Development of an *In Vitro* Three-dimensional Breast Tissue Model to Decipher the Effects of Stromal Heterogeneity on Normal and Cancerous Mammary Epithelial Cells

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DEVELOPMENT OF AN *IN VITRO* THREE-DIMENSIONAL BREAST TISSUE MODEL TO DECIPHER THE EFFECTS OF STROMAL HETEROGENEITY ON NORMAL AND CANCEROUS MAMMARY EPITHELIAL CELLS

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Presented to
the Graduate School of
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of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
Erin Jennifer McCave
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Accepted by:
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ABSTRACT

Breast cancer research aims to develop a detailed understanding of the underlying mechanisms of disease progression and to generate approaches to improve early detection, monitoring, and treatment of breast cancerous lesions. The success of current standardized breast cancer therapies varies as each breast cancer patient and tumor is different and unique whereas the standard of care is based on specific breast cancer patient cohorts. To further current clinical success, the characteristics of each tumor mass must be considered. Such an individualized or personalized approach is incompatible with current understanding of “average” breast cancer tumors and responses to treatments. Thus, the long-term objective of the present research is to develop modular breast tissue models to (1) further our understanding of individual breast cancer tumors and (2) monitor and develop customized treatment plans, thus contributing to breast tissue and breast cancer research.

The main goal of this project was to develop a three-dimensional (3D) breast tissue in vitro test system using tissue-engineering concepts. The model is uniquely different from existing models in that it accounted for extracellular matrix (ECM) heterogeneity through use of an ECM hydrogel embedded with polylactide beads. Furthermore, the 3D model was used to specifically investigate the effect of heterogeneity of the mammary microenvironment on normal human mammary epithelial cells (MCF10A) and human breast cancer cells (MCF7). Specifically, (1) polylactide beads with various physicochemical features were produced and characterized, (2) an
ECM hydrogel representing the stromal component was evaluated and selected, and (3) a 3D tissue engineered composite system with and without polylactide beads containing either MCF10A or MCF7 cells was used to investigate the effects of microenvironment heterogeneity. Development of a benchmark 3D breast tissue model, where cellular interaction can be studied in an environment that is more representative of the native tissue, helps researchers better understand cell reactions and behaviors in breast cancer. This model allows the rapid assessment of therapies as well as controlled studies of basic breast cancer processes and mechanisms. The outcome of this research was the generation of a 3D in vitro breast tissue model that better mimics specific influences of the ECM in breast tissue and breast cancer progression.

While the scientific merit of the proposed work was to advance the understanding of breast tissue development and early breast cancer stages, the goal was to reach outside the breast cancer community to share the work and progress. Further, the second objective of this research was to reach out to young scientists and engineers through an undergraduate introductory research program, highlighting interdisciplinary approaches in scientific endeavors. This initiative broadened the intellectual merit of the project and introduced ideas related to breast cancer research in other related fields of research, thereby generating additional in-depth research opportunities and advancements in the field of breast cancer research.
DEDICATION

This work is dedicated to my parents, DeWayne and Janice, my sister Megan, and to my grandmother Bernice Davidson. You were my rock throughout the process of completing my degree and I never would have been able to do it without your love, support, and encouragement. To my dearest friends Amy, Christy, Adam, Betsy, Laura, and Courtney: Thanks for keeping me on my toes and balanced throughout the process. And lastly, thanks to my church family for the unconditional love and encouragement you have shown me along the way.
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PREFACE

Breast cancer is the most prevalent malignant disease of women in the developed world, apart from skin cancer, with approximately 1 in 8 women in the United States being diagnosed with breast cancer during their lifetime [1]. Breast cancer begins when normal mammary epithelial cells grow, divide and form a cell mass, which then invades surrounding tissue and eventually leads to metastasis. Much research using breast tissue models has been conducted to understand how cells become cancerous in the mammary gland, propagate, and eventually metastasize in the body. While this research has provided a better understanding of the underlying mechanisms associated with normal mammary gland function and breast cancer, our understanding of the extracellular matrix (ECM)-cell interactions during normal breast tissue and malignant development remains unclear. Model systems currently in use include clinical and in vivo models, two-dimensional (2D) in vitro models, and three-dimensional (3D) in vitro culture models. In recent years, 3D model systems have been developed for numerous normal tissues and pathologic conditions. Ranging from the use of tissue tumor explants to cell lines in homo or heterotypic cultures, 3D in vitro culture systems provide information on the role of mesenchymal cells, the matrix composition, and density in the formation of acinus- and duct-like structures. However, the need for reliable, versatile, and reproducible 3D in vitro model systems that allow the modulation of the ECM properties remains. Therefore, 3D in vitro mammary culture may provide further understanding of breast tissue growth and malignant development. Furthermore, standardization of such culture systems may generate a reliable and reproducible model to test and monitor breast cancer treatments.
To understand how this disease develops and how normal mammary epithelial cells transform into cancerous cells, the development of a modular breast tissue model system was used to study the cellular interactions and the mammary microenvironment.

This research focuses on the development of a new 3D heterogeneous \textit{in vitro} breast tissue model. The model was engineered as a complex tissue, with normal and cancerous mammary epithelial cells embedded within a 3D matrix. Acinus- and duct-like mammary structure formation within the 3D system was assessed to determine how ECM heterogeneity affects structure formation. The matrix structure \textit{in vitro} was controlled using combinations of natural and synthetic biomaterial substrates to mimic the heterogeneous stiffness of the normal mammary tissue. In these conditions, the behavior of the normal and cancerous mammary epithelial cells in response to heterogeneity changes was monitored. The ability to control the phenotypic changes of normal and cancerous mammary epithelial cells provided a means for improved understanding of \textit{in vivo} conditions and the effect heterogeneity plays during breast formation. As the cell-cell and cell-ECM interactions [2] play a large part in breast formation and mammary tumor progression, developing a benchmark 3D breast cancer model to study these interactions provides a new research tool to further our understanding of breast development and mammary malignant growth. Such a model may also be relevant in clinical diagnostic, therapeutic, and monitoring applications. This model is not meant to replace existing models, but to radically enhance our understanding of the normal breast and breast cancer microenvironment, thereby allowing development of more efficient tools for diagnosing and treating breast cancer.
The next chapters cover the research conducted, which includes two components: development of a 3D *in vitro* breast tissue model and understanding how involvement of undergraduate students in Science, Technology, Engineering, and Mathematics (STEM) disciplines in research helps them develop a research identity. The overall research objective is to establish a new 3D breast tissue model composed of polylactide (PL) beads embedded in a hydrogel and to determine behavior of normal and cancerous mammary epithelial cells in the presence of increased microenvironment heterogeneity. Chapter 2 discusses polylactide bead fabrication and characterized. In Chapter 3, the hydrogel matrix material, representative of the stromal component of the breast, for the 3D *in vitro* breast tissue model was determined. The stromal component was selected based on cell viability and acinar-like structure formation. Portions of results in Chapter 3 were generated by an Institute for Biological Interfaces of Engineering interdisciplinary team, including Clemson University undergraduate student Devleena Kole, and were presented at the 2013 Annual Meeting and Exposition of the Society For Biomaterials: McCave EJ, Kole D, Burg KJL: Development of a Heterogeneous In Vitro Three-Dimensional Breast Tissue Model. In: Society For Biomaterials 2014 Annual Meeting & Exposition: 2014 (Denver, CO; 2014) [3]. The laboratory research concluded in Chapter 4 where the composite 3D model system was generated and evaluated to determine effects of heterogeneity on cells. Mechanical properties of the 3D breast tissue model, composed of hydrogel matrix material alone or hydrogel containing PL beads, were characterized. Further, normal human mammary epithelial (MCF10A) or human breast cancer (MCF7) cells were added to the model system and changes in cell viability,
proliferation, protein expression, and function determined. This 3D model will enhance understanding of the role of mammary microenvironment in the function of normal mammary epithelial cells and in early stages of mammary cancer progression.

The educational portion of this project, Chapter 5, focuses on research identity development through participation in research programs. Minority undergraduate students from Clemson University and the University of North Carolina Charlotte participated in one semester of the National Science Foundation: Emerging Frontiers in Research and Innovation (NSF:EFRI) – Research Experience and Mentoring (REM) program. Students were introduced to research focused on developing different 3D culture methods for normal and breast cancer research. The REM program was students’ first exposure to research and focuses on developing necessary skills for students to succeed in future research within their own majors. Students were paired with a mentor on the mentor’s project. Beyond the laboratory skills gained through the experience, different professional development topics, such as “introduction to research”, “how to conduct research”, “how to read scientific articles”, “how to produce a standard operating procedure”, and “exploration of career paths”, were discussed with the students. A joint 10-week summer Research Experience for Undergraduates (REU) was offered to four of the participating REM students and helped the students further develop their research identity by building their research skills and providing them with the opportunity to develop an independent research project. Results in Chapter 5 were generated by an Institute for Biological Interfaces of Engineering interdisciplinary team and co-written with Clemson University doctoral student Jordon Gilmore, and were presented at the
CHAPTER ONE


LITERATURE REVIEW

Anatomy of the Human Breast

The human breast is a complex tissue composed of a glandular structure (the mammary gland) located on top of the pectoral muscles of the chest [7], consisting of a nipple, lobes, and ducts, surrounded by stromal tissue, fibrous and fatty tissue. The mammary gland is a dynamic structural tissue that changes with stages in life, beginning with the formation of the lobule bud structure during neonatal development, continuing with the development of alveoli or acini during adolescent puberty, then full lobulo-alveolar development with child birth, followed by involution at the end of lactation [8]. Each breast contains 15-20 lobes of glandular tissue [7, 9] that branch into smaller lobules which are subdivided into the secretory alveoli for milk production (Figure 1.1). Each lobule has an excretory ductal system that converges to form the lactiferous ducts which exit through the nipple; just behind the nipple the lactiferous ducts widen to create reservoirs called lactiferous sinuses [7]. The stroma, i.e. the fatty and connective tissue, surrounds the lobes of the glandular tissue and is connected to the chest wall by Cooper’s ligaments which help shape the breast [7]. Lymph nodes, responsible for draining the lymph fluids from the breast, are present in five major areas surrounding the breasts, and play an important role in fighting infection by removal of bacteria, other disease-causing
organisms, and abnormal cells [9, 10]. The breast contains very little muscle and is mainly composed of stroma, which defines the shape and size of the breast [7, 9, 11].

![Diagram of breast anatomy]

**Figure 1.1** A schematic of the anatomy of the female breast. The glandular structure is surrounded by fatty connective tissue. The enlargement demonstrates the lobule structure and the normal excretory ductal structure [12].

**Ductal Tissue and Structure**

The glandular ducts consist of luminal epithelial cells associated with myoepithelial cells and surrounded by a basement membrane (BM) that connects the glandular tissue to the stroma of the breast [13]. In early mammary gland formation *in utero*, the mammary placodes formed in the ventral skin of the embryo through epithelial-mesenchyme interactions [14] create the rudimentary ductal structure of the mammary
gland. The functional unit of the gland, the acinar shaped ducts [15, 16], are lined with a single layer of luminal epithelial cells which are associated with a layer of alveolar myo-epithelial cells, which are stellate shaped and form a basket-like structure around the acinus [17]. Although approximately 20% of the luminal epithelial cells do directly contact the BM, the remainder are adjacent to myo-epithelial cells [18]. Polarity is a fundamental property of epithelium, allowing the surfaces of the cells and tissues to divide into apical and baso-lateral areas, where cell-cell contact and cell-extracellular matrix (ECM) interactions determine the asymmetric architecture and polarity, resulting in directionality of protein localization and appropriate function of the organ during lactation [19].

During adolescent puberty, the distal ends of the rudimentary ducts enlarge to form club shaped lobular structures, called terminal end buds (TEB), which contain highly proliferative cells [14]. The TEB (Figure 1.2) have a basally located monolayer of cap cells at their tip that move to the proximal part of the duct and differentiate into myo-epithelial cells [17], which act similarly to smooth muscle cells and aid in contraction during lactation [15, 20, 21]. Breast epithelial stem cells, found throughout the ductal structures, are thought to be responsible for continuous cell renewal, growth, and branching throughout the reproductive period, as well as the massive epithelial expansion seen during pregnancy [21]. As the ductal tree forms, repeated dichotomous branching proceeds, creating the tubes that deliver milk to the nipple, while lateral buds develop along mature ducts and are constrained by the lack of open territory of the surrounding stroma [22]. Clusters of lobular alveoli and the ducts that drain them form into units
called lobules [15], which continue to develop with new budding of structures with each ovulatory cycle [23].

Figure 1.2 Cross-section through end bud with accompanying diagram. End buds are bilayered structures; an outer layer of myo-epithelial progenitor cells (cap cells) overlays a multilayered mass of luminal cells fated to form the walls of the ductal lumen (L). Stained with hematoxylin and eosin. Original magnification approx. ×300 [22].

The breast tissue attains its maximum development during pregnancy and is characterized by proliferation of the ductal tree and further development of the existing lobules as a result of increased cell number due to cell division and in cell size due to cytoplasmic enlargement. The second half of pregnancy is characterized by progressive branching with less bud formation and the formation of true secretory acini (differentiated structures) [23]. Even with creation of the lobulo-alveolar structures during gestation, the onset of milk secretion by the alveolar cells does not occur without progressive biochemical and structural differentiation [15]; thus, not all lobulo-alveolar structures produce the same amount of milk during lactation.
**Stromal Tissue**

The stroma, composed of the fibrous connective tissue and adipose (fatty) tissue, comprises approximately 80% of the resting breast volume and is composed of fibroblasts, epithelial cells, adipocytes (fat cells), blood vessels, inflammatory cells, nerve cells, and a macromolecular network of proteoglycans and glycoproteins, such as laminin, fibronectin, elastin and collagen [13, 24], which contribute to the extracellular matrix (ECM) [11]. The stroma is composed of two types of ECM. The first is the basement membrane (BM), which consists primarily of collagen IV, laminins, entactin/nidogen, and heparan sulfate proteoglycans; the second is the interstitial matrix, which consists primarily of collagen I and III and fibronectin, which contribute to the mechanical strength of the tissue [25]. The stroma is thought to be critical in the characteristic shaping of the branching structure of the mammary gland [8] through localized activity of transforming growth factor beta (TGFβ). In the branching area of the ductal structure, TGFβ is absent, hence its inhibitory effects on epithelial cell proliferation and production of ECM-degrading enzymes do not occur, which allows the basement membrane to undergo remodeling and promoting cell proliferation and branching morphogenesis [24]. As the gland continues to develop, the area occupied by the epithelium increases at the expense of the surrounding stromal tissue, which does not necessarily mean the loss of stromal cell numbers, but rather a rearrangement of the existing stromal cells and tissue elements [15]. The mature mammary fat pad consists primarily of adipocytes coupled with developing epithelium that is encased in fibrous connective tissue, resulting in the specialized interlobular and intralobular stroma of the
mature gland [8]. The BM and stroma influence the apoptotic (programmed cell death) behavior of the epithelial cells during involution and during menopause, by producing enzymes that degrade and rearrange the BM of the mammary gland resulting in a pre-pregnancy-like glandular structure [8, 15, 23, 26].

**Breast Development**

The development of the breast correlates to changes in size, shape, and function which are associated with the stages of infantile growth, puberty, pregnancy, lactation, and menopause [23].

**Newborn**

Female children are born with small breasts consisting of a nipple and an undeveloped system of ducts which exit at the nipple [9]. Mammary gland parenchyma arises from a single epithelial ectodermal bud [26] where the mammary placodes, which are surrounded by a primary mesenchyme, give rise to the mammary nipple and the underlying ductal tree [8]. The placodes elongate, then sprout into the underlying stroma of preadipocytes (composing the mammary fat pad) to form the rudimentary ductal system [8, 14]. The breast of a newborn is composed of 6-10 straight ductal structures opening into the nipple; the ducts open into primitive lobules in the mammary fat pad [26]. The mammary fat pad and ductal structure continue to grow isometrically, keeping pace with the growth of the child until puberty [20, 26].
**Puberty**

With the onset of puberty, the mammary ductal development accelerates, showing growth in both the glandular tissue as well as the surrounding stroma [26]. The reproductive hormones which aid in the glandular ductal structure formation are estrogen, which promotes the growth of the gland and ducts, and progesterone, which stimulates the development of the milk-producing cells [7]. The glandular tissue increases with the growth and division of the ductal system through the TEB [8, 14, 17], or alveolar buds, which advance through and to the edge of the fat pad by use of cap cells, i.e. myo-epithelial cell precursors, which have the ability to clear a “path” through the stromal tissue in order for the ducts to advance forward, by channeling the internal force of dividing cells in a forward direction [22]. The ducts dichotomously divide as they progress through the fat pad, leaving a full ductal tree system that continues to develop with each menstrual cycle, until about the 35th year of age [26].

**Pregnancy**

During pregnancy, the breast attains its maximum development through two distinct phases: an early phase of growth, characterized by proliferation of the distal elements of the ductal tree that results in formation of new branches and new ductules, and a late phase of lobular differentiation, which occurs in the last half of the pregnancy where the lobules formed enlarge and increase in number in preparation of lactation [26].
**Lactation**

Lactation starts after post-partum withdrawal of placental lactogen and sex steroids, which appear to prevent the action of prolactin on the mammary epithelium [26]. Prolactin, released from the anterior pituitary, stimulates milk production while oxytocin, released from the posterior pituitary in response to suckling, causes milk ejection from the lactating breast [7]. During lactation, no morphological changes occur to the mammary gland, which consists of enlarged lobules and acini with dilated lumina [26]. The varied size of the lobules throughout the gland suggests varied activity throughout [26]. Milk is synthesized and stored in the lactiferous reservoirs until it is needed, although if it is stored for longer than 48 hours, milk production and secretion begins to decline [26]. Cessation of lactation following weaning is accompanied by massive apoptosis and tissue remodeling, and the gland reverts to a structure resembling that before pregnancy [15, 20].

**Menopause**

When a woman reaches menopause, usually in her 50’s, breasts undergo changes related to the loss of the reproductive hormones, estrogen and progesterone, causing the firm breast tissue to shrink and the amount of fat (adipose) tissue to increase [9]. The breasts usually become larger and the connective tissue begins to lose strength, causing the breasts to sag [10]. At this stage, it is easier for radiologists to detect breast cancer because abnormalities are not hidden in dense breast tissue [10].
Breast Cancer

Breast cancer is the second most prevalent malignant disease of women in the developed world with approximately 1 in 8 women in the United States being diagnosed with breast cancer at some point in their lives [1]. The National Cancer Institute estimated in 2010 based on NCI’s SEER Cancer Statistics Review that 207,090 females and 1,970 males in the United States would be newly diagnosed with breast cancer in 2010, while 39,840 and 390 deaths would occur in 2010 [27]. The development of the breast and understanding of ductal growth is necessary because 90% or more of mammary cancers are ductal in origin [22]. Breast tumors are caused by both genetic alterations of the normal mammary epithelial cells and epigenetic factors, such as interactions with the stromal tissue microenvironment, which play into the initiation, progression, and metastasis of the disease [28-31].

All normal cells incur many continuous mutations over their lifetime (normal rate of mutations); almost all of these mutations will have no functional effect on the cell; however, tumor (cancer) cells are usually formed when “two hits” occur somatically in the same gene [32]. Cancer stem cells have been identified, from a subset of cancer cells, as key components in solid tumor growth as these cells are self-renewing cells responsible for maintaining cancer growth and producing differentiated progeny that form the bulk of the tumor [33]. However, the search for specific genetic causes of breast cancer has elucidated important clues to how cells regulate and turn off specific genes to promote proliferation, apoptosis, differentiation, adhesion, and maintain genomic
stability, which are relevant to the initiation, progression, and potential metastasis of tumors [34].

The stromal tissue microenvironment, a source of epigenetic factors, is instrumental in tumor initiation and progression [25, 28, 30, 31, 35-37]. Changes in the microenvironment, such as ECM remodeling, angiogenesis, lymphatic infiltration, and an increase in the number of fibroblasts and myo-fibroblasts have been shown to influence tumor progression [28], while cellular signaling proteins such as cytokines, growth factors, and proteases secreted from the various cell types in the stroma affect tumor invasion and later metastasis of breast cancer primary tumors [35]. Cellular behaviors are regulated by complex molecular interactions that involve both positive and negative feedback loops, as well as high levels of cellular cross-talk [30].

The breast changes with age and reproductive history; these changes are reflected at the phenotypic level and at the genomic level [23]. This dynamic state provides a need to further study the complexity of breast tissue and the cellular interactions that occur during development as well as cancer initiation and progression and use this knowledge to develop strategies for cancer prevention and cure.

**Cell, Tissue, and Organ Culture**

Cell and tissue culture began back in the 19th century with the works of Ringer and Roux [38], while in 1907 Ross Harrison began by culturing frog neurons using the “hanging-drop” method [38, 39]. In 1912, Alexis Carrel took a small fragment from the heart of an 18-day-old chick embryo and explanted it on hypotonic plasma; passaging the tissue 18 times over a period of 3 months and observing that it not only remained viable,
but also continued its rhythmic beat [40]. In the 1950’s animal cell culture became routine in the laboratory and lead to the development of the first human cell line, HeLa, developed from a cancer patient [38, 41]. These early research endeavors have lead to the cell and tissue culture techniques that remain a laboratory staple in biomedical and biological research today.

**Types of Culture**

Tissue culture is a commonly used generic term for the *in vitro* cultivation of cells, generally consisting of heterogeneous cultures of crudely disaggregated tissues; terms such as organ culture, cell culture, primary explants, and *ex vivo* propagation all concern the *in vitro* cultivation of cells or tissues [38]. Cell culture, on the other hand, usually refers to the isolation and propagation of a homogeneous or heterogeneous population of cells (e.g., epithelial cells, fibroblasts, etc.) that originate from either primary cells, which have a finite lifespan, or cell lines, immortalized cells which are normally derived from transformed or cancerous cells [38]. Primary cells are widely used to examine the effects of toxins, infectious agents, and other cellular interactions that would not otherwise be feasible *in vivo* [38]. Cell lines are often used to investigate questions in biomedical research because of their immortality and because they are well studied and characterized; however, they generally do not have the phenotypic characteristics of the cell from which they originated [38]. Early cell lines were established from tumor tissue and, as such, possess abnormal growth characteristics [38].

Organ culture involves *ex vivo* culture of the whole organ or a significant portion of the organ which allows the retention and preservation of the original cell-cell
interactions and three-dimensional (3D) extracellular architecture [38]. Organ culture is important when an *ex vivo* system requires the original organ architecture; however, organ cultures do not normally grow rapidly and are not suitable for studies where large numbers of cells are required [38]. While tissue or organ culture have been used previously to investigate cellular interactions and determine developmental biology processes, two-dimensional cell culture is the prominent laboratory technique used, due to the ease of cell propagation on tissue plastic surfaces, ability to produce large populations of single cell cultures, and the ability to manipulate the microenvironment for research purposes.

**Cell Culture Materials**

Three-dimensional cell culture matrices, also known as scaffolds, were introduced to cell culture to overcome the 2D culture limitations [42]. These scaffolds are porous substrates that can support cell growth, organization, and differentiation on or within their structure [42] and are composed of either naturally derived materials (e.g., collagen type I, rBM) or synthetic materials (e.g., polylactide). Such materials should provide a 3D support to interact with cells to control their function, guiding the spatially and temporally complex multicellular process of tissue formation and regeneration [43]. In designing scaffolds, mimicking the natural ECM, an intricate interwoven fiber meshwork of collagen and elastic fibers embedded in a highly hydrated gel-like material of glycosaminoglycans, proteoglycans, and glycoproteins [42], is attractive, but other chemical, physical, and mechanical properties of the scaffold material influence cellular affinity and interactions. While all these properties must be taken into consideration,
scaffold fabrication remains highly challenging as the natural in vivo remodeling process that is part of the tissue formation process does not take place in vitro [44].

**Natural Materials**

There are many naturally derived materials that have been and are currently being used in epithelial and breast cancer model systems. Some of the common materials used are collagen (most often type I, III, and IV), rBM (e.g. Matrigel™), and decellularized mammary gland ECM (MGEM). Emerman and colleagues used floating collagen membranes as a substrate for culturing mammary epithelial cells in order to evaluate the effects of substrate-induced cell shape on differentiation [45, 46]. Most of these materials are naturally found in the ECM and are derived from animal ECM. While these materials are natural, provide the necessary structure and properties for cells, and direct the macroscopic process of tissue formation, they are not always preferred for tissue engineering applications and 3D model systems due to their quick remodeling, stimulation of inflammatory response, and high cost.

**Synthetic Materials**

Many different synthetic materials have been used as substrates for tissue engineering applications and 3D models. Presently, materials such as poly(lactide-co-glycolide) (PLG) [47, 48] as sintered microspheres [49, 50] and porous scaffolds [51, 52], hyaluronic acid-based (HA) spongy scaffolds [53-56], gelatin sponges [57], polyethylene terephthalate (PET) nonwoven fabrics [58], poly(ester amide) (PEA) nonwoven scaffolds [59], poly(ethylene glycol) diacrylate hydrogels [60], collagen-chitosan hydrogels [61]
and many other materials have been used as tissue engineering scaffolds. Each scaffold is chosen based on certain criteria: rigid enough to structurally support the tissue, but not so stiff that it causes differences in appearance, and biodegrades at a rate consistent with tissue growth and proliferation.

**Types of Culture Models**

Numerous model systems have been developed in order to evaluate normal mammary epithelial tissue formation and function as well as tumor formation and progression. Normal human mammary epithelial cells have been cultured in order to compare their behavior with that of breast cancer cells, to investigate molecular mechanisms underlying any determined differences, and also to examine the effect of manipulating the microenvironment on cellular behavior [62]. Currently, animal or human in vitro models, in vitro cell culture systems, and animal genetic knockout mice are used to investigate many different in vivo tissue processes [63].

**2D vs. 3D**

Two-dimensional models were first developed, but do not represent the three-dimensional nature of tissues and organs; however, our ability to understand tissue formation, function, and pathology has often depended on 2D monolayer culture, organ culture, or animal model systems [64, 65]. Monolayer culture is normally performed on 2D surfaces such as micro-well plates, tissue culture flasks, Petri dishes, or glass slides [42] and has been used to evaluate cell morphology, growth, differentiation, gene expression, and toxicity [45, 46, 66-69].
Even though monolayer culture has provided substantial understanding of normal cell morphology and phenotypic expression and allowed evaluation of therapeutic cancer drugs [70-72], there are limitations. Cells dissociated from their normal tissues and maintained on tissue culture plastic or glass substrates rapidly lose their normal and differentiated characteristics despite the presence of nutrients from medium and essential hormones [45]. Another major limitation of 2D monolayer culture is the lack of stroma, which is important when modeling breast cancer since stroma of the mammary gland accounts for more than 80% of the resting breast volume [2]. The ECM and its components are pivotal in determining cell phenotype and function, especially in the mammary gland [73].

Three-dimensional cultures provide a well-defined geometry, which makes it possible to directly relate structure to function, they can be composed of representative cell combinations (for example, proliferating, non-proliferating, and necrotic cells), and they can support co-cultivation of multiple cell types in order to study the interaction between cell types and their surrounding matrix [2]. Overall, 3D models have emerged as powerful tools to investigate fundamental cellular and biophysical mechanisms that have not been readily amenable to traditional genetic or biochemical analysis [74].

**Animal Models**

A number of different approaches have been taken to model early breast cancer progression though the use of murine models. Research methods include the induction of premalignant lesions, the generation of genetically engineered mice that are susceptible to neoplasia or preneoplasia, or the use of epithelial cells that have been spontaneously
transformed, transduced with oncogenic viruses, or transfected with activated oncogenes to derive altered cells that mimic premalignant lesions when tested in immune deficient mice [75]. Smith and colleagues have used murine models to investigate DNA division in label-retaining epithelial cells in the mouse mammary gland through transgenic mammary implants [76-78]. Animal models frequently provide definitive tests of the importance of specific molecules and processes; however, there can be discrepancies between conclusions due to the distinct differences between human and animal tissues and the chemicals that could interfere with the function of specific proteins [64]. Although animal models are able to provide us with relevant biological interactions and help in the understanding of developmental biology, they are costly and complex with problems of unpredictable characteristics and ethical approval, thus other physiological 3D model systems are more desirable [65].

**Normal Mammary Epithelial and Tumor Models**

Normal human and murine mammary epithelial cell culture models have been established in order to uncover the key components of initiation and maintenance of cell differentiation, which include both the cell and the surrounding microenvironment [69]. While these models have investigated the correlation between cell shape and growth and differentiation [45, 46, 66], the role the ECM plays to influence cell phenotype [67, 69, 79, 80], and the epithelial-stromal interactions [13, 25, 81], natural materials (e.g. collagen, rBM) are used in these culture models to maintain a morphology reminiscent of the *in vivo* conditions [69]. The natural materials used in these culture conditions provide the necessary structure to form polarized luminal ductal and acinar structures, but are
limited by the components present in the model system; thus, interpretations of data from these systems need to be weighed against the limitations of the particular system [74].

Cancer models have significantly enhanced our understanding of carcinoma biology in four areas: the formation and maintenance of a hollow glandular lumen and its disruption by cancer genes, the regulation of apicobasal polarity in normal and cancerous epithelium, the discovery that cell-cell and cell-matrix adhesion pathways can interfere with the phenotypic expression of the tumorigenic state, and the emerging importance for tensional force in driving 3D tissue architecture and homeostasis [74]. While these four areas are of great importance in understanding tumor formation and cancer progression, others have used 3D models to evaluate the cytotoxicity and toxicology of cancer drugs [82-84].

Overall, current 2D and 3D models have allowed insight into the development and function of normal mammary epithelial tissue, tumor formation and cancer progression, as well as evaluation of effective therapeutic agents in cancer treatment; however, there are limitations as to what cellular and matrix components are used, what these models can accomplish, and how well they represent the true in vivo breast conditions.

**Adipose Tissue Models**

Engineered adipose tissue, composed of the patient’s own cells, a suitable scaffold, and appropriate growth factors, was initially developed for use in breast and soft tissue reconstruction after tumor resection [85-87]. Preadipocytes and mesenchymal stem cells have been seeded on PLG scaffolds [47, 48, 51, 52], various hydrogel materials [61,
87], and porous polymers [56-58, 88] in order to produce a defined volume of adipose tissue for implantation into a defect site. While these tissue engineering strategies have been used for reconstruction, they are inherently useful in breast tissue model systems as the breast, in its entirety, is composed of the epithelial parenchyma surrounded by intrastitial and interstitial ECM, all embedded in the mammary fat pad. Numerous natural, synthetic, and hybrid materials have been used to act as adipose surrogates; they have predominantly been used to replace adipose volume and not function [85]. To date, epithelial culture systems have been used to recreate the cardinal features of glandular epithelium in vivo and represent a valuable tool for modeling breast cancer initiation and progression in a structurally appropriate context [74], but have not been used to determine how adipose tissue influences mammary gland structure and development. Improvement upon the current model systems is needed for translational results and further understanding.

**Tissue Test Systems**

A three-dimensional model system that is used in applications where a whole tissue is represented can also be referred to as a tissue test system. Tissue test systems are developed to represent the in vivo conditions, provide a tunable system to investigate certain aspects of the environment, and also allow precise data collection. In any 3D model system, the specific cellular and matrix microenvironment provided to the cells can substantially influence the experimental outcome [64]; thus, the model/tissue test system should be designed to provide the necessary cellular and micro-environmental components to best represent the natural in vivo conditions of the breast. Selection of the
cell type(s), scaffold material or components, and micro-environmental cues, such as
hormones, growth factors, etc., all play a considerable role in the development of the
overall tissue test system. Current 3D model systems have limitations as to how they
perform and the data that can be obtained, therefore development of a tunable system
would be helpful to understand all aspects of the tissue and its function in vivo.

Normal Breast Tissue and Breast Cancer Models

Studying normal breast tissue biology, architecture, and development to
understand how human breast cells grow, interact, and undergo programmed cell death
began with 2D culture of cells [45, 46, 66-68, 89, 90] coupled with the development of
cell lines to be used for research purposes. Two-dimensional models have allowed a
better understanding of the effect of the surrounding environment on cell growth [66, 67],
proliferation, interaction [90], differentiation [45, 46, 67], and apoptosis. Further
investigation of human breast development, the pathological progression of breast cancer,
and therapeutic drug evaluation continued to be answered through use of 2D models such
as the NCI60 cell line assay [41, 70, 71]. Although these models were able to identify key
biological development processes and interactions of mammary epithelial cells, the 2D
models do not represent the complete in vivo conditions of the normal and cancerous
breast. The mammary gland is a complex tissue; thus, in order to evaluate the complex
phenotypic alterations of the mammary epithelial cells and the surrounding
microenvironment [91], 3D models have been developed. The advent of 3D cell culture
models has allowed investigators to make significant progress toward characterizing the
factors involved in the establishment and maintenance of epithelial architecture.
The function of an organ relies upon the organ’s constituent cell types and overall organization; i.e., the structure of a tissue or organ is critical for its function [92]. In developing 3D models and tissue test systems, achieving and maintaining the remarkable level of tissue organization, mammary epithelial cells (MECs) and their surrounding ECM components must integrate their structure in a highly concentrated fashion [93]. Tissue engineering, i.e. the construction of tissues using cells and biomaterial “scaffolds” or “matrices” as foundational building blocks, has been of interest for many years for mammary reconstruction following mastectomy or lumpectomy, and has been investigated more recently for use in building 3D mammary tissue models [6]. Many different natural and synthetic materials, cell types (primary cells or cell lines), and microenvironments have been used in order to engineer normal breast tissue and breast tumor tissue. Many of the tailored models that have been use to investigate normal and malignant breast tissues are outlined further in order to describe the specific niches researchers have thoroughly explored in order to fully understand the biological principles of the mammary gland tissue and the breast environment.

**Ductal Structure Formation, Terminal End Bud Motility, and Epithelial Cell Polarity**

Ductal structure formation, terminal end bud motility, and epithelial cell polarity are all dependent on the microenvironment and interactions of the epithelial cells with each other and the basement membrane. A great deal of breast biology and breast cancer research on ductal structure formation has included mammary epithelial cells or breast cancer cells embedded in natural materials such as collagen Type I, reconstituted basement membrane products (rBM, e.g. Matrigel™), or a combination of the two, to
represent the naturally-occurring ECM of the breast [79-81, 94]. Dhimolea and colleagues found that flexible Type I collagen matrices supported polarized acini and branching ducts when human breast epithelial (MCF10A) cells and human mammary fibroblasts obtained from reduction mammoplasties (RMF) cells were suspended in the gels in co-culture [79]. Krause and colleagues co-cultured MCF10A and RMF cells embedded in a Type I collagen gel, which resulted in the development of branched ducts, but when rBM was added to the collagen in a 1:1 ratio, branching ducts and alveoli were produced [81]. Dréau and coworkers found that the behavior of 3D cultures of normal murine mammary gland (NMuMG) cells, cultured either alone or in combination with mouse mesenchymal stem cells (D1), were dependent on the “matrix” or surrounding biomaterial environment consisting of agarose, collagen, or Matrigel™ alone or in combination [95]. The number of acinar structures was significantly higher in cultures grown in combination matrices of agarose with Matrigel™ or collagen I when compared to cultures grown in Matrigel™ or collagen I alone. No tubular structures were formed when agarose was included in the matrix, regardless of the combination. These works highlight the process of ductal formation, demonstrating how epithelial cells react in different gel environments, highlighting the fact that the surrounding microenvironment plays a particular role in normal ductal formation and overall regulation of ductal branching and lobular formation in the breast. The downfall of these studies is that the researchers do not include all the components found in the normal and cancerous breast environments. If researchers are to investigate the development of the normal breast, they would need to include adipose tissue components which also influence the developing
ductal tree. Without the adipose and stromal cell components and their signaling factors and influence within the model system, the results from these tissue models do not fully represent the native tissue. Model systems are just beginning to use all of these components in one system, Wang and colleagues cultured human breast epithelial cells in a tri-culture with stromal and adipose cells in a silk scaffold in which the cells displayed more differentiated morphological phenotype and functional activity compared to mono- or co-culture of the cells. [96] This study in fact demonstrates how current model systems are deficient and what they should strive for in the future.

The TEB, at the tip of the ductal structure in the normal breast, initiates invasion into the surrounding fat pad of the breast [97]. Proliferation of TEB cells results in ductal elongation, while clefting results in bifurcation or branching of the ducts [97]. Ductal formation and TEB motility is guided by changes in the surrounding ECM components, specifically the fat pad and fibroblasts surrounding the epithelial cells [13]. The TEBs include a cell population with high rates of mitosis which are known to include endocrine and local growth-regulatory signals, stromal-epithelial interactions, ECM remodeling, and dynamic adhesions within the end buds that maintain the bilayer structure [22]. As TEBs advance through into the fat pad, remodeling of the surrounding ECM is necessary for the ductal structure to advance, branch, and expand. Remodeling of the ECM usually occurs through systematic crosstalk between the adipose cells, fibroblasts, and cap cells of the TEB and includes signaling proteins such as BMP, Wnt, and EGF [97]. Metalloproteinases (MMPs) the principal matrix-degrading enzymes, are regulated by tissue inhibitors of metalloproteinase (TIMPs) [13].
Luminal epithelial cells are polarized glandular cells with specialized apical and basolateral membrane domains [98]. The permanent loss of polarity in tumors disrupts tissue structure, compromises the segregation of signaling effectors, and exacerbates the increased cell proliferation that is induced by other oncogenic signals [19]. In normal breast development, luminal epithelial cells orient themselves through cues from myo-epithelial cells and the surrounding BM, although the exact signaling mechanisms are not fully understood. Experiments have focused on polarity through the formation of the mammary ductal structure and also how a lack of polarity and BM deposition is characteristic of tumor cells [98]. Gudjonsson and colleagues used breast luminal epithelial and myo-epithelial cells derived from reduction mammoplasty biopsies and residual tissue from breast carcinoma mastectomy specimens to investigate the differences in normal and myo-epithelial cells and their interactions with luminal epithelial cells to recapitulate polarity [21]. Double-layered breast acini formed when normal luminal and myo-epithelial cells were cultured together, but when normal luminal and cancerous myo-epithelial cells were cultured together only some acini formed, due to a lack of laminin-1 production from the cancerous myo-epithelial cells, demonstrating the importance of laminin-1 production and the role of the myo-epithelial cells in maintenance of polarity in normal breast and how myo-epithelial cells may function as structural tumor suppressors [21]. In contrast, Liu and colleagues investigated the potential connection of cellular proliferation and polarity to tumor expansion and invasion; these researchers demonstrated that although cell proliferation and polarity defects are separable states, both are required for the development of a malignant
phenotype [19]. These studies highlight the importance of polarity in the normal breast as well as in tumor initiation, progression, and metastasis. The models used here to investigate polarity and the influence it has in the cancerous breast are lacking the cellular and structural components that would be seen in the cancerous environment. Polarity models should not only include the normal and cancerous epithelial cells, but fibroblasts, macrophages, inflammatory cells, and endothelial cells. The tumor microenvironment is a heterogeneous environment that is most notably characterized by an increase in fibroblast and myo-fibroblast activity, increased angiogenesis, increased infiltration of inflammatory cells and remodeling of the extracellular matrix adjacent to the cancerous cell. [28] Without the influence and inclusion of these cell types and structural components within the current models, we will not be able to completely understand how polarity affects tumor initiation, progression, and metastasis.

Overall, understanding the developmental biology of ductal structure formation, terminal end bud motility, and polarity within the normal breast environment is crucial to advancing technology for breast cancer detection, prevention, and cures.

**Hormones, growth factors and signaling molecules**

The development of the mammary ductal structures involves a complex interplay between epithelium and mesenchyme; the branching of the mammary ducts is dependent on circulating hormones for stimulation and synchronization with reproductive events, but is also influenced by local factors to provide signals that influence glandular growth, differentiation, and morphogenesis [99]. Extracellular signals are transmitted across the cell membrane via the transmembrane receptors which recognize ECM molecules;
changes in these receptors, triggered by ligand binding, in turn cause rearrangement of the cytoskeletal network and intracellular cascade of signal transduction, leading to changes in gene expression, and therefore the growth and differentiation state of the cells [69].

During normal breast development, estrogen and progesterone are required for proliferation and morphogenesis of the normal mammary gland; estrogen drives ductal development during puberty, whereas estrogen plus progesterone mediate the proliferative and morphological changes of ductal side-branching and alveologenesis that occur at sexual maturity and during pregnancy [100]. Both epithelial and stromal cells express the estrogen receptor (ER)-α [92], which is a crucial regulator of branching in the virgin gland [101]. The hormone progesterone, in combination with the prolactin receptor (PRL), promotes differentiation of the alveoli, which are the structures that synthesize and secrete milk during lactation during pregnancy [101]. In vivo studies by Smith and coworkers examined two populations of slowly dividing (label-retaining) cells, ER-α-positive and progesterone receptor (PR)-positive, which were equally represented among the body cells of the TEBs and within the epithelial of the subtending ducts of the FVB/N mice [77]. In the normal mammary glands of both mice and women, ER-α-positive cells are not normally proliferative, but this association is lost in breast cancer [100, 101], thus providing a breast cancer indicator for which one can test. Additionally, prolactin was examined by Gill and colleagues using immunohistological processes to compare normal, benign, and malignant breast tissues, where PRL was expressed at different sites within normal, benign, and malignant breast epithelial cells, expressed in over two thirds of the
breast carcinomas, and was correlated with strong staining for ER-α-positive but not other prognostic factors [102]. These studies have all looked at the influence of hormones within the glandular structure; however, most of these studies are conducted in animal models or through histological evaluation of biopsied tissue. Animal models, while useful, are not the best representation for extrapolation to human condition. Rodents typically possess very little subcutaneous adipose tissue [51] and the mammary glands are not structurally the same; in many rodents and other mammals the glands are relatively thin and flat. [15] In summary, to advance the understanding of hormonal influence and interactions within the normal and cancerous breast environments, use of animal models that are better representative of the human condition and development of in vitro models is crucial.

While estrogen and progesterone and their receptors are necessary in normal mammary gland development, there are growth factors that are also important. Hepatocyte growth factor (HGF) is a mesenchyme-derived growth factor that is synthesized in the stroma in vivo and stimulates the proliferation, motility, and morphogenesis of nearby epithelium [103]. Haslam and colleagues investigated cell proliferation of luminal and myo-epithelial cells of the mammary gland in organoid culture; cell proliferation did not increase with progestin (R5020) or 17β-estradiol (E2) alone or R5020+E2, but did increase when HGF was added to E2 and further increased in combination with R5020 forming tubules off the organoids [103]. There is also evidence of bidirectional crosstalk between epidermal growth factor receptor (EGFR) and β1-integrin in human mammary tumor cultures [65]. Wang and colleagues cultured human
mammary epithelial cells (HMT-3522) and human mammary tumor cells (T4-2) in 3D rBM in order to evaluate the bidirectional cross-modulation of β1-integrin and EGFR signaling, which does not occur in 2D culture [94]. The group found that regulation of EGFR (as well as β1-integrin) is linked to tissue morphogenesis, that EGFR and β1-integrin pathways are coupled, and that as long as the pathways are not altered or deleted, aberrant behavior can be corrected, thus restoring normal function to tumor cells in a 3D BM. These studies demonstrate the importance that growth hormones and their receptors play in the overall development of the normal mammary gland as well as tumor initiation and progression.

Some of the most researched signaling molecules in the breast are the transforming growth factor β (TGFβ) family, composed of three isoforms (1, 2, and 3), which have been identified as multifunctional cytokines with pivotal roles in several cellular functions including cell growth, differentiation, extracellular matrix production and degradation, motility, and regulation of the immune system [24, 104]. Osin and colleagues used histological staining to determine where TGFβ was found in fetal and infant breasts; they found that TGFβ was localized to the ECM, suggesting that TGFβ plays an important role in inhibiting epithelial proliferation at specific sites and thereby influencing morphogenesis [105]. TGFβ inhibits branching morphogenesis during puberty, blocks formation of alveoli and secretion of milk during pregnancy, and promotes apoptosis during involution [92]. Beyond TGFβ and its effects in normal and stromal microenvironments, there are many other growth factors, signaling molecules and hormones that influence normal breast development and tumor formation.
While these studies are great beginning points and have given us insight into the crosstalk and signaling that occurs in the normal and tumor microenvironment, there is still much technical insight to be gained. Further development is needed for model systems to include the stromal components, i.e. fibroblasts, adipocytes, and other cell types found within the microenvironment being studied in order for researchers to better understand the underlying mechanisms influenced by hormones, growth factors, and other signaling proteins.

**Stroma**

The stroma is composed of multiple cell types that co-exist within the ECM network, where the cellular components evolve with the developmental stages of the mammary gland [13, 24]. Therefore, it is expected that the variations seen in epithelial-stromal interactions and ratios that occur during the different stages of breast development will influence the components of cellular microenvironment (growth factors, hormones, and ECM) and, in turn, influence gene expression that may account for the susceptibility or risk to develop breast cancer [99, 106]. While research has focused on epithelial-stroma interactions and how they influence the proliferation, the differentiation, and, at times, the quiescence of the epithelial cells and their progenitors, there is still information about the cellular interactions and their influence on cellular behavior in the normal breast and breast cancer environment that is unknown.

*In vitro* model systems have been developed, through the use of tissue engineering methods [107], in order to better understand the development and structure of the gland parenchyma and how the stroma influences development as well as
influences breast cancer initiation and progression. While normal formation of the mammary gland depends on the coordination of physical and biochemical signals from the normal microenvironment (defined by neighboring cells, surrounding ECM, and local soluble factors) [93], tumor initiation and progression are marked by an imbalance in the microenvironment. In normal mammary tissue, where multiple cell types co-exist, homeostasis and involution are driven by programmed cell death (PCD, or apoptosis regulation) through cell-cell and cell-ECM adhesion as well as the 3D tissue architecture [108].

Stromal interactions

Normal growth, function, and homeostasis of breast epithelial cells depend on intricate interactions between the numerous surrounding stromal cells within the mammary gland [109]. These cells secrete multiple cellular products, such as growth factors and ECM components, which influence normal epithelial cell behavior, while alterations in regular communication between these cells, i.e. an abnormal stromal environment, can lead to the progression or expansion of malignant growth [73, 109].

Within the stroma, fibroblasts and the molecular signals they produce seem to play a critical role in normal and malignant epithelial cell behavior. Fleming and colleagues demonstrated that fibroblasts in the surrounding intralobular and interlobular do not show genetic differences, but they do influence the progression of malignant growth [109]; therefore, these researchers suggested that co-culturing fibroblasts with cancer cells would be necessary to examine tumor progression and metastasis. Recently, Rodrigues-Lisoni and colleagues co-cultured Hep2 cancerous cells with fibroblasts in
order to determine how cancer-stromal interactions influenced gene expression and found that the molecular crosstalk between neoplastic and the surrounding tissue induced several stromal changes, including neoangiogenesis and immune/inflammatory reaction, as well as new ECM formation and the activation of fibroblast-like cells (i.e. desmoplasia) [110]. Although fibroblasts seem to be important to stromal interactions with mammary epithelial cells, ECM components also regulate epithelial cell behavior.

Cell adhesion plays an important role in a variety of basic biological processes, including guiding cells into their appropriate locations in the body, providing cell anchorage, and controlling cell proliferation, differentiation, and apoptosis [111]. The identification of cellular transmembrane receptors for specific sequences present on ECM molecules established that ECM molecules affect cellular behavior. These molecules include integrins, cell-surface proteoglycans, and other receptors [24]. Integrins are a family of heterodimeric transmembrane proteins composed of α and β subunits that contribute to the ligand specificity and contain potential binding sites for ligands [111]. These ligands specifically bind to protein sequences, for example, the RGD sequence found in many of the ECM components [24, 111], and regulate cell behavior through signaling pathways. There is also evidence that in mammary epithelial cell cultures, ECM must suppress growth before signals for differentiation can be received [24]. Bissell and colleagues have conducted many studies to determine how these ligands and cell adhesion receptors influence the luminal epithelial cell polarity and ductal formation and development [80, 94, 98].
ECM Architecture, Stiffness, and Mechanical Forces

The stroma and cellular architecture, stromal components, ECM stiffness, and mechanical forces regulate the formation and branching of the mammary gland parenchyma. Each of these influences epithelial and stromal cell phenotypes, thus impacting the development, regulation, and function of the mammary gland and surrounding stroma as well as tumor formation and metastasis.

The ECM architecture and epithelial cytoskeletal architecture are imperative in normal cellular function, while loss of ECM architecture is inherent in involution of the mammary gland and impacts cell migration. All cells contain a cytoskeleton which is important in orchestrating cellular events such as cell motility, protein trafficking/secretion, and mitosis [112]; this cytoskeleton is composed of actin microfilaments, microtubules, and intermediate filaments (IFs; see Figure 1.3), which determine the mechanical properties of adherent cells [113]. Researchers have investigated how the intermediate filaments, specifically vimentin in fibroblasts, influence cell stiffness and migration [112-115]. While cellular stiffness and cell-cell junctions (e.g. E-cadherin in adherens junctions, see Figure 1.3 [97]) increase the stiffness of a given tissue, the ECM architecture of the BM and the supporting connective tissue has been determined to influence cellular behavior as well. The ECM BM is integral to the polarity of luminal epithelial cells [18] and maintaining tissue function of the mammary gland [108], while collagen stiffness regulates cellular contraction, matrix remodeling, and cellular migration [116, 117].
Figure 1.3 Scheme of dynamic reciprocity between cells and their microenvironment. Cell-cell and cell-ECM interactions cause a cascade of biochemical and mechanical signaling to the nucleus, which in turn affects the cellular micro-architecture and gene expression (adapted from [92] and [118]).
Stiffness of the mammary gland parenchyma and surrounding stroma plays an integral role in cell migration and gene expression. Hadjipanayi and colleagues investigated the migration of cells over a collagen matrix with a graded directional stiffness, where it was determined that cells migrate to stiffer surfaces and that the speed of migration increases on softer matrices [116]. Karamichos and colleagues used collagen matrix stiffness to assess the gene expression of matrix metalloproteinases (MMPs), which are produced in order to degrade ECM proteins for cell migration, and found that with an increase in stiffness there was an increase in MMP-2 production [117]. Research conducted by Hadjipanayi and Karamichos involves the use of dermal fibroblasts in collagen matrices and while it does not directly include breast cells, it is useful in understanding how cells migrate. Improvements in these methods might include starting with the replacement of dermal fibroblasts with epithelial cells and stromal cells within the collagen matrix, but then progressing to use of a system that incorporates the entire cancerous environment to better understand not only how tumors attract different cells, but also how metastatic cancer cells migrate to other tissues. Research of normal and tumor tissue requires the development of 3D models that reflect the tissue stiffness and components in order to best represent the native tissue. Samani and colleagues have used mechanical testing of normal breast tissue and breast tumor tissues in order to determine the stiffnesses necessary for development of future 3D models [119]. While these researchers have made progress toward understanding the processes involved in cell migration and how matrix stiffness influences normal cells and tumor cells, there is still
more to investigate and understand with the use of 3D model systems that can be tailored to a specific research question.

When modeling epithelial-stromal interactions, it is important to recognize that many studies exclusively employ epithelial cells and lack any stromal components [91]. While these studies have been able to give researchers insight into cellular behavior, the mammary stroma comprises over 80% of the cellular population of the in vivo gland, thus studies should use co-culture of epithelial cells and their stromal counterparts to investigate the relationships and subsequent behaviors [91].

**Tumor Microenvironment**

Tumors persistently shape their microenvironment, thereby establishing an abnormal ecosystem [120]. The tumor microenvironment is represented by an increase in fibroblast and myofibroblast activity, an increase in angiogenesis, an infiltration of inflammatory cells, and ECM remodeling adjacent to the cancerous cells [28]. While all of these components contribute to tumor progression, macrophages, recruited to the tumor by various signals, begin to release chemotactic factors that in turn recruit monocytes to the area that mature into tumor-associated macrophages (TAMs) which in turn promote neoplasia and angiogenesis [121]. In support of this theory, Robinson and colleagues found that the macrophage density increased with the grade of the tumor (in situ to invasive), that vessel density was higher in in situ and invasive tumors, and that there was a lack of epithelial cells in any proximity to either tumor cells or macrophages [1]. However, their results were not conclusive; thus, better understanding of the macrophage and its influence in the tumor microenvironment is necessary and could be
achieved through heterogeneous model systems. Further research studying the tumor microenvironment by Rodrigues-Lisoni and colleagues focused on gene expression influenced by the cancer-stroma interactions using conditioned medium (HCM – Hep2 Conditioned Medium, FCM – Fibroblast Conditioned Medium) from Hep2 cancer cells and fibroblasts. These researchers measured the gene expression and its influence on angiogenesis, the immune/inflammatory response, ECM formation, and fibroblast activation in culture [110]. They found that FCM inhibited Hep2 cell line proliferation and induced apoptosis, suggesting that factors secreted by fibroblasts include proteins that interfere in cell growth and death of neoplastic cells, whereas fibroblasts treated with HCM down-regulated genes corresponding to biological processes such as cell proliferation, transport, transcription and translation, apoptosis, and protein and RNA metabolism [110]. While this study shed light into gene expression of tumor cells and the influence of soluble paracrine factors produced in vitro by stromal cells, the study lacked the 3D nature of the body, as all culture was completed in well plates. Further studies should be conducted in 3D to better understand gene expression and how it influences the tumor microenvironment and progression of a tumor.

The tumor microenvironment is driven by the soluble factors and crosstalk occurring between the multiple cell types present within and surrounding a tumor. While studies have begun to address the individual interactions that occur between tumor cells and the surrounding vasculature cells, inflammatory and immune cells, fibroblasts, and macrophages, they have yet to piece this heterogeneous microenvironment together. Also, a majority of these studies have been conducted on tissue biopsies. Modular model
systems could be developed to further enhance the understanding of the tumor microenvironment, especially for each pathological state, by seeding relative cell densities of the representative cell populations within the model systems. By incorporating the different types of cells beyond epithelial cells, researchers create a microenvironment more representative of the pathological state.

**Cytotoxicity and Toxicology**

Cytotoxicity and toxicology models started in 2D using assays to determine how cancerous cells reacted to drug therapies. The National Cancer Institute developed the NCI60 cell line, which includes 60 different human tumor strains, for cancer cell microarray testing to evaluate cellular behavior including early clinical trials of drugs [72] and gene expression [71]. With the advent of 3D models and the better representation of the normal tissue environment in 3D compared to 2D cell culture, current toxicology models use tumor cells grown in natural and synthetic materials. Research groups have used polymeric substrates, such as electrospun polycaprolactone, poly(ethylene glycol) diacrylate hydrogels, and polylactide-co-polyglycolide microspheres or porous disks, to create 3D engineered models of adipose tissue to assess the cytotoxicity of breast cancer drugs [47, 52, 56, 83, 87, 88]. Horning and colleagues used polylactide microspheres seeded with MCF-7 cells in order to evaluate the efficacy of cancer drugs in 3D and 2D *in vitro* models, determining that drug efficacy is significantly lower in 3D compared to 2D culture and suggesting that the role of the natural 3D cellular architecture affects drug uptake by the cells and lowers the distribution throughout the tissue, thus lowering the efficacy of the drug response [83].
Although this study demonstrated why a third dimension is necessary when evaluating toxicity of cancer drugs, their study was not comprehensive and representative of the natural environment, i.e. incorporating all the cellular components present in the body. Other limitations of this study were that it not only used different substrates in their 2D and 3D comparison, but they used a set cell concentration within the culture environments instead of seeding for confluency (i.e. seeding based on surface area), which could influence results as cells could be confluent in 2D culture, but may not be in 3D culture inhibiting a true comparison of results. Similarly, Dhiman and colleagues evaluated the cytotoxicity of tamoxifen on 3D cultured MCF-7 cells on a chitosan matrix; the 3D culture provided a better understanding of the carbohydrate metabolism, cytotoxic effect of tamoxifen, and the kinetics and uptake of cathepsin D in breast cancer cells [82]. Limitations of this study by Dhiman were the use of cell lines instead of primary cells, the fact that different substrates were used in 2D compared to 3D and that material characterization was not conducted to determine if the substrate could influence results. Such 3D cancer models can be used to evaluate new anticancer drugs and to provide better understanding of the signaling factors that influence cancer cell growth, but 3D models that address the above limitations need to be developed before in vitro cytotoxicity and toxicology models will be comparative to human response in vivo.

**Adipose Tissue Engineering**

The majority of mammary gland and breast cancer research focuses on epithelial cells and the surrounding ECM interactions, further, adipose tissue and its influence in mammary gland development and tumor initiation and progression has rarely been
investigated. Tissue engineering of adipose tissue using natural or synthetic scaffold paired with stem cells or preadipocytes has been the focus of adipose research for soft tissue defects of late [56, 122-124].

Stem cells or preadipocytes have previously been used to produce mature adipose tissue for applications in replacing soft tissue due to trauma, diseases, or congenital abnormalities [87, 124, 125]. For example, Halbieb and colleagues seeded preadipocytes on a hyaluronic acid-based scaffold and found that hyaluronic acid-based scaffolds appear to be suitable three-dimensional carriers for culture and in vitro differentiation of preadipocytes [56], however, material characterization was not completed on the scaffold material to determine the degradation rate, stiffness, or other material properties which influence how cells react within the 3D system. Similarly, Patrick and colleagues seeded PLG polymer disks with preadipocytes to demonstrate the potential of using preadipocytes as a cell source in cell-seeded polymer scaffolds for tissue engineering [52]. This study was further developed to investigate the long-term feasibility of these cell-seeded scaffolds for clinically translatable, tissue engineered constructs for reconstructive, correctional, and cosmetic indications by implanting the scaffolds subcutaneously on the back of rats [51]. Although these preliminary studies were able to prove that preadipocytes would proliferate and mature throughout the polymer scaffold, in the long-term study the lack of vascularization and rat microenvironment around the implants may have contributed to a decrease in adipose tissue after 2 months in vivo. Reinforcing the limitations of rodent models in bioengineering research, where rodent models typically have very little subcutaneous fat [51] which could be why the implanted
constructs decreased in the amount of adipose tissue present after 2 months. Beyond designing engineered adipose tissue for soft tissue defects and reconstruction application, the ability to understand adipose differentiation is important. Kang and colleagues used an adipose tissue model of preadipocytes seeded on fibrous polyethylene terephthalate (PET) polymer matrices to understand the mechanisms behind adipose tissue differentiation [58]. The researchers determined that the PET nanoscale fibers provided a microenvironment for cell differentiation and spatial organization, thus mimicking the morphology of ECM in adipose tissue. Preadipocytes have not been the only cell type used in adipose tissue engineering; many studies have used mesenchymal or embryonic stem cells in adipose tissue engineering applications [47, 48, 57, 87, 88]. Recently, Kang and colleagues used murine embryonic stem cells seeded on an electrospun polycaprolactone (PCL) matrix using an adipogenic cocktail to produce functional fat cells [88]. The 3D geometry of the electrospun matrix provides environmental cues for adipogenesis and 3D structural features and functionality that cannot be obtained in 2D cell culture. Although all of these studies have focused on developing methods in which to engineer adipose tissue for soft tissue defects and breast reconstruction and they do not address the fact that the microenvironment of adipose tissue is highly vascularized and contains much more than just adipose cells, they are still useful in helping to better understand the adipose microenvironment and challenges of developing a comprehensive model system for the normal breast and breast cancer. Some of the limitations of the previously mentioned studies that need to be addressed are that the material needs to allow for anchorage of the cells and provide support during tissue development, while
upholding the mechanical properties of normal adipose tissue through material characterization. The substrates used also have to allow for spatial organization of the preadipocytes in order to fully differentiate into mature adipocytes. Hopefully, further studies will expand on the knowledge gained from these adipose tissue engineering studies in order to build thorough tissue models for use in normal breast and breast cancer research in the future.

Whereas synthetic polymer substrates may fully represent the tissue ECM, the 3D geometry provides environmental cues for adipogenesis that cannot be obtained in 2D (monolayer) culture [88]. The substrates provide the necessary mechanical stability, attachment capability, and biocompatibility to produce function-specific tissue [83]. Epithelial cells are anchorage-dependent cells that require a substrate to ensure proper cell morphology and functionality. In fact, while synthetic polymeric materials with the appropriate binding sites may be suitable for adipocytes, natural materials generally facilitate polarity in epithelial cell cultures. However, use of synthetic polymeric matrices seeded with preadipocytes, or stem cells, combined with epithelial cells and fibroblasts (which would not normally be incorporated for tissue reconstruction following breast cancer), and embedded in a collagen-rBM gel has the potential to produce engineered 3D models that better represent the architecture and microenvironment of the breast [6].

Overall, the unique mammary gland microenvironment influences mammary tissue homeostasis through cues from hormones, soluble factors, stroma, and physical stress and strain [74]. Tissue engineering and model systems can help better represent the in vivo conditions of the mammary gland and the surrounding stroma; indeed, the
microenvironment and ECM are key components of 3D in vitro mammary systems that can be tailored to study normal epithelial cell-stromal interactions, gland development, and breast cancer initiation, progression, and metastasis.

Epilogue

Approximately 1 in 8 women in the United States will be diagnosed with breast cancer at some point in their lives [1]. Breast cancer begins when normal mammary epithelial cells begin to grow and divide out of control, forming a cell mass which then invades surrounding tissue and eventually, if not caught and diagnosed, leads to metastasis. In an effort to understand normal mammary gland development and function and how breast cancer begins, much research has been conducted to understand how cells become cancerous in the mammary gland, how they propagate, and how they eventually metastasize throughout the body. The complexity of the tumor microenvironment is immense and much information is still necessary for better understanding how the relationship between stroma and carcinoma cells can be used for diagnostic and prognostic evaluation and a target for therapy [110]. While this research has helped us understand some of the underlying mechanisms associated with normal mammary gland function and breast cancer, there is still much we do not understand; the diagram below (Figure 1.4) details the pros and cons of the current model systems and the needs to be addressed in the future.
Figure 1.4 Types of models available for normal breast and cancer research. Pros and cons are listed for each model, also included are a list of suggested 3D model improvements.

Normal breast and breast cancer models have been developed to investigate many aspects of form, function, and interaction within the breast microenvironment, beginning with 2D and animal models and progressing to 3D models. Two-dimensional cell culture models are easy to generate and assess, but these models lack the realistic third dimension of the body. While these models are able to provide a better understanding of the normal interactions and development of the mammary gland, as well as tumor formation, progression, and microenvironment, they are not able to mimic the cellular conditions of the human body and do not normally include all the components (i.e. multiple cell types, cellular and acellular ECM components, spatial architecture)
representative of the normal breast or tumor environment. The ultimate goal is to develop a culture system in which cells respond to various extracellular signals in a manner that is physiologically relevant; the first step toward achieving this goal is to allow the cells to maintain a morphology reminiscent of their counterparts in vivo [69]. Two-dimensional models, while appropriate for pilot studies and initial investigative research, do not mimic the 3D tissue microenvironment and provide different results compared to animal and 3D models.

Animal models are used in research to provide an environment which is spatially and biochemically more relevant to the human biology than the 2D in vitro models. In cancer research, animal models have been and are used to investigate cell characteristics, cellular components comprising the tumor microenvironment, and tumor formation. While they provide insights and data that cannot be reproduced in 2D model systems, they still have inherent limitations. For example, when tumors are grown in immune deficient or compromised mice, there is an accelerated development of the mammary gland structure which correlates with the development of the human mammary gland, but the glandular components, development of the ductal structure, and percentage of fat in the mammary pads is not representative of the human environment. Also, the absence of a normal immune system in those mice prevents any analysis of the key role of the immune system in both mammary gland physiology and mammary tumor progression. Also, rodents have a much smaller percentage of fat compared to humans, thus the influences that may be seen from the mammary fat pad of a human cannot be fully represented in rats and mice. Further, animals may have other signaling molecules,
hormones, and other factors than humans. These may influence or conflict with the signaling factors and biomaterials that are being studied in the experiments. Finally, conduction of in vivo animal studies can be costly and limit time points investigated. Beyond that, the animal model variabilities (e.g., genetic, experimental, methodological) can also influence data obtained highlighting the need for 3D alternative models. In contrast, 3D test systems can be precisely replicated for experiments,

While each of the above models is useful to better understand normal breast and cancer development, each has a specific niche and limitations. Two-dimensional models were first developed as rudimentary systems in which to investigate cell response. While these models have been extensively used to date, one must be aware of the drawbacks. Animal models, although used less frequently, have provided perspective and expanded understanding of the mechanisms involved in breast and cancer development. Initial experimentation will continue in 2D and animal models as proof of concept, to test biocompatibility, or to benchmark against but 3D models must be developed to add crucial missing information regarding normal and cancerous breast tissue.

In the past two decades, researchers have realized the importance of 3D model systems especially, for cellular function and signaling. Even though the use of a 3D model inherently adds complexity to the culture system, a 3D system more closely represents the tissue conditions. Currently, multiple 3D models are available including using scaffold materials, either natural or synthetic, in or on which the cells are grown. There is a range of materials that have chemical and structural properties that can be tailored to the specific application. For example, most epithelial cell cultures are
conducted in rBM, laminin, or collagen in order to mimic the ECM present in the breast. Subsequently, many adipose tissue models have used synthetic materials (i.e., polylactide, PLG, PCL) in different forms to provide structural support for the stem cells or preadipocytes. Cell type and selection is another key parameter to consider in 3D models; while cell lines are great to study initially to determine how the cells react within the modeled environment, these cells typically are transformed and have lost many of their phenotypic characteristics, thus the 3D models using cell lines may only partially mimic the tissue of interest. These multiple parameters of a 3D model system should be critically considered when investigating biological responses within 3D models of the mammary gland environment and of breast cancer progression.

Beyond epithelial cell and scaffold selection, 3D models currently are missing other molecular, cellular, and structural components. Most 3D models still use a single cell population and very few include multiple cell types. In order to encompass a more relevant range of cellular components of the mammary gland or tumor, the 3D model system must include epithelial and myo-epithelial cells, fibroblasts, adipose cells, inflammatory cells, and vascular cells. All of these cells add to the overall microenvironment of the breast, influencing gene expression, signaling, and even apoptotic behavior of the cells, thus 3D models should strive to include multiple cell types to better represent the heterotypic environment present in the normal mammary gland as well as in tumors. Further, research is necessary to advance our knowledge of the microenvironment in normal mammary gland and during breast tumor development.
Development and use of 3D model systems has increased in the past decade. However, there is still a need to further enhance established 3D model systems by developing more modular model systems, reducing the production costs and the utilization costs, and increasing their result productivity. Globally, tissue cultures improved from cell culture conducted in petri dishes years ago to today’s state of cell culture conducted within 3D model systems. The latter approach is currently used to investigate the development of the normal and cancerous mammary gland, to understand the interactions that occur through cell-cell and cell-ECM interactions, to improve detection methods for cancer, and finally, to develop improved treatment methods for breast cancer patients.
CHAPTER TWO

DEVELOPMENT OF A TUNABLE POLYMERIC MATERIAL FOR A 3D BREAST TISSUE MODEL

Introduction

Three-dimensional cell culture matrices, also known as scaffolds, were introduced to cell culture to help overcome 2D culture limitations [42]. These scaffolds are substrates that support cell growth, organization, and differentiation on or within their structure [42] and are composed of either naturally derived materials (e.g. collagen type I, rBM) or synthetic materials (e.g. polylactide). Such materials provide a 3D support to interact with cells to control their function, guiding the spatially and temporally complex multicellular process of tissue formation [43]. In designing scaffolds mimicking the natural ECM, an intricate interwoven fiber meshwork of collagen and elastic fibers embedded in a highly hydrated gel-like material of glycosaminoglycans, proteoglycans, and glycoproteins [42] is attractive, but other chemical, physical, and mechanical properties of the scaffold material influence cellular affinity and interactions. While all these properties must be taken into consideration, scaffold fabrication remains highly challenging as the in vivo remodeling process that is part of the tissue formation process to a large extent not take place in vitro [44].

Synthetic absorbable polymers allow cells to attach, spread, proliferate, and differentiate to form tissue [42]. As with all biomaterials, the nature of the scaffold itself is of utmost importance in the design of tissue model systems. The physical and chemical
properties of the material used in such a system affect its cellular affinity [126], absorption or degradation characteristics, and mechanical stability; these characteristics ultimately determine the suitability of a tissue engineering scaffold. Absorbable polymers such as polylactide (PL), polyglycolide, polycaprolactone, and their copolymers have been used over the past decades in medical applications, such as bone plates and screws [127], films [128, 129], and biodegradable sutures, and have been investigated for uses in tissue engineering. The word “polylactide” refers to a family of polymers that have varying degradation profiles and characteristics, depending on the structure and molecular weight of the polymer. Poly-L-lactide and poly-D-lactide are crystalline (i.e. generally comprised of ordered, more densely packed molecular chains), while copolymers of poly-D,L-lactide are normally amorphous (i.e. generally comprised of disordered, less densely packed molecular chains) [44]. These synthetic, absorbable polymers, typically characterized by their degradation profiles and initial molecular weight, are selected for use based on their mechanical and chemical properties depending on the target application.

Processing of degradable materials plays a large part in the initial physical and chemical properties of these scaffolds and, accordingly, influences cell adhesion, migration, and differentiation. Therefore, activities focused on developing processing methodologies and techniques, understanding the relationships among device composition, structure, and the resulting degradation properties are crucial to understand and control cell–surface interactions in a 3D tissue engineering scaffold [130]. Beads have been produced using phase separation, solvent evaporation, solvent extraction, spray
drying, and cold precipitation techniques [131, 132]. Most commonly used are single or double emulsion technique (also called “oil-in-water” and “water-in-oil-in-water”, respectively) [131], which have been previously used to produce absorbable beads of polylactide, polyglycolide, polycaprolactone, and their copolymers for drug delivery applications. A single emulsion technique consists of dropping a solution of a hydrophilic polymer dissolved in a solvent (oil phase) into an aqueous solution containing a hardener such a polyvinyl alcohol (water phase) and stirring at a high rate to create hollow beads. Double emulsion techniques can be employed to encapsulate drugs or proteins in the polymer prior to bead formation; the beads can later be delivered in a controlled manner upon polymer degradation/absorption in the body [132]. Beads of injectable diameter, ranging from approximately 1 µm in diameter [133] to approximately 100 µm [134] have been reported [135-139]. The bulk and surface properties that are influenced by the processing variables impact cellular behavior. Some of the processing variables that affect bead properties include solvent type, solution volumes, processing vessel size, stir speed, and polymer composition [132]. Thus, when designing beads for tissue engineering applications, modulating processing techniques and methodologies to tune the scaffold is critical.

Although both single emulsion and double emulsion techniques have been used to generate beads for drug delivery applications [131, 132, 140, 141], both techniques are being explored to produce larger, tunable beads of suitable size for cell seeding [47, 48, 50, 126, 136, 142, 143]. With the prevalence of tissue engineering and the clinical familiarity of degradable polymers, such as polylactides, understanding how polymer
properties are influenced by processing is important in developing scaffolds that support cells and maintain a microenvironment for viable tissue growth. To date, little work has been conducted to address the impact of polymer processing and the resulting structural changes on the performance of these materials with respect to cellular behavior [144]. In fact, most work focuses on cell response to absorbable materials with little to no regard for the dynamic material characteristics. Accordingly, this study was designed to assess specific processing variables in the production of polylactide beads and how changes in those variables affected bead formation and properties, specifically bead size, degradation, molecular weight, and crystallinity. Understanding how processing affects the material characteristics provides a way to produce polylactide beads that can be tuned for specific needs within the 3D breast tissue model.

Materials and Methods

Polylactide (PL) beads were produced in separate batches using a single emulsion technique. Three processing methods, Bead Fabrication Procedure 1, 2, and 3, were used to produce polylactide (PL) beads (Table 2.1). Each fabrication method had four separate conditions and within each condition four different batches of beads was produced. In Bead Fabrication Procedure 1, the effect of processing tank size and stir paddle size was observed. Processing tanks of 1L, 2L, and 6L volume were used with either a square stir paddle measuring 6.9 cm x 6.9 cm (large, Figure 2.1B) or an oval swivel blade paddle measuring 5.0 cm x 1.3 cm (small, Figure 2.1A). In Bead Fabrication Procedure 2, the effect of varying PL solution amounts, ranging from 10 mL in the smallest tank to 60 mL in the largest tank, was studied. Finally, in Bead Fabrication Procedure 3, the
concentration of polyvinyl alcohol (PVA) was varied in the 2L tank using 20 mL of PL solution and the swivel blade paddle, ranging from 0.05 to 0.50%.

Table 2.1 Specific parameters for Bead Fabrication Procedures. For every condition the stir speed (300 RPMs), stir time (90 minutes), and rinse times (10 minutes) were similar. All beads were washed in 2% Isopropanol solution over night on a shaker plate, placed on filter paper within a buchner funnel, then rinsed 3x with dH2O. The beads were dried using a vacuum system for 24 hours and then stored in a desiccator.

<table>
<thead>
<tr>
<th>Fabrication Procedure</th>
<th>Vessel Size (L)</th>
<th>Paddle Size (cm)</th>
<th>PL Soln. Volume (mL)</th>
<th>PVA Soln. Conc. (%)</th>
<th>Rinse Soln. Volume (mL)</th>
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<tr>
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<td>II. Varied by PL Solution Amount</td>
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<td>III. Varied by PVA Concentration</td>
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As-received PL beads and post-processing beads were characterized using several methods, including imaging, gel permeation chromatography (GPC), differential scanning calorimetry (DSC), and lactic acid analysis. Degradation of produced beads was
evaluated by immersing beads in phosphate buffered solution (PBS) while crystallinity and thermal characterization properties were determined before immersion in PBS, and throughout the 49-day study.

**Polylactide Bead Processing**

**Bead Fabrication Procedure I**

Polylactide (PL; NatureWorks LLC, Minnetonka, MN) pellets (2 g) were dissolved in 20 mL of dichloromethane (10% w/v; Mallinckrodt Baker, Inc, Phillipsburg, NJ) for 24 hours in scintillation vials (VWR International, West Chester, PA). A 0.3% (w/v) aqueous PVA solution (weight average molecular weight 13,000-23,000 Daltons (Da); Sigma-Aldrich, St. Louis, MO) and a 2% (v/v) isopropanol solution (Honeywell, Morristown, NJ) were prepared in 1 L pyrex bottles (Corning, Corning, NY). To make a 0.05% PVA stir solution in the 6L tank, 1 L of aqueous PVA solution was added to the tank, followed by 5 L of distilled H₂O (dH₂O), to achieve a final volume of 6 L. The PVA solution was stirred at 300 RPM for 10 minutes using the square stir paddle. Using a 20 mL Luer-Lok™ syringe (BD, Franklin Lakes, NJ) with an attached 16-gauge needle (BD, Franklin Lakes, NJ), the polymer solution was injected into the PVA solution while stirring at 300 RPM with the needle tip submerged in the liquid at a 45° angle. The beads were stirred for 90 minutes at 300 RPM to ensure complete solvent extraction. The stirring was halted and the PVA solution drained until 1 L remained in the 6L tank. Subsequently, 2 L of 2% isopropanol solution was added and the beads were rinsed by stirring for 10 minutes. The remaining solution was drained and the beads were removed from the tank and deposited in a 250 mL glass bottle (Corning, Corning, NY) with 100
mL fresh 2% isopropanol. The bottle with beads was placed on a shaker plate for 24 hours at 150 RPM, at which time the beads were placed on filter paper in a Buchner filter funnel (VWR International, West Chester, PA), rinsed with dH₂O three times, dried in the chemical hood (Hamilton Concept from Fisher Hamilton LLC, Two Rivers, WI) under constant air flow produced by the suction of the house vacuum for 24 hours, and stored in a desiccator.

Figure 2.1 (A) Bead processing tank set-up with small paddle. (B) Large paddle.

Four batches of polylactide beads were produced consecutively on the same day to ensure similar processing conditions. For the 1L and 2L tank set-ups the processing was the same with the exception of solution volumes and paddle used (Table I). The solution volumes used for the 1L and 2L tanks were 150 mL and 300 mL of PVA
solution, 750 mL and 1500 mL of dH₂O, and 400 mL and 600 mL isopropanol, respectively. The square stirrer paddle and the swivel blade paddle were used in the 2L tank, while only the swivel blade paddle was used in the 1L tank.

**Bead Fabrication Procedure II**

The protocol for Bead Fabrication Procedure I was repeated using varying PL solutions of 10, and 20 mL for the 1L tank and 20 and 60 mL for the 6L tank.

**Bead Fabrication Procedure III**

The protocol for Bead Fabrication Procedure I was repeated using varying PVA solution concentrations of 0.05, 0.1, 0.25 and 0.50% for the 2L tank with the swivel blade paddle.

**Bead Diameter Measurements**

A Lumenera 2-3C 3.3 MP camera (Lumenera Corp, Ottawa, ON, Canada) mounted on a stand and MatLab 7 (MathWorks, Natick, MA) were used to capture images of the PL bead batches produced by Fabrication Procedures I, II and III. AxioVision 4.7 software (Carl Zeiss AG, Germany) was used to measure the average diameter of 300 beads per batch. To account for different shapes and sizes, two measurements in perpendicular directions were taken for each bead. The mean and standard deviation of the bead diameters were calculated for all batches.

**Acellular Degradation Study**

The degradation properties of the beads produced by each of the processing methods were studied by immersing beads produced in 2 mL Dulbecco’s Phosphate
Buffered Saline (PBS; Sigma, Milwaukee, WI) solution in each well of a 24-well cell culture plate. The well plates were stored in an incubator (37°C, 5% CO₂). Throughout the study, aliquots of the PBS solution were taken weekly and analyzed using an YSI Biochemistry analyzer for lactic acid content; subsequently, the PBS solution was replaced. At each time point, after aspiration of the PBS, beads were collected from the well plates using a spatula and placed on filter paper in a Buchner filter funnel. The beads were rinsed with dH₂O three times, dried in the chemical hood under constant air flow produced by the suction of the house vacuum for 24 hours, and stored in a desiccator until characterization was completed. Beads were characterized through molecular weight analysis, thermal analysis, and crystallinity determination.

**Molecular Weight Analysis**

Molecular weight was measured using gel permeation chromatography (GPC; Waters, Milford, MA). A Waters Breeze (Milford, MA) system equipped with a Refractive Index (RI) detector was used to assess PDL beads produced. The fluid pump for each was set to a rate of 1 mL/min. Polystyrene standards (Polysciences, Inc., Warrington, PA) with narrow weight average molecular weights (M₇), including 1,000,000, 400,000, 233,000, 104,000, 50,000, 23,000, 4000, and 436 Da. were used. Samples were prepared in high performance liquid chromatography grade chloroform (Honeywell, Morristown, NJ) at a concentration of 1 mg/mL and filtered through a 0.2μm Teflon membrane filter (VWR International, West Chester, PA).

Number average (Mₙ) and weight average (M₇) molecular weights values were determined from the retention curves using Breeze 2 Software. The polydispersity index
(PD), which is the molecular weight average to molecular number average ratio ($M_w/M_n$), was calculated for each bead type produced.

**Thermal Analysis and Crystallinity Determination**

A TA Instruments DSC 2920 (New Castle, DE) differential scanning calorimeter controlled by TA Instruments Thermal Advantage Software was used to thermally analyze PDL beads prior to immersion in PBS as well as at time points of 21 and 49 days. Beads were removed from the PBS at 21 and 49 days and placed on filter paper in a Buchner funnel and dried in the chemical hood under constant air-flow for 24 hours. Each sample ($n=3$), weighing approximately 5 mg, was heated from 0˚C to 200˚C in nitrogen at a rate of 10˚C per minute. Thermal transitions were analyzed using TA Instruments Universal Analysis 2000, and percent crystallinity was calculated using the following equation:

$$\frac{\Delta H_f - \Delta H_c}{\Delta H_f^{\text{theoretical}}} \times 100 = \%\text{Crystallinity}$$

where $\Delta H_f$ is the measured heat of fusion, $\Delta H_c$ is the measured heat of crystallization, and $\Delta H_f^{\text{theoretical}}$ is the theoretical value for the heat of fusion of polylactide (92.9 J/g) [145]. Thermal glass transition temperatures ($T_g$) were taken at the onset, inflection, and end points; the inflection values are reported.

**Statistics**

JMP software (SAS Institute Inc., Cary, NC) was used to run a one-way Analysis of Variance (ANOVA, $\alpha=0.05$) to analyze differences comparing all pairs using post-hoc
Tukey-Kramer HSD for bead measurements, molecular weight analysis, and thermal analysis of polylactide beads produced in Bead Fabrication Procedures I, II, and III.

Results

Bead Diameter Measurements

Bead Fabrication Procedure I determined how changes in the size of the vessel and the type of paddle used affected PL bead size and shape. PL beads with average diameters of 403±47, 544±96, 864±168, and 807±120 µm were produced in the 6L tank, 2L tank with large paddle, 2L tank with small paddle, and 1L tank (Figure 2.2A), respectively. The shape of the beads changed depending on the vessel size and paddle size used to produce beads. The 6L tank produced the smallest size beads and also the beads that were most uniformly round (Figure 2.2D, 6L20). There was a significant size difference (p<0.01) between beads produced using the large vs. small paddle in the 2L tank. Beads produced in the 1L tank were similar in size to those produced in the 2L tank with the small paddle, however, comparatively fewer beads were abnormally shaped (e.g. peanut, dumbbell, tailed).

Bead Fabrication Procedure II determined how increasing or decreasing the amount of PL solution in the processing protocol affects bead size. Beads with average diameters of 320±63, 403±47, 807±120, and 896±79 µm were produced in the 6L tank using 60 and 20 mL of PL solution and the 1L tank using 20 and 10 mL of PL solution (Figure 2.2B), respectively. Beads produced in the 6L tank and the 1L tank, regardless of how much PL solution was added to the process, showed no significant differences
between conditions within the same vessel. Comparing beads produced in the different vessels, the 6L60 beads were significantly different from both the 1L10 (p<0.001) and the 1L20 (p<0.01) beads.

Lastly, in Bead Fabrication Procedure III, the concentration of the PVA solution was varied to look at the changes in bead size and shape. The most notable difference in beads produced in Bead Fabrication Procedure III is that as the PVA concentration increased, the average bead diameter decreased (Figure 2.2C) from 864±168 to 628±33 µm and the different shapes (e.g. dumbbell, oval, tailed) were no longer present (i.e. the beads were all round) after the PVA concentration rose above 0.25% (Figure 2.2D images 2L20s, 2Lp1, 2Lp25, and 2Lp5).
Figure 2.2 Average diameter measurements of beads produced in Bead Fabrication Procedure I (A), II (B), and III (C) with representative images of PL beads (D; scale bar = 1000µm), * p<0.05, ** p<0.01, *** p<0.001. Beads produced by Bead Fabrication Procedure I (6L20, 2L20B, 1L20, and 2L20s) demonstrate how vessel size and paddle size affect bead size and shape. Beads produced in Bead Fabrication Procedure III demonstrate how beads produced in each condition decrease in size as PVA concentration increases. As PVA concentration increases shape of beads becomes more round. Beads made with 0.05% and 0.1% PVA concentration have varying shapes, e.g. oval, peanut, dumbbell, and tailed beads.
Acellular Degradation Study

A bolus release of lactic acid was seen for all conditions through Day 14 and a much slower release of lactic acid through Day 49 (Figure 2.3) during the degradation study. Beads with the highest release of lactic acid were 2Lp5 beads followed by 2L20s and 1L10 beads. Beads with the lowest release of lactic acid over the 49-day period were 6L20 beads. There was no correlation between the amount of lactic acid released and processing method or size of the bead.

Figure 2.3. Lactic acid release for each condition over the 49-day degradation study showing a bolus release of lactic acid within the first 14 days.
Molecular Weight Analysis

The same as-received PL pellets were used to produce beads for all Bead Fabrication Procedures. After processing, the molecular weight of the PL beads was not different from the as-received PL pellets. The as-received PL and Day 0 beads had similar average molecular weight ($M_w$) and polydispersity index (PD) values. All beads produced by Bead Fabrication Procedure I showed a significant difference ($p<0.0001$) from as-received PL after Day 21. At Day 49, 6L20 beads were significantly different ($p<0.0001$) from 2L20s and 1L20 beads, while 2L20B beads were significantly different ($p<0.05$) from both 6L20 and 1L20 beads. Significant differences ($p<0.05$) in PD were only seen at Day 49 between 6L20 and 1L20 beads. For Day 21 and Day 49 time points, beads produced in Bead Fabrication Procedure II were all significantly different ($p<0.0001$), except for 6L60 beads ($p<0.01$) at Day 21. At Day 49, no significant differences were seen when the amount of PL solution was increased in the 6L tank or decreased in the 1L tank. Bead Fabrication Procedure III beads showed a significant difference of $p<0.0001$ at both Day 21 and Day 49 for all bead types compared to as-received PL.
Figure 2.4 Comparisons of weight average molecular weight (A) and polydispersity index (B) of beads produced in each Bead Fabrication Procedure as a function of immersion time (* p<0.05, ** p<0.01, and *** p<0.0001).
**Thermal Analysis and Crystallinity Determination**

Processing affected the glass transition temperature ($T_g$, Figure 2.5A), crystallization temperature ($T_c$, Figure 2.5B), and melting temperature ($T_m$, Figure 2.6A) of the beads. As beads degraded, $T_g$ and $T_m$ gradually increased to values closer to as-received PL over the 49-day degradation period, while the $T_c$ only increased through day 21 and then stabilized. The $T_g$ of the as-received PL was significantly higher compared to all conditions, regardless of the processing method. At Day 21 $T_g$ differences between the as-received PL were only seen in Bead Fabrication Procedure II with 1L10 beads ($p<0.05$) and in Bead Fabrication Procedure III with 2Lp1 beads ($p<0.01$). No $T_g$ significant differences were seen between any bead types and the as-received PL at Day 49, however, significant differences, $p<0.05$, were seen at Day 49 in both Bead Fabrication Procedure II between 6L60 and 6L20 beads and Bead Fabrication Procedure III between 2L20s beads and 2Lp1 and 2Lp25 beads.

Crystallization temperatures (Figure 2.5B) were only compared across bead types, as the as-received PL did not have a $T_c$. In Bead Fabrication Procedure II there were no significant differences seen when the PL solution volume increased. In Bead Fabrication Procedure III, as the PVA concentration was increased the $T_c$ decreased. The only significant difference was seen at Day 0, where 2L20s had a significantly higher $T_c$ compared to concentrations of 0.1% PVA and higher. The largest differences in $T_c$ were seen in Bead Fabrication Procedure I, where the vessel and paddle size changed between conditions, indicating that this variable in processing is very important. At Day 0, 1L20 had the lowest $T_c$ and 6L20 the highest. A significant difference, $p<0.0001$, was seen in
6L20 and 2L20B compared to all other conditions, whereas, beads produced with the smaller paddle (2L20s and 1L20) only showed a significant difference of \( p < 0.01 \) compared to each other. As the beads degraded all conditions showed increased in \( T_c \). By Day 21, 6L20 was the only condition to have a \( T_c \) significantly higher than both 1L20 (\( p < 0.01 \)) and 2L20s (\( p < 0.0001 \)). At Day 49, the \( T_c \) had much less variation within each condition of bead produced and thus there were significant differences seen based on which paddle was used to process the beads with.
Figure 2.5 Comparisons of glass transition (A) and crystallization temperatures (B) of beads produced as a function of immersion time (* p<0.05, ** p<0.01, and *** p<0.0001). Asterisks in a color other than black were used to differentiate between groups, e.g. in Procedure I had a statistically higher Tg compared to all other conditions, indicated by three black asterisks.
Figure 2.6 Comparisons of melting temperatures (A) and crystallization percentage (B) of beads produced in each Bead Fabrication Procedure as a function of immersion time (Statistical significance indicated by: * p<0.05, ** p<0.01, and *** p<0.0001). Asterisks indicating statistical significance using a color other than black were used to differentiate between groups.
The as-received PL had a significantly higher $T_m$ compared to all conditions in Bead Fabrication Procedure I, II, and III (Figure 2.6A), however, no significant differences were seen between the conditions at Day 0 due to large standard deviations for all conditions except the as-received PL. Comparing conditions from Bead Fabrication Procedure I at Day 21, 2L20B had a significantly higher $T_m$ compared to both 2L20s ($p<0.01$) and 1L20 ($p<0.05$). Comparing conditions from Bead Fabrication Procedure III, at Day 21 2Lp5 had a significantly higher $T_m$ compared to 2L20s ($p<0.0001$) and both 2Lp1 and 2Lp25 ($p<0.01$) indicating that even though 2Lp5 beads were produced in the highest concentration of PVA at 0.5%, a known plasticizer, the plasticizer is no longer present in the polymer after 21 days as shown by the increase in both $T_g$ and $T_m$.

Lastly, percent crystallinity was compared based on bead fabrication procedure. No differences were seen in beads produced in Bead Fabrication Procedure II, but significant differences were seen in both Bead Fabrication Procedure I and III. The beads with the highest percentage of crystallinity from Bead Fabrication Procedure I were the 2L20s beads (22.9%) which was significantly higher compared to 1L20 (20%, $p<.01$), 2L20B (14.4%, $p<0.0001$), and 6L20 (7.4%, $p<0.0001$) after processing. At Day 21 and 49, beads produced using the small paddle had crystallinity percentages that were significantly higher ($p<0.0001$) than those produced using the large paddle, but no significant differences were seen between conditions made with the same paddle. When the PVA concentration was altered in Bead Fabrication Procedure III, the beads produced had similar percentages of crystallinity because they were all produced in the same vessel.
with the same paddle. However, at Day 0 the 2Lp5 beads had the highest crystallinity percentage of all beads (28.5%), which was significantly higher (p<0.01) than 2L20s beads. At the end of the degradation study, 2L20s beads were 15.5% crystalline and were significantly lower (p<0.01) compared to beads produced with a PVA concentration higher than 0.1%.

In summary, the processing parameters that influence bead characteristics the most are the ratio of paddle size to vessel size and PVA concentration; however, the one material property not affected by processing is the molecular weight (Figure 2.7).

Figure 2.7 Processing affects bead size, shape and thermal transitions, but not molecular weight as seen in Bead Fabrication Procedure I (A) and III (B) comparing bead size, molecular weights, and micrographs (scale bar = 1000 μm) of beads produced in each.
Discussion

Fabrication of degradable polymer scaffolds for tissue engineering, not only for therapeutic applications but also for diagnostic applications, remains highly challenging [44]. Processing methodologies and techniques have been developed to produce scaffolds with varying compositions, surface modifications, and properties to target the location and use of the scaffold materials. Degradation is one of the major characteristics of these tissue engineered scaffolds and systems [129], where the degradation process is influenced by the size of the scaffold, the starting molecular weight of the material, and the crystallinity. The majority of manuscripts detailing the use of polymeric tissue engineered scaffolds report the as-received molecular weights; complete material characterization after processing and sterilization generally is not conducted [49, 146, 147]. Characterization of the polymer post processing and post sterilization is crucial to provide a better understanding of the likely in vitro and in vivo performance of a given scaffold. Indeed subtle changes in material properties can cause radical changes in cell behavior [130]. Understanding the relationship between processing and post-processing material characteristics allow for tuning the production process to generate a scaffold suitable for a specific application.

When producing scaffolds of designated shape and diameter, changes in processing should not alter the final product. Production of the PL beads in Bead Fabrication Procedure I demonstrate that processing changes i.e., paddle size and vessel size (Figure 2.7A) caused notable differences in the diameter of the beads produced. The beads produced in the 2L tank with the large paddle (2L20B) were on average 540 µm in
diameter. In contrast, when the large paddle was substituted with a small paddle (2L20s) the beads produced had an average diameter of 864 µm. Thus, although the same protocol was followed, the final materials produced were significantly different.

That observation suggested that other simple processing parameters routinely reported in processing protocols [132], such as solvent type, solution volumes, and polymer composition may also impact the final product. To assess this, Beads produced in Bead Fabrication Procedure II addressed whether the PL solution volume affected bead size. Bead diameters were not significantly different between beads produced in the 6L tank or the 1L tank regardless of the PL solution volumes. Stir solution concentrations were investigated by altering the PVA concentration. Increasing the concentration of the PVA solution decreased both the diameter of the beads and the number of non-spherical beads produced. While bead diameter measurements can be used to detect changes in processing, the size of the beads also impact material properties including degradation.

Single emulsion methods used here were quite reproducible from batch to batch, however, a large distribution of bead diameters within each batch occurred in many of the conditions tested. Since degradation rate correlates to material mass, and therefore, bead size (i.e. diameter), a high within-batch variability corresponds to a variety of degradation profiles. This variability could be favorable for tissue-engineering applications, especially where a bulk release of by-products could be used to develop a microenvironment that is more representative of the breast cancer microenvironment. Bead size is a variable that may be readily tuned when designing scaffold materials. Not only does the diameter of
the bead determine its suitability in a given application, but it also impacts the degradation profile of the system.

Although size and shape of a tissue engineering scaffolds are important, the degradation profile is an additional property that requires careful consideration with respect to the intended application. The mechanical properties of a tissue-engineering scaffold change with the degradation profile. Practically, a scaffold should be treated before and after processing to remove lower molecular weight species. PL is a material that degrades by bulk degradation; thus, another bolus release of lactic acid will be released as the material fully degrades. Hence the cell-material interactions will vary with time and release of by-products. Some applications may require pre-degradation to ensure accelerated rates and full degradation of the scaffold [148], while other applications like use in a 3D in vitro model may need polylactide release at specific times to best represent the breast environment at a specific time. As our results indicate, even small material and processing changes affected the overall degradation profiles. Lactic acid measurements from aliquots of the PBS solution containing immersed acellular beads gave an indication of bead degradation. As the PL polymer chains in the beads were hydrolyzed into lactic acid monomers, the amount of lactic acid measured in the PBS immersion solution increased (Figure 2.3). With all bead types an initial bolus release of lactic acid within the first 14 days was observed, then the lactic acid release slowed considerably. Longer duration studies would enhance understanding of the lactic acid release profile and should include evaluation of the cellular response.
Polylactides, thicker than approximately 0.80mm [149], undergo bulk degradation once implanted into the human body, where the molecular weight of the polymer starts to decrease. However, the volume of the polymer generally does not decrease until the molecular chains are reduced to a size where they can freely diffuse out of the polymer matrix [150-152]. Regardless of the thickness of the device, the first by-products to emerge will be those with the lower molecular weight [151]. As the PL beads degraded and concurrently diffused lactic acid in the PBS solution, a corresponding decrease in molecular weight of the beads was expected. Instead, here the beads produced demonstrated an increase in $M_n$ and $M_w$. This may in part indicate the removal of impurities and lower molecular weight species from the as-received material, resulting in an increase in molecular weight and a decrease in polydispersity index.

The molecular weight of a polymer influences how quickly the scaffold will degrade when used in different applications. The as-received material, processing vessel size, paddle type, and processing speed all influence the molecular weight of the beads; hence, each parameter should be adjusted to obtain the targeted outcome. For example, scaffolds used for injectable applications should have small diameters; thus, beads produced by emulsion solvent methods could be produced with a large vessel and paddle size and high spinning speeds. Another application may require beads with an accelerated rate of degradation; in this case processing PL beads using a small vessel may be more appropriate [148].

Crystallinity is another property of an absorbable polymeric scaffold that influences degradation, mechanical stability, and cellular behavior. Crystalline surfaces in
general have higher surface energies compared to amorphous materials; surface energy can impact protein absorption patterns and conformations, which stimulate specific cellular behaviors [128, 130, 153]. The PL beads had a lower average \( T_g \) (Figure 2.4A) than the as-received PL, likely due to the PVA and any remaining moisture in the beads acting as a plasticizer within the beads. As the beads degraded, the PVA was released from the beads and the \( T_g \) increased back to a temperature similar to the as-received PL. The significant differences seen in \( T_g \), \( T_c \), \( T_m \), and crystallinity overall for the PL beads produced were caused by very tight standard deviations which caused the slightest change in temperature to be significant from the last. For example, the range of temperatures for \( T_m \) was 148-151°C.

The decrease in crystallinity seen in the beads produced in the Bead Fabrication Procedures can be explained by the polydispersity changes in the polymer chains over the 49-day study. In Bead Fabrication Procedure I for example, the \( M_n \) of the 1L20 and 2L20B beads decreased by almost 22 and 30\%, respectively, while the \( M_w \) only decrease by 8 and 21\%, respectively. The chain lengths stayed relatively constant in the 1L20 beads and showed a slightly larger decrease in the 2L20B beads, while the number of chains decreased at similar rates. This suggests that as a relatively high number of shorter chains are removed, the longer chains do not have the opportunity to quickly rearrange, and thus a decrease in crystallinity is seen, albeit a less pronounced decline in the 1L20 beads compared to the 2L20B beads. Because crystallinity affects the surface energy of the material and thus cellular behavior, it is crucial to purposefully select processing parameters that will guide cellular response to implanted materials.
Processing materials is a challenge, as so many factors influence the physicochemical and mechanical properties of a given material. Results from this study showed that, of the variables studied in this research, the bead processing parameters of vessel size/paddle area and PVA concentration have the largest influence on bead diameters. While these are the main factors, they are not the sole factors. Paddle speed, solvent and solution concentrations, as well as polymer molecular weight, crystallinity, and polydispersity, all play a part in the resultant beads and their designated mechanical and physiochemical properties. A small change in processing can cause drastic changes in the final product, thus knowledge of how these parameters influence the outcome characteristics is crucial and can help enormously in producing tissue-engineering scaffolds that have tailored properties for their application.

**Summary**

While the focus of producing suitable degradable biomaterials for use in tissue engineering applications is at the forefront of materials research, development of methodologies and techniques to be used and the effects processing has on the bulk and surface properties of the material have yet to be thoroughly explored and detailed. Here we determined that both the vessel size/paddle size and PVA concentration have a significant effect on both bead size and shape. Thus, these two processing variables should be controlled regardless of the intended application. These observations highlight how each handling and/or processing step affects absorbable systems, both their chemical and physical properties and their function. Future investigations should assess the bulk
properties of multiple scaffolding materials and define how processing parameters can be modulated to generate tunable “platform” systems.
CHAPTER THREE

Portions of results in this chapter were generated by an Institute for Biological Interfaces of Engineering interdisciplinary team, including Clemson University undergraduate student Devleena Kole, and were presented at the 2013 Annual Meeting and Exposition of the Society For Biomaterials: McCave EJ, Kole D, Burg KJL: Development of a Heterogeneous In Vitro Three-Dimensional Breast Tissue Model. In: Society For Biomaterials 2014 Annual Meeting & Exposition: 2014 (Denver, CO; 2014) [3].

GENERATION OF THE STROMAL COMPONENT OF A 3D BREAST TISSUE MODEL USING EXTRACELLULAR MATRIX MATERIALS

Introduction

The human breast is a complex tissue composed of a glandular structure (the mammary gland) [7], consisting of a nipple, lobes, and ducts, surrounded by stromal tissue, fibrous and fatty tissue. The mammary gland is a dynamic structural tissue that changes, beginning with the formation of the lobule bud structure during neonatal development, continuing with the development of alveoli or acini during adolescent puberty, then full lobulo-alveolar development with child birth, followed by involution at the end of lactation [8]. The stroma, i.e. the fatty and connective tissue, surrounds the lobes of the glandular tissue [7]. The breast contains very little muscle and is mainly composed of stroma, which defines the shape and size of the breast [7, 9, 11].

The stroma is not an inert tissue. The composition and organization of the ECM and cellular components evolve with the developmental stages of the mammary gland [13, 24]. Thus, the variations in epithelial-stromal interactions and ratios that occur during the various stages of breast development influence the components of cellular microenvironment (growth factors, hormones, and ECM) and also cell activities.
including gene expression accounting for the susceptibility or risk to develop breast cancer [99, 106]. While research has focused on epithelial-stroma interactions and how they influence the proliferation, the differentiation, and, at times, the quiescence of the epithelial cells and their progenitors, there is still much that is unknown. In vitro model systems have been developed, through the use of tissue engineering methods (reviewed in [107]), to better understand the development and structure of the gland parenchyma and how the stroma influences development and even breast cancer initiation and progression.

Tissue engineering, i.e. the construction of tissues using cells and biomaterial “scaffolds” or “matrices” as foundational building blocks, has been of interest for many years for mammary reconstruction following mastectomy or lumpectomy [154, 155], and has been investigated more recently for use in building 3D mammary tissue models [107]. The intricacies of mammary tissue have provided numerous challenges in tissue engineering. The mammary gland is a complex tissue comprised of epithelial parenchyma embedded in an array of stromal cells that regulate its proliferation, differentiation, and survival [8]. The fibrous connective tissue of the stroma, also known as the ECM, is a 3D network that surrounds the cells and is of two types. The first constitutes the basal membrane (BM), which interacts directly with the epithelium and consists primarily of globular collagen IV, laminin, entactin/nidogen, and heparan sulfate proteoglycans. The second forms the interstitial matrix, which consists of fibrous collagen (usually Type I and III) and fibronectin [25]. The latter contributes to the mechanical strength of the tissue [25]. Luminal epithelial cells line the ducts and are surrounded by a layer of myoepithelial cells that attach to the BM [93], which acts as a mechanical barrier between the
epithelial-lined ductal structure and the surrounding connective and fat tissue. The ECM, largely through its dynamic chemical and mechanical characteristics, is able to regulate cell shape, proliferation, polarity, differentiation, transcription, synthesis, and secretion for a variety of cell types [106].

Epithelial cell monolayers have traditionally been used to study breast cancer, yet two-dimensional (2D) cultures only partially reproduce the structure or the function of the mammary epithelium \textit{in vivo} [81]. Thus, 3D cultures have been developed to better represent the \textit{in vivo} environment. Much of breast biology and breast cancer research has included mammary epithelial cells or breast cancer cells embedded in ECM component mixtures such as collagen Type I, reconstituted basement membrane products (rBM, e.g. Matrigel™), or a combination of the two, to mimic the breast ECM [79-81, 94]. Dhimolea and colleagues found that flexible Type I collagen matrices supported polarized acini and branching ducts when human breast epithelial (MCF10A) cells and human mammary fibroblasts obtained from reduction mammoplasties (RMF) cells were suspended in the gels in co-culture [79]. Krause and colleagues co-cultured MCF10A and RMF cells embedded in a Type I collagen gel, which resulted in the development of branched ducts, but when rBM was added to the collagen in a 1:1 ratio, branching ducts and alveoli were produced [81]. Dréau and coworkers [95] found that the behavior of 3D cultures of normal murine mammary gland (NMuMG) cells, cultured either alone or in combination with mouse mesenchymal stem cells (D1), were dependent on the “matrix” or surrounding biomaterial environment consisting of agarose, collagen, or Matrigel™ alone or in combination. The number of acinar structures was significantly higher in
cultures grown in combination matrices of Matrigel™ and collagen I when compared to cultures grown in Matrigel™ or collagen I alone. No tubular structures were formed when agarose was included in the matrix, regardless of the combination. These works highlight the process of acinar and ductal formation, demonstrating how epithelial cells react in different gel environments, highlighting the fact that the surrounding microenvironment plays a particular role in normal ductal formation and overall regulation of ductal branching and lobular formation in the breast.

The objective of this work was to develop a heterogeneous in vitro 3D breast tissue model. This model will be used as a proof of concept of a modular research model system. Gels of different compositions and concentrations, seeded with mammary epithelial cells, were used to develop the stromal component of the 3D breast tissue model. Three hydrogel matrices, Agarose, Collagen Type I, and Matrigel™, were assessed for use as the 3D tissue model stromal component. Cell viability and generation of acinar-like structures was assessed following seeding with MCF10A cells. The most appropriate hydrogel was selected. This study, first determined the most suitable component(s) for the base of the 3D model and second defined the optimal hydrogel concentration for the 3D system.

Materials and Methods

Cell Culture and 3D Constructs

Normal breast epithelial cells (MCF10A; ATCC, Manassas, VA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Atlanta Biologicals®, Lawrenceville, GA)
supplemented with 10% Fetal Bovine Serum (FBS; Corning, Manassas, VA), 1% Fungizone (Life Technologies, Grand Island, NY), 5% antibiotic/antimycotic (Life Technologies) and Clonetics® MEGM® SingleQuots® (Lonza, Walkersville, MD) supplements including: 2.0 mL BPE, 0.5 mL hEGF, 0.5 mL hydrocortisone, 0.5 mL insulin, and 0.5 mL GA-1000. While in 2D culture, medium was changed every 2-3 days. Once cells were approximately 75% confluent, they were trypsinized (Corning) spun down, and resuspended at 6x10⁶ cells/mL. To generate the 3D culture systems three hydrogel materials: agarose (Lonza, Rockland, ME), collagen Type I (BD Biosciences, Bedford, MA), and Matrigel™ (BD Biosciences) at 1.6% (w/v), 1.6 mg/mL, and 1.6 mg/mL, respectively, were used either alone or in combination as the conditions for the 3D constructs based on previous work by Swamydas et al. [95]. An initial layer (150 µL) of the 3D system components were plated in 8-well chamber slides (Nunc, Rochester, NY) and gelled in an incubator (37°C, 5% CO₂) for 30 minutes to provide a base layer in the system to prevent cells from attaching to the bottom of the well. Then a top layer of 300 µL was mixed with 20 µL of cell suspension (6x10⁶ cells/mL) and plated on top of the base layer. Constructs were placed in the incubator and medium added after 24 hours. Medium was changed every 3 days. MCF10A cells were grown for 10 days within each system and evaluated for cell viability and acinar-like and ductal-like structure formation.

To determine the concentration of the base model 3D system, MCF10A cells (1.2x10⁵ cells/well) were mixed with collagen Type I and Matrigel™ (1:1 v/v) at 1.6, 2.4, 3.2, and 4.0 mg/mL concentrations, using the above method of a bi-layer design. Medium was added 24 hours after seeding and changed every 3 days, harvesting the constructs at
day 10. Repeating Live/Dead and histological analysis of the gels to determine the optimal concentration.

**Live/Dead Assay**

Cell viability was assessed using a LIVE/DEAD® cell viability kit (Invitrogen, Eugene, OR) according to the manufacture’s protocol. A Live/Dead assay solution using a ratio of 2 mL of Dulbecco’s Phosphate Buffered Saline (PBS; Sigma, St. Louis, MO) to 1 µL of 4mM calcein-AM stock and 4 µL of 2 mM EthD-1 stock was prepared. After 30 min incubation with the Live/Dead assay solution the solution was removed and gels were fixed in 4% paraformaldehyde (Sigma) for 30 minutes. Constructs were rinsed twice with PBS to remove any remaining paraformaldehyde. A Zeiss Axiovert 40 (Carl Zeiss MicroImaging, Gottingen, Germany) inverted fluorescent microscope was used to capture representative fluorescent images of cells within the 3D constructs. Images were analyzed using Image J (National Institutes of Health) cell count plug-in.

**Cell Cluster Measurements**

Phase contrast images of the 3D constructs containing cells were taken at the same time as the Live/Dead assay. Images were captured on a Zeiss Axiovert 40 inverted microscope. Using the outline tool in the Zeiss AxioVision software (Version 6.4, Carl Zeiss) borders were drawn around the cell clusters and the corresponding area of the cell clusters was calculated.
**Histological Analysis**

Histological 3D culture samples were fixed in 4% paraformaldehyde and frozen sectioned. Hematoxylin and Eosin (H&E, Richard Allan Scientific, Thermo Fisher Scientific, Kalamazoo, MI) staining was used to evaluate the acinar and ductal-like structure formation within the 3D constructs. A semi-quantitative analysis on a scale of 0-3 with 0 representing clusters with 2 or fewer cells, 1 representing clusters with 2-5 cells, 2 representing 5 or more cells [156] with partial polarization, and 3 representing clusters with 5 or more cells with full polarization (full acinar structure formation) was used to evaluate acinar structure formation in different construct conditions and concentrations. A similar scale was used to evaluate ductal-like structure formation.

Immunofluorescence was used to evaluate the expression of E-cadherin (e.g. confirmation of acinar-like structures and polarization of these cells in the clusters) and Ki-67 (i.e., cell proliferation). Slides were rehydrated using a series of rinses (2X each) in distilled H₂O (MilliQ, Darmstadt, Germany), PBS, and 0.2% Tween 20 (Bio-Rad, Hercules, CA) in PBS. A 1:1 solution of 5N hydrochloric acid (HCl; Ricca Chemical Company, Arlington, TX) and PBS was placed on the samples for 15 minutes to permeabilize the cells. Slides were rinsed 2X with 0.02% Tween 20 and then blocked using 10% goat serum (Sigma) for 30 minutes. The first primary antibody, E-cadherin (1:1600 in 1% goat serum, Pierce™ Thermo Scientific, Rockford, IL), was placed on the samples at incubated at room temperature (RT) for 2 hours. The primary antibody was removed and the secondary antibody, Alexafluor® 594-conjugated AffiniPure F(ab')2 Fragment Goat anti-rat IgG (H+L, 1:200; Jackson ImmunoResearch Laboratories, Inc.,
West Grove, PA), was added to the samples and incubated overnight at 4°C. Alexafluor®
594 was removed and a second primary, Ki-67 (1:1000, Pierce™) was added for 2 hours
at RT. Alexafluor® 488 goat anti-rabbit IgG (H+L, 1:200; Life Technologies, Eugene, OR) was incubated on the samples overnight at 4°C. Samples were rinsed twice with
PBS and ddH₂O. Slides were mounted with ProLong Gold® antifade reagent with DAPI
(Life Technologies). Slides were allowed to dry and imaged using a Zeiss Axiovert 40
inverted fluorescent microscope. E-cadherin was evaluated semi-quantitatively using a
scale of 0-3, 0 indicating no expression and 3 indicating expression within cell clusters
containing 5 or more cells. Single cells and cell clusters containing 4 or fewer cells were
excluded from the analysis. Expression of Ki-67 was evaluated by counting the number
of cells that were expressing the Ki-67 protein. A total cell count was taken in order to
determine the percentage of Ki-67 positive cells.

Statistical Analysis

JMP software (SAS Institute Inc., Cary, NC) was used to run a one-way Analysis
of Variance (ANOVA, α=0.05) to analyze differences comparing all pairs using post-hoc
Tukey-Kramer HSD for differences in 3D construct conditions as well as between 3D
construct component concentrations for cell viability, cell cluster measurements, and
histological analysis.

Results

To determine the most suitable stromal component(s) to use in the 3D breast
tissue model, Agarose, Collagen Type I, and Matrigel™ were used either alone or in
combination and seeded with MCF 10A cells. This combination of hydrogel material and cells formed the stromal component constructs. A series of analyses were used to optimize the type of hydrogel material(s) and the concentration of those components to use in the 3D breast tissue model starting with cell viability. Cell cluster measurements and histological analyses were completed on those constructs not eliminated by cell viability.

**Figure 3.1** Optimization of the component(s) and concentration to be used for the 3D breast tissue model stromal component.

**Part I – Determination of Stromal Component(s)**

**Live/Dead Analysis**

Live/Dead analyses indicated that differences in stromal conditions led to alterations in cell viability and morphology. Conditions that decreased cell viability below 75% included Agarose alone (A), Agarose/Collagen (AC), and Collagen alone (C).
However, the effects of these stroma conditions on cell viability and morphology were not significantly different from one another. The stromal component condition produced from a 1:1:1 ratio of Agarose, Collagen, and Matrigel™ (ACM) had an average MCF10A cell viability of 74.18±10.69%. All other stromal component conditions had a cell viability over 75%, including Agarose/Matrigel™ (AM), Collagen/Matrigel™ (CM), and Matrigel™ alone (M) with 85.3±6.1%, 88.0±7.9% and 93.1±4.4%, respectively. AM, CM, and M conditions were not significantly different from each other, but were significantly different from ACM, C, AC, and A stromal component conditions (Figure 3.2). The average for Agarose/Collagen/Matrigel (ACM) was below 75%, but could not be excluded confidently as determined through statistical analysis. Cell viability for AM, CM, and M conditions were not significantly different from each other, but CM and M were significantly different (p<0.0001) from ACM, C, AC, and A stromal component conditions. The ACM condition was statistically different from both AC and AM at p<0.01.
Figure 3.1 Average percentage of cell viability (** p<0.01, *** p<0.0001). Conditions with cell viability less than 75% were excluded from further analysis.

Histological cell analyses indicated multiple cells clustered together in all conditions, with larger cell clusters being present in M constructs. The only condition to show cells spread across a distance, which indicates ductal-like structure formation, in the cell viability assay occurred in CM constructs (Figure 3.3). Conditions that contained Agarose had fewer cells in a single plane compared to conditions that did not contain Agarose.
Figure 3.3 Live/Dead microphotographs depict cell viability in each condition (magnification = 50x, scale bars = 200µm); (A) Agarose condition – lowest cell viability, (CM) Collagen/Matrigel™ condition showing the ductal-like structures, (M) Matrigel™ condition – highest cell viability. Cells were imaged in multiple planes within the constructs to get a representative cell cluster count for cell viability.

After exclusion of conditions with a viability of 75% four stromal component conditions, ACM, AM, CM, and M, were further analyzed for cell cluster measurements and histological analysis to determine the most appropriate stromal components for the model.

*Cell Cluster Measurements*

Cell cluster measurements were divided into categories of single cells/small clusters (0-125 µm$^2$), medium sized clusters (126-500 µm$^2$), large clusters (500-2000 µm$^2$), and extra-large clusters (>2000 µm$^2$). These structures were confirmed using H&E staining. The greatest number of cell clusters (n=4285) were found in M constructs, but the cell clusters formed at the intersection of the top and bottom layers of the construct leaving all the cell clusters in focus at once. All other construct conditions, AM, ACM, and CM, had cells and clusters that were distributed throughout the 3D constructs. The CM constructs (n=693) and AM constructs (n=477) had the largest percentage of clusters
(Figure 3.4) that fell within the category of large clusters with 48.4±6.5% and 48.5±6.5% respectively, which was significantly higher (p<0.01) than both ACM and M construct conditions. The ACM constructs had the lowest percentage (18.9±2.6%) of large clusters.

![Graph showing percentage of cell clusters by size in conditions with cell viability above 75%](image)

**Figure 3.4** Percentage of cell clusters by size in conditions with cell viability above 75%.

**Histological Analysis**

Histological sections of the constructs were stained with H&E to assess the presence of acinar and ductal-like structures. Ductal-like structures were observed in CM conditions. H&E staining also showed that cells and cell clusters were not distributed
throughout M based constructs, where cells settled on the bottom layer of the gel constructs.

Immunofluorescence staining was used to evaluate cell proliferation (Ki-67) and acinar polarity (E-cadherin) in the stromal component constructs. Regardless of stromal component conditions, low percentages of Ki-67 positive cells indicative of low cell proliferation were measured (Table 3.1). ACM constructs had the lowest cell proliferation after 10 days in culture with 0.40% and M constructs the highest with 1.25%. E cadherin staining was conducted on cell clusters with 5 or more cells, scored on a scale of 0-3. M constructs had the highest semi-quantitative score and ACM with the lowest (Table 3.1). Agarose containing constructs, ACM and AM, had very few cell clusters containing 5 or more cells, 0 and 16 clusters respectively, whereas the Matrigel™ containing constructs had more cell clusters of 5 or more cells. Some M clusters contained over 10 cells per cluster and many of the clusters were polarized with cell nuclei oriented around the outer edge of the clusters with an open center (Figure 3.5).

**Table 3.1** Histological analysis of the effects of stromal component conditions on both cell proliferation and cell cluster polarity as determined by Ki-67 and, E-cadherin staining, respectively.

<table>
<thead>
<tr>
<th>Cond</th>
<th>Ki-67 Counts</th>
<th>E-cadherin Scoring Categories</th>
<th>Total Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#Cells</td>
<td># Pos</td>
<td>% Pos</td>
</tr>
<tr>
<td>ACM</td>
<td>250</td>
<td>1</td>
<td>0.40%</td>
</tr>
<tr>
<td>AM</td>
<td>473</td>
<td>4</td>
<td>0.85%</td>
</tr>
<tr>
<td>CM</td>
<td>655</td>
<td>8</td>
<td>1.22%</td>
</tr>
<tr>
<td>M</td>
<td>1036</td>
<td>13</td>
<td>1.25%</td>
</tr>
</tbody>
</table>
Figure 3.5 E-cadherin staining showing polarity of cell clusters for ACM, AM, CM, and M (nuclei in blue and E-cadherin in red). Magnification = 100x, scale bars = 100 µm.

**Part II – Determination of Stromal Component Concentration**

From Part I of the study, constructs of Collagen/Matrixel™ were selected for the stromal component of the 3D breast tissue model. Next, the concentration of the stromal component must be determined. Initial concentrations of 1.6, 2.4, 3.2, and 4.0 mg/mL were considered. Concentrations with cell viability below 75% were eliminated and then cell cluster measurements and histological analysis were conducted to inform the decision of the appropriate concentration for the 3D breast tissue model.
**Live/Dead Analysis**

Cell viability based on the stromal component concentration was evaluated with a Live/Dead analysis. Collagen/Matrigel™ constructs produced with concentrations of 4.0 mg/mL led to the lowest cell viability i.e., below 75% and significantly lower (p<0.0001) than the cell viability observed in Collagen/Matrigel™ at concentrations of 1.6, 2.4, and 3.2 mg/mL, respectively. The 2.4 mg/ml Collagen/Matrigel™ construct reliably (low variability) produced led to the highest cell viability (78.3±3.4%, Figure 3.6).

![Cell viability graph](image)

**Figure 3.6** Cell viability within 3D construct produced from different concentrations, ranging from 1.6-4.0 mg/mL of Collagen Type I and Matrigel™ in a 1:1 ratio.
Cell Cluster Measurements

The 1.6 mg/mL concentration of Collagen and Matrigel™ had a significantly higher (p<0.01) percentage of cell clusters in the 501-2000 µm² range, with 66.2±8.2% (n=935) of clusters falling within this range. A lower percentage of large area clusters formed for 2.4 and 3.2 mg/mL constructs, 29.0±1.4% (n=1393) and 18.8±5.6% (n=165), respectively.

Figure 3.7 Cell cluster measurements for remaining concentrations with cell viability above 75%.
Histological Analysis

Hematoxylin and Eosin staining showed that Collagen/Matrigel® constructs with a concentration of 2.4 mg/mL had both acinar and ductal-like structures whereas only acinar-like structures were observed in constructs generated using CM at 1.6 or 3.2 mg/mL concentrations (Figure 3.8). Of note, many of the cells and cell clusters were found as a single layer at the intersection of the top and bottom layers of the construct in the 1.6 mg/mL concentration. In contrast, the constructs generated with CM at 2.4 and 3.2 mg/mL concentrations had more uniformly distributed cells and cell clusters. However, in the CM 2.4 mg/mL constructs the ductal-like structures mainly formed at the intersection of the bottom and top layers. The construct generated from CM 3.2 mg/mL concentration led to cell cluster formation uniformly throughout the construct.
Figure 3.8 H&E staining of 1.6, 2.4, and 3.2 mg/mL stromal component concentration constructs (top row: magnification = 50x, scale bars = 200 µm; bottom row: magnification = 200x, scale bars = 50 µm).

Immunofluorescence staining was used to evaluate cell proliferation (Ki-67) and acinar polarity (E-cadherin) in the remaining CM constructs produced at concentrations of 1.6, 2.4, and 3.2 mg/mL. Regardless of stromal component conditions, low percentages of Ki-67 positive cells indicative of low cell proliferation were measured (Table 3.2). CM constructs of 3.2 mg/mL concentration had the lowest cell proliferation after 10 days in culture with 0.82% and 1.6 mg/mL concentration constructs the highest with 1.18%. E cadherin staining was conducted on cell clusters with 5 or more cells, scored on a scale of 0-3. Constructs produced from 3.2 mg/mL components had the highest semi-quantitative E-cadherin score, 88.4, closely followed by the 2.4 mg/mL concentration at 76.1, while the 1.6 mg/mL concentration had the lowest score at 40.4 (Table 3.2). Constructs of 1.6 mg/mL had many cell clusters that formed along the
intersection of the bottom and top layers of the construct (Figure 3.9). These cell clusters did not demonstrate as much polarization as the 2.4 and 3.2 mg/mL concentrations cell clusters containing 5 or more cells. In the 2.4 and 3.2 mg/mL concentration constructs many of the clusters were polarized with cell nuclei oriented around the outer edge of the clusters with an open center (Figure 3.9).

Table 3.2 Histological analyses of stromal component concentrations

<table>
<thead>
<tr>
<th>Conc</th>
<th>Ki-67 Counts</th>
<th>E-cadherin Scoring Categories</th>
<th>Total Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># Cells</td>
<td># Pos</td>
<td>% Pos</td>
</tr>
<tr>
<td>1.6</td>
<td>844</td>
<td>10</td>
<td>1.18%</td>
</tr>
<tr>
<td>2.4</td>
<td>780</td>
<td>9</td>
<td>1.15%</td>
</tr>
<tr>
<td>3.2</td>
<td>736</td>
<td>6</td>
<td>0.82%</td>
</tr>
</tbody>
</table>

Figure 3.9 DAPI staining from Ki-67/E-cadherin staining showing polarity of cell clusters found in 1.6, 2.4, and 3.2 mg/mL concentration constructs (magnification = 100x, scale bars = 100 µm).

Based on consistency, a cell viability greater than 75%, cell cluster measurements that indicate the formation of cell clusters containing 5 or more cells, and histological analysis of the cell clusters constructs formed using CM at a concentration of 2.4 mg/mL is optimal for use in our 3D breast tissue model.
Table 3.3 An overview of why the 2.4 mg/mL Collagen/Matrigel™ concentration was selected for the 3D breast model stromal component.

<table>
<thead>
<tr>
<th>Collagen/Matrigel™ Concentration</th>
<th>1.6 mg/mL</th>
<th>2.4 mg/mL</th>
<th>3.2 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Viability</strong></td>
<td>Cell viability above 75%</td>
<td>Highest cell viability (78.35%) with lowest variability</td>
<td>Cell viability above 75%</td>
</tr>
<tr>
<td><strong>Cell Cluster Measurement</strong></td>
<td>Significantly higher (p&lt;0.01, 63.7%) percentage of cell clusters in the 501-2000 mm² range</td>
<td>29% of cell clusters fell in the 501-2000 mm² range</td>
<td>14.9% of cell clusters fell in the 501-2000 mm² range</td>
</tr>
<tr>
<td><strong>Acinar Structure Formation</strong></td>
<td>Acinar structures formed mostly at the intersection between construct layers</td>
<td>Only concentration to have both acinar and duct-like structure formation</td>
<td>Acinar structure formation found uniformly throughout constructs</td>
</tr>
</tbody>
</table>

Discussion

The objective of this study was to optimize the stromal component(s) and concentration for the 3D model system to best represent the normal microenvironment of the breast. The stromal components of Agarose, Collagen Type I, and Matrigel™ were chosen based on their prevalent use in 3D model systems [79-81, 94, 95, 150]. All three materials chosen have limitations and challenges. Agarose is stiffer than the other materials and only allows limited cell movement and growth. Collagen is remodeled quickly and alterations in the gel structure and stiffness are observed in short periods of time. Lastly, Matrigel™ is a complex ECM with somewhat variable composition derived from tumors, which may include components that adversely affect cell growth within the
3D stromal constructs. Thus we tested different combinations of Agarose, Collagen Type I, and Matrigel™ in varying ratios and densities to produce different constructs.

In producing the 3D constructs, we added a bottom layer of the component(s) to prevent cells from settling out of the construct and attaching to the bottom of the 8-well chamber slides. If the cells settled out of the constructs, then in Chapter 4, the polylactide beads that are added to the 3D constructs would also settle out providing an environment that was more 2D than 3D for the cells cultured. Also, in preliminary studies, if constructs were produced without the bottom layer they would detach and begin floating in 3-5 days. This is problematic because it affects the mechanics of the microenvironment and the mechanical forces the cells sense in the constructs [157]. While a detached gel model is successful in modeling breast cancer [45, 46, 67, 79], our model was intended to stay anchored in the 8-well chamber slides. To prevent this from happening a thin bottom layer was added to the constructs to ensure attachment of the constructs for 10 days. Dhimolea and colleagues found that after 10 days in culture, MCF10A cells contraction of collagen gels decreases and the risk of the gels floating reduces greatly [79].

Monitoring cells in 3D presented some unique challenges. For example, Live/Dead analysis of the cells required longer incubation time as the dye solution had to diffuse throughout the constructs before cells could be effectively stained. One of the key technical limitations of the microscopic analyses of the 3D cell culture conditions is the monitoring of 3D structures spanning multiple microscopy planes. Similar challenges are inherently present in the histological analyses of those 3D structures. Approaches to address those challenges have been reviewed [158]. Here, serial histological sections
provided additional information regarding cell location and organization in the conditions tested.

Based on the cell viability criterion (i.e., above 75%), observations in Part I of this study led to the removal of constructs generated from A, C, and AC from further consideration. As expected, constructs containing Agarose led to low cell viability partly because they only allow limited movement and growth of the cells. Collagen I based constructs also led to low cell viability (below 75%). Indeed, while Collagen Type I is one of the main components of the breast, it has been shown that cell viability is reduced in constructs made solely from Collagen Type I [159]. Matrigel™ containing constructs performed as described previously in many of the assays conducted [95, 160]. Matrigel™ is used in many 3D cultures because of the cellular response to the material [94, 160, 161]. While the Matrigel™ used in the constructs tested here is growth factor reduced, it is nevertheless an ECM secreted by tumor cells, with inherent variability in the composition of ECM components and factors presents [161]. Here, Matrigel™ alone based constructs were found to be a challenging 3D environment mostly due to the lack of thickness the construct provides (Figure 3.5) that prevented incorporation of 300-425μm diameter polylactide beads (see Chapter 4).

The remainder of the analyses were performed on constructs selected on the basis of cell viability, all of them containing Matrigel™. Cell cluster measurements revealed that AM and CM based constructs led to the formation of the highest percentage of cell clusters with area measurements in the large range of 501-2000 μm². While M based constructs led to many more clusters than any other construct (4,285 vs. 425 to 700,
respectively), it led to the lowest percentage of large-range cell clusters. In contrast with other constructs, in the M based constructs, cells formed a compact cell layer facilitating the microscopic monitoring of both single cell and cell clusters. In AM and CM based constructs single cells and cell clusters were dispersed in multiple focal planes and averaged between 10 and 50 cell clusters per stack of fields of view.

H&E staining of the ACM, AM, CM, and M based constructs confirmed that in M based constructs the cells seeded onto the top of the bottom layer and grew at the interface between the 2 layers of the constructs. In all other constructs cells dispersed throughout. ACM based constructs had smaller cell clusters, likely due to the presence of Agarose that form a stiffer matrix holding cells in place and preventing matrix remodeling as mammalian epithelial cells typically do not express the enzyme (agarase) to break down agarose [160]. Agarose molecules within the construct limit interactions and remodeling of collagen and other matrix compounds present in Matrigel™ [160]. Agarose also by limiting cell interactions and signaling by ECM compounds prevented cell proliferation as suggested by low Ki-67 staining. The most Ki-67 positive cells were observed in M constructs and can be contributed to Matrigel™ composition that provides a 3D microenvironment for cell growth and proliferation, but also acted as a 2D substratum allowing cells to concentrate in limited area thereby increasing inter-cell signaling resulting in both proliferation and acinar structure formation. This interpretation is supported by the observations that in M based constructs cells also formed the most E-cadherin positive cell clusters compared to other constructs tested.
Next, the optimal Collagen/Matrigel™ (CM) concentration to be used in constructs was determined. Initial constructs with CM concentrations of 1.6 to 4.0 mg/mL were derived from previous work by Swamydas and colleagues [95]. The initial constructs using a CM concentration of 1.6 mg/mL was selected based on the stiffness of the material and its ability to incorporate polylactide beads without breaking apart or not covering the beads completely as it settled while gelling. CM constructs with concentrations greater than 4.0 mg/mL are significantly more stiff [162]. However, such stiffness is significantly higher than those measured in normal breast tissues and thus were excluded from the present study. Three criteria for the stromal component concentration of the constructs were used to determine the optimal constructs: (1) cell viability within the constructs, (2) cell formation of acinar and ductal-like structures, and (3) proper polarization of the formed structures.

Live/Dead assays demonstrated that the cell viability significantly decreased in CM-based construct generated with 4.0 mg/mL compared to CM based constructs made with either 1.6, 2.4 or 3.2 mg/mL. The observed decrease in cell viability likely has multiple causes including possibly the consequence of a decreased nutrient diffusion but more likely the absence of appropriate microenvironment cues [92].

As the concentration of CM increased in the constructs tested, a decrease in the size and number of clusters was observed. However, in the 1.6 mg/mL CM derived constructs, cells settled to the bottom of the top layer producing a dense layer of cells at the interface between the two layers of the construct. In those conditions, larger cell clusters formed at that location whereas fewer cell clusters were present throughout the
rest of the construct. In the CM constructs derived from 2.4 and 3.2 mg/mL concentrations, cell clusters were smaller, but more dispersed throughout the top layer of the construct. Additionally, in the 2.4 mg/mL CM derived constructs, ductal-like structures were observed mostly at the interface between the bottom and top layers (as seen in Figure 3.8). Some ductal-like structures had cell nuclei that oriented around an open middle that carried over a distance greater than 3-4 cells. In both H&E and fluorescent histological sections, these structures were shaped like commas, where at least one end was tapering while either the center had more space or the opposite end was rounded out similar to a terminal end bud.

The histological analysis conducted confirmed the cell viability and cell cluster measurement results. Ki-67 expression was not expected to be prevalent by day 10 within the constructs as most cells in clusters would be in the process of differentiating or if they were located at the center of a cluster, they may be in the process of apoptosis. Single cells that were isolated would not be proliferating unless they had the right cues from nearby cells and cell clusters. E-cadherin expression should only be observed in cell clusters that are in the process of becoming polarized or differentiating. Results showed that E-cadherin expression increased as the concentration of Collagen and Matrigel™ increased. One explanation might be that in order for cell polarization and differentiation within the cell clusters the cells must be in a stiffer environment or provided signals from adjoining cells through cell-cell junctions.

Based on the data gathered in the present study, the optimal 3D matrix (construct) for modeling breast tissue can be generated using a ratio 1:1 of Collagen I and
Matrigel™ at a concentration of 2.4 mg/mL. This CM derived construct provided MCF10A cells a microenvironment compatible with cell survival, proliferation and differentiation into organized acinar and ductal-like structures.

**Summary**

Optimal MCF10A cell growth occurs in 3D constructs derived from combined collagen I and Matrigel™. Therefore, these components will serve as the stromal matrix of the heterogeneous 3D breast tissue models (see Chapter below). The next studies further our understanding of how combinations of matrix components and polylactide microspheres modulate cell viability. Additionally, normal and cancerous cell behavior is evaluated in a heterogeneous breast tissue model that incorporates polylactide beads in CM-based constructs.
CHAPTER FOUR

A COMPOSITE 3D BREAST MODEL TO DETERMINE THE EFFECTS OF HETEROGENEITY ON NORMAL AND CANCEROUS BREAST EPITHELIAL CELLS

Introduction

Much research using breast tissue models has been conducted to understand how cells become cancerous in the mammary gland, propagate, and eventually metastasize in the body. While this research has provided a better understanding of the underlying mechanisms associated with normal mammary gland function and breast cancer, our understanding of the extracellular matrix (ECM)-cell interactions during normal breast tissue and malignant development remains unclear. Model systems currently in use include clinical and in vivo models, two-dimensional (2D) in vitro models, and three-dimensional (3D) in vitro culture models. In recent years, 3D model systems have been developed for numerous normal tissues and pathologic conditions. Ranging from the use of tissue tumor explants to cell lines in homo or heterotypic cultures, 3D in vitro culture systems provide information on the role of mesenchymal cells, the matrix composition, and density in the formation of acinus- and duct-like structures. However, the need for reliable, versatile, and reproducible 3D in vitro model systems that allow the modulation of the ECM properties remains.

In the normal breast, cellular and ECM components change with development over time. In breast cancer, changes in the microenvironment are indicated through
increased cell populations as well as changes in the ECM. The tumor microenvironment is a heterogeneous environment that is most notably characterized by an increase in fibroblast and myofibroblast activity, increased angiogenesis, increased infiltration of inflammatory cells, and remodeling of the extracellular matrix adjacent to the cancerous cell [28]. Without the influence and inclusion of these cell types and structural components in current 3D models, it is not possible to completely understand how polarity affects tumor initiation, progression, and metastasis.

Normal human and murine mammary epithelial cell culture models have been established to uncover the key components of initiation and maintenance of cell differentiation. Those components include both the mammary and stroma cells and the proteins of the surrounding ECM [69]. In these models the correlation between cell shape and growth and differentiation [45, 46, 66], the role the ECM plays to influence cell phenotype [67, 69, 79, 80], and the interaction between epithelial and stromal cells [13, 25, 81] have been investigated. In most of these 3D culture systems, matrix materials (e.g., collagen, reconstituted basement membrane) allow the formation and maintenance of morphology and functions mimicking those observed in vivo [69]. In particular, these materials support the generation of polarized luminal ductal and acinar structures. However, the use of these matrix materials is challenging, in part because of composition inconsistency as well as variability between lots, and makes the interpretations of observations made in those system more difficult [74].

Cancer models have significantly enhanced our understanding of carcinoma biology in four areas: the formation and maintenance of a hollow glandular lumen and its
disruption by cancer genes, the regulation of apical-basal polarity in normal and cancerous epithelium, the discovery that cell-cell and cell-ECM adhesion pathways interfere with the phenotypic expression during the tumorigenic progression, and the emerging importance of ECM tensional force driving 3D tissue architecture and homeostasis [74]. While these four areas are of great importance in understanding tumor formation and cancer progression, others have also used 3D models to evaluate the cytotoxicity and toxicology of cancer drugs [82-84]. While these models help to further our understanding of breast cancer and its progression, they have yet to investigate the effects of heterogeneity on normal and breast cancer cells.

Overall, current 3D models have allowed insight into the development and function of normal mammary epithelial tissue, tumor formation and cancer progression, as well as evaluation of effective therapeutic agents in cancer treatment. However, there are limitations as to what cellular and matrix components are used, what these models can accomplish, and how well they represent the true in vivo breast conditions. Furthermore, standardization of such culture systems may generate a reliable and reproducible model to test and monitor breast cancer treatments. Thus, a new modular 3D breast tissue model will be developed. The 3D breast tissue model system will be customizable to answer specific questions about the normal mammary microenvironment and its influence in cancer progression.

The development of a composite 3D breast tissue model will allow for determination of the effects of heterogeneity on normal mammary epithelial and cancerous epithelial cells. The mechanical stiffness of the 3D model system, generated
with or without beads embedded in the hydrogel, will be evaluated at the macro- and micro-mechanical level using an Instron mechanical tester and an atomic force microscope (AFM), respectively. MCF10A and MCF7 cell viability, proliferation, and function will also be evaluated in these 3D model systems and the effects of stiffness (i.e. inclusion of the beads) determined.

Materials and Methods

Cell Culture and 3D Breast Model Systems

Normal breast epithelial cells (MCF10A; ATCC, Manassas, VA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Atlanta Biologicals®, Lawrenceville, GA) supplemented with 10% Fetal Bovine Serum (FBS; Corning, Manassas, VA), 1% Fungizone (Life Technologies, Grand Island, NY), 5% antibiotic/antimycotic (Life Technologies) and Clonetics® MEGM® SingleQuots® (Lonza, Walkersville, MD) supplements including: 2.0 mL BPE, 0.5 mL hEGF, 0.5 mL hydrocortisone, 0.5 mL insulin, and 0.5 mL GA-1000. MCF7 cells (ATCC) were cultured in DMEM supplemented with 10% FBS, 1% Fungizone, 5% antibiotic/antimycotic. While in 2D culture, medium was changed every 2-3 days. Once cells were approximately 75% confluent, they were trypsinized (Corning) spun down, and resuspended at 6x10^6 cells/mL. To make the 3D breast model systems Collagen Type I (BD Biosciences, Bedford, MA) and Matrigel™ (BD Biosciences) at 2.4 mg/mL were used in a ratio of 1:1 as the stromal component for the 3D models based on previous work by Swamydas et al. [95]. An initial layer (150 µL) of the 3D system components were plated in 8-well
chamber slides (Nunc, Rochester, NY) and gelled in an incubator (37°C, 5% CO₂) for 30 minutes to provide a base layer in the system. Then a top layer of 300 µL was pipetted into a 1.5 mL microcentrifuge tube either without cells (control gels) or with 20 µL of MCF10A or MCF7 cell suspension (model gels) and incubated for 24 minutes. The microcentrifuge tubes were removed from the incubator and 17 mg of beads were added to half of the model gels containing no cells, MCF10A cells, or MCF7 cells. The beads were mixed into the gels using 1000 µL pipette tips that were modified to have a wider opening, pipetting the beads and gel mixture to mix thoroughly. The model gel contents were then transferred to the 8-well chamber slides and plated on top of the base layer. Gels were placed in the incubator and respective cell medium added after 24 hours. Medium was changed every 3 days. All 3D models were grown for 10 days then evaluated for cell viability, acinar- and ductal-like structure formation and measurements, and macro- and micro-mechanical changes, as well as histological analysis of cell proliferation, cell-cell adhesion, cell-ECM adhesion, and migration markers.

**Live/Dead Assay**

Cell viability was assessed using a LIVE/DEAD® cell viability kit (Invitrogen, Eugene, OR) according to the manufacture’s protocol. A Live/Dead assay solution using a ratio of 2 mL of Dulbecco’s Phosphate Buffered Saline (PBS; Sigma, St. Louis, MO) to 1 µL of 4mM calcein AM stock and 4 µL of 2 mM EthD-1 stock was prepared. After a 30-minute incubation with the Live/Dead assay solution and 10-minute incubation with Hoechst (Invitrogen) the solution was removed and gels were fixed in 4%
paraformaldehyde (Sigma) for 30 minutes. Gels were rinsed twice with PBS to remove any remaining paraformaldehyde. A Zeiss Axiovert 40 (Carl Zeiss MicroImaging, Gottingen, Germany) inverted fluorescent microscope was used to capture representative fluorescent microphotographs of cells within the 3D constructs. Microphotographs were analyzed using Image J (National Institutes of Health).

**Cell Cluster Measurements**

Phase contrast microphotographs of the 3D constructs containing cells were taken at the same time as the Live/Dead assay. Microphotographs were captured on a Zeiss Axiovert 40 inverted microscope. Using the outline tool in the Zeiss AxioVision software (Version 6.4, Carl Zeiss) borders were drawn around the cell clusters and the corresponding area of the cell clusters was calculated.

**Macro- and Micro- Mechanical Analysis**

Macro-mechanical analysis of the 3D breast models was conducted using an Instron mechanical tester (Instron, Norwood, MA). Acellular model systems were produced as stated above in 8-well chamber slides. The chamber slides were then placed on a platform (made in house, using acrylic, to fit over the chuck) on the Instron machine. A 50 N load cell (Instron) and a circular compression platen (diameter = 6 mm) was used to determine how the inclusion of beads within the model changes the mechanical properties. The compression platen was lowered into the sample at 3mm/min until the Instron registered a compressive load of 0.05 N, indicating full surface contact with the models. After contact, the compression platen continued lowering into the samples at 3
mm/min until a strain of 25% was reached based on the average initial thickness of the model gels. Bluehill 2 software (Instron) was used to calculate the 3-15% chord modulus of the gels.

Micro-mechanical analysis of the 3D models was conducted using an atomic force microscope (AFM). Acellular model systems were produced in a similar fashion above, but plated into custom made molds representing an 8-well chamber slide that was 2 mm thick so that the AFM tip could be lowered into the model systems without interference. An MDF-3D-BIO™ AFM (Asylum Research, Santa Barbara, CA) with spherical AFM tips (5 mm radii, spring constant 0.08 N/m; NanoandMore, Lady's Island, SC) was used. Each sample was tested at four points. The elastic modulus was calculated by fitting a Hertz model, as defined in Lance et.al. [162].

**Histological Analysis**

Histological 3D culture samples were fixed in 4% paraformaldehyde and frozen sectioned. Hematoxylin and Eosin (H&E, Richard Allan Scientific, Thermo Fisher Scientific, Kalamazoo, MI) staining was used to evaluate the acinar-like and ductal-like structure formation within the 3D constructs. A semi-quantitative analysis on a scale of 0-3 was used to evaluate acinar structure formation with cell clusters containing at least 5 cells [18] in different construct conditions and concentrations. A semi-quantitative analysis on a scale of 0-3 and reported with a score between 0 and 300, with 0 representing clusters with 2 or fewer cells, 1 representing clusters with 2-5 cells, 2 representing 5 or more cells with partial polarization, and 3 representing clusters with 5 or more cells with full polarization (full acinar structure formation) was used to evaluate
acinar structure formation in different construct conditions and concentrations [163]. A similar scale was used to evaluate ductal-like structure formation.

Immunofluorescence was used to determine the expression of E-cadherin (i.e. the presence of acinar-like structures and polarization of these cells in the clusters), c-Met (migration), Integrinβ1 (cell-ECM adhesion), and Ki-67 (cell proliferation). Frozen section from 3D cultures for each condition tested were first rehydrated using a series of rinses (twice each) in distilled H$_2$O (MilliQ, Darmstadt, Germany), PBS, and 0.2% Tween 20 (Bio-Rad, Hercules, CA) in PBS. Then sections were incubated 15 minutes in 1:1 solution of 5N hydrochloric acid (HCl; Ricca Chemical Company, Arlington, TX) and PBS to permeabilize cells. After rinsing twice with 0.02% Tween 20 and then blocking using 10% goat serum (Sigma) for 30 minutes, slides were incubated at room temperature (RT) for 2 hours with E-cadherin (1:1600 in 1% goat serum, Pierce™ Thermo Scientific, Rockford, IL). Following rinsing step, slides were incubated overnight at 4°C with Alexafluor® 594-conjugated AffiniPure F(ab’)2 Fragment Goat anti-rat IgG (H+L, 1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Next, after a rinsing step, samples were incubated at RT for 2 hours with either Ki-67 (1:1000, Pierce™), c-Met (1:1000, Pierce™), or Integrinβ1 (1:1000, Pierce™). After a subsequent rinsing step, slides were incubated overnight at 4°C with Alexafluor® 488 goat anti-rabbit IgG (H+L, 1:200; Life Technologies, Eugene, OR). After rinses in PBS and ddH$_2$O, samples were mounted in ProLong Gold® antifade reagent with DAPI (Life Technologies). Slides were allowed to dry and imaged using a Zeiss Axiovert 40 inverted fluorescent microscope. E-cadherin and Integrinβ1 expressions were evaluated semi-
quantitatively using the following 0-3 scale with 0 indicating no expression and 3 indicating positive expression, within cell clusters containing 5 or more cells. Single cells and cell clusters containing 4 or fewer cells were excluded from the analysis. Expression of Ki-67 and c-Met was evaluated based on expression in single cells.

**Statistical Analysis**

JMP software (SAS Institute Inc., Cary, NC) was used to run a one-way Analysis of Variance (ANOVA, $\alpha=0.05$) to analyze differences comparing all pairs using post-hoc Tukey-Kramer HSD for cell viability, cell cluster measurements, mechanical properties, and histological analyses.

**Results**

The objective of the study was to investigate whether increased heterogeneity generated by the inclusion of polylactide beads in Collagen/Matrigel™ matrices, within the 3D models, affected both normal and cancerous mammary epithelial cells.

**Live/Dead Analysis**

In CM matrix containing or not polylactide beads, MCF10A or MCF7 cells were seeded and cell viabilities were determined. MCF10A cells showed a significantly higher cell viability compared to MCF 7 cells in either CM matrix or CM matrix + polylactide beads ($p<0.0001$, Figure 4.1). In CM matrix, the cell viability of MCF10A cells was 62.1±20.1%, while the percentage of viable MCF7 cells was 50.1±19.5%. When beads were incorporated into the CM matrix, the MCF10A cell viability increased to 69.1±18.9% while the MCF7 cell viability decreased to 38.3±20.1%. Thus, the addition
of polylactide beads significantly decreased MCF7 cells viability (p<0.05, Figure 4.1). When MCF10A cells were seeded onto CM matrix containing polylactide beads, the cells attached and grew on the beads (Figure 4.1B), whereas MCF7 cells in similar conditions did not. The coverage of the polylactide beads embedded in the CM matrix by MCF10A differed. Nevertheless, MCF10A cells completely coated many of the smaller beads (~200-250 µm Figure 4.1B). As most of the beads were lost during the sectioning process, no histological analysis was performed.
Figure 4.1 (A) Comparison of cell viability in the model systems produced with and without polylactide beads; * p<0.05, *** p<0.0001. (B) Fluorescent image of a 3D Hoechst stained Collagen/Matrigel™ gel embedded with polylactide beads. Arrow points to a polylactide bead surrounded by MCF10A cells; scale bar = 200 µm. (C) Section of an MCF10A model containing beads; nuclei of cells stained with DAPI. Scale bar = 100 µm.
Figure 4.2 Phase contrast microphotographs of the model systems containing MCF10A (left column) and MCF7 cells (right column) with (bottom row) and without (top row) polylactide beads (magnification = 200x, scale bars = 50 μm). In all models without beads, single cells and acinar structures formed throughout (black arrows) while ductal-like structures (orange arrow) formed mostly in models with MCF10A cells.

Throughout the different planes of the CM matrix + beads many different structures formed. Using phase microscopy, 3D cell structures formed were evaluated. In CM matrices throughout the matrix, MCF10A cells were present either as single cells, acinar structures, or ductal-like structures (Figure 4.2, upper left). Many of the ductal like structures spanned multiple planes and seemed to orient directionally (not shown) within
the gels. In the CM matrix + beads, cells were concentrated mostly in the areas between the beads. Consequently, MCF10A cells were densely present in the CM matrix and used the beads as a 2D surface proliferating across them (Figure 4.1B). This contrasted with MCF7 cells that did not adhere to the beads but rather formed larger cell clusters (Figure 4.2, lower left).

**Cell Cluster Measurements**

Cell clusters were categorized as single cells/small clusters (0-125 µm²), medium sized clusters (126-500 µm²), large clusters (500-2000 µm²), and extra-large clusters (>2000 µm²). Seeding of MCF7 cells onto CM matrix embedded with or without beads led to the formation of MCF7 cell clusters with surface areas of 1821 and 1442 µm², respectively. In contrast, in similar conditions, MCF10A cell clusters had surface areas of 631 and 910 µm², respectively.
Figure 4.3 Percentage of cell clusters that fell within the specified cluster measurement ranges of single cells/small clusters (0-125 \( \mu \text{m}^2 \)), medium sized clusters (126-500 \( \mu \text{m}^2 \)), large clusters (500-2000 \( \mu \text{m}^2 \)), and extra-large clusters (>2000 \( \mu \text{m}^2 \)).

Macro- and Micro-mechanical Testing

Mechanical testing was conducted on acellular model systems with and without beads. Instron testing was used to determine if incorporation of polylactide beads influenced the macro-mechanical properties of the model systems. No significant differences were observed in the mechanical properties of the models when comparing models without beads to models containing beads. Determined by Instron mechanical
testing the 3% chord modulus of the models was 66.2 ±128.2 Pa (n=12) and 37.3±68.9 Pa (n=8) for models without and with beads, respectively. AFM, used to determine the micro-mechanical properties of the model systems, indicated that inclusion of beads did not affect the micro-mechanical properties of the systems. The modulus was determined to be 3.04±0.37 Pa and 2.84±0.99 Pa for the CM matrix without (n=4) and with polylactide beads (n=4), respectively.

**Histological Analysis**

MCF10A cells formed acinar and ductal-like structures as shown by H&E staining, regardless of the inclusion of beads, within the CM matrix. The number of structures formed was semi-quantified. MCF10A cells seeded in CM matrix containing beads had the highest acinar structure whereas as MCF10A developed less acinar structures when seeded in CM matrix (no beads) (score of 16.5 and 13.1, n≥6). MCF7 cells in similar conditions led to acinar structure scores of 14.4, and 13.1 (n=6) respectively. Ductal-like structure quantification of H&E stained samples also indicated that MCF10A cells seeded in CM matrix with beads led to the highest number of ductal-like structure (16.5 vs. 11.7 in CM matrix without beads). MCF7 cells also led to ductal-like structures more so in CM matrix containing beads (12.6 vs. 9.7, respectively). While these observations have a high variability (no significant differences was observed between cells or conditions for score for acinar and ductal structures), the inclusion of polylactide beads in the CM matrix tends to affect the number of acinar and ductal-like structures.
Furthermore, the expression of Ki-67, E-cadherin, Integrinβ1, and c-Met were investigated to assess cell proliferation, cell-cell attachment, cell-ECM attachment, and migration. The number of Ki-67 positive cells a marker of cell proliferation, was between 0.9 and 1.9% regardless of cell or condition tested. More proliferation was observed with MCF10A cells (1.9% and 1.7% for CM matrix with and without beads, respectively) compared to MCF7 cells in similar conditions (1.4% and 0.9% in CM matrix with and without beads, respectively). E-cadherin was analyzed in cell clusters containing 5 or more cells. CM matrix containing beads led to higher E-cadherin expression, 107 and 83 for MCF10A and MCF7 cells, respectively. In CM matrix (no beads) E-cadherin expression was lower (81 and 71 for MCF10A and MCF7 cells, respectively). No significant difference was observed between the conditions tested regardless of the embedding of beads or the cells tested. Nevertheless, these observations strongly suggest that increasing the heterogeneity of the CM matrix by the addition of polylactide beads promote acinar structure formation especially by MCF10A cells. The expression of integrin β1 and c-Met was inconclusive possibly because of limitations in the fluorescent filters used.
Figure 4.4 E-cadherin staining of model systems. First column includes nuclei stained using DAPI. Second column, same sections stained with E-cadherin primary antibody. Third column, combined images overlaying the DAPI stained nuclei and the E-cadherin images. Magnification = 100x, scale bars = 100 µm.
Discussion

Criteria for a optimized breast tissue 3D model included the ability to easily and quickly change the stromal components to mimic different stages in breast development and breast cancer progression. Further, knowing that the breast cancer environment is heterogeneous, different materials and/or cell types were incorporated within the 3D model. The rationale associated with the incorporation of polylactide microspheres in the 3D model was two-fold. First, the polylactide beads are much stiffer [164] compared to the hydrogel stromal components composed of Collagen Type-I/Matrigel™ [162]. Second, as the beads degrade they release lactic acid into the surrounding microenvironment helping mimic an acidic microenvironment normal in breast cancer. While lactic acid release was not a parameter studied in the present set of experiments designed to develop an heterogeneous 3D environment, lactic acid release profiles likely would be similar to that presented in Chapter 2. The latter demonstrates that polylactide beads can be used in multiple facets to study different effects.

During the formation of the 3D matrix, a bi-layer approach was used to prevent the polylactide beads from settling out of the gels to the bottom of the wells. To get the beads to stay suspended throughout the top layer of the constructs, the Collagen/Matrigel™ components were incubated for 24 minutes and then beads were mixed into the pre-gelled matrix. Since the gels were “pre-gelled” prior to adding the beads, when the beads were mixed in the gel separated into chunks. After the beads are mixed in, the chunks of pre-gelled components intermix within the non-gelled components limiting the space where the beads can fall through the matrix, and thus
keeping them suspended within our top layer. This method of mixing the beads into the gel needs to be optimized further as differences in incubators and mixing methods influence how well the pre-gelled phase keeps the beads in suspension.

In 3D CM matrix embedded with beads, the MCF10A cells are close enough to the beads to sense the increased stiffness leading to cell migration and attachment to the beads. In contrast, MCF7 cells did not attach to the beads in similar conditions. This is most likely due to the anchorage dependence of MCF10A a behavior that is decreased or lost in MCF7 cells.

Cell viability and cell cluster were used to ensure that the CM derived constructs generated consistently mimicked observed made earlier (see Chapter 3). Inclusion of the beads in the CM matrix did not seem to affect the cell viability. In fact an increased the MCF10A cell viability as the beads provided additional surface for cells to adhere and proliferate. Further, cell cluster observations made in the CM matrices embedded with polylactide indicated that the pre-gelling process did not affect cell viability or how cells clustered together. However, the double mixing step may have promoted the migration of cells out of the CM matrix resulting in fewer clusters of cells as compared to our previous observations where the top layer was plated and gelled without the intermediate “pre-gelling” step before adding beads (See Chapter 3).

Mechanical testing of the CM matrix with or without embedded polylactide beads was conducted. However, due to the properties of the CM matrices, they could not be removed from their plastic wells for mechanical testing. Thus, the CM matrices with or without embedded beads were tested within the vessel wells. During the testing, the
beads cut through the CM matrix due to the stiffness differential between the beads and CM matrix and the pockets of liquid within the gels. To improve the accuracy of measurements of the stiffness, an improved method of generating the CM matrix ± beads should be implemented. The steps associated with the generation of CM matrix ± beads also limited micro-mechanical testing methods. Normally when conducting AFM, gels are tested in an unconfined manner, but due to the properties of the gels only confined tests could be run. Further, the beads used in the CM matrix were on average 300 µm in diameter, so the CM matrix had to be at least 500 µm thick to ensure that the beads were suspended in the matrix. At this thickness, it was not possible to image through the matrix while taking measurements. This made it impossible to determine exactly where measurements were being taking within the matrix. To make more definitive conclusions about how the incorporation of the beads affected the stiffness of the microenvironment detected by the cells, more measurements at specific locations within the 3D microenvironment should be undertaken.

The formation of cell clusters within the 3D conditions tested either acinus- or duct-like structures was demonstrated using H&E staining and DAPI nuclear staining. MCF10A cells in the 3D condition tested formed cell clusters and cell clusters with 5 or more cells started to form a polarized structure with hollow center. In contrast, when MCF7 cells were cultured in those 3D conditions clustered cells rarely formed polarized structures. Indeed, some of the MCF7 cell clusters had over 20 cells but no organized structure. Further, MCF10A cells cultured in 3D CM matrix with beads led to clustered cells expressing the greatest amount of E-cadherin suggesting proper polarity of acinar
structures. While E-cadherin expression as seen in MCF7 cell clusters generated in similar 3D culture conditions, the expression was not as localized between the cells contrasting with the observation made in MCF10A cell clusters.

The embedding of beads to increase matrix heterogeneity led to increased cell density in the matrix areas between the beads. These increased cell densities were associated with the formation of larger clusters. This could be in part due to increased signaling between cells through increased growth factor concentrations and/or direct cell-to-cell contacts. Additionally, this could also be explained in part by the variation in the mechano-transduction generated within the 3D CM matrix by the addition of beads. Indeed, beads have a higher mechanical stiffness compared to Collagen and Matrigel™ and, thus the cells may be responding to this localized stiffness increase. Further research should be conducted to confirm the importance of mechano-transduction in the cell behavior.

**Summary**

A tunable 3D breast tissue model was generated that incorporate polylactide beads to assess the effects of matrix heterogeneity on the behavior of both normal mammary epithelial and cancerous epithelial cells. Further work should focus on refining the process in which beads are suspended in the Collagen/Matrigel™ matrix and on the optimization of the number of cells to be seeded in the 3D model. Additional validation of the heterogeneous 3D CM matrix + bead environment as breast tissue matrix is required.
CHAPTER FIVE


MINORITY, UNDERGRADUATE STUDENT’S DEVELOPMENT OF A RESEARCH IDENTITY

Introduction

The increasingly technical global economy and rapidly changing national demographics have presented the US with a critical workforce shortage in the educational areas of Science, Technology, Engineering, and Mathematics (STEM) [165]. As the country attempts to maintain its leadership position in research, development, and innovation, studies reported in the literature have made clear that US production of STEM graduates needs improvement. Employment in STEM fields grew by 23% between 1994 and 2003, compared to only 17% for non-STEM fields; nonetheless, the US is now struggling to meet the rapidly increasing demand for STEM workers [166]. The continued need to remain globally competitive and the fact that 39% of people in the US under 18 are persons of color (U.S. Census 2000) underlie the urgent need for
colleges and universities to improve their efforts to graduate minority students in STEM disciplines [167].

Along with an increased interest in undergraduate degree attainment, there is significant interest in increasing the number of graduate degrees awarded in STEM, particularly to underrepresented minority students [168]. STEM education researchers have commonly defined underrepresented minorities (URMs) as African American, Hispanic/Latino, or Native people, including Native American, Alaska Native, Native Hawaiian, and Pacific Island individuals [168]. The drive to increase the number of graduate degree recipients is directly relevant to research and innovation goals and national economic interest [168]. One of the strategies employed for increasing the number of URM students in STEM has been the introduction and promotion of undergraduate research programs. Both federal and private agencies have committed to investing significant funding into these programs, as they have been reported to increase student intention of enrolling in graduate or professional schools [169, 170]. The National Science Foundation (NSF), through the Emerging Frontiers in Research and Innovation (EFRI) program is one of these program examples. The NSF has awarded supplemental funding for Research Experience and Mentoring (EFRI-REM) outreach programs with a focus on developing research in STEM disciplines. The REM program provides a training program for researchers and supports the national priority to attract and retain a diverse STEM population.

Many researchers have explored potential causes for minority student underrepresentation in the STEM disciplines. Issues such as preparedness deficiencies,
stereotype threats, familial or societal expectations, or low esteem have been presented as potential reasons for low interest, aspiration, admission, retention, and persistence in STEM of ethnic minority students [171-178]. Diminished pursuit of graduate studies for URM students were thought to be largely related to financial hardship post baccalaureate; however, further research has shown that URMs in STEM also may not see graduate or professional schools as significantly beneficial to career aspirations and interest [179].

Undergraduate research programs have been shown to be effective in fostering the interest, skills, and aspirations that may develop into pursuit of graduate / professional school and potential research and innovation careers [169, 170]. The concept of “communities of practice” described by Wenger supports the idea that participation in different communities and experiences affects participant identity development [180]. The National Science Board members, in their report “Moving Forward to Improve Engineering Education”, propose participation in research experiences, specifically in the freshman and sophomore years, as a desirable means to engage URM students in the community of STEM [181]. These experiences aim at introducing students to STEM and broadening their education while improving retention. One aspect that has been highlighted by undergraduate research experts is identity development within the context of STEM [169]. Attention to identity has increased, specifically within the sciences [182], as work continues toward increasing the STEM population and workforce. Investigators have suggested that participation in an undergraduate research program results in domain identity related to the area of participation [183]. It is this identity development process that fosters feelings of preparedness for future research and creation
of a research identity integrated with a student’s STEM domain-specific identity. Domain-specific identity, comprising three dimensions of student beliefs - interest, performance/competence, and recognition, has been used to observe math, physics, and general sciences identities [184-189].

It has been suggested that engineering research is advanced by an increasingly diverse population of STEM researchers aiming to complete interdisciplinary research objectives. Diversity of thought and perspective is a prerequisite to addressing the world’s complex problems. There is a significant need for training and development of diverse populations to answer evolving research questions. To develop researchers one must understand how their identity, which is based on a researcher’s belief about his/her performance, competence, recognition by others, and interest, influences their feelings of preparedness for research experiences. It is our assertion that students that feel more prepared for research experiences are more likely to participate in future research experiences.

Our study focused specifically on a research training opportunity funded by the National Science Foundation (NSF) Emerging Frontiers in Research and Innovation (EFRI) program. We hosted an undergraduate research program to introduce underrepresented, early-stage undergraduates to research using our tissue-engineering laboratory as the backdrop. The program was designed as a first step toward full engagement in undergraduate research, i.e., to lower the perceptual barriers to participation, provide tools, and promote confidence to pursue rigorous research. We assessed how URM student participation in an introductory, interdisciplinary tissue
engineering research experience prepared the students for future research endeavors in their own majors. Student experiences highlighted in this study pertain to their participation in an NSF-EFRI Research Experience and Mentoring (REM) program during the academic year and subsequent research or professional experiences the following summer. These research or professional experiences included Research Experience for Undergraduates (REU) programs, cooperative education (co-op) experiences, institutionally-funded research programs, and other summer employment opportunities. The purpose of this study was to understand how a student’s perception of preparedness is influenced by the student’s science and engineering identity, based on their participation in interdisciplinary research.

Methods

Clemson University (CU) and the University of North Carolina at Charlotte (UNCC) receive NSF:EFRI funds that allow CU engineering researchers and UNCC biology researchers to work together to build and analyze breast cancer benchtop tissue test systems. The NSF distributed a competitive call for supplemental funding proposals to funded EFRI grantees, termed Research Experience and Mentoring; the goal of the opportunity was to further the progress in EFRI topic areas while broadening participation of underrepresented groups in STEM fields [190]. CU and UNCC successfully competed and were awarded REM funding for the 2012-2013 academic year. The objective of the CU-UNCC NSF:EFRI-REM program was to introduce URM undergraduate students, especially those at an early transition point in their academic career, to a positive introductory research experience that would inspire confidence and
create credentials for future research opportunities. Students with no prior research experience were specifically recruited. Each student participated in 1 semester of the NSF:EFRI-REM program, either in the fall or spring, and each had the opportunity to apply to participate in a 10-week REU program encompassing experiences at both universities. All REM participants were encouraged to apply for other REU programs across the nation, other summer research experiences, or co-operative education experiences.

During the school year, URM students were recruited through CU and UNCC-supported offices with focus on underrepresented student recruitment and retention in STEM. Students were encouraged to apply for the REM opportunities and were selected based on their interest in the program, their ability to communicate how this experience might influence them, and their academic progress (including performance in STEM classes). The principal investigators, graduate student and staff mentors reviewed applications, and the undergraduate students, termed by NSF the Research Participants (RPs), were invited to participate in the REM program. Each semester, the REM program began with a Research Studio lasting approximately 8 hours before students began the laboratory experience. The Research Studio began with a pre-survey regarding research experiences and then included an introduction of tissue test systems and overall EFRI project goals, completion of laboratory safety training, an introduction to research ethics, technical writing, and basic laboratory practices, participation in a team building exercise, discussion of the projects to which each student would be exposed, and discussion of the expectations for and of RPs. Once RPs completed the Research Studio, each RP was
paired with a graduate student mentor and the mentor’s project. After completion of the Research Studio, each student was required to spend 3 hours on lab/research-related activities each week during the semester. Weekly professional development exercises introduced the RPs to a variety of research-related skills and topics. Students ended the semester with a rapid fire podium presentation and poster presentation at Networking Day, a day where all students, graduate student mentors, faculty mentors, and external support mentors gathered to discuss research activities and outcomes of the REM program. A research experience post-survey was given, following the 15-week semester, to assess the student experience.

The joint EFRI:REU began in late May for a 10-week period and included two RPs from CU and two RPs from UNCC. The first 5 weeks were spent in the engineering laboratories at Clemson University, and the second 5 weeks in cancer biology laboratories at the University of North Carolina at Charlotte. Each REU weekday consisted of approximately 8 hours of lab/research-related activities. All EFRI:REU RPs gave poster presentations overviewing their research at the end of the REU and all EFRI:REU students were invited to apply to participate in/present at the NSF and American Association for the Advancement of Science-sponsored Emerging Researchers National Conference in STEM in Spring 2014.

Each academic semester, eight RPs participated in the REM program, four at each university. The demographics of the population were determined by information submitted in the REM applications, including gender, ethnicity, college level, major (with concentration), and minor. Of the 16 RPs in the REM program, three were male and
thirteen female. Students self-indicated their ethnicity on the application as: Hispanic or Latino (regardless of race), American Indian or Alaska Native, Asian American, Black or African American, White, or Native Hawaiian or Other Pacific Islander. The population included two Hispanic or Latino students, thirteen Black or African American students, and one Asian American student. RPs included thirteen sophomores, one freshman, and two juniors. Clemson University RPs were students seeking either engineering or science degrees while University of North Carolina at Charlotte RPs were students pursuing science degrees. Out of the 16 participants, six were obtaining engineering degrees in industrial engineering, computer engineering, environmental engineering, and chemical engineering, while two had yet to declare an area of focus and were still in the general engineering program. The other 10 students were pursuing science degrees; specifically, chemistry (1) and biology (9). Of the 16 students that participated in the REM program, two from each university were selected to participate in the summer REU program. All of the REU participants were female and three of the four were science majors. The REU RPs, all females, identified their ethnicities as Asian American (1), Hispanic/Latino (1), and Black or African American (2).

The REM program pre- and post-survey (Table 5.1) was administered to CU students only and was used to assess how the REM program influenced students’ feelings of preparedness for future research programs and to assess skills gained through the program. Pre- and post-surveys included: participant demographics (i.e. name, educational aspirations, major), research preparedness items (Q1-Q5, Table 5.1), research skills assessment items (Q6-Q10, Table 5.1), and short answer questions pertaining to
laboratory practices and attributes. Items Q1-Q5 were evaluated on a Likert-type scale, with 1 indicating strong disagreement and 5 indicating strong agreement. Questions Q6-Q10 were answered as yes/no and evaluated in binary format, where yes = 1, no = 0. An Analysis of Variance ($\alpha=0.05$) was used to analyze pre- to post- response as a whole as well as with respect to academic term.

**Table 5.1 Pre- and Post-Preparedness Survey Items**

<table>
<thead>
<tr>
<th>Item #</th>
<th>Research Survey Items</th>
<th>Research Preparedness Items</th>
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<tbody>
<tr>
<td>Q1</td>
<td>I feel prepared to participate in a research program at the college level</td>
<td></td>
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<tr>
<td>Q2</td>
<td>Research is an important part of my undergraduate education</td>
<td></td>
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<tr>
<td>Q3</td>
<td>Continuing research as a graduate student would be beneficial to me and my career goals</td>
<td></td>
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<tr>
<td>Q4</td>
<td>I feel comfortable speaking about or presenting scientific research in an academic setting</td>
<td></td>
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<tr>
<td>Q5</td>
<td>I am familiar with ways to find research opportunities</td>
<td></td>
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<tr>
<td>Q6</td>
<td>Have you ever applied to participate in a research program at the college level (other than to this program)?</td>
<td></td>
</tr>
<tr>
<td>Q7</td>
<td>Have you ever participated in a research program at the college level?</td>
<td></td>
</tr>
<tr>
<td>Q8</td>
<td>Have you ever used scientific journals as part of a research project?</td>
<td></td>
</tr>
<tr>
<td>Q9</td>
<td>Have you ever given a research presentation (oral or poster)?</td>
<td></td>
</tr>
<tr>
<td>Q10</td>
<td>Have you ever attended or participated in a research conference or meeting?</td>
<td></td>
</tr>
</tbody>
</table>

An identity survey, given to REM participants from both CU and UNCC, was used in order to assess identity development after participation in the REM program. Former REM RPs were given an identity pre-survey in May before they started their summer activities. Eleven of the 16 participants completed the pre-survey. Students that completed the pre-survey were given a follow-up identity post-survey the first week of the fall semester following the various summer activities. Ten post-surveys were completed; five by science majors and five by engineering majors. The summer
experiences of these 10 RPs included REU (4), co-operative education experience (2), summer research experience (2), and non-research related activities (2). The identity survey questions were adapted from the Sustainability and Gender in Engineering (SaGE) survey [184-186, 188, 189]. Questions for engineering and research identity were adapted from these valid and reliable survey items with the help of experts in engineering education research. The survey items were divided into three identities; science, engineering, and scientific research. The same questions were asked to investigate each identity, substituting the word science, engineering, or scientific research in each item. Each question was evaluated on a Likert-type scale, the far left of the scale anchored as “Strongly Disagree” (1.0) and the far right of the scale anchored as “Strongly Agree” (7.0). Questions in the survey pertaining to preparation were categorized based on the type of future experience, and included research, non-research, and graduate research questions. Statistical analysis of the data was conducted using Analysis of Variance (ANOVA, $\alpha=0.05$) to determine statistical differences between majors, for both pre-survey and post-survey responses, and within majors (pre- to post-response).

Results and Discussion

REM Program Survey

The REM survey results indicated an overall positive increase in student survey responses pertaining to preparedness in research and its influence on future research activities. The results suggest the students felt more prepared to participate in research and felt more comfortable with some of the tasks (e.g. presenting research) required when
completing research activities. Significant increases were seen in two of the responses from pre- to post-survey, specifically “I feel prepared to participate in a research program at the college level” and “I am familiar with ways to find research opportunities” (Figure 5.1A), with p-values of 0.0172 and 0.0075, respectively. Further, a significant increase (p=0.0089, Figure 5.1B) was seen in response to the question “Have you ever used scientific journals as part of a research project”.

Figure 5.1. REM research program survey results. Asterisk indicates significant differences between pre- and post-survey items at p<0.05.
The NSF:EFRI–REM program resulted in a significant increase in students’ feeling of preparation for future research experiences. By introducing students early in their career to research activities, we begin to give them the tools necessary to succeed in future research endeavors. Teaching skills that will be used in both academic and research settings allows students to gain confidence in themselves and their abilities to succeed in similar situations, thus preparing them for STEM careers.

Identity Survey

Results from pre- and post- identity surveys suggest that science and engineering identities are related to each other, as well as to the development of research identity. The analyses shown below in Tables 5.2, 5.3, and 5.4 compare survey item responses for science majors versus those of engineering majors. For example, the first line of Table 5.2 indicates that pre-survey responses for science majors yielded a mean (µ) response of 6.80, while engineering majors had a mean response of 7.0. These responses were related to the question, “To what extent do you disagree or agree with the following statement? I am interested in learning more about science.” The difference in science majors’ and engineering majors’ pre-responses yielded a non-significant p-value of 0.3466 after ANOVA testing. Similarly, post-responses also had a non-significant difference (p=0.1720) with means of 7.0 and 6.4 for science and engineering, respectively. Analyses completed comparing pre- to post-responses within majors were conducted but are not included in table format. Only two of the survey items were significant; descriptions of these items are included below.
Table 5.2: Self-Reported Interest Items Comparing Science and Engineering Majors.
Symbols \( \mu \) and \( \sigma \) represent the mean and standard deviation of the population, respectively.
P-Values highlighted indicate significant differences between majors.

<table>
<thead>
<tr>
<th>Survey Item</th>
<th>Pre-Summer Experience</th>
<th>Post-Summer Experience</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>( \sigma )</td>
<td>( \mu )</td>
</tr>
<tr>
<td>To what extent do you disagree or agree with the following statements?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I am interested in learning more about science</td>
<td>6.80</td>
<td>0.45</td>
<td>7.00</td>
</tr>
<tr>
<td>I enjoy learning science</td>
<td>7.00</td>
<td>0.00</td>
<td>6.60</td>
</tr>
<tr>
<td>To what extent do you disagree or agree with the following statements?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I am interested in learning more about engineering</td>
<td>5.00</td>
<td>1.73</td>
<td>7.00</td>
</tr>
<tr>
<td>I enjoy learning engineering</td>
<td>4.40</td>
<td>0.89</td>
<td>6.80</td>
</tr>
<tr>
<td>To what extent do you disagree or agree with the following statements?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I am interested in learning more about scientific research</td>
<td>6.60</td>
<td>0.55</td>
<td>6.40</td>
</tr>
<tr>
<td>I enjoy learning scientific research</td>
<td>6.60</td>
<td>0.55</td>
<td>6.40</td>
</tr>
</tbody>
</table>

Two questions addressed the aspect of domain-specific interest. The questions “I am more interested in learning more about …” and “I enjoy learning …” revealed significant differences between the science and engineering majors when the topic was engineering, for both the pre-survey and post-survey responses (Table 5.2). While the science and engineering majors’ means for both the pre-survey and post-survey are nearly equal for science and scientific research identity items, the engineering identity items reveal a significant difference. Engineering students identified much more interest in engineering topics as compared to the science students.

Questions were posed about RP competence in the three areas of science, engineering, and research. While the survey items addressed competence, performance
was not included in this analysis as there were no grades assigned to student research outcomes. Regarding competence (Table 5.3), it was found that science students felt significantly less confident in their ability to understand science outside the classroom after their summer experience. This could be, in part, because more in-depth research and summer experiences broadened the students’ perspectives to what is required to understand science and conduct scientific endeavors outside the classroom.

The other area of significance of note within competence from Table 5.3 is in the differences of “understanding engineering”, “understanding concepts studied in engineering”, and “being able to overcome limitations and setback/obstacles in engineering”. Significant differences were seen by science students in all of these categories except “I am confident that I can understand engineering in the laboratory”. The results may be explained, in part, by the fact that three of the five science majors who completed the surveys participated in the joint summer EFRI:REU program. The summer EFRI:REU incorporated an engineering component and thus many of the science majors were exposed to engineering problems. The engineering students were significantly more confident in every one of these categories after their summer experiences. This result was expected, as all but one of the engineering RPs that completed the survey were involved in summer research that focused on some aspect of engineering, most of them in areas of their own majors. These RPs gained experience and knowledge in their specific engineering areas and thus would have strengthened identity in the area of competence. The engineering question that did not result in significant differences when comparing majors both pre- and post- summer experience was
“Engineering makes me nervous”. However, the science students, when comparing their pre- to post-summer experience responses with respect to science, did indicate significantly less (p = 0.0046, data not shown in table format) nervousness post-summer.

One of the major foci for this study was the development of feelings of preparedness for future research opportunities. Results shown in Table 5.3 below indicate that both engineering and science majors are relatively confident in their level of preparedness for future research. This is signified by means above 6.0 for nearly every preparedness item. There was no significant difference between engineering and science majors in terms of preparedness, suggesting the program helped to develop confidence in research preparedness across the spectrum of represented majors. The mean confidence level of science majors with respect to preparedness items was also slightly higher (though not significant), again indicating that perceived research outcomes may be more closely related to the skillset students identify with science.
Table 5.3: Self-Reported Competence Items Comparing Science and Engineering Majors.

Symbols $\mu$ and $\sigma$ represent the mean and standard deviation of the population, respectively.

P-Values highlighted indicate significant differences between majors.

<table>
<thead>
<tr>
<th>Survey Item</th>
<th>Pre-Summer Experience</th>
<th>Post-Summer Experience</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>$\sigma$</td>
</tr>
<tr>
<td>To what extent do you disagree or agree with the following statements?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I am confident that I can understand science in class</td>
<td>6.40</td>
<td>0.55</td>
</tr>
<tr>
<td>I am confident that I can understand science in the laboratory</td>
<td>6.20</td>
<td>0.45</td>
</tr>
<tr>
<td>I am confident that I can understand science outside of class</td>
<td>6.40</td>
<td>0.55</td>
</tr>
<tr>
<td>I understand concepts I have studied in science</td>
<td>6.60</td>
<td>0.55</td>
</tr>
<tr>
<td>Science makes me nervous</td>
<td>4.20</td>
<td>1.79</td>
</tr>
<tr>
<td>I can overcome limitations in science</td>
<td>5.60</td>
<td>0.89</td>
</tr>
<tr>
<td>I can overcome setbacks/obstacles in science</td>
<td>5.60</td>
<td>0.89</td>
</tr>
<tr>
<td>To what extent do you disagree or agree with the following statements?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I am confident that I can understand engineering in class</td>
<td>4.40</td>
<td>0.55</td>
</tr>
<tr>
<td>I am confident that I can understand engineering in the laboratory</td>
<td>5.20</td>
<td>1.30</td>
</tr>
<tr>
<td>I am confident that I can understand engineering outside of class</td>
<td>4.40</td>
<td>0.89</td>
</tr>
<tr>
<td>I understand concepts I have studied in engineering</td>
<td>4.40</td>
<td>0.89</td>
</tr>
<tr>
<td>Engineering makes me nervous</td>
<td>4.60</td>
<td>0.89</td>
</tr>
<tr>
<td>I can overcome limitations in engineering</td>
<td>4.00</td>
<td>0.71</td>
</tr>
<tr>
<td>I can overcome setbacks/obstacles in engineering</td>
<td>4.00</td>
<td>0.71</td>
</tr>
<tr>
<td>To what extent do you disagree or agree with the following statements?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I am confident that I can understand scientific research in class</td>
<td>6.40</td>
<td>0.55</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th></th>
<th>6.40</th>
<th>0.55</th>
<th>6.00</th>
<th>0.00</th>
<th>0.1411</th>
<th>6.60</th>
<th>0.55</th>
<th>6.20</th>
<th>0.84</th>
<th>0.3972</th>
</tr>
</thead>
<tbody>
<tr>
<td>I am confident that I can understand scientific research in the</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>laboratory</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I am confident that I can understand scientific research</td>
<td>6.00</td>
<td>0.71</td>
<td>5.60</td>
<td>0.55</td>
<td>0.3466</td>
<td>6.40</td>
<td>0.55</td>
<td>6.00</td>
<td>1.00</td>
<td>0.4554</td>
</tr>
<tr>
<td>outside of class</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I understand concepts I have studied in scientific research</td>
<td>6.20</td>
<td>0.45</td>
<td>5.80</td>
<td>1.10</td>
<td>0.4714</td>
<td>6.60</td>
<td>0.55</td>
<td>5.80</td>
<td>0.84</td>
<td>0.1114</td>
</tr>
<tr>
<td>Scientific research makes me nervous</td>
<td>4.80</td>
<td>1.30</td>
<td>4.00</td>
<td>2.00</td>
<td>0.4751</td>
<td>3.40</td>
<td>1.52</td>
<td>3.40</td>
<td>1.82</td>
<td>1.0000</td>
</tr>
<tr>
<td>I can overcome limitations in scientific research</td>
<td>5.80</td>
<td>0.45</td>
<td>6.00</td>
<td>1.22</td>
<td>0.7404</td>
<td>6.20</td>
<td>0.84</td>
<td>6.40</td>
<td>0.55</td>
<td>0.6666</td>
</tr>
<tr>
<td>I can overcome setbacks/obstacles in scientific research</td>
<td>5.80</td>
<td>0.45</td>
<td>6.40</td>
<td>0.55</td>
<td>0.0943</td>
<td>6.40</td>
<td>0.55</td>
<td>6.40</td>
<td>0.55</td>
<td>1.0000</td>
</tr>
</tbody>
</table>

To what extent do you disagree or agree with the following statements? I feel prepared to participate in...

| Academic research program (e.g. REU, research experience)         | 6.40 | 0.55 | 6.00 | 0.71 | 0.3466 | 6.80 | 0.45 | 6.40 | 0.89 | 0.3972 |
| offered during the summer                                        |      |      |      |      |        |      |      |      |      |        |
| Academic research programs offered during the academic year      | 6.40 | 0.55 | 6.20 | 0.45 | 0.5447 | 6.80 | 0.45 | 6.60 | 0.55 | 0.5447 |
| Non-academic research program (e.g. scientific or engineering   | 6.20 | 0.45 | 6.40 | 0.55 | 0.5447 | 6.60 | 0.55 | 6.40 | 0.89 | 0.6811 |
| based co-operative education experience or internship) offered   |      |      |      |      |        |      |      |      |      |        |
| during the summer                                               |      |      |      |      |        |      |      |      |      |        |
| Non-academic research programs offered during the academic year  | 6.20 | 0.45 | 6.20 | 0.45 | 1.0000 | 6.40 | 0.55 | 6.20 | 0.84 | 0.6666 |
| Continued research at the graduate level                        | 6.40 | 0.55 | 5.75 | 0.96 | 0.2381 | 6.40 | 0.55 | 5.80 | 1.10 | 0.3052 |

The third aspect of identity, recognition, reveals some of the stark differences between science students and engineering students with respect to how they and others recognize them in the communities of practice of science, engineering, and research. Recognition plays a crucial role in how people see themselves fitting into a Community of Practice and a lack of recognition has been shown to deter students from pursuing certain career paths [191].
Before the summer experience, science students reported significantly higher (p=0.0039) recognition from their mentor(s) as compared to engineering students, whereas in every other aspect of science identity (i.e. recognition of self and recognition by parents, friends, advisor(s), and faculty), there were no significant differences by major in either the pre- or post-summer experience items. Engineering identity of science majors was significantly lower (Table 5.4) compared to the engineering majors both pre- and post-summer experience, except for recognition by their mentor(s) in the pre-survey. The higher recognition by mentor(s) of the science students in this category could be due to the fact that two of the five science students who completed the surveys participated in the engineering REM program instead of the science REM program, thus their mentor(s) were of engineering backgrounds instead of biology. The last significant difference of note was between majors evaluating the survey item “Others ask me for help in scientific research”. The science student responses, in the pre-survey, reveal significantly higher (p=0.0438) recognition with respect to others asking their help compared to engineering majors. This difference is most likely influenced by the coursework completed by each student. Many of the engineering students, at this point in their degree progress, have just begun to enroll in science-related classes, whereas science degree-seeking students enrolled in general science classes immediately upon matriculation as they are required to take many more science classes compared to engineering students. Further, engineering students are less likely to take a biology class compared to science students, as most engineering degrees require many more physics classes and physics is not, at this point, classified as a general science class for engineering majors.
Table 5.4: Self-Reported Recognition Items Comparing Science and Engineering Majors.

Symbols $\mu$ and $\sigma$ represent the mean and standard deviation of the population, respectively.

P-Values highlighted indicate significant differences between majors.

<table>
<thead>
<tr>
<th>Survey Item</th>
<th>Pre-Summer Experience</th>
<th>Post-Summer Experience</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>$\sigma$</td>
</tr>
<tr>
<td>To what extent do you disagree or agree with the following statements?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I see myself as a science person</td>
<td>6.60</td>
<td>0.55</td>
</tr>
<tr>
<td>My parents see me as a science person</td>
<td>6.60</td>
<td>0.55</td>
</tr>
<tr>
<td>My friends see me as a science person</td>
<td>6.80</td>
<td>0.45</td>
</tr>
<tr>
<td>My faculty advisor sees me as a science person</td>
<td>6.20</td>
<td>1.30</td>
</tr>
<tr>
<td>My mentor(s) see me as a science person</td>
<td>6.60</td>
<td>0.55</td>
</tr>
<tr>
<td>My professor(s) see me as a science person</td>
<td>6.00</td>
<td>1.22</td>
</tr>
<tr>
<td>Others ask me for help in science</td>
<td>6.00</td>
<td>0.71</td>
</tr>
<tr>
<td>To what extent do you disagree or agree with the following statements?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I see myself as an engineering person</td>
<td>3.80</td>
<td>1.64</td>
</tr>
<tr>
<td>My parents see me as an engineering person</td>
<td>2.80</td>
<td>1.10</td>
</tr>
<tr>
<td>My friends see me as an engineering person</td>
<td>2.75</td>
<td>1.50</td>
</tr>
<tr>
<td>My faculty advisor sees me as an engineering person</td>
<td>2.40</td>
<td>1.14</td>
</tr>
<tr>
<td>My mentor(s) see me as an engineering person</td>
<td>3.80</td>
<td>2.17</td>
</tr>
<tr>
<td>My professor(s) see me as an engineering person</td>
<td>3.20</td>
<td>1.64</td>
</tr>
<tr>
<td>Others ask me for help in engineering</td>
<td>3.00</td>
<td>1.22</td>
</tr>
<tr>
<td>To what extent do you disagree or agree with the following statements?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I see myself as a scientific research person</td>
<td>5.80</td>
<td>0.45</td>
</tr>
<tr>
<td>My parents see me as a scientific research person</td>
<td>6.20</td>
<td>0.45</td>
</tr>
<tr>
<td>My friends see me as a scientific research person</td>
<td>6.40</td>
<td>0.55</td>
</tr>
</tbody>
</table>

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One of the major outcomes of this analysis was the indication that science RPs did not identify as engineers, either before or after participation in various summer experiences. This result was consistent across all explored aspects of identity: interest, competence, and recognition. This result was also statistically significant across most survey items concerning engineering identity, with science RPs reporting statistically lower means than those of their engineering RP counterparts. For the RPs surveyed, this result suggests a distinction between science and engineering for students majoring in science. When comparing science major responses with regard to science identity to corresponding engineering identity items, a significantly higher mean response (statistics not shown in table) can be seen for science responses. This further supports the assertion that these science RPs have very strong viewpoints on the components of science identity and its distinction from engineering identity components.

In contrast to these results, engineering RPs indicated comparable levels of science identity as reported by their science RP colleagues. It can be seen across each measured component of identity that engineering student and science student responses to science-focused identity items resulted in non-significant differences in most cases. It is our assertion that these results indicate an intersectionality of science identity and engineering identity for engineering students. These students do not see the two fields of
study as inherently different as do the science students. This idea is supported by the work of Godwin and coworkers, in which both science and physics identities were shown to support or contribute to the development of engineering identity [186]. These results suggest that for these engineering students, the components contributing to a strong science identity are the same as, or necessary for the development of, the components of their engineering identities.

These contrasting results are interesting, considering the implications derived from the research identity items explored in this study. For the most part, research identity items yielded non-significant differences between science and engineering majors for both pre- and post-survey results. However, closer examination of the mean values of these items reveal that, although not significant, science majors consistently reported slightly higher responses than engineering majors with respect to research identity items. Because these results are not statistically significant and because of the limited sample size, one cannot definitively conclude science majors report higher research identity than engineering majors. However, the consistency of the responses across all areas of identity suggests that science identity may be more closely linked to research identity for these students. Interestingly, the lack of significant difference also suggests that engineering students also readily identify with components of research. Two explanations may clarify this result. First, engineering students may identify with research through some set of components common to both engineering and science identity. This explanation supports the previous assertion there is significant intersectionality between the science and engineering identities of engineering students.
Second, engineering students may identify with research through their identification with science. This idea supports the previous statement that the most direct link to research identity may be through a strong science identity, but science and engineering identities are indeed separate. Figure 5.2 below illustrates these two potential explanations.

**Figure 5.2** Potential explanations for research identity data. 1) Significant intersectionality between engineering and science identities, with science identity being most directly linked to research identity. 2) Engineering and science identity are distinctly different, but connected. Science identity is most directly linked to research identity.

It is also important to note some outcomes of this work not specifically related to the analysis. Science student post-survey results indicated a significantly higher response to the item, “I see myself as a research person” when compared to pre-survey results. This result indicates a significant growth in the self-recognition component of research identity for this group of students. It was the goal of this work to improve research identity development in these students; therefore, this result was a positive outcome of the study. Corresponding engineering student results for this item indicate comparable
pre- and post-results without a statistically significant difference. This result reinforces the previous assertion that students of both majors more closely associate research with science at this stage in their academic development. It is our hypothesis that the differing natures of summer experiences for the engineering students responding to this survey may have played a role in research identity indication. We also hypothesize that students overwhelmingly consider research to be an academic exercise; therefore, students participating in more industry-focused experiences may not have associated their specific summer activities with research.

The results suggest that engineering students identify less with research, as compared to science students, and subsequently feel less prepared to conduct research; however, participation in an interdisciplinary experience increases their indication of academic research preparedness. The results show, for the population studied, that participation in a research program, such as REM and summer REUs, increases URM student research identity which, in turn, could help increase diversity of the research population.

**Limitations and Future Directions**

While this work is a good starting point to better understand minority undergraduate students’ perceptions of science, engineering and research identity and preparedness to conduct research, it is evident that the programs, and therefore the surveys, were limited by the small sample size. While this study was intended to assess how students participating in the program identify within science, engineering, and research, further, in depth work assessing engineering and research identity is necessary
to better understand how federally-funded and related programs impact students and the future of STEM fields. Some limitations of the study related to the identity survey items include the adaptation of items and missing data. The survey items have been validated and proven reliable for science and math identity through the SaGE study [189]. Further, missing data responses were dealt with by deleting entire responses for missing pre- or post-results. As for the REM program survey, each student’s perception of the questions may have skewed their response to the questions and thus the results of the survey items.

Future work in this area of study should focus on capturing a larger, more representative population of undergraduate researchers. A longitudinal study would be insightful to follow up this work in order to see how the identities of science, math, engineering, and research change and morph over time with each RP’s experiences and beyond, as he/she becomes part of the STEM community. Future work comparing research experiences of URMs to those of non-URM students would add insight regarding the relationship between ethnicity, gender, or experience level and research identity, or regarding the influence of cultural differences (e.g. between English speaking countries, other Western countries, and Eastern European or Asian or African countries) on identity development. Further work must be conducted to establish the validity and reliability of research identity survey items. Based on current literature, science, math, and physics identities factor into the development of engineering identity [186]. Future research may explore the relationship of these already validated identities with research identity, or may explore the connection of engineering identity to research identity. This
could be done through interviews to better understand student perceptions of research identity and their feelings of preparedness for research.

**Summary**

The overall motivation for this work is to increase the number of underrepresented minority students pursuing STEM careers which may lead to the fulfillment of research and innovation goals for the United States in years to come. It is our position that participation in undergraduate research programs fosters the development of research identity in both science and engineering students and will allow students to feel more prepared to pursue further research opportunities. The program highlighted in this work combined “hands-on” experience with faculty and graduate student mentoring to develop this research identity. Interest, competence, and recognition are critical factors in the development of any type of identity. Survey tools used in this study sought to explore the effect of participation in this program on those factors in identity development. Results showed that science students and engineering students may see their respective areas of study in different lights than their counterparts, but also they see research and its connection to their established academic identities as different. Science majors seemed to identify highly with only science, while engineering students identified with both science and engineering identity items. Science identity seemed to be the most direct link to the development of research identity in these students. Based on the results from this study, we consider these programs to be a positive and impactful experience for underrepresented minority students interested in research careers.
CHAPTER SIX

CONCLUSIONS

The overarching objective of this work had two components; the first was technical work focused on developing a 3D in vitro breast tissue model and the second, to understand how undergraduate students in Science, Technology, Engineering, and Mathematics (STEM) disciplines who participate in research develop a research identity. In Chapter 2, it was determined that processing parameters affect the final material properties of polylactide beads. Both the vessel size/paddle size and PVA concentration have a definitive effect on bead size and shape. These are two processing variables that can and should be controlled for the intended application. In choosing beads for the 3D breast tissue model, beads with a diameter of 250-325 mm were selected because 1) the larger the beads have more mass and anything over 400 mm would not suspend in the model and 2) the smaller beads have a more uniform shape providing a more consistent model.

Collagen/Matrigel™ of 2.4 mg/ml was chosen to represent the stromal component of the 3D breast tissue model based on their performance reported in Chapter 3. This combination provided a MCF10A cell viability above 75%, production of both acinar and ductal-like structures and cell cluster measurements consistent with a cluster of 5 or more cells providing the most representative model of the normal breast tissue.

To produce a heterogeneous 3D breast tissue model, polylactide beads were suspended in the 2.4 mg/ml Collagen/Matrigel™ system. Cell viability, cell cluster
measurements, macro and micro mechanical properties, histological analysis of cell proliferation, cell-cell adhesion, and acinar and ductile-like structure formation were analyzed to assess the feasibility of producing a model system to look at the influence of heterogeneity on normal and cancerous mammary epithelial cells. This work demonstrates that a heterogenous 3D breast tissue model can be produced incorporating degradable, synthetic materials. Further, inclusion of these materials affected acinar and ductile-like structure formation, for example, cancerous epithelial cells formed larger clusters of disorganized cells, which is more consistent with a breast cancer environment.

The overall motivation for the educational component of this work is to increase the number of underrepresented minority students pursuing STEM careers. This work suggests that participation in undergraduate research programs foster the development of research identity in both science and engineering students during their first two years of undergraduate study. The students felt more prepared to pursue further research opportunities after this initial experience. During the program, students received mentoring from faculty and graduate students aiding in their development of a research identity. These conclusions are drawn from the results of a survey tool designed to assess, interest, competence, and recognition, three critical factors in the development of engineering, science, and research identity. One difference that was found between engineering and science students was that the science majors seemed to identify highly with only science, while engineering students identified with both science and engineering identity items. Correlations revealed that science identity was connected to the development of research identity in these students. Based on the results from this
study, we consider these programs to be a positive and impactful experience for underrepresented minority students interested in research careers.
CHAPTER SEVEN

RECOMMENDATIONS FOR FUTURE WORK

1. Monitoring of 3D structures for this study was challenging. Use of an inverted fluorescent microscope works well for histological sections but when imaging full 3D gels, in the future the use of a confocal microscope is recommended. This will allow the capture through multiple microphotographs of the 3 dimensions of the acinar and ductal-like structure formation.

2. Some specific histological staining could be conducted on whole constructs allowing analysis of full structures instead of sectioning the structures. However, this approach is limited to few stains beside specific immunohistochemistry using fluorescent probes. Although, this may allow more accurate cell cluster counts and measurements and provide a more comprehensive understanding of cell viability and cell organization with H&E stains and routine stain, serial sections remains the standard with efforts to develop computer software approaches allowing section compilation and 3D rendering.

3. In the studies conducted constructs were seeded with approximately 120,000 cells per well, While these numbers were based on values found in the literature, using a ratio of cells to a specific volume or area in the matrix could provide a better model. Whether increasing the number of cells seeded in the 3D construct would facilitate both physical and chemical cell interactions and potentially promote the generation of breast like structures remains to be investigated.
4. In the process to form the heterogeneous 3D breast CM matrices, the suspension of polylactide beads in the Collagen/Matrigel™ was challenging. In the future, a gelation method that decreases the amount of time, e.g. crosslinking of the matrix materials, would prevent the polylactide beads from settling out of the matrix and provide a more uniform distribution of the beads throughout the construct. In addition, mechanical testing and histological analyses were affected by the need for specific vessels to contain the 3D constructs and the difficulties sectioning 3D matrix containing beads. By changing the consistency of the model constructs, more accurate mechanical testing could occur. Future research should address those limitations.

5. Polylactide beads were incorporated into the 3D CM matrices to provide both heterogeneity and an acidic environment normal within the breast tumor microenvironment. Polylactide beads release lactic acid into the surrounding matrix as they degrade; however, pH change in the matrix surrounding the beads was not evaluated. Further work should explore the effects of pre-degrading the beads, the pH changes that occur due to bead degradation, and the effects of the acidic environment within heterogeneous 3D CM matrices.

6. The 3D breast tissue model produced here needs to be validated to ensure the model system represents the desired select aspects of the normal breast environment, but also can be tuned to represent the different environments seen in breast cancer disease progression. Currently the model has been developed to
represent aspects of the normal mammary epithelial environment, however, further benchmarking must be done to ensure it is truly representative.
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APPENDICES
Appendix A
Polylactide (PL) Bead Processing – 1L Beaker

Equipment Required
- 1L Beaker
- Plastic tubing for transferring beads to rinse bottles
- IKA Works overhead stirrer with paddle stir rod
- Syringe (glass not required) with 16 gauge needle
- Orbital shaker
- Glass flasks with ceramic insert and filter paper for drying beads with house air

Working Solutions
- 10% (w/v) PL Solution: 2g PL pellets in 20 mL dichloromethane (Mallinckrodt #4879). Leave overnight on orbital shaker at 150 rpm for pellets to go into solution. This particular concentration was optimized for Cargill amorphous PLLA pellets, so it may differ from the most desirable concentration for the particular batch you are using. Two vials of this solution have been used to successfully make one batch of beads.
- 0.3% (w/v) PVA Solution: 3g PVA (MW of 13-23k – Aldrich #36-317-0) in 1000 mL distilled water. Stir on low heat until PVA dissolves in water. Cool to room temperature before using to process beads.
- 2% (v/v) Isopropanol Solution: 20 mL Isopropanol (VWR #VW5520-3) in 980 mL distilled water. Be sure to have 3 bottles of this solution prepared before beginning.

Procedure
1. Beaker set-up. Place 1 L beaker on stir platform.
2. Set stirring conditions. Add 150 mL of 0.3% PVA solution to beaker. Make the solution 0.05% by adding an additional 750 mL of distilled water to the beaker. Position stir rod on overhead stirrer in center of beaker so that the stir rod sits at the 300 mL mark on the beaker. Stir at 200 rpm for 5 minutes to ensure thorough mixing.
3. Attach needle to syringe. Remove plunger from syringe. Pour 20 mL of PL solution into syringe quickly but carefully. Avoid pouring the solution down the side of the syringe – try to pour down the center. Solution on the sides, near the top of the syringe, will cause it to seize in a short time making bead production extremely stressful on your thumb, hand, and wrist. After emptying contents into
syringe, insert plunger. While maintaining slight pressure, turn the syringe over and let the air bubbles in the solution float to the top. Remove the air bubbles from the syringe by applying gently pressure. Be sure to have a paper towel handy to avoid squirting PL solution all over the hood.

4. Submerge needle of syringe at a 45° angle near (but not in) the stirring vortex at the center of the beaker. Try to apply consistent pressure to syringe and dispense entire contents of syringe into the stirring PVA solution.

5. Stir beads in the solution at 300 rpm for 90 minutes. These settings should only be used as a guide. Ultimately, the decision is up to you to determine if your beads appear to be small enough. Lowering the speed below this range will cause the beads to increase in size while increasing the speed will produce beads of smaller size.

6. Turn the overhead stirrer OFF. Allow beads to settle in beaker. Using the house vacuum and “bioreactor” bottle top remove the 0.05% PVA solution from the beaker leaving approximately 200 mL.

7. Add 400 mL of 2% Isopropanol solution. Turn overhead stirrer ON, and stir again in the 175-200 rpm range for 5-10 minutes.

8. Turn the overhead stirrer OFF. Allow beads to settle on bottom of beaker. Again using the house vacuum, remove the remaining solution.

9. Move beads to a 250 mL pyrex bottle by pouring the beads into bottle from beaker. You may need to use a squirt bottle full of Isopropanol solution to help move beads from beaker to bottle.

10. Add 50 mL of fresh 2% Isopropanol solution to 250 mL pyrex bottle.

11. Place bottle on orbital shaker overnight (24 hours) at 150 rpm.

12. Place ceramic insert into top of glass flask. Insert 3” filter paper into ceramic insert. Remove the beads from the bottle that was on the orbital shaker, and place them on the filter paper. Turn the house air on low flow to slowly dry the beads. Remove the beads from the filter paper and store them in 20 mL glass scintillation vials under vacuum.
Appendix B

Immunofluorescence Staining

Objective: Use immunofluorescence staining to assess expression of primary and secondary antibodies on fixed, frozen sections embedded in OCT.

Materials:
- 0.2% tween PBS
- 10% goat serum in PBS
- 1% goat serum in PBS
- 1:1600 rat anti-Ecadherin in 1% goat serum
- 1:200 goat anti-rat Alexa 594 (spun down @ 1000 rpm for 1 min)
- 1:1000 rabbit anti-Ki67, 1:1000 rabbit anti-CD29, or 1:1000 rabbit anti-met in 1% goat serum
- 1:200 goat anti-rabbit Alexa 488 (spun down @ 1000 rpm for 1 min)
- ddH2O
- PBS
- Pap pen
- Paper towels
- Waste container
- Sectioned samples, embedded in OCT

Protocol:
1. Draw around each slide using a pap pen to contain rinses to slide
2. Rinse twice with ~1 mL ddH2O per slide for 2 minutes to remove OCT. Pour ddH2O into waste container.
3. Rinse once with ~1 mL PBS per slide for 2 minutes. Empty into waste container.
4. Add ~1 mL 1:1 HCl:PBS to each slide for 30 minutes. Empty into waste container.
5. Rinse twice with 0.2% tween PBS for 2 minutes. Empty into waste container
6. Block with 10% goat serum for 45 minutes at room temperature (RT).
7. Add rat anti-Ecadherin primary antibody solution for 2 hours at RT.
8. Rinse with 0.2% tween PBS for 2 minutes. Empty slide(s) in waste.
10. Rinse once with PBS for 2 minutes. Empty slide(s) into waste.
11. Add second primary antibody (e.g. rabbit anti-Ki67) for 2 hours at RT. Protect from light.
12. Rinse with PBS for 2 minutes. Empty slide(s).
14. Rinse twice with ddH2O for 2 minutes. Empty slide(s).
15. Mount coverslip with Prolong Gold Antifade Reagent + DAPI and store away from light.