Thomas Jefferson University

Annual Progress Report: 2010 Formula Grant

Reporting Period

January 1, 2011 – June 30, 2011

Formula Grant Overview

Thomas Jefferson University received $3,085,950 in formula funds for the grant award period January 1, 2011 through December 31, 2014. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Network-Directed Therapy of Prostate Cancer - Although considerable progress has been made in the understanding of cancer genes, only a handful of molecular therapies have become standard of care for cancer patients in the last thirty years. The management of prostate cancer remains challenging, and confusion still exists regarding the molecular networks that drive the disease, the reliability of biomarkers, and the appropriateness of treatment options. The present application will address these problems taking an innovative approach. This project will focus on a single gene network, the survivin pathway, which plays a critical role in prostate cancer. This will involve analysis of tumor initiation and metastasis, *in vitro* and *in vivo*, supported by mechanistic studies on receptor ligation, gene expression, and intracellular signaling pathways.

Anticipated Duration of Project

1/1/2011 - 12/31/2012

Project Overview

In addition to its essential roles in the regulation of mitosis and inhibition of apoptosis in tumors, we recently found that survivin acts as a novel metastasis-promoting gene, *in vitro* and *in vivo*. This involves a complex mechanistic pathway in which survivin expression in tumor cells results in a broad gene expression program, with upregulation of a distinct gene signature comprising various adhesion molecules, most notably fibronectin. Accordingly, survivin-expressing cells exhibited increased cell migration and invasion *in vitro* and in various xenograft and transgenic mouse models, *in vivo*. Initial dissection of the molecular requirements of survivin-directed tumor cell invasion suggests a primary role of NFκB-dependent transcription, which has been implicated in *fibronectin* gene expression. Therefore, *a unifying hypothesis can be formulated that survivin promotes metastasis, in vivo, via upregulation of gene expression, and potentially independently of its functions in the control of mitosis and inhibition of apoptosis*.

This pathway may be particularly important for prostate cancer progression, where survivin has

been recognized as a marker of aggressive disease, and a validated target for novel molecular therapies. This project will integrate basic and translational studies to define the role of survivin as a metastasis-promoting gene in prostate cancer. Overall, this project will provide a comprehensive and mechanistic map of a new pathway of tumor cell invasion, and will provide a broad molecular foundation for the late-preclinical and clinical studies of targeting the survivin networks that constitute the focus of the present application. The specific aims are as follows: (AIM 1) To characterize the cellular requirements of survivin-directed tumor cell invasion. (AIM 2) To elucidate the mechanism of survivin-directed gene expression. (AIM 3) To dissect the role of survivin as a metastasis-promoting gene, *in vivo*.

Principal Investigator

Lucia R. Languino, PhD
Professor
Thomas Jefferson University
223 South 10th Street, BLBS 506
Philadelphia, PA 19107

Other Participating Researchers

None

Expected Research Outcomes and Benefits

This laboratory has uncovered a novel pathway of prostate cancer progression centered on the signaling properties of survivin and $\alpha_v\beta_6$ integrin. Therapeutic targeting of this pathway using molecular and genetic disease models that approximate the human disease, combined with mechanistic dissection of its molecular requirements, may open concrete new prospects for the rational treatment of patients with advanced and metastatic prostate cancer.

Summary of Research Completed

Our project focuses on the analysis of a novel pathway, which is likely to play a crucial role in prostate cancer metastasis. This pathway is mediated by the $\alpha_v\beta_6$ integrin, a cell adhesion receptor, and survivin, an inhibitor of apoptosis.

We recently found that survivin acts as a novel metastasis-promoting gene, *in vitro* and *in vivo*, in addition to its essential roles in the regulation of mitosis and inhibition of apoptosis in cancer cells. Survivin's ability to act as a novel metastasis-promoting gene, *in vitro* and *in vivo* involves a complex mechanistic pathway whereby its expression in tumor cells results in a broad gene expression program, with upregulation of a distinct gene signature comprising various adhesion molecules, most notably fibronectin.

During this reporting period we have continued to characterize the pathway described above and have obtained the following results.

a) Since the $\alpha_v\beta_6$ integrin and the $\alpha_v\beta_3$ integrin are both up-regulated in prostate cancer, we transfected the $\alpha_v\beta_6$ integrin or the $\alpha_v\beta_3$ integrin in LNCaP prostate cancer cells. Survivin regulation by the $\alpha_v\beta_6$ integrin in prostate cancer cells was analyzed at the protein levels by immunoblotting analysis. The results show that $\alpha_v\beta_6$ integrin regulates survivin at the protein levels and that the effect is specific since the $\alpha_v\beta_3$ integrin does not increase survivin levels. We also found that the $\alpha_v\beta_6$ integrin promotes expression of survivin, but not of bcl-2 or XIAP, which are also inhibitors of apoptosis. Although the mechanism of regulation of survivin may occur at the transcriptional, post-transcriptional, translational or post-translational level, the observed increase in survivin is likely to be a reflection of a change in RNA levels since this molecule is known to be predominantly regulated at the RNA level.

Methods: 1) Cells*:* LNCaP cells were from ATCC. LNCaP stable cell lines expressing full length β_3, β_6 or empty vector have been described previously by our group in "Manes, T., Zheng, D.Q., Tognin, S., Woodard, A.S., Marchisio, P.C., and Languino, L.R. ‹v®3 integrin expression up-regulates cdc2, which modulates cell migration. *J Cell Biol.* 2003; 161:817-826." 2) The immunoblotting analysis was performed as described by our group in "Goel HL, Fornaro M, Moro L, Teider N, Rhim JS, King M, Languino LR. Selective modulation of type 1 insulin-like growth factor receptor signaling and functions by β_1 integrins. *J Cell Biol.* 2004;166:407-418."

b) We then tested whether $\alpha_v\beta_6$ integrin increases levels of survivin expression through modification of cell morphology (Figure 1). The results show that $\alpha_v\beta_6$ integrin expression does not affect cell morphology as shown in Fig 1 A, or expression of other integrin subtypes as shown in Fig 1 B.

Methods: 1) Cellular morphology was visualized using an Olympus IX71 inverted microscope with IPLab V3.55 (Scanalytics, Inc, Rockville, MD). 2) FACS analysis was performed to determine surface expression of integrins using Abs specific to β_1 (TS2/16), to β_5 (P1F6), to β_6 (10D5), and to α_v (VNR147) on cell surface. Abs including 12CA5, IC10 or non-immune mug were used as negative controls.

c) Given the known ability of $\alpha_v\beta_6$ integrin to mediate cellular response to cytokines and growth factors, we tested the response of prostate cancer cells to TGF-beta, a pro-metastatic cytokine. Using two different prostate cancer cell lines, PC3-1 and PC3-2, we show that TGF-beta significantly up-regulates the $\alpha_v\beta_6$ integrin although only one cell line, PC3-1, responded to this stimulation (Figure 2). This result is consistent with the observation that PC3-1 cells are more aggressive and more metastatic than PC3-2 cells. Given the observations described above that the $\alpha_v\beta_6$ integrin controls survivin levels and is upregulated by TGF-beta, and the previous knowledge that TGF-beta is a pro-metastatic cytokine, we concluded that this pathway is likely to be important for the metastatic process of prostate cancer cells.

In conclusion, based on the data generated during this funding period, we hypothesize that the TGF-beta/ $\alpha_v\beta_6$ integrin/survivin pathway controls fibronectin levels and promotes metastasis.

Manuscripts related to the findings described above are in progress.

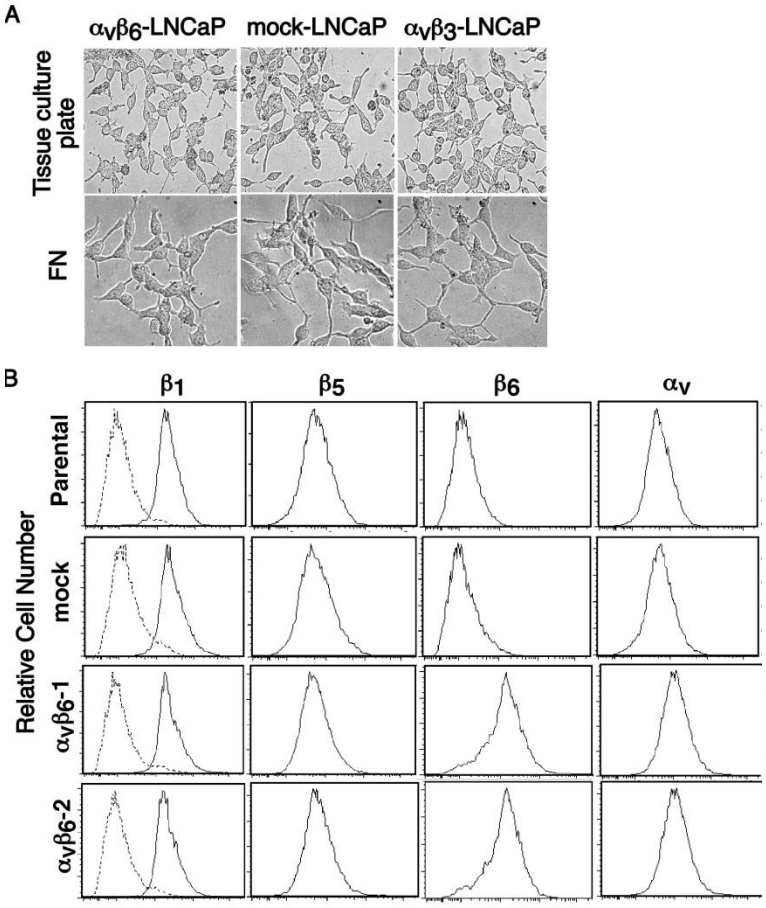

FIGURE 1. **$\alpha_v\beta_6$ integrin expression does not affect cell morphology or expression of other integrin subtypes.** (A) $\alpha_v\beta_6$-LNCaP, mock-LNCaP and $\alpha_v\beta_3$-LNCaP cells were cultured in either culture plates or FN (5 mg/ml)-coated culture plates in culture medium as mentioned in Supplemental Experimental Procedures. After 72 hr, cellular morphology was visualized using an Olympus IX71 inverted microscope with IPLab V3.55 (Scanalytics, Inc, Rockville, MD). (B) Integrin expression in parental-LNCaP, mock-LNCaP and $\alpha_v\beta_6$-LNCaP (clone $\alpha_v\beta_6$-1 and $\alpha_v\beta_6$-2) cells was analyzed by FACS using specific Abs (solid line) to the β_1, β_5, β_6, α_v integrin subunits. The Abs specific to integrin subtypes were TS2/16 to β_1, P1F6 to β_5, 10D5 to β_6, and VNR147 to α_v. Dotted-lines show Ab isotype controls.

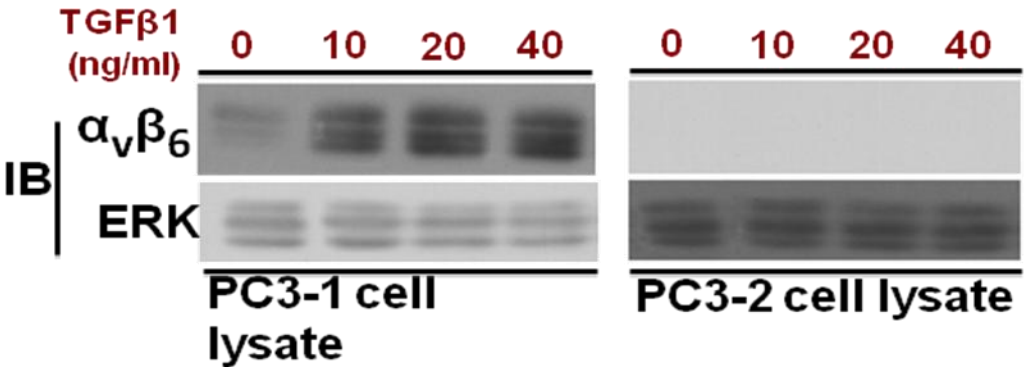

FIGURE 2. **$\alpha_v\beta_6$ is differentially expressed in PC3-1 or PC3-2 cells.** PC3-1 or PC3-2 cells are cultured in 10% FBS RPMI1640 medium, starved in serum-free culture medium for 24 hr before being incubated with TGFβ1 for 48 hr. Serum-free cultures were used as control. Cell lysates were analyzed by immunoblotting using 2A1 mAb to $\alpha_v\beta_6$. pAb (C18) to ERK was used as a loading control.

Research Project 2: Project Title and Purpose

Cell Cycle Regulation in Prostate Cancer - In the United States, prostate cancer (PCa) is the most frequently diagnosed malignancy, and the second leading cause of cancer death, in men. The morbidity associated with this tumor type is attributed to ineffective means in combating advanced disease. Local PCa can be definitively treated by radical prostatectomy or radiation therapies. However, disseminated PCa is largely resistant to standard cytotoxic chemotherapeutics. The androgen receptor (AR) is the therapeutic target of disseminated disease. As such, first line therapies for disseminated disease are centered on the addiction of this tumor type to androgen. This project will utilize the combined knowledge of AR and cell-cycle based cytotoxic action, to design strategies for maximal PCa cell death.

Anticipated Duration of Project

1/1/2011 - 12/31/2014

Project Overview

This project builds on previous data, which show that AR activity can be effectively manipulated to sensitize cells to taxane-based cell death. The data demonstrate that the ability of AR to induce G1 progression is critical for selected cell-cycle based cytotoxic interventions. This concept will be challenged in this project, which will utilize the combined knowledge of AR and cell-cycle based cytotoxic action, to design strategies for maximal PCa cell death.

Goal: Delineate mechanisms to enhance PCa cell death through coordinated manipulation of taxane sequence, AR activity, and p53 status.

Aim 1: Determine the impact of taxanes during intermittent hormone therapy.

Aim 2: Determine the p53 requirement for androgen-enhanced taxane cytotoxicity.

Objective: These principles will challenge the impact of sequence and timing on taxane-based action during intermittent hormone therapy and the contribution of p53 status to this response.

Principal Investigator

Karen Knudsen, PhD
Associate Professor
Thomas Jefferson University
233 S. 10th Street
Bluemle 1040
Philadelphia, PA 19107

Other Participating Researchers

None

Expected Research Outcomes and Benefits

This laboratory has shown that the G1-S progression function of AR sensitizes PCa cells to the cytotoxic effects of taxanes. This suggests that this function of AR could be manipulated during intermittent hormone therapy, to enhance therapeutic response and prolong or prevent tumor progression. This project addresses this innovative hypothesis directly, by dissecting the optimal cycling sequence of taxanes and AR ablative strategies using model systems reflective of naïve tumors. This research will distinguish the responses of wild-type and mutant AR. Moreover, the contribution of p53 status to this process will be determined, as taxane-induced p53 tumor suppressor activation is enhanced by active AR. Thus, the project will determine which cohorts of patients (dependent on p53 and AR status within the tumor) may most benefit from combinatorial therapy. Lastly, the ability of pharmacological agents that activate endogenous p53 to replace or enhance the ability of AR to sensitize cells to taxanes will be determined, thus potentially identifying a novel mode of adjuvant intervention with the taxanes. Project outcomes have the capacity to markedly alter/improve therapeutic strategies to treat the most deadly forms of PCa before the stage of hormone resistance is reached.

Summary of Research Completed

<u>Aim 1 Progress</u>
<u>Goal:</u> Determine the impact of taxanes during intermittent hormone therapy.

We have had strong progress for discerning mechanisms to enhance the response to taxanes. As shown in Figure 1, use of PARP1 inhibitor dramatically increased the response to taxanes. Unexpectedly, the PARP1 inhibitor was observed in these studies to have a single agent effect on prostate cancer cell growth as well. Thus, the impact of PARP1 is currently being investigated, so as to more fully understand the underpinning means by which PARP1 inhibitors sensitize prostate cancer cells to taxanes. Early studies to date suggest that PARP1 influenced androgen receptor (AR) activity. Specifically, new data generated as part of this aim demonstrate that AR activity is suppressed in the presence of a PARP1 inhibitor (Figure 2).

Aim 2 progress

Goal: Determine the p53 requirement for androgen-enhanced taxane cytotoxicity.

Progress. We have just begun these studies. As the overarching goal is to discern the impact of p53 status on therapeutic response, we initiated studies to assess the kinetics of p53 activation after exposure to ionizing radiation. These studies were key, as radiation is given to patients with locally advanced disease as a standard of care. Patients progressing after treatment of locally advanced disease will be given taxane therapy – thus, it is imperative to discern the impact of tumor suppressor status (including RB and p53) on the response to both taxanes and ionizing radiation. As shown in Figure 3, p53 activation occurs with rapid kinetics after exposure to therapy, and results in upregulation of the cyclin-dependent kinase inhibitor p21cip1. These results are striking, as p21cip1 and resultant alterations in cell cycle position have significant effects on the cellular response to taxanes.

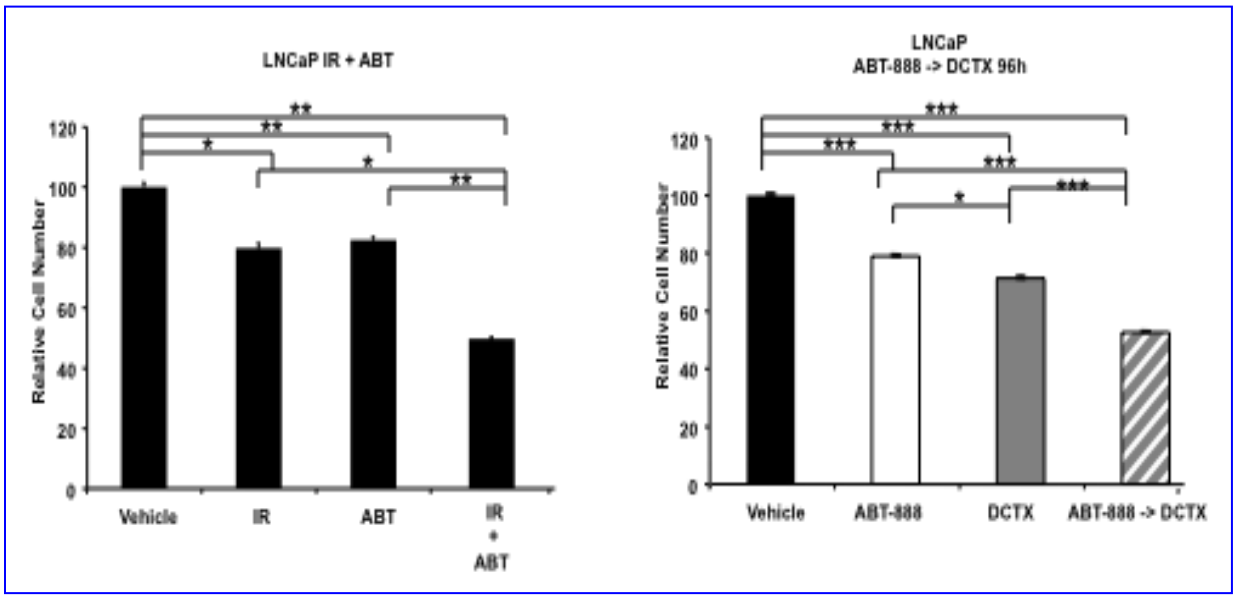

Figure 1. PARP1 inhibitors sensitize prostate cancer cells to both ionizing radiation, IR (left panel) and the taxane docetaxel, DCTX (right panel). Cell number 96h after exposure to indicated treatment in the LNCaP cell lines. ABT-888 (2.5uM), IR (ionizing radiation, 2Gy), ABT-888+IR (2.5uM + 2Gy), DCTX (1nM docetaxel), and ABT-888+DCTX (2.5uM+1nM docetaxel). n=3 independent experiments in triplicate, with vehicle control set to 100.

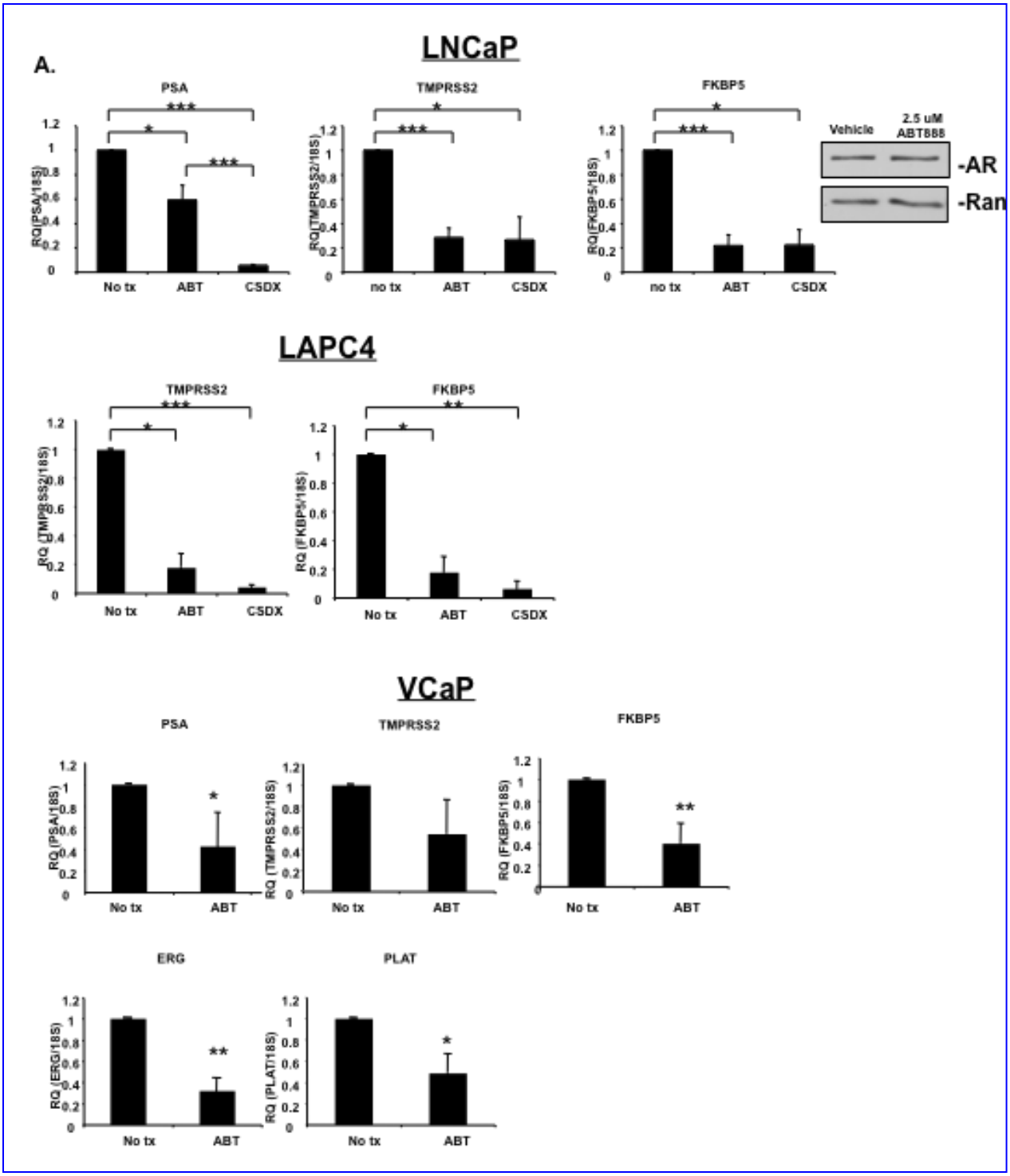

Figure 2. PARP1 functions to suppress AR signaling. Cells were seeded in steroid-proficient media, treated for 24h with either ethanol control (0.01%), ABT-888 (2.5uM), or Casodex (1uM). Cells were harvested, and qPCR analysis for indicated AR target genes was performed. n=3 independent experiments in triplicate. Results normalized to 18S, and represented as means±SE. Experiments were peformed in LNcaP (panel A), LAPC4 (panel B), and VCaP (panel C) cells.

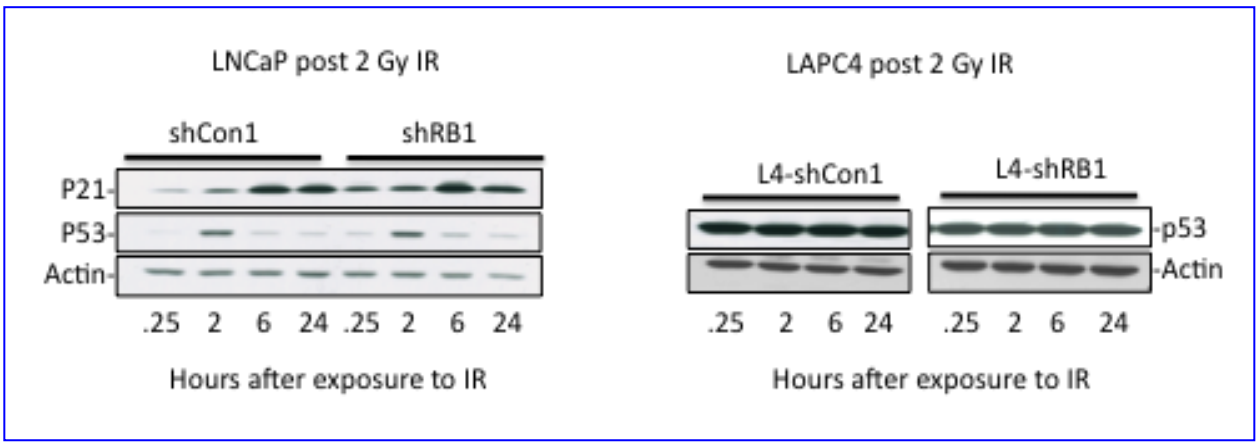

Figure 3. Ionizing radiation elicits induction of p21 that is dependent on active p53 status in prostate cancer cells. Prostate cancer cells with wild-type p53 (left) or mutant p53 (righ) were treated with 2Gy ionizing radiation. Subsequently, cells were harvested and monitored for p53 and p21 expression. As expected, p53 activity is enhanced in cells proficient for this tumor suppressor (manifest as transient accumulation of the protein). No such accumulation was observed in LAPC4 cells, and p53 activation was indistinguishable in cells proficient for the RB tumor suppressor (shCon1) vs. those with RB depleted (shRB1). Importantly, p53 activation resulted in sustained upregulation of the cyclin dependent kinase inhibitor p21.

Research Project 3: Project Title and Purpose

Increasing Colorectal Cancer (CRC) Screening in Primary Care among African Americans - Determine the impact of behavioral interventions on CRC screening, and the use of CRC screening tests among African American patients, in primary care practices 12 months after enrollment in the study.

Anticipated Duration of Project

1/1/2011 - 6/30/2013

Project Overview

This project will build on a current trial being conducted in primary care practices affiliated with Thomas Jefferson University (TJU) and the Albert Einstein Health Network (AEHN) in Philadelphia. A randomized, controlled study of behavioral interventions, intended to boost colorectal cancer (CRC) screening, assesses the status of patients six months after randomization. We will extend the screening observation period after random assignment from 6 to 12 months per patient. The additional time will allow for the complete ascertainment of CRC screening use and specific screening procedures (e.g., colonoscopy screening). In this project, we will also develop and pilot test new patient recruitment procedures. Patients will be able to register in their primary care practices to be contacted by study personnel, relative to enrollment. Furthermore, we will audio record patient navigation telephone calls for analysis. The analyses will seek to identify barriers to and facilitators of successful navigation. Focus groups will be

conducted with study participants who screened (one group for women and one group for men), and those who did not screen (one group for women and one group for men), within the 12-month observation period. Two additional focus groups (one with Hispanic men and one with Hispanic women who are patients in AEHN practices) will also be conducted. These focus groups will be conducted to learn what factors influenced response to intervention. Patient-related screening costs, and information on insurer payments related to increases in CRC screening rates will be analyzed. Finally, we will translate intervention materials into Spanish to broaden the reach of intervention contacts. Results of these analyses will support the design of an enhanced CRC screening intervention system.

Principal Investigator

Ronald E. Myers, PhD
Professor and Director
Thomas Jefferson University
Department of Medical Oncology
Division of Population Science
1015 Walnut Street, Suite 1014
Philadelphia, PA 19107

Other Participating Researchers

Randa Sifri, MD – employed by Thomas Jefferson University

Expected Research Outcomes and Benefits

The project will provide measures of CRC screening, and the use of specific screening tests, within 12 months after intervention. Additional analyses will be performed to determine important mediators and moderators of CRC screening among study participants; develop effective patient recruitment strategies, and identify barriers to and facilitators of response to CRC screening interventions in population subgroups. Findings from these analyses, along with results of patient-specific cost analyses will help to inform the development of an improved CRC screening navigation system for use in primary care. The project will also result in the production of CRC screening intervention materials for use with Hispanic patients.

Summary of Research Completed

The purpose of this project *is to determine the impact of behavioral interventions* on CRC screening and the use of different CRC screening tests among African American patients in primary care practices *12 months after enrollment in the study*.

<u>Recruitment</u>
Participation in the 12-month chart audit and the focus group portion of this study is contingent upon participation in the American Cancer Society-funded randomized trial.

Accrual
As of July 1, 2011, a total of 871 patients were eligible for a 12-month chart audit.

Outcomes
A 12-month endpoint chart audit has been completed for 289 participants.

We have also designed a new approach to patient recruitment that involves in-office recruitment. Here, a brief slide presentation will be shown on waiting room televisions that are part of the AEHN Healthy Advice Channel. This presentation will briefly describe the study and will direct interested patients to the reception desk. Patients will be given an educational booklet and a registration card to be completed and returned to project personnel. A study research assistant will then contact respondents to assess eligibility, obtain consent for participation, and administer the baseline survey. An amendment to initiate this process has been submitted to IRBs at TJU and AEHN. We have established a professional services agreement (PSA) with a professional focus group company. Project personnel are working with the company to develop a focus group discussion guide. We are also working with AEHN on the plans to recruit focus group participants.

To date, we have audiorecorded a total of 27 patient navigation sessions for use in data analysis. Finally, we have translated a CRC screening informational booklet into Spanish.

Research Project 4: Project Title and Purpose

Targeting Endoplasmic Reticulum Stress (ERS) Signaling for Overcoming Lung Cancer Resistance - Lung cancer patients die as a consequence of therapeutic resistance and treatment failure. One of the important features of cancer resistance is the inability to undergo apoptosis. Ongoing research from this laboratory supports the idea of inducing ER stress-mediated autophagy as a way of combating resistant lung cancer cells. Therefore we will investigate the underlying mechanisms and preclinical concepts of using ER stress activators for enhancing radiotherapy in treating lung cancer.

Anticipated Duration of Project

1/1/2011 - 12/31/2014

Project Overview

Tumor cells, as a result of transformation or tumor microenvironment, have dysregulated signaling pathways that control cell survival or death. This enables the cells to evade programmed cell death (apoptosis) following treatment with cancer therapies. In every tumor, there is a population of cells that harbors multiple genetic defects in a major form of apoptosis. These cells are enriched following cytotoxic chemotherapy such as cisplatin and become a source of failure due to therapeutic resistance. This laboratory has identified an alternative death program, autophagy (self-eating), which can be triggered in cells that are defective in apoptotic signaling and that overcome tumor resistance to ionizing radiation (IR). The translational potential of this concept uses model systems of several chemical agents that increase IR-induced

cell death, even in the presence of defective apoptotic machinery. In these systems, the radio-sensitizing mechanism is dependent upon autophagy mediated by PERK-dependent endoplasmic reticulum stress (ERS) signaling. This project will extend this work using *in vitro* and *in vivo* cancer models, and lung cancer cell clones that are resistant to cisplatin and IR.

The hypothesis is that in tumor cells lacking functionally intact apoptosis, ERS regulates the IR response through autophagic pathways. To validate this theory, both cancer cells with known apoptotic gene defects and cancer cells fully resistant to cisplatin will be used to determine the key molecules that regulate ERS and autophagy signaling. The following four aims are proposed:

(1) Determine if the regulation of ERS is a significant molecular target of the cellular damage response that occurs during IR-induced autophagy;
(2) Determine the mechanism by which caspases 3/7 regulate ERS signaling in the IR response;
(3) Determine whether ERS inducers increase IR sensitivity in *in vitro* cancer models;
(4) Determine whether ERS inducers increase IR sensitivity in *in vivo* cancer models.

Principal Investigator

Adam P. Dicker, MD, PhD
Professor and Chair
111 South 11th Street
Department of Radiation Oncology
PhiladelphiaPA19107
Thomas Jefferson University

Other Participating Researchers

Bo Lu, MD, PhD – employed by Thomas Jefferson University

Expected Research Outcomes and Benefits

Cancer mortality is due to ineffective cancer therapies. Lung cancer alone is responsible for more deaths than most other cancers combined and remains a huge challenge to the general public, treating physicians and our health care system. While prevention and early detection are important, the vast majority of lung cancer patients will present with advanced, incurable disease requiring systemic medical therapy and local regional radiotherapy. The unfortunate fact is that most of these treatments will fail, presumably owing to the intrinsic resistance of tumor cells to apoptotic cell death. This project will *to determine whether ERS signaling is regulated by molecules that orchestrate apoptotic cell death, and whether ERS can be targeted to induce alternative programmed cell death, i.e., autophagic cell death*. This concept will be tested in cisplatin-resistant lung cancer cells, which, owing to their extreme resistance to apoptosis, are the potential source of recurrence of locally advanced lung cancer after treatment with chemoradiation. The hope is that this research strategy will eventually improve the outcomes of lung cancer patients.

The overarching theme of this research is that, when apoptosis is dysfunctional, IR–induced cell

death is mediated by PERK-dependent ERS signaling. This project will test the novel idea that downstream pro-apoptotic molecules (caspases) regulate ERS signaling, triggering an alternative cell death program (autophagy). The feasibility of targeting ERS will be tested to sensitize lung cancer cells that are resistant to cisplatin and IR. Using these novel lung cancer cell models, we will identify the associated molecular biomarkers and define methods to overcome therapy resistance.

Summary of Research Completed

We have established and characterized lung cancer cell lines that are resistant to therapy and apoptosis during the reporting period. Results from the study are accepted for publication and summarized here:

Purpose: The development of drug-resistant phenotypes has been a major obstacle to Cisplatin (CDDP) use in non-small cell lung cancer (NSCLC). We aimed to identify some of the molecular mechanisms that underlie CDDP resistance by using microarray expression analysis.

Methods and Materials**:** H460 cells were treated with cisplatin. Differences between cisplatin-resistant lung cancer cells (CDDP-R) and parental H460 cells were studied using Western blot, MTS and clongenic assays, *in vivo* tumor implantation, and microarray analysis. CDDP-R cells were treated with human recombinant insulin-like growth factor binding protein-3 (IGFBP-3) and siRNA targeting insulin-like growth factor-1 receptor (IGF-1R).

Results: CDDP-R cells illustrated greater expression of the markers CD133 and ALDH, more rapid *in vivo* tumor growth, more resistance to cisplatin- and etoposide-induced apoptosis, and greater survival after treatment with cisplatin or radiation than the parental H460 cells. Also, CDDP-R demonstrated decreased expression of IGFBP-3 and increased activation of IGF-1R signaling as compared to parental H460 cells in the presence of IGF-1. Human recombinant IGFBP-3 reversed cisplatin resistance in CDDP-R cells, and targeting of IGF-1R using siRNA resulted in sensitization of CDDP-R-cells to cisplatin and radiation.

Conclusions: The IGF-1 signaling pathway contributes to CDDP-R resistance to cisplatin and radiation. Thus, this pathway represents a potential target for improved lung cancer response to treatment.

These cell models will be used for the proposed aims.

Publication

Yunguang Sun, Siyuan Zheng, Christina Speirs, Zhongming Zhao and Bo Lu. Gene profiling of lung cancer stem-like cells reveals IGF pathway as a promising target for therapeutic resistance. *Int J Radiat Oncol Biol Phys.*

Research Project 5: Project Title and Purpose

New Stem/Progenitor Cell-Related Biomarker(s) for Predicting DCIS Prognosis and

Progression - Ductal Carcinoma *In Situ* (DCIS) is characterized by a clonal proliferation of malignant epithelial cells confined within the lumens of mammary ducts, without invasion into adjacent stroma. Since DCIS represents a group of biologically and morphologically heterogeneous diseases, its classification and management remains very challenging. Breast conserving therapy with or without radiotherapy is the accepted treatment for most cases of DCIS. However, the local recurrence rate with such therapy ranges from 10 to 40% with half of these patients developing invasive carcinoma. Previous studies have attempted to identify prognostic molecular markers. Many of the molecules thought to play critical roles in progression of invasive breast cancer have not been shown to be independent prognostic markers for local recurrence or progression of DCIS. Therefore, there is a critical need to identify novel predictors of DCIS progression and potential targets for therapy.

Anticipated Duration of Project

1/1/2011 - 12/31/2012

Project Overview

Breast cancer is a serious health threat and a common cause of death and disability. The understanding of this cancer is incomplete; however, certain abnormalities of the breast can be identified, such as ductal carcinoma *in situ* (DCIS), that commonly lead to invasive cancer. Not all of these early lesions lead to invasive breast cancer and this is a major source of concern for patients. Currently, women with these lesions typically receive toxic therapies with potentially serious side-effects, although many may not need or benefit from this treatment. In this study, a promising molecular marker of these early lesions will be examined, namely SPRR1A, that may predict which patients in this early stage of DCIS, will go on to develop invasive breast cancer.

SPRR1A is a novel stem/progenitor cell marker that has not yet been implicated in the pathogenesis of human breast cancer. Our unpublished preliminary results directly show that SPRR1A is upregulated during the transition from pre-malignancy (hyperplasia and DCIS) to invasive ductal carcinoma (IDC).

The two <u>Specific Aims</u> of the project are:

1) <u>Examine the Role of SPRR1A in the Human Breast Cancer Onset and Progression, from Pre-Malignancy and DCIS to Invasive Ductal Carcinoma.</u> The expression of SPRR1A in human pathology specimens will be examined, and its expression will be correlated with other biomarkers (ER, PR, HER2), patient progression, survival, and recurrence data. The possible use of SPRR1A as a diagnostic predictive biomarker for breast cancer progression from pre-malignancy will be assessed.

2) <u>Determine the Functional Role of SPRR1A in Human Breast Cancer, using Xenograft Models.</u> Our unpublished preliminary results show that SPRR1A is highly expressed in malignant breast cancer cell lines, such as MCF7 cells, but fails to be expressed in "normal" immortalized cell lines, such as MCF10A cells. Thus, we will determine if over-expression of SPRR1A is sufficient to allow MCF10A cells to form tumors in nude mice. Conversely, it will

be determined whether the expression of SPRR1A siRNA can prevent MCF7 tumor formation in nude mice.

Principal Investigator

Michael P Lisanti, MD, PhD
Professor and Chair
Thomas Jefferson University
233 S 10th street BLSB Rm939
Philadelphia, PA 19107

Other Participating Researchers

Philippe G Frank, PhD – employed by Thomas Jefferson University

Expected Research Outcomes and Benefits

Advanced medical technologies and therapeutic strategies are necessary for the successful detection, diagnosis, and treatment of breast cancer. This project will use novel technologies (tissue microarrays (TMA) and automated quantitative bioimaging (AQUA)) to identify new therapeutic and prognostic markers for human breast cancer. More specifically, the activation status of a new signaling pathway will be studied, which has been implicated in breast cancer pathogenesis, using both mouse animal models and cells in culture. For this purpose, the association of SPRR1A expression with pre-malignant lesions and progression from pre-malignancy to full-blown breast cancer will be studied.

Summary of Research Completed

Previous studies have shown that the small proline-rich repeat protein 1A (SPRR1A) is highly expressed in stem cell-enriched structures, the terminal end buds and Cav-1 null mammary lesions. However, the implication of SPRR1A in cancer "stemness" and in breast cancer malignancy remains unknown. To address this issue, we first evaluated the expression of SPRR1A in the mammary glands from MMTV-Wnt-1 transgenic mice, which exhibit a remarkable expansion of the mammary stem cell compartment. We found that SPRR1A is upregulated in pre-neoplastic mammary lesions and in mammary tumors of mammary glands from Wnt-1 Tg as compared to mammary glands from wild-type, INK4 KO and PyMT mice. To establish a cause-effect relationship, we stably downregulated SPRR1A in MDA-MB-231 cells, a triple-negative breast cancer cell line. SPRR1A downregulation in MDA-MB-231 cells impaired mammosphere formation and reduced the growth of MDA-MB-231 tumors using a xenograft mouse model (See Figures 1 and 2). Therefore, targeting cancer cell "stemness" may represent a novel therapeutic strategy for preventing DCIS progression.

Related to these studies, we have also recently published the following paper:

Ketones and lactate increase cancer cell "stemness," driving recurrence, metastasis and poor clinical outcome in breast cancer: achieving personalized medicine via Metabolo-Genomics.

Martinez-Outschoorn UE, Prisco M, Ertel A, Tsirigos A, Lin Z, Pavlides S, Wang C, Flomenberg N, Knudsen ES, Howell A, Pestell RG, Sotgia F, Lisanti MP. Cell Cycle. 2011 Apr 15;10(8):1271-86.

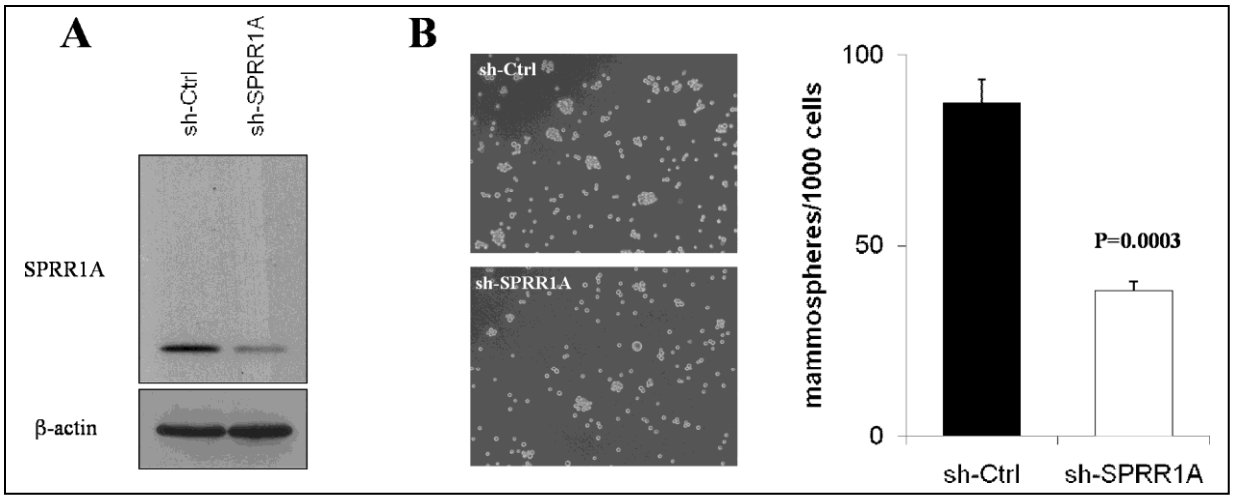

Figure 1. SPRR1A **promotes mammary epithelial tumor stem cell expansion. A) Validation of SPRR1A down-regulation in MDA-MB-231 cells** by Western blot. To confirm expression levels in SPRR1A transduced MDA cell lines, cells were cultured and lysates were prepared and subjected to immunoblot analysis with SPRR1A antibody. Decreased levels of SPRR1A are observed in sh-SPRR1A cell lines compared to sh-Ctrl. Beta-actin is shown as loading control. B) Mammosphere production by MDA-MB-231 cells with down-regulation of SPRR1A. Note that downregulation of SPRR1A significantly reduces (p=0.0003) mammosphere formation in MDA-MB-231 cells. P-values are indicated in the graphs, as determined by the Student's t-test.

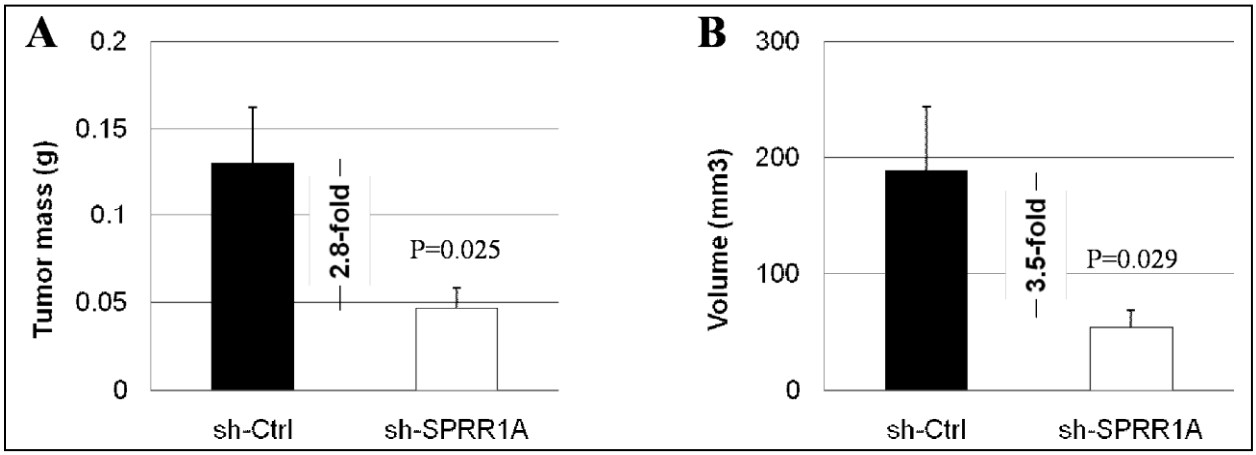

Figure 2. SPRR1A promotes breast tumor growth. SPRR1A-knockdown MDA-MB-231 cells were injected into the flanks of nude mice, along with their respective controls. After 3 weeks post-injection, the tumors were excised and tumor mass and volume were determined. Relative to controls, down-regulation of SPRR1A expression in MDA-MB-231 cells significantly reduced tumor growth, resulting in 2.8-fold decrease in tumor mass (A) and 3.5-fold decrease in tumor volume (B). P-values are indicated in the graphs, as determined by the Student's t-test.

Research Project 6: Project Title and Purpose

Phenotypic and Molecular Characterization of Occult AML - The high incidence of relapse in acute myelogenous leukemia (AML) observed after induction of complete remission (CR) is thought to be due to the persistence in a protective niche in the bone marrow (BM). The residual AML cryptic cells are a rare population with self-renewal potential, representing Minimum Residual Leukemic Myeloblasts (MRLM). Homing of these AML cells in the BM is largely mediated through the interaction of the chemokine receptor CXCR4 expressed by AML cells with its ligand CXCL12 expressed by BM stromal cells. The objective of this project is to establish a rationale for mobilization of AML occult cells from the BM by treating patients in CR with a CXCR4 antagonist and demonstrating that AML precursor cells will be detected in bone marrow and peripheral blood as early predictor or relapse using cytogenics, multiparameter flow cytometry and next generation DNA sequencing.

Anticipated Duration of Project

1/1/2011 - 12/31/2014

Project Overview

The overall hypothesis of this proposal is that inhibition of CXCR4 could induce mobilization into peripheral blood of AML stem cells (AML-SC) hosted in the BM that constitute minimal residual disease during hematologic remission in patients with AML following stem cell transplantation (SCT). The objective of this project is to establish CXCR4 as a target for mobilization of AML occult cells in patients in CR after chemotherapy and allogeneic SCT and

to establish the phenotypic and molecular signature of these AML myeloblats and AML-SC. Bone marrow and peripheral blood samples will be collected in a longitudinal study at 28, 90 and 180 days following SCT.

In Specific Aim 1, the identification of (recipient) leukemic myeloblasts will be performed using routinely performed techniques, including morphologic analysis and detection of cytogenetic abnormalities present at the time of the initial diagnosis and/or sex chromosome conversion following SCT using fluorescence *in situ* hybridization (FISH).

In Specific Aim 2, the identification and quantification of leukemic myeloblasts will be achieved by multiparameter flow cytometric detection of the abnormal phenotype present at the time of diagnosis using a panel of antibodies specific for myeloid markers and non-myeloid differentiation markers, as well as antigens associated with AML-SC such as CD34, CD117 (c-kit), CD123 (IL-3R alpha chain), CD47, and CXCR4.

In Specific Aim 3, the quantification of leukemic myeloblasts will be based on the analysis of hematopoietic chimerism using molecular genetic analysis of short tandem repeats (STR) alleles from donor and recipient genotypes. This will be achieved with the PowerPlex 16 assay routinely used to monitor chimerism following SCT. This assay consists of a set of multiplex PCR amplification reactions and analysis of the length of the DNA fragments will be performed on an ABI3130 Genetic Analyzer. However, this traditional method offers a limited sensitivity due to the limited threshold of detection of traditional sequencers. The sensitivity of detection and the accuracy of quantification of chimerism will be extended by using next generation sequencing (Roche 454) to analyze the products of the PowerPlex 16 assay.

These specific aims will provide the tools to address the question as to whether mobilizing AML

cells into peripheral blood and thereby sampling the entire marrow organ would be a reasonable strategy to detect residual or recurrent AML at an earlier time point than conventional marrow analysis.

Principal Investigator

Stephen C. Peiper, MD
Peter A. Herbut Professor and Chair
Jefferson Medical College of Thomas Jefferson University
1020 Locust Street
279 JAH
Philadelphia, PA 19107

Other Participating Researchers

Jean-Marc Navenot, PhD, Neal Flomenberg, MD – employed by Thomas Jefferson University

Expected Research Outcomes and Benefits

Acute myelogenous leukemia (AML) is a heterogeneous disease, both phenotypically and at a genomic level. Although chemotherapy is frequently capable of achieving complete remission (CR), most adults with AML will relapse, even after allogeneic hematopoietic stem cell transplantation (SCT). It is widely accepted that relapse is due to the persistence in the bone marrow (BM) of a rare population of AML cryptic cells with self-renewal potential representing Minimum Residual Leukemic Myeloblasts (MRLM). These AML stem cells (AML-SC) have the capacity to initiate leukemia when transplanted in immune deficient mice and share functional and phenotypic features with normal hematopoietic stem cells (HSC). The expression of the chemokine receptor CXCR4 by AML-SC and of its ligand SDF-1/CXCL12 by BM stromal cells is responsible for their homing into a BM niche that protects them from obliteration by chemotherapy.

CXCR4 antagonism by blocking antibodies or by the small molecule antagonist plerixafor was shown in mouse models to prevent homing of AML-SC to the BM, to induce their mobilization into peripheral blood and to sensitize AML cells to chemotherapy, thus identifying CXCR4 as a potential target for the eradication of minimum residual disease (MRD) in AML. This project will use highly sensitive methods to establish the phenotypic and molecular signature of AML occult cells and will build a foundation for the future use of CXCR4 as a target for mobilization of AML-SC from the BM in patients with impending relapse of AML. The capacity to mobilize residual myeloblasts and AML-SC in peripheral blood would provide an early and more reliable detection of residual or recurring leukemia compared to conventional BM sampling by effectively investigating the entire BM organ, thus allowing a better prediction of relapse and possibly improving the efficacy of therapeutic intervention.

Summary of Research Completed

The objective of the research project is to identify residual leukemic myeloblasts and leukemia stem cells in the peripheral blood of patients with Acute Myelogenous Leukemia (AML) after heterologous Stem Cell Transplant (SCT). This requires the development of methods capable of detecting with high sensitivity an extremely small number of AML cells from the patient in blood samples consisting mostly of cells from the donor.

We have focused on the strategy introduced in Specific Aim 3 which consists of using genetic markers to discriminate the hematopoietic cells coming from the recipient from those coming from the donor. This strategy is based on the concept that emergence of recipient's cells after SCT represents an early indication of impending hematologic relapse. For these studies of mixed chimerism, we proposed to adapt the PowerPlex® 16 assay (Promega) to the Roche 454 platform of next-generation sequencing. The PowerPlex® 16 assay consists of 16 polymorphic loci containing variable numbers of tandem repeats of 4 or 5 base pairs. Pairs of primers specific for each locus are used to amplify by PCR, using genomic DNA as a template, a fragment of DNA that comprises the repeat region. The length of that fragment depends on the number of repeats and is determined by analyzing the PCR product on a DNA sequencer such as the ABI 3130 Genetic Analyzer. Separate analysis of genomic DNA from recipient and donor origin identifies the loci that can be used to discriminate the recipient and the donor (i.e. the loci where donor and

recipient have at least one different allele). Although very specific, this technology offers limited sensitivity for the detection of the minor alleles in a mixed sample (at least 2%).

Roche 454 next generation sequencing (NGS) offers the possibility to analyze large numbers of individual sequences in a single run. This allows the detection and quantification of rare genetic variants and should lend itself to the analysis of mixed populations of polymorphic alleles. The possibility to use 454 NGS to analyze mixed chimerism using the PowerPlex® 16 assay was determined in five de-identified AML patients who had relapsed following SCT. Sequential archived DNA samples previously studied using the PowerPlex® 16 assay were obtained from each patient at different time points (2 to 4) after SCT. Each case included at least one sample obtained shortly after SCT and one sample obtained after clinical relapse. When available, 1 or 2 additional DNA sample(s) collected before relapse were included. Two or 3 informative loci were used for each patient.

The preparation of the samples for 454 sequencing included 4 steps:

1) Primers specific for each locus and identical to those included in the Promega PowerPlex® 16 kit were used to amplify genomic DNA using the reagents and conditions recommended by Promega. PCR products were analyzed on a Bioanalyzer 2100 (Agilent).

2) For each patient, PCR products were purified and quantified. For each time point, the PCR products corresponding to the different loci (2 per patient) were mixed in equimolar amounts and used to prepare a library for which PCR products were end-repaired and ligated to an oligonucleotide incorporating an adaptor sequence followed by a specific sequence (MID) that functions as a barcode to identify the sample during sequencing. Each time point was prepared using a different MID in order to conduct multiplexed sequencing and analyze separately the data for each time point. After ligation, the products were purified from the unligated oligonucleotides, quantified and the different time points were mixed in equimolar quantities to prepare the library.

3) The library was diluted so that, statistically, each sequencing bead was conjugated to only 1 DNA fragment. After addition of DNA to the sequencing beads, DNA was clonally amplified by emulsion PCR (emPCR) in which each DNA bead is included into an individual micro-reaction chamber within the emulsion. After emPCR, the emulsion was broken and the beads carrying some DNA were magnetically separated from the empty beads.

4) The DNA beads were loaded onto a pico-titer plate according to Roche-454 protocol and the plate was installed on a 454 Junior sequencer. Reads were sorted according to their MID and analyzed using CLC Genomics Workbench (CLC bio). Mapping was performed against all the existing alleles of each locus.

The number of usable reads for each sequencing reaction ranged from 62,000 to 148,000, distributed between the 2 loci and the 2-4 time points. A total of 6 loci were used for 5 patients (D5S818, D7S820, D8S1179, D13S317, D16S539 and TPOX) and, in most cases, the results were in agreement with the PowerPlex® 16 data (Figure 1). In the post-relapse samples, a decrease of the donor-specific allele(s) was observed compared to the pre-relapse samples while

a simultaneous increase of the recipient-specific allele(s) was noted. However, for each sample, a percentage of the reads had a number of repeats that did not correspond to alleles present either in the donor or the recipient. These reads are related to the "stuttering" of the polymerase that occurs in presence of repeats. Most of the stutters are 1 repeat shorter than the template (down-stuttering) and some are 1 repeat longer (up-stuttering) (see for instance patients AML-BME-007 and AML-BME-009, Fig 1). A calculation of the percentage of stuttering can be introduced but this problem could compromise the sensitivity of detection of rare events that could otherwise be achieved by 454 sequencing.

If the problem cannot be resolved, alternative approaches will be used. First, Roche 454 recently released a kit for high-resolution HLA genotyping. Since donor and recipient of SCT are usually an incomplete match, this system could be used to discriminate the cells of donor and recipient origin. Another approach could be the search for Single Nucleotide Polymorphisms (SNPs) that would be different between donor and recipient. Since no data is available for the patient or the donor, both DNA would have to be screened first in order to identify informative SNPs. When this is done, 454 sequencing should be able to detect a small percentage of the recipient's allele.

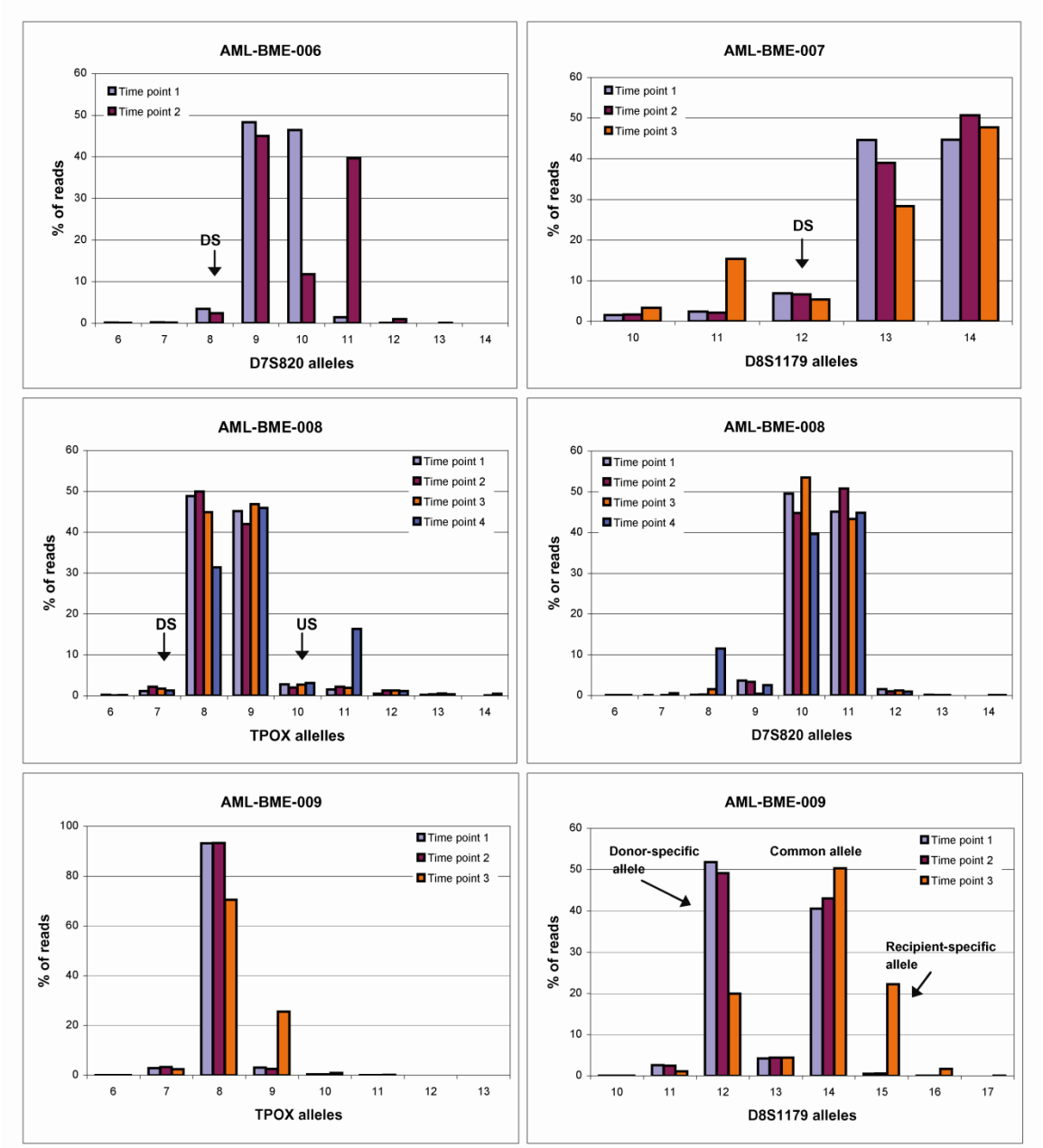

Figure 1: Adaptation of the PowerPlex 16 Assay to the Roche-454 NDG platform. Data for patients 006, 007, 008 and 009 illustrate the capacity to effectively detect and quantify alleles specific for the donor and the recipient. For each patient, the last time point is after clinical relapse. Data indicate the presence of down-stuttering (DS) and up-stuttering (US) corresponding to reads with lower or higher repeat numbers compared to the actual alleles present in the sample. Data are in accordance with PowerPlex 16 data indicating percentages of recipient cells of 86% for patient 006, 40% for patient 007, 32% for patient 008 and 50% for patient 009.