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MicroRNA Mediated Negative Regulation of Caveolin-1 as a Biological Mechanism Driving Breast Cancer Disparities

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By

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Abstract

In South Carolina, mortality differences between African American (AA) and Caucasian-American (CA) women with breast cancer are amongst the highest in the country. Increasing evidence suggests that the racial disparity exists independent of socioeconomic and standard of care issues and could also be attributed to poorly understood inherent genetic and molecular characteristics within racial specific tumors. Caveolin-1 (Cav1) is a scaffolding protein with a tumor suppressor role. Loss of Cav1 in the tumor stromal compartment has emerged as a novel biomarker for predicting poor clinical outcome in all of the most common subtypes of breast cancer. While the loss of stromal Cav1 is becoming well established as a marker of poor outcome in breast cancer, the mechanism of this loss is still unknown. We propose that differences in microRNA mediated stromal Cav1 loss between AA and CA women are driving the racial disparity in breast cancer. MicroRNAs (miRNAs) are short, endogenous, non-coding RNAs that function as negative regulators of gene expression. miR-510 is an oncogenic miRNA that has been shown to be elevated in breast tumors. Cav1 is a predicted target of miR-510; therefore, miR-510 mediated negative regulation may be a novel mechanism of Cav1 loss in the tumor stroma. Our research shows that Cav1 is directly targeted by miR-510 by luciferase reporter assay and that overexpression of miR-510 leads to downregulation of Cav1 protein expression, specifically in the stromal compartment. This may be racially significant as our supporting studies also show that miR-510 levels are elevated and Cav1 levels are reduced in AA breast cancer patients compared to their CA counterparts. Data from our *in vivo* studies shows that tumors grown from co-injection of MDA-MB-231 cells plus miR-510 derived fibroblasts resulted in faster tumor growth and larger tumors at the end of the study than tumors grown from injection of MDA-MB-231 cells alone, suggesting that miR-510 derived fibroblasts enhance

tumorigenicity when co-injected with breast tumor epithelial cells. Our results suggest that the difference in miR-510 mediated regulation of stromal Cav1 is driving racial disparity in breast cancer.

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List of Abbreviations

AA: African-American

BC: Breast Cancer

BSA: Bovine Serum Albumin

CA: Caucasian-American

CAF: Cancer-associated Fibroblast

Cav-1: Caveolin-1

DAB: Diaminobenzidine

DCIS: Ductal Carcinoma In-situ

ESA: Epithelial Specific Antigen

ECM: Extracellular Matrix

EMT: Epithelial-Mesenchymal Transition

FSP1: Fibroblast Specific Protein 1

GOF: Gain of Function

IF: Immunofluorescence

IHC: Immunohistochemistry

K18: Cytokeratin

LCIS: Lobular Carcinoma In-Situ

LOF: Loss of Function

miRNA: MicroRNA

PBS: Phosphate Buffer Solution

qPCR: Quantitative Real-Time PCR

RT: Room Temperature

αSMA: alpha-Smooth Muscle Action

SDM: Site-Directed Mutagenesis

UTR: Untranslated Region

VEGF: Vascular endothelial growth factor

Background/Review of Literature

Breast Cancer: Breast cancer is the most commonly diagnosed cancer in women. In 2015, an estimated 231,840 new cases of invasive breast cancer are expected to be diagnosed in women in the United States, with 60,290 new cases of non-invasive (in situ) breast cancer diagnosed. Breast cancer is also the second-leading cause of death among women; in fact, 40,290 women in the U.S are expected to die from the disease in 2015 [1]. A number of risk factors can contribute to breast cancer development, including lifestyle, environmental, and genetic factors. Some controllable risk factors include, diet, exercise, alcohol consumption, smoking and weight. Evidence shows that women are more likely to develop breast cancer if they have poor diets low in fresh fruits and vegetables, live primarily sedentary lifestyles lacking exercise, frequently consume copious amounts of alcohol, use tobacco products, or are overweight [2]. Exposure to estrogen through hormone replacement therapies and oral contraceptive use can also contribute to cancer growth [2; 3]. Environmental factors, such as radiation exposure and exposure to various pollutants and pesticides are also thought to increase risk [2; 3]. One of the most evident genetic factors contributing to breast cancer development is gender. Although men can also develop the disease, women are at a much higher risk due to the increased prevalence and activity of progesterone and estrogen in their bodies. Risk even increases with age; in fact, two out of three women with invasive cancer are diagnosed after age 55 [3]. A personal history or family history of breast cancer, especially in the immediate family, also increases the risk of developing the disease. Other personal risk factors can include an early onset of menarche, a pregnancy later in age or no pregnancies at all, dense breast tissue, which can make it more difficult to detect abnormalities, or late menopause [3]. Race is also a risk factor associated with breast cancer, as Caucasian-American (CA) women are more likely to be

diagnosed with the disease, but African-American (AA) women suffer from higher mortality rates [2]. While a variety of non-biological factors contribute to the development of breast cancer and a racial disparity, this project focuses strictly on biological factors and seeks to uncover a biological mechanistic link driving higher mortality rates in AA breast cancer patients, as shown in Figure 1.

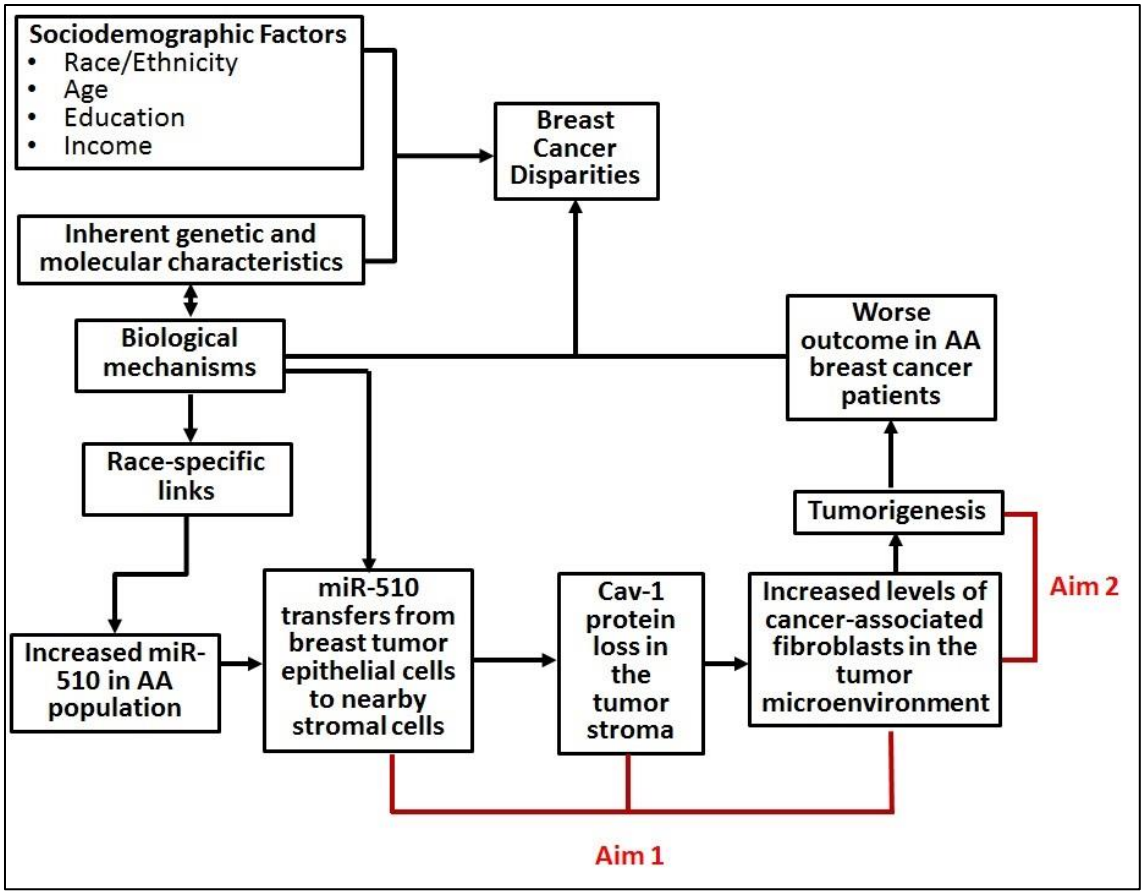

Figure 1: Conceptual Model. Breast cancer disparities are attributed to a combination of sociodemographic factors and inherent biological factors. Disparities also exist independently of sociodemographic factors. Aim 1 of this project examines a biological mechanism contributing to breast cancer disparities, while Aim 2 examines functional outcome of this mechanism.

Mammary gland development mostly occurs postnatally, with cell differentiation taking place at multiple stages, including pregnancy, lactation, and the involution cycle [4]. The mammary gland is composed of multiple cell types that interact with each other and with the microenvironment to facilitate proper development and function of the breast. The milk-transporting ducts are lined by an inner layer of secretory luminal epithelial cells and are surrounded by myoepithelial cells and the basement membrane [4]. These same cell-cell and cell-microenvironment interactions that facilitate normal breast development are also subject to mutations and abnormal epithelial proliferation that lead to breast tumorigenesis. Breast carcinomas most often arise from the milk-producing lobules of the breast or from the milk-transporting ducts. Abnormal cells contained in the lobules are known as lobular carcinoma in situ (LCIS), while abnormal cells contained in the ducts are known as ductal carcinoma in situ (DCIS), as these cancers have not yet infiltrated beyond the original cell layer. There are a number of histopathological changes that occur during the progression of in situ to invasive breast carcinoma and finally, metastasis [4]. Studies have shown that progression to invasive breast carcinoma is promoted by stromal fibroblasts, whose numbers increase as the tumor progresses, and is inhibited by normal myoepithelial cells, whose numbers decrease as the tumor progresses, through their combined effects on the basement membrane [4; 5].

Breast Cancer Disparities: Significant racial disparities exist for African-American (AA) women compared to Caucasian-American (CA) women, especially in our state of South Carolina. Studies including data from SC residents with BC show that AA women exhibit more advanced stage, more aggressive histologic features, and worse survival than their counterparts, even if detected at earlier stages [6; 9]. In general, AA women develop breast cancer 5-10 years earlier than their

counterparts [7]. A study conducted in Columbia, SC determined that tumors from AA women with BC in SC are significantly larger than in CA women at every decade of age [9]. Data from this same study shows that ER negative status and high-grade, a highly unfavorable combination for prognosis, is twice as common in AA women as in CA women in the state of SC [9]. According to the American Cancer Society, CA women in the US have a greater incidence of breast cancer, with 128.1 per 100,000 compared to AA women at 124.3 per 100,000 [Figure 2]. However, even with a lower incidence, AA women are still more likely to die from the disease, with 31.0 deaths per 100,000 compared to 21.9 deaths per 100,000 in CA women. Even more shocking than the elevated mortality rate is the survival rate of AA women diagnosed with breast cancer. According to the Center for Disease Control, the current 5-year relative survival rate is 77% for AA women compared to 90% among CA women. Previous data also reveals that AA women have a higher recurrence rate regardless of age and tumor size [8].

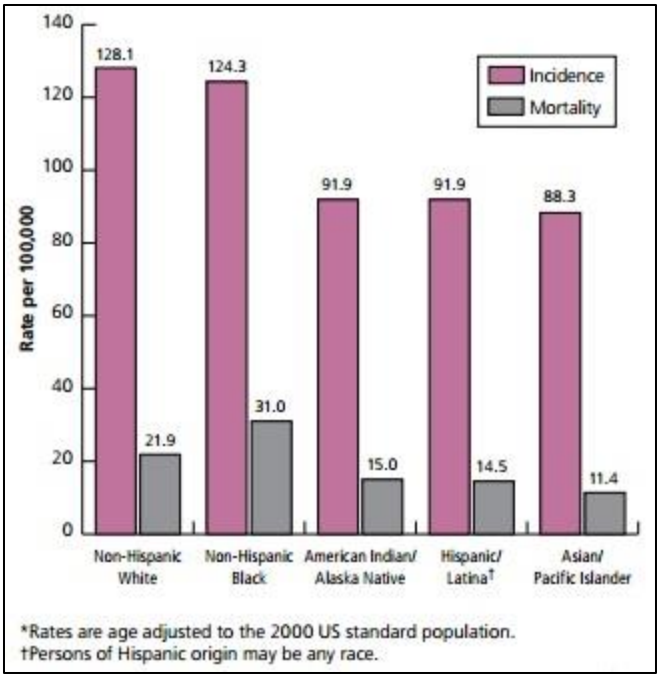

Figure 2: Breast cancer incidence and mortality rates by race and ethnicity, 2008-2012. Adapted from ACS Breast Cancer Facts and Figures 2015-2016.

Biological Factors and Mechanisms in Cancer: While strong evidence exists linking non-biological factors to racial disparities in breast cancer, studies show there is evidence for biological factors, as well [Figure 1]. After adjusting for clinical, demographic, and treatment variables, does the survival disparity still exist between AA and CA women? The Southwest Oncology Group (SWOG) conducted a study of approximately 20,000 various cancer patients from 1974-2001 through 35 randomized phase III clinical trials. The patients were first grouped by cancers of similar histology and stage, followed by an assessment of overall survival after controlling for socioeconomic status, prognostic, and treatment variables. Interestingly, the study concluded that even after controlling for these factors, AA patients had statistically significantly worse survival in sex-specific cancers (breast, ovarian, and prostate), including both early stage pre/postmenopausal breast cancer [10]. This data strongly suggests that inherent biological and molecular characteristics are predisposing AA women to worse breast cancer survival outcomes independent of standard of care issues. Genetic and molecular contributors are thought to include hormonal factors, inherited single-nucleotide polymorphisms associated with inflammatory processes, and communications between cell types in the mammary gland [9; 10].

The contribution of biological mechanisms promoting cancer disparities is not clear, thus the study of these mechanisms remains a challenge. However, a few biological mechanistic links have been identified in other sex-specific cancers, previously mentioned to have statistically significant worse survival in AAs. AA prostate cancer patients exhibit more aggressive cancers and higher mortality rates compared to CA prostate cancer patients [11-15]. Foster and colleagues examined advanced glycation end product (AGE) metabolites in clinical specimens of AA and CA prostate cancer patients. AGEs are reactive metabolites formed during glycation and

contribute to a number of diseases, including diabetes and cardiovascular disease [14; 15]. AGE functions as a ligand activator for RAGE (receptor for AGE), which is an oncogenic transmembrane receptor that promotes inflammatory responses [15]. Activation of the AGE-RAGE inflammation signaling axis promotes inflammation and contributes to tumorigenesis and worse outcome in AA prostate cancer patients [14; 15]. In both low-grade and high-grade serum, AA patients had significantly higher circulating AGE metabolite levels, which also showed a strong positive correlation with AGE levels observed in the tumor [14]. This race specific, tumor-dependent pattern of AGE accumulation represents a biological mechanistic link between cell metabolism and cancer [14; 15].

Another racial disparity in both incidence and survival outcomes exists in AA vs. CA women for endometrial cancer [16; 17]. Allard and colleagues identified a gene influencing this disparity through transcript expression analysis in normal and tumor tissue samples from AA and CA endometrial cancer patients [16]. Phospho serine phosphatase like (PSPHL) was found to be the most differentially over-expressed gene in AA endometrial cancer patients, along with an additional splice variant clone. Ultimately, PSPHL may be able to serve as a prognostic marker in endometrial cancer, and its effect on biologic function requires further investigation.

Zhao and colleagues also identified a racial disparity in AA compared to CA women with early breast cancer. This team performed miRNA expression analysis amongst these racial groups and found that 31 miRNAs were significantly differentially expressed between CA cases and CA healthy controls, and 18 miRNAs were significantly differentially expressed between AA cases and AA healthy controls [18]. Interestingly, they only found two differentially expressed miRNAs

overlapping between CA and AA study subjects, suggesting potential racial differences in circulating miRNA expression in early breast cancer patients.

MicroRNAs: MicroRNAs (miRNAs) are small, non-coding RNAs approximately 19-24 nucleotides long that affect expression of protein-coding genes primarily through binding to the 3' UTR of their mRNA targets [31]. MiRNAs can regulate gene expression through translational repression of their target genes or by degradation of the mRNA transcript [31]. Interestingly, one miRNA has the ability to regulate expression of hundreds of genes, and a single transcript can be targeted by multiple miRNAs [46]. They are also involved in a number of cellular and physiological processes, including tissue development, cellular differentiation and proliferation, signaling pathways, and apoptosis [32; 46]. Most miRNAs reside between genes (intergenic) and are transcribed as primary miRNA (pri-miRNA) transcripts by RNA polymerase II. miRNAs can also reside within introns of protein coding or non-protein coding genes (intronic) and must be excised through splicing [47]. The biogenesis of miRNAs begins in the nucleus, where miRNAs are transcribed by RNA polymerase II or RNA polymerase III to produce the pri-miRNA transcript. The pri-miRNA is then cleaved by the Microprocessor, which includes Drosha and DGCR8, resulting in the precursor miRNA (pre-miRNA) hairpin. The pre-miRNA is exported out of the nucleus by Exportin 5 to be further processed by the Dicer complex. Dicer cleaves the loop, and one strand of the miRNA duplex is loaded onto Argonaute to form the miRNA-RISC complex. The now mature miRNA is then capable of down-regulating gene expression through mRNA decay or translation inhibition [Figure 3].

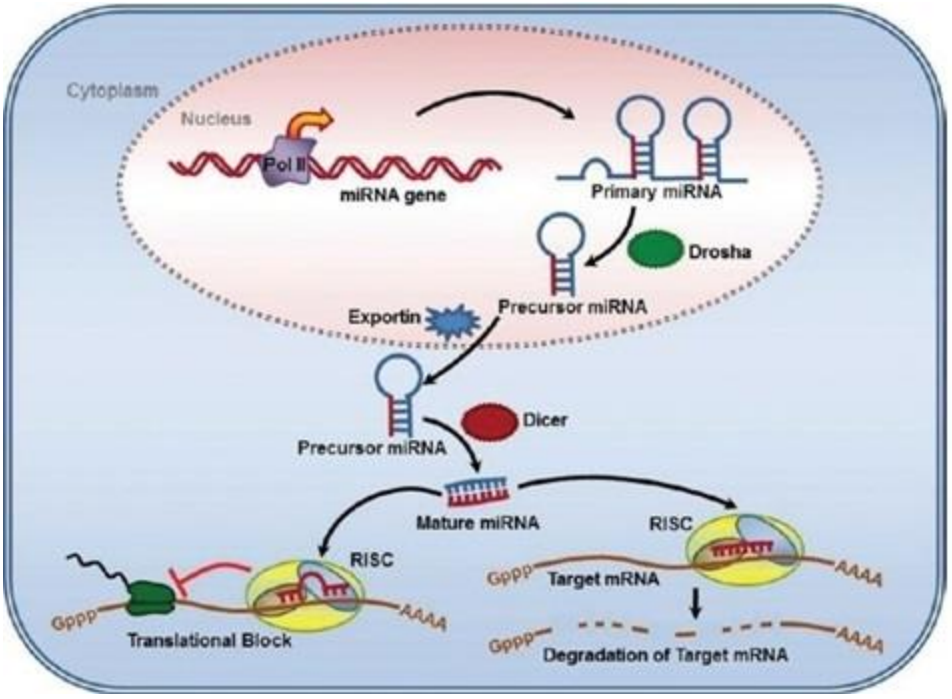

Figure 3: microRNA biogenesis.
Adapted from Joshi, et al 2011.

Mounting evidence indicates that miRNAs also play a role in carcinogenesis acting either as oncogenes or tumor suppressors [31; 32; 46; 48]. About 50% of annotated human miRNAs are detected in cancer associated regions and fragile sites [32; 48], with 72.8% of breast cancers exhibiting miRNA abnormalities [49]. Recently, it was discovered that extracellular miRNAs circulate in the blood of both healthy and diseased patients, and they are both stable and detectable in human serum and plasma [48; 50]. This allows miRNAs to be measured repeatedly and non-invasively and has sparked extensive research in miRNAs as therapeutic targets, as well as potential biomarkers of prognosis and diagnosis. MiRNA secretion and its role in intercellular communication have also recently gained increased attention. Studies have provided evidence that microvesicles (up to 1 μm) released from many cell types can transfer miRNAs to neighboring or distant cells, and these exogenous miRNAs function similarly to endogenous miRNAs to regulate target gene expression and recipient cell function [48; 50; 51]. Therefore, secreted miRNAs may serve as a novel class of signaling molecules for mediating cell-to-cell communication.

miR-510: MiR-510, the miRNA of interest in this study [Figure 1], is located in the intergenic region of chromosome Xq27.3, a region that is reported to be amplified in cancer [54]. While few studies have investigated miR-510 in cancer, our lab has shown that miR-510 levels are significantly elevated in breast cancer samples compared to matched non-tumor samples [31]. Furthermore, our published studies have shown that over-expression of miR-510 in the non-invasive MCF7 breast cancer cell line results in increased migration, invasion, and colony formation in soft agar and a morphology change similar to that observed in cells undergoing

EMT [52]. Further research is necessary to investigate the role of miR-510 in the development and progression of breast cancer.

MiR-510 has also been shown to act as a prognostic marker in ovarian cancer, another sex-specific cancer previously mentioned to be disparate after controlling for socioeconomic status, prognostic, and treatment variables. Zhang and colleagues concluded that the expression of miR-510 was significantly higher in low grade serous carcinoma and clear cell carcinoma tissues, compared with high grade serous carcinoma and normal ovarian tissues [53]. Therefore, miR-510 may potentially serve as a novel biomarker for clinical outcome prediction in epithelial ovarian cancer.

Caveolin-1: Our lab chose to examine Caveolin-1 protein and its relationship with miR-510 [Figure 1], because considerable amounts of literature show that Caveolin-1 expression has prognostic significance specifically in breast cancer [35; 37; 38; 39; 40; 41]. Caveolin-1 (Cav-1) is a membrane-bound scaffolding protein and the structural/functional element of Caveolae. Caveolae are small invaginations of the plasma membrane about 50-100 nm in diameter, and they serve as signaling regulators for a variety of pathways [33]. Cav-1 is a homo-dimer that enables anchorage to the plasma membrane through a central hydrophobic domain, which is flanked by hydrophilic N- and C-terminal cytosolic domains [35]. Cav-1 is thought to play a role in tumorigenesis through various functions, including lipid transport, membrane trafficking, gene regulation, and signal transduction [36]. Located on the cell surface in most cell types, Cav-1 expression is most abundant in terminally differentiated cells such as epithelial and endothelial cells, fibroblasts and adipocytes [33; 34]. Terminally differentiated cells are often

regulated by pathways that act to prevent cell-cycle progression; therefore, abundant Cav-1 expression in these cell types suggests that Cav-1 acts as a tumor suppressor by regulating pathways involved in cell-cycle progression, such as the RB/E2F pathway [24; 44]. This data is further supported by studies that have shown that Cav-1 negatively regulates cell proliferation (i.e. inhibition of pro-proliferative signaling pathways initiated by receptor tyrosine kinases or serine-threonine protein kinases [44]), exhibiting another role of tumor suppression [34; 38]. The Cav-1 gene, CAV1, maps to a suspected tumor suppressor locus (D7S522/7q31.1) that is frequently deleted in a number of carcinomas, including breast cancers [33].

Cav-1 in Breast Cancer: Cav-1 expression in the stromal compartment is a strong predictor of clinical outcome in breast cancer patients [38]. Ductal carcinoma in situ (DCIS) that exhibit loss of stromal Cav-1 are more likely to progress to invasive carcinomas, suggesting that Cav-1 loss regulates tumor progression [39]. Numerous studies have demonstrated that loss of stromal Cav-1 is significantly associated with larger tumor size, higher grade, higher nodal stage, higher recurrence rate, metastasis, and reduced progression-free survival [35; 38]. Overall, breast cancer patients with a loss of stromal Cav-1 show a 20% 5-year survival rate, compared to the 80% 5-year survival of patients with high stromal Cav-1 expression [40]. Interestingly, researchers have also shown that positive expression of stromal Cav-1 is associated with improved outcome in breast cancer, including a higher survival rate [37; 38; 41]. While strong evidence exists showing the significance of stromal Cav-1 loss in breast cancer outcome, it is important to note that there is no known association between Cav-1 expression in the epithelial compartment and clinical outcome [35; 37; 38; 41].

The effects of Cav-1 loss have been studied extensively utilizing in vivo models. A study by Williams and colleagues showed that loss of Cav-1 expression accelerates the development of dysplastic mammary lesions in tumor-prone transgenic mice [42]. Polyomavirus middle T antigen transgenic mice (PyMT) provide an ideal model to study the effect of a given gene on early tumorigenesis, because multifocal dysplastic foci develop in the mammary epithelium of PyMT mice as early as 3 weeks of age. Figure 4 shows identical magnifications of Cav-1 positive mice and Cav-1 null mice at 3 weeks, carmine-stained and whole mounted. The primary duct (PD), which originates from the nipple area, is visible in the top left corner and terminal end buds (TEB) are also visible. What is important to note is the dramatically increased size and number of the dysplastic foci in the mammary glands of Cav-1 null mice compared to the Cav-1 positive mice. At 4 weeks, the mammary epithelial tree extended to the region of the lymph node in both genotypes, and the dysplastic areas in the mammary glands of Cav-1 null mice coalesced to form larger singular masses [42].

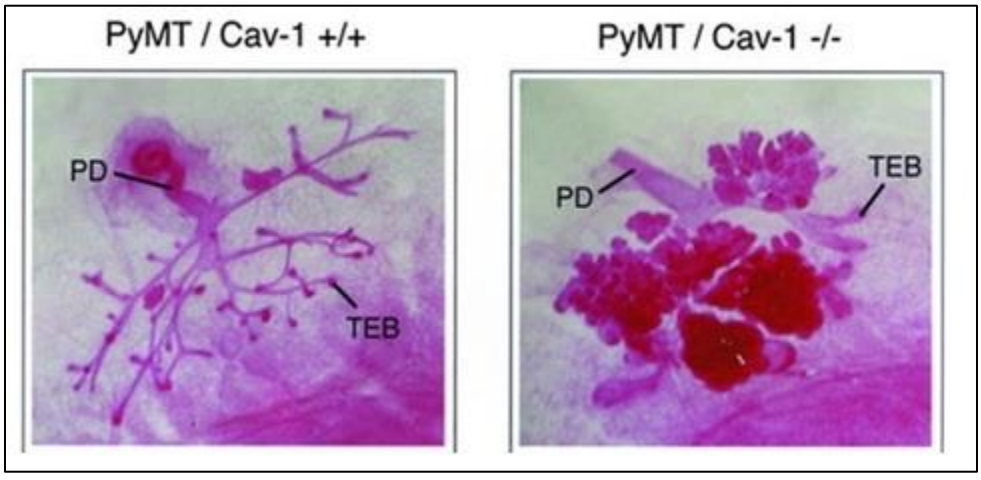

Figure 4: Foci development in mammary glands of tumor-prone transgenic mice at 3 weeks of age, carmine-stained and whole mounted. Adapted from Williams, et al 2003.

Tumor Microenvironment: The tumor microenvironment is the stroma hosting malignant breast cancer cells, which includes the extracellular matrix (ECM) and various stromal cell types: leukocytes, fibroblasts, myoepithelial cells, and endothelial cells [4;19]. The tumor stroma changes during malignancy and eventually promotes growth, invasion, and metastasis. Specifically, we see infiltration of inflammatory cells, angiogenesis, and ECM remodeling [21]. Studies show that the cellular microenvironment is dramatically different at the molecular level between normal breast tissue and breast carcinomas, with differences already evident at the in situ stage before invasion even takes place [4; 5; 18; 20]. In DCIS, myoepithelial cells are phenotypically altered and their numbers decreased [Figure 5]. As breast cancer progresses, the number of stromal fibroblasts increases, as well as numbers of myofibroblasts, lymphocytes, and endothelial cells [4; 21].

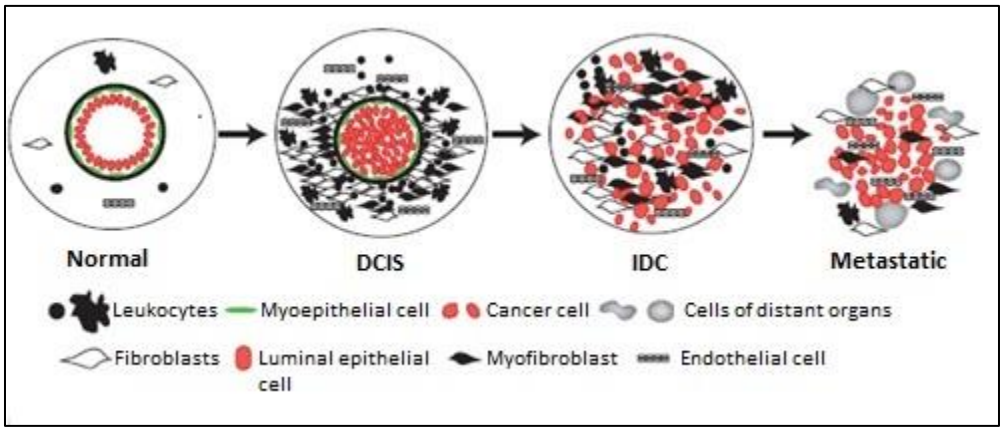

Figure 5: Micro environmental alteration during tumor progression. Adapted from Polyak K & Kalluri R 2010.

Cancer-Associated Fibroblasts: Down-regulation of Cav-1 is a mechanism implicated in the oncogenic transformation of fibroblasts to cancer-associated fibroblasts (CAFs) [37; 42]. One of the most prominent cell types in the tumor stroma is the activated fibroblast, or CAF. Normal fibroblasts are responsible for the development of most of the connective tissue components and the synthesis and turnover of the ECM [22; 23; 25]. In addition, they control tissue homeostasis and actively participate in wound healing and senescence [23; 25]. But unlike normal fibroblasts, CAFs inhabit the tumor itself or infiltrate the tumor mass, and promote tumor progression by encouraging tumor growth, inflammation, and metastasis through various mechanisms [21; 23; 25]. CAFs can promote tumor growth through release of growth factors, such as hepatocyte growth factor (HGF) and transforming growth factor beta (TGF-β), or vascularization through vascular endothelial growth factor (VEGF) [21]. They can also promote tumor invasion through modulation of matrix metalloproteinase (MMP) activity, metastasis by inducing epithelial-to-mesenchymal transition (EMT), and prevent cancer cell apoptosis [19; 25; 30]. In co-culture experiments, CAFs have been shown to enhance proliferation and migration, while in vivo studies have demonstrated their ability to promote angiogenesis [23; 24]. CAFs are also said to be perpetually activated, as they never revert to a normal phenotype or undergo apoptosis or spontaneous quiescence as would a wound-healing fibroblast [24; 26]. Although many plausible sources of CAFs have been identified by researchers, they can be viewed as normal fibroblasts altered by continuous exposure to cancer cells that promote tumorigenesis [Figure 1].

Epithelial-stromal Crosstalk: Epithelial-stromal communication though the ECM is crucial for proper development and function of the mammary gland. However, disruption of the cell-cell

interactions between compartments can both induce and promote breast cancer [19; 27; 28]. Evidence has demonstrated that compared to normal mammary gland stroma, the tumor microenvironment shows disruption of epithelial-fibroblast cell communication in terms of cell proliferation and extracellular matrix remodeling [2; 30]. While studies show that the mammary stroma provides instructive and permissive signals that allow the mammary epithelium to proliferate and invade, oncogenic signaling occurs reciprocally and creates advantageous conditions for malignant cells [22; 27].

Hypothesis: miRNAs secreted from epithelial tumor cells to nearby stromal cells is a mechanism leading to Cav-1 reduction, resulting in increased levels of cancer-associated fibroblasts in the tumor microenvironment, and thereby promoting tumor progression and worse outcome in AA breast cancer patients [Figure 1].

Specific Aim 1: To assess whether miR-510 expression in epithelial cells results in the reduction of Cav-1 in fibroblasts *in vitro*.

Rationale: There is a well-established correlation between reduced stromal Cav-1 expression and outcome in breast cancer patients; however, the mechanism of loss is still unclear. Accumulating evidence has demonstrated that interactions between malignant epithelial cells and stromal fibroblasts contribute to tumor initiation and progression [28]. The two compartments communicate through release of various soluble factors to create advantageous conditions for the tumor microenvironment [22; 28]. A proposed mechanism of this epithelial-stromal communication is tumor-derived microvesicles, shown to be released from epithelial tumor cells [28; 51]. These microvesicles serve as intercellular transmitters, capable of carrying miRNA, mRNA, proteins, DNA, and lipids [50; 51; 55]. Once assimilated by cells of the tumor microenvironment, the contents of these vesicles may modulate the molecular profile and function of recipient cells, leading to tumorigenesis [55]. Specifically, miRNAs have been shown to be transferable by microvesicles, such as exosomes, and functional in recipient cells [50; 51]. miRNA target prediction software (TargetScan) predicted Cav-1 to be a direct target of miR-510. Therefore, this aim will first establish Cav-1 as a novel direct target of miR-510 and then will explore the possibility that miR-510 secreted from epithelial cells in microvesicles negatively regulates Cav-1 expression in neighboring stromal cells.

Preliminary Data

miR-510 levels are elevated in human breast cancer patients. Previous work in our lab has shown miR-510 to be significantly elevated in human breast tumor samples compared to matched non-tumor samples, suggesting an important role in breast cancer progression within an individual patient [Figure 6A]. Preliminary experiments in the lab also investigated the potential disparate expression of miR-510 by examining serum samples of AA and CA women. Analysis of serum miR-510 was performed utilizing qPCR, and the results show a statistically significant increase of miR-510 in the serum of AA women with BC when compared to AA women with benign disease [Figure 6B]. However, this increase in serum miR-510 levels was not significant in CA women with BC compared to benign disease. We also observed a trend in the elevation of miR-510 levels in the serum of AA women with BC compared to CA women with BC however due to the small sample size this did not reach statistical significance. The trends we observed suggests miR-510 may be elevated in AA women with BC. This will have to be further confirmed in a large scale study, but it does provide evidence that miR-510 expression may be racially disparate.

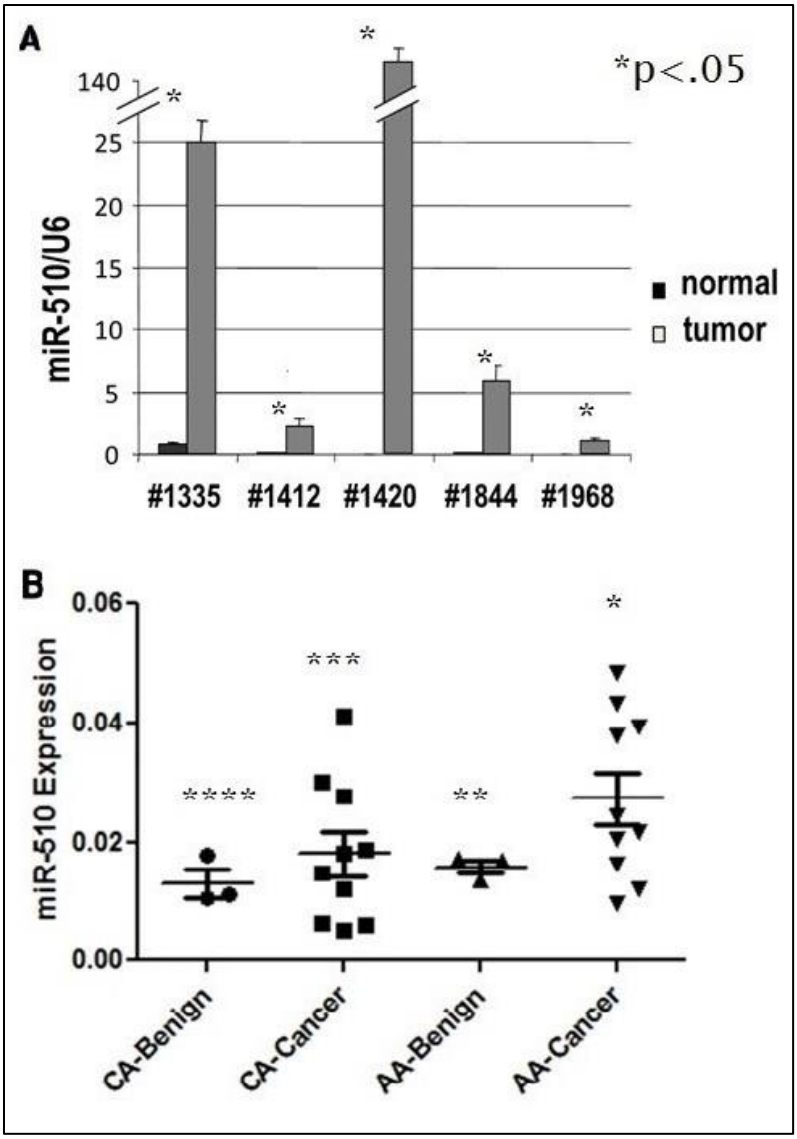

Figure 6: miR-510 expression in breast cancer patient samples. qPCR assessing normalized miR-510 expression in breast tumors compared to matched non-tumor samples (A). qPCR quantitation of miR-510 expression in serum of CA vs. AA women with breast cancer (B). (****CA-Benign v. CA-Cancer = .29 *** CA-Cancer v. AA-Cancer = .12 ** AA-Benign v. AA-Cancer = .04 *AA-Benign v. CA-Benign = .40)

Cav-1 levels are disparate in AA breast and prostate cancer patients. Data mining revealed that Cav-1 mRNA levels are lower in AA women with breast cancer (tumor epithelial or stromal not specified) when compared to CA women in data sets from Oncomine [Figure 7A]. These findings suggest loss of stromal Cav-1 may be the mechanism leading to poorer breast cancer survival in AA women and warrants further investigation of its role in promoting breast cancer disparity. Clinical studies suggest that AA prostate cancer patients manifest a more aggressive form of the disease compared with CA prostate cancer patients. Cav-1 IHC from this study shows that Cav-1 levels are elevated in the stroma of white patients, while levels are elevated in the epithelial cells of AA patients [Figure 7B]. Subsequent studies have shown an inverse correlation between epithelial and stromal expression, suggesting perhaps that stromal Cav-1 is lower in AA prostate patients, which leads to a poorer outcome [45].

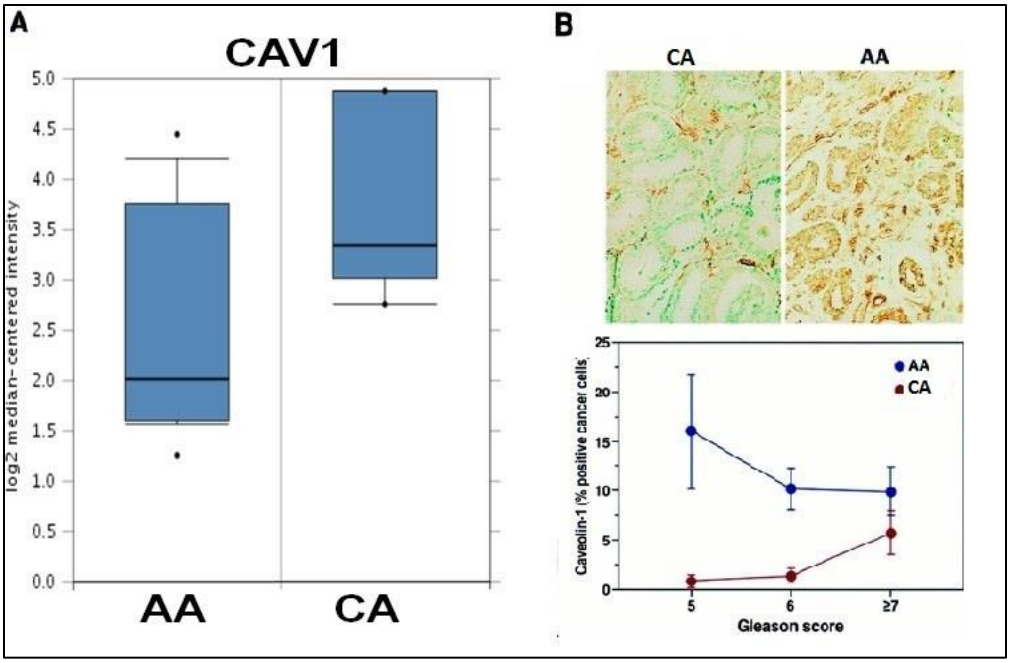

Figure 7: Cav-1 levels are disparate in AA breast and prostate cancer patients. Cav-1 mRNA expression is lower in AA women compared to CA women with breast cancer. Adapted from Chang Data Set, Oncomine (A). Cav-1 IHC shows that Cav-1 levels are elevated in the stroma of CA prostate cancer patients, while levels are elevated in the epithelial cells of AA patients. Adapted from Yang, et al 2011 (B).

Specific Aim 1 Tasks:

Task 1: Perform miR-510 gain of function (GOF) studies in cell lines to define:

- If miR-510 overexpression correlates with loss of Cav-1 protein/mRNA expression in MDA-MB-231 tumor epithelial cells
- If miR-510 overexpression correlates with loss of Cav-1 protein/mRNA expression in WPMY1 stromal fibroblasts

Task 2: Clone the 3'UTR of Cav-1 in luciferase reporter vector

Task 3: Perform dual luciferase assay to determine:

- If Cav-1 mRNA is a direct target of miR-510

Task 4: Perform site directed mutagenesis to mutate the seed site for miR-510 in Cav1 3'UTR

Task 5: Perform dual luciferase assay to determine:

- If miR-510 directly targets CAV1 3'UTR through the predicted site

Task 6: Perform *in vitro* co-culture studies with MDA-MB-231 tumor epithelial cells and WPMY1 stromal fibroblasts to determine:

- If miR-510 is secreted from MDA-MB-231 epithelial cells
- If miR-510 is taken up by WPMY1 stromal fibroblasts when cultured together with MDA-MB-231 tumor epithelial cells
- If transfer of miR-510 from MDA-MB-231 tumor epithelial cells results in a loss of Cav-1 in the WPMY1 stromal fibroblasts

Task 7: Perform migration assay in co-cultured WPMY1 stromal fibroblasts to determine:

- If WPMY1 stromal fibroblasts co-cultured with miR-510-expressing MDA-MB-231 tumor epithelial cells are more aggressive compared to those co-cultured with a scrambled control expressing MDA-MB-231 tumor epithelial cells.

Task 8: Perform exosome isolation from *in vitro* co-culture studies to determine:

- If MDA-MB-231 tumor epithelial cells transfer miR-510 into neighboring WPMY1 stromal fibroblasts via exosomes

Specific Aim 1 Methods:

Cell Culture: MDA-MB-231 and MCF10a breast epithelial cell lines, as well as NIH-3T3 mouse stromal cells, were utilized for miR-510 overexpression. WPMY1 prostate stromal cells were also utilized for miR-510 overexpression in lieu of a primary breast stromal cell line. HEK-293 cell lines were utilized for the dual luciferase assay. All cell lines were cultured in a 37°C, 5% CO2 incubator in their respective media. MDA-MB-231, 3T3, WPMY1, and HEK-293 cells were incubated in DMEM/High Glucose media (HyClone, South Logan, UT), with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT) and 1% Penicillin/Streptomycin mixture (Fisher Scientific, Hampton, NH). MCF10a cells were incubated in DMEM/F media (HyClone, South Logan, UT), with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT) and 1% Penicillin/Streptomycin mixture (Fisher Scientific, Hampton, NH). Media on the cells were changed every 2-3 days and cells are passaged by using 0.05% trypsin (HyClone, South Logan, UT) when plates were ≤90% confluent.

GOF studies: MDA-MB-231 and MCF10a epithelial cells, as well as NIH-3T3 and WPMY1 stromal fibroblasts are plated at a density of 1 x 10^5 cells/mL in 2ml in a 6 well plate. After 24 hours, the cells are transfected with 200 µL of OPTI-MEM, either 1µg pEZX Scr vector (control), 0.5µg or 1µg pEZX miR-510 vector, and 3 µL of XTREME Gene HP DNA Transfection Reagent per µg of DNA, followed by a 15 minute incubation period. After 48 hours, the cells are collected for RNA and protein extraction.

Quantitative Real Time PCR (qPCR), Reverse Transcription and downstream analysis: RNA is extracted using the RNeasyPlus Mini Kit from Qiagen as per the manufacturer's instructions. For

microRNA analysis, 100 ng total RNA is reverse transcribed using miR-510 specific primers using the Applied Biosystems reverse transcription kit as per the manufacturer's instructions. qPCR is performed with 1 µl of reverse transcribed cDNA using the TaqMan Assay from Applied Biosystems as per the manufacturer's instructions on the Roche LightCycler 480. For mRNA analysis, 1 µg total RNA is reverse transcribed in a 20 µl reaction using iScript (Bio-Rad). qPCR for gene expression is performed with 5 µl of a 1:20 dilution of reverse transcribed cDNA using the universal probe library (UPL) system from Roche (Nutley, NJ) in a LightCycler (see Table 1). The cycling conditions are performed as per the manufacturer's instructions. Triplicate reactions are run for each cDNA sample. The relative expression of each gene is quantified on the basis of Ct value measured against an internal standard curve for each specific set of primers using the software provided by the instrument manufacturer (Roche, Nutley, NJ). The data is normalized to GAPDH.

Table 1: iScript (BioRad) Genes and UPL System Data

Gene	UPL Number	5' Primer Sequence	3 Primer Sequence	Amplicon Length
CAV1	60	acagcccagggaaacctc	ggatgggaacggtgtagagat	103 nt
COLLAGEN1	50	caggcaaacctggtgaaca	ctcgccagggaaacctct	89 nt
EPCAM	3	ccatgtgctggtgtgtgaa	tgtgttttagttcaatgatgatcca	111 nt
FSP1	24	gctcaacaagtcagaactaaaggag	gcagcttcatctgtccttttc	78 nt
GAPDH	60	agccacatcgctcagacac	gcccaatacgaccaaatcc	66 nt

Protein Extraction and Western Blot Analysis: Protein is extracted by lysis of cell pellets with RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5 % sodium deoxycholate, 1 % Triton X 100 and protease inhibitor cocktail) and protein concentrations are determined by performing a BSA assay as per the manufacturers' instructions (Pierce). 25 ug protein of each sample is then run on an SDS-PAGE gel and transferred to a PVDF membrane using the Bio-Rad western blotting system. Membranes are probed with Cav-1 (Cell Signaling Technology, 1:2500 dilution) and GAPDH (Santa Cruz Technology, 1:1000 dilution) antibodies.

CAV1 3'UTR Identification and Cloning: Bioinformatic tools are available on the web that predict the potential candidate mRNA targets of any given miRNA. However, these putative targets must be validated through experimental approaches. We consulted the miRNA target prediction websites, TargetScan (http://targetscan.org) and Miranda (http://www.microrna.org), linked on miRBase (http://mirbase.org) for miR-510 to score for putative targets of miR-510. Both TargetScan and Miranda microRNA target prediction databases list CAV1 as a potential direct target of miR-510. CAV1 3'UTR luciferase reporter vector purchased from Genecopoeia.

Site directed mutagenesis (SDM): The miR-510 seed site within the 3'UTR of CAV1 is mutated using the site directed mutagenesis kit from Stratagene as per the manufacturer's instructions. Potential clones are sequenced to verify the correct mutation.

Dual Luciferase Assay: The CAV1 3'UTR luciferase reporter clone along with an empty vector (EV) control are transiently transfected into HEK-293 cells with and without miR-510 expression, and then 48 hours later the luciferase activity is measured. In addition, we will mutate the seed

sequence of miR-510 in the Cav-1 3'UTR by site-directed mutagenesis (Stratagene) and confirm that the predicted miR-510 binding site is the actual binding site for miR-510.

Co-culture experiments: MDA-MB-231 tumor epithelial cells are cultured in a non-contact manner with WPMY1 stromal fibroblasts using a transwell migration system [Figure 8]. WPMY1 stromal fibroblasts are plated in the bottom of a 6-well dish at a density of $2.0x10^5$ cells/2mL in complete media and breast epithelial cells, either expressing miR-510 or a scrambled control, are seeded at a density of $2.0x10^5$ cells/1mL into the upper chamber of a Transwell insert with a pore size of 0.4 μM. The MDA-MB-231 tumor epithelial cells in the upper chamber are unable to move through the pores, however microvesicles derived from the MDA-MB-231 tumor epithelial cells are able to move through the pores and into the lower chamber where they can interact/fuse with the WPMY1 stromal fibroblasts. After 48h, the fibroblasts in the lower chamber are collected for RNA and protein analysis. A transwell insert containing media only serves as an additional negative control.

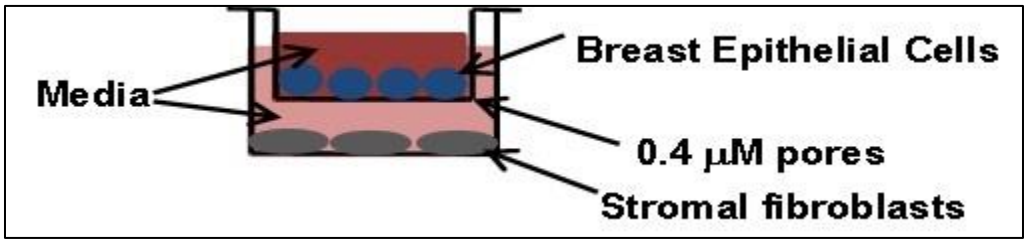

Figure 8: Schematic representation of co-culture model

Conditioned media experiment: WPMY1 stromal fibroblasts plated in a 6-well dish at a density of $2.0x10^5$ cells are incubated with 2mL/well of conditioned media from MDA-MB-231 tumor epithelial cells for 24h to assess whether miR-510 is present in the media from MDA-MB-231 tumor epithelial cells in the absence of any neighboring cell stimulation and RNA and protein analysis performed as described above.

Migration Assay: MDA-MB-231 tumor epithelial cells are cultured in a non-contact manner with WPMY1 stromal fibroblasts using a transwell migration system [Figure 8]. WPMY1 stromal fibroblasts are plated in the bottom of a 6-well dish at a density of $2.0x10^5$ cells/2mL in complete media and breast epithelial cells, either expressing miR-510 or a scrambled control, are seeded at a density of $2.0x10^5$ cells/1mL into the upper chamber of a Transwell insert with a pore size of 0.4 μM. After 48h, the fibroblasts in the lower chamber are collected and counted. The fibroblasts collected from the lower chamber are then seeded at a density of $5.0x10^4$ cells/500μL in serum free media into the upper chamber of a new Transwell insert with a pore size of 0.8 μM. 500μL of complete media is added to wells in a 24-well dish. Inserts are placed into each well and incubated at 37°C for 4h. After 4h, the inserts are cleaned and placed in new wells with 500 μL of 3.7% formaldehyde for 15 minutes, followed by two washes with PBS for 3 minutes each. Inserts are then placed in new wells with 2% crystal violet for 30 minutes. Inserts are washed in Millipore water and air dried. Migrated cells are quantified by averaging the count of migrated cells in 5 planes.

Exosome isolation: MDA-MB-231 tumor epithelial cells are cultured in a non-contact manner with WPMY1 stromal fibroblasts using a transwell migration system [Figure 8]. WPMY1 stromal

fibroblasts are plated in the bottom of a 6-well dish (notched for use with cell culture inserts to collect ample media for exosome isolation) at a density of $5.0x10^5$ cells/2mL in complete media with exosome-depleted FBS. Breast epithelial cells, either expressing miR-510 or a scrambled control in serum-free media, are seeded at a density of $5.0x10^5$ cells/1mL into the upper chamber of a Transwell insert with a pore size of 0.4 µM. The MDA-MB-231 tumor epithelial cells in the upper chamber are unable to move through the pores, however microvesicles derived from the MDA-MB-231 tumor epithelial cells are able to move through the pores and into the lower chamber where they can interact/fuse with the WPMY1 stromal fibroblasts. After 48h, the fibroblasts in the lower chamber are collected for exosome isolation, using the exosome isolation kit from PureExo. Following collection of 2mL of cell culture medium, the cell media is centrifuged at 2,000 RPM for 10 minutes at 4ºC to remove cells or debris. The cell-free culture media is then transferred to a new glass tube and prepared mixture A/B/C from the kit is added, followed by a vigorous 30 second vortex and 30 minute incubation at 4ºC. Following incubation, the top of 2 layers is aspirated and discarded without disturbing the bottom fluff layer. The fluff layer is transferred to a new micro centrifuge tube and spun at 5,000 RPM for 3 minutes. A new 3-layer separation occurs, the top and bottom layers are removed, and the remaining middle fluff layer is transferred to a new micro centrifuge tube. After centrifugation at 5,000 RPM for 3 minutes, the tube cap is left open to air dry for 5-10 minutes at RT. 200µL of PBS is added to resuspend the collected fluff. The tube is left on a horizontal shaker for 10 minutes, followed by pipetting up and down vigorously. The tube is spun at 5,000 RPM for 5 minutes and the supernatant carefully transferred into provided PureExo Column. The column is spun at 1,000 RPM for 5 minutes to collect all flow-through. The flow-through is the isolated exosome suspended in PBS. MicroRNA is then extracted from exosomes using the microRNA

extraction kit from Qiagen as per the manufacturer's instructions. qPCR, Reverse Transcription, and downstream analysis are performed as described as above.

Statistical Analysis: For statistical testing, two-sided paired Student's *t*-tests were done using Excel spreadsheet. *p* values are given for each individual experiment, but in general, $p < 0.05$ was considered statistically significant. Error bars represent standard deviations of three independent experiments unless indicated otherwise.

Specific Aim 1 Results:

Cav-1 is a direct target of miR-510.

In order to determine if CAV1 is a direct target of miR-510, HEK-293 cells were transiently transfected with a luciferase reporter vector containing the wild type CAV1 3'UTR (CAV1 UTR) or mutant CAV1 3'UTR with deletion of the bioinformatically predicted miR-510 seed site (CAV1 SDM) and either miR-510 or a scrambled control (scr). Dual luciferase assays were performed 48h after transfection and we observed a 66% reduction in luciferase activity in the cells transfected with both the CAV1 3'UTR and miR-510 when compared to the scr control [Figure 9A]. In the cells transfected with the mutant CAV1 construct, a much lower reduction (~20%) in luciferase activity was observed in the cells co-transfected with miR-510 when compared to the scr control. qPCR for miR-510 serves as a control and shows a successful transfection of miR-510 into HEK-293 cells [Figure 9B]. These data suggest that miR-510 directly binds to 3'UTR of CAV1 and that this occurs through the predicted seed site, shown in Figure 9C.

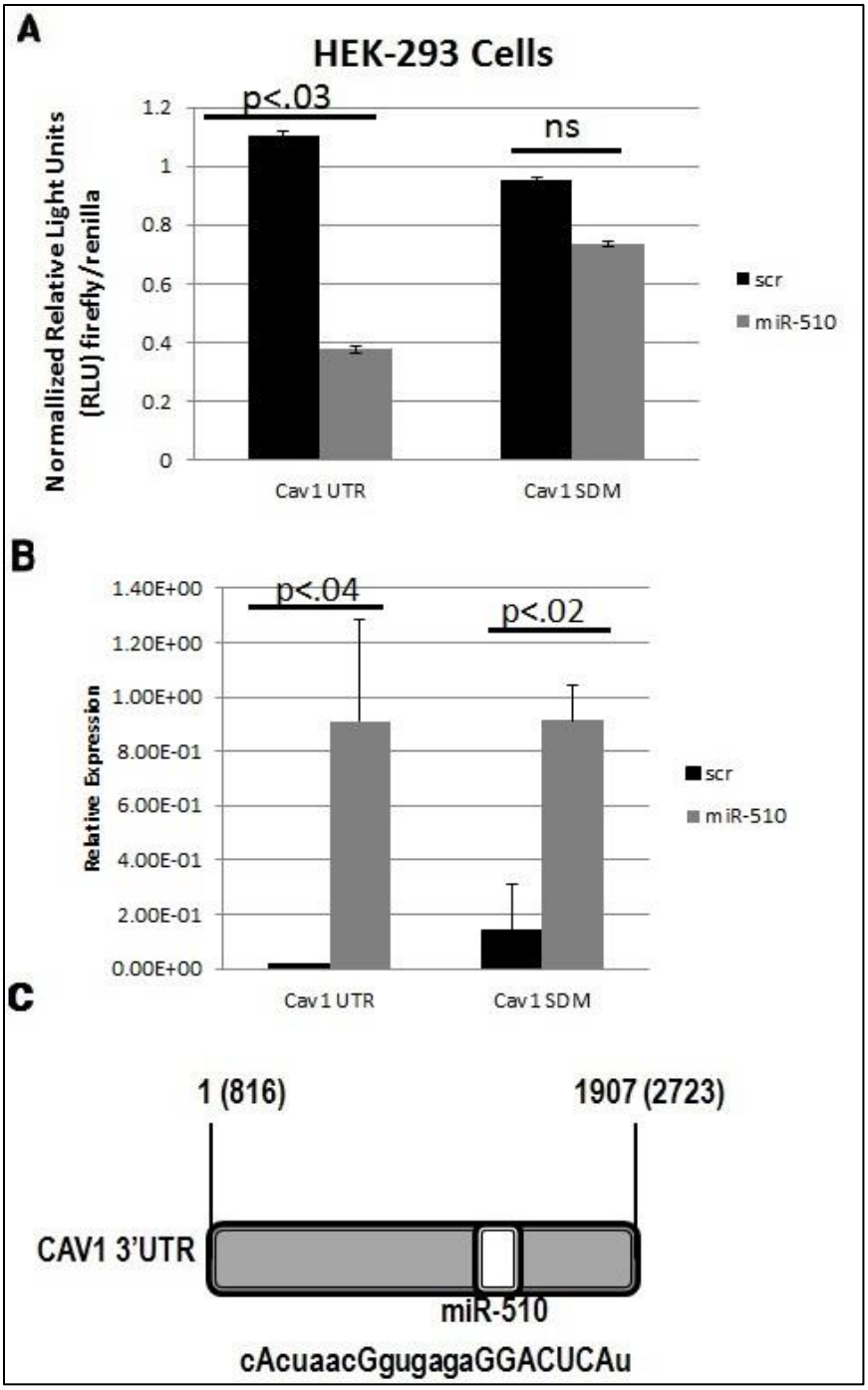

Figure 9: Dual luciferase assay. HEK-293 cells transfected with luciferase construct containing Cav-1 3'UTR mutated at miR-510 seed sequence (A). qPCR quantitation of miR-510 expression in cultured HEK-293 cells (B). Predicted seed site for miR-510 on 3'UTR of CAV1 (C).

miR-510 expression modulates Cav-1 expression specifically in stromal cells.

To assess whether miR-510 negatively regulates protein expression *in vitro*, western blot analysis was performed on epithelial cell lines and stromal cell lines following transfection of miR-510. MDA-MB-231 and MCF10a epithelial cells overexpressing miR-510 do not show a reduction in Cav-1 protein expression levels [Figure 10A; Figure 10B], suggesting that it does not negatively regulate Cav-1 in these cells. However, Cav-1 expression in breast tumor epithelial cells is not associated with poor survival in breast cancer patients, suggesting the mechanism of miR-510 regulation of Cav-1 in breast epithelial cells does not contribute to poorer prognosis. WPMY1 and 3T3 stromal fibroblasts transfected with miR-510 show a reduction in Cav-1 protein expression levels [Figure 10C; Figure 10D], providing encouraging evidence that upregulation of miR-510 may be the mechanism of Cav-1 loss in stromal cells. qPCR for Cav-1 mRNA in all cell types shows no significant change in Cav-1 mRNA expression [Figure 10].

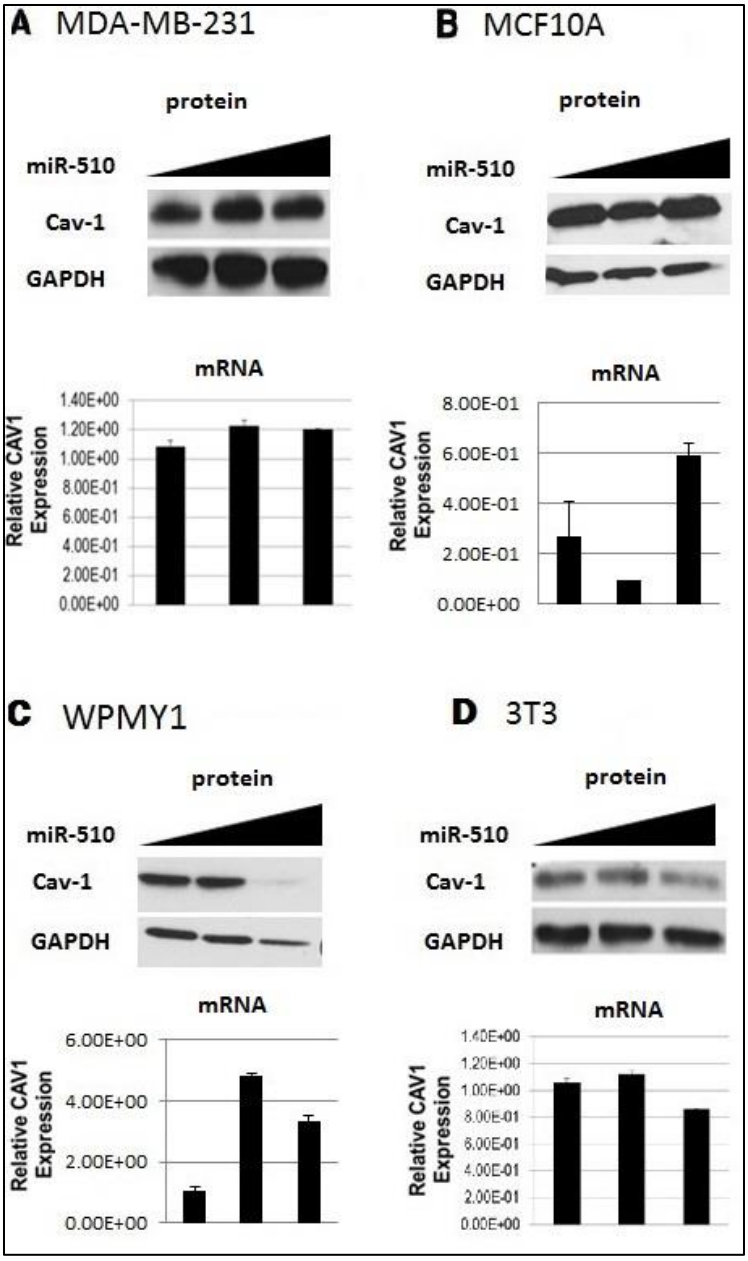

Figure 10: miR-510 expression modulates Cav-1 expression in stromal cells, but not in epithelial cells. Western blot analysis for Cav-1 protein in epithelial cells transfected with miR-510 shows no reduction in Cav-1 protein expression. qPCR quantitation for Cav-1 mRNA shows no significant change in Cav-1 mRNA expression (A,B). Western blot analysis for Cav-1 protein in stromal cells transfected with miR-510 shows a reduction in Cav-1 protein expression. qPCR quantitation for Cav-1 mRNA shows no significant change in Cav-1 mRNA expression (C,D).

miR-510 levels are elevated in fibroblasts co-cultured with, or treated with conditioned media from, miR-510 expressing epithelial cells. To determine whether miR-510 expressed in epithelial cells is transferred to fibroblasts we performed co-culture experiments with MDA-MB-231 breast tumor epithelial cells either stably expressing miR-510 or scr control in the upper well of a transwell insert and WPMY1 normal fibroblasts in the bottom [Figure 11A]. Transwell inserts with no cells (just media) in the upper chamber were included as an additional negative control. 24 hours after cells were cultured together, WPMY1 fibroblasts were collected and we performed qPCR for miR-510 expression. We observed a significant increase in the levels of miR-510 in the WPMY1 fibroblasts co-cultured with miR-510 expressing MDA-MB-231 epithelial cells when compared to both the scr and media alone controls [Figure 11B]. We observed no difference between WPMY1 fibroblasts co-cultured with scr control MDA-MB-231 epithelial cells and media alone. To assess whether miR-510 is present in the media from MDA-MB-231 tumor epithelial cells in the absence of any neighboring cell stimulation, conditioned media from miR-510 expressing MDA-MB-231 epithelial cells was applied to WPMY1 fibroblasts for 24h. WPMY1 fibroblasts were collected and we performed qPCR for miR-510 expression. We observed a significant increase in the levels of miR-510 in the WPMY1 fibroblasts treated with conditioned media from miR-510 expressing epithelial cells [Figure 11C].

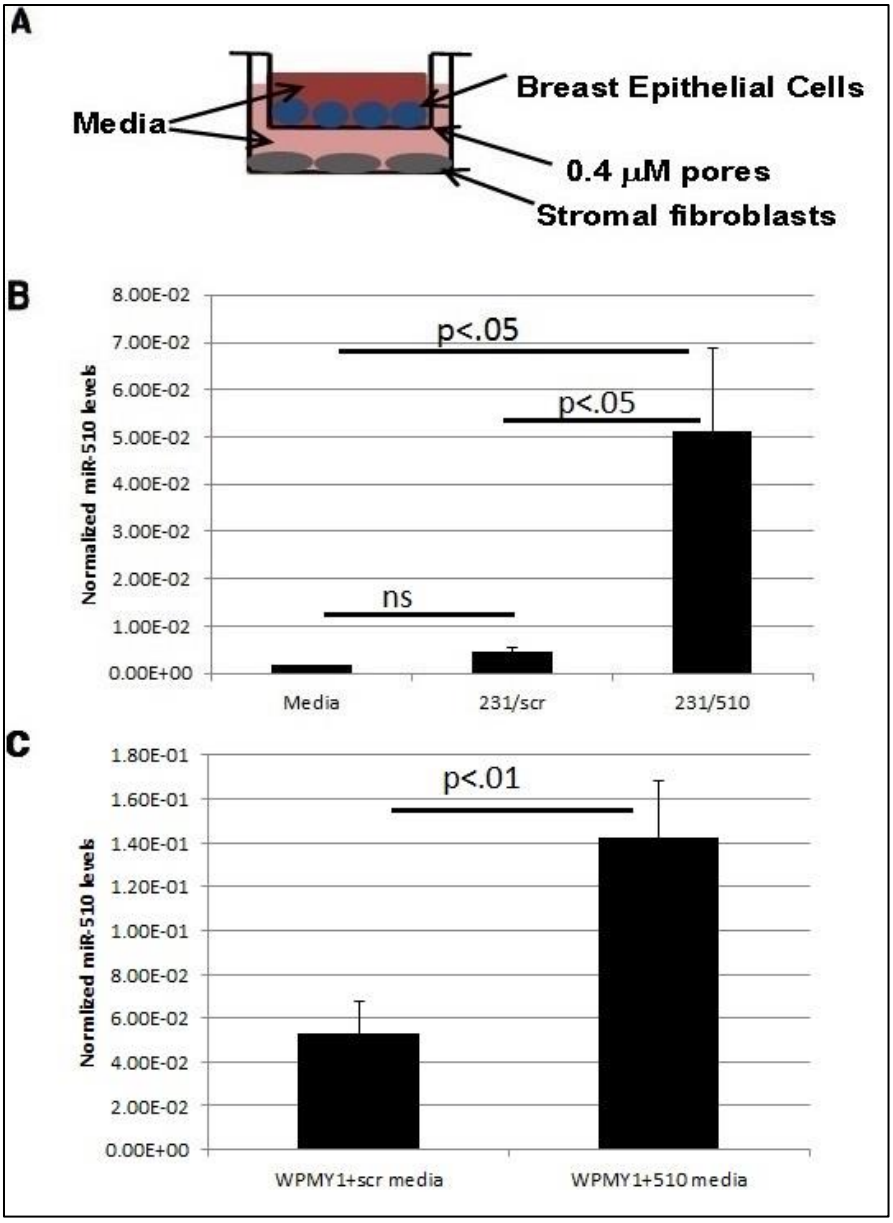

Figure 11: miR-510 levels are elevated in fibroblasts co-cultured with, or treated with conditioned media from, miR-510 expressing epithelial cells. Schematic representation of co-culture experiment (A). qPCR of miR-510 expression in co-cultured WPMY1 fibroblasts (B). qPCR of miR-510 expression in WPMY1 fibroblasts treated with conditioned media from miR-510 expressing epithelial cells (C).

Cav-1 protein is reduced in fibroblasts co-cultured with, or treated with conditioned media from, miR-510 epithelial cells. To determine whether Cav-1 protein expression is modulated in fibroblasts by epithelial derived miR-510, we performed western blot analysis on WPMY1 fibroblasts co-cultured with miR-510 expressing MDA-MB-231 cells as described above. We observed a reduction in Cav-1 protein in fibroblasts co-cultured with miR-510 epithelial cells [Figure 12A]. To determine whether the presence of stromal cells is required to signal the epithelial cells to secrete miR-510, we performed western blot analysis on WPMY1 fibroblasts treated with conditioned media from MDA-MB-231 epithelial cells [Figure 12B]. We observed a decrease in Cav-1 protein levels in cells treated with conditioned media from miR-510 expressing breast tumor epithelial cells when compared to the scr control suggesting that the cells do not need to be cultured together for epithelial cells to secrete miR-510.

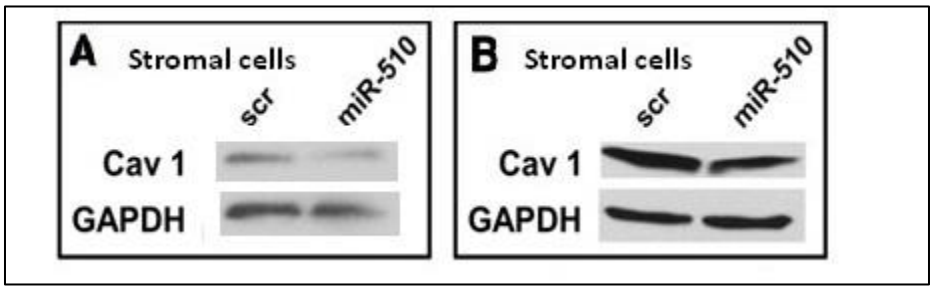

Figure 12: Cav-1 protein is reduced in fibroblasts co-cultured with miR-510 epithelial cells and in fibroblasts treated with conditioned media from miR-510 expressing epithelial cells. Western blot analysis for Cav-1 in WPMY1 stromal fibroblasts co-cultured with miR-510 overexpressing MDA-MB-231 tumor epithelial cells shows decrease in Cav-1 protein expression (A). Western blot analysis for Cav-1 in WPMY1 stromal fibroblasts treated with conditioned media from miR-510 overexpressing MDA-MB-231 tumor epithelial cells shows decrease in Cav-1 protein expression (B).

Fibroblasts co-cultured with miR-510 expressing epithelial cells are activated to a CAF phenotype. To determine whether fibroblasts co-cultured with miR-510 overexpressing breast tumor epithelial cells are 'activated', we performed co-culture experiments as described above, followed by qPCR for COL1A1 (collagen) and FSP1 (fibroblast specific protein), genes that are used as CAF markers [56]. We observed a significant increase in the levels of both collagen and FSP1 (~12 fold) in the WPMY1 fibroblasts that were co-cultured with miR-510 expressing MDA-MB-231 epithelial cells when compared to the scr control [Figure 13].

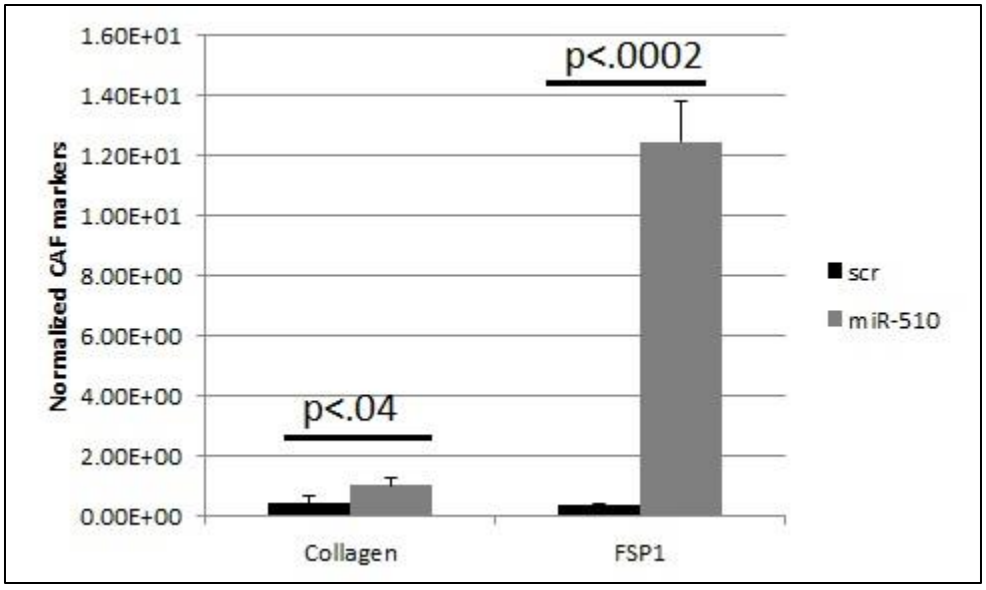

Figure 13: Stromal fibroblasts are activated to a CAF phenotype when co-cultured with mir-510 overexpressing epithelial cells. qPCR quantitation for CAF markers collagen and FSP1 shows increase in WPMY1 fibroblasts co-cultured with MDA-MB-231 epithelial cells overexpressing miR-510.

Fibroblasts co-cultured with miR-510 expressing epithelial cells show increased migration.

To determine if the 'activated' WPMY1 stromal fibroblasts are more aggressive compared to the non-activated scr controls, we performed a migration assay on WPMY1 fibroblasts that had been co-cultured with miR-510 expressing MDA-MB-231 breast tumor epithelial cells or scr controls for 24 hours [Figure 14]. We observed a 60% increase in the migratory capacity of the 'activated' fibroblasts indicating that these stromal fibroblasts are more aggressive.

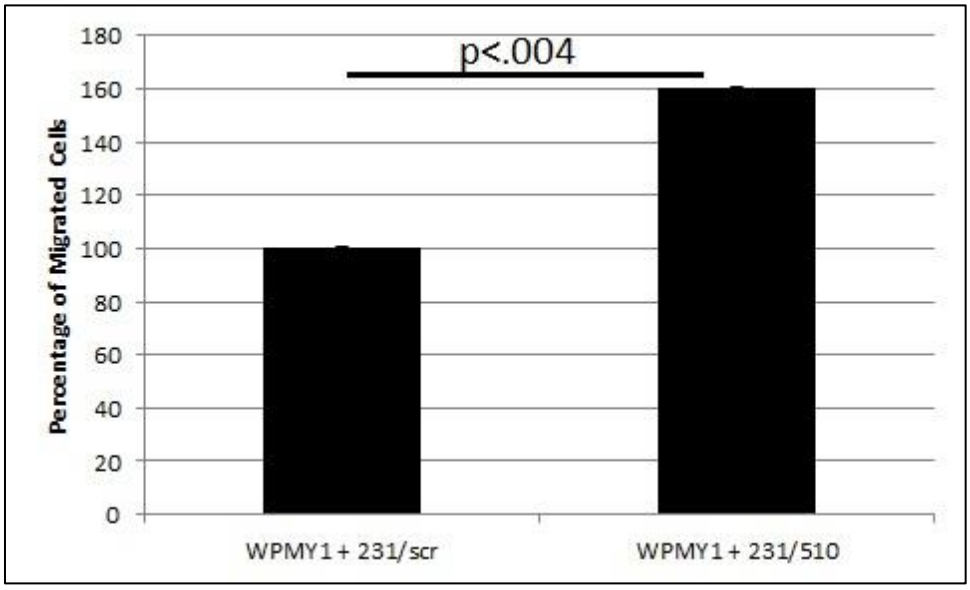

Figure 14: Migration assay. WPMY1 stromal fibroblasts co-cultured with MDA-MB-231 tumor epithelial cells show 60% increase in migration.

miR-510 is not transferred from tumor epithelial cells to nearby stromal cells via exosomes.

To determine if epithelial derived miR-510 is transferred to neighboring fibroblasts via exosomes, we isolated exosomes from the co-culture studies as described above and performed qPCR for miR-510. We expected to see high expression of miR-510 in the isolated exosomes, providing evidence that miR-510 is being transferred via exosomes. However, qPCR for miR-510 revealed that no miR-510 was detectable in our isolated exosomes. To verify that we had isolated intact exosomes, we performed qPCR for miR-21 as this is a miRNA that has been shown to be present in exosomes isolated from MDA-MB-231 conditioned media [57]. We were able to show that miR-21 was present in exosomes isolated from WPMY1 fibroblasts co-cultured with either miR-510 expressing breast tumor epithelial cells and scr controls [Figure 15].

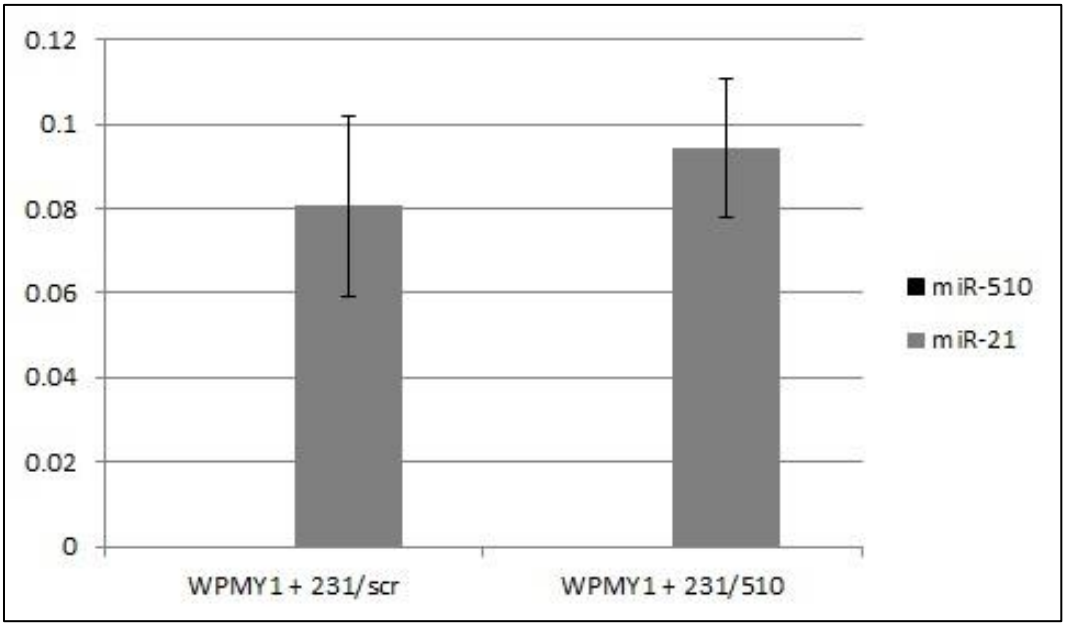

Figure 15: miR-510 is not detectable in isolated exosomes from co-culture study. qPCR for miR-510 and miR-21 in isolated exosomes.

Specific Aim 2: To test the hypothesis that elevated miR-510 expression in breast epithelium promotes tumorigenesis through the negative regulation of Cav-1 expression in the stroma

Rationale: Cav-1 behaves as a transformation suppressor in fibroblasts [44]; therefore, downregulation of Cav-1 is a mechanism implicated in the oncogenic transformation of fibroblasts to CAFs [38; 43]. A study conducted by Sotgia and colleagues provided the first molecular genetic evidence that loss of Cav-1 expression directly contributes to the CAF phenotype [44]. Sotgia and colleagues prepared mammary stromal fibroblasts (MSFs) from wild-type and Cav-$1^{-/-}$ mice and then subjected them to genome-wide transcriptional profiling. Interestingly, the Cav-$1^{-/-}$ mammary stromal fibroblast transcriptome significantly overlapped with that of human breast CAFs, as they identified a list of 55 genes that were commonly up-regulated in both human breast CAFs and Cav-$1^{-/-}$ mammary stromal fibroblasts [Figure 16]. Both show a nearly identical profile of RB/E2F (retinoblastoma/ E2 promotor binding factor)-regulated genes that are up-regulated, which is consistent with RB inactivation [24; 44]. When RB is hypo-phosphorylated in quiescent or differentiated cells, it interacts with the E2F family to repress the transcription of genes necessary for cell cycle progression [24]. When researchers examined the state of phosphorylation in human breast CAFs and Cav-$1^{-/-}$ MSFs, RB was hyper-phosphorylated compared to normal fibroblasts, resulting in RB inactivation [24; 44]. This study concluded loss of Cav-1 expression may be a critical initiating event leading toward the CAF phenotype, as the replacement of Cav-1 expression in CAFs is sufficient to revert their hyper-proliferative phenotype and prevent RB hyper-phosphorylation [24; 44]. Therefore, this aim will explore if elevated expression of miR-510 *in vivo* results in a reduction of Cav-1 and determine its effects on tumor derived fibroblasts and tumorigenesis.

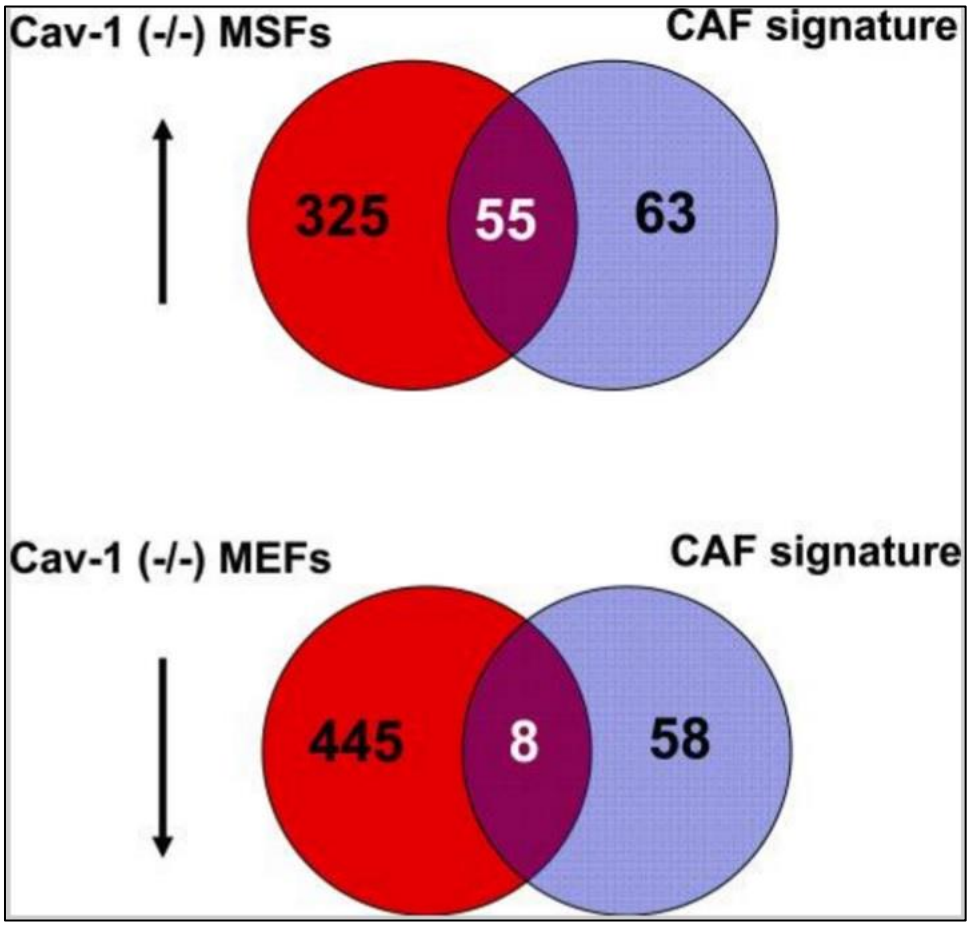

Figure 16: CAF versus Cav-$1^{-/-}$ mammary stromal fibroblast (MSF) gene signatures. Venn diagrams summarizing the similarities and differences between gene transcript changes in human breast CAFs and Cav-$1^{-/-}$ mammary stromal fibroblasts. Adapted from Sotgia F, et al 2009.

Preliminary Data

miR-510 promotes tumor growth in vivo. To assess the functional effects of miR-510 *in vivo*, nude mice were injected orthotopically with MDA-MB-231 cells stably infected with miR-510 or scr control. Tumor growth was monitored twice weekly for approximately 30 days. Tumors from both miR-510 expressing and scr controls appeared to initiate at a similar time frame. Published data from our lab shows that by three weeks the miR-510 expressing tumors were growing more rapidly, and by the end of the study, the miR-510 expressing tumors were larger than the scr controls [52; Figure 17]. Following this previous study, our current study asked the question, "Do miR-510 overexpressing tumors result in more aggressive fibroblasts in the tumor microenvironment than scr control tumors?"

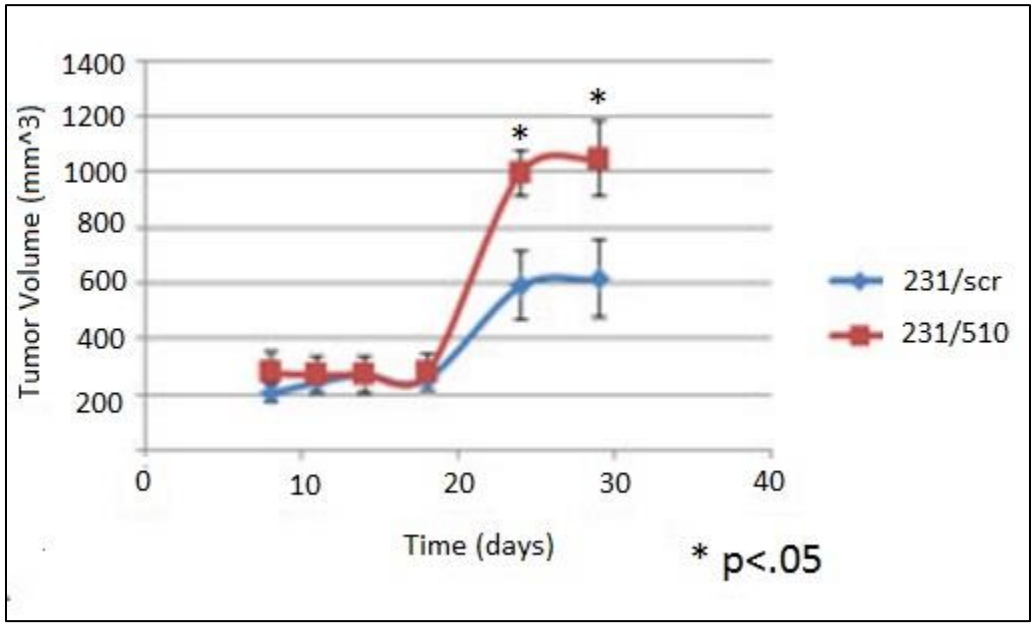

Figure 17: miR-510 promotes tumor growth *in vivo.* Average tumor volumes of mice orthotopically injected with MDA-MB-231 cells stabling infected with miR-510 or scr control show that cancer cells overexpressing miR-510 result in faster tumor growth compared to scr control tumors. Adapted from Guo Q, et al 2013.

Specific Aim 2 Tasks:

Task 1: Orthotopically inject 6 week old nude mice with MDA-MB 231/scr and MDA-MB 231/510 cells and allow to grow to maximum size (~1500mm^3)

Task 2: Harvest tumors and isolate CAFs

Task 3: Assess enriched population of CAFs by qPCR to identify:

- CAF associated markers: COL1A1 and FSP1

Task 4: IF staining of isolated CAFs to determine:

- If miR-510 expressing CAFs show a reduction of Cav-1 protein

Task 4: Orthotopically co-inject scr or miR-510 CAFs and MDA-MB-231 cells into nude mice and monitor tumor growth/size over time to assess:

- If miR-510 derived CAFs results in more aggressive tumor growth

Task 5: IHC of Cav-1 in scr CAF:231, 510 CAF:231, MDA-MB-231 alone tumors to determine:

- If expression of miR-510 results in a reduction of Cav-1 *in vivo*

Methods

Xenograft Assay (in vivo): MDA-MB-231 cells either expressing miR-510 or scrambled control are orthotopically injected into 6 week old nude mice and allowed to grow until the tumors reach a size of ~1.5 cm. Tumors (two per mouse from 68 mice) are harvested from euthanized mice immediately following isoflurane asphyxiation according to IACUC protocols. CAFs are isolated as described below. Following CAF isolation, 5 x 10^4 MDA-MB-231 cells are co-injected orthotopically into 6 week old nude mice, with 1.5 x 10^5 CAFs isolated from either miR-510 expressing or scrambled control tumors, and tumor size/volume is monitored bi-weekly by calipers. Orthotopic injections into 32 total mice of 2 x 10^5 MDA-MB-231 cells, miR-510 or scrambled CAFs alone serve as additional controls. Animals are euthanized at day 64 and solid tumors, lungs and other organs (kidney, liver) are excised to assess for macro metastasis.

CAF Isolation: Tumors are cut into small 1-2 mm pieces followed by digestion in a 0.5mg/ml Collagenase I (Sigma) solution of aMEM for 35 minutes at 37°C in a shaking incubator. The digested tumor suspension is vortexed on high for 30 seconds before filtration through a 40µM cell strainer. The filtered solution is centrifuged at 1600 rpm for 5 minutes and the pellet resuspended in 5ml 0.1% BSA/PBS. The resuspended pellet is centrifuged at 1600rpm for 5 minutes and the resultant pellet resuspended in 20% FBS/aMEM + Pen/Strep (P/S) and plated. 48 hours after plating the cells are trypsinized with 1 ml of 0.25% trypsin-EDTA for approximately 3 minutes with rocking to remove tumor cells. To stop the action of the trypsin, 5 ml of CAF media (20% FBS/aMEM + P/S) is added and then immediately removed. The plate is washed twice with either CAF media or HBSS (no AA). Finally, 10 ml of CAF media is added to the

plate and returned to the incubator to recover for 24 hours before using the cells. The expected yield is approximately 150,000 CAFs/tumor.

Immunofluorescence Assay: 200 µL of fibronectin is placed on a coverslip in a 12-well plate 2 hours prior to plating cells. 5 x 10^4 CAFs in 0.5mL CAF media are plated. Growth media is removed by aspiration and washed twice in 1 x PBS. Cells are fixed in 1 mL of 3.7% formaldehyde in 1 x PBS pH7 and incubated at room temperature (RT) for 15 minutes, followed by 3 washes with cold 1 x PBS. Cells are permeabilized with 1 mL of 0.1% Triton X-100 in 1 x PBS for 20 minutes, followed by 2 washes with cold 1 x PBS. 2 mL of 2% BSA in 1 x PBST is added for 24 hours to reduce background signal. A 1:100 dilution of primary antibody Cav-1 (560 fluorescent wavelength) in 2% BSA in PBST is applied and incubated for 2 hours at RT. Excess antibody is washed 3 times with 1 x PBST for 10 minutes with gentle agitation. To reduce background signal from secondary antibody, 2 mL of 1% BSA in 1 x PBST is added for 2 hours. A 1:100 dilution of secondary antibody, α-rabbit, in 1% BSA in 1 x PBST is applied, followed by incubation at RT for 30-40 minutes. Cells are washed 2 times with 1 x PBS (5 minutes each). 1 µL of HOECHST (1:10000 dilution) is added, and cells incubated for 8 minutes at RT. Excess antibody is washed 3 times with 1 x PBST for 5 minutes with gentle agitation. Slides are mounted with Prolong Gold-Antifade reagent. 5 images per sample are taken on a Nikon A1r confocal microscope with 1 representative image selected.

Tissue sectioning: MDA-MB-231 scr and miR-510 tumors from the *in vivo* study are stored in 70% ethanol at 4°C. The tumors are dehydrated by passing through 95% ethanol twice and then 100% ethanol twice, for 1 hour each. Tumor samples are then placed into 100% toluene twice

for 4 hours each. Next the tumors are placed in a 50:50 mixture of toluene/paraffin overnight at 60°C. The 50:50 mixture of toluene/paraffin is removed and 100% paraffin is added to the samples, twice for 2 hours each, at 60°C. The samples are embedded in cassettes with the EG1160 paraffin embedding station (Leica, Buffalo Grove, IL). The tumors are then sectioned (5 microns thickness) with Jung RM2055 microtome (Leica, Buffalo Grove, IL) and mounted onto Superfrost Plus glass slides (Fisher, Hampton, NH) for immunohistochemistry (IHC).

Immunohistochemistry (IHC): Tumors from the *in vivo* experiment are fixed and embedded in paraffin blocks. The tumors are then sectioned (5 microns) and mounted onto glass slides for immunohistochemistry (IHC). For IHC, slides are incubated in a 60°C oven for 1 hour and then placed in xylene (Fisher, Hampton, NH) for 10 minutes and then rehydrated with serial incubations in decreasing ethanol concentrations (100%, 95%, 70% and 50%) for 10 minutes each. Next, the slides are subjected to heat-induced antigen retrieval by heating slides in a vegetable steamer with 1% citrate buffer solution (Vector Labs, Burlingame, CA) for 30 minutes. Slides are further washed with 1X PBS twice for 3 minutes each and then incubated with 0.3% hydrogen peroxide (Fisher, Hampton, NH) to sequester endogenous peroxidase activity for 30 minutes. Next, the slides are washed with 1x PBS-Tween (0.05%) for 5 minutes before blocking with 2.5% horse serum (Vector Labs, Burlingame, CA) at 25°C in an incubation chamber. A 1:250 dilution of primary antibody, Cav-1, is then applied and incubated overnight at 4®C. Slides are washed 3 times with 1 x Phosphate Buffer Solution (PBS), and then incubated with a dilution of 1:200 secondary antibody (Vector Labs, α-rabbit) at RT for 30 minutes. The slides are then incubated with Diaminobenzidine (DAB) for 1 minute and then counterstained with hematoxylin. Slides are then dehydrated with serial incubation in increasing concentrations of

ethanol (50%, 70%, 95% and 100%) and xylene for 5 minutes each. Finally, slides are mounted with Permount (Fisher, Hampton, NH) and a glass coverslip (Fisher, Hampton, NH). 5 images per tumor are taken on a Nikon 90i light microscope at 20x magnification with 1 representative image selected. For Cav-1 quantitation, images are scored based on intensity of staining from 1 representative image each (0 for no staining, 1 for low staining, 2 for moderate staining, 3 for high staining, and 4 for very high staining). These scores are plotted.

Results

Fibroblasts were isolated from miR-510 expressing and scr control breast tumors. In order to isolate an enriched fibroblast population, mice were injected with MDA-MB-231 cells overexpressing miR-510 or MDA-MB-231 cells expressing a scr control, and tumor growth was monitored as they grew to maximum size (1500mm^3) [Figure 18]. Following isolation of fibroblasts from those miR-510 and scr expressing tumors, we performed qPCR for fibroblast marker FSP1 and epithelial cell marker ESA [Figure 19A]. qPCR shows that the extracted fibroblasts express fibroblast marker FSP1 and show little to no expression of epithelial cell marker ESA. We also performed qPCR for miR-510 in isolated fibroblasts [Figure 19B]. The isolated fibroblasts express miR-510, providing evidence for an indirect transfer of miR-510 from tumor epithelial cells due to the lack of genomic mouse miR-510.

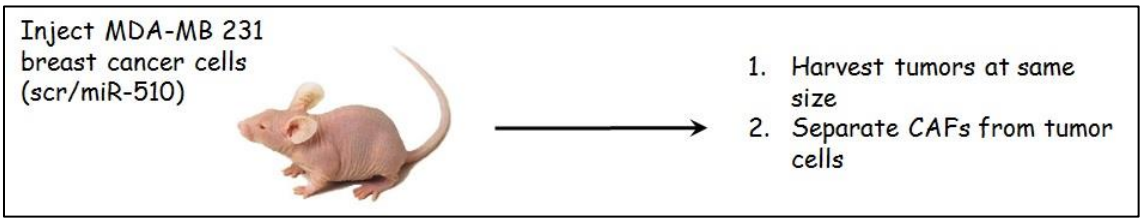

Figure 18: Schematic representation of mouse injections for enriched fibroblast isolation

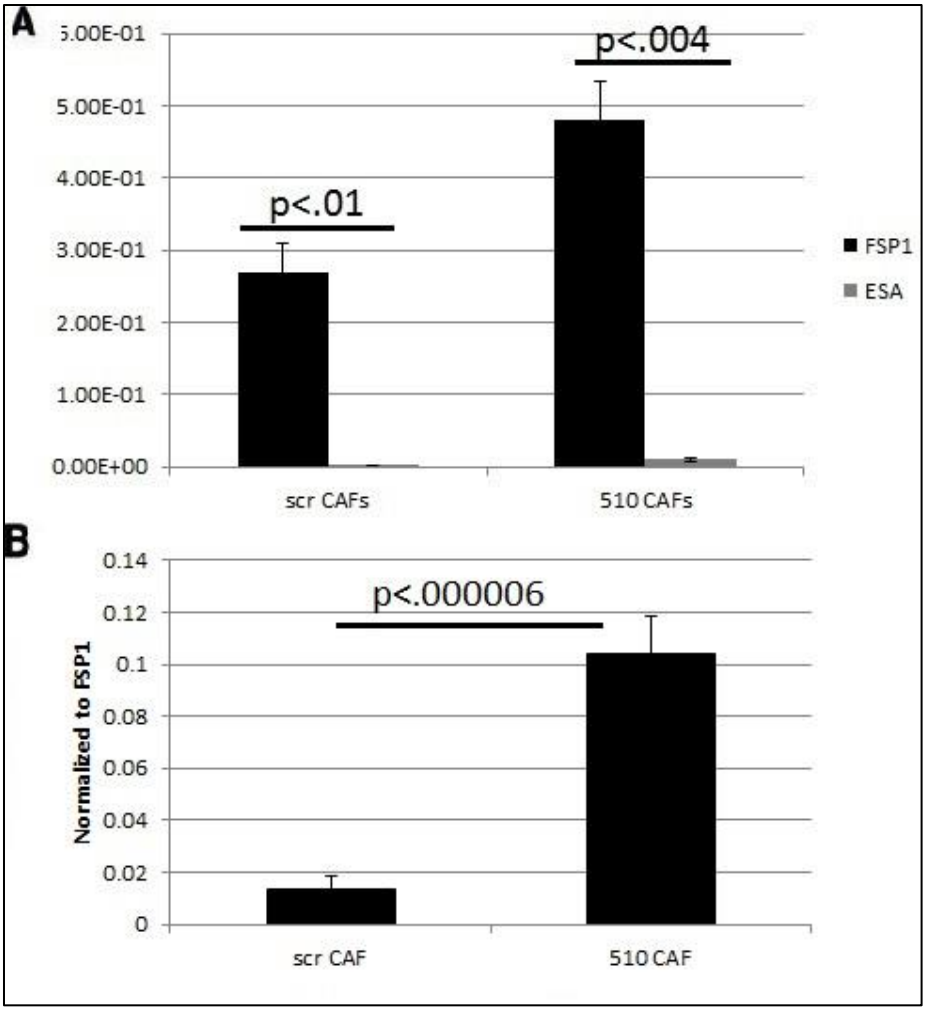

Figure 19: Fibroblasts were isolated from miR-510 expressing and scr control tumors. qPCR for fibroblast marker FSP1 shows elevated FSP1 in miR-510 expressing CAFs and little expression of epithelial marker ESA (A). qPCR for miR-510 shows extracted fibroblasts from miR-510 expressing tumors express miR-510 (B).

Cav-1 is reduced in miR-510 expressing CAFs.

To determine if miR-510 expressing CAFs show a decrease in Cav-1 expression, we performed IF staining on our enriched CAF population. Total number of cells, including epithelial cells and CAFs, were counted in 5 fields for scr CAFs and 510 CAFs. Table 2 shows percentage of CAFs in total cell population, as well as percentage of CAFs with Cav-1 positive staining. Representative images of staining intensity reveal CAFs isolated from miR-510 expressing breast tumors show a reduction in Cav-1 protein expression compared to CAFs from scr control tumors [Figure 20].

Table 2: Quantitation of IF CAFs

	CAFs/ Total Cell population	**Cav-1 positive staining**	**p value**
scr CAFs	53%	88%	p<.04
510 CAFs	43%	47%	

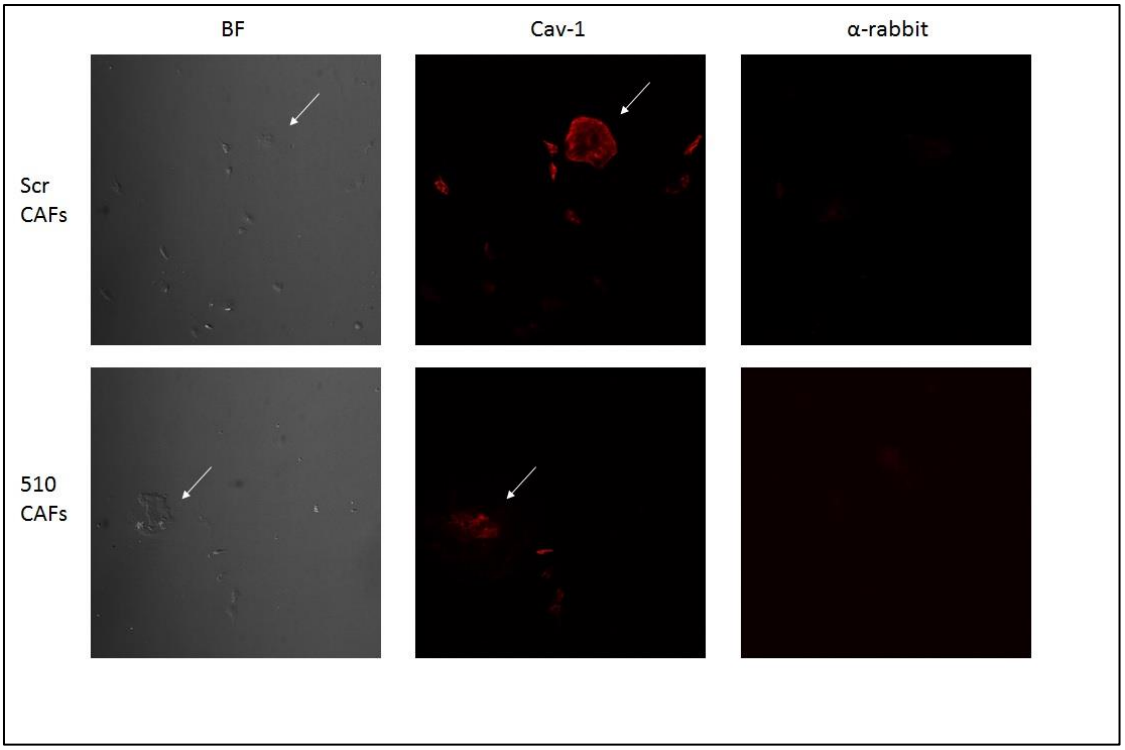

Figure 20: Representative images of Cav-1 staining intensity of CAFs. Cav-1 protein is reduced in CAFs derived from miR-510 expressing tumors compared to CAFs derived from scr control tumors. Images are 20x magnification.

Fibroblasts extracted from miR-510 expressing tumors are activated to a CAF phenotype.

Following isolation of fibroblasts from miR-510 and scr expressing tumors as described above, we performed qPCR for activated fibroblast marker COL1A1 (collagen) [Figure 21]. Extracted fibroblasts from miR-510 expressing tumors are activated to a CAF phenotype, as shown by increased expression of activated fibroblast marker COL1A1 (collagen). This data is also supported by qPCR for fibroblast marker FSP1 and epithelial cell marker ESA, showing that the extracted fibroblasts express fibroblast marker FSP1 and show little to no expression of epithelial cell marker ESA [Figure 19A].

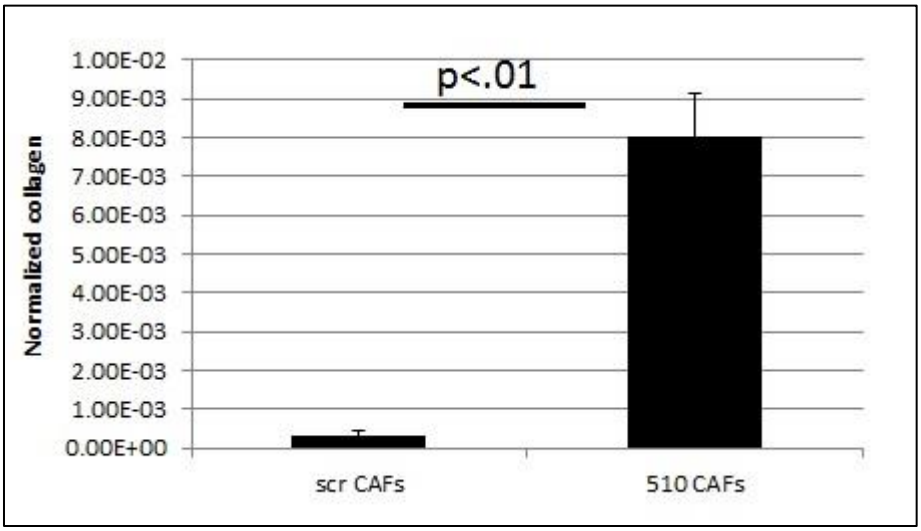

Figure 21: Isolated fibroblasts from miR-510 expressing tumors are activated to a CAF phenotype. qPCR shows increased expression of collagen (activated fibroblast marker).

miR-510 expressing CAFs result in more aggressive tumor growth.

In order to determine whether miR-510 expression results in a more aggressive CAF, 6 week old nude mice were orthotopically injected with: (1) CAFs isolated from scr control tumors + MDA-MB-231 cells (scr CAF:231); (2) CAFs isolated from miR-510 expressing tumors + MDA-MB-231 cells (510 CAF: 231); and (3) MDA-MB-231 epithelial cells alone (231) [Figure 22]. CAFs derived from miR-510 and scr control tumors were also injected alone to serve as additional controls. Tumor volume was monitored twice weekly throughout the study for 64 days. Comparing the tumors resulting from the 231 only controls with the 510 CAF:231 tumors, the 510 CAF:231 tumors grew faster and were much larger at the end of the study [Figure 23A]. Tumor weight showed the same trend as tumor volume [Figure 23B]. The scr CAF:231 tumors did not lead to a more aggressive tumor growth when compared to the 231 tumors alone suggesting that the scr CAFs were not aggressive enough to drive tumor growth. These results suggest that miR-510 expression in breast tumor epithelial cells leads to a more aggressive CAF in the tumor microenvironment and more aggressive tumor growth.

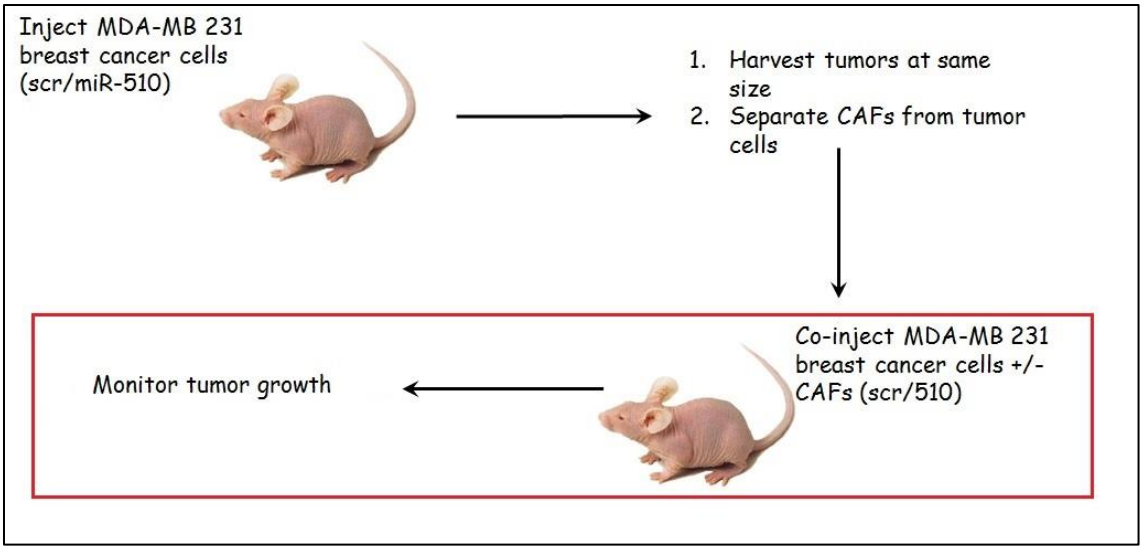

Figure 22: Schematic representation of CAF co-injections

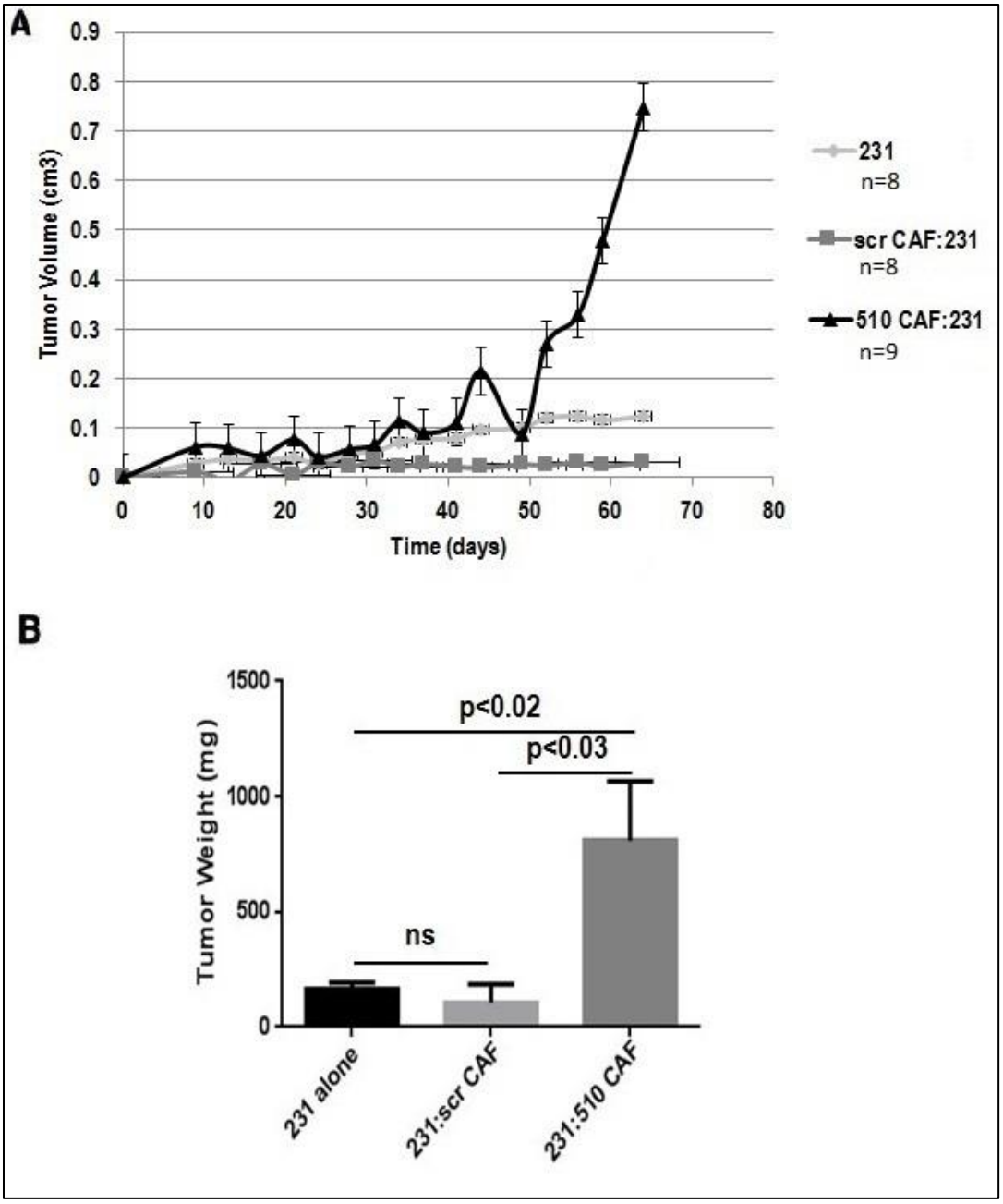

Figure 23: MDA-MB-231 tumor growth and weight. Average tumor volumes of mice orthotopically injected with MDA-MB-231 cells alone or co-injected with miR-510 expressing CAFs or scr control CAFs. Normalized to CAF only injections (A). Average tumor masses of experimental groups at the conclusion of the study (B).

Cav-1 is reduced in 510 CAF:231 tumors.

In order to assess the levels of Cav-1 *in vivo* we performed IHC staining of tumors extracted from the mice described above. Cav-1 IHC of the tumor sections shows an overall reduction of Cav-1 expression in 510 CAF:231 tumors compared to scr CAF:231 and 231 tumors [Figure 24]. Stromal vs. epithelial staining is not specified. Quantitation of tumor Cav-1 protein expression with representative tumors from each experimental group is shown [Figure 25]. Tumors were grouped together based on Cav-1 IHC score and plotted. This data suggests that miR-510 modulates Cav-1 expression *in vivo*.

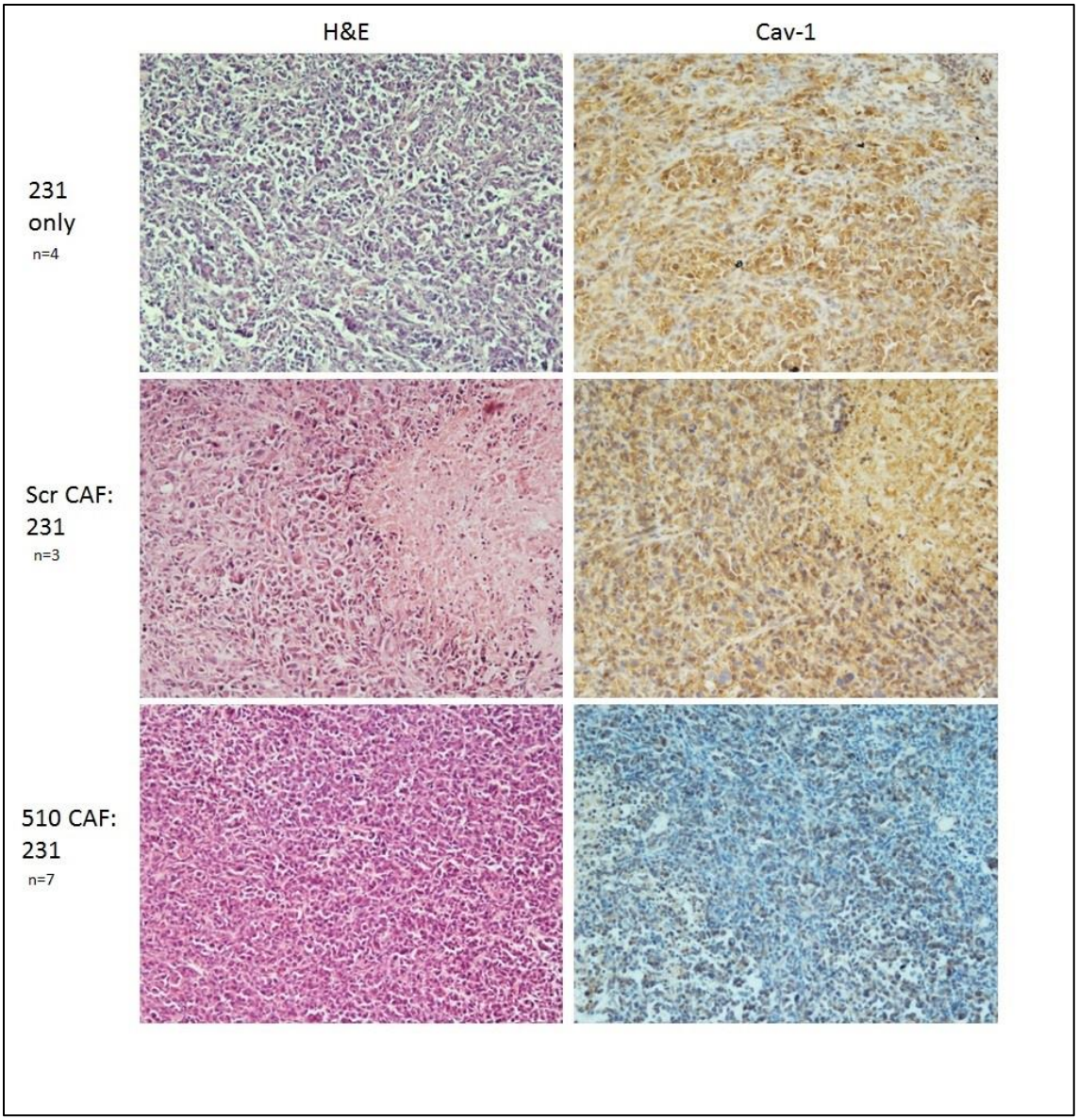

Figure 24: IHC of tumors from *in vivo* study. Representative images of hematoxylin & eosin and Cav-1 staining in MDA-MB-231 only, scr CAF:231, and 510 CAF:231 tumors. 20x magnification.

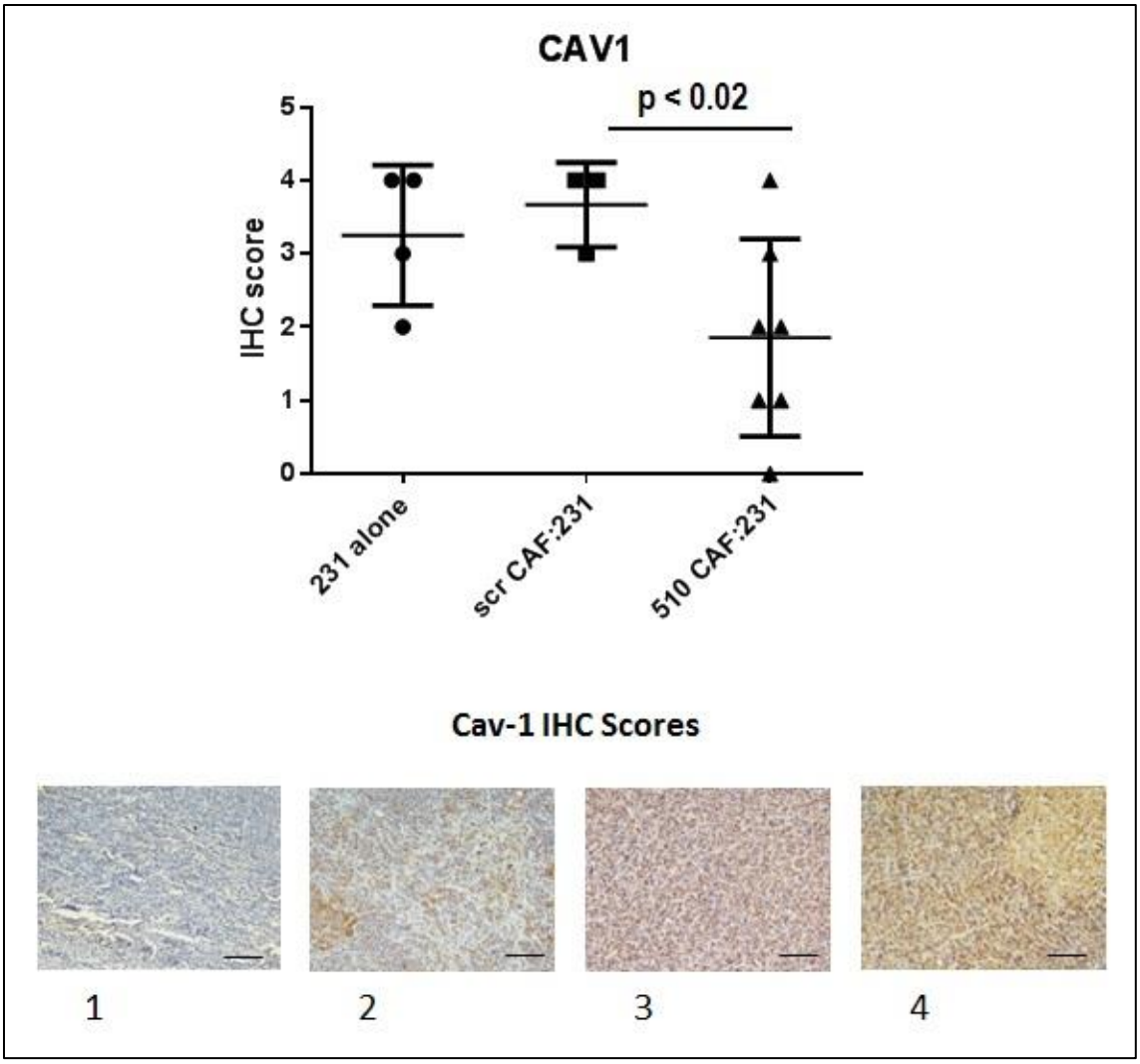

Figure 25: Cav-1 expression in tumors from *in vivo* study. Quantitation of Cav-1 IHC in tumors from *in vivo* study with tumors grouped based on Cav-1 IHC score. Representative images of Cav-1 IHC Scores are shown at 20x magnification.

Discussion

In our state of South Carolina, mortality differences between AA and CA breast cancer patients are amongst the highest in the country. Growing evidence shows that racial disparity in breast cancer exists due to molecular differences in tumor biology in addition to socioeconomic and standard of care issues [6; 9; 10]. A greater understanding of the genetic and biological differences associated with breast cancer will significantly impact AA communities due to the higher deaths associated with the disease in this population. Sparse information exists regarding the molecular mechanisms that promote this breast cancer health disparity. Our studies have identified a potential race specific mechanistic link between miR-510 and Cav-1 [Figure 1].

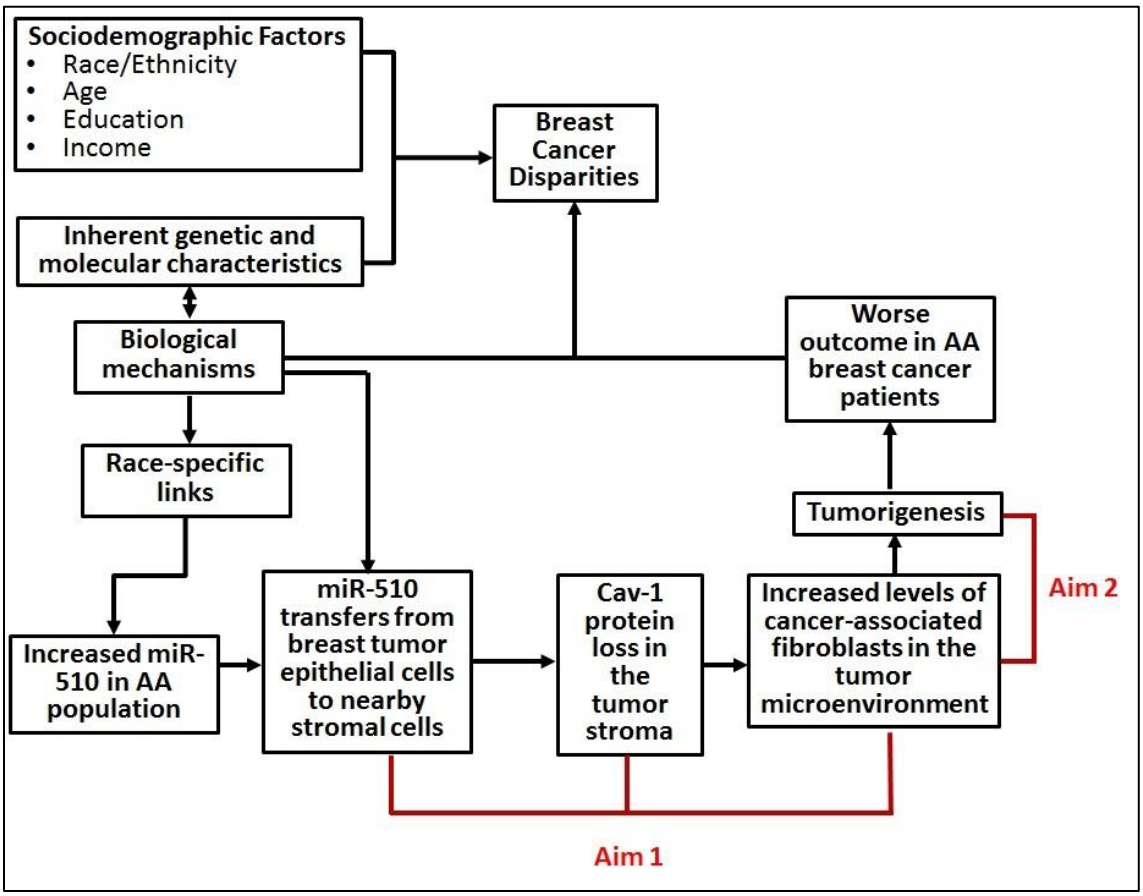

Figure 1: Conceptual Model. Breast cancer disparities are attributed to a combination of sociodemographic factors and inherent biological factors. Disparities also exist independently of sociodemographic factors. Aim 1 of this project examines a biological mechanism contributing to breast cancer disparities, while Aim 2 examines functional outcome of this mechanism.

Results showing negative Cav-1 regulation by miR-510 in fibroblasts is promising, suggesting that miR-510 is a mechanism of stromal Cav-1 loss in breast cancer. Interestingly, miR-510 overexpression in MDA-MB-231 and MCF10a epithelial cells does not have an effect on Cav-1 protein expression. However, there is no known association between Cav-1 expression in the epithelial compartment and clinical outcome [34; 36; 37; 40], which reinforces the idea that stromal Cav-1 expression is a primary determinant of clinical outcome in breast cancer patients. These *in vitro* experiments [Figure 10] could be strengthened by repeating them in AA and CA cell lines. We would expect to see a greater reduction of Cav-1 protein in AA cell lines compared to CA, providing more relevance to the disparity component of this project. Cav-1 expression may also be disparate between races, as data mining on Oncomine revealed that Cav-1 mRNA levels are lower in AA with breast cancer (tumor epithelial or stromal not specified) when compared to CA women. In addition, miR-510 expression in breast cancer patients is disparate between races, as we observed an elevation in miR-510 levels in serum samples extracted from AA breast cancer patients when compared to their benign counterparts. This difference was not observed in CA breast cancer patients. Limitations to the miR-510 serum data include a small sample size. However, these findings suggest loss of stromal Cav-1 mediated by miR-510 may be the mechanism leading to poorer breast cancer survival in AA women and warrants further investigation of its role in promoting breast cancer disparity. We are active collaborators with Dr. Ashley Evans-Knowell at South Carolina State University, and her group is investigating the correlation between miR-510 and Cav-1 expression in tissue from AA and CA breast cancer patients. These data will allow us to determine whether a true negative correlation exists in breast cancer patients and specifically in AA patients.

We hypothesized that tumor derived microvesicles deliver miR-510 from epithelial cells to recipient fibroblasts in the tumor microenvironment, resulting in a loss of stromal Cav-1 protein expression, and that this may provide a biological mechanism promoting breast cancer disparity [Figure 1]. To address this, we performed qPCR for miR-510 in CAFs isolated from miR-510 expressing tumors. This upregulation of miR-510 in the isolated mouse-derived CAFS provides evidence of an indirect transfer of miR-510 from human epithelial cells to resident fibroblasts as mice do not have miR-510 encoded in their genome. Tumor derived microvesicles can also result in circulating miRNAs in the blood and can serve as accessible biomarkers for diagnosis and prognosis [47; 49]. In addition, they may define a novel area of potential therapeutic intervention to reduce cancer disparity. This study shows that co-culture of miR-510 expressing tumor epithelial cells with fibroblasts *in vitro* leads to downregulation of Cav-1 in the fibroblasts. We have also shown that conditioned media from miR-510 expressing tumor epithelial cells leads to Cav-1 downregulation in stromal fibroblasts, demonstrating that cell-to-cell contact is not required for miR-510 transfer. We examined miR-510 levels in exosomes isolated from the media of co-culture studies to determine if exosomes were the transport vesicle for miR-510. However, miR-510 was not detectable in isolated exosomes, demonstrating this this in not their method of transfer. However, qPCR for miR-21 was positive, demonstrating that our exosome isolation was successful. In the future, those exosomes could be further verified by electron microscopy or characterized by exosome protein markers using Western blot analysis.

Although exosomes are the most widely studied of extracellular vesicles, studies show that malignant tumor cells release a heterogeneous population of microvesicles [54]. Therefore; it is necessary to further study alternative tumor-derived microvesicles in which miR-510 could be

transferred. Exosomes and microvesicles are terms often used interchangeably, as they are both membrane-bound vesicles capable of miRNA transfer. But they can be characterized according to different properties, including biogenesis and physical properties. Exosomes are a homogenous population with a size of 40-100nm in diameter and are derived from the endocytic recycling pathway, while microvesicles are a heterogeneous population with a size of 50-1000nm in diameter and are produced directly from outward budding and fission of the plasma membrane [55]. Moving forward, our lab could perform a microvesicle preparation utilizing stromal fibroblasts from our co-culture model, followed by characterization and identification of those microvesicles, and then probe for miR-510 by qPCR.

Our alternative hypothesis is that a release of cytokines or growth factors from epithelial tumor cells leads to an upregulation of miR-510 in neighboring stromal cells. Studies have reported that tumor-derived microvesicles showed the presence of chemokine (a subpopulation of cytokines) receptors CCR6 and CD44v7/8, signaling factors TGFβ1/2, and contained mRNA for growth factors including VEGF and interleukin-8 (IL-8) [56; 57]. Interestingly, TGFβ1/2 and VEGF released from tumor cells is implicated in the oncogenic transformation of fibroblasts to CAFs [56]. Following exhaustion of probing for miR-510 in microvesicles from our co-culture studies, these signaling pathways could be studied for their contribution to breast cancer disparities.

Results from our *in vivo* studies was supportive of our hypothesis. We were able to successfully isolate a cell population enriched for CAFs. We used qPCR to first validate that our extracted fibroblasts expressed fibroblast markers, then to show a transfer of miR-510 from miR-510 expressing tumors to the CAFs, and finally to verify that our extracted fibroblasts had been

activated to a CAF phenotype. IF for enhanced CAFs also showed a decrease in Cav-1 expression, supporting our hypothesis that miR-510 negatively regulates Cav-1. Future studies will co-stain with antibodies against αSMA and wide spread cytokeratin to identify activated fibroblasts versus epithelial cells.

Tumors from miR-510 CAF:231 co-injections resulted in faster tumor growth and larger tumors at the end of the study than tumors derived from injection of MDA-MB-231 cells alone. Interestingly, we observed macro metastasis in the liver of one of the experimental mice that correlated with the largest tumor size. Unexpectedly, we observed tumor growth in some of our control CAF only injections. Therefore, we normalized our experimental co-injection groups to CAF only injections in order to accurately reflect tumor growth. We suspect that our CAF only tumors grew due to poor experimental design, as CAF only controls were injected on the same mice as experimental co-injections. It is possible that the enriched CAF populations had a small percentage of contaminating epithelial cells and that paracrine factors released by malignant tumors caused these cells to grow. The scr CAF:231 co-injection tumors did not grow as well as the MDA-MB-231 tumors alone, suggesting that the scr CAFs were not aggressive enough to drive tumor growth.

IHC revealed an expected overall decrease of Cav-1 expression in 510 CAF:231 tumors at 20X magnification. Future studies will stain with antibodies against epithelial and stromal cells in order to determine Cav-1 loss in specific tumor compartments. Future studies will also examine potential increases in angiogenesis and vascularization in 510 CAF: 231 co-injection tumors compared to controls.

Future Direction

Next steps in this project will be to continue to investigate the racial disparity of miR-510 and Cav-1 expression in breast cancer patients. Our preliminary data have established in a cohort of breast cancer serum samples that miR-510 levels are elevated in AA women when compared to their benign counterparts, but tissue expression of miR-510 and CAV1 will show if a negative correlation exists in patients. In collaboration with SCSU, our lab will continue to add data from patient samples. Based on our hypothesis, we expect to see an inverse correlation between miR-510 and Cav-1 in both the AA and CA samples. However, we expect to see higher miR-510 and lower Cav-1 levels overall in the AA samples or a greater number of patients with the inverse correlation in the AA group of patients. We may also expect to see higher miR-510 levels in the CAFs within the stroma of these tissue samples. In-situ hybridization of miR-510 will allow us to examine its expression in specific tumor cell populations (stromal/epithelial). The results generated so far have provided encouraging evidence and collaboration with Dr. Evans-Knowell will help determine the role of miR-510 in promoting stromal loss of Cav-1and its implications in breast cancer disparity.

While we are confident that we have identified a potential biological mechanistic link driving stromal Cav-1 loss and worse outcome in AA breast cancer patients, we are unsure of how miR-510 is transferring to stromal cells from epithelial cancer cells. Our greatest challenge moving forward will be to determine in which microvesicle miR-510 is transferred. We must also explore the alternative hypothesis that a growth factor or released cytokine is upregulating miR-510 expression. Therefore, we must consider exploring the role of miR-510 on various pathways, including VEGF or TFGβ1/2.

Study Significance

The goal of this project is to provide novel insight into racial specific cancer associated miR-510 and Cav-1 expression levels in breast cancer that could lead to more effective individualized screening and treatment options. Tumor-derived microvesicles that result in circulating miRNAs in the blood may even be able to serve as accessible biomarkers for diagnosis and prognosis. This project could have a significant impact on the development of novel strategies for the racial specific treatment of breast cancer, thereby decreasing the gap in breast cancer mortality rates between AA women and CA women. By exploring its role in regulation of stromal Cav-1, miR-510 may provide a novel pathway that is driving the racial disparity in breast cancer. Future studies could assess the utilization of miR-510 as a therapeutic target, preventing the loss of stromal Cav-1 and thereby improving breast cancer prognosis. This research has great translational significance, providing a potential means to reduce racial disparity in breast cancer and to help relieve the burden of this disease, specifically in the AA community.

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