University of Pittsburgh

Annual Progress Report: 2009 Formula Grant

Reporting Period

July 1, 2010 – June 30, 2011

Formula Grant Overview

The University of Pittsburgh received $9,897,440 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

Application of Adult Stem Cells for Tissue Regeneration - Degenerative skeletal diseases, like osteoarthritis, are the primary cause of disability, particularly in the elderly. The purpose of this research project is to develop novel tissue regenerative approaches for the treatment of such diseases. The included studies will investigate the regenerative activities of human adult stem cells, specifically the nature of the optimal physical and biological microenvironment that supports their activities. This knowledge will guide the design of biomaterial scaffolds and the selection of bioactive factors for effective stem cell-based therapy for the engineering, regeneration, and functional restoration of diseased or injured skeletal tissues.

Anticipated Duration of Project

1/1/2010 - 12/31/2013

Project Overview

Degenerative skeletal diseases are the primary cause of disability in the elderly. Cell-based therapy is a promising treatment approach for the engineering and regeneration of the diseased or injured tissues. Adult stem cells, known as mesenchymal stem cells (MSCs), possess the ability to differentiate into musculoskeletal cells and represent a promising cell type for skeletal tissue engineering. Successful application of MSCs in cell-based therapy requires a full understanding of the mechanisms that regulate their biological activities, specifically proliferation and differentiation. Three interconnected projects are described here. The first project focuses on elucidating the mechanism of action of selected genes previously identified as potential "stemness genes" (i.e., genes that encode proteins that act to maintain the potency of the MSCs). These genes include both secreted factors and extracellular matrix components and will be analyzed in terms of their control of MSC proliferation and differentiation and their cellular signaling pathways. Functional molecules identified in this study will be considered as ligands for incorporation into a 3-dimensional (3D) matrix (e.g., using nanostructured biodegradable polymer fibers to construct a bioactive scaffold for MSCs). Using bioreactor technology, culture

conditions will be optimized to induce the cell-seeded constructs to differentiate into a hyaline cartilaginous tissue with biological and mechanical characteristics approximating those of native articular cartilage. The utility of the engineered constructs for cartilage repair will be tested in animal models of traumatically induced lesions in the femoral condylar articular cartilage. In summary, these studies will identify the key molecules important for MSC activity and exploit the identified pathways to produce optimal 3D scaffolds for cartilage engineering that will be tested for cartilage repair in clinically relevant animal models. Positive results from these proof-of-concept studies will yield a rational basis for the design of clinical trials using this technology.

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Expected Research Outcomes and Benefits

The new discipline of regenerative medicine aims to restore form and function to the patient via promotion of the regenerative activities of tissues and organs. Cell-based therapy is one of the most promising approaches in regenerative medicine. Adult human mesenchymal stem cells (MSCs) are able to differentiate into multiple cell types and represent a highly promising cell type in cell-based therapy. The studies included in this project take an integrated approach to probe the molecular mechanisms that maintain MSCs in an active state and ready for differentiation. The information, gained from identifying the signaling pathways and bioactive factors involved, presents a platform to design and/or select specific molecular moieties for MSC activation. In the tissue engineering study, by incorporating selected moieties as ligands to functionalize an electrospun nanofibrous polymeric matrix, a bioactive 3-dimensional (3-D) scaffold for MSCs will be constructed. Upon chondrogenic induction, these MSC-seeded constructs are expected to yield structurally and biologically improved engineered cartilage tissue. The preclinical study will use articular cartilage injury animal models to directly assess the use of engineered cartilage in repairing trauma-induced cartilage degeneration. The investigation, thus, has the potential to yield findings that will lead, with further clinical trials, to the development of an innovative clinical approach—based on autologous MSCs and bioactive scaffolds—to treat degenerative joint diseases, like osteoarthritis, which are a primary cause of disability.

Summary of Research Completed

During this reporting period, we have made significant progress toward elucidating the mechanisms of action of potential stemness genes and exploiting those pathways to design and engineer 3-D bioactive scaffolds for MSCs.

Expression and Activity of Caveolin-1 in Human Mesenchymal Stem Cells

Caveolae are cholesterol-rich plasma membrane invaginations that can affect the organization and function of cell signaling components. The main protein component of caveolae is caveolin. caveolin-1 is a 22 kilodatlton (KDa) scaffolding protein that interacts with multiple signaling proteins in caveolae and is known to inhibit cell proliferation, promote cell senescence, and inhibit canonical Wnt/β-catenin signaling. Lack of caveolin-1 expression in mice has been associated with expansion of stem cell populations in the gut, brain, and mammary gland. Our research is based on the hypothesis that caveolin-1 may also affect human MSC proliferation and differentiation. Our previous preliminary studies suggested that caveolin-1 knockdown in adult human bone marrow-derived MSCs increased their osteogenesis *in vitro*, and this increase was measured by alkaline phosphatase (ALP) activity and alizarin red staining of MSCs (derived from four individual patients) transfected with caveolin-1-specific small interfering ribonucleic acid (siRNA) and treated with osteogenic medium. During this reporting period, we extended these studies to include three additional patients. Furthermore, we have begun to examine the effects of caveolin-1 knockdown on MSC proliferation and gene expression and to characterize the expression of caveolin-1 in untransfected MSCs incubated in regular and osteogenic growth medium.

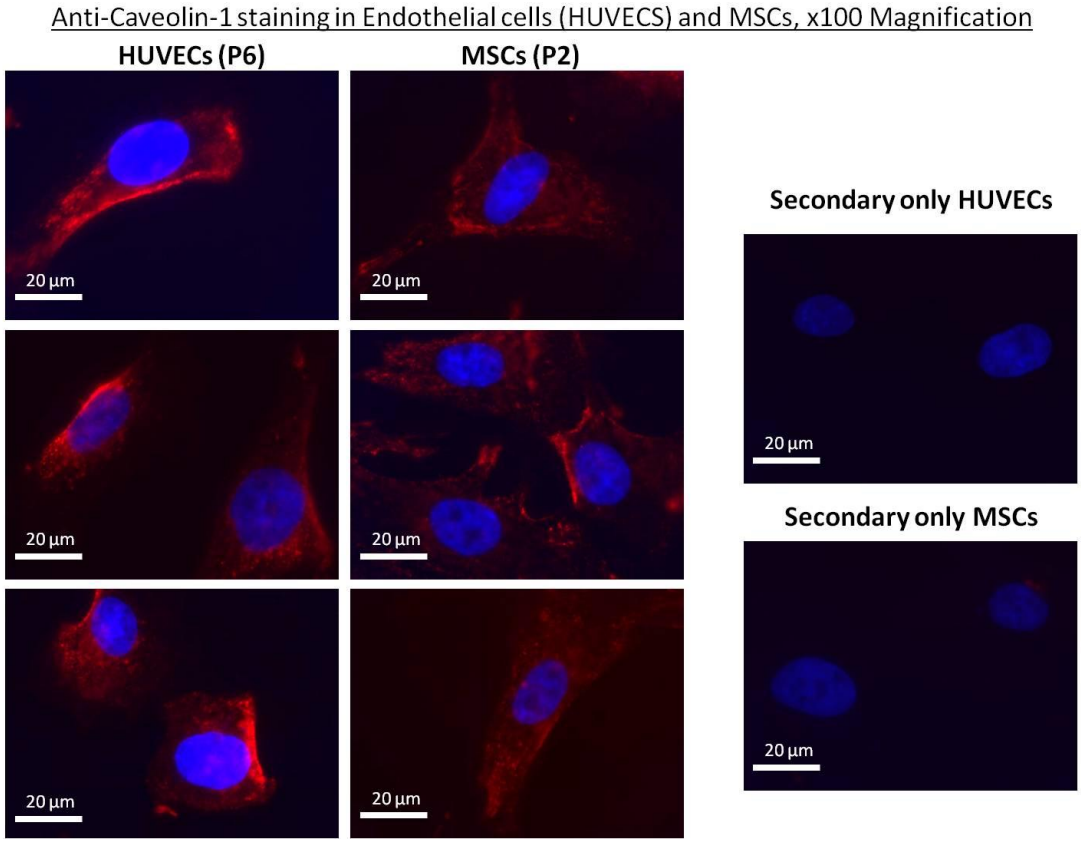
Methods

We disrupted caveolin-1 expression in MSCs via siRNA transfection. After transfection, MSCs were placed in normal osteogenic growth medium, which was changed every three days thereafter. Knockdown in caveolin-1 siRNA-treated cells relative to controls was confirmed by real-time polymerase chain reaction (RT-PCR) and Western blotting. Osteogenic differentiation of MSCs was assessed by measuring ALP activity and matrix mineralization using alizarin red staining after 4 and 21 days, respectively, in osteogenic medium. MSC proliferation was measured by cell counting on days 1, 5, and 10 of culture in normal growth medium. The effect of caveolin-1 on MSC gene expression was assessed with an RT^2 Profiler PCR Arrays (Qiagen/SABiosciences) assay run on an Applied Biosciences (ABI) PCR Machine 7900HT. The caveolin-1 expression pattern was examined in untransfected MSCs by staining cells with an anti-human caveolin-1 antibody (BD Biosciences) and visualizing with fluorescence and confocal microscopy. Endothelial cells, which are known to express caveolin-1, were used as a positive control for immunofluorescent staining. The location of caveolin-1 expression in MSCs was probed by ultracentrifuging the cell lysates on a sucrose gradient and running the resultant fractions on Western blots.

Results

We found that the immunofluorescent staining pattern of caveolin-1 in human MSCs was similar to that in endothelial cells (Figure 1). Further, after MSC cell lysates were separated on a sucrose gradient, caveolin-1 protein localized to the density fraction corresponding to that known to contain lipid rafts. This finding suggests that caveolin-1 may be localized to caveolae in the cell surface membrane, as in other cell types. We will confirm this hypothesis by immunogold labeling and electron microscopy.

Figure 1



Previously, we observed that a reduction in caveolin-1 expression increased ALP activity induced by osteogenic medium in four of four patients studied. We have now updated this observation to include seven of seven patients studied.

We previously found that matrix mineralization induced by osteogenic medium was strongly enhanced in one patient after caveolin-1 knockdown. We have since confirmed this finding in a second patient, but our results remain variable for the other five patients studied. This variation may be related to the extent and duration of transient caveolin-1 knockdown. We are currently addressing this variation by stable silencing of caveolin-1 expression using lentiviral short hairpin RNA (shRNA). The stable cell lines generated using shRNA will be used to further probe the role of caveolin-1 in other aspects of MSC biology.

The steady state expression of caveolin-1 increased in untransfected MSCs incubated in osteogenic medium, suggesting that the contribution of caveolin-1 activity to MSC osteogenesis is likely to be complex. Caveolin-1 knockdown increased MSC proliferation in three of three female patients studied but did not occur or reach significance in two other male patients. Interestingly, preliminary gene expression studies suggest that POU5F1/Oct4 expression is reduced in male, but not female, MSCs in which caveolin-1 expression is reduced. Knockdown of caveolin-1 expression may, therefore, reduce renewal capacity and increase differentiation capacity of male MSCs. Further investigations are required to confirm this hypothesis and uncover the mechanism behind this phenomenon.
Manipulation of caveolin-1 expression in human MSCs appears to affect the self-renewal and differentiation propensity of these cells, a finding that could have important therapeutic applications. However, manipulation of caveolin-1 expression may have different consequences in male and female cells, and this possibility would be an important factor to investigate and consider in future studies.

Human Mesenchymal Stem Cells in Bone and Tendon Interaction

In this subproject, we investigated whether MSCs play a role in the osseointegration activity between bone and tendon. Modulating this activity is of significance to improving anterior cruciate ligament reconstruction. Primary tenocytes and calvarial osteocytes were co-cultured for seven days. Samples were collected for RNA isolation; and specific genes associated with bone-tendon interface, including collagen type II, aggrecan, and cartilage oligomeric matrix protein (COMP), were examined using quantitative RT-PCR (qPCR). After seven days of co-culture, there was no increase in cartilage marker gene expression (Figure 2).

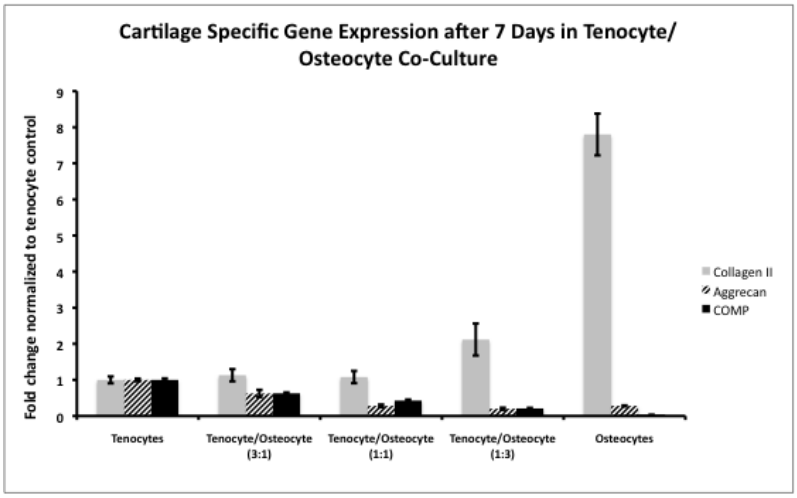


Figure 2: Co-culture of primary tenocytes with different ratios of primary calvarial osteocytes does not induce expression of cartilage specific genes. Primary tenocytes and primary calvarial osteocytes were isolated from White Leghorn chicks at incubation day 14 and co-cultured (1:1 and 1:3) for seven days. Samples were collected for RNA isolation, and cartilage (interface)-specific genes (collagen type II, aggrecan, and COMP) were examined using qPCR.

Mechanobiology of Cyclic Hydrostatic Pressure in Chondrogenic Mesenchymal Stem Cells

To improve MSCs for cartilage tissue regeneration, their natural tendency to undergo a hypertrophy-like program during chondrogenesis must be suppressed. During development, mesenchymal cells express β-catenin during condensation, but that expression completely

disappears during chondrogenesis until the chondrocytes begin to undergo hypertrophy. We seek to determine the role of β-catenin in MSC chondrogenesis.

β-catenin inhibition was assessed using a small molecule inhibitor as well as an siRNA construct against β-catenin. To evaluate the small molecule XAV-939, adult MSCs were transfected with viral luciferase reporter constructs driven by a promoter region containing several β-catenin/TCF/LEF (T-cell factor/lymphoid enhancer factor-1) consensus binding sites. MSC pellets were formed and cultured for 11 days in chondrogenic medium (CM) containing several concentrations of XAV-939. On days 2 and 11, pellets were collected and analyzed for luciferase expression using a Promega GloMax Luminometer. To assess β-catenin inhibition by siRNA, MSCs were transfected with several concentrations of β-catenin siRNA via lipid-based transfection. MSC pellets were cultured for 11 days in standard growth medium. On days 2 and 11, the RNA was collected and analyzed for relative β-catenin expression. The results from these two studies are being analyzed and will provide the groundwork to study the role of β-catenin inhibition on MSC chondrogenesis and hypertrophy.

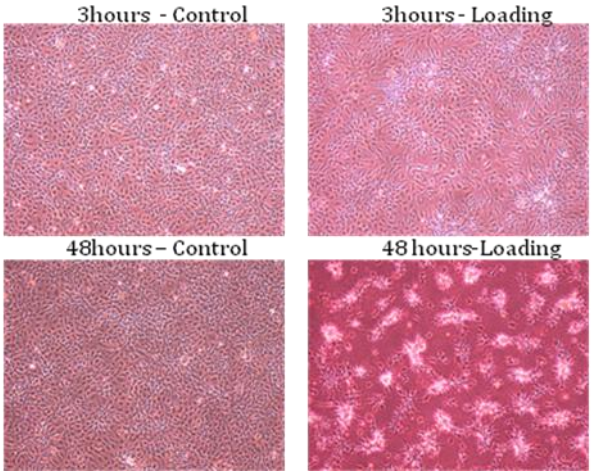
Functional Analysis of Candidate Stemness Factor CCL-2 on Mesenchymal Stem Cells

MSCs are potentially powerful therapeutic agents, but the regulation of their stem-like state is incompletely understood. We are investigating possible mechanisms of a candidate "stemness gene," identified in a previous gene profiling study. Stemness genes are operationally characterized by their decreased expression when cells differentiate and increased expression when cells are maintained in an undifferentiated state. Among those recognized factors are two secreted agents: interleukin-6 (IL-6) and chemokine (C-C motif) ligand-2 (CCL-2). Our laboratory previously demonstrated the ability of IL-6 to affect the proliferation, survival, and differentiation capacity of MSCs; therefore, we sought to examine the role of CCL-2 in regulating bone marrow-derived MSC differentiation. We confirmed a reduction in CCL-2 secretion with MSC differentiation but found little difference in the ability of MSCs to differentiate along adipogenic and osteogenic pathways in the presence of exogenous CCL-2. While there was some evidence that CCL-2 affected MSC proliferation and promoted IL-6 secretion, high patient-to-patient variability made identifying a clear role for this chemokine difficult.

Epigenetic Changes during Mechano-Mediated Injury of Chondrocytes

We are interested in the molecular mechanisms responsible for the effects of injurious mechanical strain on chondrocytes that may lead to posttraumatic cartilage degeneration. Chondrocytes isolated from adult bovine articular cartilage were cultured on Bioflex plates and subjected to biaxial tensile strain (16 percent, 0.5 Hz) using a custom-manufactured vacuum-operated loading device. This cyto-mechanical loading system has been established in our laboratory as a model of chondrocyte injury, resulting in chondrocyte phenotype changes similar to those observed in osteoarthritis. Cell shape changes can be noticed after three hours of loading and continue to be observed over a 48-hour time course (Figure 3).

Figure 3: Morphology of chondroctyes after injurious mechanical loading



Histone deacetylase (HDAC) inhibitors have been recently reported to have a chondroprotective role. Therefore, we treated bovine chondrocytes with HDAC inhibitors under both normal and loading conditions. RNA was isolated after 48 hours of loading, followed by 24 hours of treatment with HDAC inhibitors. Cartilage catabolic markers such as matrix metalloproteinase (MMP13) were decreased by the treatment with HDAC inhibitors in a dose- response manner.

Cells were plated in parallel and treated with various concentrations of HDAC inhibitors. After 24 hours of treatment, we analyzed acetylated histones (H3) and found increased levels of Acetyl-H3 with increasing concentrations of the inhibitors.

Regulation of Adult Stem Cells: Partial Reprogramming and Effect of Hypoxia

Adult and embryonic stem cells both exhibit desirable tissue regenerative properties. Many studies have shown that *in vitro* culture of stem cells changes their properties, in some cases making them less "stem-like" and less useful for regenerative medicine or tissue engineering projects. We previously investigated the partial reprogramming of adult stem cells using gene transfer of reprogramming transcription factors to enhance their proliferation and differentiation capacity. During this reporting period, we began to investigate the effects of that reprogramming process on one of the pathways critical for adult stem cell proliferation and differentiation—canonical Wnt signaling. Other groups have shown in osteoblasts that *in vivo* aging of the stem cell pool or *in vitro* expansion of adult stem cells can shift β-catenin signaling from activating/enhancing canonical Wnt signaling to instead activating FOXO (forkhead box O) signaling as a response to accumulated metabolic stress. We examined activity of TCF/LEF (the transcription factor activated by Wnt/beta-catenin) and FOXO1 and FOXO3 in late passage versus early passage adult stem cells to determine whether the same phenomenon is observed in these cells as in osteoblasts. We are also performing functional knockdowns of FOXO1, FOXO3, and β-catenin in these cells to determine the effects on their capacity to respond to metabolic stress. Our next step is to examine the activity of these transcription factors in late passage cells that have undergone reprogramming, with the expectation that reprogramming resets the cells' capacity for metabolic stress to some degree.

We have also begun to examine the effect of ambient oxygen levels on stemness preservation in adult stem cells. Several groups have shown that, while extreme hypoxia can be toxic to cells, some degree of hypoxia recapitulates physiologic and pathologic environments adult stem cells may experience *in vivo*. We have determined that in most adult stem cell samples, low oxygen tension greatly enhances stem cell colony formation; this enhancement could be due to increased proliferation, decreased cell death, or increased migratory ability of the stem cells, which would allow them to spread and form a greater number of distinct colonies. We have found that low oxygen tension indeed enhances proliferation, with increased cell metabolism, increased mitotic index, and increased cell number. However, we also found that the rate of cell death is slightly higher at five percent oxygen tension. We examined gene expression and activity of MMPs in these cultures, which displayed a matrix-stabilizing profile under normoxic and hypoxic conditions. However, MMPs critical for cell migration were slightly up regulated at low oxygen tension, suggesting that this is a potential factor in increased colony formation and meriting further investigation. We are currently investigating the effects of oxygen tension on differentiation of these cells, and preliminary results show that oxygen tension is critical not only during the differentiation process but also as a preconditioning factor while the cells are expanded prior to differentiation. For chondrogenesis, the effects of preconditioning seem to be as important as the environment during chondrogenic differentiation. We have also observed that adipogenic differentiation is greatly enhanced at 21 percent oxygen tension relative to 5 percent oxygen tension, with even control stem cell populations displaying greater adipogenesis than populations receiving supplemented media at 5 percent oxygen tension.

Research Project 2: Project Title and Purpose

Targeting Nrf2 for Cancer Prevention - Cancer prevention involving reduction or elimination of human exposure to environmental carcinogens may not always be possible. The overriding goal of this project is to provide the mechanistic framework to facilitate the efficient translation of the most effective small molecule activators of the Nrf2 signaling pathway into use as protective agents in human populations exposed to environmental toxicants like aflatoxins. To facilitate this goal, we need better understanding of the molecular mechanisms of action of the chemo preventive agents, further validation of intermediate biomarkers, and assessment of efficacy in animal models with close relevance to human carcinogenesis.

Anticipated Duration of Project

1/1/2010 - 12/31/2013

Project Overview

Inhibiting cancer development by administering anticarcinogenic agents may offer practical alternatives for reducing human cancer burden. The Keap1-Nrf2-ARE signaling pathway has been identified as a target for chemoprevention. The Nrf2 transcription factor regulates an integrated cell survival response that can be triggered by multiple classes of cancer chemopreventive agents (e.g., dithiolethiones, isothiocyanates, triterpenoids). In this project, both pharmacologic and genetic approaches will be used to probe the molecular mechanisms of action of these chemopreventive agents, assess their efficacy in animal models with close

relevance to human carcinogenesis, and use these interventions to validate intermediate biomarkers. Two aims will be pursued.

First, advanced methods of isotope-dilution mass spectrometry will be used for quantitation of biomarkers of the human carcinogen aflatoxin in liver, blood, urine, and feces to develop for the first time a comprehensive mass balance for the fate of the ultimate carcinogen, exo-aflatoxin-epoxide, in rats. Unique to this project, the predictive value of these biomarkers for individual risk of liver cancer will be assessed. Biomarkers will be quantified longitudinally during a bioassay for protection against hepatocarcinogenesis by an exceptionally potent triterpenoid activator of Nrf2 signaling. We will also evaluate the predictive value of monitoring DNA circulating in plasma for mutations in target oncogenes using quantitative short oligonucleotide mass spectrometry.

In the second aim, we will evaluate the similarities and distinctions of chemical class, species, and genetic activation of Keap1-Nrf2 signaling by comparing the gene expression patterns in rat and mouse liver following treatment with lead compounds of three different chemical classes of Nrf2 activators at doses equi-effective for inhibition of aflatoxin-induced preneoplastic lesions and by comparing the gene expression patterns in liver of mice in which either Nrf2 or its repressor, Keap1, has been genetically disrupted. The effect of these genetic and pharmacologic interventions on aflatoxin disposition will be determined using the mass spectrometry-based analysis of its biomarkers. Collectively, these studies will further the goal of effectively using activators of Nrf2 signaling as protective agents in human populations exposed to environmental toxicants.

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Expected Research Outcomes and Benefits

This project will provide a comprehensive analysis of the molecular and biochemical actions of several distinct chemical classes (dithiolethiones, isothiocyanates, triterpenoids) of anticarcinogenic compounds. Results from this project will allow for the prioritization of lead compounds within these classes for subsequent clinical evaluation in human populations, especially those at elevated risk for unavoidable exposures to chemical carcinogens. Members of each of these chemical classes have been utilized in clinical trials in humans, so there are realistic possibilities for continued development as practical cancer preventive agents. These

studies will also rigorously establish the utility of targeting the Nrf2 transcription factor pathway as a means for cancer prevention. Central to the project, highly quantitative, exquisitely sensitive, mass spectrometry methods will be used for analyzing candidate biomarkers reflecting both the fate and disposition of an important environmental carcinogen, aflatoxin. In addition, mass spectrometry in the form of "short oligonucleotide mass analysis" will be used to measure genetic changes in tumor DNA shed into the circulation using biospecimens provided to the study team from long-term animal bioassays. Collectively, these studies will provide a longitudinal evaluation of biomarkers during the course of cancer development induced by exposure to environmental agents. Results from this project will provide an analytical template for the assessment of early indices of environmental carcinogenesis in humans at risk for exposures to food-, air- and water-borne carcinogens.

Summary of Research Completed

In the previous reporting period, we acquired and installed a Thermo TSQ Vantage mass spectrometer with an ion max source heated electron spray ionization (H-ESI-II) probe coupled to an Accela ultra-high performance liquid chromatography (HPLC) system with autosampler and Quick Quan software. During the current reporting period, our laboratory personnel were trained on the instrument, which is now being used for this project's specific aims.

We have begun to quantify biomarkers of the human carcinogen aflatoxin in liver and urine using isotope-dilution mass spectrometry (MS) (Figure 1). To facilitate our methods development, we used an acute exposure model in male F344 rats, a strain exquisitely sensitive to aflatoxin hepatocarcinogenicity. The animals were dosed by gavage with 20 µg aflatoxin B1 (AFB1), placed in metabolism cages, and sacrificed 24 hours later. We measured hepatic burden of deoxyribonucleic acid (DNA) adducts (AFB-N7-guanine and formamidopyrimidine [FAPy]) along with urinary excretion of the N7-guanine adduct and aflatoxin mercapturide (AF-NAC), an epoxide-derived detoxication product. Animals were either pretreated with vehicle or the putative chemopreventive agent CDDO-Im (30 µmole/kg body weight) every other day prior to carcinogen challenge. As shown in Table 1, substantive levels of the DNA adducts were detected in both the liver and urine of vehicle-AFB1 treated rats. The appearance of DNA adducts in the urine reflects both spontaneous depurination and enzyme-mediated repair of the hepatic adducts. The excreted DNA adducts serve as useful biomarkers of a biologically effective dose of the carcinogen.

As is evident from the data in Table 1, pretreatment of animals with CDDO-Im [1-(2-cyano-3,12-dioxooleana-1,9{11}-dien-28-oyl)imidazole], a potent small molecule activator of Nrf2 signaling, leads to dramatic reductions in the formation of aflatoxin DNA adducts in the target organ and in its subsequent elimination in urine. Equally striking, pretreatment with CDDO-Im leads to a 20-fold increase in the elimination of AF-NAC. This profoundly altered disposition of the reactive epoxide intermediate likely reflects Nrf2-mediated induction of glutathione transferases, enzymes key to the formation of mercapturic acids. Shunting of the aflatoxin toward the mercapturic acid pathway spares DNA from electrophilic modification.

Prior to embarking on studies comparing the pharmacodynamic action of a series of chemical activators of Nrf2 signaling (e.g., CDDO-Im, sulforaphane) in rodents, we compared the global

gene and protein expression patterns resulting from either pharmacologic or genetic activation of the Nrf2 pathway in normal human mammary epithelial cells (MCF10A). Microarray and stable isotope labeling with amino acids in culture (SILAC) technologies were used. The phytochemical sulforaphane (SFN) was used as the pharmacologic activator, while Keap1 (kelch-like erythroid cell-derived protein [ECH]-associated protein) expression was disrupted by Keap 1 siRNA (siKeap1), in turn leading to diminished proteolytic degradation of the transcription factor Nrf2 and, hence, enhanced Nrf2 signaling. Sulforaphane is an isothiocyanate found in cruciferous vegetables, with particularly high levels in three-day-old broccoli sprouts. SFN is an attractive chemopreventive agent because it is safe and can be distributed easily and widely as a broccoli sprout extract. We are currently evaluating broccoli sprout extracts in clinical trials targeted at populations at high risk for exposure to aflatoxins and subsequent development of liver cancer.

MCF10A and MCF12A cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 minus L-Lysine and L-Arginine for SILAC. The media were supplemented with 5 percent horse serum, 20ng/ml epidermal growth factor, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, and 10 µg/ml insulin at 37 degrees Celsius in a humidified environment with 5 percent carbon dioxide (CO_2). For light medium, $^{12}C_6$ L-Lysine:2HCL and $^{12}C_6$ L-Arginine-HCL were supplemented, and $^{13}C_6$ L-Lysine:2HCL and $^{13}C_6$ L-Arginine:HCL were supplemented for heavy medium. To knock down KEAP, 30nM non-targeting control (NTC) or KEAP1 siRNA was transfected into $2x10^6$ MCF10A or MCF12A cells in 10 cm dishes using Lipofectamine™ RNAiMax reagent according to the manufacturer's reverse transfection protocol. NTC siRNA was transfected into cells grown in light medium, and KEAP1 siRNA was transfected into cells grown in heavy medium. RNA was collected 24 hours after transfection. For SFN treatment, 2.5 $x10^6$ cells per 10 cm dish were treated with 15 µM SFN or acetonitrile vehicle 24 hours after plating, and RNA was collected 24 hours after treatment. Vehicle-treated cells were grown in light medium, and SFN-treated cells were grown in heavy medium. The microarray experiments were performed in quadruplicate. For the SILAC samples, MCF10A and MCF12A cells were exposed to SFN or KEAP1 siRNA as described, and lysates were collected 48 hours later. For microarrays, total RNA was isolated from cells using TRIZol reagent and purified by the Qiagen RNeasy kit. Agilent whole human genome chips (G4112F) were used. Data were imported into GeneSpring, and differentially- expressed genes were determined. Quantitative polymerase chain reaction (qPCR) was used to confirm altered gene expression. For SILAC analysis, after 48 hours of siRNA exposure or SFN treatment, MCF10A cells were washed with ice-cold phosphate buffer solution (PBS) six times, and protein was extracted in 8 M urea with protease inhibitors. Whole cell lysates were sonicated and centrifuged at 13,000 revolutions per minute (rpm) for 10 minutes at 4 degrees Celsius, and the protein concentration was measured using the bicinchoninic acid assay. Three hundred µg of protein were resolved on a polyacrylamide gel and stained with colloidal Coomassie stain. Thirty-six protein bands were excised and destained, reduced, alkylated with iodocetamide, and dehydrated. The extracted peptides were dried and reconstituted in 0.1 percent formic acid and analyzed using the 6520-accurate-mass qTOF mass spectrometer. Data-dependent acquisition was achieved using the MasssHunter workstation data acquisition software. Spectral acquisition parameters included MS mass range m/z 350-1,700; MS/MS mass range m/z 100-1,700; acquisition rate for MS and MS/MS 3 spectra/s and maximum of 3 precursors per cycle. MS/MS data were processed to generate mascot generic

format files and then the data were searched against Human RefSeq 35 protein sequence database. The workflow for these experiments is summarized in Figure 2.

The main focus for the microarray pathway analyses were those transcripts shown to be regulated by both SFN treatment and KEAP1 knockdown. When these overlapping transcripts were analyzed by the Ingenuity Pathways Analysis (IPA) program, the top three pathways to emerge were NRF2-mediated oxidative stress response, metabolism of xenobiotics, and glutathione metabolism. Like the microarray analysis, when the genetic and pharmacologic SILAC experiments were analyzed by IPA, the top pathways regulated were NRF2-mediated oxidative stress response, metabolism of xenobiotics, and glutathione metabolism. Key xenobiotic metabolism genes regulated by SFN treatment and/or *KEAP1* knockdown in both microarray and SILAC experiments included *AKR1* subfamily members *NQO1, CBR1, ALDH3A1,* and *EPHX1*. The antioxidant genes *TXNRD1, FTH, BLVRA,* and *TXN* were also coordinately regulated in the genetic and pharmacologic experiments using microarray and SILAC. The genes *NQO1*, *AKR1C1, HMOX1, GPX2*, *TXNRD1*, *TXN, FTH*, *FTL, GSR*, and *PRDX1* have been shown to have antioxidant response elements (AREs). Strikingly, the most highly up-regulated transcripts and proteins were the AKR1 subfamily members. AKR1C1 protein increased 16- and 5-fold following SFN treatment and KEAP1 knockdown by SILAC, respectively. Western blot analysis showed that AKR1C1 was dramatically up regulated by the pharmacologic and genetic approaches (Figure 2 *right*), as were other proteins identified as up regulated in the SILAC experiment. AKR1C1 also had high transcript levels compared to the other xenobiotic metabolism and antioxidant genes. The mass spectra from the SILAC analysis of AKR1C1 are shown in Figure 3. Based on our preclinical studies, AKR1C1, AKR1C3, AKRB10, NQO1, and ALDH3A1 are candidate biomarkers to assess the pharmacodynamic action of SFN in human tissues.

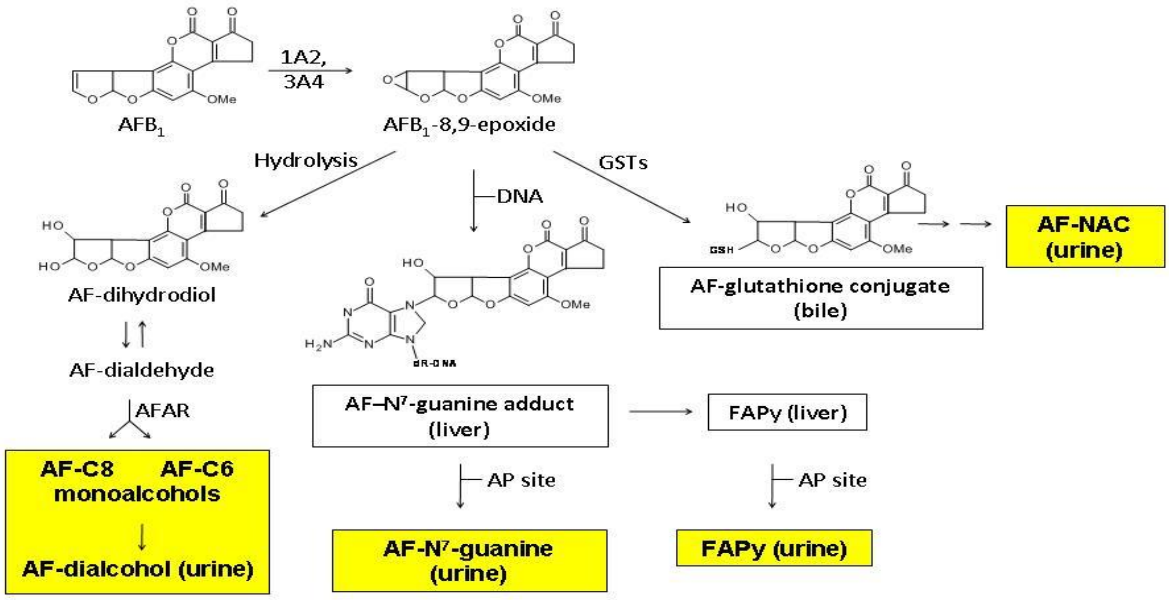

FIGURE 1. Scheme for the metabolism of the hepatocarcinogen AFB1. Metabolites labeled in boxes will be quantified by isotope dilution mass spectrometry in the target organ liver as well as in biofluids.

TABLE I. Levels of aflatoxin metabolites following acute exposure.

Aflatoxin metabolite	**Vehicle (n=5)**	**CDDO-Im (n=5)**
Hepatic N^7-guanine adduct levels (pmol/mg DNA)	10.70 ± 1.45	1.07 ± 0.28
Hepatic FAPY adduct levels (pmol/mg DNA)	15.37 ± 2.73	2.42 ± 0.59
Urinary N^7-guanine levels (pmol/mg creat)	35.2 ± 3.71	6.35 ± 1.18
Urine N-acetyl-cysteine levels (pmol/mg creat)	189.30 ± 13.20	4085.07 ± 1308.32

Data are represented as mean ± SEM.

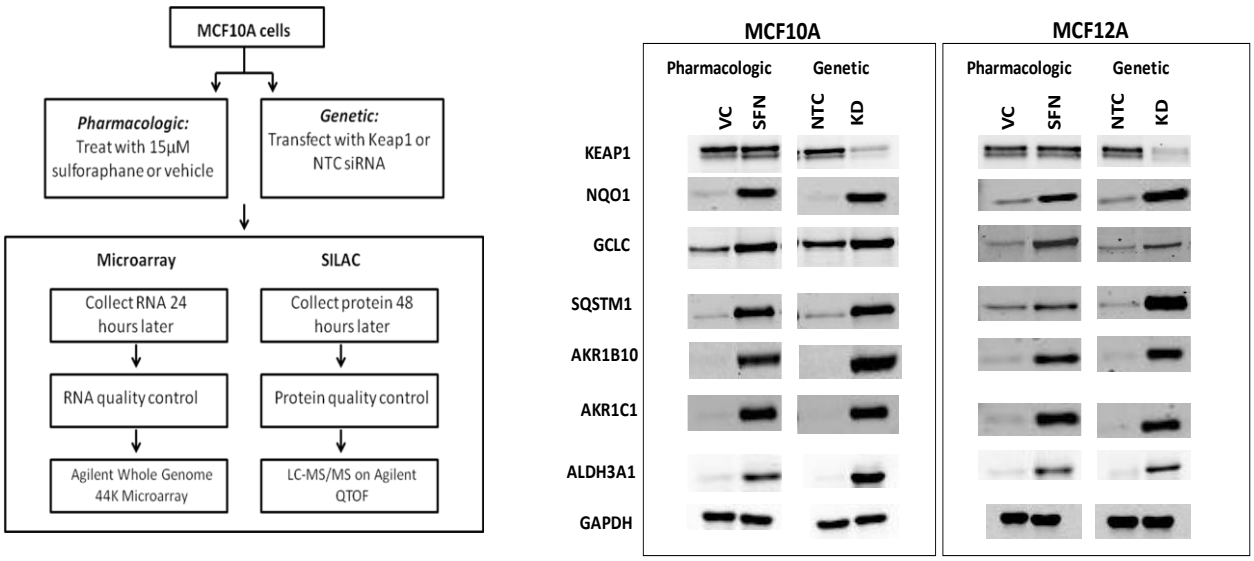

FIGURE 2 (*left*). Workflow for microarray and SILAC experiments. NTC, non targeting control. (*Right*). Western blots for proteins of interest from microarray and SILAC experiments showing elevation of protein levels with SFN treatment and KEAP1 knockdown.

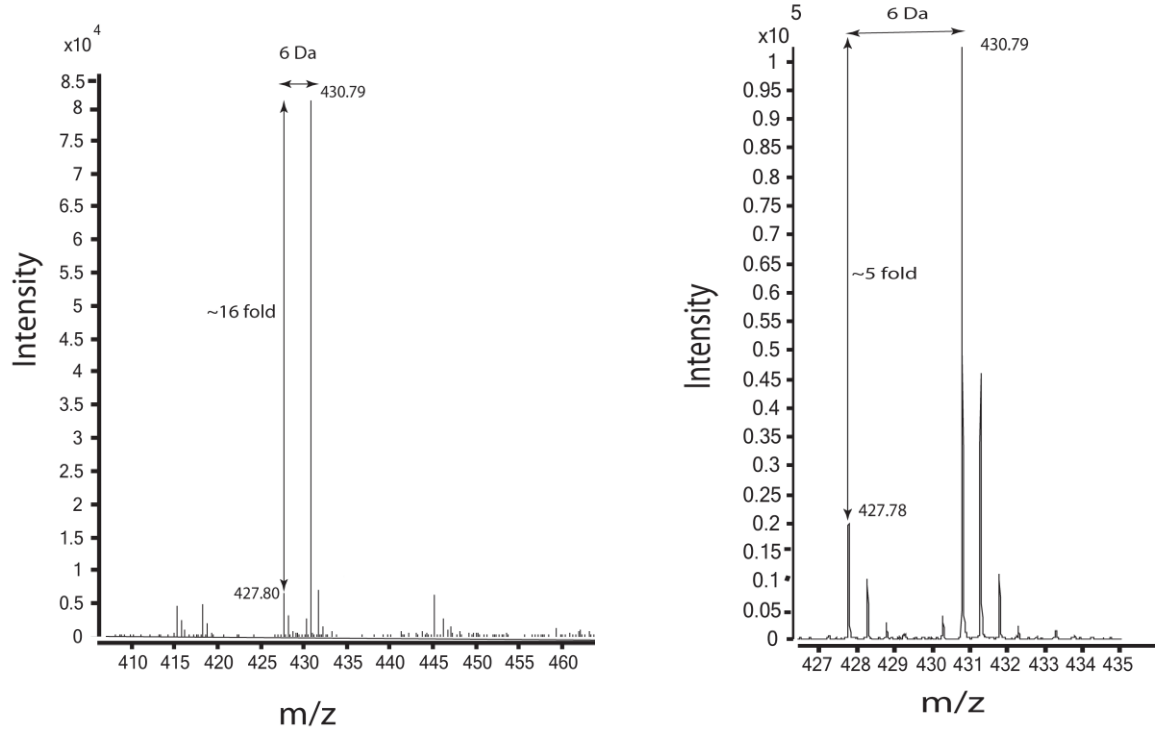

FIGURE 3. Mass spectra from SILAC analysis of candidate biomarker proteins. (*Left*). AKR1C1 following SFN treatment. (*Right*) AKR1C1 following KEAP1 knockdown. Peptide: $TPALIALR^{+2}$.

Research Project 3: Project Title and Purpose

Establishing New Biobehavioral Paradigms for Translational Research in Cancer Prevention and Control - The possibility that "mind-body" effects may contribute to the risk of developing cancer and of experiencing debilitating cancer- and treatment-related symptoms has been examined in numerous studies over the past three decades, with mixed results and considerable controversy. These desultory results stand in stark contrast to the steady progress that has been made in biomedical cancer research, which is grounded in a translational approach where basic laboratory findings from tissue culture and animal models are brought to bear on critical clinical issues. The purpose of this project is to bring this translational approach to mind-body research. Both arms of the project use animal models; one will investigate vomiting and other aversive effects of chemotherapy, while the other explores animal and tissue culture models of human breast cancer progression.

Anticipated Duration of Project

1/1/2010 - 12/31/2011

Project Overview

Despite widespread acceptance by the lay public and some promising findings in the research literature, the contribution of "mind-body" effects to the war on cancer has yet to be well established. More than three decades of research exploring how such effects influence the central issues in cancer prevention and control—the risk of developing cancer and the risk of experiencing debilitating cancer- and treatment-related symptoms—has yielded mixed results. The desultory progress in this area of research stands in stark contrast to the steady gains that have been made in biomedical cancer research overall. In part, the slow pace of progress in this research area can be attributed to the unique challenges of the cross-disciplinary approaches needed to explore the biobehavioral mechanisms involved in mind-body effects; however, there is another possible explanation that has yet to receive appropriate attention. Biomedical research in cancer has long been grounded in a translational perspective, in which basic laboratory findings from tissue culture and animal models provide empirically guided input to human studies of cancer etiology and treatment. Mind-body research, in contrast, has commonly begun with human correlational or interventional research approaches, largely without the benefit of directly relevant preclinical findings. The overarching long-term objective of this project is to lead the way in bringing this powerful biomedical approach to mind-body research in cancer. To that end, we propose to build on existing preclinical models to develop new paradigms for exploring biobehavioral mechanisms of mind-body effects. Aim 1 examines the side effects of chemotherapy, using a species (the musk shrew) that, unlike rodents, is capable of vomiting and can, thus, allow investigation of that particularly aversive side effect, as well as other well known aversive consequences of chemotherapy (e.g., anorexia, fatigue); Aim 2 examines the incidence and progression of cancer using (1) transgenic mice with genetic modulations known to affect susceptibility to mammary cancer and (2) human breast cancer cell lines.

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Expected Research Outcomes and Benefits

While there has been considerable interest in the impact of mind-body effects in the prevention of cancer and the control of aversive symptoms of cancer and its treatment, research results over the past three decades have been mixed. The substantial potential of harnessing the power of the mind to influence the body has yet to be realized, perhaps in part because we have moved too quickly to human studies when we do not yet understand the underlying biological pathways linking what happens in the mind/brain to cancer and its treatment. More traditional biomedical science approaches to cancer have shown the power of preclinical research models using tissue culture and animal research to provide theoretical breakthroughs that can translate into novel interventions to reduce the risk of cancer and improve response to treatment in humans. This project is intended to begin to address those missing links for mind-body research by developing and applying new preclinical research strategies. It is anticipated that the two lines of research in this project will yield two major benefits. First, they will provide specific data concerning the role of the mind/brain in the experience of side effects of chemotherapy and in the development of breast cancer that will speak to basic science researchers in a language they understand and may, thus, bring basic science perspectives to bear on mind/body research. Second, they will serve as a model of the power of such preclinical research for other investigators interested in mind/body effects and may, thus, engender additional interdisciplinary research that will provide a map to guide more successful interventions with humans based on the solid ground of basic research.

Summary of Research Completed

Aim 1 (Chemotherapy Side Effects):
Effect of Cisplatin on Emesis
In the last reporting period, we described the development of a computer algorithm to detect emetic episodes from the musk shrew, an insectivore that is capable of vomiting (unlike rodents such as mice and rats). During the past year, we extended this work to analyze behavioral patterns that are temporally associated with emesis. This work has now been accepted for a conference presentation (Horn et al., 2011a) and has been submitted for publication (currently in the revision stage). Here we applied a statistical method, t-pattern

(temporal pattern) analysis, to determine patterns of behavior linked to emesis. Musk shrews were injected with the chemotherapy agent cisplatin (a gold standard in emesis research) to induce acute (< 24 hour) and delayed (> 24 hour) emesis. Emesis and other behaviors were manually coded and automatically tracked from video files (Table 1). In general, t-pattern analysis revealed that musk shrews show a large number of non-random patterns of behavior associated with emetic episodes, including sniffing, changes in body contraction, and locomotion. There was some evidence that locomotion was inhibited by the occurrence of emesis. However, eating was not significantly associated with emesis-related behavioral patterns. Eating and drinking and other larger body movements, including rearing, grooming, and rotation, were significantly less common in emesis-related behavioral patterns in real versus randomized data (Fig. 1). These results lend preliminary evidence for the expression of emesis-related behavioral patterns of sickness, including reduced ingestive behavior, grooming, and exploratory behaviors. In summary, this statistical approach to behavioral analysis in a pre-clinical emesis research model could be used to assess the more global effects and limitations of drugs used to control nausea and its potential correlates, including reduced feeding and activity levels.

Study 2: Effects of contextual fear conditioning on musk shrews

In the last reporting period, we assessed the effects of restraint stress on shrews. To develop a stress paradigm that more closely mimics the experience of cancer patients who undergo chemotherapy, we used a learning paradigm to produce stress responses in shrews. Two experiments were performed to confirm electrical shock-induced contextual conditioning.

Unconditioned effects of electrical current on movement. Four shrews were tested individually in a behavioral chamber (Med Associates Inc.; mouse module) followed by a step-wise increase in electrical current (increments of 0.1 mA every 30 seconds) applied to the floor grid for three seconds (AM Systems pulse stimulator). Behavior was recorded using a high-resolution digital video recorder (Sony, DCR-SR300) positioned above the test arena. Movement detection and analysis were conducted off-line using automated software (EthoVision XT 7; Noldus; Fig. 1). Shrews consistently vocalized with multiple squeaks and locomoted at levels of 0.6 to 0.7 mA (Fig. 2). This level of electrical current is similar to the intensity used in studies of contextual conditioning in mice.

Effects of pairing electrical shock and context on movement. To confirm that electrical shock (0.7 mA, 3 seconds) can be an unconditioned stimulus for contextual learning in shrews, we used a protocol similar to those previously used in rodent studies. Fourteen naïve shrews were randomly assigned to two groups (each n = 7): (1) no-shock controls and (2) three shocks (3 second duration, 60 second inter-shock interval). Shocks began three minutes after an animal was placed in the contextual chamber, and shrews were returned to the home cage 60 seconds after the last shock. No-shock controls experienced the same amount of time in the contextual chambers. One day after training, animals were exposed to the chamber (15 minutes) without shock, and behavior was recorded using a video camera and analyzed with computer software. Measures included locomotion and immobility. Immobility, a measure of freezing behavior, was set at a limit of ≤ 2 percent change in body contour based on manual observation of freezing. Musk shrews with just one session pairing the chamber with electrical shock showed less movement and more immobility (freezing) when re-exposed to the chamber compared to the no-

shock control animals (Fig. 3). Electrical shock paired with a context produced effects in musk shrews similar to rodents, including that shocked animals moved less when re-exposed to the chamber. These preliminary data confirm that musk shrews can be trained to show conditioned responses to a context. We plan to further refine this paradigm by varying the shock number, interval, and intensity during training to maximize this conditioned effect. We will then explore the effects of contextual conditioning with electric shock on behavioral measures of anxiety, including a battery of measures used in rodent studies (e.g., elevated plus maze, open field, and light-dark box).

Study 3: Effect of Stress on Cisplatin-Induced Emesis, Feeding, Drinking, and Activity
This study has not yet been initiated. We are awaiting results from additional testing of the fear conditioning paradigm before conducting this subproject. We hope to carefully assess the outcomes of the contextual fear paradigm with classical anxiety tests, including the open field, light-dark box, and elevated plus maze.

Study 4: Transneuronal Tracing of Pathways from the Intestine and Stomach
Sensory pathways from gut to brain that are responsible for stimulating vomiting and perceiving nausea are not completely understood. A variety of gastric stimuli produce emesis in musk shrews, including distension and chemical irritation. The aim of this study was to define the sensory component of the gut-brain axis of the shrew using transneuronal viral tracing. The H129 strain of herpes simplex virus-1 was previously used in rats to define ascending gut-brain sensory circuits. In the current study, H129 was microinjected into the ventral stomach wall in shrews and rats to compare results between species (Fig. 4). Five days later, brain and spinal cord tissues were collected. In both shrews and rats, immunohistochemistry revealed transported virus within key circuit nodes of ascending visceral sensory pathways, including the spinal cord dorsal horn, nucleus of the solitary tract, parabrachial nucleus, reticular formation, and hypothalamus. Overall, these results support the continued use of transneuronal viral tracing as a useful strategy to reveal the synaptic organization of the gut-brain axis of the musk shrew. We have presented this information at a conference (Horn et al., 2011b) and are in the process of writing a manuscript for publication.

<u>Aim 2 (Cancer Development and Progression):</u>

Study 1: To Investigate Possible Stress-Induced Differences in Angiogenic Processes in Mouse Mammary Tissue Collected from Glands with and without Tumor
During this reporting period, we have focused on determining differences in angiogenesis in mammary tissue sections obtained for our initial study of environmental stress effects in mouse mammary tumor virus (MMTV)-neu mice. Paraffinized tissue blocks were produced containing the left and right mammary gland from each mouse, regardless of whether a tumor was present. This design resulted in two mammary tissue blocks from each mouse (one from the left gland and one from the right gland), with some blocks containing tumor and some being tumor free. One section from each tissue block was stained with hematoxylin and eosin and re-evaluated by a board-certified veterinary pathologist to classify whether each section contained tumor or hyperplasia or was histologically normal.

To determine the level of angiogenesis in the tissue sections, microvessel density (MVD) was determined on each tissue section. Each tissue section was stained with an antibody directed against CD31, an endothelial cell marker. Each slide was scanned using the Aperio ScanScope XT, resulting in an image of the entire slide, which can be viewed using Aperio ImageScope Software. The software allows the user to zoom in on any area of the slide (to a maximum of 20 X), take pictures, and annotate. Figure 5 shows an example of a single scanned slide (a hematoxylin and eosin [H&E] of a mammary tissue). In this figure, the green rectangle denotes the area shown in Figure 6, which represents a 20 X image of this area. The capability of scanning whole slides and using the Aperio software to view, annotate, and record images allows us to more easily perform immunohistochemistry analyses. Figure 7 shows an example of immunohistochemistry of the mammary sections using the CD31 antibody for MVD analyses. As seen in Figure 7, the antibody directed against CD31 clearly identifies the blood vessels (indicated by solid lines), while the mammary ducts (which are lined with epithelial, not endothelial, cells) are not stained (indicated by dashed lines). We have completed all CD31 staining and are performing MVD analyses, which require an operational definition of what constitutes a blood vessel. The published literature does not agree on this point, as some laboratories require the presence of a lumen while others do not. We have decided to determine the MVD for five distinct areas in any tumor and 10 distinct areas in tumor-free areas (focusing on regions that contain mammary ducts). We will use the following definition of a blood vessel in these analyses: (1) CD31-positive cells must be present; (2) a lumen may or may not be present, and (3) a vessel must not be in an area containing a transversely-cut mammary duct. For this last requirement, we have found that the majority of blood vessels are located around the mammary ducts. Therefore, any vessels found to be present around a mammary duct cut transversely may represent the same vessel that is simply wound around the duct and, as a result, could give a false positive.

Study 2: To Explore Possible Differences between Stressed and Control Mice in Expression of Proinflammatory Cytokines and Signaling Pathways Involved in Tumorigenesis in Mouse Mammary Tissue Collected from Glands with and without Evidence of Tumor

Part of our goal in this project is to determine differences between stressed and control mice in expression of proinflammatory cytokines. We have spent considerable effort to optimize our staining with different antibodies. In the this reporting period, we focused on identification of cells that express the beta-2 adrenergic receptor (a receptor for the stress hormones epinephrine and norepinephrine). As shown in Figure 8, and recently presented at a peer-reviewed meeting (Shuda et al., 2011), we have documented expression of beta-2 adrenergic receptor in our mouse mammary tissue. We are currently performing multistaining using Q-dot technology to identify proinflammatory cytokines and the cell types expressing these cytokines. In addition, we are also determining associations between cytokine expression and beta-1 and beta-3 adrenergic expression.

Study 3: To Test the Hypothesis that Stress-Related Proinflammatory Cytokines and Hormones Will Have Significant Molecular Level Effects on Breast Cancer Cell Lines in a Tissue Culture Model

We have obtained five breast cancer cell lines (MCF10A, MCF7, MDA-MB-231, MDA-MB-468, and SKBR3) and are currently performing growth curve analyses of each line in dose

response experiments using beta-2 adrenergic agonists. We are also determining the ability of the adrenergic receptor agonists to stimulate cyclic adenosine monophosphate (cAMP) signaling. We have hypothesized that stimulation by adrenergic receptor agonists will not result in increased cell growth but will result in increased cAMP signaling as well as other downstream signaling events such as production of inflammatory cytokines and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). The downstream signaling following adrenergic stimulation will be measured after the completion of these studies.

Table 1. Musk shrew behavioral event types

Manually coded

emesis	a sequence of contractions of the abdomen (retching)
eat	putting the head into the food container for ≥ 2 s and visible movement of head and jaw
drink	placing the snout on the sipper tube for ≥ 1 s
sniff	moving the snout with elongation of the body
rear	standing on back legs, against the wall, sometimes jumping

Automatically tracked

Locomotion

dchi,	distance moved, body center, high
dclo	distance moved, body center, low
mc	movement (velocity, begin > 2 cm/s, end < 1.75

Turning

rot,	rotation-clockwise (a turn of 360 degrees),
rotc	rotation-counter clockwise
tanhi,	turn angle of the nose, high
tanlo	turn angle of the nose, low
tachi,	turn angle of the body center, high
taclo	turn angle of the body center, low

Movement in place

con,	contracted,
norm,	normal,
long	elongated body contour
immob,	immobile (< 1%),
mob,	mobile (> 1%, < 8%),
mobhi	and highly mobile (> 8%; change in body contour)
dnhi,	distance moved, nose, high
dnlo	distance moved, nose, low

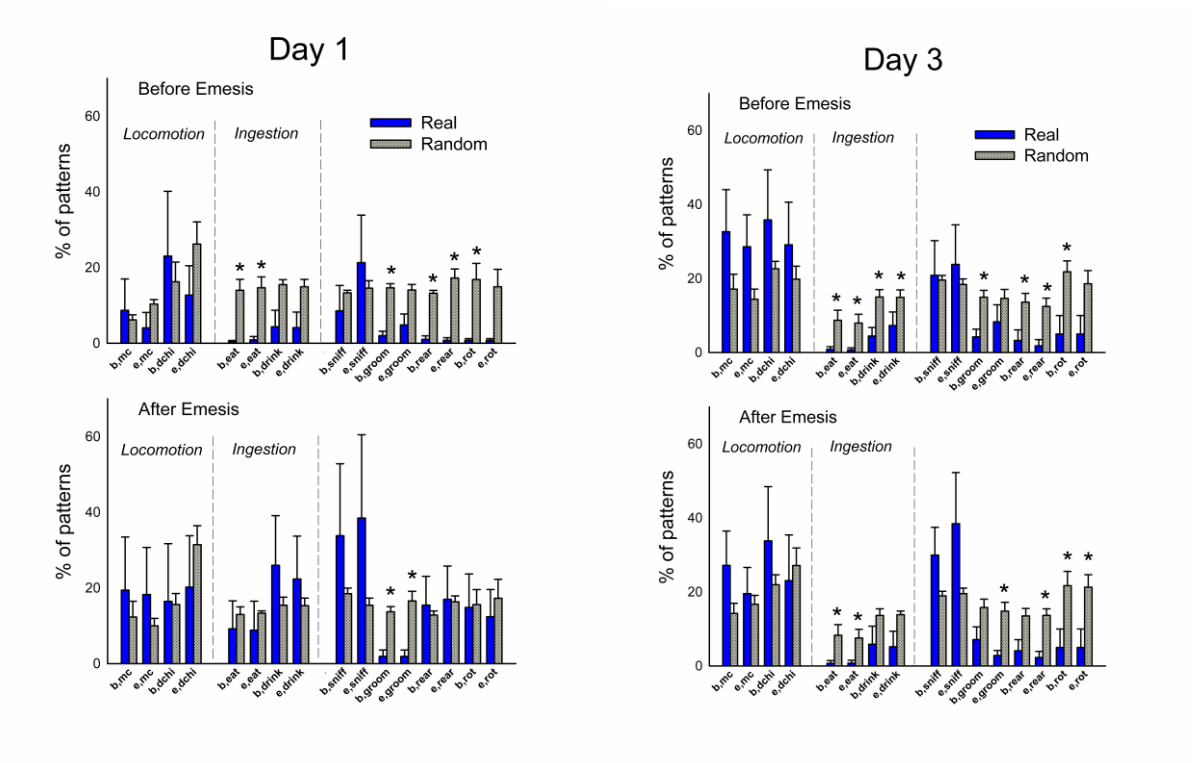

Fig. 1. Comparison of event types in emesis-related t-patterns detected on Days 1 and 3 after injection with cisplatin, 30 mg/kg, ip, n = 6. Results represent the mean ± SEM. * = $p < 0.05$, two-tailed t-test, real versus random data. See Table 1 for listing of event types. Each event type has a "b" for beginning and "e" for ending, for example, "b,mc" = beginning of movement.

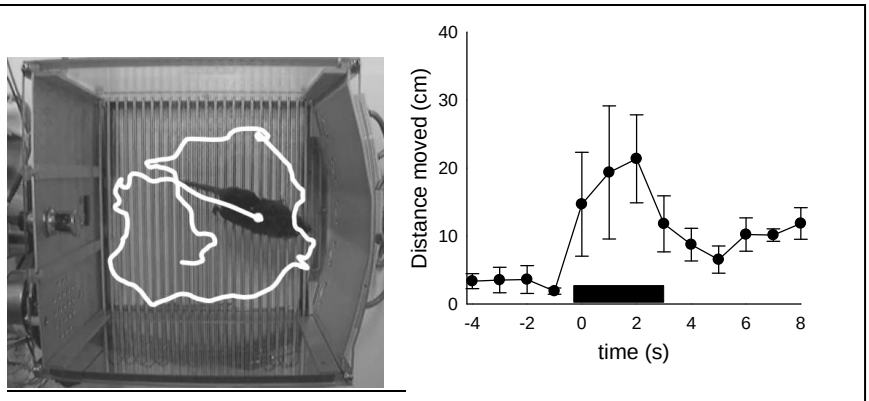

Fig. 2: *Left*: Chamber (18 x 22 cm) with a musk shrew. White line indicates the path of the animal tracked by computer software. *Right*: Effect of 0.6 mA on movement. Black bar = duration of shock.

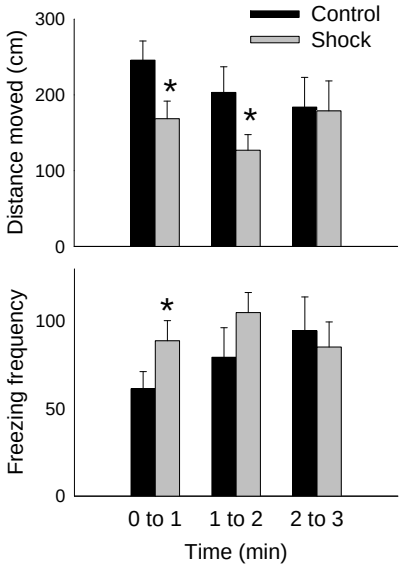

Fig. 3: Top: Distance moved (cm). Bottom: Freezing (immobility) frequency (≤ 2% change in body contour from previous video frame, analyzed at 12 frames per second). * = p< 0.05.

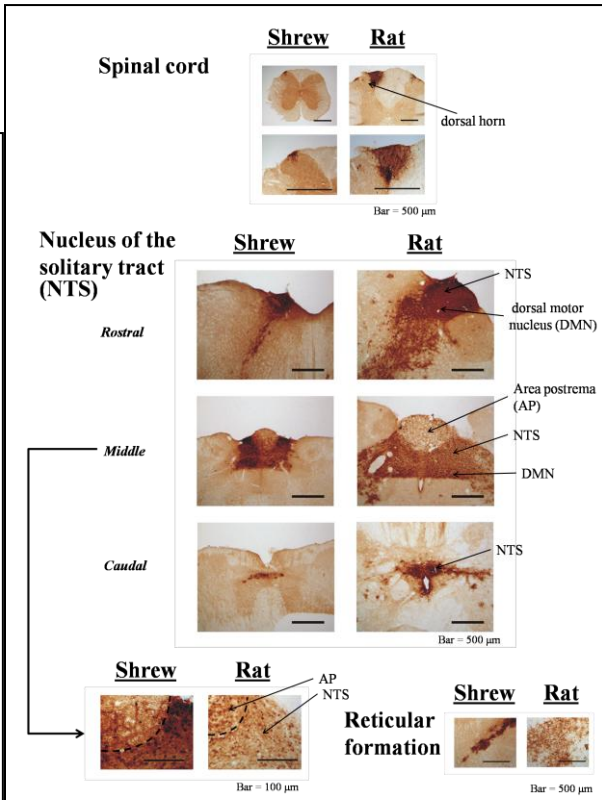

Fig. 4. Comparison of H129 staining in the hindbrain of the musk shrew versus the rat. H129 was injected into the ventral surface of the stomach 5 days before tissue collection.

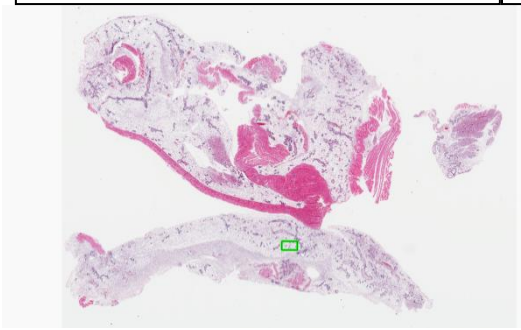

Figure 5

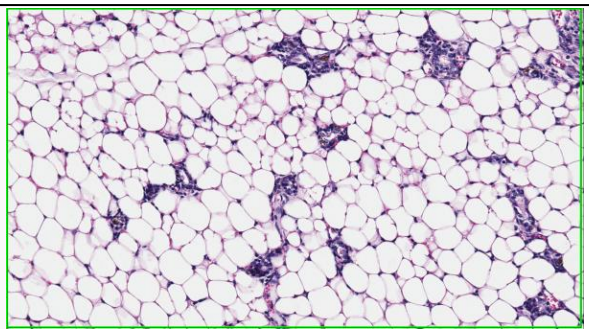

Figure 6

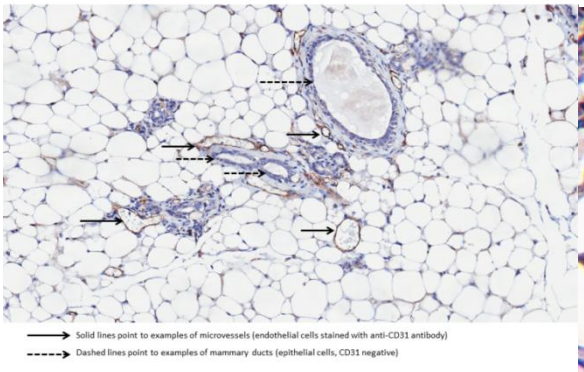

Figure 7

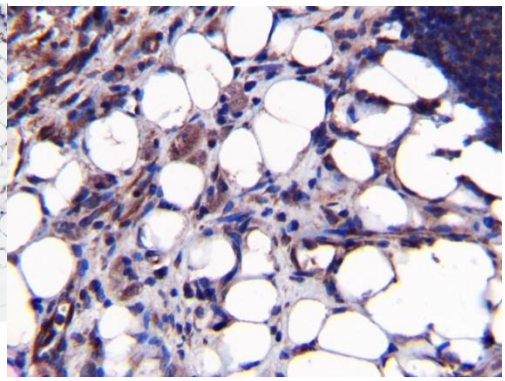

Figure 8

Published Abstracts

Horn, C.C., Henry, S., Meyers, K., and Magnusson, M.S. (2011a). "Behavioral patterns associated with chemotherapy-induced emesis: A potential signature for nausea in musk shrews", in: *Society for Neuroscience Annual Meeting.* (Washington, DC).

Horn, C.C., Meyers, K., Dye, M., Pak, D., Rinaman, L., and Yates, B.J. (2011b). "Transneuronal viral tracing of sensory pathways from the stomach to the brain in the musk shrew, a small animal model for vomiting research.", in: *Experimental Biology Annual Meeting* (Washington, DC).

Shuda, Y., Bovbjerg, D.H. and Jenkins, F.J. "The Utility of the Nuance™ Imaging system to visualize multiple markers on individual cells with bright-field and fluorescent immunohistochemistry for biobehavioral and other cancer research studies" in Proceedings of the 23rd Annual UPCI Retreat (Pittsburgh, PA).

Research Project 4: Project Title and Purpose

Clinical Trials in Multiple Myeloma - The University of Pittsburgh Cancer Institute's (UPCI) Clinical Research Service (CRS) provides valuable resources for researchers and clinicians seeking to improve clinical research as a means to elevate patient care standards and treatment efficacy. This project will examine two therapeutic trials in patients with multiple myeloma: (1) a safety and efficacy trial to determine a maximum dose of bendamustine combined with lenalidomide to establish a treatment modality for relapsed, refractory multiple myeloma; and (2) a randomized clinical trial to determine whether there is ultimately a need for autologous peripheral blood stem cell transplant in patients with newly diagnosed myeloma who are receiving lenalidomide and low-dose dexamethasone.

Anticipated Duration of Project

1/1/2010 - 12/31/2011

Project Overview

The first clinical trial will to seek to determine the maximum tolerated dose (MTD) of bendamustine and lenalidomide in combination with a fixed dose of dexamethasone for patients with refractory or relapsed multiple myeloma and to establish the dose of each drug recommended for a future Phase II protocol with the combination. It will also explore anti-tumor activity of the combination of bendamustine plus lenalidomide and dexamethasone and evaluate toxicity, time to progression, and overall survival.

The second clinical trial will evaluate the complete response rate in newly diagnosed multiple myeloma patients receiving autologous peripheral blood stem cell transplant after undergoing four cycles of lenalidomide and low-dose dexamethasone (Arm A) versus those receiving six to eight cycles of lenalidomide and low-dose dexamethasone until plateau of best response (Arm B). The secondary objectives are to estimate duration of complete response, objective response rate (complete response rate plus partial response rate), progression-free survival, overall survival, time to progression, and toxicity of the two treatments.

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Other Participating Researchers

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Expected Research Outcomes and Benefits

In the first clinical trial, the combination of lenalinomide and bendamustine may result in additive/synergistic anti-myeloma effects without cross-resistance. This drug combination might be especially useful for patients who are suffering from pre-existing neuropathy and not eligible for treatment with bortezomib. Also, in patients who have had bortezomib-induced neuropathy, this regimen might be very effective and still feasible. In the future, this combination might be used up front to avoid the development of neuropathies that can lead to severe debilitation in patients with multiple myeloma.

The second project is a randomized clinical trial designed to determine whether there is ultimately a need for autologous peripheral blood stem cell transplant in newly diagnosed myeloma patients who are receiving lenalidomide and low-dose dexamethasone. Due to the fact that lenalidomide alone can achieve similar response rates as transplant, more and more patients treated with lenalidomide refuse transplant after achieving complete response (CR). It will be important to define the role of transplant in newly diagnosed patients, as transplant may result in a longer duration of remission, even if transplant and lenalidomide alone achieve similar initial relative response.

Summary of Research Completed

UPCI 07-089: Phase I Study of Bendamustine in Combination with Lenalidomide (CC-5013) and Dexamethasone in Patients with Refractory or Relapsed Multiple Myeloma
Enrollment for this study was completed during the reporting period. Thirty-six participants have been screened, with seven screen failures, for a total of 29 participants enrolled in the trial. Preliminary results were presented at the 52nd American Society of Hematology Annual Meeting and Exposition (December 2010) and are summarized below. Final analyses are currently underway. Participants will be followed for progression and survival.
Background: Lenalidomide is an analog of thalidomide that has significant clinical activity in combination with dexamethasone in patients with relapsed or refractory multiple myeloma (MM). Bendamustine is a bifunctional alkylating agent that is approved for the treatment of chronic lymphocytic leukemia and indolent non-Hodgkin's lymphoma that has progressed during

or relapsed within six months following a rituximab-containing regimen. This multicenter Phase I trial is the first to investigate the combination of bendamustine, lenalidomide, and dexamethasone. Our primary objective was to determine the maximum tolerated dose (MTD) and safety profile of bendamustine and lenalidomide when administered with dexamethasone for patients with relapsed or refractory MM.

Methods: Patients aged > or = 18 years with confirmed, measurable symptomatic MM that was refractory to or progressed after one or more prior therapies were treated with bendamustine by intravenous infusion on days 1 and 2, oral lenalidomide on days 1-21, and oral dexamethasone on days 1, 8, 15, and 22 of each 28-day cycle. Treatment was continued until a plateau or best response, as determined by the International Myeloma Working Group uniform response criteria, was reached. Study drug doses were escalated through 3 levels (Table 1) in a 3+3 dose-escalation scheme. The MTD was defined as the dose level at which < or = 1 of 6 patients experienced dose-limiting toxicity (DLT) during the first cycle of therapy when the next higher dose level is associated with DLTs in > or = 2 patients. After determining the MTD, an expansion cohort of 12 additional patients at the MTD will be treated to better evaluate toxicity and clinical activity. Secondary endpoints included preliminary efficacy as evidenced by objective response, time to disease progression, and overall survival.

Table 1. Dose escalation schema

Dose Level	Bendamustine, mg/m^2	Lenalidomide, mg	Dexamethasone, mg
1	75	5	40
2	75	10	40
3	100	10	40

Results: Twenty-six patients with a median age of 63 years (range, 38-81 years) were enrolled. The mean number of prior therapies was three (range two to seven); 81 percent of the patients had prior lenalidomide, 48 percent had prior thalidomide, and 29 percent had both. The MTD was identified at dose level 2: 75 mg/m^2 bendamustine and 10 mg lenalidomide. Four DLTs were recorded: at dose level 2 (n=6), one patient with grade 4 neutropenia; at dose level 3 (n=6), two patients with grade 4 neutropenia and another with delayed platelet recovery from grade 3 thrombocytopenia. Currently, no patients have been enrolled in the expansion cohort. Twenty-one of 26 patients received at least two cycles and were included in the response assessment. A partial response (PR) or better was observed in 63 percent (n=12) of the patients, including 16 percent (n=3) achieving a very good PR (VGPR). In addition to these 12 patients, another three (15 percent) had a minor response (25-49 percent reduction in M-protein). Stable disease was observed in 32 percent (n=6), and only 5 percent (n=1) had disease progression. The median time to next treatment was 8.1 months (range, 1.9-27.3 months). Other grade 3/4 adverse events occurring after the first cycles of treatment included prolonged QTc in one patient.

Conclusions: This is the first Phase I trial testing the combination of bendamustine, lenalidomide, and dexamethasone for relapse and refractory MM. This regimen is well tolerated, even in older patients up to 81 years. With a PR/VGPR rate of 63 percent, this combination is a highly active regimen, even in heavily pretreated MM patients, and its side effect profile makes it an attractive treatment option for MM patients, especially those with pre-existing therapy-related peripheral sensory neuropathy.

UPCI 07-134: A Randomized Clinical Trial of Lenalidomide (CC-5013) and Dexamethasone With and Without Autologous Peripheral Blood Stem Cell Transplant in Patients With Newly Diagnosed Multiple Myeloma
This study is ongoing. Fifty-one subjects have been screened to date, with 44 eligible to commence on the randomized treatment arm. Twenty-one subjects have been randomized and treated with lenalidomide (CC-5013) and dexamethasone with autologous peripheral stem cell transplant (PSCT) (Arm A), and 23 have been randomized to lenalidomide (CC-5013) and dexamethasone (Arm B). With compelling results of a national cooperative clinical trial, the study has been modified to include peripheral stem cell harvesting in Arm B and maintenance lenalidomide (CC-5013) in Arm A and Arm B. Interim analysis is forthcoming.

Research Project 5: Project Title and Purpose

Improving the Therapy of Women with Metastatic Breast Cancer - While great advances in breast cancer therapy have been made in recent years, therapy for metastatic breast cancer (MBC) is not adequate. We propose three trials that can have a direct impact on the treatment of MBC. The first trial seeks to determine whether changes in positron emission tomographic (PET) scanning very early in the treatment of MBC can be used to predict response and outcome to therapy. The second and third trials investigate the activity of a novel agent (ABT-888), which inhibits a protein active in DNA repair in response to stress, in the treatment of BRCA 1/2 (breast cancer genes 1 and 2) positive metastatic breast cancer and other cancers.

Anticipated Duration of Project

1/1/2010 – 12/31/2011

Project Overview

This project consists of three clinical trials that will have a direct impact on the treatment of women with metastatic breast cancer (MBC).

The first trial, UPCI 07-067, will seek to determine whether changes in a positron emission tomography (PET) scan performed two weeks after the initiation of therapy for metastatic breast cancer can predict response of the cancer to therapy. Thirty women with MBC will have a baseline PET-CT scan. The patients will then be treated with nab-paclitaxel chemotherapy weekly on days 1, 8, and 15 of a 28-day cycle. The patients will receive a research PET-CT scan on days 7 and 15 of cycle 1. A glycolytic index (*G*) will be calculated based on the uptake of 18-fluorodeoxyglucose (18-FDG) in the total volume of tumor present in scans on days 7 and 15. Patients will be treated with nab-paclitaxel until progression, and response rate (RR) and progression free survival (PFS) will be calculated. The primary endpoint of the trial is to determine whether the glycolytic index (*G*) calculated on day 7 and day 15 of chemotherapy is predictive of RR and PFS.

The second trial, UPCI 07-015, is a phase I dose escalation study of ABT-888. ABT-888 is one of a new class of drugs that inhibit poly (ADP-ribose) polymerase (PARP), a protein important in

the repair of DNA damage induced by chemotherapy. This protein is found to be elevated in BRCA 1/2 positive breast cancer, as well as basal type breast cancer and other tumors. The primary aim of this trial is to determine the maximally tolerated and effective dose of ABT-888 in patients with these advanced cancers. A secondary aim is to determine the level of PARP inhibition and DNA damage in tumor samples, peripheral blood mononuclear cells (PBMCs), and other tissues obtained from the subjects participating in the study. Up to four patients will be treated in each cohort with escalating doses of oral ABT-888. One patient in each cohort will be BRCA 1/2 positive. There are six dose escalations allowed. If there is no serious toxicity in the cohort one month after the last patient is treated, the next level cohort will be started. At the dose-limiting toxicity cohort, six patients with BRCA1/2 mutations will be treated. Blood samples and hair follicles will be tested for DNA damage at various points during the trial.

The third trial, UPCI 08-121, has an identical design to UPCI 07-015, except that the ABT-888 is combined with the chemotherapy agents paclitaxel and carboplatin.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

UPCI 07-067: We expect to determine in this study whether a glycolytic index (G) calculated from a PET-CT within one to two weeks of initiating therapy is predictive of response to chemotherapy two to four months later. This information will be invaluable in that decisions about whether chemotherapy is successful can be made at a much earlier time, sparing the patient unnecessary toxicity if the therapy is not working and allowing transition to another therapy much earlier in the patient's disease course.

UPCI 07-015 and UPCI 08-121: The major achievements of these trials are determinations of the proper doses of ABT-888 to proceed to further clinical trials. Additionally, we will learn more about this compound's ability to affect PARP activity and DNA repair. These drugs will likely prove to be valuable new tools to treat women with breast cancer harboring mutations in the BRCA1 or BRCA2 gene.

Summary of Research Completed

UPCI 07-067:
Accrual for this trial is underway, with five patients accrued to date.

UPCI 08-121:
For this study, we have enrolled 48 patients, both with and without mutations in BRCA1/2. We have completed seven of nine planned dose levels, and the maximum tolerated dose has not yet been determined. We anticipate enrolling two additional dose escalation cohorts. We have begun to analyze the correlative data in conjunction with pathology. A number of patients who have been part of the study for longer than six months have achieved clinical benefits, including prolonged stable disease and tumor shrinkage. Pharmacokinetic and pharmacodynamic studies are ongoing.

UPCI 07-015:
More than 70 patients have been accrued to this trial, 12 of whom have breast cancer. This study is nearing completion, and maximum tolerated doses of chemotherapy in combination with the PARP inhibitor have been determined: paclitaxel, 200 mg/m^2; carboplatin, AUC 6; and ABT-888, 80 mg twice per day. Based on the success of this study and the number of patient responses (including two sustained complete responses), a study of weekly paclitaxel, carboplatin, and ABT-888 has been launched (UPCI 09-082). This treatment regimen has potentially greater application in a breast cancer population, with weekly paclitaxel being standard of care in both early stage and advanced breast cancer. There are planned Phase II outlets in both the metastatic and neo-adjuvant settings.

Research Infrastructure Project 6: Project Title and Purpose

Relocation of a 9.4 Tesla Magnetic Resonance Imager - The project involves the relocation of a 9.4 Tesla magnetic resonance (MR) imaging unit to provide a University of Pittsburgh team of structural biology and biophysics researchers a cohesive research space in which they can improve and advance their collaborative efforts. Existing, unoccupied space in the Biomedical Science Tower 3 (BST3) that is adjacent to the structural biology magnet facility will be renovated to accommodate the MR imager. After these preparations have been made, the MR unit will be moved from its current location in the McGowan Institute for Regenerative Medicine to the BST3, about 2.3 miles away.

Summary of Research Completed

This project was deleted. No health research grant funds were spent on this project.

Research Infrastructure Project 7: Project Title and Purpose

Research Infrastructure: Neurological Surgery Laboratory Renovations - Neurological surgery research is a growing area of translational research and is rapidly expanding at the University of Pittsburgh. This infrastructure project will facilitate the renovation of neurological surgery laboratories in Scaife Hall on the main campus of the University of Pittsburgh. These new

laboratories will enable more efficient use of space for the expanding department and will promote collaboration and shared resources both within and outside the Department of Neurological Surgery.

Anticipated Duration of Project

3/16/2011 – 6/30/2013

Project Overview

Scaife Hall, home to the University of Pittsburgh School of Medicine, is approximately 56 years old. The fifth floor, where the Department of Neurological Surgery is housed, suffers from aging infrastructure, deteriorating interior construction, and an outmoded layout of cloistered laboratories with no natural lighting and limited opportunities for collaborations or sharing of resources. Approximately 9,300 square feet of outdated laboratory space will be renovated into modern, generic laboratory space that will accommodate new researchers and equipment and will help enhance the research activities of the department, which is under the direction of a new chair, Robert Friedlander, MD. The renovation of this space into a suite of modern and attractive laboratories will assist Dr. Friedlander in recruiting top-tier faculty, improve space usage efficiency and work flows, and reduce maintenance burden and costs necessary to meet the needs of new faculty and emerging research directions.

Principal Investigator

Arthur S. Levine, MD
Senior Vice Chancellor for Health Sciences
University of Pittsburgh
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3550 Terrace Street
Pittsburgh, PA 15261

Other Participating Researchers

None

Expected Research Outcomes and Benefits

The renovation of this space into a suite of modern and attractive laboratories will assist Dr. Friedlander in recruiting top-tier faculty to the department of Neurological Surgery, improve space usage efficiency and work flows, and reduce maintenance burden and costs necessary to meet the needs of new faculty and emerging research directions.

Summary of Research Completed

No grant funds were expended on this project during the reporting period.