OBESITY-ALTERED ADIPOSE STEM CELLS PROMOTE BREAST CANCER METASTASIS AND RADIATION RESISTANCE

AN ABSTRACT

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OF PHILOSOPHY BY

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ABSTRACT

Obesity is an established risk factor for the development of breast cancer in postmenopausal women. The incidence of obesity has been steadily increasing around the world and, presently, two-thirds of the adult population in the United States is overweight or obese. The clear link between obesity and breast cancer development and progression has led to numerous studies investigating the role of adipose tissue and adipose stem cells (ASCs) on breast cancer. ASCs play an important role in the tumor microenvironment. ASCs are biologically altered by obesity and become obese ASCs (obASCs). Previous studies have shown that obASCs secrete higher levels of leptin, a protein that upregulates ERα and aromatase thereby increasing estrogen bioavailability. This promotes estrogen receptor positive (ER+) breast cancer (BC) tumor growth and metastasis. In Aim 1, we evaluate the effect of obASCs on ER+BC outside of the ERα signaling axis using breast cancer models with constitutively active ERα through clinically relevant mutations (Y537S and D4358G). In Aim 2, we evaluate the effect of obASCs on triple negative breast cancer (TNBC). TNBC is a clinically aggressive subtype of BC with high rates of metastasis, recurrence and therapeutic resistance. In Aim 3, we evaluate the effect of obASCs on ER+BC response to radiation therapy (RT). Obesity is associated with poorer responses to chemotherapy and radiation for breast cancer, which leads to higher mortality rates for obese women who develop breast cancer.
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1 Introduction

The prevalence of obesity has dramatically increased over the past four decades. Previous research has linked this increase in obesity to the rise of cancers such as post-menopausal breast cancer, esophageal cancer, liver cancer and pancreatic cancer [1, 2]. Here we will focus on breast cancer (BC). Obese postmenopausal women have a 20%-40% increase in risk of developing breast cancer compared to normal-weight women [3]. Of breast cancer cases approximately 71% percent are estrogen receptor positive (ER$^+$), 12% are triple negative, and 17% are HER2$^+$ [4]. Obesity is associated with an increased risk of development of all subtypes of postmenopausal breast cancer, though given the higher rates of ER$^+$ breast cancer in postmenopausal women, clinicians are more likely to see obesity related ER$^+$ breast cancer cases [5]. In addition to an increased risk of breast cancer for obese postmenopausal women, obese women with BC, regardless of subtype, are more likely to have a larger primary tumor, have increased risk of metastatic disease, do not respond as well to standard therapies and are more likely to die from their cancer [6, 7]. The epidemiologic data demonstrating a link between obesity and breast cancer is surmounting; however, the cellular mechanisms to correlate the interaction between the two are still not fully understood. Here we use adipose stem cells (ASCs) to investigate how obesity-alterations in the tumor microenvironment (TME) promote tumorigenesis, metastasis, and therapeutic response. The goal of this research is to address the gap in knowledge of the mechanism(s) through which obesity-altered adipose stem cells (obASCs) promote breast cancer.
2 Chapter 1: Adipose stem cells

Stem cells are capable of asymmetric cell division promoting self-renewal and multi-lineage differentiation potential [8]. Due to the controversy over the use of embryonic stem cells, recent interest has grown surrounding the potential of adult stem cells. Adult stem cells have been identified in most adult organs and tissues including bone, cartilage, skeletal muscle and adipose tissue [9-14]. Although adult stem cells have a lesser capacity for self-renewal and differentiation than embryonic stem cells, there is a rapidly growing body of evidence that suggests that the therapeutic potential of adult stem cells is much more significant than was once thought [15, 16].

Adipose tissue serves as a reservoir for mesenchymal stem cells, which we will refer to as adipose stem cells (ASCs) in this thesis. Adipose tissue is a multifaceted organ with many functions including endocrine functions with the secretion of various adipokines, structural and lipid storage functions and an immunology function as there are many immune cells and immunomodulatory cells, namely ASCs, resident in adipose tissue [15]. ASCs can differentiate into mature cells of the adipogenic, osteogenic, chondrogenic, and myogenic lineages [17]. Adipose tissue is harvested and enzymatically digested to isolate the stromal vascular fraction (SVF). SVF is composed of many cell types: ASCs (15-30%), endothelial cells (10-20%), pericytes (3-5%), and immune cells (25-45%) [18, 19]. After SVF isolation, the heterogeneous cell composition can be cultured, and the plastic adherence capacity of ASCs allows for the acquisition of
homogenous populations of ASCs. Human ASCs can be phenotypically identified as CD45⁻CD235a⁻CD31⁻CD34⁺ and cultured ASCs can be phenotypically identified as CD13⁺CD73⁺CD90⁺CD105⁺CD31⁻CD45⁻CD235a⁻ [18]. ASCs do not express human leukocyte antigens including HLA-DR molecules and co-stimulatory molecules that are important for immune recognition of "self" versus "non-self" that would trigger significant immune responses, making ASCs potential candidates for not only autologous therapy but possibly allogeneic therapy [17, 20].

Adipose tissue confers an advantage because stem cell yield from is 100-500 times higher per tissue volume than from bone marrow rendering ASCs a more attractive candidate for therapeutic use [21, 22]. Harvesting adipose via liposuction can yield large quantities of fat tissue with minimal risk to the patient. This procedure is very commonly performed, but the removed adipose tissue/lipoaspirate is discarded as medical waste [20, 22]. The American Society of Plastic Surgeons 2015 annual report registered a total of 222,051 liposuction procedures in 2015 alone [23]. Thus, there is a vast number of potential ASCs going to waste.

Many reasons that make ASCs such an attractive cell type for regenerative medicine applications are: their multipotent potential for regenerative uses; ease of isolation; plentiful source; either autologous or allogeneic use; and their innate ability induce angiogenic traits. ASCs have been demonstrated to mediate robust anti-inflammatory and immunomodulatory effects, which have led to various preclinical studies and clinical trials to investigate therapeutic efficacy. Tissue inflammation activates ASCs to produce anti-inflammatory cytokines and angiogenic factors [17, 18, 24]. The immunomodulatory function of ASCs results in an environment where ongoing
inflammation is minimized, and a regenerative environment is promoted to restore homeostasis. The regenerative and anti-inflammatory potential of these cells have led to studies using these stem cells across various disciplines, examples of which are covered in this section.

2.1 ASCs and regenerative medicine

2.1.1 ASCs as Therapy for Cardiac Disease

ASCs are multipotent and retain the ability to differentiation into mesodermal tissues. The potential to regenerate cardiomyocytes with ASCs has drawn substantial research attention due to the increasing incidence of myocardial infarctions (MI) and the poor prognosis associated with scar tissue formation in the myocardium post-MI [25-29].

Ischemic heart disease (IHD) is the leading cause of death worldwide. Much of the burden of IHD is due to the inability of the cardiac tissue to regenerate after cardiac events. Instead, infarctions lead to an inflammatory response, which ultimately results in necrosis of the infarct zone, rendering the region unable to participate in electrical conduction or cardiac muscle contraction. Currently, there are no effective ways to regenerate cardiac muscle tissue. ASCs have been studied for their ability to regenerate cardiac tissue and/or improve cardiac function in multiple model systems. The first study to demonstrate mesenchymal stem cells’ ability to differentiate into cardiomyocytes by Rangappa et. al. used rabbit ASCs [30]. Another group demonstrated differentiation of human ASCs after exposure to rat cardiomyocyte proteins [31]. These studies examined structural and functional capacities of cardiomyocytes to characterize differentiation; however, no in vivo experiments were preformed.
Valina et al. used a porcine model to show that intracoronary administration of autologous ASCs 15 minutes after reperfusion improved various cardiac functions post-infarction [32]. The results showed significantly greater capillary density in the infarct border zone, wall thickness (WT) and left ventricular ejection fraction (LVEF), all in the ASC-treated group compared to the control 30 days post-procedure. A rat model was also used to assess the benefits of ASCs in the setting of chronic heart failure. The ASC-treated group showed a significantly improved LVEF and reduced infarct area [33].

A Phase 1/2 clinical trial has also investigated the safety of SVF in patients with post-MI IHD. A total of 28 patients participated in the study. After noticing similar results as in the aforementioned studies (significantly increased LVEF and increased WT) and better patient performance in the six-minute walk test at the 3-, 6- and 12-month follow up compared to baseline (before cell transplantation), with no significant adverse effects, the authors concluded that the intra-myocardial injection of 30 million cells was safe and efficacious [34]. Despite a limited number of patients tracked at the various time points and lack of a control group, these results provide some support that SVF may benefit patients with decreased ventricular performance due to MI-induced scarred cardiac tissue.

2.1.2 ASCs and Biomaterials

ASCs represent a cell source that has the potential to transform the field of tissue engineering and regenerative medicine. Seeding biomaterials, like poly(lactic-co-glycolic) acid (PLGA), Poly(vinyl alcohol) PVA, decellularized extracellular matrix or chitosan with ASCs has been used to assist in healing and regeneration of muscle, cartilage, functional fat tissue, tendon, and bone [21, 35-40].
Once implanted, one of the most important considerations for any tissue-engineering product is the supply of nutrients to the scaffold and removal of cellular waste from the scaffold, either through transport through the biomaterials via diffusion and/or by encouraging neovascularization within the tissues. In the field of tissue engineering a major roadblock is blood vessel formation, especially for thick or dense materials. Substantial research is required to overcome these barriers by utilizing the innate proangiogenic traits of ASCs to induce blood vessel formation to supply engineered tissues with the necessary route for nutrients and waste transport.

Harnessing and enhancing the angiogenic traits of ASC using biomaterials is of interest to the regenerative medicine field. It was demonstrated that the ASC encapsulated in larger PLGA spheroids were found to upregulate angiogenic growth factors and adipogenesis \textit{in vitro}, and allowing of the scaffolds to recapitulate significant vascular ingrowth \textit{in vivo} in a nude mouse model [41]. Similarly, other groups have also been working towards capitalizing on the angiogenic and/or adipogenic capabilities of ASC in tissue engineering application [39, 42]. ASC have also been encapsulated in thermosensitive hydrogels, like chitosan/ gelatin mixtures, to create an injectable for therapeutic angiogenesis for ischemic materials by allowing for more prolonged survival of dissociated ASC [43]. Utilizing materials like these with pre-seeded ASC, it has been found to increase angiogenic growth factor concentration in the growth media \textit{in vitro}, allowing for a more considerable amount of tubule formation in the hydrogel when co-cultured encapsulated with endothelial cells. Data from \textit{in vivo} studies demonstrate higher densities of capillaries were found when applying the encapsulated ASCs in a chick embryo chorioallantoic membrane assay [43]. Like adipose tissue, other soft tissue
applications have benefitted from ASCs. Choi et. al. used an elastin-like polypeptide
matrix with ASCs where the hydrogel would coagulate in the wound site. This is
beneficial because it could mold to any wound shape, enabled retention of the ASCs at
the wound site to promote regeneration, and activated wound clotting to promote faster
regeneration [36]. Conductive biomaterials were used with ASCs to help stimulate
vascular smooth muscle repair. These scaffolds with stimulation improved ASC viability
and differentiation towards smooth muscles cells improving their utility in vascular tissue
engineering applications. Electrical stimulation systems may be a means to enhance
differentiation for tissue engineering applications [40]. Alternatively, decellularized
porcine small intestinal submucosa with human ASCs have been shown to be an effective
biological scaffold for hernia repair in rat models. Treating the scaffold with fibronectin
before ASC seeding improved ASC attachment and histology demonstrated the presence
of the stem cells in the scaffold up to one month post-op [44]. The retention of stem cells
in these living scaffolds makes them attractive candidates for long-term sustainable
regenerative therapies.

2.2 ASCs as Immunomodulators

2.2.1 ASCS as Therapy for autoimmune diseases

Lymphocytes are integral cells of the immune system. More specifically, CD4+ helper T (Th) cells can be divided into subsets that distinguish the effector cells, Th1, and Th2 cells, and the regulatory T cells (Tregs) that maintain the balance between autoimmunity and immune tolerance, respectively. These effector T cells play a critical role in promoting autoimmune diseases, especially Th1 cells, which have been further delineated to include Th17 cells. Secreted pro-inflammatory cytokines by Th1 and Th17
cells perpetuate antigen-specific responses. During autoimmunity, Th1 and Th17 aberrantly recognize self-antigens and drive an immune response that propagates a cascade of pathologic events [45-47].

The immunomodulatory capacities of ASCs have been investigated to regulate the Th1/Th2 balance and promote Tregs to restore immune tolerance in autoimmune diseases. ASCs secrete anti-inflammatory cytokine interleukin-10 (IL-10) that enhances Tregs' activity, which responds by further secreting and amplifying IL-10 signaling [48, 49]. Tregs and associated IL-10 attenuate the activities of Th1 and Th17, which, in turn, reduce the recruitment of additional pro-inflammatory immune cells to sites of pathology [47, 48]. These anti-inflammatory and immunomodulatory effects of ASCs have been demonstrated in several preclinical models of autoimmune diseases.

**Multiple Sclerosis.** Multiple sclerosis (MS) is an autoimmune disease against integral components of the central nervous system (CNS) that leads to neurodegeneration and inflammation. Using the murine experimental autoimmune encephalomyelitis (EAE) model of MS, ASCs have demonstrated attenuation of disease that leads to comprehensive improvements. Treatment with ASC before the onset of EAE led to amelioration of the disease course by robust immunomodulation that countered Th1-mediated pathology. Collectively, infusion of ASCs resulted in reductions in tissue damage, and cellular infiltrates and preservation of myelin in the CNS, which ameliorated symptoms of this disease [50-53]

**Rheumatoid Arthritis.** Another common autoimmune disease, rheumatoid arthritis (RA), is characterized by Th1-mediated tissue damage and inflammation within joints. Th17 cells have also been correlated with the production of granulocyte-
macrophage-colony stimulating factor (GM-CSF) that leads to the recruitment and subsequent infiltration of cells causing an inflammatory milieu and tissue damage [54, 55]. Using a mouse model of RA, treatment with ASCs diminished this pathogenic signaling while increased the Tregs. The production of IL-10 and generation of antigen-specific Tregs was attributed to treatment with ASC, which suggests re-establishment of immune tolerance. These studies demonstrate the immunomodulatory potency that attenuated pathogenic processes and countered autoimmunity that led to reduced incidence and severity of experimental arthritis [45, 46, 52, 55].

**Diabetes Mellitus Type 1.** Type 1 diabetes mellitus (T1DM) is caused by autoimmune destruction of insulin-producing cells of the pancreas that results in hyperglycemia and abnormal glucose metabolism due to insulin deficiency [56]. In a preclinical model of T1DM, intravenous administration of ASCs reportedly reduced fasting blood glucose levels, increased expression of insulin protein, and suppressed islet injury [57]. Another interesting approach using ASCs for treatment of T1DM led to promising results. ASCs were transduced to express insulin and subsequently transplanted into the pancreas of T1DM animals. By harnessing the anti-inflammatory effects and the production of insulin, treatment with transduced ASCs lowered blood glucose levels and decreased glucose tolerance while improving the overall appearance of the animals [58].

### 2.3 ASCs in Neurodegenerative diseases

Neurodegenerative diseases involve several pathophysiological mechanisms beyond the loss of neurons that determine the course and severity of illness, including neuroinflammation, mitochondrial dysfunction, and protein aggregation. While the brain
is thought to be immune privileged there are several cell types that mediate debris clearance and regulate the environment of the CNS. Microglia are tissue-resident macrophages, and whether polarized to the classical pro-inflammatory or alternative anti-inflammatory activation states, can fight against or contribute to the hallmarks of neurodegenerative pathology. ASCs have been gaining attention as therapeutic candidates due to their ability to secrete neurotrophic and immunomodulatory mediators, restore mitochondrial function, promote neurogenesis, modulate glial activation states, enhance protein clearance, and fight neuroinflammation in neurodegenerative pathologies. The following section will summarize recent findings on the efficacy of ASCs in treating three distinct neurodegenerative diseases: Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis (ALS).

2.3.1 Alzheimer’s disease

Alzheimer's disease (AD), the most common cause of dementia amongst the aging population, is characterized by amyloid-beta (Aβ) plaques and neurofibrillary tangles, limbic system neurodegeneration, and progressive cognitive decline [59]. Using a mouse model of familial early-onset AD, ASCs were tested for their preventive and therapeutic effects by administering therapy at pre- and post-symptomatic time points. Both groups showed improved performance on the Morris Water Maze (MWM) task, a significant reduction in Aβ plaque formation in the cortex, and reduced protein levels of Aβ and amyloid precursor protein (APP) with enhanced levels of Aβ-degrading enzymes [60]. In another AD mouse model, ASC treatment led to increased secretion of anti-inflammatory cytokines, enhanced expression of Aβ-degrading enzymes, and improved performance on learning and memory tasks [61]. Additionally, mice showed increased
brain levels of the anti-inflammatory cytokine IL-10, which polarizes microglia towards the alternative activation phenotype, and several angiogenic and neurotrophic factors [60-62].

Other studies demonstrated that ASCs might attenuate symptoms of AD by promoting neurogenesis. Intracerebral injection of ASCs gave rise to significantly higher numbers of newly generated bromodeoxyuridine (BrdU)-positive cells than vehicle-treated controls in both the dentate gyrus and subventricular zone, some of which differentiated into mature neurons [63]. One proposed mechanism for this enhanced neurogenesis is the leptin secreted from ASCs, as this hormone alone has been shown to promote neurogenesis and reduce neurodegeneration in an AD mouse model [64]. Together, these studies highlight the capacity of ASCs to promote enhanced clearance of harmful protein aggregates, stimulate neurogenesis, and modulate the neuroinflammatory environment by secreting immunomodulatory cytokines in Alzheimer's disease models.

### 2.3.2 Parkinson’s Disease

Parkinson's disease (PD) is the second most common cause of neurodegeneration and is characterized by progressive dopaminergic neuronal loss in the substantia nigra pars compacta (SNpc) and progressively worsening motor symptoms. ASCs have been investigated in PD animal models to determine if and how they may attenuate neurodegenerative damage. In a rat model of PD, ASCs injected directly into the SNpc exerted neuroprotective effects via enhanced secretion of soluble growth factors [65]. Additionally, ASC treated rats had significantly decreased numbers of activated microglia in the lesioned brain areas compared to untreated controls, suggesting that these soluble factors are also impacting the neuroinflammatory aspects of PD [65]. ASCs
also enhanced both acute and long-term neurogenesis in rat models of PD, which correlated with amplified secretion of anti-inflammatory cytokines and brain-derived neurotrophic factor (BDNF) [66]. Despite this observed benefit, ASCs were unable to protect dopaminergic neurons from acute damage, and the newly created neurons are not able to functionally replace lost dopaminergic neurons [66]. However, in a mouse model of PD long-term dopaminergic cell survival was significantly higher with ASC treatment [67].

2.3.3 Amyotrophic Lateral sclerosis

ALS is a debilitating neurodegenerative disease characterized by the rapid and progressive loss of upper and lower motor neurons, muscle wasting, and death for most patients within five years of diagnosis [68]. In recent years ASC therapies have been investigated in animal models for their potential benefit in ALS, with some successes. In ALS mouse models, both single and repeated daily injections of ASCs resulted in preserved motor neuron survival, delayed disease progression, and fewer reactive astrocytes in the spinal cord that can contribute to neuronal cell death [69, 70]. Similar results were seen when ASCs were given before symptom onset, suggesting ASCs may have preventive capability [71]. The injected ASCs persisted in the CNS in their undifferentiated state, indicating that the therapeutic benefit resulted from soluble factors secreted by the transplanted cells rather than engraftment and differentiation [71]. Several lines of evidence further support the notion that ASCs exert their beneficial effects via secretion of paracrine factors. ASCs co-cultured with ALS mouse astrocytes demonstrates increased production of angiogenic and neurotrophic factors, upregulation of a critical suppressor of glutamate excitotoxicity and inhibition of apoptotic signals [72-
When neural stem cells from ALS mice are co-cultured with ASC-derived exosomes, small vesicles secreted from the cells, aberrant protein aggregation was suppressed while imbalanced mitochondrial protein levels were restored [75].

2.4 Clinical applications of ASCs

To report on completed and ongoing clinical trials studying the utility of ASCs in the treatment of human disease, information was collected from the clinical trial database (clinicaltrials.gov) [76]. The parameters of this search included studies using "adipose stem cells" and/or "stromal vascular fraction" that were ongoing or completed and excluded trials that had been withdrawn, were terminated, were not yet recruiting, or had unknown status. The details of the clinical studies were evaluated to include reports of using the stromal/stem progenitors from adipose tissue and/or SVF, excluding duplicates and studies of other mesenchymal stem cells, i.e., BM-MSCs. As of 2018, there have been 165 clinical trials involving ASCs and/or SVF in the treatment of human disease. These studies have taken place in various fields with most of the trials focused orthopedic applications (n=39) followed by autoimmune disorders (n=26). Figure 1 details the areas in which ASCs are administered as a potential therapy in clinical trials found from the search. These clinical trials are conducted around the world with the majority taking place in the United States (n=54) followed by South Korea (n=25) and Spain (n=21).
Figure 2.1 Clinical trials utilizing ASC or SVF.
The distribution of medical areas in which clinical trials utilizing ASC or
SVF have been or are being conducted with the highest occurrence of
trials in orthopedics. The distribution of medical areas in which clinical
trials utilizing ASC or SVF have been or are being conducted with the
highest occurrence of trials in orthopedics.
3 Chapter 2: ASCs and Cancer

It is well established that the tumor microenvironment (TME) plays an important role in cancer development and progression. The TME is composed of neoplastic cells, endothelial cells, pericytes, adipocytes, fibroblasts and other connective tissue cells, extracellular matrix (ECM) components, multiple stem and progenitor cells, and a diverse array of innate and adaptive immune cells [77]. The relative composition of the TME can vary depending on tumor cell type, location within the body, and comorbid conditions [77]. Numerous studies have demonstrated that paracrine and autocrine signaling within the TME are multidirectional, influencing tumorigenicity, aggressiveness of the cancer, and resistance to therapy. For example, breast cancer cells can secrete cytokines that alter the expression profile of ASCs in the TME. Subsequently, these altered ASCs secrete various adipokines, cytokines, chemokines, and growth factors that increase breast cancer cell proliferation, ultimately forming a positive feedback loop [77]. Understanding the mechanisms behind cell-cell communication in the TME is critical to understanding the drivers of tumorigenesis and metastasis. The impact of obesity on the TME, with particular focus on the interaction between ASCs and cancer cells, is the focus of this section.

3.1 Obesity as a risk factor for cancer

Several risk factors contribute to the risk of developing cancer, one of the primary factors is obesity, which is commonly defined as a body mass index (BMI) greater than or equal to 30 Kg/m² [78]. Rates of obesity are increasing worldwide, with an estimated
641 million obese adults in 2014 [79]. Furthermore, approximately 40% of adult women and 35% of adult men are considered to be obese [5, 80]. The percent of the population that is considered to be super obese or morbidly obese (BMI>40) has increased to 5.5% for men and 9.9% for women in the United States by 2014 [80]. Obesity is identified as a risk factor for thirteen cancers including adenocarcinoma of the esophagus, colorectal cancer, multiple myeloma, ovarian cancer, liver cancer, and postmenopausal breast cancer [81].

Obesity-related alterations to adipose tissue are conducive to promoting tumor growth, invasion, metastasis, recurrence, and mortality [82]. Excess adiposity leads to tissue hypoxia, which triggers the release of hypoxia-induced factor 1-alpha (HIF-1α) and increased angiogenesis [83]. Furthermore, circulating levels and tissue-expression levels of pro-inflammatory markers increase dramatically with increasing BMI [84]. Adipokine expression levels also change significantly with increasing adiposity which leads to increased levels of leptin and decreased levels of adiponectin [84]. Numerous studies have demonstrated chronic, low-grade inflammation as the connection between obesity and tumorigenesis, as it allows for increased multidirectional crosstalk within the TME [82]. This chronic low-grade inflammation also has been shown to alter the biology of adipose stem cells (ASCs), which are an important, but understudied cell player in the TME.

### 3.2 Adipose stem cells and cancer

As described above, ASCs are multipotent, mesenchymal lineage stem cells that can differentiate into osteocytes, chondrocytes, adipocytes, myocytes, and neuron-like cells [24, 85]. Furthermore, ASCs are thought to be immune-privileged as they do not
express MHC class II or costimulatory molecules [15, 20]. MHC class II molecules are a class of major histocompatibility complex molecules important in initiating immune responses, found only on antigen-presenting cells. Although there is data in human and rodent studies indicating that allogeneic cells can elicit antibody responses in vivo demonstrating that allogeneic, not syngeneic ASCs induced antibody production [86, 87]. ASCs are potent immunomodulators in inflammatory environments and promote wound healing and regeneration [15, 20, 88]. Upon their recruitment to the TME, ASCs secrete cytokines and growth factors such as leptin, IL-6, CCL5, and PDGR [89-92].

Obesity has been shown to alter ASCs. Some biological differences in ASCs from lean or obese donors include a change in obesity-altered ASCs (obASCs) secretome to be more proinflammatory and have a decreased capability to differentiate. For example, when comparing ASCs derived from healthy weight versus obese adipose tissue donors, obASCs demonstrate an increased expression in proinflammatory factors including IL-1, IL-6, IL-12, PDGF-A, TNF-α, LIF, ICAM-1, and G-CSF [16]. Additionally, obesity-altered ASCs (obASCs) have a decreased ability to differentiate into fat compared to lnASCs [93]. Obesity also correlates negatively with ASC viability [94]. Furthermore, Cozzo et al. demonstrated that relative to lnASCs, obASCs are recruited at higher levels to breast tumors in both humans and mice [82].

3.2.1 Adipose stem cells to cancer associated fibroblasts

Obesity results in ASC hyperplasia, which alters the ASC gene expression profile to release factors that promote a chronic low-grade inflammatory state and enhance tumor growth [92, 95, 96]. After ASCs are recruited to the tumor site by soluble factors secreted by tumor cells, ASCs can be induced to transition into carcinoma-associated fibroblast-
(CAF) -like cells, and then integrate into the tumor stroma [89, 97-99]. CAFs have been shown to promote angiogenesis, ECM remodeling, inflammation, and metabolism reprogramming in the TME [99].

When ASCs differentiate into CAFs, they express alpha-smooth muscle actin (α-SMA), fibroblast activation protein alpha (FAP1), and fibroblast-specific protein (FSP). They also produce ECM proteins including collagen and fibronectin. Together, these ECM remodeling factors increase matrix rigidity, which has been shown to promote the proliferation and metastasis of breast and colorectal cancer cells [82, 100, 101]. CAFs can also secrete matrix metalloproteinases that alter the malignant potential of cancer cells, as well as secrete numerous growth factors and cytokines that promote angiogenesis [99]. Overall, numerous studies have shown that the secretory profile of CAFs results in increased invasiveness, chemoresistance, and cancer recurrence [102].

Recently, our research group demonstrated that obesity supports a more rapid conversion of ASCs to CAFs, thus enhancing the proliferative rate, phenotype, and gene expression profile of breast cancer cells [103]. Comparing ASCs derived from either lean or obese subjects (lnASCs or obASCs respectively), obASCs expressed higher levels of CAF markers at earlier time points following exposure to breast cancer cells. When co-cultured with breast cancer cells, obASCs also expressed higher levels of cytokines compared to lnASCs. Furthermore, the induction level in breast cancer cells exposed to obASCs was more robust compared to that of lnASCs. These findings provide further insight into the increased rates of morbidity, mortality, and incidence in obese breast cancer patients [104].
3.2.2 ASCs in prostate cancer

Several studies have shown that the periprostatic adipose depot contributes to prostate cancer (PCa) malignancy [105-107]. This may be due to the recruitment of ASCs from periprostatic adipose tissue to the prostate by chemokines secreted by PCa cells [82]. One study showed that PCa patients had significantly higher numbers of ASCs in periprostatic adipose tissue compared to nearby visceral tissue [105]. Similar to breast cancer, PCa cells recruit ASCs to the TME through the CXCL12/CXCR4 axis [108]. Within the PCa TME, ASCs increase vascularity and promote tumor growth through upregulation of fibroblast growth factor 2 (FGF2) [108]. Upon priming with PCa conditioned media or PCa derived exosomes, ASCs form prostate-like neoplastic lesions in vivo and aggressive tumors in secondary recipients [109]. PCa derived exosomes, containing miR-130b, induce ASC neoplastic reprogramming through upregulation of hRAS and kRAS, and the downregulation of TSG PDCD4 [109, 110].

Several studies have shown that increased adiposity plays a crucial role in the aggressiveness of PCa [111]. ASCs are found at higher frequencies in the circulation of overweight or obese PCa patients compared to lean PCa patients [105]. obASCs are also recruited to the prostate tumor stroma at higher levels than lnASCs, which is attributed to the differential secretion of CXCL1 and CXCL8 by PCa cells [112]. In cell culture models, CXCL1 and CXCL8 chemoattract ASCs by signaling to CXCR1 [112]. While CXCL8 expression is obesity independent, CXCL1 expression is obesity dependent, and CXCR1 is expressed at higher levels in obASCs than lnASCs in the tumor stroma [112]. Together, these results highlight how BMI-dependent differences in multidirectional paracrine signaling between ASCs and tumor cells influence the TME.
3.2.3 ASCs in Colon Cancer

The paracrine signaling effects of ASCs on colorectal cancer (CRC) have also been well described in the literature [113, 114]. Wei et al. demonstrated that ASCs release the cytokine IL-6 to act on CRC cells through the JAK/STAT pathway to increase sphere generation and cell growth and upregulate self-renewal gene expression [113]. IL-6 also increases VEGF production in fibroblasts, promoting angiogenesis in the growing tumor [115]. Stromal-derived factor-1 has also been shown to increase CRC cell migration, neovascularization and supported tumor growth [116]. Li et al. demonstrated that senescent ASCs have a more significant impact on CRC cell proliferation than non-senescent ASCs [114]. Senescence can induce a senescent associated secretory phenotype, which can serve as a tumor-supporting cell in the TME. One example of this is secretion of Galectin 3 from senescent ASCs which activate the MAPK pathway in the CRC cells [114]. Another group showed that neuregulin 1 via the HER3 receptor acts as a paracrine signal from ASCs that promotes CRC invasion, survival, and tumorigenesis [117].

Other studies have demonstrated that the paracrine signaling between mesenchymal cells and CRC cells is bidirectional. Chen et al. provided evidence that CRC cells induce ASCs to take on a CAF-like phenotype via the Wnt pathway [118]. Wnt levels are higher in CRC than in normal colon epithelium [119]. Although interference with the Wnt pathway has also been shown to decrease CRC proliferation in mono in vitro cell culture, the impact that Wnt has on CAF differentiation provides another means by which Wnt plays a part in CRC progression [119]. These CAFs in turn increase secretion of a variety of paracrine signals including FGF10 and VEGFC as well
as metastatic factors such as matrix metalloproteinases (MMPs) to increase the invasive and colony forming abilities of cancer cells, as well as increase tumor size [118]. Another recent study demonstrated that CRC cells induce lipolysis in adipocytes altering the metabolic pathways in the TME [120]. CRC cells take up these fatty acids resulting in the upregulation of autophagy and mitochondrial FAO via the activation of AMPK, which enhances CRC cells’ resistance to low-nutrient conditions [120].

3.2.4 ASCs in Ovarian Cancer

Ovarian cancer is a particularly troubling cancer because of its tendency to metastasize and seed the peritoneum conferring worse prognosis with a five-year survival rate of 47% [121]. Many studies have shown that ASCs promote a more aggressive metastatic phenotype of ovarian cancer and suggest that the proximity of this cancer to peritoneal and abdominal fat may increase the effect ASCs play in the TME. Ovarian cancer cell co-culture with ASCs has been shown to increase proliferation, migration, and spheroid formation through various mechanisms [122]. Kim et al. provided evidence that ASCs increase ovarian cancer migration by secreting IL-6 thereby activating the Jak2Stat3 pathway [123]. Chu et al. demonstrated that ASCs also increase MMP expression, particularly MMP2 and MMP9, which degrade the extracellular matrix (ECM) and promote invasion in ovarian cancer cells [124]. This group used a xenograft model to determine if these in vitro findings translated in vivo and found that ovarian cancer xenografts with ASCs demonstrated increased tumor metastasis and growth in the peritoneal cavity. ASCs taken from patients with omental metastasis demonstrate reverse Warburg effect, providing lactate substrate to cancer cells for oxidative phosphorylation [125]. Omental ASCs taken from patients with omental cancer metastasis also
demonstrate an increased effect on cancer cell migration as well [125]. Further, it has been shown that obesity status and fat location of ASCs also has an impact on the extent that ASCs increase ovarian cancer progression [125]. Obese and visceral ASCs have been shown to increase the growth of ovarian tumors [122]. ObASCs also increase vascularity and the number of inflammatory cells in ovarian tumors [122].

3.2.5 ASCs in other malignancies

ASCs have been shown to play a role in various other cancers such as multiple myeloma (MM), osteosarcoma (OS), cervical cancer, bladder cancer, and gastric cancer. In MM, ASCs from obese donors promoted growth and metastasis of MM cell lines. This group found that obASCs had an altered cytokine/adipokine profile, which led to increased cell adhesion and MMP2 expression in MM [94]. This work is consistent with studies evaluating the effect of ASCs on other malignancies such as breast cancer [91, 92]. Additionally, leptin stimulates proliferation of MM cells and decreases the efficacy of chemotherapies in this malignancy so it is possible that leptin from ASCs in the TME promotes MM progression [126]; however, more work is needed to identify ASC pathways and drivers of MM progression. Studies evaluating OS, a primary malignancy of bone that mostly affects children and young adults, have seen similar effects [127]. Wang et al. found that ASCs promote OS growth and metastasis through upregulation of MMP2 and MMP9 through STAT3 activation. STAT3 inhibition attenuated these effects, decreased MMP2/9 expression and prolonged survival in mice [127]. Although the upstream activators of STAT3 were not identified, STAT3 is activated by many of the aforementioned paracrine factors from ASCs. More work in OS is needed to identify secreted factors that are contributing to these outcomes. Limited studies have been done
to evaluate the effect of ASCs on cervical cancer, however one study by Tian et al. found that S100A7, described above for its role in breast cancer proliferation and metastasis, has also been shown to enhance cell migration, invasion, metastasis and the epithelial to mesenchymal transition (EMT) of cervical cancer [128]. ASC secretion of two pro-inflammatory cytokines IL-8 and IL-6, described below, was increased when co-cultured with bladder cancer cells [129]. This is believed to have played a role in the observed reduced ECM adhesion, as well as increase in the cell viability, invasion, and migratory ability [129]. Finally, ASCs have also been shown to increase gastric cancer cell growth, migration, and progression through the SDF-1/CXCR4 axis [130]. While there is limited research investigating the role of ASCs in these less common malignancies, the consistency of results across these various cancers further strengthens the need to study the role of ASCs in the TME to identify pathways through which ASCs promote tumor growth and metastasis to identify potential therapeutic targets.

3.2.6 ASCs and breast cancer

Numerous studies have demonstrated a positive correlation between adult BMI and incidence of breast cancer, especially for postmenopausal breast cancer and estrogen receptor positive (ER+) breast tumors [81]. Obesity has also been associated with poor prognosis, increased risk of recurrence, resistance to chemotherapy, and worsened outcomes for both pre- and postmenopausal women with breast cancer [131-133]. Given these statistics, it is not surprising that obesity is the leading risk factor for postmenopausal breast cancer deaths in the U.S. [134]. Studies have investigated the link between increased adiposity and breast cancer by examining the effects that ASCs have on the TME [135, 136]. Groups have shown that secretion of various factors including
leptin, insulin-like-growth factor 1 (IGF-1), and S100A7, a small calcium-binding protein, promote breast cancer [92, 137, 138]. It is clear that the secretome of ASCs strongly influences BC behavior.

Strong et al. found that leptin from obASCs promoted ER\textsuperscript{+}BC growth and metastasis compared to ASCs from donors in the healthy weight BMI category (BMI 18.5-24.9) (InASCs) and ER\textsuperscript{+}BC alone. Leptin upregulates aromatase and ER\textalpha likely targeting the estrogen axis [92].

Other secreted factors also have an impact. While breast cancer stimulated-ASCs secrete numerous cytokines to promote proliferation, a recent analysis revealed that an increase in chemokine C-X-C ligand 5 (CXCL5) was most noticeable and that neutralization of CXCL5 reversed the stimulatory effects that ASCs had on BCC proliferation [139]. Further, Sakuai et al. have demonstrated that crosstalk between these cells within the TME is promoting proliferation and migration. This group found that BCCs stimulate ASCs to secrete cytokines by upregulating the expression of S100A7 [137]. Moreover, knockdown of S100A7 significantly suppressed ASC-stimulated BCC proliferation and migration [137].

Cytokines, which are secreted by ASCs, act on BCCs through various pathways including protein kinase B (AKT), extracellular signal-regulated kinases 1/2 (ERK1/2), and Janus kinase-signal transducer and activator of transcription 3 (JAK2-STAT3). For example, BCCs enhance the secretion of stromal cell-derived factor-1 (SDF-1) from ASCs, which, in turn, binds to the chemokine C-X-C receptor 4 (CXCR4) to enhance the motility, invasion, and metastasis of BCCs [90]. CXCR4 interacts with its ligand, CXCL12, to promote breast cancer metastasis [140]. More specifically, the
CXCR4/CXCL12 signaling axis can activate the PI3K/Akt (Phosphatidylinositol-4, 5-bisphosphate 3-kinase/Protein kinase B) intracellular signaling pathway, which increases Vascular endothelial growth factor (VEGF) to induce angiogenesis and tumor progression [141].

3.3 Influential pathways in ASCs and Cancer

3.3.1 The Warburg Effect

ASCs may contribute to cancer by altering the metabolism in the TME. Cells usually produce energy from mitochondrial cell respiration and oxidative phosphorylation. However, tumors primarily use glycolysis and produce high levels of lactate even during aerobic conditions. This is known as “the Warburg effect” or aerobic glycolysis [142]. The drivers of the Warburg effect remain unclear. Recent evidence points to a reverse Warburg effect, where stromal cells increase glycolysis in the TME to produce glycolytic products, such as lactate, which are taken up by adjacent cancer cells and used for oxidative phosphorylation and increased ATP production [143]. These stromal cells provide the same glycolytic substrates such as lactate and pyruvate for oxidative phosphorylation to the cancer cells after undergoing myo-fibroblastic differentiation [144]. The reverse Warburg has been shown using omental derived ASCs and ovarian cancer and is implicated in the increased metastatic potential of ovarian cancer cells after exposure to ASCs [125].

3.3.2 Extracellular matrix

ASCs have been shown to express extracellular matrix (ECM) remodeling proteins as well as upregulate expression of these proteins by cancer cells themselves.
ECM remodeling proteins such as MMP-2, MMP-9, and TIMP-1 are associated with tumor progression and metastasis [92, 124, 127]. Specifically, MMP-2 and MMP-9 are proteolytic enzymes that digest type IV collagen, a major constituent of the basement membrane; invasion through the basement membrane is an essential step in metastasis particularly for epithelial tumors (carcinomas) [145-147]. Tissue inhibitors of matrix metalloproteinases (TIMPs) not only inhibit MMPs and can play an inhibitory role in cancer, but also have been shown to promote some aspects of cancer [148, 149]. For example, TIMPs have been shown to increase cell proliferation and promote angiogenesis [149, 150].

3.3.3 IL-6

IL-6 is a cytokine produced by ASCs implicated in tumor progression in multiple cancers, as mentioned above [123, 136]. Further, it should be noted that increased IL-6 is found in the blood serum of obese patients and correlates clinically with a poor prognosis for breast cancer patients [151]. The binding of IL-6 to its common receptor subunit gp130 activates the JAK/STAT pathway, which along with various other roles, has been shown to play an important role in cancer cell migration, proliferation, cell survival and therapeutic resistance [152-154]. This cytokine is seen to be playing a role in ASC related cancer progression [91, 155]. However, most research focuses paracrine effects of IL-6 secreted by ASCs and acting on cancer cells, an unexplored pathway in the context of cancer is the autocrine effects of IL-6 or IL-6 from other cells in the TME affecting the ASCs themselves that may change the ASC behavior in the TME.
3.3.4 Leptin

**Leptin and Obesity.** Serum leptin concentrations are directly related to the severity of obesity [156]. Hyperleptinemia contributes to the chronic low-grade inflammation observed in obesity through increases in the expression of interleukin-6 (IL-6), tumor necrosis factor (TNF-α), and reactive oxygen-species (ROS) production in adipose tissue [157, 158]. A recent study showed that ER\(^+\) breast cancer samples taken from obese patients had significantly higher leptin expression than in overweight and control samples [159]. This study also reported a statistically significant increase in the level of leptin in plasma samples from the TME of obese patients with ER\(^+\) breast cancer compared with peripheral plasma samples. Furthermore, the study found that obese ER\(^+\) breast cancer tissue overexpressed the leptin gene as compared to lean breast cancer tissue. Taken together this data indicates that increased leptin levels may contribute to the increased incidence of breast cancer in obese patients [157].

**Leptin and CCN5.** Emerging evidence suggests that CCN5 mediates leptin-dependent growth and progression of ER\(^+\) BCCs [160]. CCN5, an ECM-associated cysteine-rich protein that belongs to the CCN (Cyr61, CTGF, -Nov) family, inversely correlates with the aggressiveness of numerous cancers [161, 162]. Leptin suppresses CCN5 in ER\(^+\) BCCs through the JAK/STAT3-Akt signaling pathway, which results in leptin-induced cell viability, EMT, migration, and sphere formation [160]. Treatment of BCCs with CCN5 protein impairs these leptin-induced pathological events [160]. Thus, CCN5 depletion in ER\(^+\) BCCs could be the estrogen-independent driving force that leads to more aggressive cancer types.
**Notch/IL-1α/Leptin signaling (NILCO).** Obesity-altered adipose stem cells produce increased levels of leptin, which leads to BC tumor growth and progression [92]. Another leptin crosstalk signaling pathway implicated in cancer progression is the crosstalk between Notch, IL-1, and leptin known as Notch, IL-1, Leptin crosstalk outcome (NILCO). While NILCO has not been directly associated with ASCs in the TME because the cell derivatives of these secreted factors in the TME have not been investigated, this crosstalk signaling has been shown to promote VEGF expression as well as proliferation and migration of breast cancer [163]. Expression of NILCO factors is associated with metastasis, therapeutic resistance, and poor prognosis in cancer and it has been shown that cancer cells and ASCs crosstalk through these factors which leads to tumor progression [163-165]. NILCO has been shown to be a major crosstalk pathway linking obesity and cancer progression and has been implicated in breast, endometrial and pancreatic cancers [166-168]. NILCO can mediate cancer stemness leading to therapeutic resistance, promote tumor growth and metastasis, and as noted above play a role in tumor angiogenesis [163, 169, 170]. ASCs may be a mediator in this crosstalk signaling given that they are known to secrete both leptin and IL-1 [16, 91, 92]; however, the cellular mediators of NILCO in the TME are still under investigation.

### 3.3.5 Exosomes and miRNA

Emerging topics that also may play a role in ASC-tumor crosstalk include exosomes and miRNAs. Exosomes are microvesicles produced by cells between 30-100 nm. Exosomes act as a signaling intermediate between cells. They can contain mRNA, IncRNA, miRNA, enzymes, proteins, and other packaged cellular material that is released from a parent cell and taken up by surrounding cells, which can utilize enclosed contents.
As described above, miR-130b packaged in exosomes from prostate cancer cells has been shown to play a role in ASC-prostate tumor crosstalk [110]. Additionally, it has been shown that exosomes derived from bone marrow-derived mesenchymal stem cells promote tumor growth of gastric carcinoma cell line SCG-7901 [171]. Exosomes and their packaged contents including miRNA likely play an essential role in the crosstalk between ASCs and cancer; however, more studies are needed to thoroughly investigate this rapidly evolving field.
4 Chapter 3: Breast Cancer and Obesity

Breast cancer (BC) is the most prevalent cancer among women in the United States [4, 172]. According to the American Cancer Society, 1 in every 8 women living in the U.S. will be diagnosed with breast cancer at some point in her lifetime [4]. As of 2016, there were over 3.5 million women alive in the U.S. with a history of BC or currently undergoing BC treatment [172]. Breast cancer ranks second only to lung cancer as the leading cause of cancer death among women [172]. In 2014 alone, 236,968 women in the U.S. were diagnosed with breast cancer and 41,211 women in the U.S. died from breast cancer [173].

The relationship between obesity and breast cancer is complicated by menopausal status. Most studies have shown that premenopausal women have an inverse relationship between BMI and breast cancer [76, 174, 175]. There is data suggesting that weight gain in adulthood and central obesity (measured by waist circumference) increase the risk of premenopausal BC [176, 177]. Further, obesity is associated with increased risk of inflammatory breast cancer in both pre- and postmenopausal women [178]. Inflammatory breast cancer is the most lethal form of breast cancer. The largest effect of obesity on breast cancer has been demonstrated in postmenopausal women. Most large epidemiological studies have found that overweight and obese women are at increased risk of developing breast cancer [76, 179]. This has been found to be true across breast cancer subtypes (ER/PR+, TNBC, HER2 enhanced) and obesity associated most strongly
with postmenopausal hormone receptor positive breast cancer risk in most prospective cohort studies [180-183]. Additionally, obesity is associated with worse outcomes, increased rates of metastasis, higher TMN stage at time of diagnosis, and larger tumor size at diagnosis for all subtypes of postmenopausal breast cancer [6, 184, 185]. Obesity is a risk factor for the development of postmenopausal breast cancer for all BC subtypes. Obesity is associated with more aggressive tumors that are more likely to metastasize and less likely to respond to therapies. This leads to increased mortality rates for obese women with breast cancer [7]. However, while the population based evidence is clear, the cellular and molecular basis for these outcomes is unclear and warrants intense investigation. Here we use mechanistic studies to evaluate the obesity-mediated cellular and molecular alterations in the tumor microenvironment that promote aggressive breast cancer phenotypes to address this lack of knowledge.
5 Chapter 4: Hypothesis

Increased adiposity in obesity increases the number of adipose stem cells (ASCs) and alters their biology [186]. ASCs from obese individuals have altered cytokine signaling factors including increased IL-1, IL-6, IL-12, PDGF-A, TNF-α, LIF, ICAM-1, and G-CSF [16, 96]. We have previously reported that ASCs from obese human lipoaspirate (BMI>30) promote tumorigenesis and metastasis in ER positive breast cancer through a leptin mediated pathway. Aromatase, responsible for the biosynthesis of estrogen, is a downstream target of leptin; therefore, increased leptin production by obASCs promotes ER positive breast cancer through estrogen-mediated pathways [91, 92, 187]. In these aims, we plan to use TNBC and ER⁺ breast cancer with clinically relevant ERα mutations that lead to constitutive ERα activity. We hypothesize that obASCs promote BC metastasis through non-estrogen pathways. Additionally, we propose to evaluate the effect of obASCs on ER⁺ BC radioresistance. Obese breast cancer patients are more likely to have local recurrence of BC after whole breast radiation. We hypothesize that obASCs play a role in this radioresistance. Here we propose that obesity-associated dysregulation fosters a pro-tumor ASC that promotes metastasis of TNBC and ER⁺ breast cancer through non-estrogen pathways and promotes radiation therapy resistance through NOTCH-IL-6-Leptin networks. We hypothesize that obASCs promote tumor metastasis through non-estrogen depend pathways and promote radiation resistance in breast cancer models.
6 Chapter 5: Specific Aims

6.1 Specific Aim 1

Evaluate the effects estrogen on obesity-altered adipose stem cells’ promotion of tumor growth and progression of estrogen receptor positive breast cancer. 

Hypothesis: obASCs promote ER\(^+\) tumor metastasis through non-estrogen pathways. In this aim we will use MCF7 cells with a CRISPR/Cas9 mutation in ER\(\alpha\) (Y537S) that results in constitutive ER\(\alpha\) activity and a patient-derived xenograft (PDX) model where the patient acquired the same mutation. This is one of the most common mutations in ER\(\alpha\) seen clinically in breast cancer patients because it confers resistance to endocrine therapies. Using ER\(\alpha\) breast cancer with this mutation in the presences or absence of obASCs will allow us to determine if obASCs promote ER\(\alpha\) breast cancer tumor growth and metastasis through ER\(\alpha\) dependent mechanisms or if obASCs activate tumorigenesis and metastasis in spite of constitutive ER\(\alpha\) activity.

6.2 Specific Aim 2

Characterize the effects of lean and obese adipose stem cells on tumor growth and progression of triple negative breast cancer (TNBC) and evaluate leptin as a potential mechanism. Hypothesis: We hypothesize that obASCs will promote metastasis, but not tumor growth of TNBC through leptin signaling. We will utilize TNBC cells
lines, as well as a PDX model (TU-BcX-2K1) to evaluate the effects of lnASCs compared to obASCs on TNBC tumorigenesis and metastasis.

6.3 Specific Aim 3

Determine the effects of lean and obese adipose stem cells on radiation resistance of breast cancer and evaluate a mechanism of radiation resistance.

Hypothesis: We hypothesize that obASCs promote resistance of ER$^+$ breast cancer to radiation therapy through Notch-Leptin-IL-6 crosstalk. We will utilize ER$^+$ breast cancer co-cultured with obASCs and a cesium gamma irradiator to deliver doses of radiation and study the effects of radiation on ER$^+$ breast cancer after co-culture with obASCs and radiation to determine if obASCs have an effect on the efficacy of radiation therapy.
Chapter 6: Evaluate the effects of estrogen on obesity-altered adipose stem cells’ promotion of tumor growth and progression of estrogen receptor positive breast cancer.

7.1 Background

Breast cancer (BC) is the most common cancer and the second leading cause of cancer death in women [172]. An estimated 246,000 patients were diagnosed with new cases of BC in 2016 [172]. Among many influential factors, obesity has been shown to increase the rates of many types of cancer [188]. Studies have shown a positive correlation between adult body mass index (BMI) and the incidence of postmenopausal breast cancer, specifically ER⁺ breast cancer [188, 189]. Obesity has also been found to increase both breast cancer recurrence and mortality [190]. Obesity is defined as having a BMI of ≥ 30 kg/m² [191]. Rates of obesity have been increasing in the United States since the 1970s [192]. It is estimated that 21% of the world’s female population will be obese as of 2025, illustrating a critical need to interrogate the relationship between obesity and BC [79].

Adipose stem cells (ASCs) play a key role in the tumor microenvironment (TME). They have been shown to support angiogenesis via recruitment of blood resident endothelial progenitors and promote inflammation [193]. ASCs have the ability to increase tumor growth, as well as the motility and invasive capacity of cancer cells via
the SDF-1/CXCR4 axis [90, 194]. ASCs also induce an epithelial-to-mesenchymal (EMT) transition in cancer cells through PDGF signaling dependent manner [195]. Additionally, cancer cells can induce a change in ASCs to cancer-associated fibroblasts (CAFs), which, in turn, increase secretion of factors that further enhance tumor proliferation, invasion, and metastasis [194, 196]. Obesity increases the rate of CAF conversion, leading to enhanced proliferation and increased invasive capability of cancer cells [103].

It has previously been shown that ASCs from obese donors promote BC tumor growth and metastasis when compared to those cultured alone or with lean donor ASCs (BMI<25) (lnASCs) [197]. Studies have demonstrated that obesity-altered ASCs (BMI>30) (obASCs) increase the proliferation and tumor size of estrogen receptor positive (ER⁺) BC and increase lung and liver metastasis. Leptin, an adipokine abundantly secreted by obASCs relative to lnASCs, promotes ER⁺BC growth and metastasis by increasing expression of ERα and aromatase [92]. Previous reports show that knocking down leptin in obASCs reduces, but does not completely ameliorate the effect obASCs have on each of these processes. This suggests that obASCs may also act through non-estrogen pathways to promote ER⁺BC tumorigenesis and metastasis [197]. In this study we use an ER⁺BC cell line (MCF7) with the Y537S mutation inserted via CRISPR in ERα and a patient derived xenograft model in which the patient developed the same Y537S mutation. Y537S results in constitutive ERα activity [198]. This clinically relevant mutation that develops de novo in BC and results in BC that is resistant to endocrine therapies that target ERα [199]. By employing these genetically
modified breast cancer cells (BCCs) in our system we can study the effect obASCs have on ER⁺BC outside of the ERα-leptin signaling axis.

7.2 Materials and Methods

7.2.1 Human Subjects

All protocols were reviewed and approved by the Pennington Biomedical Research Center Institutional Review Board and all human participants provided written informed consent (PBRC #23040). Human ASCs were isolated from 12 Caucasian females (2 groups, 6 donors per group) undergoing elective liposuction procedures, as previously described [92]. The mean BMI for each of the two donor groups was as follows: Obese (32.7 ± 3.7) and Lean (22.7 ± 1.9). The mean age of the subjects for each group of donors was as follows: Obese (42.5 ± 8.9) and Lean (38.8 ± 7.0). No statistical significance in age was observed between the donor groups.

7.2.2 Cell Culture

ASCs were isolated, cultured, and characterized as previously described [92]. MCF7 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). MCF7-Y537S cells were obtained from the Department of Surgery and Cancer at Imperial College London (London, England) and cultured as previously described [200]. Cells were cultured in complete culture media (CCM), which consisted of α-minimal essential media (αMEM; Gibco; Grand Island, NY, USA), 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville GA, USA) 100 units per mL penicillin/100μg/mL streptomycin (P/S; Gibco), and 2 mM L-Glutamine (Gibco). Cells were grown at 37°C with 5% humidified CO², CCM was changed every three to four
days, and split 1:4 to 1:6 when they reached 90% confluency as previously described [92].

7.2.3 RT-qPCR

BCCs (5x10⁴) were plated in the bottom of a 6-well plate (Nunc) and six pooled donors of obASCs (BMI>30) or lnASCs (BMI<25) were seeded at a density of 5x10⁴ cells in a 0.4µm pore Transwell (Corning Inc., Corning, NY, USA). Cells were allowed to attach overnight. After 24 hours, Transwell inserts containing ASCs were transferred to wells harboring BCCs for 96 hours. RNA was isolated using Qiazol (Qiagen, Valencia, CA, USA) and an RNeasy Mini Kit (Qiagen). RNA was converted to cDNA using RT² First Strand Kit (Qiagen). RT² Profiler™ PCR Array Human Breast Cancer with RT² SYBR Green qPCR Mastermix was used to identify breast cancer genes/pathways upregulated by obASCs (Qiagen).

7.2.4 Conditioned media proliferation assay

Lean and obese ASCs plated on a 150 mm² dish were allowed to reach 70% confluence. Plates were washed with sterile PBS and medium was replaced with serum-free αMEM for 24 hours. Media was collected and filtered through a cell strainer (0.2 µm nylon mesh; Fisher Scientific, Hampton, NH, USA) to remove cellular debris. BCCs were plated at 200 cells per well of a 96-well plate in triplicate in CCM and allowed to adhere overnight. Cells were then washed with PBS and 200 µL of lean or obese ASC CM, or serum-free αMEM was added. Proliferation assay was conducted with 10% Alamar blue reagent (Invitrogen, Carlsbad, CA, USA) per manufacturer’s instructions. Proliferation quantification was done by measuring relative fluorescent units (RFU) (excitation 530-560 nm; emission 590 nm).
7.2.5 Migration Assay

CCM or 0.5 x 10^6 ASCs in CCM were plated in the bottom of a 6 well plate and allowed to adhere overnight. 0.5 x 10^6 breast cancer cells were seeded in Transwells (0.4 μm pore; Corning) and allowed to adhere overnight. After 24 hours Transwells were transferred to wells with CCM or ASCs in CCM and cultured for three days. Transwells were then fixed and stained with Crystal Violet (3% in methanol) for 30 minutes, washed with deionized water, and migrated cells were counted manually.

7.2.6 Orthotopic Xenografts

Four to six week old SCID/beige (CB17.Cg-PrkdscidLystbg-J/Crl) ovariectomized female mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Mice were divided into six groups of five animals: MCF7-Y357S only, MCF7-Y357s plus obASCs (n = 6 donors), MCF7-Y357S plus lnASCs (n = 6 donors), MCF7-Y357S plus estradiol, MCF7-Y357S plus obASCs and estradiol (n = 6 donors), and MCF7-Y357S plus lnASCs and estradiol (n = 6 donors). Where indicated, estradiol pellets were implanted subcutaneously in the lateral area of the neck (0.72 mg, 60-day release; Innovative Research of America, Sarasota, FL, USA) as previously described [92].

MCF7-Y357S cells (10^6) alone or MCF7-Y357S cells (10^6) in combination with ASCs (10^6) suspended in a total volume of 50 μL of sterile PBS were mixed with 100 μL of reduced growth factor Matrigel (BD Biosciences, Bedford, MA, USA). Cells were injected subcutaneously into the fifth mammary fat pad on both sides as previously
described [92]. All procedures in animals were performed under anesthesia using a mixture of isoflurane and oxygen delivered by nose cone. Tumor size was measured every three days using digital calipers and calculated as previously described [92]. At necropsy, animals were euthanized by cervical dislocation after exposure to CO₂. Lungs were removed and fixed in 10% neutral buffered formalin and paraffin embedded for metastatic analysis.

All procedures involving animals were conducted in compliance with State and Federal law, standards of the US Department of Health and Human Services, and guidelines established by Tulane University Institutional Animal Care and Use Committee (IACUC). All protocols were approved by the Tulane IACUC.

### 7.2.7 Patient Derived Xenografts

The PDX models used in this study WHIM20 (isolated from a patient who developed the Y537S mutation) and WHIM43 (D451G mutation) were obtained from Washington University in St. Louis. Tumor tissue was isolated and obtained in compliance with NIH regulations and institutional guidelines and approved by the Institutional Review Board at Tulane University. All animal procedures were reviewed and approved by Tulane University IACUC. SCID/beige (CB17.Cg-Prkdc<sup>scid</sup>Lyst<sup>bg-1</sup>/Crl) 4-6-week-old female mice were obtained from Charles River Laboratory. Intact tumor pieces were removed and sliced with a scalpel to 3mm x 3mm and coated with 100 uL phenol-free growth factor reduced Matrigel (BD Biosciences). In indicated groups, 10<sup>6</sup> pooled donors (n=6) of lnASCs or obASCs were resuspended in Matrigel and coated the tumor. Where indicated, estradiol pellets were implanted subcutaneously in the lateral area of the neck (0.72 mg, 60-day release; Innovative Research of America). Tumors
were implanted into the fifth mammary fat pad bilaterally under isoflurane and oxygen anesthesia delivered by nose cone and animals were given 5mg/kg/day meloxicam for three days post-surgery. Tumors were measured by digital caliper every three to four days. At endpoint (tumors reach 750-1000 mm$^3$) blood and lungs were collected for analysis. PDX derived cells were cloned out from PDX tumors.

### 7.2.8 Flow Cytometry

To identify circulating tumor cells whole blood was collected with 0.5M EDTA (Gibco). Samples were incubated with 0.008% NH$_4$CL (ThermoFisher, Waltham, MA, USA) for red blood cell lysis and washed with PBS. Cells were then blocked with 1% BSA and 1% anti-CD16/anti-CD32 (EBioscience) in PBS and stained with antibodies against HLA1 ( Invitrogen), CD24 (EBioscience), CD44 (EBioscience). Samples were analyzed with a Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA) with Kaluza software (Beckman Coulter). A minimum of 10,000 events were captured and analyzed.

### 7.2.9 Statistical Analysis

Analysis was performed using Prism (Graphpad Software, San Diego, CA, USA). All values are presented as means ± standard error. Statistical differences among two or more groups were determined by ANOVA, followed by post-hoc Tukey tests versus the respective control group. Statistical differences between two groups were performed by Student’s t-test. Statistical significance was set at P <0.05.
7.3 Results

7.3.1 Obesity-altered adipose stem cells promote metastasis but not tumor growth of breast cancer with mutant ERα

Previous studies have reported that obASCs promote growth and metastasis of MCF7 xenografts [91, 92]. We demonstrate that obASCs enhance growth of MCF7+estrogen pellet xenografts and have increased metastatic index (Fig. 7.1); however, MCF7-Y537S xenografts and WHIM20 patient-derived xenografts that have the Y537S mutation do not have enhanced growth in the presence of obASCs compared to lnASCs or control xenografts with no stem cells (Fig. 7.2A). obASCs do increase the metastatic index significantly in MCF7-Y537S xenografts with 3.30% ± 0.76 (Mean ± SEM) area occupied by metastases in the obASC group compared to 0.69% ± 0.16 with lnASCs and 1.04% ± 0.21 with control (Fig. 7.2B). Similarly, WHIM20 PDX demonstrated 2.36% ± 0.09 area occupied by metastases in the obASC group compared to 1.70% ± 0.08 with lnASCs and 0.95% ± 0.27 with control (Fig. 7.2B). We used flow cytometry to evaluate the presence of circulating tumor cells in the WHIM20 PDX model and found that mice with PDX tumors grown with obASCs had no significant difference in human (HLA1+) circulating tumor cells (CTCs); however CTCs from tumors grown with obASCs demonstrated a trend of enrichment for the breast cancer stem cell marker CD44+CD24− (Fig. 7.2C). MCF7-Y537S and WHIM20 xenografts+estrogen pellets show a similar trend to xenografts without estrogen pellets where there is no effect of obASCs on tumor growth, but obASCs promote tumor metastasis and CTCs (Fig 7.3).
Figure 7.1 obASCs promote tumor growth and metastasis of WT ER⁺ BC.
Average tumor volume of ER WT cell line MCF7 xenograft was consistent for all groups across the time course of the experiment. Evaluation of metastasis revealed increased metastatic index (percent area of lungs occupied my metastasis) of MCF7 tumors grown with obASCs with and without estrogen pellets. Caliper measurements were taken every three to four days until tumor volume reached 750-1000 mm³. Values reported are the mean (n=5 mice/group). Bars, ± SEM. *p<0.05, ** p<0.01, ***p<0.001.
Figure 7.2 obASCs promote metastasis but not tumor growth of constitutively active ERα xenograft models - MCF7-Y537S and WHIM20 PDX

A) Tumor volume was tracked over time after day of injection (Day 0). There is no change in tumor volume when BC was implanted in the presence of lnASCs or obASCs compared to BC alone. B) Area of the lung occupied by metastasis (metastatic index) was evaluated at endpoint.
Groups where BC was implanted with obASCs had higher levels of metastasis compared to BC alone or grown with lnASCs. C) Circulating tumor cells were analyzed in animals harboring patient derived xenograft (WHIM20) at endpoint using flow cytometry. There was no change in human “HLA1+” cells across groups; however analysis of circulating tumor cells enriched for the cancer stem cell marker CD44+CD24- was increased in PDX+obASCs compared to PDX alone. Caliper measurements were taken every three to four days until tumor volume reached 750-1000 mm$^3$. Values reported are the mean (n=5 mice/group). Bars, ± SEM. *p<0.05, ** p<0.01, ***p<0.001.
Figure 7.3 obASCs promote metastasis, but not tumor growth of ER MUT xenografts in the presence of estrogen pellets.
A) Average tumor volume of ER MUT tumors (MCF7-Y537S and WHIM20 PDX) over the time course of the experiment is shown. B) Metastatic index of orthotopic cell line xenograft and patient derived xenograft grown alone or with InASCs or obASCs demonstrates a trend of increased metastasis in tumors grown with obASCs. C) Circulating tumor cell analysis of PDX groups demonstrates increased human (HLA1+)
circulating cells when tumors were grown with obASCs and increased human cancer stem cells (CD44+CD24-) circulating in mice with PDX+obASCs. Values reported are the mean (n=5 mice/group). Bars, ± SEM. *p<0.05, ** p<0.01, ***p<0.001.
7.3.2 *In vitro* obASCs promote proliferation and migration of ER WT and ER MUT cells

To evaluate *in vivo* the effects we saw *in vivo*, we used conditioned media (CM) from ASCs and measured BCC proliferation over time. Secreted factors from obASCs promote proliferation of ER$^+$BCCs *in vitro*. While obASC conditioned media (CM) had a greater effect on proliferation of MCF7 than MCF7-Y537S, we saw a trending increase in proliferation when estrogen is constitutively active (Fig 7.4A). This demonstrates that obASCs exert some effect through the estrogen receptor on ER dependent breast cancer cells. CM from obASCs did significantly increase proliferation of PDX-derived cells with constitutive ER activity (Fig. 7.4A). To study the metastatic phenotype *in vitro*, we used a migration assay and saw that obASCs significantly promoted migration of ER$^+$BCCs and a PDX-derived cell line irrespective of ER WT or MUT status (Fig. 7.4B). Specifically, in ER WT BC (MCF7) an average of 60.7 cells $\pm$ 5.0 (Mean $\pm$ SEM) migrated to obASCs compared to 4.3 $\pm$ 3.8 cells to lnASCs and 10.0 $\pm$ 7.2 cells migrated to CCM. ER MUT BC cell line (MCF7-Y537S) showed a similar trend with an average of 32.3 cells $\pm$ 5.8 (Mean $\pm$ SEM) migrated to obASCs compared to 11.0 $\pm$ 2.5 cells to lnASCs and 9.7 $\pm$ 1.5 cells migrated to CCM and ER MUT PDX derived cells (33.3 $\pm$ 3.8 to obASCs, 5.7 $\pm$ 0.9 to lnASCs, and 6.7 $\pm$ 2.4 to CCM) (Fig. 7.4B). This further supports the hypothesis that obASCs are promoting metastasis in the TME outside of the estrogen-signaling axis.
Figure 7.4 In an estrogen-depleted environment, obASCs promote proliferation and migration of ER WT and ER MUT BCCs in vitro.

A) Conditioned media collected from ASCs after 24 hours promotes proliferation of ER WT (MCF7) and ER MUT with constitutively active ERα (MCF7-Y537S and PDX-derived WHIM43) cells. B) obASCs promoted increased migration of BT20, MCC1806 and MCF7, patient derived xenograft TU-BcX-2K1 derived cells through a 0.4 um membrane. Values reported are the mean of three independent experiments each performed in triplicate. Bars, ± SEM. *p<0.05, **
p<0.01, ***p<0.001.
7.3.3 obASCs upregulate more breast cancer related genes in ER WT compared to ER Mutant cells; however, obASCs up regulated SERPINE1 and ABCB1 in both ER WT and MUT

We used Qiagen RT$^2$ Profiler™ PCR Array Human Breast Cancer to determine genes and pathways altered in ER WT and MUT cells after 96-hour Transwell co-culture with obASCs. We used a cutoff of 2-fold expression change and found that obASCs upregulate 22 genes in ER WT cells (MCF7) and 9 genes were downregulated by obASCs. In comparison, ER MUT (PDX-derived WHIM43 cells) had 5 genes upregulated by obASCs and 4 genes downregulated after co-culture with obASCs (Fig. 7.5, Table 1). Of the 22 genes upregulated by co-culture with obASCs, obASCs upregulated estrogen related genes that have been shown to promote tumor growth, such as ESR2; as well as cell-cycle related genes associated with proliferation, such as CDKN1C and CDKN2A. ER WT and MUT cells upregulated 2 genes: SERPINE1 (2.51 fold in ER WT and 4.47 fold in ER MUT) and ABCB1 (6.48 fold in ER WT and 4.99 fold in ER MUT) (Fig. 7.5, Table 1). In light of our results showing that obASCs promote metastasis but not tumor growth in ER MUT BC, it is likely that these overlapping gene changes are associated with the increased metastasis we found in ER WT and ER MUT BC when exposed to obASCs. These two genes could be a common mechanism outside of the estrogen axis through which obASCs promote metastasis of ER WT and ER MUT tumors.
Figure 7.5 PCR array demonstrates that obASCs upregulate two common genes in ER WT and ER MUT cells.
PCR array demonstrates that obASCs upregulate above a cutoff of 2x: 20 BC related genes in ER WT (MCF7) cells compared to 5 genes ER MUT (WHIM43) and down regulated 11 BC related genes in ER WT compared
to 4 in ER MUT. Two genes were up regulated in both ER WT and ER MUT: SERPINE1 and ABCB1. PCR arrays were conducted (n=1) for each condition.
Table 1: PCR array fold changes of genes up regulated and down regulated by obASCs in ER WT (MCF7) and ER MUT (PDX-derived WHIM43) cells.
This table demonstrates the specific genes and fold changes in gene expression after 96-hour transwell co-culture with pooled donors of obASCs. PCR arrays were conducted with an n=1 for each condition.

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<th>Fold change</th>
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7.4 Discussion

Obesity is recognized as a leading preventable cause of cancer [201]. Obesity has also been shown to alter the way ASCs interact with cancer cells within the TME [187]. However, there is little research on obesity-specific cancer therapies that target the TME, despite the crucial role obesity-associated cytokines play in cancer development and progression. The current study aimed to reveal cross-talk between obASCs and ER\(^+\)BCCs outside of estrogen-dependent signaling, to identify novel pathways through which obesity promotes BC metastasis.

obASCs have been shown to promote tumor growth and metastasis of MCF7 through increased secretion of leptin, which upregulates aromatase and ER\(\alpha\). In this study, we show that obASCs exert effects on BC through non-estrogen signaling pathways. We found that obASCs promote metastasis, but not tumor growth of ER\(^+\)BC with mutations in ER\(\alpha\) that result in constitutive ER\(\alpha\) activity. We found that obASCs promote a metastatic phenotype \textit{in vitro}, which correlates with increased circulating tumor cells and circulating cancer stem-like cells in PDX models and increased lung metastases in cell line and PDX models. To evaluate genes and pathways commonly activated in ER WT and ER MUT cells we used an array of 84 breast cancer related genes and found that obASCs upregulated ABCB1 and SERPINE1 in both ER WT and ER MUT breast cancer.

ABCB1, also known as multidrug resistance 1 or P-glycoprotein, is an efflux transporter with an ATP binding cassett [202, 203]. Overexpression of ABCB1 in breast cancer is associated with poor response to first line chemotherapies because ABCB1 can efflux many drugs used in the treatment of breast cancer such as taxanes, anthracyclines,
and vinca alkyloids [204]. There are many different signal transduction pathways and transcription factors that can lead to ABCB1 transcription and ultimately chemoresistant tumors including: Ras [205-207], cyclic adenosine monophosphate (cAMP)/Protein kinase A (PKA) pathway [208, 209], protein kinase C [210, 211], phosphatase and tensin homologue (PTEN) [212, 213], phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway [214, 215], and p53 [216, 217]. These signaling pathways could be activated by any number of known secreted growth factors and cytokines from obASCs to upregulate ABCB1. ABCB1 is not known to play a role in the metastatic phenotype of breast cancer, but is associated with a more aggressive cancer that is drug resistant leading to worse outcomes.

SERPINE1, also known as plasminogen activator inhibitor-1, which was also upregulated in both ER WT and ER MUT BC by obASCs is associated with tumor progression and invasion [218]. SERPINE1 is an inhibitor of urokinase plasminogen activator (uPA), which is itself an extracellular matrix-degrading protease associated with cancer invasion [219, 220]. Based on its ability to suppress uPA, it was previously hypothesized that SERPINE1 would be tumor inhibitory; however, studies have now demonstrated that SERPINE1 plays a role in neoangiogenesis in the tumor microenvironment and thereby plays a role in tumor progression, invasion, and metastasis [221, 222]. SERPINE1 in keratinocytes in a wound healing environment have been deemed the “molecular switch” from proliferation to migration by Simone et al. [223]. Because obASCs upregulated SERPINE1 in ER WT and MUT breast cancer and promoted metastasis, but not tumor growth in both xenograft models and a PDX model, we hypothesize that SERPINE1 could be a key mediator in obesity-altered ASCs
promotion of metastasis. Future studies are needed to fully investigate this hypothesis as well as evaluate the factors secreted by obASCs that upregulate SERPINE1 in order to develop therapeutic strategies to block this obesity-mediated promotion of a metastatic disease.
8 Chapter 7: Characterize the effects of lean and obese adipose stem cells on tumor growth and progression of triple negative breast cancer (TNBC) and evaluate leptin as a potential mechanism.

8.1 Background

Obesity is an important risk factor for breast cancer in postmenopausal women as it increases both the incidence and also the mortality rate [1]. Overall, obese patients are diagnosed with larger primary tumors and increased incidence of lymph node metastasis for all subtypes of breast cancers [201]. Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer-related deaths in women in the United States. With obesity rates in the United States rising, an increase in obesity-associated cancers including breast, endometrial, pancreatic, and colon cancers has also been noted [2]. With the number of obese breast cancer patients rising, there is a critical need to understand the link(s) between obesity and breast cancer progression.

Triple-negative breast cancers (TNBCs) are a clinically aggressive breast cancer subtype associated with higher mortality. Patients with TNBC are likely to be younger when they develop breast cancer and are more likely to have a recurrence and metastasis within the first three years [224, 225]. TNBCs are associated with the BRCA1 mutation and there are higher rates of TNBC in African American women [4]. TNBCs
are defined by the lack of targetable receptors (estrogen receptor (ER), progesterone receptor (PR), and Her2 (EGFR2) amplification). Commonly used small molecule targeted therapies have been developed for these receptors, and are used to treat other cancer subtypes. TNBCs cannot be treated with this approach, resulting in higher rates of recurrence and metastasis and making non-TNBCs more challenging to treat with targeted therapies. Patients with TNBC have worse 5-year survival rates than patients with other BC subtypes [226]. The lack of known targetable receptors severely limits therapeutic options, which contributes to the aggressive clinical behavior of TNBCs [227, 228].

Metastasis accounts for 90% of tumor-related deaths and an estimated 20-30% of women diagnosed with invasive breast cancer will have recurrence [229]. Metastasis is driven by many different mechanisms. Epithelial-to-mesenchymal transition (EMT) is one proposed mechanism responsible not only for the acquisition of metastasis but can also contribute to resistance to chemotherapy and radiation [230]. During EMT, luminal cells lose epithelial characteristics and gain mesenchymal features, phenotypes that are based on molecular characteristics of the cells as well as cell morphology. This transition facilitates progression to a more invasive and clinically aggressive phenotype.

Obesity is as an independent modifiable risk factor for TNBC that increases the risk of disease progression [231]. The prevalence of obesity has dramatically increased over the past four decades, which has directly correlated to the increased incidence of obesity-associated cancers [232]. Obesity is defined as a body mass index (BMI) >30, and women with a high BMI have an increased incidence and mortality of breast cancer [233]. Additionally, postmenopausal women have a 20%-40% increased risk of
developing breast cancer compared to women with a BMI in the healthy weight category [3]. While the link between obesity and breast cancer has been under intense investigation, the cellular mechanisms that are responsible for this association are not fully understood.

Prior studies have determined that obesity biologically alters adipose stem cells (ASCs), a stem cell population resident in adipose tissue that is recruited to sites of inflammation including tumors [16, 96, 234]. ASCs from obese individuals (obASCs) have a markedly altered biological profile compared to those from lean individuals (lnASCs) and have been shown to produce significantly increased levels of the adipokine leptin [91, 92]. Analysis of cytokine expression demonstrated a pro-inflammatory profile in obASCs compared to lnASCs [16]. Our group has previously reported that ASCs from human lipoaspirate from obese donors (BMI>30) promote tumor growth and metastasis in ER+ breast cancer through at least in part a leptin-estrogen pathway [91, 92]. Aromatase, responsible for the biosynthesis of estrogen, is a downstream target of leptin [91, 92]. Therefore, we concluded that increased leptin production by obASCs promotes ER+ breast cancer through estrogen-mediated pathways [91, 92, 187]. The interaction(s) between obASCs and TNBC has not been fully characterized, but we hypothesize that obASCs will promote TNBCs through pathways other than estrogen.

Patient-derived xenograft (PDX) models have emerged as a novel translational tool for cancer research with the potential to more accurately recapitulate the molecular and behavioral aspects of cancer in the laboratory setting [235, 236]. Traditional in vivo models of breast cancer specifically, the use of immortalized cell lines and orthotopic xenografts, are limited by the lack of tumor heterogeneity. These models use
immortalized cell lines that have been used for years, resulting in very high passages in culture and constitute a homogenous, clonal population of breast cancer cells. The long-term culture and passaging of the immortalized cell lines results in irreversible alterations to genetic information and characteristics. Furthermore, these orthotopic xenograft cell line models do not retain tumor tissue architecture or tumor stromal components, which are crucial elements in both tumorigenesis and metastasis. The incorporation of PDX models in our research more directly facilitates the translation of our findings into clinical observations.

Although there is a known association between obesity and TNBC, we are among the first to characterize the interaction between obASCs and TNBC. Here, we interrogate this interaction and demonstrate that obesity-altered ASCs promote metastasis of TNBCs through leptin signaling which has implications for discovering novel therapeutic options in a malignancy that has an urgent need for novel targeted therapies.

8.2 Materials and Methods

8.2.1 Adipose Human Subjects

All protocols were reviewed and approved by the Pennington Biomedical Research Center Institutional Review Board. Subjects provided informed written consent (PBRC #23040). ASCs were isolated from normally discarded adipose tissue from 12 Caucasian females (2 groups, 6 donors/group) undergoing elective liposuction procedures, as previously described [91, 92]. ASCs were isolated from lipoaspirate of subcutaneous adipose tissue isolated from obese women (BMI >30) or lean women (BMI<25). Lipoaspirate was washed with phosphate buffered saline (PBS), incubated at 37°C in a rocking incubator at 100 rpm for 1 hour in 0.1% collagenase type 1 (Sigma, St.
Louis, MO, USA) and 1% powdered bovine serum albumin (Sigma) dissolved in 1ml/g tissue in PBS. Digested tissue was then centrifuged to remove lipids, primary adipocytes, and collagenase solution leaving behind the stromal vascular fraction in the cell pellet. Cells were resuspended in complete culture media (CCM), which consisted of α-minimal essential media (αMEM; Gibco; Grand Island, NY, USA), 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville GA, USA) 100 units per mL penicillin/100ug/mL streptomycin (P/S; Gibco), and 2 mM L-Glutamine (Gibco) and plated on T175 culture flasks. Media was replaced every three to four days until the cells achieved 70% confluence. At 70% confluence cells were harvested with 0.25% trypsin/1mMEDTA (Gibco) and cryopreserved in liquid nitrogen. The average BMI for the donor groups is: lnASCs (22.7 +/- 1.9; n=6) obASCs (32.7 +/- 3.7; n=6).

8.2.2 Cell Culture: ASCs

Frozen vials of ASCs were thawed and cultured on 150 cm² dishes (Nunc, Rochester, NY, USA) in 20 mL CCM and incubated at 37°C with 5% humidified CO₂. After 24 hours plates were washed with PBS and viable cells continued growing with media changes every three to four days. For all experiments, sub-confluent cells (<70% confluent) between passages 2 and 6 were used. ASCs were characterized as previously described [92]. Stable transfection of obASCs with a construct targeting leptin and a construct targeting a non-human gene as a negative control was performed as previously described [91].

8.2.3 Cell Culture: breast cancer cell lines

BT20, MDA-MB-231, MDA-MB-468, MCF7, HCC1806 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). BT20, MDA-
MB-231, MDA-MB-468, and MCF-7 cells were cultured in α-MEM CCM, and HCC1806 were cultured in RPMI (Gibco) with 10% FBS, 1% L-Glutamine, and 1% P/S as per ATCC recommendations. Cells were grown at 37°C in 5% humidified CO₂, with media changed every 3-4 days, and passaged when cells reached 80-90% confluence.

8.2.4 Conditioned media proliferation assay

ASCs were plated on a 150 cm² dish and allowed to reach 70% confluence. Then, plates were washed with sterile PBS and medium was replaced with serum-free αMEM for 24 hours. Media was collected and filtered through a cell strainer (40 µm nylon mesh; Fisher Scientific, Hampton, NH, USA) to remove cellular debris. ASC donors were kept separate and media of six pooled donors was combined for each condition. When culturing TNBC cell lines with ASC conditioned media (ASC CM), TNBC cells were plated at 200 cells per well of a 96-well plate in triplicate in CCM and allowed to adhere overnight. Cells were then washed with PBS and 200 µL lean or obese ASC CM, or serum-free αMEM was added. Proliferation assay was conducted with 10% Alamar blue reagent (Invitrogen, Carlsbad, CA, USA) per manufacturer’s instructions. Proliferation quantification was done by measuring relative fluorescence (excitation 530-560 nm; emission 590 nm).

8.2.5 Migration Assay

CCM or 0.5 x 10^6 ASCs in CCM were plated in the bottom of a 6 well plate and allowed to adhere overnight. 0.5 x 10^6 breast cancer cells were seeded in transwells (.4 µm pore; Corning) and allowed to adhere overnight. After 24 hours transwells were transferred to wells with CCM or ASCs in CCM and cultured for three days. Transwells
were then fixed and stained with 3% crystal violet in methanol for 30 minutes, washed with deionized water, and imaged. Cells were counted with ImageJ.

8.2.6 RT-qPCR

Six pooled donors of lean or obese ASCs were seeded on top of a transwell migration chamber (4µm pore) (Corning Inc., Corning, NY, USA). Breast cancer cells were plated in six well plates in CCM. Cells were allowed to adhere overnight. Transwell inserts containing ASCs were then transferred to wells with breast cancer cells, or as a control breast cancer cells were cultured alone for three days. After three days, breast cancer cells were collected for analysis. RNA was isolated with Qiazol reagent (Qiagen, Valencia, CA, USA) followed by RNeasy columns (Qiagen) and purified by DNase 1 (Qiagen). VILO cDNA synthesis kit (Invitrogen) was used to synthesize cDNA from 1 µg of cellular RNA. Quantitative real time PCR was performed using EXPRESS SYBR Green qPCR SuperMix (Invitrogen). All qPCR data was calculated and reported as the ΔΔCt values that were normalized to the control group for quantitative comparison of mRNA expression levels.

8.2.7 Orthotopic xenograft model

SCID/beige (CB17.Cg-Prkdc<sup>scid</sup>Lyst<sup>bg-1</sup>/Crl) female mice (4-6-week-old) were obtained from Charles River Laboratory (Wilmington, MA, USA). All protocols involving animals were conducted in compliance with State and Federal law and approved by Tulane University Institutional Animal Care and Use Committee (IACUC). Mice were divided into three groups, with five animals per group: BT20 alone, BT20 with six pooled donors of lnASCs , or BT20 with six pooled donors of obASCs . Cells
(1x10⁶ per injection) were suspended in 50ul of PBS and 100ul phenol free growth factor reduced Matrigel (BD Biosciences, MA, USA) and injected bilaterally into the fifth mammary fat pads. Animals were anesthetized with isoflurane gas and oxygen delivered by nose cone. Tumor size was measured every three to four days using digital calipers and calculated as previously described [91]. At necropsy, tissue was collected for further analysis.

8.2.8 Tissue Histology

Harvested tissue was formalin-fixed paraffin embedded (FFPE) and sectioned at a thickness of 5 µm. For hematoxylin and eosin (H & E) staining, slides were deparaffinization and rehydrated and stained with hematoxylin and eosin (Thermo Scientific). For immunohistochemistry, tissue was deparaffinized and rehydrated with Histochoice through descending grades of alcohol to water. 1x citrate buffer pH of 6 (Sigma) was used for heat-mediated antigen retrieval. Tissues were blocked with 1% BSA in TBS-T at room temperature for 30 minutes in a humidified chamber and stained with primary antibodies against Ki-67 (Abcam, Cambridge, UK) diluted 1:200 in 1% BSA in TBS-T or CD31 (Abcam) diluted 1:50 1% BSA in TBS-T overnight in a humidified chamber at 4°C. Sections were washed with TBS and incubated with HRP conjugated secondary for one hour at room temperature in a humidified chamber. ImmPACT DAB reagent (Vector Labs, Burlingame, CA, USA) was used per manufacturer’s instructions to for colorimetric reaction. Slides were washed with PBS and counterstained with hematoxylin or light green. Sections were then dehydrated through ascending grades of alcohol to water and cover slipped using Permount Mounting Medium (Fisher Scientific). Quantification of Ki67 percent positivity was
assessed using ImageScope (Aperio, Vista, CA, USA). Metastatic lesions were quantified by determining the area of tissue occupied by metastasis divided by total area of tissue and represented as metastatic index.

Double label immunofluorescence staining was performed on paraffin-embedded tissue sections according to the standard protocol of LSUHSC Molecular Histopathology and Analytical Microscopy Core. Briefly, paraffin-embedded tissue sections were deparaffinization in xylene, re-hydration through descending grades of alcohol to water, non-enzymatic antigen retrieval in citrate buffer, and then washed by PBS and followed by blocking. First primary antibody was added to the tissue sections and incubated overnight at room temperature, rinsed in PBS and a fluorescein-conjugated secondary antibody (1:200 dilution; Invitrogen) was added and incubated for an hour in the dark. After washing with PBS, a second primary antibody was added overnight, followed by rinsing with PBS and incubation with a second rhodamine-tagged secondary antibody (1:200 dilution; Invitrogen) for an hour in the dark. Primary antibodies included mouse monoclonal antibodies against Carnitine Palmitoyltransferase 1, (CPT1) (Abcam), mouse monoclonal against CD44 (Abcam) and rabbit polyclonal CD36 (Abcam). Sections were then washed in PBS and analyzed using a Confocal Microscope (Olympus FV1000).

8.2.9 Patient-derived xenograft model

The PDX model used in this study, TU-BcX-2K1, was derived from the biopsy specimen of an African-American patient that had node negative invasive ductal carcinoma at the time of biopsy. Tumor tissue was obtained through the Louisiana Cancer Research Consortium (LCRC) Biospecimen Core in compliance with NIH regulations and institutional guidelines and approved by the Institutional Review Board at Tulane
University and LCRC. All animal procedures were reviewed and approved by Tulane University IACUC. SCID/beige (CB17.Cg-Prkdc<sup>sci1Lyst<sup>bg-1/Crl) 4-6-week-old female mice were obtained from Charles River Laboratory. Intact tumor pieces were removed and sliced with a scalpel to 3mm x 3mm and coated with 100 uL phenol-free growth factor reduced Matrigel (BD Biosciences). In indicated groups, 10<sup>6</sup> pooled donors (n=6) of lnASCs or obASCs were resuspended in Matrigel and coated the tumor. Tumors were implanted into the fifth mammary fat pad bilaterally under isoflurane and oxygen anesthesia delivered by mask and animals were given 5mg/kg/day meloxicam for three days post-surgery. Tumors were measured by digital caliper every three to four days. At endpoint (tumors reach 750-1000 mm<sup>3</sup>) blood, lungs, and tumor were collected for analysis.

8.2.10 Flow Cytometry

To identify circulating tumor cells whole blood was collected with 0.5M EDTA (Gibco). Samples were incubated with 0.008% NH<sub>4</sub>Cl for red blood cell lysis and washed with PBS. Cells were then blocked with 1% BSA and 1% CD16/CD32 in PBS and stained with antibodies against HLA1 (Invitrogen), CD24 (EBioscience), CD44 (EBioscience), CD326 (EBioscience), CD11b (EBioscience), CD86 (EBioscience), CD206 (EBioscience). Samples were analyzed with a Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA) with Kaluza software (Beckman Coulter). A minimum of 10,000 events were captured and analyzed.
8.2.11 Statistical analysis

All data are represented as mean ± SEM. Analysis of Variance (ANOVA) was used for comparison of three groups with Tukey post-hoc analysis. Statistical significance was set at p<0.05. Analysis was performed using GraphPad Prism software.

8.3 Results

8.3.1 ASC secretome enhances proliferation and migration of TNBCs

Adipose stem cells secrete numerous immunomodulatory and growth factors [16]. We have previously reported that obASC conditioned media enhanced the proliferation of the ER\(^+\) breast cancer cell line MCF7 [92]. To evaluate the effect of the ASC secretome on TNBC proliferation TNBC cell lines (BT20, HCC1806, MDA-MB-468, MDA-MB-231) were cultured in ASC conditioned media (CM) from both lnASCs and obASCs and compared to TNBC proliferation rates in serum free (SF) medium as a control. BT20 cultured with obASC CM produced 4683 ± 579 relative fluorescent units (RFU) with Alamar blue on day four compared to 3237 ± 378 (mean ± SEM) with lnASC CM and 739 ± 41 RFU with SF medium (Fig. 8.1). ASC conditioned media similarly stimulated proliferation of TNBC cell lines: HCC1806, MDA-MB-231, and MDA-MB-468 (Fig. 8.1).
Figure 8.1 Secreted factors from adipose stem cells promotes proliferation and migration of breast cancer.
Conditioned media collected from ASCs after 24 hours promotes proliferation of MDA-MB-231, MDA-MB-468, BT20, HCC1806 TNBC cell lines and TU-BcX-2K1 derived cells. Values reported are the mean of three independent experiments each performed in triplicate. Bars, ± SEM. *p<0.05, ** p<0.01, ***p<0.001. *-obASC vs. control, #-lnASCs vs. control.
Additionally, a migration assay was performed in a transwell system with stem cells plated in the well and cancer cells seeded in the transwell insert. The non-invasive ER\(^+\) breast cancer (MCF7) and Basal-A TNBC (BT20 and HCC1806) cell lines had significantly increased migration to the obASCs (BT20: 131.3 ± 10.9 migrated cells) compared to lnASCs (BT20: 40.9 ± 5.4) or CCM (BT20: 42.5 ± 4.3) (Fig. 8.2). TNBC PDX-derived cells from TU-BcX-2K1 migration was also evaluated. PDX cell migration was significantly increased to obASCs (31.2 cells ± 8.6; mean ± SEM) compared to lnASCs (16.2 cells ± 8.6) or CCM (0.3 cells ± 0.2) (Fig. 8.2). These data indicate that the obASC secreted factors promote a migratory breast cancer phenotype.
Figure 8.2 Secreted factors from adipose stem cells promote migration of breast cancer.

obASCs promoted increased migration of BT20, MCC1806 and MCF7, patient derived xenograft TU-BcX-2K1 derived cells through a 0.4 um membrane. Values reported are the mean of three independent experiments each performed in triplicate. Bars, ± SEM. *p<0.05, ** p<0.01, ***p<0.001.
8.3.2 obASCs alter the gene expression profile of luminal/basal-A breast cancer cell lines

TNBC cell line BT20 was co-cultured for 96 hours with lean or obesity-altered ASCs using transwells and changes in expression of mRNA of genes associated with breast cancer progression was quantitated using RT-qPCR. The genes represent pathways such as epithelial-to-mesenchymal transition (EMT), inflammatory signaling, and cancer stem cells (CSCs). The genes analyzed were: MYC, OBR, UPAR, AP2, DICER, CFOS, DROSHA, JUND, SMAD3, SERPINE-1, MMP2, IL-6, TWIST1, SNAI2, ACTA2, CDH1, CDH2, VIM, ZEB1, ZEB2, FRA1, PLAU, SNAI1, PTGS2, CCL2, CCL8, CCL5, TGFB, CXCl0, CCL17, CD14, CD24, CD44, CD90, CD133, ALDH1A. Transwell co-culture with obASCs resulted in significant increase mRNA expression of many of these genes. Expression of three genes involved in metastasis (SERPINE1, SNAI2, and TWIST1) was significantly up regulated in BT20 and expression was also evaluated in non-invasive breast cancer cell lines HCC1806 and MCF7 as well as TU-BcX-2K1 PDX-derived cells. Specifically, obASCs up regulated the expression of Serpine1 3.79 fold ± 0.18 in BT20 over control while lnASCs increased expression 1.56 fold ± 0.02. TWIST1 was also up regulated 6.86 fold ± 1.08 with exposure to obASCs while expression in BT20 after culture with lnASCs was 0.79 fold ± 0.02 of control. ObASCs up regulated SNAI2 expression 3.30 fold ± 0.34 over control while expression in BT20 after culture with lnASCs was 0.86 fold ± 0.04 of control. Exposure of PDX-derived cancers cells to obASCs resulted in the increased expression of Serpine1 8.61 fold ± 0.19 over control while lnASCs did not significantly affect fold change of Serpine1 (1.08 times ± 0.06). Similarly, obASCs increased expression of TWIST1 2.60 fold ±1.40 and SNAI2 6.11
fold ±1.15 over control PDX derived cells. Obesity-altered ASCs similarly increased expression of these genes in MCF7 and HCC1806 while ASCs from lean adipose tissue did not promote expression of these genes (Fig. 8.3).
Figure 8.3 Transwell co-culture of breast cancer cells with obASCs increases expression of metastatic genes. TNBC cell line BT20 gene expression was screened for changes in epithelial to mesenchymal transition (EMT), inflammatory, and cancer stem cell (CSC) genes after 96-hours of transwell co-culture (shown in heat map). Selected genes (Serpine1, TWIST1, and SNA12) were evaluated across four cell lines: BT20, HCC1806, MCF7, and TU-BcX-
2K1 PDX-derived cells. Values reported are the mean of three independent experiments each performed in triplicate. Bars, ± SEM. *p<0.05, ** p<0.01, ***p<0.001.
8.3.3 Obesity-altered ASCs do not enhance tumor growth but promote metastasis of TNBC orthotopic xenograft

The TNBC cell line, BT20, was used in an orthotopic xenograft model in SCID/Beige immunocompromised mice. BT20 tumors grown in the absence of ASCs were compared to tumors grown with pooled donors of lnASCs or obASCs (n=6). No significant differences in tumor growth were observed over time across all of the groups (Fig. 8.4). Immunohistologic analysis of tumors at endpoint revealed no differences in proliferation of tumor cells as quantified by Ki-67\(^+\) cells (Fig. 8.4). Additionally, immunostaining for CD31\(^+\) as an indicator of mature endothelial vasculature revealed no differences in angiogenesis at the termination of the study across tumor groups (Fig. 8.4). H & E staining of the harvested lungs was used to evaluate tumor metastasis. There was a significant increase in the metastatic index, defined as the area of the lung occupied by metastases, in mice with BT20 tumors grown in the presence of obASCs while mice with BT20 tumors with lnASCs showed no significant change from BT20 tumors (Fig. 8.5). Together, these data from the orthotopic xenograft experiment show that obASCs, promote metastasis of a basal-A TNBC cell line, but do not affect tumor growth, proliferation or angiogenesis.
Figure 8.4 TNBC orthotopic xenograph shows no difference in tumor growth, Ki67+, and CD31+ with obASCs
Average tumor volume of TNBC cell line BT20 xenograft was consistent for all groups across the time course of the experiment. Histologic analysis of tumors at end point revealed that stem cells had no effect on proliferation (ki67+ cells) or angiogenesis (CD31+ blood vessels). Values reported are the mean (n=5 mice per group). Bars, ± SEM. *p<0.05, **p<0.01, ***p<0.001.
**Figure 8.5 obASCs promote metastasis of BT20 TNBC cell line xenografts**

Evaluation of metastasis revealed increased metastatic index (percent area of lungs occupied by metastasis) of BT20 tumors grown with obASCs. H&E staining of lungs shows metastases of various sizes in all three groups. Values reported are the mean (n=5 mice per group). Bars, ± SEM.

*p<0.05, ** p<0.01, ***p<0.001.
8.3.4 TNBC patient-derived xenograft tumor growth is unaffected by obASCs; however, obASCs promote increased circulating tumor cells and metastasis of TNBC PDX

To confirm our findings in a pre-clinical translational model, a PDX model, TU-BcX-2K1, established at Tulane University that represents an invasive ductal carcinoma was used (Fig. 8.7A). TU-BcX-2K1 intact PDX tumor pieces were implanted bilaterally into the mammary fat pads of SCID/Beige mice coated in growth factor reduced Matrigel alone, or with pooled donors (n=6) of lnASCs or obASCs resuspended in growth factor reduced Matrigel. Evaluation of tumor volume over time revealed the ASCs did not have any effect on the growth of TU-BcX-2K1 (Fig. 8.6A). Histological analysis of tumors revealed no change in proliferation as measured by Ki67+ cells or angiogenesis as measured by CD31+ vessels (Fig. 8.7B). The effects of ASCs on metastasis were examined based on the presence of circulating tumor cell (CTC) populations in peripheral blood and histologic analyses of lungs harvested from the implanted TU-BcX-2K1 experiment. Flow cytometric analysis of blood for CTCs revealed a significant increase in HLA+ “human” circulating cells in mice with PDX tumors coated with obASCs (Fig. 8.6B). The frequency of breast cancer CTCs as indicated by CD44+CD24− was also significantly increased from PDX tumors with obASCs (Fig. 8.6B). Due to the increased levels of CTCs (CD44+CD24−) in the blood of the animals in the obASC-PDX group (Fig. 8.6B), the expression of CD44 in tumor sections was analyzed using immunohistochemistry. CD44 positive cells also co-expressed fatty acid transporter marker CD36 as well as CPT1, the rate-limiting enzyme of fatty acid oxidation (FAO) pathways (Fig. 8.7B). We also evaluated the frequency of myeloid cells in circulation and
found that PDX tumors with obASCs stimulated the myeloid population in these mice and there was an increase in both M1 (CD11b+CD86+) macrophages and M2 (CD11b+CD206+) macrophages in circulation (Fig. 8.8B). Additionally, we evaluated the expression of the epithelial cell adhesion molecule (CD326) because this protein is down regulated during EMT. Analysis of the PDX tumors from the obASC group demonstrated depletion of CD326, but a significant increase in CD326+ CTCs in the blood (Fig. 8.6A, Fig. 8.8B). The migration of CD326+ cells from the tumor into the circulation taken along with our in vitro data suggests that obASCs are promoting EMT. Next, the frequency of metastases was evaluated to determine whether the increase in CTCs correlated with increased metastasis. A significant increase in the metastatic index was observed in the lungs of the mice with TU-BcX-2K1 TNBC PDX grown with obASCs compared metastasis from tumors with lnASCs or PDX alone (Fig. 8.6A, Fig. 8.8A).
Figure 8.6 Adipose stem cells do not affect tumor growth of TNBC PDX, but obASCs increase metastasis

A) Average tumor volume of TNBC PDX TU-BcX-2K1 was consistent for all groups across the time course of the experiment. Evaluation of metastasis revealed increased metastatic index of PDX tumors grown with obASCs in comparison to lnASCs or PDX only. B) Flow cytometric analysis of the blood demonstrates increased circulating HLA1+ “human cells” in the blood of the obASC group. There is an increase in cancer stem cell marker CD44+CD24- in the CTCs from the obASC group. There is an increase in circulating CD326+ (epithelial cell adhesion molecule) cells in the obASC group. Caliper measurements were taken every three to four days until tumor volume reached 750-1000 mm³. Values reported are the mean (n=5 mice/group). Bars, ± SEM. *p<0.05, ** p<0.01, ***p<0.001.
Figure 8.7 TU-BcX-2K1 PDX model.
A) Primary human TNBC was surgically removed and implanted with matrigel into SCID/Beige female mice (n=5 per group). SCID/Beige mice lack B cells, T cells and functional natural killer cells. At passage seven tumors pieces were implanted with lnASCs, obASCs, or PDX alone. B) Histologic analysis CD44+, CD36+, and CPT1 is shown. Analysis of immunostaining of tumors at end point revealed
that ASCs had no effect on proliferation (Ki67+ cells) or angiogenesis (CD31+ blood vessels). Values reported are the mean (n=5 mice/group). Bars, ± SEM. *p<0.05, ** p<0.01, ***p<0.001.
Figure 8.8 Flow cytometric analysis of PDX tumor shows obASCs promote EMT and increase classically activated macrophages and alternatively activated macrophages.

A) H&E staining of lungs shows metastases of various sizes in all three groups. B) PDX tumors were digested using 0.01% collagenase to a single cell suspension and cells were analyzed using flow cytometry. Tumors had no significant change in cancer stem cell enrichment marker CD44+CD24-, but tumors from the obASC group showed decreased expression of CD326 (epithelial cell adhesion molecule). Analysis of myeloid cells in circulation revealed that obASCs increase the circulating myeloid cells (CD11b+). obASCs
significantly increase classically activated macrophages (CD11b+CD86+) and alternatively activated macrophages (CD11b+CD206+) cells. Mean values are represented (n=5 mice/group) Bars, ± SEM. *p<0.05.
8.3.5 Leptin knockdown via shRNA abrogates obASC promotion of metastatic potential of breast cancer

It has been shown that obASCs produce higher levels of leptin and that leptin promotes growth and metastasis of ER$^+$BC through upregulation of aromatase and ER$\alpha$ [91, 92]. We hypothesized that leptin, produced at elevated levels by obASCs, is a key molecule that promotes the metastatic phenotype observed in these TNBC cell lines and PDX model. An shRNA strategy was used to knockdown leptin expression in obASCs, and a non-human gene as a negative control shRNA construct for control obASCs, as previously described [91]. RT-qPCR was used to demonstrate the reduced leptin expression as a result of stable transfection of obASCs with leptin shRNA (Fig. 8.9).
**Figure 8.9 shRNA knockdown of leptin in obASCs.**
Leptin expression in control shRNA obASCs versus leptin shRNA obASCs was compared to evaluate knockdown efficiency. Values reported are the mean of three independent experiments each performed in triplicate. Bars, ± SEM. *p<0.05, **p<0.01.
Migration assays were performed in transwells in which TNBC lines were co-cultured with leptin shRNA knockdown ASCs. Quantification of breast cancer cell migration to leptin shRNA obASCs compared to control shRNA obASCs demonstrated that breast cancer had decreased migratory ability when leptin was knocked down in obASCs (Fig. 8.10).
Figure 8.10 Leptin secreted by obASCs promotes migration of BCCs
Leptin shRNA obASCs decreased migration of BT20, HCC1806 and MCF7 breast cancer cell lines through a 0.4 um transwell membrane. Values reported are the mean of three independent experiments each performed in triplicate. Bars, ± SEM. *p<0.05, **p<0.01, ***p<0.001.
Evaluation of gene expression changes in breast cancer cells after transwell co-culture with leptin shRNA obASCs reduced the increased expression of various EMT genes increased by obASCs. ObASCs increased Serpine1 expression in BT20, MCF7, HCC1806 and 2K1 PDX-derived cells 2.02 ± 0.14, 4.63 ± 0.30, 5.78 ± 0.12, and 7.72 ± 0.11 fold respectively. The gene expression fold change in these cells after transwell co-culture with obASCs with leptin shRNA was 0.04 fold ±0.0043 (BT20), 0.19 ± 0.0013 (MCF7), 2.87 ± 0.52 (HCC1806), and 1.30 ± 0.08 (2K1 PDX). Other metastasis-related genes demonstrated similar trends, including TWIST1, CCL5, CD90, PTGS2, and IL-6. (Fig. 8.11).
Figure 8.11 shRNA knockdown of leptin in obASCs decreases pro-metastatic gene expression in breast cancer cells.

RT-qPCR of breast cancer cells after three days of transwell co-culture with control shRNA obASCs or leptin shRNA obASCs shows an increase in expression of CCL5, CD90, PTGS2, and IL-6 after co-culture with obASCs that is abrogated by leptin shRNA in the obASCs. Values reported are the mean of three independent experiments each performed in triplicate. Bars, ± SEM. *p<0.05, **
p<0.01, ***p<0.001.
Finally, our group used TU-BcX-2K1 TNBC PDX with control shRNA obASCs and shLeptin obASCs to evaluate if leptin from obASCs promoted TNBC metastasis in vivo. Evaluation of tumor growth revealed that obASCs and shLep obASCs did not affect tumor growth compared to PDX alone. When tumor volume reached 750-1000 mm$^3$ animals were sacrificed and circulating tumor cells were evaluated using flow cytometry and metastases were quantified. We found that obASCs promoted increased percentage of HLA1$^+$ “human” circulating tumor cells compared to mice with control tumors and tumors with shLep obASCs. Additionally CTC evaluation of mice with PDX tumors with obASCs demonstrated a trend of an increased percentage of human cells enriched for the cancer stem cell marker CD44$^+$CD24$^-$ compared to mice with control tumors and tumors with shLeptin obASCs. This demonstrates that leptin from obASCs promotes increased circulating tumor cells in a TNBC PDX model. Finally, we evaluated the metastatic index of the lung in animals with TU-BcX-2K1 tumors along, with obASCs or with shLep obASCs, which is calculated as percent area of the lung occupied by metastases and found that obASCs significantly increased the metastatic index compared to control tumors and tumors grown with shLep obASCs (Fig. 8.12).
Figure 8.12 Leptin knockdown abrogates pro-metastatic effect of obASCs in TNBC PDX model.
Evaluation of tumor growth over time after implantation demonstrates that there is no difference in tumor growth of TU-BcX-2K1 grown alone or in the presence of obASCs or shLEP obASCs. Evaluation of metastatic index demonstrates that obASCs promote metastasis of TNBC PDX model compared to both control and shLeptin tumors. Evaluation of circulating tumor cells reveals that there is an increased percentage of HLA$^+$ circulating tumor cells when tumors are grown with obASCs.
compared to control and shLep obASC groups. Additional evaluation of cancer stem cell markers on circulating tumor cells reveals a trend of an increased percentage of circulating cancer stem-like cells in groups of TNBC PDX compared to control and shLep obASCs. Mean values are represented (n=5 mice/group) Bars, ± SEM. *p<0.05.
8.4 Discussion

Studies focused on TNBC because it is very aggressive, highly metastatic, and is associated with obesity. The incidence of TNBC in obese women is higher than that in non-obese women [237]. Additionally, obese TNBC patients are reported to have larger tumor size, grade and stage [237]. These TNBC lacks targetable receptors for treatment with targeted therapies; therefore, discovery of novel therapies for TNBC is necessary. TNBC can be further categorized into luminal and basal-like subtypes, with basal being more clinically aggressive and conferring a worse prognosis. Luminal TNBC is characterized by the expression of cytokeratins 7 and 8 and increases in many hormonally regulated pathways, whereas basal TNBC is characterized by expression of cytokeratins 5 and 6, and high epidermal growth factor receptor expression [238, 239]. Basal subtypes are subclassified as basal-A and basal-B, with basal-B being more invasive and mesenchymal-like [240]. In this study we used less aggressive, basal-A TNBC established cell lines and a luminal ER$^+$ cell line MCF7 for comparison and observed that obesity-altered ASCs promoted a more aggressive metastatic phenotype in these cells through leptin-mediated pathways.

Previous studies demonstrate that ASCs resident in adipose tissue are recruited to the tumor and promote tumor progression through proteases, pro-angiogenic factors, and adipokine secretions [91, 92, 125, 234, 241]. In this study, the impact of obesity-altered ASCs on TNBC was investigated. Here we found that CM from lean or obesity-altered ASCs promoted BC proliferation, which suggests that the factors secreted by ASCs signal with the TNBC cells to promote
proliferation in vitro. However, in vivo tumorigenesis experiments with a basal-A TNBC cell line and TU-BcX-2K1 TNBC PDX indicated that the ASCs had no effect on tumor growth. This outcome is different from what has previously been reported on tumor growth of ER+ breast cancer and obASCs, where obASCs promoted tumor growth of ER+ breast cancer xenografts. We posit that this observation was the result, in part, of the fact that TNBC at baseline is more proliferative and aggressive than ER+ breast cancer and the non-estrogen pathways that promote tumor growth in ER+ breast cancer are already activated in TNBC.

While obASCs failed to promote tumor growth of TNBC xenograft and PDX tumor growth, the same cells promoted metastasis of both in vivo. Circulating tumor cell (CTC) analysis in our PDX model revealed a significant increase in human circulating cells (HLA1+) enriched for breast cancer stem cell marker (CD44+CD24-). This increase in circulating human CSCs correlated to enhanced metastases seen when the PDX were co-implanted with obASCs. Evidence supports the role of cancer stem-like cells in tumor metastasis [242-245]. We evaluated PDX tumors for CSC markers through IHC. We found fatty acid transporter, CD36 and the rate-limiting enzyme in fatty acid oxidation, CPT1 expression within CD44+ cells, which have been identified as subpopulations of metastasis initiating cells. Recent studies also suggest the role of extracellular lipid uptake and increased FAO in CSCs [246-248]. Altogether, these data support the finding that ObASC-PDX tumor bearing mice had increased metastasis with elevated circulating CSCs. This is significant because metastasis accounts for 90% of tumor related deaths in breast cancer patients. The promotion of EMT and
a metastatic phenotype in TNBC by obASCs could explain, at a cellular level, the worse prognosis and outcomes for obese patients with TNBC that is seen on an epidemiologic level. Evaluation of potential mechanisms that obASCs employ to promote a metastatic phenotype was performed based on expression changes of EMT, CSC- and inflammation-associated genes and migration assays. We found that obesity-altered ASCs but not their lean counterpart promoted EMT and a metastatic phenotype.

ASCs are known to be a source of leptin, a growth factor, in adipose tissue. It has previously been shown that obASC produce much higher levels of leptin than lnASCs [91, 92]. Previous reports have demonstrated leptin’s ability to promote many cancer signaling pathways including phosphatidylinositol 3–kinase protein kinase B (PI3K/AKT), mitogen-activated protein kinase (MAP-K), and Janus Kinase 2-Signal transducer and activator of transcription 3 (JAK2/STAT3) pathways that promote tumorigenesis and metastasis [91, 249-253]. Singh et. al. have previously shown that leptin upregulates expression of Serpine1 in vascular epithelial cells [254]. Serpine1 is a serine protease inhibitor shown to play a role in cancer metastasis through binding of vitronectin leading to detachment of cancer cells from the extracellular matrix [255]. Here we show leptin produced by obASCs increases expression of Serpine1 in TNBC promoting a metastatic breast cancer phenotype. Additionally, obASCs promote increased expression of IL-6. IL-6 is a pro-inflammatory cytokine that is associated with tumor progression [256, 257]. SNAI2 (snail family zinc finger 2) expression is a gene implicated in EMT progression [258]. Leptin treatment of breast cancer cells
in vitro has been shown to increase SNAI2 expression [249]. Elevated levels of Serpine1, IL-6 and SNAI2 correlate with the prometastatic phenotype of breast cancer cells induced by obASCs in vitro and the increase in metastatic lesions in the lungs of mice with TNBC tumors grown with obASCs.

In this study, obASC stably transfected with a leptin shRNA construct were used to interrogate the mechanism by which obASC-derived leptin alters TNBC biology [91]. We previously reported that obASCs co-cultured with BC cells promotes a mesenchymal and metastatic cell phenotype based on up-regulation of select EMT genes; stable knockdown of leptin through shRNA abrogated these effects. These data suggest that leptin enhances EMT in TNBC through up regulation of TWIST1, SERPINE1, SNAI2, IL-6, PTGS2, CCL5 and CD90. Beyond gene expression alterations, in a transwell migration assay we found that knockdown of leptin in obASCs decreased the migration of BC that was stimulated by obASCs. These data suggest that the high levels of leptin produced by obesity-altered ASCs promote metastasis of TNBC through the up-regulation of the expression of multiple factors that promote cancer cell migration and metastasis.
9 Chapter 8: Obesity-altered adipose stem cells promote estrogen receptor positive breast cancer radiation resistance through paracrine signaling

9.1 Background

Breast cancer (BC) is the most prevalent cancer and the second most fatal in women after lung cancer in women, claiming approximately 40,000 lives every year [173, 259]. BC prognosis has improved over the past two decades due to better screening methods and improving therapies; however, challenges associated with standard therapy remain unresolved. Radiotherapy (RT) has been a treatment for many malignancies, including breast cancer, for over a century and has been shown to substantially increase patient survival [173]. The main challenges associated with this treatment are damage to the surrounding normal tissue and radioresistance, characterized by the unresponsiveness of some cancer cells to RT. Obesity has been implicated as a risk factor in RT resistance, metastasis, and overall poor prognosis in breast cancer [260-262]. Furthermore, moderate to severe obesity increases the likelihood of BC recurrence and BC-specific mortality [262, 263]. Obesity may promote resistance to RT through metabolic dysregulation and altered signaling axis [260]. Women who receive whole breast RT were 12.6% more likely to have regional recurrence after 5 years if they had two or more risk factors for breast cancer, including obesity [264]. Obese breast
cancer patients do not respond as well to standard of care when compared to healthy weight patients. However, the mechanism(s) through which obesity alters the tumor microenvironment (TME) leading to radiation resistance have yet to be determined. It was hypothesized that obesity-altered adipose stem cells (obASCs) may be a key player in the TME player that mediates obesity driven radiation resistance.

Adipose stem cells (ASCs) are mesenchymal lineage stem cells resident in fat tissue that are known to be recruited to sites of inflammation, including tumors [15, 234]. In inflammatory environments, ASCs secrete paracrine factors such as: adipokines, cytokines, and growth factors to promote regeneration and wound healing [15, 16]. ASCs play an important role in the TME and can lead to more aggressive tumors. Obesity results in chronic low-grade inflammation of adipose tissue, which alters the biology of resident ASCs [93, 265]. ASCs from obese individuals (BMI>30) secrete more proinflammatory cytokines and leptin [16, 92, 266]. Studies have demonstrated that obASCs from obese individuals (BMI>30) have been shown to promote proliferation and metastasis of breast cancer compared to ASCs from healthy weight donors (BMI<25) (InASCs) or breast cancer alone in vitro and in vivo [91, 92]. obASCs promote estrogen receptor positive breast cancer (ER⁺BC) through leptin upregulation of ERα and aromatase [91]. Additionally, increased secretion of leptin from obASCs promotes triple negative breast cancer (TNBC) metastasis through upregulation of epithelial-to-mesenchymal transition genes [91, 92]. In this study the role obASCs play in ER⁺BCCs response to RT. These studies have focused on ER⁺BCCs, because they
account for approximately 80% of breast cancer cases [267]. We hypothesize that these factors could play a role in ER\(^+\)BCCs response to RT such that obesity-altered ASCs will promote the RT resistance in ER\(^+\)BCCs. Investigation of this interaction will provide evidence of altered stromal cell biology within the TME that promotes RT resistance and demonstrates on a cellular and molecular level the signaling pathways driving increased mortality and decreased efficacy of standard treatment seen in obese BC patients.

9.2 Materials and Methods

9.2.1 Cell culture

Breast cancer cells (BCCs) MCF7, T47D, and ZR-75 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in estrogen deprived complete culture media (CCM) made up of phenol free \(\alpha\)-MEM (Gibco, Grand Island, NY, USA) with 10% heat inactivated charcoal dextran stripped FBS (Atlanta Biologicals, Flowery Branch, GA, USA), with 1% L-glutamine (Gibco), and 1% anti-anti (Gibco). Cells were grown at 37°C in 5% humidified CO\(_2\) and media was changed every 2-3 days. Cells were passaged when plates reached 70-80% confluence.

ASCs were cultured on 150 cm\(^2\) dishes (Nunc, Rochester, NY, USA) in DMEM-F12 (Gibco), 10% lot tested heat inactivated FBS (Hyclone Laboratories, Inc., Logan, UT, USA), 1% L-glutamine (Gibco), and 1% anti-anti (Gibco). Cells were incubated at 37°C with 5% humidified CO\(_2\). Media was replaced every 2-3 days. Cells were passaged when plates reached 70% confluence. shLeptin ASCs
were made as previously described [91]. For all co-culture experiments obASCs and BCCs were cultured in estrogen depleted phenol free media. Where indicated, cancer cells were treated with 20ng/mL humanized recombinant IL-6 (ThermoFisher, Waltham, MA, USA), 20 ng/mL of humanized recombinant IL-1α (BioLegend, San Diego, CA, USA) or 0.1 µg/mL IL-6 neutralizing antibody (R&D Systems, Minneapolis, MN, USA).

9.2.2 Co-culture studies

5x10⁴ BCCs were plated in the bottom of a 6-well plate (Nunc) and six pooled donors of obASCs (BMI>30) or lnASCs (BMI<25) were seeded at a density of 5x10⁴ cells in a 0.4µm pore transwell (Corning Inc., Corning, NY, USA). Cells were allowed to attach overnight. After 24 hours, transwell inserts containing ASCs were transferred to wells harboring BCCs cells for 96 hours. ER⁺BCCs were then irradiated at (2, 5, 10 Gray (Gy)) using a Nordion Gammacell40 Cesium gamma irradiator (0.692 Gy/min). BCCs were then co-cultured with ASCs for 24 hours. BCCs were then collected for analysis.

9.2.3 Cell Cycle Analysis

MCF7 were seeded at a density of 5x10⁴ in a 6-well plate (Nunc) and six pooled donors of 5x10⁴ obASCs (BMI>30) or lnASCs (BMI<25) were seeded in a 0.4µm pore Transwell (Corning) and allowed to adhere overnight. BCCs were serum starved for 24 hours. Media was replaced with CCM and Transwells containing ASCs were introduced to BCCs for 24 hours. BCCs were then harvested and cell suspensions were pelleted and washed twice with PBS. Beckman Coulter Cell Cycle Kit was used to measure quantity of DNA on a
Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA) and modeled on ModFit LT Software (Verity Software House, Topsham, ME, USA).

**Live cell imaging**

Cytation5 (BioTek, Winooski, VT, USA) was used for live cell imaging. Cells were stained with DCFDA (Sigma-Aldrich, St. Louis, MO, USA) for oxidative stress, CellEvent Caspase 3/7 green detection reagent for apoptotic index, and purified anti-phosphorylated human γ-H2A.X (Ser139) (BioLegend) with Hoechst 33342 (ThermoFisher) as a counterstain for double stranded DNA breaks. Cell counting was performed within the Cytation5 Software (BioTek).

**9.2.4 Survival Fraction**

Viable BCCs after radiation were counted using trypan blue (Gibco). Clonogenic assay was set up and cultured in CCM for 14 days at which time the plates were washed with PBS (Sigma) and fixed and stained with 3% crystal violet in methanol (Sigma-Aldrich) for 30 minutes at room temperature on a rocker. Excess stain was removed with DI water and colonies were counted manually (minimum size 200 cells/colony). Survival fraction was adjusted for plating efficiency. Survival fraction was calculated as described by Munshi et al. [268].

**9.2.5 Xenograft Model**

Ovariectomized SCID/beige (CB17.Cg-Prkdc<sup>scid</sup>Lyst<sup/bg-1</sup>/Crl) female mice (4-6-week-old) were obtained from Charles River Laboratory (Wilmington, MA, USA). All protocols involving animals were conducted in compliance with State and Federal law and approved by Tulane University Institutional Animal Care and
Use Committee (IACUC). Animals were anesthetized with isoflurane gas and oxygen delivered by nose cone for tumor implantation. Mice were divided into four groups, with five animals per group: unirradiated MCF7, unirradiated MCF7 that had been exposed to obASCs for 96 hours via transwell co-culture, MCF7 after 2 Gy of radiation, MCF7 that had been exposed to obASCs for 96 hours via transwell co-culture after 2 Gy of radiation. Cells (1x10^6 per injection) were suspended in 50µl of PBS and 100µl phenol free growth factor reduced Matrigel (BD Biosciences, MA, USA) and injected bilaterally into the fifth mammary fat pads. 17β Estradiol 60 day slow release pellets (Innovative Research America, Sarasota, FL, USA) were implanted in between the scapula of all animals. Tumor size was measured every three days using digital calipers and calculated as previously described [91].

9.2.6 RT-qPCR

BCCs were collected and RNA was isolated with Qiazol reagent (Qiagen, Valencia, CA, USA), and purified with RNeasy columns (Qiagen) followed by treatment with DNase 1 (Qiagen). One µg of RNA was collected from each sample for further cDNA synthesis using VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). The cDNA then underwent RT-qPCR with EXPRESS SYBR Green qPCR synthesis kit (Invitrogen). RT-qPCR was performed using Bio-Rad CFX96 C1000 Thermocycler (Bio-Rad, Hercules, CA, USA) Fold change (2^{−ΔΔCT}) values were calculated and RT-qPCR results were graphed in GraphPad Prism (La Jolla, CA, USA).

9.2.7 Mammospheres
After co-culture and a 2 Gy dose of gamma radiation BCCs were lifted and plated in ultra-low attachment surface cell culture plates (Corning) in mammosphere media (DMEM/F-12 (Gibco) + 10% B27 (Gibco) + 10µg/mL EGF and FGFβ (Invitrogen) added every 3 days). Spheres per well were counted after 7 days.

**9.2.8 Statistical analysis**

All values are presented as means ± standard error of the mean (SEM). Analysis of Variance (ANOVA) with Tukey post-hoc analysis was used to determine statistical differences among three or more groups. Statistical significance was set at p<0.05. Data was analyzed using GraphPad Prism software (GraphPad).

**9.3 Results**

**9.3.1 Obesity-altered ASCs promote radiation resistance of ER⁺BCCs lines**

Time-lapse imaging of MCF7, ZR-75, and T47D cells lines alone or co-cultured with six pooled donors of lnASCs or obASCs and gamma irradiated at a dose of 2 Gy demonstrates that ionizing radiation (IR) induces apoptosis more efficiently in BCCs alone compared to BCCs that had previously been transwell co-cultured with ASCs. Further, obASCs conferred the largest radioprotective effect (Fig. 9.1).
Figure 9.1 Decreased apoptosis of BCCs co-cultured with obASCs before radiation.
Quantification of time-lapse imaging for 10 hours after co-culture and radiation demonstrates decreased apoptotic index (CellEvent fluorescent green positive cells) of ER$^+$ BCC lines after radiation if co-cultured with obASCs. Breast cancer cell (BCC) lines and ASCs were co-cultured via transwell for 96 hours. BCCs were irradiated at a dose of 2 Gy and then live cell time-lapse microscopy was used to quantify apoptotic cells after radiation. Hours 0 and 10 are shown.
To evaluate the tumorigenic potential of the viable cells after radiation, the survival fraction of MCF7 was assessed at increasing doses of gamma radiation ranging from 0 Gy, 2 Gy, 5 Gy, and 10 Gy doses. The data indicate that viable BCCs after transwell co-culture with obASCs had a significantly increased survival fraction after radiation compared to BCCs alone at both 2 Gy and 5 Gy of radiation exposure and a trend of higher survival at 10 Gy and BCCs co-cultured with lnASCs had significantly increased survival at 5 Gy compared to control (Fig. 9.2).
Figure 9.2 obASCs increase the survival fraction of MCF7
Survival fraction of MCF7 after co-culture and radiation revealed increased survival fraction at 2 Gy and 5 Gy of cells co-cultured with obASCs.
To evaluate the relapse potential of MCF7 co-cultured and irradiated at 2 Gy, an in vivo tumorigenic assay was performed by injecting irradiated MCF7 cells that had been cultured in isolation or together with obASCs into ovariectomized immunocompromised mice. MCF7 cell exposed to obASCs prior to irradiation displayed significantly increased tumorigenic potential and tumor weight at endpoint compared to MCF7 that were irradiated, but not co-cultured (Fig. 9.3). Additionally, tumor growth and weight at endpoint of MCF7 co-cultured with obASCs and irradiated showed comparable tumorigenicity to non-irradiated cells controls or non-irradiated co-cultured cells (Fig 9.3).
Figure 9.3 obASCs are radioprotective for ER$^+$ BCC

*In vivo* tumorigenesis assay demonstrated that MCF7 co-cultured with obASCs and irradiated prior to injection had increased tumorigenic potential compared to cells that were not co-cultured before radiation. Tumor weight at endpoint demonstrates that tumors burden was larger when cells were exposed to obASCs than control. Unirradiated cells injected into the mammary fat pads reveals that MCF7 co-cultured with obASCs prior to injection did not affect tumor growth of MCF7 xenografts. Data from animal experiments represent an n=5 animals per group.
with bilateral tumors. Bars represent mean ± SEM.
9.3.2 obASCs promote radiation resistance through decreased oxidative stress in ER⁺BCCs and promoting a higher percentage of cells in S-phase

To assess potential mechanisms of survival following IR, the extent of oxidative stress in BCCs was evaluated after co-culture and 2 Gy IR and found that co-culture with obASCs significantly reduced the oxidative stress in ER⁺BCCs (201.3±61.0 positive cells (Mean±SEM)) compared to lnASCs (1068.0±66.9) or non-co-cultured cells (1707±204.3) (Fig. 9.4A). Next the levels of DNA damage were evaluated, as this is a consequence of oxidative stress, using γ-H2AX foci/nuclei as a marker of double stranded breaks after radiation. No significant difference in double stranded DNA damage was found after co-culture and radiation across groups (Fig. 9.4A). To evaluate an alternative mechanism from protecting against DNA damage through which obASCs may be promoting radioresistance, we evaluated percentage of BCCs in the various phases of the cell cycle after co-culture with lnASCs or obASCs because S phase is associated with radiation resistance [269-271]. We cultured cells in serum free media for 48 hours to synchronize cells. Then BCCs were cultured in CCM for 24 hours or a transwell insert containing lnASCs or obASCs in CCM was introduced to BCCs for 24 hours. Cell cycle analysis was performed using PI and flow cytometry and we found that cells cultured with obASCs had significantly increased percentage of cells in S phase (44.72±2.16; mean±SEM) compared to BCCs+lnASCs (31.37±0.30) and BCCs+CCM (29.34±1.89) (Fig. 9.4B). These data demonstrate that obASCs have a radioprotective effect by increasing the percent of cells in S
phase and reducing oxidative stress in BCCs after radiation, but did not affect DNA damage in BCCs after radiation.
Figure 9.4 obASCs decrease oxidative stress in breast cancer cells after radiation but have no effect on DNA damage

A) DCFDA, which becomes fluorescent by reacting with oxidative species, was used to quantify number of cells
undergoing oxidative stress after radiation. Quantification demonstrates that there is a significant decreased in cells that were co-cultured with obASCs undergoing oxidative stress. Evaluation of double stranded DNA breaks via immunofluorescent γ-H2AX staining with Hoechst 33342 nuclear counterstain revealed no difference in DNA damage after radiation between groups. B) Cell cycle analysis using propidium iodide (PI) staining and ModFit software demonstrates percent of cells in the different phases of the cell cycle. Quantification reveals increased percent of diploid cells in S phase after transwell co-culture with obASCs. Values reported are the mean of three independent experiments each performed in triplicate. Bars, ± SEM. *p<0.05, ** p<0.01, ***p<0.001.
9.3.3 obASCs produce increased leptin, which promotes radiation resistance through IL-6 upregulation

Our team has previously shown that obASCs produce increased levels of leptin compared with lnASCs [91]. To determine if leptin from obASCs promoted survival following IR, BCCs were co-cultured with obASCs that had stable knockdown of leptin via shRNA or obASCs with control shRNA constructs. Survival fraction of MCF7 BCCs cultured alone, with obASCs, or with shLep obASCs demonstrate that obASCs confer a significant survival benefit over non co-cultured cells at 2 and 5 Gy. MCF7 co-cultured with shLep obASCs had decreased survival fraction compared to obASCs; however were still radioprotective at 5 Gy compared to MCF7 cultured alone (Fig. 9.5).
Figure 9.5 Leptin from obASCs promotes radioresistance. Stable knockdown of leptin in obASCs through shRNA abrogated the pro-survival effect of obASCs on BC after radiation (* ctrl vs. obASCs; # obASC vs. shLep obASCs).
To evaluate a potential mechanism, the expression of both IL-6 and IL-1α, which have been shown to promote stemness and radiation resistance, were assessed by RT-qPCR [272, 273]. IL-1α expression was undetectable in control and co-cultured BCCs (40 cycles, data not shown). However, a significant difference in expression of IL-6 was identified in ER⁺BCCs from three different cell lines (MCF7, ZR-75, and T47D). ER⁺BCCs were cultured alone or co-cultured with lnASCs, obASCs, or shLep obASCs. Across all three cell lines, BCCs co-cultured with obASCs demonstrated an upregulation of IL-6 expression compared to non-co-cultured control cells, BCCs cultured with lnASCs, or BCCs cultured with shLep obASCs (Fig. 9.6).
Figure 9.6 obASCs produce increased leptin, which leads to upregulation of IL-6. obASCs up regulated IL-6 expression in ER$^+$ BCCs while lnASCs and shLep obASCs did not upregulate IL-6.
Survival fraction of MCF7s treated with a physiologic dose (20ng/mL) of recombinant IL-6 prior to radiation demonstrated similar survival benefit to obASCs and had a significant increase in survival fraction at 2 and 5 Gy compared to untreated MCF7 (Fig. 9.7). Further, addition of an IL-6 neutralizing antibody to the co-cultures abrogated the survival benefit of obASCs and obASCs conferred significant survival compared to both control and obASCs+IL-6 neutralizing antibody at a dose of both 2 and 5 Gy and trended to have survival benefit at 10 Gy (Fig. 9.7).
Figure 9.7 IL-6 promotes radiation resistance.

obASCs upregulated IL-6 expression in ER+ BCCs while lnASCs and shLep obASCs did not upregulate IL-6. Cells treated with 20ng/mL of recombinant human IL-6 for three days and irradiated have increased radiation resistance. IL-6 neutralizing antibody added to co-culture with obASCs also abrogated the pro-survival effect of obASCs. Values reported are the mean of three independent experiments each performed in triplicate. Bars, ± SEM. *p<0.05, ** p<0.01, ***p<0.001.
9.3.4 Leptin from obASCs promotes a cancer stem-like phenotype through NOTCH signaling

BCCs from three separate cell lines (MCF7, ZR-75, and T47D) were cultured alone, with obASCs, or with shLep obASCs and analyzed for changes in mRNA expression of genes associated with the NOTCH signaling pathway. Genes screened include: DLL1, JAG1, JAG 2, NOTCH1, NOTCH2, NOTCH3, NOTCH4, DLL3, DLL4, CDKN1A, and WNT4. BCCs co-cultured with obASCs had significant upregulation of NOTCH signaling pathway members; however, BCCs co-cultured with shLep obASCs did not demonstrate this enrichment for NOTCH signaling. Specifically, all three ER⁺BCCs showed significant upregulation of NOTCH1 (1.45, 5.68, 8.34-fold)(MCF7, T47D, ZR-75), DLL1 (7.08, 5.89, 2.76-fold)(MCF7, T47D, ZR-75) JAG1 (2.28, 13.42, 8.63-fold)(MCF7, T47D, ZR-75) and JAG2 (15.11, 8.49, 15.16 Fold)(MCF7, T47D, ZR-75) as well as significant upregulation of other NOTCH players that are cell line depended demonstrating an overall enrichment of NOTCH signaling by obASCs that is not up regulated by shLeptin obASCs (Fig. 9.8). NOTCH signaling is associated with a cancer stem-like phenotype, therapeutic resistance, and increased mortality [274, 275].
Figure 9.8 Leptin produced by obASCs upregulates gene expression of NOTCH signaling components in ER⁺ BC.
Co-culture with obASCs up regulated NOTCH signaling pathway players; however, co-culture with shLep obASCs does not up regulate NOTCH signaling. Values reported are the mean of three independent experiments each performed in triplicate.
Bars represent mean ± SEM. (*=obASCs compared to control)
*p<0.05, ** p<0.01, ***p<0.001.
To determine if leptin from obASCs induced persistent stem-like change in BCCs, mammosphere formation was evaluated after co-culture and 2 Gy IR. Mammosphere formation is an *in vitro* assay to assess tumorigenesis potential and cancer stemness. We found that obASCs significantly increased sphere forming ability of MCF7, T47D and ZR-75 following 2 Gy IR, however BCCs co-cultured with shLep obASCs before IR showed no significant difference in sphere forming ability compared to non-co-cultured BCCs (Fig. 9.9).
Figure 9.9 obASCs promote a cancer stem-like phenotype through leptin.
BCCs have increased ability to form mammospheres after co-culture with obASCs followed by 2 Gy radiation compared to non-co-cultured cells or cells co-cultured with shLep obASCs. Values reported are the mean of three independent experiments each performed in triplicate. Bars, ± SEM. *p<0.05, ** p<0.01, ***p<0.001.
9.4 Discussion

Cancer stem cells (CSCs) are widely thought to represent the tumorigenic cell population responsible for therapeutic resistance [276-278]. CSCs and CSC-like cells, that is, those expressing stem-cell markers and displaying stem cell properties, have been observed to be chemoresistant and radioresistant in a variety of cancers [279-281]. The mechanism through which CSCs acquire this radioresistant phenotype is still a subject of active investigation [279]. Here we demonstrate that obASCs upregulate IL-6 and NOTCH signaling in BCCs in part through leptin, thereby promoting a CSC phenotype, which promotes RT resistance of ER+ breast cancer (Fig 9.10).
obASCs are recruited from normal adipose tissue to the TME where they secrete increased levels of leptin. Leptin from obASCs upregulated NOTCH signaling and IL-6 expression in ER$^+$ BCCs leading to radiation resistance.
This preclinical study is important because it demonstrates that normal tissue surrounding a tumor can affect tumor biology and ultimately ascribe therapeutic resistance. These data provide cellular evidence of leptin-IL6-NOTCH signaling from obASCs promoting radiation resistance. This provides a possible mechanism contributing to the poor response of obese women to standard therapies resulting in increased morbidity and mortality. More studies in this field are warranted; however, these data suggest development and implementation of precision therapies targeting this pathway in obese patients receiving RT for breast cancer.

CSCs promote self-renewal through activation of several pathways including NOTCH, Hedgehog and Wnt [282]. It has been reported that NOTCH drives self-renewal through Hes5 [283]. Several NOTCH receptor-ligand parings are up regulated in BCCs following radiation exposure in a dose-dependent pattern [282]. In non-small cell lung cancers, tumors with high NOTCH activity are more proliferative and radioresistant than tumors with normal NOTCH activity [164, 284]. Furthermore, patients whose tumors show high NOTCH activity have worse prognoses than those with otherwise normal NOTCH activity [164]. Increased NOTCH signaling also plays a significant role in chemoresistant pancreatic cancer, where down regulation of this same pathway has been shown to reduce the cancer’s invasiveness and partially reverse the EMT phenotype [285]. In addition to poor prognosis and association with metastasis, NOTCH is essential for angiogenesis and growth of breast cancers, a pathway in which Leptin also plays a role [163].
Leptin is an adipokine found in higher levels in obese as compared to lean individuals [286]. Leptin-induced NOTCH signaling contributes to BC proliferation, metastasis, and correlates to BC development in the context of obesity [286]. In breast and pancreatic cancer cells, leptin upregulates NOTCH receptors, ligands, and targets [286, 287]. Leptin has pro-tumorigenic effects, such as increasing cancer cell proliferation, self-renewal, angiogenesis, and survival [286, 287]. Leptin has also been shown to induce resistance to both Fulvestrant and Tamoxifen in ER⁺BCCs [286].

The broad acting inflammatory cytokine IL-6 has been demonstrated to play a key role in numerous cancers [272, 288, 289]. High levels of IL-6 have been implicated in promoting epithelial-mesenchymal transition and stem cell-like properties in cancer cells [288]. Elevated IL-6 levels in BC patients’ tumors and serum are associated with a poor prognosis [272, 290]. In addition, IL-6 promotes BC bone metastasis via the NOTCH signaling pathway [291]. IL-6 induces STAT3 activation, which, in turn, promotes proliferation, inhibits apoptosis, and contributes to radiation resistance [289]. Research shows that hormone resistant prostate cancer cells have increased IL-6 expression and activated STAT3 [289]. IL-6 overexpression is also positively linked to radiation resistance [289]. A study by Wu et al. demonstrated that IL-6 inhibition of radioreistant prostate cancer sensitized the cancer to radiotherapy [289]. Inhibition of STAT3 has also been shown to reverse the radioreistant phenotype in breast cancer cells [292].
Chapter 9: Conclusions

10.1 Summary of Aims

10.1.1 Aim 1: obASCs promotion of metastasis is not dependent on upregulation of estrogen signaling or estrogen related genes in ER+BC

obASCs promote tumor growth and metastasis of ER WT BC, and promote metastasis of ER MUT BC. These data demonstrate that there are independent pathways promoted by obASCs that affect tumor growth and metastasis. The pathways that promote tumorigenesis are ER dependent; however, in BC where these pathways are constitutively activated obASCs promote metastasis, but have no affect on tumor growth. When 84 key breast cancer related genes were evaluated in BCCs after Transwell co-culture with obASCs we found that obASCs upregulate more genes in ER WT than ER MUT cells. Interestingly, there were two genes upregulated in both cell types: ABCB1, a gene associated with multidrug resistance, and SERPINE1, a gene associated with an invasive metastatic phenotype. Future studies should aim to investigate the dependency of obASC promotion of metastasis on SERPINE1 expression as well as investigate the expression of SERPINE1 in human tumors from lean and obese women to see if these findings are translational.

10.1.2 Aim 2: obASCs promote metastasis of TNBC, but not tumor growth through leptin production
Obesity alters ASCs to acquire tumorigenic characteristics, which are then recruited to the tumor microenvironment and secrete increased levels of leptin, which results in enhanced metastasis of TNBC through leptin-mediated pathways. Here it is shown that leptin from obASCs increases expression of SERPINE1, SNAI2, IL6, TWIST1, PTGS2, which are some key players in the EMT and CSC programs in cells in MCF7, BT20, HCC1806, and TU-BcX-2K1 TNBC PDX derived cell lines. The results of these studies provide evidence on a cellular level of obesity-mediated promotion of TNBC metastasis via increased levels of leptin produced by obesity-altered ASCs. These data suggest that leptin may be a novel prognostic marker for the worse outcomes seen in clinic for obese patients with TNBC. Future studies that interrogate leptin and its downstream mediators as potential targets for precision medicine in obese TNBC patients are necessary.

10.1.3 Aim 3: Leptin from obASCs promote radiation therapy resistance for ER⁺BC

Obesity-altered ASCs (obASCs) in the tumor microenvironment (TME) promote breast cancer cells to have a stem cell-like phenotype. Stem cell characteristics result in increased proliferation, metastasis, and greater resistance to conventional therapeutic measures such as chemotherapy and radiotherapy. One well-studied pathway implicated in the stem cell phenotype is the NOTCH signaling pathway. This study demonstrates that exposure to obASCs confers significant radiotherapy resistance to BCCs via increased leptin, which upregulates the NOTCH pathway and IL-6 in BCCs resulting in a radiation resistant cancer stem-like BCCs. These data show a direct link between upregulation of IL-6 and
NOTCH signaling pathway intermediates and ER$^+$ BCC survival after ionizing radiation therapy. Obesity is a risk factor for breast cancer and for increased mortality due to worse patient responses to standard therapies. Here, evidence of a cellular intermediate and paracrine-signaling pathway that may contribute to worse outcomes for obese breast cancer patients is provided, which could be targeted with precision medicine to improve prognoses for the obese patient population.

10.2 Obesity, ASCs, and Breast Cancer

Obesity rates are reaching new extremes. The most recent data suggest that 40% of adult women and 35% of adult men are obese (defined as BMI 30-40) and the percent of the population that is “super obese” (BMI>40) has reached 5.5% for men and 9.9% for women [80]. The prevalence of obesity has dramatically increased over the past four decades and has directly correlated to the increased incidence of obesity-associated cancers. Specifically, obese women have an increased incidence and mortality of breast cancer. Postmenopausal women have a 20%-40% increase in risk of developing breast cancer compared to normal-weight women [3]. With obesity rates rising and obesity being defined as a risk factor for the development of postmenopausal breast cancer, it follows that we will see an increase in obese breast cancer patients. This is noteworthy because obese breast cancer patients do not respond as well to the current standard of care for breast cancer, are more likely to have metastatic disease, and ultimately are more likely to die from their cancer. This demonstrates the critical need for studies, like the above described, that investigate obesity-alterations in the tumor microenvironment that alter tumor biology and tumor response to standard
therapies.

Increased adiposity in obesity increases the number of ASCs and alters their biology [186]. ASCs from obese individuals have altered cytokine signaling factors including increased IL-1, IL-6, IL-12, PDGF-A, TNF-α, LIF, ICAM-1, and G-CSF [16, 96]. ASCs are recruited to tumor sites where they secrete cytokines and growth factors that enhance tumor growth and progression [187]. In these studies, we have demonstrated that obesity-mediated dysregulation of ASCs fosters a pro-tumor ASC that promotes metastasis of TNBC and ER⁺BC and promote radioresistance of ER⁺BC. These data are consistent with previous studies that have described that the activation of ASCs in the presence of breast cancer contributes to pathogenesis [136, 293].

These results demonstrate that leptin is a key mediator of the biological alterations seen in BC exposed to obASCs. Studies from our group have demonstrated that obASCs secrete an increased level of leptin. Here we have shown that knockdown of leptin in obASCs abrogates the increased metastasis promoted by obASCs in TNBC xenografts. Additionally, obASCs have been shown to upregulate IL-6 and NOTCH signaling leading to a radioresistant, cancer stem-like phenotype in breast cancer. When leptin is knocked down in obASCs, upregulation of these genes and radiation resistance is not seen. The mechanism through which leptin is exerting these effects is an important future direction worthy of investigation, because leptin is known to signal through various signaling pathways, but the mechanism through which leptin promotes metastasis is not directly known and may have wide implications for a variety of cancers.
10.2.1 Leptin in Cancer.

According to a recent analysis of many different cancer types, increased leptin and leptin receptor (LEPR) expression is associated with worse prognosis, increased morbidity, mortality, metastasis, and cancer recurrence in patients in many types of cancer including bladder, lung, breast, prostate, testicular, ovarian, large B-cell lymphoma, mesothelioma, pancreatic, kidney, colorectal, liver, acute myeloid leukemia, and thyroid [294]. LEPRs are also less abundant in benign or normal tissues than in tumor tissues [295].

Leptin is one factor through which ASCs may influence breast cancer progression. Leptin is an adipokine secreted in ASCs that has been shown to induce proliferation in ER$^+$ breast cancer cells from the MCF-7 cell line in vitro [92]. Leptin expression is more robust in obASCs compared to lnASCs and has also been shown to significantly increase in the presence of estrogen in vitro [92]. When leptin expression was inhibited in vitro in either lnASCs or obASCs co-cultured with the MCF-7 line, breast cancer cell invasion was significantly reduced only in the obASC group [91]. This suggests that leptin may induce ER$^+$ breast cancer cell proliferation through an estrogen-mediated leptin-response pathway, and that leptin reduction may reduce the aggressiveness of ER$^+$ breast cancer in obese patients.

Leptin indirectly increases estrogen synthesis and estrogen receptor alpha (ER$\alpha$) activity by upregulating the expression and activity of aromatase in ER$^+$ breast cancer cells (BCCs) [250]. Activation of ER$\alpha$ increases estrogen-induced proliferation of ER$^+$ BCCs [296]. Leptin also enhances ER$^+$ BCC proliferation by
inducing the expression of Janus kinase/signal transducer and activator of transcription-3 (JAK/STAT3) and extracellular signal-related kinases 1 and 2 (ERK1/2) [159, 297]. Leptin can upregulate E-cadherin expression in ER+ BCCs as well, which promotes tumor cell proliferation and homotypic tumor cell adhesion [298]. Moreover, beta-catenin, a subunit of E-cadherin, is required for leptin-induced EMT in BCCs [299]. Leptin can also stimulate angiogenesis in ER+ BCCs by increasing the expression of VEGF [300]. Leptin can also promote lymphangiogenesis by upregulating VEGF-C expression in chondrosarcomas [112]. Moreover, this study showed that leptin-induced VEGF-C is mediated by the FAK (Focal adhesion kinase), PI3K and Akt signaling pathway [158]. Lastly, leptin has been shown to increase cell viability by suppressing apoptosis in BCCs [160]. At this point we are unable to say which pathway(s) is utilized by leptin to promote a pro-metastatic microenvironment. Future word is needed to understand this relationship.

10.3 Future directions

The complexity of the tumor microenvironment with biochemical and cellular crosstalk is complicated; however, it is important that we do not underestimate the role of tumor-supporting cells in tumor development, metastasis, and relapse. These data, consistent with the literature, demonstrate pathways implicated in ASC promotion of BC metastasis and radiation resistance: leptin, SERPINE1, NOTCH, and IL-6 signaling. Studies evaluating the role of ASCs in numerous different tumor types highlights the common pathways activated by ASCs that are tumor supporting. Here we have highlighted several important
pathways ASCs are known to upregulate to promote tumorigenesis and/or progression, including the role that stromal cells can play in the Warburg effect in the TME and alter the metabolic function of cancer cells. Further, ASCs promote ECM remodeling and increased production of MMPs by cancer cells, which enables cancer cells to migrate and invade through the ECM of origin and metastasize to other areas. ASC-derived exosomes promote tumor growth and package miRNAs that have been shown to promote tumor growth and/or metastasis. In reviewing the literature regarding ASCs and cancer, we see emerging pathways implicated across tumor type including metabolic changes, leptin signaling, and IL-6 signaling. There is surmounting evidence that leptin can promote tumor growth and metastasis through various signaling pathways and it is known that ASCs altered by obesity produce higher levels of leptin than ASCs from lean adipose tissue. This results in greater activation of these pathways and ultimately a greater promotion of cancer from obASCs, which correlates to the increased incidence of obesity-related cancers and worse outcomes for obese cancer patients.

With evidence that ASCs promote cancer through the pathways presented above, the next steps are to develop strategies to block these interactions. To develop therapeutic targets against the ASC-tumor crosstalk, there is a need for research applying existing therapies that target these pathways to block the tumor promotion driven by the ASC secretome. Additionally, there is still much left to be characterized regarding ASC promotion of breast cancer. We should continue to expand the scope of inquiry to include emerging, but less well-characterized,
signaling molecules such as miRNAs and exosomes whose roles have not been fully evaluated to date.
11 List of publications


Matossian MD, Burks HE, Bowles AC, Elliott S, Barnes VT, Sabol RA,

12 Biography

Rachel A. Sabol was born in Bozeman MT. She is the oldest child of Renee T. Sabol and Joseph W. Sabol II. Laurel is her only sibling. Rachel received her bachelors in Cell and Molecular Biology with a minor in Spanish from Tulane University, completing this degree in three years. She received a Masters in Neuroscience before beginning her MD/PhD program also at Tulane University. She joined the lab of Bruce A. Bunnell in June 2016, who supported her doctoral studies in conjunction with the Physician Scientist Program. She will return to complete the final two years of medical school in May before perusing a residency in Radiation Oncology.
13 References

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