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14. ABSTRACT

Angiogenesis is the growth of new capillary blood vessels, and is a critical component of solid tumor growth. We characterized molecular changes between human breast tumor vessels and normal vessels to identify genes that may serve as therapeutic targets. We developed a method for rapid immunohistochemistry (IHC) and laser capture microdissection (LCM) of vascular cells from frozen human breast tumors and normal breast tissue for genomic analysis. We found SFRP2 to have 6 fold increased mRNA expression in breast tumor vessels, and confirmed localization of SFRP2 to endothelium using IHC with antibodies to SFRP2 on paraffin-embedded breast tumors. SFRP2 protein expression in endothelium was significantly higher in breast tumors than normal (13/15 or 87% versus 4/10 or 40%, p=0.03). We found that SFRP2 stimulates angiogenesis ex vivo and in vitro through a calcineurin/ NFAT pathway. A polyclonal antibody to SFRP2 inhibited malignant endothelial tube formation, demonstrating the contribution of SFRP2 to angiogenesis. We found that tacrolimus, a calcineurin/ NFAT inhibitor inhibits SFRP2 induced endothelial tube formation, and inhibited malignant endothelial tumor growth in mice. Based on its expression and function, we have discovered that SFRP2 is a novel therapeutic target for the treatment of breast cancer.

15. SUBJECT TERMS
Angiogenesis, Breast Cancer, Endothelial Cells, Gene Expression Profiling, Microarray

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Introduction:

Angiogenesis is the growth of new capillary blood vessels, and is a critical component of solid tumor growth. Inhibition of angiogenesis with the humanized monoclonal antibody against VEGF, bevacizumab, has demonstrated clinically significant improvements in outcomes in patients with metastatic breast cancer(1) and represents the first successful proof-of-concept trial for antiangiogenic therapy. However, there is still a need for additional antiangiogenic agents that target pathways other then VEGF because 1) patients with metastatic disease still die of cancer, 2) only 30% of patients with cancer respond to bevacizumab, and 3) tumors that do respond to bevacizumab may eventually become resistant. Since the majority of available anti-angiogenic agents are VEGF inhibitors, we wanted to look beyond VEGF to identify and characterize the next generation of angiogenesis targets.

Given that tumor endothelial markers (TEM)s differ *between* tumor types(2), and that breast cancers are molecularly heterogeneous, we sought to determine whether TEMs differ *within* the different molecular subtypes of breast cancer. Gene expression studies using DNA microarrays have identified several distinct breast cancer subtypes(3) that differentiate breast cancers into separate groups that differ markedly in prognosis(4). The intrinsic subtypes include 2 main subtypes of estrogen receptor (ER) negative tumors: Basal subtype (ER negative and Her2/neu negative) and Her2/neu subtype (Her2/neu positive and ER negative); and an ER positive (luminal subtype)(4). Our goal for our DOD Physician-Scientist Traning Award was to identify TEMs overexpressed in human breast cancer and elucidate their subtype specificity, which may be important in patient selection for vascular targeting agents. (5). For this purpose we set out to characterize molecular changes between breast tumor vessels and normal vessels to identify differentially expressed genes that may serve as therapeutic targets. This was achieved with a novel method that we developed for rapid immunohistochemistry (IHC) and laser capture microdissection (LCM) of vascular cells from frozen human breast tumors, where the RNA was of high quality and sufficient for genomic analysis(6). We found 55 genes with > 4 fold increased expression in breast tumor endothelium. We have focused our studies on SFRP2, which belongs to a large family of secreted frizzle-related proteins, which had had 6 fold increased gene expression in breast tumor endothelium compared to normal. We confirmed localization of SFRP2 protein to endothelial cells of human breast tumors using immunohistochemistry, and subsequently demonstrated that SFRP2 protein expression was significantly higher in breast tumor endothelium than normal, and was highly expressed in luminal A, basal, and Her2/neu subtypes of breast tumors (luminal B tumors were not evaluated)(6). This suggests that SFRP2 may be a target in the three main molecular subtypes of breast cancer.

Body:

SPECIFIC AIM #1: IDENTIFICATION OF CANDIDATE GENES INVOLVED IN BREAST CANCER-INDUCED ANGIOGENESIS USING LONG OLIGO-SPOTTED DNA MICROARRAYS. *We will use an inductive approach to look for genes involved in angiogenesis that may or may not have been previously characterized. To achieve this goal, we will employ laser capture microdissection (LCM) of endothelial cells from normal human breast tissue and invasive ductal carcinoma. We will then extract and amplify RNA, and proceed with tumor genomic profiling using our custom long oligo-spotted DNA microarrays. The most promising candidates will be referred for further study in Specific Aim #2 and identified by phenotype-expression pattern correlations. Differential expression of candidate genes will be confirmed by RT-PCR, and localization will be determined by immunohistochemistry in Specific Aim #3.*

This aim is completed and the goals have been accomplished:This has been published Bhati, R.; Patterson, C.; Fan C.; Livasy, C; Ketelsen, D.; Reynolds, E.; Tanner, C.; Perou, C.; Klauber-DeMore, N., Molecular Characterizations of Human Breast Cancer Vascular Cells. *Amer Journal of Pathology.* 172(2):1381-1390, 2008.

SPECIFIC AIM #2. PHENOTYPE-EXPRESSION PATTERN CORRELATIONS. In this aim, we will embark on a genome-wide screen for changes in gene expression from RNA extracted from endothelial cells from Specific Aim #1 using microarray technology. We will utilize this technology to determine how changes in gene expression differ between quiescent and stimulated endothelium. One approach we will take for data analysis will rely on assembly of gene expression profiles using computational methods. In particular, we will use the UNC Microarray Database (UNC MD) to determine the gene expression profiles from treated and untreated cells. UNC MD provides tools for hierarchical clustering, k-means clustering, and self-organizing maps. The most promising gene candidates will be characterized further in Specific Aim #3.

This aim is completed and the goals have been accomplished:

SPECIFIC AIM #3: MOLECULAR CHARACTERIZATION OF CANDIDATE GENES THAT REGULATE BREAST TUMOR ANGIOGENESIS. *The broad goal of this aim will be to establish molecular relationships between gene expression patterns and angiogenesis by functionally characterizing differentially regulated genes. These studies may allow us to assign new functions to known genes, or to study uncharacterized genes with functions that are particularly relevant to angiogenesis.*

SFRP2 is a novel angiogenesis factor: Based on the expression of SFRP2 in vascular endothelium, we hypothesized that SFRP2 stimulates angiogenesis. The process of angiogenesis occurs as an orderly, tightly regulated series of events, characterized by four major steps: selective degradation of the basement membrane and surrounding extracellular matrix, endothelial cell proliferation and migration, and finally the formation of vascular tubes.

Chick Chorioallantoic Membrane (CAM) Assay: To determine whether SFRP2 induces angiogenesis *ex vivo*, we incubated fertilized chickens eggs (NC State University Chicken Research Farm) at 100°F on an egg turner for 4 days. On day 4, the eggs were cracked into sterile Petri dishes and incubated at 99°F 3%CO2 65% humidity. For application of drug onto the CAM, Whatman grade 1 filter paper was cut into circles with a 6mm diameter paper punch and autoclaved. To decrease inflammatory effects of the disk, the discs are soaked in 1ml of 3.0mg/ml cortisone acetate in absolute ETOH and air dried for 60 min in laminar flow hood. On day 8, disks are placed on outer third of CAM, 2-3 mm from a vessel. Control PBS 7 µl is added to the discs for the control CAMS, and SFRP2 100 ng/ 7 µl PBS is added to the disks for the treated CAMS (n=13 control disks and n= 23 SFRP2-treated disks). The CAMs are evaluated under stereomicroscope on day 3 after disk placement. Pictures are taken with a Wild M-4 70 Macrosystem, and angiogenesis is quantified using Metamorph Software with an angiogenesis module. SFRP2 induced angiogenesis on the CAM (Fig 1).

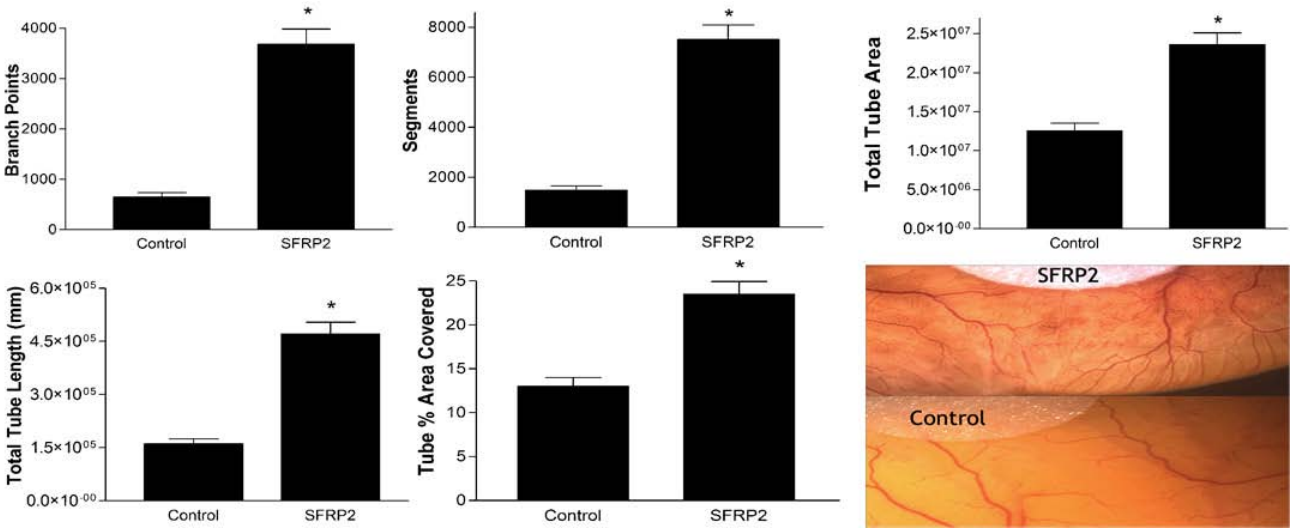

Figure 1. SFRP2 induces angiogenesis on the CAM. N=13 disks in control and n=23 disks in SFRP2 treated groups, *p<0.0001.

Scratch Wound Assay: The migration properties of SFRP2 on mouse endothelial cells (MEC) cells were evaluated using a scratch wound assay. Mouse endothelial cells were plated at 10,000 cells/well into a 96 well plate and allowed to become confluent in DMEM with 10%FBS. The cells were quiesced in DMEM for 18 hours. The wound was formed using a 1 ml pipette tip and a 0.3pM-300pM dose curve of mouse recombinant SFRP2 (US Biologicals, Swampscott) was added to the cells. Each concentration was performed in triplicate and the experiment was repeated three times with similar results. Migration was measured from 16 to 32 hours. Migration distance was measured at each time point. Statistical differences between SFRP2 and control were evaluated with an unpaired two-tailed Student's *t*-test, with p<0.05 being significant. SFRP2 increased endothelial cell migration in the picomolar concentration (Fig. 2).

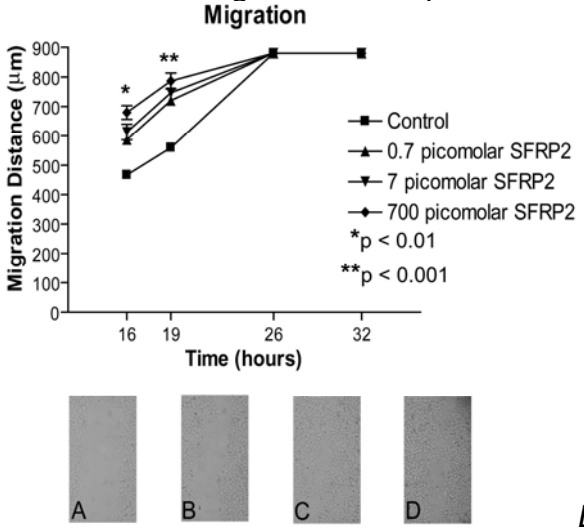

Figure 2. SFRP2 increases endothelial cell migration in a wound scratch assay.
The rate of wound closure is shown for controls and increasing concentrations of mouse recombinant SFRP2. The values are the means ± SEM, n=3 for each concentration. SFRP2 increased MEC cell migration at low concentrations ranging from 0.7pM-700pM.

Endothelial cell apoptosis: Human coronary artery endothelial cells (HCAEC) were used for apoptosis assays because we were not able to induce apoptosis in the MEC cells. HCAECs were grown in 10-cm dishes (Becton Dickinson, Franklin Lakes, NJ) with endothelial cell basal medium-2 (EGM-2) BulletKit media (Clonetics, San Diego, CA) until 80% confluent. Medium was then replaced with optimal medium according to different assays. The hypoxic condition was

created by incubating HCAECs in EGM-2 media without BullietKit growth factors at 37°C in a hypoxia chamber with an atmosphere of 5% CO_2/95% N_2. The oxygen level into the chamber was controlled to 1.0%. Apoptosis was determined by measuring the activity of cleaved caspase 3 by using a caspase-specific fluorogenic substrate according to the protocol for the Caspase 3 Assay Kit (Sigma). HCAECs were lysed after treatment with concentrations of SFRP2 (70pM and 700pM) for 36 h under hypoxia. Then, 5 µl of cell extract is incubated in reaction buffer at room temperature for 1 h. The enzyme-catalyzed release of 7-amino-4-methyl coumarin (AMC) was measured by a fluorescence microplate reader. We found that SFRP2 protected against hypoxia induced endothelial cell apoptosis ($p<0.02$) (Fig. 3). This is consistent with other studies in different cell types: SFRP2 decreased susceptibility to UV-induced apoptosis in primary culture of canine mammary gland tumors(7), and SFRP2 decreased apoptosis in cardiomyocytes exposed to hypoxia(8).

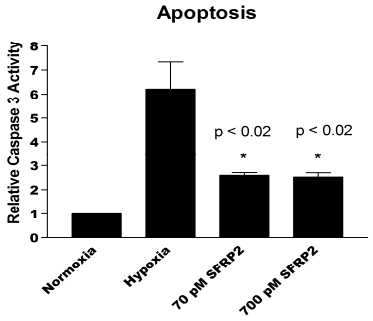

Figure 3. SFRP2 Inhibits Hypoxia Induced Apoptosis in HCAEC cells. Sfrp2 decreases caspase 3 activity *in vitro*. Cleaved caspase 3 activity as measured by a fluorometric assay demonstrates decreased caspase activity in hypoxic mouse endothelial cells after Sfrp2 treatment. The activity was calculated as fold changes compared to normoxia. Each condition was performed in quadruplicate, *$p<0.02$.

Western blot for beta catenin and NFAT in MEC cells: To evaluate whether SFRP2 is stimulating angiogenesis via the canonical Wnt signaling pathway, we compared protein levels of dephosphorylated nuclear beta catenin in control and SFRP2 treated endothelial cells. To evaluate the role of the non-canonical Wnt/ Ca++ pathway in SFRP2 induced angiogenesis, we compared nuclear dephosphorylated NFATc3 protein levels control and SFRP2-treated endothelial cells..MEC cells were plated in 12-well plates and allowed to attach overnight. The next day, the media was changed and added to the wells with and without SFRP2 (700 pM). Cells are incubated for 1, 2, 4, 8 and 16 hours, and the nuclear proteins were extracted by using NE-PER^{TM} nuclear and cytoplasmic extraction reagent from PIERCE (Pierce Biotechnology) as described in the manufacturer's manual. The Western blot analysis was performed using standard methods, with primary antibody to the dephosphorylated (active) b-catenin antibody or NFATc3. There was no change in nuclear ß-catenin in the SFRP2- stimulated endothelial cells ($p=0.4$, Fig. 5), suggesting that the angiogenic property of SFRP2 is not mediated through the canonical Wnt signaling pathway. We found that NFATc3 was increased at 30 minutes in the nuclear fraction of SFRP2-treated endothelial cells (Fig 5).

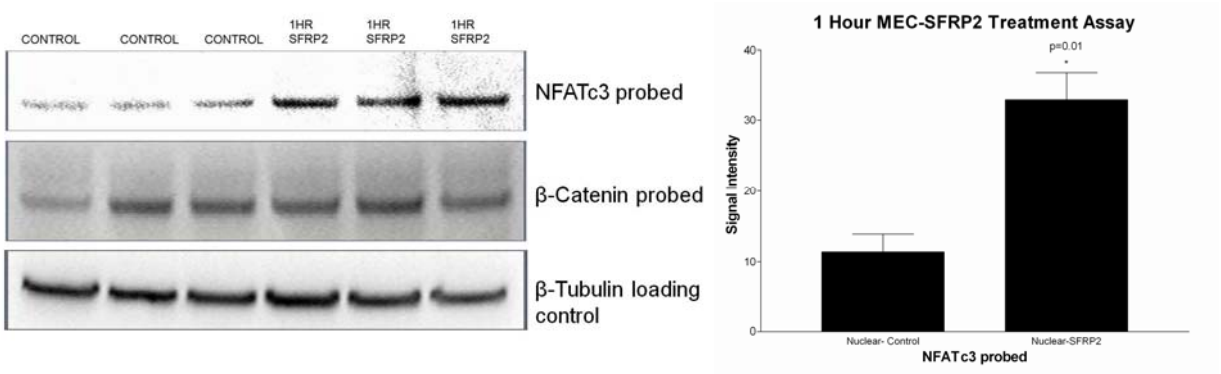

Fig 5. Western blot analyses of nuclear fractions of MEC cells were treated with and without SFRP2 (700 pM) for 1hour.

*A mouse model of angiosarcoma overexpresses SFRP2 protein***:** In order to study whether inhibition of SFRP2 will inhibit tumor growth, we set out to identify a tumor model that overexpresses SFRP2. To do this we studied a transformed mouse endothelial cells line. Ms1 cells were generated by immortalizing murine endothelial cells by expressing the temperature-sensitive large T antigen(9) (gift of Dr. Jack Arbiser, Emory University). Upon implantation into mice, these cells form dormant hemangiomas(9). Ms1 cells were then transfected with Ras (SVR cell line), and this cell line forms angiosarcomas when injected into nude mice(9). We collected protein lysates from MS1 and SVR cell lines and, using western blot analyses probing for SFRP2, found that SFRP2 was increased in SVR cells (Fig. 6). Since this cell line forms aggressive angiosarcomas, it is an ideal mouse model to study whether inhibitors of SFRP2 will inhibit tumor growth.

Tube formation assay: The tube formation properties of SFRP2 on MEC cells were evaluated using an endothelial cell tube formation assay. ECMatrix (Chemicon) was thawed, diluted and solidified in a 96 well plate according to the manufactures instructions. $1x10^4$ cells / well in 150 µl of DMEM (cellgro) with 10%FBS (HyClone) and a concentration range (3-3000pM) of SFRP2 (US Biologicals) were seeded onto the matrix and returned to 37^oC, 5% CO_2 for 8 hours. Images were acquired using the Nikon Eclipse TS100 microscope at 4x magnification with a Nikon CoolPix 995 digital camera. Results were quantified by counting the number of branch point. To evaluate whether Tacrolimus inhibits SFRP2 induced tube formation, MEC cells were treated as above with SFRP2 30nM with and without Tacrolimus (1 uM -100 uM) for 8 hours and branch points were determined as described above. To evaluate whether inhibitors of SFRP2-mediated angiogenesis would inhibit the growth of SVR tumor cells, SVR cells were treated with Tacrolimus (1 uM -100 uM) or with a rabbit polyclonal antibody to SFRP-2 (H-140) (Santa Cruz Biotechnology, Santa Cruz, CA, catalogue # sc-13940) in the tube formation assay.

MEC endothelial tube formation was induced by SFRP2 in a concentration-dependent manner at 8 hours (p=0.0006 at 7nM) (Fig. 6). To further evaluate whether the angiogenic effects of SFRP2 were mediated through NFAT, we treated endothelial cells in a tube formation assay with SFRP2 (30nM) with and without the calcineurin inhibitor Tacrolimus. Tacrolimus (1µM) inhibited SFRP2 induced tube formation by 64%± (0.002) (Fig. 6). Tacrolimus was not cytotoxic to MEC cells, as only 5% of tacrolimus-treated cells took up trypan blue dye (data not shown). Tube formation in SVR angiosarcoma cells were also inhibited by Tacrolimus (Fig. 6), and SVR tube formation was inhibited

by a polyclonal antibody to SFRP2, suggesting that SFRP2 is required for tube formation in this angiosarcoma tumor cell line (Fig. 6).

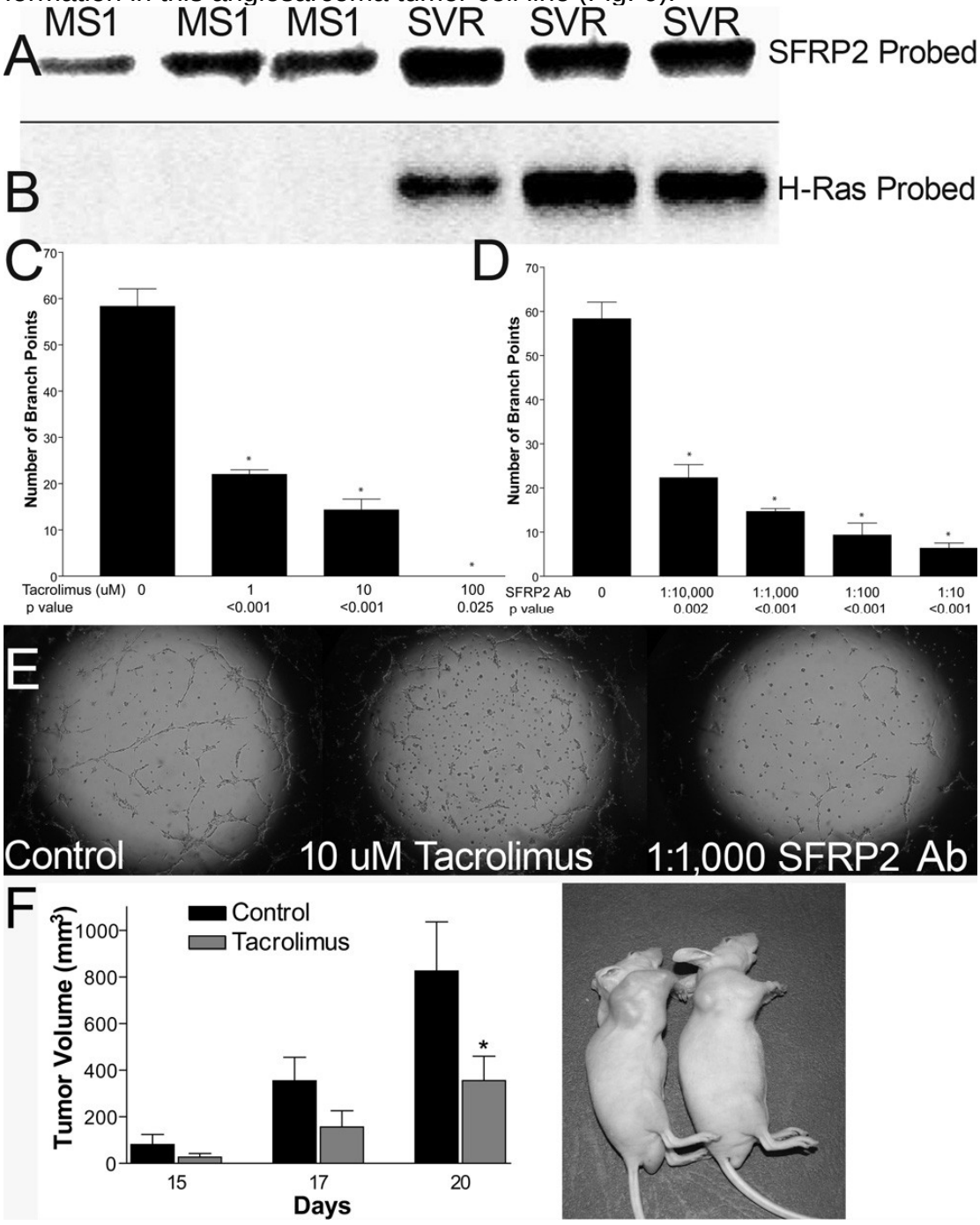

Figure 6. A)SVR angiosarcoma cell line overexpresses SFRP2 by western blot analyses. C, E) Tacrolimus inhibits SVR tube formation in vitro. D, E) A polyclonal antibody to SFRP2 inhibits SVR tube formation in vitro. F) Nude mice were injected with 1 x 10^6 SVR tumor cells as described in Material and Methods. Tacrolimus 3 mg/kg (n=4) or control buffer (n=4) was injected i.p daily starting on day x. Tacrolimus inhibited tumor growth on day 19 by 57% (*p=0.04).

KEY RESEARCH ACCOMPLISHMENTS:

•We have developed a novel protocol that allows for rapid immunohistochemistry and laser capture microdissection, while maintaining RNA integrity.

•We have confirmed the identity and purity of the endothelial cells.

•We have performed long-oligospotted microarrays and significance analysis of microarrays and have identified differential expression of genes between 5 breast tumor endothelium and 5 normal endothelial, and identified 7 genes encoding membrane proteins and 5 genes encoding secreted proteins with greater than 4 fold differential expression.

We have validated localization to tumor endothelium and increased protein expression between breast tumors and normal breast for SFRP2 and FAP with IHC.

We have identified SFRP2 as a novel angiogenesis factor that stimulates angiogenesis via the calcineurin/ NFAT pathway.

We have discovered that tacrolimus, an FDA approved drug for transplant rejection, inhibits the growth of a malignant endothelial cell tumor in mice.

Training Accomplishments:

•EDITORIAL BOARD MEMBERSHIPS

Cancer
Annals of Surgical Oncology
International Journal of Neoplasia
World Journal of Surgical Oncology
Journal of Surgical Oncology

Reviewed applications for Avon-NCI Progress for Patients Program Review July, 2006

Friends for an Earlier Breast Cancer Test Medical Advisory Board 2005-present

•APPOINTMENT TO NATIONAL COMMITTEES:

CALGB Breast Surgery Subcommittee member 2005-present
Society of Surgical Oncology Grants and Fellowship Committee 2008-2011

•APPOINTMENT TO LEADERSHIP POSITIONS WITHIN UNC:

Co-Program Leader, Breast Cancer, Protocol Office Executive Committee 2005-present

•STUDENTS MENTORED

Faculty Mentor for Tach Bhati, third-year surgical resident participating in the NIH T32 Training Grant. July 2004-June 2006

Faculty Mentor for Kirby Tanner, second-year medical student participating in summer research project at UNC. May 2006-August 2006

Faculty Mentor for Andrew Courtwright- 4th year medical student who spent one year in my lab

Faculty Mentor for Shara Rhanin, Ph.D., postdoctoral student.

REPORTABLE OUTCOMES:

Funding obtained based on work supported by this award:
ACTIVE:
10/01/06-7/31/11
National Cancer Institute
Spore in Breast Cancer, (Earp III)
Role on Project: Principle Investigator Project #4
$500,000
11/1/04-11/1/09
National Cancer Institute 1 K08 CA098034-01A2
Principle Investigator: Nancy DeMore
"Genetic Regulation of Human Breast Cancer Dormancy
$652,214
10/01/05-09/30/07
National Institute of Health 1 R13 DK073239-01 (Juliano)
Major Challenges in Clinical Medicine: An Overview
for Basic Scientists
Role: Mentor
8/01/05-7/31/06
National Cancer Institute
Spore in Breast Cancer,
Principle Investigator: Earp III
Role on Project: Developmental Project
$20,000

Patent Filed *Discovery of Novel Targets for Angiogenesis Inhibition,* Report of Invention Ref. # OTD07-008, patent pending. Inventors: Nancy Klauber-DeMore, MD, Cam Patterson, MD, PhD., Rajendra Bhati, MD 9/25/2006.

Presentations:

1. Bhati, R.; Patterson, C.; Fan, C.; Livasy, C.; Ketelsen, D.; Zhiyuan, H., Reynolds, E.; Tanner, C.; Perou, C, **Klauber-DeMore, N.,** Molecular Characterization of Human Breast Cancer Vascular Cells. *15th SPORE Investigators Workshop*, Baltimore, MD, July 9, 2007

2. Bhati, R.; Patterson, C.; Perou, C.; Livasy, C.; Fan, C.; Ketelsen, D.; Reynolds, E.; Tanner, C.; **Klauber-DeMore, N.** Discovery of Novel Tumor Endothelial Markers in Breast Cancer. *Society of Surgical Oncology, Washington D.C,* March 16, 2007.

3. **Klauber-DeMore, N**, Patterson, C, Fen, C, Livasy, C, Bhati, R, Ketelsen, D, Perou, C. Heterogeneity of Angiogenesis in Breast Cancer. *Keystone Symposia: Host Cell Interation and Response to the Cancer Cell,* Keystone, Colorado, January 22, 2007.

4. Bhati, R., Ketelsen, D., Perou, C., Patterson, C., **Klauber-DeMore, N**. Laser Capture Microdissection of Endothelial Cells from Human Breast Tumors for mRNA Analysis. "Anti-Angiogenesis and Drug Delivery to Tumors: Bench to bedside and Back" *American Association for Cancer Research*, November, 2005.

5. Bhati, R., Ketelson, D., Perou, C., Patterson, C., **Klauber-DeMore, N**. Laser Capture Microdissection of Endothelial Cells from Human Breast Tumors for mRNA Analysis. *13th SPORE Investigators' Workshop*, July 11, 2005

6. **Klauber-DeMore, N**., Bhati, R., Ketelson, D., Perou, C., Patterson, C. Laser Capture Microdissection of Endothelial Cells from Human Breast Tumors for mRNA Analysis. Era of Hope 2005 *Department of Defense Breast Cancer Research Program*, June 9, 2005.

Publications:

1. Bhati, R.; Patterson, C.; Fan C.; Livasy, C; Ketelsen, D.; Reynolds, E.; Tanner, C.; Perou, C.; Klauber-DeMore, N., Molecular Characterizations of Human Breast Cancer Vascular Cells. *Amer Journal of Pathology.* 172(2):1381-1390, 2008.

CONCLUSIONS:

During the first year of this grant we overcame a major obstacle to the feasibility of this project, which is obtaining intact RNA from a small number of endothelial cells after being subjected to immunohistochemistry. During the second year of this grant we have confirmed the purity and identity of the endothelial cells we have microdissected, and successfully performed RNA amplification and microarray analysis. We have compared gene expression profiles between breast tumor and normal endothelium and have identified differentially expressed genes. During the third year of the grant we have localized protein expression of 4 gene transcripts to tumor endothelium and found increased protein expression for two of the proteins, SFRP2 and FAP, in breast turmo endothelium compared to normal. Our next step is to evaluate the remaing 8 genes with high differential expression that encode membrane and secreted proteins with IHC on human breast cancers. During the fourth year of the grant we have discovered a novel angiogenesis factor, SFRP2, and a novel potential use of an FDA approved drug, tacrolimus, at inhibiting tumor growth. The long term goal is to evaluate the role that these genes play in angiogenesis, and if functionally active, develop monoclonal antibodies to them to inhibit angiogenesis and tumor growth.

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