The Age-Dependent Characterization of the ER-Alpha Positive Breast Cancer Tumor Microenvironment

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THE AGE-DEPENDENT CHARACTERIZATION OF THE ER-α⁺ BREAST CANCER TUMOR MICROENVIRONMENT

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural Mechanical College
In partial fulfillment of the
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in

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by

Katie Marie Hamel
B.S., Louisiana State University, 2011
March 2021
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To my parents, who have made countless sacrifices to ensure I have every opportunity to pursue and accomplish my goals. With their unwavering support, they instilled a strength in me to never relinquish my dreams and to continue even during trying times.

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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................... ii

ABSTRACT ........................................................................................................................................ iv

CHAPTER 1. BACKGROUND AND SIGNIFICANCE ........................................................................ 1

CHAPTER 2. CHARACTERIZATION OF YOUNG ASCs .............................................................. 21

CHAPTER 3. INVOLVEMENT OF STROMAL AGE IN BREAST CANCER ESTROGEN SIGNALING ......................................................... 29

APPENDIX A. SUPPLEMENTAL TABLES ................................................................................... 44

APPENDIX B. PERMISSION REQUESTS .................................................................................. 46

REFERENCES ............................................................................................................................... 51

VITA ............................................................................................................................................... 80
In disease states such as cancer, endocrine and paracrine signals from adipose tissue contribute to cancer progression and drug resistance. Young individuals diagnosed with estrogen receptor-alpha positive (ER-α+) breast cancer have an observed increase in resistance to endocrine therapies. This suggests that an alternative estrogen signaling pathway is active within these tumors. Despite this, the effects of stromal age on the endocrine response in breast cancer is not well known. Here, we review and highlight the involvement of the stromal age in both tumorigenesis and physiological wound healing.

To identify specific differences between young and aged ER-α+ breast tumors, RNA sequencing data was obtained from The Cancer Genome Atlas (TCGA). Analysis revealed enrichment of matrix and paracrine factors in young (<40 years old) patients compared to aged (>65 years old) tumor samples. Analyzing cell infiltrate of young and aged ER-α+ breast tumors revealed significant differences in several immune cell populations, however, there were no differences in MSC and adipocyte infiltrate between young and aged tumors. Based on these results, we next sought to determine if stromal cells exhibited age-dependent differences. To determine if the age of tumor stroma differentially regulated the ER-α+ tumor microenvironment (TME), adipose-derived stem/stromal cells (ASCs) from healthy young and aged donors were evaluated for alterations in matrix production and paracrine factors. Results demonstrated that young and aged ASCs were neither phenotypically different, nor did they demonstrate alterations in matrix production. Analysis of paracrine factors demonstrated that young and aged ASCs had differences in pro-inflammatory cytokines. Paracrine factors from young ASCs enhanced the ER-α regulated genes progesterone receptor (PR) and stromal-derived factor 1 (SDF-1) in the MCF-7 ER-α+ breast cancer cell line. Additionally, western blot analysis demonstrated increased activation of p-ER ser167 in the MCF-7 cell line treated with young ASC paracrine factors. These results are important in understanding the mechanisms of estrogen receptor signaling in young breast cancer patients, as well as unveiling underlying factors that contribute to the unique TME in young breast cancer patients.
CHAPTER 1. BACKGROUND AND SIGNIFICANCE

1.1 Breast Cancer and Tumor Microenvironment

Breast cancer is a heterogeneous disease that includes several biological subtypes that exhibit different clinical behaviors and treatment responses\(^1\). These tumors express the estrogen receptor (ER) and/or progesterone receptor (PR). Together, these comprise the luminal subtype and represent approximately \(\sim 70\%\) of all breast cancer cases\(^2, 3\). Hormone receptor positive breast cancer can be further subdivided into luminal A and luminal B. Luminal A accounts for 30-40\% of all invasive breast cancers, whereas luminal B accounts for 20-30\%\(^4\). Other subtypes of breast cancer include human epidermal growth factor receptor 2 (HER2) enriched and basal-like/triple negative (Table 1.1). The presence of PR in breast tumors is an important indicator for endocrine therapy responsiveness and ER function\(^5, 6\).

Table 1.1. Classification of molecular subtypes of breast cancer. Table reproduced from data from Fragomeni \textit{et al.}, 2018.

<table>
<thead>
<tr>
<th>Molecular Subtype</th>
<th>ER</th>
<th>PR</th>
<th>HER 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>Positive</td>
<td>And/or Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Luminal B</td>
<td>Positive</td>
<td>And/or Positive</td>
<td>Or Positive Or Negative</td>
</tr>
<tr>
<td>HER2</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Triple Negative or Basal-Like</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

1.1.1 ER-\(\alpha^+\) Breast Cancer: Biology and Treatment Options

Estrogen plays an important role in the development and progression of breast cancer. ER is one of the most significant prognostic biomarkers in breast cancer and possesses a dual role as both a nuclear hormone receptor and transcription factor. ER has two subtypes: ER-\(\alpha\) and ER-\(\beta\). ER-\(\alpha\) is present mainly in mammary glands, uterus, bone, male reproductive organs, and liver. On the other hand, ER-\(\beta\) is expressed mainly in the bladder, colon, and the immune system\(^7\). Both subtypes are expressed in adipose, prostate, and ovarian tissue. The activation of the ER-\(\alpha\) receptors are responsible for most estrogen effects on normal breast tissue and cancerous breast tissue, leading to hormone-dependent tumor growth\(^8, 9\). ER-\(\alpha\) can be activated via classical estrogen ligand-binding or through a complex network of tyrosine kinase membrane receptors and their downstream targets (\textbf{Fig 1.1}). The classical mechanism of estrogen signaling is also known as direct genomic signaling. Upon binding to
estradiol, the nuclear estrogen receptor ER-α behaves as a transcription factor and translocates from the cytoplasm to the nucleus\textsuperscript{10, 11}. The complex then binds to estrogen response elements (EREs) on or within close proximity to promoters on chromatin\textsuperscript{12}.

Figure 1.1. Estrogen receptor (ER) signaling pathway. (a) Classic ER signaling (b) The ER activation through receptor tyrosine kinases (RTKs) (c) Nongenomic functions of ER. Figure reproduced with permission from Macmillan Publishers Ltd.: Musgrove and Sutherland, 2009.

Receptor tyrosine kinases (RTKs) including epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R), and HER2 can stimulate ER translocation, thus regulating gene expression and controlling cell growth, proliferation, and survival\textsuperscript{13}. The non-genomic function of ER-α and its ability to bind to and activate transcription factors adds to the complexity of ER-α signaling. The non-genomic actions of estrogen lead to a more rapid signaling response. After the discovery of the G Protein-Coupled Estrogen Receptor (GPER1), a membrane bound estrogen receptor that induces a fast estrogen-mediated activation of extracellular signal-regulated kinases (ERKs), the mechanism of estrogen expanded beyond direct target gene transcription and protein synthesis\textsuperscript{14, 15}. The non-genomic actions of estrogen involve the activation of several protein-kinase signaling cascades including: the phospholipase C (PLC)/protein kinase C (PKC) pathway, RAS/RAF/MAPK cascade, phosphatidylinositol 3 kinase (PI3K)/AKT
kinase cascade, and cAMP/protein kinase A (PKA) signaling pathway\textsuperscript{16-21}. These signaling pathways subsequently activate transcription factors such as the nuclear factor-kappa B (NF-\(\kappa\)B) complex, which translocates to the nucleus and to their cognate DNA binding sites. Estrogen receptors can also be activated in the absence of estrogens or receptor agonists via a ligand-independent signaling mechanism\textsuperscript{22-25}. Activation occurs via phosphorylation of specific residues (i.e. serine or tyrosine) on the receptors or by association with coregulators. The coregulators encompass a variety of regulatory molecules including PKA, PKC, MAPK cascade components, inflammatory cytokines (IL-2), cell adhesion molecules (Heregulin), cell cycle regulators (RAS), and peptide growth factors (EGF, IGF-1, TGF\(\beta\))\textsuperscript{25}. ER-\(\alpha\) activation and signaling is best interpreted through ER-\(\alpha\) response genes. PR is an important target gene of ER-\(\alpha\) and thus behaves as a downstream indicator of estrogen action\textsuperscript{26}. Similar to ER-\(\alpha\), there is also extensive crosstalk between PR and signal transduction pathways that are not only required for mammary gland development, but are also often elevated in breast cancer. Historically, PR was thought to be a tightly regulated target of ER-\(\alpha\); however, studies have shown that growth factors such as IGF-1 downregulate PR activity through the PI3K/AKT/mTOR signaling pathway independent of ER-\(\alpha\) activity\textsuperscript{27}. Furthermore, the downregulation of PR activity signifies increased growth factor activity\textsuperscript{28}. The ER-\(\alpha\) independent function of PR and the complex crosstalk of multiple signal transduction pathways with ER-\(\alpha\) and PR increase the difficulty to provide targeted therapy for breast cancer patients.

In ER-\(\alpha^+\) breast cancers, active ER-\(\alpha\) signaling drives cell proliferation, thus making ER-\(\alpha\) heavily exploited as the target for treatment. Inhibitors of the ER-\(\alpha\) pathway are categorized based on their mechanism of action: 1) compounds that decrease endogenous estrogen production (aromatase inhibitors-AI and gonadotropin-releasing hormone agonists or 2) compounds that directly antagonize ER-\(\alpha\) (selective estrogen receptor modulators and selective estrogen receptor degraders-SERMs and SERDs)\textsuperscript{29}. Despite their positive therapeutic effect on ER-\(\alpha^+\) breast cancers, many of the ER-\(\alpha\) antagonists have become ineffective due to endocrine resistance. A large number of patients with ER-\(\alpha^+\) breast cancers do not respond to endocrine therapy either due to \textit{de novo} or acquired resistance\textsuperscript{13, 30-32}. As described above, many different factors including growth factors, cytokines, and estrogens may contribute to and induce ER-\(\alpha\) signaling cascades. The complexity of endocrine signaling makes it difficult to define, identify the root causes, and generate alternative treatment options. Clinical studies have described several mechanisms for the resistance to endocrine therapies\textsuperscript{33, 34}. The variance in mechanisms makes the treatment strategy increasingly difficult and dependent on the context. The impact of the tumor microenvironment (TME) plays a significant role in the development and progression of breast cancer and is a contributing source for many of signaling molecules that can modulate ER-\(\alpha\) signaling. It should be considered as a key player in the development of endocrine response and resistance in breast cancer patients.
1.1.2 The Tumor Microenvironment (TME)

The TME is a heterogeneous network consisting of different cellular types and extracellular components. The cellular and extracellular network communicates with the tumor cells, creating an intricate signaling system. The “seed and soil” hypothesis suggests that the host microenvironment (the soil) is required for optimal growth of the tumor cells (the seed)\(^{35}\). This hypothesis has driven a shift from primary focus on the tumor cells to the characterization and analysis of the interactions of the tumor stroma with the tumor cells. The communication between the host stroma and tumor cells drastically impacts tumor growth and progression. Dvorak describes tumors as “wounds that do not heal” and emphasizes the similarities of normal wound healing to tumor stromal generation\(^{36}\). Similar to the phases of wound healing, tumor stromal generation exhibits neoangiogenesis, infiltration of fibroblasts and immune cells, and remodeling of the extracellular matrix (ECM) (Table 1.2). Tumors recruit supporting cells from the local host stroma which promote ECM remodeling, cellular migration, neoangiogenesis, invasion, drug resistance, and evasion of immunosurveillance through the production of growth factors, chemokines, and cytokines\(^{37}\). Supporting cells include fibroblasts, myofibroblasts, endothelial cells, adipocytes, mesenchymal stem cells (MSCs), and various immune cells\(^{38-40}\). The infiltration of the cellular recruits is in response to tumor-driven inflammation\(^{41}\). Chronic inflammation aids in shaping the TME and has been referred to as a host reaction to the tumor; however, it is more appropriate to characterize this response as a tumor promoting reaction\(^{41}\). A contributing factor to the inflammatory nature of the tumor infiltrates is the self-induced hypoxic environment. Early in tumor development, tumor cells activate hypoxia-responsive genes, which in turn stimulate the influx of inflammatory cells into the TME. Hypoxia activates the NF-κB signaling pathway, which plays a key role in signaling of cancer cells and tumor-infiltrating leukocytes\(^{42-44}\). NF-κB activation leads to the secretion of tumor necrosis factor-alpha (TNF-α) and other pro-inflammatory cytokines that drive the expression of cytokine genes responsible for cell proliferation. In response to the pro-inflammatory cytokine cascade, tumor and stromal cells produce a wide array of biological mediators that help maintain the cell proliferation and differentiation, matrix remodeling, neoangiogenesis, and cell migration/recruitment necessary for tumor growth. MSCs, one of the supporting cell populations of the tumor stroma, are of particular interest in the development and progression of breast cancer because of their release of cytokines and growth factors that enhance the inflammatory nature of tumors.

Some MSCs, such as ASCs, have a natural proclivity to induce inflammation because their tissue of origin, adipose tissue, actively participates in physiologic and pathologic processes including immunity and inflammation. As the largest endocrine organ, adipose tissue releases a variety of pro-inflammatory and anti-inflammatory factors including the adipokines leptin (LEP) and adiponectin (ADIPOQ), as well as cytokines and chemokines such as TNF-α, interleukin 6 (IL-6), and monocyte chemoattractant protein 1 (MCP-1)\(^{45}\). Further discussion of the effect of MSCs on breast cancer development and progression is presented in section 1.2.

The TME and its stromal cell population heavily influences breast cancer initiation and growth; however, several key elements are missing from the picture. Included within this is the effect of patient demographics. Studies have examined the role of body mass
index (BMI) on the TME and breast cancer progression, but have yet to consider the role of other patient demographics including age on the nature of the TME.\textsuperscript{46, 47} The characterization of MSCs based on age and the role it plays in breast cancer development and progression has yet to be fully elucidated.

1.2 Breast Cancer and Stem Cells

As previously described in section 1.1, the tumor stroma is composed of a heterogeneous cell population that includes MSCs. The secretion of factors such as MCP-1 and SDF-1 from the tumor's cancer and inflammatory cell population recruits MSCs to the TME. After recruitment to the site of inflammation, MSCs become part of the tumor stroma and facilitate tumor growth. Studies have shown that MSCs not only stimulate tumor growth, but also promote angiogenesis and increase cancer cell invasion.\textsuperscript{48-50} Breast cancer cell-activated MSCs secrete a large panel of cytokines, chemokines, and growth factors that in turn enhance breast cancer cell proliferation in an MSC/breast cancer cell reciprocal feedback loop.\textsuperscript{51} The dynamic relationship between MSCs and cancer cells not only influences tumor growth, aggressiveness, and metastatic spread, but may also function as barrier for therapeutic intervention in breast cancer patients.

1.2.1 Adipose-Derived Stem/Stromal Cell Biology

ASCs, a subset of mesenchymal stem cells isolated from adipose tissue, have gained clinical interest due to both their abundant and readily available resources, and because of their similarity to other established stem cell lines.\textsuperscript{52} ASCs possess the ability to self-renew and differentiate toward adipogenic, osteogenic, and chondrogenic lineages in response to specific stimuli.\textsuperscript{52-55} ASCs promote damaged tissue recovery by proliferating and differentiating cells; however, their capacity to supply growth factors and cytokine to tissue repair may be of even greater importance.\textsuperscript{56} Studies have shown that ASCs are superior in secretion of bioactive factors that may stimulate cell proliferation, differentiation, and migration of cell types including fibroblasts, endothelial cells, and epithelial cells.\textsuperscript{57} ASCs release a wide range of trophic factors including chemokines, growth factors, pro-inflammatory cytokines, anti-inflammatory cytokines, and pro-
angiogenic/anti-apoptotic/anti-scarring factors\textsuperscript{56, 58} (Fig 1.2, Table 1.2). Hsiao et al. found that out of all MSC populations, ASCs were the most optimal for promoting angiogenesis and had higher levels of insulin like growth factor 1 (IGF-1), vascular endothelial growth factor D (VEGF-D), and hepatocyte growth factor (HGF)\textsuperscript{59, 60}. These growth factors have both angiogenic and anti-apoptotic properties and are associated with endothelial cell growth and survival\textsuperscript{61}. In addition to growth factors, ASCs also secrete a variety of cytokines and chemokines. Many of the secreted cytokines including interferon gamma (IFN-\(\gamma\)), IL-1\(\beta\), IL-17, IL-8, and TNF-\(\alpha\) are pro-inflammatory in nature\textsuperscript{62}. Other MSC-secreted inflammatory chemokines including MCP-1 and CCL5 promote tumor aggressiveness and support tumor-supporting effects in TME cells. Furthermore, these chemokines play a pivotal role in the growth and metastasis of cancers, including breast cancer\textsuperscript{60, 63-67}. Previous studies have shown that stromal-derived factor 1 (SDF-1), another ASC-secreted chemokine, is not only an important mediator of ASC chemotaxis,
but it is also contributes to primary breast tumorigenesis and promotes hormone independent growth of the hormone-dependent breast carcinoma cell line MCF-7. In contrast to a tumorigenic role, IL-8, MCP-1, CCL5, and SDF-1 are also targeted to improve wound healing. IL-8 acts as a potent neutrophil attractant during the early stages of wound healing. ASCs also release CCL5 and MCP-1, which recruits macrophages to the wound site. In the proliferation phase of wound healing, ASCs secrete SDF-1 to recruit more ASCs to the site, which then differentiate into endothelial cells and fibroblasts to form the granulation tissue. Similar to ASCs, “tumor wounds” secrete cytokines and chemokines that aid in myeloid cell recruitment. After reaching the tumor site, many of these myeloid cells differentiate into immunosuppressive and angiogenic phenotypes that help to facilitate tumor growth. This prevents tissue restoration and allows the tumor to remain in the proliferative phase of the wound healing process. Analogous to a tumor, the chemokine and cytokine milieu released by ASCs can also instigate the formation of a non-healing “tumor wound”. The dual nature of ASCs as both regenerative and pro-oncogenic has earned them their reputation as a “double-edged sword”. Despite their

Figure 1.2. Paracrine effects of cultured MSCs. The secretion of a broad range of bioactive molecules is now believed to be the main mechanism by which MSCs achieve their therapeutic effect and it can be divided into six main categories: immunomodulation, anti-apoptosis, angiogenesis, support of the growth and differentiation of local stem and progenitor cells, anti-scarring and chemoattraction. Figure reproduced with permission from da Silva Meirelles et al., 2009.
therapeutic potential, ASCs also pose a risk for the development and progression of tumors, as well as the recurrence of different types of cancer. Studies have shown that ASC paracrine signaling plays a crucial role in the breast cancer TME and aids in tumor progression.

1.3 Cancer and Wound Healing

1.3.1 Parallels between Cancer and Wound Healing

Aging entails the progressive, generalized impairment of physiological function, resulting in a poor response to environmental challenges and expanding risk of disease and death. Although susceptibility to chronic diseases such as cancer and impaired wound healing increases with age, the potency of wound and cancer response appears to be far greater in younger individuals. Studies have shown that younger individuals experience more aggressive cancers with lower survival rates in both breast and colorectal cancers. The physiological wound healing response occurs via a tightly regulated series of overlapping phases involving numerous cell types, tissues, secretory factors, and proteolytic enzymes. Tumors, which can be characterized as an unconventional, non-healing wound, hijack the body’s wound response to create an ideal environment for tumor growth and progression. Studies have shown that all major pathways activated in wound healing are also active in cancers. A study by Chang et al. revealed that the gene expression pattern of serum-treated fibroblasts exhibiting a wound healing response parallels that of human carcinomas. Molecular features that characterize the wound-like phenotype are observed at an early clinical stage, persist during treatment, and predict risk of metastasis and death in breast, lung, and gastric carcinomas. Riss et al. have shown that 77% of genes expressed in a model of renal repair and regeneration were also expressed in renal cancer. Many of these genes are active pathways common to cancer and repair including cell proliferation, growth, metabolism, and defense. In a study by Groessl et al., cancer-associated fibroblasts from breast cancer biopsies displayed a wound healing signature. Multiple sources have emphasized that inflammation, an essential phase of the wound healing response, is a key player in cancer progression. An overabundance of specific inflammatory cell types, as discussed in detail below, results in the transformation of an acute wound into a chronic wound, and non-cancerous tissue into tumors. Similar to a wound, tumors also secrete various trophic factors that aid in the recruitment of multiple cell types including MSCs.

1.3.2 Stromal Contribution to Cancer and Wound Healing

BMSCs and ASCs respond to damaged tissues and organs and secrete cytokines, chemokines, and growth factors to mediate the physiological wound healing response. The therapeutic effect of MSCs during wound repair can be attributed to the release of these trophic factors, which promote angiogenesis, cell recruitment, differentiation, proliferation, and ECM formation. Kilroy et al. characterized the cytokine profile of MSCs showing that the use of the pro-inflammatory ligand lipopolysaccharide (LPS), an immune stimulating glycolipid produced by gram-negative bacteria. Results from this
study demonstrated that MSCs released angiogenic HGF and VEGF, and hematopoietic (IL-7, GM-CSF) cytokines, in addition to secreting a number of pro-inflammatory cytokines (IL-6, IL-8, IL-11, TNF-α)\textsuperscript{104}. The paracrine behavior of MSCs also poses both immunomodulatory and immunosuppressive effects that impacts the wound healing process. As a response to both injury and inflammation, the release of prostaglandin E2 (PGE2) by MSCs results in the upregulation of interleukin 10 (IL-10) and decrease in TNF-α and interleukin 12 (IL-12) secretion from dendritic cells, leading to a shift from a more pro-inflammatory Th1 subtype to an anti-inflammatory Th2 subtype\textsuperscript{105}. MSCs attenuate the release of inflammatory mediators from macrophages and modulate the proliferation, differentiation, and immunoglobulin secretion of B cells\textsuperscript{105-107}. The release of cytokines that both support and delay tissue recovery pose many questions concerning the role of MSCs in wound healing and tumorigenesis. Evidence has shown that MSCs not only aid in the healing of conventional wounds, but are also linked to non-healing tumor wounds. Similar to wound response, MSCs respond to tumors by homing to the tumor site to mediate the physiological wound healing response. Both newly recruited and resident MSCs work together to accelerate the tumor “wound healing”\textsuperscript{108}. Similar to their role in physiological wound healing, MSCs assist in regulating tumor-associated immune responses. After instruction by the TME, MSCs secrete cytokines and chemokines that exaggerate tumor-associated inflammation via the recruitment of multiple immune cell types\textsuperscript{109-113}(Table 1.3). In addition to inducing inflammation, MSCs can also suppress the adaptive immune response in the TME through the release of effector molecules including nitric oxide (NO) and indoleamine 2,3-dioxygenase (IDO)\textsuperscript{114-118}. Although there is a substantial body of work dedicated to the role of BMSCs in wound healing and tumorigenesis, ASCs and their impact on wound healing and tumorigenesis will be the sole focus of this dissertation. For more information regarding BMSCs, please refer to the following articles\textsuperscript{119-121}.

**Table 1.3. The effect of MSCs on immune cell infiltrate.**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MSC effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>Suppress responsiveness, decrease IFN-γ secretion, increase IL-4 secretion, increase T\textsubscript{regs}</td>
</tr>
<tr>
<td>B cells</td>
<td>Regulate proliferation, differentiation, and function</td>
</tr>
<tr>
<td>NK cells</td>
<td>Decrease IFN-γ secretion</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Skew to anti-inflammatory M2 phenotype</td>
</tr>
</tbody>
</table>
1.3.3 The Impact of Aging on Cancer and Wound Healing

Age has been hypothesized as a significant contributing factor in both wound healing and cancer, yet few studies have focused on the characterization of MSCs on the basis of age. As illustrated by Dvorak, the tumor response has striking similarities to the physiological response to tissue injury. Based on this relationship, age could play a crucial role in both responses. Young individuals exhibit a more exuberant wound healing response compared to aged. The impact of aging is observed in all phases of the wound healing process. The disruption of any step in one of the phases in wound repair can lead to a 20-60% delay in healing. Fully understanding the dynamic interplay of stromal age and wound healing response could offer insight into why young patients with some cancer subtypes present with a poorer prognosis and greater mortality than aged. In this dissertation, we investigated the role of MSCs in the physiological wound healing process, their response to non-healing tumor wounds, and the impact of age on the MSC response to both wound types.

1.4 ASC Response to Tumors and Wounds

1.4.1 Physiological Wounds Vs. Tumor Wounds

The wound healing process is characterized by a dynamic series of overlapping phases involving numerous cell types, tissues, cytokines, chemokines, growth factors, and proteolytic enzymes. Cellular activities including proliferation, migration, and ECM synthesis are tightly regulated in the wound healing phases of hemostasis (coagulation), inflammation, proliferation (formation of granulation tissue), and tissue remodeling (scar formation). Hemostasis is characterized by fibrin clot formation, platelet activation, and release of inflammatory mediators. The release of numerous cytokines, chemokines, and growth factors including PDGF, transforming growth factor A1 (TGF-A1), and TGF-2 promotes the migration of inflammatory cells such as leukocytes, neutrophils, and macrophages to the wound site. The inflammatory phase, characterized by the infiltration of neutrophils and macrophages, is crucial in supplying growth factor and cytokine signals that are responsible for cell migration and subsequent tissue repair.
Figure 1.3. Cytokines released during the phases of acute wound healing are also present during cancer hallmark acquisition. (A) Tissue injury activates platelet recruitment to the site of injury, where a temporary fibrin clot stops blood vessel hemorrhage. Platelets release a heterogeneous mix of growth factors and cytokines (PDGF, TGF-β, IL-8, SDF-1, CXCL4, bFGF, VEGF) that aid in the repair process. (B) The inflammatory phase begins with the influx of neutrophils followed by macrophages to the wound bed. Neutrophils begin the phagocytosis of debris in the wound and release chemokines (MCP-1 and CCL5) that recruit macrophages to the wound. With the reduction of neutrophils around days 2-4, macrophages become the dominant inflammatory cells in the wound. They not only protect the wound from foreign microorganisms, but they also release growth factors, chemokines, and cytokines (VEGF, bFGF, PDGF, TNF-α) that aid in wound repair. (C) Around days 3-10, fibroblasts are recruited to the wound, where they contribute to the formation of a temporary ECM. (D) Several months to years after injury, cells leave the wound or undergo apoptosis, ECM is broken down by matrix metalloproteinases (MMPs) and metalloproteinase tissue inhibitors (TIMPs). Type III collagen that was deposited during the proliferation phase is degraded and replaced by a more permanent Type I collagen. Growth factors and cytokines highlighted in the 4 phases of wound healing are also actively involved in the hallmarks of cancer outlined by Hanahan and Weinberg (2011). Parallels in secreted factors of wound healing are observed in the following cancer hallmarks: tumor and repair promoting inflammation, sustaining proliferative signaling, and angiogenesis. The color scheme linking the association between the hallmarks of cancer and specific paracrine factors can be referenced using the figure legend. This figure was created using BioRender.com.
Neutrophils release a variety of inflammatory cytokines including TNF-α, IL-1β, and IL-6, IL-8, and MCP-1, which not only amplify the inflammatory response but also stimulate the release of VEGF and IL-8 to further enhance the repair response. Macrophages are described as key regulators in the response because of their roles in debris removal, promotion and conclusion of inflammation, and secretion of cytokines and growth factors for recruitment and activation of other cells involved in the repair process. The proliferative phase entails the replacement of temporary fibrin matrix with granulation tissue via fibroblast-driven ECM deposition. Growth factors produced by remaining inflammatory cells and migrating epidermal and dermal cells maintain cell proliferation and initiate cell migration to the wound bed. In response to the hypoxic wound environment, a robust angiogenic response is initiated and sustained by the production of VEGF, fibroblast growth factor 2 (FGF2), and PDGF by platelets and resident cells. In the final phase of remodeling, ECM components undergo multiple steps of degradation and synthesis to restore normal tissue architecture. The vascularization process initiated during the previous phase provides a favorable environment for continued epidermal and dermal cell migration and proliferation. This leads to wound re-epithelialization and restoration of epidermal integrity. Fibroblasts proliferate within the wound and synthesize ECM, which is initially composed of collagen III, fibronectin (FN1), fibrin, and hyaluronic acid. The initial matrix is then substituted with ECM mainly composed of collagen I. After wound contraction and matrix remodeling, fibroblasts undergo apoptosis and leave a relatively acellular scar that is comparable to the unwounded skin. The previous overview of the wound healing process strictly applies to acute wounds. Unlike acute wounds, chronic wounds exhibit prolonged or excessive inflammation, inability of dermal and epidermal cells to respond to reparative stimuli, or persistent infections (Fig. 1.4). Chronic wounds, including diabetic ulcers, pressure ulcers, and vascular ulcers, are all characterized by a chronically inflamed wound bed and failure to heal. Although the differences between ECM composition of acute and chronic wounds is both minimal and controversial, studies have shown that chronic wounds are characterized by either prolonged or poor expression of fibronectin, chondroitin sulfate, and tenascin. This leads to impaired cell proliferation and migration. Glycation, the attachment of sugars to matrix proteins, also contributes to matrix instability and disrupts interactions between collagen and its binding partners as observed in aging. In chronic wounds, an excessive recruitment of inflammatory cells that produce various reactive oxygen species (ROS) results in the damage of ECM structural elements and cell membranes, leading to premature cell senescence. Increased levels of matrix metalloproteinases (MMPs) also contributes to the degradation of ECM and inhibition of new ECM deposition. In conjunction with pro-inflammatory cytokines, ROS induces the production of enzymes that degrade and inactivate ECM components and growth factors necessary for normal cell function. Although chronic wounds exhibit an observed increase in growth factors compared to acute wounds, both their quality and bioavailability are compromised in chronic wounds. In addition to excessive inflammation chronic wounds also display impaired angiogenesis and neovascularization, which subsequently leads to insufficient oxygen and nutrient supply to cells within the wound bed. Similar to a physiological wound, tumors activate the same multi-step process to aid in growth and progression.
Normal tissues are comprised of two components, the parenchyma and stroma. The stroma, a mixture of fixed tissue cells, inflammatory cells, blood vessels, matrix proteins, and proteoglycans, provides support to the parenchyma. Tumor composition is analogous to normal tissue, organized into the parenchyma (malignant cells) and stroma. Solid tumors such as carcinomas or sarcomas attain semblance to healing wounds by exploiting the host vascularized tissue stroma for survival, growth, and metastasis\textsuperscript{131-135}. Troester et al. have shown that a wound response signature is activated in histologically normal tissue of breast cancer patients\textsuperscript{90}. The overlapping phases of blood clotting, inflammation, extracellular matrix alterations, angiogenesis, and tissue remodeling are expressed in normal tissue adjacent to breast cancer and in tumors, further solidifying similarities between tumors and physiological wounds\textsuperscript{84, 136}. The hallmarks of wound healing also parallel some of the hallmarks of cancer\textsuperscript{86}. Out of the ten hallmarks of cancer, three of the hallmarks have close ties with the four phases of wound healing. Secretory factors observed in wound healing are also observed in cancer. Many of these factors are observed in multiple phases of wound healing and actively participate in multiple wound healing phases including TGF-\(\beta\), VEGF, PDGF, basic fibroblast growth factor (bFGF), TNF-\(\alpha\), and IL-8 (Fig 1.3). One type of tumor that embodies the nature of malignant tumors and chronic wounds is the keloid scar. Keloid scars are characterized as benign human tumors without malignant potential that exhibit elevated matrix deposition and chronic inflammation\textsuperscript{137}. Previously, keloids were characterized by an overabundance of disorganized type I and III collagen bundles\textsuperscript{138}. Fibroblasts in keloids exhibit an altered phenotype of intrinsic or growth factor stimulated collagen, fibronectin, elastin, and proteoglycan accumulation\textsuperscript{139}. Current studies have shown that new keloid lesions demonstrate elevated matrix protein expression of collagens (COL1A1, COL6A1, COL10A1, COL11A1, COL12A1), FN1, and fibrillin-2 (FBN2) compared to non-lesion tissue\textsuperscript{140, 141}. Many of these same matrix components are observed to be elevated in triple negative breast cancer (TNBC) compared to matched non-diseased breast adipose tissue. TNBC is void of ER, PR, and HER2, characterizing it as a more aggressive breast cancer type. Previous studies have evaluated the matrix composition of TNBC compared to distal adipose tissue and observed elevated protein levels of COL6A1, COL11A1, COL12A1, FN1, and FBN2, with a minor increase in COL1A1\textsuperscript{140}. Similar data was observed by others who profiled 13 TNBC samples compared to adjacent adipose tissue\textsuperscript{140}. Another link between keloids and cancer progression is plasminogen activator inhibitor 1 (PAI-1). In in-vitro 3D culture, keloid fibroblasts show elevated collagen accumulation and altered fibrin degradation. PAI-1, a major inhibitor of plasminogen activators that are responsible for the conversion of plasminogen to plasmin, is thought to be linked with poor fibrin degradation observed in keloids\textsuperscript{99}. For proper repair in the wound healing process, the temporary fibrin matrix must be degraded and replaced by fibroblast-synthesized collagen. In addition to organ fibrosis, PAI-1 also plays a role in tumor progression. Not only is it highly expressed in tumor biopsies, but it is also prognostic for disease progression and relapse in certain cancer types, one in particular being breast cancer\textsuperscript{142}. PAI-1 can induce tumor vascularization, promote cell dissemination, and tumor metastasis\textsuperscript{142}. Keloid-derived fibroblasts have been shown to exhibit elevated expression of transforming growth factor-\(\beta\)1 (TGF\(\beta\)-1) and transforming growth factor-\(\beta\)2 (TGF\(\beta\)-2)\textsuperscript{141, 143}. TGF\(\beta\) has profound effects on PAI-1 upregulation in tissue and organ fibrosis. TGF\(\beta\) increases production of ECM molecules by cells and
slows down their removal by upregulating protease inhibitors (TIMP1 and PAI-1) and downregulating protease (MMP1 and uPA) expression\textsuperscript{139}. Elevated TGF\(\beta\)-1 in hepatocellular carcinoma and breast, lung, and prostate cancer patients correlates with poor outcome\textsuperscript{144}. In breast cancer, TGF\(\beta\)-1 exhibits a dual role as both tumor suppressive and oncogenic in nature. In early stages of breast cancer, TGF\(\beta\)-1 shows tumor suppressive effects by inhibiting epithelial cell cycle progression and promoting apoptosis. However, in the late stages of breast cancer, TGF\(\beta\)-1 is correlated with increased tumor progression, higher cell motility, cancer invasiveness, and metastasis\textsuperscript{145}. Interestingly, with respect to patient age, the expression TGF-\(\beta\), a growth factor responsible for the stimulating the synthesis of collagen and promoting wound healing, is markedly reduced in aged individuals compared to young\textsuperscript{146, 147}. Similar to TNBC, keloid scarring has a higher prevalence in African Americans and in individuals between the age of 10 and 30\textsuperscript{148, 149}. Age and race appear to be common factors between keloid scarring and the more aggressive cancer subtypes such as TNBC. Data suggests that breast cancer in young women (<40 years old) have a poorer prognosis and higher mortality compared to breast cancers diagnosed in older women\textsuperscript{150, 151}. Furthermore, young African American women (<40 years old) are more likely to be diagnosed at younger ages with a more

Figure 1.4. Chronic wounds exhibit similarities to tumors. Acute wounds exhibit adequate angiogenesis that aids in fibroblast proliferation, re-epithelialization, and neutrophil infiltration. On the other hand, chronic wounds exhibit poor angiogenesis, elevated neutrophil infiltration, persistent bacterial infections, and decreased fibroblast proliferation. Higher infiltration of inflammatory cells leads to the excessive secretion of inflammatory markers, which leads to growth factor and ECM degradation. The chronic inflammation also prevents macrophage polarization from an inflammatory to anti-inflammatory phenotype, which prevents resolution of the wound and maintains it in a non-healing state. Similar to chronic wounds, tumors also exhibit higher neutrophil and macrophage infiltrate, increased levels of pro-inflammatory cytokines, and ECM degradation as a result of inflammation. Unlike chronic wounds, tumors increase collagen synthesis. This figure was created using BioRender.com.
aggressive TNBC subtype\textsuperscript{152, 153}. The commonalities between keloid scarring and TNBC may offer more insight into the role of age in tumorigenesis. To date, commonalities between age and TNBC has been established; however, the extent to which age impacts the ER-\(\alpha^+\) subtype and TME has yet to be fully elucidated.

1.4.2 ASCs: Their role in the physiological wound and TME

Adipose tissue is a viable source of stromal/stem cells that exhibit both multipotency and immunomodulatory characteristics\textsuperscript{154-156}. The regenerative capacity of ASCs is harnessed through their secretome\textsuperscript{157, 158}. ASCs secrete a wide variety of growth factors, cytokines, adipokines, neurotrophic factors, adipokines, and angiogenic factors such as PDGF, FGF, VEGF, HGF, SDF-1, IL-6, IL-8, transforming growth factor alpha (TGF-\(\alpha\)) and angiopoietin\textsuperscript{159-162}. In vivo, ASCs reside in the stem cell niche, where they are surrounded by the ECM and other supporting cells\textsuperscript{163}. The stem cell niche or more commonly referred to as the microenvironment, modulates the ability of ASCs to differentiate, proliferate, and migrate as they aid in the restoration of cellular age defects and tissue repair\textsuperscript{164}. In response to injury, ASCs have been shown to shift the inflammatory phenotype of local immune cells to a more anti-inflammatory phenotype via soluble factors\textsuperscript{165}. ASCs alter the inflammatory profiles of macrophages, T cells, B cells, and dendritic cells, which in turn furthers the proliferative and remodeling phases of the wound healing process\textsuperscript{166, 167}. Many of these soluble factors described above are also associated with expressing and/or secreting multiple growth factors, cytokines, chemokines, and inflammatory markers that are linked to cancer development and progression\textsuperscript{168-176}. ASCs promote the recovery of wound blood via the secretion of angiogenic factors. VEGF is the most important angiogenic growth factor because of its ability to promote endothelial progenitor cell mobilization, recruitment, and migration, which accelerates angiogenesis in the wound\textsuperscript{177}. Heo et al. determined that TNF-\(\alpha\)-activated ASCs produce pro-inflammatory cytokines IL-6 and IL-8 aid in angiogenesis and epithelium regeneration in wound repair\textsuperscript{178, 179}. Interestingly, high serum levels of both IL-6 and IL-8 correlated with strong tumor invasion and poor prognosis in breast cancer\textsuperscript{180}. In addition to VEGF, many angiogenic factors including PDGF and angiopoietin are secreted throughout several phases of the wound healing process\textsuperscript{181}. Although beneficial during wound repair, both VEGF and PDGF aid in endothelial cell proliferation and vascularization of tumors\textsuperscript{181}. TNF-\(\alpha\), a pro-inflammatory cytokine involved in systemic inflammation, is secreted by both ASCs and tumor cells. TNF-\(\alpha\) is upregulated in the inflammatory phase of wound healing. ASCs stimulate macrophages to secrete TNF-\(\alpha\), which further aids in activation, proliferation, apoptosis, and differentiation of other macrophages. TNF-\(\alpha\) secreted by ASCs has been associated with the development of the tissue architecture necessary for tumor growth and metastasis\textsuperscript{182}. In addition, long term exposure of hormone receptor positive (HR\(^+\)) breast cancer cells to TNF-\(\alpha\) results in epithelial to mesenchymal transition (EMT) and a more aggressive phenotype\textsuperscript{182}. In physiological wound healing, ASC-secreted IL-6 aids in macrophage recruitment and M1 to M2 polarization\textsuperscript{164}. Studies have exhibited that chronic inflammation associated with excessive IL-6 secretion facilitates tumor development\textsuperscript{182}. MCP-1, another pro-inflammatory cytokine produced by ASCs, promotes macrophage recruitment during wound healing. Similar to previously mentioned mediators, MCP-1 is also involved in
breast cancer tumor progression and metastasis\textsuperscript{182}. LEP, an adipokine secreted by ASCs, stimulates angiogenesis, cell proliferation and differentiation, and migration of keratinocytes to enhance wound healing\textsuperscript{182}. LEP also plays a crucial role in tumorigenesis. In HR\textsuperscript{+} breast cancer, LEP is correlated with higher recurrence rates and increased invasiveness\textsuperscript{76}. LEP also increases TNF-\(\alpha\) expression, ROS production, MCP-1 expression, and endothelial cell proliferation and migration, all of which increase cancer cell growth and mobility\textsuperscript{182}. As previously mentioned in terms of keloid formation, PAI-1 contributes to proper wound healing through the breakdown of the fibrin clot. Studies have shown that ASC-secreted PAI-1 has been associated with tumor cell invasion and metastasis, as well as a poor prognostic indicator in breast and colon cancers\textsuperscript{182}. Circulating levels and protein content of growth differentiation factor 11 (GDF11), a member of the TGF-\(\beta\) superfamily, is affected by pathological conditions and age\textsuperscript{183}. It has a role in multiple phases of the wound healing process by increasing cell proliferation and migration, angiogenesis, and ECM production\textsuperscript{164}. Studies on the effect of GDF11 and cancer progression are inconclusive. Some studies have shown that GDF11 induces tumor suppressive properties, while others indicate GDF11 promotes tumorigenesis\textsuperscript{183}. Similar to GDF11, TGF-\(\beta\)1 is active in multiple steps of the wound healing process. TGF-\(\beta\)1 plays the role of a paracrine mediator that activates fibroblasts, macrophages, and ASC secretions\textsuperscript{164}. In breast cancer, TGF-\(\beta\)1 enhances progression of breast malignancies into more malignant phenotypes\textsuperscript{76}. Increased expression of the chemokine SDF-1 by ASCs amplifies ASC migration to the wound site\textsuperscript{164}. SDF-1 has also been noted as an important factor in the spread of breast cancer cells\textsuperscript{76}. SDF-1 and its receptor, chemokine receptor type 4 (CXCR4), play an important role in HR\textsuperscript{+} breast cancer\textsuperscript{70}. The expression of SDF-1-CXCR4 is associated with the EMT phenotype. Furthermore, crosstalk between ER-\(\alpha\), SDF-1, and CXCR4 contributes to hormone independence, making it increasingly difficult to treat with endocrine therapy\textsuperscript{70}. Although these specific soluble mediators have roles in both wound healing and tumorigenesis, the question arises concerning how their roles are altered by aging. Many of the regenerative qualities of ASCs including proliferation and differentiation capacity change with age. The ASC secretome has been harnessed as a viable treatment option for chronic non-healing wounds, however studies have yet to characterize the ASC secretome as a function of age\textsuperscript{184}. Several of the aforementioned mediators including PDGF, VEGF, IL-6, TNF-\(\alpha\), SDF-1, TGF-\(\beta\)1, and LEP are affected by aging\textsuperscript{125}(Table 1.4). Although little evidence suggests ASC-secreted PDGF is impacted by age, studies have shown that aging results
Table 1.4. Paracrine Factors Observed in Wound Healing and Tumorigenesis. Asterisk (*) indicates information was provided by a review article.

<table>
<thead>
<tr>
<th>Paracrine Factor</th>
<th>Role in Wound Healing</th>
<th>Role in Tumorigenesis</th>
<th>ASC Mediated Cancer Type</th>
<th>Differentially Expressed with Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Upregulated in inflammatory phase, ASCs stimulate macrophages to secrete TNF-α</td>
<td>Development of tissue architecture for tumor growth and metastasis, EMT with long-term exposure to TNF-α</td>
<td>ERα breast cancer (Strong et al. 2015), Breast cancer (Schweizer et al. 2015)</td>
<td>✓</td>
</tr>
<tr>
<td>IL-6</td>
<td>Regulates chronic inflammation, macrophage recruitment, macrophage polarization</td>
<td>Chronic inflammatory environment can lead to tumor development, enhances migration and invasiveness of tumor cells</td>
<td>Breast Cancer (Schweizer et al. 2015), Saloo et al. 2019, Bladder cancer (Bla et al. 2019)</td>
<td>✓</td>
</tr>
<tr>
<td>IL-8</td>
<td>Stimulates angiogenesis, granulocyte recruitment</td>
<td>EMT, neutrophil migration</td>
<td>Breast cancer (Schweizer et al. 2015), Strong et al. 2015, Melanoma (Presner et al. 2018)</td>
<td></td>
</tr>
<tr>
<td>CCL5</td>
<td>Macrophage recruitment</td>
<td>Immune cell recruitment, stimulates angiogenesis, modulates ECM, tumor cell proliferation, enhances tumor cell migration and invasiveness</td>
<td>Breast cancer (Schweizer et al. 2015)</td>
<td></td>
</tr>
<tr>
<td>MCF-1</td>
<td>Macrophage recruitment</td>
<td>Involved in tumor progression and metastasis</td>
<td>Bladder cancer, Breast cancer (Schweizer et al. 2015), Melanoma (Presner et al. 2018)</td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>Keratinocyte migration, fibroblast migration and fibrinolysis, myofibroblast differentiation</td>
<td>Tumor cell migration and metastasis, macrophage migration and polarization, inhibits fibroblasts, cell adhesion</td>
<td>Breast cancer, Cervical cancer (Strong et al. 2015)</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>Stimulates collagen deposition, angiogenesis, and epithelialization</td>
<td>Stimulates angiogenesis, enhances tumor cell invasiveness and migration</td>
<td>Melanoma (Presner et al. 2018), Breast cancer (Schweizer et al. 2015), Scott et al. 2019</td>
<td>✓</td>
</tr>
<tr>
<td>PDGF</td>
<td>Stimulates chemotaxis, proliferation, and new gene expression in macrophages and fibroblasts</td>
<td>ECM remodeling</td>
<td>Breast cancer (Scott et al. 2019)</td>
<td>✓</td>
</tr>
<tr>
<td>GDF11</td>
<td>Activates fibroblasts in hemosiderosis and proliferation phases, activates platelets in clot formation, increases angiogenesis/cell proliferation/cell migration/ECM production</td>
<td>Indicates tumor suppressive and oncogenic properties</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>SDF-1</td>
<td>Chemotaxis of ASCs</td>
<td>SDF-1, CXCR4 expression associated with EMT phenotype, contribution to hormone independence via SDF-1, CXCR4-Erα cross-talk</td>
<td>ERα breast cancer (Schweizer et al. 2015)</td>
<td>✓</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>ECM production and remodeling</td>
<td>Promotes EMT, increases tumor cell mobility and metastasis, ECM remodeling</td>
<td>Breast cancer (Schweizer et al. 2015)</td>
<td>✓</td>
</tr>
<tr>
<td>LEP</td>
<td>Stimulates angiogenesis, cell proliferation and differentiation, keratinocyte migration</td>
<td>Increases TNF-α and ROS production, increases MCP-1 expression, increases endothelial cell proliferation and migration, increases tumor cell invasion and metastasis</td>
<td>ERα breast cancer (Scott et al. 2019)</td>
<td>✓</td>
</tr>
</tbody>
</table>
in the enhanced release of PDGF, TGF-β, and TGF-α from α-granules\textsuperscript{125}. Increased age also correlates with a decline in phagocytic activity of wound macrophages, leading to an accumulation in IL-6 and TNF-α\textsuperscript{125}. In contrast, Pandey \textit{et al.} revealed that aged ASCs exhibit lower expression of TNF-α relative to young ASCs. This suggests that the impact of aging on each cell type is distinct. Aging may have different effects on immune cells compared to tissue-derived cells such as ASCs. VEGF, an essential regulator of angiogenesis, has exhibited a decline in expression with aging\textsuperscript{125}. In contrast, studies have shown that ASC-secreted VEGF levels is unaffected by age when active in bone healing\textsuperscript{185}. Aging also results in a decrease in SDF-1 expression in wound healing, however there is no evidence suggesting that age affects ASC production of SDF-1\textsuperscript{125}. Hypoxia plays a significant role in the physiologic wound healing process\textsuperscript{186}. With aging, wound response to hypoxia diminishes, thus responsiveness to TGF-β1 also diminishes. In normal tissues, TGF-β1 increases the deposition of collagen by stimulating its synthesis and minimizing its degradation. In aged wound tissue, this ratio of synthesis to degradation is imbalanced\textsuperscript{125}. The adipokine LEP not only promotes wound healing, but it also plays an oncogenic role in breast cancer\textsuperscript{187, 188}. In a study by Pandey \textit{et al.}, LEP expression in aged ASCs was significantly lower compared to young ASCs\textsuperscript{189}. With elevated levels in young ASCs and links to both wound healing and tumorigenesis, LEP could be a potential target underlying amplified tumor aggressiveness observed in young patients. Based on this investigation, paracrine factors have an extensive role in wound healing and tumorigenesis regardless of ASC involvement. We have knowledge that ASCs secrete the paracrine factors listed in Table 1.4, however, we can only speculate that ASCs release these factors in response to wound repair and tumorigenesis. ASCs are also recognized as playing a pivotal role in both wound healing and tumorigenesis. The extent to which age impacts the role of ASCs in both of these pathologies is not well understood. Studies on aging and cutaneous wound healing have highlighted changes in many of these secreted factors. While these factors are ASC associated, these changes have not yet been linked specifically to ASCs.

### 1.4.3 Adipose-Derived Stem/Stromal Cells and ER-α\textsuperscript{+} Breast Cancer

As previously described in section 1.1.2, the interaction between ASCs and the TME has adverse effects on breast cancer development and progression. Based on a review by Freese \textit{et al.}, numerous studies have been conducted to determine the role of ASCs in human breast cancer development, growth, progression, and metastasis\textsuperscript{169}. The duality of ASCs as both regenerative and tumorigenic in nature makes their role in tumor development extremely complex. Many studies have suggested that the ability of ASCs to release pro-migratory factors may influence their role in cancer\textsuperscript{169, 190-193}. Others have suggested ASCs play an important role in pathologic angiogenesis and formation of a blood supply for the growing tumor\textsuperscript{194}. Crosstalk between ASCs and breast cancer cells diminishes the efficacy of hormonal therapy\textsuperscript{195}. To date, the full impact of ASCs on the TME is yet to be established and may have confounding results due to differences in patient demographics that influence ASC function.

Patient characteristics such as BMI can alter ASC function and the stimulatory effect on ER-α\textsuperscript{+} breast cancer. In non-cancerous tissue, obesity increases the expression of pro-inflammatory factors such as IL-6, IL-1, IL-12, PDGF, TNF-α, leukemia inhibitory
factor (LIF), and granulocyte-colony stimulating factor (GSF)\textsuperscript{196}. Strong \textit{et al.} discovered that LEP from obese ASCs (BMI greater than or equal to 30 kg/m\textsuperscript{2}) promoted ER-\(\alpha^+\) cancer growth and metastasis compared to those of a healthy BMI (18.5-24.9 kg/m\textsuperscript{2})\textsuperscript{197, 198}. LEP indirectly increases estrogen synthesis and ER-\(\alpha\) activity by upregulating aromatase expression and activity in ER-\(\alpha^+\) breast cancer cells\textsuperscript{199}. ASCs exhibit an innate ability to drive ER-\(\alpha^+\) breast cancer through aberrant paracrine signaling. In the absence of cell-cell contact, ASCs are able to increase the proliferation of MCF-7 cells\textsuperscript{70}. This evidence suggests ASCs not only behave as protagonists in ER-\(\alpha^+\) breast cancer, but also complicate treatment efforts. Their homing and immunomodulatory functions in observed in the wound healing and tissue regeneration process also appears to prime and transform sites into suitable environments for tumor growth\textsuperscript{199, 200}. As presented in this dissertation, ASCs may play a similar role in ER-\(\alpha^+\) breast cancer and wound healing response. The body of evidence investigating ASCs from this perspective is still sparse; however, there is considerable amount of evidence focusing on BMSCs and their role in wound healing and cancer progression\textsuperscript{108}. Many of the cytokines and growth factors that have been presented in section 1.4.2 as potential links between ASCs and the tumor wound have been previously cited in epithelial stem cells lines\textsuperscript{201}. With this said, more short-term experiments focusing on the ASC secretome’s impact on wound healing and tumorigenesis need to be conducted to bridge this gap in knowledge. Rather than generalizing, studies need to focus on specific cancer types and specific ASC demographics.

1.5 Overview of Research

This dissertation is divided into three specific aims that focuses on the characterization of stromal age and breast cancer, specifically the impact of adipose-derived stem/stromal cells on regulation of endocrine signaling. These aims are designed to test the overall hypothesis that the young ASC secretome contributes to elevated estrogen signaling in ER-\(\alpha^+\) breast cancer.

\textbf{Aim 1: Characterization of ER-\(\alpha^+\) breast cancer tumors of young and aged patients.} This aim is designed to determine differences in young and aged breast cancer tumors based on gene expression and resident cell population. Data acquired from The Cancer Genome Atlas (TCGA), GenEx Miner, Kaplan Meier plotter was used to investigate gene expression as a function of tumor age. TCGA and GenEx Miner was used to compare the gene expression of ER-\(\alpha^+\) breast cancer tumors from aged and young patients. Kaplan Meier plotter was used to determine the impact of significant genes observed in the former platforms on the survival rate of ER-\(\alpha^+\) breast cancer patients. xCell cell type enrichment analysis was implemented to characterize the cell infiltrate of young and aged tumors.

\textbf{Aim 2: Characterization of young ASCs.} This aim was designed to evaluate young ASC phenotype. To determine the impact of ASCs on the stromal environment, we compared the proliferation, differentiation, matrix gene expression, and cytokine expression of young and aged ASCs. The proliferation and differentiation capacity of young and aged ASCs was assessed on tissue culture plastic. Matrix gene expression of
young and aged ASCs was evaluated using qRT-PCR. The secretome of young and aged ASCs was compared using cytokine array analysis.

**Aim 3: Identify the role of the ASC secretome on ER-α⁺ breast cancer estrogen activity.** This aim was designed to assess how the young ASC secretome affects ER-α⁺ breast cancer. The MCF-7 ER-α⁺ breast cancer cell line was exposed to conditioned media (CM) acquired from young and aged ASC donors and assessed for differences in proliferation and estrogen signaling. Proliferation of MCF-7 cells stimulated with young and aged CM was evaluated using Crystal Violet staining. Differences in estrogen signaling was compared in MCF-7 cells stimulated with young and aged CM using qRT-PCR and Western Blot Analysis.
CHAPTER 2. THE CHARACTERIZATION OF YOUNG ASCs

2.1 Introduction

Aging is notably characterized as a progressive, generalized impairment of function, resulting in a poor response to environmental challenges and expanding risk of disease and death. Two of the many chronic diseases associated with aging are cancer and impaired wound healing. Wound healing occurs via a complex, coordinated series overlapping phases involving numerous cell types, tissues, cytokines, chemokines, growth factors, and proteolytic enzymes. The cellular activities including migration, proliferation, phagocytosis, and ECM synthesis are tightly regulated. The wound repair program follows the chronological order of hemostasis, inflammation, proliferation, and tissue remodeling to ensure optimal repair. Tumors, an unconventional, non-healing wound, hijack the body’s wound repair program, creating an ideal environment for tumor growth and progression. Similar to a wound, tumors secrete factors that aid in the recruitment of multiple cell types including MSCs.

MSCs, derivatives of mesenchymal tissues including bone marrow and adipose tissue, display a propensity to migrate toward damaged tissues and organs where they then secrete cytokines to mediate wound healing. The therapeutic effect of MSCs can be attributed to the release of these cytokines that promote new vessel formation, recruit endogenous cells, and direct cell differentiation, proliferation, and ECM formation during wound repair. Kilroy et al. characterized the cytokine profile of MSCs (mean age 37.8 yo +/- 10.6, BMI 25.4 kg/m²) showing that lipopolysaccharide (LPS)-stimulated MSCs not only release angiogenic (HGF, VEGF) and hematopoietic (IL-7, GM-CSF) cytokines, but also secrete a number of pro-inflammatory cytokines (IL-6, IL-8, IL-11, TNF-α)104. The release of cytokines that both support and delay tissue recovery pose many questions concerning the role of MSCs in wound healing. Evidence has shown that MSCs not only aid in the healing of conventional wounds, but are also linked to the “wounds that never heal” observed in cancer. Age is a significant factor in both wound healing and cancer, yet few studies have focused on the characterization of MSCs on the basis of age. Previous studies have demonstrated that key demographic factors such as age and diseased states can alter stem cell biology. Modifications to the underlying biology of stem cells can result in an altered response to diseased states such as non-healing wounds and cancer. In this study, we investigated the effect of age on ASC function. We compared the proliferation, differentiation, matrix deposition, and paracrine secretions of aged and young ASCs. Through an age-dependent characterization of ASCs, we intended to determine which ASC characteristics may impact the features observed in young ER-α breast cancer tumors.
2.2 Materials and Methods

Cell Culture:
Cell Source: Human adipose-derived stem/stromal cells (ASCs) were obtained from Obatala Sciences (New Orleans, LA). Young donors (24-34 years old, mean age 27.8 +/- 4.1 SEM) and aged donors (60-70 years old, mean age 62 +/- 7.8 SEM) matched according to corresponding body mass indices. All donors were female and Caucasian (Table S1).

ASC Culture: Cultures of ASCs (3,000 cells/cm²) were maintained with Alpha-MEM with L-glutamine (Cat #: 12561-056, Gibco) supplemented with 10% fetal bovine serum (FBS, Cat #: 43602-500, JR Scientific) and 1% Antibiotic-Antimycotic (Anti-Anti, Cat #15240062, Gibco) until attained 80% confluence. The procedure implemented for ASC expansion was described previously by Bunnell et al. Cells were then seeded at 2,000 cells/cm² in 6-well plates, 3,000 cells/cm² in 96-well plates, 3,000 cells/cm², and 2,000 cells/cm² in 60 x 15 mm plates for qRT-PCR, immunostaining, proliferation, and differentiation assays. For coated ECM, aged and young ASCs were seeded at 3,000 cells/cm² on laminin (Cat #: 354533, Corning) and fibronectin (Cat #: 354532, Corning) coated 25 cm² flasks and maintained at 5% CO₂ at 37°C until attaining 80% confluent and cells were collected for qRT-PCR.

Adipogenic Differentiation: Cultured ASCs were detached, counted, and seeded in 6-well plates at 2,000 cells/cm². Three days after plating, cells were induced using adipogenic differentiation medium (Cat #: OS-002-01, Obatala Sciences), changing medium every 3 days for 14 days. Cells were then washed 3x with Phosphate Buffer Solution (PBS, Cat #: 10010-023, Gibco) and fixed for 1 h with 10% formalin. Fixative was then removed and cells were washed 3x with PBS prior to staining with 0.5% Oil Red O. After staining for 1 h, wells were washed 3x with PBS and imaged using an inverted fluorescence microscope (Olympus IX-81, Olympus Corporation) and NIS Elements Microscope Imaging Software (Nikon).

ASC Proliferation: Cultured ASCs were detached, counted, and seeded in 96-well plates at 3,000 cells/cm². Three days after plating, wells were maintained with culture medium (Alpha MEM with L-glutamine, 10% FBS, and 1% Anti-Anti) or induced with osteogenic or adipogenic differentiation medium. Cell density at days 1, 3, 7, and 10 was assessed using the Cell Counting Kit-8 (CCK-8, Dojindo). Absorbance was measured using a cell imaging multimode reader (Cytation 3, BioTek) and Gen5 Microplate Reader and Imager Software (BioTek).

Immunofluorescent Staining: Cultured ASCs were seeded in 96-well plates at 3,000 cells/cm² and maintained in culture medium for 24 h. Cells were then fixed with 10% formalin for 15 min, washed with PBS 3x, and permeabilized with 0.5% (v/v) Triton 100-X for 10 min. After removing the detergent, cells were washed 3x with PBS and blocked with 0.1% (w/v) BSA in PBS for 1 h. With the removal of the blocking agent, cells were stained with 100 µL of Alexa Fluor 488 phalloidin for 30 min at room temperature (Cat #A12379, Invitrogen). This was proceeded by washing 3x with PBS and staining with 100
µL of DAPI diluted 1:300 in 0.1% (w/v) BSA for 10 min at room temperature (Cat#: 157574, MP Biomedicals). Cells were then washed 3x with PBS and imaged using an inverted confocal fluorescence microscope (Leica SP8, Leica Microsystems) and Leica Application Suite X (LASX, Leica Microsystems).

Quantitative Real-Time Polymerase Chain Reactions (qRT-PCR): ASCs were collected and total RNA was extracted with Quick RNA MicroPrep kit (Cat #: R1051, Zymo Research) as per manufacturer protocol. cDNA was synthesized using qScript cDNA SuperMix (Cat #: 101414-106, VWR), as per manufacture protocol with 1µg RNA per sample. Primer was obtained using Primer-BLAST software (NCBI). Primer sequences are presented in Table S2. Quantitative real-time PCR (qRT-PCR) was performed using the BioRad iCycler (BioRad, v4.006). Expression was computed using the comparative cycle threshold (Ct) method using the reference gene GAPDH. Normalization of young donor gene expression was to aged donor gene expression designated as 1.

Cytokine array analysis: ASCs were cultured until attained 80% confluency in 10% Alpha-MEM with L-glutamine (Cat #: 12561-056, Gibco) supplemented with 10% fetal bovine serum (FBS, Cat #: 43602-500, JR Scientific) and 1% Antibiotic-Antimycotic (Anti-Anti, Cat #15240062, Gibco). Cells were washed with sterile phosphate buffer saline (PBS, Cat #: 10010023, Gibco). ASCs were then cultured in phenol-free Dulbecco’s modified Eagle’s medium (Cat #:31053-028, Gibco), 5% charcoal stripped FBS (Cat #: SH3006803, GE Healthcare), 1% penicillin/streptomycin (P/S, Cat #: 15140-122, Gibco), 1% MEMAA, 1% NEAA, 1% Glutamax (Cat #: 35050-061, Gibco), and 1% sodium pyruvate for 24 h. The conditioned media was collected, filtered, and stored at -20°C for cytokine array analysis. The cytokine array analysis procedures followed the manufacturer’s protocol (Cat #: ARY005B, R&D Systems, Minneapolis, MN).

Biostatistics: All values are presented as means ± standard error (SEM). Statistical analyses among two groups were performed using the Student’s t-test. Statistical significance was set at p<0.05. Analysis was performed using Prism 8 (GraphPad Software, San Diego, CA).
2.3 Results

Adipose-derived stem/stromal cell differentiation, proliferation, and morphology is consistent amongst young and aged.

Figure 2.1. Young and aged ASCs exhibit comparable morphology, differentiation capacity. (A,B) Aged (A) and young (B) ASCs were maintained in stromal medium for 7 days, stained with Alexa Fluor 488 Phalloidin, and counterstained with DAPI. (C,D) Adipogenic-differentiated aged (C) and young (D) ASCs were maintained 14 days in adipogenic differentiation medium. Oil Red-O was used to stain for lipid rich vacuoles. (E,F) Aged and young ASCs proliferation cultured in basal (E) and adipogenic (F) medium was assessed at days 1, 3, 7, and 10 using the Cell Counting Kit-8 (CCK-8).

In order to determine the multi-lineage differentiation capacity of young and aged ASCs, ASCs were differentiated into adipogenic lineages using lineage specific induction factors. The number of aged ASCs capable of adipogenic differentiation parallel that of young ASCs (Fig 2.1C and 2.1D). Phalloidin staining of aged and young ASCs showed no visible difference in morphology (Fig 2.1A and 2.1B). To test the proliferation of young and aged ASCs, ASCs were maintained in basal medium or differentiated into adipogenic lineages. Proliferation of ASCs in basal (Fig 2.1E) and adipogenic (Fig 2.1F) was measured at days 1, 3, 7, and 10. Results revealed no significant difference in the proliferation of young and aged ASCs in a stem or differentiated state.
Adipose-derived stem/stromal cell extracellular matrix gene expression is not altered by donor age.

To delineate differences between young and aged ASC extracellular matrix and intracellular signaling gene expression, ASCs were maintained in basal medium until attaining 80% confluency and collected for qRT-PCR analysis. There were no significant changes in ECM gene expression observed in young ASCs. Many ECM genes examined in qRT-PCR displayed high cycling and thus were no included the final analysis. Both COL4A2 (10.95 +/- 10.62 SEM) and LAMA3 (6.82 +/- 4.72 SEM) were elevated in young ASCs; however, expression was variable within each cohort (Fig 2.2).

Figure 2.2. Young ASCs displayed similar expression of matrix compared to aged. Young and aged ASCs were cultured in basal medium and collected at 80% confluence for qRT-PCR analysis. Primers were chosen based on pooled young and aged ASC gene expression and TCGA data. N=3 biological replicates. Mean value represented by vertical bar.
Age alters paracrine signaling of adipose-derived stem/stromal cells.

Since there were no observed differences in ASC matrix expression between young and aged ASCs, we next sought to determine if paracrine factors were altered between young and aged ASCs. To compare the production of paracrine factors from aged and young ASCs, ASC cell supernatants were collected, mixed with select antibodies, and incubated on the array. Chemiluminescence results revealed elevated levels of proteins MCP-1, CCL5, SDF-1, CD40, CXCL1, IFN-γ, IL-8, IL-10, IL-16, IL-32a, and TNF-α in young ASC conditioned media (Fig 2.3). Many of these paracrine factors are associated specifically with the inflammatory phase of the wound healing process. Furthermore, many of these are also elevated in young tumors, suggesting young ASCs display similar responses to wounds and tumors (Table 2.1).
Table 2.1 Comparison of young tumors and young ASC cytokine expression and their role in wound healing.

<table>
<thead>
<tr>
<th>Secreted factor by ASCs</th>
<th>Role in wound healing</th>
<th>Elevated in young tumors</th>
<th>Elevated in young ASCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>Macrophage chemotactant Possible macrophage polarization</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Macrophage polarization</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-10</td>
<td>Anti-inflammatory cytokine</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-8</td>
<td>Neutrophil recruitment</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-6</td>
<td>Induction of inflammatory cells, leukocyte recruitment, promotes granulation tissue formation</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-4</td>
<td>Dampen inflammatory phase, promote collagen synthesis</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-32a</td>
<td>Neutrophil and monocyte stimulation, monocyte differentiation</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-16</td>
<td>N/A</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-2</td>
<td>N/A</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-17a</td>
<td>N/A</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-18</td>
<td>N/A</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Conflicting—shown to accelerate vascularization but also delays wound healing</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Immune cell activation, neutrophil recruitment, cell clearance via apoptosis, MMP-2 activation</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>CCL5</td>
<td>Macrophage recruitment</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CD40L</td>
<td>T&lt;sub&gt;1&lt;/sub&gt; response, B cell isotype class switching/anti-secretion/memory B cell differentiation</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Keratinocyte migration, fibroblast migration and fibroblast-myofibroblast differentiation</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Gm-CSF</td>
<td>Induction of keratinocyte proliferation, promote epithelial cell migration</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
2.4 Discussion

Aging is characterized by both intrinsic and extrinsic changes to the human anatomy, one in particular being modifications to the ECM. As a component of the microenvironment, the ECM not only alters MSC behavior, but also undergoes age-associated changes that are linked to multiple pathologies including cancer\textsuperscript{205}. Although it has been extensively shown that the ECM undergoes compositional changes with age, results have been variable regarding the impact of age on MSC driven changes in ECM composition. Uncertainty exists regarding age-related changes in MSC collagen synthesis. Sun et al. found no difference in ECM total protein concentration synthesized by aged and young murine bone marrow derived stem cells (BMSCs)\textsuperscript{206}. Similarly, our study shows no significant differences in ECM gene expression between aged and young ASCs. Furthermore, ASCs cultured on fibronectin and laminin proteins revealed no significant difference between aged and young. This suggests that age may not play a role in ASC ECM deposition. This study also revealed similarities between aged and young ASC proliferation and differentiation. Refuting evidence exists on the effect of age on ASC differentiation and proliferation. Kornicka et al. and Choudhery et al. both confirmed that ASC colony forming units (CFUs) and doubling time was affected by age\textsuperscript{207, 208}. In contrast, Dufrane et al. showed ASC expansion does not change based on donor age. Khan et al. observed a decline in osteogenic potential in aged donors, whereas Shi et al. reported no change with age\textsuperscript{43, 44}. Pandey et al. also showed that ASCs from younger donors had a significantly elevated potential to differentiate toward adipogenic and osteogenic lineages\textsuperscript{189}. These discrepancies suggest that a 2D platform may not provide clinically relevant insight into the role aging plays on ASC behavior. Traditionally, the hallmark of MSCs (and ASCs) was the ability to differentiate into various cell types for regenerative repair of damaged tissue\textsuperscript{54, 209, 210}. However, many studies suggest that the treatment modality of MSCs occurs through the expression of cytokines and chemokines\textsuperscript{211}. Numerous studies have focused on the age-related changes in ASC proliferation and differentiation; however, very few studies have delved into the effect of age on ASC paracrine function. Our study has shown an elevated level of cytokine and chemokine expression in young ASCs. Many of these factors including CXCL1, SDF-1, CCL5, IL-8, IL-10, and TNF-\textgreek{a} are associated with various phases of the wound healing process. The ASC secretome has been harnessed as a cell-free treatment option for many conditions including wound healing. Although studies have shown that ASC paracrine activity has beneficial effects on non-healing wounds, evidence also indicates the secretome presents adverse effects on pathologies including cancer. Furthermore, no study to date has focused on the impact of ASC age on paracrine activity and its influence on disease progression. Since there are observed differences in aged and young ASC paracrine function, we will evaluate the extent to which those differences influence the stromal environment of ER-\textgreek{a}+ breast cancer tumors.
CHAPTER 3. INVOLVEMENT OF STROMAL AGE IN BREAST CANCER
ESTROGEN SIGNALING

3.1 Introduction

In the United States, approximately 1 in 8 women will develop invasive breast cancer\(^{212}\). The risk of breast cancer increases with age, a large percentage occurring in women 50 and older. Fewer than 5% of breast cancers occur in women under the age of 40; however, breast cancer is the leading cause of cancer death amongst women between 20-39 yo\(^{213}\). Roughly 11 per 100,000 breast cancer patients between the ages of 15-39 years old have estrogen receptor-alpha (ER-\(\alpha\)) positive tumors, making endocrine therapy the treatment of choice. Although initially successful, many patients develop resistance to treatment, resulting in the reappearance of cancer\(^{13}\). Young individuals that have luminal ER-\(\alpha^+\) breast cancer have an observed increase in resistance to endocrine therapies such as tamoxifen\(^{214}\). Currently, there is neither specific focus evaluating mechanisms for resistance to therapy in young ER-\(\alpha^+\) patient populations nor are current therapies designed to combat endocrine resistance evaluated in young ER-\(\alpha^+\) breast cancer patients\(^{214, 215}\). Recently, attention has been directed to tumor microenvironment as an active participant in tumorigenesis and metastasis, as well as the progression toward hormone independence and endocrine therapy resistance\(^{37, 216-218}\). Tumors are composed of a diverse population of stromal cells such as ASCs, endothelial cells, fibroblasts, adipocytes, and immune cells\(^{36, 219}\). The interaction between tumor cells and the microenvironment can be characterized as reciprocal. This interplay between normal cells, cancer cells, and the extracellular matrix contributes to the hallmarks of cancer including immunomodulation, angiogenesis, invasion and metastasis, and apoptotic resistance\(^{87}\). Similar to the wound healing process, the host response leads not only to the modification of secreted proteins from tumor cells and stroma, but also activates surrounding cells including ASCs\(^{220-224}\). ASCs, a multipotent cell population found in breast tissue, are an integral part of the tumor microenvironment. Although elicited for their multi-lineage differentiation and proactive role in tissue regeneration, ASCs are linked to breast cancer progression\(^{54, 207, 219, 225, 226}\). ASCs express and secrete multiple growth factors, cytokines, chemokines, and inflammatory biomarkers such as SDF-1 and IGF-1 that aid in cancer development and progression\(^{168, 227, 228}\). These signaling molecules also enhance breast cancer progression and regulate resistance to endocrine therapies in ER-\(\alpha^+\) breast cancer. Studies show that ASCs create a pro-inflammatory tumor microenvironment that promotes tumorigenic activity through the release of cytokines such as TNF-\(\alpha\), IL-6, and IL-8\(^{192}\). In addition, donor characteristics alter the ASC phenotype. Specifically, ASCs derived from obese donors are demonstrated as having elevated levels of LEP and contribute to endocrine resistance in ER-\(\alpha^+\) breast cancer through LEP.

The microenvironment or stem cell niche not only supports surrounding cell populations, but also provides diverse biophysical and biochemical cues for regulating cell behavior\(^{229}\). The ECM, a heterogeneous, non-cellular component of the microenvironment, guides the behavioral patterns of stem cells including self-renewal, quiescence, migration, differentiation, and apoptosis\(^{230-233}\). Aging of cells within this
specialized microenvironment or age-dependent modifications in the acellular components of the microenvironment can result in stem cell dysfunction\textsuperscript{234}. As a component of the microenvironment, the ECM also undergoes age-related changes that compromises its mechanobiological role\textsuperscript{235}. Studies have also shown that age-associated changes in ECM composition subsequently alters stem cell behavior\textsuperscript{236}. Although the ECM alters the regenerative potential of stem cells, it is questionable as to whether or not it is permanent. When aged MSCs are seeded young MSC-derived ECM, the aged MSCs express greater pluripotency\textsuperscript{237}. This further demonstrates the significance of the microenvironment and its potential as a medium to rejuvenate aged stem cells.

Age-related changes in the ECM not only mediates cell proliferation, differentiation, inflammation, and apoptosis, but also impacts the recruitment and functional integration of ASCs\textsuperscript{236}. As a result, processes such as wound healing become less effective. Wound healing is a complex process involving the active participation of multiple cell types, biochemical mediators, cytokines, growth factors, and ECM components\textsuperscript{238, 239}. The dynamics of a tumor parallel those of the wound healing process. Similar to a wound, tumors attract a diverse population of stromal cells including stem cells and immune cells\textsuperscript{219, 240}. Although the stem cell response becomes damaging in light of tumor function and progression, it contrastingly exhibits regenerative properties for chronic wounds\textsuperscript{241-244}. Current research has yet to focus on the impact of stromal age on the development of endocrine resistance. In the present study, we assessed the effect of ASC age on the MCF-7 cell line in order to discover an underlying mechanism for the development of endocrine resistance.

3.2 Materials and Methods

Cell culture: MCF-7 human breast adenocarcinoma cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM, Cat #: 11965-092, Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Cat #: SH30087.03, GE Healthcare), 1% Non-essential amino acids (NEAA, Cat #: 11140-050, Gibco), Minimal essential amino acids (MEMAA, Cat #: 11130-051, Gibco), sodium pyruvate (100 mM, Cat #: 11360-070, Gibco), L-glutamine (Cat #: 12561-056, Gibco), Antibiotic-Antimycotic (Anti-Anti, Cat #: 15240062, Gibco), and insulin (4 mg/mL, Cat #: 12585-014, Gibco) at 37°C in humidified 5% CO\textsubscript{2}. MCF-7 cells were seeded at 3,000 cells/cm\textsuperscript{2} in T150 flasks until attained 80% confluence. Human adipose-derived stem/stromal cells (ASCs) were obtained from Obatala Sciences and Dr. Frank Lau. Young donors (24-34 years old) and aged donors (50-69 years old) were grouped by a specific age range (Table 1.5). Cultures of ASCs (3,000 cells/cm\textsuperscript{2}) were maintained with Alpha-MEM with L-glutamine (Cat #: 12561-056, Gibco) supplemented with 10% fetal bovine serum (FBS, Cat #: 43602-500, JR Scientific) and 1% Antibiotic-Antimycotic (Anti-Anti, Cat #: 15240062, Gibco) until attained 80% confluence. The procedure implemented for ASC expansion was described previously by Bunnell et al\textsuperscript{204}.

Conditioned media: ASCs were cultured until attaining 80% confluence in 10% Alpha-MEM with L-glutamine (Cat #: 12561-056, Gibco) supplemented with 10% fetal bovine serum (FBS, Cat #: 43602-500, JR Scientific) and 1% Antibiotic-Antimycotic (Anti-Anti, Cat #: 15240062, Gibco). Cells were washed with sterile phosphate buffer saline (PBS,
ASCs were then cultured in phenol-free Dulbecco’s modified Eagle’s medium (Cat #: 10010023, Gibco), 5% charcoal stripped FBS (Cat #: SH3006803, GE Healthcare), 1% penicillin/streptomycin (P/S, Cat #: 15140-122, Gibco), 1% MEMAA, 1% NEAA, 1% Glutamax (Cat #: 35050-061, Gibco), and 1% sodium pyruvate for 24 h. The conditioned media was collected, filtered, and stored at -20°C. MCF-7 cells were treated with 0.25% Trypsin-EDTA (Cat #: 25200056, Gibco) and seeded at 50,000 cells/cm² in 10 cm dishes. Cells were maintained in 10% DMEM at 37°C in humidified 5% CO₂ until 80% confluent. Once confluent, cells were maintained in conditioned media obtained from aged and young ASCs for 72 h or 15 min. Cells were then collected and stored at -80°C for RNA extraction and western blot analysis.

Proliferation Assay: MCF-7 cells were seeded at 50 cells/cm² in 96-well plates. Cells were maintained in 10% DMEM at 37°C in humidified 5% CO₂ and treated with conditioned media after 24 h. At days 0, 1, 5, and 7, cells were fixed with 95% methanol for 10 min. They were washed 3x with PBS prior to crystal violet staining. Cells were stained with 3% crystal violet solution and incubated at room temperature for 1 h. The wells were washed with water then allowed to dry prior to imaging. After dissolving in 33% acetic acid, absorption was determined at 595 nm using the Cytation 3.

Differentiation Assays: ASCs were cultured until attained 80% confluency in 10% Alpha-MEM with L-glutamine supplemented with 10% FBS and 1% Anti-Anti. Adipogenic differentiation medium (AdipoQua, Obatala Sciences, New Orleans, LA) was introduced for 3 days and alternated with MCF-7 conditioned medium for 3 days for an 11-day period. Cells were then collected and stored at -80°C for RNA extraction and qRT-PCR analysis.

Quantitative Real Time PCR (qRT-PCR): RNA was extracted from cell pellets using QIAshredders (Cat #: 79656, Qiagen) and RNeasy Mini Kit (Cat #: 74106, Qiagen) as per manufacturer instructions. Complementary DNA (cDNA) was synthesized using qScript cDNA SuperMix (Cat #: 101414-106, VWR), and 1µg total RNA. Quantitative PCR was performed using the BioRad iCycler (BioRad, v4.006) and Quanta SYBR green (VWR, Radnor, PA) as per manufacture’s protocol. Expression was computed using the ΔΔ(Ct) method using the reference gene GAPDH (ASCs) and β-Actin (MCF-7s). Primer sequences are presented in Table S2. Normalization was to aged ASC gene expression designated as 1.

Western blot analysis: MCF-7 cells were seeded at 50,000 cells/cm² in 10 cm dishes. Cells were maintained in 10% DMEM at 37°C in humidified 5% CO₂ until 80% confluent. At 80% confluency, 10% DMEM was removed and either 5% charcoal stripped media, 5% charcoal stripped media with estrogen (Cat #: E2758-1G, Sigma Aldrich, St. Louis, MO, 1 µg/mL), or conditioned media from aged and young ASC donors was added to the dishes. After 15 min at 37°C in humidified 5% CO₂, the media was removed, cells were washed, and cells were collected on ice and stored at -80°C. Cell pellets were lysed 100 µL of mammalian protein extraction reagent (M-PER, Cat #:78501, ThermoFisher Scientific, Waltham, MA) supplemented with 1x Halt protease and phosphatase inhibitor (Cat #:78444, ThermoFisher Scientific). BCA protein assays were run on the cell extracts and 1µg of total protein was mixed with a master mix (Cat #:SM-W004, ProteinSimple,
San Jose, CA) to give a final concentration of 0.2 mg/mL total protein. Samples were heated at 95 °C for 5 min. Samples, blocking solution, primary antibodies (ER-α, Santa Cruz # sc-543, 1:50; actin, Novus Biologicals # NB600-501H, 1:500; phospho-ER-α serine 167, Cell Signaling #64508 1:25; phospho-ER-α serine 118, Cell Signaling #2511 1:25; phospho-ERK1/2, Cell Signaling #4307 1:25; total ERK, Cell Signaling #4695 1:50; phospho-AKT, Cell Signaling #4060 1:25; total AKT, Cell Signaling #4691 1:50), horseradish peroxidase-conjugated secondary antibodies (Cat#: DM-002, ProteinSimple), chemiluminescent substrate (Cat#: DM-002, ProteinSimple), and separation and stacking matrices (Cat #: SM-W004, ProteinSimple) were loaded into designated wells in a 384-well plate according to the manufacturer’s instructions. After plate loading, fully automated electrophoresis and immunodetection took place in the WES capillary system (ProteinSimple). Proteins were separated by molecular weight at 375V for 25 min, and primary and secondary antibodies incubated for 30 minutes. Chemiluminescence was captured by a charge-coupled device camera, and the digital image analyzed using Compass software (ProteinSimple). The amount of each protein was calculated based on peak area. A virtual image is shown based on peaks from the electropherogram.

xCell Cell Type Enrichment Analysis: Immune and stromal cell infiltrate of young and aged ER-α⁺ was analyzed using the xCell webtool. Ductal and lobular neoplasms were selected from the case set of the TCGA-BRCA project (phs000178.v11.p8) derived from the NIH Genomic Data Commons (GDC) Data Portal. Based on the clinical information provided with the TCGA-BRCA project, tumors were grouped into young (<45 years old) and aged (>65 years old) categories. These selected cases were matched with pre-calculated TCGA data provided by the xCell webtool.

Breast cancer data sources and analysis: Invasive breast carcinoma data set (IlluminaHiSeq) from The Cancer Genome Atlas (TCGA) research network was evaluated for differences in gene expression between young and aged patients based on ER status. Data base for Annotation, Visualization and Integrated Discovery v6.8 (DAVID v6.8) was used to identify enriched functional-related gene groups found of interest in the TCGA dataset. The Breast Cancer Gene-Expression Miner v4.4 (bc-GenExMiner v4.4) was used to perform an age-dependent comparison in gene expression of significant genes from the TCGA data. Original data analyzed in bc-GenExMiner was acquired from the NCI GDC Data Portal. The population selected was based solely on age, not receptor status. The Kaplan-Meier Plotter was used to assess the effect of targeted genes on the survival of ER-α⁺ breast cancer patients. Cell enrichment analysis of young and aged ER-α⁺ breast tumors was performed using the xCell webtool.

Biostatistics: All values are presented as means ± standard error (SEM). Statistical analyses among two groups were performed using the Student’s t-test. Statistical significance was set at p<0.05. Analysis was performed using Prism 8 (GraphPad Software, San Diego, CA).
3.3 Results

*Tumor micro-environment composition is altered in young ER-α+ breast cancer compared to age.*

According to the NCI Surveillance, Epidemiology, and End Results Program (SEER), HR+ breast cancer patients ages 15-39 years old have a lower survival rate (87.7%) compared to ages 65-74 years old (95.6%) and 75+ years old (94.2%). To better identify distinguishing features and underlying mechanisms for lower survival rates observed in young ER-α+ patients, previously aligned and published data derived from The Cancer Genome Atlas (TCGA) IlluminaHiSeq invasive breast cancer was analyzed for differences between aged and young patient populations. Tumor samples were separated based on ER-α+ receptor status and age (young individuals <40 years old and aged individuals >65 years old).

![Diagram of NF-κB Signaling Pathway](image)

Figure 3.1. Tumor microenvironment is differentially regulated in young and aged ER-α+ breast cancer patients. DAVID functional annotation tool was used to determine enriched pathways of elevated genes in the TCGA data set. Results revealed significant p values (p<0.05) for matrix, focal adhesion, cytokine, PI3K/AKT, and NF-κB pathways. TCGA data was filtered based on fold changes ≥1.4.

Results demonstrated that key pathways enriched in young ER-α+ breast cancer samples were those associated with the extracellular matrix (COL6A6, ITGA1, ITGA10, ITGA2, ITGA4, LAMA1, LAMA2, LAMB3, LAMC1, LAMC2, LAMC3), focal adhesions, cytokine-cytokine interactions (CCL19, CCL21, CCR4, CCR6, CXCL5, CXCL9, CD27, IL12B, IL20), PI3K/AKT, and NF-κB (TNFRSF13B, TNFAIP3, TNFSF11) (Fig 3.1). Specifically, tumors from young individuals had higher levels of matrix genes and these genes correlated with poor patient survival.

In depth analysis of genes enriched in young (<40 years old) ER-α+ breast cancer compared to aged ER-α+ (>65 years old) demonstrated that in addition to matrix, increased cytokines associated with a pro-inflammatory phenotype (CCL5, IL12B, IL-20, IL-11, IL-19) and growth factors (FGF-1, PDGFB, SDF-1, IGF-1) were also detected.
increase in pro-inflammatory cytokines and growth factors suggests the tumors may be eliciting support from surrounding cell types, specifically stem cells\textsuperscript{220, 254}. Comparison of breast tumor cell infiltration revealed significant differences (p<0.05) in CD8+ T-cells, T-helper 2 (Th2) cell, and M2 macrophages between young and aged ER-α+ breast tumors (Fig 3.2A). Young tumors exhibited higher xCell scores for CD8+ T-cells (0.02± 0.003) and Th2 cells (0.04 ± 0.004), but a lower score for anti-inflammatory M2 macrophages (0.01 ±0.0008). In contrast to immune cell infiltration, both MSC and adipocyte xCell scores were similar for young and aged ER-α+ breast tumors (Fig 3.2B).

Figure 3.2. Immune cell infiltrate differs between young and aged ER-α+ breast cancer patients. (A) xCell cell type enrichment analysis of young (<45 years old) and aged (>65 years old) ER-α+ breast cancer patients revealed significant p values (p<0.05) for M2 macrophages, CD8+ T cells, and Th2 cells. Both CD8+ T cells and Th2 cells are enriched in young tumors while M2 macrophages exhibit a lower quantity compared to aged. (B) Both adipocytes and MSCs exhibit similar quantities in young and aged ER-α+ breast tumors.

With an increase in specific stromal components in both the TCGA and enrichment data, we next sought to determine the role of these specific components in patient survival. Although selected genes exhibited increased expression in young in the TCGA data, they were not individually associated with poor patient survival in the Kaplan Meier plots (Fig 3.3).
Breast tumors exhibit age-dependent differences in specific stromal components.

The Breast Cancer Gene-Expression Miner (bc-GenExMiner) was used to evaluate the age-dependent expression of genes linked to paracrine signaling. Targeted genes in age groups <40 years old, 40-70 years old, and >70 years old were compared to determine significant differences in gene expression between 0-40 years old and 70-90 years old was observed in CXCL1 and COL5A2. On the other hand, TCGA data comparing young and aged HR+ tumors revealed significant differences in multiple genes (Table 3.1). Many of these observed paracrine factors are pro-inflammatory, suggesting an inflammatory response may play a role in the young patient tumor microenvironment.

Figure 3.3. High expression of targeted genes exhibits no impact on patient survival. Kaplan Meier plots of elevated genes in young ER+ breast tumors were used to evaluate the effect of expression on patient survival. All plots displayed did not indicate a correlation between high expression of targeted genes and poor patient survival.
Table 3.1 Comparison of bc-Genex Miner and TCGA Paracrine Factor Expression in Young Tumors.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Bc-Genex Miner</th>
<th>P-value</th>
<th>TCGA</th>
<th>P-value</th>
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</tr>
<tr>
<td>SDF-1</td>
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<td>N/A</td>
<td>Increased</td>
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</tr>
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<td>N/A</td>
<td>Increased</td>
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<tr>
<td>COL5A2</td>
<td>Increased</td>
<td>0.051</td>
<td>Increased</td>
<td>0.006</td>
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</tbody>
</table>

bc-GenExMiner p-values represent the comparison between 0-40 and 70-90 years old. TCGA p-values represent the comparison between <45 years old and >65 years old.
Young stem cells induce endocrine response in ER-α+ breast cancer.

Since age affects ASC paracrine signaling, we then assessed the effect of the ASC secretome on ER-α+ breast cancer. To evaluate the effect of the secretome on proliferation, MCF-7 cancer cells were treated with CM obtained from aged and young ASCs. Crystal violet staining revealed increased proliferation of MCF-7 cells treated with CM from young ASCs (684.5 +/- 69.78 SEM) compared to those treated with aged CM (480.4 +/- 158.2 SEM) at day 7(Fig 3.4). The proliferation of MCF-7 cells treated with young, CM showed significant change from day 1 to day 7 (p<0.05) whereas those treated with aged, conditioned medium showed no significance in proliferation. To determine if the increased proliferation was mediated through endocrine signals, ER-α response genes were evaluated via qRT-PCR. Results show that the ER-α regulated genes PGR and SDF-1 were increased by 1 (+/- 0.44 SEM) and 1.25 (+/- 0.35 SEM) respectively in MCF-7 cells treated with young ASC CM when compared to those treated with aged CM (Fig 3.5). Protein expression of upstream ER-α regulators and their ER-α phosphorylation sites was evaluated to further determine the role of the ASC secretome on MCF-7 endocrine signaling. After stimulation with conditioned medium for 15 min, MCF-7 cells were collected for western blot analysis. Results revealed an increase at p-ER S167 in MCF-7 cells exposed to young CM, suggesting that ASC signaling is mediated through the estrogen receptor. To confirm these results, MCF-7 cells were treated with tamoxifen for 4h prior to stimulation with aged or young CM. Cell were collected after 24h and ER-α response genes were evaluated with qRT-PCR. While elevated in the presence of young CM, ER-α shows loss of signal in the presence of tamoxifen. These results suggest that ER-α plays a key role in stem cell signaling. To further validate the effect of stem cell signaling on ER-α activity, MCF-7 cells were treated with aged and young CM followed by treatment with adipogenic differentiation media for 11 days. qRT-PCR results revealed no difference in gene expression between the treatment groups.
expression of ER-α, PGR, and SDF-1 between aged and young. The loss of stemness and commitment to an adipogenic phenotype results in comparable ER-α expression in aged and young.

Figure 3.5. Young ASC secretome enhances estrogen signaling in MCF-7 cell lines. (A) MCF-7 cells were cultured with young and aged ASC CM and collected for qRT-PCR. Both PGR and SDF-1 expression were significantly different (p<0.05) from aged expression. (B) MCF-7s were cultured with young and aged ASC CM and evaluated for total and phosphorylated AKT, ERK, and ER protein expression using Western blot analysis. Results show an increase at p-ER-S167 in MCF-7s stimulated with young CM. (C) MCF-7 cells were treated with tamoxifen 4 h before being treated with either young or aged ASC CM. MCF-7 cells were collected after 24 h for qRT-PCR to evaluate ER regulated gene expression. Results confirm that ASC signaling is mediated by ER. (D) Differences in gene expression between young and aged is lost with adipogenic differentiation.
3.4 Discussion

The microenvironment plays a pivotal role in directing cell behavior. Strong evidence supporting the significance of the microenvironment on cell behavior is demonstrated in heterochronic parabiosis studies. The breast cancer microenvironment is defined as a combination of cells within the tumor and its stroma, the ECM, and surrounding signaling molecules. In recent years, an overwhelming amount of evidence supports that the tumor microenvironment, and more specifically components of the ECM, contribute to breast cancer progression and metastasis. Furthermore, studies have shown that the tumor microenvironment actively participates in the progression toward hormone independence and endocrine therapy resistance. Age-related physical and compositional changes in the ECM is linked to tumor progression. Although it is well-documented that specific extracellular matrix gene profiles are associated with the prognosis and treatment of breast cancer, little evidence is known about how local cellular function, specifically the ASC secretome, contributes to endocrine resistance in young ER-α+ breast cancer patients. The extent to which ASCs, a resident of the breast cancer microenvironment, affect breast cancer progression and response to treatment has yet to be fully elucidated. Previous studies indicate that ASC age has no bearing on ECM dynamics. Although active in ECM production, ASCs also have both a paracrine and immunomodulatory function. In regenerative medicine, ASCs are no longer solely used for cell restoration. Their secretion of trophic factors has been harnessed as a viable approach for tissue regeneration. Although beneficial in terms of regenerative medicine, the paracrine actions of ASCs may enable tumor formation, growth, and maintenance. The expression of paracrine factors between young and aged BC tumors is strikingly different. Chemokines including CXCL1 and IL-8 both exhibited elevated expression in young ASCs in the cytokine array analysis. Increased CXCL1 protein expression is associated with increased tumor growth and pulmonary metastasis in MDA-MB-231 breast cancer cells. Furthermore, increased expression of CXCL1 RNA is associated with a decreased survival of patients with ER-α+ breast cancer. IL-8, a pro-inflammatory chemokine that promotes neutrophil chemotaxis and degranulation, is typically upregulated in breast cancer and associated with poor prognosis. The monocyte attracting chemokine CCL5 was also upregulated in young ASCs. Studies have shown that an overexpression of CCL5 in breast tumor cells results in increased invasiveness. SDF-1, a chemokine naturally secreted by MSCs, is an important mediator of MSC chemotaxis and primary breast tumorigenesis. SDF-1, another cytokine elevated in young ASCs, has been shown to promote hormone independent growth in the hormone dependent MCF-7 line. As an ER-α-regulated gene, SDF-1 is suggested to play a role in the progression of breast carcinoma cells. The amount of SDF-1 secretion by MSCs has shown variance based on microenvironmental factors, one of which could be age. SDF-1 signaling also contributes to chemotherapy resistance in cancer cells by modulating survival pathways such as AKT, NOTCH, and the BCL-2 family. Although tumor expression of specific cytokines, chemokines, and growth factors is correlated with poor patient survival, Kaplan Meier plots of TNF-α, IL-16, IL-32, SDF-1, IGF-1, and CD27 do not reflect those same findings. Rather than examining singular genes, further analysis these genes and patient survival must be performed using gene signature cohorts or groups of genes.
Breast cancer is composed of a large cellular array: ASCs, adipocytes, fibroblasts, immune cells, and endothelial cells. The analysis of cell infiltrate in young and aged breast tumors has revealed significant differences between several immune cell populations including CD8+ T cells, M2 macrophages, and T\(_h\)2 (Fig 3.6A and B). Previous studies have shown that CD8+ T cells, T-helper 1 (T\(_h\)1) cells, M1 macrophages, and natural killer (NK) cells exhibit anti-tumor activities, whereas T-regulatory (T\(_{\text{reg}}\)) cells and M2 macrophages exhibit immune-inhibitory, pro-tumor activities\(^{279}\). Interestingly, M2

---

Figure 3.6. Young ER-\(\alpha^+\) breast tumor stroma exhibits significant differences in specific matrix components and immune cell types. (A) Young tumor stroma (<45 years old) revealed significantly higher levels of matrix components (fibronectin, integrin \(\alpha\) and \(\beta\), collagen III, collagen V, collagen VI, collagen VII, and laminins A, B, and C) and immune cell types (CD8+ T cells and T\(_h\)2 cells) (p<0.05). (B) Aged tumor stroma (>65 years old) revealed a significantly higher M2 macrophage infiltrate (p<0.05) compared to young stroma. The young and aged tumor stroma exhibit comparable numbers for MSC and adipocyte infiltrate; however, young MSCs may exhibit a greater potency and stimulate a rapid estrogen signaling pathway via cytokine and growth factor secretions. This contributes to the increased ER-\(\alpha\) signaling and increased proliferation observed in MCF-7 cells stimulated with young CM compared to those stimulated with aged CM.
macrophage infiltrate was significantly lower in young compared to aged tumors, and CD8+ T cells were significantly higher in young compared to aged tumors. Although Jin and Hu determined that CD8+ T cells are associated with disease-free survival in young breast cancer patients compared to aged, Ali et al showed that CD8+ T cells infiltrate predicts a more favorable outcome for ER+ tumors than ER- tumors. Based on the literature, there is still no conclusive evidence identifying significant biological differences and T-cell immune infiltration between young and aged ER+ breast cancer patients. With discrepancies in immune cell infiltration as prognostic indicators, other stromal cells have been exploited as possible prognostic indicators for breast cancer. Although MSC infiltration in young and aged breast tumors were not significantly different, MSCs may influence age-dependent tumorigenesis in an alternative manner. Rather than directly infiltrating the tumor, it is suggested that MSCs prime the site for tumor cell growth via secretion of high levels of multiple growth factors, cytokines, and chemokines. Younger patients may have MSCs that secrete higher volumes of pro-tumorigenic factors compared to aged, thus resulting in poor prognoses in young patients with specific subtypes including ER+ and TNBC. Patient age correlates with stem cell potency, inflammatory secretion, and differentiation capacity. Studies on BMSCs noted that aged stem cells had a lack of receptors for cytokines and chemokines, including TNFR and loss of these receptors resulted in an inability to respond to immune signals from the surrounding environment. Together this suggests that young stroma, specifically young ASCs, may be more reactive to cytokine signals in a tumor environment than aged stroma. Prior studies evaluating differences in young and aged ASCs demonstrate that young ASCs have increased levels of pro-inflammatory signaling (MCP-1, IL-8, TNF-α) and adipogenic markers (PPARγ) compared to aged ASCs. TNF-α is critical for ER-α+ breast cancer progression and released in response to estrogen stimulation.

TNF-α is not only secreted by tumor cells, but is also produced by ASCs. TNF-α production by tumors has been linked with poor prognosis and loss of hormone responsiveness. The interaction of TNF-α with TNFRs activates downstream targets including the pro-inflammatory transcription factor NF-κB. NF-κB consists of a family of transcription factors that contribute to inflammation, immunity, cell proliferation, differentiation, and survival. The interaction of NF-κB with ER-α is one of the mechanisms of estrogen signaling. ER-α acts through several different mechanisms. Direct genomic signaling is the traditional mechanism of estrogen signaling. The binding of the dimerized estrogen receptors leads to nuclear translocation and binding to estrogen response elements (EREs) sequences of target genes. Estrogen can also induce rapid, non-genomic signaling in which estrogen induces gene transcription and protein synthesis by alternative mechanisms. The activation of signaling cascades including the PKC pathway, RAS/RAF/MAPK pathway, PI3K/AKT pathway, and the cAMP/PKA pathway leads to subsequent activation and binding of a variety of transcription factors (i.e. C/EBPβ, NF-κB, STAT) to alternative DNA response elements. Adding to the complexity of estrogen signaling, estrogen receptors can also be activated in the absence of estrogens or other receptor agonists. Ligand-independent estrogen receptor activation occurs via the phosphorylation of specific residues (i.e. serine or tyrosine) in the receptors themselves or by coregulators such as PKA, MAPK, PKC, inflammatory cytokines (i.e. IL-2), and peptide growth factors (i.e. IGF-1, TGFβ, EGF). Evidence has demonstrated that
the crosstalk between ER-α and many of the aforementioned growth factors and inflammation-associated transcription factors including NF-κB results in acquired endocrine resistance\textsuperscript{287-295}.

Although ER-α has been shown to repress the transcriptional activity of NF-κB, reports have also exhibited the existence of positive crosstalk between ER-α and NF-κB\textsuperscript{296-298}. Studies have shown that constitutive activation of NF-κB in breast tumors is correlated with more aggressive ER-α\textsuperscript{+} tumors, the development of resistance to endocrine therapy, and progression to estrogen-independent growth\textsuperscript{299-303}. It has also been reported that cytokines and chemokines released in the cancer-induced inflammatory environment activates NF-κB-associated pathways that desensitize SERM response\textsuperscript{304}. Long term antihormonal therapy alters ER-α driven regulation of metabolism and inflammation which also results in the constitutive activation of NF-κB\textsuperscript{305-307}. The constitutive activation of the canonical NF-κB pathway is not only triggered by ER-α, but also by TNF-α. TNF-α activation of NF-κB results in the transcription of cytokines including IL-8, IL-1β, and a positive feedback loop resulting in NF-κB dependent TNF-α production\textsuperscript{33}. Both IL-8 and TNF-α exhibit increased expression in both young ASCs and tumor samples from young breast cancer patients. As previously mentioned in section 1.4, NF-κB expression is elevated in older donors; however, commonly associated molecules including IL-1α, TNF-α, iNOS, IL-8, and IκB were significantly downregulated in older donors\textsuperscript{189}. Furthermore, older donors exhibited elevated concentrations of NF-κB in the cytosol whereas younger donors displayed an accumulation of NF-κB in the nucleus. An accumulation of NF-κB subunits in the cytosol suggests decreased transcriptional activity, thus verifies the decreased pro-inflammatory cytokines and molecules observed in older donors.

A link between ER-α and NF-κB possibly lies at the Serine 167 (Ser-167) residue on ER-α. NF-κB is normally sequestered in the cytoplasm of most cells and bound to the inhibitory proteins IκB. IκB kinase α (IKKα) phosphorylates IκB proteins, leading to the degradation and subsequent activation of NF-κB gene expression. A study by Bennett \textit{et al.} has shown that increased IKKα levels correlate with reduced cancer-specific survival and reduced recurrence-free survival in ER\textsuperscript{*} breast cancer\textsuperscript{308}. In addition to IKKα, the non-canonical kinase IKKε has been identified as an important mediator of inflammatory and oncogenic signaling. IKKε as a breast cancer oncogene has been amplified and overexpressed in over 30\% of breast carcinomas and breast cancer cell lines\textsuperscript{309}. It has been shown that IKKε phosphorylates Ser-167 of ER-α, and activation of ER-α contributes to tamoxifen resistance in breast cancer. Western blot analysis results from this study indicate increased expression of p-SER-167 in MCF-7 cells stimulated with young ASC conditioned media. Ser-167 is located within the hormone-independent transcriptional activation function 1 (AF-1) region, suggesting the phosphorylation of this site behaves independently of estrogen-ER-α interactions\textsuperscript{310, 311}. Future studies should further explore the role of the NF-κB pathway on ASC-mediated estrogen-independent signaling in young ER-α\textsuperscript{+} breast cancer patients.

In addition to TNF-α, CD40 ligand (CD40L) may contribute to the onset of endocrine resistance. CD40L, an activator the noncanonical NF-κB pathway, showed elevated expression in young patient tumors. CD40L binding to its receptor CD40 results
in the transcription of SDF-1, a cytokine that is elevated in both young patient tumors and young ASCs. In addition to TNF-α, CD40L expression may have an underlying role in NF-κB activation and subsequent endocrine resistance in young ER-α⁺ breast cancer patients.

Through this study, we have observed that the ASC secretome enhances estrogen independent signaling in the ER-α⁺ MCF-7 cell line. There is also limited evidence that the aged ASC secretome impacted MCF-7 cells in a manner comparable to the young ASC secretome.
APPENDIX A. SUPPLEMENTAL TABLES

Table S1. Young and aged ASC donor demographics.

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Table S2. qRT-PCR oligonucleotide primers.

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APPENDIX B. PERMISSION REQUESTS

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VITA

Katie Hamel, born in Covington, Louisiana, completed her Bachelor of Science in Biological Sciences from Louisiana State University (LSU) in May 2011. She graduated with Upper Division Honors Distinction and was awarded multiple university-based scholarships including Tiger Athletic Award Scholarship, Flagship Ph.D. Graduate Assistantship, and Graduate School Dean’s Travel Award. Katie plans to graduate with her Ph.D. in Biological Engineering from LSU in May 2021 and continue to work in stem cell biology at Obatala Sciences. Her research focused on breast cancer and adipose-derived stem/stromal cell interactions in Dr. Elizabeth Martin’s lab.