Thomas Jefferson University

Annual Progress Report: 2006 Formula Grant

Reporting Period

July 1, 2010 – December 31, 2010

Formula Grant Overview

The Thomas Jefferson University received $3,674,642 in formula funds for the grant award period January 1, 2007 through December 31, 2010. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Identification of a New Class of Genes that Contributes to the Development of Breast Cancer - The proposed research will identify a new class of genes that contributes to the development of breast cancer. The researcher's studies of over 2,000 human breast cancer samples have shown the loss of the gene Dachshund predicts a substantially higher risk of death. Breast cancer cellular migration and metastasis are blocked by Dachshund and breast cancer cells are reverted to a precancerous phase. The researcher will characterize the function of this new class of genes to regulate breast cancer development. The researcher has developed a new mouse model which will allow the researcher to remove the Dach1 gene in breast cancer. These mice will allow the researcher to prove Dachshund can block the development of breast cancer. These mice will be valuable to test new therapies for breast cancer.

Duration of Project

7/26/2007 – 12/31/2010

Project Overview

Human breast cancer resistant to current forms of therapy kills women each year. Resistance of ErbB2-mediated breast cancer to current therapies arises in women treated with current therapy. New molecular targets governing breast tumor onset and progression are urgently needed. DACH1 represents a novel tumor suppressor pathway. Reduced DACH1 expression correlates with poor prognosis in human breast cancer. Reintroduction of DACH1 into human mammary epithelial cells transformed with distinct oncogenes (Ras, ErbB2, d-Myc) reverts the transformed phenotype in 3D cultures. Revision of the tumor phenotype and the associated genotype led to the DACH1 gene being referred to as a "commandeering" tumor suppressor. DACH1 is structurally distinct to the current known tumor suppressors. This research has identified key functional domains of DACH1 required for inhibition of tumor growth. These residues may represent excellent new targets for breast tumor therapy. As DACH1 functions as a cell fate determination factor, understanding the mechanisms by which DACH1 inhibits breast

tumorigenesis may have important implications for the role of cell fate in tumorigenesis.

Aim 1. Determine the mechanisms governing DACH1//Eya/Six inhibition of breast tumor cell proliferation. The researcher will determine the DACH1 binding proteins governing breast tumor growth. The researcher will define endogenous gene targets of DACH1 using ChiP on chip assays. This will include: 1) determining the components of DACH1/Eya complex inhibiting breast tumor growth; 2) determining the mechanisms by which the DACH1DS domain regulates cyclin D1 and $p21^{CIP1}$; 3) determining the mechanisms by which the DACH1 DS domain is recruited in local chromatin to the cyclin D1 promoter; and 4) identification of chromatin regions occupied by DACH1 and its associated proteins.

Aim 2. Determine the role of DACH1 as a suppressor of mammary tumor onset, progression and metastasis using $DACH1^{fl/fl}$ mice. To determine the role of Dach1 in mammary tumorigenesis; 1) the researcher will deploy inducible DACH1 SIRNA to access DACH1 function suppressor in oncogene collaboration assay. 2) The researcher will determine Dach1 tumor suppressor function using mammary epithelial cell-targeted $Dach1^{fl/fl}$ transgenic mice crossed with MMTV-ErbB2/MMTV-Cre transgenic mice. 3) In order to link the findings of these studies to human breast cancer the research will conduct analysis of human breast cancer samples.

Principal Investigator

Richard G. Pestell, MD, PhD
Thomas Jefferson University
Kimmel Cancer Center
Bluemle Life Sciences Building
233 South 10th Street, Room 1032
Philadelphia, PA 19107

Other Participating Researchers

None

Expected Research Outcomes and Benefits

Completion of these studies will define for the first time a comprehensive analysis of a completely novel model of tumor suppression. The *in vivo* significance will be established using a state-of-the-art molecular genetic approach. The $Dach1^{fl/fl}$ mice and the inducible siRNA viral vectors are innovative and will establish the importance of endogenous Dach1 in tumor suppression. The mechanistic studies, using ChIP on chip, provide the highest level of resolution known, to establish the mechanisms by which DACH1 regulates gene expression. The ChIP on chip analysis are not open ended but are highly focused on a specific question using tiled arrays which are available. The studies of human breast cancer use tissue arrays that are well annotated and previously published by us. Collectively the studies of human and murine breast cancer will provide an integrated understanding of the function of DACH1 in human breast cancer and potentially a new avenue for targeted therapies of human breast cancer.

Summary of Research Completed

Substantial progress has been made on this project during the last six months that focused on the molecular mechanisms by which the RDGN governs tumorigenesis.

Aim1: The goal was to determine the DACH1-binding proteins and to determine the components of DACH1/Eya complex inhibiting breast tumor growth. To address this, we conducted proteomic analysis of DACH1 binding proteins using cultured tumor cells. These studies identified the DACH1 associated protein CA150 as a DACH1 binding protein. We conducted migration and invasion assays to assess the role of CA150 in DACH1-mediated function and demonstrated an essential role for CA150 as a co-integrator of DACH1 mediated transcriptional function. As we had identified using in silico analysis and using ChIP-Seq (1.4 below) that DACH1 binding sites were highly homologous to Forkhead binding sites, we analyzed the role of CA150 in FKHR function and showed that CA150 serves as a rate limiting co-integrator for the DACH-Forkhead pathways.CA150 is also known as TCERG1.
In these studies we showed that DACH1 repressed, whereas FOX proteins enhanced, both DRE and FOXA-responsive element-driven gene expression. Reduced DACH1 expression using a shRNA approach enhanced FOX protein activity. As DACH1 antagonized FOX target gene expression and attenuated FOX signaling, we sought to identify limiting co-integrator proteins governing DACH1 signaling. Proteomic analysis identified transcription elongation regulator 1 (TCERG1) as the transcriptional co-regulator of DACH1 activity. The FF2 domain of TCERG1 was required for DACH1 binding, and the deletion of FF2 abolished DACH1 trans-repression function. The carboxyl terminus of DACH1 was necessary and sufficient for TCERG1 binding. Thus, DACH1 represses gene transcription through direct DNA binding to the promoter region of target genes by recruiting the transcriptional co-regulator, TCERG1.

To determine at the genome wide level the gene promoters occupied by DACH1. We conducted cyclic amplification and selection of targets and thereby identified a DACH1 DNA-binding sequence that resembles the FOX (Forkhead box-containing protein) binding site. Genome-wide in silico promoter analysis of DACH1 binding sites identified gene clusters populating cellular pathways associated with the cell cycle and growth factor signaling. ChIP coupled with high-throughput sequencing mapped DACH1 binding sites to corresponding gene clusters predicted in silico and identified as weight matrix resembling the cyclic amplification and selection of targets-defined sequence. DACH1 antagonized FOXM1 target gene expression, promoter occupancy in the context of local chromatin, and contact-independent growth. Attenuation of FOX function by the cell fate determination pathway has broad implications given the diverse role of FOX proteins in cellular biology and tumorigenesis. (Published Results in J Biol ChemDec 17; 285(51):40342-50. Epub 2010 Oct 18).

Aim 2: The aim was to "link the findings of these studies to human breast cancer." These studies were particularly informative and may have direct importance in management of a subset of human breast cancer, known as the basal breast cancer phenotype. We conducted analysis of human breast cancers that had been subclassified based on their molecular genetic profile using genome wide microarray analysis. Currently human breast cancer is classified into five distinct molecular genetic categories. One of these genetic subtypes is known as the basal phenotype. This type of cancer has a particularly poor prognosis and is more frequent in African American

women. The basal breast cancer subtype is enriched for breast cancer stem cell markers. We examined the expression of DACH in the distinct molecular genetic subtypes of human breast cancer and showed that DACH1 expression was lost distinctly in the basal phenotype. We next examined a series of human breast cancer cell lines, and showed that the breast cancer cell lines that were enriched for breast cancer stem cells, also showed reduced expression of DACH1.

Breast tumor-initiating cells (TIC) may contribute to tumor progression and therapy resistance. We showed that, endogenous DACH1 was reduced in breast cancer cell lines with high expression of TIC markers and in patient samples of the basal breast cancer phenotype. Re-expression of DACH1 reduced new tumor formation in serial transplantations in vivo, reduced mammosphere formation, and reduced the proportion of CD44(high)/CD24(low) breast tumor cells. Conversely, lentiviral shRNA to DACH1 increased the breast (B)TIC population. Genome-wide expression studies of mammary tumors demonstrated DACH1 repressed a molecular signature associated with stem cells (SOX2, Nanog, and KLF4) and genome-wide ChIP-seq analysis identified DACH1 binding to the promoter of the Nanog, KLF4, and Lin28 genes. KLF4/c-Myc and Oct4/Sox2 antagonized DACH1 repression of BTIC. Mechanistic studies demonstrated DACH1 directly repressed the Nanog and Sox2 promoters via a conserved domain. Collectively these studies demonstrated that endogenous DACH1 regulates BTIC in vitro and in vivo and show an important link between DACH and human breast cancer genetic subtypes. (Results published in J Biol Chem 2011 Jan 21;286(3):2132-42. Epub 2010 Oct 11.).

Research Project 2: Project Title and Purpose

Role of the MYC Oncoprotein in Human Cancer - Human cancer is caused by genetic mutations that occur in otherwise normal genes. The most commonly mutated gene in cancer is the MYC oncogene. MYC is mutated so frequently because it is a master regulator of the cell division. MYC accomplished this by controlling the activity of ~3000 other genes. While this number is too large to provide useful information about new drug targets or therapeutic strategies, recent data suggest that only a small subset of these 3000 genes play a direct role in cancer. Using new technology that allows the examination of all genes in a cell at once, the MYC targets actually required for transformation are being defined. The researcher has begun to decipher how these few critical targets participate in converting a normal cell to malignancy. Studies such as these should allow the rational design of new therapeutic strategies.

Duration of Project

1/1/2007 - 12/31/2010

Project Overview

Genetic alterations targeting the *myc* oncogene are the most common transforming events in human cancer. These alterations cause overexpression of the transcription factor MYC. A thorough understanding of the MYC transcription network is critical to progress against cancer. Despite intensive study, the essential genes lying downstream of MYC remain poorly understood. Of the 629 reported MYC target genes, only 6 have documented roles in MYC-mediated transformation. An expression-profiling screen that utilized a discrete mutant of

MYC was used to selectively identify those targets relevant to transformation. This screen identified 40 novel MYC targets and studies have begun to validate their importance in MYC-driven tumorigenesis. For example, shRNA-mediated depletion of the novel target MTA1 blocks the increased invasiveness of tumor cells caused by MYC. MTA1 reportedly controls the epithelial-to-mesenchymal transition; however its link to MYC was previously unknown. Additional targets from the screen (BAG-1, POLRMT and CD30), were characterized at the molecular level and are now being characterized to determine the biological function they contribute during MYC-mediated transformation. *The aim of this study is to understand the biological roles played by MTA1, BAG-1, POLRMT and CD30 in MYC-driven tumors.* Together, these studies are likely to identify new cellular functions for MYC, including regulating invasiveness, blocking the anti-tumor immune response and inhibiting the apoptotic stimuli generally associated with oncogene overexpression. Using well-defined systems in which human cells can be transformed by the MYC oncoprotein *in vitro*, each of the targets identified will be dissected in order to understand the precise advantage they provide to MYC-driven tumor cells.

Principal Investigator

Steven B. McMahon, PhD
Thomas Jefferson University
Room 609 Bluemle Bldg.
Kimmel Cancer Center
233 South 10th Street
Philadelphia, PA 19107

Other Participating Researchers

Xiaoyong Zhang, PhD, Maya Varthia, BS - employed by Thomas Jefferson University

Expected Research Outcomes and Benefits

An overwhelming number of deaths from human cancer can be directly attributed to MYC overexpression. As the design of rationally targeted therapies becomes more commonplace, a complete knowledge of the MYC pathway will be critical. The recent identification of target genes activated by MYC has yielded a number of exciting candidates for therapeutic intervention. The best characterized of these targets, MTA1, displays the property that its induction is absolutely required for MYC mediated transformation. Thus the screen significantly enriched the pool of transformation-relevant MYC targets. The identification of MTA1 itself also provides novel insight in the MYC pathway. In general, MYC activation has been considered to be an early, initiating event in human cancer. The demonstration that MYC regulates a step later in the process (i.e., the acquisition of invasive growth capacity) may have important clinical ramifications. For example, this knowledge may impact decisions about when to administer some of the anti-MYC therapies that are now being developed. The other targets from this original screen appear to have an equal likelihood of providing fundamental knowledge critical to understanding and treating cancer. BAG-1 for instance may help explain the lingering enigma of why MYC can induce transformation in some settings and cell death in others. This

specific knowledge may be useful for reactivating the cell death pathway in the many cases of cancer where MYC is overexpressed. The ability to selectively activate the intrinsic cell death pathway in MYC expressing tumor cells but not normal cells would be of obvious therapeutic advantage.

Summary of Research Completed

The MYC oncoprotein is among the most commonly over-expressed proteins in human cancer. However, efforts to target MYC therapeutically have been unsuccessful. Many transforming proteins, including MYC, E2F, Ela etc., can induce robust apoptosis when expressed in specific cellular contexts. This ability of potent oncoproteins to induce cell death under certain conditions, has been termed "intrinsic tumor suppression". This property provides complex multi-cellular organisms with a mechanism for the selective elimination of malignant cells, thereby protecting the organism as a whole. In principle, successful cancer therapies could be based on reactivation of this apoptotic potential in human tumors where MYC is overexpressed. To date, these efforts have been hampered by our limited understanding of the biochemical distinction between cells where MYC drives malignant transformation and those where it induces apoptosis. Recent evidence that MYC levels in normal cells are well below those required for apoptosis suggests the possibility that clinical activation of MYC-induced apoptosis in cancer patients might have a viable therapeutic index.

MYC functions in large part as a transcriptional regulator, capable of modulating the expression of several thousand downstream genes. Current models suggest that deciphering the functional relevance of individual downstream targets of MYC will allow advances in our understanding of this previously intractable pathway. In an effort to clarify the identity of essential nodes within this large set of targets, we conducted an unbiased screen focused on the selective identification of the subset of downstream MYC targets that transmit critical effector functions. Via this screen, we identified MTA1 as a novel MYC target. Functional characterization further showed that MTA1 induction by MYC is essential for MYC-driven increases in the invasive growth of mammalian cells. These observations are consistent with previous studies implicating MTA1 in metastasis. We report here the identification of the BAG1 gene in this same screen for critical MYC effectors. MYC directly binds the BAG1 locus and transcription of BAG1 is regulated by MYC in all human and mouse cell backgrounds examined, including a murine tumor model of MYC-induced lymphoma.

The *BAG1* locus encodes a family of co-chaperone proteins important for stabilizing a number of pro-survival client proteins in a wide variety of cell types. BAG1 is frequently overexpressed in human cancer and high levels of expression have been associated with poor prognosis. Evidence from genetic studies in mice has shown that a 50% reduction in BAG1 gene dosage results in increased apoptosis in tumor cells and a nearly complete inhibition of lung adenoma progression, without any effect on cell cycle progression. Given the important role of BAG1 in controlling cell survival pathways, we assessed whether induction of BAG1 by MYC might participate in controlling the decision between transformation and apoptosis. Remarkably, blocking BAG1 induction by MYC was sufficient to convert cells from MYC-driven proliferation to MYC-induced apoptosis. The identification of a single node regulating the apoptosis/survival balance in MYC expressing cells provides a novel point of attack for targeted therapies in cancer.

While the relevance of our findings in H1299 cells and MCF7 cells is supported by studies implicating MYC expression as important in both lung and breast cancer in humans, a more direct assessment of the correlation between MYC and BAG1 expression *in vivo* was warranted. For this purpose, BAG1 mRNA expression was quantitated in tumors from a well-established mouse model of MYC-driven mammary cancer. In these animals, MYC expression is mammary gland-specific and also regulated by a doxycycline-responsive promoter. Tumors were induced in 8 mice beginning at 6 weeks of age, by activating MYC expression with doxycycline treatment. After appearance of palpable mammary tumors, 4 mice were withdrawn from doxycycline for 96 hours, while MYC activation was maintained in the other 4 mice. Tumors were then harvested and mRNA levels for BAG1 determined by qRT-PCR (Figure 1). This analysis demonstrated that BAG1 transcript levels tightly correlate with MYC activity in an *in vivo* setting, with a significant decrease in BAG1 mRNA observed in mice where MYC activity was inhibited for even a few days. A tight correlation between MYC activity and BAG1 transcript levels was also observed in a mouse model of MYC-driven lymphomagenesis (Figure 2). Thus, data from independent *in vivo* models are consistent with the *in vitro* data demonstrating that BAG1 mRNA is regulated by MYC. Furthermore, the conservation of *BAG1* as a MYC-regulated gene in both humans and mice suggests that the link may be of functional importance.

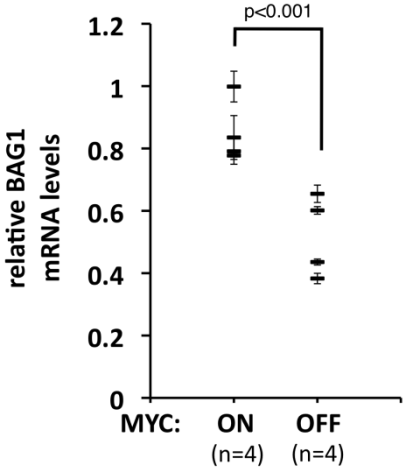


Figure 1. BAG1 levels are regulated by MYC *in vivo*. Mammary tumors were induced in six-week-old female bitransgenic MMTV-rtTA; TetO-MYC mice via administration of 2 mg/mL doxycycline in drinking water. After mammary tumor formation, doxycycline was withdrawn from the water of 4 mice (MYC OFF) while the other mice were maintained on doxycycline (MYC ON). Ninety-six hours after doxycycline withdrawal, tumors were harvested and mRNA from tumors was converted to cDNA followed by quantitative RT-PCR analysis. BAG1 levels were normalized to actin. Error bars represent standard deviation of quadruplets from each sample. p value was calculated using Student's t-test (two-sided, t = -25.67).

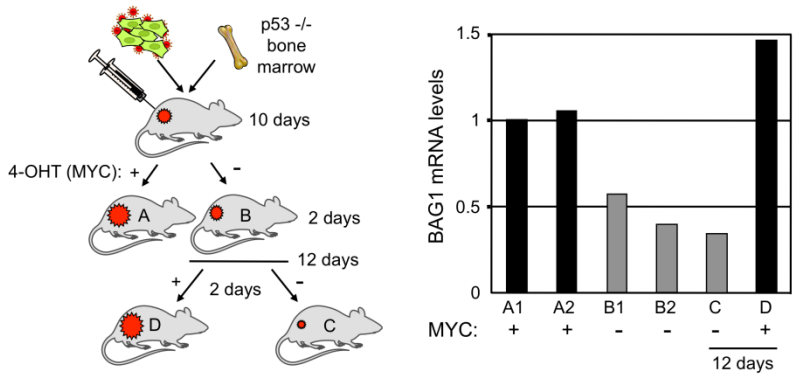


Figure 2 BAG1 levels are regulated by MYC in a murine tumor model.
(A) Tumors were induced via inoculation of cells from a transplantable B cell lymphoma originally developed by infection of bone marrow from a p53-/- mouse with the MYC/ER retrovirus. In total, these cells were transplanted into the flanks of 6 naïve mice. After 10 days of 4-OHT treatment, mice developed aggressively growing lymphomas. At this point, two mice were continued on 4-OHT treatment (mice A1 and A2) while 2 mice were withdrawn from treatment for 2 days (mice B1 and B2), and the remaining 2 mice were withdrawn for another 10 days (mice C and D). Subsequently, one of the remaining mice was treated with 4-OHT (mouse D) while the final mouse was not (mouse C). (B) Tumors were harvested and mRNA analyzed for BAG1 levels by quantitative RT-PCR, at the times indicated in (a).

Research Project 3: Project Title and Purpose

Stat5 Interaction with Androgen Receptor in Growth Promotion of Prostate Cancer - This project may validate Stat5a/b as a novel therapeutic target for androgen-independent prostate cancer. The outcome of the studies will increase the knowledge about the molecular mechanisms underlying androgen-independent growth of prostate cancer. The proposed studies may validate active Stat5a/b in prostate cancer as a biomarker for poor response to androgen deprivation therapy. This project has a high impact on prostate cancer patients and for prostate cancer research. This is because there are currently no effective pharmacological therapies for hormone-refractory prostate cancer. Stat5a/b may provide a molecular target for new pharmacological therapy development. Specifically, pharmacological inhibition of Stat5a/b can be achieved by small-molecule inhibitors for dimerization or DNA binding of Stat5a/b.

Duration of Project

1/1/2007 – 12/31/2010

Project Overview

This project aims to determine whether transcription factor Stat5a/b promotes androgen-independent growth of prostate cancer through functional interaction with androgen receptor (AR). The rationale behind this proposal centers on the researcher's finding of a critical role of Stat5a/b for cell viability of human prostate cancer cells. Also, inhibition of Stat5a/b blocks

prostate xenograft tumor growth in mice. Androgen-receptor signaling, in turn, is known to be necessary in prostate cancer development, and androgen receptor signaling promotes proliferation and survival of prostate cancer cells.

The researcher hypothesizes that Stat5a/b increases transcriptional activity of AR, and that AR stimulates transcriptional activity of Stat5a/b in prostate cancer cells. The researcher proposes that the functional interaction of Stat5a/b and AR promotes prostate cancer cell viability *in vitro* and prostate tumor growth *in vivo.* The researcher will test these hypotheses in human prostate cancer cells in culture and in human prostate cancer tumor models in nude mice using the researcher's special molecular tools to manipulate Stat5a/b and that active Stat5a/b predicts poor response of primary prostate cancer to androgen deprivation therapy. The hypothesis of this project is supported by strong preliminary data. The researcher's new data support a novel concept that Stat5a/b and androgen receptor functionally synergize in prostate cancer cells. Specifically, the researcher's preliminary data indicate that: 1) Stat5a/b promotes transcriptional activity of androgen receptor, and 2) androgen receptor increases transcriptional activity of Stat5a/b. No previous published work exists on functional interaction of Stat5a/b and androgen receptor in prostate cancer.

The project is innovative since the concept of functional interaction between Stat5a/b and AR in promotion of androgen-independent growth of human prostate cancer is entirely novel. Specifically, the researcher proposes that transcription factor Stat5a/b is a co-activator for AR in human prostate cancer cells. These concepts are new and no published work exists on these topics.

Principal Investigator

Marja T. Nevalainen, MD, PhD
Thomas Jefferson University
Blumle Life Science Bldg, Suite 309
233 South 10th Street
Philadelphia, PA 19107

Other Participating Researchers

Feng Shen, PhD - employed by Thomas Jefferson University

Expected Research Outcomes and Benefits

The researcher has recently established transcription factor Stat5a/b as a critical protein for survival of human prostate cancer cells. Stat5a/b is constitutively activated in human prostate cancer but not in adjacent normal epithelium, and activation of Stat5a/b is associated with high histological grade of prostate cancer. The researcher has shown that active Stat5a/b in primary prostate cancers predicts early disease recurrence. The researcher's preliminary data show that Stat5a/b is highly active in the majority of recurrent clinical human prostate cancers.

This project is important for several reasons: 1) The outcome of the studies may validate Stat5a/b

as a novel therapeutic target for androgen-independent prostate cancer. 2) The outcome of the studies will increase the knowledge about the molecular mechanisms underlying androgen-independent growth of prostate cancer. This project has high impact on prostate cancer patients and for prostate cancer research. This is because there are currently no effective pharmacological therapies for hormone-refractory prostate cancer. Stat5a/b may provide a molecular target for new pharmacological therapy development. Specifically, pharmacological inhibition of Stat5a/b can be achieved by small-molecule inhibitors for dimerization or DNA binding of Stat5a/b.

Summary of Research Completed

The molecular mechanisms underlying progression of prostate cancer to the castrate-resistant state are poorly understood. Identification of molecular changes that lead to castrate-resistant growth of prostate cancer cells is critical for the development of better therapeutic interventions for primary and advanced prostate cancer. Castrate-resistant prostate cancer is characterized by continued expression of androgen receptor (AR) and androgen-regulated genes, suggesting that the AR signaling pathway remains active despite low levels of circulatory androgens.

In this funding period, additional molecular mechanisms underlying the transcriptional synergy between Stat5a/b and androgen receptor in human prostate cancer cells were identified.

Stat5a/b regulates androgen receptor protein expression in human prostate cancer was discovered during this project. LNCaP human prostate cancer cells were cultured in the presence or absence of androgens (DHT, 0.1 nM) or human prolactin (Prl; 10 nM) and infected with lenti-virus expressing Stat5a or Stat5b shRNA for 72 h. Androgen receptor (AR) expression and Stat5a/b expression were determined by Western blotting with actin immunoblotting as the loading control.

As demonstrated in Fig. 1a, when Stat5a expression was inhibited by lenti-viral expression of Stat5a shRNA, AR levels were significantly decreased in LNCaP prostate cancer cells. In addition, lenti-viral expression of Stat5b shRNA decreased AR expression, although to a lesser extent than Stat5a shRNA (Fig.1.b). These results suggest that Stat5 regulates AR expression in human prostate cancer cell line LNCaP and that the Stat5a isoform of Stat5 may have a predominant role in the regulation of AR levels.

We investigated further whether AR expression levels correlate with Stat5a/b expression in clinical human prostate cancers. We analyzed 443 prostate cancer samples from 126 prostate cancer patients for androgen receptor and Stat5a/b expression by immunohistochemistry using specific anti-AR and anti-Stat5a/b antibodies. The immunohistochemical stainings were scored by two independent evaluators. Mixed effects regression analysis was used to test whether AR score differed by the level of Stat5a/b in the prostate cancer samples. Stat5 level was included as a fixed categorical effect and a random intercept term was included to account for correlation among samples from the same subject. Interestingly, the statistical analysis indicated that AR protein levels in clinical prostate cancers were associated with Stat5a ($p=0.038$), Stat5b ($p=0.012$) and Stat5ab ($p<0.0001$). In conclusion, the higher the Stat5a/b protein levels were in human prostate cancers, the higher were also the AR protein levels in the same samples.

Fig. 1.a.

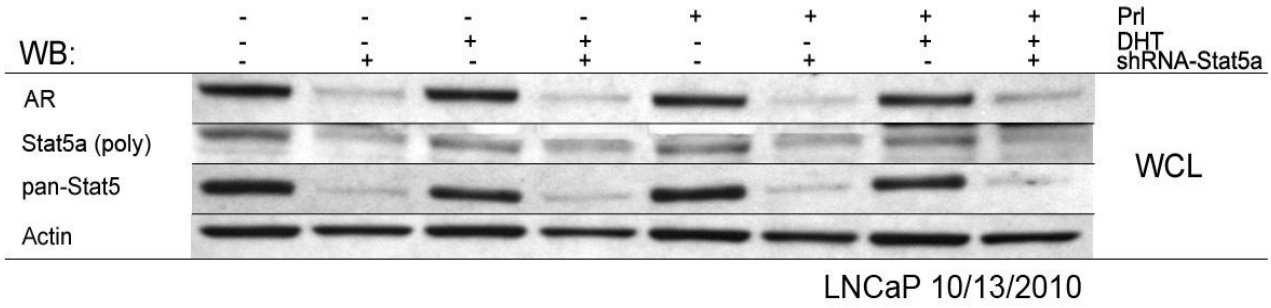

WB:
Prl
DHT
shRNA-Stat5a
AR
Stat5a (poly)
pan-Stat5
Actin
WCL
LNCaP 10/13/2010

Fig. 1.b.

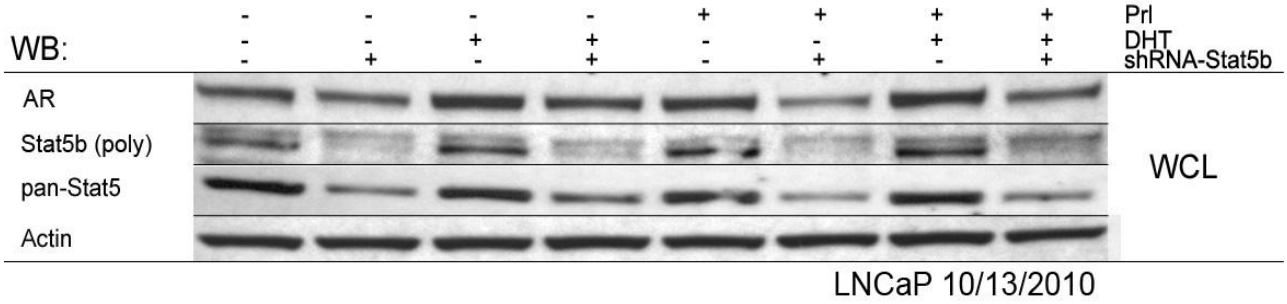
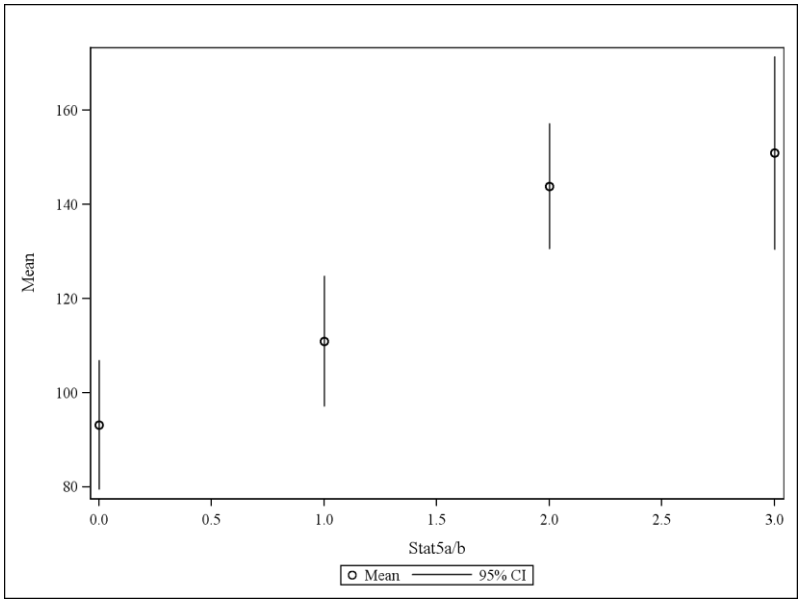

WB:
Prl
DHT
shRNA-Stat5b
AR
Stat5b (poly)
pan-Stat5
Actin
WCL
LNCaP 10/13/2010

Fig. 2.

Stat5a	Mean AR	95% CI		Stat5b	Mean AR	95% CI		Stat5ab	Mean AR	95% CI
0	114.8	(103.7,125.9)		0	113.0	(101.8,124.1)		0	93.2	(79.4,107.0)
1	135.6	(114.8,156.3)		1	131.4	(112.3,150.5)		1	111.0	(97.1,124.9)
2	132.1	(115.9,148.2)		2	137.7	(121.1,154.4)		2	143.9	(130.5,157.2)
								3	151.0	(130.4,171.5)



Research Project 4: Project Title and Purpose

Multidisciplinary Systems Approach to Predicting Response to Combination Therapy - This project is focused on developing the mathematical and experimental framework to determine the molecular mechanisms for the efficacy of cancer therapies based upon the combination of existing drugs. This model is important for not only predicting whether two drugs in combination will have synergy, but will also be used for determining the protein biomarkers of response for patients. The aim of this project is to find a mathematical model based on fuzzy logic that could accurately predict a patient's response to drugs in a clinical setting as it would facilitate the individualization of patient treatment and help to find some prognostic and predictive markers of breast cancer.

Duration of Project

1/1/2007 - 12/31/2010

Project Overview

Objective: to develop a mathematical model that will accurately predict the response of

chemotherapeutic drugs alone and in combination in breast cancer cell lines and xenograft mouse models.

Develop a fuzzy-logic based signaling model that describes the complex interactions of the altered pathways due to combination therapy using HDAC inhibitors and anti HER2/neu therapies. The researcher will use a combination of these drugs at different doses as well as siRNA to perturb the molecular pathways in the SK-BR-3 cell line. Protein-protein interactions will be determined by co-immunoprecipitation and mass spectrometry and the researcher will utilize a combination of proteomics techniques to determine changes in protein expression. The researcher will pay particular attention to the differential expression of the phosphorylated proteins. Recently developed computational techniques will be used to describe signaling pathways.

Specific Aim 1. Define response as the phenotypic change in cell death and growth. Determine response upon treatment of the HDAC inhibitors TSA, valproic acid, and MS-275 in combination with anti-HER2/neu therapy in the SK-BR3 breast cancer cell line.

Specific Aim 2. Determine global changes in protein expression resulting from drug treatments described in Specific Aim 1.

Specific Aim 3A. Use the data from Specific Aims 1-2 to develop a fuzzy logic based signaling model of cellular response; 3B Use the model developed in Specific Aim 3A to identify key interacting proteins of interest, modify their expression using siRNA, and determine response to drugs to validate and refine the model.

Specific Aim 4. Test the protein interaction model developed using a mouse xenograft model. Determine the differentially expressed proteins in the tumors and serum during treatment.

Principal Investigator

Andrew A. Quong, PhD
Thomas Jefferson University
815 Bluemle Life Sciences Building
233 South 10th Street
Philadelphia, PA 19107

Other Participating Researchers

Wen-Shuz Yeow, PhD - employed by Thomas Jefferson University

Expected Research Outcomes and Benefits

The proposed research aims at establishing a research program aimed at the rational design of combination therapy for breast cancer. Although this program is initially focused on breast cancer, the computational and experimental tools that will be developed will be useful for describing signaling pathways underlying not only other diseases but cellular processes in

general. In the long run, the benefits of having this toolset are the ability to examine the molecular signature of a patient's disease and help with the design of an individualized therapy. As part of this, the model would also provide a molecular signature of response that can be used to more quickly monitor a patient to see if they are responding to the treatment. Such an approach is needed in cancer therapy where commonly used agents are ineffective in many patients.

Summary of Research Completed

Specific Aim 1. Define response as the phenotypic change in cell death and growth**.**

A number of experiments were designed to determine the change in proliferation of hormone responsive breast cancer cells to two drugs with chemo-preventative potential: tamoxifen and metformin Table 1 summarizes the analysis of drug combination data to determine if there is evidence for synergy or antagonism. The measured response (i.e. absorbance measured in the MTT assay) at each time point for each combination dose is listed in the measured column. Data listed in Table 1 indicate that tamoxifen and metformin are antagonistic at a majority of the dose combinations tested at both time points.

Specific Aim 2. Determine global changes in protein expression resulting from drug treatments described in Specific Aim 1.

A quantitative proteomics experiment was designed to investigate molecular mechanisms of action for tamoxifen and metformin and to describe the mechanisms that contribute to the antagonistic behavior observed in the data described in Specific Aim 1. A label-based proteomic method was used to measure protein expression in the control and tamoxifen treated cells at all three time points and the metformin treated cells at 72 hours. The 121 Da iTRAQ reagent was used to label an internal reference standard of proteins. Differential expression of proteins in control vs. drug treated conditions at each time point was identified from normalized protein expression values using the two-sample t-test with a significance p-value threshold of 0.1. Approximately 50 proteins were differentially expressed in each condition tested (i.e. tamoxifen vs. control, 24, 48 and 72 hours; metformin vs. control, 72 hours). A total of 131 proteins were identified as differentially expressed in tamoxifen treatment at all time-points.

Functional enrichment analysis of differentially expressed proteins implicated that tamoxifen treatment affects a number of biological processes including metabolism, cell cycle, DNA repair, and protein translation and degradation. A significant number of proteins involved with metabolism and cell division are differentially expressed following treatment with tamoxifen or metformin. Many of these proteins are down-regulated in comparison with control treatment. This observation is consistent with the anti-proliferative effect of these drugs and suggests that the identified differentially expressed proteins are biologically relevant. However, functional enrichment analysis does not indicate mechanisms by which antagonism between tamoxifen and metformin might result. From our experience, functional enrichment analysis of protein expression data is limited by the relatively small number of detected proteins in each pathway. Based on these results, we contend that the statistical methods developed for functional enrichment analysis of gene expression microarray will have limited utility in quantitative

proteomics analysis until the number of proteins detected can be increased significantly. These results demonstrate the need for alternative methods to gain insight into biological mechanisms and generate testable hypotheses on the basis of quantitative proteomics data.

Specific Aim 3A. Use the data from Specific Aims 1-2 to develop a fuzzy logic based signaling model of cellular response; 3B. Use the model developed in Specific Aim 3A to identify key interacting proteins of interest, modify their expression using siRNA, and determine response to drugs to validate and refine the model.

A fuzzy logic framework previously reported was used to generate a computational model of the mechanism of action of tamoxifen based on the expression data of differentially expressed proteins identified in Specific Aim 2. Fuzzy logic models of protein expression are represented as directed graphs, in which nodes represent proteins and edges symbolize co-regulatory or mechanistic relationships. Fuzzy models relating the expression of proteins that demonstrate differential expression patterns in breast cancer cells treated with tamoxifen were generated. A number of the derived interactions reflect known biological relationships between proteins.

Identify relationships that are indicative of known biology. These examples indicate that the relationships derived using the fuzzy logic algorithm are biologically relevant. Figure 1 shows the network of co-regulatory relationships derived by integrating the fuzzy logic models of expression of each protein. Nodes (i.e. proteins) are identified by their IPI accession number as in Table 2. Edges (i.e. co-regulatory relationships) are annotated with an arrow head such that input proteins point to modeled proteins. The network consists of a connected graph of 126 nodes and 377 directed edges. Nodes that are highly connected in the network represent proteins that influence the expression of many other proteins. We hypothesized that these highly connected nodes are likely to represent proteins that are important regulators of the response to tamoxifen treatment in hormone responsive breast cancer cells. To identify these key regulatory proteins, we ranked nodes according to their outdegree, the number of edges that point from the node to another node. Table 2 lists the IPI accession number, protein description, and gene symbol for these proteins along with the fold-change of expression in tamoxifen treatment and metformin treatment. In the future, we will investigate the role of these proteins in the mechanisms of action of tamoxifen and metformin experimentally.

Table 1. Effect of combinations of metformin and tamoxifen on proliferation of MCF7-Bus cells.

		Day 2			Day 4		
Met Dose (mM)	Tam Dose (μM)	Measured	Predicted	Difference	Measured	Predicted	Difference
0	0	1.033			1.352		
0.5	0	0.886			1.241		
1	0	0.781			1.113		
5	0	0.718			0.933		
10	0	0.691			0.815		
20	0	0.587			0.581		
0	0.5	0.778			1.235		
0.5	0.5	0.74	0.668	**0.071**	1.269	1.135	**0.134**
1	0.5	0.787	0.589	**0.198**	1.211	1.018	**0.193**
5	0.5	0.737	0.541	**0.196**	0.942	0.853	**0.089**
10	0.5	0.878	0.521	**0.357**	0.927	0.745	**0.182**
20	0.5	0.681	0.443	**0.238**	0.685	0.531	**0.154**
0	1	0.935			1.416		
0.5	1	0.898	0.802	**0.096**	1.472	1.3	**0.171**
1	1	0.78	0.707	**0.073**	1.203	1.166	**0.037**
5	1	0.943	0.65	**0.293**	1.067	0.977	**0.089**
10	1	0.797	0.625	**0.172**	1.095	0.853	**0.242**
20	1	0.741	0.532	**0.209**	0.788	0.608	**0.18**
0	5	0.843			1.144		
0.5	5	0.912	0.724	**0.188**	1.222	1.051	**0.171**
1	5	0.834	0.637	**0.196**	1.471	0.942	**0.528**
5	5	0.791	0.586	**0.205**	1.052	0.79	**0.262**
10	5	0.722	0.564	**0.158**	0.961	0.69	**0.272**
20	5	0.582	0.48	**0.102**	0.74	0.492	**0.249**
0	10	0.77			1.109		
0.5	10	0.707	0.661	**0.046**	0.894	1.018	**-0.125**
1	10	0.682	0.582	**0.099**	0.856	0.913	**-0.057**
5	10	0.646	0.535	**0.111**	0.761	0.765	**-0.005**
10	10	0.711	0.515	**0.196**	0.772	0.668	**0.104**
20	10	0.647	0.438	**0.208**	0.524	0.476	**0.047**
0	20	0.725			0.791		
0.5	20	0.714	0.623	**0.091**	0.677	0.726	**-0.049**
1	20	0.716	0.548	**0.167**	0.687	0.651	**0.035**
5	20	0.608	0.504	**0.103**	0.391	0.546	**-0.154**
10	20	0.552	0.485	**0.066**	0.299	0.477	**-0.177**
20	20	0.403	0.413	**-0.009**	0.27	0.34	**-0.069**

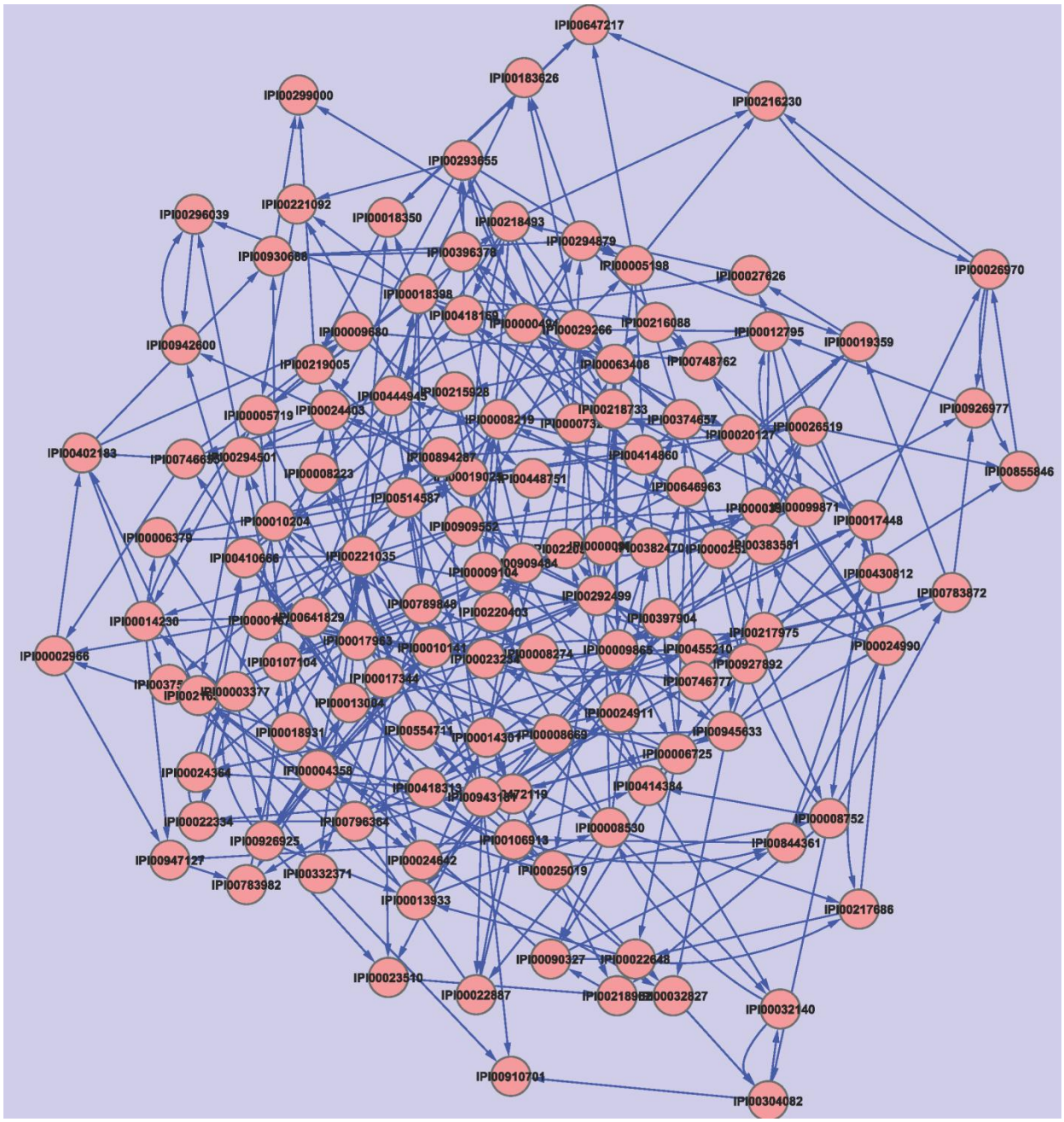


Figure 1. Network of inferred co-regulatory relationships between proteins that show differential expression in tamoxifen treatment vs. control.

Table 2: Highly connected proteins with differential expression patterns in tamoxifen and metformin treatments compared to control.

IPI	Gene Symbol	Protein Name	Fold Change	
			Tam Day 3	Met Day 3
IPI00019025	PARD6B	PARTITIONING DEFECTIVE 6 HOMOLOG BETA.	1.189	0.571
IPI00024990	ALDH6A1	METHYLMALONATE-SEMIALDEHYDE DEHYDROGENASE [ACYLATING], MITOCHONDRIAL.	1.055	0.878
IPI00218493	HPRT1	HYPOXANTHINE-GUANINE PHOSPHORIBOSYLTRANSFERASE.	0.885	1.009
IPI00292499	HSPA14	HEAT SHOCK 70 KDA PROTEIN 14.	1.091	0.809
IPI00418169	ANXA2	ISOFORM 2 OF ANNEXIN A2.	0.889	1.129

Research Project 5: Project Title and Purpose

Cyclin D1 Regulation of Cellular Migration and Invasion - These studies aim to further understanding of how cyclin D1 collaborates in cellular invasion, migration and transformation. The abundance of the cyclin D1 gene product is rate-limiting in progression through the G_1 phase of the cell-cycle and is required for transformation by Ras, ErbB2, Src, Stats, Wnt and mutations of the *Apc* gene. The researcher's laboratory has shown that cyclin D1 promotes DNA synthesis and cellular survival. Recent studies from our laboratory using *cyclin D1*$^{-/-}$ mice and cyclin D1 siRNA have demonstrated that cyclin D1 regulates cellular migration and invasion. The proposed studies will determine the mechanism by which cyclin D1 regulates migration in cells and *in vivo*. Several novel technologies developed in this laboratory will be used in the proposed studies to provide high-resolution analysis of the mechanisms governing this process.

Duration of Project

1/1/2007 – 12/31/2009

Summary of Research Completed

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