ABSTRACT

NORDBERG, RACHEL CHRISTINE. Translating Human Adipose Stem Cells for Musculoskeletal Applications: A Tissue Engineering, Mechanistic, and Electrical Cell-Substrate Impedance Spectroscopy Approach. (Under the direction of Dr. Elizabeth Loboa-Polefka).

Human adipose stem cells (hASC) are an attractive cell source for tissue engineering, regenerative medicine and immunomodulatory applications due to their relative ease of harvest, proliferative capacity, and multipotent differentiation potential. However, in order to translate hASC into widespread clinical use, there are a number of obstacles that must be addressed. This body of work describes novel tissue engineering techniques for musculoskeletal repair using hASC, elucidates relevant mechanisms in cartilage and bone biology, and studies age-grouped hASC donor-to-donor variability in osteogenic differentiation using electrical cell-substrate impedance spectroscopy.

Within our first study to advance the translation of hASC therapy, we describe a new method for enhanced cellular infiltration in meniscal allografts. Medial menisci were decellularized and a needle-punched method was used to enhance porosity. After 28 days of in vitro culture, we demonstrate needle-punching enhances hASC infiltration, which could improve long-term efficacy of meniscal transplantation procedures by helping to maintain the meniscus in vivo.

With the understanding gained in the meniscal study, we used cartilage extracellular matrix to drive differentiation in a 3D biopotted scaffold that induces site-specific hASC differentiation. Our scaffold was fabricated using 3D bioprinting of biodegradable polycaprolactone (PCL) with either 20%TCP tricalcium phosphate (TCP) or decellularized cartilage extracellular matrix (dECM) to induce site-specific osteogenesis and
chondrogenesis, respectively. In addition, histological analyses of full osteochondral scaffolds showed site-specific tissue characterization using a single adult stem cell source. In future *in vivo* studies, this approach holds great potential to treat OA patients in a highly personalized manner using a patient’s own hASC donor cells.

In order to better understand the signaling mechanisms that drive hASC differentiation and musculoskeletal development within our scaffold system, we next investigated relevant musculoskeletal mechanisms. The goal of the first mechanistic study was to determine how LRP4, LRP5, and LRP6 within canonical Wnt-signaling are regulated in simulated microgravity and cyclic hydrostatic pressure in order to elucidate the mechanisms of cartilage degeneration. LRP5 is demonstrated to be upregulated in both simulated microgravity and hydrostatic pressure and in the articular cartilage of hind limb unloaded mice. Further elucidation of this mechanism could provide significant clinical benefit for the identification of pharmaceutical targets for the maintenance of cartilage health.

After identifying an important mechanism for regulating cartilage homeostasis we next studied a signaling mechanism relevant to hASC osteogenic differentiation. We show that Corin is highly upregulated throughout osteogenic differentiation and demonstrate that calcium accretion and metabolic activity are decreased when Corin is knocked down via siRNA. Interestingly, Corin knockdown also significantly increased VEGF mRNA expression during osteogenesis, suggesting that Corin is also involved in the regulation of angiogenic mechanisms. Overall this study suggests that Corin is a key regulator of osteogenesis in hASC, likely through crosstalk with vascular pathways.
One of the major commonalities throughout all hASC studies is that there is major donor-to-donor variability. In order to better understand this variability, we investigated the use of electrical cell-substrate impedance spectroscopy (ECIS) to track complex bioimpedance patterns of hASC throughout proliferation and osteogenic differentiation. Superlots comprised of hASC from young, middle-aged, and elderly donors were seeded on gold electrode arrays to track complex impedance measurements throughout proliferation and osteogenic differentiation. We show that stages of osteogenic differentiation can be tracked via ECIS. In addition, hASC from younger donors require longer time to differentiate than hASC from older donors. This is the first study to use ECIS to predict osteogenic potential of multiple hASC populations and to show that donor age may temporally control onset of osteogenesis. Overall, the findings presented in this dissertation are critical for the translation of safe and effective hASC therapies for musculoskeletal repair.
Translating Human Adipose Stem Cells for Musculoskeletal Applications: A Tissue Engineering, Mechanistic, and Electrical Cell-Substrate Impedance Spectroscopy Approach

by
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BIOGRAPHY

Rachel Nordberg grew up in Eden Prairie Minnesota. She has two younger siblings, Alison and Jimmy. Rachel was a competitive swimmer and played the cello throughout her childhood. She graduated Eden Prairie High School in 2008. After high school, she moved to Bethlehem Pennsylvania to attend Lehigh University. At Lehigh she was actively involved with Engineers Without Borders, tutoring, and playing for various sports teams. She graduated with her B.S. in bioengineering in 2012. Rachel obtained her M.Eng. in Biomedical Engineering from Cornell University in 2013 before entering into the Joint Biomedical Engineering Program at North Carolina State University and the University of North Carolina Chapel Hill for her doctoral studies.
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CHAPTER 1

Introduction

1.1 Motivation

Tissue engineering has been a growing field of interest over the past few decades due to its tremendous potential to change the standard of treatment for many medical conditions ranging from osteoarthritis to paraplegia to dental repairs. In addition, tissue engineering could potentially provide patient specific treatments using a patient’s own cells. This study specifically focuses on the abundantly available human adipose derived stem cells (hASC), which can be obtained from fat pad resection or liposuction procedures. hASC are multipotent and can differentiate down the osteogenic, chondrogenic, and adipogenic lineages, making them an ideal cell source for musculoskeletal applications in particular. However, despite the excitement that tissue engineering and the potential use of adipose stem cells have generated within the research community, tissue-engineering technologies have been slow to translate into the market. The motivation for this research was to further develop strategies for musculoskeletal tissue engineering using hASC, elucidate relevant cellular mechanisms of musculoskeletal development, and determine factors that cause donor-to-donor variability between hASC populations.

1.2 Objectives

This dissertation explores the many facets of translating hASC therapies. First, Chapter 2 will further introduce hASC and describe in-depth the many aspects that must be considered to bring hASC therapy “from bench to beside.” In addition, Chapter 2 will discuss the state of hASC clinical trials and the industry that is emerging in this field.
Chapters 3 and 4 will describe novel tissue engineering strategies for musculoskeletal tissue engineering using hASC. Specifically, in Chapter 3 we will describe a method for treating allograft menisci for improved hASC infiltration, which could improve long-term compatibility with the host and extend the life of the transplant. In Chapter 4 we develop a 3D bioplopted osteochondral scaffold that promotes site-specific chondrogenesis and osteogenesis from a single hASC cell source. The objective of these two chapters is to further advance the development of musculoskeletal tissue engineering therapies using hASC.

In order to translate hASC therapies effectively, a deeper understanding of the underlying molecular mechanisms of musculoskeletal development and maintenance is needed. Chapters 5 and 6 focus on signaling mechanisms that are highly involved within musculoskeletal development. Specifically, Chapter 5 describes how low density lipoprotein receptor-related proteins (LRPs) within the canonical Wnt-signaling pathway respond to mechanical stimuli in cartilage tissue. Elucidation of this mechanism will better inform functional tissue engineering using mechanical stimuli and help elucidate how LRPs are involved in maintenance of cartilage tissue. Chapter 6 focuses on Corin, a gene that our lab has previously identified as one of the most highly upregulated genes during osteogenic differentiation of hASC. Here, we better characterize the role that Corin plays within hASC and provide evidence that Corin crosstalks with vascular pathways during osteogenic differentiation. The objective of these two chapters is to further investigate these mechanisms to help optimize the development of tissue engineering therapies and provide necessary mechanistic information for regulatory agencies as hASC therapies enter human patients.
Finally, a major barrier to clinical translation of hASC is that cells from different donors have varying capacities to proliferate and differentiate. In Chapter 7 we provide new insight into variability between hASC isolated from different donors. Specifically, electrical impedance spectroscopy is used to noninvasively track osteogenic differentiation of age-grouped donors in real-time, showing that age-grouped hASC have distinct complex impedance patterns. The objective of this chapter is to better understand the biology that causes variability between hASC populations and to provide quantitative quality control standards for hASC populations in stem cell manufacturing and bone tissue engineering applications.

1.3 Dissertation Findings

This research has resulted in the following three publications:


Three additional publications are in preparation:

5. **Nordberg R**, Mellor L, Loboa E. LRP receptors in canonical Wnt signaling are responsive to microgravity and cyclic hydrostatic pressure and regulate cartilage homeostasis. In preparation for submission to *Osteoarthritis and Cartilage*.

CHAPTER 2

Current research and progress in translation of human adipose derived stem cells

Human adipose stem cells (hASC) are an attractive cell source for tissue engineering, regenerative medicine and immunomodulatory applications due to their relative ease of harvest, proliferative capacity, and multipotent differentiation potential. Before hASC can be used in widespread clinical applications however, standardization of current practices is essential. Topics such as heterogeneous cell populations, donor-to-donor variability, animal product free culture, industrial production, storage, preservation, shipping, and procedure variability need to be addressed while adhering to strict quality control measures. Despite these challenges, hASC are rapidly entering clinical trials. This chapter summarizes the current state of hASC translation and critical steps needed to most efficiently and safely bring hASC therapies to patients.

An abbreviated version of this chapter has been published in Stem Cells Translational Medicine with co-author Elizabeth G. Loboa (1).

2.1 Introduction

Human mesenchymal stem cells are multipotent adult stem cells that have been isolated from almost every major tissue in the body. Within the last quarter of a century, human mesenchymal stem cells have garnered much attention for use in tissue engineering, regenerative medicine, and immunomodulatory applications. The term hMSC is used to refer to human mesenchymal stem cells or human multipotent stem cells, and often is within the
context of bone marrow MSC (BM-hMSC) [1]. BM-hMSC were the first hMSC to be isolated and have thus received much attention in basic and translational research. However, human adipose stromal/stem cells (hASC), as defined by the International Society of Cellular Therapy (ISCT) and International Federation for Adipose Therapeutics and Science (IFATS) [2]. hASC have been demonstrated to possess similar therapeutic potential but have several distinct translational advantages owing to the fact that hASC are derived from a generally undesired and excess tissue source: fat tissue. This allows hASC to be obtained in large quantities from a minimally invasive procedure. Recent advancements in hASC research have capitalized upon these advantages to position hASC on the threshold of clinical translation. In the past five years the number of clinical trials using hASC has rapidly risen – from 18 to 152 studies (January 1, 2010 to March 9, 2015; clinicaltrials.gov). These clinical trials address a wide range of conditions including fistula, musculoskeletal disorders, ischemia, soft tissue damage, host versus graft disease, and many more. In recent years, there have been a number of reviews that have discussed the clinical translation of hASC [3-5]. Herein, we offer a new perspective to this literature by discussing the current topics in hASC research, technology development, clinical trials, and industry that must be considered to effectively translate hASC to widespread clinical use.

2.2 Current topics in hASC translation

2.2.1 Standardization in hASC research

Although there has been impressive progress in hASC research and translation since hASC were first isolated in the early 2000s, there are still major challenges in the standardization of hASC research. In 2013, a major step towards standardization of hASC
research was taken when the International Federation for Adipose Therapeutics and Science and the International Society of Cellular Therapy (ISCT) released a joint statement to define both adipose tissue-derived SVF and culture expanded adipose tissue-derived stromal cells [2]. The three criteria that IFATS and ISCT use to define hASC are plastic adherence, a specific surface antigen expression profile greater than 80% expression of CD13, CD29, CD44, CD73, CD90, and CD105 and less than 2% expression of CD31, CD45, CD235a, and a capacity for trilineage differentiation into osteoblasts, adipocytes, and chondroblasts. This definition has been adopted by many hASC investigators and may serve to standardize the diverse field of hASC research. Beyond the international standardization of hASC research, the field must also work towards international harmonization of translational aspects of hASC therapy. As materials and biologics from hASC therapies begin to cross international borders, national and international regulatory agencies will need to coordinate to define policies that can safely and effectively moderate the global effort towards hASC translation.

2.2.2 Heterogeneous cell population

It is important to consider that hASC populations are inherently heterogeneous cell populations, since there are numerous cell subpopulations within any given adipose tissue. The heterogeneous cell population of the SVF may be beneficial to hASC translation because it allows for access to various cell types, which may be advantageous in different applications. However, the heterogeneous cell population will also complicate quality control when ensuring that hASC populations administered to a patient are the desired cell composition. Human ASC are traditionally isolated via a collagenase digestion of liposuction aspirates (Figure 2-1), as we have previously reviewed(2). Cells released from the tissue are
centrifuged to separate mature floating adipocytes from the pelleted cells termed the stromal vascular fraction (SVF). The SVF has an approximate composition of 15-30% stromal cells, 3-5% pericytes, 10-20% endothelial cells, 10-15% lymphocytes, 5-15% monocytes, 10-15% granulocytes, and less than 0.1% hematopoietic stem and progenitor cells (3). Therefore, it is not surprising that flow cytometry has been used to isolate subpopulations within the SVF with varying differentiation capacities (4). Plastic adherence is the traditional method by which hASC are selected for and enriched from the SVF cell populations. Cellular phenotyping of freshly isolated SVF has demonstrated multiple cell populations, and includes differentiated cells. It has been shown that after 3 days of adherent culture, the composition of the cell population shifts to a more homogenous cell population that predominantly lacks markers of differentiated cells (5). After culture, the plastic adherent cells are then defined as the hASC population, although this is also a heterogeneous cell population (3). Adipose multilineage-differentiating stress-enduring (adipose-Muse) cells are a recently discovered subpopulation within hASC (6). Subpopulations that exhibit varying degrees of chondrogenic or osteogenic differentiation potential have also been identified (7). As the research on hASC progresses, it is likely that more subpopulations will be distinguished from the heterogeneous hASC population.
Figure 2-1 General procedure for isolating the SVF and hASC. In order to isolate the SVF, adipose tissue is digested enzymatically and centrifuged. The pelleted cells are the SVF, which can be culture expanded to obtain a population of hASC.

In order to reduce such cellular heterogeneity, researchers have developed methods to rapidly sort and purify hASC. Fluorescence activated cell sorting (FACS) has been used to sort hASC for enriched adipogenic, (4) osteogenic, (7, 8) and chondrogenic (7) differentiation capacity. Magnetic activated cell sorting (MACS) has been used to enrich for hASC that differentiate into endothelial cells for use in vascular applications (9). However, both FACS and MACS require the use of animal-derived antibodies, which is undesirable for regulatory purposes. A double lentiviral transfection method was used by Bai et al. to fluorescently label and enrich hASC that were potential cardiac progenitors (10). However, the use of
lentiviral transfections is again not ideal from a regulatory standpoint. Yet another approach that has been used to purify hASC is membrane filtration. The permeate solution of hASC purified through a hybrid poly(lactic-co-glycolic acid/silk) filter was demonstrated to have significantly higher osteogenic differentiation potential than the unfiltered cell solution (11). Alternatively, the recovery solution of hASC filtered through a polyurethane membrane showed a 3-4.5 fold increase in the concentration of cells expressing stem cell markers, depending on surface modifications to the polyurethane (12). Once further refined, these methods and others will allow researchers to more precisely control hASC heterogeneity and potentially select for ideal sup-populations for each therapeutic application.

2.2.3 Donor-to-donor variability

Donor-to-donor variability has been highly documented by our group and others between hASC populations. We have shown that pre-, peri-, and postmenopausal age grouped hASC superlots exhibit high donor-to-donor variability and that hASC from older donors exhibit an increased proclivity to osteogenic differentiation but a reduced expansion capacity (13). Sen et al. demonstrated that hASC isolated from 12 patients showed significant variation in adipogenic differentiation as measured by ability to accumulate lipid and express the adipocyte specific gene product adipocyte protein 2 (14). Aust et al. reported dramatic variability in hASC yield between donors ranging from 158,000 to 1,134,000 cells per milliliter liposuction aspirate with a mean of 404,000 and a standard deviation of 206,000 cells per milliliter tissue (15). Fraser et al. also demonstrated significant variability in hASC yield between patients regardless of tissue harvest method (16). We have also demonstrated donor-to-donor variability in response to mechanically induced osteogenesis. (17)
the overwhelming evidence that donor-to-donor variability presents a major barrier to clinical translation, there has been limited research to address this problem or understand its underlying cause.

Studies that have compared tissue isolation sites have identified factors that may play a role in donor-to-donor variation. Findings from Fraser et al. that adipose tissue recovered from the hip had a statistically different progenitor content than adipose tissue isolated from the abdomen of the same donor suggest that factors such as blood vessel density, fatty acid content, and/or insulin sensitivity, all of which have been shown to be site-dependent, may partially account for variations between hASC populations (16). Work by Prunet-Marcassus et al. further supports the theory that tissue vascularization may play a role in determining hASC differentiation capacity by showing that hASC isolated from brown adipose tissue exhibited significantly reduced plasticity when compared to white adipose tissue (18). However, there has not been a study to examine how patient-to-patient variations in adipose tissue vascularity correlates to proliferation and differentiation capacity.

Others have attempted to determine cell markers that are associated with donor cell variability. Suga et al. sorted hASC into CD34+ and CD34- factions and demonstrated that CD34- cells differentiated more readily than CD34+ cells but CD34+ cells had a greater proliferative capacity (19). Forty-nine cell surface markers have been identified on hASC that showed significant donor-to-donor variability, including CD34 (20). Taken together, these results suggest that CD34 may be associated with donor cell variations in proliferation and differentiation. It has also been demonstrated that hASC positive for c-Kit exhibit
increased telomerase activity and differentiation potential (21). However, c-Kit positive cells only account for approximately 0.5% of the hASC population, preventing cell marker sorting from being an effective strategy for obtaining enough cells for clinical applications. As evidenced by these studies, cell markers may elucidate how cell heterogeneity affects donor-to-donor variability.

Another approach that some researchers have taken to better understand donor-to-donor variability is to look for correlations between patient demographics and chemically induced osteogenic differentiation and proliferation. Frazier et al. found a negative correlation between body mass index (BMI) and hASC proliferation capacity in cryopreserved ASC populations (22). Choudhery et al. reported that hASC expansion capacity and frequency is negatively correlated to donor age (23). Contrarily, Aust et al. found hASC cell yield had a significant negative correlation to donor BMI but not age (15). However, pediatric hASC have been reported to exhibit enhanced expression of pluripotent transcription factors and an elevated risk of differentiation into an undesired lineage, again suggesting that donor age may play a role in donor-to-donor variability (24). Gender has also been shown to influence donor-to-donor variability. It has been reported that hASC isolated from male donors were better osteogenic differentiators than hASC isolated from female donors (25). Although these studied demonstrate that demographics impact donor-to-donor variability, other factors such as ethnicity, smoking status, and other lifestyle factors have not yet been fully investigated. As hASC therapies enter clinical use, it will be essential to better understand the mechanisms underlying donor-to-donor variability and to develop methods for coping with this variability so that all patients can receive treatment.
Another critical concern for the development of clinical grade hASC products is the elimination of animal-derived reagents in the processing of hASC. Most traditional methods of culturing hASC require the addition of fetal bovine serum (FBS) to the growth media. While this is economical for basic research, the use of animal-derived products is highly undesirable for translational purposes due to concerns with safety and reproducibility. The use of animal products introduces the risk of immune response, anaphylaxis, and cross-species transmission of bacteria and viruses (26). To complicate matters further, proteins from FBS are internalized within the cells during the culture process; thus washing steps alone cannot sufficiently remove the xenogenic risk (27). Further, batch-to-batch variations in FBS protein content and concentrations can lead to variable cell responses, which is unacceptable for Good Manufacturing Practice (GMP) standards (28). Therefore, autologous serum or serum free media have been studied for hASC culture in order to circumvent the problems of animal serum use.

Human serum could be an advantageous alternative to the use of FBS when culturing cells since it can be isolated autologously from the patient, mitigating the risk of an immune response or disease transmission. Kocaoemer et al. demonstrated that hASC cultured in human serum or thrombin-activated platelet-rich plasma exhibited an increased proliferation rate but similar differentiation characteristics when compared to hASC cultured in FBS (29). Additionally, Kim et al. showed that culturing hASC in human serum promoted engraftment in an in vivo model (27). Recently, a protocol was developed for hASC expansion and differentiation that completely eliminated the use of all animal derived products and replaced
FBS with human serum albumin. Again, cells grown in human serum proliferated faster but maintained differentiation capabilities (30). However, the use of human serum must be further characterized before it can be used in standard clinical procedures. Lindroos et al. demonstrated differential expression of genes when hASC were cultured in FBS or human serum. Human serum culture resulted in an overexpression of cell cycle genes and FBS resulted in an overexpression of genes associated with differentiation (31). Although the use of human serum may reduce safety concerns, reproducibility is still a challenge due to donor-to-donor variability in human serum.

Alternatively, serum-free media allows for fully defined and consistent media, which is desirable for translational purposes. Although Parker et al. found that serum free media increased the doubling time of hASC from 1.86 days to 5.79 days when compared to a 0.5% human serum formulation, the differentiation capacity and cell marker expression profile was maintained (32). Lindroos et al. demonstrated that hASC grown in StemPro® serum free media had a higher proliferation rate but a comparable differentiation potential and surface marker expression profile relative to hASC grown with human serum or FBS (26). Serum replacements have also been evaluated for the culture of hASC. It has been shown that some serum replacements reduce hASC expansion and differentiation capacities when compared to animal serum, but a “knockout serum replacement” that exhibited comparable properties to FBS was also reported in that same study (33). Fully defined serum-free media formulations optimized for each hASC application will be highly advantageous due to scalability for production purposes and reproducibility for regulatory purposes.
To completely avoid the use of all animal products, animal-derived Trypsin and Collagenase must also be eliminated from the hASC cell culture protocol (34). There are a few animal component free dissociation reagents available on the market today including TrypLE Select (Invitrogen) and TrypZean (Sigma-Aldrich) (35, 36). Although Collagenase is traditionally used to isolate cells from liposuction aspirate, previous research has isolated cells from the fluid portion of the lipoaspirate, circumventing the necessity to use Collagenase (34, 37). However, collagenase and trypsin free methods need to be further optimized before widespread use in clinical applications.

2.2.5 Industrial production

Traditional cell culture techniques use static tissue culture flasks to expand cells. This method is simple and relatively inexpensive on a small scale, but presents major problems on the industrial scale. For example, the methods for MSC expansion described by Hanley et al. require the use of 256 T-175 cm² tissue culture flasks, which is both time consuming and expensive (35). Traditional cell culture requires highly trained personnel. A closed system bioreactor would greatly reduce the required resources for cell expansion and could be operated by individuals without extensive cell culture experience. In addition, the automated nature of bioreactors assists in the implementation of the Food and Drug Association (FDA) guidelines for “process analytical technology,” a framework for controlling and regulating the manufacturing process of pharmaceutical products.

Dynamic bioreactors have a number of additional advantages over static culture for large-scale cell production. Bioreactors allow for reproducible, steady state cultures that can be
tightly monitored by sensors and controlled by actuators. Continual mixing prevents gradients of nutrients or waste from forming. Bioreactors have the ability to introduce oxygenation systems instead of simply relying on diffusion (38). While static culture using traditional tissue culture flasks requires a substantial amount of incubator space, which is costly and time consuming to maintain, bioreactors can produce the same cellular yield at a fraction of the space using an automated system. Disposable single-use bioreactors are an especially attractive option for autologous stem cell expansion because they reduce costs and prevent cross contamination between batches, desirable for Good Manufacturing Practice (GMP) compliance (39).

For the expansion of hASC, adherent culture is necessary. Microspheres can be used to increase the surface area for adherent cell growth in many bioreactors. For more information of culturing stem cells on microspheres, Sart et al. has reviewed the properties and applications of currently available microspheres (40). There are several bioreactors on the market today that allow for the expansion of adherent cells. Bioreactors that would be particularly useful for the expansion of hASC include roller bottle, packed plate, packed bed, hollow fiber bioreactors, and wave bioreactors (41). Roller bottle bioreactors have been used extensively for the commercial production of proteins and viral vaccines (42). However, roller bottle bioreactors are not closed-system and thus may not possess the same advantages as some other bioreactors described here. Packed plate bioreactors, such as the Integrity® Xpansion™ offered by ATMI, minimize the spacing between tissue culture plates and contain the expansion process into one disposable system. Stirred tank bioreactors are typically used for non-adherent cell culture but can be modified by the addition of a “packed
bed” of microspheres, such as in the CelliGen® BLU Bioreactor from Eppendorf. Hollow fiber bioreactors are designed so that semi-permeable fibers deliver nutrients and remove byproducts throughout the length of a cell-culture compartment. Hollow fiber bioreactors may be advantageous as they provide uniform exchange of nutrients and waste, minimize shear stress applied to the cells, and allow for three-dimensional cell culture of hASC, preferable in many tissue-engineering applications (43). The wave bioreactors could also be used to culture hASC if modified with microcarriers. It has been previously shown that the WAVE bioreactor by GE Healthcare Life Sciences can be used to culture MSC on a large scale (44). The development of efficient adherent bioreactors will be critical to obtaining enough cells for effective therapeutic therapies.

Bioreactors can also be used to apply biomimetic mechanical loading to hASC to promote differentiation down specific lineages. We have previously demonstrated that hASC can be induced down an osteogenic lineage using a bioreactor that applies 10% cyclic tensile strain (17, 45-48). The application of fluid shear stress by pulsating fluid flow has also been indicated to promote hASC down an osteogenic lineage (49, 50). To induce a chondrogenic lineage, we and others have shown that hydrostatic pressure can be used to stimulate hASC (51-53). As research in biomimetic loading progresses, it is likely that bioreactors will be developed to apply physiologic mechanical stimuli to hASC for functional tissue engineering and potentially other specific therapeutic applications.
2.2.6 Storage, preservation, and shipping

Although hASC can be used from freshly isolated tissue samples, long-term storage of cells obtained from liposuction procedures is desirable to allow patients to draw upon stored cell populations for later procedures. Storage of hASC is also desirable to allow for a backup source of cells in the event a tissue engineered construct fails or is contaminated. Even if the patient has finished their initial procedure successfully, many patients may desire to bank their cells for the length of their lifetime, in the event new medical conditions arise. Several stem cell banking companies already exist. Celltex Therapeutics, Biolife Cell Bank, and American CryoStem Corporation are examples of stem cell banking companies that specialize in adipose derived stem cell banking specifically.

Cryopreservation is the most commonly used preservation technique. In traditional methods for cryopreservation, cells are suspended at a concentration of 1 million cells per milliliter in cryopreservation media containing dimethyl sulfoxide and aliquoted into cryovials. The vials are frozen at a rate of approximately -1°C per minute using a controlled-rate freezer until -80°C is reached (35, 54). The cryovials are then transferred to a liquid nitrogen container for long-term storage (54). Cryopreservation presents some challenges to the clinical utilization of hASC. In order to obtain FDA approval, it is desirable that no animal serum is used in clinical storage solutions. Cryopreservation may also decrease the viability of hASC; the challenge of location and duration of storage is not trivial. Although cryopreservation may be a viable option for long-term storage of cells, there has been some debate over the effectiveness of hASC after cryopreservation. While it was reported that 6 months of hASC cryopreservation did not alter cell surface markers, proliferation capacity, or differentiation
potential, (55), a recent study on the closely related bone marrow derived mesenchymal stem cells suggests that immunomodulatory properties of the cell population are reduced after cryopreservation (56). Further studies will be required to determine if hASC retain full treatment efficacy after cryopreservation. There has been little research on other methods of storing hASC, although some groups have studied alternate methods for storing bone marrow derived MSC. Gordon et al. demonstrated that MSC maintained viability and proliferative capacity after air-drying and shipping overnight (57). Zhang et al. showed that freeze-drying could also be a potential method for the preservation of MSC (58). It remains to be determined what the optimal method is for preserving hASC while conserving effectiveness long-term.

As hASC are translated to the clinic, it is very unlikely that the cell isolation, expansion, manipulation, and/or storage will be carried out at the same locations as tissue collection or end-product administration. Instead, it is likely that tissue and hASC will be shipped to and from external GMP facilities for processing. Shipping could introduce both variations in temperature and abnormal vibration patterns that may affect hASC viability or proliferative capacity (34). However, there is currently very little information on the effects of shipping on the viability and characteristics of hASC.

2.2.7 Clinical procedure variability

Other processes that must be standardized for clinical translation include the clinical procedures that are used to both isolate donor adipose tissue and administer the final hASC treatment to the patient. The method by which adipose tissue is isolated from the patient has
been shown to affect cell yield. Fraser et al. demonstrated that manual syringe aspiration yielded a higher cell recovery than a mechanical suction pump (16). While Smith et al. did not find significant changes in cell viability using different harvest and processing techniques, the group suggested that minimal processing may improve results (59). Mojallal et al. demonstrated a higher cellular yield when the negative pressure for the procedure was -350mmHg compared to -700mmHg, whether or not the procedure was machine-assisted (60). Fisher et al. found no difference in yield between ultrasound- and suction-assisted liposuction, but post harvesting with cotton-gauze or filtration to remove the liquid portion significantly increased tissue retention upon implantation (61). Alternatively, it has been shown that excised fat may be favorable to fat from liposuction due to increased progenitor yield (62). In order to most efficiently translate hASC, an optimized standard procedure for tissue harvest should be developed.

In a related topic, the method of hASC administration will also influence the effectiveness of any given treatment. Today, many hASC procedures in clinical trials use an intravenous administration method. However, systemic intravenous administration may not deliver the desired number of hASC to the target tissue. The “pulmonary first-pass effect” is a major concern for systemic hASC administration; a majority of the administered cells accumulate in the lungs (63). In the case of cardiac conditions, cells have also been delivered via intracoronary injection (64) and direct myocardial injection (65). Cell assisted lipotransfer, which is adipose tissue enriched by the addition of hASC, is another method that has been used for many soft tissue applications (66). The optimal delivery method of hASC for each
target condition should be further investigated to treat patients in the most effective and efficient manner.

2.2.8 Regulatory Concerns

Regulatory guidelines for hASC products have been ambiguous as companies have begun to enter the hASC industry. To address this problem, in December 2014 the U.S. Food and Drug Administration (FDA) released a draft guidance document for industry outlining criteria to determine if a product derived from adipose tissue is regulated as a drug, device, and/or biological product [35]. This document, currently in draft form, outlines the four criteria that a product derived from adipose tissue must meet to avoid premarket review. The four criteria for such a product are that it must: 1) be minimally manipulated, 2) be intended for homologous use, 3) not be combined with most other agents, and 4) be derived from autologous or a first- or second-degree blood relative (unless it does not have a systemic effect or depend upon activity of living cells). Adipose tissue is defined by the FDA as a structural tissue, and hence if this document is approved it will require adipose therapies that are intended to serve functions other than structural to undergo the entire FDA premarket approval process. This is of particular concern to breast augmentation or reconstruction therapies that currently transplant subcutaneous adipose tissue to the breast, which has other functions than structural (i.e. lactation). There has been a wealth of evidence that adipose tissue serves numerous functions other than structural including participating in endocrine, hematopoietic, immunological, and regenerative functions within the human body [36,37]. The FDA is currently revising this guidance document and is expected to include these topics within the final document.
2.2.9 Quality control

Another important regulatory concern is the standardization of quality control practices in hASC therapies. In order to ensure that a safe and effective hASC product is administered to the patient, quality control must occur throughout hASC processing. After an adipose tissue sample is taken, it is desirable to perform an initial test to determine if the tissue is an acceptable donor source for the desired procedure. Karyotyping the sample to screen for any major chromosomal abnormalities has been suggested [38]. Other testing procedures that are often performed upon initial isolation are colony forming unit assays and flow cytometry cell marker analyses based on recommendations of IFATS and ISCT, as previously discussed [2].

During the expansion process, some suggest performing a phenotypic analysis, cytogenetics, sterility, tri-lineage differentiation potential testing, and colony forming unit assays after each split [38]. This testing should also be performed after removing cells from cryopreservation. Before administering the cell product to a patient, testing often includes all or a subset of the following tests: cell number, viability, purity and identity based on cell markers, cytogenetics, bacterial and fungal sterility, endotoxin, and mycoplasma [38-40]. Quality control must also be considered for both manufacturing facilities and reagents [41].

Additional information on such cGMP facilities and regulations for cell therapy has been previously reviewed [42]. As hASC enter clinical translation, standard quality control measures must be developed to ensure that hASC products are safe and effective for patient treatment.
2.3 Trends in translation

“While there has been a wealth of innovative and important research in hASC biology within the last decade, it has become apparent that to effectively translate hASC within the near future there will be a heavy reliance on technologies that can simplify and engineer around the gaps in our hASC understanding. We highlight four recurrent themes in translational technology as they pertain to hASC; automated closed-system operations, biosensors and real-time monitoring, biomimetics, and rapid manufacturing.

Automated closed-system devices will become an essential component of translating hASC. They greatly reduce the required resources for in vitro cell handling and effectively minimize human error. In addition, automated closed system operations assist in the implementation of the Food and Drug Association (FDA) guidelines for “process analytical technology,” a framework for controlling and regulating the manufacturing process of pharmaceutical products. Currently, there are two major functions of automated closed-system devices: isolation or expansion. Automated closed-system isolation devices allow clinicians to isolate a patient’s cells and re-administer the cells back to the patient within the same surgery. Several companies already manufacture such devices to isolate the stromal vascular fraction (SVF) from adipose tissue [6] and the SVF has been used in a number of clinical trials for soft tissue repair. In a clinical trial encompassing both breast augmentation and facial reconstruction, the Tissue Genesis Isolation System was used to isolate the SVF. Forty-two patients were successfully treated by cell-assisted lipotransfer (CAL), a procedure that enriches traditional lipotransfer methods with the addition of the SVF [7]. Cytori Therapeutics and collaborators have also conducted a number of clinical trials using their...
Celution® System. In a breast reconstruction study, they reported that SVF enriched fat grafts did not elicit any serious adverse effects and showed satisfactory aesthetic results in 57 out of 67 patients [8]. The clinical trials conducted by both Tissue Genesis and Cytori Therapeutics demonstrate that closed-system machines can isolate SVF reliably. Yet another form of automated closed-system devices are bioreactors used for cell expansion. There have been a number of recent studies focused on optimizing bioreactors for hASC expansion [9,10]. In the future, automated closed systems for isolation and expansion will likely be combined into the same device.

A critical component to the design of closed system devices is the ability to monitor internal conditions through the use of biosensors and real-time monitoring technologies. Biosensors are already incorporated into commercial stirred tank bioreactors to ensure batch control for commercial fermentation or pharmaceutical applications. However, the use of cell lines from different donors presents a new layer of complexity to the biomanufacturing process. It is known that hASC isolated from different donors have differing proliferation and differentiation potentials [11,12]. For this reason, there has been increasing interest in quantifying and monitoring variability between cell lines in order to generate reproducible results from a variable input. A technology that has potential to be integrated into hASC expansion to monitor donor cells is Raman spectroscopy, which can be used to non-invasively quantify biochemical changes within a cell line. It has been shown that cell-source-dependent variations in bone formation capacities can be monitored using Raman spectroscopy [13]. We have also shown that Raman spectroscopy can be used to non-invasively measure lipid production during hASC adipogenesis within as little as one day.
after the onset of adipogenesis [14]. Mass spectroscopy also holds promise for these types of applications. Mass spectroscopy has previously been used to monitor the proteome [15] and secretome [16] of hASC and also has the potential to monitor the hASC metabolome in real-time. The use of mass spectroscopy to track hASC allows for use of a minimal amount of conditioned media to provide a rapid, comprehensive, and potentially quantitative means of assessing hASC throughout expansion and differentiation. Finally, electrical impedance spectroscopy holds similar promise having also been used to track adipogenic and osteogenic differentiation of hASC in real-time [17]. The likely integration of biosensors into closed-system devices will allow for real-time monitoring and, if needed, correction of conditions within such devices for desired hASC response.

Biomimetics is essential to effective in vitro hASC technologies, in particular when extended in vitro culture or manipulation is required. Soluble chemical signals have long been used to differentiate and manipulate stem cells. However, it has become increasingly clear that mechanical and other physical stimuli also play a key role in directing stem cell fate. There has been a wealth of recent research on the use of biomimetic mechanical loading to direct hMSC and hASC fate. We and others have used biomimetic magnitudes of cyclic tensile strain [12,18-20] or fluid shear stress to promote osteogenesis [21,22] hydrostatic pressure to promote chondrogenesis [23], oscillatory shear stress to alter actin organization and differentiation potential [24] and unloading to promote adipogenesis [25,26] or to maintain stemness of hASC spheroids [27]. Electrical stimulation has been shown to enhance hASC differentiation for cardiac, [28] neuronal, [29] and osteogenic applications [30] applications. The use of biomimetics to direct stem cell fate will likely be incorporated into automated
closed system devices through physiologic chemical, mechanical, and electrical stimuli to further optimize hASC performance for specific applications.

Another theme that can be observed throughout current translational tissue engineering and regenerative medicine research is the use of rapid manufacturing technologies such as 3-D printing. Advantages of these techniques include automation, ease of generating patient-specific designs, reduced manufacturing costs, tunability, and three-dimensional tissue architecture. There are numerous options and considerations for generating bioprinted tissues [31], many of which have not yet been used in hASC applications. However, a few recent publications have incorporated hASC into rapid manufacturing technologies. Patient-specific reconstruction of mandibular ameloblastoma resection defects using computer-aided additive manufacturing of beta-tricalcium phosphate constructs seeded with a hASC biologic component was recently reported [32]. Rapid manufacturing has also been used for cartilage applications and it has been demonstrated that hASC seeded on 3-D printed chitosan scaffolds could be induced to differentiate down the chondrogenic lineage [33]. In an effort to generate a more biologically relevant scaffold, Pati et al. recently demonstrated that hASC and other cell lines could be printed in a cell-laden extracellular matrix bioink to generate adipose, cartilage, and heart tissue [34]. These rapid manufacturing technologies are expected to gain even more momentum as hASC translation moves forward into patient specific applications in the coming years.

While there have been many advances in hASC research in the past few years, fully successful hASC translation still requires significantly more innovation. The entire process
of an autologous hASC therapy and potential barriers to translation is illustrated in Figure 2-2. Research and development of technologies that simplify, standardize, and enhance quality control within this process will be particularly instrumental in facilitating hASC translation.

**Figure 2-2** The steps of adipose stem cell therapy. Two modes of hASC therapy are highlighted and examples of some critical issues at each step are shown (but by no means all inclusive). In Mode 1 of hASC therapy, standardized methods should be developed to pre-screen each patient for hASC therapy candidacy and to determine the best method of adipose tissue acquisition (whether resection, liposuction, or alternative). Since Current Good Manufacturing Practice (cGMP) facilities for hASC may be located off-site, technologies for shipping hASC should be optimized. Cell isolation technologies should maximize cellular yield. There will be many issues to consider when manipulating cells including the high level of hASC variability between donors, and the inherently heterogeneous cell population. The development of closed-system devices that continually monitor cells and adjust culture conditions to deliver a consistent hASC output may be especially useful in achieving this goal. In addition, if the patient would like to bank cells for future procedures, long-term storage methods must be validated for safety and efficacy. An ideal mode of hASC administration is both condition-specific and patient-specific. After a hASC treatment, standard methods are needed to monitor a patient for adverse side effects. In Mode 2, hASC are isolated, processed and administered back to the patient at the point of care. This method will require the optimization of closed system isolation devices and the determination of whether the SVF or hASC will be the final cell therapy delivered back to the patient.
2.4 The emerging hASC industry

With the transition of hASC from the lab bench to widespread clinical application, there is naturally the emergence of an hASC based industry. The beginnings of such an industry can already be identified from clinical trial and patent data. As of March 9, 2015, after steadily rising for the past decade (Figure 2-3A), 152 results could be found on clinicaltrials.gov with the search term ‘adipose stem cell.’ The majority of these trials are in Phase 1 (18%), Phase 2 (13%), or Phase 1/2 (41%) (Figure 2-3B). Most of the studies are in the process of recruiting, although 37 trials have already been completed (Figure 2-3C). The total enrollment capacity of these 152 trials includes 11,162 patients. Of the current clinical trials, 49% are sponsored by the private sector and 51% are sponsored by non-commercial entities.

Clearly, there is significant interest from the private sector in hASC research. As of March 9, 2015, there were 25 companies that were the primary sponsor of hASC clinical trials or patents specifically pertaining to hASC registered with the U.S. government, as illustrated in Figure 2-3D. These companies include a mixture of established companies expanding into hASC research and new companies specifically focused on hASC therapy. One of the advantages to industrial translation of hASC over other stem cell sources is that it is possible to perform simple, low-risk procedures such as isolating fat tissue from a patient and re-transplanting it into a patient within the same procedure. This has provided a relatively low risk entry point to firms looking to enter the field. However, as regulatory agencies implement higher regulatory standards on non-homologous and allogenic hASC products, entry into this field is becoming more complex. Nevertheless, if the wide range of clinical trials is any indication of the coming industry trends, the industry is likely to expand and
diversify in the coming years. For now, it is clear that hASC companies have emerged across the globe and there is no sign of this trend slowing down.

Figure 2-3 hASC clinical trials and the emerging global industry. A. The number of hASC clinical trials registered on clinicaltrials.gov has been gaining momentum for the past decade. As of March 9, 2015 most trials were still in B. Phase 1 and 2 and C. in the process of recruiting. D. In addition to clinical trials, there has been an emergence of a global industry. The twenty-five mapped companies throughout the globe are current leaders within hASC commercialization as identified by hASC-related clinical trials and patents. These companies were identified from either the 152 previously mentioned clinical trials or a U.S. patent search (ABST/(adipose AND stem AND cells) or TTL/(adipose AND stem AND cells)) on http://patft.uspto.gov on March 9, 2015. General mesenchymal stem cell patents and patents held by non-commercial institutions were not included within this industry map, although a number of universities and hospitals also held clinical trials and patents.” (1)
2.5 Focuses in hASC clinical trials

Of the clinical trials discussed in the previous section, certain conditions have garnered especial attention. Crohn’s disease fistulas was one of the first and most researched conditions that has been treated with hASC in clinical trials. In 2005, a Phase I clinical trial reported a 75% healing rate in the treatment of 8 fistulae with autologously derived hASC (67). A Phase II clinical trial in 2009 reported 71% healing in patients treated with hASC and fibrin glue as compared to 16% healing in those treated with glue alone (68). However, in 2012, a Phase III clinical trial reported no significant difference observed between patients treated with fibrin glue alone and fibrin glue in conjunction with hASC (69). In 2013, a clinical trial administered hASC via a local injection. The results suggested that this method is also safe and effective for the treatment of Crohn’s fistula (70). In addition to healing fistulae for Crohn’s disease, hASC might be a viable option for the treatment of other related conditions. A case report demonstrated that hASC are capable of re-epithelialisation and re-vascularization of tracheal fistulae (71).

Soft tissue repair and regeneration has been a popular subject of hASC clinical trials. The injection of autologous lipoaspirates was demonstrated to facilitate progressive regeneration in all treated patients suffering from severe radiation symptoms or irreversible functional damage from radiation in one study that was targeted towards breast cancer survivors (72). In a procedure called cell-assisted lipotransfer, hASC are used in combination with traditional lipotransfer methods to reduce variability in graft survival from necrosis. Cell-assisted lipotransfer has been used to successfully treat patients for both facial lipoatrophy and cosmetic breast augmentation (66, 73). In a clinical trial encompassing both breast
augmentation and facial reconstruction, the Tissue Genesis Isolation System was used to isolate the SVF and 42 patients were successfully treated by cell-assisted lipotransfer (74). Cytori Therapeutics and collaborators have also conducted a number of clinical trials using their Celution® System. In a breast reconstruction study, hASC enriched fat grafts did not elicit any serious adverse effects and showed satisfactory aesthetic results in 57 out of 67 patients (75). In addition to repairing soft tissue damage, the clinical trials conducted by both Tissue Genesis and Cytori Therapeutics demonstrated that closed-system machines could be used to isolate hASC in a highly defined manner.

Clinical trials for the treatment of musculoskeletal damage have also been underway. In a recent study, 18 patients with osteoarthritis of the knee were injected with hASC. Patients that were injected with a high dose of 1x10^8 cells demonstrated a decrease in defect size and increased cartilage regeneration without adverse effects (76). A case study successfully produced ectopic bone for maxillary reconstruction using autologous hASC in a beta-tricalcium phosphate scaffold dosed with BMP-2 (77). As this technology has advanced over the last few years, researchers recently published a similar study in which an hASC seeded beta-tricalcium phosphate scaffold with BMP-2 was implanted into three patients from the outset of the procedure, thus eliminating the need to generate the bone ectopically. Three patients were successfully treated using this in situ bone formation technique (78). In a case report, calvarial defects were repaired with milled cancellous bone that was injected with autologous hASC and fibrin glue. After a three-month follow up, new bone formation was evident from CT-scans and the defects were almost completely healed (79). The use of hASC
for musculoskeletal repair has been highly studied in preclinical animal models and is likely to become a major sector of hASC therapy in the future.

The immunomodulatory properties of hASC have also been assessed in clinical trials. Studies on the salvage therapy of steroid-refractory graft-versus-host disease have been highly promising for patients who negatively respond to hematopoietic stem cell transplantation. A case study demonstrated that two pediatric patients were treated successfully using hASC infusion after failing to respond to standard therapy (80). In another small study, 5 out of 6 patients showed remission from graft-versus-host disease after an infusion with hASC (81). A case study demonstrated that hASC effectively treated pure red cell aplasia, another hematopoietic stem cell transplantation complication, in two patients (82). These studies demonstrate the potential that hASC have for immunomodulatory applications.

Clinical studies have also assessed the safety of hASC in a variety of other conditions. In addition to the previously mentioned studies, the safety of hASC was recently assessed in a clinical trial in which patients with idiopathic pulmonary fibrosis received three infusions of hASC into the lungs. The patients that received the treatment did not exhibit any adverse effects, demonstrating the safety of this procedure, although efficacy has yet to be determined (83). In the case of spinal cord injuries, no adverse side effects were evident in any spinal cord injury patients after a 3-month follow up of an intravenous hASC injection (84). Although further long-term studies will be required to definitively assess the side effect and tumorigenicity of hASC in human patients, the current body of literature on hASC clinical trials supports that the adverse side effects are minimal for hASC procedures.
2.6 Conclusions and future directions

The translation of hASC from laboratory research to wide spread clinical use is progressing rapidly as evidenced by the expanding industry with hASC clinical trials and intellectual property. However, before hASC therapy is truly to be considered a safe and effective treatment for any condition, more standardization must be achieved within the field and several basic-science topics within hASC research must be further investigated. These include but are not limited to cell population heterogeneity, donor-to-donor variability, and culture with animal-product free methods. Further, significant effort must be dedicated to topics at an industrial level such as production, preservation, banking, shipping, and quality control. Finally, clinical methods relating to hASC procedures must also undergo standardization. By focusing on these topics, the hASC research community will be able to see a more rapid translation of hASC to treat the many patients that could benefit from hASC based therapies.
In the prior chapter, we described the considerations that must go into the translation of adipose stem cell therapies. hASC can be administered therapeutically using variety of methods including direct injection of the stromal vascular fraction, direct injection of processed hASC, incorporation of hASC into manufactured tissue engineered constructs, and seeding hASC onto naturally derived extracellular matrix. In this chapter, we describe a method for incorporating hASC into a decellularized meniscal allograft. The goal is to improve hASC infiltration into the decellularized meniscus so that it can be repopulated with a patient’s autologous hASC.

The work in this chapter has been published in the Journal of Orthopaedic Surgery and Research with co-authors Adisri Charoenpanich, Christopher E. Vaughn, Emily H. Griffith, Matthew B. Fisher, Jacqueline H. Cole, Jeffrey T. Spang, and Elizabeth G. Loboa(85).

3.1 Introduction

“The meniscus plays a crucial role in knee joint function by providing joint stability and allowing shock absorption, load transmission, and stress distribution within the knee joint. Meniscal tears are the most common knee injuries with an annual reported incidence of 60-70 per 100,000 persons (86, 87). With limited natural repair capabilities, surgical treatment is very common. Over 1 million surgeries involving the meniscus are performed annually in the
United States (88). Although meniscus repair is preferred, not all meniscus tears can be repaired, such as those that occur in the avascular inner-third and complex tears that compromise the structural integrity of the meniscus. If repair is not possible, a meniscectomy is commonly used to alleviate symptoms. However, partial or total removal of the meniscus has detrimental effects on the knee joint, and these treatments increase the contact stresses on the articular surface of the knee joint (89). In a knee with a meniscectomy, the contact area between the tibia and the femur is reduced by 50 percent(90). Long-term, there is a high risk of osteoarthritis development after meniscectomy procedures (91).

Currently, the standard treatment for a symptomatic patient who has undergone a substantial meniscectomy is meniscal allograft transplantation (92). In general, the current body of literature is supportive of meniscal allografts and many patients have experienced reduced pain and improvements in joint function (93). In addition, meniscal allografts can restore biomechanics of the knee after meniscectomy (94). Meniscus allograft transplants have shown favorable results in terms of clinical improvement by reducing pain and improving function in both short- and medium-term follow-up, (2 and 5 years, respectively) and even, in some cases, at long-term (>10 years) follow-up (95-98). The survival rate for a 10-year follow-up in cryopreserved and fresh-frozen meniscal allograft transplants is between 50 and 70%, with defined failure as tearing and/or sub/total destruction requiring repair/partial meniscectomy or removal of the allograft (95, 98-100). A magnetic resonance imaging study on the width and thickness of fresh frozen meniscal transplants showed that shrinkage in the width (89%) and increase in the thickness (115%) will be observed within the first year of transplantation (101). Furthermore, long-term effectiveness in patients who participate in
contact sports is unclear; some of the current studies advise against participation in sports with strenuous cutting and twisting (93). Moving forward, research must address how to better integrate the meniscal allografts into the host knee and prevent degradation over time due to lack of cellular incorporation after implantation (102, 103).

Previous human retrieval studies and animal studies have reported incomplete cellular incorporation, absence of cell proliferation, and a microscopic immune response (104-106). The high tissue density of the meniscus has been postulated to result in low cellular incorporation in meniscal allografts (107). Without successful ingrowth and cellular repopulation, meniscal allografts lack the capacity to remodel and perform necessary internal maintenance. To improve the success rate of allograft transplants, previous investigators have used animal models to evaluate whether removal of donor cellular components and/or increasing porosity of a transplanted meniscus will allow for the incorporation of a biologically active substrate (growth factors, platelets or mesenchymal stem cells (MSC)) (108-112). Importantly, the incorporation of MSC has been suggested to enhance meniscal regeneration and healing (109, 111).

To date, little research (113, 114) has focused on the possibility of modifying the human meniscus with human stem cells to enhance the meniscus as a potential scaffold; and/or to determine biological responses of human stem cells seeded on a human meniscal allograft. In this study, human menisci were decellularized to remove donor cells, and meniscus porosity was enhanced via both chemical and mechanical factors. Needle punching was implemented
to increase porosity and improve cell penetration. Because of their chondrogenic differentiation potential, autologous availability, and immunocompatibility (1, 115-119), human adipose derived stem cells (hASC) were utilized to investigate their potential for cell seeding, viability, and migration within the allograft derived meniscal scaffold. Material property testing was performed to determine the effects of chemical and mechanical decellularization and porosity enhancement on meniscus mechanical properties both pre- and post-hASC recellularization. We hypothesized that increasing the meniscal porosity would improve cellular incorporation of human adipose stem cells without compromising the bulk biochemical or biomechanical integrity of the allograft.

3.2 Methods

3.2.1 Meniscus acquisition and experimental groupings

Gamma sterilized, frozen human menisci attached to a hemiplateau were provided by the International Institute for the Advancement of Medicine, a subsidiary of the Musculoskeletal Transplant Foundation (Edison, NJ). Demographic information of menisci used in this study is included in Table 2-1. The menisci were examined prior to testing and did not exhibit any overt structural defects. Menisci were then removed in their entirety from the hemiplateau prior to laboratory work.

For this study, 20 medial menisci from 12 donors were separated into five experimental groups for an n=4 in each grouping. The groups consisted of 1) control native menisci (Control), 2) decellularized menisci (Decell), 3) decellularized menisci seeded with hASC (Decell + hASC) 4) decellularized needle-punched menisci (Decell + NP), and 5)
decellularized needle-punched menisci seeded with hASC (Decell + NP + hASC). For the control group, only one medial meniscus was used per donor; for the other four groups, both medial menisci were used, split between either the needle-punched or non-needle-punched groups (one for decellularized and one for decellularized + hASC). Following decellularization and needle punching, the four experimental conditions were cultured for four weeks after their respective treatments. After four weeks of culture, samples were dissected for histological, biochemical, and mechanical analysis.

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Age</th>
<th>Gender</th>
<th>Right Meniscus</th>
<th>Left Meniscus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>Female</td>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>41</td>
<td>Male</td>
<td>-</td>
<td>Control</td>
</tr>
<tr>
<td>3</td>
<td>46</td>
<td>Male</td>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>38</td>
<td>Male</td>
<td>-</td>
<td>Control</td>
</tr>
<tr>
<td>5</td>
<td>47</td>
<td>Male</td>
<td>Decell+hASC</td>
<td>Decell</td>
</tr>
<tr>
<td>6</td>
<td>45</td>
<td>Male</td>
<td>Decell</td>
<td>Decell+hASC</td>
</tr>
<tr>
<td>7</td>
<td>39</td>
<td>Male</td>
<td>Decell+hASC</td>
<td>Decell</td>
</tr>
<tr>
<td>8</td>
<td>26</td>
<td>Female</td>
<td>Decell</td>
<td>Decell+hASC</td>
</tr>
<tr>
<td>9</td>
<td>43</td>
<td>Male</td>
<td>Decell+NP+hASC</td>
<td>Decell+NP</td>
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<tr>
<td>10</td>
<td>38</td>
<td>Male</td>
<td>Decell+NP</td>
<td>Decell+NP+hASC</td>
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<tr>
<td>11</td>
<td>41</td>
<td>Male</td>
<td>Decell+NP+hASC</td>
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<tr>
<td>12</td>
<td>41</td>
<td>Male</td>
<td>Decell+NP</td>
<td>Decell+NP+hASC</td>
</tr>
</tbody>
</table>

3.2.2 Decellularization and porosity enhancement of human meniscus

Chemical decellularization of the menisci was performed to remove donor cells, as described previously (108). In brief, whole meniscus was placed in deionized water at 37°C for 48 hours, followed by a 24-hour enzymatic digestion in 0.05% trypsin EDTA, a 24-hour trypsin neutralization in complete growth medium (CGM) (α-MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% Penicillin Streptomycin), and a 48-hour incubation in 2% Triton X-100 and 1.5% peracetic acid. The menisci were then subjected to three washes in deionized water. This approach has previously been shown to remove cell debris fully, increase porosity, and maintain mechanical properties and glycosaminoglycan (GAG).
content in ovine menisci (108). To enhance porosity further in the needle-punched groups, a 28 G microneedle was used to punch pores manually through the meniscus in both a superior-to-inferior and inferior-to-superior pattern (1mm spacing, full thickness punches).

3.2.3 Cell seeding and cell culture

Human ASC were isolated from liposuction aspirates of female patients undergoing voluntary liposuction procedures using an approved IRB protocol at the University of North Carolina at Chapel Hill (IRB 04-1622), as described previously by our laboratory (13). CGM was used to expand hASC. An age-grouped (age: 24-36, 5 donors), hASC superlot was generated and verified as representative of individual donors, as described previously (2). The hASC superlot was seeded at a density of 250,000 cells per gram of meniscal tissue. To enhance cell attachment and integration throughout the meniscus, samples were sealed in sterile heat-sealed bags for seeding and centrifuged 5 times at 2,000 rpm for 1-minute intervals, with gentle resuspension of unattached cells after each cycle to increase cell-meniscus contact. After centrifugation, bags were left on an orbital shaker overnight in incubator before the menisci were transferred to specimen cups for remainder of cell culture. Cell-seeded menisci were cultured in an incubator for up to four weeks in CGM at 37°C and 5% CO₂. After four weeks, cell viability was verified via Calcein AM for visualization (Molecular Probes, Eugene, OR).

3.2.4 Scanning electron microscopy analysis

To detect changes in ECM after treatments, scanning electron microscopy (SEM, JEOL JSM-6400F, MiQro Innovation, Québec, Canada) was used to visualize specimens before and after
decellularization. Menisci were fixed with 2.5% glutaraldehyde, followed by serial ethanol dehydration. They were then critical point dried in liquid CO₂ and gold-platinum sputter coated for scanning electron microscopy analyses.

3.2.5 Histological analyses

Cross-sectional samples of each meniscus were taken for histological analyses (Figure 3-1). To verify the decellularization process, cell-seeded menisci were dissected and fixed in 10% formalin for 24 hours, followed by two washes in phosphate buffered saline (PBS), ethanol dehydration, and paraffin embedding. Menisci were sectioned into 10-µm thick slices with a Leitz 1512 rotary microtome (Leica, Wetzlar, Germany), and sections were stained with 4,6-diamidino-2-phenylindole (DAPI) to identify residual nuclear debris. Additional sections were stained with fast green/safranin O with a hematoxylin counter stain to visualize collagen organization, proteoglycans, and cellular infiltration in both the “red-red zone” and “white-white zone” of the menisci.

![Figure 3-1](image)

**Figure 3-1** Diagram of meniscus sample preparation. 5-mm plugs were taken from the anterior and posterior for material property testing. Biochemical samples were taken beneath the surface of the meniscus. Histological samples were taken in cross sections and imaged in the “red-red zone” towards the exterior of the meniscus and at the “white-white zone” towards the interior of the meniscus.
3.2.6 Biochemical Analysis

Menisci were dissected with a scalpel to obtain four samples (approximate size of 1mm$^3$) from beneath the surface of each meniscus (Figure 3-1). Samples were lyophilized for 48 hours and massed to obtain dry weight of the tissue. Samples were papain digested for 16 hours, and DNA content was quantified using a Hoechst 33342 assay (Thermo Fisher, Waltham, MA).

3.2.7 Mechanical Testing

Full-thickness cores were taken from the anterior and posterior regions of the meniscus (Figure 3-1), in a direction perpendicular to the femoral surface, using a 5-mm diameter cylindrical biopsy punch. To provide parallel surfaces for even load distribution during testing, the superior and inferior edges of each sample were trimmed using a HM525 NX cryostat (Thermo Fisher, Waltham, MA). The samples were stored at -20°C until testing and then thawed individually in PBS at room temperature just prior to testing. The initial thickness and diameter were each measured in three locations on the sample using digital calipers, and the mean values were calculated.

Samples were submerged in a PBS bath within a custom fixture, and the static and dynamic mechanical properties were measured in unconfined compression (120) between two rigid, impermeable platens using an axial testing system (Bose EnduraTEC ELF3220, EnduraTEC Systems Corp., Minnetonka, MN) equipped with a 500-g load cell (Model 31 Miniature Load Cell, Sensotec, Columbus, OH). Several compression tests were performed in succession, as follows. A compressive tare load of 0.025 N was applied in load control for 5 minutes, and
then a new sample thickness was calculated based on the initial thickness and the change in actuator displacement under the creep tare load. A stress relaxation test was performed to 10% strain (based on the post-creep thickness), applied at a rate of 0.01 mm/s and held for 40 minutes to ensure the sample reached equilibrium. Sinusoidal cyclic loading was then applied using a magnitude of 10 ± 1% strain (9-11% strain) for 10 cycles each at frequencies of 0.1, 1, and 10 Hz. A second stress relaxation test was performed to 20% strain, applied at a rate of 0.01 mm/s and held for 40 minutes, followed by a second set of sinusoidal cyclic loading to 20 ± 1% strain (19-21% strain) for 10 cycles each at frequencies of 0.1, 1, and 10 Hz. Load and displacement were recorded for all tests.

Testing data were processed in MATLAB (MathWorks, Natick, MA). Stress and strain were calculated from the original cross-sectional area and post-creep thickness, respectively. Using the stress relaxation data sets, equilibrium stress was computed for 10% and 20% strain, defined when the mean stress change per minute was less than 1%. Compressive equilibrium moduli were determined from the equilibrium stress and applied strain values. Dynamic compressive moduli were measured at each frequency as the mean slope of the stress-strain curves for all cycles plotted together at each strain level.

3.2.8 Statistics

Statistical analysis was performed following biochemical and mechanical testing. Biochemical and mechanical testing data were analyzed using a linear mixed-effects model, and a significance level of 0.05 (SAS 9.4, SAS Institute Inc., Cary, NC). A random blocking factor was introduced to account for correlations between the two menisci taken from the
same donor that were divided between treatments. Treatment groups were divided evenly across the blocks to make a complete block design. For biochemical data, the average DNA content of all four samples from within a menisci used and the treatment group was treated as a fixed effect. Orthogonal contrasts were used to test: 1) the effect of decellularization on non-needle punched menisci (control vs. decellularized); 2) the effect of decellularization on needle punched menisci (control vs. decellularized needle-punched); 3) the effect of reseeding non-needle punched menisci (decellularized vs. decellularized + hASC); and 4) the effect of reseeding needle punched menisci (decellularized needle-punched vs. decellularized needle-punched + hASC).

For mechanical data, treatment group was treated as a fixed effect, and anatomical location (anterior, posterior) was treated as a repeated measure. Orthogonal contrasts were used to test: 1) the effect of decellularization (all treatments vs. control); 2) the effect of needle punching (decellularized vs. decellularized needle-punched, decellularized + hASC vs. decellularized needle-punched + hASC); and 3) the effect of seeding with hASC (decellularized vs. decellularized + hASC, decellularized needle-punched vs. decellularized needle-punched + hASC).

3.3 Results

3.3.1 Decellularization of Human Menisci

The general shape and architecture of the menisci were maintained after decellularization (Figure 3-2A,B). Electron microscopy revealed that chemical decellularization removed the donor cell membrane but did not unpack the collagen bundle (Figure 3-2C,D). Successful
removal of donor nucleic acid was observed with negligible DAPI/nuclear staining in the
decellularized menisci relative to the intact menisci (Figure 3-3).

**Figure 3-2** Gross inspection of human meniscus A. before decellularization and B. after
decellularization showed that overall shapes were maintained for decellularized compared to
intact meniscus. Scanning electron micrographs of C. control meniscus at 500x
magnification, D. decellularized meniscus at 500x, showing that the extracellular matrix
retained its structure throughout the decellularization process (scale bars = 50 µm).

**Figure 3-3** Confirmation of the decellularization process. Cellular and nuclear content was
tested via DAPI staining. Bright, punctate spots (arrows) indicating nuclei A. can be seen
before decellularization but B. are absent after decellularization (blue background staining of
tissue is still present). High magnification images of nuclear staining in both C. control and
D. decellularized menisci. (All scale bars = 100 µm).
3.3.2 Viability of hASC on Decellularized Menisci

Live staining indicated that hASC remained viable in both decellularized and needle-punched decellularized menisci (Figure 3-4A,B). SEM revealed elongated hASC aligned parallel to the native collagen fibers of the decellularized menisci (Figure 3-4C,D).

**Figure 3-4** The seeded hASC remained viable and aligned along the fibers of the meniscus. Live staining (green) of A. hASC-seeded decellularized meniscus and B. needle-punched decellularized meniscus (scale bars = 200 µm). Scanning electron micrographs revealed that hASC were well organized and aligned parallel to the collagen fiber extracellular matrix of the decellularized meniscal allograft, as shown at C. 200x (scale bar = 100 µm) and D. 2000x (scale bar = 10 µm).

3.3.3 Histology

Histological analyses of the hASC-seeded meniscal allografts indicated that cells were able to proliferate on the periphery of the menisci, but decellularization alone did not allow for cells to migrate further than the surface of the menisci (Figure 3-5A,B). With needle punching, cells were able to migrate through the pores deeper into meniscal tissue (Figure 3-5C,D).
Figure 3-5 Safranin-O staining of reseeded meniscal samples. hASC seeded on a non-needle-punched decellularized meniscus within the A. exterior “red-red zone” and B. interior “white-white zone” of the meniscus. hASC seeded on a needle-punched decellularized meniscus on the C. exterior “red-red zone” and D. interior “white-white zone” of the meniscus. Collagen is stained in blue, and nuclei (arrows) are stained in dark brown. Greater cellular infiltration was observed in needle-punched samples. All scale bars = 200 µm.

3.3.4 Biochemical analysis

Biochemical quantification of DNA content between treatment groups (Figure 3-6) showed a 54% reduction in DNA in the non-needle punched decellularized menisci when compared to the control (0.156 ± 0.0265 vs. 0.0719 ± 0.0139 µg DNA/µg tissue, p=0.001). When the menisci were needle punched, there was a 61.5% reduction in DNA content (0.156 ± 0.0265 vs. 0.0602 ± 0.0101 µg DNA/µg tissue, p=0.0005). When menisci were not needle punched, reseeding with hASC did not increase DNA content within the menisci (0.0719 ± .0139 vs. 0.0699 ± 0.0176 µg DNA/µg tissue, p=0.6853). However, when menisci were needle punched, reseeding with hASC significantly increased DNA content within the menisci (0.0602 ± 0.0101 vs. 0.0901 ± 0.0217 µg DNA/µg tissue, p=0.0009).
Figure 3-6 Subsurface DNA content of menisci normalized to dry weight after 4-weeks of culture. DNA content of menisci was significantly reduced in decellularized menisci (** p<0.01 Decell vs. Control and Decell + NP vs. Control). Recellularization did not increase DNA content in menisci that had not been needle-punched (n.s. p>0.05 Decell vs. Decell + hASC). However, needle-punched menisci had increased DNA content when reseeded (** p<0.01 Decell + NP vs. Decell + NP + hASC).

3.3.5 Material Properties

Anterior and posterior values of the equilibrium moduli (10% strain, 20% strain) and dynamic moduli (10% strain at 0.1/1/10 Hz, 20% strain at 0.1/1/10 Hz) were not significantly different (per paired t-tests), and the mean values are reported here for simplicity, although both values were used in the mixed-model analysis.

Decellularization did not significantly affect the equilibrium modulus, as the values in the four treatment groups were similar to those in the control group at both 10% strain (32.2 ±
12.0 kPa vs. 34.9 ± 25.3 kPa, p = 0.71) and 20% strain (30.3 ± 15.1 kPa vs. 31.4 ± 26.3 kPa, p = 0.88, Figure 3-7A). Dynamic modulus tended to be lower for menisci in the treatment groups than in the control group for both 10% strain (mean 219.4 ± 94.0 kPa vs. 408.4 ± 352.9 kPa) and 20% strain (mean 426.8 ± 245.1 kPa vs. 748.6 ± 658.4 kPa), but these differences were not statistically significant at 0.1 Hz (10% strain: p = 0.059, 20% strain: p = 0.11), 1 Hz (p = 0.069, p = 0.12), 10 Hz (p = 0.092, p = 0.13), or for all frequencies combined (p = 0.066, p = 0.11, Figure 3-7B).

Figure 3-7 Decellularization, needle punching, and reseeding with hASC had no significant effect on meniscal material properties, assessed with A. equilibrium modulus (mean over anterior and posterior samples) and B. dynamic modulus (mean over all frequencies for anterior and posterior samples).
Neither needle punching nor reseeding with hASC significantly altered either the equilibrium or dynamic moduli beyond the effects of decellularization. For needle-punched menisci only, reseeding with hASC tended to reduce the equilibrium modulus at 10% strain, although this effect was not significant (p = 0.088).

### 3.4 Discussion

Ideally, a meniscal allograft would be populated with a patient’s own autologous cells that could respond to mechanical stimuli and maintain the meniscus throughout the patient’s life. However, current meniscal allografts have high-density collagen networks that are difficult for cells to penetrate \textit{in vivo}. In the current study, we have investigated the potential to improve human meniscal allografts by introducing additional porosity to decellularized human menisci and seeding with hASC. We hypothesized that increased porosity would allow for increased autologous cellular infiltration into the scaffold.

Following a previously studied method of meniscal decellularization (108) we first verified that the menisci were decellularized and supported hASC cell growth. The meniscal allografts maintained their structure throughout the decellularization process, confirmed both on a macroscopic scale via gross observation and at the extracellular matrix level via SEM. In accordance with previous studies, decellularized menisci lacked defined nuclear staining, demonstrating the absence of cellularity. Immunohistochemistry of fresh, cryopreserved, and frozen human menisci have shown that the presence of human leukocyte antigen and A, B, and H blood group antigens can complicate a meniscus allograft transplant (106, 121). In this study, we have successfully removed donor cells while maintaining the overall structure of a
human meniscal allograft. Reseeding the decellularized allograft with hASC showed promising results, as cells remained viable and even organized parallel to the structure of the collagen fiber extracellular matrix within the meniscal allograft. Viability of the hASC were maintained on both intact and needle-punched menisci. The ability of hASC to align parallel to the native collagen fibers in the meniscal allograft is promising in that it may allow for future collagen production by hASC within a meniscal allograft. Cell orientation has been previously shown to determine alignment of a cell-produced collagen matrix (122, 123).

Histological evidence showed an increase in cellular infiltration in needle-punched menisci compared with non-needle-punched menisci. The increased porosity introduced channels through which the hASC can migrate into the interior of the scaffold, which is critical for the living scaffold to integrate fully and maintain tissue homeostasis. hASC seeded on scaffolds that lacked needle punching clustered on the surface of the meniscus, but did not penetrate into the interior of the scaffold. Altogether, this demonstrates that the introduction of additional porosity is necessary for allogenic meniscal transplants to permit the infiltration of autologous cells.

Biochemical analysis of DNA content further elucidated the extent of decellularization and recellularization in the allografts. DNA content was significantly decreased (p<0.01) in both menisci with no needle punching and needle-punched menisci. Although the menisci still contained some nuclear material we obtained 54% reduction in DNA content, which was highly consistent with the 55% reduction that had previously been reported using this decellularization method in ovine menisci (108). It has been postulated that nuclear material
remains in the menisci through hydrostatic interactions with the tissue, even when cellular content is absent (108). A slightly lower DNA content was observed in the needle-punched menisci with a 61% reduction. Since needle-punching took place after the decellularization process, this suggests that the needle punching allowed residual DNA fragments to wash out during the 4-week incubation period. During the recellularization process, no increase in DNA content was observed in the non-needle-punched samples. However, the needle-punched menisci did significantly increase (p<0.01) in DNA content when reseeded. Since the biochemical samples were taken from the interior of the meniscus, this suggests that cells were unable to migrate into the non-needle-punched menisci but were able to penetrate into the needle-punched menisci. Although DNA content did not reach levels of native tissue in the reseeded needle-punched menisci, future optimization of this method could lead to more efficient infiltration of hASC. Herein, we demonstrated that the seeded hASC can migrate into the interior of the needle-punched allograft. Cellular content in the central zones may increase with longer culture or after in vivo implantation, which will be the subject of future investigations.

Both static and dynamic compressive material properties were not altered significantly by treatments used to prepare the meniscal allografts. Relative to control samples, menisci that were decellularized, needle punched, and/or reseeded with hASC had similar equilibrium and dynamic moduli in unconfined compression, regardless of the anatomical location (anterior, posterior), strain level (10%, 20%), or dynamic testing frequency (0.1, 1, 10 Hz). A previous study in fresh human meniscus showed that dynamic compressive modulus was positively correlated with glycosaminoglycan content and negatively correlated with water content.
Therefore, the trend for decreased modulus with decellularization may indicate a slight loss in collagen content, increase in water content, or both within the allografts (124). The moduli values in this study were similar to those reported in other studies of axial unconfined compression in human meniscus (124, 125). While some studies have measured differences in moduli for different locations within the meniscus (anterior, central, posterior), others have measured similar properties in anterior vs. posterior samples (125), as shown here. Our material property testing results are consistent with a recent study in which it was shown that laser drilled menisci exhibited a slight decrease in Young’s modulus and instantaneous stress but remained within a physiological range (126).

A limitation of the current study is that only one needle-punching configuration (1mm$^2$ spacing with a 28 G microneedle) is tested. Future studies should optimize spacing and needle diameter to allow cellular infiltration while minimizing disruption of the natural meniscal architecture. In addition, future in vivo studies must be carried out to assess the potential for clinical translation. We expect that the hASC introduced into the scaffolds would allow for enhanced remodeling of the meniscus once the scaffold is transplanted in vivo.

3.5 Conclusions

In this study, we observed the increase in hASC infiltration into human meniscal allograft, without significant alterations in the compositional or mechanical integrity of the scaffold. Since hASC can be easily isolated in large quantities for autologous use, the hASC could be an ideal cell source to repopulate an allograft scaffold before transplantation into the patient.
Successful tissue engineering utilizing hASC would allow meniscal allograft transplantation to be transformed by improving and extending the life of the transplant, leading to a large clinical advancement in the treatment of patients with meniscal deficiency.” (85)
CHAPTER 4
Investigation of multi-material multi-phasic 3D-bioplotted scaffolds for site-specific chondrogenic and osteogenic differentiation of human adipose derived stem cells for osteochondral tissue engineering

In the previous chapter, we discussed a method for incorporating hASC into decellularized mensical tissue. The natural ECM from the menisci was able to support hASC as they infiltrated into the scaffold. In this chapter, we describe a method incorporates decellularized ECM into a full-thickness bioplotted osteochondral scaffold. Site-specific chondrogenic and osteogenic differentiation of hASC was achieved. Chondrogenesis was promoted by coating PLA with dECM from articular cartilage and osteogenesis was achieved by incorporating 20% TCP within the scaffolds. In addition, an electrospun tidemark was used to prevent the cell from migrating across the tissue boundary, which is important to prevent ossification of the articular cartilage. The goal of this study was to create a novel scaffold that can induce site-specific differentiation of human adipose derived stem cells (hASC) to generate a full osteochondral tissue.

The work in this chapter is in preparation to be submitted to Tissue Engineering with co-authors. Liliana F. Mellor, Pedro Huebner, Michael A. Taylor, William Efird, Julia Thom Oxford, Jeffrey Spang, Rohan A. Shirwaiker, and Elizabeth G. Loboa.

4.1 Introduction
Osteoarthritis (OA) is a degenerative joint disease that limits mobility of the affected joint due to the degradation of articular cartilage and changes in the adjacent subchondral bone.
OA has been estimated to affect approximately 27 million people in the United States, and the prevalence is only expected to grow(127). There are significant challenges with treating cartilage and osteochondral degradation due to the limited regenerative capacity of articular cartilage, lack of understanding of the molecular mechanisms that trigger early changes in cartilage homeostasis, and lack of tools to detect the disease at early stages. To date, there is no cure for OA and therapeutic treatments are limited to controlling pain and inflammation of the joint.

Current clinical treatment alternatives for cartilage repair include autologous chondrocyte implantation (ACI), microfracture, and mosaicplasty(128). ACI involves a biopsy of the patient’s articular cartilage from a healthy joint, isolation and expansion of the isolated chondrocytes for several days in culture, followed by injection of chondrocytes into the defect site. While this process avoids issues with the immune response, the chondrocytes de-differentiate and lose their phenotype after few passages and become more fibroblast-like (1) resulting in implantation of cells that behave less like chondrocytes and more like fibroblasts, and compromising the chemical and mechanical properties of the repair site. This fibrocartilaginous tissue eventually degenerates(129). Microfracture involves drilling small holes through the articular cartilage to the subchondral bone allowing mesenchymal stem cells to migrate into the defect for cartilage repair(130). This technique results in fibrocartilage formation with very little collagen II and aggregan expression(131) and therapeutic effects are only sustained for approximately one year(130). Mosaicplasty, where plugs of autologous osteochondral tissue are taken from a healthy joint, is limited by tissue harvest and associated with donor site morbidity(132). Because of the limitations with
current treatment options, tissue engineering approaches using stem and/or primary cells have become an active area of research.

One of the critical challenges from a clinical application perspective is creating an engineered tissue that can mimic the complex multi-phasic nature of osteochondral tissue (133). The majority of stem cell based approaches for cartilage or osteochondral tissue engineering have used bone marrow derived mesenchymal stem cells (MSC) and have focused on cartilage regeneration (132, 134-142) instead of full osteochondral tissue regeneration (143-146). Attempts to engineer osteochondral tissue have usually approached the problem by generating either a multiphasic scaffold or gradient systems to generate bone, cartilage and the transitional zone of calcified cartilage that joins the two tissues. Biphasic scaffolds have been created by assembling two separate scaffolds of engineered bone and cartilage tissue (147-149). A disadvantage of this method is the lack of mechanical integration at the junction, leading to delamination of the layers. One approach to avoid delamination is to develop a monolithic scaffold with two phases that can be cultured as a unit to induce osteogenesis and chondrogenesis simultaneously. In this method, there are challenges of promoting both chondrogenic and osteogenic factors within the same culture system (150), which can be addressed by introducing biomimetic factors (151, 152) to the scaffold that induce either cartilage or bone formation, as desired.

In addition to biphasic systems, a few studies have attempted to achieve a graded transition zone of bone to cartilage throughout the depth of an engineered osteochondral tissue (152-157) as an attempt to improve load-bearing capabilities in vivo and reduce shear stresses at
the biomaterial interfaces(158). Some of the technical and culturing challenges include identifying the ideal cell source, optimizing growth factors and signaling molecules to induce site-specific differentiation, inhibiting hypertrophy, and developing a simple and reliable manner to manufacture scaffolds of varying size to fit a specific injury site. After implantation, the primary challenges include blood vessel invasion into the cartilage region that results in endochondral ossification, and proper integration with the host tissue(159).

The objective of this study was to investigate the use of a single cell source, human adipose derived stem cells (hASC) within an integrated multi-material, multi-scale triphasic 3D scaffold created using a combination of 3D bioplotting and electrospinning to achieve complete osteochondral tissue generation. Human ASC, which are more abundant and accessible than human bone marrow derived hMSC (35-37), are capable of both osteogenic and chondrogenic differentiation and can express bone and cartilage extracellular matrix (ECM) constituents (34,35). Although some reports indicate that hASC chondrogenesis may be inferior to hMSC chondrogenesis (38,39), we have shown the ability to overcome this by altering growth factor supplementation (40). Our triphasic 3D scaffold consists of physiologically inspired chemical cues to induce site-specific hASC differentiation in a manner that recapitulates the depth-dependent properties of native osteochondral tissue. The integrated 3D scaffold includes a deep layer of a composite of polycaprolactone (PCL) and β-tricalcium phosphate (TCP) to induce osteogenesis, a superficial layer of PCL combined with decellularized articular cartilage ECM (dECM) hydrogel to induce chondrogenesis, and an intermediate overlaid electrospun layer of PCL to mimic the native tidemark of osteochondral tissue to separate the two phases and potentially prevent blood vessel invasion.
into the cartilage layer in vivo (Figure 4-1). Decellularized ECM hydrogels derived from a variety of tissues, including cartilage, are known to be chemoattractive, promote constructive remodeling, and provide tissue-specific differentiation cues for progenitor cells (42-46).

Figure 4-1 Scaffold design for osteochondral tissue generation using a single cell source. In order to engineer a full osteochondral tissue using a single cell source, site-specific chemical cues were incorporated within the scaffold to induce site-specific differentiation of human adipose derived stem cells (hASC). To achieve chondrogenesis, hASC were seeded within a 3D bioplotted polycaprolactone (PCL) scaffold containing decellularized bovine cartilage extracellular matrix (dECM). An electrospun layer of PCL was created and used to recreate the tidemark. To achieve osteogenesis, hASC were seeded within a 3D bioplotted scaffold comprised of 80% PCL and 20% β-tricalcium phosphate (TCP). Full composite scaffold shown on top right (scale bar = 5 mm).

4.2 Materials and Methods

We first conducted analyses of hASC osteogenesis and chondrogenesis on separate phases of 3D bioplotted PCL-TCP and PCL-dECM scaffolds, respectively, and then assessed the site-specific hASC differentiation characteristics of integrated multi-material triphasic scaffolds resembling full thickness osteochondral tissue.

4.2.1 Preparation of PCL-TCP composite

The PCL-TCP composite (80-20% by weight) used as the raw material for 3D bioplotting of the osteogenic scaffold phase was created by mixing the appropriate proportion of PCL (Mn:
80,000, ~ 3 mm pellets; Sigma-Aldrich, St. Louis, MO) and TCP (Riedel-de-Haën AG, Seelze, Germany), and heating the mixture at 180°C for 60 minutes with intermittent stirring in a temperature-controlled oven until a homogeneous, single phase mix was obtained.

4.2.2 Preparation of dECM hydrogel

Decellularized ECM derived from bovine hooves (Micro Summit Processors, Micro, NC) was used in a hydrogel form as a constituent of the 3D biopotted scaffolds for the chondrogenic phase. Cartilage was harvested from the hooves, frozen and decellularized by suspending in a solution of deionized water and 0.5% penicillin/streptomycin and mixing for 24 hours. The decellularized cartilage was then frozen in 1X PBS and lyophilized for 24 hours. Finally, the lyophilized cartilage was pulverized in a mill using a number 40 size mesh to obtain the decellularized cartilage ECM powder.

ECM gels were prepared at an initial concentration of 25 mg/mL. Digestion of the ECM took place in a 2 mg/mL solution of pepsin in 0.1 M HCL. 4 mL of this solution was added to 100 mg of cartilage ECM powder. The ECM was digested in the pepsin solution for 48 hours. Next, 400 uL of 1 M NaOH was added to the solution in order to bring the pH to 7.4. Finally, 489 uL of 10X PBS was added to the solution to make the concentrations of NaOH, HCl, and PBS equal to each other. The final concentration of the gel was calculated to be 20.5 mg/mL.

4.2.3 Proteomic analysis of decellularized ECM

Proteins were extracted using RIPA buffer protocol (Millipore, Billerica, MA, USA). Protein concentrations were determined using a BCA protein assay (Thermo Fisher Scientific).
Twenty micrograms of total protein from each sample was digested in solution with Trypsin/Lys C mix (Promega) following the manufacturer’s instructions. Briefly, protein samples were precipitated with cold acetone and dissolved in 8M urea solution. After reduction with dithiothreitol and alkylation with iodoacetamide, samples were diluted with 50 mM Tris-HCl pH 8.0, to reduce urea concentration to 1 M. Trypsin/Lys C Mix was added at a 20:1 protein:protease ratio (w/w). Samples were digested overnight at 37 °C. Digestion was terminated with trifluoroacetic acid. Resulting peptide mixtures were cleaned using a C18 reverse-phase spin column (Thermo Fisher Scientific), dried under vacuum, and reconstituted in 5% acetonitrile, 0.1% formic acid in water for LC-MS/MS analysis.

LC–MS/MS analysis of peptides was conducted on a Velos Pro Dual-Pressure Linear Ion Trap mass spectrometer equipped with a nano electrospray ionization source and coupled with an Easy-nLC II nano LC system (Thermo Scientific, Waltham, MA, USA). Five microliters of peptide mixture was loaded onto a C18 reverse-phase column (10cm x 75µm, 3 µm, 120 Å). Each sample was analyzed three times. A linear gradient with two mobile phases (A: 5% acetonitrile, 0.1% formic acid, 94.9% water, 0; B: 80% acetonitrile, 0.1% formic acid, 19.9% water) at a flow rate of 300 nL/min was used to separate peptide mixtures. The gradient began at 0% B, increased linearly to 50% B over 180 min and then to 100% B over 16 minutes, and maintained at this percentage for 14 min as a washing step. Eluted peptides were ionized in a nano ESI source with a spray voltage of 2.2 kV. Full scan MS spectra were acquired from m/z 300-2000. Collision-induced dissociation (CID) was used to fragment the precursor ions. MS/MS spectra were acquired in the data-dependent acquisition mode for the ten most abundant precursor ions in the preceding full MS scan.
Peptide spectral matching and protein identification were achieved by database search using Sequest HT algorithms in Proteome Discoverer 1.4 (Thermo Scientific, Waltham, MA, USA). Raw spectrum data were used to search against the UniProtKB/Swiss-Prot protein database for bovine. Main search parameters included: trypsin, maximum missed cleavage site of two, precursor mass tolerance of 1.5 Da, fragment mass tolerance of 0.8 Da, and variable modification of oxidation/hydroxylation of methionine, proline, and lysine (+15.995 Da). Decoy database search was performed to calculate false discovery rate (FDR). Proteins containing one or more peptides with FDR ≤ 0.05 were considered positively identified and reported. Total number of peptide spectral matches (PSMs) for each protein reported by Protein Discoverer 1.4 was used for quantification.

4.2.4 3D bioplotting of osteogenic and chondrogenic scaffold phases

The base computer assisted design (CAD) models of the scaffolds were created in Solidworks (Dassault Systèmes SOLIDWORKS Corp., Waltham, MA) as discs (Ø14.5 x 2 mm) to fit a standard 24-well culture plate. The STL file, sliced into 6 layers, was positioned onto the bioplotting stage (BioplotterRP software, EnvisionTEC GmbH, Gladbeck, Germany). To begin scaffold fabrication on the 3D-Bioplotter (EnvisionTEC GmbH), the strand-pore geometry previously determined to be favorable for hASC (160) and experimentally optimized process parameters were assigned (Table 4-1) in VisualMachines software. 6.8 g of material (80% PCL-20% TCP composite for osteogenic scaffolds; pure PCL for chondrogenic scaffolds) was pre-heated in the high temperature printhead and the scaffolds bioplotted, one at a time. Post-fabrication, all scaffolds were weighed (AR2140 digital balance, Ohaus Corp., Parsippany, NJ) and characterized for strand width and
interstrand spacing (50X magnification, KH-7700 microscope, Hirox, Hackensack, NJ). For each scaffold, for each dimension metric, measurements were taken at 8 randomly selected locations. All scaffolds were sterilized for 30 minutes in 70% ethanol, and washed three times with sterile 1X PBS and once with complete growth medium (CGM, defined below).

<table>
<thead>
<tr>
<th>Scaffold design</th>
<th>PCL-TCP</th>
<th>PCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strand lay orientation (°, layer 1/2/3)</td>
<td>0/120/240</td>
<td>0/120/240</td>
</tr>
<tr>
<td>Strand width (mm)</td>
<td>0.378 ± 0.040</td>
<td>0.351 ± 0.033</td>
</tr>
<tr>
<td>Interstrand spacing (mm)</td>
<td>0.602 ± 0.041</td>
<td>0.632 ± 0.055</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>0.1537 ± 0.0242</td>
<td>0.1180 ± 0.0164</td>
</tr>
</tbody>
</table>

Table 4-1 Scaffold design and 3D biplotting parameters

Extrusion temperature (°C) | 130 | 160 |
Preheat interval (min) | 25 | 45 |
Nozzle diameter (mm) | 0.4 | 0.4 |
Extrusion pressure (N/mm²) | 0.5 | 0.5 |
Nozzle speed (mm/s) | 0.7 | 0.4 |
Pre-flow delay (s) | 1 | 1 |
Post-flow delay (s) | 0 | 1 |
Wait time between layers (s) | 0 | 3 |

To prepare the PCL-dECM scaffolds for chondrogenic analyses, the decellularized bovine cartilage ECM hydrogel was neutralized to pH 7.4 by adding 1 M NaOH, and 500 uL of the solution was added onto each sterilized 3D bioplotted PCL scaffold. The hydrogel settled between the pores of the scaffold, creating a denser framework for cartilage formation. The hydrogel-infused scaffolds were placed in an incubator (37°C, 5% CO₂) overnight to allow
the gel to solidify, and were then placed in 4-well plates prior to seeding with human adipose derived stem cells (hASC).

4.2.5 Characterization of calcium release from PCL-TCP scaffolds

PCL–TCP composite scaffolds were placed into separate wells in a 24 well plate (n = 3 per time point). The scaffolds were treated for 30 minutes with 70% ethanol to mimic the sterilization process used in later cell culture experiments. Ethanol treatment also increased the hydrophilicity of the scaffold for the Ca$^{2+}$ release experiments. The scaffolds were then fully submerged in 0.5 mL of phosphate buffered saline (PBS) and maintained in an incubator (37°C, 5% CO$_2$) for the duration of the experiment. PBS was collected at time points of 1, 2, 7, 14, and 28 days and stored at -25°C until assayed. A Calcium LiquiColor® Assay (StanBio, Boerne, TX) was used to quantify calcium content of each sample. Using the measured weight of the scaffolds along with the molar mass of calcium ions (Ca$^{2+}$ 40.08 g•mol$^{-1}$), orthophosphates (PO$_4^{3-}$ 94.97 g•mol$^{-1}$), and TCP [Ca$_3$(PO$_4$)$_2$ 310.18 g•mol$^{-1}$] the weight of Ca$^{2+}$ doped within each scaffold was calculated to be 11.9 µg.

4.2.6 Human ASC isolation, expansion, and in vitro culture of scaffolds

Excess adipose tissue was collected from five female pre-menopausal donors (ages 24 to 36) in accordance with an approved IRB protocol at UNC Chapel Hill (IRB 04-1622), and hASC were isolated from the tissue as previously described by our lab and others(2, 13). Cells were expanded in complete growth medium (CGM) comprised of alpha-modified minimal essential medium (α-MEM with L-glutamine) (Invitrogen, Carlsbad CA), 10% fetal bovine serum (FBS) (Premium Select, Atlanta Biologicals, Lawrenceville GA), 200 mM L-
glutamine, and 100 I.U. penicillin/ 100 µg/ml streptomycin (Mediatech, Herndon VA). Human ASC were cultured (37°C, 5% CO₂) until reaching 80% confluency changing the media every 3 days, and then passaged using trypsin-EDTA (Invitrogen). A superlot was generated by pooling equal numbers of cells from the five individual donor cell lines into a single culture vessel as previously described by our lab, and characterized for multilineage differentiation potential by culturing for 14 days in osteogenic differentiation medium (Minimum Essential Medium, alpha modified supplemented with 10% FBS, 2mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 50 lM ascorbic acid, 0.1 lM dexamethasone, and 10mM β-glycerolphosphate) and adipogenic differentiation medium (Minimum Essential Medium, alpha modified supplemented with 10% FBS, 2mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 1 µM dexamethasone, 5 µg/ml insulin, 100 µM indomethacin and 500 µM isobutylmethylxanthine), ensuring the superlot differentiation was representative of an average of the five cell lines(13). All experiments were run using superlot passage 6 or lower.

To assess osteogenesis, PCL-TCP scaffolds (n = 8) were seeded with hASC at a density of 250,000 cells per scaffold and incubated for 28 days in CGM. To assess chondrogenesis, 250,000 hASC in 1ml of CGM were seeded onto each PCL-dECM scaffold (n = 9) and cultured for 21 days either with or without chondrogenic growth factors (TGFβ-1, 10 ng/ml and BMP6, 10ng/ml) (151, 161).
4.2.7 Osteogenic differentiation assays

Osteogenic differentiation was assessed via calcium staining and endogenous alkaline phosphatase activity (ALP). Scaffolds were fixed 30 minutes in formalin at day 28 and stained via Alizarin Red (Sigma-Aldrich, St. Louis, MO) for calcium accretion. Endogenous ALP activity was assessed at days 14 and 28. Scaffolds were collected in 1 ml RIPA buffer (Fisher). ALP was quantified with the Alkaline Phosphatase Liquicolor Test (Stanbio, Boerne, TX), using the P-nitrophenylphosphate methodology, as described previously(162). ALP activity was normalized to total protein content quantified using a Pierce Micro BCA Protein Assay Kit (Fisher).

4.2.8 Safranin-O staining

Safranin-O staining was used to determine proteoglycan content of the seeded scaffolds. Samples were first fixed in 10% formalin and rehydrated through a series of alcohol solutions. Scaffolds were first stained with Weigert’s Iron hematoxylin stain (Sigma-Aldrich, St. Louis, MO) for 10 minutes then rinsed under running tap water. Scaffolds were then stained with fast green for 5 minutes and washed in 1% acetic acid, followed by an 8 minute safranin-O stain and dehydration through a series of alcohol washes.

4.2.9 Alcian Blue staining

Alcain Blue staining was used to determine sulfated glycosaminoglycan (sGAG) content of the seeded scaffolds. After fixation and dehydration as described above, scaffolds were stained in an Alcian blue solution (Sigma-Aldrich, St. Louis, MO) for 30 minutes, running tap water for 2 minutes, then rinsed with distilled water. Scaffolds were then stained with
0.1% Nuclear Fast Red (Sigma-Aldrich, St. Louis, MO) for 5 minutes, tap water for 1 minute, and dehydrated through a series of alcohol washes.

4.2.10 Quantitative RT-PCR

Quantitative real-time RT-PCR was used to determine relative mRNA expression changes of chondrogenic-specific genes in seeded scaffolds with dECM or without dECM. Total RNA was extracted directly from the scaffolds after 28 days in culture (n=4 per condition), following the TRIZOL protocol (Life Technologies). Briefly, the scaffolds were submerged in 1 ml of TRIZOL followed by a chloroform (Ricca Chemical Company) extraction. The precipitated solution was incubated with an equal volume of isopropanol (Sigma-Aldrich) and 1 µL glycolblue (Life Technologies) at room temperature for ten minutes. The solution was centrifuged and the precipitate was washed once with 75% ethanol (Koptec) and centrifuged again to collect the RNA containing pellet. The RNA was resuspended in DEPC treated water (Mediatech) and the yield was calculated using a NanoDrop 2000 Spectrophotometer (Sigma Aldrich). To quantify mRNA expression, 120 ng of total RNA was reverse transcribed into cDNA using a high capacity first strand cDNA synthesis kit (OriGene) as per manufacturer’s instructions. qRT-PCR was performed using 1 µL cDNA, 10 µL SYBR Green (Applied Biosystems), 7 µL DEPC treated water (Mediatech), and 1 µL forward and reverse primer (IDTDNA) for the following genes: **b-actin** ‘5-CACTCTTCCAGCCTTCCCTTC-3’, ‘3-TGTAGGCCTTCTGGGATG-5’, **Collagen1a2** ‘5-AGAGTGGAGCAGTGGTTA-3’, ‘3-GAGATGACCGCTTTGGGAC-5’, **Aggrecan** ‘5-CAGGCAGATCACTTGGGAT-3’, ‘3-
ACATTAGGGTCGATGATCCCTC-5’, Sox9 ‘5-TGACCTATCCAAGCGCATTAC-3’, ‘3-
ATTTGGGAGAAGTCTCGTTCG-5’.

qRT-PCR was performed using an ABI 7000 Sequence Detection system (Applied Biosystems). Samples were assayed in triplicate in one run (40 cycles) per gene. qRT-PCR data were analyzed using the ΔΔCT method as described previously (17) with β-actin as the housekeeping control. Relative quantification values are presented as fold changes in gene expression relative to the control group.

4.2.11 Fabrication and in vitro hASC culture of integrated full osteochondral scaffolds

Electrospun PCL matrices, designed to mimic the function of the tidemark layer, were separately fabricated prior to 3D bioplotting of the full thickness multiphasic scaffold. PCL (Mn: 80,000, Sigma-Aldrich) was dissolved in chloroform and dimethylformamide (3:1 ratio by volume) to create a 12% solution. The solution was mixed continuously at 80°C for 4 hours. The PCL solution was electrospun immediately after preparation at a feed rate of 0.7 µl/hour using 15 kV and then cut into discs (Ø14.5 mm X 30 µm) to match the 3D biopotted scaffolds.

The integrated triphasic scaffolds (Ø14.5 x 6 mm) were designed to resemble the overall organization of the osteochondral complex. The material composition, strand-pore geometries, and process parameters for the bone (PCL-TCP), cartilage (PCL-dECM), and tidemark (electrospun PCL) phases were as described above. To create the scaffolds in a single build, the PCL-TCP phase (Ø14.5 x 4 mm, 12 layers) was biopotted first.
Immediately upon completion of the topmost PCL-TCP layer, the process was briefly halted, and the pre-fabricated electrospun PCL disc (Ø14.5 mm X 30 µm) was overlaid on top of the partial scaffold. 3D bioplotting was then continued with the appropriate height offset adjustment to create the PCL phase (Ø14.5 x 2 mm, 6 layers).

A two-day hASC seeding protocol was used to seed the full thickness scaffolds (n = 3). On the first day, the scaffolds were sterilized with successive 70% ethanol and PBS washes. The chondrogenic phase was prepared by submerging the 3D bioplated PCL in neutralized dECM solution and incubating at 37°C for 1 hour. After the hydrogel had solidified, the scaffold was flipped chondrogenic PCL phase up, seeded with 250,000 hASC, and incubated overnight (37°C, 5% CO₂). On the second day, the scaffolds were flipped PCL-TCP phase up, and seeded with an additional 500,000 hASC. The total number of cells used in these scaffolds was tripled in the full osteochondral scaffolds because they were three times thicker than the individual chondrogenic and osteogenic scaffolds. Each complete, osteochondral scaffold was incubated in 3 ml CGM for 28 days. Media was changed and scaffolds were flipped every two days.

4.2.12 Full thickness characterization

After 28 days of culture, full thickness scaffolds were fixed for 30 minutes in formalin. Scaffolds were sectioned with a scalpel into 1 mm thick sections for histological analyses. Sections were stained via Alizarin Red, Alcian Blue, and Safranin-O/fast green, as described above.
4.2.13 Statistical Analyses

Statistical analyses to analyze the ALP and qRT-PCR data were performed using Prism (version 5.0a, GraphPad Software). Differences were determined using a t-test (ALP) or one-way ANOVA with Tukey post-hoc test. A level of \( p < 0.05 \) was considered significant.

4.3 Results

4.3.1 PCL–TCP scaffolds induced osteogenic differentiation of hASC

Controlled release assays of calcium demonstrated that calcium was released from the PCL-TCP composite scaffolds throughout the 28 day incubation period, with a large initial release within the first 24 hours (Figure 4-2A).

To determine how the calcium release from the scaffolds affected the seeded hASC, assays were performed to assess osteogenic differentiation. Our results demonstrated that hASC cultured on PCL-TCP scaffolds accreted calcium while hASC cultured on PCL scaffolds alone did not accrete calcium. Acellular PCL-TCP controls showed mild Alizarin Red staining due to the TCP found within the scaffold, but seeded PCL-TCP stained dark red throughout the scaffold, indicating hASC-mediated calcium accretion (Figure 4-2B). The porous structure of the scaffold was filled in the seeded scaffold, but not in the unseeded scaffold, indicating a deposition of ECM by hASC in the seeded scaffold. Scaffolds without TCP showed no Alizarin Red staining. In addition, enzymatic ALP activity, a hallmark of osteogenic differentiation(162), was increased in PCL-TCP composition scaffolds when compared to PCL scaffolds alone. This trend was observed at both the 14 and 28-day time points.
Figure 4-2 Osteogenesis of human adipose derived stem cells (hASC) on 3D-biopotted PCL/TCP scaffolds. A. The calcium release profiles of osteogenic scaffolds (20% TCP-80% PCL) were evaluated out to 28 days. A burst release profile was initially observed, releasing 0.54 µg (4.5% of total calcium ions) within the first 24 hours; and, 1.04 µg (8.7% of total calcium ions) after 28 days (bars represent standard error of the mean; different letters indicate statistical difference (p<0.05)). B. Alizarin Red staining indicated an increase in calcium content within the 20% TCP scaffolds seeded with hASC (scale bars = 200 µm). C. Although not statistically significant, an increase in endogenous ALP activity was observed at both the 14 (p=0.11) and 28 day (p=0.07) timepoints.

4.3.2 PCL-dECM scaffolds induced chondrogenic differentiation of hASC

The decellularized ECM hydrogel used to induce hASC chondrogenic differentiation was analyzed using proteomic analysis to ensure that the primary ECM proteins were preserved after the decellularization process (163) (Figure 4-3). We identified 775 unique peptides corresponding to proteoglycans, collagens and other extracellular matrix proteins. Figure 4-3 shows the relative abundances of the most prevalent proteins.
Proteomic analysis of decellularized bovine cartilage extracellular matrix (dECM). Proteomic analysis of the decellularized matrix identified several cartilage proteins based on 775 unique peptides from articular cartilage. A. Proteoglycans. The most prevalent include fibromodulin, biglycan, PRELP, chondroadherin, decorin, aggrecan, link protein, lumican, and perlecan. B. Collagens. The most prevalent identified include COL2A1, COL6A3, COL9A1, COL12A1, COL6A2, COL6A1, COL15A1, COL11A2, and COL11A1. C. Noncollagenous ECM proteins. The most prevalent include fibronectin, Cartilage Oligomeric Matrix Protein (COMP), matrilin 3 (MATN3), cartilage intermediate layer protein 2 (CILP2), tenascin C (TNC), thrombospondin type 1 (THBS1), thrombospondin type 4 (THBS4), and clusterin.

Human ASC were then seeded on 3D printed scaffolds with or without dECM and evaluated for proliferation and chondrogenic differentiation (Figure 4-4). Because the hydrogel settled between the pores of the scaffold, preventing cells from falling through during seeding, cell proliferation was more uniform in dECM scaffolds relative to non-dECM scaffolds. Chondrogenesis was greater in dECM scaffolds as assessed by safranin-O staining. Human
ASC chondrogenesis was further evaluated by qPCR with similar results (Figure 4-5). When hASC were seeded on PCL-dECM scaffolds for 14 and 28 days, they exhibited increased expression of sox9, and decreased collagen I expression relative to hASC seeded on non-dECM scaffolds. The downregulation of collagen I at 14 days in PCL-dECM was statistically significant (p=0.0004). Further, after 28 days in culture, aggrecan expression in PCL-dECM scaffolds was increased (Figure 4-5).

![Figure 4-4](image)

**Figure 4-4** Chondrogenic differentiation of human adipose derived stem cells (hASC) using decellularized bovine cartilage extracellular matrix (dECM) on 3D bioplotted scaffolds. Top: Human ASC seeded for 28 days in complete growth medium on 3D bioplotted scaffolds without dECM. Bottom: Human ASC seeded under the same conditions on 3D-bioplotted scaffolds with dECM. Cell proliferation was more uniform on PCL-dECM scaffolds as shown by DAPI staining, and chondrogenesis was higher on PCL-dECM scaffolds as shown by safranin-o staining.
4.3.3 Full thickness osteochondral scaffolds exhibited site-specific tissue differentiation

Histological staining of the full-thickness scaffolds revealed site-specific osteochondral tissue characteristics. The PCL-TCP region of the scaffold stained dark red via Alizarin Red, indicating calcium accretion within this region of the scaffold. The PCL-dECM portion of the scaffold stained via both safranin-O, indicated the presence of proteoglycans, and Alcian
Blue, indicating the presence of sulfated glycosaminoglycans. The bone-like portion of the scaffold and cartilage-like portion of the scaffold transitioned cleanly from one phase to the other at the electrospun tide mark. (Figure 4-6).

![Image of Alizarin Red, Safranin-O, and Alcian Blue staining](image)

**Figure 4-6** Site-specific differentiation of hASC in full osteochondral scaffolds. Human ASC were cultured for 28 days in full osteochondral scaffolds. Alizarin red shows ossification of the deep bone layer, while safranin-O and alcian blue stainings show increased chondrogenesis in the superficial cartilage layer (dashed line represents the electrospun tidemark layer; scale bar = 1 mm).

### 4.4 Discussion

The objective of this study was to develop a tissue engineered osteochondral construct that closely resembled the native architecture of osteochondral tissue using a single cell source of abundant and accessible hASC. A triphasic scaffold was created that not only supported hASC viability and proliferation but also induced desired site-specific osteogenic and chondrogenic differentiation of the hASC by incorporating chemical cues within the different layers of the scaffold. Full thickness osteochondral scaffolds comprised of three biomimetic layers: cartilage, tidemark, and bone, were successfully manufactured. The superficial cartilage region was created by 3D bioplotting a porous PCL scaffold that was then infused
with dECM to provide a denser network for cartilage formation and to induce hASC chondrogenesis via natural cartilage ECM factors contained in the dECM. Proteomic analyses of the dECM showed that several cartilage ECM proteins were still conserved in the pulverized matrix after undergoing the decellularization process. A thin electrospun PCL layer was inserted under the cartilage layer to mimic the natural tidemark found in osteochondral tissue. We believe that the incorporation of this layer is important because it prevents cell migration between the cartilage and bone layers (164), and it could also potentially act as a barrier to prevent blood vessel invasion into the cartilage layer when implanted in vivo. Finally, a PCL-TCP scaffold was utilized under the electrospun layer to induce osteogenesis of seeded hASC via extracellular calcium released from the scaffold (151, 165, 166). With this triphasic design, hASC seeded on and within the construct were induced to differentiate down site-specific chondrogenic (top) or osteogenic (bottom) lineage pathways as hypothesized, creating a complete biomimetic osteochondral tissue plug. The full thickness scaffold demonstrated site-specific bone and cartilage characteristics that remained separate throughout a full month of cell culture.

We have demonstrated that we can direct chondrogenic differentiation of hASC using dECM, even in the absence of chondrogenic growth factors. We compared chondrogenic differentiation of hASC in 3D bioplotted PCL-dECM and non-dECM scaffolds with and without chondrogenic growth factors (10 ng/ml of both TGF-β1 and BMP6). We found that dECM in the scaffolds induced similar chondrogenesis of hASC regardless of addition of growth factors to the media (data not shown). We also found that the 3D bioplotted PCL-TCP scaffolds promoted osteogenic differentiation of seeded hASC. Both an increase in
hASC-mediated calcium accretion and endogenous alkaline phosphatase activity was observed within hASC that had been stimulated via the addition of 20% TCP within the PCL scaffolds. Our results in this study are in accordance with our previous studies, which have also demonstrated that 20% TCP electrospun scaffolds promoted osteogenic differentiation of hASC (151, 166, 167). These findings allowed for incubation of the full osteochondral scaffolds in complete growth medium alone (without any additional osteogenic and/or chondrogenic growth factors) to induce both hASC chondrogenesis and osteogenesis in desired site-specific locations using only the chemical cues within the layers of the scaffold.

In this study we take advantage of recent advances in biomanufacturing to generate scaffolds that are thick enough to repair full-thickness osteochondral defects, unlike electrospun scaffolds. The calcium release profiles of the bioplotted PCL-TCP scaffolds demonstrate that calcium is released at a much slower rate than electrospun scaffolds. Specifically, we have previously demonstrated a burst release of 50% calcium within the first 24 hour in electrospun scaffolds (166). Here, we only witnessed a release of 0.54 µg (4.5% of total calcium ions) with the first 24 hours, and a sustained release over the course of 28 days. Even after 28 days, only 1.04 µg (8.7% of total calcium ions) had been released from the scaffolds, suggesting that calcium-based signaling would be sustained long term in hASC seeded upon these scaffolds, which could be favorable for implantation of the tissue engineered constructs in vivo.

This study is the first to incorporate TCP and dECM in a single 3D-bioplotted scaffold to induce site specific differentiation of hASC as a single cell source to generate a full
osteochondral construct. Our novel approach holds great potential to treat OA patients, and offers a less invasive alternative to full joint replacement as no healthy cartilage tissue is removed from the patient. Our technique also minimizes tissue rejection by using an abundant and accessible source of autologous stem cells, and our biofabrication techniques allow for a precise, customizable methodology to rapidly produce the scaffold.
CHAPTER 5

LRP receptors in canonical Wnt signaling are responsive to microgravity and cyclic hydrostatic pressure and regulate cartilage homeostasis.

In the previous chapter, we describe a method for producing an osteochondral scaffold for repair of cartilage defects. The next step in this research is in vivo implantation of this scaffold into the knee joint of a large animal model. Within the synovial joint, the construct will be subject to significant mechanical loading, and signaling pathways that are activated by mechanical stimulation will regulate cellular behavior. The Wnt signaling pathway is a major regulator of both bone and cartilage homeostasis. However, little is know about how Wnt signaling responds to mechanical stimulation within articular cartilage. In this chapter, we examine how LRP receptors within the canonical Wnt signaling pathway respond to mechanical stimulation. The goal is to elucidate how LRPs are regulated and how this may play a role in modulating cartilage homeostasis.

The work in this chapter is in preparation to be submitted to Osteoarthritis and Cartilage with co-authors Liliana F. Mellor and Elizabeth G. Loboa.

5.1 Introduction

Mechanical loading is essential for the maintenance of musculoskeletal homeostasis. It is well known that mechanical loading stimulates bone formation but the absence of loading, such as in patients on prolonged bed-rest or astronauts on long-term space missions, leads to loss of bone mass (168-170). Cartilage has also been demonstrated to be highly
mechanoresponsive. Excessive repetitive loading of cartilage has been associated with chondrocyte death and cartilage degeneration (171, 172). Obesity is recognized as a major risk factor of osteoarthritis, in part due to increased axial loading patterns on the hip and knee joints (173). However, insufficient cartilage loading can also lead to degenerative conditions. Patients on bed-rest experience loss of cartilage thickness after only 14 days (174). Muscle weakness has also been associated with the progression of osteoarthritis (175, 176). Taken together, research suggests that moderate loading patterns from normal daily activities such as walking promote cartilage health. However, the mechanisms by which chondrocytes respond to mechanical stimuli are not clearly understood.

Cartilage and bone are developmentally linked through processes such as endochondral ossification and share common signaling pathways including canonical Wnt-signaling. Canonical Wnt-signaling has been shown to regulate long-bone development and endochondral ossification, and it is associated with various pathologies of the musculoskeletal system (177). In active Wnt-signaling, Wnts bind to the frizzled and low-density lipoprotein receptor-related protein (LRP) co-receptors. This activates a signaling cascade, preventing β-catenin from degrading within the cytoplasm thus allowing β-catenin to translocate and accumulate within the nucleus (178). Nuclear β-catenin initiates the transcription of many genes responsible for bone and cartilage homeostasis.

Within the canonical Wnt-signaling pathway there has been considerable research focused on the LRP receptors. LRP5 and LRP6, specifically, are crucial to transduce canonical Wnt-signaling (177). In bone, LRP5/6 receptors have been shown to be critical for maintaining the
optimal balance between bone formation and resorption (170). LRP receptors are also important for signaling in cartilage and, as in bone, have been indicated to transduce Wnt signaling and induce nuclear β-catenin localization. LRP5 has been found to be upregulated in osteoarthritis (179). However, LRP6 loss-of-function mutation has been associated with an increased progression of osteoarthritis (180), illustrating the delicate balance within the Wnt-signaling pathway that must be achieved in order to maintain proper cartilage function. A lesser-known LRP is LRP4. LRP4 has also been implicated in regulation of Wnt-signaling and has been indicated to induce extracellular matrix production in cartilage (181). However, the exact mechanism by which LRP4 acts remains elusive, as it appears to have a role distinct from LRP5/6.

The mechanosensing properties of canonical Wnt-signaling have been primarily studied within the context of bone biology. There has been significant evidence that Wnt-signaling is responsive to mechanical stimuli and is a primary mechanism through which osteocytes sense and react to mechanical cues (182). It has been shown that Wnt inhibitors Sclerostin and DKK1 are elevated in patients on bed-rest (183). Further, Sclerostin downregulation is required for bone to respond to mechanical loading (184). However, it is currently unclear how Wnt-signaling and LRP receptors participate in chondrocyte mechanosensitivity.

The goal of this study was to determine how LRP receptors are regulated in simulated microgravity and cyclic hydrostatic pressure in order to elucidate the mechanisms by which mechanical stimulation regulates cartilage homeostasis. Since canonical Wnt-signaling and LRPs are involved in the maintenance of bone homeostasis and LRPs have been associated
with cartilage pathologies, we hypothesized that LRP expression is responsive to mechanical stimuli and that LRPs modulate the expression of cartilage matrix degrading proteases.

5.2 Methods

5.2.1 Rat chondrosarcoma cell culture

Rat chondrosarcoma cells (RCS) were cultured in standard RCS media (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin). When pellet culture was utilized, RCS were resuspended at a density of 250,000 cells per pellet in 15 ml conical tubes. The RCS were centrifuged at 300 g for 5 min to form a pellet, and incubated for 2 days with loose tube caps in order for the cell pellets to coalesce.

5.2.2 Stimulation in simulated microgravity and cyclic hydrostatic pressure

Pellets were subjected to either simulated microgravity or cyclic hydrostatic pressure and compared to control pellets, which were continuously incubated at 37°C, 5% CO₂ without stimulation. Simulated microgravity was achieved by culturing cell pellets in a rotating wall vessel (RWV) bioreactor developed by NASA, which rotates at a constant speed to maintain pellets in free-fall resulting in a randomized gravitational vector. The forces generated by this vessel produce vector-averaged forces comparable with that of near-earth free fall orbit(185, 186). The RWV bioreactor was set to a speed of 11RPM and kept in a standard 37°C, 5% CO₂ for the duration of the experiment. Air bubbles that accumulated within the vessel were removed daily.
For cyclic hydrostatic pressure stimulation, the pellets were placed in sterile heat-sealed bags with 10 ml of standard RCS media and loaded in a custom cyclic hydrostatic pressure system designed by our lab, as described previously (17, 51). Briefly, the heat-sealed bags were placed in a stainless steel pressure vessel (Parr Instruments, Moline, Illinois) filled with mineral oil. The pressure vessel was connected via high-pressure stainless steel tubing to a hydraulic cylinder mounted to an MTS 858 Mini Bionix II load frame. Loading operations were controlled using a MTS TestStar control program (MTS System Corp, Eden Prairie, MN). Loading was applied at a magnitude of 7.5 MPa and frequency of 1Hz, 4 hours per day for up to 14 days. After daily loading, the heat-sealed bags were removed from the apparatus and maintained in a standard incubator at 37°C. Pellets were transferred in new bags with fresh media every 3-4 days.

5.2.3 Mouse hindlimb unloading procedures

In order to determine if LRP5 is responsive to mechanical stimulation in vivo, hindlimb unloading (HLU) was used to reduce mechanical loading within the synovial joints of male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) for 14 days. Protocol was carried out as described previously, in collaboration with the Penn State College of Medicine. All procedures were approved by the Penn State Institutional Animal Care and Use Committee (Protocol #2013-033). Briefly, mice were anesthetized and tape strips were secured to the tails. The tape allowed the animal to be attached to tethers that ran along the top of the cage. The tethers were adjusted to support the mouse at 30° of elevation. Control mice were kept in identical housing units without the tethers. After 14 days, mice were sacrificed.
5.2.4 Histology

After 3 and 14 days of culture, pellets were fixed in 10% buffered formalin for 1 hour. Mouse femoral heads from the HLU study were also fixed in formalin for 1 hour and decalcified before processing. Fixed pellets and femoral heads were paraffin embedded and sectioned at the North Carolina State University College of Veterinary Medicine histology facilities. Sections were taken with a thickness of 10 µm. Pellet sections were stained via Hematoxylin and Eosin (H&E) to visualize cellular organization. Sulfated glycosaminoglycans were stained via Alcian Blue/Nuclear Fast Red. Proteoglycans were stained via Safranin-O/hematoxylin with a fast green counterstain. In addition, sections were taken from both pellets and mouse femoral heads for immunofluorescent LRP5 staining.

5.2.5 Immunofluorescence

Fluorescent immunostaining was used to determine protein-level LRP5 and active β-catenin expression within the pellets, and LRP5 expression in mouse femoral heads of HLU treated animals. Both pellet and femoral head sections were deparaffinized in SafeClear II (Thermo Fisher, Waltham, MA), hydrated in ethanol series, and treated with Antigen Retrieval Reagent-Universal (R&D Systems, Minneapolis, MN) following manufacturer’s protocol. The samples were then blocked with a 0.2% Triton X-100/5.0% BSA stock solution for 40 minutes.

Primary antibody dilutions were prepared in a PBS solution containing 0.2% Triton X-100, and 0.5% BSA. Pellets were incubated in goat polyclonal antibody to LRP5 (1:500 dilution,
Abcam, Cambridge, United Kingdom) and mouse monoclonal antibody to active β-catenin (1:300 dilution, Millipore, Billerica, MA). The mouse femoral head sections were incubated in a goat polyclonal antibody to LRP5 solution (1:500 dilution). Sections were incubated overnight at 4°C in the primary antibody solution and then rinsed three times in PBS. Secondary antibody solutions were prepared using chicken anti-mouse Alexa Fluor 488 (1:1000 dilution, Molecular Probes, Eugene, Oregon), donkey anti-goat 594 (1:1000 dilution, Molecular Probes), and DAPI (1:1000, Molecular Probes, Eugene, Oregon). The samples were incubated in secondary antibody solutions for one hour at room temperature followed by three more PBS washes. Prolong Gold Mounting Media (Molecular Probes, Eugene, Oregon) was used to mount coverslips on the slides. The slides were dried in the dark for 24 hours and imaged on a Leica DM5500B Fluorescent Microscope using the compatible LAS-AF software.

5.2.6 siRNA Knockdown

For knockdown experiments, Stealth RNAi™ siRNA Negative Control Med GC, and LRP4, LRP5, and LRP6 Stealth RNAi™ siRNA (Thermo Fisher Scientific Waltham, MA) were used. In order to achieve the greatest knockdown efficiency, siRNA transfection was optimized using BLOCK-iT™ Alexa Fluor® Red Fluorescent Control (Thermo Fisher, Waltham, MA), as per manufacturer’s protocol. Variables assessed included siRNA concentration (20-80 nM), transfection reagent (Lipofectamine® 2000 (Thermo Fisher Scientific Waltham, MA) vs. Lipofectamine® RNAiMAX (Thermo Fisher Scientific, Waltham, MA)), transfection reagent concentration (1µl/ml-5µl/ml), and transfection type (standard vs. reverse). Highest Alexa Fluor® Red Fluorescent intensity, indicating highest
transfection rate, was achieved using a 1:1:50:250 ratio of Lipofectamine® 2000: 25 nM siRNA: OptiMEM (Thermo Fisher Scientific, Waltham, MA): antibiotic-free RCS medium. Standard vs. reverse transfection did not affect knockdown efficiency, and hence reverse transfection was utilized to reduce overall experimental timelines.

Using our optimized ratio, the following protocol was used to knockdown LRP expression. 4μl of siRNA oligomers, 4μl of Lipofectamine® 2000, and 200 μl OptiMEM were combined and incubated for 20 minutes within individual wells of a 12-well plate. RCS were trypsinized and resuspended at a concentration of 150k/ml. Of this RCS suspension, 1ml (150k RCS cells) was added to each well. After a 24-hour incubation, the transfection media was replaced with standard RCS media formulation. RNA was collected after 48 hours. When the knockdown was evaluated via PCR 48-hours post transfection, a 49% knockdown efficiency was observed.

5.2.7 Application of Wnt-regulating treatments

In order to determine how sclerostin levels alter LRP expression in RCS cells, RCS cells were cultured in standard RCS medium supplemented with recombinant SOST protein (R&D Systems, Minneapolis, MN). RCS were seeded at a density of 375,000 cells per well in a standard six well plate and were incubated for 24 hours in standard RCS media. Media formulations were then switched to contain 25ng/ml, 100ng/ml, or 250ng/ml SOST protein and cultured for an additional 48 hours.
5.2.8 Total RNA extraction and RT-PCR

RNA extraction was carried out using a Trizol (Invitrogen, Carlsbad, CA) extraction method using the manufacturer’s protocol. RNA concentration and quality were assessed using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). The RNA was reverse-transcribed using Marligen’s First-strand cDNA Synthesis System (Origene, Rockville, MD). RT-PCR was performed with an ABI Prism 7000 system (Applied Biosystems, Carlsbad, CA) using SYBR Green (Life Technologies, Grand Island, NY) for fluorescent detection. Primers were designed using the Integrated DNA Technologies (Coralville, IA) website. Primer sequences used were:

18s, 5’AAGACGAACCAGAGCGAAAG3’
3’TCTATGGGCAGCATCAAGGCT5’;

LRP4 5’GCAGCAAGAGGAAGGTACTAAT3’,
3’TCTATGGTTTTACGACTGGGC5’;

LRP5 5’CCATACAGGCCCTACATCATCATT3’,
3’GATGGACCTGAACCTTAAGCCTG5’;

LRP6 5’GGGAGAAGTGCCAAGATAGA3’,
3’CTAATACTCCTCGCCTTGCAA5’;

MMP-1 5’GCTACACATGGTACACTGAAGA3’,
3’TGTTGGCTGGATGGGATTT5’;

MMP-7 5’CAGTTCTGTGATGTACCTACC3’,
3’CAGCTTGTCTCCTTTCCATATAAC5’;

MMP-13
5’CCCTGATGGTTTCCATCTTACC3’, 3’ATCTCCTGGACCACATAGAGGAGAC5’;

ADAMTS5 5’CTTCGCTTCGCTGAGTAGATT3’, 3’CGTCAGCAGGTAGTGCTTTA5’;

5.2.9 Statistical Analyses

All gene expression data were analyzed using one-way ANOVA with Tukey post-hoc analyses. A level of p < 0.05 was considered significant. Significance is denoted through study as *p<0.05, **p<0.01.
5.3 Results

5.3.1 Cartilage matrix deposition is promoted by hydrostatic pressure but inhibited by simulated microgravity

Histological staining of RCS pellets at 3-day and 14-day time points showed that hydrostatic pressure promoted matrix deposition and cartilage tissue development. H&E staining (Figure 5-1) showed that pellets maintained in simulated microgravity or control conditions did not develop matrix by day 3 and did not uptake nuclear Hematoxylin stain. Small, amorphous cellular patterning was observed at day 3 in both control and microgravity conditions. However, pellets that were stimulated by cyclic hydrostatic pressure exhibited robust nuclear staining by day 3, with large round nuclei. By day 14, all pellets showed nuclear staining. Again, pellets stimulated via cyclic hydrostatic pressure had large, round nuclei while control and simulated microgravity pellets had smaller irregular nuclei. The nuclei in simulated microgravity were the most tightly packed, while the nuclei in hydrostatic pressure had the greatest spacing between nuclei, indicating greater matrix deposition in loaded conditions.

These results were further confirmed by staining for Alcian Blue for sulfated glycosaminoglycans (s-GAGs), and Safranin-O/fast green for proteoglycans. Alcian Blue (Figure 5-2) did not stain for g-GAGs in day 3 pellets. Nuclear fast red showed some nuclear staining in RCS pellets maintained in microgravity and control conditions at day 3, unlike hematoxylin. Pellets maintained in simulated microgravity had more concentrated nuclear staining at the pellet surface. Robust nuclear staining was again observed in day 3 cyclic hydrostatic pressure conditions. At day 14, all pellets stained for Alcian Blue; but more robust staining was observed on control and hydrostatic pressure pellets than pellets in
simulated microgravity. Safranin-O (Figure 5-3) did not stain for proteoglycans in day 3 pellets, but robust staining was observed by day 14. The control pellets showed the darkest Safranin-O staining at day 14, but nuclei were most spread apart in the cyclic hydrostatic pressure conditions as observed in the H&E staining.

**Figure 5-1** Hematoxylin and Eosin staining of RCS pellets at days 3 and 14. At day 3, the RCS pellets in simulated microgravity and unloaded control conditions do not display nuclear staining and cells appear to be densely packed with a shrunken phenotype. RCS that have been stimulated via cyclic hydrostatic pressure have a large, round phenotype typical of healthy chondrocytes. By day 14, RCS in all conditions have nuclear staining and have deposited extracellular matrix (ECM). The RCS exposed to cyclic hydrostatic pressure have deposited the most ECM on a per cell basis, while RCS in simulated microgravity have produced the least ECM. Again, the RCS in cyclic hydrostatic pressure show a large round phenotype. All scale bars = 50 µm.
Figure 5-2 Alcian Blue staining on RCS pellets at days 3 and 14. Nuclear staining patterns follow those observed via H&E staining. No s-GAG (blue) staining is observed at day 3, but by day 14 all samples stain for s-GAGs. s-GAG staining intensity is reduced in microgravity conditions and approximately equivalent in unloaded control and cyclic hydrostatic pressure. All scale bars = 50 µm.

Figure 5-3 Safranin-O staining on RCS pellets at days 3 and 14. Nuclear staining patterns follow those observed via H&E staining. No proteoglycan (red) staining is observed at day 3, but by day 14 all samples stain for proteoglycans. Proteoglycan staining intensity is the highest in the control condition, and comparable in the simulated microgravity and cyclic hydrostatic pressure conditions. All scale bars = 50 µm.
5.3.2 LRP expression patterns are modulated by mechanical stimulation

Expression of LRP4, LRP5, and LRP6 in response to simulated microgravity and cyclic hydrostatic pressure was evaluated at day 3 and day 14 via quantitative real time RT-PCR (Figure 5-4). LRP4 did not respond to mechanical stimulation at either time point. LRP6 was slightly elevated in microgravity at day 3 (1.5 fold) and both microgravity (1.7 fold) and hydrostatic pressure (2.4 fold) at day 14, although not statistically significant. LRP5 was highly upregulated in simulated microgravity at day 3 (9.9 fold, p<0.01) and trended towards upregulation (3.7 fold) in cyclic hydrostatic pressure. At day 14, again LRP5 expression was elevated in both simulated microgravity (3.5 fold) and cyclic hydrostatic pressure (4.4 fold). Expression of LRP5 in microgravity at day 3 was significantly higher than the upregulation in hydrostatic pressure at day 3 (p<0.05) and both day 14 conditions (p<0.05 in both).

Figure 5-4 Expression changes of LRP4, LRP5, and LRP6 in response to simulated microgravity and cyclic hydrostatic pressure at 3 days and 14 days. LRP4 shows little response to loading or unloading at either time point. At day 3, LRP5 is highly upregulated in simulated microgravity (p<0.01), and trends towards upregulation in cyclic hydrostatic pressure. At day 14, LRP5 expression is elevated in both simulated microgravity and cyclic hydrostatic pressure. LRP6 expression trended towards elevation in both simulated microgravity and cyclic hydrostatic pressure and day 14, although not statistically significant. All data was normalized to the day 3 unloaded control data represented by the dashed line (*p<0.05, **p<0.01)
To evaluate protein-level changes within the Wnt-signaling pathway, 14-day pellets were stained for LRP5 and active β-catenin (Figure 5-5). LRP5 expression was elevated in both simulated microgravity and cyclic hydrostatic pressure when compared to the unstimulated controls. LRP5 expression was greater in hydrostatic pressure than simulated microgravity. Active β-catenin, which corresponds to activated canonical Wnt-signaling, followed the same trend as LRP5 expression with highest expression in cyclic hydrostatic pressure and lowest expression in the unstimulated controls.

![Figure 5-5](image.png)

**Figure 5-5** LRP5 and active β-catenin after 14 days of stimulation. Both active β-catenin and LRP5 staining intensity are elevated in simulated microgravity and cyclic hydrostatic pressure when compared to the unloaded control. Highest expression is observed in cyclic hydrostatic pressure. All scale bars = 50 µm.

In order to determine if LRP5 was also responsive to loading *in vivo*, we stained the femoral heads of mice subjected to either normal loading or hindlimb unloading (Figure 5-6). LRP5 expression was higher within the articular cartilage of mice subjected to hindlimb suspension than in that of the control mice.
Figure 5-6 LRP5 expression was elevated in articular cartilage of mouse femoral heads after 14 days of hindlimb suspension. Greater staining intensity is observed within the chondrocytes of the suspended mice. All scale bars = 50 µm.

5.3.3 LRP knockdowns result in altered expression of cartilage degrading proteases

In order to assess the downstream effect of altered LRP expression, siRNA was used to knock down expression of LRP4, LRP5, and LRP6 (Figure 5-7). Although not statistically significant, trends were observed within this data set. MMP1 expression was elevated in all LRP knockdowns. MMP7 expression also increased when LRPs were knocked down, especially in the LRP4 knockdown. MMP13 expression increased with LRP5 knockdown but decreased with LRP6 knockdown. All LRP knockdowns resulted in a slightly decreased ADAMTS5 expression.
Figure 5-7 Expression of cartilage degrading proteases after LRP4, LRP5, LRP6 siRNA knockdown. Although not statistically significant, LRPKD increased MMP1 and MMP7 expression in all three LRP knockdowns. MMP13 did not exhibit an overall trend. ADAMTS5 expression was reduced in all three LRP knockdowns. All data was normalized to the control knockdown data represented by the dashed line.

5.3.4 Sclerostin modulates expression of LRP receptors in a dose-dependent manner

To determine the effect of SOST on LRP expression, RCS pellets were cultured in the presence of 25ng/ml, 100ng/ml, or 250ng/ml of recombinant SOST. Interestingly, a dose-dependent effect was observed (Figure 5-8). 25ng/ml SOST inhibited expression of all three LRP receptors but 250ng/ml SOST increased expression of all three LRP receptors. 100ng/ml expression levels fell between the 25ng/ml and 250ng/ml and were similar to control levels. Of the three LRP4s tested, LRP4 was most responsive to SOST addition and
exhibited a reduction to 0.47 fold expression with the addition of 25ng/ml SOST (p<0.01). Higher LRP4 levels were observed when SOST concentrations were increased to 100ng/ml and 250ng/ml, with statistical significance observed between 25ng/ml and 250ng/ml (p<0.01). Although not statistically significant, LRP5 and LRP6 followed the same trend of low expression when cultured in 25ng/ml SOST and higher LRP expression patterns in 100ng/ml and 250ng/ml SOST.

**Figure 5-8** Expression of LRP4, LRP5, LRP6 was modulated by stimulation with exogenous SOST. Expression of all LRPs was decreased when stimulated with 25ng/ml SOST, but higher concentrations of SOST returned LRPs to control or slightly elevated expression levels. All data was normalized to the control 0ng/ml SOST data represented by the dashed line (**p<0.01).

### 5.4 Discussion

The objective of this study was to investigate the role of LRP receptors in the maintenance of cartilage homeostasis in response to mechanical stimuli. We hypothesized that LRP expression is modulated by mechanical stimuli and that LRPs control MMP expression levels. To test our hypothesis, we stimulated RCS pellets in simulated microgravity and cyclic hydrostatic pressure and evaluated LRP expression patterns via qRT-PCR and fluorescent immunostaining. Our *in vitro* data demonstrated that LRP5 is regulated by
mechanical stimuli, being upregulated in both simulated microgravity and cyclic hydrostatic pressure. To further investigate this effect in vivo, we used a HLU mouse model to confirm LRP expression is modulated by mechanical loading patterns. In vivo, HLU yielded higher LRP5 expression than the ground control, reflecting our in vitro observations. To our knowledge, this is the first report of LRP5 response to mechanical loading and unloading conditions in chondrocytes.

We also evaluated the overall changes of cellular organization and matrix composition in response to mechanical stimuli. Evaluation of RCS matrix deposition within the present study demonstrated that simulated microgravity inhibits matrix deposition. At day 3, both the simulated microgravity pellets and control pellets exhibited minimal histological staining, while the pellets stimulated via cyclic hydrostatic pressure had robust nuclear staining and showed cellular organization more closely resembling native cartilage than the other two conditions. This suggests that cyclic hydrostatic pressure promoted the RCS cells to begin cartilage tissue formation at the early time point of 3 days, which is consistent with previous research by our group and others that suggests cyclic hydrostatic pressure favors cartilage formation in human derived adipose stem cells (51, 187-191).

By day 14, RCS pellets maintained in all three conditions displayed nuclear, Alcian Blue, and Safranin-O staining. However, different tissue-level characteristics were observed between the samples. Greatest spacing between nuclei was observed within the cyclic hydrostatic pressure samples and the smallest spacing was observed within the simulated microgravity. Alcian Blue stain was more robust in the control and hydrostatic pressure
pellets than in the simulated microgravity pellet, further indicating that simulated microgravity inhibits matrix deposition. This is consistent with previous research that has demonstrated simulated microgravity inhibits cartilage matrix deposition during chondrogenesis of human mesenchymal stem cells (192). The control RCS pellets showed more robust Safranin-O staining than either simulated microgravity or cyclic hydrostatic pressure. This again suggests that simulated microgravity inhibits matrix deposition, but the effect of hydrostatic pressure on matrix deposition remains unclear.

LRP5 was the most mechanoresponsive LRP at the time points and conditions evaluated in this study. Interestingly, LRP5 was upregulated in both simulated microgravity and cyclic hydrostatic pressure when compared to unloaded controls, as confirmed by both RT-PCR and immunostaining. In addition, LRP5 expression was modulated by loading conditions in the in vivo model. LRP5 has been shown to be necessary for skeletal mechanotransduction (193). In addition, LRP Knockout mice have been shown to have greater sensitivity to disuse (194). To our knowledge, this is the first study to suggest that LRP5 also plays a role in cartilage mechanobiology. Within cartilage biology, activated Wnt signaling has a catabolic effect on the tissue (195). Hence, greater LRP expression would potentially lead to cartilage degradation. Within the hind limb unloading model, we saw an increase in LRP5 expression in unloaded tissue, which is in accordance with our previous research that has demonstrated that cartilage thickness is decreased in hindlimb unloaded mice (195). In addition, our RCS pellets in simulated microgravity saw an increase in LRP5 expression and downstream active β-catenin expression, which corresponded to decreased Safranin-O and Alcian Blue staining.
Within this study, LRP5 expression was elevated in hydrostatic pressure but the downstream effect on matrix deposition remains inconclusive.

The progression of arthritic conditions is characterized by increased levels of MMPs, which have been previously demonstrated to respond to mechanical stimuli and are able to degrade and remodel cartilage ECM (196). Wnt signaling has been previously indicated to regulate MMP expression (197). In this study we expected that reduced LRP expression would modulate expression patterns of MMP1, MMP7, MMP13, and ADAMTS5. Generally, LRP4/5/6 knockdown increased MMP1 and MMP7 expression and decreased ADAMTS5 expression. While general trends were observed upon LRP knockdown, statistical significance was not observed. This could be in part due to biological redundancies between LRP5 and LRP6 (198). Future studies should explore combination LRP knockdowns to further explore how LRPs modulate MMP expression within cartilage.

We next examined how SOST, a Wnt-inhibitor, regulates LRP expression in cartilage. Previous studies have shown SOST participates in the mechanobiology of both bone and cartilage. In one study, SOST had reduced expression in bone subjected to high mechanical strain (199). In another study, the down regulation of SOST was shown to be necessary for the increased bone formation due to mechanical loading (184). We have shown SOST is increased in chondrocytes in microgravity conditions (195). This is consistent with studies in bone, which have shown that mechanical unloading of bone increases SOST expression (200). While this is unfavorable to bone density maintenance, in cartilage this response acts as a defense mechanism to modulate Wnt signaling and prevent excessive cartilage
degradation. In this study, we demonstrate that the addition of exogenous SOST to chondrocytes in microgravity modulates LRP4/5/6 expression. While lower concentrations of SOST were found to repress LRP expression, high concentrations restored LRP expression patterns to non-treated levels. SOST has been previously demonstrated to repress LRP5 and LRP6 expression in ovine chondrocytes using the same range of SOST concentrations (201). Unlike the current study, the previous study found LRP5 and LRP6 repression was enhanced with higher SOST concentrations. This discrepancy could potentially be the result of cell sourcing from different animal models, which may respond to concentrations of SOST at different magnitudes. Nevertheless, results from this study suggest that SOST modulates LRP expression, which further supports future research on SOST as a pharmaceutical target for osteoarthritis treatment.

A limitation of the current study is that all experiments were carried out using small animal chondrocytes. Small animal models have different magnitudes of physiological loading, and hence small animal chondrocytes may not respond to mechanical stimuli at the same magnitudes as chondrocytes from large animal and human models. Future studies should determine how the mechanical regulation of LRP expression described in the present study translates into large animal models and human cell lines.

In conclusion, we have shown that mechanical stimulation of chondrocytes regulates matrix deposition and Wnt-signaling expression characteristics. Specifically, simulated microgravity decreases matrix deposition. Both simulated microgravity and hydrostatic pressure promote increased LRP5 expression, and LRP5 expression is increased in an in vivo mouse hindlimb
suspension model. Further, exogenous SOST modulates the expression of LRPs, which suggests a potential mechanism for pharmaceutical treatment of osteoarthritis. Understanding exactly how Wnt-signaling is influenced by mechanical stimuli is essential to identifying and optimizing the best pharmaceutical intervention strategies for prevention and treatment of degenerative cartilage diseases such as osteoarthritis.
CHAPTER 6

Corin, an atrial natriuretic peptide-converting enzyme, regulates osteogenic differentiation of human adipose stem cells.

In the previous chapter we described new findings on the mechanosensitivity of the Wnt-signaling pathway within cartilage. While Wnt-signaling is a major regulator of cartilage and bone biology, there are a multitude of pathways that regulate musculoskeletal development and maintenance. In order to determine which pathways are the most relevant to hASC osteogenic differentiation, we previously performed microarray studies to determine which genes were upregulated during osteogenesis. The Corin gene was identified as one of the most highly upregulated genes during chemically induced osteogenic differentiation. The goal of this chapter is to further elucidate the mechanism by which corin, traditionally studied within cardiac research, contributes to bone development.

The work in this chapter is in preparation to be submitted to PNAS with co-author Elizabeth G. Loboa.

6.1 Introduction

Human adipose derived stem cells (hASC) have great potential for use in bone tissue engineering applications due to their osteogenic differentiation potential, autologous availability, and immunocompatibility (1, 115-119). However, the molecular mechanisms that underlie osteogenic differentiation within hASC are poorly understood.
We previously performed microarray analyses of hASC during osteogenic differentiation and identified Corin as one of the most highly upregulated genes in osteogenic differentiation (46). Corin has also been found to be upregulated in differentiation of human bone marrow derived mesenchymal stem cells (hMSC) (202). Corin has been studied extensively within the vascular system, and is known to regulate blood pressure and sodium homeostasis by activating Atrial natriuretic peptide (ANP) and Brain natriuretic peptide (BNP) (203, 204). ANP and BNP have previously been shown to modulate angiogenesis and vascular remodeling (205, 206). In addition, BNP has been linked to regulating endochondral ossification (207) and bone mineral density (208). Interestingly, Corin is expressed in the prehypertrophic zone of developing bone, indicating that it may play a role in the regulation of endochondral ossification in developing bone tissue (209). Previous studies have reported that serum corin levels are significantly reduced in patients with osteopenia and osteoporosis (210). However, to date there has not been an in-depth study to determine Corin’s role in skeletal biology and osteogenesis.

In the present study, we hypothesized that Corin is a critical regulator of osteogenic differentiation potentially through cross talk with angiogenic signaling pathways. To test our hypothesis, we (a) monitored Corin expression throughout hASC osteogenic differentiation, and (b) knocked down Corin expression in hASC to determine the effect of Corin knockdown on hASC osteogenic potential and angiogenic signaling.
6.2 Materials and Methods

6.2.1 Cell Isolation and Culture

Human ASC were obtained from waste adipose tissue derived from three female donors (ages 25-36) undergoing elective abdominoplasty surgeries (IRB exemption protocol #10-0201), at University of North Carolina hospitals (Chapel Hill, NC). Isolation of hASC was performed as described previously (2, 13). Cells were maintained in complete growth medium (CGM, Eagle's Minimum Essential Medium, alpha-modified supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin) and osteogenic differentiation was induced using osteogenic differentiation medium (ODM, Eagle's Minimum Essential Medium, alpha-modified supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, 50 µM ascorbic acid, 0.1 µM dexamethasone, and 10 mM β-glycerolphosphate).

6.2.2 siRNA Knockdown

For knockdown experiments, Stealth RNAi™ siRNA Negative Control Med GC and Corin Stealth RNAi™ siRNA (Thermo Fisher Scientific Waltham, MA) were used. In order to achieve the greatest knockdown efficiency, siRNA transfection was optimized using BLOCK-iT™ Alexa Fluor® Red Fluorescent Control (Thermo Fisher, Waltham, MA), as per the manufacturer’s protocol. Variables assessed included siRNA concentration (20-80 nM), transfection reagent (Lipofectamine® 2000 (Thermo Fisher Scientific Waltham, MA) vs. Lipofectamine® RNAiMAX (Thermo Fisher Scientific, Waltham, MA)), transfection reagent concentration (1µl/ml-3µl/ml), and transfection type (standard vs. reverse). Highest Alexa
Fluor® Red Fluorescent intensity, indicating highest transfection rate, was achieved using a 2:3:200:800 ratio of 25 nM siRNA: Lipofectamine® 2000: OptiMEM (Thermo Fisher Scientific, Waltham, MA): antibiotic-free CGM medium. Standard vs. reverse transfection did not affect knockdown efficiency, and hence reverse transfection was utilized to reduce overall experimental timelines.

Using our optimized ratio, the following protocol was used to knockdown Corin expression. 5 µl of siRNA oligomers, 7.5 µl of Lipofectamine® 2000, and 500 µl OptiMEM were combined and incubated for 20 minutes in each well within a 6-well plate. hASC were trypsinized and resuspended at a concentration of 25k/ml in antibiotic free CGM. Of this cell suspension, 2ml (50k hASC cells) were added to each well. After a 24-hour incubation, the transfection media was replaced with standard CGM or ODM media formulations. When the knockdown was evaluated via PCR 72-hours post transfection, an average of 58% knockdown efficiency was observed.

6.2.3 Osteogenic differentiation evaluation

Osteogenic differentiation was assessed via calcium accretion at 14 days of differentiation. After 14 days of culture in ODM, samples were scraped and suspended in 0.5N hydrochloric acid for calcium quantification. Samples were incubated at 4°C overnight on an orbital shaker and then quantified via calcium liquicolor assay (Stanbio, Boerne, TX) as per the manufacturer’s protocol. Protein was also collected after 14 days of culture in CGM and ODM for quantification and endogenous alkaline phosphatase (ALP) activity evaluation, respectively. Samples were scraped and suspended in 0.5ml RIPA buffer. ALP activity was
quantified with the Alkaline Phosphatase LiquicolorH Test (Stanbio Laboratory, Boerne, TX), using the P-nitrophenylphosphate methodology (162). Protein content was quantified using the BCA absorbance assay (Thermo Fisher Scientific Waltham, MA), as per manufacturer’s protocol.

Gene expression was quantified after 3 days of culture in ODM via quantitative real time RT-PCR. RNA extraction was carried out using a Trizol (Invitrogen, Carlsbad, CA) extraction method using the manufacturer’s protocol. RNA concentration and quality were assessed using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) and reverse-transcribed using Marligen’s First-strand cDNA Synthesis System (Origene, Rockville, MD). Expression of bone markers Osterix (Hs01866874_s1) and Runx2 (Hs00231692_m1) and angiogenic marker VEGF-A (Hs00900055_m1) were evaluated using Taqman Gene expression assays. All gene expression profiles were normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Assay HS99999905_M1) in an ABI Prism 7000 system.

6.2.4 Tube forming assay

Preliminary Matrigel (Corning Inc., Corning, NY) tube forming assays were used to determine the angiogenic potential of human bone marrow derived stem cell (hMSC) Corin KD lines. hMSC have also been shown to exhibit Corin upregulation during osteogenic differentiation(202) and show differentiation potential similar to that of hASC (211) and hence were used for preliminary analyses within this study. Stable Corin knockdown hMSC had been previously generated via lentiviral KD and selected with anti-puromycin ability
according to manufacturer’s protocol (Santa Cruz Biotechnology). To perform tube forming assays, wells in 96-well plates were coated with 50 µl Matrigel and incubated for 1 hour at 37°C to solidify. Each well was seeded with 1.7k of either control KD or Corin KD hMSC in EBM-2 endothelial medium (Lonza, Basel, Switzerland). After 7 days of incubation, samples were imaged for sprouting assessment.

6.2.5 Statistical Analysis

Paired student t-tests were used to compare Corin KD vs. Control KD data. Corin time course data were analyzed using one-way ANOVA with Tukey post-hoc analyses. Data were log transformed before running analyses. A level of p<0.05 was considered significant. Significance is denoted throughout this study as *p<0.05, **p<0.01.

6.3 Results

6.3.1 Corin expression was increased during osteogenic differentiation

Corin expression was highly upregulated throughout osteogenic differentiation in all three donors (Figure 6-1). Comparison of CGM and ODM Corin expression levels showed significantly higher expression in ODM at all time points in all three donors (p<0.05). Donor-to-donor variability was observed within the Corin expression profiles of each donor. Donor 1 expression peaked at day 7, donor 2 expression peaked at day 10, and donor 3 expression peaked at days 10 and 14.
6.3.2 Corin knockdown modulated osteogenic differentiation potential

Corin KD modulated the osteogenic potential of hASC. Calcium accretion was decreased after 14 days of osteogenic differentiation when Corin expression was knocked down (Figure 6-2). This trend was observed in all three donors, although only statistically significant in Donor 2 (p<0.01). Total protein content assessed at day 14 was significantly decreased in both CGM and ODM of all three donors (Figure 6-3). Endogenous ALP activity, a marker for osteogenic differentiation, was decreased in all donors after 14 days of osteogenic differentiation, and was statistically significant in Donor 2 (p<0.05) and Donor 3 (p<0.01) (Figure 6-4A). When ALP data was normalized to total protein content, the trend remained although statistical significance was lost (Figure 6-4B).

Figure 6-1 Corin mRNA expression was highly upregulated throughout culture in ODM in all three donors. All data was normalized to day 7 CGM, and statistics were run on log transformed data with a significance level set to p<0.05. Different letters indicate statistical difference.
Figure 6-2 Corin KD decreased calcium accretion in all donors after 14 days of culture in ODM (**p<0.01).

Figure 6-3 Corin KD decreased total protein levels of hASC cultured in A. CGM and B. ODM after 14 days. This trend was observed in all three donors (*p<0.05, **p<0.01).
Figure 6-4 Endogenous alkaline phosphatase (ALP) was decreased in Corin KD when hASC were cultured in ODM for 14 days. A. The total ALP was decreased in all three donors. B. When data was normalized to protein content, this trend was still observed although statistical significance was lost (*p<0.05, **p<0.01).

6.3.3 Corin knockdown modulated vascular potential

Corin knockdown increased vascular characteristics of hASC and hMSC. When RT-PCR was carried out after 3 days of culture in ODM, the osteogenic markers osterix and runx2 were not modulated with any apparent trend (Figure 6-5A,B). However, Corin KD significantly increased VEGF-A mRNA expression during osteogenic differentiation in all three donors (Figure 6-5C). In addition, preliminary tube forming assays were carried out using stable Corin KD hMSC and an increase in vascular sprouting patterns were observed in Corin KD cells (Figure 6-6).
Corin KD gene expression was evaluated after 3 days of hASC culture in osteogenic differentiation medium (ODM). Osteogenic markers A Osterix and B Runx2 did not show a clear trend when Corin was knocked down. However, VEGF-A expression significantly increased with Corin KD in all three donors. Data was normalized to Control KD expression levels (dashed line) and statistics compare Corin KD vs. Control KD (*p<0.05 **p<0.01).

Preliminary data run with a stable lentiviral corin knockdown line showed that corin KD within bone-marrow derived mesenchymal stem cells induces a greater propensity to undergo vascular sprouting in a Matrigel tube forming assay.

6.4 Discussion

The objective of this study was to determine if Corin is involved within the process of hASC osteogenic differentiation and determine the mechanism(s) through which Corin may act. We hypothesized that Corin regulates osteogenic potential of hASC and crosstalks with vascular
pathways. We found that Corin is highly upregulated during osteogenic differentiation with a greater than 200-fold increase in corin expression in hASC from all donors assessed. We demonstrated that Corin knockdown decreases osteogenic potential of hASC by reducing calcium accretion and endogenous ALP activity. We also found that Corin regulates vascular characteristics of both human adipose and bone marrow derived mesenchymal stem cells and Corin knockdown yields increases in VEGF-A expression and tube-forming sprouting.

We previously reported an increase in Corin during osteogenic differentiation of hASC (46) but there has been conflicting evidence on whether Corin is expressed in skeletal tissue. Corin mRNA expression was observed via in situ hybridization in developing mouse bone. Specifically, Corin was expressed in the region adjacent to hypertopic chondrocytes and in the perichondrocytes (209). However, Corin was not detected via immunohistological analyses in human skeletal tissue (212), although developing human bone tissue has not been specifically studied. Our data suggest that Corin is likely expressed only during development, but future studies should confirm this in vivo.

In this study, we found that Corin KD reduces calcium accretion and endogenous ALP in hASC undergoing osteogenic differentiation. This demonstrates that Corin is not just passively upregulated during osteogenic differentiation and suggests that Corin plays an active role in the mediation of hASC osteogenic differentiation. In addition, protein levels were significantly decreased in Corin knockdown of hASC cultured in both CGM and ODM. It is surprising that Corin KD modulated protein content in CGM culture since Corin mRNA expression is extremely low during CGM culture. Better understanding of how Corin affects
hASC in CGM should be investigated in future studies. Corin knockdown also reduced total protein content when cultured in ODM. Since the BCA assay for protein content quantifies total protein within a sample, it is unclear what proportion of the sample is part of the cellular structure and what portion is part of the deposited ECM. Since control KD cells likely lay down more extracellular matrix, this may partially explain why statistical significance is lost when ALP activity is normalized to protein content. Overall, our data suggest that Corin is an important protein for hASC function and the reduction of Corin expression impairs osteogenic differentiation.

The mechanisms by which Corin regulates skeletal development are not clearly understood. Within cardiac research, Corin has been shown to cleave the precursors pro-ANP and pro-BNP into active ANP and BNP, respectively (203, 204, 213, 214). Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are hormones that regulate cardiac homeostasis (215). Interestingly, natriuretic peptides have been previously suggested to play a role in skeletal biology and endochondral ossification (207, 208, 216, 217). Previous research has suggested that BNP plays a role in controlling endochondral ossification and over expression can lead to skeletal overgrowth (207). In addition, N-terminal Pro-BNP serum levels have been shown to be negatively correlated with lumbar bone mineral density (208). C-type natriuretic peptide (CNP) is also critical to regulation of bone development and has been shown to regulate endochondral ossification (218). Although CNP is a mediator of endochondral ossification, to date, there has been no evidence that Corin processed pro-CNP to active CNP (213), but rather Furin is considered the major pro-CNP processor (219). However, our previous microarray data did not show an increase in Furin expression during
osteogenic differentiation (46, 220), suggesting that the CNP pathway is not responsible for the hASC osteogenic mediation witnessed in this study.

Due to Corin’s role in vascular biology and the role that natriuretic peptides have been suggested to play in endochondral ossification, we investigated whether Corin modulates vascular characteristics of hASC and hMSC. We found that Corin knockdown promotes VEGF-A expression in hASC and vascular sprouting potential of hMSC in tube forming assays. Within the literature, the Corin-ANP pathway has been previously implicated in the regulation of trophoblast invasion and uterine spiral artery remodeling (221). Whether this mechanism is used to regulate angiogenesis during endochondral ossification has yet to be determined. Interestingly, although Corin is a cardiac protein, Corin reduction leads to an increase in angiogenic potential suggesting that Corin is a regulator, rather than a promoter, of angiogenesis.

In conclusion, this is the first study to suggest that Corin is a key regulator of osteogenesis in hASC, likely through crosstalk with vascular pathways. Understanding the underlying molecular mechanisms of osteogenesis will aid in the development of optimized bone tissue engineering and regenerative medicine strategies using hASC.
CHAPTER 7

Electrical cell-substrate impedance spectroscopy can monitor age-grouped human adipose stem cell variability during osteogenic differentiation

In the prior chapter, we described the role that Corin plays in osteogenic differentiation of hASC. While understanding individual mechanisms is necessary to optimize functional tissue engineering therapies and providing safe and effective treatments to patients, in some cases it may be necessary to determine the overall osteogenic potential of an hASC population. When patients require hASC therapy, it is desirable to know whether or not they would be a good candidate for an autologous procedure. While individual genes may affect how well an hASC population differentiates, in reality it is the sum of all reactions occurring within a stem cell population that dictates its absolute osteogenic potential. It is not practical to monitor and evaluate all signaling pathways within a cell population, and so techniques must be developed that monitor collective characteristics of a cell population that reliably allow us to evaluate hASC populations. In this chapter we use electrical cell-substrate impedance spectroscopy (ECIS) to non-invasively track hASC through stages of proliferation and osteogenic differentiation in real time. The goal of this study was to use this method to evaluate differences between age-grouped hASC populations.

The work in this chapter has been published in Stem Cells Translational Medicine with co-authors Jianlei Zhang, Emily H. Griffith, Matthew W. Frank, Binil Starly, and Elizabeth G. Loboa (222).
7.1 Introduction

“Variability between stem cells isolated from different donors is a well-documented barrier to the clinical translation of autologous stem cell therapies (1, 13, 17, 23, 223). Human adipose stem cells (hASC) are an attractive autologous stem cell source for tissue engineering and regenerative medicine applications due to their relative ease of isolation, multipotent differentiation capacity, and immunocompatibility (115-119). However, as with other stem cell sources, hASC isolated from different donors exhibit dramatic variability in potential to proliferate and differentiate. We have previously demonstrated that hASC isolated from donors of different age groups have different capacities to differentiate down the osteogenic lineage (13). Other studies have documented that other demographic factors such as body-mass index (BMI) (22), and gender (25) also affect hASC proliferation and/or differentiation potential. We have shown that hASC from different donors also have a variable response to mechanical stimuli (17). However, the study of donor demographics alone cannot provide a clear understanding of the underlying causes of donor variability. Further, as hASC enter widespread clinical use, it is desirable to develop a non-invasive, quantitative method to screen hASC populations for their potential use in autologous therapies, and to monitor their differentiation status for quality control purposes.

Electrical cell-substrate impedance spectroscopy (ECIS) was originally developed by Giaever and Keese (224) and has been used in a variety of applications including monitoring cell micromotion (225), response to drugs, and assessment of barrier function (226). ECIS applies a very weak (<1μA), non-invasive alternating current to cells seeded on a gold electrode array, allowing the cell impedance to current to be monitored in real-time and
By capturing complex impedance patterns, dynamic aspects of cultured cells through its dielectric properties can be evaluated. In addition to providing impedance measurements, the data collected from ECIS allows additional morphology-related parameters to be determined. Parameters that describe cell coverage including barrier resistance ($R_b$), capacitance of the cell plasma membrane ($C_m$), and current flow beneath the cells ($\alpha$) can be calculated. ECIS has previously been used to track both human bone marrow derived mesenchymal stem cells (hMSC) undergoing osteogenic differentiation and hASC during osteogenic and adipogenic differentiation. Those studies demonstrated that hMSC and hASC have distinct impedance properties as they differentiate down adipogenic or osteogenic lineages. However, it is not known whether donor-to-donor variability can be captured via ECIS. In this study, we hypothesized that ECIS impedance properties could be utilized to quantify donor-related differences in hASC populations during both proliferation and osteogenic differentiation.

Herein, we use ECIS to detect distinct bioimpedance patterns between three age-grouped hASC superlots throughout proliferation and osteogenic differentiation. Our results demonstrate that ECIS can potentially be used to screen for osteogenic potential of hASC populations, track stages of osteogenic differentiation for quality control purposes, and to better elucidate the underlying biological causes of hASC variability between donors.
7.2 Materials and Methods

7.2.1 Superlot Generation

hASC were isolated from liposuction aspirates of female patients undergoing voluntary liposuction procedures. Complete growth medium (CGM) (Minimum Essential Medium, alpha modified (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA), 2mM L-glutamine (Corning Inc., Corning, NY), 100 U/mL penicillin, 100 mg/mL streptomycin (Penicillin-Streptomycin Solution, Corning Inc., Corning, NY)) was used to expand hASC. Age-grouped hASC superlots were created as described previously (13). Briefly, hASC were pooled in equal proportions from age-clustered donors per superlot. Age groupings consisted of young (age: 24-36, 5 donors), middle-aged (age: 48-55, 4 donors), and elderly (age: 60-81, 5 donors) patients. The superlots were previously verified as a representative average of the individual hASC behavior, by quantifying metabolic activity, calcium accretion during osteogenic differentiation, and lipid production during adipogenic differentiation (13).

7.2.2 Impedance Sensing

Using a ECIS Zθ instrument (Applied BioPhysics, Troy, NY), hASC were seeded into 8-well plates lined with a 40 working electrode array to measure in real-time the complex impedance values of proliferating and differentiating hASC. Measurements were taken every 5 minutes at frequencies ranging from 10Hz to 100KHz. To prepare plates, each well was coated with 1mg/ml collagen type I (Advanced BioMatrix, Inc., San Diego, CA) by covering the bottom of the plate and allowing it to sit at room temperature for 30 minutes before aspirating collagen solution off, and treating with l-cysteine (Applied BioPhysics, Troy, NY).
for 30 minutes in the same manner, as per recommendation of Applied BioPhysics. Cells were seeded at a density of 3,000 cells per 0.8cm² well in 400µl of CGM. Media was replaced every 48 hours until hASC reached 80% confluence (determined visually by optical microscopy), at which point cell culture medium was replaced with 400µl of either CGM or osteogenic differentiation medium (ODM) (Minimum Essential Medium, alpha modified (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA), 2mM L-glutamine (Corning Inc., Corning, NY), 100 U/mL penicillin, 100 mg/mL streptomycin (Penicillin-Streptomycin Solution, Corning Inc., Corning, NY), 50 µM ascorbic acid (Sigma-Aldrich, St. Louis, MO), 0.1 µM dexamethasone (Sigma-Aldrich, St. Louis, MO), and 10 mM b-glycerolphosphate (Sigma-Aldrich, St. Louis, MO)). The experiment was concluded once hASC had undergone the impedance drop phase.

7.2.3 Differentiation and viability characterization

Metabolic activity of the hASC populations were assessed via Alamar blue (AbD Serotec, Raleigh, NC) every 96 hours. DNA was quantified via Hoescht 33258 assay (Life Technologies, Carlsbad, CA). Osteogenic differentiation was assessed via calcium accretion at 14 and 21 days of differentiation using both a quantitative calcium liquoricolor assay (Stanbio, Boerne, TX) and Alizarin Red (Pacific Star Corporation, Houston, TX) staining as we have described previously (13, 46, 48, 165). In order to rule out the possibility that lipid accumulation influenced our impedance data, Oil Red O (Thermo Fisher Scientific, Waltham, MA) staining was carried out after 21 days of differentiation.
7.2.4 Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) Analysis

In order to determine composition of observed accreted matrix, ToF-SIMS was used to analyze matrix after 21 days of culture in ODM. ToF-SIMS is a highly sensitive surface analytical technique for acquisition of elemental and molecular information from the surface of a material with high spatial and mass resolution. ToF-SIMS analyses were conducted using a TOF SIMS V (ION TOF, Inc. Chestnut Ridge, NY) instrument equipped with a $\text{Bi}_n^m+$ ($n = 1 - 5$, $m = 1, 2$) liquid metal ion gun, $\text{Cs}^+$ sputtering gun and electron flood gun for charge compensation. Both the Bi and Cs ion columns are oriented at 45° with respect to the sample surface normal. The analysis chamber pressure is maintained below $5.0 \times 10^{-9}$ mbar to avoid contamination of the surfaces to be analyzed. For high lateral resolution mass spectral images acquired in this study, samples were sputtered for 20 cycles with $\text{Bi}_3^+$ DC beam at 10 nA, and a Burst Alignment setting of 25 keV $\text{Bi}_3^+$ ion beam was used to raster a 160 µm by 160 µm area. The negative secondary ion mass spectra obtained were calibrated using $\text{CN}^-$, $\text{PO}_2^-$, and $\text{PO}_3^-$. The positive secondary ion mass spectra were calibrated using $\text{Mg}^+$ and $\text{Ca}^+$.

7.2.5 ECIS Model

This study employed the model developed by Giaever and Keese (224). When cells are confluent on the electrodes, the ECIS model decomposes the time-course impedance data into three frequency independent parameters: 1) $R_b$ (Ω.cm²), intercellular resistance established by tight cell-cell junctions. 2) $C_m$ (µF/cm²), the average cell membrane capacitance, attributed to the charge collection by the phospholipid layers of the cellular
membrane; 3) $\alpha^2 (\Omega \cdot \text{cm}^2)$, indicating cell-substrate interaction. $R_b$ and $\alpha^2$ varied between age-grouped superlots and were included within this report.

7.2.6 Entropy calculation

We utilized the Shannon Entropy equation as a measure of signal unpredictability. The resistance portion of the impedance signal was first detrended and the signal divided into 48 hour blocks. Shannon entropy for the resistance signal was calculated using the formula,

$$H(R) = -\sum_{i=1}^{n} P(R_i) \log_2 P(R_i).$$

7.2.7 Statistics

Turning point analysis was carried out using PROC GLM in SAS (Version 9.4, Cary, NC). Changepoints were modeled as a function of age category using an analysis of variance model. Mean comparisons were made using least-squares means and post-hoc testing. The calcium accretion data were summarized at day 14 and day 21. Both sets of data were analyzed using a linear model in SAS (Version 9.4, Cary, NC) allowing for the effects of age and trial. The Alamar blue data were analyzed using a repeated measures model on the natural-log transformed percent reductions. Effects of day, age, trial, and a day by age interaction were included in the model. Post hoc pairwise comparisons were made using Tukey’s adjustment to control the Type I error rate. A repeated measures model was also fit to the entropy data, allowing for effects of cell culture type (ODM v. CGM), age group, and day. In addition, paired t-tests were calculated for the individual age groups to find the first day with a significant drop from baseline (day 0). Bonferroni’s correction was used to control the Type I error rate across all paired t-tests. The significance level was set at 0.05.
7.3 Results

7.3.1 Age-grouped superlots exhibit distinct impedance phases and time-course patterns during proliferation and osteogenic differentiation

In order to determine if ECIS could be used to rapidly detect donor-to-donor variability during proliferation and osteogenic differentiation, dielectric properties of hASC were quantified in real-time by seeding age-grouped superlots onto multi-electrode arrays and tracking complex impedance patterns at frequencies ranging from 10Hz to 100kHz throughout hASC proliferation and osteogenic differentiation. The greatest differential between hASC lines was observed at the 40kHz frequency. This frequency was therefore used for analysis purposes. We obtained ECIS curves of hASC isolated from all three superlots during expansion in complete growth medium (CGM) (Figure 7-1A). Spikes in impedance were observed at each media change every 48 hours. Due to the small surface area of the ECIS wells, the hASC are especially susceptible to delamination due to overcrowding. The young donor superlot proliferated, reached confluence and overgrowth, and delaminated at day 6 when cultured in CGM, characterized by the detachment and rolling up of the cell monolayer. Our results are consistent with the ECIS results of Bagnaninchi et al., who observed delamination of hASC maintained in growth media approximately 6.7 days after seeding(229). The middle-aged superlot, elderly superlot, and all superlots grown in ODM did not delaminate over the experimental duration. In addition, impedance curves were generated that monitored hASC differentiation in osteogenic differentiation medium (ODM) (Figure 7-1B). Elderly donor cells exhibited overall lower impedance in both growth medium and differentiation medium. Because an impedance drop was observed in elderly and middle-aged donors within the short-term 10-day differentiation
study, the young superlot study period was extended until equilibrium at 26 days (Figure 7-2A). Distinct phases in differentiation could be observed throughout osteogenesis of all age-grouped superlots: increase, primary stabilization, drop phase, and secondary stabilization. Cells remained viable throughout drop phase. Human ASC from elderly donors had a significantly shortened confluent phase, resulting in an earlier drop phase when compared to hASC isolated from younger donors. Via turning point analysis, it was found that the impedance reached a maximum value before drop phase at day 10.0 in the young superlot, day 6.1 in the middle-aged superlot, and day 1.3 in the elderly superlot (Figure 7-2B). The age-grouped time-to-maximum values were highly significant (p<0.0001). Matrix deposition was first observed via light microscopy 48 to 96 hours post impedance maximum in the hASC cultured in osteogenic differentiation medium (ie., at day 14 in the young superlot, day 8 in the middle-aged superlot, and day 4 in the elderly superlot) (Figures 7-S1, 7-S3, 7-S3). ToF-SIMS analysis revealed that the observed matrix consisted of calcium, phosphates, and organic compounds (Figure 7-3).
Figure 7-1 Real-time impedance (Avg ± SD) curves of hASC during A. culture in complete growth medium (CGM) or B. culture in osteogenic differentiation medium (ODM). Osteogenic induction occurred at day 0 when applicable. Asterisk indicates delamination of young hASC superlot cultured in CGM at day 6 media change due to cell overgrowth.
Figure 7-2 An extended impedance curve (Avg ± SD) of the young superlot (hASC from 5 female donors aged 24 to 36 years). The hASC were cultured in CGM until induction (Day 0), at which point culture medium was changed to ODM. Matrix deposition, as identified by light microscopy, was first observed during drop phase. Full light microscopy time courses can be found in Fig. S1-3. As shown through live dead imaging, cell viability was maintained throughout the impedance drop. Scale bars = 200 µm.

Figure 7-3 ToF-SIMS images of deposited matrix composition after 21 days of culture in either CGM (A-E) or ODM (F-J). The elderly superlot is represented in above images. Brighter intensity corresponds to greater ion detection. The cellular structures are visible on control samples cultured in CGM, and distinct matrix structure is observed samples cultured in ODM. Images reveal that CN- ions (A, F), PO2- ions (B, G), and PO3- ions (C, H) were observable within cellular structures in CGM, but ions cover the entire matrix surface after culture in ODM. The positive Mg+ ions (D, I) did not change significantly between control and experimental samples, but Ca+ ions (E, J) were greatly increased after culture in ODM.
7.3.2 hASC maintain viability but display changes in mineralization in correlation with osteogenic impedance phases

In order to understand the underlying cause of each impedance phase, parallel studies were carried out to track the differentiation and proliferation status of the hASC. After two weeks of culture in osteogenic differentiation medium, hASC were alive in all superlots indicating that the impedance drop had not been the result of cell death (Figure 7-4A). However, a notable difference in cell density could be observed. Young hASC had formed a dense layer of cell coverage, while elderly hASC had not grown as dense and the cells appeared larger in size. Human ASC proliferation in all conditions increased or was maintained throughout culture in both CGM and ODM (Figure 7-4B). The interactions between metabolic activity and day and age were both highly significant (p < 0.0001). Alamar blue activity data was in accordance with DNA quantification data (Figure 7-S4). Calcium accretion was tracked throughout osteogenic differentiation. Human ASC for older donors appeared to exhibit slightly higher calcium accretion at 14 days, but no difference was apparent at the 21-day time point (Figure 7-4C). When quantified via calcium liquicolor assays, the effect of age on calcium accretion was found to be statistically significant at both day 14 (p = 0.010) and day 21 (p = 0.002). The young hASC superlot had significantly lower calcium accretion at the 14 day time point than both elderly (p = 0.009) and middle-aged (p = 0.035), but at day 21 the young superlot had accreted significantly more calcium than both elderly (p = 0.005) and middle-aged (p = 0.001) (Figure 7-4D). No significant difference was found between hASC from elderly and middle-aged at day 14 (p = 0.234) or day 21 (p = 0.089). After 21 days of osteogenic differentiation, low levels of lipid deposits (<1% of cells) were observed in the
young and middle-aged superlots but no significant lipid accumulation was observed in elderly superlot (Figure 7-S5).

Figure 7-4 The viability and calcium accretion of superlots cultured in osteogenic differentiation medium (ODM) was assessed in parallel to ECIS experiments. A. Live dead imaging of age-grouped hASC superlots after 2 weeks of culture in ODM (Scale bars = 200 µm). B. Alamar Blue profiles of superlots indicated that metabolic activity of all superlots increased or was maintained throughout culture in ODM. Greater percent reduction correlates to higher metabolic activity. C. Alizarin red staining for calcium deposition of hASC after culture in ODM showed dense calcium deposition from all superlots (Scale bars = 200 µm) but with timing of calcium accretion varying between age groups. D. The calcium deposition per well (Avg ± SD) was quantified via Calcium Liquicolor at two and three-week time points and showed that the young superlot accreted significantly less calcium at 14 days but significantly more calcium at 21 days than the middle-aged and elderly superlots (* = p<0.05).
7.3.3 Distinct membrane resistance and cell-to-substrate adherence factor can be observed between age-grouped superlots

The ECIS model developed by Giaever and Keese was applied to calculate the membrane resistance ($R_b$) and cell-to-substrate adherence factor ($\alpha^2$) when the cells had completely covered the electrodes (Figure 7-5). These parameters were retrieved for all superlot cell groups and time-course changes to $Z(t,f)$ were observed during early induction and osteogenesis. $R_b$ for the younger and middle age groups continued to increase as the intercellular junctions tightened post-induction. This was not seen in the elderly group of cells as a meaningful $R_b$ was not detected. For the young superlots, peak $R_b$ of 3.1 $\Omega$.cm$^2$ was detected on Day 11 before dropping to 1.5 $\Omega$.cm$^2$ by Day 26. During this period of $R_b$ increase, $\alpha^2$ initially rose for 24 hours post induction, reached a peak of 21.6 $\Omega$.cm$^2$ and then remained steady until Day 11. It then steadily decreased to a value of 7.5 $\Omega$.cm$^2$ by Day 26. For the hASC isolated from middle-aged donors, a peak $R_b$ of 1.8 $\Omega$.cm$^2$ was reached on Day 6. Much higher $\alpha^2$ was detected for the middle-age hASC when compared to younger cells. $\alpha^2$ rose for 48 hours post induction to a peak of 36.4 $\Omega$.cm$^2$ and dropped to 6.5 $\Omega$.cm$^2$ by Day 10. Interestingly, $R_b$ was found to increase within 4hrs of induction for the younger group of cells, while it took 36 hours for $R_b$ to start increasing in hASC from middle-age donors. The intercellular junction resistance for hASC in both CGM and ODM increased at roughly the same time point irrespective of culture medium type. This indicates that induction by ODM did not affect junction formation. The peak strength of the intercellular junction was higher for the younger group of cells relative to the middle age group. In contrast, the amount of adherence of the middle-age hASC superlot during the initial phases of induction was higher. Taken together, distinct dielectric properties were observed between the superlot groups,
specifically with respect to differences in cell adhesion, strength of the intercellular junctions, and time at which connections begin to strengthen and decline during long term monitoring.

**Figure 7-5** A comparison of $\alpha^2$ and $R_b$ coefficients of age-grouped superlots tracked in real time. A. The $\alpha^2$ parameter of superlots cultured in CGM were lower in the young superlot than either the middle-aged or elderly superlot, indicating greater cell-spreading in middle-aged and elderly superlots. B. In ODM, the $\alpha^2$ parameter was higher prior to differentiation, suggesting that cell spreading decreases in late stage osteogenic differentiation. $\alpha^2$ was highly reduced in the young and middle-age superlots as culture time in ODM increased, but $\alpha^2$ in the young superlot only decreased slightly as the hASC were cultured in ODM. C. $R_b$ of the elderly superlot was greatly diminished when compared to the young and middle-aged superlots cultured in CGM, which indicates fewer cell-to-cell junctions in elderly hASC. D. In ODM, elderly superlots did not establish a $R_b$ coefficient during the duration of the study, indicating minimal cell-to-cell junctions in elderly hASC. In the young and middle-aged superlots, $R_b$ decreased after long-term culture in ODM, at approximately day 6 in the middle-aged and day 11 in the young superlot. This suggests that the number and composition of intercellular junctions are altered in late-stage osteogenic differentiation. Delamination of the young superlot in CGM is indicated by * and middle-aged and elderly superlots were ended at the **, at which point they had undergone differentiation and the impedance drop phase.

### 7.3.4 Micromotion of the hASC decreased during osteogenic differentiation

Cellular motility of hASC superlots was evaluated by analyzing the resistance signal obtained from the real component of the impedance signal. Noise in the signal is attributed to
vertical fluctuations of the cellular membrane on the electrode and is often quantified by calculating an index obtained from the power spectral density of the signal. Due to the differences in time scales during monitoring of the superlot groups, entropy in the signal data was calculated. A larger entropy measure indicates that the noise in the data is significant, and is attributed to cell motility and micromotion. When quantified, the effect of “days post osteogenic induction” on micromotion index was found to be statistically significant (p<0.0001). Human ASC micromotion decreased in all superlot types at time points that correlated to the impedance drop phase (Figure 7-6). The decrease occurred at different time points for each of the hASC types. The elderly hASC noise signal dropped progressively until the end of the experiment, beginning immediately after osteogenic induction. By day 2, the micromotion index had decreased significantly from day 0 (p<0.0001). For the middle-aged group, the micromotion first significantly increased at day 2 (p=0.002) and day 4 (p=0.003), before a drop in micromotion was observed. The drop was first apparent at day 6, although it was not significantly lower than day 0 until day 8 (p<0.0001). Micromotion of the young hASC superlot also increased at day 2 (p=0.014) and began dropping at day 12 (p=0.047). However, when accounting for multiple testing, significance from day 0 was not achieved until day 16 (p=0.0003). Figure 7-S6 shows that during proliferation in CGM, entropy measured across all superlots is high and there is no significant difference between age-grouped superlots, but it was found that the medium (CGM vs. ODM) had a statistically significant interaction with micromotion index (p=0.002).
Figure 7-6 Entropy of the impedance signal was quantified throughout culture in ODM. Entropy decreased as superlots differentiated, and correlated to the impedance drop phase. Entropy decreased significantly from day 0 at day 2 in the elderly (p<0.0001), day 8 in the middle-aged (p<0.0001), and day 16 in the young superlot (p=0.0003). This indicates that micromotion of hASC is decreased during late stage osteogenic differentiation.

7.4 Discussion

This is the first study to monitor age-grouped hASC proliferation and osteogenic differentiation using ECIS. We hypothesized that ECIS could be utilized to elucidate donor age-related differences in dielectric properties of hASC proliferation and osteogenic differentiation in real-time, thus providing a means to better understand and predict variability between hASC cell lines. We have demonstrated, for the first time, that age-grouped superlots of hASC exhibit distinct complex impedance patterns.

We further determined a general impedance pattern observed in the differentiation of all hASC superlots that consisted of four major phases: initial increase, primary stabilization,
drop phase, and secondary stabilization. Parallel tracking of hASC through traditional methods offered some explanation for these patterns. As visualized by light microscopy, hASC proliferated after initial seeding until confluence, which correlated to increasing impedance values. In addition, this phase corresponded to increasing proliferation of the hASC as measured via Alamar Blue. Once cells reached confluence, impedance stabilized for a period of time. Eventually, an impedance drop was observed, which appeared to precede matrix deposition and mineralization by 48 to 96 hours. This suggests that cells undergo morphology changes during late stage differentiation and that these changes can be detected via ECIS. This drop was not caused by cell death as cells maintained viability throughout the mineralization phase as confirmed through Live/Dead® assays. However, throughout osteogenic differentiation, morphological changes resulted in less dense cell coverage and correspondingly fewer cell-to-cell junctions, as indicated by the dropping Rb values. Since the impedance drop preceded matrix deposition, the matrix protein interference with impedance readings alone cannot explain the impedance drop. As observed in the young hASC superlot, impedance eventually reached a secondary stabilization phase, at which time the cell impedance data stabilized while cells continued to accrete matrix. To our knowledge, this is the first time it has been shown that ECIS can be used to track specific stages of hASC osteogenic differentiation.

In this study, our goal was to use ECIS to better understand variability between hASC populations. We found the greatest separation in impedance values between age groupings at 40kHz, at which the frequency is affected mostly by cell-coverage. Our observations also hold true across a wide range of frequencies. Very similar impedance trends were witnessed
in both higher and lower frequencies, and normalized curves have been included in Fig. S7. We postulate that the similarities between high and low frequencies are because cell coverage is correlated to the degree of intercellular junctions. Future studies are needed to further elaborate upon optimization of frequency range to detect specific morphological changes in differentiating stem cells.

Although matrix deposition and mineralization occur simultaneously as the hASC undergo osteogenesis, the two processes are distinct and can have temporal variation. During endochondral ossification in vivo, collagen mineralizes to form bone(230). Here, we detected mineralization slightly before matrix deposition is observed, but more robust mineralization is detected after matrix deposition has initiated. Our ToF-SIMS analysis suggested that the matrix is a combination of both organic molecules and ionic compounds. The observable matrix in this study was likely composed of large organic compounds such as collagen, as calcium ions would not be visible to the eye until large-scale crystallization occurs.

Different characteristics of the general four-phase curve were observed for each age group. Most notably, the impedance drop was observed in osteogenic differentiation of all hASC superlots but exhibited great temporal variation. Turning point analyses determined that the time-to-maximum impedance was at day 10.0 in young, day 6.1 in middle-aged, and day 1.3 in elderly hASC. This could indicate that hASC isolated from elderly donors exhibited reduced stemness when compared to the young hASC and are therefore induced down the osteogenic lineage more rapidly. Conversely, hASC isolated from younger patients require longer exposure to osteogenic factors before they begin to deposit matrix and mineralize.
These influences of donor age on hASC during osteogenic differentiation were clearly recognized in real time with the ECIS approach. Such influences are not facilely identifiable by more traditional characterization techniques since data is taken only at specific points in time rather than tracking data in real-time. For example, although the middle-aged and elderly superlots showed significant differences in our turning point analysis, calcium accretion was not statistically different at both the 14-day and 21-day time points. Total calcium accretion from a given cell population is the result of multiple factors such as length of time cells have been accreting calcium, the amount of calcium accreted on a per cell basis, and whether the cells are accreting calcium at a continuous or variable rate over the time frame of interest. Quantifying total calcium accretion at a discrete point in time does not fully elucidate the dynamic nature of differentiating hASC, demonstrating that a real-time method to track hASC differentiation such as ECIS may provide greater insight into a hASC population’s ability to differentiate.

Temporal timing of the onset of osteogenic differentiation could partially explain the observed variability between hASC isolated from different donors. Although after two weeks the young superlot had accreted less calcium than the middle-aged and elderly superlots, after three weeks of osteogenic induction the youngest superlot had accreted significantly more calcium per well than either the middle-aged or elderly hASC superlot. Again, this suggests a delayed onset of osteogenic differentiation and mineralization in the younger hASC population, allowing more time for the cells to proliferate. A previous study used ECIS to quantify impedance throughout osteogenic differentiation of hASC, but no impedance drop was reported (229). This could potentially be explained by temporal variation in the onset of
the impedance drop. The impedance drop phase of the hASC isolated from younger donors was identified between days 12 and 20 post-induction, while the previous study was only carried out to day 14 post-induction. The hASC used in that previous study may not have been exposed to osteogenic factors long enough to reach the impedance drop phase of osteogenic differentiation. In addition, low levels of lipid accumulation detected via Oil Red O could have had a slight effect on impedance data. Our data that the young and middle-aged superlots exhibited greater lipid production than the elderly superlot is in accordance with our previous research (13).

After osteogenic induction, immediate differences in $R_b$ coefficients were observed between hASC of different age-grouped superlots, suggesting that cell-to-cell junctions may help mediate hASC differentiation. In the young superlot, stronger junctions were evident in the hASC induced down the osteogenic lineage. These findings indicate that cell-to-cell junctions may play an early role in osteogenic differentiation. Adherin proteins have been previously demonstrated to correlate to differentiation stage (231). In addition, dexamethosone induced osteogenic differentiation has been demonstrated to down regulate expression of N-Cadherin and Cad11 and upregulate R-Cadherin (232); this corresponds to the $R_b$ drop that we observed during late-stage osteogenic differentiation. Since cell-to-cell junctions are a major contributor to the impedance of a cell monolayer, we would expect that as adherin expression changes throughout osteogenic differentiation that impedance would also change accordingly. Staining of Cnx43 at the time of induction (Figure 7-S8) showed punctate staining indicative of Cnx 43 in both the young and middle-age superlots. However, the elderly superlot did not exhibit this staining pattern, suggesting fewer cellular junctions.
Further studies are required to determine which junction proteins control ECIS impedance throughout the duration of osteogenic differentiation. In addition, future studies should examine the impedance properties of BMP-2 induced osteogenesis, which may yield different impedance patterns since BMP-2 induced osteogenesis upregulates N-cadherin and Cad11 expression but downregulates R-Cadherin (233). The effects of cell-to-cell junction impedance of differentiating hASC that we found are consistent with previous studies that demonstrated ASC induced down the adipogenic lineage had significantly lowered impedance values than ASC induced down the osteogenic lineage (229). Before the impedance drop phase of the middle age and elderly cells, $a^2$ for the young cells was lower than that of the middle age and elderly cells. This indicates that despite $R_b$ being high for the younger cells, the amount of adherence to the electrode was not as high. This could perhaps be explained by overcrowded younger cells, preventing tight adherence to the electrode surface.

Fluctuations in the collected impedance data were observed throughout this study (Figure 7-S9). Movement of cells on the electrode can cause fluctuations in impedance readings (224, 225). In the current study, throughout the confluent stabilization phase, there was a significant amount of noise associated with the resistance portion of the impedance data. During the impedance drop phase, there was an observed decrease in these fluctuations in the readings. To quantify the fluctuations in the data, we used signal entropy, which measures the amount of information contained within the signal (234). The higher the unpredictability of the signal data points, the higher its entropy value. It has been previously shown that mesenchymal stem cell motion is transiently upregulated in the early stages of osteogenic
differentiation and decreased in late stage osteogenic differentiation (235). This is in agreement with the fluctuations captured in our impedance data and supports our conclusion that the impedance drop corresponds to final differentiation of the hASC. Further, significant differences were observed between the entropy values of the superlots immediately upon osteogenic induction. Elucidation of these differences could be further developed as an early indicator for measuring donor-specific hASC vitality and viability for tissue engineering procedures. Future studies should examine the micromotion of the hASC by employing single electrodes and capturing changes in resistance data within 1Hz intervals and measured in periodic 1hr bursts to better understand the dynamics of hASC motility both during and after differentiation.

In this study, superlots were used instead of individual cell populations in order to determine the overall effects of age on the ability of hASC to proliferate and differentiate down the osteogenic lineage. Superlots allow for an “average” effect of a variable (i.e. age) to be studied while reducing the noise introduced by individual variability unrelated to age (13). However, for this method to be developed into a technology for screening individual cell populations, hASC from individual donors must be tested. Determining how individual hASC populations compare with the pooled results presented here should be studied in future investigations.

In conclusion, this is the first study to use real-time, noninvasive ECIS to elucidate and screen hASC proliferation and osteogenic variability between gender-matched, age-grouped donors. Importantly, different dielectric properties were observed between hASC of different
age groups. This suggests that ECIS may be used to screen hASC isolated from different donors for osteogenic differentiation potential, providing a more complete understanding of the quality of a hASC population than can be achieved by evaluating the population at a single time point. This technology could be incorporated into future bioreactors to track hASC through proliferation and differentiation to assist in quality control during stem cell manufacturing. Through real-time monitoring of the differentiation of different age group hASC superlots, we have found that hASC from older donors differentiate down the osteogenic lineage in a shorter time frame than younger superlots, offering new insight into the known variability between hASC lines. The impedance drop phase preceded the first observed matrix deposition 48 to 96 hours after maximum impedance in all hASC superlots, suggesting that ECIS can detect cell morphology changes that correspond to late-stage osteogenic differentiation. In addition, differences in micromotion could be discerned both between hASC superlots and between early and late stage differentiation, offering yet another method to evaluate and track hASC populations. The findings presented herein could be critical in developing patient-specific bone tissue engineering and regenerative medicine therapies and better translating hASC therapies to clinical applications.
7.5 Supplementary Figures

**Figure 7-S1** Light microscope time course of young hASC cultured in both CGM and ODM. Matrix deposition was first observed at day 14 culture in ODM.

**Figure 7-S2** Light microscope time course of middle-aged hASC cultured in both CGM and ODM. Matrix deposition was first observed at day 8 culture in ODM.
Figure 7-S3 Light microscope time course of elderly hASC cultured in both CGM and ODM. Matrix deposition was first observed at day 4 culture in ODM.

Figure 7-S4 Total DNA per well after 21 days of culture in ODM. DNA was quantified via Hoescht 33258 assay (Life Technologies, Carlsbad, CA). Significantly more DNA was observed in the younger superlot than middle-aged and elderly superlots. Results are in accordance with Alamar Blue data, which shows greatest percent reduction (ie. highest metabolic activity) in young superlot. The elderly superlot yielded low DNA, consistent with low percent reduction in Alamar Blue data (** = p<0.01).
Figure 7-S5 Oil Red O staining for lipid accumulation after 21 days of osteogenic differentiation in A. young B. middle-aged and C. elderly superlots. There was little lipid accumulation in any superlot. However, more lipid accumulation was observed in the young superlot, followed by the middle-aged superlot. The elderly superlot did not exhibit any significant lipid accumulation (scale bars= 50µm).

Figure 7-S6 No significant difference in signal entropy was observed between superlots cultured in CGM for the duration of the study.
Figure 7-S7 A comparison of impedance data at different frequencies shows that similar trends are observed at both high and low frequencies. Data is normalized to starting impedance value of each data set.
Figure 7-S8 Immunostaining of A. young B. middle-age and C. elderly superlots at time of induction (day 0). Stains used were DAPI (blue) (Invitrogen, Carlsbad, CA), phalloidin (green) (Invitrogen, Carlsbad, CA), and Cnx43 (red, arrows) (Abcam, Cambridge, United Kingdom). Cnx43 staining was observed in both young and middle-aged superlots. The elderly superlot did not show Cnx43 staining (scale bars= 50µm).

Figure 7-S9 A representative young superlot impedance data set. Higher signal noise was observed in the data before the impedance drop than after the impedance drop. The signal noise was quantified via signal entropy. “ (222)
CHAPTER 8

Conclusions and recommendations for future work

The results of this research show promising advancements in the development of tissue engineering therapies using hASC, elucidation of underlying mechanisms of musculoskeletal development, and identification of factors that cause donor-to-donor variability between hASC populations. There are numerous opportunities to continue this work in the future, but here we highlight three major avenues of research that we plan to follow within the immediate future due to their great scientific importance.

First, we plan to follow up our *in vitro* osteochondral scaffold study by implanting this scaffold into a large animal model for *in vivo* analysis. Specifically, we will evaluate the repair of osteochondral defects in the knees of skeletally mature Sinclair minipigs. Our study will compare defect repair between the following treatments: 1) open osteochondral lesions, 2) osteochondral plug implants, 3) acellular scaffolds, and 4) porcine ASC seeded scaffolds. We will track the treatments via CT scanning out to 4-months, at which point plugs will be taken to evaluate via histological and biomechanical analyses. This study will allow us to further evaluate the translational potential of our osteochondral scaffold for treatment of osteoarthritis or repair of critical size osteochondral defects.

The next step in our Corin investigations will be to examine the musculoskeletal characteristics of Corin knockout mice. Specifically, we will compare the long bones of 12-week old female wild type and corin KO mice. We will use histology to visualize tissue organization and determine if altered vascular patterning is evident in the absence of Corin.
Mineralization of the bone will be characterized via Alizarin Red staining and calcium liquicolor assays. In addition, mechanical properties will be assessed via a three point bending technique. This research will allow us to better understand how Corin is involved in musculoskeletal biology in vivo.

The next step within our ECIS research will be to use this technology to monitor hASC populations isolated from individual patients. We have used superlots to determine how donor age affects osteogenic potential of hASC, but in order to determine if this is valid on a donor-to-donor basis, individual cell lines must be evaluated. We are currently isolating new hASC populations with extensive demographic information. We will evaluate these individual hASC lines via ECIS and determine how demographic characteristics impact ECIS curve characteristics. In addition, the micromotion index of hASC from individual patients will be evaluated to determine if it could be a potential rapid indicator of hASC proliferative or osteogenic potential. This research will deepen our understanding of hASC donor-to-donor variability so that personalized hASC therapies can be better optimized.

Overall, the current body of research detailed within this dissertation has generated many avenues for future research. Further development of our tissue engineering strategies, elucidation of relevant signaling mechanisms, and characterization of donor-to-donor variability within hASC will help advance the translation of hASC therapies to the clinic for musculoskeletal tissue engineering applications.
REFERENCES


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