

# Thomas Jefferson University

## Annual Progress Report: 2009 Formula Grant

### Reporting Period

July 1, 2010 – June 30, 2011

### Formula Grant Overview

Thomas Jefferson University received \$3,746,521 in formula funds for the grant award period January 1, 2010 through December 31, 2013. Accomplishments for the reporting period are described below.

### Research Project 1: Project Title and Purpose

*Genetic Targets of Breast Tumor-Initiating Cells* - Breast cancer is the second most prevalent cancer-related death in women in the United States. A subpopulation of breast tumor cells have been identified and are referred to as “breast tumor-initiating cells” or cancer stem cells (CSCs). These self-renewing CSCs were first described in the hematopoietic system, and have now been putatively identified in breast, colon and brain tumors. It has been proposed that all tumor metastasis are caused by CSCs, and it is suspected that these cells contribute to therapy resistance and tumor recurrence. The focus of this project is to determine new genetic mechanisms governing breast CSC expansion and invasion, and to identify specific secreted factors regulated by gene deletion, to provide CSC-based therapies.

### Anticipated Duration of Project

1/1/2010 - 12/31/2013

### Project Overview

The goal of this project is to determine new genetic mechanisms governing CSC expansion and invasion, using transgenic mice. Over the last 15 years, this laboratory has generated mammary gland-targeted gene excision/inactivation mice, and crossed these mice to mammary gland-targeted oncogene mice. These mice and the genetic material and tissues previously derived from these animals, will be used to determine the role of six specific genes, in the expansion of CSC.

Specific Aim: Determine the role of genetic determinants in CSC expansion. The relative proportion of cells with characteristics of breast CSCs will be assessed in transgenic mice and tumor-derived cell lines by genetic deletion of Notch, Akt1, c-jun, NFκB, p21<sup>Cip1</sup> and DACH1, using multiple assays (self renewal, self surface markers, Aldh1 production, lineage repopulation).

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None

## **Expected Research Outcomes and Benefits**

Each of the six genes being examined in this project has been shown to regulate breast tumor proliferation and growth. Identification of specific secreted factors regulated by gene deletion may be used for CSC-based therapies. The transgenic mice and cell lines generated in this laboratory have the unique advantage of providing a normal immune system. Studies conducted in immune-deficient mice may ignore important effects of the immune system on tumor progression. This is particularly important in these studies, as the factors identified in breast CSCs that regulate expansion are also made by the normal immune system. This laboratory has established a subtractive proteomic approach and, based on the prior 15 years developing unique genetic mouse models, it is uniquely positioned to determine the importance of these key target genes in CSC expansion and the role of specific secreted factors in response to therapy.

This project will characterize a completely novel tumor and metastasis pathway if these functional analyses identify secreted factors and relevant antibodies which govern breast tumor proliferation and growth *in vivo*.

## **Summary of Research Completed**

In order to examine further the mechanisms by which DACH1 inhibited cellular growth and tumorigenesis, genome-wide expression studies were conducted of DACH1-transduced cells. DACH1 repressed gene expression of signaling pathways governing hematopoietic cell lineage, cellular communication, blood vessel development, and multicellular organismal development. DACH1 induced an acute inflammation response and cytokine-cytokine receptor interaction (Fig. 1A). Several recent studies have suggested the molecular circuitry controlling stem cells may be active in certain tumors. Some of the key regulators of embryonic stem cell (ES) identity, *Oct4*, *Sox2* and *Nanog* are expressed in specific tumors. Comparison of Dach1 regulated genes in Met-1 cells to gene sets associated with ES cell identity via gene set enrichment analysis, demonstrated Dach1 downregulates expression of Sox2, Oct4 gene targets, NOS targets (genes common to Nanog, Oct4 and Sox2) and a gene set overexpressed in hES cell lines (Fig. 1B). We examine whether expression of DACH1 could inhibit expression of genes associated with the cancer stem cell phenotype.

Quantitation of the relative expression of the ES cell markers (Sox2, Oct4, Nanog, KLF4 and c-Myc) was conducted using mRNA from the Met-1 cells expressing DACH1 or control (Fig. 1C). QT-PCR analysis demonstrated a reduction in the abundance of *Sox2*, *Nanog* and *KLF4*. Each of these genes promotes stem cell expansion. In order to determine the functional significance of KLF4 and Sox2 repression by DACH1, DACH1 transduced Met-1 cells were transfected with expression vectors encoding KLF4 or Sox2. A FACS analysis was conducted to examine the relative proportion of CD24<sup>-</sup>/CD44<sup>+</sup> cells. DACH1 reduced the proportion of CD24<sup>-</sup>/CD44<sup>+</sup> by ~80%. Re-expression of KLF4/Myc or Sox2/Oct4 partially reversed the phenotype (Fig. 1D, E).

*DACH1 binds promoters of genes governing progenitor cell expansion in ChIP and ChIP-Seq.*

ChIP-Seq analysis was conducted of MDA-MB-231 cells expressing DACH1 in order to determine whether DACH1 bound the promoters of stem cell regulatory genes. DACH1 occupancy was identified at the Sox2, Nanog, KLF4 and Lin28 promoters (Fig. 2A). Sox2, KLF4 and Lin28 are known to play an important role in the maintenance of stem cell pluripotency.

Comparison was made using Met-1 cells expressing Flag tagged DACH1 or control vector. ChIP of the Sox2 promoter was conducted using oligonucleotides directed to either the distal or the proximal promoter. ChIP for DACH1 at the distal promoter failed to identify chromatin associated DACH1, however oligonucleotides directed to the proximal promoter including the Sox2 binding site demonstrated the recruitment of DACH1 (Fig. 2B). Similarly, the ChIP analysis of the Nanog promoter identified DACH1 recruitment to the proximal but not distal promoter region (Fig. 2C). As DACH1 had reduced the expression of Sox2 and Nanog, studies were conducted to determine whether the *Sox2* and *Nanog* genes were directly repressed by DACH1. The promoter of the *Sox2* and the *Nanog* genes were directly repressed by DACH1 expression (Fig. 2D, E). Deletion of the DACH1 DS domain abrogated transcriptional repression DACH1 (Fig. 2D, E).

Herein, an MEC-targeted inducible transgenic inhibitor of NF- $\kappa$ B (I $\kappa$ B $\alpha$ SR) was developed in ErbB2 mammary oncomice. Inducible suppression of NF- $\kappa$ B in the adult mammary epithelium delayed the onset and number of new tumors. Within similar sized breast tumors, TAM and tumor neoangiogenesis was reduced. Coculture experiments demonstrated MEC NF- $\kappa$ B enhanced TAM recruitment. Genome-wide expression and proteomic analysis showed that I $\kappa$ B $\alpha$ SR inhibited tumor stem cell pathways. I $\kappa$ B $\alpha$ SR inhibited breast tumor stem cell markers in transgenic tumors, reduced stem cell expansion in vitro, and repressed expression of Nanog and Sox2 in vivo and in vitro. MEC NF- $\kappa$ B contributes to mammary tumorigenesis. As we show that NF- $\kappa$ B contributes to expansion of breast tumor stem cells and heterotypic signals that enhance TAM and vasculogenesis, these processes may contribute to NF- $\kappa$ B-dependent mammary tumorigenesis.

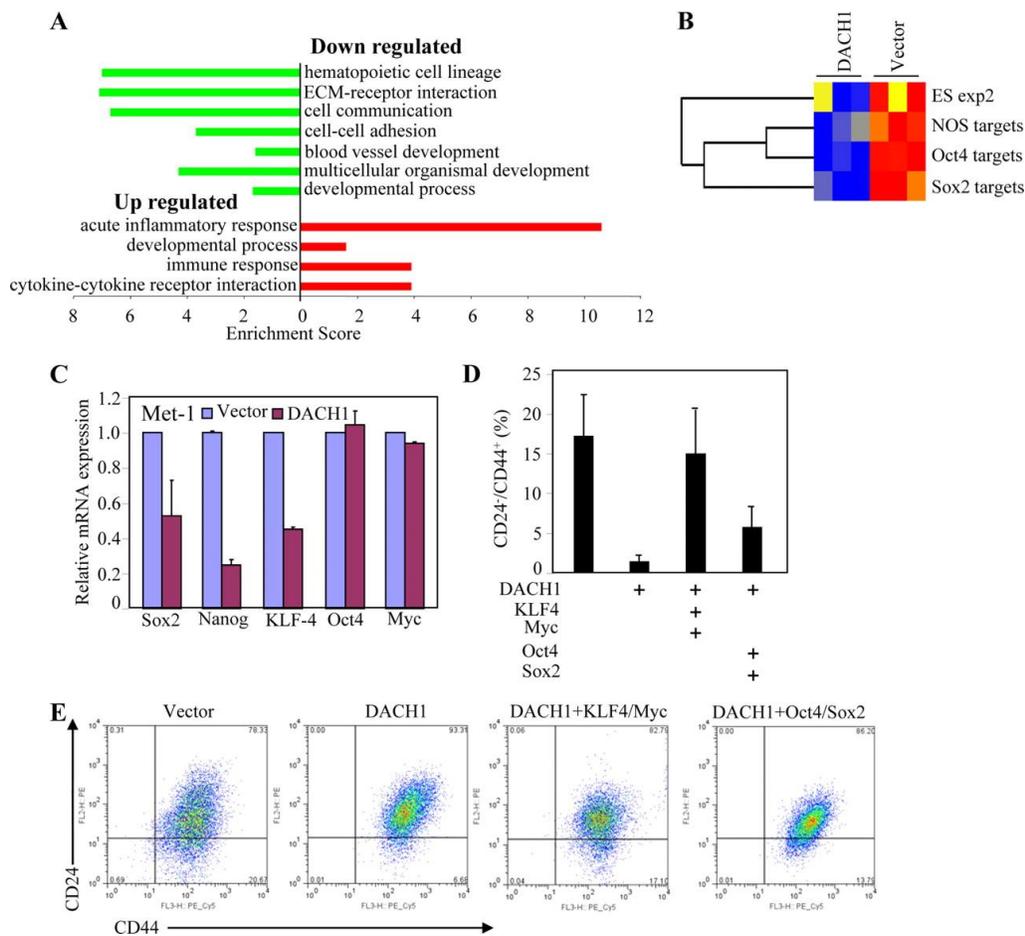
These findings were published in:

Wu, K., Jiao, X., Li, Z., Katiyar, S., Casimiro, M.C., Yang, W., Zhang, Q., Willmarth, N.E., Chepelev, I., Crosariol, M., Wei, Z., Li, A., Zhao, K., Pestell, R.G. The Cell-Fate Determination

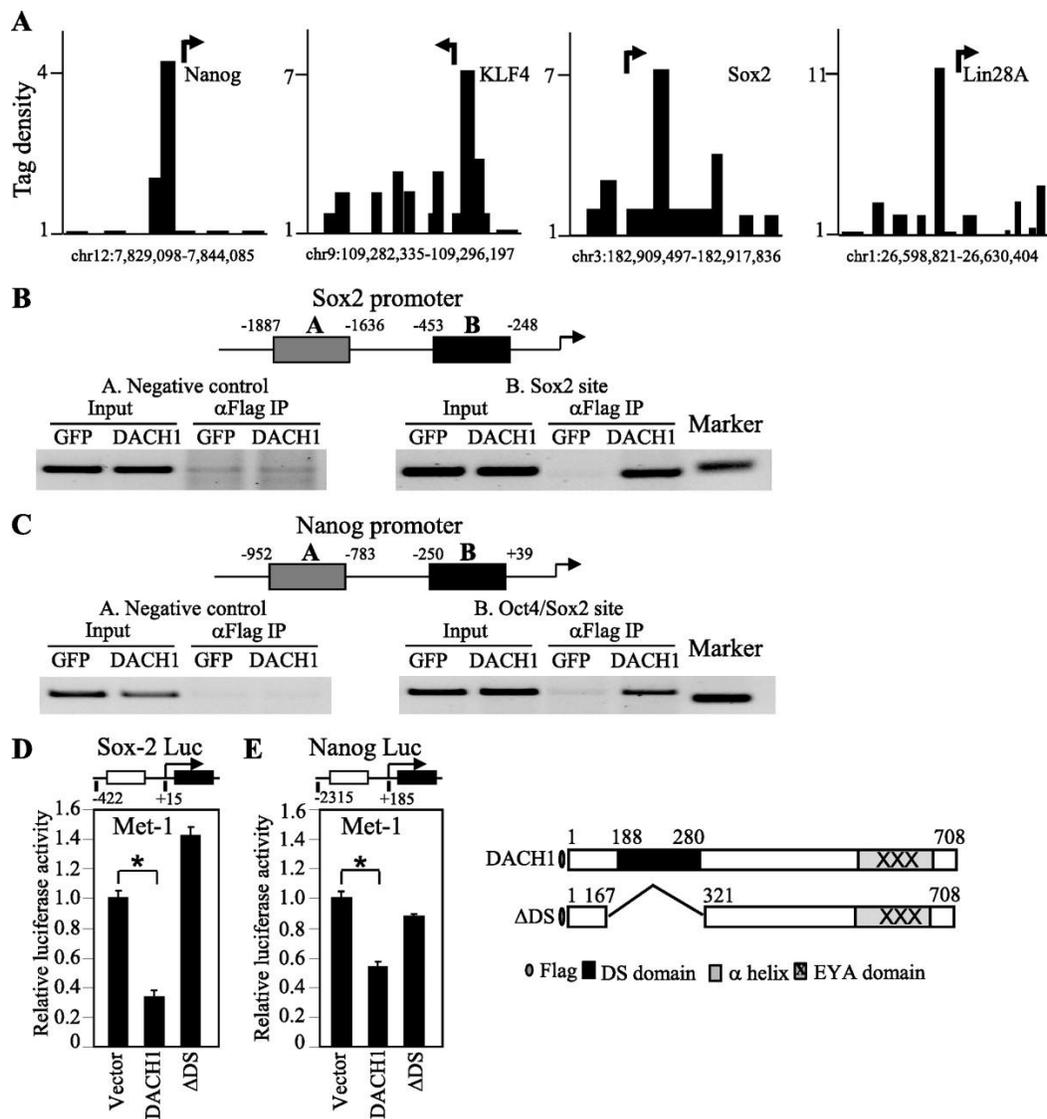
Factor Dachshund Reprograms Breast Cancer Stem Cell Function. J Biol Chem. 2010 Oct 11.

Liu , M., Sakamaki, T., Casimiro, M., Willmarth, N., Quong, A., Ju, X., Ojeifo, J., Jiao, X., Yeow, WS., Wang, C., Katiyar, S., Shirley, L., Albanese, C., Joyce, D., Pestell, R.G. The canonical NF- $\kappa$ B pathway governs mammary tumorigenesis in transgenic mice via tumor stem cell expansion . *Cancer Res* . 2010 Dec 15;70(24):10464-10473

We published a review article on this topic: Ve lasco-Velázquez MA, Popov VM, Lisanti MP, Pestell RG. The Role of Breast Cancer Stem Cells in Metastasis and Therapeutic Implications. *Am J Pathol*. 2011 May.



**Figure 1. Pathway analysis.** *A*, pathway analysis of microarray data from Met-1 cells expressing DACH1 or control vector by DAVID using Gene Ontology and KEGG pathway sets. Pathways are graphically represented by enrichment score. *Red* indicates up-regulated and *blue* indicates down-regulated. *B*, gene sets enrichment analysis of microarray from Met-1 cells using gene targets of Sox2, Oct4, or NOS (Nanog, Oct4, and Sox2), which are enriched in ES cells (*A*). *C*, real time RT-PCR detection of ES cell-related gene expression (data are mean  $\pm$  S.E.,  $n = 3$ ). *D*, quantitation of CD24<sup>-</sup>/CD44<sup>+</sup> staining of multiplicate transductions. *E*, FACS analysis of Met-1 cells transduced with viral vectors encoding KLF/c-Myc or Oct4/Sox2.



**Figure 2. DACH1 represses Sox2 and Nanog transcription.** (A) DACH1-dependent tag density at selected gene promoters. Arrow indicates the start site and direction of transcription. (B) Chromatin immunoprecipitation assays of the Sox2 and (C) Nanog promoter using Met-1 cells expression Flag-tagged DACH1 or control GFP vector. Schematic representation of oligonucleotide probes directed to the promoters. (D) Luciferase reporter gene assays of Sox-2 and (E) Nanog DACH1 wild type or a mutant of the conserved DS domain were co-transfected. The data are mean  $\pm$  SEM of  $n = 6$  separate transfections ( $p < 0.001$ ).

## **Research Project 2: Project Title and Purpose**

*Phosphorylated Proteins in Breast Cancer Prognosis* - Our overall objective is to develop techniques and protocols to measure quantitative changes in protein levels in the stromal and epithelial compartments of clinical samples to 1) identify new prognostic markers of response to

therapy and outcome and 2) to understand the role of autocrine and paracrine signaling associated with the tumor microenvironment.

### **Anticipated Duration of Project**

1/1/2010 - 6/30/2012

### **Project Overview**

In this project, we aim to classify retrospectively collected tumor samples using phosphopeptide expression profiles and apply these profiles to classify tumor types. We hypothesize that the observed heterogeneity in clinical characteristics and therapeutic response in breast cancer populations is associated with differential expression of phosphopeptides. In order to investigate this hypothesis, we have defined the following objectives:

Specific Aim 1: Optimize protocols for the extraction of total proteins and phosphoproteins from formalin fixed tissue that is compatible with the iTRAQ reagents and with Multiple Reaction Monitoring (MRM) experiments.

Specific Aim 2: Optimize the methods for laser capture micro-dissection of formalin fixed tissue using slides that have been stained with Hematoxylin and two-color Immunohistochemistry that are compatible with mass spectrometry.

Specific Aim 3: Determine the differences in both total protein expression and phosphoprotein expression in the epithelium and stroma of normal breast tissue and in tumor tissue.

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### **Expected Research Outcomes and Benefits**

Breast cancer is a heterogeneous disease in which tumors that are similarly classified by traditional diagnostic and prognostic indicators can have markedly different clinical outcomes. Although metrics such as lymph node status, histological grade, tumor size, hormonal receptor status and the presence of distant metastases are correlated with prognosis, these clinical parameters indicate little about the biology of the disease. In addition, the development and

effectiveness of targeted therapies in defined patient populations in breast cancers (e.g. Tamoxifen, estrogen receptor-positive breast cancer; Trastuzumab, *ERBB2*-positive breast cancer) makes accurate sub-classification of tumors a priority.

Altered function of signaling pathways also plays a role in clinical response. For example, constitutive phosphorylation of *AKT* in the *PI3K* pathway mediated by loss of *PTEN* or mutation of *PIK3CA* has been identified as a mechanism of Trastuzumab resistance. Phosphopeptide expression profiling provides the means to catalog expression signatures of this and other similar mechanisms of drug action and resistance, and generate a tumor sub-classification system that more accurately predicts clinical outcome.

Our expectation is that examining changes in the protein levels associated with the loss or gain of particular gene products in human tumors will provide new insight into the signaling mechanism associated with disease development, progression and response to therapy that cannot be captured in either cell culture or animal models alone.

### **Summary of Research Completed**

This project has three specific aims that examine the use of proteomics to determine if changes in phosphorylated proteins in breast tumors and if changes in total proteins in the epithelium and the stroma can be used to stratify patients to clinical response or identify new aberrant signaling pathways in the tumors.

During this reporting period, we have been evaluating alternative strategies for Aim 1 -Optimize protocols for the extraction of total proteins and phosphoproteins from formalin fixed tissue that is compatible with the iTRAQ reagents and with Multiple Reaction Monitoring (MRM) experiments.

For Aim 1, the issue that has arisen is that the current mass spectrometry instrumentation we employ does not have sufficient sensitivity for the very small samples obtained from the clinical material. In addition, the MALDI ToF mass spectrometer that we used for these studies has difficulty fragmenting the singly charged phosphopeptides. Because of these two issues we initiated the process of purchasing a new instrument and using some funds from this grant to pay for the instrument. We spent a fair amount of time benchmarking instruments and narrowed this down to a QToF instrument and we evaluated offerings from both Agilent and Waters. We have ordered the Agilent 6520 QToF with the chipcube system. The additional advantage offered by the Agilent system is the use of a microfluidics systems that uses customizable chips for additional separations. One such chemistry includes a TiO<sub>2</sub> trapping column for the isolation of phosphopeptides. The chip only requires a small amount of protein consistent with our clinical samples. We will be using this system for the analysis of the samples beginning around August 15, 2011.

We are also working extensively on Specific Aim 3 - Determine the differences in both total protein expression and phosphoprotein expression in the epithelium and stroma of normal breast tissue and in tumor tissue.

For Aim 3, we have been focusing our work on the identification of differentially expressed

proteins in the stroma and epithelial cells in the tumor. This work is ongoing and required a significant amount of time to dissect the slides. The first set of experiments did not work as well as we would like and we are repeating the experiment with additional samples. As before, we are examining eight breast cancer tumors, four of which stained positively for estrogen and progesterone receptors and are negative for Her2. The other four samples are the triple negatives and do not show positive staining for all three of the receptors. Laser Capture Microdissection is done and we will be analyzing the samples on the new mass spectrometer.

### **Research Project 3: Project Title and Purpose**

*The Role of Lipogenesis Pathway in Endometrial Cancer Progression* - The number of women with newly diagnosed endometrial cancer (EC) increased 20% from 1987; however the number of deaths posted a 168% increase during the same time period. Estimated new cases and deaths from EC in the United States in 2009 are 42,160 and 7,780, respectively. As population size affected by this disease is expected to grow, particularly in developing countries, EC will continue to be a serious public health problem. Despite the fact that over-expression of lipogenic enzymes were observed in endometrial tumors, the role of the master regulator of lipogenesis, sterol regulatory element-binding protein 1 (SREBP1), is largely unknown. This project will determine the role of SREBP1 and DACH1, a newly identified transcriptional repressor, in endometrial tumorigenesis and the mechanisms by which lipogenic pathways regulate tumor progression.

### **Anticipated Duration of Project**

1/1/2010 - 12/31/2011

### **Project Overview**

The significant increase of lipogenesis is a metabolic hallmark of rapidly proliferating tumor cells. Although most normal cells acquire the bulk of their fatty acids from circulation, tumor cells synthesize more than 90% of required lipids de novo. Consistent with a great demand for lipid synthesis, diverse human cancer cells express high levels of lipogenic enzymes, such as fatty acid synthase (FASN). Although the clinical correlations between the over-expression of lipogenic enzymes and tumorigenesis are well documented, little is known about the molecular mechanisms by which the lipogenic pathway is enhanced in tumorigenesis. The sterol regulatory element-binding protein 1 (SREBP1) and peroxisome proliferator-activated receptor gamma (PPARgamma) are master regulators of lipogenesis in diverse organisms.

Previous studies have established that FASN, one of the major transcriptional targets of SREBP1, promotes synthesis of various fatty acids, which then serve as ligands for PPARgamma activation. However, whether SREBP1, FASN, and PPARgamma contribute to endometrial carcinogenesis has not been systematically investigated. Excitingly, the preliminary data of this project suggests that DACH1, a newly identified transcriptional repressor, may negatively regulate FASN expression. DACH1 is down-regulated in endometrial cancers, and the re-introduction of DACH1 reverts tumor-associated phenotypes. In this project, we hypothesize that increased SREBP1 and loss of DACH1 are associated with endometrial cancer progression. They

may function collaboratively to promote endometrial tumorigenesis through enhanced lipogenesis.

To test this hypothesis, the correlation between cancer progression and lipogenic gene expression in human endometrial cancer samples will be determined. By using three independent endometrial tissue microarrays and frozen tissues together with experimental approaches including immunohistochemistry (IHC) staining, quantitative real-time PCR (qRT-PCR), and Western Blot, determination will be made of the correlation between 1) endometrial cancer (EC) progression and levels of the key lipogenic enzymes, such as SREBP1, FASN, and PPARgamma; 2) lipogenic gene expression and other known prognostic factors; and 3) evaluation of the diagnostic values of SREBP1 in predicting survival and metastasis in EC.

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### **Other Participating Researchers**

None

### **Expected Research Outcomes and Benefits**

Several reports have noted an enhanced lipogenic pathway in advanced stages of human cancer compared to normal tissue, including human endometrial cancer, in which the PPARgamma and SREBP1 targeted genes such as FASN and SCD1 are induced to high levels of expression. Interestingly, elevated expression of SREBP1 coincided with malignant transformation, cancer progression, and metastasis for several cancer types, particularly hormone-responsive tissues including breast and prostate cancers. The role of SREBP1 in endometrial cancer is largely unknown. Given the evidence of elevated expression of SREBP1 transcriptional target genes, one aim is to first determine the SREBP1 expression status in endometrial cancer. Increased nuclear staining for SREBP1 in higher grade (G2-G3) tumors from our pilot study suggests that enhanced SREBP1 transactivation may contribute to endometrial cancer progression through induction of lipogenic gene expression and lipogenesis.

In this project, there will be expansion of analysis to determine whether increased expression and/or nuclear translocation of SREBP1 are the preferred mechanism for lipogenesis in human endometrial cancer. This project will allow identification of the molecular mechanisms underlying lipogenesis-dependent tumor growth in endometrial cancer. This is important because the data will provide new strategies for targeting endometrial cancer progression; according to this model, targeted SREBP1 inhibition may efficiently inhibit tumor growth. Furthermore, together with known prognostic factors, SREBP1, DACH1, and PPARgamma may serve as

diagnostic biomarkers for human endometrial cancer and may be used to predict response to targeted therapies.

## **Summary of Research Completed**

### *Increased SREBP1 expression in endometrial cancer.*

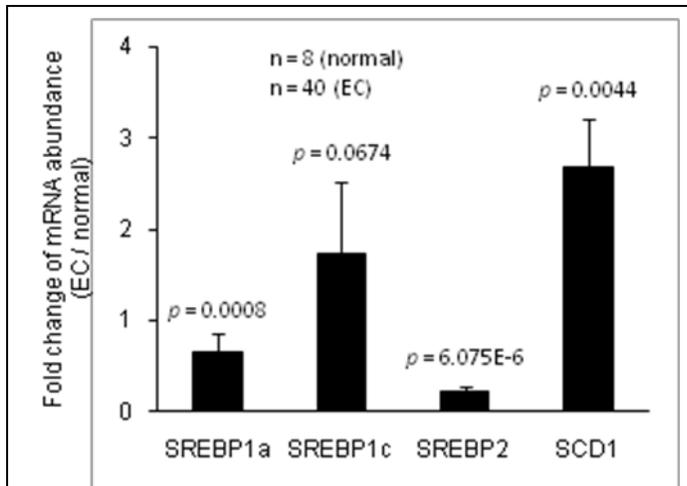
Previous studies indicated that lipogenic gene such as FASN overexpresses in endometrial cancers. The underlying mechanisms by which EC cells overexpress FASN are not well understood. SREBP1 is the major transcription factor which binds to FASN gene promoter and positively regulates FASN expression. We reasoned that elevated SREBP1 expression and/or activity may contribute to enhanced FASN expression in EC. In this study, we first determined the SREBP1 gene expression at mRNA level using quantitative real-time PCR (qRT-PCR) approach on a panel of cDNA samples prepared from endometrial cancers and normal tissues. Compared to normal tissues, slightly decreased SREBP1 mRNA abundance was seen in EC tissues (Fig. 1). Immunohistochemical (IHC) staining procedures for SREBP1 on human endometrial cancer samples have been validated (Fig. 2), showing an increase of SREBP1 protein expression.

The data shows by IHC staining that SREBP1 is over-expressed in poorly-differentiated EC. Nuclear localization of SREBP1 was frequently seen in poorly-differentiated tumors (grade 2-3), but not in well-differentiated tumors (grade 1) (Fig. 2). The majority of SREBP1 was found in the cytoplasm of epithelial cells of normal endometrium and well-differentiated tumors, while nuclear SREBP1 was detected in high-grade from moderately differentiated to poorly differentiated tumors (Fig. 2). These observations strongly support the hypothesis that activated SREBP1 may play a role in tumorigenesis and cancer progression. Furthermore, the result also suggests a posttranslational modification occurring which leads to the stabilization of SREBP1 in cancer cells.

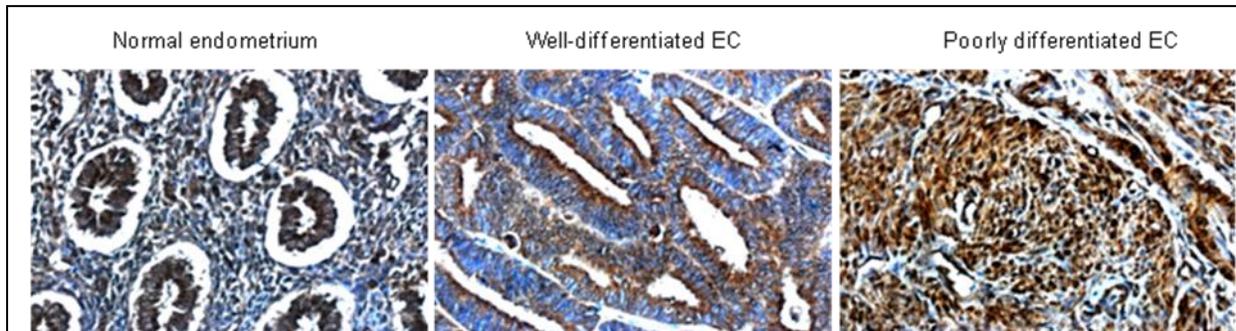
### *SREBP1 is required for cellular proliferation*

In order to determine the role of SREBP1 in cellular proliferation, we conducted experiments to knockdown the endogenous SREBP1 expression. We have performed the initial screening for SREBP1 expression in five commonly used endometrial cell lines including ECC, HEC-1-A, RL95-2, KLE, and AN3-CA. SREBP1 expression was undetectable in the well-differentiated ECC-1 cells, which provided an ideal model for determining the effect of SREBP1 by over-expression (Fig. 3). RL95-2 and AN3CA express relatively higher levels of SREBP1, shCtrl (vector control line) vs shSREBP1 (cell expressing shRNA to target SREBP1) were established (Fig. 4A). The expression of target genes including *FASN* and *SCD1* were reduced in cells transduced with shSREBP1.

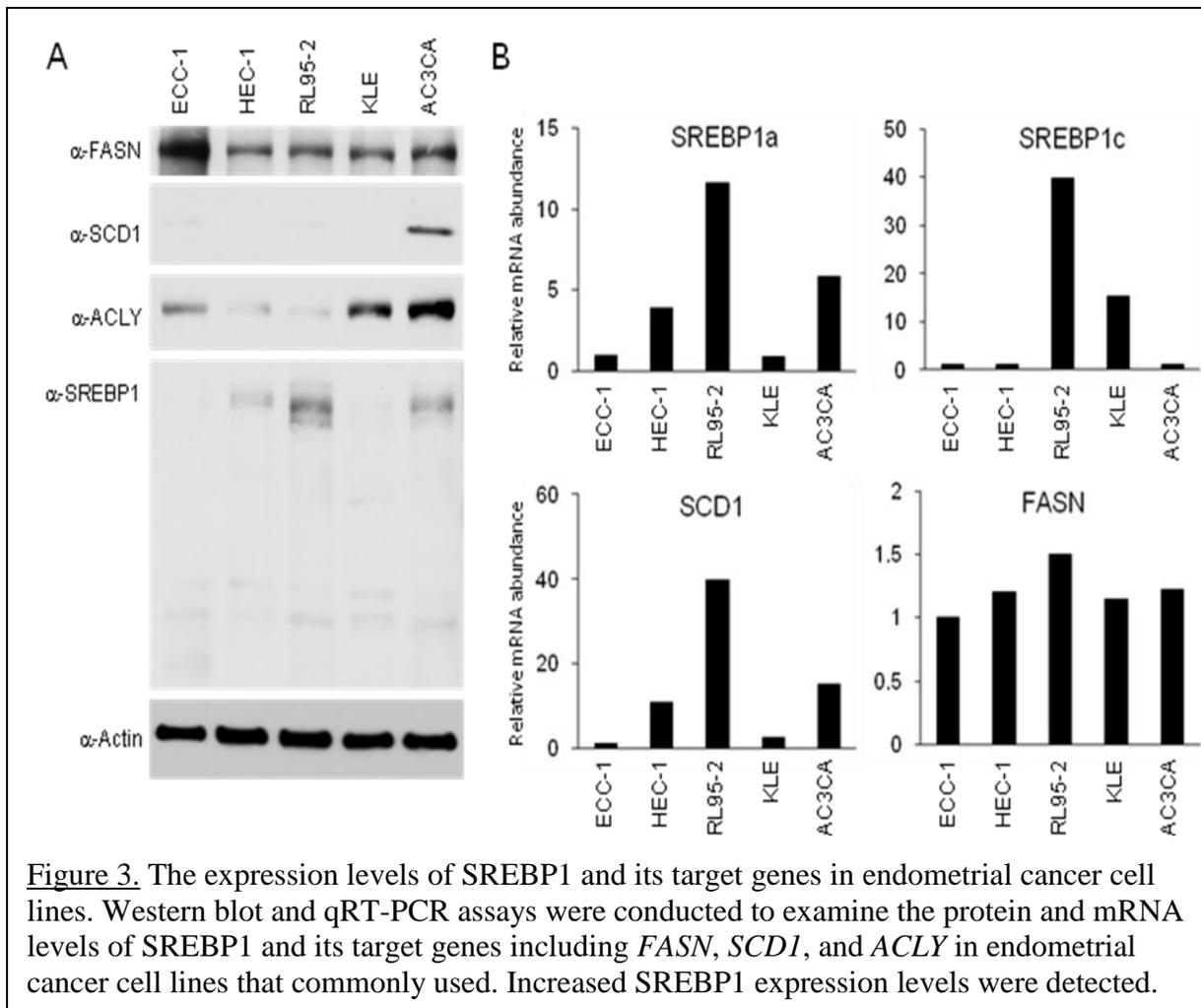
Cell proliferation is tightly controlled by mitogenic signaling and requires the activation of biosynthetic pathways for the generation of macromolecules, including proteins and lipids. Given the evidence that SREBP1 regulates lipogenesis, the metabolic process that supplies cells with lipids, we expect that the knockdown of endogenous SREBP1 will reduce cellular proliferation and cell growth, and promote cell death. As we expected, the knockdown of SREBP1 significantly reduced the cellular proliferation measured by cell viability assays.

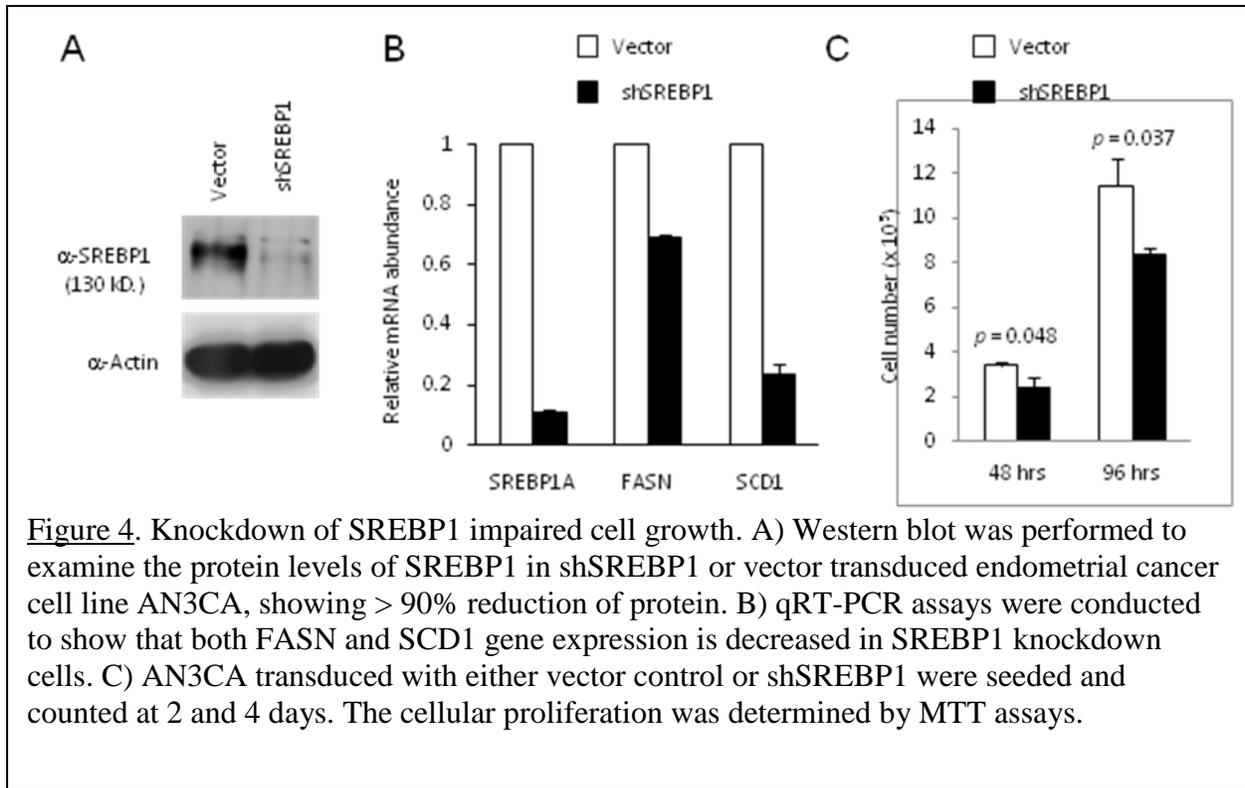


**Figure 1.** The expression of lipogenic genes in endometrial cancer. Quantitative RT-PCR assays were performed on samples derived from endometrial cancer and normal endometrium. Decreased SREBP1 and SREBP2 expression were detected.



**Figure 2.** The expression of SREBP1 in endometrial cancer. IHC staining was conducted to examine the protein levels of SREBP1 in endometrial cancer. Increased SREBP1 expression levels were detected.





**Figure 4.** Knockdown of SREBP1 impaired cell growth. A) Western blot was performed to examine the protein levels of SREBP1 in shSREBP1 or vector transduced endometrial cancer cell line AN3CA, showing > 90% reduction of protein. B) qRT-PCR assays were conducted to show that both FASN and SCD1 gene expression is decreased in SREBP1 knockdown cells. C) AN3CA transduced with either vector control or shSREBP1 were seeded and counted at 2 and 4 days. The cellular proliferation was determined by MTT assays.

**Research Project 4: Project Title and Purpose**

*Tailored Preference Intervention and Colon Cancer Screening in Primary Care* - The purpose of this project is to determine the impact of a mailed, preference-based intervention on colorectal cancer screening among African Americans.

**Anticipated Duration of Project**

1/1/2010 - 6/30/2013

**Project Overview**

Colorectal cancer (CRC) screening can reduce CRC incidence and significantly lower CRC mortality by detecting and removing polyps at an early stage of disease. Unfortunately, screening is underused by African Americans. This project will be a cohort study ancillary to an IRB-approved investigation of African Americans who are 50 to 74 years of age.

This project will assign 427 consenting participants to receive a mailed intervention that is based on the individual’s preferred CRC screening test, ascertained from a baseline survey. This approach will be referred to as a “tailored preference intervention” (TPI). Participants in the TPI group who prefer stool blood testing, will be mailed a stool blood test kit; those who prefer colonoscopy will be mailed instructions for scheduling a colonoscopy; and those who have an equal preference for the two tests will be mailed both. A generic screening reminder will be sent

to participants 45 days after randomization. An endpoint survey and endpoint chart audit will be completed six months following study group assignment. The primary aim of the project is to determine whether screening use is significantly greater in those who received a mailed stool blood test, along with instructions for colonoscopy screening.

### **Principal Investigator**

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### **Other Participating Researchers**

Randa Sifri, MD – employed by Thomas Jefferson University

### **Expected Research Outcomes and Benefits**

The primary goal is to determine if screening utilization in the Tailored Preference Intervention group over the six-month observation period is significantly greater than screening use in the Standard Intervention Group. This outcome will be a dichotomous variable based on data obtained from the endpoint chart audit and the endpoint survey. We will compute the proportion of participants who screened, and the corresponding 95% confidence interval for TPI group participants. Screening test use will encompass CRC screening tests (i.e., SBT, colonoscopy, flexible sigmoidoscopy, or barium enema x-ray) performed during the six-month observation period after randomization. A screening test occurrence will be counted if it is recorded or reported in either source, along with an associated procedure performance date that falls within the study's six-month follow-up period. Through this research, we expect to identify the intervention approach that achieves the highest level of participation.

### **Summary of Research Completed**

The “Tailored Preference Intervention and Colon Cancer Screening in Primary Care” project (SAP# 4100050910) seeks to determine the colorectal cancer screening rate for African American patients who receive a print-based tailored preference intervention. This project is part of a larger, ongoing ACS-funded randomized trial. The addition of a TPI Group to the larger study is intended to determine if screening rates are higher in the TPI Group than in the SI Group.

### **Recruitment**

Patients from primary care practices affiliated with Thomas Jefferson University (TJU) and Albert Einstein Healthcare Network (AEHN) are targeted for inclusion in the project. In accordance with an IRB-approved protocol, electronic medical records are reviewed to assess

age, past CRC screening, and medical history. Patients who are 50 to 79 years of age and have not had a recent CRC screening test are eligible to participate in the project; while those who have had a personal diagnosis of CRC, polyps or a family history of CRC are ineligible. A study invitation letter is sent to the remaining pool of potential participants. The letter states that patients who do not want to participate in the study can “opt out” of further contacts by returning an enclosed “opt out” card or by calling a toll-free number. Professional telephone interviewers contact all potential participants who do not opt out within 14 days. Interviewers assess eligibility, obtain oral informed consent, and administer a baseline survey. Survey respondents are randomly assigned to the Tailored Preference Intervention (TPI) included in this study.

### Accrual

As of July 1, 2011, following an invitation letter mailing and follow-up telephone contacts 115 patients were randomized into the TPI Group.

### Baseline Characteristics of TPI Group Participants

Of the 115 TPI Group participants, 66% were 50-59 years of age, 68% were female, 37% were married, and 39% had more than a high-school education. In terms of perceptions about CRC and screening, 97% of participants viewed screening as a salient and coherent preventive health behavior. We also found that 40% of participants were worried about CRC or screening; and 37% felt that their chances of developing colorectal cancer or polyps were high. Importantly, 94% of participants believed that CRC can be prevented or cured, and 85% thought that their doctor or health care professional supported CRC screening. The distribution of participant CRC screening decision stage at baseline was as follows: 2% had decided against screening, 16% were not considering or were undecided about screening, and 82% had decided to screen. Overall, 15% of participants indicated that they preferred to have SBT screening, 61% had an equal preference for SBT and colonoscopy screening, and 24% preferred colonoscopy screening.

### Intervention Delivery and Outcomes Assessment

Participants in the TPI Group (n=115) who reported on the baseline survey that they preferred stool blood testing were mailed an stool blood test (SBT) kit; while those who preferred colonoscopy were mailed instructions for scheduling a colonoscopy. Those TPI Group participants who had an equal preference for SBT and colonoscopy screening were mailed an SBT kit, along with instructions for scheduling a colonoscopy. A generic screening reminder has been sent to all TPI Group participants at 45 days after the initial screening kit mailing. To date, an endpoint telephone survey and chart audit have been completed for 27 participants who have come due for these assessments.

### **Research Project 5: Project Title and Purpose**

*MicroRNA Genetic Variations as Predictors of Hepatocellular Carcinoma Risk* - The project goal is to use a pathway-based polygenic approach to identify genetic variations in miRNA genes that can be used to predict the risk of hepatocellular carcinoma (HCC) in patients with chronic

Hepatitis B virus (HBV) infection. The efforts will be essential for our ultimate aim to build up an HCC risk assessment model that can be applied in clinical settings.

### **Anticipated Duration of Project**

1/1/2010 - 12/31/2011

### **Project Overview**

Hepatocellular carcinoma (HCC) is the fifth most common solid malignancy and the third leading cause of cancer mortality worldwide. Chronic hepatitis B virus (HBV) infection is the most prominent established etiologic factor for HCC. Worldwide, there are over 400 million HBV patients, over 5% of the world's total population. Approximately 20% of these patients develop HCC. Previous studies have reported non-genetic predisposition factors for HCC. However, *bona fide* genetic determinants largely remain to be identified. This proposal builds on and seeks to further extend our previous studies on the molecular epidemiology of microRNA (miRNA) in cancer susceptibility. In the proposed study, our goal is to use a pathway-based polygenic approach to identify genetic variations in miRNA genes that can be used to predict the risk of HCC in patients with chronic HBV infection. The efforts will be essential for our ultimate aim to build up an HCC risk assessment model that can be applied in clinical settings.

The specific aims of the proposed project are:

- 1) To assess the genetic susceptibility of single nucleotide polymorphisms (SNPs) of miRNA genes in the development of HCC in chronic HBV patients. We will genotype 12 common SNPs located in precursor miRNA and mature miRNA regions in 800 chronic HBV patients (200 cases and 600 controls). Our hypothesis is that genetic polymorphisms in miRNA genes modulate HCC susceptibility through their influences on the production and functions of mature miRNAs.
- 2) To develop a multivariate quantitative risk prediction model for HCC in chronic HBV patients. We will develop a multivariate risk prediction model to identify the gene-gene and gene-environment interactions that modulate the progression from HBV infection to hepatocarcinogenesis. The prediction model will integrate epidemiologic, viral, clinical, and genetic data. We hypothesize that there are joint and interaction effects between these factors in the modulation of HCC susceptibility in chronic HBV patients.

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## **Expected Research Outcomes and Benefits**

In this project, we expect to identify miRNA gene polymorphisms that contribute to the predisposition of HCC in HBV patients. Once validated, the biomarkers will become promising targets for downstream genetic fine-mapping, deep re-sequencing, and functional characterizations. Furthermore, we will incorporate findings with currently available risk factors, to develop an exploratory risk assessment model that stratifies chronic HBV patients, and identifies those with the highest potential for malignant progression. In addition, the further growth of our HBV patient cohorts will offer us the opportunity to develop a clinic-based prospective longitudinal study to evaluate the antiviral treatment efficacy and other factors that affect the risk of HCC in HBV patients.

In the United States, the incidence of new, acute, HBV infection has decreased in the past two decades, but the number of patients living with chronic HBV infection has been growing. This development is partly due to the trend of increasing immigration from countries with high HBV endemicity. Of the 41 million Americans born outside of the United States, approximately 1.5 million have HBV infection. The direct annual cost of HBV-related medical care increased from less than \$400 million to over 1.3 billion within 20 years. In addition, over 60% of current HBV patients in the United States are relatively young, with an age between 20 and 49 years. As these patients age, significant increases in HBV-induced HCC and corresponding medical costs are expected to occur. Continuous monitoring of these patients and identification of high-risk individuals among them for targeted intervention are important to the reduction of HBV-induced burden to the healthcare system of the United States. Therefore, the findings of this proposed project will be of considerable public health significance.

## **Summary of Research Completed**

During the time period between July 1, 2010 and June 30, 2011, we continued to identify more patients that were included in the study population. As of 03/2011, we have finished and validated the chart review for 1759 Korean Chronic Hepatitis B (CHB) patients, as listed in Table 1. The average follow-up time is 6.6 years. The data on age, gender, smoking, drinking, family histories of cancer, cirrhosis, and HBV are completely collected without any missing data. Moreover, we have collected several pivotal clinical variables that have been implicated in HBV-HCC, for both the initial and many follow-up visits, including AST, ALT, AFP, HBV DNA load, and HBeAg/Ab status (Table 1 lists the values at the initial visit). About 95% of these patients received nucleoside/nucleotide (NA)-based antiviral treatments only. The other 5% of patients either received no treatments, interferon treatments, or combined treatments of NA and interferons. In addition, we also collected a set of additional clinical variables (see footnotes of Table 1). The rate of missing data for these variables in the initial patients' visits ranged from 0.91% (AST) to 39.9% (LDL). The vast majority of these patients have frozen serum samples obtained at initial and many follow-up visits. More than 99% of these samples have never been thawed.

Using this patient population, we conducted several analyses to identify clinical variables that are associated with the risk of developing HCC in HBV patients. The first clinical variable we studied is alpha-fetoprotein (AFP). AFP is a serum protein generated by the yolk sac and the liver during fetal life. Serum AFP has long been used as a diagnostic marker for HCC. In addition, it has also been suggested that elevated level of serum AFP was associated with increased risk of HCC in patients with HCV-related viral cirrhosis. However, the role of AFP as a prospective predictive marker or a surveillance indicator of HCC among HBV patients has not been as extensively evaluated and thus remains controversial.

For the purpose of this study, we included all those patients who (a) were non-cancer patients who had only HBV infection at their first clinical visits, (b) had recorded AFP measurement within 12 months after their first clinical visit, (c) had been followed for a minimum of 12 months, and (d) was not diagnosed with HCC within 12 months of their first clinical visit. Statistical analyses were performed using the SAS software version 9.2 (SAS Institute, Cary, NC). The primary outcome of this analysis was the diagnosis of HCC. Incidence rates of HCC were calculated by dividing the number of incident HCC cases by the total number of person-years of follow-up. Cumulative incidence of HCC by follow-up year was derived using the Nelson-Aalen method. Kaplan-Meier analysis was used to compare the cumulative risk for developing HCC in patients with different levels of serum AFP. The log rank test was used to determine the statistical significance of associations. Cox proportional hazards model was performed to determine the association between the independent variables and the risk of developing HCC. The risk of HCC was estimated using hazard ratio (HR) and 95% confidence interval (CI) after adjustment for age, gender, smoking, drinking, cirrhosis, family history of HBV, family history of cirrhosis, and family history of cancer, where appropriate.

We found that there were a total of 3,785 person-years of follow-up in the 617 study subjects, with the average (SD) follow-up time period of 6.2 (4.7) years (range: 1.0-22.2 years). During follow-up, a total of 61 HBV patients developed HCC after 12 months. We analyzed the association of major demographic variables and baseline serum AFP value with the risk of developing HCC using multivariate Cox proportional hazard model (Table 2). We found that male patients, ever smokers, and ever drinkers exhibited an increased but non-significant risk of HCC development with an HR of 1.90 (95% CI 0.84-4.30), 1.65 (0.81-3.34), and 1.10 (0.56-2.16), respectively. Compared to patients  $\leq 39$  years old, patients who were 40-49 years old, 50-59 years old, and over 60 years old showed an increased risk for developing HCC, with an HR (95%CI) of 1.45 (0.71-2.96), 6.28 (3.12-12.63), and 2.11 (0.85-11.40), respectively. As expected, patients with cirrhosis had a statistically significantly increased risk of developing HCC (HR=8.02, 95% CI 3.38-19.05).

Furthermore, patients who reported a family history of cirrhosis also had a significantly increased risk of HCC with an HR (95% CI) of 3.20 (1.09-9.43). However, no significant associations were observed between HCC risk and self-reported family history of HBV infection (HR 0.94, 95% CI 0.52-1.69) and family history of cancer (HR 1.13, 95% CI 0.65-1.99). There were 300 patients with a baseline AFP level that was lower than the median value. In this group, 15 (5.0%) patients developed HCC in an average of 7.9 years. In comparison, 46 of 317 (14.5%) patients with an AFP level higher than the median value developed HCC in an average of 4.6 years (chi square test  $< 0.0001$ , data not shown). Multivariate Cox proportional hazard analysis

indicated that compared to patients with a lower-than-median serum AFP, those with a higher-than-median serum AFP value had a 2.73-fold (95% CI 1.25-5.99) increase in the risk for developing HCC (Table 2). The incidence rates of HCC per 100,000 person-years by baseline serum AFP level increased from 801 per 100,000 person-years for the low AFP group to 2405 per 100,000 person-years for the high AFP group (Table 2).

Finally, we conducted analyses to calculate the cumulative incidence of HCC during different years of follow-up time by serum AFP level using the Nelson-Aalen method (Table 3). We found that the cumulative incidence of HCC was significantly higher in patients with higher baseline serum AFP than those with lower AFP value, in each of the follow-up time periods examined. In patients with lower-than-median baseline AFP value, the cumulative incidence of HCC was 2.4, 3.9, 5.2, 10.0, and 17.9 at the end of 3, 6, 9, 12, and more than 18 years of follow-up, respectively. In comparison, HCC incidence was 6.6, 10.9, 15.5, 27.8, and 40.8, respectively, in patients with higher-than-median baseline AFP values (Table 3, upper panel). Very similar results were observed when the analysis was conducted within those 363 patients whose baseline AFP was measured within 6 months of first clinical visit and had been followed for more than 36 months (Table 3, lower panel).

In addition to AFP, we also identified another clinical variable, gamma-glutamyl transpeptidase (GGT) that was prospectively associated with risk of developing HCC in HBV patients in our study population. Both GGT and AFP will be incorporated in the multivariate risk prediction model proposed in Aim 2 of this study. Moreover, we have placed the order for the OpenArray Genotyping assays of the 12 miRNA-related genetic polymorphisms from Applied Biosystems. In the next 6 months, we will genotype these polymorphisms and identify the significant ones to be analyzed jointly with other major/significant demographic and clinical variables to build the risk prediction model of developing HCC in HBV patients.

Table 1. Host characteristics of 1759 Korean CHB patients

Characteristics*	Total patients (%) N=1759	
Age (years, mean±SD)	44.5±13.2	
Gender	<i>Male</i>	1141 (64.9)
	<i>Female</i>	618 (35.1)
Smoking status	<i>Never smoker</i>	1201 (68.3)
	<i>Ever smoker</i>	558 (31.7)
Drinking status	<i>Never drinker</i>	1115 (63.4)
	<i>Ever drinker</i>	644 (36.6)
Family history of HBV	<i>Yes</i>	823 (46.8)
	<i>No</i>	936 (53.2)
Family history of cirrhosis	<i>Yes</i>	288 (16.4)
	<i>No</i>	1471 (83.6)
Family history of cancer	<i>Yes</i>	571 (32.5)
	<i>No</i>	1188 (67.5)
Cirrhosis	<i>Yes</i>	498 (28.3)
	<i>No</i>	1261 (71.7)
Average follow-up years	6.6	
AST (IU/L)	69.9	
ALT (IU/L)	97.5	
AFP (IU/L)	5 categories#	
Viral DNA load (copies/mL)	5 categories#	
HBe Ag/Ab	4 categories#	
Antiviral Treatments	<i>NA-based**</i>	1668 (94.8)
	<i>Other**</i>	91 (5.2)

\* There are other variables not listed on the table, including total protein, albumin, total bilirubin, direct bilirubin, alkaline phosphatase (ALP), platelet, phlebotomize time, cholesterol, low-density lipoprotein, triglyceride, ferritin, blood urine nitrogen (BUN), and creatinine.

\*\*NA, nucleoside/nucleotide analog; Other, including interferon treatment, combined treatment or no treatment

# These variables were categorized into ranges, including <20, 20-99, 100-999, 1000-9999, >10000 for AFP; <300, 300-9999, 10000-99999, 100000-999999, and >1000000 for viral DNA load; and +/-, -/+, +/+, -/- for HBe Ag/Ab status.

Table 2. The associations of demographic variables and serum AFP value with the risk of developing HCC in HBV patients.

Variables	Number (%) of patients (N=617)	Person-years of follow-up	Number (%) of HCC cases (N=61)	Incidence rate per 100,000 person-years	HR (95% CI) *	P value
<b>Age (Year)</b>						
≤39	233(40.88)	1651	8	485	Reference	
40-49	194(34.04)	1179	17	1442	1.45 (0.71-2.96)	0.30
50-59	108(18.95)	531	26	4896	6.28 (3.12-12.63)	<0.0001
≥60	35(6.14)	148	3	2027	2.11 (0.85-11.40)	0.09
<b>Sex</b>						
<i>Female</i>	200(32.41)	1160	9	776	Reference	
<i>Male</i>	417(67.59)	2625	52	1981	1.90 (0.84-4.30)	0.12
<b>Smoking status</b>						
<i>Never</i>	412(66.77)	2544	29	1140	Reference	
<i>Ever</i>	205(33.23)	1241	32	2579	1.65 (0.81-3.34)	0.17
<b>Drinking status</b>						
<i>No</i>	376(60.94)	2369	30	1266	Reference	
<i>Yes</i>	241(39.06)	1416	31	2189	1.10 (0.56-2.16)	0.78
<b>Cirrhosis</b>						
<i>No</i>	390(63.21)	2315	7	302	Reference	
<i>Yes</i>	227(36.79)	1470	54	3673	8.02 (3.38-19.05)	<0.0001
<b>Family history of HBV</b>						
<i>No</i>	305(49.31)	1894	31	1637	Reference	
<i>Yes</i>	312(50.57)	1891	30	1586	0.94 (0.52-1.69)	0.83
<b>Family history of cirrhosis</b>						
<i>No</i>	501(81.20)	3106	45	1449	Reference	
<i>Yes</i>	116(18.80)	679	16	2356	3.20 (1.08-9.43)	0.04
<b>Family history of cancer</b>						
<i>No</i>	407(65.96)	2547	37	1453	Reference	
<i>Yes</i>	210(34.04)	1238	24	1939	1.13 (0.65-1.99)	0.67
<b>AFP</b>						
≤ <i>median</i>	300(48.62)	1872	15	801	Reference	
> <i>median</i>	317(51.38)	1913	46	2405	2.73 (1.25-5.99)	0.01

\* Adjusted for age, sex, smoking status, drinking status, cirrhosis, family history of HBV, family history of cirrhosis, family history of cancer, and AFP, where appropriate.

Table 3. Cumulative incidence of HCC by baseline serum AFP level and different years of follow-up in patients.

Years of follow-up	Baseline serum AFP( $\leq$ median) Cumulative incidence	Baseline serum AFP( $\geq$ median) Cumulative incidence
In patients with more than 12 month's follow-up*		
3	2.4	6.6
6	3.9	10.9
9	5.2	15.5
12	10.0	27.8
$\geq 18$	17.9	40.8
In patients with more than 36 month's follow-up**		
6	1.7	5.1
9	3.0	10.3
12	8.0	24.1
$\geq 18$	16.1	37.8

\* Within patients who had an AFP value within 12 months of first clinical visit and who had been follow-up for at least 12 months.

\*\* Within patients who had an AFP value within 6 months of first clinical visit and who had been follow-up for at least 36 months

### **Research Project 6: Project Title and Purpose**

*Development of a Decision Aid for Hepatitis C Testing in High Risk Populations* - More than 3 million Americans are infected with Hepatitis C virus (HCV); many have no symptoms and are unaware of their HCV status. HCV infection is a leading cause of chronic liver disease and the most common reason for liver transplantation. Specific populations that are at increased risk for HCV infection have been defined, and a reliable diagnostic test to determine HCV status exists. However, there is disagreement among experts about the utility of routine testing for high-risk individuals. In situations like this, patients may benefit from additional information to assist them in deciding to be tested. This project lays the groundwork for a larger study that applies a technique called "*mediated-decision counseling*" to assist patients in making an informed decision about HCV testing.

### **Anticipated Duration of Project**

1/1/2010 - 6/30/2012

### **Project Overview**

Medical experts disagree about the necessity of routine HCV testing for those at high-risk of infection. Decision counseling assists patients in making decisions regarding which courses of action are reasonable and acceptable. In this project, a trained professional will explain to patients the risks and benefits, as well as the expected outcomes and consequences, of HCV testing. This will enable the patient to comprehend their choices regarding whether to participate in testing, and will allow shared-decision making with a physician.

The primary objective for this project is to develop and pilot test a “decision aid” for high-risk individuals considering HCV testing. All materials will be pre-tested with representatives of the target audience prior to use in the pilot test, and key processes and outcomes will be measured. Existing materials related to HCV testing will be reviewed and altered if necessary, to fit the needs of this project. For the pilot test, individuals who present for a health care visit at Jefferson Family Medicine Associates (JFMA), will be assessed of their HCV risk using a short questionnaire. Those who are found to be at increased risk will be eligible to participate in the research project.

The Research Assistant will provide an explanation of the project to participants, and obtain informed consent prior to their scheduled appointment. The physician-patient encounter will be audio taped to assess baseline rates of informed decision making by the patient. After the physician visit, the patient will complete a brief baseline survey, and then be guided through decision counseling by a research staff member to clarify their preference for HCV testing. Within seven to ten days, patients will complete an end-point survey; roughly 90 days after the office visit, a chart review will assess HCV test performance.

There are multiple aims for this project. The first is to develop the baseline and end-point surveys, and the HCV testing decision aid for the counseling sessions. The second is to recruit high-risk individuals to participate in the pilot test of the decision counseling protocol. The third is to measure baseline rates of informed decision making during physician-patient encounters. The fourth is to determine which factors in decision-making predict HCV testing among high-risk individuals.

### **Principal Investigator**

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### **Other Participating Researchers**

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### **Expected Research Outcomes and Benefits**

Expected outcomes include the development of materials and procedures that can lay the groundwork for future projects of a larger scale. Individuals who are involved in the pilot test will benefit from knowing their HCV risk. Those who are found to be at increased risk of HCV may opt to take preventive measures, while those who are found to be infected with HCV as a result of testing will have the opportunity to begin treatment early, which could prevent long-lasting infection, liver damage and possibly death.

## Summary of Research Completed

This reporting period has been devoted to pilot testing a decision aid for individuals at risk for hepatitis C testing (Specific Aim 3). Specific Aim 1 (developing pilot study materials) and Specific Aim 2 (pre-testing pilot study materials) were met previously.

Specific Aim 3: Pilot test a “decision aid” for high-risk individuals considering HCV testing. Specific Aim 3 is ongoing at this time. During pilot study implementation, we have tried to maximize patient participation while minimizing the burden to the patients and providers at Jefferson Family Medicine, our site of patient recruitment.

If patients are eligible and wish to participate, the patient completes informed consent and sees their doctor as scheduled. Subsequent to the appointment, the patient completes the baseline questionnaire and receives an education session and decision counseling session delivered by the Research Assistant. The educational booklet is used during the session to guide the order of information and the discussion. During decision counseling, patients provide up to three factors for or against HCV testing and rated their factors based on intensity of feeling and the level of influence. A preprogrammed algorithm computed a preference score on a scale of 0 to 1 (for or against testing). Approximately 7-10 days later, the Research Assistant administers the follow-up questionnaire via telephone and determines the patient’s hepatitis C testing status through the electronic medical records system at JFMA.

A total of 80 patients have been enrolled in the study. We expect to enroll an additional 20 patients in the next few months, completing patient enrollment by September 2011. See Table 1 for data related to recruitment and enrollment activities

### *Content Coding of Informed Decision Making about hepatitis C testing*

To document the extent of informed decision making (IDM) about hepatitis C testing during the provider-patient encounter at Jefferson Family Medicine, a subset of patients have agreed to have their physician visit audio recorded. The audio recordings were coded by the research team for nine essential elements of IDM: (1) the patient’s role in decision making; (2) the impact of the decision on the patient’s daily life; (3) the nature of the decision; (4) alternatives; (5) pros and cons of the alternatives; (6) uncertainties of the alternatives; (7) provider assessment of patients’ understanding; (8) provider assessment of patient’s desire for input from others; and (9) provider solicitation of the patient’s preference. A total IDM score is computed based on the presence of each element. Coding for IDM is being guided by Dr. Clarence Braddock, who developed the coding mechanism and is an expert consultant for this study.

In order to become competent in coding IDM, two research assistants and the Principal Investigator reviewed training materials provided by Dr. Braddock. These training materials not only explained the theoretical foundation and importance of IDM, but also the methods involved in coding the audiorecordings. Training tapes of previously recorded physician-patient interactions (not about hepatitis C testing) were utilized to provide practice and ensure inter-rater reliability among coders. Coding practice occurred weekly for two months. To date, we have audiorecorded eight physician-patient interactions involving discussions about hepatitis C

testing; if all future study patients agree to audiorecording, we could have as many as 28 audiorecorded sessions to be used for documenting levels of IDM about hepatitis C testing.

**Table 1:** Summary of recruitment activities to date

<i>Number approached at Jefferson Family Medicine</i>	<i>Number screened for hepatitis risk factors</i>	<i>Number eligible to participate in the study</i>	<i>Number enrolled in the study</i>
808	218	134	80

**Research Project 7: Project Title and Purpose**

*CAV3, Myo-Epithelial Barrier Function and Breast Cancer* - Advanced medical technologies and therapeutic strategies are necessary for the successful detection, diagnosis, and treatment of human breast cancer. This project will identify new paracrine signaling mechanism(s) that contribute to breast cancer pathogenesis, using a new mouse model, the Cav-3 knock-out (KO) mice. These mice develop pre-malignant mammary lesions including mammary ductal thickening, increased primary ductal branching, and lobulo-alveolar hyperplasia. In addition, the mice will be crossed into the polyomavirus middle T-antigen transgenic mice (MMTV-PyMT) genetic background to determine the role of Cav-3 in mammary tumor formation and metastasis. This will allow the elucidation of the role of Cav-3 and myo-epithelial barrier function in ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC). The Cav-3 knock-out mice will also be valuable for testing new therapies for breast cancers.

**Anticipated Duration of Project**

1/1/2010 - 12/31/2013

**Project Overview**

Caveolin-3 (Cav-3) is a muscle-specific gene that is primarily expressed in muscle cell types, including myo-epithelial cells. Caveolins function as broad-spectrum kinase inhibitors that can induce cell cycle arrest in the G0/G1 phase of the cell cycle. In this project, the role of Cav-3 in the pathogenesis of human breast cancer will be investigated, with a focus on myo-epithelial cell functioning. Myo-epithelial cells play a tumor suppressor role as the gatekeepers of luminal mammary epithelial cells. During progression from DCIS to IDC, the protective role of the myo-epithelial cell layer is compromised.

A genetic approach will be used to study the protective role of myo-epithelial cells in this transition process using Cav-3 (-/-) null mice, which lack caveolin-3 protein expression in their myo-epithelial cell layer. These mice are predicted to develop myo-epithelial cell dysfunctions such as hyper-proliferation and defects in terminal differentiation—since Cav-3 normally functions in mediating cell-cycle arrest. As such, loss of Cav-3 may also increase the mammary stem/progenitor cell population, predisposing the mice to the development of invasive mammary lesions. Thus, the role of Cav-3 will be assessed in the tumor micro environment using Cav-3 null mice as a novel preclinical model.

The two Specific Aims of the project are:

1. Determine the role of Cav-3 and Myo-Epithelial Cells in Mammary Cell Hyperplasia, Dysplasia, and Ductal Carcinoma *In Situ* (DCIS).
2. Determine the role of Cav-3 and Myo-Epithelial Cells in Mammary Tumor Onset, Progression, and Metastasis.

### **Principal Investigator**

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### **Other Participating Researchers**

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### **Expected Research Outcomes and Benefits**

Breast cancer is a major cause of morbidity and mortality worldwide. New diagnostic and therapeutic strategies are urgently needed. The current research project will elucidate novel signaling pathways that directly contribute to the onset and progression of human breast cancers.

Recently, a novel pre-clinical mouse model was generated by this laboratory, to determine the functional role of Cav-3 and the myo-epithelial cell layer in the onset and development of mammary tumorigenesis and metastasis. The results indicate that this novel mouse model (Cav-3 KO mice) shows the onset of mammary hyperplasia, which can be considered a pre-malignant mammary abnormality.

Finally, Cav-3 KO mice will be examined to determine if they are more susceptible to the development of mammary tumors and lung metastases, by crossing these mice with a well-established mouse model of mammary tumorigenesis, namely MMTV-PyMT mice. This project will then examine if a loss of Cav-3 and abnormal myo-epithelial cell functioning exacerbates the development of DCIS and full-blown mammary tumors.

This novel mouse model could also be used for testing the efficacy of newly developed breast cancer therapies.

### **Summary of Research Completed**

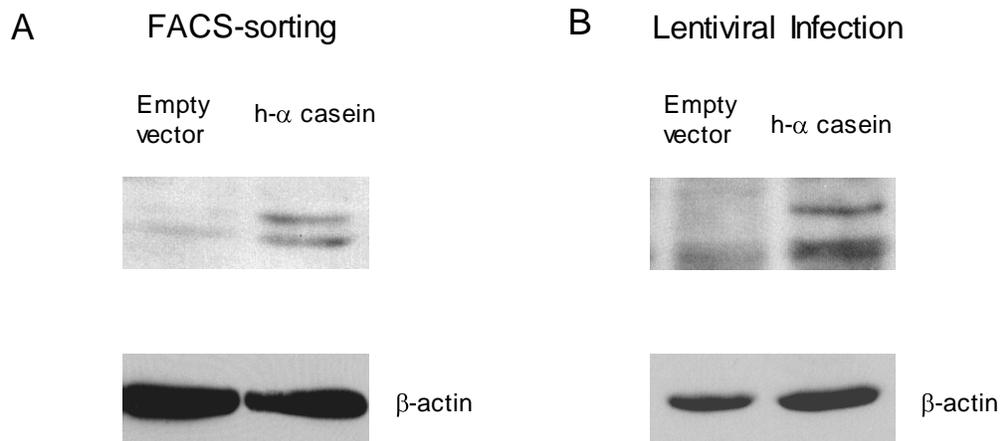
Breast cancer is a major cause of death in the United States and the Western World. Advanced

medical technologies and therapeutic strategies are necessary for the successful detection, diagnosis, and treatment of breast cancer. Here we have identified  $\alpha$ -casein as a milk protein component with protective effect against the progression of human breast cancer and lung metastasis formation. To provide evidence supporting the important role of  $\alpha$ -casein in decreasing mammary tumor development and metastasis formation, we used a novel cellular model based on Met-1 cells transfected with human  $\alpha$ -casein cDNA, injected into the flank of nude mice (See Figures 1-3). To identify possible molecular mechanism(s) underlying the anti-tumorigenic and anti-metastatic effect of  $\alpha$ -casein, we performed gene expression profiling on those cells. Findings from this analysis show that  $\alpha$ -casein gene expression signature includes up-regulation of genes associated with the activation of the immune system and inflammation process, as well as down-regulation of genes associated with cell migration and invasion, and genes that are stem cell associated. Thus, we conclude that a lactation-based therapeutic strategy would provide a more natural and nontoxic approach to the development of novel anti-cancer therapies.

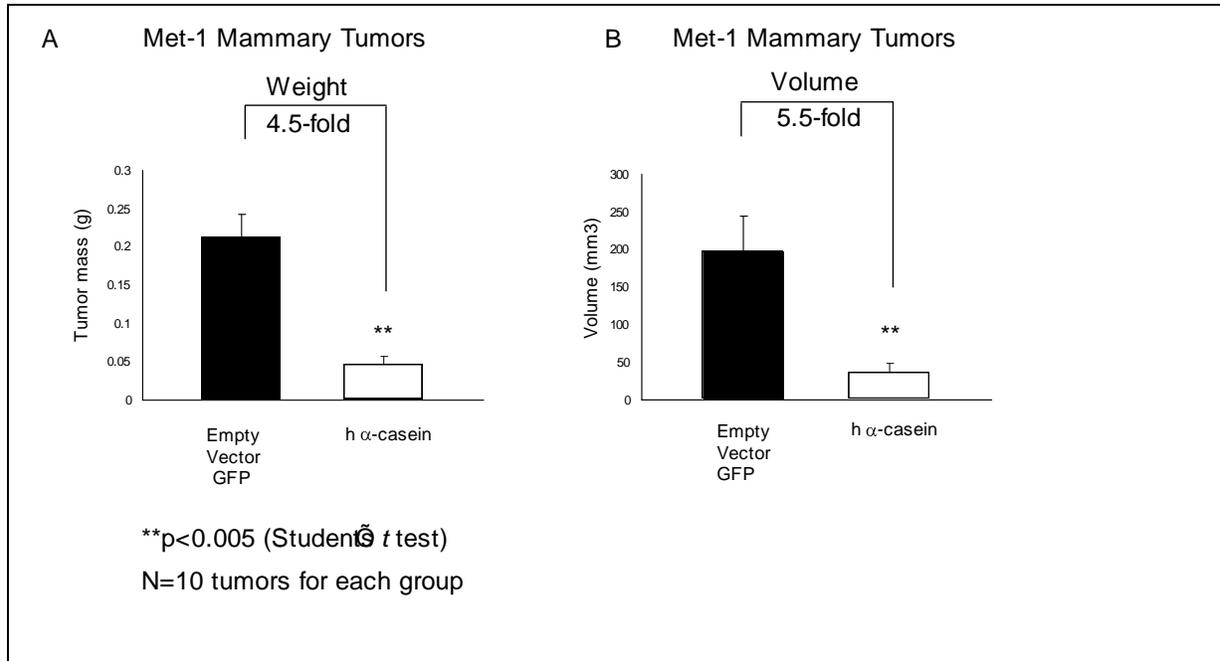
Related to these studies, we have also recently published the following papers:

Matrix remodeling stimulates stromal autophagy, "fueling" cancer cell mitochondrial metabolism and metastasis. Castello-Cros R, Bonnuccelli G, Molchansky A, Capozza F, Witkiewicz AK, Birbe RC, Howell A, Pestell RG, Whitaker-Menezes D, Sotgia F, Lisanti MP. *Cell Cycle*. 2011 Jun 15;10(12):2021-34. Epub 2011 Jun 15.

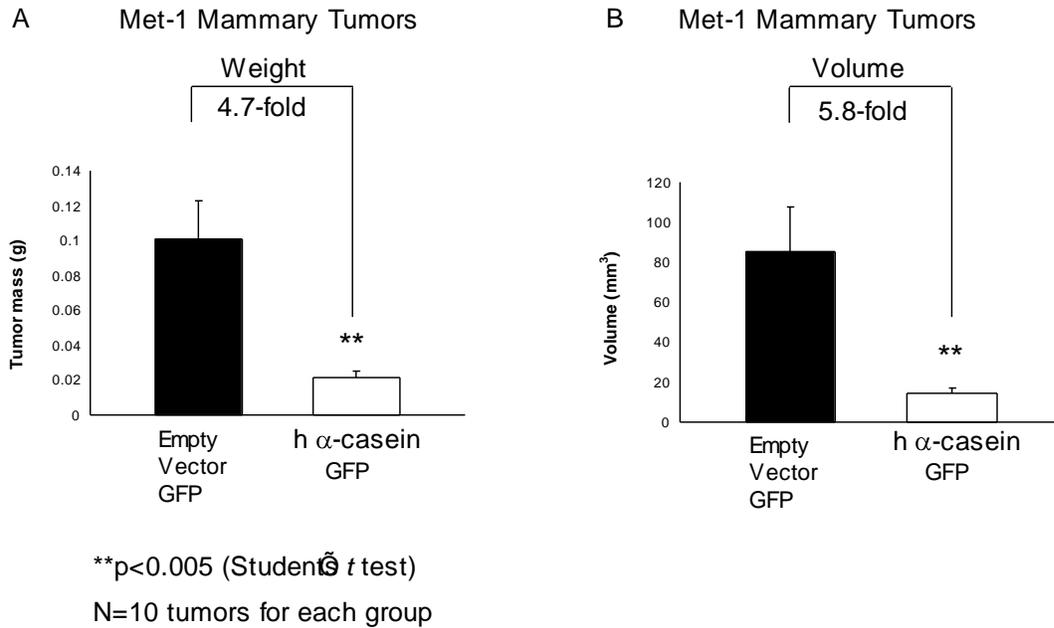
Stromal-epithelial metabolic coupling in cancer: Integrating autophagy and metabolism in the tumor microenvironment. Martinez-Outschoorn UE, Pavlides S, Howell A, Pestell RG, Tanowitz HB, Sotgia F, Lisanti MP. *Int J Biochem Cell Biol*. 2011 Jul;43(7):1045-51. Epub 2011 Feb 15.



**Figure 1. Derivation of Met-1 mammary cell lines expressing human  $\alpha$ -casein.** (A) Met-1 cells were stably transfected with empty vector (control) and vector containing human  $\alpha$ -casein, both GFP positive. After selection with G418, FACS-sorted stable cell populations GFP positive were generated and subjected to Western blot analysis. Immunoblots were probed with an anti- $\alpha$ -casein antibody to detect the transfected  $\alpha$ -casein. Note that the expression of  $\alpha$ -casein is absent in Met-1 empty vector control. (B) Western blot analysis, with antibodies directed against human  $\alpha$ -casein, was performed on Met-1 cells after selection with puromycin to confirm the efficiency of stable lentiviral infection with human  $\alpha$ -casein. As expected, recombinant  $\alpha$ -casein was absent in Met-1 empty vector control.  $\beta$ -actin was used as a loading control.



**Figure 2.  $\alpha$ -Casein decreases primary tumor formation in a xenograft model employing Met-1 cells (stably-transfected and FACS-sorted for GFP-positivity).** Met-1 cells with empty vector and human alpha-casein were injected into the flanks of athymic nude mice to evaluate their tumorigenic properties. Tumor formation was assessed by measuring weight and tumor volume, at 3-weeks after cell injection. (A) Note that Met-1/ $\alpha$ -casein reduces tumor growth of ~ 4.5-fold, relative to the empty vector control. (B) Met-1/ $\alpha$ -casein also had a significant effect on tumor volume. Indeed, Met-1/ $\alpha$ -casein cells also showed a reduced tumor volume of 5.5-fold versus control. \*\* $P < 0.005$ ,  $\alpha$ -casein versus vector alone; Student's t-test. N = 10 tumors for each group.



**Figure 3. Lentiviral delivery of the  $\alpha$ -casein cDNA reduces tumor growth.** Met-1 cells stably infected with empty vector and human alpha-casein were injected into the flanks of athymic nude mice to assay for tumor growth. Tumors were collected at 3-weeks after cell injection (n = 10 tumors for each group). (A) Met-1/ $\alpha$  casein cells showed an ~ 4.7-fold reduction in tumor growth, relative to the Met-1/empty vector control. (B) Met-1/ $\alpha$  casein cells also showed a ~5.8-fold decrease in tumor volume, as compare to the control. \*\**P*< 0.005, vector alone versus  $\alpha$ -casein (Student's *t*-test).