MOLECULAR MECHANISMS FOR THE ANGIOTENSIN-(1-7)-MEDIATED INHIBITION OF METASTATIC TRIPLE NEGATIVE BREAST CANCER

BY

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A Dissertation Submitted to the Graduate Faculty of
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<td>[D-Ala$^7$] Ang-(1-7)/D-Ala</td>
<td>[D-alanine$^7$]-angiotensin-(1-7)</td>
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<tr>
<td>α-SMA</td>
<td>Alpha-smooth muscle actin</td>
</tr>
<tr>
<td>μg</td>
<td>Micrograms</td>
</tr>
<tr>
<td>μL</td>
<td>Microliter</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
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<td>Angiotensin converting enzyme 2</td>
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<td>Ang-(1-7)</td>
<td>Angiotensin-(1-7)</td>
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<td>Ang-(1-5)</td>
<td>Angiotensin-(1-5)</td>
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<td>ARB</td>
<td>Angiotensin receptor blockers</td>
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<td>AT$_{(1-7)}$R</td>
<td>Angiotensin type (1-7) receptor</td>
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<td>AT$_2$R</td>
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<td>Adenosine triphosphate</td>
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<td>Breast cancer 1/2, early onset</td>
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<tr>
<td>CAF</td>
<td>Cancer-associated fibroblast</td>
</tr>
<tr>
<td>CAM</td>
<td>Chick chorioallantoic membrane</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CDK</td>
<td>Cyclin-dependent kinase</td>
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<td>Description</td>
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<tr>
<td>Co-Smad</td>
<td>Co-mediator Smad</td>
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<td>Cyclooxygenase-2</td>
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<td>Cyclic AMP responsive element</td>
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<td>Cyclic AMP response element-binding protein</td>
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<td>Connective tissue growth factor</td>
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<td>Ductal carcinoma in situ</td>
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<td>DNA</td>
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<td>Dual specificity phosphatase 1</td>
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<td>Extracellular matrix</td>
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<td>EMT</td>
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<td>FOXO</td>
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<td>Hepatocyte growth factor</td>
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<td>High-mobility group A2</td>
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<td>JNK</td>
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<td>kDa</td>
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<td>kg</td>
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<td>LAP</td>
<td>Latency-associated peptide</td>
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<td>L-NAME</td>
<td>N(G)-nitro-L-arginine methyl ester</td>
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<td>Definition</td>
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<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
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<td>LOX</td>
<td>Lysyl oxidase</td>
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<td>LTBP</td>
<td>Latent-TGFβ-binding protein</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MH1/2</td>
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<td>mL</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>mTORC</td>
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<td>NEP</td>
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<td>p90Rsk</td>
<td>p90 ribosomal S6 kinase</td>
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<td>Polyacrylamide gel electrophoresis</td>
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<td>Poly (ADP-ribose) polymerase</td>
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<td>PAX2</td>
<td>Paired homeobox 2</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PE</td>
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<td>PI3K</td>
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<td>PP2A</td>
<td>Protein phosphatase 2A</td>
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<tr>
<td>PPM1A</td>
<td>Protein phosphatase, Mg(^{2+})/Mn(^{2+}) dependent, 1A</td>
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<td>PPP</td>
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<td>PR</td>
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<td>PRAS40</td>
<td>Proline-rich Akt substrate of 40 kDa</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue deleted on chromosome 10</td>
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<td>PTP1B</td>
<td>Protein tyrosine phosphatase 1B</td>
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<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
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<td>RAS</td>
<td>Renin-angiotensin system</td>
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<td>Retinoblastoma 1</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>Receptor-Smad</td>
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<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RTKI</td>
<td>Receptor tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>SAE</td>
<td>Serious adverse event</td>
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<td>s.c.</td>
<td>Subcutaneous</td>
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<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<td>SD</td>
<td>Stable disease</td>
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<td>Ser/S</td>
<td>Serine</td>
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<td>SEER</td>
<td>Surveillance, Epidemiology, and End Results</td>
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<td>SEM</td>
<td>Standard error of the mean</td>
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<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
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<tr>
<td>SH2</td>
<td>Src homology 2 domain</td>
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<td>SHP-1/2</td>
<td>SH2-containing protein tyrosine phosphatase-1/2</td>
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<td>Acronym</td>
<td>Definition</td>
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<td>------------</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
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<td>TAF</td>
<td>Tumor-associated fibroblast</td>
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<td>TGF-β</td>
<td>Transforming growth factor-β</td>
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<td>TIMP-1</td>
<td>Tissue inhibitor of metalloproteinases-1</td>
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<tr>
<td>TNBC</td>
<td>Triple negative breast cancer</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor, node, and metastasis</td>
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<td>Tuberous sclerosis complex 2</td>
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<tr>
<td>Tyr/Y</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
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ABSTRACT

Approximately 40,000 women in the U.S. die from breast cancer annually. An estimated 15% of all breast cancers are triple negative, wherein tumors lack estrogen receptor and progesterone receptor expression and possess unamplified human epidermal growth factor receptor 2 (HER2) levels, contributing to an absence of effective, targeted therapeutics. Furthermore, triple negative breast cancer (TNBC) is highly-aggressive with greater patient mortality rates compared with other breast cancer subtypes, necessitating development of novel treatment options.

Angiotensin-(1-7) [Ang-(1-7)] is an endogenous peptide hormone of the renin-angiotensin system with anti-proliferative, anti-fibrotic and anti-metastatic properties. Ang-(1-7) inhibited the growth of human MDA-MB-231 and murine 4T1 TNBC cells and orthotopic tumors. Phosphorylated Akt [p-Akt(Ser473) and p-Akt(Thr308)], a major driver of cell proliferation and survival, was concomitantly reduced suggesting the heptapeptide decreases the activation of Akt to inhibit TNBC growth. The serine/threonine phosphatase responsible for Akt inactivation, protein phosphatase 2A (PP2A), is upregulated in TNBC cells and tumors treated with Ang-(1-7). This indicates the heptapeptide mediates inhibition of TNBC growth via increased PP2A to prevent Akt activity.

Metastasis remains a serious medical issue for which few therapies have been effective at treating or preventing, thus contributing to 90% of deaths in patients with solid tumors. Human TNBC has a high rate of metastasis to the lung, liver, bone and
brain, a pattern mimicked by the 4T1-Balb/c syngeneic mouse model. Ang-(1-7) reduced the growth of metastatic 4T1 tumors in the lungs of mice, evidenced by decreased lung wet weight upon sacrifice, average tumor number and average tumor area measured by hematoxylin and eosin (H&E) staining. Breast tumor fibrosis facilitates tumor growth and progression via cancer-associated fibroblast (CAF)–mediated extracellular matrix (ECM) protein production. Ang-(1-7) inhibited the growth of CAFs isolated from orthotopic 4T1 tumors as well as the fibrotic mediators transforming growth factor-beta (TGF-β), p-Smad2, connective tissue growth factor (CTGF) and tenasin C in 4T1 CAFs, metastatic 4T1 tumors, and primary orthotopic 4T1 tumors. These results are the first to show the anti-fibrotic effects of Ang-(1-7) in reducing metastatic TNBC growth, thus supporting the role for the heptapeptide as a novel treatment option for metastatic TNBC.
CHAPTER I: INTRODUCTION

1. Breast Cancer

Breast cancer claims approximately 40,000 lives each year in the U.S., with 232,000 estimated new cases for 2013, establishing breast cancer as both the most common type of cancer in women as well as the second leading cause of cancer-related deaths in women, behind lung cancer (1). One in eight U.S. women will develop breast cancer in her lifetime, with a worldwide estimate of 450,000 deaths as a result of the disease (1, 2). Despite this, death rates for women with breast cancer declined by 34% from 1990 to 2010 (3). Furthermore, 5-year overall survival rates for non-Hispanic white women and Asian-American/Pacific Islander women with breast cancer were 88.6% and 91.1%, respectively, for the years 2003-2009 (1). However, African-American women with breast cancer maintain the lowest 5-year survival rates (78.9%), likely due to a combination of genetic, environmental, and socioeconomic factors (1, 4).

Breast cancer is a heterogeneous disease encompassing a diverse array of clinical, pathological, and molecular characteristics, and affecting all races of women as well as a small percentage of men. Breast tumor development frequently originates in the lining of the mammary ducts or in the lobules of the breast, known as ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS), respectively, accounting for approximately 20-25% of all newly-diagnosed breast cancer patients in the U.S. each year (5). DCIS and LCIS are both non-invasive or pre-invasive forms of breast cancer and thus, may be easily treated by lumpectomy or mastectomy, wherein the tumor tissue or the entire breast is surgically removed (6).
DCIS and LCIS as well as all other forms of breast cancer are most commonly diagnosed through screening measures, such as mammography followed by biopsy. The number of breast cancer cases has experienced a spike in the last two decades as a result of implementation of newer recommendations for screening and self-examination, improving early stage diagnosis and patient prognosis.

The most widely-used method for classification of breast tumors and most cancer types in general is the tumor, node, and metastasis (TNM) staging system to help identify the extent or severity of the cancer (7). Tumor staging assists physicians in making prognostic predictions and formulating an appropriate treatment plan for the patient. The TNM system is based on the size of the primary tumor, the extent to which the tumor has spread to the lymph nodes, and whether metastatic or secondary tumors are present in distant sites in the body. Each of the three levels of cancer classification (T, N, M) receives a score and these factors help determine the stage of the tumor, which can range from a stage 0, meaning that the cancer is non-invasive, to a stage IV, meaning that the cancer has spread to one or many distant tissue sites in the body. For breast cancer, additional consideration is given to tumor hormonal receptor levels, human epidermal growth factor receptor 2 (HER2) expression, and the menopausal status of the patient when determining tumor stage. Both DCIS and LCIS are considered in situ tumors due to their non-invasive nature or lack of migration out of the original layer of cells in which the cancers developed. Localized cancer is defined as cancer which is limited to the organ in which it originates, without evidence of spread. Regional cancer occurs when the
Cancer has become invasive and spreads to nearby lymph nodes and/or tissues. The most aggressive type of cancer is termed ‘distant’, wherein the cancer has become highly invasive and metastatic, spreading to distant lymph nodes and/or tissue sites in the body. Tumor staging is determined using a number of different tests which include physical examinations, imaging (x-rays, computed tomography scans, magnetic resonance imaging scans, and positron emission tomography scans), laboratory tests analyzing blood and urine, biopsy for pathology reports, and surgical reports for any findings accumulated during surgery.

Breast cancers are also classified using molecular and gene expression profiling, which separate the different subtypes of breast cancer based on the presence of estrogen receptor (ER), progesterone receptor (PR), HER2, and other various markers (2, 8-10). Gene expression profiling utilizes hierarchical clustering of intrinsic genes differentially expressed in individual breast cancer patients, thus generating a division of breast cancers into 5 major subtypes: luminal-A, luminal-B, normal-like, HER2-enriched, and basal-like (8-10).

Luminal breast tumors represent approximately 60% of all breast cancers and derive from the luminal epithelium of the breast. Both luminal-A and luminal-B tumors are ER-positive, express luminal cytokeratins 8/18, the ER-activating gene and cell cycle regulator CCND1 (cyclin D1), and have frequent mutations in the gene for phosphatidylinositol-3 kinase (PI3K) p110 subunit, PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) and the MAPK genes, MAP3K1/MAP2K4.
(mitogen-activated protein kinase kinase kinase 1/ mitogen-activated protein kinase kinase 4), contributing to deregulated growth factor signaling (2, 11, 12). Despite these shared characteristics, luminal-A and -B tumors display several contrasting features. Luminal-B breast tumors have increased proliferation, higher grade, lower ER-related gene expression, and significantly poorer outcome compared to luminal-A tumors (11, 13). Both -A and -B luminal tumors show variable PR and HER2 expression; however luminal-B breast tumors may express HER2 at greater levels than luminal-A tumors (14, 15). Hormonal therapies like tamoxifen, a selective estrogen receptor modulator (SERM) that antagonizes ER in breast tissue, and aromatase inhibitors like anastrozole that prevent estrogen synthesis, have been effective in the treatment of luminal breast cancer by inhibiting estrogen/ER signaling, thereby preventing the growth-promoting effects of the hormone.

HER2-amplified breast cancers account for approximately 15% of all breast cancers and are characterized by over-expression of the *ERBB2* (erythroblastic leukemia viral oncogene homolog 2) gene (16). HER2 is a member of the epidermal growth factor (EGF) family of receptor tyrosine kinases and can form a heterodimer with ligand-bound EGF receptors to facilitate growth signaling pathways (17). HER2-enriched tumors tend to be high grade, show poor prognosis, and typically display a lack of hormone receptor expression (18). Patients with HER2-amplified breast cancer can effectively be treated with Herceptin (trastuzumab), a monoclonal antibody that binds to and blocks the HER2 receptor to prevent mitogenic signaling contributing to tumor growth. Additionally, lapatinib, a receptor tyrosine kinase inhibitor, pertuzumab, a monoclonal antibody
receptor dimerization inhibitor, and ado-trastuzumab emtansine, a chemotherapeutic-conjugated monoclonal antibody, are all also HER2-targeting agents useful as first-line therapies for HER2-amplified breast cancer (16).

Approximately 6-10% of breast cancer cases are defined as normal-like, resembling normal breast tissue samples and sharing many of the same gene expression sets as that of adipose tissue and other non-epithelial cell types including genes for glycerol-3-phosphate dehydrogenase 1 and lipoprotein lipase (9). Normal-like breast tumors also possess high basal-like and low luminal gene expression with intermediate patient prognosis (19).

Basal-like breast tumors account for 10-15% of all breast cancers and possess gene expression profiles similar to that of cells comprising the basal epithelial layer of mammary ducts and glands (8). Basal tumors express cytokeratins 5, 6, and 17, epidermal growth factor receptor (EGFR), have elevated proliferation, and a high frequency of mutations in the tumor suppressor gene TP53 (tumor protein p53), the DNA damage sensor gene BRCA1/2 (breast cancer 1/2, early onset), and PIK3CA (2, 9, 20, 21). The tumor suppressor protein p53 is a critical regulator of cell cycle progression. The p53 gene frequently undergoes deletions and loss-of-function or gain-of-function mutations in cancer, eliminating the cell cycle checkpoint and contributing to cancer cell growth (22). BRCA1 and BRCA2 genes encode tumor suppressor proteins which mediate DNA repair in the cell; thus, mutations in the BRCA1/2 gene are directly associated with carcinogenesis due to the resultant genetic instability which leaves cells vulnerable to
malignant transformation (23, 24). Basal-like breast tumors are high grade, have a high mitotic index, predominantly affect younger, premenopausal women, African-American and Hispanic women, and are highly aggressive and invasive, contributing to poor patient outcomes (20, 25). Due to gene mutation, these tumors also lack functional Retinoblastoma 1 (RB1), a protein responsible for cell cycle regulation and tumor suppressor function (26). Most basal breast tumors lack expression for ER and PR and show normal levels of HER2, often leading to their designation as triple negative. However, not all basal tumors fall into this category (18, 21, 27). Claudin-low breast cancers also represent a recently-defined subgroup of the triple negative class of breast cancers characterized by low gene expression for claudin-3, -4, and -7, tight junction proteins, as well as the cell-cell adhesion glycoprotein E-cadherin; however, these tumors are not classified as basal-like (28).

a.) Triple Negative Breast Cancer

Triple negative breast cancer (TNBC) is an invasive ductal carcinoma with a high proclivity for rapid spread, producing greater mortality rates compared with other breast cancer subtypes. The lack of hormone receptor expression and normal levels of HER2 in TNB tumors contributes to a severe lack of available targeted therapies due to the unresponsiveness of TNB tumors to standard receptor-targeting treatments. Currently, TNBC patients are treated with cytotoxic chemotherapies including doxorubicin, a DNA-intercalating agent, cyclophosphamide, a DNA-crosslinking agent, and paclitaxel, a mitosis inhibitor, which induce cell death in rapidly-proliferating cells (29-31). These
drugs are non-specific, meaning that both cancer cells and normal cells are affected, contributing to extreme nausea and vomiting, susceptibility to infection, fatigue, loss of appetite, diarrhea, and hair loss, ultimately producing poor quality of life for these patients. For early-stage TNBC, combination cytotoxic chemotherapy is administered in a standard dose-dense or metronomic schedule as a neoadjuvant or an adjuvant treatment.

Current clinical trials evaluating targeted and effective therapeutic agents for the treatment of TNBC are limited. Cytotoxic chemotherapies in various combinations and dosing regimens represent the majority of ongoing human patient studies. Despite the toxic side effects of most chemotherapy however, TNB tumors are particularly sensitive to this form of cancer treatment compared to other breast cancer subtypes (29, 32). Studies demonstrate that pathologic complete response (pCR) rates are higher in TNBC patients receiving chemotherapy than in non-TNBC patients, meaning there were no residual tumor cells by histological analysis in a greater number of TNBC patients (30, 32). However, progression-free survival (PFS) or disease-free survival in TNBC patients is significantly shorter at patient follow-up than in those patients with other subtypes of breast cancer, indicating that initial chemosensitivity is high in TNBC but does not necessarily translate to overall survival. This paradox may be due to the absence of a functional BRCA1 protein, as a result of the high prevalence of mutations in the BRCA1 gene in TNBC and the lack of a BRCA1-induced DNA repair mechanism. However, given the inherently aggressive nature of TNB tumors, this initial response to treatment likely cannot be sustained over time for reasons not yet well-understood.
Women of African-American and Hispanic descent as well as premenopausal women are disproportionately afflicted with TNBC, due to a combination of different factors. The Carolina Breast Cancer study demonstrated that 26% of African-American breast cancer patients fall into the basal-like breast tumor subtype whereas 16% of non-African-American cases were basal-like (20). In the same study, premenopausal women were more likely to have basal-like breast cancer than women who were postmenopausal (24% versus 15%, respectively). In addition, women possessing a combination of these factors are more likely to develop basal-like breast cancer. Breast cancer in these women tends to be higher grade, present at a later stage upon diagnosis, and result in poor survival, which is likely due to differences in access to screening and treatment measures, variability in the biology and genetics of the cancers themselves, and differences in lifestyle, nutrition, and environmental exposures (33-40). Management of these different factors and development of novel therapeutics directed at restoring deficient tumor suppressor levels or antagonizing proliferative signaling pathways in basal-like/TNBC is essential and should be focused on the highly-susceptible young, African-American and Hispanic patient populations.

b.) Metastasis

Metastasis is defined as the process by which cancer cells spread from their original or primary location to a secondary site in the body. Metastatic cancer is a significant problem in the clinic, accounting for approximately 90% of all solid-tumor cancer deaths (41). The metastatic process has several steps, the first of which involves the invasion of local or nearby tissue by the cancer cells. Invasive or infiltrating breast
cancers occur when the tumor cells have invaded across the endothelium of surrounding tissues (42). Following local invasion, cancer cells proceed to intravasation of the nearby lymph or blood vessels which act as highways for transport to secondary tissue sites. Cancer cells circulate via lymphatics or by hematogenous routes and eventually colonize at a distant location whereby invasion across capillary walls takes place, a process termed extravasation. The metastatic cancer cells then proliferate in the new tissue site, forming small tumors termed micrometastases. In addition, new blood vessel formation, or angiogenesis, is induced upon tumor-mediated growth factor secretion. Angiogenesis is required for the delivery of nutrients and oxygen to the growing tumors to perpetuate continued tumor growth and survival (43).

Cancer cells metastasize to several different organs in the body. TNB tumors predominantly metastasize to the lung and liver, and less commonly to the bone and brain via the bloodstream (44, 45). The propensity of TNBC for hematogenous spread rather than traveling through the lymphatic system offers an explanation for the characteristically aggressive and highly-metastatic nature of these tumors. Also, hematogenous spread gives TNBC a ‘head start’ by evading standard lymph node detection methods. While few treatments have been effective in reducing metastasis, patients with metastatic breast cancer are commonly treated with combination chemotherapy including a targeted therapeutic after ER/PR/HER2 assessment, and considered for local surgery and radiation if feasible (46).
Women with TNBC show a shorter time to relapse and metastasis than those with non-TNBC subtypes. Once diagnosed with metastatic TNBC, survival is limited to 9-14 months, whereas metastatic breast cancer patients with a non-TNBC subtype experience an average of 22 months survival (47, 48). Following diagnosis of the primary tumor, the risk of metastasis of TNBC is highest within the first 5 years and peaks at approximately 2.5-4.2 years compared with patients with alternative forms of breast cancer, where the greatest chance of metastasis generally occurs approximately 5-6 years following initial diagnosis (41, 47). Given the lack of effective targeted therapies for the treatment of TNBC, and the greater likelihood of metastatic tumor development in these patients, there is an imminent need for novel TNBC treatment strategies.

The way in which tumors respond to external stimuli and cues in the tumor microenvironment, such as the presence of extracellular matrix proteins, basement membranes, immune infiltrate, reactive oxygen species, and the amount of nutrients and oxygen, dictate the metastatic potential of the tumor (42). Increased activity of matrix metalloproteinases (MMPs) enhances the metastatic capabilities of tumors via proteolytic cleavage of the basement membrane and extracellular matrix initially acting as barriers to tumor cell invasion. Minn et al. demonstrated that TNBC cells specific for lung metastasis overexpressed genes for MMPs (*MMP1* and *MMP2*), which was also observed in primary TNB tumors growing orthotopically in mice, suggesting MMPs directly contribute to the metastasis of TNBC (49). The production of matrix proteins and reduced MMP activity is required for metastatic tumor colonization and growth once the tumor cells have colonized a secondary tissue site. Tensinal forces exerted by tumors can result
in integrin receptor clustering, conferring increased mechanotransduction signaling via ERK activation producing disruptions in tissue polarity and increased metastasis (50). Reactive oxygen species generated by infiltrating inflammatory cells and proliferating tumor cells can directly promote genomic instability of cancer cells via \textit{TP53} mutations and mediate expression of pro-metastatic genes (51). During periods of low oxygen levels in the tumor, the hypoxia inducible factor-1 (HIF-1) transcriptional complex is stabilized to promote activation of genes that facilitate angiogenesis, cell survival, and cell invasion (52). Semenza et al. demonstrated that tumors with increased HIF-1 stabilization show a greater likelihood for metastasis (53). Lysyl oxidase (LOX), a HIF-1 target, may be responsible for mediating HIF-1-directed metastatic tumor development via alterations in focal adhesion kinase (FAK) activity and collagen fibril development (54). In addition, Erler et al. showed that LOX-mediated breast cancer metastasis was associated with poor overall survival in ER-negative patients. Moreover, tumor hypoxia can induce Met expression which induces tumor cell invasion under ligand stimulation by hepatocyte growth factor (HGF) (55).

Breast cancer metastasis occurs through several signaling pathways and cross-talk mechanisms. HER2 for instance, mediates increased breast cancer metastasis and aggressive behavior upon ligand activation in tumors through activation of extracellular signal-regulated kinase (ERK) and Akt signaling pathways (56). Akt signaling undergoes activation by numerous different growth factors to promote increased tumor growth, invasion, and metastasis. Active Akt directly phosphorylates Twist1 on the serine 42 residue in human breast cancer to promote epithelial-to-mesenchymal transition (EMT).
and facilitate tumor metastasis to the lung (57). EMT is a pro-metastatic process occurring in cancer cells wherein cells lose traditional epithelial characteristics and acquire a mesenchymal phenotype. Upon activation of Snail, Twist, and Slug transcription factors, EMT promotes cancer cell loss of epithelial (E)-cadherin, gain of neural (N)-cadherin, and mediates cancer cell-stromal cell adhesions and a more invasive and migratory cell type, thus contributing to increased metastasis (42, 58). Transforming growth factor-beta (TGF-β) is a major inducer of EMT in epithelial cells mediating its effects through activation of Smad proteins (59), thus TGF-β signaling as well as Akt signaling, warrant further investigation as therapeutic targets for the treatment of metastatic breast cancer, as discussed later in this dissertation.

2. PI3K-Akt Signaling

Phosphatidylinositol-3 kinase (PI3K) and Akt participate in coordinated signaling with each other, mediating downstream events important for regulation of normal tissue growth as well as cancer growth and progression. PI3K isoforms can be divided into three classes--class I, II, and III--where class I PI3Ks are the most well-studied and are present in all mammalian cell types (60). PI3K-Akt signaling can be induced upon binding of several different growth factors to their respective RTKs or G protein-coupled receptors (GPCRs) including insulin-like growth factor-1 (IGF-1/IGF-1R) (61, 62), EGF/EGFR (63), and PDGF/PDGFR (64). PI3K transduces molecular signaling pathways upstream of Akt through the activities of its two structural subunits, a regulatory p85 subunit and a catalytic p110 subunit, of which several different isoforms also exist (p85α/β/γ/δ/ε and p110α/β/γ/δ, respectively) (60). Following ligand binding to
a receptor tyrosine kinase at the plasma membrane of a cell, the p85 subunit undergoes conformational changes which facilitate binding of the p110 subunit to lipid substrates, allowing p110 to catalyze the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3) at the plasma membrane via phosphorylation of the 3-hydroxyl group on the inositol ring (60). PIP3 facilitates recruitment of Akt (Protein Kinase B/PKB) to the plasma membrane where it co-localizes with two different protein kinases, phosphoinositide-dependent kinase 1 and 2 (PDK1/2).

Akt is a 57 kilodalton (kDa) serine/threonine kinase originally discovered in 1987 (v-Akt) as a potential gene component for the Akt8 provirus (65). Three Akt isoforms exist, namely, Akt1/2/3 or PKBα/β/γ, which share a highly-conserved domain structure composed of a pleckstrin homology (PH) domain at the N-terminus and a regulatory domain at the C-terminus or hydrophobic motif with an intervening kinase domain. Akt is maintained in an inactive state through interaction between the PH and kinase domains (66). The lipid-binding PH domain of Akt preferentially binds PIP3, which produces a conformational change in Akt to expose its two main phosphorylation residues, threonine 308 (Thr308) located in the kinase domain and serine 473 (Ser473) located in the C-terminal regulatory domain (66). The conformational changes in Akt give PDK1 and PDK2 access to the activation loop of Akt to mediate its phosphorylation at Thr308 and Ser473, respectively, thereby inducing Akt activation (66). Akt can undergo phosphorylation at either Thr308 or Ser473 for partial activation of the protein although full Akt activation requires that both Thr308 and Ser473 undergo phosphorylation. Studies have indicated that phosphorylation of Thr308 is a prerequisite step for Akt
activation, increasing activity by 100-fold, but that additional phosphorylation of Ser473 is required for maximal Akt activity (67, 68) (Figure 1).

Increasing evidence suggests that phosphorylation of Ser473 is more important in regulating specificity of downstream effector targeting than overall Akt activity. Studies showed that inhibition of phosphorylation at Ser473 resulted in reduced Akt-mediated phosphorylation of the downstream targets Forkhead box transcription factors (FOXO) 1/2a and FOXO3, but little alteration in activation of the Akt substrates glycogen synthase kinase 3β (GSK3β) and tuberous sclerosis complex 2 (TSC2) (69, 70). Additionally, the Ser473 residue of Akt can undergo phosphorylation by mammalian target of rapamycin complex 2 (mTORC2) and DNA-dependent protein kinase (DNA-PK) which act as kinases for Akt during mitogen-stimulated conditions or as a DNA damage and cell stress response, respectively (71-74). Further investigation into the specific roles of each Akt phosphorylation residue in conferring Akt function is warranted.
**Figure 1: Mode of Akt activation.** Upon growth factor binding to its cell membrane receptor, PI3K mediates the conversion of PIP2 to PIP3 at the plasma membrane. Akt is recruited to PIP3 where it undergoes phosphorylation at two primary residues, threonine 308 (p-Thr308) and serine 473 (p-Ser473), by PDK1 and PDK2, respectively, along with mTORC2. Phosphorylation at both sites of Akt confers its activation, allowing it to signal to downstream mediators to promote cancer cell proliferation and survival.
RTK

PI3K

PIP2 → PIP3

PDK1 → PDK2

Akt

Active Akt

p-Thr308

p-Ser473

mTORC2

Cell Proliferation & Survival
All three isoforms of Akt are structurally homologous and share a similar mode of activation. However, while Akt-1 and Akt-2 are ubiquitously-expressed, expression of Akt-3 in tissues is more limited (75, 76). Once Akt is activated, the kinase dissociates from the plasma membrane and translocates to the cytoplasm or nucleus to participate in numerous downstream signaling events modulating cell proliferation, survival, and metabolism via its serine/threonine kinase activity which can stimulate or inhibit downstream effectors. Substrates for Akt are defined by a minimal recognition sequence for Akt kinase activity, RXRXX(pS/pT)Ψ, where R represents arginine, X represents any amino acid, pS/pT is phospho-serine/phospho-threonine, and Ψ represents a bulky hydrophobic residue (77). The existence of this phosphorylation motif distinguishes Akt substrates from substrates unique to similar kinases of the AGC family, p90 ribosomal S6 kinase (p90Rsk) and p70S6K (78).

Akt mediates cell survival by targeting a diverse array of signaling effectors. This occurs via a shared mechanism of inhibiting the pro-apoptotic Bcl-2 homology domain (BH3)-only proteins. One example is the Akt-induced phosphorylation and inhibition of BAD, a BH3-only protein, at the Ser136 residue (78-80). Phosphorylation of the substrate generates a binding site for 14-3-3 proteins which sequester the substrate, thereby preventing its association with target proteins Bcl-2 and Bcl-XL for cell death induction (81). The FOXO family of transcription factors also serves as an Akt target for the induction of cell survival. FOXO1 is phosphorylated by Akt on three residues, Thr24, Ser256, and Ser319, resulting in a similar sequestration of the phosphorylated sites by 14-3-3 proteins as with BAD, and preventing the FOXO transcription factors from accessing
their target genes for synthesis of proteins that stimulate apoptosis and cell-cycle arrest such as BIM and Fas ligand (FasL) (78, 82-84). Furthermore, Akt phosphorylates the E3 ubiquitin ligase MDM2 (HDM2 is the human form) at Ser166 and Ser186, thereby inducing MDM2 translocation to the nucleus to inhibit the function of the tumor suppressor p53 (85, 86). Lastly, Akt promotes cell survival via phosphorylation of the apoptotic protein, pro-caspase-9, effectively blocking its protease activity (87).

Acting as a major mediator of cell growth, Akt phosphorylates several different downstream targets to regulate growth-promoting pathways. Mammalian target of rapamycin complex 1 (mTORC1) represents the predominant downstream mediator for Akt-induced cell growth by regulating initiation of translation, ribosome biogenesis, and cell cycle progression (88). Akt primarily activates mTORC1 signaling through phosphorylation and inactivation of mTORC1-regulating proteins. Tuberous sclerosis complex 2 (TSC2) and proline-rich Akt substrate of 40 kDa (PRAS40) are both negative regulators of mTORC1 signaling. Phosphorylation of Ser939 and Thr1462 on TSC2 by Akt results in its inactivation, allowing downstream mTORC1 activation and subsequent phosphorylation and activation of its effectors, eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and p70S6K, to promote cell growth (78, 89-93). PRAS40 undergoes Akt phosphorylation on its Thr246 residue, which results in its dissociation from mTORC1 to relieve its inhibitory hold on the protein, and sequestration by 14-3-3 proteins to restore mTORC1 signaling (94). Akt also phosphorylates the cyclin-dependent kinase inhibitor p21^{CIP1/WAF1} at Thr145, causing the cell growth inhibitory protein to translocate out of the nucleus to the cytoplasm, rendering it inactive (95).
Akt primarily regulates cell metabolism via phosphorylation of glycogen synthase kinase-3 (GSK-3), a protein responsible for phosphorylating and inactivating glycogen synthase, at Ser21 on GSK-3α and Ser9 on GSK-3β (96). As a result of Akt-mediated GSK3 phosphorylation, glycogen synthase does not undergo phosphorylation and is activated, allowing for synthesis of increased glycogen under insulin-Akt-activating conditions. Akt also facilitates a positive feedback mechanism on the insulin receptor for glucose transport regulation. The protein tyrosine phosphatase PTP1b is phosphorylated at Ser50 by Akt to inhibit dephosphorylation of the insulin receptor, preventing insulin signaling ablation (97).

a.) Regulation of PI3K-Akt Signaling

Regulation of Akt signaling is primarily mediated by Akt-inactivating phosphatases responsible for opposing the actions of kinases by removing activating-phosphate groups (Figure 2). Dephosphorylation of both p-Akt(Thr308) and p-Akt(Ser473) is mediated by protein phosphatase 2A (PP2A), and p-Akt(Ser473) dephosphorylation is carried out by PH domain leucine-rich repeat protein phosphatase (PHLPP), resulting in inactive Akt (98-101). PP2A is a ubiquitously-expressed tumor suppressor and protein, accounting for approximately 1% of total cellular protein, and is responsible for a large portion of serine/threonine phosphatase activity in most cells and tissues (102). PP2A belongs to the family of phospho-protein phosphatases (PPPs) which function as cell cycle regulators responsible for halting progression through mitosis via interaction with targets of cyclin-dependent kinases (CDKs) (103). PP2A is a
heterotrimeric structure composed of a 65 kDa scaffolding (A) subunit, a 55 kDa regulatory (B) subunit, and a 36 kDa catalytic (C) subunit (104, 105), present as an AC dimer associated with the B subunit (104). The A subunit binds tightly to the C subunit and functions to coordinate B subunit recruitment, primarily serving as a scaffold to which the B subunit can bind (104). The C subunit is responsible for the phosphatase activity of PP2A (102) and its deletion produces lethality in yeast and mice, demonstrating the importance PP2A (102, 106, 107). The functional specificity of PP2A is primarily determined by the B subunit, which can also modulate catalytic activity of the phosphatase in vitro (102, 104). Both the A and C subunits contain α and β isoforms and many different isoforms of the B subunit exist, each recognizing similar sequences in the A subunit for binding purposes. The variety of different combinations of PP2A subunits and isoforms allows for the potential of PP2A to transduce a variety of signaling pathways (102).

PP2A expression is tightly controlled in the cell to maintain homeostasis and regulate numerous cellular activities. In resting cells, PP2A levels are relatively high, requiring phosphorylation-mediated inactivation to execute mitogenic signaling pathways (102, 108). These findings were first demonstrated in vitro where tyrosine 307 (Tyr307) in the C-terminal sequence of PP2A underwent phosphorylation and phosphatase inactivation induced by stimulation of EGFR or IGFR kinases, an observation that was enhanced by incubation with the phosphatase inhibitor okadaic acid (109). This effect was also demonstrated in serum-stimulated fibroblasts, where Tyr307 phosphorylation of PP2A was absent in non-serum-stimulated cells (110). Furthermore, a more recent study
showed that BT-474 HER2-positive breast cancer cells treated with the growth factor heregulin had increased p-Tyr307 PP2A amounts, an effect that was blocked by incubation with the HER2 kinase inhibitor tyrphostin AG825 (111). This same study also demonstrated that phosphorylated PP2A was only detected in HER2-positive human breast tumors and absent in normal tissue. These observations suggest that growth factor receptor kinase stimulation in malignant cells and tissues may mediate downstream cell growth and survival activities by inducing phosphorylation and inactivation of PP2A. In addition, the transient nature of p-PP2A(Tyr307) indicates that mitogen-induced inactivation of PP2A is a mechanism to “flick” the activity of the phosphatase on or off during the growth cycle (108). Moreover, studies demonstrating the requirement for reductions in PP2A activity for initiation of cancer growth support the tumor-suppressive role of PP2A (112, 113).

The PHLPP family of serine/threonine phosphatases contains two isoforms: PHLPP1 and PHLPP2 (114) which preferentially act on the phosphorylated Ser473 residue of Akt to prevent downstream signaling (99, 115). PHLPP1 and 2 are widely expressed in human tissues as well as breast cancer cells (99, 116), however the two show differential specificity for Akt isoforms demonstrated by studies showing PHLPP1 binds to and dephosphorylates Akt2 and Akt3 whereas PHLPP2 binds to and dephosphorylates Akt1 and Akt3 (99, 117). PHLPP phosphatases (collectively known as PHLPP) display tumor suppressive properties; overexpression of PHLPP in breast cancer cells produced reductions in colony formation and growth in vitro (115). Breast cancer cells (21T) with increased p-Akt(Ser473) showed decreased PHLPP amounts, an effect
which was more pronounced in metastatic breast cancer cells compared with primary breast tumor cells (118), suggesting PHLPP plays an important role in reducing breast cancer aggressiveness. PHLPP phosphatases are clear regulators of Akt phosphorylation and activation however, the tumor suppressor role of PHLPP requires further investigation.

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is another negative regulator of Akt signaling. PTEN is a constitutively-active protein tyrosine phosphatase bound to the plasma membrane upstream of Akt signaling, mediating the dephosphorylation of PIP3 to generate its precursor, PIP2 through hydrolysis. PTEN is a tumor suppressor gene located on human chromosome 10q23 and serves as a major regulator of the Akt-mediated cell growth and survival signaling which is implicated in tumor growth (119-121). PTEN directly opposes the actions of PI3Ks to negatively regulate Akt activation. PTEN has protective effects in cancer, but also participates in tight regulation of metabolism through antagonism of insulin-induced anabolic effects and increased energy expenditure (122). Given the emergence of studies investigating cancer cell dependency on metabolic functions, investigators are just scratching the surface regarding the expanding influence of PTEN in the regulation of Akt signaling.
Figure 2: Mode of Akt inactivation by phosphatases. Akt signaling is inactivated by the removal of its phosphates groups at threonine 308 (Thr308) and serine 473 (Ser473) residues mediated by protein phosphatase 2A (PP2A). PH domain leucine-rich repeat protein phosphatase (PHLPP) can also dephosphorylate Akt at Ser473. Activation of Akt is also prevented by phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a phosphatase which converts PIP3 back to PIP2 upstream of Akt, inhibiting phosphorylation and activation of Akt and preventing downstream signaling to promote cell growth.
b.) Deregulated PI3K-Akt Signaling in Cancer

Over-activation of PI3K-Akt signaling contributes to elevated cell proliferation and avoidance of cell death signaling to promote tumor growth and progression. Akt is one of the most frequently activated protein kinases in cancer, and several of the signaling molecules that participate in Akt signaling are also altered in different types of cancer (123-125). Increased Akt signaling is associated with poor patient outcome in a wide range of tumor types, including breast cancer (126). The activation status of Akt or phosphorylated-Akt (phospho-Akt or p-Akt) has been used as a biomarker in tumor immunohistochemistry, where p-Akt is increased in TNBC as well as other types of breast cancer (127-129).

Despite being one of the most frequently-activated signaling pathways mediating cancer cell progression, mutations in the gene for Akt itself are uncommon; only in cases of the Akt2 isoform has gene amplification and overexpression been observed (130). Mutations in the PI3K-Akt pathway are frequent, occurring in as many as 25% of breast cancers (131). Several proteins involved in regulating Akt signaling frequently undergo mutation, including RTKs, PI3K, PTEN, and PP2A. Overexpression or activating mutations in RTKs and their growth factor ligands can lead to increased Akt signaling in cancer. In the case of EGFR/EGF, the receptor is amplified in many cancers, resulting in increased copy number (130, 132, 133). Mutations in exon 18 encoding the activation loop of PDGF-Rα confer constitutive kinase activation and subsequent Akt activation in gastrointestinal cancers (134). Increased ligand concentration and reduced receptor turnover can directly promote constitutive activation of Akt signaling as well. However,
RTK mutations found in TNBC are not common and genetic aberrations in RTKs may not serve as viable targets for TNBC treatment (135).

A high percentage of TNBC patients exhibit mutated forms of the \textit{PIK3CA} gene (2). Generally, \textit{PIK3CA} undergoes a gain-of-function missense mutation to produce a constitutively active kinase as observed in many breast cancers (136), although amplification of the gene was also observed in head and neck, gastric, and cervical cancers as well as squamous cell lung carcinoma (119, 137, 138). Overall, mutations in \textit{PIK3CA} occur in approximately 35-40\% of breast cancer cases and correlate with larger tumor size and reduced overall survival for all breast cancer subtypes (139).

Phosphatases are critical regulators of Akt activation and also frequently undergo mutations in cancer. The lifetime risk for cancer is increased in patients with \textit{PTEN} mutations and underlies the main component for aberrant Akt signaling in cancer (140). Somatic \textit{PTEN} alterations are common in breast cancer as well as endometrial, prostate, thyroid, and kidney cancers. Germline mutations in the \textit{PTEN} gene result in hamartoma tumor syndrome (PHTS), which includes a range of autosomal-dominant clinical syndromes such as Cowden disease and Bannayan-Zonana syndrome (140). In TNBC, \textit{PTEN} inactivation is common (141, 142), demonstrated by loss of heterozygosity (LOH) at chromosome 10q23 via homozygous deletions, frameshift or nonsense mutations (121). Loss of PTEN leads to increased PIP3, generating overactivated Akt signaling and growth of cancer cells. Terakawa et al. demonstrated a significant inverse correlation between PTEN loss and Akt activation (p-Akt) in endometrial cancer (143), suggesting p-
Akt may serve as a potential biomarker for PTEN dysfunction in tumors. In addition, a recent study showed that the fraction of breast cancer cases associated with a loss of PTEN was 30% and 25% in primary and metastatic breast tumors, respectively, indicating a role for Akt dysregulation in facilitating breast cancer spread and metastasis (144).

Alterations in PP2A functionality are also observed in human cancers, serving as another point of Akt dysregulation. In breast carcinomas, mutations in PP2A were found in the gene encoding the alpha/α (PPP2R1A) and beta/β (PPP2R1B) isoforms of the A subunit of the phosphatase (145). Somatic mutations affecting the α isoform were primarily nucleotide substitutions or frame-shift mutations and those mutations affecting the β isoform included exon deletions, indicating that alterations in both isoforms found in breast tumors are involved in mediating Akt hyper-activation (145-147). Aβ mutants have an impaired ability to form complexes with the PP2A-B and -C subunits, resulting in a loss of PP2A-mediated Akt dephosphorylation and inactivation (146). Haploinsufficient products resulting from Aα mutants also prevent generation of a fully-functional PP2A, thereby reducing phosphatase activity on its target, Akt, and mediating tumorigenicity (148). A loss in transcript expression for PPP2R2A, the gene encoding the B subunit of PP2A (149), was also observed in ER-negative breast tumors. Inactivation of PP2A therefore plays a crucial role in abnormal Akt signaling in cancer, and especially in breast cancer.

3. The Tumor Microenvironment
The tumor microenvironment comprises a diverse array of cell types participating in cross-talk mechanisms and contributing to the growth and progression of tumors. Immune cells, inflammatory cells, fibroblasts, pericytes, and endothelial cells are members of the tumor microenvironment whose interaction directly facilitates tumor growth, cell invasion and evasion of growth suppressors underlying the hallmarks of cancer (150). The surrounding supporting tissue defined as the epithelial parenchyma makes up the tumor stroma, a connective tissue framework acting as the extracellular matrix (ECM) or tumor matrix. The tumor stroma provides the growing tumor with a supportive tissue scaffold which furthers tumor growth through alterations in growth factor secretion and deposition of ECM proteins. The stroma-directed behavior of tumors led to development of the term ‘reactive tumor stroma’ or desmoplasia (151). ECM proteins, such as fibronectin and collagen type I, type III, and type V, provide structural integrity for the tumor matrix and are synthesized by a specific stromal cell type, the fibroblast (152, 153).

Fibroblasts are non-vascular, non-epithelial, and non-inflammatory cells serving as the main cellular component comprising connective tissue (154). Fibroblasts are as the principal source of ECM components and therefore are the major contributors of scar formation and tissue fibrosis (155). Fibroblasts that secrete ECM proteins and growth factors at an elevated level and also proliferate more than their normal counterparts are considered ‘activated’ fibroblasts (sometimes referred to as myofibroblasts) and are found at higher amounts at sites of wound healing and in fibrotic tissue (155, 156). Myofibroblasts share features with fibroblasts and smooth muscle cells and display a high
level of motility (157). These activated fibroblasts are identified by their expression of specific markers including alpha-smooth muscle actin (α-SMA) as well as by normal markers of fibroblasts such as vimentin, fibronectin, and collagen type I or III (155).

Tumors are considered ‘wounds that do not heal’ (158), where fibroblasts are perpetually activated to facilitate continuous growth of the tumor. Activated fibroblasts that participate in a tumor-supportive role are considered cancer-associated fibroblasts (CAFs) or tumor-associated fibroblasts (TAFs). Approximately 80% of stromal fibroblasts in breast tumors have acquired the activated phenotype, demonstrating the potential role of CAFs in mediating breast tumor growth and progression (159). CAFs communicate with tumor cells via cross-talk mechanisms to mediate tumor cell proliferation, migration, and invasion. Cancer cells in turn, secrete growth factors to facilitate CAF proliferation. ECM proteins produced by CAFs enhance cancer cell migration in vitro, indicating their role in facilitating eventual tumor metastasis (160). Clinically, TNBC patients show increased levels of tumor fibrosis which is also associated with the presence of distant metastasis (161). The involvement of the tumor stroma and CAFs in mediating tumorigenesis and progression poses a clear target for the development of novel cancer treatments.

a.) TGF-β Signaling

Transforming growth factor-beta (TGF-β) is the principle inducer of fibroblast activation and its expression is strongly associated with tumor fibrosis and cancer progression (162, 163). TGF-β is a pleiotropic cytokine secreted by stromal cells and
cancer cells and exists in three isoforms--TGF-β1, TGF-β2, and TGF-β3. In normal tissues, TGF-β functions as an important immunosuppressor, a negative regulator of proliferation in endothelial, epithelial, and hematopoietic cells, and controller of differentiation of immune, neuronal, mesenchymal, and epithelial cell types (164). Evidence for the role of TGF-β as a pro-oncogenic factor during breast cancer progression includes the demonstration that infiltrating breast carcinomas show high immunoreactivity for TGF-β1 (165). Furthermore, TGF-β expression correlates with the rate of disease progression as patients with ER-negative breast tumors with low expression for the TGF-β receptor II (TGFβ-RII) experience more favorable outcomes (166, 167). Moreover, human breast tumors exhibiting higher TGF-β activity are associated with selective metastasis to the lung, which is also linked to patients with ER-negative breast tumors but not ER-positive breast tumors (168). These observations clearly demonstrate that TGF-β is a prognostic indicator for TNBC.

Prior to binding to its receptors, active TGF-β is generated by proteolytic cleavage of an inactive precursor. The secreted latent form of TGF-β consists of a large complex containing the mature TGF-β1 molecule, a dimerized N-terminal remnant of the TGF-β1 precursor termed TGF-β1 latency-associated peptide (LAP) or TGF-β propeptide, and a latent-TGF-β-binding protein (LTBP) which is anchored to the ECM itself (169). All three TGF-β isoforms are secreted as a 75 kDa propeptide homodimer LAP which undergo cleavage catalyzed by furin-type enzymes to release the mature form of TGF-β from the complex, followed by its activation by proteases allowing TGF-β to bind to its receptors (169, 170-172).
The TGF-β dimer displays a high affinity for the TGFβ-RII at the cell membrane and does not initially interact with TGFβ-RI alone (173). Once TGF-β binds TGFβ-RII in the extracellular domain, TGFβ-RI is incorporated into the receptor complex where it also interacts with the TGF-β ligand to form a heterotetrameric ligand-receptor complex comprising the ligand dimer bound to the extracellular domains of both TGF-β type I and type II receptors (174). TGF-β receptors are transmembrane, serine/threonine protein kinases sharing similar properties with the activin family of receptors (175). Following TGF-β ligand binding to the extracellular domain of its receptors, TGFβ-RI undergoes phosphorylation at multiple serine and threonine residues of its GS domain in the cytoplasmic region, catalyzed by the kinase domain of TGFβ-RII, thereby activating the type I receptor (173, 174). Once activated, TGFβ-RI is able to interact with and phosphorylate its downstream effectors, the cytosolic Smad2/3 proteins, at two serine residues in the C-terminal SXS motif in the MAD-homology 2 (MH2) domain, in turn activating Smad2/3 (174) (Figure 3).

Smad proteins are a family of transcription factors that were discovered as the first intracellular mediators of TGF-β signaling and can be divided into three distinct classes: receptor-regulated Smads (R-Smads), co-mediator Smads (Co-Smads), and inhibitory-Smads (I-Smads) (174, 176, 177). R-Smads are the only type of Smad that can be directly phosphorylated and activated by the type I receptor kinase. Smad proteins share conserved MH1 and MH2 structural domains in the N-terminus and C-terminus, respectively, serving as important binding domains for signaling regulation (174).
Phosphorylation of the MH2 domain of Smad2/3 mediates direct activation of the R-Smads, which increases Smad2/3 affinity for the Co-Smad, Smad4, for subsequent binding (174). Smad2/3 activation also exposes a nuclear import region on the MH2 domain of the R-Smad allowing translocation into the nucleus upon complex formation with Smad4 (178). The Smad2/3-Smad4 complex directly binds DNA via a Smad-binding element (SBE), mediated mainly by sequence-specific binding of Smad3 and Smad4 (174, 179, 180). The Smad complex regulates target genes through interaction with members from many different DNA-binding proteins including Fast1 of the forkhead family, Mixer of the homeobox family, E-box, Jun/Fos (AP-1), Runx, cyclic AMP response element-binding protein (CREBP), and E2F (174, 181-183). Furthermore, the MH2 domain of Smad2/3 and Smad4 interacts with the transcriptional co-activators p300, ARC105, and Smif to guide gene regulation (182, 184). The large number of transcription factors and co-activators involved in Smad signaling underlies the pleiotropic nature of this signaling mechanism, allowing Smad to target a wide range of functionally diverse genes.

Connective tissue growth factor (CTGF), also known as CCN2 as a member of the family of matricellular CCN proteins, is a hallmark of fibrosis whose transcription is induced by TGF-β and Smad signaling (185-190). CTGF expression is induced by TGF-β in fibroblasts via Smad binding to elements in the CTGF promoter region (191, 192). CTGF induction promotes fibroblast proliferation, ECM protein production, cell adhesion, cell migration, and ECM contraction in fibroblasts (193-198). Many of the effects elicited by CTGF are mediated through interaction with integrins (199). In human
TNBC cells, knockdown of CTGF attenuated cell migration (199). Further studies in breast cancer cells demonstrated that CTGF enhanced cell migration using a Boyden chamber assay as well as angiogenesis as measured by a chick chorioallantoic membrane (CAM) assay (200). Clinically, elevated CTGF in primary breast tumors is associated with presentation at a later stage and positive lymph node status (201). Therefore, the CTGF has high potential for mediating the growth and progression of breast cancer.

Another important gene whose transcription is induced by TGF-β-Smad signaling is Tenascin C (202). Tenascin C is a member of a family of ECM glycoproteins and its expression is rapidly elevated in response to tissue injury and in the tumor stroma mediating inflammation, fibrosis, and tissue remodeling (203-205). Stromal cells including CAFs produce Tenascin C which interacts with integrins, similar to the mechanism of action of CTGF, to mediate its pro-fibrotic effects (205). Tenascin C is emerging as an important target for cancer treatment, due to its high expression in mammary tumors and absence in the normal adult mammary gland (203, 206). Additionally, Tenascin C expression correlates with poor patient prognosis in breast cancer as well as cancer of the bladder (207, 208). In preclinical breast cancer studies, Tenascin C blockade abrogated migration and anchorage-dependent proliferation of human TNBC cells (209). Moreover, downregulation of Tenascin C impaired the ability of breast cancer cells to spread to the lungs and form metastatic tumors. Tenascin C expression in primary breast tumors in mice also correlated with a shorter progression to metastatic lung relapse, an effect that was almost completely abolished by Tenascin C
inhibition (210). Thus, Tenascin C plays an important role in mediating breast tumor growth and spread.
Figure 3: TGF-β induces activation of Smads to mediate synthesis of pro-fibrotic proteins. Following transforming growth factor-beta (TGF-β) binding to its receptor on cancer-associated fibroblasts (CAFs), cytosolic Smad2 and Smad3 are phosphorylated and activated by the TGF-β receptor (TGFβ-R). Phosphorylated Smad2/3 (p-Smad2/3) forms a complex with the co-Smad, Smad4 followed by translocation of Smad2/3/4 to the nucleus where it mediates transcription of genes including connective tissue growth factor (CTGF) and Tenascin C. This results in an increase in fibrosis and extracellular matrix (ECM) protein deposition promoting tumor growth.
The response to TGF-β ligand binding is highly complex and context-dependent based on cell type and the subset of transcription factors present from cell to cell. As a result of this underlying complexity, cancer cells have developed methods of evading the anti-oncogenic signaling induced by TGF-β under normal conditions, by shutting down core tumor-suppressive signaling arms of the TGF-β pathway or becoming non-responsive to anti-oncogenic signals in order to serve the needs of the tumor (157). This effectively shifts the response to TGF-β to a pro-oncogenic one in cancer cells and cells comprising the tumor microenvironment, subsequently transforming TGF-β into a potent mediator of cell growth and deregulated tumor fibrosis. For example, Gomis et al. showed that the TGF-β receptor and Smads functioned normally in breast cancer cells extracted from pleural fluids of patients with metastatic disease; however, the cancer cells displayed partial or complete loss of response to the cytostatic or growth-inhibitory signals induced by TGF-β, with half of these cases lacking oncogenic c-MYC repression (211). Additionally, metastatic breast cancer cells derived from patients displayed TGF-β-mediated induction of Inhibitor of DNA Binding (ID) expression, which inhibits apoptotic gene transcription, and is normally repressed in response to TGF-β in normal epithelial cells (168). ID1 expression mediates cancer cell invasiveness and proliferation and is also associated with the unique lung metastasis gene signature found in patients with TNB tumors that have relapsed, indicating a clear pro-oncogenic switch occurring in response to TGF-β (49, 212, 213).

Cancer cells that have lost the tumor-suppressive arm of the TGF-β signaling pathway experience increases in cell growth, invasion, and immune evasion contributing
to tumor growth and spread. TGF-β is a potent inducer of epithelial-mesenchymal transition (EMT), a process wherein cells acquire a motile phenotype and express markers such as vimentin, fibronectin, N-cadherin, Twist, and Snail, similar to mesenchymal cells (59). Smad proteins mediate EMT via induction of high-mobility group A2 (HMGA2) expression which, in turn, induces expression of Snail, Slug and Twist (214). TGF-β-induced EMT gives way to the invasive and increasingly metastatic capabilities of the transformed cells, further promoting tumor progression.

b.) TGF-β Clinical Therapeutics

Despite the opportunities TGF-β presents as a novel therapeutic target for the inhibition of cancer cell proliferation, invasion, and metastasis, the widespread effects mediated through this cytokine-derived pathway pose several challenges for its clinical use. Several classes of TGF-β signaling inhibitors were developed and showed mild success in preclinical and clinical studies in cancer as well as fibrotic diseases. These therapeutic agents include inhibitors of TGF-β production (antisense oligonucleotides), anti-TGF-β antibodies, anti-TGFβ-R antibodies, TGF-β ligand traps, and TGFβ-R kinase inhibitors (157). Inhibitors of Smad activity are currently under development in a preclinical setting and have shown effectiveness in preventing TGF-β signaling (215-217). Treatment of TNB tumor xenografts in mice with the TGFβ-RII kinase antibody LY2157299 prevented tumor reestablishment after treatment with the chemotherapeutic paclitaxel (218). LY2157299 is currently under evaluation in clinical trials for patients with glioma, hepatocellular carcinoma, and pancreatic cancer (219). Trabedersen (AP 12009) is a phosphorothioate antisense oligodeoxynucleotide specific for human TGF-β2
mRNA which increased median survival for glioma patients to 39.1 months compared with 21.7 months for standard temozolomide chemotherapy in a phase IIb study (220), demonstrating clinical benefit resulting from TGF-β targeting in cancer.

Several problems must be addressed for the successful progression of TGF-β inhibitors through clinical trials. First, TGF-β targeting may lead to upregulation of compensatory mechanisms which activate Smads independent of TGF-β, as in the case of patients with TGFBR1 or TGFBR2 inactivating mutations (221). Another problem with TGF-β inhibition as a form of cancer therapy is the timing of treatment, since TGF-β is initially tumor-suppressive in the premalignant stages of tumor development; screening for patients whose tumors rely heavily on TGF-β for survival should be a priority prior to the use of a TGF-β-blocker as an appropriate treatment option. Lastly, since TGF-β elicits pleiotropic actions in normal and cancerous cell types, administering systemic TGF-β inhibition may have deleterious effects on healthy cells and tissues which require TGF-β signaling for growth and repair. The recent advances in identifying genetic signatures and molecular expression profiling in tumors may improve methods of patient enrollment for optimal benefit from receipt of TGF-β-targeting therapies. Furthermore, development of novel TGF-β targeting methods can be achieved through in-depth understanding of current physiological molecular systems, such as the renin-angiotensin system as discussed below.

4. The Renin-Angiotensin System
The control of blood pressure and electrolyte homeostasis, renal development, and vascular tone is primarily mediated by the renin-angiotensin system (RAS) in the body, which involves a number of different organ systems and enzymatic contributions acting at both the systemic and local tissue level (222). Renin is the rate-limiting enzyme of the RAS produced by the kidney to cleave angiotensinogen and form the decapeptide angiotensin I (Ang I) (223). The subsequent processing of Ang I then diverges to include the carboxypeptidase angiotensin-converting enzyme (ACE) which converts Ang I into the octapeptide angiotensin II (Ang II), or conversely, neprilysin, thimet oligopeptidase or prolylendopeptidase (PE) which catalyze the formation of angiotensin-(1-7) [Ang-(1-7)] from Ang I (222) (Figure 4). Both Ang II and Ang-(1-7) are present in the circulation at similar concentrations (224). Ang II acts on the G protein-coupled receptors angiotensin type 1 and 2 (AT1 and AT2, respectively) to regulate vascular reactivity, cell proliferation, fibrosis and inflammation (222, 225). Ang II is the most well-studied member of the RAS, primarily mediating its mitogenic effects via binding to the AT1 receptor, whereas the effects elicited following Ang II binding to the AT2 receptor are less well-characterized and are generally thought to oppose AT1 receptor-mediated effects (222). Ang II can also be degraded by PE and the monocarboxypeptidase angiotensin converting enzyme 2 or ACE2, a homolog of ACE which cleaves the carboxy-terminal amino acid of Ang II, to form Ang-(1-7) (222, 226). Ang-(1-7) is further degraded by ACE to form the inactive metabolite Ang-(1-5) (227, 228). Treatment with ACE inhibitors and AT1 receptor blockers (ARBs) shifts the balance of vasoactive peptide production, resulting in elevated levels of ACE2 and circulating Ang-
(1-7) due to prevention of the conversion of Ang I to Ang II and degradation of Ang-(1-7) (229-231).
Figure 4: Formation of the renin-angiotensin system peptides. Angiotensinogen secreted from the liver is cleaved by renin to form angiotensin I (Ang I) which can be metabolized by angiotensin converting enzyme (ACE) to form angiotensin II (Ang II) or undergo enzymatic cleavage by neprilysin (NEP), prolylendopeptidase (PE) or thimet oligopeptidase to form angiotensin-(1-7) [(Ang-(1-7)]. Ang II can be further metabolized by ACE2 to form Ang-(1-7). ACE further degrades the heptapeptide hormone to form the inactive metabolite angiotensin-(1-5) [Ang-(1-5)]. Ang II elicits biological effects through the G-protein coupled receptors AT1 and AT2, while Ang-(1-7) activates the unique AT_{(1-7)} receptor mas.
a.) Angiotensin-(1-7)

Ang-(1-7) is an endogenous, seven amino-acid peptide hormone which binds to and activates the seven-transmembrane G-protein-coupled AT$_{(1-7)}$ receptor encoded by the mas gene to oppose the actions of Ang II and promote vasodilatory as well as anti-proliferative, anti-fibrotic, anti-thrombotic, and anti-inflammatory effects (225, 232-236). Ang-(1-7) activates its receptor mas expressed by cells of several organ types and tissues, including the heart, kidney, blood vessels, testis, and brain (227, 237, 238). Ang-(1-7) functions to maintain homeostasis in various organ systems. In ischemic rat hearts, Ang-(1-7) improved cardiac function and prevented ischemia-induced cardiac arrhythmia (239-241). An oral formulation of the heptapeptide hormone also attenuated isoproterenol-induced heart function impairment and cardiac remodeling in infarcted rats (242). In addition, Ang-(1-7) was increased following rat coronary artery ligation (243), demonstrating the cardioprotective role for the heptapeptide hormone.

Ang-(1-7) counterbalances the vasoconstrictive effects of Ang II by stimulating nitric oxide (NO) release from the endothelium, as well as inducing bradykinin and prostaglandin production, to induce vasorelaxation during blood pressure regulation, an effect mediated via the mas receptor (228, 232, 244, 245). Ang-(1-7) mediates downregulation of AT1 receptors in VSMC’s (224) coinciding with the reduction of proliferating VSMCs in vitro (246). The heptapeptide hormone also prevents the Ang II-mediated proliferation of endothelial cells in culture, providing support for the counter-regulatory actions of Ang-(1-7) (247). In addition, the growth of cardiomyocytes in vitro was inhibited when incubated with Ang-(1-7), an effect that was blocked by incubation
with D-Ala (235). Furthermore, following balloon-catheter injury to rat carotid arteries in vivo, Ang-(1-7) prevented neointimal growth of VSMCs, but had no effect on the non-proliferating medial cell layer, demonstrating that the heptapeptide hormone selectively inhibits actively proliferating cells (234, 248).

Ang-(1-7) is also a potent anti-fibrotic agent opposing the fibrosis-stimulatory actions of Ang II in vitro and in vivo. For example, in adult rat cardiac fibroblasts, Ang II stimulated collagen synthesis, TGF-β1 mRNA expression, and cardiomyocyte hypertrophy, effects which were reversed with Ang-(1-7) treatment (249). In a deoxycorticosterone acetate (DOCA)-salt rat animal model of hypertension, infusion of animals with Ang-(1-7) resulted in prevention of interstitial and perivascular collagen deposition (250). Additional studies using cardiac fibroblasts isolated from rats demonstrated that Ang-(1-7) opposed endothelin-1 (ET-1) stimulation to reduce proliferation and collagen synthesis of cardiac fibroblasts, which was prevented with [D-alanine\(^7\)]-angiotensin-(1-7) (D-Ala) incubation (251). Moreover, Ang-(1-7) decreased TGF-β and p-Smad2 in skeletal muscle fibroblasts to attenuate muscle fibrosis (252). Ang-(1-7) also abrogated fibrosis in the lungs of mice through the mas receptor as shown by reductions in collagen and the fibrotic signaling mediators TGF-β and p-Smad2/3, indicating a significant role for Ang-(1-7) in targeting fibrosis (253).

The molecular mechanisms for the Ang-(1-7)-mediated effects are thought to occur primarily through increases in the second messenger cyclic adenosine monophosphate (cAMP) following activation of the mas receptor (225, 234). Inhibition
of the cAMP-dependent protein kinase A (PKA) using Rp-CAMPS in VSMCs completely blocked the Ang-(1-7)-mediated reduction in cell proliferation, indicating that Ang-(1-7) requires activation of the cAMP protein kinase via cAMP release to elicit inhibitory effects on cell growth (225). Ang-(1-7) also regulated MAPK activation to mediate reductions in cell growth. In VSMCs incubated with Ang II, pre-treatment with Ang-(1-7) reduced phosphorylated-ERK1 and ERK2 (p-ERK1/2), signaling effectors participating in the growth-promoting mitogen-activated protein kinase (MAPK) signaling pathway (225, 254). Treatment of cardiac fibroblasts from rat hearts with Ang-(1-7) also produced a reduction in p-ERK1/2 which was associated with decreased cardiac fibroblast proliferation, suggesting that Ang-(1-7) targets activated MAPKs to reduce downstream cell growth effects (251). Ang-(1-7) also reduced p-ERK1/2 in the left ventricles of rat hearts, proximal tubular cells, and endothelial cells (247, 255, 256).

Due to the decrease in phosphorylated ERK1/2 in both VSMCs and cardiac fibroblasts with Ang-(1-7) treatment and the observation that the inhibition of p-ERK1/2 by Ang-(1-7) was abrogated by treatment with an inhibitor of tyrosine phosphatases or serine/threonine phosphatases (244), studies were conducted to determine whether Ang-(1-7) upregulates a dual specificity phosphatase to mediate ERK inactivation and reduce cell proliferation and cardiac fibrosis. In both isolated rat cardiac fibroblasts and rat cardiac ventricles, Ang-(1-7) increased DUSP1 and prevented radiation-induced inflammation in rat primary astrocytes through reductions in p-ERK1/2 and upregulation of DUSP1, which was reversed by treatment with the phosphatase inhibitor sodium vanadate. These results suggest that Ang-(1-7) upregulates protein phosphatase activity to
reduce ERK phosphorylation, activation, and downstream mitogenic signaling (251, 255, 257).

The mechanism for the Ang-(1-7)-mediated upregulation of DUSP1 to attenuate activated ERK1/2 signaling is not yet well-understood. MAPK activation is regulated by cAMP-PKA signaling in a cell type-dependent manner to inhibit downstream cell proliferation (258). Additionally, the human DUSP1 gene contains binding sites for cAMP responsive element (CRE) in the promoter/enhancer region, which is responsible for increases in DUSP1 transcription (259). Ang II activation of MAPK signaling is also correlated with ECM remodeling and increases in CTGF in isolated cardiac fibroblasts, an effect that is exacerbated in ACE2 knockout mice. This suggests that antagonism of ERK activation by Ang-(1-7) may also serve as the molecular mechanism underlying the attenuation of tissue fibrosis due to Ang-(1-7) treatment (260). Therefore, these pathways may serve as potential mechanisms to explain the increase in DUSP1 and inhibition of MAPK-mediated cell growth and fibrosis by the heptapeptide hormone.

b.) Renin-Angiotensin System and Cancer

Hypertensive patients at the Glasgow Blood Pressure Clinic treated with ACE inhibitors showed a reduced risk for the development of sex-specific cancers compared with patients on other anti-hypertensive drugs such as calcium-channel blockers, beta-blockers and diuretics (261), providing the first evidence of an involvement for the RAS in cancer. Dysregulation of RAS components was demonstrated in several cancers including breast, prostate, ovarian, gut and pancreatic which all possess their own local
tissue RAS (262, 263). A variety of RAS gene polymorphisms contribute to an increased risk for the development of breast cancer in women, most commonly in the genes for ACE and the AT1 receptor which influence tumor invasiveness leading to increased cancer spread (264, 265).

As discussed above, Ang II induces proliferation of VSMC’s, cardiomyocytes and endothelial cells in normal tissues. However, Ang II can also promote proliferation of cancer cells (234, 235, 266, 267). MAPK signaling is initiated following Ang II binding to the AT1 receptor resulting in upregulated protein kinase C (PKC) followed by phosphorylation and activation of the MAPKs, extracellular signal-regulated kinases 1/2 (ERK1/2) to promote breast cancer cell proliferation (224, 268). In prostate cancer cells, Ang II increases cell proliferation with concomitant upregulation of nuclear transcript factor-κB (NF-κB) and c-myc, an effect which is blocked by a small-interfering RNA (siRNA) to the AT1 receptor (269). Additional studies using prostate cancer cells showed that Ang II activates the transcription factor paired homeobox 2 (PAX2) through increased ERK1/2 activation to mediate cell proliferation (270).

Tumor angiogenesis is also stimulated by Ang II through upregulation of the pro-angiogenic genes for vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), tissue inhibitor of metalloproteinases-1 (TIMP-1) and hypoxia-inducible factor-2α (Hif-2α), most notably in estrogen receptor negative breast cancer cells (267). In support of these findings, Ang II increases VEGF production by pancreatic ductal adenocarcinoma cells (271). Treatment of cells with inhibitors of MAPK signaling, either PD098059 or
U0126, prevents the Ang II-induced upregulation of VEGF, suggesting that MAPK activation is required for this pro-angiogenic event. Further, VEGF is increased under Ang II stimulation in ovarian cancer cells and bladder cancer cells and is mediated through activation of the AT1 receptor (272, 273).

ARBs (which block activation of the AT1R) and ACE inhibitors (which prevent formation of Ang II) may be useful agents for the treatment of cancer. In a recent clinical study, breast cancer patients taking ARBs in combination with chemotherapeutics demonstrated a significantly higher 5-year recurrence-free survival rate (82%) compared to patients not on ARBs or ACE inhibitors (71%) (274). Another clinical study showed reduced colorectal cancer incidence in hypertensive patients with long-term use of ARBs or ACE inhibitors (275). Preclinical studies demonstrated that ARBs and ACE inhibitors were effective in reducing tumor growth in models of fibrosarcoma (276), metastatic lung cancer (277, 278), ovarian cancer (279), prostate cancer (280), esophageal carcinoma (281), and breast cancer (282, 283). These findings clearly demonstrate an important role for the RAS in the regulation of cancer formation and growth. Treatment with ACE inhibitors and ARBs effectively shifts the RAS cascade to favor formation of the anti-proliferative and anti-fibrotic Ang-(1-7) hormone as opposed to increasing plasma Ang II levels. Prevention of Ang-(1-7) breakdown with the use of ACE inhibitors augments this effect, giving rise to the potential for Ang-(1-7) as a novel therapeutic for the treatment of cancer.

c.) Ang-(1-7) and Cancer
Ang-(1-7) reduces the growth and proliferation of cardiovascular cells as previously mentioned (225, 234, 235). The actions of Ang-(1-7) further oppose those mediated by Ang II to inhibit cell proliferation, fibrosis, and angiogenesis in cancer. *In vitro*, Ang-(1-7) reduced the growth of lung cancer cells by inhibiting DNA replication while also inhibiting activation of the proliferative mediator participating in MAPK signaling, ERK1/2 (326). Ang-(1-7) inhibits the migration of serum-stimulated human A549 lung cancer cells *in vitro* through reductions in activated Akt and ERK (p-Akt and p-ERK1/2, respectively) proliferative signaling (285). Ang-(1-7) treatment of lung tumor xenografts in nude mice prevented tumor growth as supported by immunohistochemical evidence of reduced Ki67 immunoreactivity as a marker of cell proliferation in Ang-(1-7)-treated tumors as well as reductions in the pro-inflammatory tumor growth-promoting prostaglandin cyclooxygenase-2 (COX-2) (236). Ang-(1-7) upregulated DUSP1 to inhibit MAPK activation and signaling in ER-positive and HER2-amplified breast tumors as a means of reducing tumor growth in the mammary fat pad of mice (233). In SCID mice pre-treated with Ang-(1-7), metastatic prostate tumor formation in the bone was completely absent in comparison to control animals (286). Osteoclastogenesis in bone marrow cells from this study was also significantly reduced in the presence of Ang-(1-7), demonstrating the potent inhibitory effect of the heptapeptide hormone on the formation of bone metastases. Furthermore, human A549 lung cancer cells stably overexpressing angiotensin-converting enzyme 2 (ACE2), the enzyme responsible for the degradation of Ang II to generate Ang-(1-7), demonstrated reduced metastatic tumor formation in the livers of mice (287). This anti-metastatic effect is likely due to the increased production
of Ang-(1-7) in these cells, providing evidence supporting the potential control of metastatic tumor formation elicited by Ang-(1-7).

Expression of the mas receptor on endothelial cells and VSMCs shows that Ang-(1-7) may also be involved in regulating angiogenesis (288). Lung tumor xenografts implanted in mice treated with Ang-(1-7) had a reduced number of blood vessels shown by a decrease CD34 immunoreactivity of endothelial cells lining blood vessels as well as a downregulation in protein and mRNA levels for VEGF (289). Ang-(1-7) also reduced endothelial tubule formation in vitro following treatment with the heptapeptide hormone, indicative of reductions in blood vessel formation (289). Additionally, in a chick chorioallantoic membrane (CAM) assay which mimics neovascularization, Ang-(1-7) treatment produces a 50% reduction in vessel formation compared with untreated controls (289).

Ang-(1-7) also mediated reductions in interstitial and perivascular fibrosis in ER-positive and HER2-amplified breast tumors with concomitant reductions in tumor-associated fibroblast proliferation, TGF-β1, fibronectin and p-ERK1/2 in tumoral fibroblasts (233). In addition, DUSP1 was increased in tumor-associated fibroblasts treated with Ang-(1-7), further supporting the role for the heptapeptide hormone as a negative regulator of MAPK signaling. These studies provide evidence for the anti-fibrotic functions of Ang-(1-7) in cancer, where the heptapeptide hormone acts specifically on the molecular mediators and ECM proteins implicated in tumor fibrosis and fibroblast cross-talk signaling, widening the therapeutic potential of Ang-(1-7).
In a Phase I Clinical Trial conducted at the Wake Forest Comprehensive Cancer Center, patients with non-resectable solid tumors were treated with Ang-(1-7) to evaluate the safety and potential effectiveness of the heptapeptide hormone (290). Out of 15 evaluable patients, clinical benefit was observed in 4 patients. Of those 4 patients, 3 were reported as having stable disease persisting for longer than 3 months and 1 patient experienced a 19% reduction in tumor size and continued on therapy for 10 months. Clinical benefit was also associated with significant reductions in plasma placental growth factor (PlGF), a pro-angiogenic molecule responsible for the promotion of tumor blood vessel growth; however, this effect was not seen in patients without clinical benefit. The Ang-(1-7)-associated decrease in PlGF levels in patients supports the role for the heptapeptide hormone as an anti-angiogenic agent, wherein its anti-tumor effects in patients are mediated, at least in part, through effects on angiogenesis. Only three serious adverse events (SAEs) possibly related to Ang-(1-7) treatment were reported in this study. One patient experienced calf pain relating to deep-vein thrombosis, strokes were observed in one patient which were not greatly improved upon removal of the treatment, and one patient experienced cranial neuropathy. These SAEs cannot definitively be attributed to the Ang-(1-7) treatment, considering the advanced stage of cancer in the patients and the chemotherapeutic treatment regimens administered prior to the start of the clinical trial which may have elicited damaging effects.

Of the 4 patients who experienced stable disease, 2 were sarcoma patients, thus providing the basis for investigating the therapeutic potential of Ang-(1-7) in sarcoma
patients in a Phase II Clinical Trial. This trial is currently ongoing in the Wake Forest University Comprehensive Cancer Center. Despite the clinical strides made by Ang-(1-7) in the treatment of cancer, there were no breast cancer patients enrolled in the Phase I study, therefore the experimental findings reported in this dissertation will potentially provide the basis for conducting a Phase I Clinical Trial for Ang-(1-7) in the treatment of breast cancer, particularly patients who have the triple negative subtype of breast cancer.

5. Hypothesis and Specific Aims

Based on the unique signaling pathways mediating TNBC growth and metastasis and the potent anti-proliferative and anti-fibrotic effects of Ang-(1-7), we hypothesized that the heptapeptide hormone would inhibit the growth of metastatic TNB tumors through regulation of Akt and TGF-β signaling. In cardiovascular studies as well as models of breast cancer of the non-triple negative subtype, Ang-(1-7) reduces MAPK signaling through decreasing activated ERK1/2 (p-ERK1/2) (233, 251, 255). These findings were associated with a significant upregulation in the MAPK phosphatase, DUSP1, resulting in attenuation of cell growth. In parallel studies in models of hypertension, Ang-(1-7) treatment increased a diverse array of other regulatory phosphatases, including PTEN (291), PTP-1b (292), and SHP-1 (293, 294). Therefore, we postulated that Ang-(1-7) may serve as a universal regulator of phosphatases, given the potent attenuating effect on kinase activity induced by the heptapeptide hormone. Previous studies conducted in our laboratory demonstrated inhibition of tumor growth following Ang-(1-7) treatment of mice with lung (236, 289), prostate (286, 295), or breast tumors (233) which correlated with reductions in cell growth and proliferation.
Thus, in Chapter 2, we hypothesized that Ang-(1-7) prevents the growth of human and murine-derived TNBC cells and tumors through reductions in phosphorylated Akt mediated by upregulation of the Akt phosphatase, PP2A.

Ang-(1-7) negatively regulated fibrosis in both cardiovascular and breast cancer studies (233, 251, 255). The heptapeptide hormone reduced the growth of orthotopic HER2-amplified and ER-positive breast tumors in mice with associated reductions in the growth of isolated CAFs. Furthermore, Ang-(1-7) treatment produced a reduction in TGF-β and fibronectin protein in CAFs. However, no studies have addressed the effects of Ang-(1-7) in metastatic breast cancer. In Chapter 3, we hypothesized that Ang-(1-7) would inhibit or reduce the growth of metastatic TNB tumors in the lungs of mice through reductions in TGF-β and p-Smad2/3 activity to prevent the downstream production of the breast tumor-promoting ECM proteins, Tenascin C and CTGF.

The use of Ang-(1-7) for the treatment of triple negative breast cancer would fill a void in currently available treatment options for TNBC patients, due to its wide-ranging effects on the growth of tumors. One major flaw with the therapies that are currently available is their inhibition of a sole molecular target, leading to inevitable upregulation of compensatory growth-promoting molecular mechanisms to overcome drug effects. Ang-(1-7), however, attenuates activation of numerous diverse signaling mediators, thereby effectively preventing the tumoral shift to alternative mechanisms of growth and avoiding drug resistance issues. Additionally, TNBC patients are primarily subject to cytotoxic chemotherapeutics contributing to poor quality of life. Ang-(1-7), on the other
hand, does not produce side effects or wound-healing problems (290, 296, 297). The benefit of Ang-(1-7) derives from its ability to reduce mitogenic signaling to basal levels without completely ablating the activity. Thus, this mechanism of action permits the normal cellular function of mitogen signaling that cells depend upon for viability. Therefore, Ang-(1-7) has the potential to serve as a first-in-class therapeutic for the treatment of TNBC and metastatic TNBC.
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Chapter II:

Angiotensin-(1-7) Attenuates Triple Negative Breast Cancer Growth Associated with a Reduction in Activated Akt

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Abstract

Triple negative breast cancer (TNBC) is an aggressive form of breast cancer which lacks expression of estrogen and progesterone receptors and expresses normal levels of human epidermal growth factor receptor (HER) 2, contributing to a lack of available targeted therapies for TNBC patients. TNBC cells are characterized by elevated Akt signaling, which promotes tumor cell proliferation and survival, and is activated by phosphorylation at threonine 308 (Thr308) and serine 473 (Ser473). Angiotensin-(1-7) [Ang-(1-7)], an endogenous, seven amino acid hormone of the renin-angiotensin system with antiproliferative properties, inhibits the growth of orthotopic TNB tumors growing in female nude mice. Mice with orthotopic human MDA-MB-231 or murine 4T1 TNB tumors were administered saline or Ang-(1-7) and phospho-Akt was measured, to determine whether the heptapeptide hormone regulated Akt phosphorylation. Ang-(1-7) inhibited activation of Akt by reducing phosphorylation at both the Thr308 and Ser473 sites in MDA-MB-231 human TNB tumors and cells as well as in the triple negative 4T1 mouse mammary carcinoma tumors and cell line. Protein phosphatase 2A (PP2A), which inactivates Akt through dephosphorylation of both Thr308 and Ser473, was significantly upregulated in MDA-MB-231 and 4T1 tumors and cells following treatment with Ang-(1-7). These results demonstrate that Ang-(1-7) reduces phospho-Akt and increases PP2A in TNBC cells, suggesting a novel molecular mechanism for inhibition of TNBC growth and progression and supporting a role for Ang-(1-7) as a novel therapeutic for the treatment of TNBC.
Introduction

Triple negative breast cancer (TNBC) comprises 10-15% of all breast tumor types, disproportionately affecting young, premenopausal women as well as African-American and Hispanic women. Breast tumors of the triple negative subtype are particularly aggressive and invasive and do not express estrogen receptors and progesterone receptors or overexpress human epidermal growth factor receptor (HER) 2 protein, contributing to the severe lack of targeted therapies and high morbidity rates for these patients (1, 2). Cytotoxic chemotherapies resulting in poor quality of life remain the standard of care for TNBC patients, demonstrating the necessity for developing novel therapeutics targeted against molecules specific to the growth-promoting events in TNBC.

Breast tumors exhibit aberrant activation of growth-promoting signaling pathways, contributing to increased cell proliferation and tumor size. Growth factors, mitogens, cytokines and hormones initiate molecular signaling mechanisms to promote blockade of cell cycle arrest checkpoints, degradation of tumor suppressors as well as increased transcription of anti-apoptotic genes and growth factor proteins. The phosphatidylinositol 3’-kinase (PI3K)-Akt/Protein Kinase B signaling pathway is upregulated in TNB tumors (3) and loss of PTEN (phosphatase and tensin homolog), the phosphatase which prevents PI3K signaling, is frequently reported (4, 5). The PI3K-Akt signaling pathway is regulated by several different cellular effectors to alter downstream mediators such as glycogen synthase kinase-3 (GSK-3), cyclin-dependent kinase inhibitors p21/Waf1/Cip1, mammalian target of rapamycin (mTOR), Bad, and the
forkhead family of transcription factors, which affect cellular transcription, translation, proliferation and survival (6-12). Akt is activated by phosphoinositide-dependent kinases (PDKs) 1/2 at two main phosphorylation residues, threonine 308 and serine 473 (Thr308 and Ser473, respectively) (13). The mTOR complex 2 (mTORC2) also phosphorylates Akt at the Ser473 site under growth-stimulated conditions (14). Maximal activation of Akt requires phosphorylation at both the serine and threonine sites (15, 16). Inhibition of Akt phosphorylation prevents downstream Akt activity, reducing cell proliferation and tumor growth. TNB tumors often have increased Akt activity (3), suggesting that inhibition of the phosphorylation and activation of Akt may serve as a target for the treatment of TNBC patients.

Phosphatases are critical regulators of protein activation, serving as modulators of protein kinases to either prevent or increase activation and subsequent signaling processes. Akt undergoes inactivation by the serine-threonine phosphatase, protein phosphatase 2A (PP2A) (17, 18), which is ubiquitously expressed and accounts for as much as 1% of total cellular protein (19). PP2A acts as a tumor suppressor by mediating inactivation of several oncogenic signaling mechanisms (19-21), and mutations in PP2A are found in human breast and lung cancers (20, 22), indicating PP2A dysregulation serves as a contributing factor to tumor growth. PP2A is a heterotrimeric molecule, consisting of scaffolding (A), regulatory (B), and catalytic (C) subunits, with the C subunit responsible for the dephosphorylation of protein substrates. Activated CIP2A (cancerous inhibitor of PP2A) as well as phosphorylation of PP2A at the Tyr307 site by GSK-3β (glycogen synthase kinase-3beta) inactivate the phosphatase (23) in response to
growth factor stimulation, which is required for the initiation and progression of many extracellular signal-regulated cascades (19, 21). Investigation of the mechanisms by which PP2A function can be restored in cancerous tissue is a promising area for the development of novel cancer therapeutics.

Angiotensin-(1-7) [Ang-(1-7)] is an endogenous, seven amino acid peptide hormone of the renin-angiotensin system exhibiting anti-proliferative properties in vascular smooth muscle cells and cardiomyocytes as well as in lung, prostate, and breast cancer cells and tumors (24-30). Ang-(1-7) increased the phosphatase activity of Src homology 2-containing protein-tyrosine phosphatase-1 (SHP-1) to prevent p38 signaling in renal proximal tubule cells and blocked the angiotensin II (Ang II)-mediated reduction in angiotensin-converting enzyme (ACE) 2 mRNA in the presence of phosphatase inhibitors (31, 32). We showed that Ang-(1-7) upregulated the mitogen-activated protein kinase (MAPK) phosphatase DUSP1 (dual-specificity phosphatase 1) in breast tumors to prevent downstream proliferative signaling with associated reductions in phosphorylated MAPKs (24). This suggests that Ang-(1-7) may inhibit tumor cell proliferation and growth through the regulation of phosphatases which dephosphorylate and inactivate protein kinases which are critical for cell growth. In this study, we determined whether Ang-(1-7) regulates the PI3K-Akt signaling pathway, to serve as a novel anti-proliferative agent for the treatment of TNBC.

Materials and Methods

Materials
Ang-(1-7) was purchased from Bachem and DMEM, RPMI, fetal bovine serum (FBS) were obtained from Life Technologies, Invitrogen. Antibodies were purchased from the indicated companies: phosphorylated-Akt [p-Akt(Ser473/Thr308)], Akt, PP2A-A and PP2A-C (Cell Signaling), Ki67 (Thermo Scientific) beta(β)-actin (Sigma) or alpha(α)-tubulin (Cell Signaling) and polyclonal and horseradish peroxidase (HRP)–conjugated secondary antibodies (GE Healthcare).

**Cell culture**

MDA-MB-231 human breast adenocarcinoma cells [American Type Culture Collection (ATCC) HTB-26], derived from a 51-year-old Caucasian female, and 4T1 mouse mammary gland tumor cells (ATCC CRL-2539) derived from a spontaneously-arising breast tumor in a female Balb/c mouse, were grown in either DMEM or RPMI respectively, containing 10% FBS and 10 nmol/L HEPES. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂/95% room air.

**Orthotopic tumor model**

MDA-MB-231 cells (2 x 10⁶/100 µl PBS) or 4T1 cells (2.5 x 10⁵/100 µl PBS) were injected into the mammary fat pad of 6-week-old female athymic nude mice and Balb/c mice, respectively. Tumors were measured twice weekly using a caliper and tumor volumes were determined using the formula [(4/3πr³)/2]. Once tumors reached a volume of 100 mm³ (MDA-MB-231) and 50 mm³ (4T1), animals were treated with saline or 1000 µg/kg Ang-(1-7) injected twice daily subcutaneously (s.c.) for 27 days in mice bearing MDA-MB-231 tumors and 24 µg/kg/h Ang-(1-7) delivered via osmotic mini-pump (s.c.,
Alzet) for 18 days in mice bearing 4T1 tumors. The mice were euthanized and tumors were harvested for experimental analysis. All animal protocols were approved by the Wake Forest School of Medicine Animal Care and Use Committee.

**Western blot hybridization**

Tumor tissue was solubilized as previously described (33) and cell monolayers were solubilized using Triton lysis buffer (29); protein content was determined using a modified Lowry method (34). Proteins were separated by polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) or nitrocellulose membranes. Incubation of membranes with Blotto (TBS with 5% powdered milk and 0.1% Tween 20) blocked non-specific binding. The membranes were incubated with primary antibodies specific to Akt (1:5,000), p-Akt(Thr308) (1:2000), p-Akt(Ser473) (1:2,000), PP2A-A (1:2,000) and PP2A-C (1:5,000) overnight at 4°C, followed by a 1-h incubation at room temperature with polyclonal HRP-conjugated secondary antibodies (1:2,000). Immunoreactive bands were visualized by chemiluminescence (SuperSignal Femto or Pico West, Pierce Technology), and band densitometry was quantified by densitometry using MCID digital densitometry software (Cambridge, UK). A monoclonal antibody to beta(β)-actin or alpha(α)-tubulin (1:5,000) was used as a protein loading control.

**Immunohistochemistry**

One half of each tumor was fixed in 4% paraformaldehyde for 24-48 h and stored in 70% ethanol until embedded in paraffin and sectioned at five microns. Tissues were
deparaffinized in xylene and hydrated in graded alcohols before antigen retrieval in citrate buffer for 40 minutes. After rinsing in deionized water, tumor sections were placed in Tris buffer and incubated with antibodies against Ki67, PP2A-A and -C (1:100) overnight in 4°C. Tumor sections were then washed in Tris buffer and probed with a biotinylated secondary antibody in Tris buffer for 20 minutes at 37°C followed by incubation with the streptavidin-alkaline phosphatase enzyme conjugate at 37°C for 20 minutes. Next, tumor sections were washed in Tris buffer and incubated with the enzyme substrate Vector Red for 5 minutes in Automation buffer. Tissues were washed in deionized water, counterstained in Mayer’s hematoxylin for 5 minutes, dehydrated in graded alcohols and cleared with Histoclear. Stained tumor sections were covered using a coverslip and permount. Using a Leica DM 4000 microscope, pictures were taken of four representative regions per tumor for every animal and the average percentage of positively-stained cells were quantified using ImageJ software (NIH).

Statistics
All data are presented as the mean ± SE. Statistical differences were evaluated by Student's t test or one-way ANOVA followed by Dunnett's post hoc test. The criterion for statistical significance was set at $P < 0.05$.

Results
Reduction of TNB tumor growth by Ang-(1-7)

Athymic mice bearing orthotopic MDA-MB-231 tumors of approximately 100 mm$^3$ in size were placed into two groups at random and administered saline or Ang-(1-7)
(saline, 111.5 ± 6.1 mm$^3$; Ang-(1-7), 110.2 ± 6.6 mm$^3$, n = 5) to determine whether the heptapeptide hormone attenuates TNB tumor growth. As shown in Figure 1A, tumor volume increased throughout the treatment period in mice receiving saline, while tumor growth in animals administered Ang-(1-7) was markedly attenuated. The percent change in volume of tumors from mice treated with saline increased 238.6 ± 39.5% from day 0 (n = 5, over the 27 day treatment period). In contrast, the tumors of mice treated with Ang-(1-7) only increased 45.8 ± 20.0% (n = 5), a reduction of greater than 192% when compared to tumors from saline-treated mice. Subcutaneous (s.c.) injection of 1000 µg Ang-(1-7)/kg twice daily was well tolerated by mice with no change in body weight, water consumption or reduced motor function. After 27 days of treatment, the mice were sacrificed and no obvious pathologic abnormalities were observed in major organs, indicating a lack of toxic side effects at the dose given.

In a similar study, female Balb/c mice were injected subcutaneously in the mammary fat pad with 4T1 TNBC cells. When xenograft tumors reached approximately 50 mm$^3$ in volume, the mice were randomized for subcutaneous infusion with either saline or 24 µg/kg Ang-(1-7) for a period of 18 days (saline, 57.3 ± 3.1 mm$^3$; Ang-(1-7), 55.0 ± 5.6 mm$^3$). Similar to the previous study with the human MDA-MB-231 xenografts, the 4T1 tumor volume of saline-infused mice increased, while the tumor volume of Ang-(1-7) administered animals was significantly attenuated during the treatment period (Figure 1B). Treatment with the heptapeptide hormone resulted in a marked reduction in average tumor volume at the end of the 18-day period (saline, 1679 ± 323 mm$^3$ versus Ang-(1-7), 900 ± 77 mm$^3$; p < 0.05, n = 5).
In order to determine whether Ang-(1-7) inhibits the growth of orthotopic TNB tumors in mice through anti-proliferative mechanisms, tumors were sectioned and probed with an antibody against the proliferative marker, Ki67. Ang-(1-7) significantly decreased the number of Ki67-positive cells by 51.3% and 82.6% in the human MDA-MB-231 and murine 4T1 TNB tumors, respectively (Figure 1C and 1D), suggesting that the heptapeptide regulates cell proliferation to mediate inhibition of tumor growth in vivo.

Inhibition of Akt activation by Ang-(1-7) in vivo

Phosphorylation of Akt was assessed in human MDA-MB-231 tumors harvested from athymic nude mice treated with Ang-(1-7) to determine whether the heptapeptide hormone reduced Akt activity in vivo. Western blot hybridization of MDA-MB-231 tumor homogenates demonstrated that Ang-(1-7) had no effect on total TNB tumor xenograft Akt compared with saline-treated controls (Figure 2A). However, the heptapeptide hormone significantly decreased p-Akt(Thr308) by 66.2% and p-Akt(Ser473) by 51.2% in tumor tissue (Figure 2A), suggesting that Ang-(1-7) reduces maximal activation of Akt by inhibiting phosphorylation at both residues. Akt activation was also assessed in orthotopic 4T1 tumors harvested from Balb/c mice; tumors from Ang-(1-7)-treated animals had a significant reduction in Akt phosphorylation at both the p-Akt(Ser473) and p-Akt(Thr308) residues, by 54.7% and 46.7%, respectively (Figure 2B and 2C). There was no difference in total Akt levels between treatment groups in the 4T1 tumors (Figure 2D), similar to the MDA-MB-231 tumors, indicating that the Ang-(1-7)-mediated reduction in p-Akt was not due to a decrease in total Akt protein.
Inhibition of Akt activation by Ang-(1-7) in vitro

Phosphorylation of Akt at both the threonine 308 and serine 473 residues confers maximal activation of Akt, increasing cell proliferation, growth, and survival. P-Akt(Thr308) and p-Akt(Ser473) were measured by Western blot hybridization in protein extracts from human MDA-MB-231 cell lysates treated with 100 nM Ang-(1-7), to determine whether the heptapeptide hormone inhibits tumor growth by preventing activation of Akt. Ang-(1-7) significantly reduced p-Akt(Ser473) by 64.6% and p-Akt(Thr308) by 44.9% after 4 h of treatment compared with controls (Figure 3A and 3B). The effect of Ang-(1-7) on Akt activation was also assessed in the parent 4T1 cell line treated with Ang-(1-7). After 4 h of incubation with Ang-(1-7), the heptapeptide hormone significantly reduced p-Akt(Ser473) by 95.1% and p-Akt(Thr308) by 84.1% compared to controls (Figure 3D and 3E), consistent with studies performed in the human MDA-MB-231 TNBC cell line. Total Akt remained unaltered in both cell lines following Ang-(1-7) treatment (Figure 3C and 3F).

Ang-(1-7) upregulates PP2A to reduce Akt activation in vitro and in vivo

Phospho-Akt is dephosphorylated and inactivated by the protein phosphatase PP2A. The scaffolding (A) and catalytic (C) subunits of PP2A were measured by Western blot hybridization in both human and murine TNBC cells and tumors, to determine whether expression of one of the PP2A subunits is regulated by Ang-(1-7) treatment. Both the A and C subunits of PP2A were significantly increased by 63.9% and 79%, respectively, in MDA-MB-231 tumors from mice administered Ang-(1-7)
compared to mice treated with saline (Figure 4A and 4B). Ang-(1-7) treatment also increased PP2A-A and PP2A-C in orthotopic murine 4T1 tumors by 43.5% and 47.3%, respectively (Figure 4C and 4D). Immunohistochemical analysis of 4T1 tumor sections from mice treated with saline or Ang-(1-7) also showed that the heptapeptide hormone significantly increased PP2A-A by 97.1% and increased PP2A-C by 95.5% (Figure 4E), with nearly undetectable PP2A immunoreactivity in tumors from untreated mice.

In MDA-MB-231 cells, PP2A-A was significantly upregulated by 62.5% and PP2A-C by 49.6% as early as 2 h after the addition of Ang-(1-7) compared to untreated controls (Figure 5A and 5B). The heptapeptide hormone also increased PP2A-A in the 4T1 cells by 69.5% and upregulated PP2A-C by 49.4% following 2 h of Ang-(1-7) treatment compared to control cells (Figure 5C and 5D), supporting the regulatory effects of Ang-(1-7) on the phosphatase both in vivo and in vitro.

Discussion

Multiple signaling pathways stimulated by both growth factors and survival factors contribute to tumor cell proliferation and progression in breast cancer. Akt signaling is frequently deregulated in TNBC, with p-Akt serving as a tumor biomarker for poor patient prognosis (3, 35, 36). In addition, the phosphorylated, maximally-active form of Akt is commonly overexpressed in invasive types of breast cancer (37). Regulation of phosphatase activities to reduce overexpressed or deregulated protein kinases thus represents a promising target to control mitogenic signaling in cancer.
The current study presents novel findings suggesting that the heptapeptide hormone Ang-(1-7) reduces the growth of TNBC cells and tumors in mice by reducing the phosphorylation of Akt. Human MDA-MB-231 and mouse 4T1 TNB tumors growing orthotopically in mice treated with Ang-(1-7) showed reduced tumor volume compared to saline-treated tumors, with an associated decrease in phosphorylated Akt at both of its primary phosphorylation residues, threonine 308 and serine 473, with no changes in total Akt. This suggests that the heptapeptide hormone inhibits maximal activation of Akt to prevent downstream molecular signaling which promotes tumor cell proliferation and growth. Ang-(1-7) similarly reduced p-Akt at both phosphorylation residues in human MDA-MB-231 and mouse-derived 4T1 TNBC cells in culture, in agreement with the in vivo results in Ang-(1-7)-treated mice.

Over one-third of all cellular proteins are phosphorylated, a process that is tightly regulated by protein phosphatases to prevent over-activation of molecular signaling pathways. However, cancer cells frequently overcome phosphatase regulation by increased expression of the activated or phosphorylated form of the protein kinase (38) or by mutations in phosphatases to reduce their ability to dephosphorylate and inactivate protein kinases (22, 39). Thus, targeting phosphatases to increase their activities may prove to be a novel strategy for cancer treatment. Ang-(1-7) increased the MAPK phosphatase DUSP1 in breast cancer-associated fibroblasts (CAFs) to reduce activation of the proliferative protein kinases ERK1/2 (extracellular signal-regulated kinases 1/2), producing decreased CAF and tumor growth (24). The heptapeptide hormone also increased SHP-1 activity in renal proximal tubule cells and SHP-2 activity in human
endothelial cells to prevent p38 MAPK and Src signaling, respectively (32, 40). Cardiovascular studies showed that Ang-(1-7) treatment of hypertensive rats increased the MAPK phosphatase DUSP1 and reduced phospho-ERK1/2 in cardiac tissues and DUSP1 was similarly increased and phospho-ERK1/2 decreased in isolated cardiac fibroblasts (41, 42). The heptapeptide hormone also upregulated DUSP1 as well as phospho-tyrosine protein phosphatase-1b (PTP1b) in the medulla oblongata of hypertensive rats to counteract MAPK and PI3K signaling (43). Our studies are the first to demonstrate the regulatory role of Ang-(1-7) in the signaling of PI3K-Akt in a TNBC model, through upregulation of the Akt phosphatase PP2A; in both human MDA-MB-231 and murine 4T1 TNB tumors and cells, Ang-(1-7) produced a significant increase in both the PP2A-A and -C subunits. This suggests that the inhibition of phosphorylation and activation of Akt by Ang-(1-7) may be mediated by upregulation of the Akt phosphatase PP2A.

Formation of the PP2A enzyme complex results from the association of three subunits -- A, B, and C -- a process in which the scaffolding A subunit initially forms a conjugate with the catalytic C subunit to generate the PP2A holoenzyme (44). One of the many isoforms of the regulatory B subunit is then recruited to the holoenzyme to ultimately form the PP2A phosphatase. Our findings demonstrate that Ang-(1-7) significantly upregulates both the A and C subunits of PP2A, suggesting that Ang-(1-7) may be involved in facilitating the prerequisite recruitment phase of PP2A upregulation, where the scaffolding A subunit forms a holoenzyme with the catalytic C subunit. This
suggests that Ang-(1-7) enhances the initial formation of the PP2A-C holoenzyme rather than solely increasing the phosphatase activity of PP2A.

Ang-(1-7) upregulates the activity of a number of protein phosphatases including DUSP1, SHP-1, SHP-2 and PTP1B (32, 40, 43) as well as PP2A. This suggests that the heptapeptide hormone may act as a universal regulator of protein phosphatases, by increasing activity of common regulatory elements for phosphatase promoters such as AP-1, CREB and Stat or facilitating binding of transcription factors to these promoter sites to ultimately increase the expression and activity of a variety of critical phosphatases including PP2A. In support of this hypothesis, Ang-(1-7) signaling results in increased cyclic adenosine monophosphate (cAMP) in vascular smooth muscle cells (26), and cAMP binds to a cAMP response element (CRE) in the promoter region of the DUSP1 gene to increase transcription of the MAPK phosphatase (45, 46) and inhibit ERK activity and cell growth (47). This suggests that Ang-(1-7) signaling may transcriptionally regulate PP2A as well as other phosphatases, to control expression, and that this process may be mediated through cAMP second-messenger signaling.

Unlike standard anti-angiogenic, anti-fibrotic and anti-proliferative therapeutics which target only a single molecule or a single process implicated in tumor growth, Ang-(1-7) reduces the effects of multiple growth factors and the downstream proliferative molecules that they regulate. The Ang-(1-7)-induced targeting of numerous signaling pathways which promote tumor growth and survival may reduce the evasive efforts of tumors to utilize alternative signaling mechanisms to overcome the growth-inhibitory
effects of the heptapeptide hormone, suggesting that tumors may be less effective in overcoming the growth inhibition by Ang-(1-7).

We showed that Ang-(1-7) increases PP2A in TNBC tumors and cells and increases DUSP1 in cancer-associated fibroblasts (24). However, phosphorylation of the kinases that are regulated by PP2A in TNBC cells (Akt) and DUSP1 in cancer-associated fibroblasts (the MAPKs, ERK1 and ERK2) is not completely ablated by the increase in phosphatase activities, but is significantly reduced. Since Akt and ERK1/2 are involved in multiple signaling pathways in both malignant and non-malignant cells, the residual protein kinase activities may allow the cells to continue to activate these pathways for normal cell function and account for the lack of significant side effects in animals or patients treated with the heptapeptide hormone (48-50).

In conclusion, we showed that Ang-(1-7) inhibits the growth of orthotopic TNB tumors of either human origin, by injection of human MDA-MB-231 into athymic mice, or of mouse origin, by injection of mouse 4T1 cells into Balb/c mice. The Ang-(1-7)- mediated inhibition of tumor growth was associated with an increase in both the structural and catalytic subunit of PP2A, an Akt phosphatase, and a decrease in the phosphorylation of Akt at both Ser473 and Thr308. A Phase I clinical trial conducted in the Wake Forest University Comprehensive Cancer Center demonstrated that, in cancer patients administered Ang-(1-7) for 5 consecutive days over 3-week cycles, 4 out of 18 evaluable patients showed clinical benefit with associated reductions in the pro-angiogenic molecule placental growth factor (PIGF) in plasma (48). Out of those 4
patients, one patient suffering from a sarcoma was continued on Ang-(1-7) treatment for 14 cycles and showed minor response to the therapy, which provided the basis for a Phase II clinical trial for Ang-(1-7) in sarcoma patients, which is currently ongoing. Our results showing that Ang-(1-7) inhibits the growth of TNBC provides the preclinical support for a clinical trial in TNBC patients treated with the heptapeptide hormone, to determine whether Ang-(1-7) may serve as a novel targeted therapeutic for TNBC patients.
Conflict of Interest

EAT and PEG hold a patent for the treatment of cancer with Ang-(1-7). The other authors disclose that they have no conflicts of interest.

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Figure Legends

Figure 1. **Ang-(1-7) inhibits the growth of orthotopic MDA-MB-231 and 4T1 tumors.** A.) Volumes of human MDA-MB-231 TNB tumors growing in the mammary fat pads of 6-week-old female, athymic nude mice treated with saline or 1000 µg/kg Ang-(1-7) injected twice daily for 27 days once tumors reached a volume of 100 mm³. Tumor wet weight was measured upon animal sacrifice; * p < 0.05, n = 4-5. Representative tumor pictures are shown. B.) Volumes of murine 4T1 TNB tumors growing in the mammary fat pads of 6-week-old female Balb/c mice treated with 24 µg/kg/h Ang-(1-7) delivered via osmotic mini-pump for 18 days once tumors reached a volume of 50 mm³. Tumor wet weight was determined upon animal sacrifice; * p < 0.05, ** p < 0.01, n = 6-7. Representative tumor pictures are shown. C.) Ki67 immunoreactivity in MDA-MB-231 orthotopic TNB tumors from mice treated with saline or 1000 µg/kg Ang-(1-7) was measured by immunohistochemistry. D.) Ki67 immunoreactivity in 4T1 orthotopic TNB tumors from mice treated with saline or 24 µg/kg/h Ang-(1-7) was measured by immunohistochemistry; * denotes p < 0.05, **denotes p < 0.01, n = 5. Representative pictures are at 20X magnification and scale bars are 10 mm.

Figure 2. **Ang-(1-7) reduces Akt phosphorylation in orthotopic MDA-MB-231 and 4T1 tumors from mice.** A.) Akt, p-Akt(Ser473) and p-Akt(Thr308) immunoreactivity in MDA-MB-231 orthotopic tumors from mice treated with saline or 1000 µg/kg Ang-(1-7) was measured by Western blot hybridization, * p < 0.05 (in triplicate), ** p < 0.03, n = 5. B.) p-Akt(Ser473), C.) p-Akt(Thr308), and D.) Akt immunoreactivity in 4T1 orthotopic tumors from mice treated with saline or 24 µg/kg/h Ang-(1-7) was measured
by Western blot hybridization, * denotes p < 0.05, **denotes p < 0.01, n = 5. Representative gel images are shown.

**Figure 3. Phosphorylation of Akt is inhibited by Ang-(1-7) in MDA-MB-231 and 4T1 cells.** MDA-MB-231 cells were treated with 100 nM Ang-(1-7), harvested at 0, 2 and 4 h and immunoreactivity was measured by Western blot hybridization for A.) p-Akt(Ser473), B.) p-Akt(Thr308), and C.) Akt; * denotes p < 0.05, ** denotes p < 0.01, n = 3-4. 4T1 cells were treated with 100 nM Ang-(1-7), harvested at 0, 2, and 4 h and immunoreactivity was measured by Western blot hybridization for D.) p-Akt(Ser473), E.) p-Akt(Thr308) and F.) Akt; ** denotes p < 0.01, *** denotes p < 0.001, Ψ denotes p < 0.0001, n = 3-4. Representative gel images are shown.

**Figure 4. Ang-(1-7) increases PP2A-A and PP2A-C protein in orthotopic MDA-MB-231 and 4T1 tumors from mice.** A.) PP2A-A and B.) PP2A-C immunoreactivity in MDA-MB-231 orthotopic tumors from mice treated with saline or 1000 µg/kg Ang-(1-7) was measured by Western blot hybridization; * denotes p < 0.05, n = 5. C.) PP2A-A and D.) PP2A-C immunoreactivity in 4T1 orthotopic tumors from mice treated with saline or 24 µg/kg/h Ang-(1-7) was measured by Western blot hybridization; **denotes p < 0.01, *** denotes p < 0.001, n = 5. Representative gel images are shown. E.) 4T1 orthotopic tumors from mice were treated with saline or 24 µg/kg/h Ang-(1-7) and immunohistochemistry was performed for PP2A-A and PP2A-C; * denotes p < 0.05, n = 5. Representative pictures shown at 20X magnification and scale bars are 10 mm.
Figure 5. PP2A-A and PP2A-C protein is increased in Ang-(1-7)-treated MDA-MB-231 and 4T1 cells. MDA-MB-231 cells were treated with 100 nM Ang-(1-7), harvested at 0, 2, and 4 h and measured for A.) PP2A-A and B.) PP2A-C immunoreactivity by Western blot hybridization, * denotes p < 0.05, ** denotes p < 0.01, n = 3-5. C.) PP2A-A, and D.) PP2A-C immunoreactivity in 4T1 cells treated with 100 nM Ang-(1-7) and harvested at 0, 2, and 4 h was measured by Western blot hybridization; * denotes p < 0.05, ** denotes p < 0.01, n = 4-5. Representative gel images are shown.
Figure 1.

A.

B.

C.

D.
Figure 2.

A. 

B. 

C. 

D.
Figure 3.

A.

B.

C.

D.

E.

F.
Figure 4.

A.  

B.  

C.  

D.  

E.  

Control  

Ang-(1-7)  

PP2A-A  

PP2A-C

PP2A-A

PP2A-C

Control  

Ang-(1-7)  

Actin  

Tubulin  

Control  

Ang-(1-7)  

Control  

Ang-(1-7)  

Control  

Ang-(1-7)  

PP2A-A Positive Cells (%)  

PP2A-C Positive Cells (%)
Figure 5.

A. PP2A-A

B. PP2A-C

C. PP2A-A

D. PP2A-C
Chapter III:

Angiotensin-(1-7) inhibits metastatic triple negative breast cancer growth in the lungs of mice associated with reductions in TGF-beta(β)-mediated extracellular matrix (ECM) protein production

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Abstract

Introduction: Triple negative breast cancer is a highly aggressive type of breast cancer which metastasizes to the lung, liver, bone and brain. Cancer-associated fibroblasts in the tumor microenvironment contribute to tumor growth by providing a collagenous tissue scaffold to support tumor stabilization and growth and secreting growth factors which increase cell proliferation. Angiotensin-(1-7) [Ang-(1-7)] is an anti-proliferative, anti-fibrotic peptide hormone which may provide targeted therapy for triple negative breast tumors.

Methods: The effect of Ang-(1-7) on the growth and production of fibrotic proteins [transforming growth factor-beta (TGF-β), phosphorylated Smad2 (p-Smad2), connective tissue growth factor (CTGF) and tenasin C] was measured in orthotopic mouse 4T1 tumors, in metastatic 4T1 tumors in mouse lungs and in cancer-associated fibroblasts isolated from 4T1 tumors.

Results: Ang-(1-7) significantly decreased 4T1 metastatic tumor growth in the lungs by decreasing tumor burden, via reductions in the number of metastatic tumors and their cross-sectional area. Ki67, a marker of tumor cell proliferation, was also significantly reduced by Ang-(1-7). The heptapeptide hormone also reduced the growth of cancer-associated fibroblasts isolated from 4T1 triple negative breast tumors, in associated with a reduction in TGF-β, p-Smad2, CTGF and tenasin C. Ang-(1-7) also decreased the profibrotic proteins TGF-β, p-Smad2, CTGF and tenasin C in both orthotopic 4T1 tumors as well as in metastatic 4T1 tumors in the lung.

Conclusions: These results suggest that Ang-(1-7) inhibits proliferation and fibrosis in triple negative breast tumors and their metastatic lesions in the lung, suggesting that the
The heptapeptide hormone may be a targeted therapeutic for the treatment of patients with both primary and metastatic triple negative breast cancer.
Introduction

Triple negative breast cancer (TNBC) is a highly aggressive and invasive type of breast cancer accounting for 10-15% of all breast cancers and disproportionately affecting pre-menopausal women, women of African-American and Hispanic descent, and those with mutations in the BRCA1/2 gene (1-3). Mortality rates are greater in TNBC patients compared to patients with other types of breast cancer, due to the higher mitotic index and histologic grade of their tumors as well as increases in mutations for the p53 tumor suppressor gene (4, 5). TNB tumors do not express receptors for estrogen or progesterone or over-express human epidermal growth factor receptor 2 (HER2), limiting treatments for these breast tumors to cytotoxic chemotherapies with adverse quality of life properties.

Approximately 90% of deaths in patients with solid tumors are due to tumor metastasis or the spread of cancer cells from their primary site of origin to a secondary site in the body, emphasizing a shift in clinical treatment from elimination of the primary tumor to improving the control of metastatic growth (7). Patients with TNBC preferentially develop metastatic tumors in their lungs, liver, brain, and bones and have a significantly shorter time to relapse or metastasis than those with other types of breast cancer (2, 8). The most likely time for development of metastasis in TNBC patients occurs within the first 5 years following the initial detection of the primary tumor, with an average time to development of 2.5 - 4.2 years compared with 5 - 6 years for non-TNBC subtypes of breast cancer (6, 9). In a study measuring the rate of TNBC metastasis in a cohort of breast cancer patients, 34% of TNBC patients relapsed to a distant site.
whereas only 20.4% of non-TNBC patients experienced distant metastasis (6). Once diagnosed with metastatic disease, survival in TNBC patients is limited to approximately 9 - 14 months, whereas survival time is extended to approximately 22 months for patients with other types of breast cancer (6, 10). Detection of metastatic tumor formation in TNBC patients is especially difficult due to the propensity of these tumors for hematogenous rather than lymphatic spread (11), thereby evading detection by monitoring of lymph nodes. The high risk of relapse and the absence of effective targeted therapeutics for the treatment of TNBC indicate an urgent need for the development of novel treatment strategies for these patients.

Metastatic tumor growth in secondary tissue sites is facilitated by cells in the tumor microenvironment, including cancer- or tumor-associated fibroblasts (CAFs or TAFs) (12, 13), which aid in the tumor desmoplasia formation that generally constitutes up to 80-90% of the breast tumor (14). These tumor-supportive stromal cells are activated by the pro-oncogenic cytokine, transforming growth factor-beta (TGF-β), leading to enhanced secretion of growth factors which perpetuate tumor cell proliferation and growth (12, 15). TGF-β is released by blood platelets, tumor cells, and various types of stromal cells and promotes the differentiation of mesenchymal precursors into fibroblasts (15). TGF-β also increases CAF production of ECM proteins including collagens, fibronectin, connective tissue growth factor (CTGF) and tenasin C, contributing to the development of a fibrotic tissue scaffold which acts as a supportive mesh for the growing metastatic tumor (12). This suggests a clear need for therapeutics that not only target infiltrating tumor cells but also activated fibroblasts to reduce breast cancer metastasis.
Angiotensin-(1-7) [Ang-(1-7)] is an endogenous, seven amino-acid peptide hormone of the renin-angiotensin system which binds to the unique G-protein-coupled AT$_{(1-7)}$ receptor mas to elicit anti-proliferative and anti-fibrotic effects in cancer (36-39). Ang-(1-7) inhibited the growth of non-triple negative BT-474 and ZR-75-1 orthotopic breast tumors in mice with associated reductions in CAF proliferation and CAF-specific extracellular signal-regulated kinase (ERK) 1/2 activities (36). Ang-(1-7) treatment also reduced the growth of cardiac fibroblasts isolated from neonatal rat hearts which was associated with an increase in the mitogen-activated protein kinase (MAPK) phosphatase, dual specificity phosphatase I (DUSP1), and a decrease in fibroblast-mediated collagen production (40), suggesting a role for the heptapeptide hormone in reducing pathological fibrosis. In a phase I clinical trial conducted at the Wake Forest Comprehensive Cancer Center, patients with solid tumors were treated with Ang-(1-7) by subcutaneous injection daily for five days over a three-week cycle (41). Four out of fifteen patients evaluated showed clinical benefit, demonstrated by a minor response (MR) or stable disease (SD) lasting longer than three months, suggesting that the heptapeptide hormone may be effective in the treatment of cancer. However, it is unknown whether Ang-(1-7) has an effect on the growth of tumors that have spread to distant sites in the body in TNBC, particularly with respect to the role of Ang-(1-7) in regulating tumor fibrosis. Thus, the aim of this study was to determine whether Ang-(1-7) inhibits the growth of TNB tumors that spontaneously metastasize to the lungs of mice and its role in regulating tumor fibrosis.
Material & Methods

Materials

The following materials were purchased from the indicated companies: Ang-(1-7) (Bachem); TGF-β (Calbiochem); DMEM/F12, RPMI, fetal bovine serum (FBS) (Life Technologies, Invitrogen); trypsin, collagenase, soybean trypsin inhibitor for fibroblast isolation (Worthington Biochemical); and hematoxylin and eosin for histochemical experiments (Newcomer Supply). Antibodies were purchased from the following companies: phosphorylated-Smad2 (p-Smad2, Millipore); TGF-β (Cell Signaling); TGF-β and CTGF (Abcam), tenascin C (Sigma); polyclonal and horseradish peroxidase (HRP)–conjugated secondary antibodies (GE Healthcare); beta (β)-actin (Sigma-Aldrich); alpha (α)-Tubulin (Cell Signaling); Ki67 (Thermo Scientific); alpha-smooth muscle actin (α-SMA), fibronectin, and vimentin (Sigma-Aldrich); mas receptor (Alomone); and secondary Alexa Fluor donkey anti-rabbit and anti-mouse antibodies (The Jackson Laboratory). 4′,6-diamidino-2-phenylindole (DAPI, Life Technologies) was used to stain nuclei.

Cell culture

4T1-Luc2 mouse mammary gland tumor cells (Caliper Life Sciences) derived from a spontaneously-arising breast tumor in a female Balb/c mouse were grown in RPMI containing 10% FBS, 100 μg/mL penicillin, 100 units/mL streptomycin, and 10 nmol/L HEPES. Isolated tumoral fibroblasts were grown in DMEM/F12 medium containing 10% FBS, 100 μg/mL penicillin, 100 units/mL streptomycin, and 10 nmol/L HEPES. Cells were grown at 37°C in a humidified atmosphere of 5% CO2/95% room air.
Isolation and Characterization of Cancer-Associated Fibroblasts (CAFs)

4T1-Luc2 cells (2.5x10^5/100 µl PBS/mouse) were injected orthotopically into the 4th inguinal mammary fat pad of 6-week-old female Balb/c mice. Tumors were measured three times weekly using a caliper to determine tumor volume calculated using the formula \( V = \frac{(4/3\pi r^3)}{2} \). Once tumors reached a volume of 100 mm³, the tumors were surgically resected, finely minced and digested with 50 µg/mL trypsin overnight at 4°C. Soybean trypsin inhibitor (100 µg/mL) was added to stop the reaction. Following centrifugation, the pellet was further digested with collagenase (85 units/mL) at 37°C for 1 h. Undigested tissue was removed with a cell strainer and fibroblast populations were isolated by differential plating as previously described (42), where fibroblasts adhere to the cell culture dish in 20 minutes and tumor cells are still in suspension. An average of 91.5% of the total amount of isolated cells which stain positively with markers associated with CAFs, as described below.

CAFs isolated from 4T1 tumors were plated in 8-well chamber slides in DMEM/F12 with 10% FBS. Cells at approximately 50% confluency were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, incubated overnight with antibodies to \( \alpha \)-SMA, fibronectin, vimentin, and \( mas \) (1:100) at 4°C in a humid chamber followed by a 1-h incubation with secondary Alexa Fluor donkey anti-rabbit or anti-mouse antibodies (1:200). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Stained cells were visualized with a Leica DM 4000 microscope (\( A_{\text{max}} = 493 \) nm Anti-Rabbit, \( A_{\text{max}} = 550 \) nm Anti-Mouse) and photographed with a QImaging Retiga 1300R camera.
4T1 CAFs were plated in 12-well plates (0.25 x 10^5 per plate) in DMEM/F12 with 1% FBS and stimulated with 1 ng/mL TGF-β in the presence and absence of 100 nM Ang-(1-7). Cell number was determined on days 0 and 7 using a hemocytometer.

**Western Blot Hybridization**

Cell monolayers (37) and tumor tissues (43) were solubilized as previously described. Protein content was determined using a modified Lowry method (44). Proteins separated by PAGE were transferred to polyvinylidene difluoride (PVDF) or nitrocellulose membranes and membranes were incubated with Blotto (Tris-buffered saline with 5% powdered milk and 0.1% Tween 20) to block non-specific binding. Membranes were incubated with primary antibodies to TGF-β (1:1,000), p-Smad2 (1:2,000), CTGF (1:5,000) or tenasin C (1:1,000) overnight at 4°C, followed by a 1 h incubation at room temperature with polyclonal HRP-conjugated secondary antibodies (1:2,000-5,000). A monoclonal antibody to β-actin or α-tubulin (1:5,000) was used as a protein loading control. Chemiluminescence was used to visualize immunoreactive bands (SuperSignal Femto or Pico West, Pierce Technology), followed by band densitometry quantification using MCID digital software.

**4T1 Orthotopic Mammary Carcinoma Model**

4T1-Luc2 cells (2.5x10^5/100 µL PBS/mouse) were injected into the 4th inguinal mammary fat pad of 6-week-old, female Balb/c mice and measured every other day with a caliper using the formula \( V = \frac{4}{3}(\pi r^3) \)/2 to calculate tumor volume. Tumors were
surgically removed once they reached a volume of 100 mm$^3$. One group of mice selected at random was treated with 24 µg/kg/h Ang-(1-7) by subcutaneous osmotic mini-pumps (Alzet) beginning one week after tumor removal, while another group served as controls. Animals were monitored for bioluminescence using Xenogen IVIS-100 imaging software, twice weekly during the treatment period. Anesthetized mice were euthanized by cervical dislocation 28 days after removal of the primary tumor or when the mice were distressed, whichever occurred first. Whole lungs were harvested and weighed prior to fixation. All procedures complied with the policies of the Wake Forest University Animal Care and Use Committee.

**Xenogen IVIS Animal Imaging**

Mice were injected intraperitoneally (i.p.) with 150 µl luciferin substrate and anesthetized with isoflurane 10 min after the injection, to allow metabolism of the substrate. Animals were placed flat on their backs in the chamber of the IVIS and imaged for times between 1 second and 5 minutes depending on the strength of the bioluminescence signal.

**Histochemistry**

Whole lungs and primary tumors were fixed in 4% paraformaldehyde for 24-48 h, stored in 70% ethanol until embedded in paraffin and serially-sectioned at five microns. Every 5$^{th}$ lung section up to 100 total sections was stained with hematoxylin and eosin (H&E) to determine the total number of tumors/section; tumor number was averaged from sections across the lung. The staining procedure was performed by first removing the paraffin by running tissues through xylene followed by hydrating the tissues in graded alcohols.
Tissues were incubated in Harris hematoxylin for 5 minutes, rinsed in deionized water and dipped into acid alcohol followed by another wash. Tissues were then soaked in Scott’s water for 1 minute, followed by incubation with 80% alcohol for 1 minute and eosin/phloxine for 1 minute. The tissues were then dehydrated in graded alcohols and cleared with Histoclear. Tissues were mounted with permount and covered with a coverslip. Images were taken using a Leica DM 4000 microscope and cross-sectional tumor area analysis was performed using Image J software (NIH); cross-sectional area was averaged from sections across the lung.

**Immunohistochemistry**

Whole lungs and primary tumors were fixed in 4% paraformaldehyde for 24-48 h, stored in 70% ethanol until embedded in paraffin and serially-sectioned at five microns. Tissues were deparaffinized in xylene and hydrated in graded alcohols before antigen retrieval in citrate buffer for 40 minutes. After rinsing in deionized water, tumor sections were placed in Tris buffer and incubated overnight at 4°C with antibodies against Ki67 (1:100) for the measurement of cell proliferation and with antibodies to TGF-β (1:200), p-Smad2 (1:100), CTGF (1:100), tenascin C (1:200), and α-SMA (1:100) to measure fibrosis. Tumor sections were then washed in Tris buffer and probed with a biotinylated secondary antibody in Tris buffer for 20 minutes at 37°C followed by incubation with the streptavidin-alkaline phosphatase enzyme conjugate at 37°C for 20 minutes. Next, tumor sections were washed in Tris buffer and incubated with the enzyme substrate Vector Red for 5 minutes in Automation buffer. Tissues were washed in deionized water, counterstained in Mayer’s hematoxylin for 5 minutes, dehydrated in graded alcohols and
cleared with Histoclear. Stained tumor sections were covered using a coverslip and permount. Four representative images from each primary tumor and images from four representative tumors per lung from each animal were taken with a Leica DM 4000 microscope and quantified using ImageJ software. The amount of immunoreactive protein is reported as the average percentage of positively-stained cells.

**Statistics**

All data are presented as the mean ± SE. Statistical differences were evaluated by Student's *t* test or one-way ANOVA followed by Dunnett's post hoc test. The criterion for statistical significance was set at *p* < 0.05.

**Results**

**Inhibition of 4T1 Metastatic Breast Tumor Growth in the Lung by Ang-(1-7)**

The mammary fat pads of female Balb/c mice were injected with 4T1 mouse TNBC cells and the tumors were allowed to grow until they reached a volume of 100 mm³, measured using a caliper. The primary tumors were surgically removed and osmotic mini-pumps containing Ang-(1-7) for the delivery of 24 µg/kg/h were implanted into the subscapular space, 7 days after tumor removal; treatment with the heptapeptide hormone was delayed 7 days to allow the initiation of metastatic tumor formation. The resected tumors were weighed to ensure even distribution of primary tumor growth between the groups and there was no difference between the primary tumors from treated or untreated mice (**Figure 1A**). Bioluminescence in the chest cavities of both treated and untreated mice was monitored weekly, as an indicator of metastatic tumor formation, and
the mice were euthanized either 28 days after removal of the primary tumor or earlier, if the mice were in distress. The average time of survival for untreated mice was 19.6 ± 2.7 days (n=9) compared to 22.6 ± 1.5 days (n=9) for mice treated with the heptapeptide hormone. Harvested lungs were weighed at the time of euthanasia; Ang-(1-7) caused a 32% decrease in lung wet weight compared to controls (Figure 1B), suggesting that the heptapeptide hormone reduced tumor burden in the lungs of mice.

Whole lung sections (every 5th section out of 100 total sections for a total of 20 sections analyzed) were stained with H&E and the average number of tumors was measured to determine the effect of Ang-(1-7) on the growth of 4T1 breast tumors in the lung. A representative image of metastatic breast tumors from untreated and treated mice is shown in Figure 2. Mice treated with Ang-(1-7) demonstrated a 66% reduction in the average number of tumors in the lungs compared to untreated mice, as shown in Figure 2A, suggesting that Ang-(1-7) reduces metastatic breast tumor burden. The total cross-sectional tumor area was also calculated in every 5th section across the lung and averaged; Ang-(1-7) reduced the cross-sectional area of lung tumors by 80% compared to the area of tumors from untreated mice, as shown in Figure 2B. Lungs from mice treated with Ang-(1-7) were stained with an antibody to Ki67, to measure cell proliferation. As shown in Figure 3, treatment with Ang-(1-7) resulted in a 43% reduction in the number of proliferating tumor cells, suggesting that the heptapeptide hormone regulates cell proliferation and growth to reduce metastatic breast tumor progression.

**Ang-(1-7) Inhibits the Growth of 4T1 Cancer-Associated Fibroblasts**
CAFs were isolated from orthotopic primary 4T1 tumors, by proteolytic digestion and differential plating, to determine whether Ang-(1-7) inhibits the proliferation of stromal cells associated with TNB tumors. The isolated stromal cells showed positive immunoreactivity for α-SMA, fibronectin, and vimentin, confirming their identity as CAFs, as shown in Figure 4A. The percentage of cells with positive immunoreactivity for each of these markers was determined in sequentially passaged cells, to ensure that the isolated cells maintained their fibroblast phenotype (Figure 4B). Only fibroblasts from passages 2-3 were used for experiments, since the cells did not maintain positive immunoreactivity for fibroblast markers after passage 3. Immunostaining for the Ang-(1-7) receptor mas was also observed in the isolated CAFs.

CAFs were incubated with 1 ng/mL TGF-β, in the presence and absence of 100 nM Ang-(1-7), and cell growth was measured on day 7, to determine the effect of the heptapeptide hormone on TGF-β-stimulated cell growth. TGF-β increased the number of CAFs 26-fold on day 7 compared to day 0 and treatment with Ang-(1-7) reduced TGF-β-stimulated cell growth by 32%, as shown in Figure 4C, suggesting that the heptapeptide hormone attenuates CAF proliferation.

Inhibition of TGF-β Signaling and ECM Protein Production by Ang-(1-7) in Cancer-Associated Fibroblasts

TGF-β stimulates the growth of CAFs and is also produced by CAFs, to increase the production of ECM proteins. TGF-β in CAFs treated with 100 nM Ang-(1-7) was measured by Western blot hybridization, to determine whether the heptapeptide hormone
reduced the production of this pro-fibrotic cytokine. Ang-(1-7) caused a time-dependent reduction in immunoreactive TGF-β which was maximal by 8 h, in which the amount of the cytokine was decreased by 81% compared with untreated controls (Figure 5A). This suggests that a decrease in TGF-β may be involved in the inhibition of CAF proliferation by the heptapeptide hormone.

TGF-β activates its receptors on the membrane of cells to initiate a signaling cascade which results in the production of ECM proteins. An initial event in TGF-β signaling is the phosphorylation of Smad2/3. CAFs were pretreated for 4 h with or without 100 nM Ang-(1-7) and stimulated with 1 ng/mL TGF-β; p-Smad was measured by Western blot hybridization, to determine the effect of the heptapeptide hormone on TGF-β signaling. TGF-β-stimulated p-Smad2 was reduced by 67% in CAFs pretreated with Ang-(1-7) compared to CAFs stimulated with TGF-β alone, as shown in Figure 5B, demonstrating that the heptapeptide hormone also reduces TGF-β-mediated cell signaling.

CTGF and tenascin C are ECM proteins that are increased by activation of TGF-β receptors, to increase tumor fibrosis. CTGF and tenascin C were also quantified in CAFs pretreated with Ang-(1-7) and stimulated with TGF-β, to determine whether the heptapeptide hormone reduces the production of ECM proteins. CTGF and tenascin C were measured by Western blot hybridization in CAFs incubated with 1 ng/mL TGF-β +/- 100 nM Ang-(1-7). Immunoreactive CTGF and tenascin C were reduced by 57% and 67%, respectively, in CAFs treated with the heptapeptide hormone (Figure 5C and 5D).
demonstrating that Ang-(1-7) reduces key proteins that regulate the production of ECM proteins in CAFs.

Ang-(1-7) Reduces Pro-Fibrotic Proteins in 4T1 Metastatic Breast Tumors in the Lung

The role of Ang-(1-7) in regulating the production of TGF-β and TGF-β-mediated cell signaling was measured in metastatic 4T1 lung tumors, by comparing TGF-β, p-Smad2, CTGF and tenascin C immunoreactivity in sections of the lungs of mice treated with the heptapeptide hormone to sections of lungs from control mice. In lung sections of mice treated with Ang-(1-7), TGF-β immunoreactivity was reduced by 61% compared to the lungs of control mice and p-Smad2 was decreased by 75%, as shown in Figure 6, suggesting that the heptapeptide hormone downregulates TGF-β and its signaling mediator p-Smad2 in 4T1 metastases in the lung as a mechanism to reduce metastatic breast tumor growth. Ang-(1-7) treatment also reduced immunoreactivity for the ECM proteins CTGF and tenascin C, by 74% and 91%, respectively (Figure 6). The Ang-(1-7)-mediated decrease in TGF-β, p-Smad2, CTGF, and tenascin C demonstrates that the heptapeptide hormone regulates expression of signaling mediators participating in fibrosis and cell proliferation to inhibit the growth of metastatic breast tumors.

Fibrotic Proteins Are Reduced by Ang-(1-7) in 4T1 Primary Breast Tumors

Identifying proteins in primary tumors that can be correlated with expression in their metastatic counterparts is critical for identifying and regulating aggressive tumor growth. 4T1 primary tumors growing orthotopically in the mammary fat pad of female
Balb/c mice were treated with Ang-(1-7) for 18 days, beginning treatment when the tumors reached a volume of 50 mm$^3$; tumor volume was significantly reduced compared with untreated controls (from 1679 ± 323 mm$^3$ in untreated controls to 900 ± 77 mm$^3$ following treatment with Ang-(1-7); p < 0.05, n = 5). Tumors from treated and untreated mice were sectioned and immunohistochemistry was performed using antibodies against TGF-β, p-Smad2, CTGF and tenasin C, to determine whether Ang-(1-7) similarly regulates these fibrotic proteins to reduce the growth of primary tumors. Ang-(1-7) reduced TGF-β by 74%, p-Smad2 by 92%, CTGF by 93% and tenasin C by 86% (Figure 7), indicating that Ang-(1-7) regulates TGF-β signaling and ECM protein expression to inhibit both primary breast tumor growth and metastatic breast tumor growth in the lung.

Ang-(1-7) Reduces the Number of Activated Fibroblasts in 4T1 Breast Tumors and Lung Metastases

Fibroblasts in the tumor microenvironment are recruited by tumors cells and activated to form myofibroblasts. A characteristic of activated myofibroblasts is their expression of α-SMA. Whole lung sections and primary 4T1 breast tumor sections from treated and untreated mice were incubated with an antibody to α-SMA, to determine whether treatment with Ang-(1-7) reduces the number of activated myofibroblasts. In both the primary tumors and metastatic tumors, α-SMA immunoreactivity was significantly reduced by Ang-(1-7) treatment (a 52% reduction in the primary tumors and a 48% reduction in the metastases; Figure 8), demonstrating that the heptapeptide
hormone reduces the number of activated myofibroblasts associated with either the primary or metastatic tumor.

Discussion

Metastasis of breast tumors is a rapidly-emerging issue in the clinic, with steadily increasing mortality rates resulting from metastatic tumor spread. TNB tumors are highly aggressive and display a short time to metastasis and relapse, with no currently available targeted treatments. Fibrosis is directly linked to tumor growth and may play an even larger role in mediating metastatic spread, given the favorable tumor microenvironment of the preferred site of metastasis, the lung (45). The present study is the first to show that Ang-(1-7) reduces the growth of TNB tumor metastases in the lung with associated reductions in TGF-β-mediated tumor fibrosis. The 4T1 orthotopic mammary carcinoma model closely mimics the breast cancer growth and metastasis observed in human patients, by metastasizing to the lung, liver, bone, and brain, and more accurately represents the targeted niche due to utilization of a syngeneic, immunocompetent mouse model (46). In our studies, 4T1 tumors were optimally grown to a volume of 100 mm³ to allow for the spread of malignant cells to distant tissue sites. Tumor removal prevented unnecessary death of the animals due to the burden of the primary tumor and mimicked clinical behavior observed for treatment of human breast cancer. Initiating Ang-(1-7) treatment one week after primary tumor removal allowed development of metastatic tumors, in order to determine whether the heptapeptide hormone reduces pre-existing metastases in the lung. Tumor burden in the lungs of mice was reduced by Ang-(1-7) treatment, demonstrated by decreases in lung bioluminescence signal, lung wet weight,
tumor nodularity of the lungs and Ki67 immunoreactivity. H&E staining of multiple sections across the lungs showed that Ang-(1-7) reduced tumor number as well as cross-sectional tumor area, confirming the inhibitory effects of the heptapeptide hormone on metastatic breast tumor formation in the lungs of mice.

Ang-(1-7) also inhibited the growth of CAFs isolated from orthotopic 4T1 mammary tumors, which were stained for CAF-specific markers to confirm their phenotype, suggesting that the heptapeptide hormone reduces metastatic tumor growth through reductions in tumoral CAF proliferation. Additionally, Ang-(1-7) treatment of CAFs isolated from 4T1 tumors resulted in a significant decrease in proteins involved in mediating tumor fibrosis, including TGF-β, p-Smad2, CTGF, and tenasin C. Thus, Ang-(1-7) not only reduces cell proliferation to inhibit metastatic tumor growth, but also attenuates tumor fibrosis. Expression of all four fibrotic markers in 4T1 metastatic tumors was reduced in lungs from animals treated with the heptapeptide hormone compared with control tissues, demonstrating that Ang-(1-7) regulates TGF-β molecular signaling in metastatic 4T1 breast tumors to inhibit activation of the downstream cellular effector, p-Smad2, and synthesis of CTGF and tenasin C ECM proteins which contribute to fibrosis-mediated tumor growth. Decreased expression of the marker of fibroblast activation, α-SMA, in metastatic 4T1 breast tumors in the lungs along with decreased CAF growth by treatment with Ang-(1-7) shows that the heptapeptide hormone reduces not only the total number of CAFs comprising the tumor stroma, but also the number of fibroblasts which are activated, ultimately contributing to decreased growth factor secretion, ECM protein synthesis and tumor cell proliferation.
Ang-(1-7) significantly inhibited expression of the fibrotic signaling molecules TGF-β, p-Smad2, CTGF and tenasin C as well as expression of α-SMA in the primary 4T1 breast tumors, suggesting the heptapeptide hormone targets the CAF population and CAF signaling to reduce metastatic tumor outgrowth. TGF-β initiates its molecular signaling in CAFs by binding to the TGFβ-RII receptor, which mediates the phosphorylation and activation of the TGFβ-RI receptor (29). The Smad family of proteins 2 and 3 (Smad2/3) are recruited to the receptor complex and are phosphorylated by TGFβ-RI, resulting in protein activation (30). Phospho-Smad2/3 (p-Smad) subsequently associates with Smad4, a co-Smad (31), and translocates to the nucleus to bind to an array of transcription factors and promote synthesis of ECM proteins including CTGF and tenasin C (32, 33). Abundant immunostaining for TGF-β was associated with infiltrative human breast carcinomas (34) as well as with human estrogen receptor-negative (ER-) breast tumors which display high rates of metastasis (35), indicating a clear role for TGF-β in mediating aggressive breast tumor growth.

ECM components are linked to breast cancer progression (16) and directly provide the mechanical forces within cells which govern the cell’s response to growth factors and cytokines (17). Both CTGF and tenasin C are considered hallmarks of fibrosis (18-21) and tumor aggressiveness (22, 23), promoting the production of additional ECM proteins, fibroblast migration, cell adhesion and cell proliferation by interacting with integrin receptors on the cell surface (24-26). Tenasin C is also elevated in response to tissue injury, mechanical stress, and inflammation in the tumor-associated
stroma (23, 24, 27). Tenascin C is absent in the normal adult mammary gland but is elevated in breast tumors, especially tumors of patients with a greater risk of metastasis and relapse (28), tenascin C expression in primary breast tumors confers a more aggressive phenotype with specific proclivity for metastatic tumor growth in the lung (28, 48), a phenomenon initially induced by TGF-β-mediated priming of primary breast tumors for metastasis to the lung (35). Regulation of the TGF-β-tenascin C activities in promoting CAF proliferation and ECM development during primary and metastatic breast tumor growth by treatment with Ang-(1-7) may be a novel mechanism by which the heptapeptide hormone reduces tumor growth. Thus, Ang-(1-7) regulates a wide range of proteins and processes which participate in fibrosis and growth of metastatic breast tumors, which includes both the fibroblasts themselves as well as their production of profibrotic mediators.

The mechanism by which Ang-(1-7) regulates TGF-β signaling in CAFs to inhibit breast tumor growth is not well-understood. However, since Ang-(1-7) was previously shown to regulate cell proliferation by upregulation of the second messenger cyclic adenosine monophosphate (cAMP) in vascular smooth muscle cells (47) and cAMP attenuates TGF-β-stimulated production of collagen and CTGF in human dermal fibroblasts and cardiac fibroblasts (49, 50), the heptapeptide hormone may regulate TGF-β molecular signaling in CAFs through increases in cAMP and cAMP-mediated cell signaling.

The phase I clinical trial conducted by the Wake Forest Comprehensive Cancer Center which evaluated the effectiveness of Ang-(1-7) in the treatment of solid-tumor
cancers did not include any breast cancer patients (41) Therefore, the reduction in the size of TNB tumors, in both mammary tissue as well as metastatic tumor growth in the lungs, provides support for a Phase I clinical trial for Ang-(1-7) in the treatment of breast cancer, and, in particular, those of the triple negative subtype, potentially providing a more effective and targeted treatment option for TNBC patients.

Acknowledgments

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Reference List


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Figure Legends

Figure 1. Lung wet weight of mice with metastatic 4T1 tumors is reduced by Ang-(1-7). A) Weight of primary breast tumors upon surgical resection from mice. B) Wet weight of lungs harvested from control and Ang-(1-7)-treated mice 28 days following removal of primary breast tumors. n = 9, * denotes p < 0.05.

Figure 2. Ang-(1-7) attenuates metastatic 4T1 tumor burden in mouse lungs. Representative H&E stained sections of 4T1 breast tumors that metastasized to the lungs of mice in control and Ang-(1-7)-treated animals. Images of H&E stained lungs were used to quantify average A) tumor number and B) tumor cross-sectional area for both control mice and mice treated with Ang-(1-7); n = 8-9, * denotes p < 0.05 and ** denotes p < 0.005. Representative pictures are at 10X magnification and scale bars are 10 mm.

Figure 3. Ang-(1-7) reduces Ki67 in metastatic 4T1 tumors. Immunohistochemistry was performed on whole lungs from mice to quantify Ki67 in 4T1 breast tumors from control and Ang-(1-7)-treated mice; n = 5-7, * p < 0.05. Representative pictures are at 20X magnification and scale bars are 10 mm.

Figure 4. Ang-(1-7) decreases the growth of TGF-β-stimulated 4T1 cancer-associated fibroblasts (CAFs). A) Representative images of CAFs isolated from orthotopic primary 4T1 breast tumors stained with antibodies for vimentin, fibronectin, α-smooth muscle actin (α-SMA), and mas. B) Positive immunoreactivity for vimentin, fibronectin, and α-SMA in isolated 4T1 fibroblasts was determined for sequential
passages 2, 3, 4, and 5 and represented as a percentage of total cells; n = 4. C) 4T1 CAFs were incubated with 1 ng/mL TGF-β in the presence and absence of 100 nM Ang-(1-7) and the number of cells was counted after 7 days. The data is presented as the percentage of the total number of TGF-β-stimulated CAFs isolated from the tumors of 5 different mice; *** denotes p < 0.001, n = 5.

Figure 5. Ang-(1-7) reduces TGF-β, p-Smad, CTGF, and tenascin C in 4T1 CAFs. A) 4T1 CAFs were treated with 100 nM Ang-(1-7), harvested at 0, 1, 2, 4, and 8 hour (h) time points, and TGF-β was measured by Western blot hybridization; n = 3, * denotes p < 0.05 and ** denotes p < 0.01. 4T1 CAFs were pre-treated with 100 nM Ang-(1-7) for 4 h, incubated with 1 ng/mL TGF-β in the presence and absence of 100 nM Ang-(1-7), and harvested after 10 additional minutes; B) p-Smad2, .) CTGF, and D) Tenascin C were measured by Western blot hybridization. n = 3; ** denotes p < 0.01 and *** denotes p < 0.001. Representative gel images are shown.

Figure 6. TGF-β, p-Smad, CTGF, and tenascin C are decreased by Ang-(1-7) in metastatic 4T1 tumors. Whole lungs from untreated (Control) or Ang-(1-7)-treated mice were sectioned and incubated with antibodies to A.) TGF-β, B.) p-Smad2, C.) CTGF, and D.) Tenasin C; n = 5-9; * denotes p < 0.05 and *** denotes p < 0.001. Representative pictures are at 20X magnification and scale bars are 10 mm. Quantified results are represented as graphs to the right of the image.
Figure 7. Ang-(1-7) reduces TGF-β, p-Smad2, CTGF, and tenascin C in primary orthotopic 4T1 tumors. Orthotopic 4T1 breast tumors from Ang-(1-7)-treated or untreated (Control) mice were harvested and incubated with antibodies to A.) TGF-β, B.) p-Smad2, C.) CTGF and D.) Tenascin C; n = 5; * denotes p < 0.05 and ** denotes p < 0.05. Representative pictures are at 20X magnification and scale bars are 10 mm. Quantified results are represented as graphs to the right of the image.

Figure 8. Activated CAFs are reduced by Ang-(1-7) in primary and metastatic 4T1 tumors. Orthotopic 4T1 breast tumors from mice treated with saline (Control) or 24 µg/kg/h Ang-(1-7) were harvested and immunohistochemistry was performed for α-SMA (left panel); n = 5-9, *** p < 0.001. Whole lungs from animals treated with saline (Control) or 24 µ/kg/h Ang-(1-7) were harvested and immunohistochemistry was performed for α-SMA (right panel); n = 5-9, *** p < 0.001. Representative pictures are at 20X magnification and scale bars are 10 mm. Quantified results are represented as graphs below the image.
Figure 1.

A. Primary Tumor Weight

B. Lung Wet Weight
Figure 2.

A.  

B.  

Tumor Number  

Tumor Area

Control  

Ang-(1-7)  

Control  

Ang-(1-7)  

10 mm  

10 mm
Figure 3.
Figure 4.

A.

B.

C.
Figure 5.

A.

B.

C.

D.
Figure 6.

A. TGF-β

B. p-Smad2

C. CTGF

D. Tenascin C
Figure 7.

A. TGF-β

B. p-Smad2

C. CTGF

D. Tenascin C
Figure 8.

Control

Ang-(1-7)

α-SMA

α-SMA

![Image of histological sections comparing Control and Ang-(1-7) effects on α-SMA expression.](image)

![Histograms showing α-SMA-positive cell percentages for Control and Ang-(1-7) conditions.](image)
CHAPTER IV: GENERAL DISCUSSION

Of the 232,000 annually diagnosed cases of breast cancer, 10-15% are classified as triple negative breast cancer (TNBC) (1, 2). These tumors are highly-aggressive and invasive, with a greater likelihood of relapse, metastasis, and death than non-triple negative subtypes of breast cancer (3, 4). Currently, there is a lack of effective targeted therapeutics available for the treatment of TNBC due to the absence of estrogen receptor (ER) and progesterone receptor (PR) expression and normal human epidermal growth factor receptor 2 (HER2) in TNB tumors. The seven amino acid peptide hormone, angiotensin-(1-7) [Ang-(1-7)], inhibited the growth of lung cancer, prostate cancer, HER2-amplified breast cancer and ER+ breast cancer (5-10). Furthermore, Ang-(1-7) attenuated fibrosis in both breast cancer and cardiac experimental models (5, 11, 12), thereby demonstrating a potential to inhibit the growth of TNBC through anti-proliferative and anti-fibrotic mechanisms. We hypothesized that the heptapeptide hormone inhibits the growth of TNBC through regulation of Akt and transforming growth factor-beta (TGF-β) growth-promoting signaling pathways.

The Effect of Ang-(1-7) on Akt Signaling in Triple Negative Breast Cancer

Previous studies showed that Ang-(1-7) inhibits the growth of breast cancer cells in vitro and orthotopic breast tumors in vivo through reductions in mitogen-activated protein kinase (MAPK) signaling (5). We also demonstrated the effects of the heptapeptide hormone in reducing phosphorylated extracellular signal-regulated kinases 1 and 2 (p-ERK1/2) implicated in aberrant cell growth in the cardiovascular setting, with associated increases in the MAPK phosphatase dual specificity phosphatase 1 (DUSP1)
Over-expression of the activated form of Akt, phosphorylated-Akt (p-Akt), which is frequently observed in TNBC as well as in non-triple negative forms of breast cancer, correlates with poor patient prognosis (13-16), suggesting that proliferative Akt signaling plays a major role in promoting TNBC cell survival and tumor growth. Therefore, we hypothesized that Ang-(1-7) reduces or inhibits the growth of TNBC cells and tumors by regulating the growth-promoting Akt signaling pathway, through reductions in phosphorylated Akt mediated by up-regulation of a phosphatase.

We demonstrated that treatment of human MDA-MB-231 and murine 4T1 orthotopic tumors growing in the mammary fat pad of mice with Ang-(1-7) produced a significant inhibition of tumor growth compared to control animals. Consistent with these findings, the wet weight of harvested tumors from animals treated with the heptapeptide hormone was reduced compared to controls, demonstrating the ability of Ang-(1-7) to regulate TNB tumor growth in vivo. The harvested tumors were sectioned and immunohistochemistry (IHC) was performed using an antibody against Ki67, a marker of cell proliferation, to determine whether Ang-(1-7) regulates tumor cell growth. Ang-(1-7)-treated tumors displayed a decrease in the number of Ki67-positive cells, which is supported by previous studies reporting the anti-proliferative properties of the heptapeptide hormone (17-21). Based upon the Ang-(1-7)-mediated reduction in Ki67 immunoreactivity, we investigated whether Ang-(1-7) regulates Akt signaling to reduce tumor cell proliferation.
Our studies demonstrate that both MDA-MB-231 and 4T1 TNBC cells and orthotopic tumors possess decreased amounts of activated Akt, with a reduction in both p-Akt(Ser473) and p-Akt(Thr308), in tumor tissue from mice treated with Ang-(1-7). Total Akt levels were unchanged with treatment, suggesting Ang-(1-7) specifically regulates the phosphorylation of Akt to prevent downstream cell proliferation. Full activation of Akt requires phosphorylation at both the Thr308 and Ser473 residues, wherein p-Akt(Thr308) increases Akt activity 100-fold and p-Akt(Ser473) confers Akt signaling specificity (22, 23). P-Akt(Ser473) is widely used as a clinicopathologic marker for advanced breast cancer (14, 24, 25), but few studies investigated the clinical significance of p-Akt(Thr308) in breast tumor samples, which is surprising given the role of p-Akt(Thr308) in conferring high Akt activity. In non-small cell lung cancer and acute myeloid leukemia, p-Akt(Thr308) is associated with poor survival (26, 27), and correlates with the Ki67 proliferation index in invasive breast carcinomas (28).

Ang-(1-7) significantly reduced both phosphorylated Thr308 and Ser473 of Akt in the TNBC cells, which suggests the heptapeptide hormone regulates both activity and specificity of Akt in TNBC. This indicates that Ang-(1-7) may be mediating upregulation of a serine/threonine protein phosphatase, similar to studies demonstrating Ang-(1-7)-mediated reductions in p-ERK1/2 were associated with increased DUSP1 (5, 12). The decreased amounts of p-Akt in TNBC cells and tumor tissues of mice treated with Ang-(1-7) correlated with increased protein phosphatase 2A (PP2A), a serine/threonine phosphatase responsible for inactivation of Akt. Ang-(1-7) upregulated both PP2A-A (the scaffolding subunit) and -C (the catalytic subunit), suggesting the heptapeptide
hormone increases AC dimer formation as well as PP2A phosphatase activity. Regulation of p-Akt and PP2A in TNBC represents a novel mechanism by which Ang-(1-7) inhibits the growth of breast cancer cells and tumors, demonstrating a promising therapeutic target for future clinical investigation.

The molecular mechanism for the Ang-(1-7)-mediated increase in PP2A has not yet been fully elucidated. Cyclic adenosine monophosphate (cAMP) regulates cell growth and proliferation by inhibition of ERK activity in a variety of cell types, including smooth muscle cells (29), fibroblasts (30, 31), and endothelial cells (32). Pharmacologic agents that increase intracellular concentrations of cAMP such as forskolin, cAMP analogs, and phosphodiesterase inhibitors reduce the serum-stimulated growth of vascular smooth muscle cells (VSMCs) in culture (33, 34). Ang-(1-7) treatment of VSMCs in vitro increased prostacyclin synthesis and cAMP production with concomitant reductions in angiotensin II (Ang II)-mediated phosphorylation of ERK1/2 and VSMC proliferation (20). In VSMCs incubated with Rp-cAMPS, an inhibitor of cAMP-dependent protein kinase A (PKA), the Ang-(1-7)-mediated inhibition of cell growth was blocked (35). Additionally, cAMP is responsible for inhibiting the phosphorylation of Akt stimulated by growth factors in rat fibroblasts (36). Moreover, the promoter/enhancer region of the DUSP1 gene contains cAMP responsive element (CRE) binding sites which are responsible for up-regulation of the phosphatase (37-39).

Treatment with forskolin, a well-known activator of adenylyl cyclase which increases intracellular cAMP, is associated with increases in PP2A activity (40-42). The
forskolin/cAMP-mediated increase in PP2A further produces anti-tumor effects in models of leukemia (43, 44). In support of these findings, protein kinase A (PKA) activates PP2A through direct phosphorylation of the B56δ subunit of the phosphatase in neuronal cells (45). Based on these reports, we postulate that Ang-(1-7) increases cAMP which activates PKA to increase PP2A activity. This process likely occurs either by direct phosphorylation and activation of PP2A by PKA or through cAMP binding to the promoter region of the PP2A gene to induce its transcription. Ang-(1-7) upregulates numerous phosphatases including DUSP1, PTEN, PTP-1b, and SHP-1, in a variety of experimental settings and disease states (5, 12, 46-49). Thus, Ang-(1-7) may serve as a potential universal regulator of phosphatases by acting through cAMP-mediated transcriptional machinery shared by most phosphatases such as cAMP response element-binding protein (CREB) or through cAMP-PKA signaling directly activating the phosphatases themselves.

The pleckstrin-homology domain leucine-rich repeat phosphatase (PHLPP) family of phosphatases are the only known phosphatases that solely target p-Akt(Ser473) to induce dephosphorylation at this residue of Akt (50, 51). Ang-(1-7) may also upregulate PHLPP to inhibit Akt activation and cell proliferation however, this has not yet been determined. Mutated, functionally-incompetent forms of PP2A or PHLPP are often found in breast tumors and correlate with poor prognosis (52-57). Thus, restoration of both Akt phosphatases in TNBC with Ang-(1-7) treatment would mitigate issues
arising from tumor insensitivity to dysfunctional PP2A or PHLPP alone. The mechanisms of PHLPP regulation are not yet well-understood. However, in neonatal rat cardiomyocytes, stimulation with forskolin increased PHLPP activity and associated reductions in p-Akt(Ser473) (58), suggesting that activation of PHLPP is dependent upon an increase in cAMP. Therefore, if Ang-(1-7) increases cAMP in TNBC cells and tumors, PHLPP may also be upregulated to prevent cell and tumor growth. This suggests that treatment with Ang-(1-7) might enhance the activity of both PP2A and PHLPP phosphatases on Akt signaling in tumors sensitive to phosphatase upregulation.

These studies suggest that the inhibition of the growth of TNBC cells and tumors is in part due to the Ang-(1-7)-mediated increase in the Akt regulatory phosphatase PP2A and the concomitant reduction in p-Akt. Furthermore, the heptapeptide hormone may potentially regulate both PP2A and another Akt phosphatase, PHLPP, through increased cAMP second messenger signaling, thereby preventing Akt activation and TNB tumor growth.

The Effect of Ang-(1-7) on TGF-β-Smad Signaling in Metastatic Triple Negative Breast Cancer

Fibrosis occurs as a result of the deposition of extracellular matrix (ECM) proteins such as collagens and fibronectin primarily secreted by fibroblasts. Elevated ECM protein deposition and increased fibrosis are common in breast tumors (59, 60), increasing tumor growth and cell migration as well as contributing to metastatic colonization and growth in secondary tissue sites. The tumor stromal fibroblasts or
cancer-associated fibroblasts (CAFs) comprise the primary cellular component mediating the production of fibrotic proteins, primarily directed by TGF-β-induced CAF activation. TGF-β binding to its receptor on CAFs induces an intracellular signaling cascade initiated by receptor-mediated phosphorylation and activation of the proteins, Smad2/3. The ECM protein, connective tissue growth factor (CTGF), is a hallmark of fibrosis induced by TGF-β/Smad signaling in fibroblasts. CTGF expression in breast tumors is associated with increased tumor metastasis in clinical breast cancer patients (61), suggesting it plays an important role in mediating tumor growth. Tenascin C is another ECM protein whose expression is induced by TGF-β/Smad in fibroblasts and is considered to be a marker of tumor aggressiveness. Tenascin C is absent in the adult mammary gland, but is highly-expressed in breast tumors in correlation with poor patient prognosis (62-64). Tenascin C may thus represent a new target for the development of anti-fibrotic and anti-tumorigenic therapeutics.

In our studies, the mammary fat pads of female Balb/c mice were injected with murine 4T1 TNBC cells to induce tumor growth. Once a size of 100 mm\(^3\) was reached, the primary tumors were removed and metastatic tumor growth was monitored, analogous to women with breast cancer who undergo surgical resection. Osmotic mini-pumps containing Ang-(1-7) were implanted subcutaneously (s.c.) one week after the primary tumors were removed, to study the effect of the heptapeptide hormone on metastatic tumor growth. We demonstrated a reduction in the growth of metastatic 4T1 TNB tumors in the lungs of mice treated with Ang-(1-7), measured by decreased lung wet weight; tumor number and tumor volume were also reduced in H&E-stained lung
sections. Tumors in the lungs harvested from Ang-(1-7)-treated animals had a decreased number of Ki67-positive cells, similar to primary orthotopic TNB tumors treated with the heptapeptide hormone, suggesting that Ang-(1-7) regulates cell proliferation as a mechanism for inhibiting tumor growth that is common to both primary and metastatic TNB tumors.

Ang-(1-7) reduces fibrotic mediators in cancer cells and tumors (5) as well as in cardiovascular cells and tissues (11, 12). We isolated CAFs from 4T1 breast tumors growing orthotopically in the mammary fat pads of female Balb/c mice, to determine whether the heptapeptide hormone elicits similar effects on fibrosis in TNB tumors. CAFs isolated from orthotopic 4T1 breast tumors showed positive immunoreactivity for alpha-smooth muscle actin (α-SMA), vimentin, fibronectin and the mas receptor, confirming their expression of activated fibroblast markers and demonstrating their capacity to respond to Ang-(1-7), respectively.

Treatment of TGF-β-stimulated 4T1 CAFs with Ang-(1-7) produced a significant reduction in cell growth compared with TGF-β alone, suggesting that Ang-(1-7) not only inhibits the growth of tumor cells themselves, but also the growth of stromal CAFs, as a mechanism of controlling tumor growth. Treatment of CAFs with Ang-(1-7) also reduced the fibroblast activator, TGF-β, as early as 1-2 hours post-treatment. This was consistent with reductions in the downstream fibrotic signaling mediator, p-Smad2, in TGF-β-stimulated CAFS pretreated with Ang-(1-7). Ang-(1-7) treatment of TGF-β-stimulated CAFs also produced a decrease in both tenascin C and CTGF, downstream
effects of the reductions in TGF-β and p-Smad2 signaling. These findings show that Ang-(1-7) specifically inhibits the growth of the CAFs as well as the CAF-mediated production of tumor-promoting ECM proteins that participate in fibrosis.

We observed a similar reduction in the number of tumor cells positive for TGF-β, p-Smad2, tenasin C, and CTGF in metastatic lung tumors from mice treated with the heptapeptide hormone. This suggests that Ang-(1-7) also reduced ECM protein deposition mediated by TGF-β and p-Smad2 signaling, to inhibit the growth of metastatic TNB tumors growing in the lungs of mice. In addition, we demonstrated that the pro-fibrotic proteins TGF-β, p-Smad2, tenasin C, and CTGF were reduced in primary orthotopic 4T1 TNB tumors growing in the mammary fat pad of mice treated with Ang-(1-7). Furthermore, we showed that Ang-(1-7) decreased the amount of α-SMA-positive cells as a measure of activated fibroblasts in both primary and metastatic TNB tumors, supporting a role for Ang-(1-7) in reducing the activation of CAFs to inhibit the growth of both the primary tumors as well as tumor growth in metastatic sites. These findings correlate with our metastatic TNB tumor data in the lungs of mice treated with the heptapeptide hormone, indicating the anti-fibrotic effects of Ang-(1-7) treatment are not limited to metastases, but also facilitate inhibition of primary tumor growth as well. These observations suggest that Ang-(1-7) regulates TGF-β fibrotic signaling as a means of abrogating the growth of the TNB tumors, at both primary and metastatic sites. However, it is not clear whether Ang-(1-7) regulates the process of CAF-mediated tumor metastasis. Further investigation in this area is needed.
These studies are the first to show that Ang-(1-7) reduces tenascin C, an ECM protein with an emerging role as a biomarker for aggressive tumor types and a facilitator of tumor metastasis. Inhibition of tenascin C may represent a novel mechanism for the Ang-(1-7)-mediated reduction in both breast tumor cell proliferation as well as the growth of metastatic tumors and tumor fibrosis. Since tenascin C is primarily expressed in breast tumors which demonstrate aggressive growth (65) and Ang-(1-7) reduces tenascin C, screening primary TNB tumors for tenascin C may be a useful tool for identifying patients with a higher likelihood for responsiveness to Ang-(1-7). Furthermore, regulation of tumor aggressiveness by reducing tenascin C with Ang-(1-7) may represent an invaluable therapeutic option for patients with metastatic TNBC.

The mechanism by which Ang-(1-7) inhibits TGF-β signaling to prevent phosphorylation and activation of Smad2/3 and production of the downstream ECM proteins, tenascin C and CTGF is not well-understood. However, studies in human dermal fibroblasts showed that cAMP inhibits TGF-β/Smad-induced production of CTGF and collagen by sequestering the transcriptional co-activators CREB-binding protein (CBP) and p300 in association with CREB, preventing the association of Smad2/3 with the transcription machinery and inhibiting ECM protein synthesis (66). Moreover, cAMP prevented the TGF-β-mediated activation of ERK1/2 to reduce collagen synthesis in rat cardiac fibroblasts (67). This suggests the heptapeptide hormone may inhibit TGF-β and Smad signaling in CAFs through cAMP signaling cascade, to prevent Smad binding to CBP/p300 transcriptional co-activators and reduce the production of the fibrotic proteins tenascin C and CTGF.
One limitation of our studies is that we do not know the source of the CAFs in the 4T1 tumors that are specifically recruited during tumor growth and progression. CAFs originate from a number of different sources including bone marrow-derived mesenchymal cells (68-70), the local tumor stromal fibroblast population (71, 72) and resident epithelial cells via epithelial-to-mesenchymal transition (EMT) (73, 74). Since the 4T1 cells are mouse-derived, we could not determine by IHC whether the CAFs originated from the tumor cells themselves or from the mouse tumor microenvironment. Since the injected 4T1 cells are luciferase-labeled, the cells could potentially be sorted based on the presence of luciferase to differentiate populations of cells derived from the tumor from those derived from the mouse. This could also be determined by injecting human TNBC cells into a mouse and identifying human and mouse α-SMA in the resultant tumors, using an antibodies specific for mouse or human α-SMA. Determination of the relative contribution of mouse- and human-derived cells to the pool of CAFs would demonstrate whether Ang-(1-7) primarily inhibits the differentiation and growth of tumor or stromal CAFs or both. In addition, identification of the CAF population that displays greater tumor growth-promoting behavior may present a novel target for controlling metastatic tumor growth.

In our studies, we demonstrated that treatment with Ang-(1-7) inhibits the growth of pre-existing metastatic TNB tumors in the mouse lung, using a model of spontaneous metastasis following resection of the primary orthotopic TNB tumor. The next question to address would be whether the heptapeptide hormone prevents the metastasis of TNBC
cells by inhibiting tumor cell colonization in the lungs or the shedding and release of primary tumor cells into the circulation. This question could be addressed by initiating treatment of mice with Ang-(1-7) immediately following resection of the primary orthotopic TNB tumor or at the time of the initial injection of cancer cells into the mammary fat pad. However, the latter option may be confounding due to the potential anti-growth effects of the heptapeptide hormone on the primary tumor itself. In prostate cancer studies from our laboratory, Ang-(1-7) pre-treatment of mice prevented metastatic tumors from forming in the bone (10), indicating that Ang-(1-7) may serve as a potential preventative treatment for metastatic cancer.
Figure 1. Summary of findings. A.) In triple negative breast cancer cells, Ang-(1-7) reduces the amount of phosphorylated Akt (p-Akt) with associated increases in protein phosphatase 2A (PP2A) to decrease cell and tumor growth. B.) In cancer-associated fibroblasts from triple negative breast tumors, Ang-(1-7) reduces the amount of transforming growth factor-beta (TGF-β) and p-Smad as well as the extracellular matrix (ECM) proteins connective tissue growth factor (CTGF) and tenascin C to reduce the growth of both primary tumors and metastatic tumors in mouse lungs.
Clinical Applications of Ang-(1-7) in the Treatment of Cancer

In a Phase I clinical trial conducted at the Wake Forest University Comprehensive Cancer Center, patients with non-resectable solid-tumor cancers were treated with escalating doses of Ang-(1-7) injected s.c. on days 1-5 of a 21-day period (75). No hypertensive events or bleeding complications were observed in any of the patients. Clinical benefit was observed in four of fifteen evaluable patients treated with the heptapeptide hormone. Ang-(1-7) reduced the pro-angiogenic molecule, placental growth factor (PlGF), in the plasma of patients who experienced clinical benefit, but not in those patients with tumor progression. The results of this study determined that the recommended dosing of Ang-(1-7) is 400 μg/kg administered daily by s.c. injection for 5 consecutive days on a 3-week cycle. This clinical trial not only determined the optimal dose to be used for subsequent phase II clinical trials for the heptapeptide hormone in cancer, but also provided further support for the anti-angiogenic role of Ang-(1-7) in cancer. Two of the four patients with clinical benefit were sarcoma patients, one of whom demonstrated minor response to Ang-(1-7) treatment evidenced by a 19% reduction in tumor size. Thus, these findings provided the basis for initiating a phase II clinical trial for Ang-(1-7) in the treatment of sarcoma which is currently ongoing.

Despite the success of the phase I study, there is still little information on the effect of Ang-(1-7) in the clinical treatment of breast cancer, as no breast cancer patients were enrolled in the Wake Forest clinical trial. A previous phase I clinical trial assessed the safety and myeloprotective effects of Ang-(1-7) in a cohort of breast cancer patients administered before and after treatment with doxorubicin/cyclophosphamide
chemotherapy (76). A maximum tolerated dose was not achieved with this study, but Ang-(1-7) did reduce the occurrence and severity of thrombocytopenia, anemia, and lymphopenia compared with the neutrophil-stimulating drug filgrastim (Neupogen). Other than these two clinical trials, no other human studies have evaluated therapeutic agents activating the mas receptor in cancer. Based on the effectiveness of Ang-(1-7) in clinical cancer patients along with the preclinical rationale provided by studies from our laboratory, our aim is to ultimately initiate a phase I clinical trial for Ang-(1-7) in breast cancer.

Ang-(1-7) provides numerous advantages over current forms of cancer treatment. The growth of actively proliferating cells is inhibited by Ang-(1-7) whereas standard chemotherapeutics often damage both cancerous and normal, healthy cells. Thus, Ang-(1-7) displays limited harmful, cytotoxic effects on undesired tissues associated with standard chemotherapies. In addition, Ang-(1-7) treatment does not increase blood pressure or heart rate and was not associated with bleeding issues which are often observed in clinical cancer patients (75). Furthermore, the activity and/or synthesis of biomolecules targeted by Ang-(1-7) are not completely abolished with treatment; the heptapeptide hormone reduces growth factors and activated mitogens to basal levels which allows for normal cellular processes requiring these molecules to still occur. This is supported by a lack of wound-healing issues in patients treated with Ang-(1-7) (75).

A major problem with many current cancer therapeutics is tumor resistance to treatment via angiogenic escape. This has been observed frequently in treatment of
cancer patients with the anti-angiogenic agent bevacizumab (Avastin) which inhibits vascular endothelial growth factor A (VEGF-A) signaling to reduce tumor blood vessel viability (77). VEGF inhibition commonly results in tumor-mediated compensatory increases in alternative pro-angiogenic molecules such as PlGF (78). Increases in PlGF levels are observed following treatment with several additional VEGF-targeting drugs including sunitinib, AMG 706, pazopanib, and sorafenib (79-82). Furthermore, the inhibition of VEGF signaling alone in tumors yields little improvement in overall patient survival (83). Ang-(1-7) targets multiple growth factor signaling pathways implicated in human cancer including both VEGF and PlGF to down-regulate a broad range of processes and events participating in tumor growth and progression (8, 75). Our findings suggest that Ang-(1-7) may sensitize tumors to treatment with VEGF inhibitors by regulating PlGF signaling, thus preventing angiogenic escape and providing greater clinical benefit (84-86).

Another major issue with current methods of cancer treatment is a reduction in therapeutic accessibility to tumors resulting from elevated tumor fibrosis. The tumor stromal cells and ECM exert physical pressures on the tumor vasculature to highly compress the blood vessels (87, 88). Vessel compression reduces vascular perfusion or blood flow as well as causes interstitial hypertension, thereby preventing maximal delivery of therapeutic agents to the tumor site and reducing potential clinical benefit. Patients with limited tumor perfusion have lower response rates to chemotherapy and shorter survival times than patients with greater tumor perfusion (89, 90). In addition, the
increased permeability and irregularity of tumor blood vessels can be attributed to the physical stress exerted by the tumor and tumor stroma.

Both stromal fibroblasts and matrix proteins contribute to tumor fibrosis-induced reductions in vessel perfusion. Diphtheria toxin-induced depletion of human CAFs in orthotopic murine mammary tumors in mice generated tumor blood vessels with larger mean diameters (88). Furthermore, inhibition of CAF proliferation in hypervascular pancreatic tumors assisted in opening compressed tumor vessels and contributing to enhanced vessel perfusion (88). Collagen fibers of the ECM are stretched and thus stiffened by the CAFs to promote matrix remodeling which applies stress on tumor blood vessels. During the promotion of vascular compression, collagen and hyaluronan, both ECM proteins produced by fibroblasts, interact to increase tumor stress exerted onto tumor vessels. Chauhan et al. shows that treatment of mice harboring pancreatic tumors with the AT1 angiotensin receptor blocker (ARB) losartan reduces isolated CAF expression of collagen I, hyaluronan, and TGF-β and promotes decompression of tumor blood vessels (87). Moreover, combination treatment of mice bearing 4T1 breast tumors with losartan and doxorubicin delayed tumor growth and increased median survival compared with monotherapy (87). Blockers of Ang II activity through ARBs or angiotensin-converting enzyme (ACE) inhibitors increase circulating Ang-(1-7) levels (91, 92). Therefore, these reports suggest a role for Ang II opposition through increased Ang-(1-7) production in mediating reductions in tumor fibrosis to increase vessel perfusion and survival benefit.
The anti-fibrotic properties of Ang-(1-7) may aid in overcoming the problem of fibrosis-induced vessel compression to improve drug delivery. The heptapeptide hormone reduces the proliferation of isolated CAFs from breast tumors, expression of the fibrotic mediators TGF-β, p-Smad, CTGF, and tenascin C in isolated CAFs, and the amount of activated fibroblasts via α-SMA expression. Furthermore, Ang-(1-7) displays anti-hypertensive properties through vasodilatory actions on the vasculature (93). Therefore, Ang-(1-7) may be potentially used to augment the delivery of anti-cancer therapeutics to the tumor for improvement of cancer treatment due to its unique anti-fibrotic and vasodilatory effects.

Ultimately, the goal is to administer Ang-(1-7) to cancer patients as a combinatorial treatment with a standard chemotherapeutic for breast cancer such as doxorubicin, paclitaxel or cyclophosphamide (4, 112, 113). This treatment strategy may allow for the reduction of chemotherapy dosages to mitigate undesired side effects, termed ‘metronomic chemotherapy’, which supports chronic, low-dose drug administration without extended rest periods (114, 115). Moreover, administering Ang-(1-7) in conjunction with a chemotherapeutic may enhance the inhibition of tumor growth due to complementary drug-mediated targeting of a wide range of tumorigenic and metastatic processes. Mice implanted with 4T1 or E0771 orthotopic breast tumors and treated with the ARB, losartan, in combination with doxorubicin displayed slower tumor growth rates compared with administration of either drug alone or saline alone (87), providing support for utilization of a doxorubicin/Ang-(1-7) treatment strategy in breast cancer. In order to maximize the clinical benefit of treatment with Ang-(1-7), tumor
samples from cancer patients may potentially undergo screening for mas receptor expression which will assist in identifying patients in which Ang-(1-7) will likely demonstrate increased effectiveness in inhibiting tumor growth and progression. Targeted therapeutics are key for controlling cancer growth, highlighting the importance of identifying the molecular pathways driving tumorigenesis in an individual patient. Personalized cancer therapy is giving greater hope to researchers, clinicians, and patients alike, relying on genetic and protein screening of tumors to determine the role of alterations in molecules implicated in the growth of the cancer (116). Understanding the role of mutations, deregulation, and dysfunction of genes and proteins in governing tumor growth in an individual helps to identify the appropriate therapeutic for treatment and improve response rates to the chosen regimen.

Drug-induced cardiotoxicity is an emerging clinical issue in cancer patients treated with chemotherapy. Anthracyclines like doxorubicin are effective anti-cancer therapeutics widely used in the clinic; however, their use is limited due to the severe resultant cardiotoxic effects (94-96). Doxorubicin-induced cardiotoxicity results in irreversible degenerative cardiomyopathy leading to congestive heart failure and eventual death in as many as 50% of patients (97, 98). In addition, onset of doxorubicin-induced cardiotoxicity can occur as soon as 2-3 days of initial administration (97). The molecular basis for these events is dependent on a range of signaling processes including increased reactive oxygen species (ROS) generation (99-101), inhibition of topoisomerase-II beta (Top2β) transcriptional effector (102), p53 activation (103, 104), and increased p38 MAPK activity (105, 106). In the cell, doxorubicin targets Top2β to form a DNA
cleavage complex for the induction of cell death. Adult mammalian cardiomyocytes express Top2β, resulting in their death following treatment with doxorubicin (102). Apoptosis signaling pathways and transcriptomic changes induced by doxorubicin are coupled with alterations in mitochondrial biogenesis yielding increased ROS production, which may be due to increases in DNA damage responders like p53, suppressing crucial activators of mitochondrial function (102, 107). Increased activation of p38 MAPK in response to elevated ROS generation further contributes to cardiomyocyte apoptosis and impaired cardiac function (106, 108). Ang-(1-7) elicits cardioprotective effects due to reductions in ROS in human endothelial cells (18), renal cortical nuclei (109), and diabetic rat kidneys (110) as well as decreases in ROS-mediated p38 activation (111). Administration of Ang-(1-7) in conjunction with a chemotherapeutic (such as doxorubicin) would allow for reduced dosing schedules due to the augmented effectiveness of tumor treatment, which may prevent the elevated dose-related cardiotoxic effects attributed to doxorubicin.

Ang-(1-7) may possess numerous advantages over current anti-cancer therapeutics and has the potential to provide a broad range of cancer patients with clinical benefit. However, Ang-(1-7) is rapidly metabolized in the plasma and is not biologically active after oral administration. Administration of Ang-(1-7) through a s.c. route increases plasma levels of the heptapeptide 2-3 fold as shown in both animal models (5, 8) and clinical cancer patients (75), similar to changes observed with angiotensin converting enzyme (ACE) inhibitor administration (117). However, the half-life of Ang-(1-7) is approximately 30 minutes in humans by s.c. administration (75, 76), thus
necessitating development of an orally-active Ang-(1-7) mimetic with increased half-life and bioavailability. Several Ang-(1-7) analogs were developed and studied. The first small molecule Ang-(1-7) mimetic was AVE-0991, a non-peptide orally-active agent that activates the *mas* receptor to mimic the effects of Ang-(1-7) (118). AVE-0991 is generated by an imidazole ring substitution and is physiologically well-tolerated. This mimetic competes with Ang-(1-7) for binding to the *mas* receptor, to mediate nitric oxide (NO) generation in endothelial cells and Chinese hamster ovary (CHO) cells (119, 120). Furthermore, AVE-0991 produces anti-diuresis in water-loaded mice (119), prevents kidney and heart damage in spontaneously hypertensive rats (SHR) (121), and improves endothelial function in rats (122). The effects elicited by AVE-0991 can be blocked by administration of the *mas* receptor antagonist D-Ala both *in vitro* (118, 119, 123) and *in vivo* (119, 122). However, AVE-0991 also binds to the AT1 and AT2 Ang II receptors with a blockade of NO production following AT1/AT2 receptor therapeutic antagonism (120). This promiscuity may result in patient side effects if not administered in a specific manner.

Another Ang-(1-7) mimetic that was developed and studied is CGEN 856S (124). CGEN 856S is a peptide derivative showing high binding specificity for *mas* with an absence of AT1 or AT2 receptor activation (124, 125). CGEN 856S does not share significant homology to angiotensins and appears more stable than Ang-(1-7), potentially due to its 24-amino acid monomeric structure (124). In isolated aortic rings from rats, CGEN-856S induced NO-dependent vasorelaxation which was blocked by administration of D-Ala, indicating a *mas*-mediated effect. CGEN-856S also attenuated isoproterenol-
induced cardiac hypertrophy in rats in correlation with reductions in collagen and fibronectin deposition in rat hearts (126), indicating the mimetic acts in a similarly cardioprotective and anti-fibrotic manner as Ang-(1-7).

A newer formulation of Ang-(1-7) which is under investigation for its efficacy as a therapeutic mimetic is a hydroxypropyl-β-cyclodextrin (HPBCD)-based oral nano-formulation of Ang-(1-7) which provides an orally-active form of Ang-(1-7) through use of cyclodextrins (127-130). Cyclodextrins are often implemented in drug development to increase drug stability, absorption across membranes, and gastric protection. This Ang-(1-7) formulation showed improvements in Ang-(1-7) effectiveness in models of diabetes (131), inflammation (130), myocardial infarction (129), thrombosis (127), and fibrosis (131). Despite the apparent effectiveness of these newly-developed Ang-(1-7) mimetics and formulations, extensive experimental investigation including additional in vivo work is needed in order to demonstrate the specificity of these potential therapeutics as well as any side effects. Furthermore, none of these agents has been tested in a model of cancer, driving researchers to consider additional ways in which the Ang-(1-7) peptide can be modified in order to maximize its therapeutic benefit in patients with cancer.

The findings presented in this dissertation along with previous preclinical and clinical studies demonstrate a clear role for Ang-(1-7) in regulating numerous pathways during cancer growth and progression. Ang-(1-7) provides a wider range of clinical benefits than current anti-cancer therapies. Thus, Ang-(1-7) has the potential to serve as
a first-in-class targeted therapeutic for the treatment of triple negative breast cancer, for
the ultimate purpose of improving the lives of this underserved population of women.


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APPENDIX

The data shown here represent supplementary findings that are relevant to the thesis, but have no logical place within the main body of text.

Methods

Cell Growth Assay

Human MDA-MB-231-LM triple negative breast cancer cells that specifically metastasize to the lung were a gift from Joan Massague (Memorial Sloan-Kettering Cancer Center), used to determine whether Ang-(1-7) had an effect on the growth of metastatic-specific triple negative breast cancer cells. Cells were seeded in 100 mm³ dishes at a concentration of 5x10⁵ cells per dish and incubated with phosphate buffered saline (PBS) or 100 nM Ang-(1-7) and the number of cells counted on days 0, 4, 7, and 10 using a hemocytometer.

Scratch Assay

Cancer-associated fibroblasts (CAFs) isolated from murine orthotopic 4T1 triple negative breast tumors were seeded in 12-well plates in the presence of 10% fetal bovine serum (FBS) DMEM/F12 media. Once the cells were 100% confluent, the media was removed and the cells were incubated in serum-free media overnight. Cells were then placed in 2 ml 1% FBS media and appropriate wells were pre-treated with 100 nM Ang-(1-7) for 4 hours. A scratch was made in each well using a 200 µl pipette tip and the media was aspirated. Wells were washed with 2 ml serum-free media and appropriate wells were each replaced with 2 ml fresh serum-free media containing either 1 ng/ml transforming
growth factor-beta (TGF-β) alone or in the presence of 100 nM Ang-(1-7) for 24 hours. Pictures were taken at 0 h and at 24 h using an Olympus QImaging camera with QCapture imaging software.

**Western Blot Hybridization**

4T1 CAF monolayers were solubilized and protein content was determined using a modified Lowry method. Proteins separated by PAGE were transferred to polyvinylidene difluoride (PVDF) or nitrocellulose membranes and membranes were incubated with Blotto (Tris-buffered saline with 5% powdered milk and 0.1% Tween 20) to block non-specific binding. Membranes were incubated with primary antibodies to p-Akt(T308), p-Akt(S473), PP2A-A (1:2,000), PP2A-C (1:5,000), DUSP1 (1:1,000), and p-ERK1/2 (1:2,000) overnight at 4°C, followed by a 1 h incubation at room temperature with polyclonal HRP-conjugated secondary antibodies (1:2,000-5,000). A monoclonal antibody to β-actin or α-tubulin (1:5,000) was used as a protein loading control. Chemiluminescence was used to visualize immunoreactive bands (SuperSignal Femto or Pico West, Pierce Technology), followed by band densitometry quantification using MCID digital software.

**Results**

In order to assess whether Ang-(1-7) has an effect on metastatic cell growth, human MDA-MB-231-LM triple negative breast cancer cells that specifically metastasize to lung tissue in an experimental mouse model were treated with the heptapeptide hormone *in vitro*. As shown in **Figure 1**, the growth of cells treated with Ang-(1-7) is
significantly reduced by 45.4% at day 10 compared with control cells. This suggests that Ang-(1-7) regulates the growth of metastatic-specific TNBC cells, and thus may play a role in mediating reductions in metastatic tumor growth or the process of metastasis itself.

In the previous chapter, we showed that Ang-(1-7) inhibits the growth of metastatic TNB tumors growing in the lungs of mice. However, it is not known whether Ang-(1-7) has an effect on TNBC cell migration or invasion, steps integral to metastasis. Furthermore, given the importance of cancer-associated fibroblasts (CAFs) in mediating tumor progression and eventual metastasis, we chose to determine the role of Ang-(1-7) during CAF migration. In order to test this, we performed an in vitro scratch assay or wound-healing assay using CAFs isolated from primary orthotopic 4T1 TNB tumors. CAFs which were incubated with transforming growth factor-beta (TGF-β), a potent inducer of fibroblast activation, showed elevated cell migration into the scratch made in the cell culture dish, as illustrated by pictures taken of the scratch at 0 h and 24 h after treatment (Figure 2A). Conversely, CAFs treated with TGF-β in addition to Ang-(1-7) demonstrated a significantly attenuated migration response compared with TGF-β incubation alone, evidenced by a 35.2% decrease in the number of cells in the denuded zone (Figure 2B). This finding suggests that Ang-(1-7) reduces CAF migration induced by TGF-β in vitro, which potentially presents a role for the heptapeptide hormone in regulating CAF and TNBC cell migration and metastasis in vivo.
Next, we determined whether Ang-(1-7) similarly regulates Akt phosphorylation and activation in the 4T1 CAFs as it does in the TNBC cells as a means of inhibiting cell and tumor growth. Ang-(1-7) treatment of 4T1 CAFs resulted in a reduction in both p-Akt(T308) (Figure 3A) and p-Akt(S473) (Figure 3B) by 38.6% and 61.4%, respectively, demonstrating that the Ang-(1-7)-mediated decrease in CAF growth may be due to attenuated p-Akt levels. We also determined PP2A amounts in 4T1 CAFs treated with Ang-(1-7) to demonstrate whether the reduction in p-Akt in CAFs treated with the heptapeptide hormone is due to upregulation of the Akt phosphatase. Ang-(1-7) significantly increased PP2A-A protein levels in the CAFs by 37.5% (Figure 4A), whereas PP2A-C levels remained unchanged with treatment (Figure 4B). Given the observed reduction in activated Akt levels in the CAFs with Ang-(1-7) treatment, this finding may potentially mean that endogenous levels of PP2A-C are sufficient to inhibit Akt phosphorylation and activation, and thus the C subunit of PP2A does not significantly increase under Ang-(1-7)-stimulating conditions.

Lastly, we assessed the effect of Ang-(1-7) on protein levels for phosphorylated extracellular signal-regulated kinases 1/2 (p-ERK1/2) and the MAPK-inactivating phosphatase dual specificity phosphatase 1 (DUSP1) in the 4T1 CAFs in order to determine whether Ang-(1-7) inhibits the activation of growth-promoting ERK1/2 via DUSP1 upregulation in the CAFs to inhibit cell proliferation. As shown in Figure 5, CAFs treated with Ang-(1-7) demonstrate a 2.5-fold increase in DUSP1 protein at 16 h (Figure 5A) which is associated with a 69.8% reduction in p-ERK1 and a 59.5% reduction in p-ERK2 protein at 16 h (Figure 5B). These observations indicate that Ang-
(1-7) mediates reductions in CAF growth by increasing DUSP1 to facilitate decreases in activated MAPKs.
Figure 1: Angiotensin-(1-7) inhibits the growth of MDA-MB-231-LM cells in vitro. Human MDA-MB-231-LM triple negative breast cancer cells that specifically metastasize to the lung were treated with PBS (Control) or 100 nM Ang-(1-7) [Ang-(1-7)] and cell number was determined on days 0, 4, 7, and 10 using a hemocytometer; n = 3, *p < 0.05, ***p < 0.001.
Figure 2: Migration of 4T1 CAFs stimulated by TGF-β is reduced by Ang-(1-7).

Cancer-associated fibroblasts (CAFs) isolated from murine orthotopic 4T1 triple negative breast tumors were seeded in 12-well plates and appropriate wells were pre-treated with 100 nM Ang-(1-7) for 4 hours. A scratch was made in each well using a 200 µl pipette tip and appropriate wells were incubated with either 1 ng/ml transforming growth factor-beta (TGF-β) alone or in the presence of 100 nM Ang-(1-7) (TGF-β+A7) for 24 hours. Pictures were taken at this time (0 h) and at 24 h. A.) Representative pictures at 0 h and 24 h time points. B.) Migration was quantified by counting the number of cells in the scratch and calculating the number as a percentage of cells treated with TGF-β alone; n = 5, * p < 0.05. Representative images are shown.
Figure 3: Phosphorylated Akt is reduced by Ang-(1-7) in 4T1 CAFs. Isolated 4T1 CAFs were seeded in 100 mm³ dishes and appropriate plates were pre-treated with 100 nM Ang-(1-7) for 4 hours. Cells were either stimulated with 1% fetal bovine serum (FBS) alone (Control) or 100 nM Ang-(1-7) alone [(Ang-(1-7))] for 10 minutes. Western blots were performed using antibodies against A.) p-Akt(Thr308) and B.) p-Akt(Ser473); n = 3-4, * p < 0.05, *** p < 0.001, Ψ p < 0.0001. Representative gel images shown.
Figure 4: Ang-(1-7) increases PP2A-A and does not alter PP2A-C protein in 4T1 CAFs. Isolated 4T1 CAFs were seeded in 100 mm\(^3\) dishes and appropriate plates were pre-treated with 100 nM Ang-(1-7) for 4 hours. Cells were either stimulated with 1% fetal bovine serum (FBS) alone (Control) or 100 nM Ang-(1-7) alone [(Ang-(1-7))] for 10 minutes. Western blots were performed using antibodies against protein phosphatase 2A (PP2A) A.) subunit A and B.) subunit C; n = 4-5, * p < 0.05. Representative gel images shown.
Figure 5. Ang-(1-7) increases DUSP1 and reduces phospho-ERK1/2 in 4T1 CAFs.

Isolated 4T1 CAFs were seeded in 100 mm$^3$ dishes, stimulated with 1% fetal bovine serum (FBS) with 100 nM Ang-(1-7) and harvested at 0, 2, 16, and 24 hour (h) time points. Immunoreactivity was measured by Western blot hybridization for A.) dual specificity phosphatase 1 (DUSP1), B.) phosphorylated extracellular signal-regulated kinase 1 (p-ERK1), and C.) p-ERK2; * p < 0.05, ** p < 0.01, n = 3. Representative gel images shown.
A.

B.

C.
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**Abstracts & Poster Presentations:**

**Alison L. Arter**, Patricia E. Gallagher, E. Ann Tallant; “Angiotensin-(1-7) Suppresses Lung Metastasis in the 4T1 Orthotopic Mammary Carcinoma Model” (2013), Wake Forest University Division of Surgical Sciences Research Day, Poster Presentation. *Received 2nd Place Award.*


**Alison L. Arter**, Patricia E. Gallagher, and Ann Tallant; “Angiotensin-(1-7) attenuates triple negative breast cancer growth and progression through regulation of protein phosphatases” (2013), Wake Forest University 13th Annual Graduate Student Research Day, Poster Presentation. *Received 1st Place Award.*


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