EVALUATION OF A BIOMATERIAL-BASED THREE-DIMENSIONAL MODEL OF BREAST CANCER METASTASIS

by

Jenna Evelynn Al-Saleh

(Under the Direction of Cheryl Gomillion)

ABSTRACT

Breast cancer metastasis (BCM) remains one of the leading causes of death in women with cancer. Research over the last several decades has indicated that the microenvironment plays a critical role in the activation of the metastatic cascade. Inflammatory cytokines secreted by adipocytes in the tumor microenvironment, such as leptin, can play key roles in metastasis and are of interest as predictive diagnostic indicators. In this work, three-dimensional (3D) chitosan-collagen scaffolds were created and evaluated to characterize their mechanical properties. Studies were performed to assess cell viability and metastatic protein expression to determine the scaffold efficacy for mimicking BCM. Leptin was then incorporated into the 3D models to study the effects of leptin on BCM. Expression of metastasis-related cytokines was measured towards building a proof-of-concept statistical model for predicting BCM based on varying levels of biomarkers, such as leptin.

INDEX WORDS: Breast Cancer Metastasis, Chitosan-Collagen Hydrogels, Logistic Regression Model, African American Breast Cancer Patients, Inflammatory Cytokines

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Jenna Evelynn Al-Saleh

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Jenna Evelynn Al-Saleh

Major Professor: Cheryl Gomillion

Committee Members: William Kisaalita
Eric Freeman

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
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DEDICATION

I want to dedicate this thesis first to my mother who believed in me when no one else did, who raised me to be the curious woman I am, and who sets an example of kindness, hard work, and love every day. To my Aunt "Uncle" Judy, thank you for being an example of an incredible woman in STEM and for your unwavering love from the moment I was born. To my little brother Mo, I am so proud of and inspired by the man you're becoming. Finally, to my sweet Bobby, thank you for your support and love through everything these last 5 years. I can honestly say I couldn't have gotten through this without you and I am incredibly excited for the adventures that await our future. I love all of you with every inch of my being.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Introduction

Breast cancer remains the second most lethal disease for female cancer patients [1]. The survival rate for breast cancer is up to 99% with early detection and treatment of breast cancer, but that number decreases significantly (27%) once breast cancer has metastasized [1-3]. This survival rate is even lower for African-American women with metastatic breast cancer (20%) [1]. While mortality rates have decreased due to early diagnosis using mammographic screening that follows adjuvant therapy, it is not possible with current technology to accurately predict risk of metastasis [3, 4]. Furthermore, earlier onset and prevalence of more aggressive breast cancers, such as inflammatory and triple-negative subtypes, also contribute to increased mortality rates in African-American breast cancer patients [5, 6]. Therefore, it is imperative that differences between not only subtypes of breast cancer, but differences between ethnicities of breast cancer patients be accurately studied as well. This would allow for personalized treatment plans to be created, decreasing the mortality gap between patients of different ethnicities.

In conjunction with racial disparities in breast cancer metastasis that may exist, the microenvironment can also possibly play roles in these differences as well. With the onset of the 'seed and soil' hypothesis, suggesting that cancer cells (the seeds) can only survive when they find a suitable environment (the soil), the idea that the tumor microenvironment plays a critical role in breast cancer metastasis was sparked [7, 8]. This microenvironment consists of stromal cells,

immune cells, and adipocytes that are inserted within an extracellular matrix (ECM), sustained via a vascular network [9, 10].

Over the years, researchers have made many attempts at studying various components of this microenvironment and its influence on breast cancer proliferation and migration. Two-dimensional (2D) monolayer cell cultures, although less expensive and easily reproducible, are unable to effectively model the microenvironment due to lack of biological and mechanical ECM properties [11]. Further, *in vivo* animal models are costly, pose issues with human clinical relevance and can present cause for ethical concerns [12]. Thus, for these reasons, scientists have worked towards creating three-dimensional (3D) breast cancer models that mimic the microenvironment, reduce cost, and increase controlled reproducibility, as well as clinical and physiological relevance [13]. By creating accurate 3D models of breast cancer metastasis, we can more easily study interactions of different behavioral and ethnicity subtypes with the microenvironment and further study specific microenvironmental aspects such as inflammation, adipokines present, ECM stiffness and structure, cell-cell interactions, and cell-ECM interactions. These findings could then help create statistical predictions of metastasis as well as personalized treatment plans for patients.

In this thesis, first, a review of the literature is provided in Chapter 2, outlining and discussing key aspects needed to understand the purposes of our work. Chapter 3 describes work for a submitted manuscript aimed at designing our biomaterial platform for mimicking the metastatic potential of cell lines from different tumor subtypes and ethnicities. The objectives of this work were as follows:

1. Develop a 3D biomaterial-based platform that mimicked breast cancer stiffness and extracellular matrix components.

2. Test these 3D hydrogel platforms to identify composition that supports cell viability and mimics *in vivo*-like tumor behavior (i.e., phenotype and metastatic behavior) most accurately.
3. Apply identified hydrogel to study cells of different subtypes and from donor sources of different ethnicities, compared to 2D controls.

In the work presented in Chapter 4, we aimed to apply our chosen biomaterial-based platform to study how the pro-inflammatory adipokine, leptin, influences breast cancer metastasis of different ethnicities and subtypes. The objectives for this work were as follows:

1. Test selected hydrogel platform to evaluate effects of 3D culture on cell viability, morphology and metastatic protein expression for cells of different subtype and from donors of different ethnicity, compared to 2D controls.
2. Study metastatic protein expression of each cell type within the 3D model to determine differences in metastatic potential between different cancer subtypes and donor source ethnicity.
3. Apply model to study the effects of leptin on metastasis of breast cancer cells of different subtype and from donor sources of different ethnicity.

Finally, in Chapter 5, concluding remarks regarding the work presented in the previous two chapters are provided and proposed future directions for the work are described. Remarks regarding the initial idea behind our work in Chapter 4 will also be discussed in Appendix A.

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CHAPTER 2

LITERATURE REVIEW

2.1 Breast Cancer Metastasis

Breast cancer remains the second leading cause of death among women with cancer [1]. The survival rate of breast cancer is up to 99% with early detection and treatment of breast cancer, but that number decreases significantly (27%) once breast cancer has metastasized (Figure 2.1) [1-3]. Therefore, mortality due to breast cancer has clearly been recognized as an issue with its metastasis to other parts of the body. While research over the last 50 years has made breast cancer a manageable disease, existing gaps in knowledge related to the genetic and environmental mechanisms for metastasis have stunted our abilities to increase metastatic breast cancer survival rates [2]. Therefore, much of breast cancer research is dedicated to understanding these mechanisms.

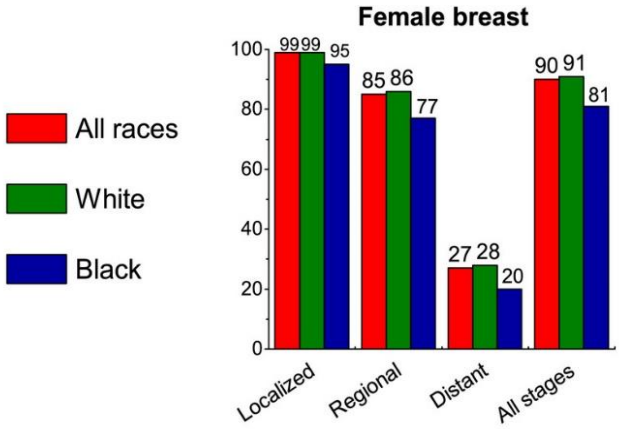


Figure 2.1. Five-year relative survival rate of females diagnosed with breast cancer from 2008 to 2014 [1].

As with all cancers, early diagnosis allows for better patient outcomes and less aggressive treatment. The rates of metastasis and mortality have decreased with the increase of early diagnosis using mammographic screening that follows adjuvant therapy, which generally consists of surgery followed by chemotherapy or radiotherapy [3, 4]. Chemotherapy has increased 15-year survival rates in breast cancer patients, but the acute and long terms effects of chemotherapy significantly affect a patient's quality of life [3]. It is not possible with current technology to accurately predict risk of metastasis nor metastatic organ site, thus, a large majority of patients receive chemotherapy and diagnosis of metastasis generally requires invasive procedures including biopsies and radiological evaluations [5]. Therefore, those who could be treated with local treatment alone including surgery and radiotherapy, will unnecessarily receive toxic chemotherapy and be subjected to invasive procedures [3-5]. Further research on the metastatic cascade could help to improve these methods.

Breast cancer is a heterogeneous disease of which there are two main types, invasive lobular carcinoma (of the breast lobules where milk is produced) and invasive ductal carcinoma (of the breast duct that brings milk from the lobule to the nipples), with ductal carcinoma being the most common type. Occasionally, a case of inflammatory breast cancer occurs as well, but will not be discussed in this review [3]. Furthermore, breast cancer is generally categorized into 3 subtypes based on status of the estrogen receptor (ER), progesterone receptor (PR) and oncogene ERBB2 (HER2) [3, 6]. The status of these receptors is indicative of how each cancer type is treated, how aggressive/metastatic the cancer is, and how each cancer type behaves [2]. ER+ tumors, also known as luminal subtypes, are the most common types of breast cancer. These tumors typically express high levels of ER and PR and are more easily treated by using hormone therapies that remove estrogen within patients [2, 4, 6]. The least common HER2 cancers are driven by the

receptor tyrosine kinase (RTK) oncogene ERBB2. These tumors are characterized by overexpression of the ERBB2 oncogene and are managed with anti-HER2 treatment. The most aggressive subtype, triple-negative breast cancer (ER-, PR-, HER2-) is a basal-like cancer for which there is currently no treatment available [6]. These cancers often result in metastasis and patients are generally managed by cytotoxic therapies [2]. Each of these cancers display different behavior *in vivo but* can all lead to metastasis.

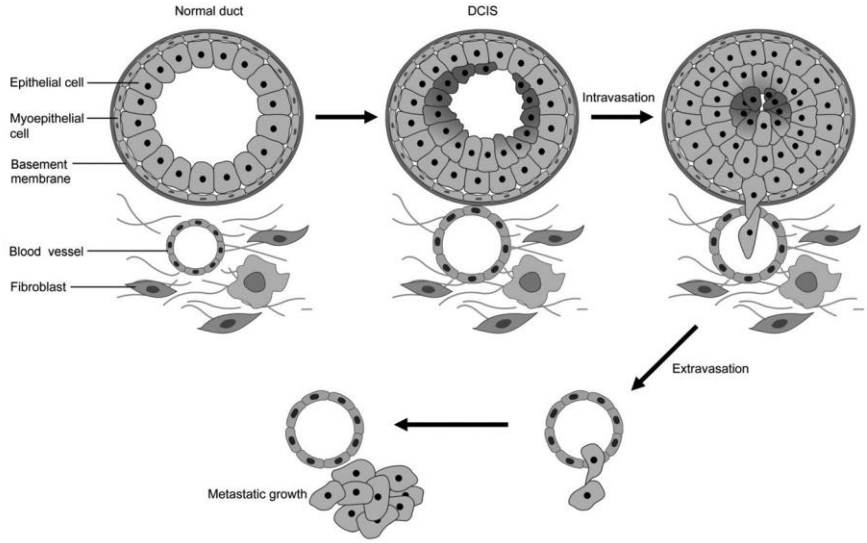
Bone, lung, liver and brain are the most common sites of metastasis, but metastatic sites can differ depending on the type of breast cancer [2, 3]. For instance, ER+ metastasis predominantly spreads to bone, and in rare cases to the brain, whereas triple-negative breast cancers spread more commonly to visceral organs such as the brain and lung [2, 7]. Likelihood of metastasis also depends on cancer type and tumor grade. For instance it is reported that tumors greater than 5 cm are more likely to metastasize than smaller tumors [8]. Currently, we do not understand why particular metastatic types spread to specific organs.

Metastasis occurs via multiple steps called the metastatic cascade (Figure 2.2). The first step of the cascade involves cells becoming locally invasive and migratory [5, 9]. This step requires cancer cells to proliferate uncontrollably as well as begin the process of epithelial-to-mesenchymal transition (EMT), which is a step that involves cancer cells becoming migratory (loss of E-cadherin and gain of N-cadherin) and secreting extracellular matrix (ECM) degrading enzymes (release of several matrix metalloproteinases or MMPs) [2, 5, 6, 9, 10]. They also induce angiogenesis via hypoxia where oxygen deprivation signals VEGF and other chemokines to create blood vessels to create transports for oxygen [6]. These traits allow for the next step of the metastatic cascade whereby cells intravasate into blood vessels. Here, cells must resist anoikis, shear forces of blood

flow, oxidative stress, and immune attack [2, 5, 10]. If successful in circulation, tumor cells can continue progression down the cascade.

After a relatively short circulation period (sometimes minutes), tumor cells find a tissue niche where they extravasate into distant organs [5, 6]. Extravasation involves many of the same players of intravasation as they are similar processes. Tumor cells can extravasate via several methods including passing through cell junctions, penetrating through a single endothelial cell or proliferating uncontrollably until rupturing the blood vessel [9, 10]. Epiregulin (EREG), MMP-1, MMP-2, cyclooxygenase-2 (COX-2), and fascin (FSCN) are genes involved in remodeling vasculature for extravasation as well as destroying vessel junctions for intravasation [2, 11]. Once there, the cells must survive the initial host immune defenses and stress of a foreign microenvironment. This is the most difficult step in the cascade as the cells are exposed to different vasculature patterns, matrix composition, stroma organization and cytokine environment [10].

Figure 2.2. Visual representation of the metastatic cascade [5].



The required genes and phenotypic characterization required for survival depends on the type of organ the cancer is invading. If successful, however, the tumor cells finally thrive, proliferate and influence their new tissue environment [2, 10]. Cancer cells will send out several

signals to the surrounding environment to aid in tumor growth, including VEGF to increase angiogenesis for oxygen and nutrient delivery to the tumor [9]. Here, cells transition to epithelial type cells via the mesenchymal-epithelial transition (MET) which allows for proliferation and attachment of the tumor cells [6].

Why breast cancer transforms from a benign tumor to a metastatic cancer is a question of uncertainty and controversy. For many years, scientist believed that cancer was the result of genetic mutation only, but in the 1890's Stephen Paget developed the seed and soil hypothesis [8, 9]. This hypothesis suggests that cancer cells (the seeds) can only survive once they find a suitable environment (the soil) [5, 9]. This ignited the idea that the microenvironment plays a critical role in determining the behavior of cancer and can influence its metastasis. For instance, bone, which is the metastatic location of more than half of all breast cancer patients [9], provides a microenvironment that is advantageous for breast cancer survival. Chemokines such as fibroblast-derived CXCL12, TGF- β, and Epithelial Growth Factor Receptor (EGFR) can signal for processes involved in breast cancer metastasis such as invasion and migration, ECM degradation and re-modeling as well as anti-apoptosis. CXCL12 is specifically expressed in bone, lung, liver and brain and in no other tissues, which provides evidence that this chemokine may be specific for breast cancer metastasis integrins [5, 9, 12]. Other parts of the microenvironment such as cell junctions, ECM proteins, and immune cells can even aid in extravasation [9]. Therefore, a whole generation of researchers are determined to study the breast microenvironment and how it interacts with breast cancer and influences its metastasis.

2.2 The Breast Cancer Microenvironment

Before we can study the role that the microenvironment plays in breast cancer metastasis, we must first understand what exactly the breast and breast cancer microenvironment is. The breast cancer microenvironment is composed of fibroblasts, immune cells, endothelial cells, mesenchymal cells, adipocytes and bone marrow derived stem cells. The cells are inserted within an extracellular matrix (ECM) that is sustained via a vascular network [5, 13]. The interaction of the ECM and stromal cells with tumor cells aids in cancer initiation, progression and metastasis [13].

Within the ECM lies several cell types that support the function of the surrounding tissues (Figure 2.3). Fibroblasts are the most abundant cell type in tumor microenvironment and provide a structural framework for the stroma [13]. They are generally inactive, except in cases of inflammation, such as with breast cancer, in which they are involved in tissue remodeling [13, 14]. Cancer-associated fibroblasts, or CAFs, aid breast cancer metastasis by producing α-smooth muscle actin (α-SMA), which aids in cell motility and tissue structure as well as other chemokines [12, 13, 15]. Mesenchymal stem cells are signaled by breast cancer cells to differentiate into CAFs to aid metastasis. Endothelial cells are involved in the angiogenesis of the growing tumor by secreting VEGF and Fibroblast Growth Factor 2 (FGF-2) [13] . Adipocytes are another cell type found in the breast cancer microenvironment [13]. These mature fat cells secrete hormones, growth factors and cytokines called adipokines [15]. These adipokines, such as leptin, adiponectin and chemokine (C-C motif) Ligand 2 (CCL2) have been associated with inflammation and breast cancer metastasis. Furthermore, cancer-associated adipocytes have been studied for their ability to influence surrounding breast cancer phenotypes during metastasis [15, 16].

Immune and inflammatory cells are generally present for all malignancies of breast cancer. M2 tumor-associated macrophages (TAMs) are immunosuppressive types of macrophages that are most abundant in the tumor [14]. The tumor microenvironment recruits macrophages into the tumor, which allows for tumor cells to produce cytokines such as VEGF, colony-stimulating factor-1 (CSF1), and CCL2 [13, 14]. TAMs have also been shown to induce EMT in cancer cells by secreting transforming growth factor-β (TGF-β) [17]. The relationship between breast cancer cells, adipocytes and inflammation will be discussed in greater detail in section 1.3.

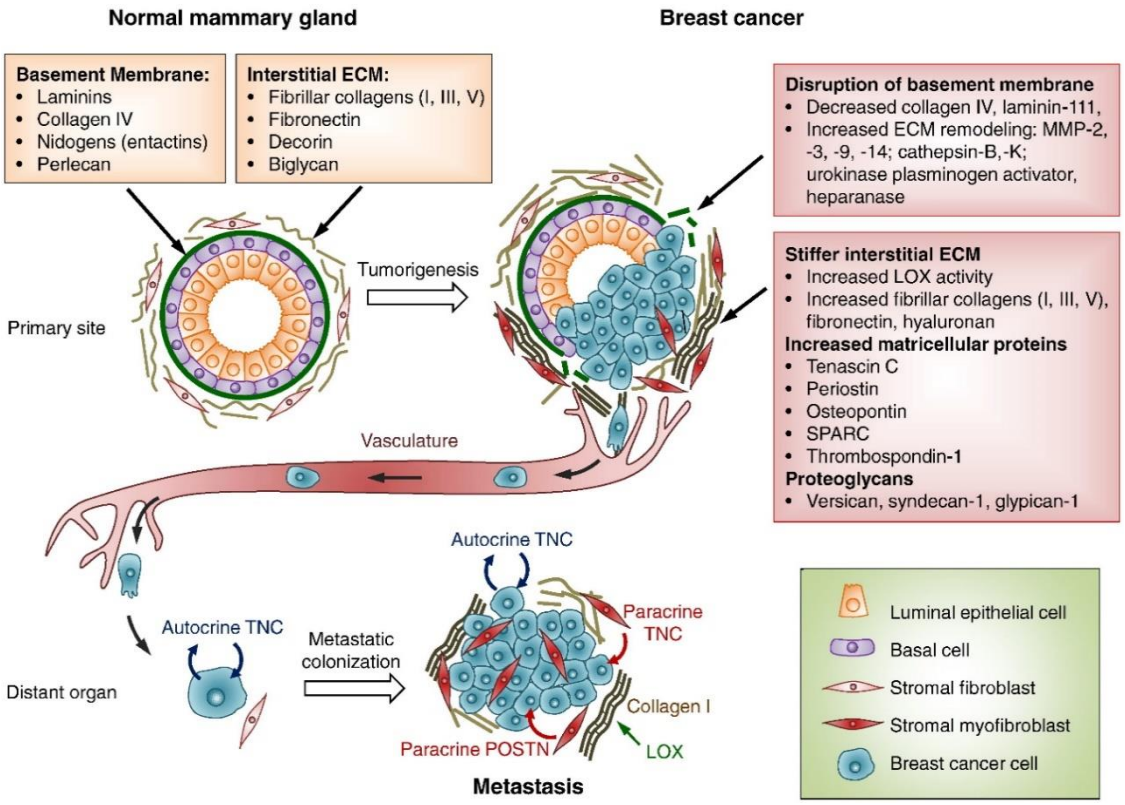


Figure 2.3. The breast cancer microenvironment influences metastasis [18].

The breast ECM is composed of a diverse set of proteins, polysaccharides and glycoproteins that have varying biochemical and physical attributes (Figure 2.3) [13, 18]. The cancer ECM shows large similarities with the ECM of tissues undergoing wound healing or remodeling [18]. The cancer ECM is comprised of two parts, the interstitial matrix that is produced

mainly by stromal cells and the basement membrane, which separates the epithelium or endothelium from stroma and is produced by epithelial/endothelial and stromal cells. The basement membrane is more compact and less porous than interstitial matrix and contains Type IV collagen, laminins, fibronectin and linker proteins such as entactins or nidogens, which connect collagen with other proteins in the matrix [13, 18, 19]. Interstitial matrix is loaded with fibrillary Type I collagens, proteoglycans, and various glycoproteins such as fibronectin and tenascin. Glycoproteins are important for proper cell-ECM attachment and breaking of those adhesions aids in extravasation [16, 19]. Glycosaminoglycans (GAGs) are large, hydrophilic and unbranched polysaccharide chains that form porous gels, occupying large amounts of space in the ECM. GAGs provide mechanical support for the tissues and allow cell-cell and cell-ECM interaction by controlling the passage of cytokines and growth factors [16]. They often bind to proteins forming proteoglycans, but both protein bound and non-protein bound GAGs play roles in metastasis [18]. Increases in Type I collagen and decreases in Type IV collagen are associated with breast cancer metastasis as the basement membrane is broken down for invasion and the interstitial matrix is reformed to support tumor cells [18, 20]. These various components make the interstitial matrix hydrated, charged, and provides the mechanical properties for the tissue, such as tensile and elastic modulus or stiffness [13]. ECM has the ability to both initiate and intercept signaling cascades within the tumor microenvironment [13]. ECM can also provide a niche for cancer stem cells and is involved in inflammation and angiogenesis pathways allowing for cancer to invade distant tissues and proliferate by creating a pro-metastatic cancer environment[20].

Tissue stiffness has also played a growing role in cancer metastasis as researchers have progressed the topic forward. Breast tissue has been measured to be between 2-90 kPa and cancerous breast tissue between 15-500 kPa [21-23]. These values can change depending on the

type of breast tissue being studied. For instance, glandular tissue is stiffer than adipose tissue. Over the years, however, breast cancer tissue has been recorded to be up to 7 times stiffer than normal mammary tissue [22, 23]. The focal adhesion complex that is composed of integrins, adaptors and signaling proteins acts as a mechanosensor that links the actomyosin cytoskeleton to the ECM [13, 24]. Many of the focal adhesion components will undergo conformational changes in response to certain applied forces or stresses. These changes can make for ideal environments for breast cancer to metastasize and invade tissues [13, 25]. Collagen organization and stiffness are also affected by modifications such as crosslinking of the matrix, which is created by tumor progression. Increased collagen formation along with straightening of collagen fibers as tumor masses grow outside the bounds of its environment, increase the mechanical strength and density of the breast tissue [18]. Acerbi *et al* demonstrated that the transition from a non-malignant to an invasive carcinoma is correlated to significant collagen deposition, fiber straightening, and bundling, which leads to stiffening of the ECM [26]. This stiffening is associated with significantly higher infiltration of TAMs, which were previously mentioned to support EMT via TGF-β [26]. Furthermore, increased stiffness promotes activation of the mitogen-activated protein kinase (MAPK) pathway that aids in unlimited cell division for cancer and the nuclear translocation of TWIST1, which promotes EMT [18, 27]. This suggests that tissue stiffness plays a role in breast cancer metastasis and should be taken into consideration when modelling the breast cancer environment.

In order to create an accurate 3D *in vitro* model of breast cancer metastasis, it is vital to understand the current literature available on the microenvironment and its influence on breast cancer metastasis. One aspect of the environment that is of interest in our research is how inflammation and the immune system effect breast cancer metastasis. This includes how

inflammatory cytokines (adipokines), such as leptin, can influence the expression of breast cancer metastasis diagnostic biomarkers and is covered in the next section.

2.3 Role of Leptin in Obesity, Inflammation and Breast Cancer Metastasis

Breast cancer has often been described as a wound that never heals. In this sense, many of the factors involved with inflammation and wound healing play similar roles in mediating breast cancer metastasis [28]. There is a link between adipocytes, inflammation and breast cancer that can be used to help create diagnostic predictions of metastasis (Figure 2.4). To understand this relationship, we must first understand inflammation before we can understand its relationship with adipocytes and breast cancer metastasis.

Inflammation is signaled by several chemokines at sites of damaged or infected tissue to begin the healing process. This includes activation and migration of several cell types including leukocytes, such as neutrophils, eosinophils and monocytes, as well as dendritic cells and mast cells. Neutrophils are the first to come to affected sites to begin the signaling cascade for required activators, mediators and healers in the forms of other cell types and their cytokines. Monocytes differentiate into macrophages, which are the main source of growth factors for endothelial and mesenchymal stem cells of the microenvironment as described previously [28]. These cells are all able to produce various cytokines, cytotoxic signals, MMPs to break down ECM, and soluble mediators of apoptosis such as TNF-α, interleukins (ILs) and interferons (IFNs) [28]. The important distinction in normal inflammation and malignant tumors is that normal inflammation is self-limiting, but in cases of malignances, the process of inflammation never ceases because self-limiting signals are either downregulated or activating signals are never are permanently turned on.

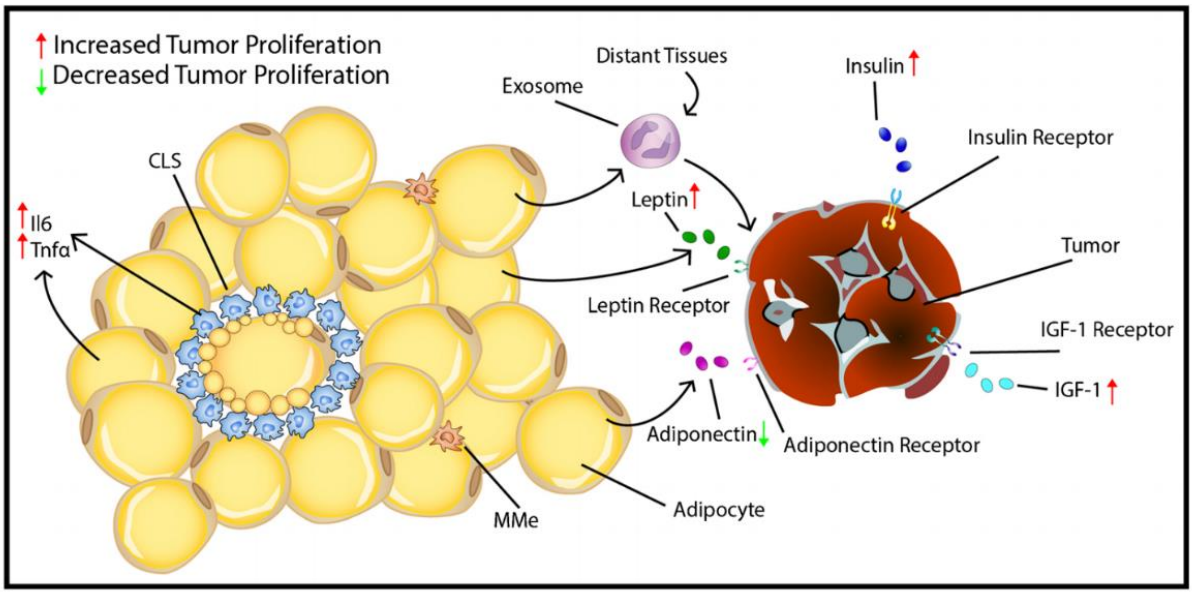


Figure 2.4. Schematic showing the relationship between obesity, pro-inflammatory cytokines and breast cancer metastasis [29].

Obesity, resulting from increased adipose tissue, has been associated with low-grade chronic inflammation, insulin resistance and secretion of pro-inflammatory cytokines that progress breast cancer metastasis [30-32]. Adipose tissue serves primarily as an energy reservoir and controls metabolic function, but it also functions as part of the endocrine system. Within adipose tissue, adipocytes can secrete several pro-inflammatory cytokines such as IL-6, TNF-α, adiponectin and leptin [32, 33]. Several immune cell types also reside within adipose tissue, but adipocytes mostly interact with macrophages [30]. These macrophages switch to a pro-inflammatory state when activated by adipocyte signaling molecules, known as adipokines. Pro-inflammatory tumor associated macrophages (TAMs) are important enablers of cancer progression, angiogenesis, and metastasis [31]. A recent study conducted by Nagahashi *et al.* discovered that obesity, inflammation and breast cancer metastasis are linked via a sphingolipid metabolite called sphingosine-1-phosphate (S1P). S1P is activated by sphingosine kinase 1 (SphK1) and is exported out of cells and signals through specific receptors (S1PR1) that regulate

cell growth, survival, immune cell trafficking, angiogenesis and invasion which are important processes for both immune and breast cancer cells [31]. SphK1 is overexpressed in breast cancer patients and S1P levels are elevated in obese patients [31]. Based on this study and others like it, research is continuing to link obesity, inflammation and breast cancer metastasis, but for the purpose of our work, we are mostly concerned with adipokines and their inflammatory influence on breast cancer metastasis.

As adipocytes comprise a majority of the breast tissue, they also comprise a large part of the breast cancer tumor environment and have been shown to influence inflammation as well as breast cancer metastasis [34]. These adipocytes produce many pro-inflammatory adipokines. One such adipokine of particular interest in this work is leptin, a hormone that plays a crucial role in metabolism, immunity and the endocrine system [35]. As a pro-inflammatory cytokine, leptin is expressed largely in the immune system. It influences cytokine secretions by macrophages and monocytes and enhances the productions of pro-inflammatory and metastatic cytokines such as tumor necrosis factor-alpha (TNF-α) and Interleukin- 6 (IL-6) [36, 37]. Leptin can therefore work with or independently from immune cells, such as macrophages, to progress tumor cell migration and invasion.

Leptin is also believed to aid with breast cancer proliferation and angiogenesis. It plays a significant role in the EMT process for breast cancer metastasis by upregulating the glucometabolic enzyme Pyruvate Kinase M2 (PKM2) that coactivates cell-cycle progression and cell motility [35]. Leptin also induces the JAK2/STAT, PI3K and MAPK pathways [36, 38], and activates Janus kinase 2 (JAK2) by phosphorylating the signal transducer and activator of transcription 3 (STAT3). STAT 3 then travels to the nucleus and regulates the transcription of VEGF and genes essential for cell proliferation, aiding cancer cell proliferation and angiogenesis

[39]. Leptin further influences the Phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways in a similar fashion, aiding in breast cancer migration, growth and proliferation [36]. In a meta-analysis of 43 studies conducted on serum leptin levels and breast cancer metastasis, it was shown that there was a significantly higher level of leptin expressed in breast cancer than in normal breast epithelium [40]. Based on several studies, normal levels of leptin are between approximately 10 and 20 ng/mL and in breast cancer patients that level increases to approximately 20-40 ng/mL [40-43]. Studying leptin could potentially link the relationship between obesity, inflammation and breast cancer metastasis.

Studying the interactions of pro-inflammatory cytokines, such as leptin, aids in understanding the tumor microenvironment which can help to create better models of breast cancer metastasis. The enables effective developments in drug screening and creating personalized medicine. How breast cancer has been modelled *in vitro* will be discussed next in the final section of this review.

2.4 Three-Dimensional Models of Breast Cancer Metastasis

Over the last several decades, our understanding of cancer has improved vastly thanks to the use and creation of clinical trials, *in vitro* cell culture models and *in vivo* animal models [44]. However, there are still limitations to these models and their utility for studying breast cancer metastasis [44]. Clinical trials can take anywhere from 10 to 15 years, are incapable of providing thorough controlled environments, are unable to provide mechanistic and biological specificity of metastasis and can pose ethical concerns [45]. *In vitro* cell culture models, while easy and quick to use, are limited by their physiological and clinical relevance to the breast tumor microenvironment [45]. Finally, animal *in vivo* models, while increasing controllability and

physiological relevance, still pose issues with clinical human relevance, are expensive and pose ethical concerns as well [46]. With the onset of tissue engineering and the discovery of how the microenvironment effects breast cancer metastasis, the research community has begun creating 3D breast cancer models that mimic specific parts of the microenvironment, reduce cost, increase controlled reproducibility and increase clinical and physiological relevance, as compared to monolayer cultures [47]. By creating these models, researchers hope to create platforms for drug testing and for studying the metastatic cascade at a biochemical and physiological level.

There are several different types of 3D models that are created depending on what aspect of breast cancer scientists are attempting to study. For studying how tumor stromal cell ECM production is affected by cancer cells, non-adherent tumor-stromal spheroid co-cultures may be the best option, as no outside biomaterial influences stromal cell protein production [45]. To increase spheroid culture number or simply add flow behavior to mimic nutrient transport, waste disposal and shear stress, 3D models such as spinner flasks or microfluidic devices can be used. Spinner flasks keep spheroids suspended in culture, while microfluidic devices allow for adhesive cell culture while allowing media to move throughout the device [45]. These are suitable models for studying nutrient or vasculature behavior within the tumor microenvironment. While these examples have aided in the push toward 3D cultures, the tumor microenvironment is complex and studying it effectively requires complex models. When wanting to study cell-cell and cell-ECM signaling, as well as matrix components such as mechanical stiffness or ECM organization, the use of tissue engineering to create *in vitro* 3D models has become more prevalent in research.

The methodology for designing and building tissues for regeneration purposes or in our case, research purposes, is called tissues engineering. There are three main components of tissue engineering: a biomaterial scaffold, cells and bioactive molecules. How and what scientists choose

to create these 3D platforms depends largely on the research goals and the step of the metastatic cascade being studied. For instance, 3D tissue engineered models have been used to mimic environments involved in bone and brain metastasis, the EMT and MET pathways, ECM stiffness, mesenchymal or stromal interactions with breast cancer, or metastatic behavior within breast tissue [48-53]. Based on goals such as these, tissue engineers generally begin with building a biomaterial scaffold that resembles the environment, including cell types and bioactive molecules, they are trying to imitate.

Scaffolds are made from either single or composites of natural (such as Matrigel™, chitosan, collagen, gelatin, silk, fibrinogen and alginate) or synthetic (such as clay, poly(ethylene glycol) (PEG), Poly(lactic-co-glycolic) acid (PLGA), and hydroxyapatite) biomaterials [48-50, 53-55]. Collagen is the most commonly used natural material for designing breast cancer metastasis scaffolds as it comprises the largest percentage of breast, bone, lung and liver ECM [46]. Collagen, however, is expensive to use, which has caused many researchers to use collagen as a component of, rather than the entire scaffold material [46, 56]. One group electrospun gelatin fibers into a woven scaffold and chemically-crosslinked the woven scaffold with collagen. They then seeded the scaffold with MCF-7 breast cancer cells (Caucasian HER2- breast cancer cell line) and exposed the scaffolds to either estrogen (EMT-induction) or progesterone (MET-induction). Using this scaffold, researchers revealed the role that estrogen plays in MCF-7 breast cancer invasion and determined that EMT-induced cells via estrogen exposure preferred their fibrous scaffold to the 2D control [46]. Studying breast cancer invasion, Liu *et al* created collagen-alginate hybrid hydrogels that had tunable stiffness and porosity. After seeding a triple negative breast cancer cell line (MDA-MB-231) as well as fibroblasts, they were able to demonstrate fibroblasts leading breast cancer migration and mimic CXCL12-CXCR4 signaling between dual tumor spheroids

[57]. These collagen hybrids allowed for researchers to maintain natural ECM protein while also allowing for manipulation of the ECM environment (increased degradation time, mechanical stiffness, protein or polymer organization, porosity) by combining collagen with another biomaterial.

The most common 3D models of breast cancer aim to study bone metastasis. Researchers have added hydroxyapatite (HA), the mineral in bone, to synthetic nanoclay composites or chitosan scaffolds and have also added bone morphogenetic protein 2 (BMP2) to their collagen or silk scaffolds [49-52]. Zhu *et al*, incorporated various sizes of HA to a porous chitosan scaffold. They then deposited bioactive molecules within the scaffold by culturing human bone marrow-mesenchymal stem cells (hBM-MSCs), creating a biomimetic environment. Three different cancer cell lines of varying aggressive behavior were then cultured, and the group showed that breast cancer proliferation increased as HA particle size decreased [49]. Studies such as these are integral in understanding that determining how these models are built depends entirely on what aspect of the metastatic cascade a researcher is interested in. While the compositions previously described are beneficial for studying bone metastasis, in our studies we aim to mimic the breast tissue and how several properties of the microenvironment (stiffness, inflammation, obesity, and natural ECM proteins) influence the metastatic potential of breast cancer lines that vary in aggressiveness and race.

Of interest in this work, is the use of chitosan as a base material for breast cancer metastasis studies. Chitosan is a natural, biodegradable polysaccharide derived by the partial deacetylation of chitin. It also shares structural similarities to glycosaminoglycans (GAGs), which are found in the natural tissue ECM [48]. GAGs play an essential role in cell signaling and cell-cell communication because of their extracellular location and similar structure in almost all animal species [58].

Chitosan's rate of degradation can be manipulated by increasing or decreasing the degree of deacetylation as well as the molecular weight of chitosan [59]. Its antimicrobial effects can be attributed to its ability to alter microbial cell wall permeability or its inhibition of microbial RNA synthesis. Moreover, chitosan scaffolds have gained popularity in mammary regeneration due to their softness and high water content [60]. For these reasons, chitosan has been studied for its use as a drug delivery vehicle, as a scaffold for bone, muscle and soft tissue regeneration and as a platform for creating tissue models [60-62].

Although not many, there are some examples of chitosan being used to study breast cancer metastasis. To study how matrix stiffness effects breast cancer, Chang *et al* created a mPEG-g-Chitosan scaffold that changed in stiffness by changing the PEGylation grafting efficiency. Polyethylene glycol (PEG) is an FDA approved synthetic polymer for biomedical use as it does not illicit negative responses in the body [48]. The group discovered that increasing stiffness increased mouse mammary carcinoma (MMC) cell proliferation and drug resistance [48]. PEG-g-Chitosan has also been tested with MMCs to create a better cancer drug screening platform in comparison to Matrigel™, the commercially available industry standard [58]. Previously, a chitosan-collagen scaffold was used to test breast cancer treatments. The study displayed that 4T1 (mouse mammary carcinoma) cells seeded on these scaffolds displayed increased resistance to irradiation and 4-hydroxycyclophosphamide (chemotherapy) [63]. Because each group used murine cancer cells, however, it is difficult to determine that the discoveries and behaviors would occur in human models. Although there are not many, studies have been performed using human cell lines on chitosan scaffolds including two studies using MCF-7 cells with chitosan or chitosan composite scaffolds as a breast cancer drug screening platform [64, 65]. While contributing greatly

to the field, these studies used their models to study cytotoxicity, not metastasis, and only used a single cell line, which limits the applicability of these studies to a broad range of patients.

There are a few 3D *in vitro* models of breast cancer metastasis and its relationship to adipocytes and/or inflammation. Yue *et al* discovered that tumor microenvironment stiffness alters stromal cell adipogenesis by designing a co-culture of 3T3-L1 (adipocyte progenitor cells) with either HCC1806 or MDA-MB-231 cells in a microarray structured hydrogel made out of either low methacrylated gelatin (softer) or high methacrylated gelatin (stiffer) (Figure 2.5) [66]. Hume *et al* and other groups have also used collagen-based scaffolds to either create a microenvironment consisting of tumor stroma (adipocytes) for drug screening studies or to study how adipocytes and/or adipokines influence breast cancer migration and drug resistance [67-69]. There are currently no models, however, that aim to mimic how specific adipocytes or inflammatory cytokines influence breast cancer metastasis and no models are being built for their potential use in creating predictive tools for breast cancer metastasis.

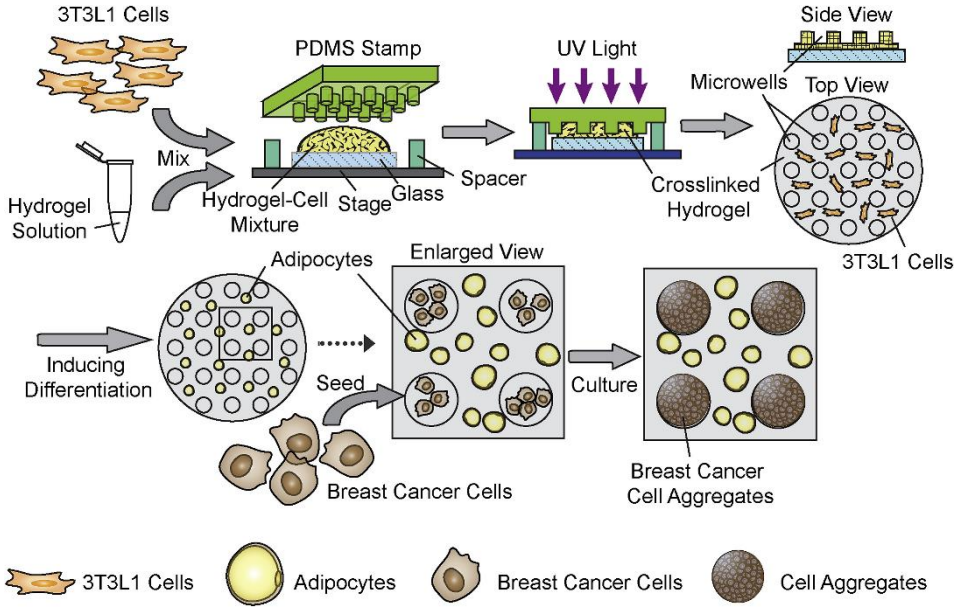


Figure 2.5. Illustration of Yue *et al*'s fabrication process for adipocyte encapsulation with hydrogel and its co-culture with HCC1806 or MDA-MB-231 tumor spheroids [66].

In these first two chapters, we have discussed breast cancer metastasis, the breast cancer environment, the relationship between obesity, inflammation and breast cancer and current 3D *in vitro* models of interest and importance for our work. The following two chapters will consist of studies related to the subjects outlined here. Chapter 3 describes creating, testing and determining an optimal model for breast cancer metastasis, and Chapter 4 highlights our application of the chosen model to determine the predictive value of leptin in breast cancer metastasis. We finish with a concluding chapter discussing final thoughts and future directions, as well as reference an appendix at the end of this work discussing options for building a statistical tool using the model platform we have created in this work.

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CHAPTER 3

CHITOSAN-BASED HYDROGELS AS A THREE-DIMENSIONAL (3D) *IN VITRO* BREAST CANCER METASTASIS (BCM) MODEL PLATFORM[1]

[1] Al-Saleh, J.E. and C.T. Gomillion. To be submitted to *Biomaterials Research*.

3.1 Abstract

Breast cancer metastasis (BCM) is detrimental for patient outcomes. Currently, the mechanisms by which cancer can bypass distant tissue defenses, invade the tissue, and proliferate once settled are unknown. To develop more effective therapeutics, it is essential to understand this process. With the inadequacies of two-dimensional (2D) monolayer and *in vivo* models limiting progress towards further understanding this disease, three-dimensional (3D) models are now being studied to elucidate knowledge of BCM not yet known. For optimal BCM models, microenvironmental factors such as surrounding matrix stiffness and extracellular matrix (ECM) proteins present, must be studied to determine their effects on BCM behavior. Thus, the creation of a comprehensive and physiologically relevant 3D model that mimics *in vivo* behavior will require inclusion of multiple components of the BCM microenvironment and confirmation of *in vivo*-like cellular responses. This will include evaluating the behavior of more than one breast cancer subtype within the 3D platform to confirm validity of the model. Therefore, the objective of this work was to create an *in vitro* 3D model mimicking the microenvironment and stiffness properties of both normal and cancerous breast tissues by examining chitosan (CH) and chitosan-collagen (CH-Col) hydrogel scaffolds with tunable stiffness. The metastatic potential of three different cell lines, including MCF-7, MDA-MB-231 and HCC1806 breast cancer cells, was examined to assess variations in cell outcomes within the model based on cell subtype or ethnicity.

3.2 Introduction

Breast cancer remains one of the leading causes of death in women with cancer [1]. The cause of death for breast cancer patients is typically not due to the tumor of origin itself, but rather its metastasis, or spreading, to distant sites of the body, including bone, lung, liver and brain as the most common secondary tumor sites [2]. In addition to the poor prognosis that can result once breast cancer metastasis is detected, there are also racial disparities in breast cancer research that must be studied to design therapies and treatments that can be effective for larger percentages of the population. For instance, African-American women are more susceptible to triple-negative breast cancer (a more aggressive subtype) due to their greater likelihood of having the BRCA1/2 mutations [3]. This could point to possible differences in metastatic behavior and potential that would change responses to therapeutics. Therefore, it is imperative that differences between ethnicities and breast cancer metastasis be accurately studied so that personalized treatment plans may be created, decreasing the mortality gap between races.

Recently, research has shifted to studying how the environment surrounding cancer cells effects the tumor cells' ability to proliferate and metastasize [4]. The breast cancer microenvironment consists of stromal cells, adipocytes and immune cells that are embedded in a vast extracellular matrix (ECM) network that is sustained via vasculature [2]. Each of these components can play a role in breast cancer proliferation, migration and invasion [5]. Two-dimensional (2D) monolayer cell cultures, although less expensive and easily reproducible, are unable to effectively model the microenvironment due to lack of biological and mechanical ECM properties [6]. Further, *in vivo* animal models are costly, pose issues with human clinical relevance and can present cause for ethical concerns [7]. Thus, for these reasons, scientists have worked towards creating three-dimensional (3D) breast cancer models that mimic the microenvironment,

reduce cost, and increase controlled reproducibility, as well as clinical and physiological relevance [4].

Tissue stiffness has been suggested to play a role in cancer metastasis, as the stiffness of normal breast tissue has been measured to be between 2–90 kPa and cancerous breast tissue stiffness between 15–500 kPa [8-10]. Increased collagen formation, along with straightening of collagen fibers as tumor masses grow outside the bounds of its environment, have been shown to increase the mechanical strength and density of the breast tissue [11]. Acerbi *et al* demonstrated that the transition from a non-malignant to an invasive carcinoma is correlated to significant collagen deposition, fiber straightening, and bundling, which leads to stiffening of the ECM [12]. This stiffening is associated with significantly higher infiltration of tumor-associated macrophages (TAMs), which support epithelial to mesenchymal transition (EMT) via transforming growth factor-β (TGF-β) [12]. Furthermore, increased stiffness promotes activation of the mitogen-activated protein kinase (MAPK) pathway that aids in unlimited cell division for cancer and the nuclear translocation of TWIST1, which promotes EMT [11, 13]. Therefore, ECM stiffness is an important factor to consider when designing 3D *in vitro* models.

Chitosan has recently been of interest as a biomaterial for tissue engineering applications. Chitosan is a natural, biodegradable polysaccharide derived by the partial deacetylation of chitin. It also shares structural similarities to glycosaminoglycans (GAGs), which are found in the natural tissue ECM [14]. Chang *et al* discovered that increasing stiffness increased mouse mammary carcinoma cell proliferation and drug resistance by creating mPEG-g-Chitosan scaffolds that change in stiffness via altering PEGylation grafting [15]. PEG-g-Chitosan has also been tested with mouse mammary carcinomas to create a better cancer drug screening platform in comparison to Matrigel®, the current commercially-available industry standard [14]. These studies, however,

used murine cell lines, which limits their clinical relevance to humans. In addition, while few in number, studies have been performed using human cell lines with chitosan scaffolds, including two studies using MCF-7 cells on chitosan or chitosan composite scaffolds as a breast cancer drug screening platform [16, 17]. These studies used the chitosan-based models to study cytotoxicity, not metastasis, and only used a single cell line for assessment. It is widely accepted that tumor cell behavior and demonstration of metastatic characteristics can vary based on breast cancer type [18]; therefore, an ideal 3D model should be able to mimic the behavior of more than one type of cancer.

For this work, we aimed to create a chitosan-collagen (CH-Col) platform that would support breast cancer metastatic behavior. A 3D CH-Col scaffold platform was developed to mimic the metastatic behavior of breast cancer, by incorporating breast cancer cells of varying subtype with different aggressive behavior and derived from donors of different ethnicities. Collagen is the most abundant protein in breast tissue ECM and is often used to recreate natural ECM properties [19]. By varying chitosan (CH) concentration, we were able to tune hydrogel stiffness and rheological properties of the hydrogel, which allowed us to study environments that mimicked both normal and cancerous breast tissue stiffness. The effects of the mechanical properties of CH and CH-Col hybrids on cell viability and metastatic potential were evaluated, and the best hydrogel for evaluating differences in metastatic potential between multiple cell types was determined. Specifically, MCF-7 (Caucasian, ER+/HER2+, mammary adenocarcinoma), MDA-MB-231 (Caucasian, ER-/PR-/HER2-, triple-negative breast cancer) and HCC1806 (African-American, ER-/PR-/HER2-, triple-negative breast cancer) tumor cells were cultured within the hydrogel platform and compared to 2D controls. Metastatic potential was determined by measuring protein expression of several known metastatic markers. In doing so, we also aimed to determine differences between MDA-MB-231 and HCC1806 cell metastatic protein expression, thereby

creating a foundation for future studies investigating variations among metastasis in patients of different ethnicities *in vitro*.

3.3 Materials and Methods

Hydrogel Fabrication

High molecular weight chitosan (HMW CH, 310–375 kDa, >75% Deacetylated) purchased from Sigma-Aldrich (St. Louis, MO) was used in these experiments. Initial hydrogel screening was performed using CH solutions ranging from 1% – 5% (w/v). Chitosan powder was dissolved in 1% (v/v) glacial acetic acid (Sigma-Aldrich) in water to obtain the specified percent solution. Hydrogels were prepared by casting in either a 6-well plate, silicone mold of 5/8-inch diameter, or 60-mm tissue culture dishes. Hydrogels for all experiments were crosslinked using a 0.5 N NaOH (Sigma-Aldrich) solution for 24 hours.

Following initial screening, 2.5% and 3.5% CH solutions were selected for all subsequent cell studies to evaluate CH and CH-Col hybrids. Briefly, to prepare CH-Col hybrids, 2.5% HMW and 3.5% HMW chitosan solutions were combined with a volume of rat tail collagen I (ThermoFisher, Carlsbad, CA) to obtain a 0.075% collagen solution, which was mixed thoroughly. The CH-Col solutions were stored at 4°C protected from light until use for analyses.

Rheological Characterization

To prepare samples for rheological analysis, approximately 2.5 mL of CH was administered to wells of a 6-well plate. A volume of 10 mL of 0.5 N NaOH solution dissolved in distilled water was added to each well for crosslinking. This was repeated for six samples of each percent solution. The resulting hydrogels were incubated in solution overnight at room

temperature. Hydrogels were then neutralized in phosphate buffered saline (PBS) for 24 hours and measured immediately after removal from the PBS solution.

Rheological properties of the hydrogels were assessed using an MCR 302 Anton Paar Rheometer (Graz, Austria). A two-plate system with a 25-mm diameter PP25/S plunger was used to perform an amplitude sweep for each of the hydrogels, based on oscillation rheometry, in which the plunger rotates on top of the gel to obtain data based on shear stress. The angular frequency was held constant at 10 radians/second, and the amplitude deflection angle was changed in intervals from 0.1% until 100% deflection was reached. The temperature was also maintained at a constant 37°C. Each hydrogel was measured with a gap of 2 mm between the plates. The storage modulus and loss modulus were measured. A total of 6 samples were measured for each type of hydrogel.

The elastic storage modulus for the fabricated hydrogels was determined by adapting Hooke's law to compare the shear storage modulus to compressive storage modulus. Thus, the compressive storage modulus was determined by using the equation:

$$E' = 2G'(1+\mu)$$

Where G' = shear storage modulus, E' = compressional storage modulus and μ = Poisson's ratio.

Scanning Electron Microscopy (SEM)

Hydrogels were fabricated for SEM using a template made from silicone sheets that were approximately 1-mm thick. The sheets were cut to fit into 100-mm Petri dishes, and using a 5/8-inch diameter hole punch, 6 holes were punched into each silicone sheet. Hydrogel solutions were added to a dish containing a silicone sheet and smoothed using a sterile surgical blade. Hydrogels were then crosslinked using 0.5 N NaOH solution for 24 hours, followed by neutralization with

PBS for 24 hours. Silicone sheets were then removed, leaving 5/8-inch diameter hydrogels enough to fit within a 24-well plate.

Hydrogels containing 2.5% CH, 3.5% CH and the 2.5% and 3.5% CH-Col were lyophilized for 24 hours. The lyophilized samples were then gold-coated using a Leica EM ACE600 coater (Leica, Buffalo Grove, IL). After coating was complete, a Teneo field emission scanning electron microscope (FESEM) (FEI, Inc., Hillsboro, OR) was used to take images of the surfaces of each hydrogel.

Preliminary In Vitro Cellular Analyses

Hydrogels were fabricated as previously described using the silicone sheet molds for templating. Prior to cell seeding, sheets were sterilized using 70% ethanol and then treated by exposing to ultraviolet light on both sides of the sheet for 30 minutes within a tissue culture dish. Commercially available breast cancer cell lines, MCF-7 and MDA-MB-231 cells, were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells ranged from passage 10 to passage 15 at time of seeding. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Waltham, MA) with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA) and 1% penicillin/streptomycin (Pen/Strep, Gibco), denoted as DMEM-Complete. For experiments to assess metastatic analyses, phenol red-free DMEM (Gibco), supplemented with 10% FBS, 1% Pen/Strep and 2% L-Glutamine (Gibco), was used.

Cell Viability

Cell viability was qualitatively assessed using a LIVE/DEAD® Viability/Cytotoxicity assay (Molecular Probes, Invitrogen, Eugene, OR). Cells were seeded in ultra-low attachment 24-

well plates (Corning, Tewksbury, MA) containing 2.5% and 3.5% CH hydrogels ± Col. Duplicates were seeded for each cell type. For each cell type, 3.8×10^4 cells were seeded per well containing hydrogel or for 2D controls on the tissue culture plate. After 3 days, media was removed from each well and the LIVE/DEAD® assay was performed based on manufacturer's instructions. A working reagent consisting of PBS containing calcein AM and ethidium homodimer-1 was added to each well, and the plate was incubated for 30 minutes at room temperature in the dark. After 30 minutes, an EVOS™ FL Imaging System (ThermoFisher Scientific) was used to visualize cells, with live cells stained green and dead cells stained red.

Metabolic Activity

AlamarBlue® assay (ThermoFisher Scientific) was used to measure cell metabolic activity as an indicator of cell viability. Cells were seeded in ultra-low attachment 24-well plates (Corning) containing 2.5% and 3.5% CH hydrogels ± Col (n = 3 per cell type). For 2D cell controls and media only controls, a regular tissue-culture treated 24-well plate was used. For each cell type, 3.8×10^4 cells were seeded per well containing hydrogels or for 2D controls. After 3 days, 50 µL of AlamarBlue® reagent was added to each well of the plates. The plates were then incubated for 4 hours at 37°C. Samples were added to a 96-well plate in triplicate and absorbance was measured using a Biotek 800 TS microplate reader (Biotek Instruments Inc., Winooski, VT) at 560 nm and 600 nm wavelengths.

Total Protein Content

A Pierce™ bicinchoninic acid (BCA) protein assay (ThermoFisher) was used to determine the total protein concentration for cells grown in culture on the hydrogels or in 2D. Cells were

seeded as previously described using ultra-low attachment 24-well plates containing 2.5% and 3.5% CH hydrogels ± Col (n = 3 per cell type) or tissue-culture treated plates for 2D controls. Cells were seeded at a density of 1.42×10^5 cells per hydrogel or control well. After 3 days in culture, protein extraction was performed using MPER Mammalian Protein Extraction reagent (Thermo Fisher) supplemented with 150 mM sodium chloride. Briefly, media was removed and added to respective microtubes. After centrifugation and PBS washes, MPER was added to both the microtubes and the hydrogels, which were incubated for 10 minutes on a shaker. Afterwards, MPER from the hydrogels was added to the microtubes and centrifuged. The supernatant was removed and frozen at -80°C until BCA analysis, which was performed according to the manufacturer's protocol. Samples were measured using a microplate reader at 562 nm. An albumin standard curve was generated to determine sample concentration. Hydrogels without cells were used as controls and subtracted from absorbance values of hydrogel samples with cells.

Effects of 3D Culturing on Metastatic Potential

Preliminary evaluation of metastatic potential was assessed by measuring the expression of matrix metalloproteinase-2 (MMP-2), a protein commonly associated with metastasis. For metastatic analyses, the protein samples collected for the BCA assay were also assessed using an enzyme-linked immunosorbent assay (ELISA) for MMP-2 (Life Technologies, Carlsbad, CA) Each sample was tested in duplicate. MMP-2 protein concentration was then normalized to total protein content for each sample condition to determine the amount of MMP-2 in each sample.

Effects of Tumor Subtype and Ethnicity on Metastatic Potential

A subsequent analysis was performed to evaluate the response of breast cancer cells of different subtype and from donors of different ethnicity when cultured in 3D. The stiffer 3.5% CH+Col hydrogels (n = 3 per cell type) or tissue-culture treated plates for 2D controls were used for this study. Hydrogels were prepared and crosslinked in 60-mm dishes as described above. Three different cell types, varying in subtype and donor ethnicity, MCF-7, MDA-MB-231, and HCC1806 cells (ATCC), were seeded in separate experiments for analysis. Cells ranged from passage 10 to passage 13 for all experiments. On the day of cell seeding, 5 mL of low-serum phenol red-free DMEM-Complete (2% FBS, 2% L-glutamine and 1% Pen/Strep) was added to 10 60-mm dishes containing crosslinked hydrogels, which were then incubated at 37°C (media aspirated after incubation). Cells were counted and 4.9 mL of cell suspension (specific numbers used for cell seeding determined based on protein yield from previous trials and are shown in Table 3.1 below) was added to each dish containing hydrogels or 2D tissue-culture treated plates. Cells were cultured for 3 days.

Table 3.1. Cell seeding densities for *in vitro* metastatic potential analyses

	MCF-7	**MDA-MB-231**	**HCC1806**
2D Cultures	1.8×10^6 cells per dish	2.6×10^6 cells per dish	2.3×10^6 cells per dish
3D Cultures	2.9×10^6 cells per dish	3.7×10^6 cells per dish	3.2×10^6 cells per dish

After culturing, protein extraction was performed using the MPER extraction protocol and reagent (also supplemented with 1% protease inhibitor (Thermo Fisher), and 1% Ethylenediaminetetraacetic acid (EDTA, Thermo Fisher)), as previously described. Total protein content was determined using the BCA assay and the protein lysates were frozen for subsequent ELISA testing. Samples were analyzed using ISO 13485 certified RayBiotech® ELISA kits

(RayBiotech, Atlanta, GA), with samples analyzed at the RayBiotech facility. Samples were tested to quantify expression of the following proteins: vascular endothelial growth factor A (VEGFA), interleukin-6 (IL-6), matrix metalloproteinase-9 (MMP-9), and transforming growth factor-beta 2 (TGF-β2). The concentration of each protein was normalized to the total protein content in each sample (pg/µg) for comparison.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 6™ (GraphPad Software, Inc.) Two-way ANOVA followed by Tukey post-tests for multiple comparisons (unless otherwise noted) were performed to determine statistical significance between individual sample groups with significance set at $p < 0.05$. Data are expressed as mean and standard deviation (SD).

3.4 Results

Rheological Characterization

Preliminary evaluation of hydrogels formed with 1–5% CH was conducted. As shown in Figure 3.1A and B, the storage modulus was higher than the loss modulus for all concentrations within the linear viscoelastic range, which stopped at a strain of approximately 1% for all hydrogels before the chemical properties of the material changed. The storage modulus was shown to be proportional to CH concentration, as the stiffness increased as the CH percentage was increased. Based on adaptation of Hooke's law, the complex elastic modulus (Young's Modulus) was determined from the storage modulus data. As shown in Figure 3.1C similar trends are observed for the Young's Modulus, where increased elastic modulus was observed as CH concentration was increased. Statistical analysis indicated that the elastic modulus was significantly different when

hydrogels of each percent solution were compared ($p<0.05$), except for comparison between the 1% and 2% CH hydrogels. Based on this data, hydrogels formed with 2% and 3% CH were suitable for a normal breast tissue model matrix, and values between 3% and 4% were suitable for a cancerous breast tissue model matrix, thus these compositions were used for subsequent experimental analyses.

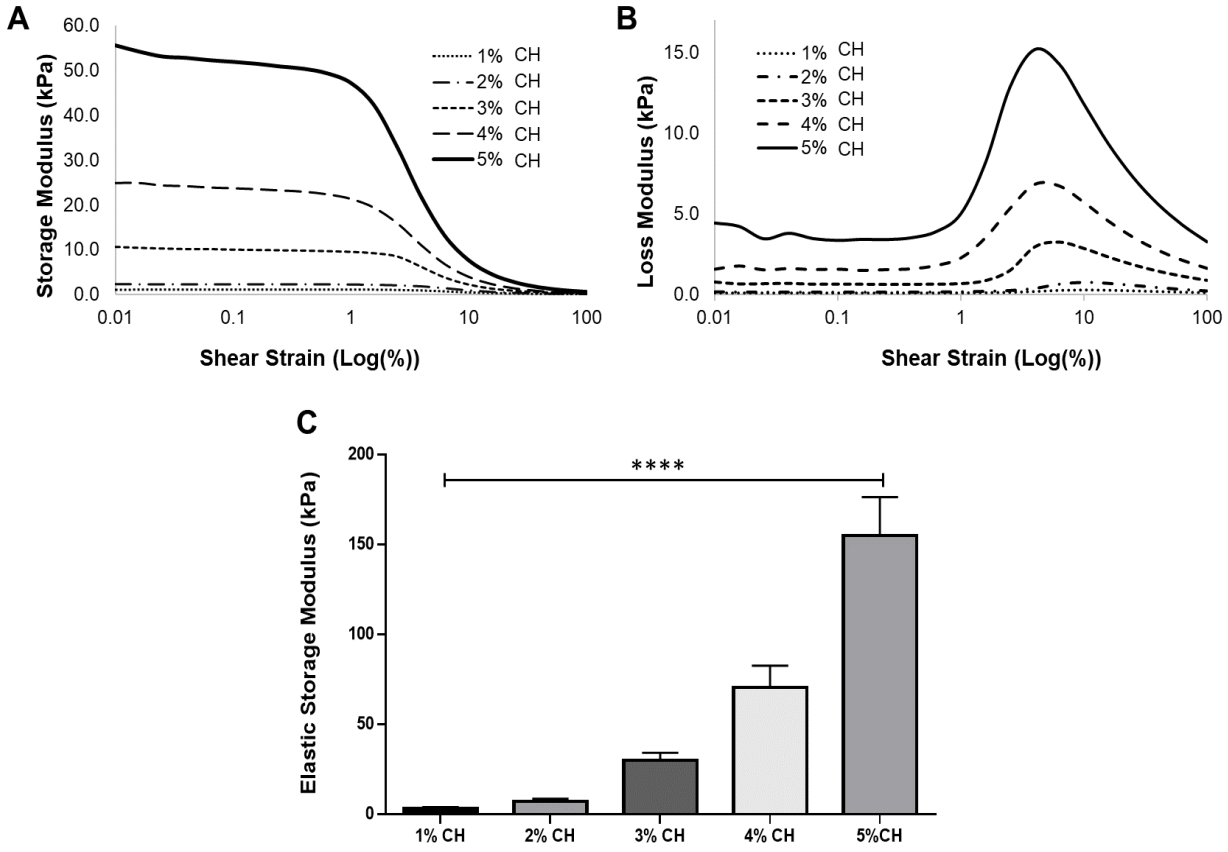

Figure 3.1. Initial rheological analysis of 1–5% HMW CH hydrogels. Storage (A) and loss (B) moduli increase as CH percentage increases. Elastic storage modulus values (C) show that lower percentages (2–3%) mimic breast tissue stiffness (7–30 kPa) and higher percentages (3–4%) mimic cancerous tissue stiffness (30–70 kPa). Data presented as mean value for each strain point (A & B) or as mean ± SD (C); n = 6 gels; p-value determined using ANOVA and Tukey post-hoc analysis; **** = $p<0.0001$.

Following identification of optimal percent solutions for modeling normal and cancerous tissue, CH-Col hybrids were formed and characterized using rheometry. Figure 3.2 shows the rheological data and elastic moduli for the selected hydrogels. The storage and loss modulus (Figure 3.2A and B) were comparable to the plain CH hydrogels when collagen was added. The complex elastic modulus for the hydrogels were determined to be 15 ± 2 kPa (2.5% CH only) and 49 ± 6 kPa (3.5% CH only). This increased only slightly to 17 ± 2 kPa and 52 ± 5 kPa, respectively when collagen was added. Statistical analysis confirmed that the addition of collagen to form CH-Col hybrids did not significantly affect the hydrogel elastic moduli ($p > 0.05$).

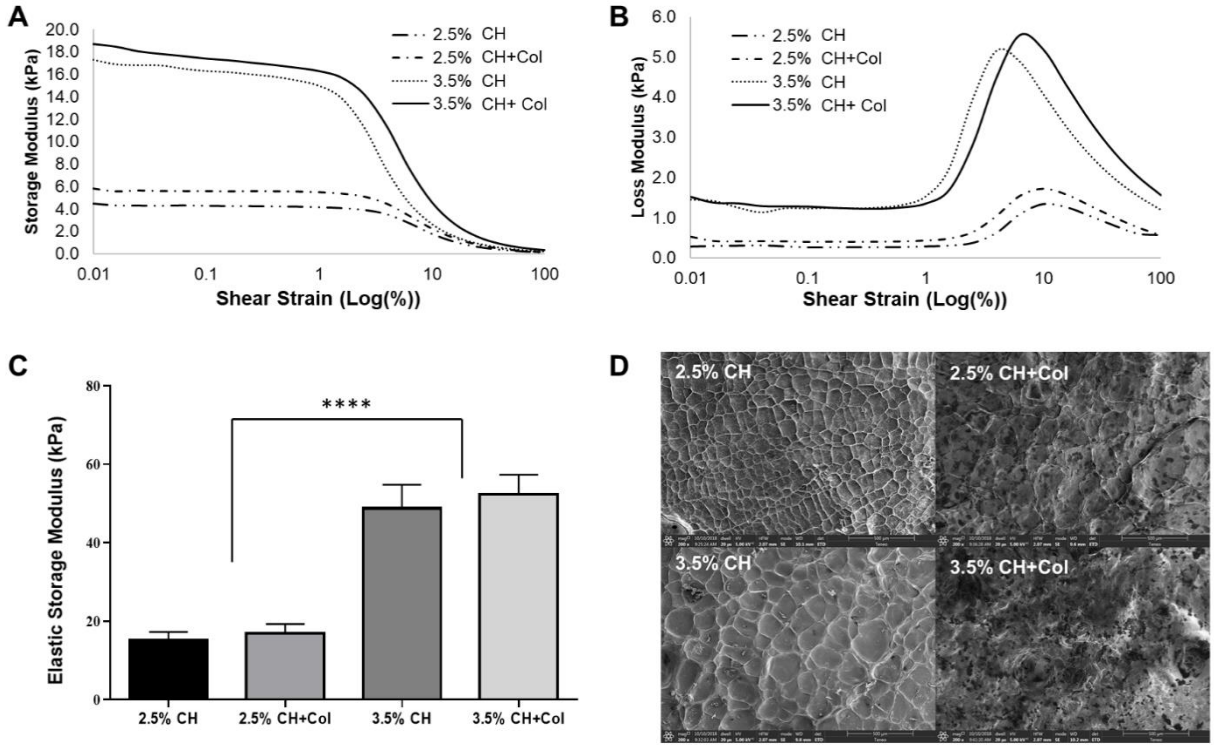

Figure 3.2. Rheological and SEM analysis comparing CH-Col hybrids to CH hydrogels. Storage (A) and loss (B) moduli plots showed that the hydrogels are more elastic than viscous until their yield point at 1% strain. Elastic storage modulus (C) evaluation showed that addition of collagen does not significantly alter hydrogel stiffness, with 2.5% Ch-Col gels exhibiting similar stiffness to normal breast tissue (17 kPa) and 3.5% Ch-Col hydrogels exhibiting comparable stiffness to tumor tissue (52 kPa). Representative SEM images (D) demonstrate the rough surface of the hydrogels, with each lacking porosity but maintaining "crater-like" surface that becomes shallower once collagen is added. Data presented as mean value for each strain point (A & B) or as mean ± SD (C); n=6 gels; p-value determined using ANOVA and Tukey post-hoc analysis; **** = $p<0.0001$.

Scanning Electron Microscopy

Representative SEM images captured for each of the samples is shown in Figure 3.2D. As shown, the hydrogels did not appear to have extensive porosity, but the surface was, rather, "crater-like" upon inspection. The 2.5% CH hydrogels had deeper craters than the 3.5% CH hydrogels. The addition of collagen to form CH-Col hybrids appears to have yielded hydrogels with ridges slightly filled out for both the 2.5% and 3.5 % CH hydrogels and the hybrid mixtures, providing a less uniform surface structure.

Preliminary In Vitro Cellular Analyses

Cell Viability

Representative images of the MCF-7 and MDA-MB-231 cells in culture are shown in Figure 3.3A and C. When cultured using the 3D platform with or without collagen, visible changes in cell morphology were observed for both cell types when compared to their respective 2D controls. Spheroids formed on the 2.5% and 3.5% CH hydrogels, but there did not seem to be distinct morphological differences between cells on the 2.5% or 3.5% hydrogels. While both cell types formed clusters when cultured on the hydrogels, the MCF-7 cells formed larger and more spheroidal shapes than the MDA-MB-231 cells. The addition of collagen resulted in greater cell spreading, as cells spread outwards, rather than forming spheroids as if they were attaching to the hydrogel. As shown in Figure 3.3B and D, when stained using the LIVE/DEAD® assay, all hydrogels were able to support viable MCF-7 and MDA-MB-231 cells, as shown by green-stained cells. Minimal dead cells were apparent, as indicated by the few red-stained cells shown.

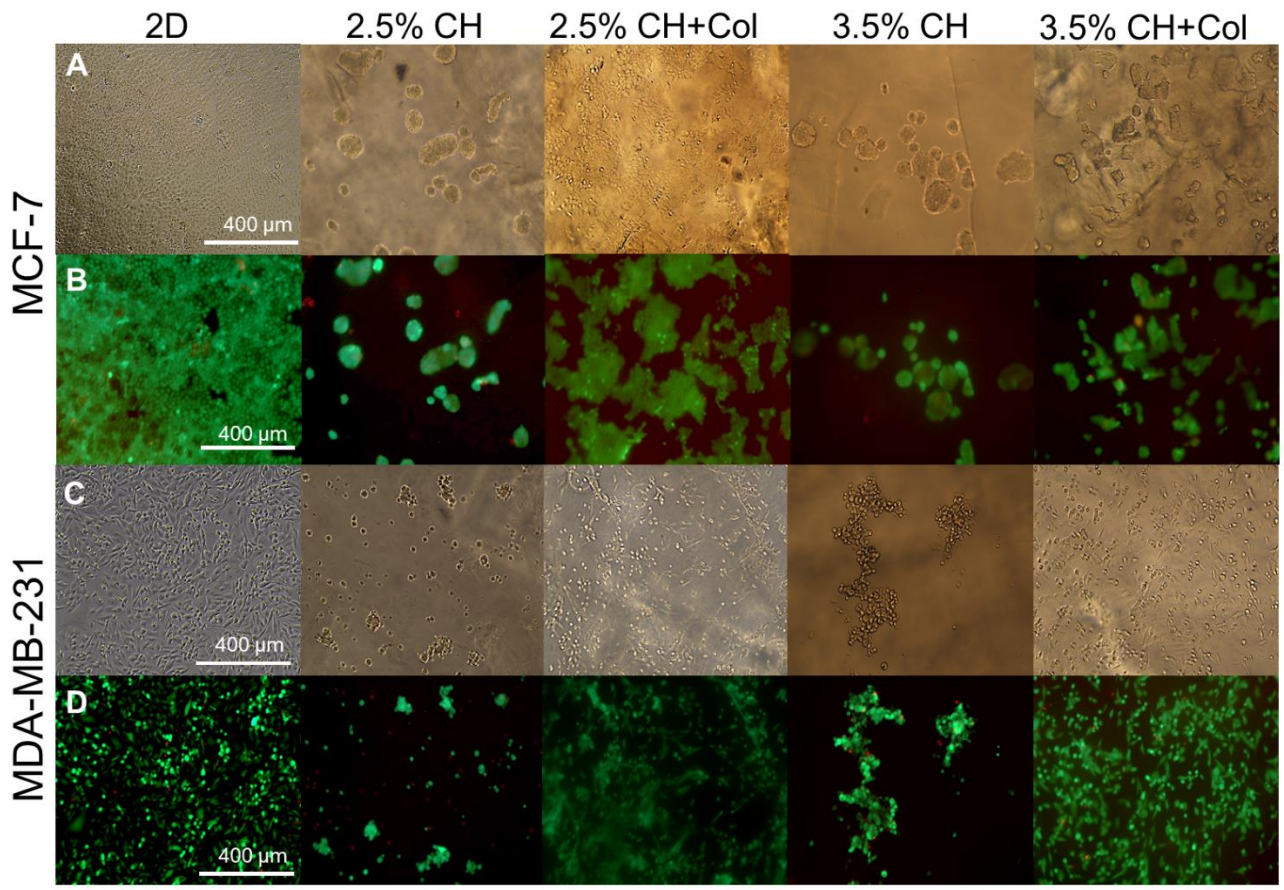

Figure 3.3. Representative cell morphology and viability staining. LIVE/DEAD® images (B and D) show almost all cells fluoresced green (live) and very few cells fluoresced red (Dead). Transmitted (A) and LIVE/DEAD® images (B) of MCF-7s show tumoroid formation on CH only gels. Transmitted (C) and LIVE/DEAD® (D) images of MDA-MB-231 cells on CH only gels show similar clustering. For both cell types (A–D) images show that cells seeded on CH-Col hydrogels displayed a morphology somewhere between that observed for cells cultured in 2D and on the CH hydrogels.

Metabolic Activity

Evaluation of metabolic activity, determined by the AlamarBlue® assay, showed a significant decrease in metabolic activity for cells cultured on all four hydrogel types when compared to 2D controls (Figure 3.4A). MCF-7 cells seeded on 2.5% CH hydrogels showed significantly lower metabolic activity than cells cultured on 3.5% CH hydrogels, while MCF-7 cells seeded on the 2.5% CH-Col hydrogels showed significantly higher metabolic activity than

those cultured on 3.5% CH-Col hydrogels. Comparison of metabolic activity also showed that for MCF-7 or MDA-MB-231 cells, culturing in the 3D platform resulted in significantly lower ($p<0.05$) metabolic activity than culturing in 2D (Figure 3.4A).

Total Protein Content

Total protein content was also evaluated for each of the conditions, as shown in Figure 3.4B. For both cell types, total protein was generally higher for cells cultured in 2D than was measured for cells in 3D culture, but significantly higher ($p<0.05$) than cells cultured using 3.5% CH hydrogels. For MCF-7 cells cultured in 2D, total protein content was also significantly higher ($p<0.05$) than 3.5% CH+Col hydrogels. These trends observed for protein content were similar to those trends observed for metabolic activity analysis shown via AlamarBlue® assay.

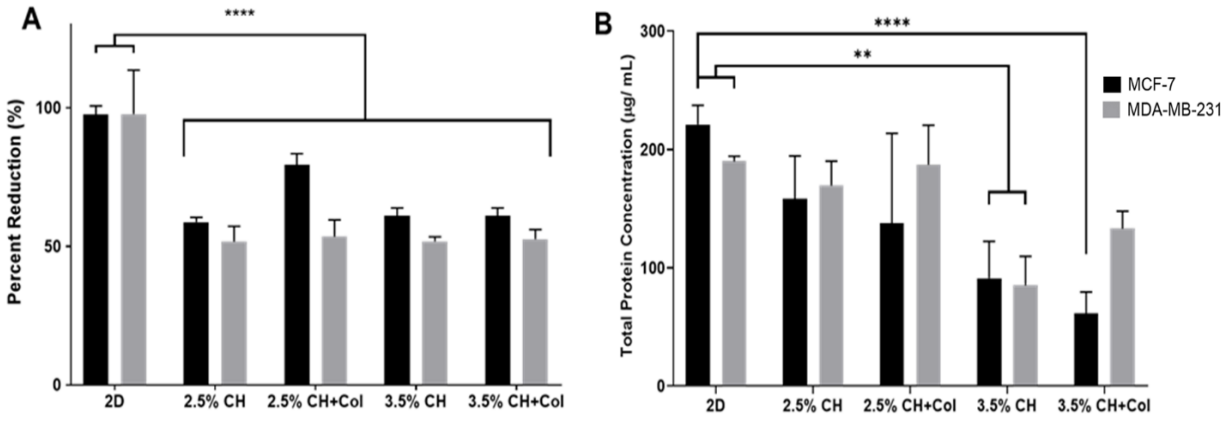

Figure 3.4. Metabolic activity and total protein quantification. AlamarBlue® assay (A) shows a significant reduction in metabolic activity for all hydrogels when compared to the 2D controls. Measurement of total protein content (B) showed significant decrease in protein expression between 3.5% CH compared to 2D controls and between MCF-7 cells seeded in 3.5% CH+Col compared to 2D control, similar to metabolic activity data shown in (A). Data presented as mean ± SD; n=3 gels; p-value determined using ANOVA and Tukey post-hoc analysis; **** = $p<0.0001$.

Effects of 3D Culturing on Metastatic Potential

MMP-2 protein concentration was measured and normalized to total protein content. As shown in Figure 3.5, approximately 0.01 to 0.07 ng MMP-2/μg total protein was measured for each condition. For MCF-7 cells, there was no difference in MMP-2 expression when cells were cultured on the 3D hydrogels or in 2D monolayer. Statistical analysis showed that for MDA-MB-231 cells, culturing of the cells on the stiffer 3.5% CH hydrogels resulted in a significant increase in MMP-2 expression when compared to the less stiff 2.5% CH hydrogels ($p<0.05$). The addition of collagen to the 3.5% CH-Col hydrogels resulted in a more significant increase in MMP-2 expression than the less stiff 2.5% CH-Col hydrogels ($p<0.01$). Based on these observations, subsequent analyses were performed to assess metastatic behavior of multiple cell types using the stiffer 3.5% CH-Col hydrogels, which was compared to cell behavior in 2D culture.

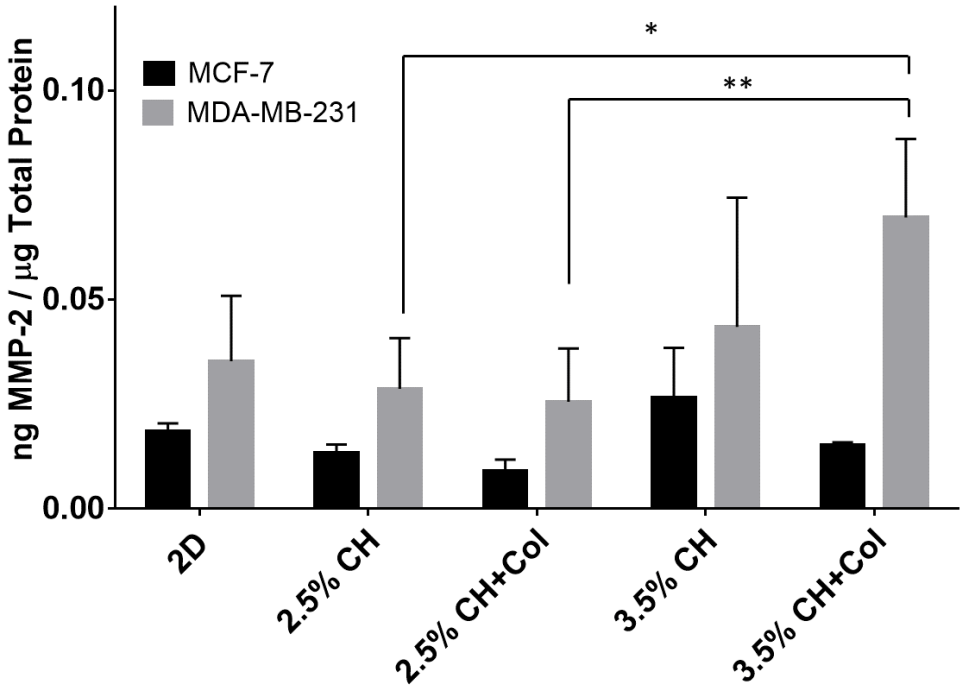

Figure 3.5. Comparison of protein expression resulting from culture with 3D hydrogels formed with varying concentrations of CH with or without collagen. Significantly higher MMP-2 expression was measured for MDA-MB-231 cells seeded on 3.5% CH + Col when compared to 2.5% CH gels with or without collagen. P-values were calculated using ANOVA and Tukey's post-hoc analysis; **$p<0.01$, *$p<0.05$; n=3.

Effects of Tumor Subtype and Ethnicity on Metastatic Potential

Figure 3.6 shows resulting ELISA protein expression data from analysis of multiple breast cancer cell types cultured in 3D using the 3.5% CH ± Col hydrogels and 2D controls. Expression of various proteins associated with metastasis (VEGFA, IL-6, MMP-9 and TGFβ2) was quantified, and their respective concentrations were normalized to total protein content. Analyses showed that there was little to no VEGFA detected for MCF-7 cells grown in 2D. When cultured in 3D, both triple-negative cell lines (MDA-MB-231 and HCC1806 cells) exhibited a significantly higher ($p<0.05$) amount of VEGFA protein than the MCF-7 cells (Figure 3.6A). Further, there was significantly higher ($p<0.05$) VEGFA expression for both triple-negative breast cancer cell lines when cultured with the 3D model platform, in comparison to their respective 2D cultures; however, there was no difference observed in VEGFA expression when comparing MDA-MB-231 and HCC1806 cells cultured in 3D (Figure 3.6A). As shown in Figure 3.6B, significantly higher ($p<0.05$) IL-6 protein expression was measured in MDA-MB-231 and HCC1806 cells when compared to MCF-7 cells. Notably, expression of IL-6 by the MDA-MB-231 cells was up to 50 times greater than that detected for MCF-7 cells. There were, however, no significant differences observed for IL-6 production for any cell types when comparing cells cultured in 2D or with the 3D model platform. MMP-9 protein was detected for each cell type when cultured in 2D and 3D, however, there was no significant difference observed for MMP-9 expression for any cell type cultured in either 2D or 3D (Figure 3.6C). Finally, evaluation of TGFβ2 expression showed that there was significantly higher ($p<0.05$) TGFβ2 expression measured for MDA-MB-231 cells than for MCF-7 or HCC1806 cells, as shown in Figure 3.6D. While not significantly different for the HCC1806 cells, both triple-negative cell types expressed higher levels of TGFβ2 than the MCF-7 cells, with the HCC1806 cells expressing less TGFβ2 than the MDA-MB-231 cells.

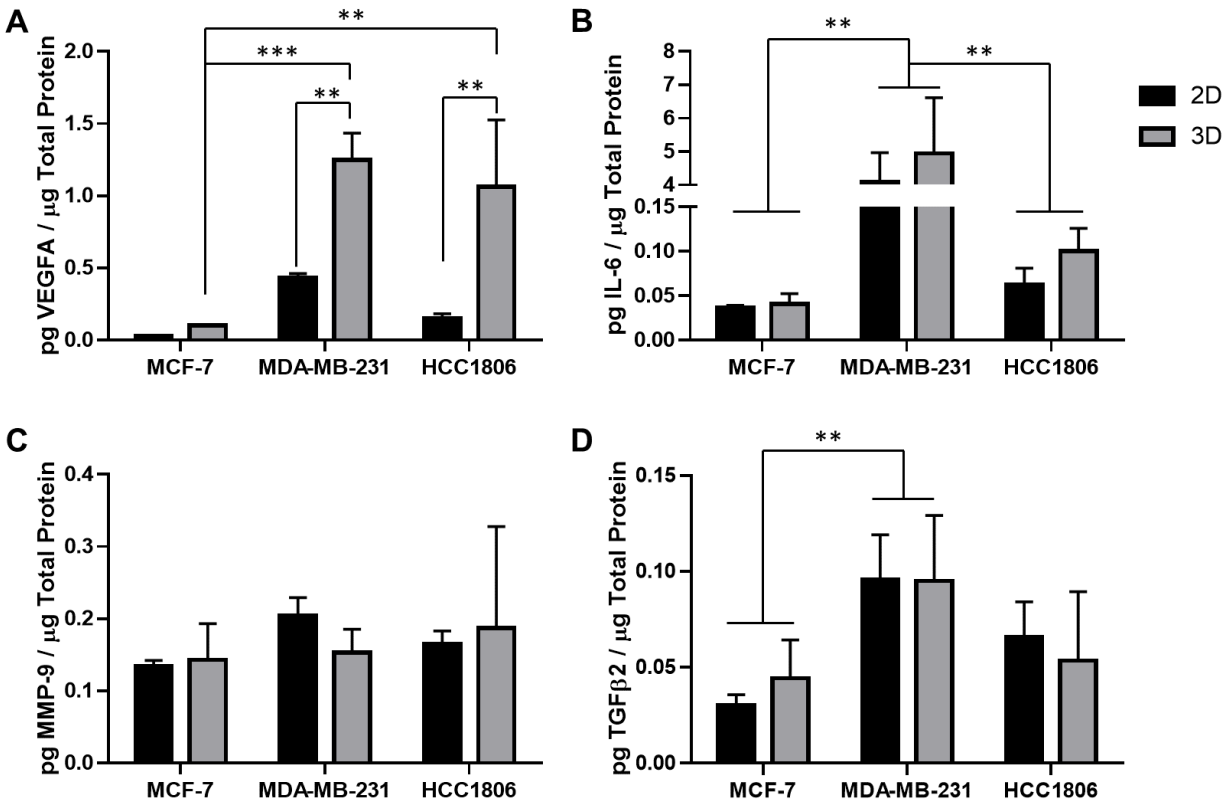

Figure 3.6. Comparison of protein expression resulting from culture of breast cancer cells, of varying subtype and donor ethnicity, in 2D and 3D. (A) Significantly higher VEGFA expression was observed for both HCC1806 and MDA-MB-231 cells when cultured in 3D, in comparison to 2D culture. VEGFA expression was significantly higher for both triple-negative cell types than MCF-7 cells. (B) IL-6 expression was significantly higher for MDA-MB-231 cells than for HCC1806 or MCF-7 cell lines. (C) No significant differences were observed for MMP-9 expression. (D) A significant increase in TGFβ2 expression was observed for MDA-MB-231 cells, when compared to MCF-7 cells. P-values were calculated using ANOVA and Sidak's post-hoc analysis; **$p<0.01$, $n = 3$.

3.5 Discussion

When modelling the breast cancer tissue microenvironment, it is important to take into consideration characteristics of the native ECM, such as mechanical properties, biological factors and ECM proteins [4]. Therefore, the goal of this work was to use chitosan, a natural biomaterial similar in structure and behavior to GAGs, to mimic breast cancer stiffness, while also incorporating a natural breast tissue ECM protein (collagen). Furthermore, these hydrogel models were used to understand metastatic disparities among tumors of varying subtype and from donors

of different ethnicities, which are commonly observed clinically [2, 20]. Understanding these differences can pave the way for personalized breast cancer treatments. Here, we discuss the creation and implementation of our model, with this proof-of-concept study, for this purpose.

Hydrogel Characterization

As previously mentioned, CH is a biocompatible and biodegradable polysaccharide that shares structural similarities to GAGs found in the ECM of natural tissues. Thus, in this work, CH was the ideal selected material for fabricating the 3D model platform. Preliminary screening of CH solutions of various concentrations was performed using rheological analysis to determine if and how the hydrogel mechanical properties would change as the concentration of CH used for each composite was varied, thus identifying which hydrogel concentration would be best suited for subsequent cell studies. Specifically, we aimed to identify which concentrations would yield 3D hydrogels with properties most resembling those of normal (2-90 kPa) and cancerous breast tissues (15-500 kPa) [8-10].

Hydrogel characterization using rheology showed that we successfully created a microenvironment that could mimic the stiffness of both normal and cancerous breast tissue. Evaluation with rheology, by performing an amplitude sweep, showed that the resulting storage and loss moduli for each sample was as expected for a hydrogel, with typical viscoelastic behavior demonstrated [21]. To determine which of the hydrogels more closely mimicked the physical properties of breast tissues, the elastic storage modulus for the fabricated hydrogels was determined by adapting Hooke's law to compare the shear storage modulus to compressive storage modulus. For rheological characterization, an amplitude sweep measures the storage and loss modulus of the hydrogel, while changing the amplitude of oscillation frequencies [22]. This is

generally a preliminary study done for most viscoelastic samples, because it determines the linear viscoelastic range of the sample, which is where the properties of the hydrogel are uniform [22]. Here, to compare our obtained data to compressive modulus, it was only necessary to measure linear properties of the hydrogels, and therefore, an amplitude sweep sufficed.

As most human breast tissues are characterized using compressive forces, the Young's Modulus is typically measured [23]. Young's Modulus is a measurable property of linearly elastic materials. Hydrogels, however, are viscoelastic [9, 21]. While we could gather just the elastic properties of the hydrogels by using atomic force microscopy, rheology, which studies the viscoelasticity of materials, gives a more thorough analysis of our hydrogels for both its viscous and elastic properties [24, 25]. The issue we faced, however, is that the output values for the elastic component of our hydrogels, the shear storage modulus, is a time-dependent value [26-28]. While this does allow for extrapolation of a stiffness value for the elasticity of our hydrogels (reported in our results as elastic storage modulus), which is similar to Young's Modulus, it does not provide an elastic value in compression for direct comparison to known Young's Moduli of human tissues.

For calculation of the elastic storage modulus based on our data, we assumed the viscous effects for the hydrogels were negligible and that the tissue was approximately incompressible, given that the loss moduli for the hydrogels were very low. This allowed us to make the assumption that Poisson's ratio is 0.5 [29] for calculating the elastic storage moduli of the 1% – 5% CH hydrogels. Comparison of the elastic storage modulus of the CH hydrogels to the Young's Modulus of normal and cancerous breast tissue was then used to determine which hydrogel best fit for our purposes for mimicking the tumor microenvironment in 3D. Upon initial analysis using 1% – 5% CH hydrogels we determined that 2.5% and 3.5% were best able to mimic breast and breast cancer tissue with an elastic modulus for 2.5% CH being approximately 14 kPa (comparable

to breast tissue) and approximately 48 kPa for 3.5% CH (suitable for stiffer cancerous breast tissue).

Next, to prepare a more physiologically similar microenvironment that would also support greater cell viability, a common ECM protein found in mammary tissue, collagen, was incorporated into the CH hydrogels. Statistical analysis showed that the addition of collagen did not significantly increase or decrease the moduli of either hydrogels, thus indicating that the hydrogels could be further optimized and tuned by incorporating native ECM proteins without effecting the desired modulus of the hydrogels for the 3D model platform. Subsequent studies to affirm the effects of culture environment stiffness were then performed using either 2.5% CH or 3.5% CH hydrogels with and without collagen incorporated for our proof-of-concept evaluation.

For biomaterial scaffolds commonly used for tissue engineering, specifically for regeneration purposes, sufficient scaffold porosity is an essential property. In particular, pores of an appropriate size can facilitate cell-cell communication, cell attachment, and nutrient transport, however, the number and size of pores must be balanced with maintenance of mechanical stability [30]. Although SEM analysis showed that our hydrogels were not porous in structure, the purposes for our hydrogels were not specifically for supporting tissue regeneration or for experimental cultures longer than 1 week. Therefore, lacking porosity does not pose a significant concern for these initial studies. In the future, should these 3D hydrogel platforms be needed for longer term use or for studying topics such as tumor angiogenesis or cell-cell communication, the inclusion of a porogen to increase overall porosity may be considered.

Preliminary In Vitro Cellular Analyses

LIVE/DEAD®, AlamarBlue®, and BCA analyses were conducted to assess cell morphology and viability, metabolic activity, and total protein content, respectively. LIVE/DEAD® images (Figure 3.3) show morphology differences observed for the 2D controls, CH only hydrogels and CH-Col hydrogels. Tumoroid formation on the CH only hydrogels may be due to lack of adhesion sites. While chitosan does have bioadhesive properties, the CH hydrogels are not as adhesive as tissue culture plates, thus cell attachment may be limited, resulting in cell aggregation; some researchers have even plasma treated chitosan to increase these properties for greater cell attachment [31]. Based on the SEM images (Figure 3.2D) to assess the structure and morphology of the hydrogels, it appears that upon crosslinking, CH hydrogels bonded to form patterned ridges. These ridges created "craters" that possibly aided in tumoroid formation but were less deep as the concentration increased. The "crater-like" structures formed around groups of cells could provide a similar environment to low attachment round-bottom tissue culture plates that are commonly used for spheroid formation [32]. For CH-Col hydrogels, cell morphology shown by LIVE/DEAD® staining appeared to be a mix between the attached cells in 2D and the tumoroid cells on the CH hydrogels. SEM images of the CH-Col hydrogels (Figure 3.2D) showed that the added collagen appeared to fill in the "crater-like" structure of the hydrogels, which could prevent isolated tumoroids from forming. Furthermore, collagen is recognizable by several cell integrins that mediate and allow cell attachment, specifically in Type I collagen, thus indicating why greater attachment and cell spreading was observed for cells culture on the CH-Col hydrogels [33-35].

When evaluating the viability of cultured cells with the LIVE/DEAD® assay, there were very few dead cells observed, which could be due to detached dead cells being removed when media was aspirated. It does appear, however, that cell viability was supported on the hydrogels

based on viable green-stained cells present for each cell type. Measurement of metabolic activity with alamarBlue® assay, however, showed that there was a significant reduction in metabolic activity by at least half when comparing 3D hydrogel cultures to those cells cultured in 2D. This may be related more to the rate of cell proliferation rather than viability, however, as the cells in the hydrogels may have proliferated slower than in 2D given exposure to a foreign environment. This is not uncommon as this behavior has been observed in other studies of *in vitro* hydrogel-based platforms [14, 36]. Further, because of the aggregation of cells observed for the 3D cultures, reduction of the alamarBlue® reagent may have been less for cells contained within the tumoroid structures that formed, contributing to the lower metabolic activity observed. For the 3D hydrogels, it does not appear that stiffness or the addition of collagen to the hydrogel had any effect on metabolic activity. These observations were supported by BCA analyses of total protein, where comparable total protein was measured for cells cultured in 3D (Figure 3.4B). During sample collection, however, researchers noted that extraction of protein lysate from 3D hydrogels was more difficult than for cells in 2D, and therefore, protein yield could have been a bit lower for cells cultured on hydrogels due to that reason. Future studies could be conducted over one to two weeks with samples collected at multiple time points to determine if proliferation would eventually be comparable for all experimental conditions and if there are possibly any differences between each of the hydrogels.

Effects of 3D Culturing on Metastatic Potential

MMP-2 is a extracellular enzyme that degrades gelatin and type IV collagen, thus aiding cell migration and angiogenesis during breast cancer metastasis [37]. Because increased collagen deposition and fiber straightening has often been associated with breast cancer stiffness, it is

possible that MMP-2 can also be more highly expressed as breast cancer stiffness increases during migration [11, 15]. Therefore, we aimed to use MMP-2 expression as an indicator of metastasis to determine which chitosan-based model would most support metastatic behavior. ELISA analysis showed no significant differences in MMP-2 protein expression for MCF-7 cells when comparing the four hydrogels or 2D control. There was, however, a significant difference in MMP-2 expression observed for MDA-MB-231 cells cultured on the 3.5% CH-Col hydrogels and both 2.5% CH and CH-Col hydrogels. Given that MCF-7 cells are a less aggressive cell line than MDA-MB-231 cells, the higher expression of MMP-2 by MDA-MB-231 cells than was observed for MCF-7 cells was as we expected and was shown in Figure 3.5. Based on the significant increase in MMP-2 for the MDA-MB-231 cells and the large difference in MMP-2 expression observed between MCF-7 and MDA-MB-231 cell lines, we determined that our 3D model with increased stiffness (3.5% CH-Col) was sufficient for supporting physiological behavior associated with metastasis, thus, we wanted to further implement the 3.5% CH-Col model to evaluate potential metastatic differences between tumor subtypes and different ethnicities.

Effects of Tumor Subtype and Ethnicity on Metastatic Potential

African-American women have worse age-adjusted survival rates of breast cancer than Caucasian women, even though they have a lower over-all incidence rate [1, 38]. This can partially be due to socioeconomic differences such as income, availability of health insurance and access to proper healthcare [39, 40]. Earlier onset and prevalence of more aggressive breast cancers, such as inflammatory and triple-negative subtypes, also contribute to increased mortality rates in African-American breast cancer patients [41, 42]. Even when controlling for these factors, however, there is still racial disparity in breast cancer outcomes between Caucasian and African-

American women [43]. These disparities could be due to several factors including genetic differences, as well as differences in cytokine expression or tumor microenvironmental factors [44]. Because there is evidence of breast cancer behavioral differences due to variations in race or ethnicity, it is essential to study these differences to increase therapeutic efficacy. Studying these differences could not only inform researchers and medical professionals why certain therapeutics may not work for patients of color, but it can also push research towards making therapeutics that do work. Therefore, we chose to use our model to study not only differences in metastatic cytokine expression between different subtypes, but also to evaluate variations in metastatic potential between different races.

Here, we used three different cell lines for our studies: MCF-7 cells (HER2+, Caucasian), HCC1806 cells (triple-negative, African American), and MDA-MB-231 cells (triple-negative, Caucasian). There have been studies that compare MCF-7, MDA-MB-231 and HCC1806 cell lines to one another, but these studies have generally evaluated drug resistance and recurrence between the cell lines [45]. No studies currently exist that study HCC1806 or any other cell lines derived from African American donors in a 3D model or comparing them to 2D cell studies. There are also very few studies that explore metastatic protein expression between cells from different ethnicities, such as the metastatic cytokine profile we show in Figure 3.6 for four different cytokines, comparing these cell types in 2D and 3D culture.

Cells cultured in 2D and 3D were evaluated to determine what effects the variations in the engineered microenvironment would have on metastasis, which was assessed by measuring the expression of certain cytokines, VEGFA, IL-6, MMP-9 and TGFβ2. Overall, evaluation of VEGFA expression showed that VEGFA expression for MDA-MB-231s and HCC1806s, both triple-negative cell types, was significantly increased when cultured in the 3D model, as compared

to 2D culture (Figure 3.6A). Evaluation of IL-6, MMP-9 and TGFβ2 showed a general increase in protein expression for cells cultured in 3D, however, the increase was not statistically significant. While not statistically significant, however, these findings and the significant increase in VEGFA suggest that our 3D hydrogel modeling approach may support increased metastatic behavior characteristic of more aggressive triple-negative breast cancer cells, thus indicating success for the model to provide a better metastatic niche, but further testing is required for confirmation of this finding.

VEGFA is a glycoprotein that stimulates the mitosis of endothelial cells and their migration. It is the predominant factor associated with angiogenesis, which is involved in early tumor progression, sustained tumor growth and early metastasis [46, 47]. Comparison of the triple-negative cell lines (HCC1806 and MDA-MB-231) showed significantly higher levels of VEGFA for both cell types in 3D, but no significant difference was observed based on the two different cell donor ethnicities (Figure 3.6A). That the triple-negative subtypes demonstrated increased VEGFA expression when cultured in 3D and the HER2+ (MCF-7) cells did not, represents what would be expected behavior *in vivo* for these specific subtypes, further suggesting physiological relevance for our modeling approach.

IL-6 is a pleiotropic cytokine involved in adipocyte signaling and the inflammatory response [48]. It has been studied as a potential prognostic factor in breast cancer due to its association with decreasing E-cadherin production (allowing for cell motility) [49, 50]. Here, the MDA-MB-231 cells showed almost a 40-fold increase in IL-6 expression when compared to both HCC1806s and MCF-7s in both 2D and 3D. This result was unexpected as clinical data shows significantly higher levels of IL-6 expressed in African-American than Caucasian women [44, 51]. There was, however, a large study performed using several breast cancer cell lines directly

extracted from patient tumors and identified by American Society of Clinical Oncology (ASCO), where a noted lack of IL-6 expression in their HCC1806 tumors was reported [52]. It is possible that to mimic studies with clinically observed IL-6 levels for African American and Caucasian women, primary cell lines directly from clinical patient samples should be used in our model system. This could remove potential influences from long term *in vitro* cell culture that might affect cell behavior and could provide us with a more accurate representation of cytokine expression. The tunability and flexibility of our modeling approach would support incorporation of patient-derived samples, further demonstrating its utility towards developing personalized therapies.

MMP-9 is associated with early onset metastasis as it is an enzyme that degrades collagen and gelatin in the basement membrane, the main barrier between *in situ* carcinoma and its extravasation [53]. Based on our study, there were no clear differences observed for MMP-9 expression for any condition. The association of MMP-9 with early metastasis may be the reason why no differences in its expression were observed here given the short-term culturing period for our study.

Finally, TGFβ2 is actually associated with breast cancer inhibition in early stages, but in later stages it is associated with malignant progression [54]. Upon inactivation of tumor suppressor genes and retrieval of oncogenic mutations, TGFβ2 is thwarted towards behaviors that increase cell motility, invasion and metastasis [55]. Here, we observed a significant increase in TGFβ2 for MDA-MB-231 cells when compared to the MCF-7 cells. Further, while not significant, the other triple-negative cell type, the HCC1806 cells also expressed higher TGFβ2 than the MCF-7 cells, such that higher cytokine production was observed for the more aggressive triple-negative cells. Comparison of the triple-negative cell lines showed that there was a slightly lower level of TGFβ2

in the African-American cell line than in the Caucasian cell line, which was also observed by a group that previously studied TGFβ2 mRNA expression between HCC1806 and MDA-MB-231 cell lines [56].

3.6 Conclusion

In this work, we characterized a chitosan-based platform that successfully incorporated natural ECM protein (collagen) while also mimicking breast cancer stiffness. We also showed novel use of our 3D model to study differences in metastasis based on tumor cell subtype and ethnicity. By increasing sample number, cell type, and measures of metastatic potential as well as possibly incorporating clinical patient-derived cell samples, we can further examine metastatic disparities between subtypes and ethnic groups. These findings could provide significant clinical information and change the scope of how breast cancer therapeutics are created. Future work includes replicating the current work with a larger sample size and increased metastatic analyses (different cytokines, wound healing assays, transwell migration assays, etc.) as well as applying this platform to possibly be used in creating predictive models of breast cancer metastasis.

3.7 References

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CHAPTER 4

CHITOSAN-BASED THREE-DIMENSIONAL (3D) MODEL TO STUDY LEPTIN AS A DIAGNOSTIC PREDICTOR OF BREAST CANCER METASTASIS (BCM)[1]

[1] Al-Saleh, J.E. and C.T. Gomillion. To be submitted to Cancer and Nutrition

4.1 Abstract

Breast cancer metastasis (BCM) remains one of leading causes of death in women with cancer. While detrimental for Caucasian women, outcomes for African American women with metastatic breast cancer are even worse, as there are disparities in breast cancer patient outcomes attributed to their specific cancer subtype and patient ethnicity. For example, African American women are more susceptible to aggressive subtypes, such as triple-negative breast cancer than Caucasian women, leading to increased deaths. In addition, researchers have realized that microenvironmental factors can also influence BCM, which has led to the development of three-dimensional (3D) models that can aid in studying specific aspects of the microenvironment and how those factors influence breast cancer behavior. Currently, there are few existing 3D models created to study how inflammation and adipokines effect BCM, especially for cancers of varying subtype or accounting for patient ethnicity. Elucidating the role of inflammatory factors and adipokines in BCM can allow for better design of personalized therapeutics, in addition to determining the potential use of these factors as diagnostic predictive markers of metastasis. In this work, a previously characterized chitosan-collagen (CH-Col) based model was used to study the effects of the adipokine leptin on metastasis of three different breast cancer cell types in 3D.

4.2 Introduction

Breast cancer metastasis (BCM) remains one of the leading causes of death in women with cancer [1]. In addition to the poor prognosis that can result once breast cancer metastasis is detected, there are also racial disparities in breast cancer research that must be studied to design therapies and treatments that can be effective for larger percentages of the population. For instance, African-American women are more susceptible to triple-negative breast cancer (a more aggressive subtype) due to their greater likelihood of having the BRCA1/2 mutations [2]. Earlier onset and prevalence of more aggressive breast cancers, such as inflammatory and triple-negative subtypes, also contribute to increased mortality rates in African-American breast cancer patients [3, 4]. Therefore, it is imperative that differences in tumor cell metastasis and the influence of patient ethnicity be accurately studied so that personalized treatment plans may be created, thereby decreasing the mortality gap between various the patient populations.

Research over the last several decades has discovered several relationships between the breast cancer microenvironment and resulting tumor cell metastasis [5, 6]. Because of these relationships, researchers have strived to recapitulate specific aspects of the microenvironment by creating three-dimensional (3D) models for studying tumor cell behavior (Figure 4.1). These models can mimic the *in vivo* environment without the associated costs and ethical concerns presented by *in vivo* animal models, while also providing physiologically-similar mechanical and biological cues that 2D *in vitro* models cannot support [7, 8]. Specifically, for mammary tissue, 3D models have been developed for studying normal tissue development, tumor formation, tumor vascularization, metastasis, and cancer cell response to therapeutics [9]. While various factors have been included in these modeling approaches (i.e., various combinations of cell types, inclusion of different ECM proteins to form surrounding matrix, etc.), the developed models that have focused specifically on elucidating the role of inflammation and adipokine signaling in the

microenvironment, have been limited. Thus, there is a need for a modeling approach to study how such factors of the microenvironment can influence breast cancer metastasis.

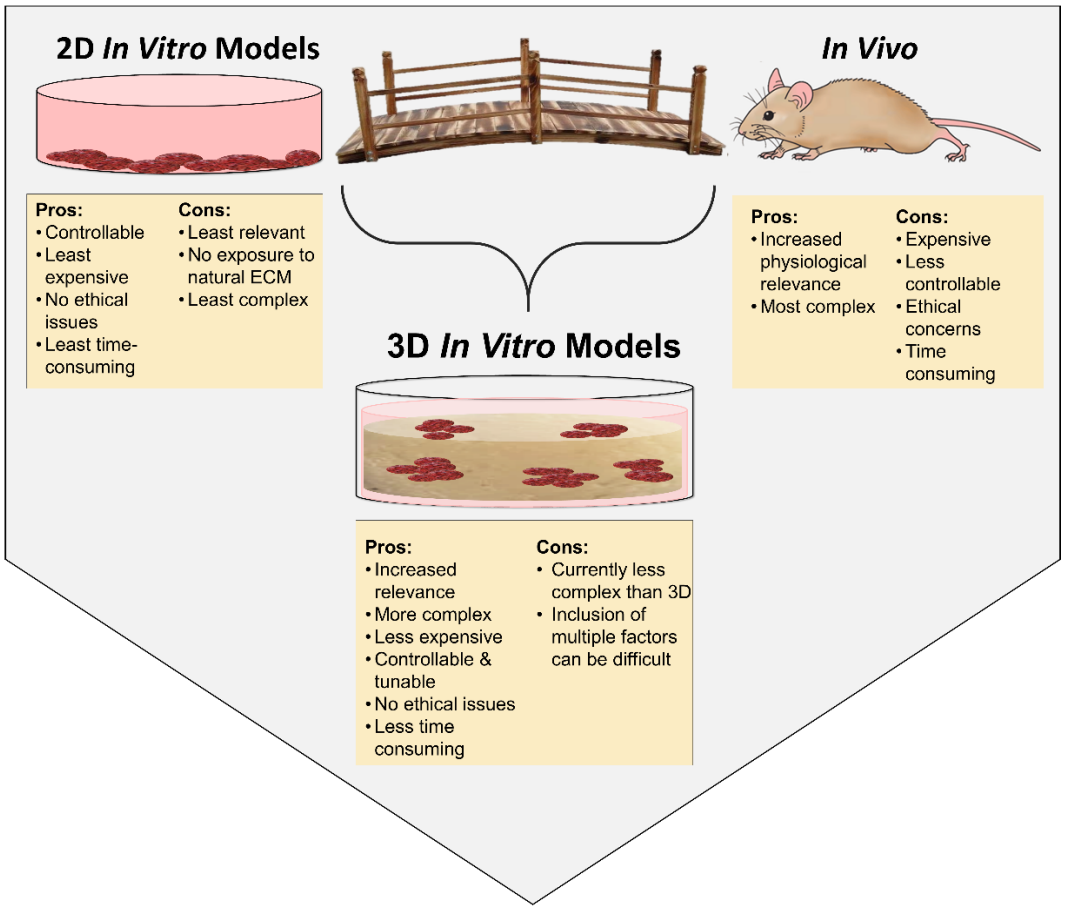

Figure 4.1. 3D *in vitro* models provide a bridge between 2D *in vitro* models (i.e., monolayer cell cultures) and *in vivo* animal models. Advantages and disadvantages of specific modeling approaches are summarized.

Many of the factors associated with obesity, inflammation and wound healing play similar roles in mediating breast cancer metastasis [10]. Obesity, resulting from increased adipose tissue, has been associated with low-grade chronic inflammation, insulin resistance and secretion of pro-inflammatory cytokines that progress breast cancer metastasis [11-13]. Within adipose tissue, adipocytes can secrete several pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), adiponectin and leptin [13, 14]. These cytokines could be studied

to not only create potential therapeutics, but also for use as predictive diagnostic markers. Specifically, of interest in our work is leptin. Leptin, a pro-inflammatory cytokine, plays a significant role in the process of epithelial-to-mesenchymal transition (EMT) associated with BCM by upregulating the glucometabolic enzyme, pyruvate kinase M2 (PKM2), that coactivates cell-cycle progression and cell motility [15]. Leptin has also been shown to induce the JAK2/STAT, PI3K and MAPK pathways responsible for inducing breast cancer cell proliferation and angiogenesis [16, 17]. Based on these reported functions for leptin, it is an important signaling molecule that should be considered and included for studies of BCM.

Previously, we reported the development and characterization of a 3D chitosan-collagen (CH-Col) hydrogel platform that mimicked tumor tissue stiffness and natural ECM composition, while supporting metastatic behavior of breast cancer cells [18]. In this work, we aimed to apply this model to study the effects of leptin on breast cancer cells of varying subtype and derived from donor sources of different ethnicities. Three different cell lines were used for this work: MCF-7 (Caucasian, ER+/HER2+, mammary adenocarcinoma), MDA-MB-231 (Caucasian, ER-/PR-/HER2-, triple-negative breast cancer) and HCC1806 (African-American, ER-/PR-/HER2-, triple-negative breast cancer). Tumor cells were cultured within the hydrogel platform and compared to 2D controls. Metastatic potential was determined using ELISAs to quantify the expression of several known metastatic markers. In doing so, we also aimed to determine if leptin effects the metastatic potential differently for cells of varying subtype and donor ethnicity, thereby establishing the potential use of our model as a platform for studying the role of inflammatory cytokines, such as leptin, and also assessing the potential of leptin for clinical use as a predictive diagnostic marker for susceptibility to BCM depending on ethnicity and type of cancer.

4.3 Materials and Methods

Hydrogel Fabrication

CH-Col hybrid hydrogels were fabricated for 3D model cultures. As previously determined, a 3.5% w/v CH solution was prepared using high molecular weight chitosan (HMW CH, 310–375 kDa, >75% Deacetylated) obtained from Sigma-Aldrich (St. Louis, MO) by dissolving 350 mg of CH powder in 7 mL of 1.43% v/v glacial acetic acid in water in a glass scintillation vial [18]. Solutions were mixed overnight on a heated stirrer at 55°C. The vials of solubilized CH were then placed on ice, and 3 mL of 2.5 mg/mL rat tail collagen I (ThermoFisher, Carlsbad, CA) was added to each vial to obtain a 0.075% v/v collagen solution that was mixed thoroughly. The CH-Col solutions were stored at 4°C protected from light until ready for use.

Hydrogels for all experiments were crosslinked using a 0.5 N NaOH (Sigma-Aldrich) solution for 24 hours in a 60-mm tissue culture dish (Corning, Corning, NY). Hydrogels were then neutralized with phosphate buffered saline (PBS, ThermoFisher) and stored at 4°C until use for cell seeding.

Cell Seeding

The 3.5% CH-Col hydrogels (n=3 per cell type) or tissue-culture treated plates for 2D controls were used for this study. Hydrogels were prepared and crosslinked in 60-mm dishes as described above. Three different cell lines, varying in subtype and donor ethnicity, MCF-7, MDA-MB-231, and HCC1806 cells (ATCC, Manassas, VA), were seeded in separate experiments for analysis. Cells ranged from passage 10 to passage 13 for all experiments. Cells were expanded prior to seeding on hydrogels in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Waltham, MA) with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA) and 1% penicillin/streptomycin (Pen/Strep, Gibco), denoted as DMEM-Complete. On the day of cell

seeding for metastatic analyses, 5 mL of low-serum phenol red-free DMEM (Gibco) containing 2% FBS, 2% L-glutamine and 1% Pen/Strep was added to ten 60-mm dishes containing crosslinked hydrogels, which were then incubated at 37°C (media was aspirated after incubation). Cells were counted and 4.9 mL of cell suspension (specific cell numbers for seeding were based on protein yields from previous trials and are shown in Table 4.1) was added to nine 3D hydrogel dishes and nine 2D dishes, with remaining dishes without cells seeded used as acellular controls. The dishes for 3D and 2D culture were divided into three groups (n=3 per group) that were treated with varying concentrations of human leptin (>97%, recombinant, expressed in *E. Coli*, lyophilized powder, Sigma Aldrich), including 0 ng/mL leptin, 10 ng/mL leptin and 30 ng/mL leptin. A volume of 100 µL of DI water was added to the 0 ng/mL plates (control), 100 µL of 0.5 µg/mL leptin in DI water was added to each 10 ng/mL plate, and 100 µL of 1.5 µg/mL leptin in DI water was added to the 30 ng/mL dishes. Cells were cultured for 3 days at standard conditions. Cultures were evaluated and representative images captured using an EVOS™ FL Imaging System (ThermoFisher).

Table 4.1. Cell seeding densities for *in vitro* metastatic potential analyses

	MCF-7	**MDA-MB-231**	**HCC1806**
2D Cultures	1.8×10^6 cells per dish	2.6×10^6 cells per dish	2.3×10^6 cells per dish
3D Cultures	2.9×10^6 cells per dish	3.7×10^6 cells per dish	3.2×10^6 cells per dish

Metastatic Protein Analyses

After culturing for 3 days, protein extraction was performed using M-PER Mammalian Protein Extraction reagent (ThermoFisher) supplemented with 150 mM sodium chloride, 1% protease inhibitor (ThermoFisher), and 1% Ethylenediaminetetraacetic acid (EDTA,

ThermoFisher). Briefly, media was removed and added to respective microtubes. After centrifugation and PBS washes, MPER was added to both the microtubes and the hydrogels, which were incubated at room temperature for 10 minutes on a shaker. MPER from the hydrogels was then added to the microtubes and centrifuged. The supernatant was removed, and samples were stored at -80°C until total protein content was determined via a bicinchoninic acid (BCA) assay using a PIERCE™ BCA assay kit (ThermoFisher), according to the manufacturer's protocol. Samples were measured using a Biotek 800 TS microplate reader (Biotek instruments Inc., Winooski, VT) at 562 nm. An albumin standard curve was generated to determine sample concentration. Hydrogels without cells were used as controls and subtracted from absorbance values of hydrogel samples with cells.

Following determination of total protein content, the remaining protein lysate for each sample was used for ELISA testing. Samples were analyzed using ISO 13485 certified RayBio® ELISA kits (RayBiotech, Atlanta, GA), with samples analyzed at the RayBiotech facility. Samples were tested to quantify expression of the following proteins: interleukin-6 (IL-6), vascular endothelial growth factor A (VEGFA), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), and transforming growth factor-beta 2 (TGF-β2). The concentration of each protein was normalized to the total protein content in each sample (pg/µg) for comparison.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 6™ (GraphPad Software, Inc.) Two-way ANOVA followed by Tukey post-tests for multiple comparisons (unless otherwise

noted) were performed to determine statistical significance between individual sample groups with significance set at $p<0.05$. Data are expressed as mean and standard deviation (SD).

4.4 Results

Evaluation of In Vitro 3D Culture on Cell Behavior

Cells cultured on the CH-Col hydrogels were evaluated to observe morphology and total protein content when cultured in 3D as compared to the 2D controls. As shown in Figure 4.2, normal cell morphology in monolayer culture is shown for each cell type. When cultured using the 3D hydrogels, slight morphological changes were observed for each cell type, where the MCF-7 cells formed visible aggregates of cells, and the MDA-MB-231 and HCC1806 cells exhibited smaller colony-like clusters. Total protein content was measured for each condition evaluated, as an indicator of cell viability (Figure 4.3). Total protein for cells cultured on the CH-Col hydrogels was significantly lower than cells cultured on the 3D hydrogels. When leptin was included in the 3D model, there was no significant difference observed in the total protein content for each condition.

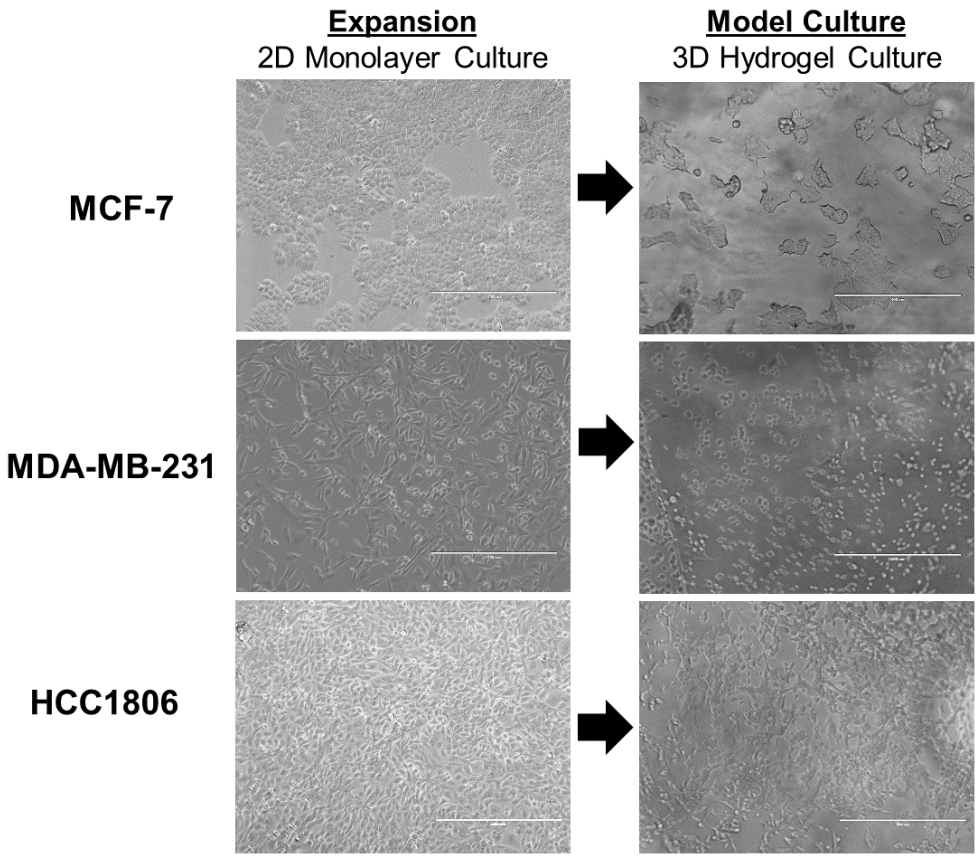

Figure 4.2. Representative images of each cell type cultured in 2D (left) and using the 3D hydrogel model platform. Morphological changes are observed for each cell type, with MCF-7 cells forming visible aggregates of cells in 3D culture and the MDA-MB-231 and HCC1806 cells exhibiting smaller colony-like clusters.

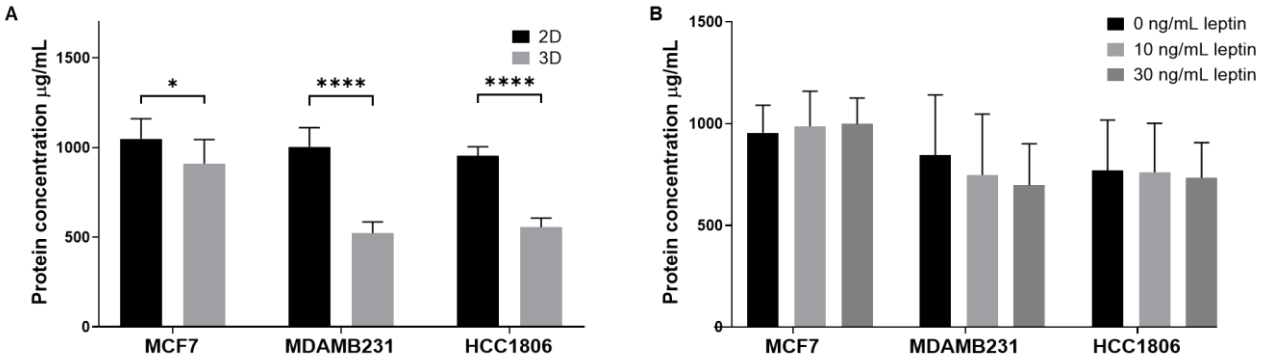

Figure 4.3. BCA assay shows 2D vs. 3D and Leptin effects on proliferation and viability. (A) Protein concentration significantly decreases in 2D compared to 3D. (B) Leptin concentration has no effect on protein production for any cell type. Statistical differences determined using two-way ANOVA with Tukey's post-hoc analysis; ****$p < 0.0001$, *$p<0.01$, $n = 9$.

Effects of 3D Culturing on Metastatic Behavior

Initial differences in metastatic potential between cells types in 2D monolayer culture and the 3D CH-Col hydrogels were evaluated to determine if our 3D model better mimics breast cancer cell behavior than 2D culture. As shown in Figure 4.4, comparison of metastatic protein expression for MCF-7 cells showed that there was no significant difference in expression for any of the selected markers when 2D cultures were compared to 3D hydrogel cultures. There was, however, a significant increase in IL-6 and VEGFA production for MDA-MB-231 cells when cultured in 3D versus 2D (Figure 4.4A and B). Similarly, HCC1806 cell types showed higher VEGFA protein expression when cultured in 3D than in 2D (Figure 4.4B). MMP2 was the only protein that showed insignificantly lower expression in 3D than in 2D. Although not significant, cells cultured in 3D produced slightly higher levels of MMP-9 (MCF-7 and HCC1806) and TGFβ2 (MDAMB231) as well (Figure 4.4D and E).

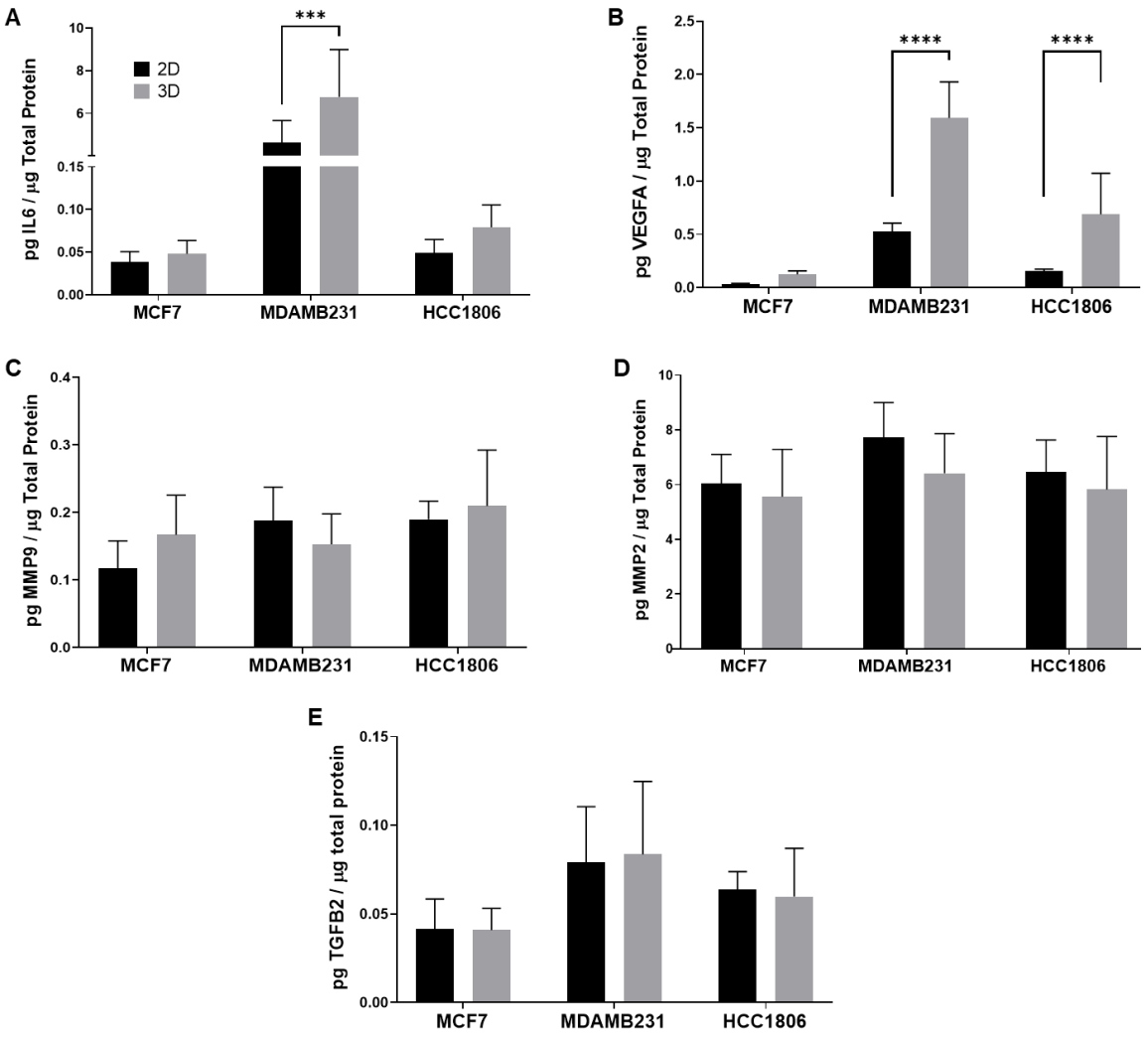

Figure 4.4. Comparison between 2D and 3D culturing for each cell type (for samples not cultured with leptin). Compared to 2D monolayer culture, MDA-MB-231 cells produced significantly more IL-6 (A) and VEGFA (B) in 3D. HCC1806s also produced significantly higher VEGFA in 3D (B). No clear differences can be seen between 2D and 3D conditions for production of MMP-2 (C), MMP-9 (D), TGFβ2 (E). Statistical differences determined using two-way ANOVA with Sidak's post-hoc analysis specific for comparing only 2D vs. 3D data sets; ****$p < 0.0001$, ***$p<0.001$, $n = 9$.

Effects of Tumor Subtype and Ethnicity on Metastatic Behavior

The expression of the selected protein markers associated with metastasis was then compared to determine any differences based on breast cancer cell subtype or ethnicity of the donor of origin for the cell lines. Specifically, samples cultured within the 3D model without leptin were

compared to determine our model's ability to mimic breast cancer behavior according to their subtype, while also seeing if the model displays any differences between triple-negative breast cancers from cells of different donor ethnicity. Figure 4.5 shows that there was significantly higher (up to 40-fold increase) expression of IL-6 in MDA-MB-231 cells (6.8 ± 2.2 pg/μg) than in HCC1806 cells (0.079 ± 0.026 pg/μg) and MCF-7 cells (0.048 ± 0.015 pg/μg). MDA-MB-231 and HCC1806 cells also expressed significantly higher amounts of VEGFA (5.6 ± 1.7 pg/μg and 5.8 ± 1.9 pg/μg) than did MCF-7 cells (0.12 ± 0.03 pg/μg). HCC1806 cells, however, expressed significantly lower levels of VEGFA than MDA-MB-231 cells, although only slightly. No clear differences were seen in MMP2 expression. Although insignificant HCC1806 cells expressed higher levels of MMP-9 than the other two cell lines, while the MDA-MB-231 cells expressed the highest amount of TGFβ2.

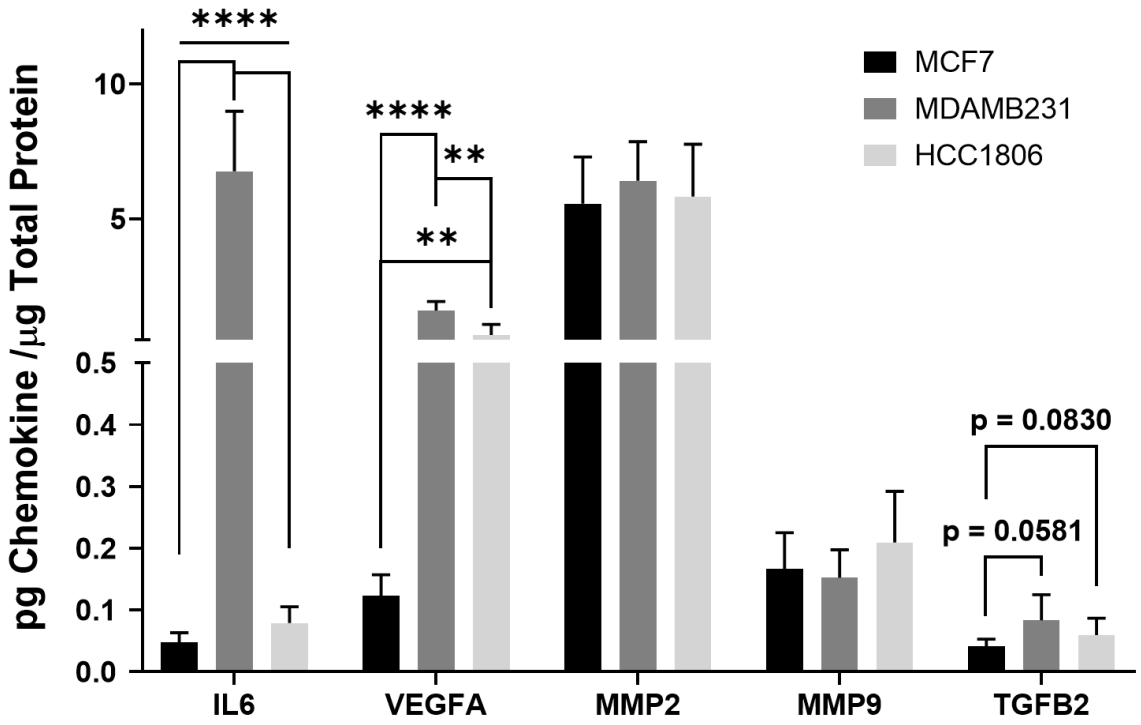

Figure 4.5. Comparison between cells of different subtype and from donors of different ethnicity seeded on 3D hydrogels (for samples not cultured with leptin). MDA-MB-231 cells produced the significantly highest amount IL-6. There are significantly different levels of VEGFA produced by all three cell types, with MDA-MB-231 cells being the highest. A similar trend can be seen in TGFβ2 production although not significantly different. No clear trend can be seen in MMP-9 and MMP-2 protein production. Statistical differences determined using Two-way ANOVA with Tukey's posthoc analysis; ****$p < 0.0001$, **$p<0.01$, $n = 9$.

Effects of Leptin on Breast Cancer Metastatic Potential

Cells cultured in 3D in the presence of leptin were evaluated to determine the effects of leptin on the expression of metastasis associated proteins for each of the three cell types. No specific trends were observed between any of the cell types for IL-6 expression, as IL-6 expression was comparable for cells even as leptin concentration increased (Figure 4.6A). There was a significant positive correlation between leptin and expression of VEGFA for MDA-MB-231 cells, where VEGFA expression increased as leptin concentration increased. However, for HCC1806 cells, the inverse was observed as VEGFA expression decreased as leptin concentration increased,

although not significant. There were no differences observed for VEGFA expression by MCF-7 cells exposed to leptin (Figure 4.6B). Further, no relationship was determined between MMP-2 or MMP-9 expression for any cell lines when exposed to leptin (Figure 4.6C and D). Although insignificant, at lower leptin concentrations, TGFβ2 expression by MDA-MB-231 cells was higher than at higher leptin concentrations, with 0.095 ± 0.034 pg/µg at 0 ng/mL leptin and 0.079 ± 0.047 pg/µg at 30 ng/mL (Figure 4.6E).

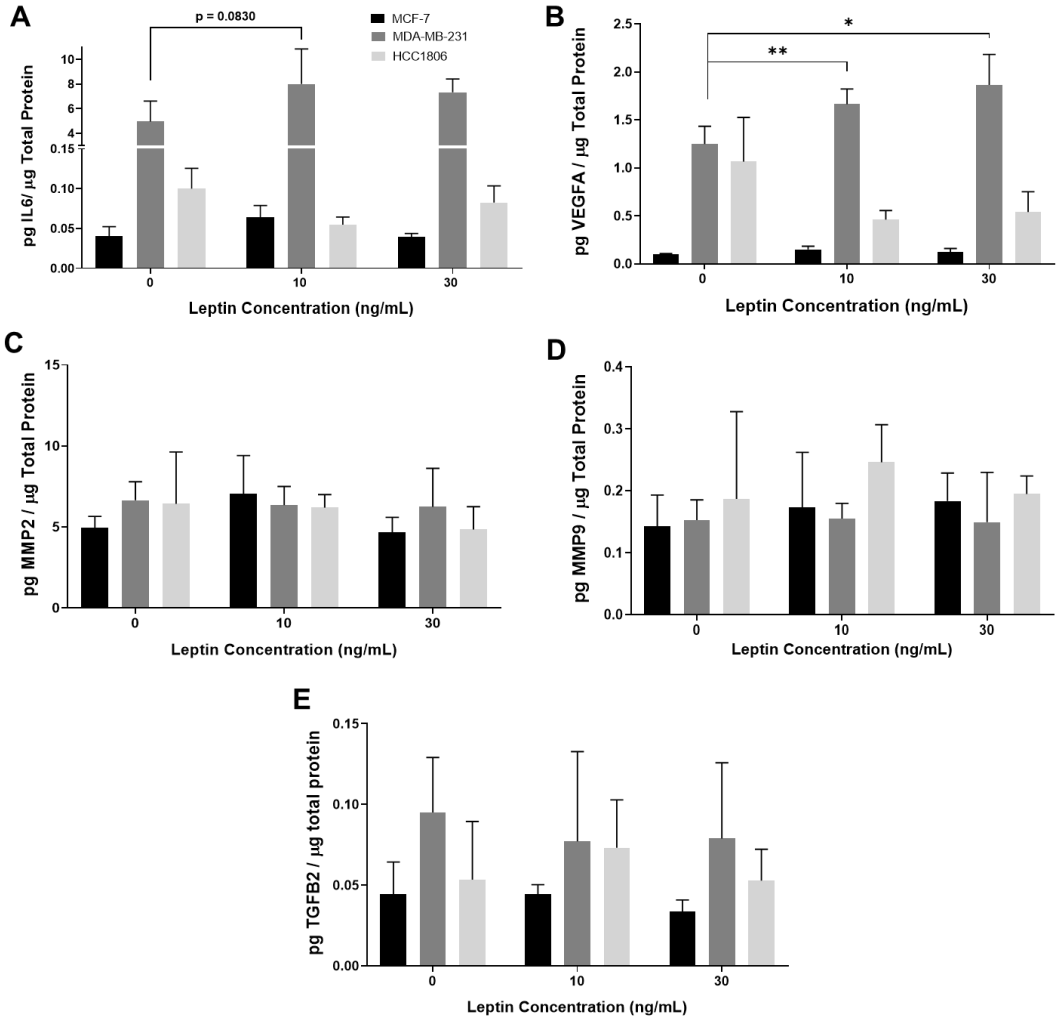

Figure 4.6. Effects of leptin on metastatic cytokine expression of different cancer cell subtypes and ethnicities seeded in 3D. A significant relationship cannot be determined between IL-6 (A), MMP-2 (C), MMP-9 (D), and TGFβ2 (E). There was a significant increase in VEGFA production as leptin concentration increased for MDA-MB-231 cells, as well as an insignificant decrease in VEGFA production as leptin concentration increased for HCC1806 cells (B). Statistical differences determined using two-way ANOVA with Tukey's post-hoc analysis; **$p<0.01$, *$p<0.05$, $n = 3$.

4.5 Discussion

Three dimensional models have been created to bridge the gap between 2D *in vitro* and 3D *in vivo* models. 2D cell culture platforms, while controllable and cost-effective, cannot provide cell-ECM and cell-cell interactions [19]. *In vivo* models on the other hand, provide what 2D cultures lack in physiological and clinical relevance, but are expensive, time-consuming and are not as easily controllable or reproducible [20]. 3D *in vitro* models, however, are less expensive, controllable, reproducible and provide the cell-ECM and cell-cell communication that 2D *in vitro* models lack [20]. These models can be tunable by altering stiffness properties, porosity, signaling molecules, cell types and source, as well as extracellular matrix material and structure. We previously demonstrated that our formed CH-Col hydrogels sufficiently mimicked cancerous breast tissue stiffness [18]. Thus, in this work, we aimed to increase the complexity of our 3D model by including a biomolecule present in the mammary microenvironment into the 3D CH-Col hydrogels. Specifically, leptin was included in the model to study its potential effects on metastasis of tumor cells of varying subtype and donor ethnicity. We attempted this by exposing three different cell lines (MCF-7, MDA-MB-231, and HCC1806 cells) cultured in 3D and 2D to varying levels of leptin, and then measuring their expression of common proteins related to metastasis. We first wanted to study protein expression between our 3D model and 2D controls to provide evidence that our platform induces metastatic-like behavior that is different than observed for monolayer 2D cultures. We then wanted to determine if differences existed between breast cancer cells of different subtypes or donor ethnicity in 3D culture, as this has not been explored before. Finally, we explored how the effects of leptin concentration on the behavior of tumor cells of different subtype and donor ethnicity in our 3D model platform.

Effects of 3D Culturing on Metastatic Behavior

Figures 4.2 and 4.3 show both morphology and cell viability of 3D hydrogels versus 2D. Aggregates of cells in 3D, although not complete tumoroids formed congregates with slightly less adhesion than in 2D. This is more like congregate that would develop in vivo and are most likely due to a mix between the slightly less adhesive properties of chitosan and the more adhesive properties of collagen [21]. Collagen is recognizable by several cell integrins that mediate and allow cell attachment, specifically in Type I collagen [22-24]. Based on total protein content, there appears to be less proliferation in 3D than 2D which could possibly suggest less cell viability although transmitted images in figure 4.2 would suggest otherwise. Cells seeded on the hydrogels may have proliferated slower than in 2D given exposure to a foreign environment. This is not uncommon as this behavior has been observed in other studies of *in vitro* hydrogel-based platforms [25, 26].

As shown in Figure 4.4, MCF-7 cells did not show any significant differences in protein expression between 2D and 3D models, although slight increases for cells seeded in 3D can be observed for VEGFA and MMP-9 expression. MCF-7 cell lines are an estrogen (ER) and progesterone (PR)-positive breast cancer cell line and are also minimally aggressive and non-invasive [27]. Based on their characterization, the fact that the MCF-7 cells do not exhibit increased metastatic behavior, even in the 3D environment that we have prepared to mimic cancerous conditions, is as expected.

Triple-negative cells (ER-, PR-, HER2-) are highly invasive and aggressive cancer subtypes [28]. Our triple-negative cell lines, HCC1806 and MDA-MB-231 cells, showed significant increases in VEGFA production when seeded in the 3D culturing environment (Figure 4.4 B). VEGFA is a glycoprotein that triggers the mitosis of endothelial cells and their migration.

It is the predominant factor associated with angiogenesis, which is involved in early tumor progression, sustained tumor growth and early metastasis [29, 30]. MDA-MB-231 cells also showed a significant increase IL-6 production when seeded in 3D as well (Figure 4.4 A). IL-6 is a pleiotropic cytokine involved in adipocyte signaling and the inflammatory response [31], and it has been studied as a potential prognostic factor in breast cancer due to its association with decreasing E-cadherin production (allowing for cell motility) [32, 33]. HCC1806 cells also showed a slight increase in IL-6 production, although this increase was not significant. Even without significance, the difference between HCC1806 in 3D versus 2D is still higher (0.03 ± 0.01 pg/μg difference) than it is for the MCF-7 cells (0.01 ± 0.003 pg/μg difference). That the triple-negative subtypes demonstrated increased VEGFA and IL-6 expression when cultured in 3D and the HER2+ (MCF-7) cells did not, represents what would be expected behavior *in vivo* for these specific subtypes, further suggesting physiological relevance for our 3D modeling approach.

When comparing 2D and 3D subsets, no specific trends or differences in expression were observed for MMP-2, MMP-9, TGFβ2 proteins (Figure 4.4 C, D, E). This could indicate that these proteins may not be essential to metastasis for these subtypes, or that our model does not mimic the portion of the metastatic cascade where these proteins are essential. It could also be that incubation time for the experiment was not long enough to see significant differences or that samples size was not large enough to see accurate differences in expression. Nonetheless, IL-6 production and VEGFA production provided evidence that our model mimics *in vivo* behavior between subtypes that is not observed with monolayer 2D culture.

Effects of Tumor Subtype and Ethnicity on Metastatic Behavior

We next compared metastatic potential between the Caucasian (MDA-MB-231) and African American (HCC1806) derived triple-negative cell lines, and the less aggressive (MCF-7) and more aggressive triple-negative (HCC1806 and MDA-MB-231) cell lines. Earlier onset and prevalence of more aggressive breast cancers, such as inflammatory and triple-negative subtypes, can contribute to increased mortality rates in African-American breast cancer patients [3, 4]. Despite this, there is little breast cancer research that incorporates women of various races, specifically women of color. This can be due to socioeconomic differences such as income, availability of health insurance and access to proper healthcare [34, 35]. Therefore, it is important to not only understand differences in subtype but to also consider race.

Here, VEGFA showed interesting trends between ethnicity and subtype in our model. While both triple-negative cell lines expressed significantly higher levels of VEGFA. HCC1806 cells displayed slight, but significantly lower VEGFA expression (0.689 ± 0.384 pg/μg) than MDA-MB-231 cells (1.592 ± 0.338 pg/μg). Although insignificant, TGFβ2 showed similar trends, with triple-negative cell lines MDA-MB-231 and HCC1806 expressing higher levels of TGFβ2 (0.084 ± 0.041 pg/μg and 0.060 ± 0.027 pg/μg, respectively) than MCF-7 cells (0.041 ± 0.012 pg/μg). In a previous study by Vol-Draper *et al*, drug resistance between MDA-MB-231 and HCC1806 cells was compared, where the viability of the same two cell lines against 6 different anti-cancer therapeutics was evaluated. In each study, the MDA-MB-231 cells were more resistant to therapy than HCC1806 cells [36]. More aggressive or metastatic breast cancer subtypes show an increased resistance to chemotherapy, such that, this data could suggest that HCC1806 cells are less metastatic than MDA-MB-231 cell lines, something not previously recognized in these cell types [37]. It could also be that these specific markers are not as essential in HCC1806 cells as

they are in MDA-MB-231 cells, further suggesting a need for therapeutic designs that target specific racial and receptor subtypes.

Regarding IL-6, MDA-MB-231 cells showed almost a 40-fold increase in IL-6 expression when compared to both HCC1806 and MCF-7 cells in both 2D and 3D (Figure 4.5). This result was unexpected as clinical data shows significantly higher levels of IL-6 expressed in African-American than Caucasian women [38, 39]. There was, however, a large study performed using several breast cancer cell lines directly extracted from patient tumors and identified by American Society of Clinical Oncology (ASCO), where the group noted a lack of IL-6 expression in their HCC1806 tumors [40]. Furthermore, with regards to VEGFA production, the same group performed a study showing that HCC1806 tumors created in mice showed significantly higher levels of VEGFA than cells directly from 2D cell culture [36]. It is possible that to mimic clinically observed levels for African American and Caucasian women, primary cell lines directly from clinical patient samples should be used in our model system. This could remove potential influences from long term 2D *in vitro* cell culture expansion that might affect cell behavior and could increase representation accuracy of cytokine expression. The tunability and flexibility of our modeling approach would support incorporation of patient-derived samples, further demonstrating its utility towards developing personalized therapies.

Of the matrix metalloproteinases assessed, we do not see a clear trend between the cell types with MMP-2. MMP-2 is an extracellular enzyme that degrades gelatin and type IV collagen, thus aiding cell migration and angiogenesis during breast cancer metastasis [41]. It is possible that this protein is not distinctive for breast cancer metastasis in these three evaluated subtypes or that longevity of experiments and sample size limited abilities to detect significant differences between the three. MMP-9, although insignificant, showed increased expression in HCC1806 cells when

compared to both MDA-MB-231 cells and MCF-7 cells. MMP-9 is associated with early onset metastasis as it is an enzyme that degrades collagen and gelatin in the basement membrane, the main barrier between *in situ* carcinoma and its extravasation [42]. Perhaps a larger sample size would have shown greater differences for this protein as well.

Effects of Leptin on Breast Cancer Metastatic Potential

Adipocytes comprise a large part of the breast cancer tumor environment and influence inflammation as well as breast cancer metastasis [43]. These adipocytes produce many pro-inflammatory adipokines. Once such adipokine of particular interest in this work is leptin, a hormone that plays a crucial role in metabolism, immunity and the endocrine system [15]. In a meta-analysis of 43 studies conducted on serum leptin levels and breast cancer metastasis it was shown that there was a significantly higher level of leptin expressed in breast cancer than in normal breast epithelium [44]. Based on several studies, normal levels of leptin are between approximately 10 and 20 ng/mL, and in breast cancer patients that level increases to approximately 20 to 40ng/mL [44-47]. Finally, we studied how leptin concentration effected the three breast cancer cell lines, using 0 ng/mL leptin as a control, 10 ng/mL leptin as normal levels, and 30 ng/mL leptin as high levels or metastatic levels.

For all conditions, there did not seem to be significant differences in metastatic protein expression for the MCF-7 cell line, no matter the leptin level, as expected for this cell type (Figure 4.6). No significant relationships were observed between leptin and IL-6 expression among the different cell types, although the closest significant relationship is between 0 and 10 ng/mL leptin for MDA-MB-231 cells (Figure 4.6 A). There appears to be a slight increase between normal leptin levels (10 ng/mL) and high leptin levels (30 ng/mL) for both MDA-MB-231 and HCC1806 cells.

Because of their insignificance, however, we cannot make any clear conclusions. We would expect, however, there to be a higher IL-6 expression with increasing leptin concentration as leptin has been known to induce IL-6 production within the breast cancer microenvironment [48].

The most prominent significant relationship is with VEGFA expression (Figure 4.6 B). Not only does VEGFA promote angiogenesis in breast cancer metastasis, but it does so in wound healing and inflammation as well, linking breast cancer and inflammation [49]. Interestingly, we see a significant increase in VEGFA production as leptin concentration is increased, as well a slight insignificant decrease in VEGFA production for HCC1806 cells. This could suggest that leptin influences these two cell lines inversely, but a definitive conclusion cannot be reached with current data.

Leptin concentration does not appear to significantly effect MMP-2 or MMP-9 production between any of the cell types, but there is a slight decreasing trend in MDA-MB-231 cell TGFβ2 expression as leptin concentration increases. TGFβ2 is actually associated with breast cancer inhibition in early stages, but in later stages it is associated with malignant progression [50]. Upon inactivation of tumor suppressor genes and retrieval of oncogenic mutations, TGFβ2 is thwarted towards behaviors that increase cell motility, invasion and metastasis [51]. Given this trend, however, it is possible that TGFβ2 is more inhibitory in this model than metastatic, which would be supported by its reduced production and leptin levels increase. Despite this possibility, these findings are statistically insignificant and limited by their sample size (n=3). Therefore, further studies are needed before these trends are confirmed.

4.6 Conclusion

In this work, we used a previously characterized 3D hydrogel platform to study metastatic protein expression between cells of different ethnicities and subtypes. Furthermore, we studied how an inflammatory adipokine, leptin, influences these expressions. We showed how our hydrogel-based model mimics *in vivo*-like behavior specific to each tumor subtype, affirmed for MCF-7 cells (less aggressive subtype) by lower protein expression in 3D culture, and for triple-negative cells (more aggressive subtypes), significant increases in IL-6 for MDA-MB-231 cells and significant increases in VEGFA for both MDA-MB-231 and HCC1806 cells cultured in 3D (Figure 4.4). Our work also exhibited differences based on cell donor ethnicity, with significant increases in MDA-MB-231 IL-6 expression compared to the other two cell types, and significant differences in VEGFA production between all three cell types (Figure 4.5). Finally, our model presented a significant increase in VEGFA production in MDA-MB-231 cells as leptin concentration increased, with a slight (insignificant) decrease in HCC1806 VEGFA expression as leptin increased (Figure 4.6). These findings suggest that metastatic phenotypic behaviors can vary for different ethnicities and subtypes, including their responses to inflammatory cytokines, such as leptin. Due to limited samples size and possibly a need for patient-sourced cell lines, we cannot confidently confirm these findings. Future work, however, will focus on use of increased sample sizes, acquisition of patient-derived cell sources, and evaluation of additional inflammatory adipokines to study this relationship further. As these studies progress, we aim to incorporate more varieties of donors and eventually be able to use this data to create a statistical predictive model of breast cancer metastasis that physicians can use to create personalized treatment plans by simply taking a panel of the patient's inflammatory adipokine levels.

4.7 References

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CHAPTER 5

SUMMARY AND RECOMMENDATIONS

In this work, we initially fabricated a tunable chitosan-collagen hydrogel that can mimic breast cancer tissue stiffness (at 52 ± 5 kPa complex elastic modulus) and extracellular glycosaminoglycan behavior (chitosan), while incorporating natural extracellular matrix protein (ECM, addition of collagen). While cell viability studies showed reduced proliferation for our model when compared to 2D controls, Live/Dead^{TM} studies showed clear viability as well as tumoroid formation on our hydrogels, exhibiting *in vivo*-like breast cancer morphology. Given that the greatest difference in MMP-2 (a collagen degradation enzyme linked to metastasis) expression, between the low-invasive cell line (MCF-7) and the highly-invasive cell line (MDA-MB-231) was showcased on the 3.5% chitosan-collagen hydrogels, that platform was used for the subsequent studies. In the final evaluation of the model, we saw that the model was able to mimic *in vivo*-like behaviors for both African American (HCC1806) and Caucasian (MDA-MB-231) donor derived triple negative cell lines as well as a Caucasian-derived HER2- (MCF-7) subtype. This was seen with no significant increases in metastatic protein expression in 2D or 3D for MCF-7 cell lines and a significant increase in Vascular Endothelial Growth Factor α (VEGFA) for triple negative cell lines in 3D. We were also able to detect differences in metastatic potential between the cells from different ethnicities with a 40-fold increase in Interleukin-6 (IL6) expression for MDA-MB-231 cells versus HCC1806 cells. Based on these analyses we were able to show that our model can

mimic metastatic behavior of three different cell lines and be used to study metastasis of different subtypes and ethnicities, a subject that has yet to be discussed in 3D modeling literature.

We then applied this model to studying how a pro-inflammatory adipokine, leptin, influences metastasis behavior of different receptor and racial subtypes. With a larger subset of data, we were able to see similar trends in metastatic cytokine expression between 2D controls and 3D hydrogels and between the three cell lines (MCF-7, HCC1806 and MDA-MB-231) as we described earlier. In our leptin studies, we see a significant positive relationship between leptin concentration and MDA-MB-231 VEGFA expression and a slight, insignificant, negative relationship between leptin and HCC1806 VEGFA production. This could suggest the leptin effects the African American and Caucasian-derived cell lines inversely. This proof-of-concept study, however, had a very small sample size (n=3). Therefore, while interesting, confident conclusions cannot be made at this time.

Based on the current work, it is recommended that these studies, specifically those including leptin, be repeated with a much larger sample size and with additional indicators of metastasis, such as other cytokines, wound healing assays, or Transwell Migration Assays, incorporated. It may also bode well to increase the difference in leptin concentrations by keeping the 10 ng/mL leptin as the normal level, but increasing the higher level to 40 ng/mL leptin. This may allow better detection of trends or determination of significant relationships. One interesting observation in our work was the very large difference in IL-6 production, with MDA-MB-231 cells showing 40-fold increases over HCC1806 cells. Clinical evidence would suggest quite the opposite. It is therefore recommended that these studies be repeated using patient-derived cell lines rather than commercially available cell lines, which could eliminate potential influences from long term two-dimensional *in vitro* cell culture that might affect cell behavior and could increase

representation accuracy of cytokine expression. Finally, expanding both the number of different cell lines of various ethnicities and subtypes could provide an interesting platform. By also incorporating other pro-inflammatory adipokines, such as tumor necrosis factor, adiponectin, and C-C motif chemokine ligand 2 (CCL2) in these studies, creates the potential for designing a statistical model that could predict breast cancer metastasis. This could allow physicians to predict metastasis likelihood in a breast cancer patient, potentially changing the course of a patient's treatment plan. This was the original idea going into our fourth chapter, which is discussed further in Appendix A. Using these recommendations based on the evidence provided in this work, researchers could potentially use this tunable chitosan-based platform to mimic specific aspects of the breast cancer microenvironment and create therapeutics and treatment plans personalized to specific patient ethnicity and receptor subtypes. This can narrow the gap in racial mortality rates and create better outcomes for a larger breast cancer patient population.

APPENDIX A

STATISTICAL MODELS OF BREAST CANCER METASTASIS

The original purpose of our work in Chapter 4 was to study the effects of serum level pro-inflammatory cytokines on several breast cancer cell lines using a platform that accurately mimics breast cancer behavior *in vivo*. The concept behind this was that via large data inputs collected using our 3D platform, we would be able to create an accurate statistical model that predicted the likelihood of breast cancer metastasis (BCM) based on how powerful each adipokine is in influencing behaviors of known cancer cell lines. In the long term, physicians could collect a breast cancer patient's blood panel that measures serum adipokine levels, input this data into our model and a prediction on the likelihood of BCM would be outputted. This would help to fill in a diagnostic gap seen in breast cancer clinics that prevents researchers from being able to create effective and preventative treatment plans, a topic we will discuss in this appendix.

Over the last few decades, researchers have developed novel imaging techniques such as Positron Emission Topography (PET), Nuclear Magnetic Resonance (MR) and Computed Tomography (CT) to diagnose both breast cancer and distant metastases [1]. These techniques, however, can only be used to detect metastasis that is already occurring, can produce false positives, and can be invasive [1]. This calls for a better method that not only detects BCM before it occurs, but that is also cheaper, more accurate and less invasive.

Currently, research is trying to push forward by using serum markers to detect BCM [1, 2]. Several researchers have noted certain biomarkers correlating to BCM using clinical data [1-5],

but there are few researchers that have taken these correlations a step further. That is, using these markers to predict BCM. To take these correlations and then design statistical models surrounding them would allow us to predict whether a breast cancer patient is at risk for metastasis. Furthermore, as these models are refined, we can then use it to predict metastasis site locations such as bone, liver, lung or brain. Using this information, we can personalize the therapy and treatment in prevention of specific metastases.

There are very few statistical models being used as predictors for BCM, but the most prominent model to date was created by Klimov *et al*. This research group collected clinical data on 322 women with Triple Negative Breast Cancer and screened 133 biomarkers specific for breast cancer metastasis to the bone, liver, lung and brain [6]. Using student t-tests and cox univariate analysis, the group created a model to predict location and risk of metastases. While this model is a respectable and well-designed analysis, the use of clinical data required the group to take into consideration several factors including age, chemotherapy status and tumor size and so metastasis could not be predicted by biomarkers alone. Another group, Delpech *et al*, used similar t-tests and cox analyses to predict bone-specific metastases from clinical data [7]. This group, however, used several factors in their model including CT scans, mammograms, PET scans, biomarkers and age in their data set rather than using a single blood test. While these statistical models provide incredible milestones in predicting BCM there are few statistical studies done because collecting clinical data can take years [8]. For instance, it took Klimov *et al* 9 years before they began creating their model and Delpech and colleagues had to log through years of data retrospectively. Tseng *et al*, developed a model that can predict metastasis three months in advance, but requires both serum markers and clinicopathological data and its tested accuracy is based on a relatively small study population (69 metastatic and 136 nonmetastatic patients) in Taiwan that took 13 years to acquire

[9]. There is also less control over variables when using clinical data because of the large possibility of human error in data collection and the numerous biological factors in cancer expression and behavior that can differ from patient to patient. While no model is perfect, the time it takes for data collection and the ability to control experimental data could be improved by moving data collection from *in vivo* to *in vitro*.

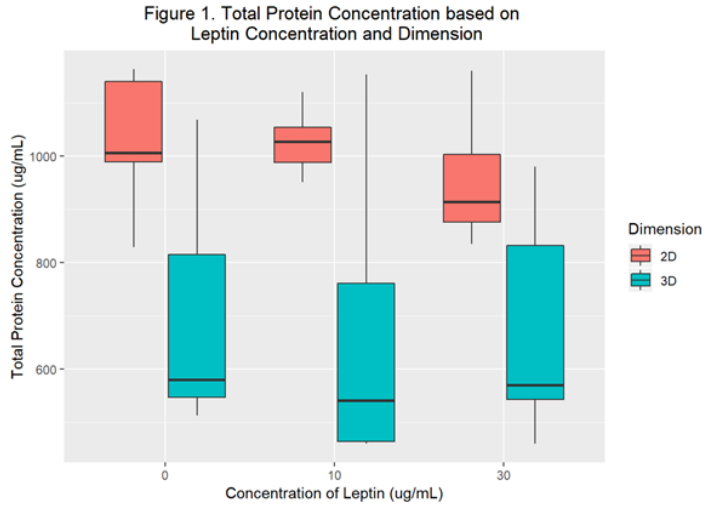
In the past, using *in vitro* data to study BCM allowed for simple biochemical analysis but lacked clinical relevance as cells behave differently in two-dimensional tissue cultures than they do *in vivo* [8] . With the onset of tissue engineering, however, scientists have been creating three dimensional models that mimic the breast cancer microenvironment, making these models mimic more closely BCM *in vivo* behavior We believe that by using these three-dimensional models, we can gather accurate data to fit a statistical model in a more controlled environment than the human body. The data collected from these models can be collected faster and cheaper than if we were to use human subjects.

When we set out to do this project, we aimed to create a statistical model based on data collected from our three-dimensional *in vitro* BCM models outlined throughout this work. Preliminary data showed that we could mimic the physiological stiffness of breast tissue using chitosan hydrogels and has shown evidence that breast cancer cells behave according to what we would expect *in vivo*. We believed that by using more aggressive metastatic breast cancer cell lines of different ethnicities (MDA-MB-231 and HCC1806) and comparing them to non-metastatic breast cancer cell lines (MCF-7), we could see correlations between biomarker concentration exposure and their behavior. We then wanted to test their metastatic behavior (to confirm that they behave the way we would expect *in vivo*) by running various metastatic analyses including wound healing assays, Boyden chamber assays, ELISA protein assays and RT-PCR on metastatic gene

expression. These analyses would then help us to first verify the expected behavior of the cell lines and furthermore classify likelihood of metastatic behavior. We would then use biomarker data to build our model predicting which combination of factors influences metastasis the most.

From initial data collection, we would build our model based on observational statistics as well as simple ANOVA and t-test statistics that provide a basis on relationships we see between cell-type (MCF-7, MDA-MB-231, HCC1806), biomarkers (Leptin, CCL2, CXCL5, etc.), and metastasis (wound healing assay data, Boyden Chamber assay data, metastatic chemokine protein and RNA expression). All statistics including our statistical model would be engineered on a program called R (The R Foundation©). R is a free programming software and language that allows for users to develop anything from basic statistical testing to advanced statistical algorithms and data analysis. An example of coding for descriptive statistics in R can be seen in figure A2. Using R, we wanted to build a cumulative logistic model which estimates the relationship between a valued and ordered categorical dependent variable and a set of independent variables [10]. In our case, we could evaluate the most important biomarker predictions and define their relationship to how likely a patient will have metastasis. The potential issues with designing this model would be that value must be assigned to each of our metastasis indicators as to how much those indicators contribute to a 'degree of metastatic potential'. To avoid false modelling, accurate and in-depth evidence and support for the values given for each degree must be provided and widely accepted by the scientific community. Another potential issue in designing the model would be the possibility that initial statistics may show that individual biomarkers alone do not have a significant effect on metastasis. This pitfall could be circumvented by also doing *in vitro* studies based on biomarker composites as literature has shown that some markers effect metastasis directly, while others effect metastasis only in their relationship to other markers.

```
library(ggplot2)
ggplot(BCData, aes(factor(Leptin), TotalProtein)) + geom_boxplot(aes(fill =Dimension))+ggtitle(
"Figure 1. Total Protein Concentration based on \n Leptin Concentration and Dimension")+xlab("Co
ncentration of Leptin (ug/mL)")+ylab("Total Protein Concentration (ug/mL)")
```




```
ggplot(BCData, aes(factor(Leptin), TotalProtein)) + geom_boxplot(aes(fill =CellType))+ggtitle("F
igure 2. Total Protein Concentration based on \n Leptin Concentration and Cell Type")+xlab("Conc
entration of Leptin (ug/mL)")+ylab("Total Protein Concentration (ug/mL)")
```

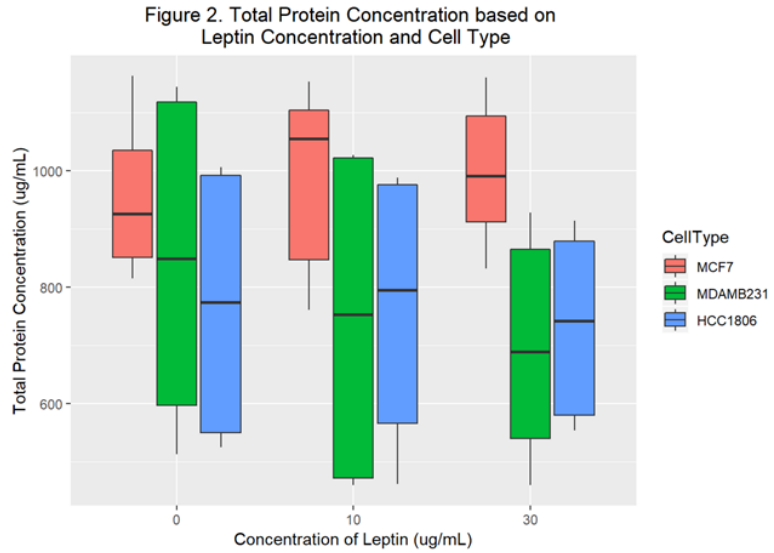


Figure A1. Example of R code and descriptive boxplots for statistical modelling. Boxplots and code used to determine relationships between total protein concentration and dimension (Figure 1) or cell type (Figure 2).

Prior to beginning this project, we had already conducted a simple multiple linear regression model predicting the percentage of chitosan we would need to create to get a desired elastic modulus that is similar in strength to breast tissue (Figure A1). The difference between this model and our desired cumulative logistic regression model being that the output value is not user

assigned and the input variables are based on raw data rather than evaluated data [11]. The statistical analysis going into creating this model, however, is much like the process we would perform using our metastatic data.

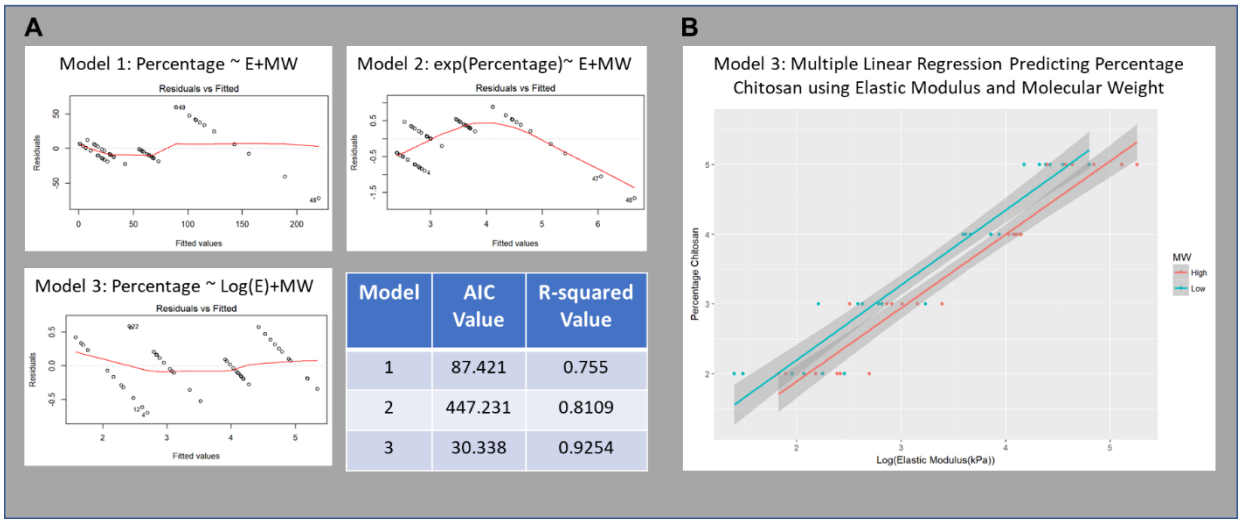


Figure A2. Evaluating and determining the best model to predict the percentage chitosan to create. (A) Residual plots as well as AIC and R-squared values determine the best model to choose. Visual fit, low AIC and high R-squared values guided choosing (B) model three as the best predictive fit.

The data in the model presented in Figure A1 includes 6 data points within each categorical subset. We tested both high and low molecular weight chitosan and 2% through 5% solutions for each weight. Analysis begins with descriptive statistics such as boxplots, dot plots, bar graphs and histograms as well as testing for normality (not shown). ANOVA and Tukey multiple comparisons showed significant differences in elastic modulus between each percentage of chitosan (except between the 2% and 3% solutions) as well as between the high molecular and low molecular weight solutions.

From this data we built three models and evaluated each of them. Figure A1(A) shows the residual plots of each of these models. The curved pattern detected in the first and second models shows a non-linear relationship, making model 3 the most linear fit. Figure A1(A) also shows the R-squared and Akaike's Information Criterion (AIC) values for each of the three models. R-

squared values determine how well the data fit the model and how well the model can predict future relationships. AIC values take both the fit of the data and the number of parameters into account (the simpler the model, the better) [12]. A higher R-squared and a lower AIC value indicates a better model which is which we chose model 3, graphically seen in Figure A1(B). If we were to build a cumulative regression model, the same evaluation and parameters would be set to determine the best fit model.

Based on the timeframe of this work, we knew we would not be able to fully study the sample size and adipokine profile needed to create a substantially effective statistical model. We did, however, aim to set a basis for which future work could be built upon in order to create this predictive model. For instance, in our work we have shown that it is possible that leptin has a positive relationship to the metastatic behavior of MDA-MB-231 and even possibly a negative relationship with HCC1806 cells based on VEGFA expression. We also show that the specific matrix metalloproteinases studied (MMP-2 and MMP-9) may not be effective enough markers to continue with in the future. Based on the current work, we believe there is enough evidence to continue this project and are hopeful of discovering significant relationships that can be used in creating this statistical model in the future.

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