University of Pittsburgh

Annual Progress Report: 2009 Formula Grant

Reporting Period

January 1, 2010 – June 30, 2010

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 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

Development of Adult Stem Cell-Based Cartilage Tissue Engineering

Objective

Mesenchymal stem cells (MSCs) derived from multiple adult tissue sources, such as bone marrow, represent a promising cell type for articular cartilage regeneration because they pose no donor site morbidity, have high expansion potential, and are able to differentiate down the chondrocyte lineage. However, *in vitro* induction of MSC chondrogenesis generally results in a hypertrophic phenotype that is unsuitable for articular cartilage. In addition, implanted MSC-derived chondrocytes remain susceptible to arthritic changes induced by the underlying inflammation and arthritic disease, necessitating chondroprotection from the resultant catabolic- and hypertrophic-like phenotype. Enhancing control over stem cell signaling, differentiation, and phenotype stability remains the challenge for the production of tissue engineered constructs and development of stem cell-based therapies. The goal of this project is to develop novel approaches to control and maintain the articular phenotype in primary and MSC derived chondrocytes. Two approaches are being tested: (1) cyclic hydrostatic loading and (2) targeted gene therapy using parathyroid hormone-related peptide (PTHrP) over-expression.

Methods

a. Cyclic hydrostatic loading. Previous observations have suggested that cyclic hydrostatic loading may suppress expression of some of the gene products associated with hypertrophy. In our study, adult human bone marrow-derived MSCs were induced to undergo chondrogenesis as high density pellet cultures and subjected to cyclic hydrostatic loading (0.8 Hz with a peak of 10 MPa, 4 hour-cycle). The MSCs were previously transfected with luciferase reporter constructs containing either a TCF/LEF, AP1, NFAT, or NFκB promoter to examine the extent of Wnt signaling upon loading.

b. PTHrP gene therapy. Human PTHrP plasmid constructs (1-141 or 1-173) were transfected into bovine articular chondrocytes, which were then seeded at high density (40,000 cells/cm^2) on flexible bottom plates coated with bovine plasma fibronectin. One day later, cells were subjected to injurious cyclic tensile strain (CTS; 0.5 Hz and 16% elongation) for 48 hours. Cell response to CTS was measured on the basis of nitric oxide (NO) and prostaglandin E2 (PGE2) production and expression of chondrocyte markers of catabolism and anabolism, as a function of PTHrP levels.

Results

a. Cyclic hydrostatic loading. Initial results showed that exposure to loading resulted in reduced expression of TCF/LEF-, AP1-, and NFAT-promoter-reporters, monitored on the basis of luciferase activities, while NFκB promoter activity was elevated. These results suggest that adult MSCs respond to physical stimulation, likely via regulation of Wnt signaling, to influence the hypertrophic program during their chondrogenic differentiation and maturation. We are currently testing this mechanistic scheme using signaling inhibitors and developing methods to enhance the load-mediated effects via stimulation of Wnt signaling.

b. PTHrP gene therapy. CTS was injurious to the articular chondrocytic phenotype, resulting in a gene expression profile resembling that of arthritic chondrocytes, including increased levels of matrix remodeling enzymes, collagenases (MMP-3, MMP-13 and aggrecanases, ADAMTS-4,5),

inflammatory factors (COX2, iNOS), as well as increased protein production of NO and PGE2 (Figure 1). In addition, CTS increased collagen type X mRNA expression, a marker of hypertrophy. Cells transduced to overexpress either PTHrP isoform inhibited the CTS-induced collagen type X gene expression (Figure 2). However, PTHrP 1-141 augmented CTS-induced NO and PGE2 gene expression and protein production (Figure 3), and neither PTHrP isoform had any significant effect on apoptotic genes (not shown). Results from the present study, thus, suggest that overexpression of PTHrP in chondrocytic cells prevents mechanical stress-induced hypertrophic-like changes and that therapeutic PTHrP gene transfer may be applicable for chondroprotection of newly formed cartilage, including tissue regenerated from the application of adult MSCs. We are continuing to investigate the feasibility of such an approach by designing and constructing inducible and multi-vector gene therapy systems for MSC-based tissue engineering.

Partial Reprogramming of Adult Stem Cells

Objective

Adult stem cells and embryonic stem cells (ESCs) both exhibit desirable tissue regenerative properties. We have applied reprogramming techniques to partially reprogram adult stem cells and to create a stem cell able to heal many tissue types like an embryonic stem cell, but without the risk of tumor formation posed by induced pluripotent stem cells (iPSCs). Recently developed reprogramming techniques alter the transcriptional program of a cell and change its fate from terminal differentiation to induced pluripotency, mimicking the phenotype of an ESC. Partial reprogramming is the process of moving an adult cell along the pathway from limited multipotency toward pluripotency, without returning it to the completely pluripotent state of an ES cell. We aim to test the hypothesis that partial reprogramming of MSCs will extend the longevity of their stemness and expand their potency to accommodate more lineages by altering their transcriptional program. The long-term objectives of this study are: (1) to enhance the functional characteristics of adult stem cells to create a more malleable tool for regenerative medicine and (2) to investigate the molecular basis of these functional changes to provide a knowledge base for further refinement of cell-based regenerative medicine methods.

Methods and Results

We have recently developed a modified gene delivery scheme using a lentiviral vector-based transcription factor cocktail consisting of c-myc, oct4, klf-4, and sox2 for transient, rather than stable, gene transduction. During the current research period, we have been developing techniques for identifying partially reprogrammed stem cells (pRSCs) through clonal assays (Figure 4). Specifically, we have begun to characterize these cells using gene reporters and surface markers to generate a selection profile. In the past, we have profiled MSCs at various passage levels to identify candidate markers associated with MSCs with high and low potencies. We have also been carrying out profiling studies on iPSCs and transduced MSC-pRSC populations. Markers we have tested include: MSC surface markers Stro-1, CD146, CD73, CD90, CD105, and CD44; HSC markers CD34 and CD45; and ESC surface markers SSEA-3, SSEA-4, TRA-1-60, TRA-2-49, and TRA-1-81 (Figure 5). We are also profiling intracellular markers in transduced MSC-pRS populations, including oct4, sox2, c-myc, and nanog (Figure 6). Our preliminary results indicate that pRSCs exhibit the standard complement of MSC surface markers, TRA-1-81, and SSEA-4 at two weeks post-lentiviral transduction. Our results also

indicate that levels of surface marker expression, rather than presence or absence of a particular marker, may be most predictive of pRSC status.

Caveolin-1 and Regulation of Osteogenic Differentiation of Adult Stem Cells

Objective

This project aims to investigate the cellular mechanisms that regulate the guided differentiation of MSCs along specific cellular lineages. Caveolae are a special type of lipid raft, 50–100 nm invaginations of the plasma membrane with a unique composition of proteins and lipids, and are involved in various cell signaling functions. Caveolin-1, a 21 kD protein, is the principal protein component of caveolae and mediates many of the functions of caveolae, including ligand-receptor signaling. We postulate that Caveolin-1 has the potential to affect MSC proliferation and differentiation by interacting with cell signaling proteins at the cell membrane.

Methods

The effect of Caveolin-1 on MSC differentiation was investigated by disrupting Caveolin-1 expression in adult human bone marrow-derived MSCs (male, 2; female, 2) using small interfering RNA (siRNA). After siRNA transfection, MSCs were changed to control growth medium or osteogenic medium, with medium change every three days thereafter. Knock down of Caveolin-1 expression in Caveolin-1 siRNA-treated cells relative to cells transfected with a non-targeting control siRNA was confirmed by real time polymerase chain reaction (RT-PCR) and western blotting. Osteogenic differentiation of MSCs was assessed by measuring alkaline phosphatase (ALP) activity and matrix mineralization using alizarin red staining after 4 and 21 days, respectively, in control and osteogenic cultures.

Results

Preliminary results suggest that knock down of Caveolin-1 expression enhances osteogenesis in bone marrow-derived MSCs of both male and female patients. After four days of growth in osteogenic medium, ALP activity was slightly but significantly increased in Caveolin-1 siRNA transfected cells compared to control siRNA transfected cells in all samples studied. Matrix mineralization was slightly enhanced in Caveolin-1 siRNA transfected cells after 21 days of growth in osteogenic medium. In one sample from a male patient, matrix mineralization was strongly enhanced in Caveolin-1 transfected cells and further enhanced if Wnt3a was added to the osteogenic medium, suggesting that canonical Wnt/β-catenin signaling may be involved in this process. In conclusion, disruption of Caveolin-1 expression may enhance osteogenesis in human MSCs.

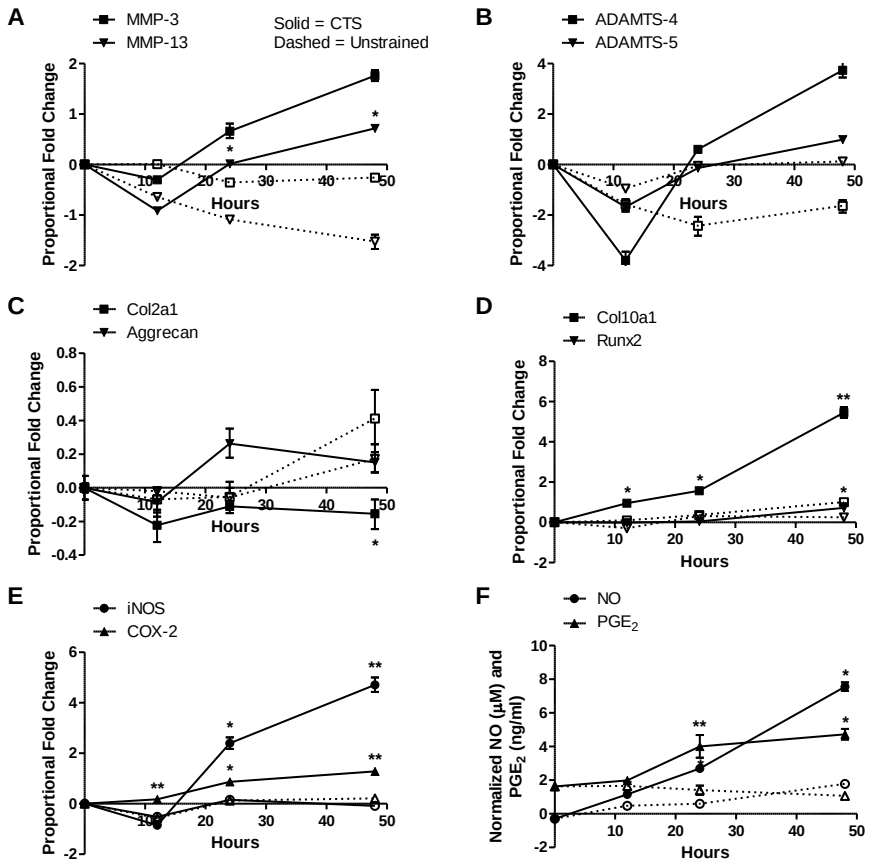

Figure 1. Injurious effects of 16% cyclic tensile strain (CTS) on chondrocytic cells. CTS increased expression of catabolic genes (A) MMPs and (B) ADAMTS, (D) hypertrophic genes, and (E) iNOS and COX-2, and decreased expression of anabolic genes, such as (C) collagen type II (Col2a1). CTS increased secretion of (F) NO and PGE2, which correlates with gene expression profiles of iNOS and COX-2 (C). Solid line = CTS; Dotted line = Unstrained control. * = P<0.05 and ** = P<0.01 versus control.

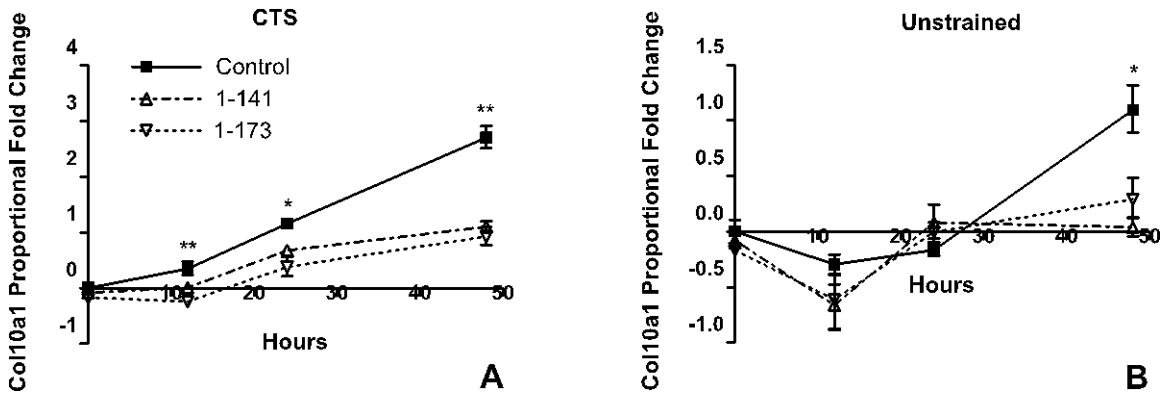

Figure 2. Over-expression of PTHrP inhibits expression of collagen type X (Col10a1) gene expression. Chondrocytic cells expressing PTHrP constructs show suppressed (A) CTS-induced Col10a1 gene expression and (B) Basal Col10a1 gene expression. PTHrP over-expression had no significant effect on matrix remodeling genes. Solid line = CTS; Dotted line = Unstrained control. * = P<0.05 and ** = P<0.01 versus PTHrP 1-141 and 1-173.

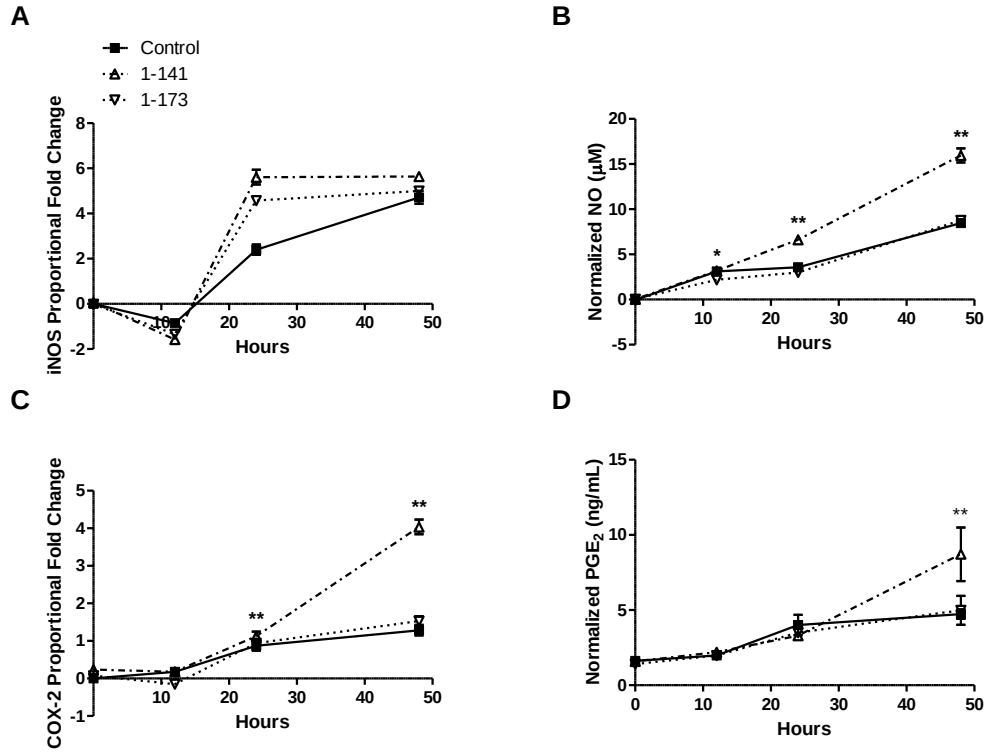

Figure 3. Effects of PTHrP over-expression on CTS-induced iNOS and COX-2 gene expression and NO and PGE_2 production. Chondrocytic cells over-expressing PTHrP 1-141 showed augmented gene expression of (A) iNOS and (C) COX-2, and production of their respective inflammatory mediators, (B) NO and (D) PGE_2. Cells expressing PTHrP 1-173 did not show significantly changed iNOS and COX-2 gene expression or production of NO and PGE_2 when compared to control. Solid line = CTS; Dotted line = Unstrained control. * = P<0.05 and ** = P<0.01 versus control.

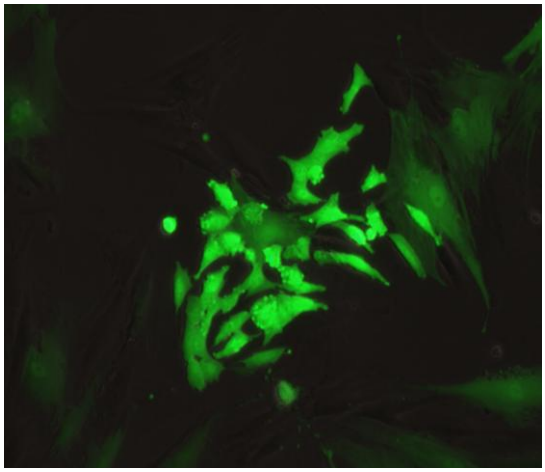

Figure 4. Lentiviral transduction of MSCs with oct4, sox2, nanog, klf4, and c-myc (plus a GFP reporter) results in colonies of partially reprogrammed cells, which assume a smaller phenotype relative to non-reprogrammed MSCs.

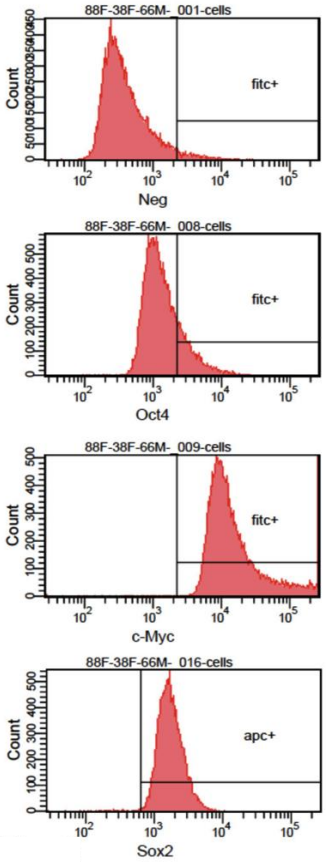

Figure 5. Surface staining of pRS population for MSC, HSC, and ESC markers.

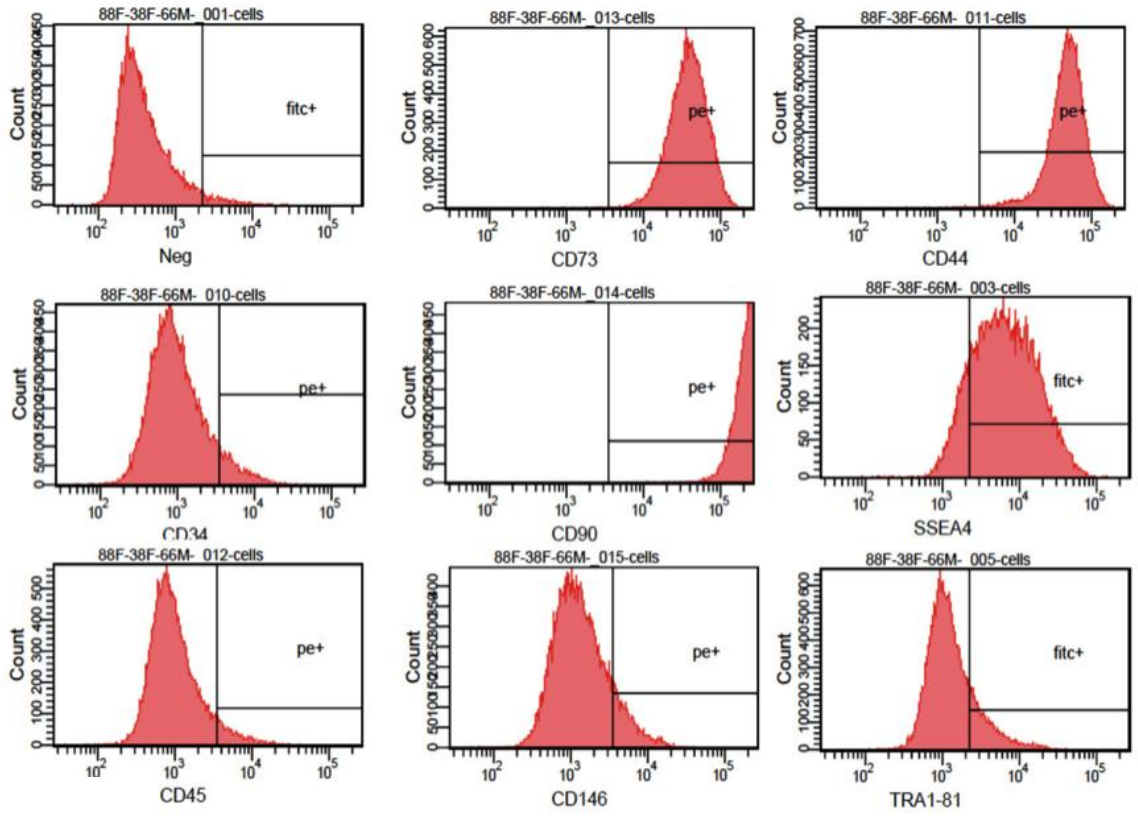

Figure 6. Intracellular staining of pRS populations.

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

Dr. Kensler left his previous position at Johns Hopkins University and joined the University of Pittsburgh this year. His lab relocated to newly renovated laboratory space in the Thomas E. Starzl Biomedical Science Tower on 1 April 2010. Central to this project was the installation of a Thermo TSQ Vantage mass spectrometer with an ion max source and H-ESI-II probe, coupled to an Accela ultra-HPLC system with autosampler and Quick Quan software. Additionally, Li Yang, PhD, a recent graduate of the University of Nebraska with extensive experience in mass spectrometry, was recruited to the laboratory. She arrived on 1 June 2010 and will hold primary responsibility for the proposed biomarker analyses. Laboratory members are now undergoing training by Thermo engineers in the optimal utilization of the instrument. Development of the necessary internal standards for some of the biomarkers is now in progress.

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)

Study 1: Effect of cisplatin on emesis

Musk shrews (a mouse-sized insectivore) are commonly employed to investigate the effects of cancer chemotherapy on vomiting because laboratory rodents lack this response. We have developed a computer algorithm in Matlab (Mathworks; Natick, MA) to automatically track emesis in short- and long-term video files. In our first experiment, we injected 10 male musk shrews with the chemotherapy agent cisplatin using an emetic threshold dose (20 mg/kg, intraperitoneal [ip]) and videotaped their behavior for two hours (h). Emesis was scored independently by two observers, and these results were compared to those generated from the computer algorithm. Half of the shrews developed vomiting and the second half served as non-vomiting controls. For computer detection, the body contour in each video frame was normalized to a parameterized shape template and projected to a feature space maximizing the shape variations in the consecutive frames during retching. These analyses show that musk shrew emetic episodes occur in bouts of ~1 second with retching occurring at a speed of approximately 8 Hz (much faster than reported from an anesthetized preparation (described in Andrews et al., *Eur J Pharmacol* 1996; 307(3)305-13), which indicates that the current approach is a very sensitive method for behavioral detection of emesis (this is the first report of the measurement of individual retches in a free moving musk shrew). The emesis events were detected following three consecutive retches occurring above a detection threshold. This method detected 91% of the emetic episodes detected by the two observers. The accuracy of the algorithm is limited by the amount of data we have collected. As we include more emetic

events, the algorithm, which supported supervised detection, can be trained to be more accurate.

In our most recent study, we injected four male musk shrews with 30 mg/kg cisplatin (ip) and video recorded their behaviors for 24 h prior to injection and 72 h post-injection, an approach similar to a published report designed to capture the acute and delayed emetic effects of cisplatin treatment (Sam et al., *Eur J Pharmacol* 2003; 472(1-2):135-45). Figure 1 shows an example of emesis detection from one of these animals. This figure demonstrates the acute and delayed phases of cisplatin-induced emesis that are reported in animal and human studies. The computer algorithm detected fewer emetic events compared to manual scoring; however, we anticipate that we will be able to train the algorithm to achieve better detection with the additional data that we collect in future experiments.

We are now positioned to apply this automated method to more rapidly complete the current study using saline, cisplatin, cyclophosphamide, and staphyloccus enterotoxin A treatments to induce emesis.

Study 2: Effects of restraint stress on open field activity

Anxiety and distress appear to potentiate nausea and vomiting induced by chemotherapy treatment in cancer patients. To test the interaction between stress and emesis in the musk shrew, we assessed the behavioral responses to restraint stress in an open field test. We predicted that, like rodents, musk shrews with prior restraint would spend less time in the center region of an open field. Musk shrews were restrained in a small aerated cylinder (0, 20, 60, or 120 minutes; n = 8 in each condition) and were then released in the middle of an open field (60 x 60 x 60 centimeters) where their behavior was video recorded for 10 min. Movement detection and analysis were conducted using computer software (Noldus). A center region was specified as 36% of the total central area of the open field, and the following measures were determined automatically: locomotion, time and frequency (in center, immobile, mobile, highly mobile), and velocity. Mobility (a change in body contour) limits were set as ≤ 2% change = immobile, > 2% and < 15% = mobile, > 15% = highly mobile. Mobility is a measure of movement that can potentially occur in one location, e.g., turning or elongation. There were no significant effects of restraint on velocity and total movement (or high mobility). However, animals restrained for 120 min versus animals without prior restraint spent less time in the center region during the last 5 min of the test period and were also more mobile (see Figure 2). Manual scoring indicated that these mobility events correspond to rearing on the side of the chamber. This study suggests that musk shrews, like rodents, respond to restraint stress by decreasing the time spent in the center of the open field and indicates that restraint stress produces a behavioral profile in musk shrews that might be used in studies to determine how stress responses interact with other behavioral effects of chemotherapy, including vomiting and reduced food intake.

Study 3: Effect of restraint stress on cisplatin-induced emesis, feeding, drinking, and activity

This study has not yet been initiated.

Study 4: Transneuronal tracing of pathways from the intestine and stomach

Four shrews and four rats received multiple injections of the H129 strain of herpes simplex virus-1 into the ventral wall of the stomach. Animals were euthanized at 5 days after injection of

virus. Brains were removed and cut at 50 microns and processed for immunohistochemistry using an anti-herpes simplex virus-1 antiserum. We have observed stained cell bodies in the brains of these animals, which indicate that the virus-infected neurons connect the gut to the brain. We now plan to conduct a more extensive study to carefully track the progression of viral infection from the brainstem to the forebrain.

Aim 2 (cancer development and progression)

Study 1: To investigate possible stress-induced differences in angiogenic processes in mouse mammary tissue collected from glands with and without tumor

During this initial phase of the funded research project, we have focused on the logistical aspects of initiating the three proposed studies. In addition to recruiting and training necessary support staff, we have conducted systematic literature reviews to update proposed methodologies in the context of emerging findings from other laboratories. Based on that review process, we have developed panels of specific antibodies selected from different commercial suppliers that can be used together for quantification of immunofluorescent labeling of the biological markers proposed for concurrent assessment in this study using Quantum-dot (Q-dot) technology. We have also received training on the use of the dedicated imaging equipment and computer software that is necessary to collect, transfer, store, and analyze the data. The Nuance system detects and removes autofluorescence from paraffin section preparations and detects and quantifies the amount of emission from up to eight different chromagens in a single section, even when the stains are bound to the same cell. Preliminary studies have been conducted to determine optimal dilutions and staining conditions for the Q-dot approach that we will use in this study.

Initial optimization results are shown in Figures 3 and 4 below. These control images demonstrate reliable staining of one of the proposed panels of antibodies using spleen as a convenient tissue in which monocytes are expected to be present. As seen in Figure 4, monocytes are also clearly evident in mammary tissue from the MMTV-neu mice.

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.

In Study 1, our initial focus was on the development and optimization of panels of specific antibodies selected from different commercial suppliers that can be used together for quantification of immunofluorescent labeling of the biological markers proposed for concurrent assessment using Q-dot technology. Here, the focus is on proinflammatory cytokines and selected related signaling pathways. It is hypothesized that, at the molecular level, the physiological response to chronic stress will result in detectable increases in expression of phosphorylated AKT, nuclear factor (NF)κB, and mitogen-activated protein kinase (MAPK).

Initial optimization results are shown in Figure 5. These control images demonstrate reliable staining of one of the proposed panels of antibodies in mammary tissue from the mouse mammary tumor virus (MMTV)-neu mice.

We have also received training on the use of the dedicated imaging equipment and computer software that is necessary to collect, transfer, store, and analyze the data. Preliminary studies have been conducted to determine optimal dilutions and staining conditions for the Q-dot approach we will use in this study.

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.
In preparing to conduct the research proposed for Study 3, we have conducted a systematic review of the literature to determine current knowledge of stress responsive elements (e.g., beta adrenergic receptors) on widely available human breast cancer cell lines with well characterized genotypes and phenotypes that will permit molecular analyses of treatment effects on the hypothesized pathways. It is hypothesized that stimulation of the beta-adrenergic receptor will have significant effects on breast cancer cell lines at the molecular level, including increased AKT phosphorylation and NFκB, MAPK, and signal transducer and activator of transcription 3 (STAT-3) activation. We are encouraged that functional adrenergic receptors have been documented on several well established breast cancer cell lines in a recent paper that also provided evidence of an association with heightened human epidermal growth factor receptor 2 (HER2) expression in breast tissue samples obtained from breast cancer patients (Shi et al., *Breast Cancer Res Treat* March 2010 [epub ahead of print]) . We now plan to conduct our proposed study in breast cancer cell lines that are documented to bear beta-adrenergic receptors and to be HER2 positive.

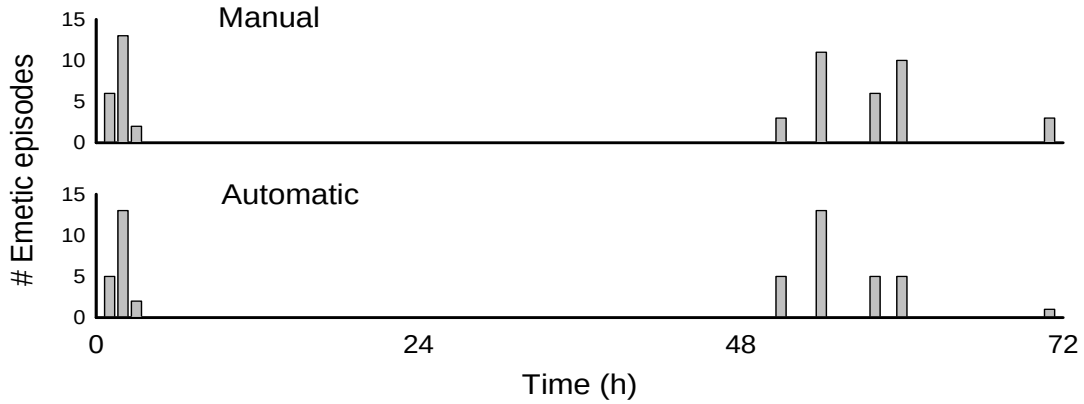

Figure 1. Video analysis of emetic events for 3 days after injection of cisplatin (30 mg/kg, ip). Events were scored manually (above) or automatically using a computer algorithm (below). The acute effects of cisplatin are shown in the first few hours after injection and the delayed effects occur after 48 hours.

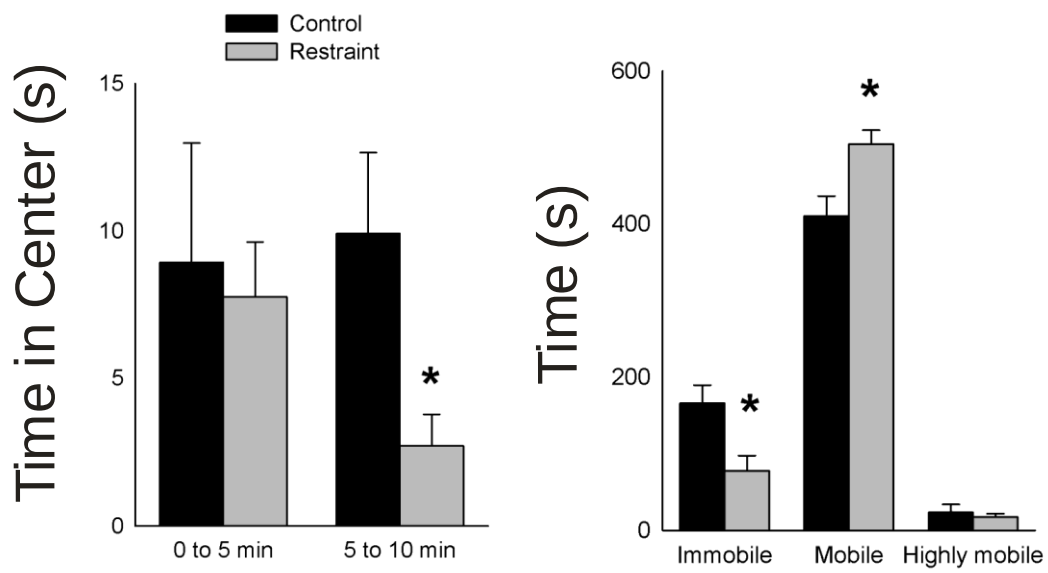

Figure 2: Effects of 120 min of restraint versus no restraint control. *Left:* time in center. *Right:* time as immobile, mobile or highly mobile. * $p < 0.05$, t-test, 2-tailed.

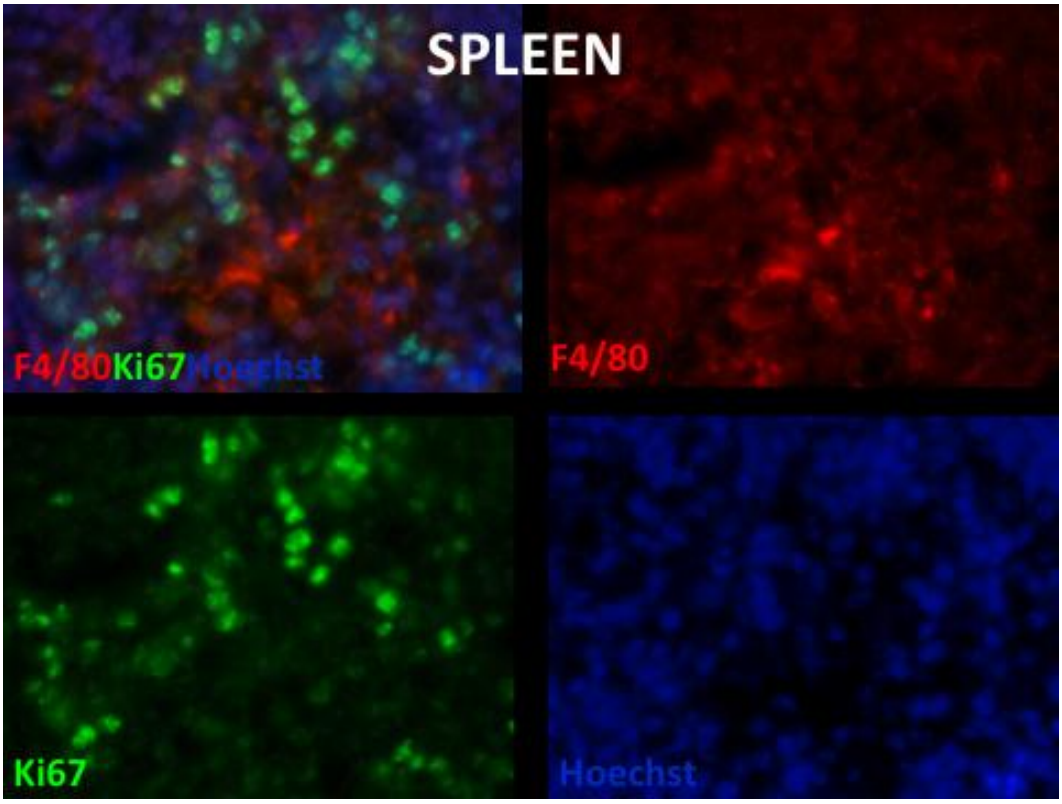

Figure 3: Q-dot Immunofluorescence of Control Spleen

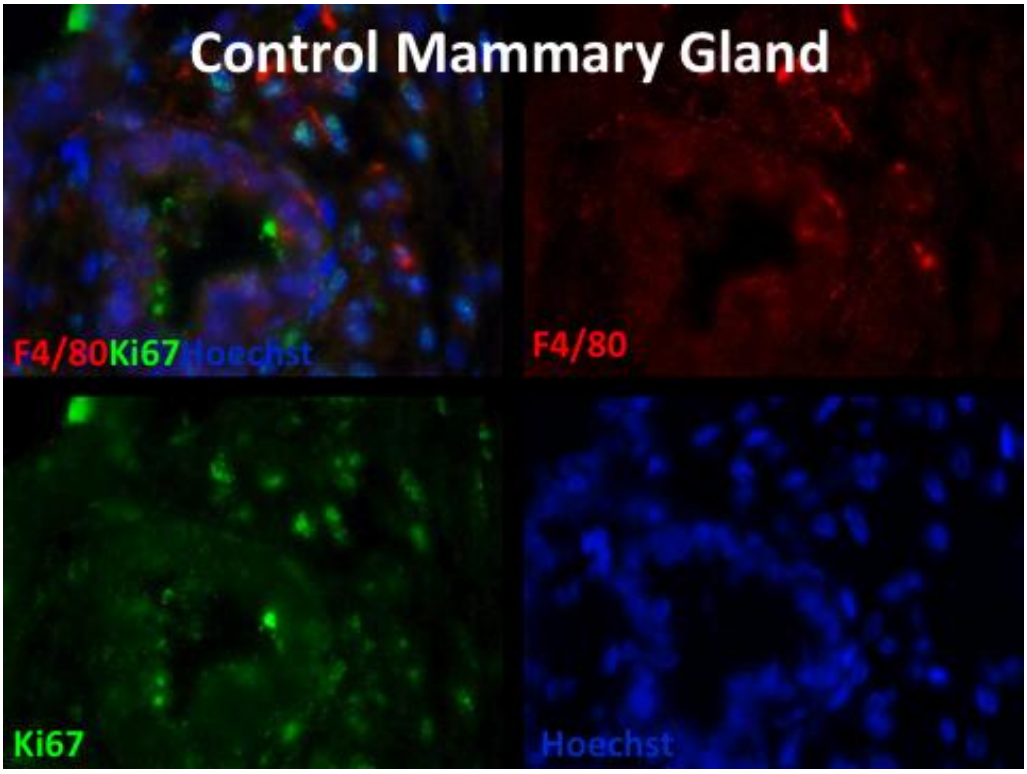

Figure 4: Q-dot Immunofluorescence of Control Mammary Tissue

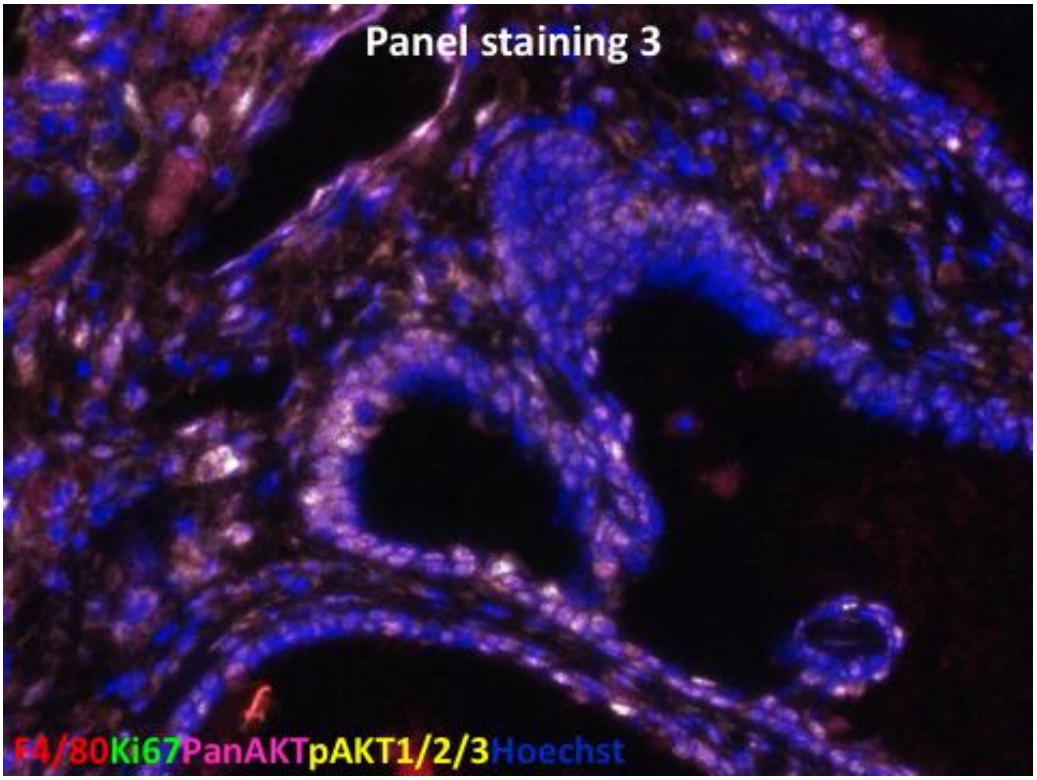

Figure 5: Representative Staining of Mammary Tissue from MMTV-neu Mice.

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

None

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
This study is ongoing. Twenty-eight participants have given informed consent, with 23 eligible to begin the treatment regimen. The maximum tolerated dose (MTD) of Bendamustine has been determined at 75mg/M^2, with enrollment continuing to establish the MTD of each drug for a future phase 2 trial, anti-tumor activity of the drug regimen, toxicity, time to progression, and overall survival.

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. Forty-two participants have given informed consent, with 37 eligible to begin the randomized treatment arm. Eighteen participants have been randomized and treated with Lenalidomide (CC-5013) and dexamethasone with autologous peripheral stem cell transplant (PSCT) [Arm A], and 19 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.

Principal Investigator

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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: This trial has been approved by the Institutional Review Board (IRB), and accrual will begin on 7/1/10.

UPCI 07-015: This trial has accrued 33 of a planned 45 participants, with no unusual or unexpected toxicities found. Accrual is ongoing.

UPCI 08-121: This trial has accrued 10 of a planned 15 participants, with no unusual or unexpected toxicities found. Accrual is ongoing.

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.

Anticipated Duration of Project

1/1/2010 – 6/30/2012

Project Overview

This research infrastructure project is designed to relocate a 9.4 Tesla magnetic resonance (MR) imager. The project requires a number of stages. First, the space in the Biomedical Science Tower 3 (BST3) into which the magnet will be relocated must be prepared. Because the space was designed to house a structural biology laboratory but, to date, has not been occupied, the floor of the space is at bedrock. Therefore, an elevated floor must be constructed to accommodate both the research space and the investigators' access to the space from the main hallway. Construction will also include needed laboratory fit-out.

Once the laboratory space has been prepared, the MR imager will be removed from the McGowan Institute for Regenerative Medicine (MIRM) and transported 2.3 miles to the space in BST3. Upon arrival, special features of the structural biology space in BST3 will assist in getting the magnet into the building. The exterior window at street level was designed so that it can be removed to allow a heavy-duty truck-based crane to lower the magnet into the space. Then, a built-in ceiling-mounted crane in the main magnet space can pick up the MR imager and move it within the space.

Principal Investigator

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

This project involves the relocation of a 9.4 Tesla magnetic resonance imager and will produce several outcomes and benefits. The first portion of the project will create new research laboratory space when an unfinished section of the BST3 basement—which currently also houses a large structural biology laboratory space—undergoes final construction and fit-out. In addition, relocating the MR unit will free up needed research space at the McGowan Institute for

Regenerative Medicine for the co-location of collaborators. Finally, creating a cohesive space for structural biology research will strengthen existing collaborations and encourage new collaborative efforts among researchers in the health sciences.

Summary of Research Completed

Our research infrastructure plans have now changed, and the relocation of the 9.4 Tesla magnetic resonance imager will no longer take place. The project's funds will be reallocated to another infrastructure initiative. We will submit a reallocation request to PA DOH as soon as we determine which infrastructure project best meets our research objectives and the goals of the CURE program.