetic Changes in Myeloid Differentiation and Acute Myeloid Leukemia - The purpose of this project is to identify changes in epigenetic mechanisms controlling myeloid differentiation and transcription in order to predict and prevent the progression of acute myeloid leukemia

(AML), a highly malignant blood cancer. Recent development of high-throughput "next-generation" genomic sequencing technologies such as ChIP-sequencing provided a dramatic breakthrough in whole-genomic mapping of epigenetic markers such as histone methylation and acetylation and their abnormalities. Here we propose to conduct a detailed whole-genomic characterization of the localization and expression of one such marker, histone H3 methylation, which is altered in AML cells, and to identify clusters of genes involved in epigenetic changes marked by this histone modification and transcriptionally deregulated in leukemia.

Anticipated Duration of Project

1/1/2010 – 6/30/2011

Project Overview

Acute myeloid leukemia (AML) is a highly malignant blood cancer with an estimated 12,810 new cases in 2009 and the lowest survival rate (23.4%) among the four major types of leukemia. This disease is caused by a combination of genetic rearrangements and epigenetic changes. Based on our previous studies of developmentally-regulated epigenetic and transcriptional changes associated with myeloid differentiation and leukemia as well as other data known from the literature, we hypothesize that epigenetic changes manifested in mis-localization of a repressive histone modification, histone H3 dimethylation at lysine 9 (H3K9me2) may interfere with transcription during myeloid differentiation and promote AML.

In this project we will conduct a detailed whole-genomic characterization of the localization and expression of H3K9me2 and identify clusters of genes involved in epigenetic changes marked by this histone modification. This project has two specific aims.

<u>Specific aim 1</u> is to map genome-wide topography of histone H3(K9) demethylation and its transitions in the process of normal myeloid differentiation. We will use ChIP-sequencing to map this histone modification over the genomes of normal myeloid precursors and differentiated granulocytes. Our analysis should reveal sites of H3K9 dimethylation involved in normal myeloid differentiation.

<u>Specific aim 2</u> is to determine spatial and functional relationship between histone H3(K9) methylation and transcriptional changes in AML. This work includes an innovative application of the cutting-edge genomic sequencing and bioinformatics analysis to study association of chromatin modification topographies with transcription in leukemia and is expected to reveal new chromosomal loci altered in AML that can be served for the disease prognosis as well as targets for its epigenetic therapy.

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Expected Research Outcomes and Benefits

The initial causes of several known forms of AML are different and often include cytogenetically distinct chromosomal mutations that result in uncontrolled proliferation and incomplete differentiation of the affected cells. There is also a growing line of evidence that leukemia and many other cancers originate from epigenetic changes that often include deregulated silencing and mis-localized heterochromatin resulting either in inactivated tumor suppressors or activated genes promoting tumor growth.

Our studies should reveal: a) which particular genes or chromosomal loci in the whole human genome are affected by spontaneous epigenetic changes associated with particular forms of AML and with individual patients, and b) which genes and chromosomal loci can be modified by epigenetic protein factors and/or epigenetic therapy. Since several chromatin remodeling and gene regulating agents such as inhibitors of DNA methylation 5-azacytidine and decitabine and histone deacetylase (HDAC) inhibitors are already used for "epigenetic therapy" of AML, identification of their potential genomic targets may lead either to development of new chromatin-targeting therapeutic approaches promoting normal myeloid differentiation or to understanding the adverse effect(s) of these drugs on hematopoietic system.

Summary of Research Completed

In this reporting period, we conducted pilot experiments on testing the spatial and functional relationship between histone H3(K9) methylation and transcriptional changes in AML. AML-derived myeloid progenitors (predominantly myeloblasts) were isolated from acute leukemia human bone-marrow samples by Ficoll-Pacque density gradient centrifugation and cryopreserved at the Claxton laboratory (IRB protocol 2000-186). Cryopreserved AML cell samples were thawed and immediately fixed by 1% formaldehyde. Two different AML samples (each containing ~1x108 cells) were taken for the first set of experiments. Nuclei were isolated, digested with micrococcal nuclease to ~180bp (optimal for SOLiD) chromatin, solubilized by sonication, and immunoprecipitated with two types of antibodies against H3K9me2: Abcam, ChIP Grade, ab1220 and Millipore 05-1249 to compare their efficiency with AML samples. We also conducted control experiments with no specific Abs added or with non-specific Abs both

showing negligible immunoprecipitation. We also prepared samples of total DNA from the same AML samples to use as the non-fractionated DNA references. Chromatin Immunoprecipitation (ChIP) procedure and DNA isolation were carried out as we described before (Istomina et al., Mol Cell Biol. 2003;23:6455-6468). 250-800 ng of DNA were isolated from each sample. These DNA samples were submitted for genomic sequencing on the Applied Biosystems SOLiD genomic sequencer at Penn State Genomics Core facility.

Research Project 6: Project Title and Purpose

Bridging the Gap between Label-Retention and Mammary Stem Cell Properties - Breast cancer patients frequently suffer relapse after prolonged periods of clinically undetectable disease. Whether dormant breast cancer is comprised of quiescent cells versus cycling cells, whose proliferation is offset by cell death, remains unknown. When modeling dormant breast cancer by reversing Wnt1-initiated mammary tumorigenesis in transgenic mice, we found that minimal residual disease (MRD) lesions harbor both latent malignant potential and mammary gland reconstitution capacity. Moreover, our preliminary data demonstrate that MRD lesions harbor a slow-cycling mammary epithelial cell (MEC) population. We propose to demonstrate that these quiescent cells are enriched for expression of validated mammary stem cell markers.

Anticipated Duration of Project

7/1/2010 - 6/30/2011

Project Overview

A diverse set of observations suggest that stem cells within normal and malignant mammary epithelium may be capable of entering into and emerging from prolonged periods of quiescence. However, it is not yet clear whether mammary stem cells cycle infrequently relative to other mammary epithelial cells (MECs).

We hypothesize that both normal and malignant mammary epithelium harbor a relatively quiescent MEC compartment with stem cell-like regenerative capacity. Here, we propose to demonstrate that these quiescent cells are enriched for expression of validated mammary stem cell markers by completing two specific aims. In Specific Aim 1, we will generate normal and neoplastic mammary tissue in transgenic mice and identify the relatively quiescent label-retaining cells (LRCs) using an H2B-GFP reporter transgene. In Specific Aim 2, we will determine whether H2B-GFP LRCs possess stem cell-associated biological features.

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Expected Research Outcomes and Benefits

The cell-of-origin that gives rise to breast cancer remains elusive. One influential model of breast carcinogenesis posits that mutations responsible for initiating tumorigenesis accrue in a long-lived, slow-cycling mammary stem cell compartment. Remarkably, though great strides have been made in delineating the mammary epithelial cell developmental hierarchy, it remains unclear whether mammary stem cells are actively cycling or relatively quiescent. Our studies will identify slow cycling mammary epithelial cells in transgenic mice and determine whether these relatively quiescent cells express the cell surface markers associated with mammary stem cells.

Summary of Research Completed

The project has not yet begun.

Research Project 7: Project Title and Purpose

Neural Control of Breathing by a Signal Encoding Venous Pressure: Implication in Heart Failure - Respiratory symptoms such as chronic hyperventilation are dominant expressions of cardiac insufficiency. Hyperventilation induced hypocapnia also has the potential to disrupt sleep architecture and to provoke central sleep apnea, further limiting quality of life in these patients. Distension of the venous system, a common feature of cardiac failure, leads to the activation of neural afferent fibers located in the adventitia of the post capillary bed, which in turn increases breathing. The proposed studies are intended to establish, in an animal model, the involvement of such a mechanism at rest and during muscle activity.

Anticipated Duration of Project

7/1/2010 – 6/30/2011

Project Overview

Profound changes in the control of breathing accompany the syndrome known as ‘heart failure’ (HF). The mechanism(s) responsible for the excessive stimulation of breathing in HF which precipitate hypocapnia, Cheyne-Stokes breathing and dyspnea, are far from being understood. Given HF is a condition which affects millions of people in the United States, there is an urgent need for research which will aid in the development of effective therapies for treating or reducing the respiratory-related consequences of this disease state. Our research group has proposed that, in the absence of pulmonary oedema, the excessive ventilation that is observed in HF patients is caused by an increase in systemic venous pressure. My experiments will specifically determine whether an increase in systemic venous pressure, leads to a stimulation of

breathing, independently of changes occurring in the systemic pulmonary and systemic arterial circulation. I will also determine the mechanism through which breathing is stimulated. Using a novel, spontaneously breathing, anesthetized sheep preparation, I will employ a combination of venous occlusion and veno-venous shunt techniques in order to measure the ventilatory response to changes systemic venous pressure.

Specific Aim 1: To determine the magnitude of the increase in pulmonary ventilation caused specifically by an increase in pressure (load) in the systemic venous system. I will use a veno-venous circulatory bypass with a venous reservoir bag to isolate the contribution of increased venous pressure in the hind limb tissues, and determine the effects of the resulting increase in systemic venous pressure on ventilation. I predict that an increase in systemic venous pressure achieved only via increasing the load imposed upon venous return will stimulate breathing and lower $PaCO_2$.

Future direction: The next step will be to investigate the mechanism through which an increase in systemic venous pressure causes an excessive level of pulmonary ventilation. These experiments will directly test the hypothesis that the stimulation of breathing which results from an increase in systemic venous pressure arises from a stimulation of mechanosensitive afferents in the muscle tissue that respond to changes in vascular distension. Infusions of various agents into the hind limbs will be used to block the activation of mechanogated ion channels on group III and IV muscle afferents and transient receptor potential vanilloid receptors.

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Expected Research Outcomes and Benefits

Approximately 5 million Americans suffer from heart failure. Somewhat surprisingly, it is pulmonary symptoms that have the most impact on the quality of life of these patients. People with heart failure breathe more in comparison to people without heart failure, and they often complain of feeling ‘breathless’, or ‘unable to catch their breath’. At present, in many patients, little can be done to decrease this “feeling” since many various and different, yet poorly understood, mechanisms can account for these respiratory symptoms. We have good reason to believe that one of these mechanisms is an increase in the pressure that blood exerts in the venous system. Heart failure causes an increase in the pressure that is present in the veins because the heart is less efficient, which causes a tendency for blood to ‘back up’ in the

circulation. The experiments I describe in this project will determine how much this increased pressure in the veins can stimulate breathing and may help to explain the resulting sensation of breathlessness in heart failure. Should we find that this mechanism is an important one, then we can work upon developing new treatment strategies that will greatly improve the quality of life in people living with heart failure.

Summary of Research Completed

The project has not yet started.

Research Project 8: Project Title and Purpose

Influence of Brain Astrocyte Glutamate Concentrations on Neurotransmission - The most abundant type of cell in the brain is the astrocyte. Astrocytes may control neurotransmitter traffic by lowering the concentration of neurotransmitter glutamate in synaptic spaces. Vesicular glutamate released from presynaptic neurons into the synaptic space stimulates depolarization and electrical signal transduction by post- synaptic neurons. The synaptic space barrier is composed of membranes from both presynaptic and post-synaptic neurons and astrocytes. Astrocytes rapidly remove glutamate from the synaptic space which would otherwise prolong signal transmission and cause excitotoxicity. The study examines the properties of the glutamate transporter in the astrocyte membrane to evaluate the influence of the intracellular astrocyte glutamate on inward transport and examines how cytosolic concentrations of glutamate are regulated by astrocytic enzymes.

Anticipated Duration of Project

1/1/2010 – 12/31/2010

Project Overview

Glutamate in the brain acts as a source of nutrient energy and as an excitatory neurotransmitter. This project focuses on mechanisms that ensure optimal use of glutamate as a neurotransmitter, by providing optimal maintenance of low glutamate concentrations in astrocytes. Astrocytes form part of the boundaries of the synaptic spaces. Na^+-linked glutamate transporters in the astrocyte plasma membranes provide the energy for the "pump" that keeps glutamate in the low µmolar range in the synaptic space. We will test the hypothesis that maintenance of low concentrations of glutamate in the astrocyte cytosol augments the capacity of the astrocytes to transport glutamate from the synaptic space and into astrocytes.

First, we will measure the rate of [U-^{14}C] glutamate entry into cultured astrocytes as a function of astrocytic, cytosolic glutamate. The external, [U-^{14}C] glutamate will be held constant at 2µM. The intracellular glutamate will be varied by preliminary incubations of cultured cells with unlabeled glutamate (these concentrations will be varied from 0-20mM). Glutamate-loaded cells will be rinsed in glutamate-free Krebs Ringer bicarbonate solution. Glutamate will not leak out because of the presence of the Na^+-linked glutamate transporter in the plasma membrane. The rate of transport of glutamate into the cells may be faster when intracellular glutamate is low and

we expect to be able to determine the cytosolic glutamate concentration that results in half maximal inhibition of ^{14}C-glutamate entry into the cells.

Secondly, we will investigate the ability of astrocytic enzymes such as malic enzyme (ME1) and phosphoenolpyruvate carboxykinase (PEPCK2) to maintain low levels of glutamate in astrocytes. These enzymes are expressed mainly in brain astrocytes and they irreversibly convert malate to pyruvate. Since glutamate is rapidly converted to malate in astrocytes, these enzymes act as glutamate drainage sinks. The activities of ME1 and PEPCK2 probably play a significant role in maintaining low astrocyte glutamate levels.

We plan to use gene silencing techniques to lower expression of these two enzymes individually and together. The necessary siRNAs are available commercially. Cells will be exposed to the inhibitory nucleotides either separately or together. We expect that they will lower astrocyte glutamate levels to concentrations that stimulate entry of external glutamate into the astrocytes. If that happens, we will conclude that these enzymes are important for brain function and search for drugs that influence their activity *in vivo*.

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Expected Research Outcomes and Benefits

In this project we will learn how cytosolic glutamate levels are regulated in astrocytes and whether low cytosolic concentrations are critical for neurological health. Three enzymes in astrocytes lower astrocytic glutamate pool sizes. These are glutamine synthetase (GS), ME1, and PEPCK2. Inhibition of glutamine synthetase will slow neurotransmission, lower glutamine levels, increase astrocytic glutamate, and deplete neuronal glutamate but will not induce glutamate excitotoxicity, seizures or neuronal cell death. Therefore, our focus is on ME1 and PEPCK2.

Disruption of glutamate uptake from synaptic spaces, perhaps due to excess glutamate within astrocytes, may play a role in neurological disorders including cerebral ischemia, neurodegeneration, migraine, cerebral edemia, and hepatic encephatopathy. Astrocytic dysfunction has also been cited as a cause of seizures in the brain. Excitotoxicity of glutamatergic neurons has been implicated in Alzheimer's disease, possibly induced by

malfunction of the astrocytes and of the glutamate glutamine cycle. If trials using siRNA to slow ME1 and PEPCK2 in the cultured astrocytes succeed and lower astrocyte glutamate levels, we will search for drugs that inhibit the activity of these enzymes in the brain such as the PEPCK inhibitor, sulfoacetate, which may be effective in treatment of the neurological diseases cited above.

In future studies, we may also evaluate the roles of these enzymes in neurological health by generation of mice with disruptions of the ME1 or PEPCK2 genes. Determination of the resistance of these mice to neurological stresses such as chemically induced seizures or cerebral ischemia will reveal the importance of efforts to find ways to lower astrocytic glutamate *in vivo*.

Summary of Research Completed

Glutamate in the brain acts as a source of nutrient energy and as an excitatory neurotransmitter. This project focuses on mechanisms that ensure optimal use of glutamate as a neurotransmitter, by providing optimal maintenance of low glutamate concentrations in astrocytes. Astrocytes form part of the boundaries of the synaptic spaces and one of their important functions is to remove the excitatory glutamate from the space where it activates post synaptic glutamate receptors. Removal stops the signal after it has been sent.

We plan to test the hypothesis that excess concentrations of glutamate in the astrocytic cytosol may interfere with regulation of neurotransmission. We want to develop techniques to vary astrocytic cytosolic glutamate and determine the influence of intracellular glutamate on influx of glutamate from the external media. Preliminary data suggested that glutamate levels within the astrocytes have more influence on removal of glutamate from the cytosol than glutamate outside the astrocytes.

Therefore, we have initiated studies of intracellular astrocyte metabolism. The activities of malic enzyme (ME1) and phosphoenolpyruvate carboxykinase are key factors in regulation of glutamate concentrations in astrocytes. These enzymes are expressed predominantly in brain astrocytes and they irreversibly convert malate to pyruvate. Since glutamate is rapidly converted to malate in astrocytes, these enzymes act as glutamate drainage sinks. The activities of ME1 and PEPCK2 probably play a significant role in maintaining low astrocyte glutamate levels after the glutamate excitatory signal is sent.

Once we have found effective ways to alter glutamate concentrations within astrocytes over a wide range, we intend to measure the rate of [$U^{14}C$] glutamate transport into isolated cultured astrocytes as a function of intracellular glutamate. Therefore, preliminary studies have been aimed at regulation of ME1 and PEPCK2 activities. These two astrocytic enzymes are likely key factors in regulating astrocytic intracellular glutamate. Since both enzymes consume malate and oxaloacetate and produce pyruvate and CO_2, we expect that reduction in their activities will result in higher than normal levels to citric acid cycle intermediates and glutamate compared to control cells.

We are currently using gene silencing techniques to lower expression of these two enzymes. We have obtained the siRNA for ME1 from Qiagen. We used the same siRNA sequence as that

which was successfully used by Pongratz (Pongratz et al, 2007) who used it to decrease ME1 expression in INS1 cells. Currently we are transfecting the primary astrocytes with siRNA in order to halt transcription of the ME1.

Materials and Methods

Methods/Primary Astrocyte culture
We have successfully learned to culture and study primary astrocytes. Primary astrocytes were prepared by a modification of the method of McCarthy and de Vellis (1978, 1980) from the newborn rats. All animals were treated in accordance with Institutional Animal Use and Care Committee of Pennsylvania State University College of Medicine. Briefly, 12-18 hour old Sprague-Dawley rat pups were anesthetized and then decapitated. The brain was removed and placed in CMF-HBSS solution (Gibco, Rockville, MD, USA). The meninges were carefully removed, and cells were separated by dicing the tissue and treating it with 0.25% trypsin-EDTA (Gibco) for 30 min. Following incubation in DNAse for 5 min, the brain homogenate was passed through 75µm Nitex screens (SEFAR America, Depew, NY, USA) and resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Cells were plated in poly-D lysine (Sigma)-coated flasks. The medium was changed after 24hour and every other day thereafter. Cells were allowed to grow for 9 days, and astrocytes were removed by shaking at 260 rpm for 18h to loosen contaminating cell types.

Results/Cell Transfection
Initial studies of cell transfection using siRNA purchased from Qiagen have commenced. 21-Nucleotide siRNA sequences (Qiagen) targeting the specific cytosolic malic enzyme genes were used for transfection. siRNA were synthesized by Qiagen with 19- base pair ribonucleic acid sequences with 5/ dideoxythymidine overhangs of the following sequences. ME1a and ME1b are isoforms of siRNA for cytosolic malic enzyme with the sequences "AACCAGGAGATCCAGGTCCTT" and "AAGCCAAGAGGCCTCTTTATC" respectively. The siRNA duplex consisted of the Sense strand and its complimentary antisense strand to provide a 2-nucleotide dithymidine overhang at each 3'terminus. siRNA constructs (scramble, ME1a, ME1b) were introduced into primary astrocytes using Lipofectamine 2000 and OptiMEMI reduced serum medium (Invitrogen) at a different concentration. For each transfection sample siRNA – Lipofectamine 2000 complexes were made in Opti-MEM I reduced serum medium.

Next we will begin the studies to determine if ME1 levels in the cultured astrocytes can be lowered with the siRNA constructs and what the influence of lowered ME1 is on intracellular glutamate.

Research Project 9: Project Title and Purpose

Identification and Function Study of Inflammatory Bowel Disease-associated DNA Methylation - Inflammatory bowel disease (IBD), with Crohn's disease (CD) and ulcerative colitis (UC) as its two major forms, is a complex human disease. In the US, about 1.4 million people are affected with IBD. Currently, there is no known specific cause and no medical cure for the disease. Although more than 32 genes have been identified as IBD-associated genes, the total number of

genetic factors discovered so far can only account for less than 20% of the overall genetic risk. It is clear that other factors make a significant contribution to the disease process. In this project we will examine the role of DNA methylation in IBD. DNA methylation is a modification of DNA molecules and has emerged as an important player in human development and diseases.

Anticipated Duration of Project

7/1/2010 – 6/30/2011

Project Overview

We hypothesize that DNA methylation plays a role in IBD pathogenesis. The objectives of this project are to identify DNA methylation CpG sites in IBD which would further add to the understanding of underlying pathophysiology and to provide a potential clinical application in terms of disease diagnosis.

Specific Aims: 1**)** To identify IBD-specific DNA methylation in naive B cell and mucosal tissues with a HumanMethylation27 BeadChip microarray, and 2) To demonstrate the effect of DNA methylation on gene expression and thus potential contribution to disease pathophysiology.

Research Design and Methods: The generally accepted mechanism of IBD pathophysiology is that there is a dysbiosis causing an inappropriate immune response in a genetically susceptible individual. Naive B cells and mucosal tissues will be used in this study, as both play vital roles in the immune response. Furthermore, they are easily accessible via blood draws and tissue biopsies. In Aim 1, 96 samples (48 B cells and 48 mucosal tissues) will be used for methylation identification. In Aim 2, 200 samples (100 B cells and 100 mucosal tissues) will be used for the study on the correlation of DNA methylation with gene expression. For comparison, the study samples will include 1) naïve B cells and EBV-transformed B cell lines from IBD (CD and UC) and non-IBD siblings, 2) diseased and non-diseased mucosal and intestinal tissues classified by a pathologist from IBD (CD and UC) patients, and 3) unrelated healthy controls. In Aim 1, genomic DNA will be isolated and converted by bisulfite with Qiagen kits. The converted DNA will then be used for a HumanMethylation27 BeadChip assay. The BeadChip covers 27,578 CpG sites of 14,000 genes which have been validated in human studies. In Aim 2, we will target 4 genes (P2RX7, PPARG, RAP1A, and CRK) that are identified as having IBD-associated methylation in our preliminary study using the Illumina Goldengate Beadsarray. The levels of gene expression will be studied at mRNA transcription by RT-PCR and real-time PCR, and at protein translation by Western blot in the above clinical samples; and the correlation of methylation with gene expression will be statistically analyzed. All the human tissues are ready for the project, and our lab is well experienced in conducting such experiments.

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Expected Research Outcomes and Benefits

Specific Aim 1. We will identify inflammatory bowel disease-associated DNA methylation sites and investigate the distribution of DNA methylation in patients with inflammatory bowel disease. The knowledge gained from these results will contribute to understanding the cause of IBD from modifications of human inherited molecules. The identified DNA methylation could be used as biomarkers for disease diagnosis and also provide target molecules for drug discovery and in developing strategies for clinical therapy.

Specific Aim 2. We will show that the levels of DNA methylation are correlated with gene expression in clinical samples in 4 target genes. These results will demonstrate that DNA methylation can affect gene expression, and thus contribute to IBD pathophysiology. This will also lead to further study on potential mechanisms of methylation related to the cause and/or severity of IBD. Determining how to control the methylation status of specific IBD genes will benefit disease prevention and treatment through regulation of the methylation process (methylation or demethylation), with drugs currently available.

Summary of Research Completed

The project has not yet started.

Research Project 10: Project Title and Purpose

Allogeneic CMV CTL for Refractory Glioblastoma Multiforme - The purpose of this project is to determine the safety as well of the clinical and immunological effects of infusing allogeneic, cytomegalovirus (CMV) specific cytotoxic lymphocytes (CTL) in patients with refractory, CMV positive glioblastoma multiforme (GBM). Patient tumors will be tested for the presence of CMV pp65 and IE-1, and if the tumors are positive and the patients meet other eligibility criteria they will receive CMV CTL from an HLA partially matched donor. We will determine the duration and magnitude of CMV specific T cell function and donor cell microchimerism, the latter using PCR assays. We will also determine the incidence of clinical responses to this intervention, and

correlate these clinical responses with donor cell chimerism and CMV specific immunity post-infusion.

Anticipated Duration of Project

1/1/2010 - 6/30/2011

Project Overview

Glioblastoma multiforme (GBM) is the most aggressive primary brain tumor affecting adults, with a median survival of approximately one year, and over 95% of patients surviving less than two years. Recently, several investigators have detected CMV proteins pp65 and IE-1 on GBM tumors, but not from normal brain tissue. While CMV is not thought to be oncogenic, the presence of these highly immunogenic CMV antigens indicates that there is the potential to intervene with CMV antigen specific immunotherapy. Since many patients with refractory GBM may have significantly compromised cellular immunity, using CMV specific cytotoxic T lymphocytes (CTL) from healthy donors would expedite the culture of CTL for these patients.

The purpose of this study is to examine the clinical and immunological effects of allogeneic CMV specific CTL for patients with refractory GBM. Patients with GBM will have their tumors tested for the presence of CMV pp65 and IE-1, and if they meet other eligibility criteria will have related donors screened for human leukocyte antigen (HLA) typing and CMV serology. We will administer HLA haplo-identical, CMV pp65/IE-1 specific CTL to patients with GBM whose tumors are refractory to primary therapy and test positive immunohistochemically for one or both of these antigens. Subjects will receive a lympho-depleting regimen consisting of single fraction total body irradiation (100 cGy) and cyclophosphamide to promote donor CTL chimerism. We will recruit 10 subjects to determine the safety of this approach, the incidence of tumor responses, and the duration and magnitude of donor CTL chimerism and CMV specific immune responses post-infusion. Since over 50% of individuals with disease progression or relapse would be expected to succumb from their disease within 2 months, a positive outcome will be defined as $\geq$ 50% of these subjects having disease stabilization, a partial response, or a complete response at 2 months post infusion. We will determine microchimerism post-infusion using a quantitative PCR assay for mismatched HLA alleles, and assess CMV specific immune function by performing cytotoxicity assays and cytofluorometry for intracellular cytokine production, correlating these findings with clinical responses.

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Expected Research Outcomes and Benefits

This project will test a novel treatment strategy in patients with a highly lethal brain tumor. It is possible that allogeneic, CMV specific CTL could recognize and kill CMV positive GBM cells in patients whose tumors test positive for CMV pp65 or IE-1. First, we will determine the safety of infusing CMV antigen specific T cells into patients with therapy resistant glioblastoma multiforme (GBM). We will also determine the incidence of clinical responses to this form of therapy, and will correlate the duration of donor cell chimerism post-infusion with tumor responses. We expect that this form of therapy should be well tolerated, as allogeneic, antigen specific CTL have been used in other patient populations, namely those with EBV related lymphomas. We also expect that a number of these subjects may have detectable donor cells post-infusion, but the duration of this finding is unknown. It will be considered highly significant if some of these patients, who would otherwise be expected to die, experience several months of progression free survival. Since the median progression free survival for patients with refractory GBM is only two months, a significant finding would be having over half of the CTL recipients experiencing either stable disease, partial, or complete responses at two months post-infusion. This study will provide important information regarding the feasibility of this approach. If a correlation is seen between donor cell persistence and clinical responses, a future strategy would be to intensify the pre-CTL preparative regimen, or to increase the cell dose.

Summary of Research Completed

Immunohistochemical analysis of paraffin embedded tissue sections was performed on 49 newly diagnosed GBM tumors, the largest series reported to date. We confirmed the presence of CMV pp65 on 25/49 (51%) and of IE1 on 8/49 (16%) of these tumors (Table 1). While pp65 and IE1 are generally found in the nucleus of cells that are permissibly infected by CMV, GBM in this series had mostly cytoplasmic staining, with only 16% having nuclear staining for one or both of these antigens. We infected GBM cell lines with a laboratory strain of CMV, and found that most of the staining was cytoplasmic, with some perinuclear localization of IE1. To test the potential for CMV infected GBM cells to be recognized by CMV pp65 and IE1 specific cytotoxic T lymphocytes (CTL), we used CMV infected GBM cell lines in cytotoxicity assays with human leukocyte antigen (HLA) partially matched CMV CTL. Lysis of CMV infected GBM tumor cells was accentuated by pre-treating these cell lines with either the demethylating agent decitabine (DAC) or interferon-γ (IFN-γ), both of which were shown to increase MHC Class I and II expression on tumor cells *in vitro*. These studies confirm the presence of CMV pp65 or IE1 on approximately half of GBM, with the possibility that CMV positive tumor cells can be recognized by CMV pp65/IE1 specific T cells.

Immunohistochemical analysis of patient tumors. We analyzed 49 paraffin sections of primary GBM from newly diagnosed patients at Penn State Hershey Medical Center for the presence of CMVpp65 and IE1 by immunohistochemistry. There were 26 males and 23 females, with a mean age of 67.6 years (range 38-79 years). Figure 1 depicts the expression of both pp65 and

IE1 from two representative tumors testing positive for these antigens. There was no staining from CMV non-infected tissues or with isotype control antibodies. These tumors had both cytoplasmic and nuclear staining for these antigens, as reported by other groups. The incidence of CMV pp65 and IE1 in our subjects was 25/49 (51%) positive for pp65 and 8/49 (16%) positive for IE1. Not all cells within a given tumor were positive for pp65 or IE1, possibly reflecting variability in the infection of GBM cells. In nearly all of the tumors, the presence or absence of staining for pp65 correlated with IE1, but 1 specimen was positive for IE1 and negative for pp65.

CMV infection of GBM cell lines. Cells that are permissively infected by CMV, such as SF, have prominent nuclear staining for these antigens, but several CMV positive GBM in this series had prominent staining for pp65 and IE1 in the cytoplasm. CMV pp65 and IE1 nuclear staining was only seen in 16% of GBM with immunohistochemical evidence for these proteins in this series. To determine the extent to which pp65 and IE1 are expressed after CMV infection of GBM cells, CMV strain AD169 was used to infect two GBM cell lines and the expression of pp65 and IE1 were analyzed by fluorescent microscopy. Approximately 30% of the cells were positive for pp65 and IE1 at three days post-infection. Figure 2 depicts staining for both antigens from two CMV infected GBM tumors, showing that most staining for pp65 was cytoplasmic, whereas there was perinuclear localization of IE1 in some tumor cells. These findings confirm the fact that GBM cells can be infected with CMV, and both antigens can be expressed.

CTL killing of CMV-infected GBM cells. Several tumor cell lines, including GBM, have been shown to have decreased expression of MHC Class I and II molecules, which could preclude tumor cell recognition by CMV pp65/IE1 specific CTL. DAC, a demethylating agent, and IFN-γ have also been shown to increase the expression of tumor antigens and MHC Class I and II molecules on tumor cells. We cultured GBM tumor cell lines with IFN-γ or DAC, and CMV infected and non-infected GBM cells were used as target cells in CRA with partially HLA matched CMV specific CTL derived from healthy donors (Figure 3). Donor 1 CMV CTL recognized B cell blasts (BB) infected with vaccinia encoding pp65 as well as BB from a partially matched donor sharing HLA A 0201 (Figure 3a). Donor 1 CMV CTL recognized the pp65 peptide pulsed tumor cell line T98G (sharing HLA A0201 with this donor), demonstrating the ability of a GBM cell line to present CMV antigens. We also tested the capacity of these CTL to lyse CMV infected T98G, and found that there was enhanced cytotoxicity when the tumor cell was treated with IFN-γ or DAC, which is presented in Figure 3b. Cytofluorometric analysis of this GBM tumor cell line following exposure to DAC and IFN-γ is depicted in Figure 4, showing increased expression of MHC class I and II antigens. IFN-γ treatment had a greater effect on MHC Class II expression on this tumor cell line.

Patient accrual. Thus far we have accrued 18 subjects with GBM to the screening portion of the trial, three of whom have progressed and are therefore potentially eligible for the treatment phase. We are currently collecting T cells from a donor for our first patient to be treated on this study. This subject will likely be treated with CMV CTL in late June 2010. There are three other subjects with progressed disease at this point in various stages of evaluation. One note is the fact that of the approximately 50% of subjects whose tumors tested positive for pp65 or IE1, we would expect that most would develop disease progression at some time during the upcoming

year. Therefore between the subjects already screened and future patients, we will be able to meet study accrual goals (10 patients total to be treated with CTL).

Table 1 Expression of CMV pp65 and IE-1 on Glioblastomas

	All tumors, n	Negative, n(%)	Positive, n(%)
CMVpp65	23	10(43%)	13(57%)
CMV IE1	23	13(57%)	10(43%)

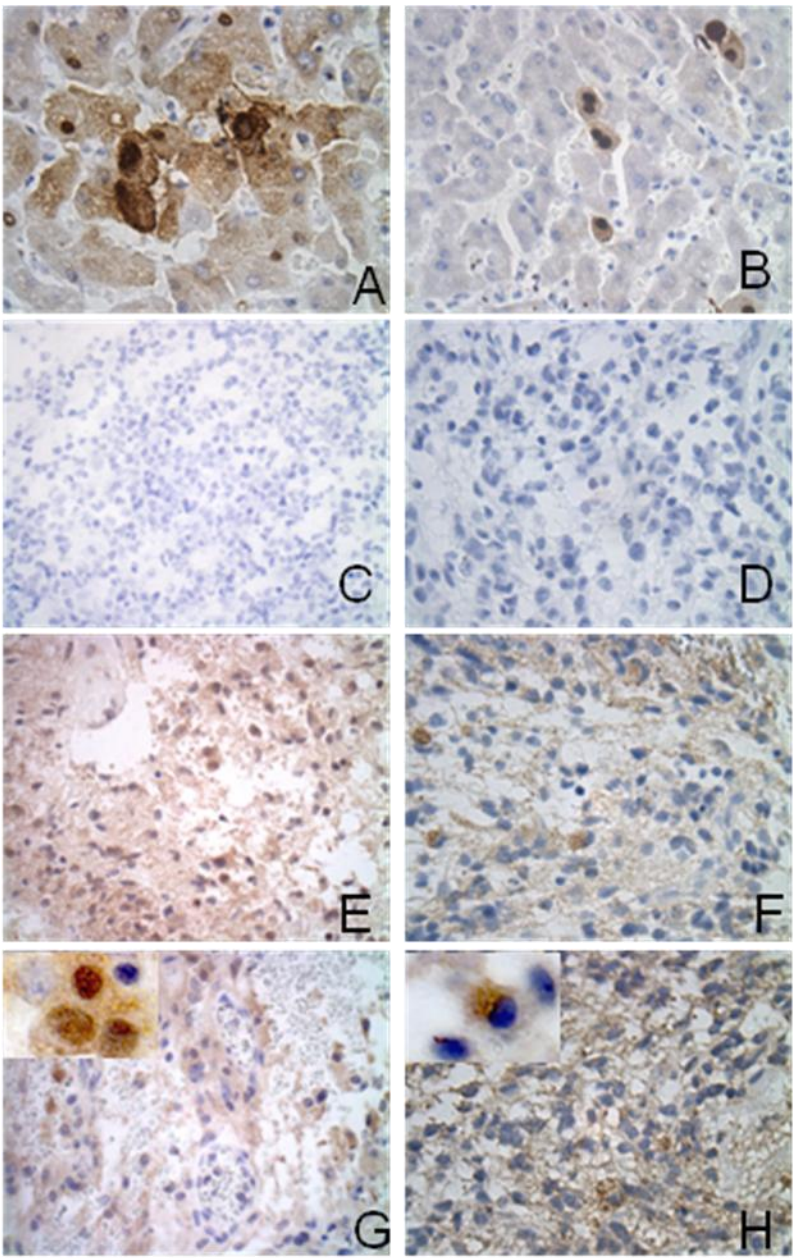

Figure 1. Immunohistochemical detection of CMV pp65 and IE-1 in glioblastomas (GBM) and control tissues. CMV infected liver stained with (A) pp65 and (B) IE-1; GBM stained with (C) pp65 and (D) IE-1 isotype control antibodies; (E, and G) GBM 1 and 2 stained with pp65; (F and H) GBM tumors 1 and 2 stained with IE1 .

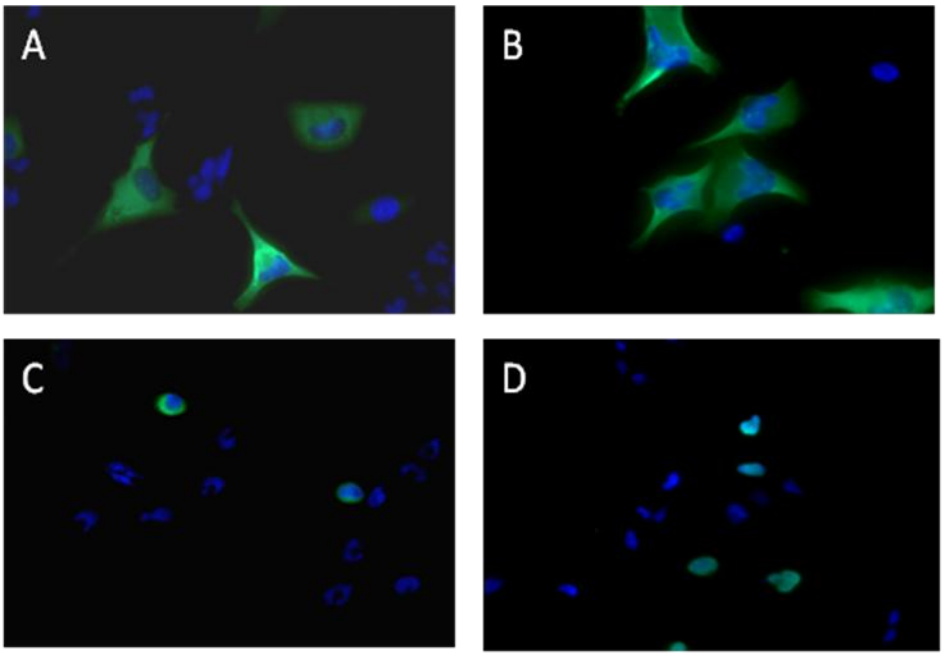

Figure 2. Immunofluorescence analysis of pp65 and IE-1 in CMV infected glioblastoma (GBM) cell lines. Nuclei are counterstained with a blue fluorescent dye. GBM cell lines (A) T98G and (B) U251 stained with an anti-pp65 monoclonal antibody, showing cytoplasmic localization of pp65 (green). Cell lines T98G (C) and U251 (D) were also stained with a murine anti-IE-1 (green), which showed some peri-nuclear localization.

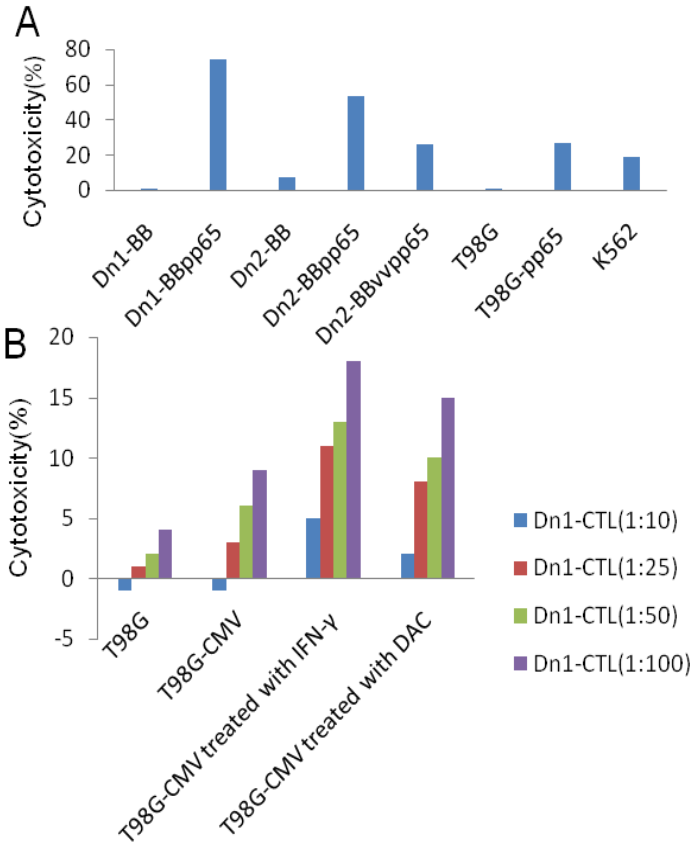

Figure 3 A: Cytotoxicity data from ^{51}Cr release assay: CMV pp65 specific CTL from a healthy donor 1(HLA-0201)(Dn1) against autologous B blasts(BB) pulsed with pp65 PepMix, an allogeneic (HLA 0201)(Dn2) BB pulsed with pp65, as well as a glioblastoma cell line (T98G, also HLA 0201) pulsed with pp65 peptides at target to effector ratio of 1:25. **B:** T98G cells infected with CMV and tested in ^{51}Cr release assays at different target to responder (CTL) ratios (1:10, 1:25, 1:50, 1:100). T98G cells infected with CMV were treated with IFN-γ (100U/ml) or 5-Aza-2'-deoxycytidine (DAC)(1uM/ml) for 3 days. CMV pp65 specific CTL from HLA-0201 posiitive Dn1 were able to kill CMV infected glioblastoma cells, and the cytotoxicity was increased by treating these cells with IFN-γ and DAC.

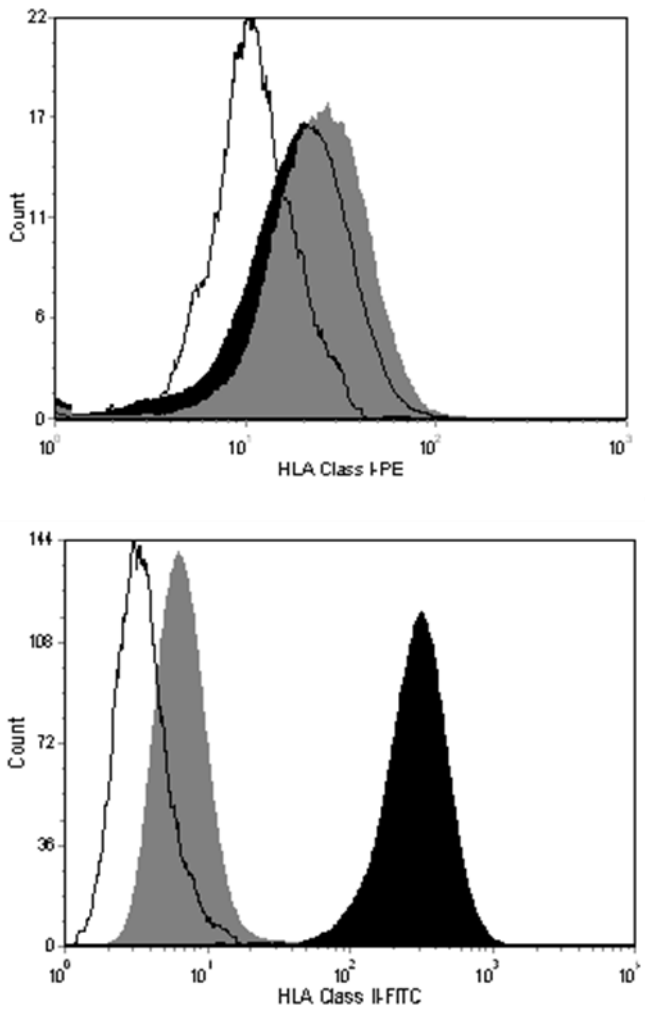

Figure 4. Antigenic potentiation of glioblastoma (T98G) by IFN-r and DAC upregulation of HLA class I and Class II. Untreated T98G (no fill), those cells treated with IFNr (black), treated with 5-Aza-2'-deoxycytidine (DAC) (gray) were stained with anti-HLA class I-PE (A) and anti-HLA class II-FITC (B)

Research Project 11: Project Title and Purpose

Molecular Mechanisms of Olanzapine's Metabolic Side Effects in an Animal Model - Understanding the mechanism of the metabolic side effects of atypical antipsychotics is very important to the patients taking these drugs and their families. This research will focus on how atypical antipsychotics lower free fatty acids and will provide data supporting our hypothesis that it both impairs lipolysis leading to increased adiposity and simultaneously increases lipid oxidation leading through substrate competition to hyperglycemia and apparent insulin resistance.

Anticipated Duration of Project

1/1/2010 - 12/31/2010

Project Overview

The long-term goals of this project are to elucidate the mechanisms of the metabolic side effects of atypical antipsychotics. These drugs (e.g., olanzapine) are used to treat psychoses and other psychiatric disorders. Over the last 15 years their use has grown significantly in adults and soared in children. The sharp rise in atypical antipsychotic use is troublesome because of their propensity to cause serious metabolic side effects including obesity, diabetes their co-morbidities. We hypothesize that these serious side effects are preceded by causal changes in peripheral circulating factors along with carbohydrate and lipid metabolism that drive the insulin resistance, hunger, adiposity and obesity-comorbidities. Our focus will be on those aspects that have been confirmed as metabolic side effects in humans. Interesting preliminary data generated in a high fidelity rodent model indicate that increased adiposity in males may develop in response to tissue specific changes in insulin sensitivity or lipolysis leading to energy repartitioning that directs fuel away from muscle and towards fat. Hunger seems to involve leptin lowering. Hyperglycemia appears to involve alterations in fuel selection that leads to metabolic inflexibility, mitochondrial overload by increased lipid metabolism. We will test these hypotheses and address the mechanism(s) that underlie the acute endocrine and metabolic changes brought about by olanzapine in the preclinical model.

The aims are Aim 1. Determine the effect of olanzapine on FFA oxidation (measure FFA uptake and Acyl carnitine species in vivo) and uncover the role of malonyl CoA in this effect and the underlying mechanism (AMPK activation, ACC inhibition, reduced anaplerosis). Aim 2. Determine the role of cAMP in the anti in vivo lipolysis effect of atypical antipsychotics. By the conclusion of this work, we will have developed an approach to rapidly predict the likelihood of obesity-related side effects in emerging compounds before they are tested in humans. This will aid in the design of next generation drugs with reduced side effects and may also reveal new therapeutic targets for the treatment of obesity and diabetes.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

In this project, we will focus on how atypical antipsychotics lower FFAs in humans. Atypical antipsychotics also cause insulin resistance, obesity and diabetes in humans, and those conditions are associated with elevated not lower FFA. We have found that fasting plasma FFAs were lower in a chronic model using male Sprague Dawley rats. They were similarly reduced acutely, indicating a rapid and body composition independent effect, as has been true, for changes in glucose infusion rate during an euglycemic hyperinsulinemic clamp. In these studies we will examine the mechanism of this effect. Specifically we will examine how atypical antipsychotics impair lipolysis and concurrently elevate FFA oxidation. These could suggest strategies to interfere with these side effects such as blocking fat oxidation. These findings may also provide a potential mechanism explaining the acute hyperglycemia/ insulin resistance (substrate competition), a strategy for reversing the metabolic side effects and may also have implications for the mechanisms underlying the cardiovascular side effects of these drugs. We will also further elaborate a non invasive pre-clinical screening strategy for these side effects by examining the effects of drugs acutely in mice by monitoring changes in fed RQ values after drug administration.

Summary of Research Completed

The Schizophrenia Bulletin study provides evidence for a very rapid effect of olanzapine on malonyl CoA and anaplerotic intermediates that frequently serve as intracellular signals for metabolic regulation and fuel selection consistent with hypothesis. Together both studies provide strong support for our original idea that these drugs were causing peripheral energy repartitioning. As we look back at the research that has emerged in the last two years on this subject, these two papers are highly significant. They provide new molecular leads and direction for our proposed studies on the molecular mechanism underlying the metabolic side effects of the atypical antipsychotics.

The Schizophrenia Bulletin study shows how rapidly olanzapine turns over in rats (about 2 hr vs. about 50 h in humans) so we are developing a continuous infusion model. To guide us in developing a dosing protocol, we developed a very sensitive UPLC-Mass spectrometry assay for olanzapine (see fig 1). We will also use this new assay (tail vein blood) to guide our dosing protocol in order to keep the drug within therapeutic concentrations of 70-350nM as described by Maria Buse's group (Robinson KA, *Prog Neuropsychopharmacol Biol Psychiatry* 30:93-98, 2006).

One conclusion of our Schizophrenia Bulletin study was that atypical antipsychotics with metabolic side effects affected intermediary metabolism leading to elevated FFA metabolism and decreased carbohydrate metabolism. Two possible overall mechanisms are possible to explain this. The first is that olanzapine raises plasma glucose and lowers FFA in response to a stimulatory effect on fat metabolism. For example, olanzapine could directly lower malonyl CoA through various means. In this scenario, olanzapine increases fat metabolism and this results in a compensatory decrease in glucose utilization and increased lipid metabolism as reflected in a lower respiratory exchange ratio (RER) (see olanzapine's effect on RER in Fig 2). Alternatively the atypical antipsychotics choke off glucose and amino acid metabolism and this

leads to lower malonyl CoA levels and thereby leads secondarily to an inappropriate albeit necessary metabolic switch to lipid utilization. These two possibilities could be tested by partially blocking FFA metabolism. In the first scenario a block in FFA metabolism would cause no effect on VO2 (capacity of an individual's body to transport and utilize oxygen), lessen or prevent the drop in RER and normalize the circulating FFA and glucose concentrations. No effect would be observed on body temperature. In the second scenario, blocking fat metabolism after olanzapine prevented glucose and amino acid metabolism, would lead to dire consequences. With all of the major catabolic pathways blocked, VO2 and body temperature should drop. If the block of FFA metabolism (CPT1) was complete, we would expect rapid morbidity. If it was a partial block of FFA metabolism, the remaining energy metabolism would still be based on FFA utilization. Thus RER would remain low and might be extended for longer periods because the metabolism of olanzapine is energy dependent.

Thus we performed studies in mice with the irreversible non-competitive carnitine palmitoyltransferase-1 (CPT1) inhibitor, tetradecylglycidic acid (TDGA). Two 30 mg/kg doses of TDGA are typically used for a complete inhibition of CPT1 in rats and mice, whereas a single dose results in a sustained partial block. At the beginning of the dark cycle when animals begin to eat more than the light cycle and their RER increases, we injected 30 mg/kg TDGA or its vehicle. At around 9 PM when the RER was maximal, we provided a low dose of olanzapine (2 mg/kg) by oral gavage. RER, VO2, food intake and body temperature were monitored.

As can be seen from Figs 2-4, the results of this experiment were more consistent with scenario 2. Olanzapine rapidly dropped energy expenditure and body temperature with little effect on the RER. The RER was actually more sustained. However it should be noted that this RER is for a rapidly declining VO2. Two doses of TDGA had no effect on these parameters when provided alone. However after 2 mg/kg or higher doses of olanzapine, rapid lethality resulted.

The protocol for Figs 2-4 is now being repeated with other atypical antipsychotics and other CPT1 inhibitors. In other experiments, we will take plasma for FFA and glucose determinations. The results now help us provide focus for mechanistic studies which will focus on glucose transport or the initial steps of glycolysis as a target of these drugs.

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2. Albaugh VL, Vary TC, Ilkayeva O, Wenner BR, Maresca KP, Joyal JL, Breazeale S, Elich TD, Lang CH, Lynch CJ: Atypical Antipsychotics Rapidly and Inappropriately Switch Peripheral Fuel Utilization to Lipids, Impairing Metabolic Flexibility in Rodents. *Schizophr Bull*, 2010

FIGURES

We have now developed a UPLC-MS assay for Olanzapine in rodent plasma

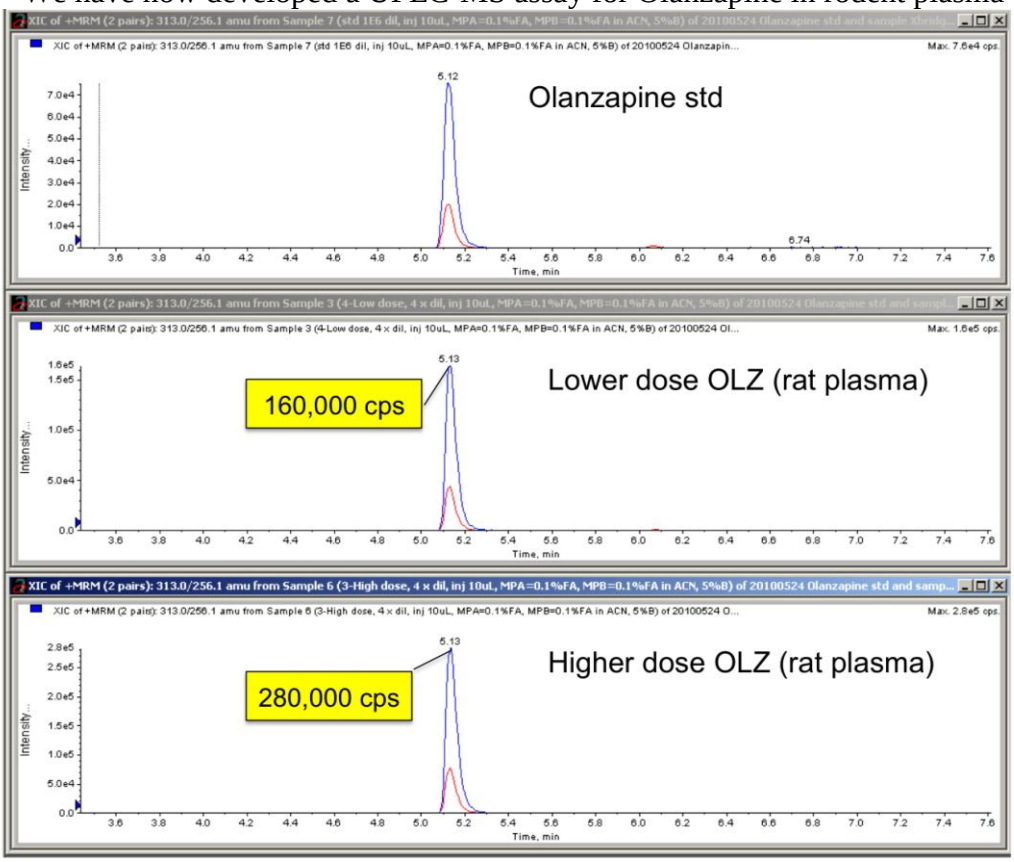

Fig 1. UPLC mass spec assay of olanzapine. Blue and red peaks at around 5.1 min elution times are different product ions from the olanzapine. This is another way to prove that the peak is olanzapine and not something else since they are lined up with elution time. One of the standards is shown plus diluted plasma from animals provided a higher and lower dose infusion during studies piloting dose protocol

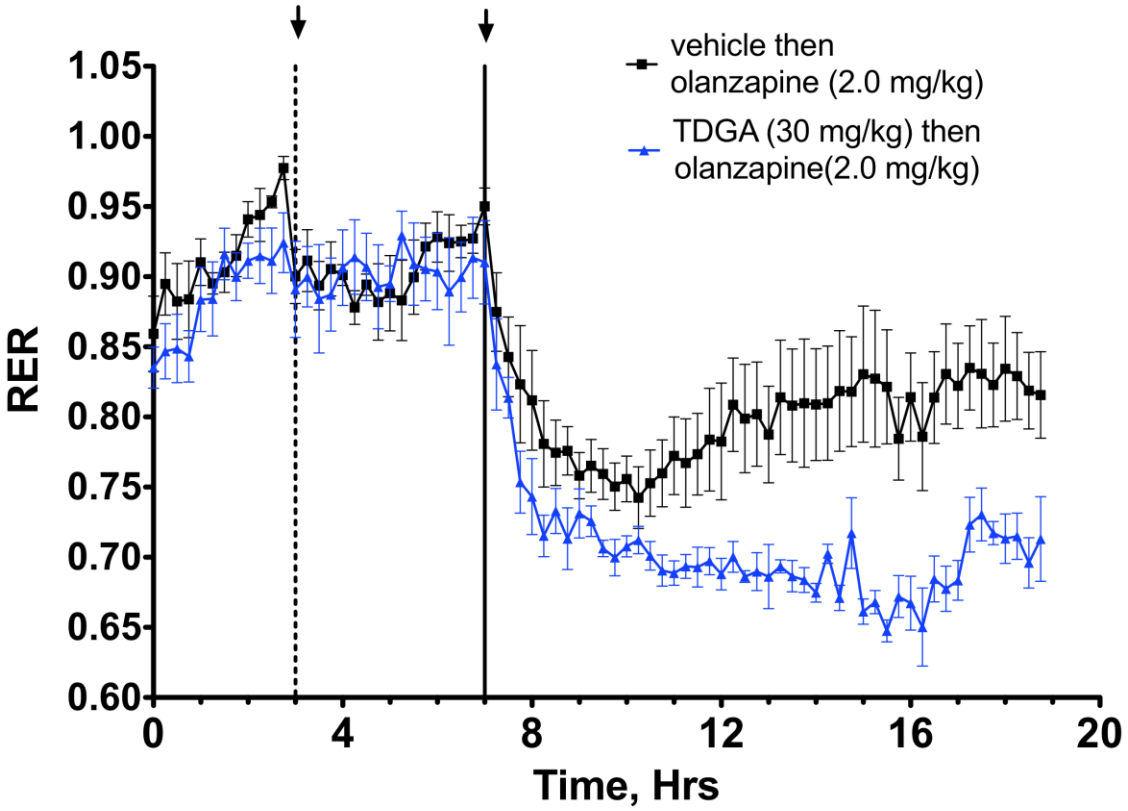

Fig 2. Effect of CPT1 blockade on olanzapine stimulated changes in RER.

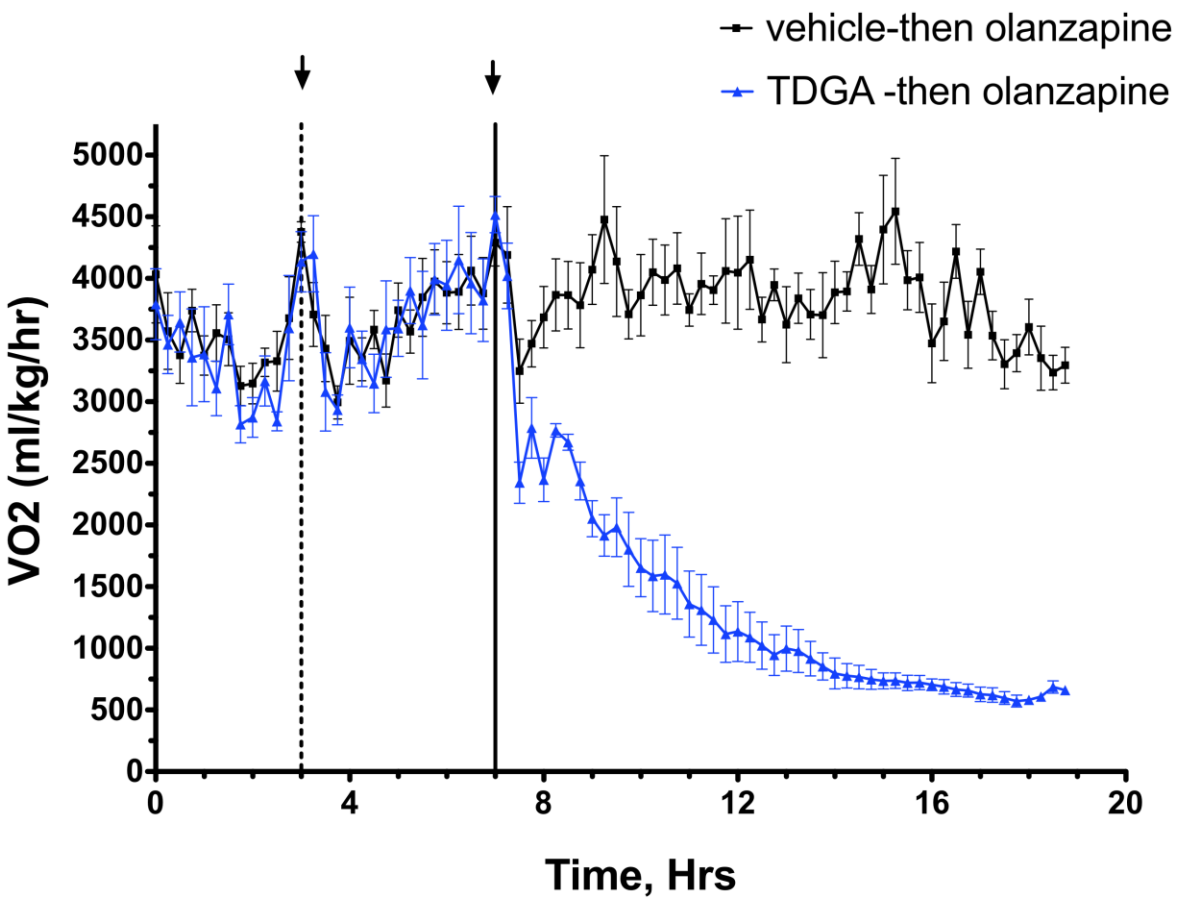

Fig 3. Effect of CPT1 blockade on olanzapine stimulated changes in VO2.

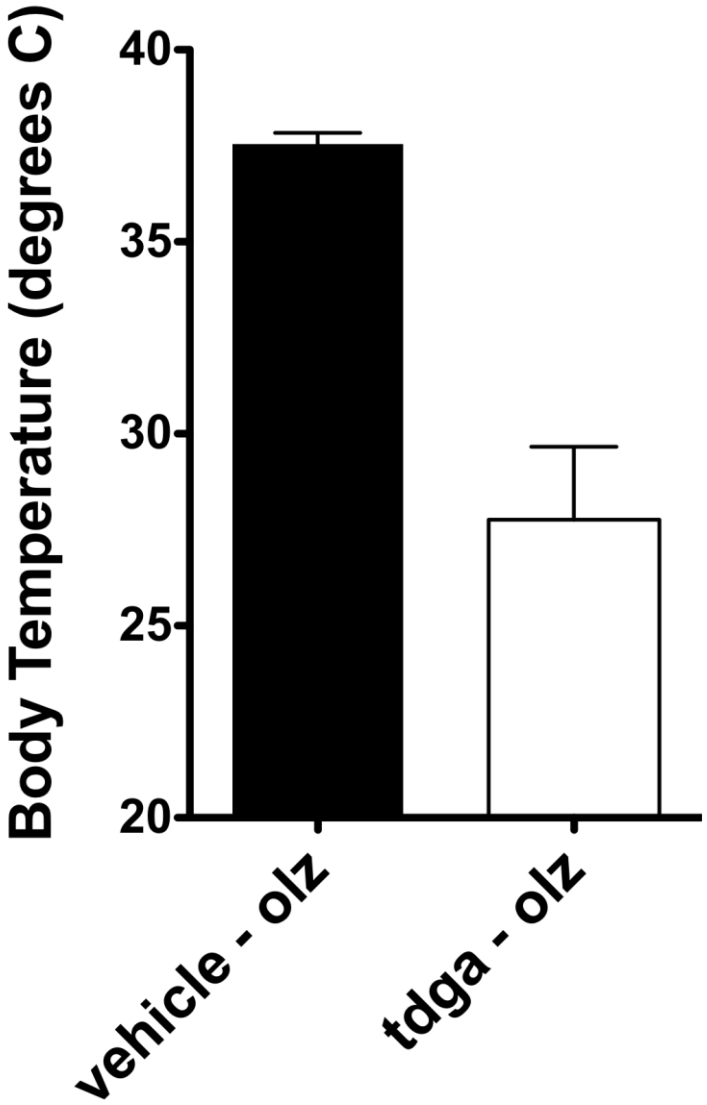

Fig 4. Effect of CPT1 blockade on olanzapine stimulated changes in rectal body temperature.

Research Project 12: Project Title and Purpose

Genetic Analysis of Papillomavirus Virion Morphogenesis - The central hypothesis of this project is that the capsid proteins of human papillomavirus (HPV) have specific functional domains for morphogenesis of infectious viral particles in a differentiation-dependent manner. When completed, this project will provide new insight into the molecular mechanisms of virion morphogenesis in a system capable of reproducing the natural complex process and differentiating environment of native HPV virion (NV) morphogenesis and infection.

Anticipated Duration of Project

1/1/2010 – 6/30/2011

Project Overview

The long-range goal of this project is to define the genetic and molecular events of human papillomavirus (HPV) virion morphogenesis. Specific Aim #1 is designed to biochemically assess the stability and structure of NVs derived from temporally distinct organotypic tissues. Specifically, NVs will be purified on Optiprep density gradients and assessed for gross morphological changes via transmission electron microscopy (TEM), and analyzed for differences in stability via partial chemical reduction and tryptic digests followed by Western blot analyses of L1 protein expression. To probe for distinct capsid assembly stages, we will utilize redox-altering reagents, which have been shown to alter DNA encapsidation efficiencies and infectivity titers of NVs and pseudovirions (PsV), and pulse tissues with these chemicals at various time points to determine when DNA encapsidation and formation of mature NVs finalize. Specific Aim #2 is designed to genetically analyze the role of capsid protein cysteine residues in capsid assembly. We propose to test cysteine mutants by purifying them on Optiprep density gradients followed by observation of gross morphological differences via TEM. Then, we will test their stabilities by partial chemical reduction and tryptic digest experiments followed by Western blot analysis of L1 expression. Specific infectivity of each mutant NV will also be assessed and we will determine how each cysteine substitution affects the tissue-dependent capsid assembly processes described in Specific Aim #1.

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Other Participating Researchers

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Expected Research Outcomes and Benefits

Our expertise with the organotypic virus culture system, which is capable of supporting the complete human papillomavirus (HPV) life cycle, puts us in a unique position to initiate the first studies to directly compare capsid assembly of *in vitro*-derived papillomavirus particles with HPV virions produced in stratified and differentiated epithelial tissues (i.e. native virions). Our studies will provide a foundation to investigate whether the molecular requirements for the production of *in vitro*-derived papillomavirus particles are the same as native virions. We will test hypotheses developed from atomic structures, in addition to genetic, molecular, and biochemical studies of *in vitro*-derived papillomavirus particles using native virions from our organotypic virus culture system. Our studies will provide direction for future atomic structure studies of *in vitro*-derived papillomavirus particles, therefore, developing a synergy between atomic structure and genetic analysis of native virions. When completed, this project will provide new insights into the molecular mechanisms of HPV virion morphogenesis in a system capable of reproducing the natural complex process and differentiating environment of HPV morphogenesis and infection. Further, such an understanding of HPV capsid assembly within human tissue will facilitate the future production of therapeutics which will inhibit the spread of HPV infection.

Summary of Research Completed

In this study, we sought to determine individual temporal roles for the highly conserved HPV16 L1 cysteine residues: C428, C175, and C185 in capsid assembly within the context of stratifying and differentiating human epithelial tissue – HPV's natural host environment. Previous studies have depicted that C428S mutations in HPV16 VLPs lead to the complete loss of capsid integrity, being disrupted into their respective capsomeres. These studies have been extended to HPV16 PsV whereby C428S mutations also lead to the failure of capsids to form and then subsequently mature, suggesting a role for this residue in initial assembly and perhaps maturation. A role for C428 in the formation of a critical interpentameric disulfide bond is supported by biochemical and cryo-electron microscopy data of non-reduced L1 dimers from HPV18 VLPs whereby an interpentameric disulfide bond between C175 and C429 is formed. A recent high-resolution cryo-electron microscopy image of native BPV confirms the presence of a C171-C426 interpentameric disulfide bond. However, the C175S substitution in both HPV16 VLPs and PsV does not lead to complete disruption of capsids to capsomeres as is seen with the C428S mutant. Instead, long tubular structures are formed, as seen via electron microscopy (EM), and aberrant dimer formation is observed via Western blot analysis. This result suggests that interpentameric contacts continue to form even without C175.

Thought to reside on an external loop neighboring C175, it has been suggested that C185 might form similar interpentameric contacts with C428 with only minor structural rearrangements of this loop. While the most recent high-resolution cryo-electron microscopy image reconstruction of native BPV virions only depicted C175-C428 interpentameric disulfide bonds and not C185-C428 interactions, this might be due to the low abundance or asymmetry of C185-C428 disulfides which are averaged out of the reconstruction. It may also be possible that C185-C428 interpentameric disulfide bonds occur only transiently during the regulated and temporal process

of stratification and differentiation of human epithelial tissue. If this is the case, C185-C428 disulfides might serve as a transition stage that facilitates final C175-C428 disulfide bonds.

Our data supports that all three conserved cysteine residues: C428, C175, and C185 are critical for proper assembly and maturation of native HPV16 virions. While no individual substitution led to the complete disruption of particles as was previously seen with HPV16 VLPs and PsV, each substitution undermined particle formation efficiency and stability – making these virions more susceptible to intrusion of endonucleases into the capsid lumen, and less able to withstand the high shear forces of ultracentrifugation. Further, all three substitutions led to a deficiency in capsid maturation when exposed either to the natural, tissue-spanning redox gradient or through artificial treatment with GSSG (GSSG data not shown).

In Fig. 1A-C, it is apparent that the C175S mutation severely limits the number of endonuclease-resistant genomes that are present within 10-day CVPs, while C185S and C175,185 mutations do not appear to limit wild-type levels. We hypothesize that the substitution of C175 for serine might allow for the formation of improper C185-C428 interpentameric disulfide bonds, which leads to structural deficiencies. This is supported by previous studies utilizing C175S mutant HPV16 VLPs and PsV which lead to the production of abnormal tubular structures via EM and the production of aberrant L1 dimers, respectfully. Accordingly, loss of C185 allows for preferable C175-C428, or unknown intra- and/or interpentameric interactions with C175 to occur. It is possible that at 10-days C175 might be precluded from forming a disulfide with C428, but C185-C428 disulfide bonds can form in the absence of C175. This rationale is supported since the C175,185S double mutation leads to near wild-type levels of endonuclease-resistant genomes within 10-day CVPs. At 10-days, HPV16 virions are within the reducing environment of the suprabasal nuclei. As published previously, in this environment, a large degree of disulfide bonding would not be expected to occur. However, some level of disulfide bonding between the high molar concentration of neighboring L1 pentamers should be expected.

While 10-day capsid assembly events remain unclear, analysis of 20-day CVPs reveals the temporal importance of C428 and C185. While numbers of endonuclease-resistant genomes remain low in 20-day C175S CVPs as they had in 10-day CVPs, C428S and C185S mutations only now begin to affect yield of endonuclease-resistant genomes. This observation suggests that either, C185 and C428 form a disulfide during maturation within tissue, or that C185 is critical for the transition to more extensive C175-C428 disulfide bonds. Due to previous structural analyses of capsids which have depicted a C175-C428 disulfide bond, we hypothesize that a transient C185-C428 disulfide bond occurs which primes final C175-C428 disulfide bonds.

Further supporting the role of a complex interplay of disulfide bonding between C428, C175, and C185, is our finding that C428S, C175S, C185S, and C175,185S 20-day CVPs have a significant collapse in detectable endonuclease-resistant genomes compared to their 10-day counterparts. This suggests that each of these residues is important in the efficient accumulation of properly formed capsids (Fig. 1C). While variations in specific infectivity of 10 and 20-day mutant virions were observed when performing infections directly from CVPs, infectivity was all but lost post-Optiprep gradient centrifugation (Fig. 3A-E). This result suggests that the formation of critical interpentameric disulfide bonds is not extremely important for infectivity (Fig. 2A-D), but adds

to the efficiency of particle assembly and to the fitness of the individual particle when it is subjected to environmental stress.

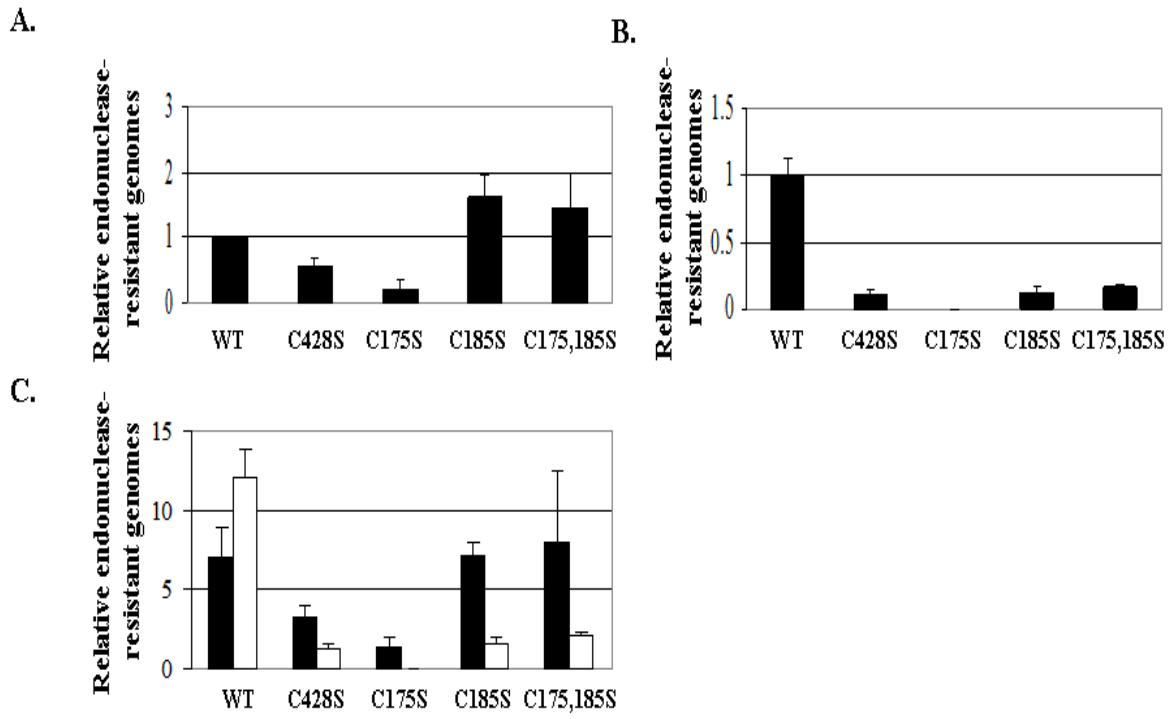

Figure 1. Endonuclease-resistant genomes in 10 and 20-day wild-type and mutant CVPs. (A) SYBR green-based DNA encapsidation assay relative quantification of 10-day wild-type (WT), C428S, C175S, C185S, and C175,185S CVPs. WT is set to 1.0. (B) SYBR green-based DNA encapsidation assay relative quantification of 20-day wild-type (WT), C428S, C175S, C185S, and C175,185S CVPs. WT is set to 1.0. (C) Side-by-side comparison of 10 (black bars) and 20-day (white bars) raw data from A and B. Relative y-axis values represent copy number controls used in the standard curve. The results are means and standard errors for at least three independent experiments.

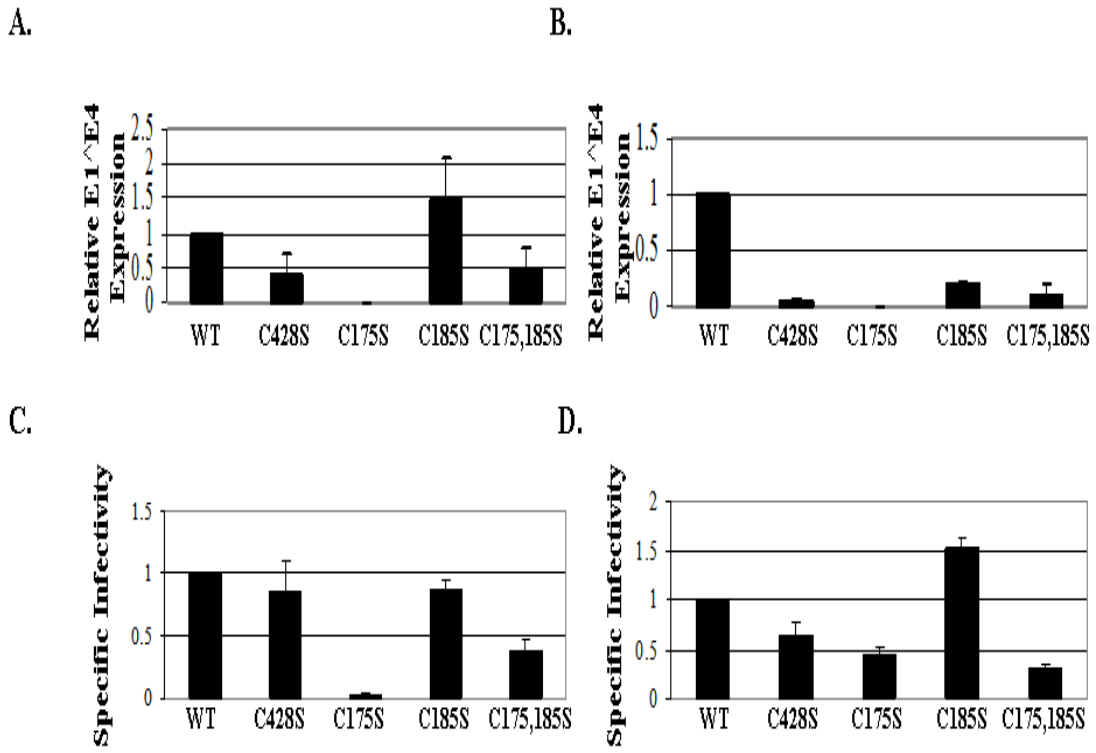

Figure 2. Relative and specific infectivity of 10 and 20-day wild-type and mutant virions. (A) Relative RT-qPCR infectivity assay of 10-day wild-type (WT), C428S, C175S, C185S, C175,185S CVPs. WT is set to 1.0. (B) Relative RT-qPCR infectivity assay of 20-day wild-type (WT), C428S, C175S, C185S, C175,185S CVPs. WT is set to 1.0. (C) Specific infectivity of 10-day wild-type (WT), C428S, C175S, C185S, C175,185S CVPs. Relative endonuclease-resistant genome data in Fig. 4A was used to normalize relative infectivity data in A. WT is set to 1.0. (D) Specific infectivity of 20-day wild-type (WT), C428S, C175S, C185S, C175,185S CVPs. Relative endonuclease-resistant genome data in Fig. 4B was used to normalize relative infectivity data in B. WT is set to 1.0.The results are means and standard errors for at least three independent experiments.

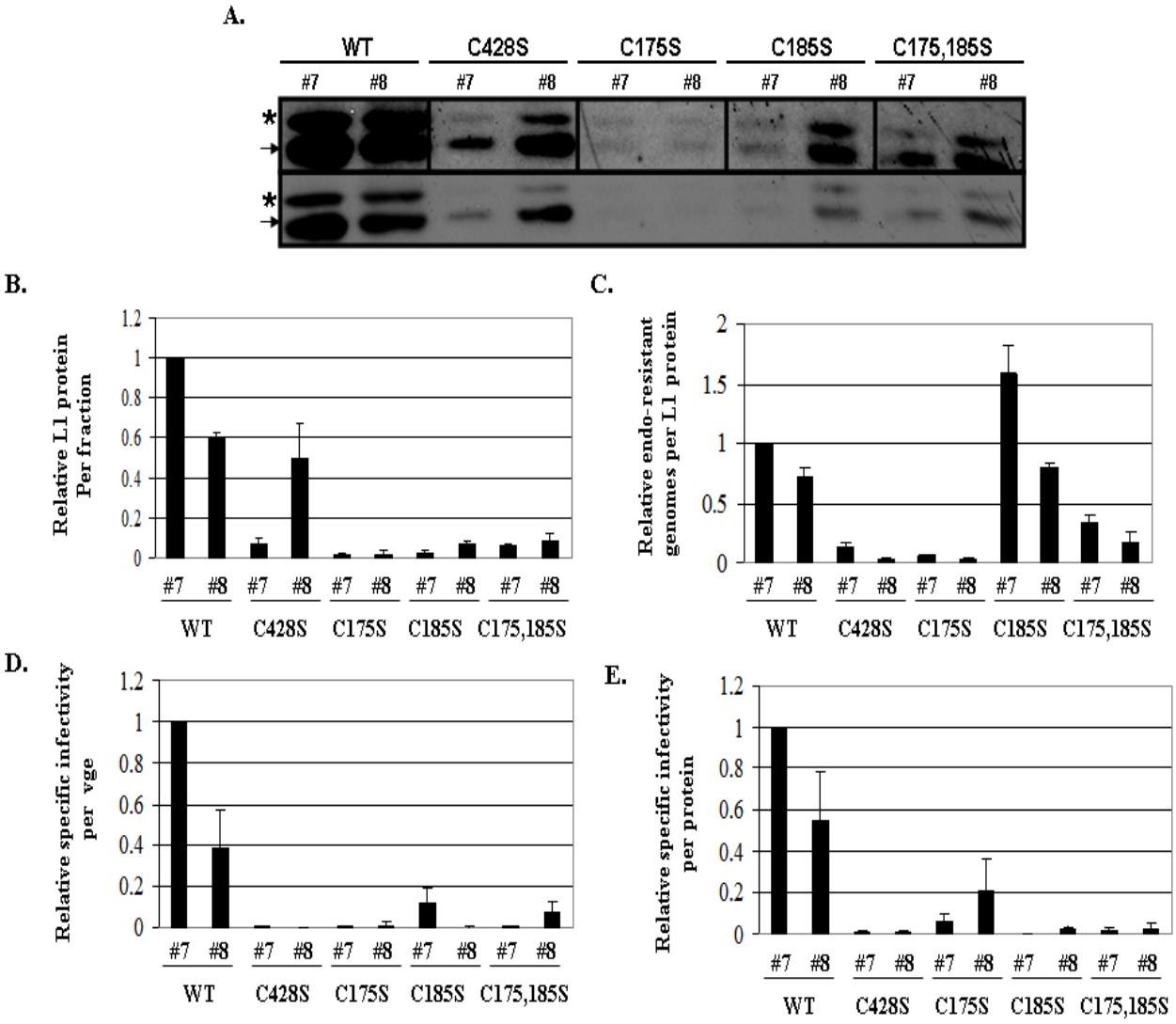

Figure 3. Analysis of infectious Optiprep fractions. (A) Western blot analysis of L1 in highly infectious Optiprep fractions #7 and #8 from Optiprep-fractionated 20-day wild-type (WT), C428S, C175S, C185S, and C175,185S CVPs. Higher and lower molecular weight species of L1 are indicated. Both species were identified as HPV16 L1 by mass spectrometry analysis. (B) Densitometric analysis of the faster migrating L1 species (i.e. 55 kD) in three independent Western blots as shown in A. WT fraction #7 was set to 1.0. (C) Relative endonuclease-resistant genomes per fraction, normalized to the relative amount of L1 protein within fractions. WT fraction #7 was set to 1.0. (D) Specific infectivity of 20-day wild-type (WT), C428S, C175S, C185S, C175,185S virions within fractions #7 and #8. Relative endonuclease-resistant genomes per fraction were used to normalize relative infectivity data within fractions. WT is set to 1.0. (E) Specific infectivity of 20-day wild-type (WT), C428S, C175S, C185S, C175,185S virions within fractions #7 and #8. Relative L1 protein content per fraction was used to normalize relative infectivity data within fractions. WT is set to 1.0.The results are means and standard errors for at least three independent experiments.

Research Project 13: Project Title and Purpose

Epidermal Stem Cell Properties in Mice with Altered Polyamines - Our research studies the role of the polyamine pathway in early skin cancer development. Since skin cancers are the most common form of malignancies world-wide, identification of possible targets for prevention is highly relevant to public health. Understanding the role of the polyamine pathway is also of great importance because the polyamine biosynthesis inhibitor difluoromethylornithine (DFMO) is a promising chemopreventive agent of human skin cancer.

Anticipated Duration of Project

1/1/2010 - 12/31/2010

Project Overview

Our project will use subpopulations of primary keratinocytes derived from several transgenic mouse models to identify the downstream targets of polyamines that alter keratinocyte stem cell expansion.

Previous work in our lab has shown that the upregulation of ornithine decarboxylase (ODC) through overexpressing constitutively active MEK in the skin results in expansion of epidermal stem cells, activation of progenitor cells, and increased skin tumorigenesis. Double transgenic mice overexpressing Antizyme (AZ), an endogenous inhibitor of ODC, and MEK showed a reduction in the MEK-induced phenotypes, including stem cell expansion and tumor formation. These data strongly support the hypothesis that polyamines are critical regulators of stem cell expansion and activation of progenitor cells. However, the downstream targets of polyamines remain largely undefined. We hypothesize that MEK plays a critical role in stem cell activation by altering transcription in response to carcinogens, and these changes are mediated in a polyamine-dependent manner. Our aim will examine the transcriptional profiles of $CD34^{+}/\alpha_6 integrin^{high}$ (immature stem cell enriched) and $CD34^{+}/\alpha_6 integrin^{low}$ (committed progenitor cell enriched) populations of cells isolated from AZ, MEK, and MEK/AZ mice. With these experiments, we hope to identify a pattern of gene expression in the keratinocyte stem cell population that responds to changes in intracellular polyamines. Our studies may thus lead to the identification of new targets for chemoprevention of skin cancer.

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Expected Research Outcomes and Benefits

Although it is known that the mammalian epidermis is maintained by self-renewal of stem cells, the underlying mechanisms, and how these mechanisms change in cancer, are not yet well-defined. Therefore, increasing our understanding of the mechanisms responsible for stem cell proliferation and their differentiation into mature cells could lead to important new cancer therapies and modes of prevention. The ability to prevent the onset of non-melanoma skin cancer in high-risk groups by topical application of a drug like the ODC inhibitor DFMO would be of great interest to the general public.

Summary of Research Completed

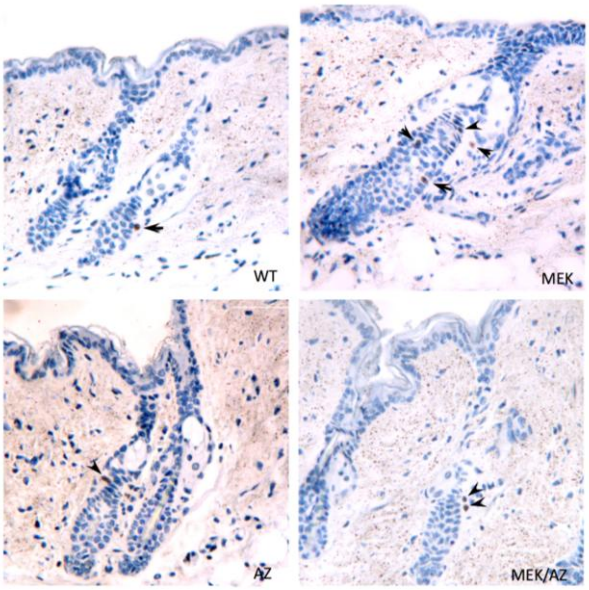
Our ultimate goal is to examine the transcriptional profiles of stem- and progenitor- cell enriched populations isolated from the skin of AZ, MEK, and MEK/AZ mice. To identify stem cells within the epidermis and hair follicles, we first determined the number of bulge-region label retaining cells (LRCs). Even though stem cells within the bulge are relatively quiescent, they still undergo cycling. These bulge region stem cells are characterized by their ability to retain BrdU over a prolonged period due to their slow cycling time. To mark LRCs, mice of all genotypes (N=3 for each group) were injected s.c. with 50 µg/g two times per day on post-natal (PN) day 3, PN4 and PN5, followed by a chase period of 8 weeks. This time point was chosen because it has been shown previously that a long chase period results in distinct label retention only in cells from the bulge region of the hair follicle. Mice were sacrificed and full-thickness skin was isolated, embedded in paraffin, and analyzed for BrdU incorporation by immunohistochemistry using an anti-BrdU primary antibody. Follicular LRCs were quantified as the number of BrdU-positive cells per 10 hair follicles, with 4 different skin sections tabulated per mouse.

The results show that MEK overexpression significantly increased the number of LRCs within the bulge (Figure 1A, B). This supports our hypothesis that overexpression of this Ras downstream effector leads to the expansion of epidermal stem cells. K5-AZ mice showed no difference in the number of bulge region LRCs compared to wild-type mice, while K5-AZ/K14-MEK mice had an intermediate phenotype with significantly more LRC than wild-type but significantly less than K14-MEK mice (Figure 1A, B). This is in keeping with our expected results, suggesting AZ expression limits epidermal stem cell expansion in K14-MEK mice, and that this contributes to the tumor suppressor effects of AZ.

To confirm that follicular BrdU-positive cells are stem cells, we are analyzing serial sections of hair follicles stained with α6-integrin and CD34, which have been established as markers of bulge region cells thought to represent stem cells. To obtain a quantitative analysis of the follicular bulge cells present in mice from each of our genotypes of interest, the number of cells positive for α6-integrin and CD34 is determined by fluorescence-activated cell sorting (FACS) analysis. Primary keratinocytes are isolated from 1-3 day old mice using standard techniques. Briefly, epidermis and dermis are incubated in 0.25% trypsin overnight at 4ºC. After removal of the dermis, the epidermis is trypsinized to create a single-cell suspension. One million cells of each genotype are labeled with an FITC-conjugated anti-α6-integrin antibody (PharMingen) and

a Phycoerithrin-conjugated anti-CD34 antibody (PharMingen) for 30 min at room temperature. Cells are analyzed by FACS analysis in the Flow Cytometry Core Facility at the Penn State College of Medicine and sorted according to their expression of α6-integrin and CD34. Cells identified as α6-high, CD34-high represent basal bulge cells that are enriched in stem cells. These experiments are ongoing. Once this technique is optimized, these isolated cell populations will be used for transcriptional analysis.

A



B

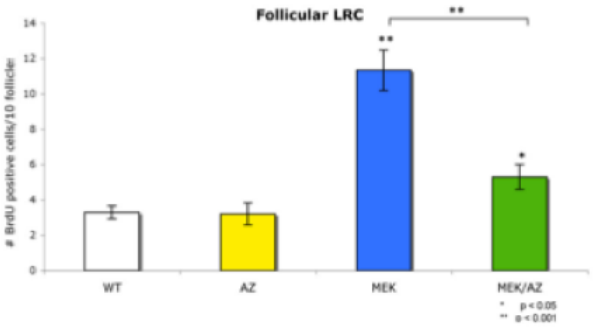


Figure 1. MEK expression expands the number of stem cells within the hair follicle bulge, and this is partially reversed by the co-expression of AZ.

Research Project 14: Project Title and Purpose

Topical Application of Isoselenocyanates for the Prevention of Melanoma – Malignant melanoma is the most fatal and invasive form of skin cancer. The cause of 60% of melanomas is yet to be determined, making development of effective targeted chemopreventive agents especially important. In spite of the widely appreciated magnitude of the problem, there is still a critical gap in knowledge regarding key deregulated signaling pathways causing melanoma and chemopreventive agents targeting these gene defects to prevent this disease. Therefore, the purpose of this project is to develop *a rationally designed chemopreventive agent targeting the Akt3 signaling pathway*. As a direct outcome of the investigation, we expect to validate the effectiveness of novel synthetic agents derived from naturally occurring chemopreventive agents as a topical agent that prevents early melanocytic lesion development.

Anticipated Duration of Project

7/1/2010 – 6/30/2011

Project Overview

New strategies are urgently needed to prevent development of melanoma. Currently, surgical excision is the mainstay for eliminating early melanocytic lesions or preventing development of more aggressive cancer. Despite these preventive strategies and increased use of sunscreen, incidence and mortality rates of melanoma continues to rise by about 4% per year in the US and worldwide. This makes melanoma the cancer with the second fastest rate of increase in this country.

Recently, we identified elevated Akt3 activity occurring in ~70% of sporadic melanomas compared to normal cells. Functionally, active Akt3 reduces responsiveness of early pre-malignant melanoma cells to agents that would normally kill via apoptosis, thereby promoting melanoma development. Unfortunately, no agents are available to inhibit the Akt3 signaling cascade in early melanoma cells. Use of sunscreen or body lotions containing topical agents with chemopreventive efficacy could be potentially useful for preventing cancer development. Therefore, novel topical chemopreventive agents with negligible systemic toxicity to prevent melanocytic lesion development would be highly significant. In order to meet this unmet medical need, potent anti-melanoma agents called isoselenocyanates (ISC) have been developed from naturally occurring isothiocyanates (ITC), which are derived from plants. In these agents, sulfur has been replaced with selenium and the alkyl side chain has been lengthened from 1 or 2 to 4 or 6.

The *central hypothesis* for this project is that targeting Akt3 signaling in early melanoma lesions would be an effective chemopreventive approach for preventing melanoma lesion development. The *hypothesis will be tested* by determining the chemopreventive utility of novel synthetic isoselenocyanate derivative as a topical agent that inhibits Akt3 signaling in a preclinical mouse model of melanoma. The *approach to* be used involves, evaluating effectiveness for retarding melanoma development in culture and animal models of this disease. Accomplishing these goals would be highly significant, providing novel insight into the chemopreventive potential of

targeting a major signaling pathway promoting melanoma development, and provide solid rationale for initiating clinical trials with potent selenium-containing topical chemopreventive agent with significant potential to prevent melanocytic lesion in melanoma patients by targeting Akt3 signaling.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

Currently, no effective chemopreventive agents exist to prevent /retard development of early into more advanced melanomas in the skin. Research, models and technology contribute to emerging novel concepts that suggest development of unique synthetic chemopreventive agents that target Akt3 signaling to prevent early melanoma development within skin.

This research is expected to yield several outcomes: First, demonstrate that inhibition of Akt3 signaling in reconstructed human skin retards development of early melanocytic skin lesions; and second, validate that topical application of a selenium analog of isothiocyanate targets Akt3 signaling and prevents melanoma development in a transgenic melanoma mouse model. Thus, a novel topical chemopreventive agent inhibiting early melanomas would be identified, contributing a needed tool to the relatively small arsenal of chemopreventive agents currently available for preventing this disease.

For melanoma patients and the general public, these discoveries have the potential to significantly impact human health by decreasing the number of persons having melanoma, thereby directly decreasing mortality rates. The positive impact for reducing melanoma incidence and mortality rates would be significant.

Summary of Research Completed

The project has not yet started.

Research Project 15: Project Title and Purpose

Activation of STAT3 Protects Liver from Ischemia Reperfusion Injury - Liver resection is the only curative option for liver tumors and liver transplantation is the only viable treatment for end stage liver disease. Warm ischemia/reperfusion (I/R) is a common clinical problem during both types of surgeries. Warm ischemia also occurs in trauma and shock. The liver is the most commonly injured abdominal organ in blunt and penetrating abdominal trauma. The management of trauma and shock frequently involves exposing the liver to varying periods of warm ischemia followed by reperfusion. I/R injury is significantly associated with morbidity and mortality in such conditions. Prevention or minimization of liver I/R injury is the clinical priority for our study, because there is still no safe and promising strategy to protect liver from I/R injury.

Anticipated Duration of Project

1/1/2010 – 12/31/2010

Project Overview

Signal transducers and activators of transcription 3 (STAT3) is a major signaling molecule for a variety of genes in response to cell stimuli and plays a key role in many cellular processes such as cell growth and apoptosis. Recent studies have suggested that STAT3 activation is involved in protection of hepatocyte from I/R injury. Our goal is to understand the mechanisms of STAT3 in I/R injury. In this project, we hypothesize that STAT3 is an essential factor in protecting liver and in survival from I/R injury. Using STAT3 hepatocyte-specific deficient mice and their wild type control, we will examine this hypothesis in response to partial and total liver ischemia.

Specific Aim 1: To demonstrate that STAT3 has hepatoprotective role from partial hepatic injury. A persistent 90 minutes partial liver ischemia followed by reperfusion will be performed in both STAT3 hepatocyte deficient mice and wild-type littermates. STAT3 expression, serum transaminase level, liver degeneration (apoptosis) and regeneration (cell cycle reentry) will be monitored by molecular biochemical and immunohistochemical assays. Animals will be sacrificed at 6 hours (injury phase) and 48 hours (repair phase) after reperfusion. Liver enzyme level and liver pathology will be compared between STAT3 deficient mice and wild-type littermates. Histological changes will be examined using liver I/R injury score.

Specific Aim 2: To demonstrate that STAT3 is essential for animal survival from total warm ischemic injury. Total liver ischemia behaves differently from partial liver ischemia. It has been shown that all animals survived 60 minutes of total ischemia, whereas all died after 90 minutes of total ischemia. An intermediate time period of 75 minutes of total liver ischemia followed by reperfusion will be performed in control and hepatocyte specific STAT3 deficient mice. A poor survival outcome is expected in the STAT3 deficient group compared to control group.

This study will provide direct evidence whether STAT3 activation is the essential process in liver protection and survival from I/R injury. In this application, we will be able to show the mechanisms of STAT3 on I/R injury not only at the cellular level but also at the in vivo level.

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Expected Research Outcomes and Benefits

To minimize blood loss during liver surgeries, hepatic vascular inflow is temporarily clamped (ischemia) and de-clamped after resection of tumors (reperfusion). This procedure is known as Pringle maneuver and it is a well-established and routine procedure during liver surgeries. However, Pringle maneuver causes I/R injury, which may cause liver failure.

Liver transplantation has significantly progressed during the past decades and it is the only viable treatment for end stage liver disease. More than 6,000 liver transplantations were performed in United States in 2008. In liver transplantation, the liver receives ischemic damage in cold preservation and the liver is exposed to reperfusion injury after implantation. I/R injury in liver transplantation is strongly associated with a non-functional liver, which is a life threatening condition and requires emergent liver re-transplantation.

I/R injury is a common clinical problem encountered during liver resection and liver transplantation. Warm I/R injury also occurs in trauma and shock. This study will provide direct evidence whether STAT3 activation is the essential process in liver protection and survival from I/R injury. We may be able to identify methods to minimize or prevent I/R injury as a result of this study.

Summary of Research Completed

Specific Aim 1: Genotyping and grouping: The genotyping of STAT3 (F/F) mice and TTR-Cre (+/-) mice was confirmed at postnatal 21 days by PCR analysis. Male homozygous and wild-type littermates were used for this study

Mice were divided into two groups between STAT3 deficient and controls, with 5 animals in each group. 70% partial liver ischemia was performed as follows: Mice were anesthetized (i.p.) with a mixture of 100mg/kg Ketamine and 10 mg/kg Xylazine. A midline incision was made on the abdomen. The liver was exposed with retractors placed in the flanks. The blood supply to medial largest lobe and lateral lobe of the liver (70% of liver) was temporally interrupted

(ischemia) by applying a microclamp (FD562; Aesculap, South San Francisco, CA) to the vascular pedicle. The blood supply was resumed by removing the microclamp after 90 minutes of ischemia (reperfusion). Mice were sacrificed at 6 hours after ischemia/reperfusion.

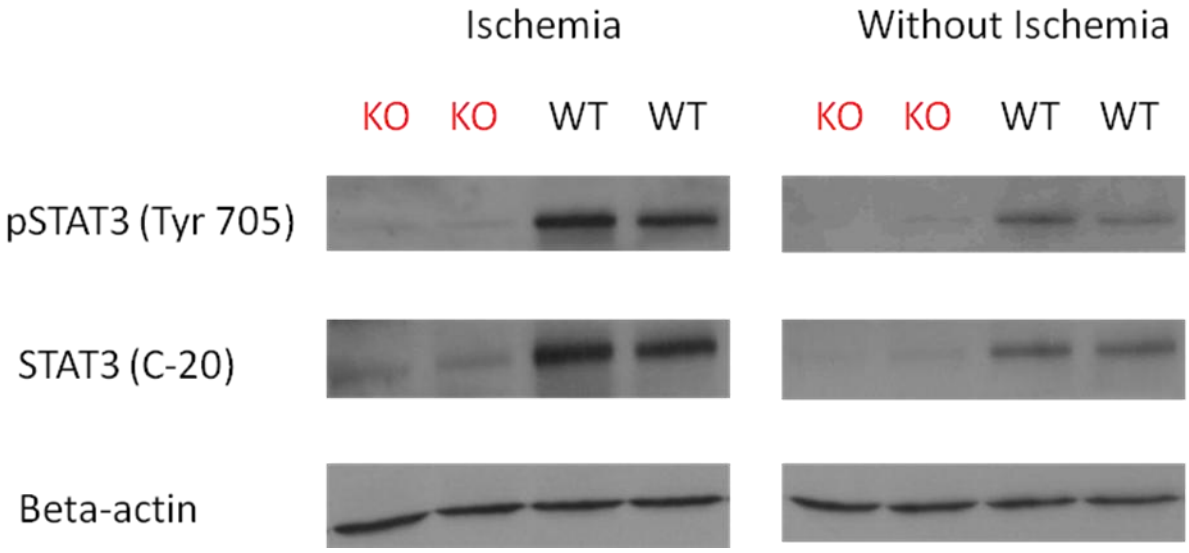
Total and phosphorylated STAT 3 expression was studied in liver at 6 hours after I/R. As shown in Figure 1, I/R injury induced significant expression of STAT3 in control groups. On the other hand, no expression of STAT3 was confirmed in STAT3 deficient mice (Figure 1).

Alanine aminotransferase (ALT) levels were compared between control and STAT3 deficient mice at 6 hours after I/R. Control group had ALT levels = 1636 ± 1571 u/L whereas STAT3 deficient group ALT levels = 808 ± 457u/L. We evaluated 3-4 mice in each group and there was no significant difference with this small number of samples.

Samples of liver tissue were also evaluated for pathology. Histological changes at 6 hours after reperfusion were evaluated for the degree of cytoplasmic vacuolation, congestion and necrosis and scored from 0 to 4 based upon Suzuki criteria of I/R injury. STAT3 deficient mice group appeared to have more liver damage compared to mice in the control group as shown in Figure 2. Statistical analysis of these data is pending.

Apoptotic change of hepatocytes is an established parameter for evaluation of liver damage after I/R injury. TdT-dUTP terminal nick end-labeling (TUNEL) analysis was used to detect apoptosis on liver tissues at 6 hours after reperfusion. STAT3 deficient mice group appeared to have more apoptotic hepatocytes compared to control group as shown in Figure 3. Statistical analysis of the related data is pending.

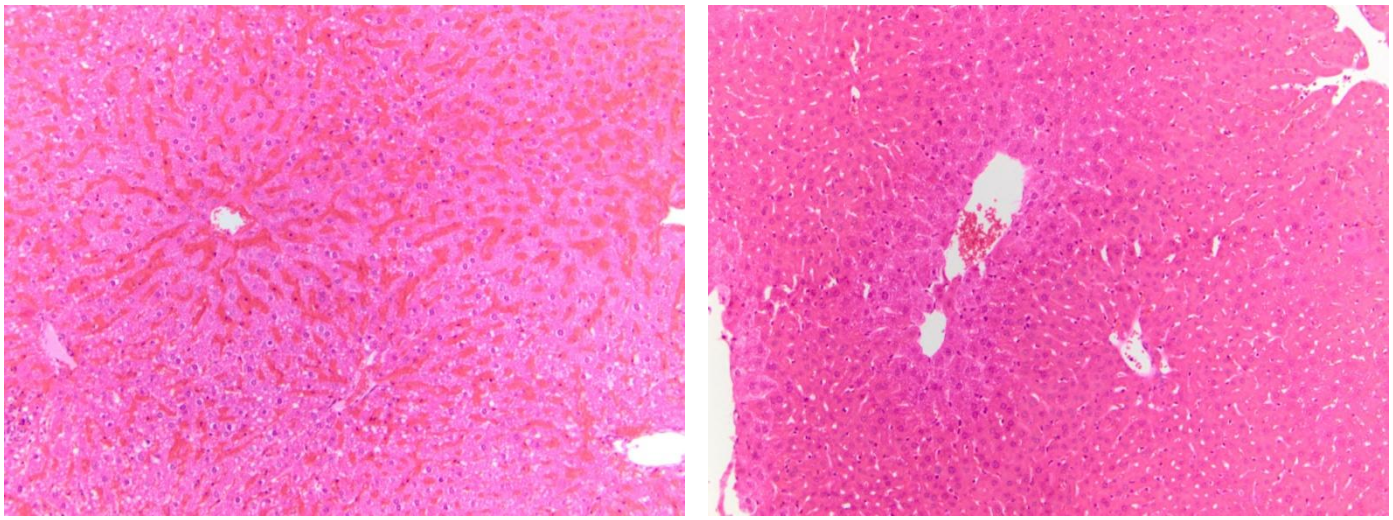
Figure 1



KO: knockout mice, WT: wild type, pSTAT3- phosphorylated STAT3

Figure 2

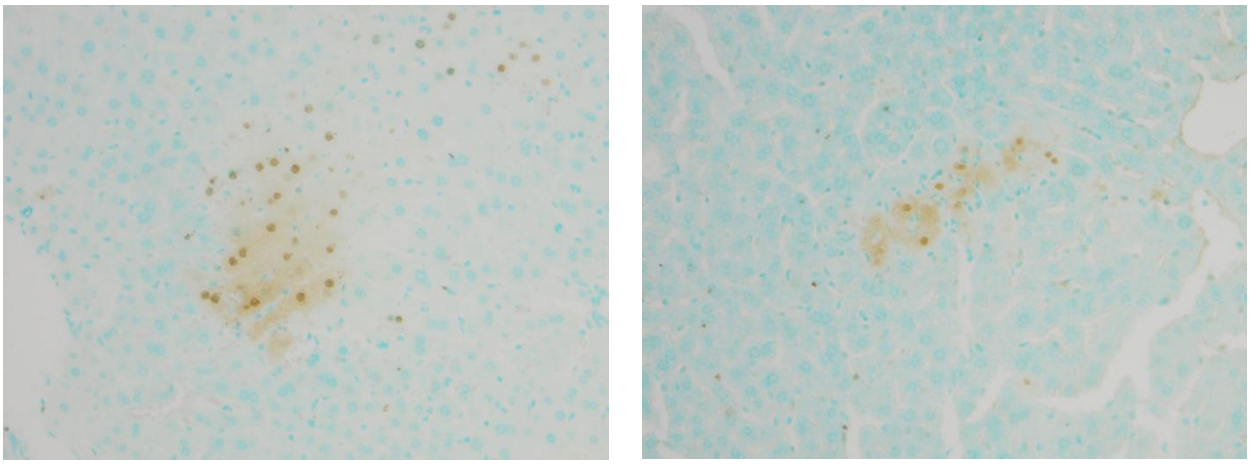
STAT 3 Control



STAT 3 KO groups had significant congestion compared with wild type groups

Figure 3

STAT 3 KO Control



Apoptotic hepatocytes were observed at 6 hours after ischemia reperfusion in both groups. Further analysis requires more sample size and statistical analysis.

Research Project 16: Project Title and Purpose

Multifunctional Nanoparticles for Melanoma and Brain - Malignant melanoma and glioblastoma multiformi (GBM) are invasive and deadly forms of skin and brain cancer respectively with no effective therapy to treat advanced disease, leading to poor survival rates. A P01 Program Project Grant is proposed consisting of 5 projects using "Multifunctional Nanoparticles" to develop a new category of agents to treat these cancers. This project will focus on developing preliminary data to be used in supporting the Program Project application and to illustrate advanced integration and improved synergy between the 5 projects incorporated in the Program Project Grant (PPG).

Anticipated Duration of Project

7/1/2010 - 6/30/2011

Project Overview

The purpose of this project will be to develop preliminary research data in the following areas: Development of liposomes targeting genes, which when targeted together, will lead to synergistically acting cancer inhibition and, into which plasmids can be loaded to express lost genes in cancer cells. This project will also undertake siRNA screens to identify genes when targeted with H-ferritin will synergistically inhibit cancer cells. The project will further undertake studies to develop a cell culture based blood brain barrier model and study liposome movement across the barrier. Finally, this project will collect preliminary data in a study that focuses on the liposome uptake to circulating tumor cells in a flow system. The preliminary data obtained from this study will be used in a Program Project application. The P01 application will focus on Melanoma and GBM since both are deadly and invasive diseases frequently

metastasizing to, or occurring in, the brain respectively. Furthermore, for both cancers, lack of effectiveness of untargeted therapies has led to the conclusion that targeted therapeutics are needed that inhibit multiple proteins or pathways causing these diseases. To accomplish this objective, multifunctional nanoparticles will serve as a single platform for delivering agents which will target multiple pathways that can also be used for imaging the cancer. As a direct result of this realization, the members of this PPG have chosen this platform to address important biological questions related to each disease. The PPG is centered on a funded R01 project (project 1), which focuses on developing synergistically acting nanoparticles targeting multiple proteins in order to more effectively treat melanoma. It should be noted that this R01 project was, in its initial phase, supported by a Pennsylvania Department of Health Research Grant. The use of multifunctional nanoparticles is a unifying theme focusing on developing synergistically acting nanoparticles targeting multiple proteins in order to more effectively treat melanoma. Use of multifunctional nanoparticles is also a unifying theme to solve important biological problems in melanoma and brain cancer. Interaction and sharing between projects will results in a cooperative and synergistic team effort.

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Expected Research Outcomes and Benefits

This project will be focused on obtaining preliminary data to be used in a P01 application. More specifically, it will be using Pennsylvania Department of Health Funds to develop research data that will support the liposome development for the respective projects of the P01application. Preliminary research data will also be used to illustrate high levels of integration and synergy between the 5 projects incorporated in the proposed PPG application. This, according to the NIH, based upon a NIH visit (12/17/2009) and presentation of the proposed PPG, will significantly increase the probability of having this PPG funded. Funding of this PPG, over the long-term, will have a significant positive impact on the currently poor prognosis faced by advanced-stage melanoma and glioblastoma patients and will contribute to the availability of more effective therapeutic agents, which would increase the length and quality of life for these patients. Therefore, the positive impact on melanoma and glioblastoma therapeutic development will be significant. Expected deliverables of this PPG are:

1. Novel synergistically acting nanoparticles targeting multiple kinase targets to more effectively kill melanoma, which do not currently exist.
2. Novel nanoparticles that synergistically increase the sensitivity of glioblastoma cells to BCNU chemotherapy, which does not currently exist.
3. Novel nanoparticles for imaging and treating melanoma lymph node, which do not currently exist.
4. Novel imaging and therapeutic nanoparticles for measuring the efficacy of ultrasound for permeating the blood-brain barrier and shrinking glioblastoma or metastasized melanoma tumors, which do not currently exist.
5. Novels nanoparticles for targeting circulating tumor cells to disrupt extracellular interactions in order to decrease metastasis, which do not currently exist.

Summary of Research Completed

The project has not yet started.

Research Project 17: Project Title and Purpose

Parathyroid Hormone and Prostate Cancer Colonization of Bone - There is a fundamental gap in understanding how osteosclerotic prostate cancer (PCa) metastases colonize bone, and the contribution of bone formation and parathyroid hormone (PTH) to this process. Our long term goal is to understand what regulates PCa colonization and establishment in bone, and to aid development of treatments that prevent bone metastases. The objective of this project is to identify the requirement(s) for PTH-enhanced bone formation in osteosclerotic PCa colonization and establishment in murine bone. Our central hypothesis is that osteosclerotic PCa colonization of bone, from the vasculature, requires enhanced bone formation. The rationale for this project is that once we know how enhanced bone formation facilitates the favorable interaction(s) between osteosclerotic PCa cells and bone, novel treatments can be developed to prevent such interactions.

Anticipated Duration of Project

4/21/2010 - 6/30/2011

Project Overview

The broad, long term objective of this project is to identify the requirement(s) for PTH-enhanced bone formation in osteosclerotic PCa colonization and establishment in murine bone. There are two specific aims of this project: 1) Identify the effect of intermittent parathyroid hormone (PTH)-zolendric acid administration on SCID/Beige bone; 2) Isolate and characterize bone marrow endothelial cells from SCID/Beige bone.

Under aim I our working hypothesis is that PTH, zolendric acid co-administration is anabolic to bone. To test this hypothesis we will employ the following approach. Twenty-four, 8-week old male SCID/Beige immune-compromised mice will be divided into four groups of six. Each group will be treated as follows: *Group one,* vehicle (control); *group two,* human PTH 1-34 (40

μg/kg per day); *group three*, zolendric acid (ZA) (3 μg/day); *group four*, human PTH 1-34 combined with zolendric acid (ZA). Mice in each group will be treated intermittently with 3 rounds. An intermittent treatment round is defined as: one subcutaneous injection each day for 5 days followed by two days of no injections. Thus, 3 treatment rounds span a total of 21 days.

Under aim II our working hypothesis is that human prostate cancer cell adhesion to murine bone marrow endothelial cells is enhanced following intermittent exposure to PTH, *in vitro*. To test this hypothesis we will employ the following approach. Six, 8-week old, male SCID/Beige immune-compromised mice will be used for the isolation and characterization of bone marrow vascular endothelial cells from the femur and lumbar spine. For endothelial cell isolation we propose to employ immunomagnetic selection using Dynabeads (Invitrogen) conjugated with mouse CD31, according to Fei et al., 1990, and Dong et al., 1997. Murine bone marrow endothelial cells (mBMECs) will be characterized: 1) morphologically; 2) by their ability to form tube-like structures when seeded on Matrigel; 3) by FACS analysis for their expression of von Willebrand factor, CD31, CD34, VCAM-1, and VE-cadherin, and PTH 1R. Cultures will be expanded for use in cell-cell adhesion assays.

The successful completion of these aims will establish the feasibility to conduct future experiments that seek to identify: 1) the requirement for enhanced bone formation in the colonization and establishment of human osteosclerotic PCa cells in murine bone; 2) the requirement for enhanced bone resorption in the colonization and establishment of human osteosclerotic PCa cells in murine bone; 3) mechanisms of osteosclerotic PCa cell colonization and establishment in murine bone.

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Expected Research Outcomes and Benefits

Expectations for aim I: Our hypothesis predicts that bone formation parameters (osteocalcin, number of ALP positive osteoblast, bone mineral density, trabecular thickness, and separation) will be enhanced by co-administration of PTH with zolendric acid relative to PTH alone. We predict ZA administration will reduce the number (TRAP staining) and activity (serum TRAcP 5b) of osteoclast in SCID/Beige bone and serum. Such outcomes will allow us to more directly test the role of osteoblast activity and bone formation, enhanced by PTH, in the colonization and establishment of osteosclerotic prostate cancer in bone.

Expectations for aim II: Our hypothesis predicts that the PTH 1R is expressed by mBMECs, and that incubation with PTH, intermittently, will enhance the ability of osteosclerotic prostate cancer cells to adhere, *in vitro*. In contrast, chronic PTH treatments will down regulate PTH 1R expression and correlate with attenuated prostate cancer cell adhesion *in vitro*. Besides direct in-growth into bone, it is well established that prostate cancer cells find their way to secondary targets of metastasis via the circulation. Bone marrow endothelial cells are thus a key cell type for prostate cancer cells to interact with prior to invading bone.

Prostate cancer metastasis to bone has been difficult to prevent or eradicate. What facilitates the favorable interaction between PCa cells and bone is unknown and is a critical barrier to solving the problem. Knowledge of how circulating osteosclerotic PCa cells metastasize to bone is critical for the development of therapeutics aimed at preventing their establishment in bone.

Summary of Research Completed

Below we report on the isolation and characterization of bone marrow endothelial cells from SCID/Beige bone.

General approach.
Isolation of murine bone marrow endothelial cells (mBMECs): Cells were flushed from the marrow cavities of femurs with RPMI media using a 26G needle. A single cell suspension was obtained by moving the aspirate through a 22G needle, followed by collagenase digestion. After 24 hrs in culture, non-adherent cells were poured off. Adherent cells were detached from the plate using 0.5% EDTA in phosphate buffer solution, washed, counted then incubated with CD146 conjugated magnetic beads (Miltenyi Biotec) to isolate endothelial cells. In contrast to human cells that express CD146 on T cells or follicular dendritic cells, murine CD146 expression has only been found on endothelial cells and a subset of $NK1.1^{+}$ cells (at low levels). Figure 1 depicts immunolabeling of paraformaldehyde fixed (2%) selected cells, after 4 days in culture, using CD146- and CD49b-FITC conjugated antibodies. Panel A, CD146 labeled cells (**green** signal). Panel B, CD49b, an NK cell marker (no signal). The blue signals in both panels denote nuclei (DAPI).

The summary of FACS (Figure 2) is provided to complement Figure 1 above by demonstrating that isolated mBMECs express CD146 and CD31 at the cell surface. Here cells were immunomagnetically selected with CD146 coated beads following overnight culture on gelatin coated surfaces. FITC-conjugated antibodies specific for CD146 (blue tracing) and CD31 (green tracing) were employed. These observations suggest CD146 but not CD31 is highly expressed on the surface of isolated cells, and that CD31 may not be ideal for *ex vivo* isolation of mBMECs.

Figure 3 depicts isolated cells after 2 weeks in culture on gelatin coated surfaces. We have observed that the cells proliferate in the presence of GM-CSF (10 ng/ml, data not shown), as suggested by Fei et al., 1990. In figure 3, cells, organized in cobblestone-like monolayers, were briefly fixed with 2% paraformaldehyde, permeabilized with methanol, and then probed with nothing (bright field, BF), or antibodies specific for CD146 (green), von Willebrand Factor (vWF, red), CD31(green), CD34 (red), or metabolically labeled with acetylated low density lipoprotein conjugated to Alexa-488 (AcLDL, green). Finally, all cells were counter stained with

DAPI (blue). Images: BF - von Willebrand factor were captured at 200x; CD31 - AcLDL were captured at 400x. AcLDL metabolic labeling (overnight) was performed to assist with discerning cellular morphology. Together, these images suggest that the cells isolated from murine bone marrow: 1) possess typical endothelial cell morphology; 2) are uniformly rounded in shape; 3) possess a cytoplasm with numerous vacuoles; 4) possess one or occasionally two nuclei; 5) express protein markers typically employed to identify endothelial cells. Moreover, since these cells are negative for CD49b (Figure 1) and are immunoreactive for vWF, they are unlikely to be NK cells or pericytes.

To determine if CD146 isolated mBMECs express PTH 1R, cell lysates were analyzed by Western blot employing PTH 1R specific antibodies as we have done previously (Gomes et al., 2009). In Figure 4, lane 1 represents PTH 1R expression in human osteosarcoma (HOS) lysate (positive control), and lane 2 PTH 1R expression in mBMEC lysate. The results suggest that after one week in culture mBMECs express the 66 kDa PTH 1R

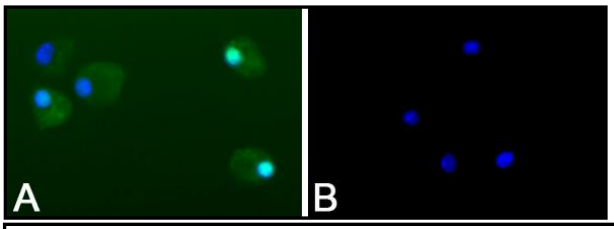


Figure 1. mBMECs express CD146 but not CD49b.

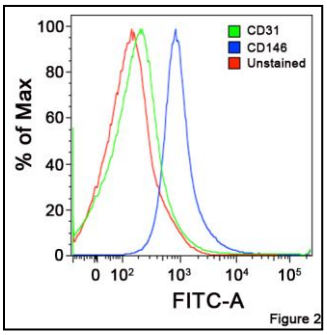
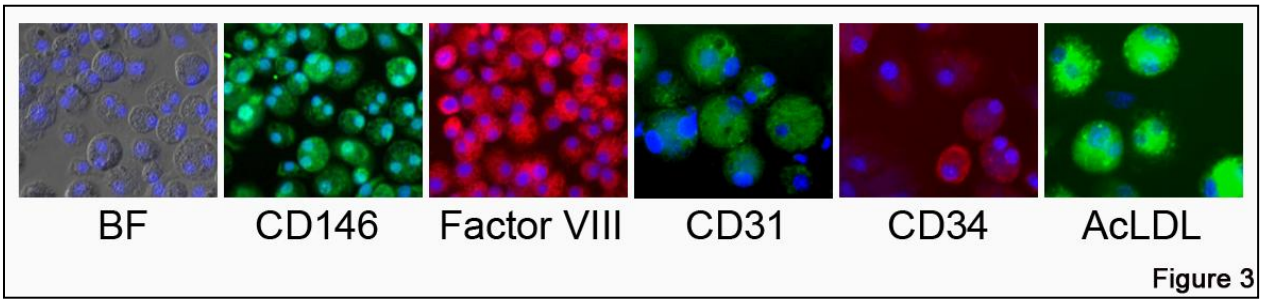
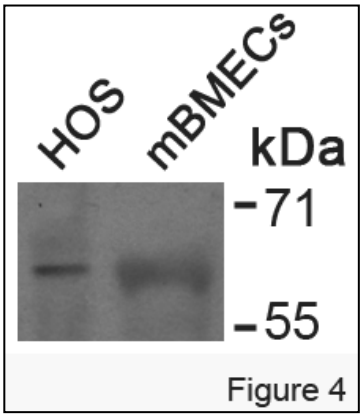


Figure 2.





Research Project 18: Project Title and Purpose

Perlecan Regulation of Synovial Hyperplasia - In patients with arthritis, synovial hyperplasia (SH) gives rise to pannus formation, invasion and erosion of articular cartilage, leading to joint destruction and chronic pain. There is a fundamental gap in understanding what regulates SH. The objective of this proposal is to identify a role for the HSPG, perlecan, in modulating SH.

Anticipated Duration of Project

4/21/2010 – 6/30/2011

Project Overview

The long term goal of our laboratory is to understand the role of basement membrane and extracellular matrix molecules in regulating articular cartilage health, and use this knowledge in the treatment and prevention of joint disorders. The objective of this project is to identify a role for the heparan sulfate proteoglycans (HSPG), perlecan (Pln), in modulating SH. Our central hypothesis is that Pln regulates the bio-availability and activity of heparin-binding growth factors important for initiation and development of SH. This hypothesis is predicated upon our strong preliminary data and a published report demonstrating: 1) Pln expression is enhanced in diseased synovial tissues; 2) Fibroblast growth factor binds to purified forms of full length and recombinant domain I of Pln; 3) binding interactions are heparan sulfate dependent. Our rationale for this project is that Pln, a regulator of heparin-binding growth factor bio-availability, is a likely candidate involved in pathomechanism(s) of SH. Yet, a role for Pln in regulating SH has not been examined. Guided by a dedicated team, and strong preliminary data, our central hypothesis will be tested pursuing the following specific aim: Determine if perlecan regulates synovial hyperplasia by modulating fibroblast growth factor bio-availability and activity. Under this aim: 1) human Pln isolated from synovial tissues will be employed in binding studies with exogenous fibroblast growth factors to identify how binding differs in diseased tissues; 2) primary synoviocytes, isolated from patients, will be employed to identify the effect of exogenous Pln +/- FGFs on synoviocyte survival, proliferation and migration; and 3) primary synoviocytes cultured in the presence of exogenous Pln +/- FGFs will be employed to identify requirement(s) for Pln's modulation of FGF-induced signaling. The planned project is innovative in its approach as no published studies have demonstrated interactions between human synovial tissue derived Pln and FGFs and/or their receptors. This research is significant, because it is expected to advance our understanding of how HSPGs bind to and modulate the activity of heparin-binding growth factors in synovial tissues, knowledge needed to develop novel pharmacologic strategies that aim to regulate such factors in slowing or preventing cartilage degradation.

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Expected Research Outcomes and Benefits

Upon completion of the planned studies, we expect to know if Pln isolated from RA synovial tissues has the capacity to bind and modulate FGF activity, *in vitro*. From the studies planned in *sub-hypothesis I*, we expect that Pln isolated from all tissues will bind heparin-binding FGFs in a heparan-sulfate dependent manner; however, Pln isolated from RA synovium will be poorly sulfated and demonstrate weak binding of FGFs relative to Pln from non-diseased and OA synovial tissues.

From the studies proposed in *sub-hypothesis II*, we expect the exogenous addition of Pln/FGF mixtures to synoviocytes in culture will enhance cell survival, proliferation, and migration. However, enhanced cell survival, proliferation, and migration are expected to be greater for Pln isolated from RA tissues (RA>>OA> non-diseased). FGF sequestration will be greatest for Pln isolated from non-diseased tissues.

From the studies proposed in *sub-hypothesis III*, we expect exogenous Pln, isolated from RA tissues, will poorly modulate FGF induced phosphorylation of FGFRs relative to Pln from OA and non-diseased tissues. We also anticipate that RA tissue derived Pln/FGF mixtures will enhance synoviocyte survival by activating the AKT pathway.

Together, our expected observations would suggest that Pln isolated from RA tissues has lost much of its ability to bind and sequester FGFs, and thus regulate activity. If supported, our hypothesis would suggest that approaches seeking to restore and/or mimic Pln's ability to bind and sequester FGF activity could slow or attenuate the development of SH.

Summary of Research Completed

Herein, we report on sub-hypothesis I: Perlecan derived from human synovium binds heparin-binding fibroblast growth factor(s) in a heparan sulfate dependant manner.

General approach
Perlecan was purified from human synovial tissues collected from knee joints of patients 65 years and older, removed during total knee arthroplasty as a consequence of trauma (non-diseased (ND); n=5), from RA (n=5), and from osteoarthritis (OA; n=5) using techniques

established in our laboratory. Purified perlecan was then employed in an immuno-blotting approach to assess FGF binding as follows: Perlecan (200 ng) coated in a 96-well high binding ELISA plate was overlaid with growth factors (FGFb and VEGFs). Bound growth factors were determined using anti-FGF and anti-VEGF antibodies followed by incubations with peroxidase-conjugated secondary antibodies, TMB substrate incubation. Color development was quantified by absorbance (450 nm). For inhibition assays, growth factors were pre-incubated with heparin for 1 h prior to overlay onto perlecan.

Results

1. We have collected 25 synovium specimens from patients (63-79 yrs of age).
2. Synovial perlecan (SnPln) has been purified from 5 samples each ND and OA. RA samples have proved more challenging to acquire.
3. Our observations suggest that perlecan isolated from OA synovium is poorly glycosylated relative to human perlecan derived from ND tissue.
4. SnPln is decorated with both chondroitin and heparan sulfate chains.

Figure 1 illustrates that perlecan purified from highly vascularized synovium poorly binds FGFb (panel A vs. B, grey bar). Recombinant PlnDI (C) was employed as a positive control. *****, different from $VEGF_{189}$.

Figure 2 suggests that FGFb and VEGF binding to perlecan derived from synovial tissues is heparan-sulfate dependent, and that very low concentrations of heparin are required to disrupt FGFb and $VEGF_{165}$ binding to perlecan from highly vascularized synovium.

In summary, these observations support our hypothesis and suggest that perlecan present in hypervascular synovium is under sulfated and poorly binds FGFb.

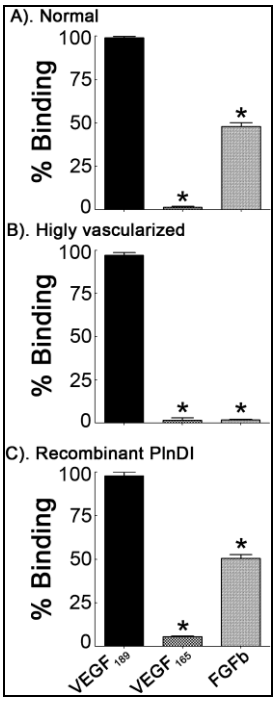

Figure 1

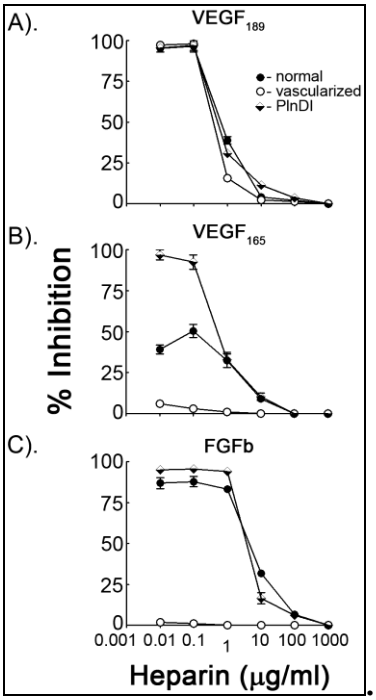

Figure 2.

Research Project 19: Project Title and Purpose

A Multi-Center Randomized Single Blind Trial of Intravenous Fluids during Labor - To determine if intrapartum use of a glucose-containing solution will improve the outcome of labor and reduce operative deliveries.

Anticipated Duration of Project

4/21/2010 - 6/30/2013

Project Overview

This will be a multi-center, prospective, single-blind randomized trial of two types of IV fluids for maintenance hydration during labor to determine if intrapartum use of a glucose-containing solution will improve the outcome of labor. This study will randomize 1524 women at greater than or equal to 36 weeks in spontaneous labor attempting vaginal delivery and will be conducted at 4 sites: Reading (Reading Hospital), Allentown (Lehigh Valley), Hershey (Penn State College of Medicine), and York (York Hospital). The primary analysis will use an intent-to-treat approach to examine differences between the two IV fluid treatments in the proportion of deliveries requiring cesarean section. We assume the proportion of deliveries requiring cesarean section will be 0.24 in the arm receiving the non-glucose-containing (i.e., lactated Ringer's) solution and 0.18 in the arm receiving the glucose-containing (i.e., D_5 lactated Ringer's) solution (i.e., a 25% relative reduction in the proportion of cesarean deliveries). We anticipate a 5% dropout rate for this study. Secondary efficacy parameters will include incidence of ketosis and influence of ketonuria on the primary outcome, incidence of prolonged labor, need for oxytocin augmentation, incidence of operative vaginal delivery, incidence of chorioamnionitis, indications for cesarean birth, maternal nausea/vomiting in labor, reduced umbilical artery pH/fetal acidemia, increased umbilical artery CO_2/fetal hypercarbia, neonatal hypoglycemia, neonatal intensive care unit admission, and difficulties with initiating breast feeding. A total of 28 months will be required to complete the study. This will be accomplished by enrolling 15 women per center per month over the two-year enrollment period, and for 3 months to clean and analyze the final data.

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Expected Research Outcomes and Benefits

We propose that even in the presence of adequate hydration, inadequate carbohydrate replacement in labor may contribute to prolongation of labor and increased need for operative delivery. Glucose is the main energy substrate for the pregnant uterus. Physiological requirements for glucose during labor are estimated to be around 10 grams per hour. Adequate supplies of glucose are needed to maintain exercise tolerance and muscle efficiency, which are important factors in the progress of labor. However, there are no data to precisely quantify the glucose needs of the human uterus during labor.

Dysfunctional labor or dystocia, which is the leading indication for primary cesarean delivery, is primarily caused by uterine forces insufficiently strong or inappropriately coordinated to efface and dilate the cervix. Dystocia can be a result of inadequate voluntary muscle effort in the second stage of labor. Supplying carbohydrate fuel for working uterine and skeletal muscle may improve progress in labor and, therefore, diminish the need for cesarean delivery. Importantly, with dysfunctional labor, there are increased risks for other intrapartum interventions and complications such as high doses of Pitocin, chorioamnionitis, postpartum hemorrhage and fetal compromise.

Our expected outcomes are that - Hydrating patients with an intravenous fluid during labor containing glucose will :1) Lower operative deliveries, i.e. cesarean section; 2) Reduce the incidence of dysfunctional labor; and 3) Reduce the incidence of fetal and maternal morbidity related to dysfunctional labor or operative delivery.

Summary of Research Completed

This project is in its early stages and we are still making minor modifications and troubleshooting. The Penn State site and the Reading Hospital site are actively recruiting. Lehigh Valley Hospital is in the final stages of IRB approval and anticipates beginning recruitment by December 2010. The York Hospital Site is still considering participation. We have taken this time period to set up monthly phone conferences, annual face to face meetings, developed and completed case report forms (CRFs), set up a central web based data entry system that can be accessed by all sites (using a WEB UQ database) and created and distributed Randomization envelopes and packets to the active sites. We have ordered supplies (i.e., recruitment brochures, envelopes, IV selection tags, labels). We have mailed out to each site their start up packet for recruitment and created necessary databases with our biostatistician team. Residents at each site have undergone a formal orientation on how to present the study to patients. The residency program at Penn State instituted a policy that new residents will have the CITI training as part of their Orientation requirement. The Clinical Trials Registration has been completed. We are now beginning to focus on recruitment strategies.

Research Project 20: Project Title and Purpose

Identification and Characterization of Regulatory Factors of the Human Telomerase Gene - Normal human somatic cells age due to the fact that telomere is limiting, and telomerase is stringently repressed in these cells. On the other hand, telomerase is activated in cancer cells. Our research is to reveal how telomerase is repressed during development and activated in cancer cells. Specifically, we have identified several candidate genes involved in telomerase regulation and plan to understand how telomerase is regulated by these genes. This study will lead to a better understanding of cancer and age-related diseases, and ultimately better therapeutic strategies for these diseases.

Anticipated Duration of Project

4/21/2010 – 6/30/2012

Project Overview

Our long-term objective is to determine the mechanisms of hTERT gene repression during development and differentiation. The human telomerase reverse transcriptase (hTERT) gene, encoding the rate-limiting subunit of human telomerase, is highly expressed in pluripotent stem cells, but stringently repressed in most adult somatic cells. Despite intensive investigation in the past decade, the mechanism of its repression in somatic cells, including cis-regulatory elements and trans-acting factors involved in hTERT repression remain to be elucidated. Our data indicated that the hTERT locus was embedded in a condensed chromatin domain in many somatic cells, including both telomerase-positive and -negative cells. Such a compacted domain was not detected in the mouse TERT locus, which is more loosely regulated than the hTERT gene. We hypothesize that (1) establishment of an appropriate chromatin environment and local nucleosomal architecture is essential for hTERT repression in somatic cells and (2) de-repression or partial de-repression of the hTERT promoter leads to telomerase activation during cellular immortalization. To study the mechanisms of hTERT repression, we developed a novel technical platform, the recombinase-mediated BAC targeting or RMBT, for targeted integration of a single-copy bacterial artificial chromosome (BAC) reporter into specified chromosomal sites. Using this technique, we demonstrated that chromosomal integration of a BAC construct containing the hTERT locus resulted in the establishment of a surrogate chromatin setting in which the hTERT promoter was tightly repressed and recapitulated its endogenous counterpart in human fibroblasts. In this project, our specific aim is validation and characterization of novel repressors of the hTERT gene. In a recent screen for regulatory factors of the hTERT gene, we identified several candidates that are likely involved in the repression of the hTERT promoter. We plan to validate their regulation of the endogenous hTERT gene and determine their roles in telomerase regulation during cell differentiation.

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Expected Research Outcomes and Benefits

Telomeres - the ends of linear chromosomes- are important determinants of human aging. Telomerase, the enzyme that elongates telomeres, is expressed in about 95% of cancer cells but is rarely found in normal human cells. In fact, ectopic expression of hTERT, the catalytic subunit of telomerase, is able to stabilize the telomere, immortalize human cells, and convert pre-neoplastic cells into tumorigenic cells. Therefore, understanding the mechanisms on how telomerase is suppressed in normal cells and activated in immortal and cancer cells will elucidate one of the key steps during the multi-step tumorigenesis. The knowledge will also help us to understand how we age and benefit the treatment of many age-related diseases.

Summary of Research Completed

Determine the roles of a novel pathway in the repression of hTERT gene.

Our data in the application to NIH (2R01GM071725-06A1) demonstrated that knockdown of GeneA in the epigenetic gene repression pathway by siRNAs significantly induced hTERT transcription from both chromatinized BAC reporter and endogenous *hTERT* locus, suggesting this pathway plays a critical role in hTERT repression. Thus, we have examined GeneB, GeneC, and GeneD, all known to function in this pathway, for their roles in the regulation of hTERT gene expression. A mixture of three siRNA duplexes against each of the four genes was transfected into a 3C167b (Tel^+) line containing an integrated H(wt) reporter. As demonstrated in the parent application, GeneA siRNAs induced hTERT transcription from the H(wt) reporter. Similarly, the siRNA duplexes against GeneB also significantly activated hTERT promoter in the H(wt) reporter (Figure 1), suggesting that GeneB was likely involved in hTERT repression. Although the initial experiment showed that siRNAs against neither GeneC nor GeneD affected hTERT transcription, additional experiments are being performed to determine whether their mRNAs or proteins are knocked down by these siRNA duplexes. In addition, it is also possible that both GeneC and GeneD need to be knocked down simultaneously in order to de-repress the hTERT promoter due to their functional redundancy.

In short, our data has provided additional evidence that the epigenetic gene repression pathway plays an important role in the repression of *hTERT* gene in somatic cells. The discovery of the involvement of this pathway will represent a significant advance in our understanding of hTERT

silencing in somatic cells and will also help to elucidate the differential regulation of *TERT* genes in human and mouse cells.

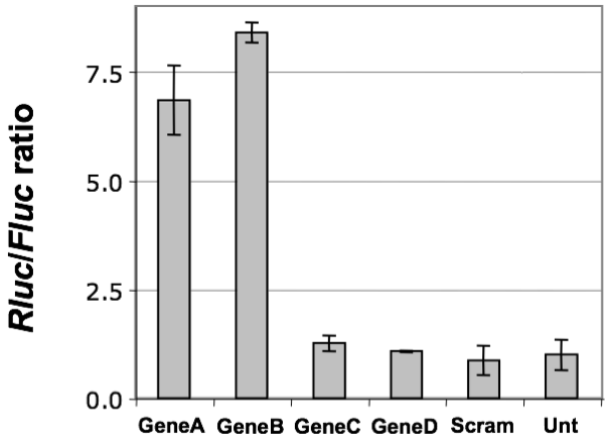

Figure 1. Effects on transgenic hTERT promoter activity by siRNAs against novel regulators. Mixtures of three siRNA duplexes against each gene were transfected into Tel^{+}/H(wt) cells using Lipofectamine RNAiMax reagent (Invitrogen) and luciferase activities were determined three days post-transfection. Data shown are averages of triplicate transfections in a 96-well plate and the experiment has been repeated multiple times.