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Development of Naturally Derived Extracellular Matrix Materials for Translational Adipose Tissue Engineering

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Publication Date
2013

Peer reviewed|Thesis/dissertation
Development of Naturally Derived Extracellular Matrix Materials for Translational Adipose Tissue Engineering

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Bioengineering

by

Dennis Adam Young

Committee in charge:

Professor Karen L. Christman, Chair
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Professor Anne M. Wallace

2013
The Dissertation of Dennis Adam Young is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2013
DEDICATION

For my incredible family, who have always supported me in everything that I do, even if it’s on the other side of the country.
EPIGRAPH

“…he also went into a pit on a snowy day and killed a lion.”

~ I Chronicles 11:22
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ACKNOWLEDGEMENTS

I would like to acknowledge the many people and organizations that contributed to my development and to the completion of this dissertation. First of all, I would like to thank my advisor, Dr. Karen Christman. She has given me invaluable support and advice over the past five years. I did not come to grad school planning to write a dissertation on adipose tissue, but Dr. Christman allowed me to indulge my curiosity and supported me as I turned a small inkling of an idea into an entire graduate thesis, even though it was outside the typical theme of our lab. She has provided countless opportunities for me to grow as a scientist and provided endless recommendation letters and nominations to make sure I felt recognized for my hard work. I would also like to thank the rest of my thesis committee, for recommendation letters, manuscript revisions, and for just generally setting aside time in their busy schedules to listen to me talk about fat for a while. They have all had a significant impact on my development as a student and scientist.

I would also like to thank all of the support I have received from the current and past members of the Christman lab, who have all served as my family away from home out here in San Diego. Drs. Jennifer Singelyn, Jessica DeQuach, and Aboli Rane have all been there to show me the ropes of grad school and to encourage me when I felt like I could never get anything to work in lab. Dr. Singelyn also played a significant role in my development outside of the lab, encouraging me to run for BEGS president even when I felt like I had no idea what was going on. I gained just as much from those experiences in BEGS as I did in the lab sometimes, and I will be forever grateful to her for encouraging me in that leadership position. I also would not have been able to survive grad school without my classmate, labmate, and conference accomplice, Dr. Sonya
Sonnenberg. She has been there since the beginning, helped keep me sane throughout the roller coaster of grad school, and supported me in more ways than I can describe. I am also extremely thankful for the support of Dr. Greg Grover, Nikhil Rao, Sophia Suarez, Todd Johnson, and Jean Wang for helping me with countless tasks around the lab, providing advice, and for buying me a beer when days got tough. I am also in debt to Rebecca Braden, Pam Schup-Magoffin, and Diane Hu for all of the help they provided with paperwork, animal surgeries, orders, and general lab inquiries over the past 5 years, and always with a smile. I also need to thank the many others in the lab that have contributed to various experiments and helped develop my patience: Dina Ibrahim, Jessica Hsieh, Anthony Monteforte, Shirley Zhang, and Vaibhav Bajaj. Lastly, I would like to thank my family, from immediate family to all of my aunts, uncles, and cousins, for all of the emotional, mental, and prayer support they have provided over the past five years; I would not be where I am today without them.

I am also grateful for the support of many others that helped in collection and analysis of data for this dissertation: Dr. Paul Chasan and the staff of Ranch & Coast Plastic Surgery Clinic; Drs. Todd McAllister and Nicolas L’Heureux and the entire staff at Cytograft Tissue Engineering; Kim McIntyre and the staff of the SCRM Histology Core; Eric O’Connor and Christine Domingo and the staff of the UCSD Stem Cell Core; and Dr. Kent Osborn and Dr. Nissi Varki. I also need to acknowledge the professional support that I received from countless individuals, but most notably Lisa Dieu and Dr. John Watson. Lastly, I owe a deep debt of gratitude to programs that provided financial support for my dissertation, including the NSF Graduate Research Fellowship Program, the NSF/ASEE Engineering Innovation Fellowship Program, and the *Acta Biomaterialia*
Student Award. Funding for these projects was also provided in part by the NIH Director’s New Innovator Award Program, part of the NIH Roadmap for Medical Research, through grant numbers 1-DP2-OD004309-01 and 1-DP2-OD006460-01.


Chapter 3, in part, has been submitted for publication as: D. Adam Young, Yu Suk Choi, Adam J Engler, and Karen L. Christman. Stimulation of adipogenesis of adult adipose-derived stem cells using substrates that mimic the stiffness of adipose tissue. 2013.


Chapter 6, in part, has been submitted for publication as: D. Adam Young, Brian Mailey, Jennifer Baker, Anne M. Wallace, and Karen L. Christman. Adipose tissue engineering and stem cells. 2013.

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ABSTRACT OF THE DISSERTATION

Development of Naturally Derived Extracellular Matrix Materials for Translational Adipose Tissue Engineering

by

Dennis Adam Young

Doctor of Philosophy in Bioengineering
University of California, San Diego, 2013

Professor Karen L. Christman, Chair

Adipose tissue engineering strives to develop novel materials and methods for repairing and regenerating fatty tissue deficits. However, current options available in the clinic for the replacement of lost adipose tissue are limited to small volumes and often are either quickly resorbed by the body or initiate a negative inflammatory response in vivo;
none of them stimulate new fat formation. These limitations highlight the need for innovative biomaterials that not only integrate well with the surrounding tissue, but also encourage natural fat regeneration to restore adipose function. In this dissertation, we will discuss the development of an injectable hydrogel derived from human liposuctioned fat. This unique approach of using lipoaspirate as a starting material produces a scaffold that provides a template for native adipogenesis when injected subcutaneously. Animal testing confirmed this hypothesis, showing the adipose extracellular matrix (ECM) hydrogel not only had comparable biocompatibility to a clinical standard but was also able to facilitate new vasculature and adipose tissue to develop in the injection region. Accordingly, we investigated the mechanisms that direct adipose differentiation of adult stem cells in vitro. We discovered that substrates that closely recapitulate the biochemical and biomechanical characteristics of adipose tissue were able to stimulate adipogenesis of adult stem cells, which likely was a contributing factor in the positive results we saw in vivo with the adipose ECM hydrogel. We next sought to develop a second injectable ECM material for adipose repair that did not require the use of potentially damaging decellularization agents. For this approach, we utilized human fibroblasts to produce particles of ECM that could be suspended in saline and delivered subcutaneously. These particles were also able to stimulate new adipose formation in vivo, further highlighting the ability of ECM-based materials to facilitate adipogenesis. Finally, this dissertation closes with a look toward the future and discusses key areas that need to be addressed within the field, suggesting a set of design parameters for guiding the development of new adipose tissue engineering strategies.
CHAPTER 1:

Introduction to adipose tissue engineering
1.1. Introduction

Traditional research regarding adipose tissue has focused on the obesity epidemic and growing prevalence of diabetes. However, more recently, researchers have begun to recognize the need of certain patients to actually gain or regenerate lost adipose tissue and, therefore, have started to apply the principles of tissue engineering to achieve this goal. In addition to the more common elective cosmetic procedures, there is a population of patients that would benefit from adipose regeneration due to congenital defects, trauma, or surgical resections. This patient population includes third-degree burn victims who have lost subcutaneous fat layers, children with hemifacial lipoatrophy or other congenital defects that cause underdeveloped features, and women undergoing lumpectomies following breast cancer treatment. These patients, among others suffering from congenital and post-traumatic defects, highlight the growing need for biomaterials that can not only replace lost or damaged adipose tissue, but also encourage its natural regeneration and continual integration with surrounding tissue throughout the lifetime of the patient.

1.1.1. Adipose structure and function

Adipose tissue is a loose connective tissue that accounts for roughly 18% of the average human body mass.[1] It serves a variety of functions within the body, including energy metabolism, insulation, cushioning, and secretion of multiple cytokines.[1-3] There are two main types of adipose tissue, each serving different functions. White adipose tissue, or WAT, is the most predominant type, being localized within the subcutaneous space of the skin, around the organs of the trunk (known as visceral fat), or
in the breast and intramuscular regions. WAT serves as an energy storage depot and endocrine organ in the subcutaneous and visceral locations. When localized around an organ, it serves more as a specific nutrient store for highly metabolically active organs like the heart.[4] A second type of adipose tissue, known as brown adipose tissue or BAT, functions much differently from WAT. BAT generates thermal energy from oxidative phosphorylation of nutrients instead of converting it into the energy transport molecule, adenosine triphosphate (ATP).[5] Because of this characteristic, BAT is abundant in infants, which have not yet developed the mechanism for shivering to produce warmth. However, during development BAT is replaced with WAT, and has only recently been identified in small quantities in human adults, most commonly in the neck and surrounding the aorta and vertebrae.[5]

Adipose tissue is a well vascularized and innervated tissue composed of several cell types surrounded by a basement membrane-like extracellular matrix (ECM). A majority of the tissue is composed of mature fat cells, known as adipocytes, which actively store triglycerides in their large lipid vacuoles (Fig 1.1A). The majority of the cytoplasm is displaced by this lipid vacuole, pushing the nucleus to the edge of the cell and giving the tissue a characteristic honeycomb appearance in histology.[6] Other cell types include endothelial cells, fibroblasts, smooth muscle cells, and a population of adult stem cells, which will be discussed in more detail in the following section.[6] These cells release a variety of cytokines and adipokines, including but not limited to leptin, tumor necrosis factor alpha (TNFα), adiponectin, and adipisin.[3] The ECM of adipose tissue accounts for roughly 3% of the mass of the tissue and its composition is similar to that of basement membrane, constituted primarily of collagens, laminin, elastin, and various
proteoglycans such as perlecan and heparan sulfate.[7] The tissue is organized into discrete lobules of adipocytes. Each adipocyte is surrounded by a reinforced basement membrane composed of a thin layer of collagen type IV and a thicker layer of collagens I, III, V and VI, as well as laminin and several glycoproteins.[7] The tissue is further reinforced by long bundles of collagen I fibers, which are aligned with the major arteries and veins of the tissue.[8]

1.1.2. Adipose-derived adult stem cells

Within adipose tissue resides a small population of multipotent progenitor cells known as adipose-derived stem cells or ASCs (Fig 1.1B). These cells are also referred to as adipose-derived regenerative cells (ADRCs), adipose-derived stromal cells (ADSCs), or adipose mesenchymal stem cells (AdMSCs). ASCs were first added to the stem cell repertoire in the early 2000s, when a team of researchers at UCLA established both the multipotent differentiation capacity and clonogenicity of a specific cell group within processed lipoaspirate; two of the required characteristics for a cell to earn the label as a “stem” cell.[9] ASCs behave similarly to the more commonly investigated bone marrow-derived mesenchymal stem cells (MSCs), but do have their own specific cell surface receptor profile and have different doubling times and differentiation efficiencies.[10] ASCs function in the body to repair many tissues following injury and are capable of regenerating the various components of the tissue that surround them. They exhibit multipotent plasticity, possessing ability to differentiate towards several different cell types including osteogenic, chondrogenic, adipogenic (Fig 1.1C), myogenic, and vasculogenic lineages.[11] Some laboratory experiments have also suggested putative
ability for ASCs to adopt characteristics of other complex tissues such as the heart, liver, and brain,[12-14] however the exact mechanisms and whether differentiation into these cell types occurs remain intensely debated. The presence and activity of telomerase within ASCs has been disputed, though, and they do exhibit some degree of cell senescence, drawing questions regarding their progenitor status. However, their multipotency and upregulated gene expression of stem cell markers OCT4 and SOX2 have justified their classification as adult stem cells.[15, 16]

The identification of an endogenous stem cell population within adipose tissue over the last decade has also brought significant attention to the field of adipose tissue engineering. ASCs are the predominant cell type used in cell-based adipose regeneration strategies, with only limited examples of embryonic stem cell use and lessening emphasis on bone marrow-derived adult stem cell use. ASCs are most commonly isolated from human liposuctioned fat (Fig 1.1D) or other discarded sources of adipose tissue resulting from cosmetic or reconstructive surgeries, such as abdominoplasty or breast reduction. This generates a plentiful and readily available source from which to derive ASCs, which are present in higher density and, in the case of liposuctioned fat, are obtained in a much more minimally-invasive manner than bone marrow-derived MSCs.[10] In fact, this ease of harvest has led several companies to begin developing point-of-care devices for the rapid isolation of ASCs at the patient’s bedside. Furthermore, the availability of these cells within the clinic has led many surgeons to already consider utilizing them to treat their patient’s autologously.
Adipose tissue and adipose stem cells. Under the microscope, adipose tissue is easily identified by its signet ring, or honeycomb, appearance when stained with H&E (A). Adipocytes are filled with large lipid vacuoles, which push the nucleus to the side of the cell, thus creating this signet ring effect. Adipose tissue also contains a significant number of adipose-derived adult stem cells (ASCs), seen here under a brightfield scope (B). These are multipotent cells capable of differentiating into a variety of cell types, most notably new adipocytes, which are easily identifiable by the accumulating lipid droplets (C). ASCs can be readily obtained from human liposuctioned fat in clinically relevant numbers (D). Scale bars = 100 µm in B, 50 µm in C and D.

1.1.3. Clinical options for replacing lost adipose tissue

There are several choices in the clinician’s toolbox for treating small volume adipose deficits or augmentations, but few for large volume repair and none offer an ideal solution in either case. Most commonly utilized are the hyaluronic acid-based soft tissue fillers. While these products have seen extensive usage and an extended safety profile in the clinic, they often lose their effectiveness after 6-9 months and require repetitive treatments for a consistent long-term effect. Accordingly, clinicians are seeking innovative solutions for adipose repair that harness the body’s natural repair mechanisms instead of the temporary benefits offered by the non-regenerative filler materials currently on the market. Therefore, the restoration of soft tissue defects with autologous
tissue remains the core challenge for plastic and reconstructive surgeons. Over the past century, advances in the field of tissue engineering and regenerative medicine have generated new options for treating challenging wounds and solving difficult reconstructive problems. These include dermal matrix systems and irradiated biological human and animal tissue scaffolds.[17, 18] A promising advance came with the identification of multipotent ASCs in adult human fat, as described by Zuk et al. in 2001.[19, 20] This finding was followed by a sharp rise in popularity of autologous fat grafting with a 10-fold increase in 2010 compared to 1997.[21] Fat grafting is a clinical procedure in which adipose tissue is removed from one location of the body, typically via liposuction procedures, and placed beneath the skin in another location of the same patient where more volume is desired. The initial success of these procedures has generally been attributed to the presence of endogenous ASCs in the grafted tissue. Fat-filling procedures now rank 9th among the most popular surgical cosmetic procedures.[21]

1.1.4. Autologous fat transfer

Autologous fat transplantation is a minimally invasive technique used for multiple applications in aesthetic and reconstructive surgery.[22-24] Adipose tissue is biocompatible, long lasting, readily available and has a natural appearance. Short recovery times and the office-based nature of injections have further contributed to its popularity. The uses of fat grafting have evolved from that of small volume utility, as in soft tissue fillers, to large volume applications as a primary mode of reconstruction.[23] This introduction of large volume grafting has provided a new option for total facial
rejuvenation or breast augmentation.[25] Today, fat grafting is part of every plastic surgeon’s repertoire and considered an adjunct or primary technique for facial and breast aesthetic and reconstructive surgery.[23, 26-29]

The efficacy of fat grafting is measured by long-term soft tissue retention. Percent of graft survival varies among patients and fat grafting methods.[30] A wide range of retention rates have been reported in the literature from autologous fat grafts, with up to 70% of the initial fat-filling tissue volume being reabsorbed.[31, 32] Ongoing investigations aim for determining optimal methods for harvest, injection and understanding how individual patient factors might affect overall outcomes.[30] Currently, a meticulous technique with an exact attention to volume dispersion is felt to be a key factor for influencing engraftement.[23] Expert recommendations for optimizing engraftment includes concentrating the fat per unit volume of graft delivered per area of native tissue.[23-25, 33] This technique maximizes surface area of grafted fat into host tissue and provides maximum nutritional support to the autologous implant. However, the exact mechanisms mediating fat graft survival and resorption remain unclear. While the positive outcomes of these grafts have been partly attributed to the presence of ASCs, the variability in ASC density between patient grafts likely also contributes to the procedure’s inconsistency.[34] This variability in graft retention, combined with the clinical time required to prepare the tissue for re-injection, argue for a new treatment alternative that still harnesses the benefits of native tissue but offers off-the-shelf availability and more consistent performance.

1.2. Current materials used in adipose tissue engineering
Over the past decade, the development of various biomaterials for adipose tissue engineering has seen exponential growth. Synthetics and biopolymers alike have been investigated for their ability to interact with subcutaneous adipose tissue in animal models or promote adipogenesis of stem cells \textit{in vitro}. While much of this work has been pursued using structured three-dimensional scaffolds, we will focus here solely on injectable materials for adipose engineering (those interested in a more predefined-shape approach are referred to a concise review by Flynn and Woodhouse in 2008 [35]).

Injectable materials for tissue engineering offer the significant advantage of adapting to complex voids and thus allowing greater patient customization. This is especially beneficial for procedures attempting to replace excised malignant tissue. The initial fluid-like behavior of injectable materials allows immediate scalability for a range of defect shapes or sizes and facilitates the fine contouring of facial features. Furthermore, materials for adipose regeneration will generally be placed subcutaneously, just below the surface of the skin. Injectable materials allow for minimally-invasive delivery, which reduces surgical complications of implantation and prevents excessive scarring. For these reasons, our focus was narrowed to developing injectable materials for adipose tissue engineering.

\textbf{1.2.1. Commercially available injectable soft tissue fillers}

Over the past decade, soft tissue filler procedures have increased nearly 200% with over one and a half million procedures in 2010 alone, as reported by the American Society of Plastic Surgeons [36]. Accordingly, the number of soft tissue fillers on the market has also magnified. The most prevalent products feature derivatives of calcium
hydroxylapatite, polylactic acid (PLA), collagen, or hyaluronic acid (HA). Other notable materials include polymethylmethacrylate (PMMA), polyacrylamide, and dextran [37]. PLA and hydroxylapatite both have established some of the longer persistence profiles, boasting subcutaneous durations out to 12 or even 18 months. However, they have also been frequently associated with granuloma formation in certain locations and foreign body reactions [38]. Calcium hydroxylapatite, marketed under the trade name Radiesse, also has the disadvantage of being radiopaque, which would be particularly undesirable in breast reconstruction following cancerous lumpectomy. Collagen-based injectables have recently experienced a precipitous decline in usage over the past few years, despite once dominating the soft tissue filler market. Their reduced usage can be partly attributed to their relatively rapid resorption within 6 months, but is also due to the rise of hyaluronic acid-based fillers. HA is a naturally occurring polysaccharide with an excellent immune profile and minimal allergy concerns [39, 40]. Currently, there are over 10 different derivatives of HA-based fillers that have received approval from the Food and Drug Administration; most commonly used in the clinic today are Juvederm and Restylane. These fillers have a demonstrated longevity of up to 1 year and few adverse side effects [40]. However, despite these positive results, HA remains classified as a temporary soft tissue filler. As with all of these materials, repeated injections are required to maintain the desired outcome. Furthermore, these commercial products do not induce adipose tissue regeneration, serving only as temporary fillers, and thus highlight the need for biomaterials that will encourage sustained adipose formation in the injection region.
1.2.2. Synthetic injectable materials for adipose tissue engineering

Synthetic polymers have become a popular choice for tissue engineers for a variety of applications as they offer stringent control over composition and mechanical properties of the implanted material. As a result, multiple synthetic polymers have seen use in adipose tissue engineering, ranging from polylactic acid (PLA) and polyethylene glycol (PEG) derivatives to polyethylene terephthalate (PET) and polytetrafluorethylene (PTFE) [41-44]. Several of these materials have already been approved by the Food and Drug Administration for various in vivo uses and are able to maintain the shape and volume of the scaffold in vivo, a highly desirable trait for adipose tissue engineering. However, synthetic materials lack any form of innate bioactivity to encourage adipogenesis on their own and many are not compatible with in situ gelation because of potentially toxic byproducts, which places constraints on injectable delivery. These drawbacks necessitate undesirable surgical implantation of the scaffold and often lead to fibrous encapsulation of the material and a foreign body response [45, 46]. As a result, degradable polymers, such as poly(lactic-co-glycolic acid) (PLGA), have gained considerable attention in the field because they could help reduce the immune response by allowing cellular infiltration and a positive resolution to the immune response. An interesting study by Brandl et al described the incorporation of a collagenase-sensitive substrate into the backbone of a PEG hydrogel, allowing it to be enzymatically degraded. When NIH 3T3-L1 preadipocytes were cultured on the degradable hydrogel, the authors noticed significantly higher levels of intracellular triglycerides and a more mature adipocyte phenotype compared to those cultured in non-degradable hydrogel controls.
These results highlight the potential importance of material degradation on adipose regeneration.

Researchers have also harnessed the tunable degradation of synthetic polymers for use in cell and drug delivery to adipose tissue. Choi et al. were able to impart injectable functionality to PLGA by producing 250 µm diameter microspheres that could be delivered subcutaneously [48]. These particles were able to support proliferation and adipogenic differentiation of human ASCs. Furthermore, in vivo studies revealed that the PLGA microspheres were able to encourage significantly more adipose tissue formation after 2 months when combined with adipocyte-differentiated ASCs than when the cells were injected on their own [49]. In addition to being a cell delivery vehicle, PLGA particles have also been investigated for their ability to deliver growth factors and other bioactive molecules that encourage in vivo adipogenesis. Basic fibroblast growth factor (bFGF) has been shown to increase the proliferation and adipogenic potential of ASCs in vitro [50]. When PLGA particles were used to encapsulate and provide sustained release of FGF in vivo, studies saw improved cell survival and neovascularization in the injection region [51]. However, it should be noted that synthetic polyesters that degrade via hydrolysis, such as PLGA, have been shown to produce acidic byproducts during both in vitro and in vivo degradation. While these degradation products do not cause systemic toxicity, they can produce dramatic changes in pH of the local microenvironment if accumulated. Due to bulk hydrolysis of PLGA microspheres, several groups have reported an average pH of 3.5 within degrading microspheres, reaching as low as 1.5 in the center of larger particles (~40 µm diameter) [52, 53]. While diffusion of the by-products typically equilibrates the pH of the local environment over time, this drastic
drop in pH could denature a protein payload or have adverse effects on cells or surrounding tissue. Despite these limitations, injectable synthetic polymers still hold promise for various functions in adipose tissue engineering, playing either a structural role by providing mechanical support to the implant or a supplemental role by delivering cells or bioactive molecules to the injection region.

1.2.3. Injectable biopolymers for adipose tissue engineering

Natural biopolymers have seen widespread use in adipose tissue engineering research and are currently dominating the clinical market for subcutaneous fillers. These materials offer the advantages of bioactivity and cell adhesion, accompanied by a limited immune response for most patients. Collagen was one of the first dermal fillers approved by the Food and Drug Administration and has been extensively researched in tissue engineering as a stiff sponge [54, 55]. However, in an injectable form, collagen has seen rapid resorption and limited vascular formation following subcutaneous injection [56, 57]. Other injectable biopolymers, such as alginate and fibrin, have produced only a slightly more favorable outcome. Marler et al described the development of an RGD-modified version of alginate that was able to maintain 58% of its original volume after 2 months in vivo and up to 88% when fibroblasts were added [58]. While no adipogenesis was reported in these studies, future versions of the RGD-modified alginate that also incorporated ASCs saw some lipid-filled adipocytes, vascular ingrowth, and limited fibrous encapsulation in the injection region [59]. Similar results have been seen with fibrin gels. Injections of fibrin alone were resorbed within 4 weeks in vivo but were able to maintain 50% of their original volume when delivering adipocyte-differentiated ASCs
Interestingly, when a polyglycolic acid (PGA) support structure was implanted subcutaneously to form a protective cage around the ASC-fibrin injection, the implant experienced relatively little volume loss and a significant increase in the number of new mouse- and human-origin adipocytes (Fig 1.2) [62]. While the subcutaneous environment is relatively static compared to the cyclic loads experienced by bone and cartilage tissues, the skin does exert a compressive force on any material placed subcutaneously. Perhaps these PGA cages bear the compressive load of the surrounding dermal tissue and thus protect the biopolymer hydrogel until it can establish adequate integration with the host tissue. These results suggest that maintaining the volume of an injectable scaffold in vivo plays a significant role in facilitating the formation of new adipose tissue. It is likely that this volume retention is highly dependent on the structural integrity of the injected material to withstand the compressive forces of the subcutaneous environment, however, the optimal strength for this interaction has yet to be identified.

Overall, the inadequate structural strength and rapid resorption of the unmodified hydrogel versions of these biopolymers have resulted in their predominant use as delivery vehicles for cells and growth factors. Porous collagen microparticles, known as CultiSphers, have been shown to support the proliferation and adipogenic differentiation of ASCs, and has the potential to be delivered subcutaneously if suspended in a biocompatible liquid [63]. Other biological materials, such as microscale particulate forms of chitosan, have also been shown to support ASC attachment and proliferation and can deliver the cells via subcutaneous injection [64]. Alternatively, gelatin and alginate have been used to encapsulate bFGF for sustained delivery in vivo. Similar to the results seen with degradable PLGA, the sustained-release of bFGF or FGF-1 from gelatin and
alginate beads increased neovascularization and volume retention [65, 66]. The prolonged diffusion of bFGF from fibrin gels, which is speculated to occur over the course of 7 days from a 0.5 mL injection, has also been shown to increase adipogenesis of simultaneously injected ASCs [62].

Hyaluronic acid (HA) is currently the most utilized biopolymer in adipose tissue engineering, seeing usage both in the clinic and research laboratories. While native HA has an abbreviated persistence profile comparable to the previously discussed biopolymers, it does have an available carboxylic acid side chain in its disaccharide backbone that makes it particularly amenable to chemical modification. This allows HA to be uniquely functionalized for a variety of applications by tailoring its degradation, strength, and interaction with other materials. Multiple implantable versions of HA have been created using PEG-diacrylate or benzyl esterification to crosslink the HA into a stiffer scaffold, but these have only produced limited adipogenesis and were not injectable, and therefore will not be discussed here [67-69]. Focusing on injectable versions, several derivatives of hyaluronic acid have been generated via amidation of the carboxylic acid to attach long, linear carbon chains and thereby increase the viscosity of HA gels. Unfortunately, these materials are relatively quickly broken down in vivo by endogenous hyaluronidases [70]. These studies suggested that chemical crosslinking is necessary for HA to function as a subcutaneous filler; a concept supported by the universal occurrence of crosslinking among commercial HA products. Tan et al recently introduced a “double-crosslinked” version of HA capable of in situ crosslinking and gelation. This study combined an amine-functionalized HA with an aldehyde-functionalized HA that spontaneously crosslink via a Schiff-base linkage, while
concurrently using genipin, a biocompatible crosslinker derived from gardenia fruit, to create a second crosslinking action [71]. This allowed for broad tuning of the degradation and elastic modulus of the material, but has yet to be tested for adipogenesis. Alternatively, Chung et al used 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC) to covalently link HA to the surface of PLGA microspheres. When these particles were combined with primary ASCs, cell/microsphere aggregates developed that ranged in size up to 1 mm [72]. Despite some loss in volume, these aggregates did encourage the accumulation of some lipids upon subcutaneous injection and could be a possible cell delivery vehicle for adipose tissue engineering. Non-chemically modified versions of HA gels have also been created by blending HA with either collagen or thrombin and platelet-rich plasma (PRP). These blends showed good biocompatibility and cell attachment, but no adipose tissue formation was reported and both materials suffered from rather quick resorption [73, 74]. While each of these versions of HA has improved the longevity and cellular interaction of the injected material, there remains limited evidence to suggest HA alone is capable of being more than just a temporary filler material.

More recently, synthetic materials have been combined with native biopolymers, such as collagen and hyaluronic acid, to create cell-adhesive, injectable synthetic polymers. These materials take advantage of the adjustable mechanical properties of a synthetic material while also incorporating natural cell binding domains of biopolymers. In 2007, Vashi et al published a study where they blended collagen with Pluronic F127, a non-ionic poloxamer surfactant. The results showed that the Pluronic F127 hydrogel did not inhibit adipogenesis of mesenchymal stem cells grown in adipogenic induction media
Because the length of the co-polymer blocks that comprise Pluronics can be customized to impart a variety of mechanical properties to the material, the addition of cell-adhesive collagen generates a set of ASC-compatible hydrogels that do not interfere with adipogenesis; however, these materials have not yet been investigated for in vivo adipogenesis. A thermoresponsive version of hyaluronic acid was later developed by Tan et al by chemically linking it to poly(N-isopropylacrylamide) (PNIPAAm). This material has a lower critical solution temperature (LCST) of 30 °C and supported ASC growth for roughly one month. It also gelled upon subcutaneous injection, however, there was no reported cellular infiltration or neovascularization [76]. Further expanding on these biohybrids, Hillel et al recently published a report on an in situ crosslinked PEG-HA composite (Fig 1.3). The material blends PEG diacrylate with eosin Y and commercial formulations of HA, and then uses a light emitting diode (LED) to photocrosslink the components after subcutaneous injection [77]. These materials were evaluated in both rodent and human trials and demonstrated biocompatibility and excellent volume retention. However, some chronic inflammation was seen and a thin pseudocapsule had formed around the injections. No neovascularization was noted within the implants and they were also not assessed for adipose formation [77]. Together, these studies indicate a trend towards developing materials that attempt to harness each of the positive attributes of both synthetic and natural polymers to achieve a mechanically stable and biocompatible material for injectable adipose tissue engineering.
Figure 1.2: Fibrin injections for adipose tissue engineering. Cho et al showed improved adipogenesis at 6 weeks when a PGA support structure was implanted subcutaneously to protect fibrin + preadipocyte injections from tissue constriction (Groups 1 & 2). Addition of bFGF to fibrin + preadipocyte injections further enhanced lipid accumulation (Groups 1 & 3). Group 4 also shows the relative inability of fibrin + preadipocyte injections to produce fatty tissue on their own. Scale bar = 200 µm (A). The relative amount of adipose tissue present was quantified using an Oil Red O stain to verify that the support structures significantly increased adipogenesis (B). Reprinted in part with permission from [62].
Figure 1.3: Injection of photoactivated PEG-HA hydrogel. Hillel et al were able to create an *in situ* crosslinking injectable filler by combining hyaluronic acid (HA) with PEG-diacrylate and the photoinitiator, eosin Y. This mixture of components was injected transdermally (A) and could be massaged into a desired shape (B). The material was then crosslinked by shining an array of light emitting diodes (LEDs) emitting light at a wavelength of 520 nm (C). This light was shown to penetrate up to tissue depths of 4 mm, and a 2 minute exposure time was sufficient to activate the eosin Y and photocrosslink the composite implant. Reprinted with permission from [77].

1.2.4. Injectable extracellular matrix based materials

The relative success of autologous fat transfers in the clinic has altered the focus for materials development within adipose tissue engineering, shifting design criteria away from materials that only temporarily fill subcutaneous space, and toward those that potentially encourage the development and ingrowth of new adipose tissue. Despite the
necrosis and resorption issues associated with fat grafting, new adipose tissue has been seen to develop in the injection region [78]. This implies that the extracellular composition of the fat graft may be contributing to the perceived benefit of the procedure, and emphasizes the need to explore injectable materials that more closely mimic the adipose microenvironment. Matrigel is a complex mixture of proteins and growth factors derived from the extracellular matrix of Engelbreth-Holm-Swarm mouse sarcoma cells, which have been considered a basement membrane tumor [79]. Some of the main components of Matrigel, such as laminin and type IV collagen, are also present in adipose tissue [80]. Several studies have demonstrated Matrigel to be highly adipogenic in vivo when injected alongside bFGF in mice (Fig 1.4) [81-83]. These experiments reported extensive neovascularization and adipose development that was specifically derived from host cells rather than exogenously implanted ones. Furthermore, controls consisting of bFGF alone or within a collagen gel did not promote adipogenesis [83]. While Matrigel is not a viable option for clinical usage due to its sarcoma cell origin, these results suggest that materials with complex combinations of extracellular matrix components could be critical to the regeneration of adipose tissue.

Decellularization of tissues has recently emerged as a popular research proclivity in the field of regenerative medicine and offers the possibility of producing a scaffold that closely mimics the physical and chemical cues seen by cells in vivo [84, 85]. Through decellularization, all cells and cellular immunogenic content is removed from an organ in order to isolate the tissue-specific ECM components of that organ as an intact scaffold. Materials produced in this manner often have positive angiogenic and chemoattractant properties [86-89]. While many tissues share similar ECM elements, each tissue has its
own complex composition and concentration of chemical constituents [90], which are known to regulate numerous cell processes including attachment, survival, migration, proliferation, and differentiation [91-95]. Furthermore, our lab has recently shown that decellularized tissues are capable of promoting the maturation of tissue-matched progenitor cells in cardiac and skeletal muscle tissues [96, 97].

![Figure 1.4: bFGF required for matrigel-induced adipogenesis in vivo.](image)

Figure 1.4: bFGF required for matrigel-induced adipogenesis in vivo. Subcutaneous injections of Matrigel and 1 µg/mL bFGF were performed in mice and H&E stained sections were analyzed over a 10-week period by Kawaguchi et al [83]. After 1 week, neovascularization could be seen in the injection region (A), and by 2 weeks fibroblast-like cells had invaded and differentiated into adipocytes (B). At 5 weeks, significant adipocyte formation and maturation was seen in the injection region (C), which persisted through the entire 10-week duration of the study (D). However, when bFGF was not included in the Matrigel injections, limited adipogenesis was seen (E). Copyright 1998, National Academy of Sciences, USA.

1.3. Scope of the dissertation

From these studies, it is becoming apparent that a synergistic relationship exists between angiogenic growth factors and certain extracellular matrix components that promotes de novo development of adipose tissue. In this dissertation we will discuss the
development of a novel tissue engineering scaffold derived from human liposuctioned adipose tissue. This processing provides an injectable hydrogel for adipose regeneration that mimics the native microenvironment of fatty tissue and promotes both neovascularization and adipogenesis in a subcutaneous animal model.

Chapter 1 provides an overview of adipose tissue and the current methods and materials available in the clinic for treating adipose deficits. It also reviews the current scientific advances being made towards developing new materials for adipose tissue engineering.

Chapter 2 describes the creation of a method to decellularize human adipose tissue and the characterization of the resulting adipose extracellular matrix. These experiments also demonstrate the initial cytocompatibility of the adipose ECM hydrogel and its injectable delivery in an animal model.

Chapter 3 investigates the mechanisms that direct adipogenic differentiation of human ASCs. We found that by recreating the biomechanics of adipose tissue in vitro, we were able to stimulate spontaneous adipogenesis through reduced cellular aspect ratio and intracellular tension. To our knowledge, this was the first demonstration of human ASC differentiation down an adipocyte lineage without the use of chemical stimulants.

Chapter 4 investigates the in vivo function of several adipose matrix hydrogels, both with and without embedded ASCs. The results from this study not only highlight the improved biocompatibility of various adipose matrix hydrogels compared to a clinical standard, but also reveal that the adipose matrix hydrogels are capable of stimulating neovascularization and adipose regeneration in vivo.
Chapter 5 outlines the development of a second injectable ECM-derived material for adipose tissue engineering that is particle-based instead of a hydrogel. This production avoids the use of decellularization agents that may disrupt the conformation of the ECM proteins. Indeed, this study shows that the ECM particles are biocompatible and well integrated with the surrounding tissue in vivo, but more importantly, are also capable of stimulating new adipose formation as well.

Chapter 6 summarizes the work of this dissertation and addresses some of the limitations that must be addressed in the field of adipose tissue engineering. Lastly, it suggests a set of design criteria, based on this work and the work of others, to guide the development of future materials for adipose regeneration.

The author of this dissertation is the primary author of this manuscript.
CHAPTER 2:

Development of an injectable hydrogel scaffold
from human lipoaspirate
2.1. Introduction

Researchers have investigated several traditional biomaterials for replacing lost or damaged adipose tissue, however each one faces significant drawbacks, as it was not originally tailored for adipose tissue. As was discussed in Chapter 1, common synthetic polymers have proven insufficient to cause natural regeneration of adipocytes and face some degree of fibrous encapsulation in animal models [46]. Natural biopolymers, such as collagen and hyaluronic acid, have also been molded into gels and cross-linked scaffolds. These materials improve biocompatibility but struggle to resist rapid resorption [54, 67]. Clinical trials of hyaluronic acid scaffolds have shown maintained shape and cellular infiltration, but the implants suffered from limited integration and an absence of mature adipocytes within the material [67].

In addition to an inability to adequately induce adipogenesis, these three dimensional scaffolds also require surgical implantation. To minimize the invasive delivery of materials for adipose regeneration, several natural and synthetic polymers with injectable functionality have been investigated for in vivo adipogenic potential. Recently, collagen and hyaluronic acid have emerged as popular injectable soft tissue fillers and are the major components of several commercially available products. Collagen has a low incidence of allergic reaction but, in an injectable form, can be rapidly resorbed and encourages only limited adipogenesis [37, 98]. Hyaluronic acid has shown improved angiogenesis and adipogenesis; however, it too faces rapid resorption in vivo [70, 74]. Tan et al recently introduced a modified version of hyaluronic acid linked to poly-(N-isopropylacrylamide) that self-assembles at body temperature, but it has yet to be tested for adipogenic potential [76]. Despite the availability of several injectable
materials, there has yet to be identified an engineered material that avoids immune complications and encourages new fat formation. Moreover, no injectable material has been designed to mimic the native adipose extracellular matrix (ECM). Several clinicians have pursued autologous alternatives by using free fat transfer to augment soft tissues [38, 99]. These “lipotransfer” treatments, as discussed previously, inject liposuctioned fat back into a patient through a cannula inserted into the subcutaneous space. Lipotransfer provides a material that contains many of the natural components of adipose tissue and consequently has promoted adequate integration with host tissue. However, the inability to control the composition or mechanics of lipoaspirate results in unpredictable implant contours and resorption.

Decellularization of tissues has recently emerged as a popular topic in the field of regenerative medicine and offers the possibility of producing a scaffold that closely mimics the physical and chemical cues seen by cells in vivo [84, 85]. Materials produced in this manner often have positive angiogenic and chemoattractant properties [86-89]. A couple tissues have been decellularized for use in adipose regeneration studies with promising results, including skeletal muscle and placental tissue [100, 101]. However, these scaffolds do not directly match the composition of the native adipose ECM. While many tissues share similar ECM elements, it is becoming evident that each tissue has its own complex composition and concentration of chemical constituents [90], which are known to regulate numerous cell processes including attachment, survival, migration, proliferation, and differentiation [91-95]. It follows that the use of decellularized adipose tissue would provide the best matrix for adipose regeneration.
Recently, a couple groups have investigated the potential to generate an acellular material from human adipose tissue [102, 103]. While successful in removing a majority of the cellular content, these methods resulted in three-dimensional scaffolds. These products would necessitate surgical implantation and limit customization for varying dimensions in the subcutaneous space. Thus, there exists a need for an acellular, injectable material that will satisfy complex contours while also closely mimicking the complexity of natural adipose ECM. Processing of adipose ECM removed via liposuction could eliminate the necrosis and variability associated with current lipotransfer procedures. In this study, we set out to fabricate an injectable scaffold from decellularized human lipoaspirate that could be both collected and delivered in a minimally–invasive manner. Herein, we assess different decellularization and processing techniques, characterize the biochemical composition and mechanical properties of the resultant scaffold, and demonstrate cell compatibility.

2.2. Methods

2.2.1. Collection of source material and cell isolation

Fresh human lipoaspirate was collected from female patients, ranging from 39-58 years of age with an average age of 43, undergoing elective liposuction surgery under local anesthesia at the La Jolla Plastic & Reconstructive Surgery Clinic (La Jolla, CA) with the approval of the UCSD Institutional Review Board. Adipose-derived mesenchymal stem cells (hASCs) were first isolated from the tissue according to established protocols.[11, 104] Briefly, the tissue was digested in 0.075% collagenase I (Worthington Biochemical Corp., Lakewood, NJ) for 20 minutes and the resulting
suspension was centrifuged at 5000 x g. The hASC-rich pellet was resuspended in 160 mM ammonium chloride to lyse blood cells and again centrifuged at 5000 x g. The remaining cells were filtered and resuspended in Growth Medium (Dulbecco’s modified essential medium/Ham’s F12 (DMEM/F12, Mediatech, Manassas, VA), 10% fetal bovine serum (FBS, Gemini Bio-Products, Sacramento, CA), and 100 U. penicillin/100 µg/mL streptomycin) and cultured overnight on standard tissue culture plastic at 37°C and 5% CO₂. After 24 hours, non-adherent cells were removed with two rinses in 1x phosphate-buffered saline (PBS) and the remaining cells were serially passaged as hASCs. Growth Medium was changed every 3-4 days. When cells reached 80% confluence they were washed with 1x PBS and released from the tissue culture surface using 0.25% Trypsin/2.21 mM EDTA (Mediatech, Manassas, VA). The cells were resuspended, counted, and plated in new flasks with fresh Growth Medium. The lipoaspirate not used for cell isolation was immediately stored at -80°C and kept frozen until further processing.

2.2.2. Decellularization and delipidization of human lipoaspirate

Frozen lipoaspirate was slowly warmed to room temperature and washed in 1x PBS for 2 hours under constant stirring. The PBS was then strained and the washed adipose tissue was placed in either 1% sodium dodecyl sulfate (SDS) in DI water or 2.5 mM sodium deoxycholate in 1x PBS. Both of these detergents have been previously shown to be effective decellularization agents [105-107]. The tissue was stirred in detergent for 48 hours and subsequently thoroughly rinsed with DI water. Each group of decellularized tissue was then placed in 2.5 mM sodium deoxycholate in 1x PBS
supplemented with 500 units of porcine lipase and 500 units of porcine colipase (both from Sigma-Aldrich, St. Louis, MO) to remove remaining lipids. This enzymatic digestion was continued until the tissue became visibly white, approximately 24-48 hours depending on the patient, or for a maximum of 72 hours if there was no change in color. Finally, the tissue was rinsed with DI water for 2 hours to remove excess detergents and frozen at -80°C overnight. Prior to freezing, representative samples were embedded in Tissue Tek OCT compound for histological analysis. Following the decellularization procedure, the frozen “adipose matrix” was then lyophilized and milled using a Wiley Mini Mill.

2.2.3. Evaluation of decellularization and delipidization

To examine the extent of decellularization of the adipose tissue, both fresh and decellularized samples that had been embedded in OCT were sectioned into 20 µm slices and stained with hematoxylin and eosin (H&E) for histological analysis. Decellularization was confirmed by staining slides with Hoechst 33342, a fluorescent nuclear stain. The tissue sections were fixed in acetone, rinsed, and stained in Hoechst dye at 0.1 µg/mL for 10 minutes. The sections were then rinsed, mounted with Fluoromount (Sigma-Aldrich, St. Louis, MO), and imaged with a Carl Zeiss Observer D1. Decellularization was further quantified using a commercially available DNEasy kit (Qiagen, Valencia, CA). Samples of lyophilized adipose matrix were weighed and DNA was extracted according to manufacturer’s specifications. DNA content (µg/mg dry weight ECM) was estimated from absorbance readings at 260 nm using a BioTek Synergy H4 microplate reader (Winooski, VT) and normalized to initial dry weight of the
As a control, lyophilized calf skin collagen (Sigma-Aldrich, St. Louis, MO) was included in the assay.

Lipid removal from the tissue was assessed by staining with Oil Red O dye (Sigma-Aldrich, St. Louis, MO), as previously described [108]. Sections of fresh tissue and decellularized tissue, both before and after lipase treatment, were fixed with 3.2% paraformaldehyde for 1 hour and rinsed in DI water and then 60% isopropanol. Oil Red O stain was prepared at 5 mg/mL in 100% isopropanol and diluted 3:2 with DI water to make a working solution prior to use. Fixed tissue sections were stained in Oil Red O working solution for 15 minutes, rinsed in 60% isopropanol and then DI water, and mounted with 10% glycerol in 1x PBS. Images of the staining were taken using a Carl Zeiss Imager.

2.2.4. Digestion and gelation of decellularized adipose matrix

Dry, milled adipose matrix was further processed using 0.1 M HCl and 3200 I.U. porcine pepsin (Sigma-Aldrich, St. Louis, MO), following a modified version of previously established protocols for different tissues [105, 109]. The pepsin was first solubilized in 0.1 M HCl and added to the adipose matrix at a ratio of 1 mg pepsin for every 10 mg lyophilized ECM. The adipose matrix was digested for 48 hours at room temperature under constant stirring. Subsequently, the pH was raised to 7.4 using 1 M NaOH and the matrix was diluted to 15 mg/mL using 10x PBS so that the final solution contained 1x PBS. This digest was kept on ice until used for characterization assays or gelation studies in vitro or in vivo. To induce gelation in vitro, the digested, neutralized adipose matrix was warmed to 37°C in a humidified incubator with 5% CO₂. In vitro
gels were characterized using an AR-G2 rheometer (TA Instruments, New Castle, DE) with a 20 mm diameter parallel plate configuration. Gels produced from tissue decellularized with SDS and with sodium deoxycholate were tested at 37°C under a constant 2.5% strain at an oscillating angular frequency of 1 rad/s.

2.2.5. Characterization of adipose matrix

Peptide content of the digested adipose matrix was assessed using SDS-PAGE. Samples were run on a NuPAGE® Novex Bis-Tris gel (Invitrogen, Eugene, OR) at 12% w/v in NuPAGE MOPS SDS running buffer (Invitrogen) and compared to rat tail collagen type I (2 mg/mL; BD Biosciences, San Jose, CA). Samples were prepared under reducing conditions with NuPAGE LDS Sample Buffer (Invitrogen) and run in an XCell Surelock MiniCell (Invitrogen) at a constant 200 V. Peptide bands were visualized using Imperial Protein Stain (Pierce, Rockford, IL). Novex® Plus2 Pre-stained Standard (Invitrogen) was used as a protein ladder. Sulfated glycosaminoglycan content of the adipose matrix was quantified using a colorimetric Blyscan assay (Biocolor, Carrickfergus, United Kingdom) according to manufacturer’s instructions. Samples from different batches of adipose matrix were tested in triplicate and absorbance was recorded at 656 nm using a BioTek Synergy H4 microplate reader (Winooski, VT).

Immunofluorescent staining was used to identify specific proteins within the adipose matrix. Sections of both fresh lipoaspirate and adipose matrix were fixed with acetone and blocked with staining buffer (0.3% Triton X-100 and 2% goat serum in PBS). Samples were then stained with primary antibodies against collagen I, collagen III, collagen IV, and laminin (1:100 dilution, Abcam, San Francisco, CA). AlexaFluor 488
(1:200 dilution, Invitrogen) served as a secondary antibody. Both primary and secondary antibodies were individually omitted on control slides to confirm positive staining. Slides were mounted with Fluoromount (Sigma-Aldrich) and images were taken with a Carl Zeiss Observer D1.

Scanning electron microscopy was used to visualize the microstructure of adipose matrix hydrogels. Gels were formed by warming digested adipose matrix to 37°C in a humidified incubator with 5% CO₂ overnight. Gels were immersed in 2.5% gluteraldehyde for 2 hours and then dehydrated in a series of 15-minute ethanol rinses (30-100%) according to previously published protocols [88, 90, 109]. The gels were then critical point dried using CO₂ and coated with chromium using an Emitech K575X sputter coater. Scanning electron microscope images were taken using a Philips XL30 field emission SEM.

2.2.6. In vitro cytocompatibility assessment of adipose matrix

Digested adipose matrix was diluted to 5 mg/mL using 0.1 M acetic acid and added to the bottom of wells of a 48-well tissue culture plate. The plate was kept at 4°C overnight to adsorb the matrix to the tissue culture plastic. Control wells were either left as normal tissue culture plastic or coated with 1 mg/mL calf skin collagen solubilized in 0.1 M acetic acid. The leftover coatings were then aspirated and the wells were washed twice with 1x PBS. Passage 1 hASCs were seeded at 5 x 10⁴ cells/cm² in Growth Medium. Media was changed every 2-3 days. After 1, 7 and 14 days, cells were stained with a fluorescent Live/Dead Viability/Cytotoxicity Kit (Invitrogen, Carlsbad, CA). A solution of 4 µM calcein and 2 µM ethidium homodimer (EthD-1) was prepared in PBS.
The solution was added to the cells and allowed to incubate for 30-45 minutes at room temperature. The cells were subsequently rinsed twice with PBS and then observed under a fluorescent microscope to examine the viability of the cells.

Total DNA content was assessed at each time point as well using the Quant-IT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA) to determine cellular proliferation. Briefly, the cells were rinsed twice in PBS and frozen at -20 °C for up to 1 week to aid cell lysis. Cellular DNA was then resuspended in 1x TE Buffer and incubated with a fluorescent PicoGreen Reagent for 30 minutes. Fluorescence was measured using a BioTek microplate reader with an excitation wavelength of 480 nm and emission wavelength of 520 nm. dsDNA was quantified by relating the sample absorbance to the absorbance measured for standards of known DNA concentration.

hASC morphology was visualized at each timepoint. Cells were washed with 1x PBS and fixed in 4% paraformaldehyde for 15 minutes. The cells were washed again and staining buffer (0.3% Triton X-100 and 1% bovine serum albumin in PBS) was added for 30 minutes to block non-specific binding. Cells were then incubated in AlexaFluor 488 Phalloidin (Invitrogen; 1:40 dilution in staining buffer) for 20 minutes to label F-actin and Hoechst 33342 (1 µg/mL in water) for 10 minutes to label nuclei. Images of the cells were taken using a Zeiss Observer D1.

2.2.7. Subcutaneous injection and gelation of solubilized adipose matrix

All animal procedures were performed in accordance with the guidelines established by the Committee on Animal Research at the University of California, San Diego and the American Association for Accreditation of Laboratory Animal Care. Male
athymic mice (nu/nu) received an overdose of sodium pentobarbital and kept on heating pads. Digested and neutralized adipose matrix was drawn into a syringe using a 25 G needle. Six injections (100 µL each) were made subcutaneously into the dorsal region of the mouse. After 15 minutes, the injected material was excised and fresh frozen in TissueTek OCT compound. This tissue was then sectioned into 20 µm slices, stained with H&E for histological analysis, and examined using a Carl Zeiss Imager A1.

2.2.8. Statistical analysis

All data is presented as the mean ± standard deviation. Both the Blyscan and DNEasy assays were performed in triplicate and the results averaged. Significance was assessed using one-way analysis of variance (ANOVA) and post hoc analysis using either Dunnett’s test or Tukey’s test.

2.3. Results

2.3.1. Isolation of adipose ECM from human lipoaspirate

Fresh-frozen lipoaspirate was decellularized and delipidized within 4 days using a combined detergent and enzymatic digestion protocol. These methods were successfully repeated on samples from multiple patients, with the only variability arising in lipase digestion time (24-48 hours) due to initial lipid content. Average yield was 625 ± 96 mg of dry adipose ECM per 100 cc of lipoaspirate (n=8). We compared the use of either SDS or sodium deoxycholate, for decellularization, in combination with lipase and colipase for deplidization. Decellularization was confirmed by absence of nuclei with
H&E and Hoechst 33342 in both the SDS and sodium deoxycholate groups (Fig 2.1).

While histological analysis demonstrated similar removal of cellular contents, a DNEasy kit revealed that SDS was more efficient in decellularizing the adipose ECM (Fig 2.2), with significantly less DNA per mg of lyophilized ECM compared to the sodium deoxycholate group, and more closely approaching the collagen control.

After decellularization, removal of lipids was achieved through the addition of lipase and colipase for 24-48 hours, producing a white ECM compared to the characteristic yellow tint of adipose tissue. As seen in Figure 1, Oil Red O staining of tissue sections revealed substantial levels of oils within fresh tissue, however treatment with lipase effectively removed lipids within the decellularized ECM, as evidenced by an absence of red staining. Decellularized tissue that was not treated with lipase only slightly reduced lipid levels compared to fresh lipoaspirate, even after 1 week of processing.
Figure 2.1: Production of decellularized and delipidized lipoaspirate. Human lipoaspirate was processed to remove both cellular and lipid content. Raw lipoaspirate (A,D,G,J) was decellularized for 48 hours in SDS or sodium deoxycholate to produce a lipid filled, acellular matrix (B,E,H,K). Removal of lipids using lipase produced a white ECM, free of cellular and lipid content (C,F,I,L). H&E staining (D,E,F) and Hoechst staining (J,K,L) confirmed the absence of nuclei after processing. Oil red O staining (G,H,I) confirmed the removal of lipids. Scale bars = 100 µm.
Figure 2.2: Quantification of remaining DNA. A DNeasy assay quantified the remaining nuclear content after decellularization and delipidization of the lipoaspirate. Treating the tissue with SDS resulted in significantly lower levels of DNA compared to sodium deoxycholate. * p < 0.0001.

2.3.2. *In vitro* characterization and gelation of adipose matrix

Following decellularization and delipidization, the isolated adipose ECM was lyophilized, milled into a fine powder (Fig 2.3A), and then solubilized with pepsin to generate a liquid injectable form of adipose matrix (Fig 2.3B). The presence of lipids in the matrix prevented complete lyophilization and efficient solubilization. Groups that did not employ lipase and colipase during the decellularization process remained oily after lyophilization and could not be milled nor fully digested, resulting in a highly particulate suspension that could not be pushed through a 25 G needle. These groups also exhibited inconsistent gelation *in vitro* and *in vivo* (Fig 2.3C). However, groups that were delipidized produced a dry matrix following lyophilization that could be easily milled into a fine powder. SDS-PAGE analysis of digested adipose matrix revealed multiple peptides and low molecular weight peptide fragments. Peptide bands characteristic of collagen were present within the digest, in addition to multiple peptides below 39 kDa (Fig 2.4). Specifically, collagens I, III, and IV were all present in immunofluorescent stains of adipose tissue both before and after processing (Fig 2.5). Collagens I and III
were more prevalent, however this could be the result of cross-reactivity of the antibody between isoforms. Laminin was also expressed at both time points, however to a lesser extent after decellularization (Fig 2.5). Control slides showed negligible background staining when primary or secondary antibodies were omitted. Glycosaminoglycan analysis estimated an average of $2.18 \pm 0.32$ µg of sulfated GAG per mg dry adipose ECM, with no significant difference between tissue decellularized with SDS versus sodium deoxycholate.

Upon adjusting the pH and temperature of the liquid adipose matrix to physiologic conditions (pH 7.4, 37°C), the solution self-assembled into a gel (Fig 2.3C). SEM analysis revealed the gels were nanofibrous scaffolds with an average fiber diameter of 100 nm and interconnecting pores (Fig 2.6). Storage moduli were determined at 1 rad/s and ranged from 5-9 Pa for tissue processed with SDS and from 7-18 Pa for tissue processed with sodium deoxycholate.

![Image](image_url)

**Figure 2.3: Digestion and gelation of adipose matrix.** Decellularized and delipidized lipoaspirate produced a dry, white powder (A) that was reduced to liquid form using pepsin and HCl (B). This liquid adipose matrix could be induced to self-assemble (C) when placed under physiologic conditions (37°C and 5% CO$_2$).
Figure 2.4: SDS-PAGE analysis of peptide content. As compared to a collagen control (C), gel electrophoresis revealed collagen as well as multiple lower molecular weight peptides present within adipose matrix that had been decellularized using SDS (A) or sodium deoxycholate (B). Protein ladder was run in lane D with peptide weights listed in kDa.
Figure 2.5: Immunofluorescent staining of adipose matrix. Fluorescent antibody staining of both fresh human lipoaspirate and adipose matrix decellularized with SDS showed retention of collagens I, III, and IV. Laminin was also present in both cases, but there was some loss of content as a result of the decellularization. Scale bar = 100 µm.
2.3.3. Adipose matrix coatings support hASC culture in vitro

To investigate the ability of the adipose matrix to support cell adhesion and survival, patient-matched hASCs were cultured either on adipose matrix-coated tissue culture plates or collagen coated plates, and maintained in growth media. On adipose matrix-coated plates, hASCs readily adhered to the surface, displaying a healthy, fibroblast-like phenotype within 24 hours (Fig 2.7) [9, 110]. Live/Dead staining revealed negligible cell death on the adipose ECM after 14 days (Fig 2.7A-C). This level of viability was consistent regardless of the surface coating (Fig 2.7D). Furthermore, DNA quantification indicated that cellular growth was not hindered by the adipose ECM (Fig 2.7E). hASC proliferation continued for 2 weeks on the adipose ECM and was not significantly different from normal proliferation on uncoated or collagen coated surfaces.
Figure 2.7: *In vitro* culture of hASCs on 2D adipose matrix. Live/Dead analysis after 14 days in culture revealed negligible cell death of hASCs (Green = alive, red = dead) seeded on normal tissue culture plastic (A), calf skin collagen (B), or decellularized adipose matrix (C). Cells growing on the adipose matrix also exhibited a healthy fibroblast-like phenotype (D, Green = F-actin, blue = nuclei). PicoGreen analysis at various timepoints indicates that the adipose ECM promoted normal proliferation over 2 weeks in culture (E). Each group increased significantly between time points but no significant difference was found between groups at each timepoint. * p<0.0001 for Day 7 values for each group compared to Day 1 values. † p<0.0001 for Day 14 values for each group compared to Day 7 values. Scale bars = 100 µm.

### 2.3.4. Gelation of adipose matrix *in vivo*

Liquid adipose matrix was injected subcutaneously in mice to investigate *in vivo* self-assembly (Fig 2.8A). The adipose matrix liquid formed a compact, white bolus when injected subcutaneously using a 25G needle (Fig 2.8B). Within 15 minutes, the bolus had solidified into gel that maintained its shape when excised (Fig 2.8C). Immediately following injection, the bolus could be pinched or molded to create elongated structures prior to gelation. H&E analysis of excised tissue showed an acellular, porous matrix in close contact with subcutaneous adipose tissue (Fig 2.8D).
Figure 2.8: *In vivo* gelation of adipose matrix hydrogels. Liquid-form adipose matrix was injected subcutaneously into nude mice using a 25G needle (A). The liquid ECM formed a solid bolus beneath the skin within 15 minutes (B). Gels held their shape when excised (C) and were analyzed with H&E (D). This staining showed an acellular matrix (m) in close contact with native fat (f). Scale bar = 50 µm.

2.4. Discussion

While several three dimensional scaffolds have been proposed for adipose tissue regeneration, injectable fillers offer unique characteristics that are specifically advantageous for application in adipose tissue. Because adipose regeneration is typically associated with enhancement or contouring of natural features to improve aesthetics, the minimally-invasive delivery of an injectable material is desirable to reduce scarring at the surgical site. Furthermore, the collection of source material from liposuction, as opposed to surgical excision of whole fat pads, compliments this minimally-invasive approach by limiting donor site damage. Injectable materials also allow for contouring of complex features within the face, a common area of desired adipose regeneration. Solid scaffolds cannot offer this level of customization. Consequently, the ideal scaffold for adipose tissue engineering would allow for injectable delivery, match the chemical complexity of
the native microenvironment, and promote natural regeneration of the tissue as it is resorbed.

We present here the production of decellularized and delipidized adipose ECM from human lipoaspirate using a combined detergent and enzymatic method. Our results indicate that decellularized lipoaspirate retains a complex composition of proteins, peptides, and glycosaminoglycans (GAGs). Immunofluorescent staining indicated the preservation of multiple collagen isoforms, a major component of native adipose ECM. Despite a slight reduction in content compared to native tissue, laminin was also expressed within the decellularized adipose ECM. Adipose ECM has been previously reported to contain many of the components of basement membrane, including collagens I, IV, and VI, laminin, and fibronectin [111, 112]. Excessive oils within the lipoaspirate prevented accurate calculation of the GAG content of native adipose tissue using a Blyscan assay. However, there are reports of multiple GAGs and proteoglycans present in the secretome of mouse 3T3-L1 adipocytes, such as perlecan, mimecan, and decorin [111, 113, 114]. We likewise found native GAGs retained within the material. Currently, a wide range of values have been reported in literature for GAGs retained within solubilized versions of decellularized tissues. Singelyn et al reported 23.2 ± 4.63 µg GAG per mg solubilized myocardial ECM, but Stern et al were unable to detect any GAGs within their solubilized skeletal muscle ECM [105, 115]. Clearly there exists extensive variability in ECM composition among tissue types and decellularization protocols. While this decellularization protocol likely causes a reduction in protein and GAG concentration compared to native tissue, this assortment of native biochemical cues mimics the microenvironment of adipose tissue, unlike existing soft-tissue fillers, and
could provide adipose specific cues for cell migration, survival, and differentiation. Sulfated GAGs are recognized for their ability to sequester growth factors and subsequently present them to cells [116-118], and thus their presence within the matrix provides a possible avenue for bioactive molecule delivery both in vitro and in vivo. In addition, PAGE analysis of the injectable adipose matrix confirmed the presence of peptides with a molecular weight at 16 kDa and below, which have been previously shown with other decellularized matrices to have chemoattractant potential [86].

We investigated SDS and sodium deoxycholate to decellularize the lipoaspirate as they have previously been shown to effectively decellularize multiple tissues [84]. When applied to fresh tissue, these ionic detergents disrupt the cell and nuclear membranes and entrap the freed nuclear contents into micelles, which are then washed away [84, 119]. Through gross and histological observation, it appeared that both SDS and sodium deoxycholate adequately removed all cellular debris. However, by quantifying the extent of decellularization with DNEasy, SDS proved to have a significantly lower amount of contaminating DNA. There remains debate over what level of DNA qualifies the material as “decellularized.” Gilbert et al suggest that there may exist a threshold DNA concentration below which no immune response will be triggered [120]. It is possible that the detergents also degrade the structure of DNA and other nuclear proteins to an extent that they are no longer recognized as foreign antigens. In fact, many commercially available acellular matrices have been found to contain some degree of cellular contaminants despite their successful use in clinical treatment [120]. Apart from decellularization efficiency, the two detergents appeared to perform at a similar degree. They both produced similar gel electrophoresis bands and GAG content, indicating that
neither detergent had a more pronounced deleterious effect on the ECM. Both methods also produced gels that showed a similar range of storage moduli, which align with previously published reports for the modulus of self-assembling collagen gels [121, 122].

Adipose tissue was, unsurprisingly, adept at trapping lipids within its ECM, resulting in multiple complications during processing into an injectable scaffold. While detergents could sufficiently eliminate free lipids surrounding the tissue, a large proportion of oily residue remained trapped on and within the adipose matrix. These sequestered lipids inhibited consistent lyophilization, milling, and solubilization of the adipose matrix. To eliminate lipids from the decellularized adipose matrix, we have produced a method inspired by the body’s natural lipid metabolism mechanism [123]. Lipase is a naturally occurring esterase produced in the pancreas to digest dietary fats within the small intestine. It specifically targets the ester bond of triglycerides, separating the compound into glycerol and fatty acids, which are readily emulsified by bile salts, such as sodium deoxycholate [124]. Lipase is also actively involved in the breakdown of triglycerides from adipose stores for energy homeostasis [125]. SDS has, however, been shown to cooperatively bind with lipase and irreversibly inhibit its activity [126]. This finding was confirmed in our research and necessitated that sodium deoxycholate be used during lipase digestion, regardless of the initial decellularization detergent (data not shown). Additionally, Labourdenne et al demonstrated that bile salts can partially inhibit lipase activity, but this inhibition can be overcome by the addition of colipase [127]. They reported that colipase increased lipase activity by 10-15 fold. In our investigation, we found that exposing the adipose matrix to lipase in excess of 72 hours resulted in significant protein degradation and an inability to self-assemble
following solubilization (data not shown). For this reason, we incorporated colipase to keep enzymatic digestion times to a minimum.

Detergent-based decellularization methods have received criticism for their potential to degrade the extracellular matrix during processing. To avoid the use of detergents, several groups have investigated the direct injection of lipoaspirate via “lipotransfer” operations or the injection of homogenized lipoaspirate emulsifications [34, 38, 99, 128, 129]. However, none of these studies attempted to remove cells or lipids from the injected material. While autologous lipid injection should not initiate a foreign antigen response initially, apoptotic cells within the implant could serve as nucleation sites for calcification [130]. Implant calcification has also been associated with the presence of cell membrane phospholipids [131]. Additionally, emulsions of lipids or cellular contents would create heterogeneity within an injectable material, yielding unpredictable material behavior in vivo and limited contouring capability. The sequelae of cellular and lipid remnants in an injected soft tissue filler argue in favor of decellularization despite the possible degradation of proteins. Our results indicate that decellularized adipose matrix retains much of the protein complexity of native tissue alongside the complete removal of lipids from the material. This removal of both cellular and lipid content reduces concerns surrounding implant immune rejection and calcification.

We have demonstrated here that human lipoaspirate can be effectively decellularized and subsequently solubilized to produce a self-assembling subcutaneous filler. While not every component of native adipose ECM was fully retained, this adipose matrix is comprised of a complex arrangement of natural proteins and polysaccharides
that more closely mimics the *in vivo* microenvironment than currently approved fillers such as collagen and hyaluronic acid. Furthermore, this material could be used as a delivery vehicle for incorporating adipose derived stem cells in a regenerative treatment. It has been postulated that the success of lipotransfer treatments can be attributed to the presence of a small population of resident hASCs within the injected material [99]. Using solubilized adipose matrix as a delivery vehicle, these cells could be delivered in a concentrated and more consistent manner.

Patient-matched hASCs readily proliferated on 2D adipose matrix coatings and showed positive viability. These systems could allow for the investigation of the influence of multiple physical and biochemical parameters on hASC differentiation. Several groups have reported control over adipogenesis using various chemical additives and paracrine signals [132-134]. However, there has been growing literature indicating that the surrounding microenvironment has a significant impact on stem cell fate as well. Here we demonstrate proof-of-concept for generating a scaffold derived from human lipoaspirate. However, the material has not yet been tested in long-term *in vivo* studies. Analysis of degradation, immune response, and *in vivo* function must all be addressed to fully characterize the benefit of this novel biomaterial. Furthermore differentiation studies using hASCs are required to examine the adipogenic potential of this adipose matrix. These experiments are essential to assess whether the adipose matrix is as an efficient scaffold for adipose tissue engineering, providing more utility than currently available soft tissue fillers. Decellularized adipose matrix could provide the biochemical cues seen by hASCs *in vivo*, yet allow the specific control over extraneous conditions offered by an *in vitro* setting. Thus, this material has the potential to be both an
injectable scaffold for adipose tissue engineering, and a platform for discovering the controlling mechanisms behind adipogenesis.

The author of this dissertation is the primary author of this manuscript.
CHAPTER 3:
Examining the *in vitro* mechanisms behind stem cell adipogenesis
3.1. Introduction

As has been shown in Chapters 1 and 2, materials currently available for adipose replacement do not mimic native adipose tissue and thus offer few cues for natural regeneration. Accordingly, many of these materials are either rapidly resorbed or encapsulated in fibrous tissue and fail to integrate with the surrounding tissue [37, 135]. Thus there remains a significant clinical need to develop materials for the treatment of burns or tumor resection that positively interact with the surrounding tissue to not only fill the void left by the damaged tissue, but also facilitate the natural remodeling and regeneration of adipose tissue. Clinicians have begun to address this issue by offering a “lipotransfer” procedures, or the direct implantation of liposuctioned adipose tissue back into the subcutaneous space of a different area of the body, as discussed previously [38, 99]. The variable outcomes of these procedures have led the field to begin mixing lipotransfer material with autologous, adipose-derived adult stem cells (ASCs). These multipotent cells have been shown to possess the ability to differentiate down multiple lineages, including adipogenic, chondrogenic, osteogenic, and myogenic pathways [9]. The success of ASCs within a lipotransfer graft has been partly attributed to their ability to both encourage neovascularization and also produce new adipocytes within the implant [34]. However, adipogenic differentiation of ASCs in vitro has occurred predominantly via soluble factors within the culture media, which typically include insulin, dexamethasone and various other steroids [11]. Chemically-based differentiation protocols provide a reductionist approach to pinpoint the effect of specific induction pathways, but they likely stimulate few of the pathways utilized in vivo when ASCs differentiate. On the other hand, lipotransfer procedures with ASCs are relatively
uncontrolled and outcomes can greatly vary between clinicians. Identification of materials, which could encourage ASC maturation prior to implantation to produce a more uniform population of ‘primed’ cells or which could be implanted with ASCs to guide their maturation, would improve retention and standardize outcomes of adipose defect treatments.

Because of the inability of current materials to offer encouragement for natural fat regeneration, many investigators have begun to turn to natural alternatives that better recapitulate the extracellular cues present within adipose tissue. The process of decellularization offers the possibility of isolating tissue specific extracellular matrix (ECM) components by removing the cellular content from whole organs or tissues. This produces a material composed of various proteins and proteoglycans characteristic of the tissue of interest, but devoid of the immunogenic cellular components [85]. These ECM-based materials have already been seen to stimulate stem cell differentiation for a variety of organs and facilitate constructive remodeling in vivo [87, 96, 97, 105, 136-138]. Recently, several groups have published methods for decellularizing human adipose tissue. Adipose ECM can be prepared in a variety of forms, from solid scaffolds to injectable gels and powders, and encourages adipogenic differentiation of ASCs [103, 129, 139-142]. While these materials mimic the ECM composition of adipose tissue, ECM mechanical cues are also a major contributor to stem cell differentiation. Biomechanical cues can stimulate mesenchymal stem cell differentiation toward a variety of cell lineages by mimicking the stiffness of the desired tissue [143, 144]. For example, a substrate that mimics the stiffness of healthy muscle or bone encourages ASCs to express myogenic or osteogenic transcription factors, respectively, in the absence of
specific exogenous growth factors [144]. ECM stiffness can even regulate differentiation in the presence of a mixture of soluble factors for osteogenic and adipogenic differentiation; softer substrates encourage bone marrow-derived mesenchymal stem cells to favor adipogenesis despite the presence of osteogenic inductive factors while stiffer substrates favor osteogenesis despite the presence of adipogenic factors [145].

These data suggest the important regulatory potential for matrix-based cues, which could be used in vitro to differentiate ASCs prior to their introduction with other tissue-engineered products in a reconstructive therapy. Thus our goal for this study was to recreate the adipose microenvironment in vitro by combining both biochemical and biomechanical adipose-specific cues to stimulate adipogenesis of ASCs. By recapitulating both the stiffness and ECM composition of adipose tissue, we can present natural stimuli to the ASCs to encourage adipogenic differentiation. Furthermore, identification of an “adipogenic stiffness” could provide an important design criteria for the future development of materials meant to encourage adipogenesis of ASCs.

3.2. Methods

3.2.1. Isolation of adipose stem cells

Human adipose tissue was collected from patients undergoing elective liposuction surgery at the Ranch & Coast Plastic Surgery Clinic (Del Mar, CA). All procedures involving tissue from human patients were reviewed and approved by the UCSD Institutional Review Board. Tissue was collected from female patients ranging in age from 34-52, with an average age of 42. Human adipose-derived adult stem cells (ASCs) were isolated from the lipoaspirate following a previously established protocol [104].
Briefly, the lipoaspirate was digested for 20 minutes in 0.075% collagenase I (Worthington Biochemical) at 37°C. The resulting suspension was centrifuged at 5000 x g to obtain an ASC-rich pellet. The cell pellet was resuspended in 160 mM ammonium chloride buffer to lyse red blood cells and again centrifuged at 5000 x g. The new cell pellet was resuspended in growth media (DMEM/F12 plus 10% Fetal Bovine Serum, 100 I.U. penicillin, and 100 µg/mL streptomycin) and passed through a 40 µm cell strainer. The remaining cells were plated on standard tissue culture plastic overnight at 37°C and 5% CO₂. After 24 hours, the non-adherent cells were removed with two rinses with 1x PBS, and then serially passaged at 70% confluence. Growth media was changed every 3-4 days. The remaining lipoaspirate was frozen at -80°C until further processing.

### 3.2.2. Adipose decellularization

Human liposuctioned adipose tissue was decellularized following our previously published protocol to isolate adipose extracellular matrix [139]. Briefly, the tissue was rinsed in 1x phosphate-buffered saline (PBS) to remove blood and free lipids. The cellular content of the tissue was then removed by rinsing with 1% sodium dodecyl sulfate (SDS). Residual SDS was removed by brief washes in DI water and then 0.01% Triton X-100. The tissue was then delipidized using a solution of 2.5 mM sodium deoxycholate with a 1:1 ratio of lipase to colipase enzymes (all chemicals were obtained from Sigma-Aldrich unless otherwise noted). The remaining white tissue was then rinsed overnight in DI water followed by a brief rinse with isopropanol. The resulting decellularized adipose ECM was then milled using a Wiley mini-mill to produce a fine powder. To facilitate use in cell culture, the adipose ECM was reduced to a liquid form.
by digesting in a solution of 0.1 M HCl and 1 mg/mL pepsin as previously described [139]. After digestion, the liquid adipose matrix was brought to physiological pH with sodium hydroxide on ice, then diluted to 100 µg/mL in 50 mM HEPES buffer at pH 8.5.

3.2.3. Fabrication of polyacrylamide gels

Polyacrylamide gels were produced using a constant 8% acrylamide solution. Glass coverslips were first activated by treating them for 10 minutes in a UV/Ozone ProCleaner (Bioforce Nanosciences) and then functionalized with 3-(Trimethoxysilyl)propyl methacrylate (20.3 mM in ethanol, 3 min). Coverslips were briefly rinsed with 100% ethanol and water and then dried. Solutions of 8% acrylamide and varying percentages of bis-acrylamide (Fisher BioReagents) were diluted in 1x PBS and the reaction was catalyzed by adding 10% ammonium persulfate and 1/1000 volume of N,N,N’,N’-tetramethylethylenediamine. This solution was then sandwiched between the methacrylate-functionalized coverslip and a DCDMS-coated glass slide. After reacting for 30 minutes, the polyacrylamide gels were rinsed three times with 1x PBS and allowed to hydrate in PBS overnight. Gels were then sterilized in a tissue culture hood by exposure to 254 nm, 30 W UV light for 4 hours. For protein attachment, the surface of the polyacrylamide gel was reacted with the photoactivated, bi-functional crosslinker Sulfosuccinimidyl 6-(4’-azido-2’-nitrophenylamino)hexanoate (sulfo-SANPAH. Pierce, 0.2 mg/mL in 50 mM HEPES pH 8.5) under 365 nm UV light with a surface intensity of 0.85 mW/cm² for 10 minutes. Solutions of 100 µg/mL adipose matrix, in 50 mM HEPES buffer at pH 8.5, were added on top of the gels and allowed to attach for 1 hour at 37°C. To confirm protein attachment, adipose matrix was first labeled with sulfo-NHS-
functionalized biotin (Pierce). After attaching the adipose matrix, the polyacrylamide gels were then reacted with neutravidin-horseradish peroxidase complex and visualized with 3,3’-Diaminobenzidine (DAB) substrate kit (Pierce).

3.2.4. Atomic force microscopy

Polyacrylamide gel stiffness was confirmed by atomic force microscopy (AFM; MFP3D, Asylum Research) as detailed previously [146]. Briefly, a pyramidal probe, 0.08 N/m force constant with a 35° half angle (PNP-TR-SPL, Nanoworld), was used to indent each gel in triplicate over 5 random regions of the gel to assess heterogeneity. Probe indentation velocity was fixed at 2 µm/s with the trigger force of 2 nN. Elastic moduli were determined by the Sneddon cone model with a sample Poisson ratio of 0.5 fit over a range of 10%-90% indentation force. Custom software written in Igor pro 6.22 was applied to analyze elastic modulus via Sneddon’s model [147]. Four polyacrylamide gels of each bis-acrylamide percentage were measured to determine the average elastic modulus for that group.

3.2.5. Cell culture

For media differentiation studies, the liquid adipose matrix was added to tissue culture polystyrene wells at a concentration of 100 µg/mL and incubated at 37°C for 1 hour to allow protein adsorption, as previously described [139]. Human ASCs were then seeded in the functionalized wells in growth media at a density of 1 x 10⁴ cells/mL and allowed to attach overnight. The next day, the media was aspirated and either fresh
growth media was added, or adipogenic media (AM: DMEM/F12 plus 10% FBS, 500 µM isobutylmethyl xanthine, 1 µM dexamethasone, 10 µg/mL insulin, 200 µM indomethacin, 100 I.U. penicillin, and 100 µg/mL streptomycin). Each respective media was then changed every 2 days until completion of the study. For stiffness differentiation studies, human ASCs were seeded in growth media on top of adipose matrix-functionalized polyacrylamide gels in a 24-well plate at a density of 1 x 10^4 cells per well and allowed to attach overnight. The next day the media was aspirated and either fresh growth media was added, or growth media supplemented with 0.25 µg/mL cytochalasin-D. Each respective media was then changed every day until completion of the study. All ASCs used in this study were between passages 1 and 3, and cells from at least 3 different patients were used to repeat each study. Brightfield images of the cells were taken every 2 days using a Nikon Eclipse TS100 microscope fitted with an Infinity 2 camera.

3.2.6. Staining

Oil Red O staining was used to visualize adipogenic differentiation. Cells were rinsed twice with 1x PBS and then fixed for 1 hour in 4% paraformaldehyde. Oil Red O was dissolved at 5 mg/mL in isopropanol and then mixed 3:2 with water to create an Oil Red O working solution. Cells were stained for 1 hour in Oil Red O working solution, and then rinsed once with 60% isopropanol and multiple washes with water. Cells cultured on tissue culture plastic were also counterstained with hematoxylin to improve visualization. Images of the cells were taken using a Nikon Eclipse TS100 microscope fitted with an Infinity 2 camera.
Cytoskeletal organization within the cells was identified using a fluorescent stain for F-actin. Cells were fixed in 4% paraformaldehyde for 10 minutes, then incubated in staining buffer (1% bovine serum albumin and 0.3% Triton X-100 in PBS) for 30 minutes to block non-specific binding. AlexaFluor 488 phalloidin was diluted 1:40 in staining buffer and added to the cells for 20 minutes. Nuclei were stained with Hoechst 33342 for 10 minutes. After rinsing, the cells were imaged on a Carl Zeiss Observer D1.

3.2.7. Image analysis

Brightfield images of the cells were taken every 2 days using a Nikon Eclipse TS100 microscope fitted with an Infinity 2 camera. Multiple images were taken in random locations within each well from 4 to 6 wells of each experimental condition, for a total of 12-18 images. All cells that completely fit within the field of view were analyzed. AxioVision software was used to quantitatively analyze the cells. For cell area measurements, a perimeter was drawn around the edges of each cell and the area calculated by AxioVision software. For aspect ratio calculations, the longest pair of two perpendicular lines that crossed at the nucleus were drawn. The aspect ratio was then calculated as the length of the longest line in the pair divided by the length of the shortest line.

3.2.8. Real-time polymerase chain reaction

Gene expression was analyzed using real time polymerase chain reaction (RT-PCR). Total RNA was isolated from cells using the Qiagen RNEasy kit, following the
manufacturer’s protocol. Extracted RNA was quantified using a Thermo Scientific NanoDrop 2000c. Superscript III reverse transcriptase (Life Technologies) was used to convert the RNA template into cDNA, following the manufacturer’s protocol. The reaction was conducted with an Applied Biosystems 2720 Thermal Cycler with the following temperature profile: 65°C for 5 minutes, 25°C for 5 minutes, 50°C for 50 minutes, and 70°C for 15 minutes. For PCR, cDNA was combined with SYBR Green Master Mix (Life Technologies) and 2 µM of forward and reverse primers. Three different adipogenic genes were used to assess differentiation: peroxisome proliferator-activated receptor gamma (PPARγ), C/CAAT enhancer binding protein alpha (CEBPα), and fatty acid binding protein 4 (aP2) along with the housekeeping gene gluteraldehyde phosphate dehydrogenase (GAPDH). Primer sequences are listed in Supplementary Table 1. The reaction was conducted using an Applied Biosystems 7900 HT with the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds followed by 1 minute at 60°C. All experimental conditions and primers were included on the same PCR plate for each repetition of the experiment. Applied Biosystems SDS 2.3 software was used to analyze amplification curves. Gene expression was first normalized to GAPDH, then to the control group (cells grown on standard tissue culture plastic in growth media), and plotted as fold change.

3.2.9. Statistical analysis

All data is presented as the mean ± standard error of the mean. PCR data was performed with technical duplicates and experimental triplicates. Statistical significance was assessed with Prism software, with a p-value < 0.05 considered significant. A
student’s t-test was used for media differentiation studies and a one-way analysis of variance (ANOVA) for all others. A Dunnett post hoc analysis was performed for PCR data, using ASCs cultured on tissue culture plastic in only growth media as a control, and a Tukey post hoc analysis was performed for all AFM and imaging data.

3.3. Results

3.3.1. Cytoskeletal reorganization during differentiation

Preadipocytes rearrange their microfilaments during maturation to accommodate the accumulation of lipids within growing lipid vacuoles [148, 149]. To investigate this phenomenon in vitro, human ASCs were cultured on tissue culture plastic in either standard growth media or adipogenic media for 14 days, and morphological changes associated with adipogenesis were quantified. ASCs cultured in growth media maintained an elongated, fibroblast-like shape, characteristic of naïve ASCs (Fig 3.1A). However, when cultured in adipogenic media the ASCs immediately began changing shape and within just a few days had adopted a more contracted morphology (Fig 3.1B). By 7 days, the cells in adipogenic media still maintained the same overall area as those cultured in growth media, but had lost their characteristically elongated shape and adopted a significantly reduced cellular aspect ratio (Fig 3.1C,D). These morphological changes were consistent through day 14, when lipid vacuoles had developed within the intracellular space of ASCs cultured in adipogenic media. No lipid vacuoles were present in any cell cultured in growth media on tissue culture plastic. These results indicate that lipid accumulation within differentiating ASCs is preceded by a significant reduction in
cellular aspect ratio, changing from an elongated shape to a more rounded morphology, despite maintenance of cell area.

Figure 3.1: Morphological changes associated with adipogenic media. ASCs that were cultured in growth media (A) exhibited no lipid development compared to the significant lipid accumulation noted in cells cultured in adipogenic media (B) at Day 14. While no changes in cellular area were noted between the two experimental groups (C), the cells in adipogenic media did show a significantly reduced cell aspect ratio compared to those in growth media (D). Scale bar = 5 µm. * indicates p < 0.001 using a t-test to compare cells in growth media to those in adipogenic media.

3.3.2. Modulating cell morphology and differentiation via substrate stiffness

To determine if mimicking biomechanical properties of adipose tissue in vitro could induce adipogenesis in the same manner as with adipogenic media, we cultured ASCs on polyacrylamide gel substrates, which can vary stiffness over several orders of magnitude [144, 146], in the absence of chemical induction factors. Human adipose tissue has been shown to have a Young’s modulus of roughly 2 kPa [8], and thus we fabricated polyacrylamide gels using a constant 8% acrylamide monomer solution with varying bis-acrylamide monomer ranging from 0.03% to 0.5% to produce gel substrates with a stiffness range of 2 to 40 kPa (Fig 3.2) that remained constant across the surface of each gel. Since previous studies have shown the importance of adipose specific ECM cues, these gels were then functionalized with adipose matrix to further recapitulate the adipose microenvironment [103, 140]. After functionalization, ASCs readily adhered to the polyacrylamide gel surface.
Within 24 hours of seeding, ASCs adopted drastically different morphologies that were sustained throughout the study. Cells on the soft 2 kPa gels, which mimicked the stiffness of adipose tissue, maintained a compact, rounded shape (Fig 3.3A). ASCs on 20 and 40 kPa gels and on tissue culture plastic demonstrated increasing levels of cellular spreading (Fig 3.3B-D). Each increase in substrate stiffness resulted in a significant increase in average cell area, with the ASCs cultured on tissue culture plastic having the largest area (Fig 3.3E). Cell aspect ratio followed a similar trend for cells on the polyacrylamide gels. The rounded shape of the ASCs on the 2 kPa gels had a significantly smaller aspect ratio than all other groups (Fig 3.3F). The ASCs cultured on standard tissue culture plastic, however, had a slightly smaller aspect ratio than ASCs on 40 kPa gels. These morphological differences at 24 hours were maintained throughout the 6 days of the study, at which point the cells were stained with Oil Red O to examine differentiation. Multiple cells, in each field of view using a 20x objective, stained positively for lipid vacuoles, compared to no evidence of staining in any of the other groups (Fig 3.4A-D). This suggested adipogenic differentiation of the cells on the substrate that mimicked adipose stiffness, even in the absence of media additives. These findings were further supported by quantitative RT-PCR analysis of PPARγ, C/EBPα, and aP2 gene expression, which are early, mid, and later markers of adipogenesis, respectively (Fig 3.4E). ASCs cultured on 2 kPa gels in growth media exhibited significant upregulation of adipogenic markers compared to cells cultured on tissue culture plastic. Though ASCs cultured on 20 kPa gels also showed significant upregulation of C/EBPα expression compared to control cells, no other adipogenic markers were upregulated. Furthermore, gene expression for ASCs on 40 kPa gels was
not statistically different from those in the control wells. These results imply that human ASCs can be directed to differentiate towards an adipogenic lineage by mechanical stimulus alone. However, it does not identify whether this encouragement was a result of just the underlying substrate stiffness or the impact of that substrate on cellular morphology.

Figure 3.2: Polyacrylamide gels of varying stiffness. Polyacrylamide gels were produced by mixing 8% acrylamide monomer with varying concentrations of bis-acrylamide. AFM analysis revealed that the gels exhibited significantly increased elastic moduli as bis-acrylamide concentration increased. * indicates p < 0.001 compared to all other concentrations.
Figure 3.3: Morphological changes associated with ASCs cultured on polyacrylamide gels. ASCs were attached and viable on polyacrylamide gels with stiffnesses ranging from 2 kPa (A), 20 kPa (B), and 40 kPa (C), and compared to those on standard tissue culture plastic (D). When cultured in growth media, the ASCs displayed increasing cell spreading as the substrate stiffness increased (A-D, Green = F-actin, Blue = nuclei). Accordingly, cell area increased with increasing stiffness (E). Cell aspect ratio also increased with substrate stiffness, with ASCs on 40 kPa gels having the largest aspect ratio and ASCs on 2 kPa gels having a significantly reduced aspect ratio (F). Scale bar = 5 µm. * indicates p < 0.01 compared to all other substrates.
Figure 3.4: Soft substrates encourage adipogenesis of ASCs. ASCs cultured in growth media for 6 days on 2 kPa gels (A) exhibited positive lipid accumulation, as identified by the red stain Oil Red O, compared to no staining observed for cells cultured on 20 kPa gels (B), 40 kPa gels (C), or tissue culture plastic (D). Examination of gene expression via PCR confirmed that ASCs on 2 kPa gels indeed expressed significantly upregulated levels of the adipogenic markers PPARγ, CEBPα, and aP2 compared to cells cultured on tissue culture plastic. Scale bar = 5 µm. * indicates p < 0.05 compared to tissue culture plastic control group.

3.3.3. Substrate stiffness stimulates adipogenesis by reducing cell aspect ratio

To determine the extent to which cytoskeletal-mediated morphology regulates adipogenesis, we supplemented growth media with cytochalasin-D, which inhibits actin polymerization. ASCs were first allowed to attach to polyacrylamide gel substrates in growth media, and were then cultured in cytochalasin-supplemented growth media for 6 days. Within 6 hours of adding the cytochalasin-supplemented growth media, the cells on all polyacrylamide gels adopted a more rounded morphology, regardless of the underlying stiffness (Fig 3.5A-C). Even cells that were cultured on tissue culture plastic began retracting their extensions (Fig 3.5D). After 6 days in the cytochalasin-supplemented growth media, the actin filaments of all cells exhibited punctuated intracellular staining, in contrast to the bright filaments seen in cells cultured with normal
growth media (Fig 3.3). Inspecting cell morphology also revealed that addition of cytochalasin-D significantly reduced the cellular area and aspect ratio of cells on 20 kPa and 40 kPa gels compared to the same gels in normal growth media (Fig 3.5E,F). However, cytochalasin-treated cells on tissue culture plastic exhibited a reduction in aspect ratio but no change in cell area (Fig 3.5E,F). Oil Red O staining of these cells suggested the cytochalasin treatment also caused an increase in adipogenesis for all of the cells. Positive lipid vacuoles were identified in cells from all groups that received the cytochalasin-supplemented growth media (Fig 3.6A-D). Quantitative RT-PCR analysis showed induction of PPARγ, C/EBPα, and aP2 expression for cytochalasin-treated cells compared to ASCs cultured in normal growth media on tissue culture plastic. Cytochalasin-treated ASCs on 2 kPa gels showed further upregulation of both PPARγ and aP2 compared to all other substrates (Fig 3.6E). Collectively, this data supports the assertion that mimicking the soft stiffness of adipose tissue can induce the adipogenic differentiation of adult stem cells through restricting the aspect ratio of the cell.
Figure 3.5: Effect of cytochalasin-D on cell morphology. Addition of the actin polymerization inhibitor, cytochalasin-D, caused the cells to adopt a more rounded morphology on 2 kPa (A), 20 kPa (B), and 40 kPa (C) gels and tissue culture plastic (D), and disrupted intracellular actin filaments (A-D, Green = F-actin, Blue = nuclei). Disrupting these actin filaments with cytochalasin-D resulted in significantly reduced cellular area for cells on 20 and 40 kPa gels compared to growth media values, but had no effect on ASCs on tissue culture plastic (E; black columns; cytochalasin-D media; grey columns: normal growth media). More importantly, however, it caused a significant reduction in cell aspect ratio for ASCs on 20 and 40 kPa gels and those cultured on standard tissue culture plastic compared to values in growth media (F, black columns; cytochalasin-D media; grey columns: normal growth media). There were no significant differences in aspect ratio between substrates in cytochalasin media. Scale bar = 5 µm. * indicates p < 0.001 compared to other groups in cytochalasin-supplemented media. # indicates p < 0.001 compared to same group in normal growth media.
Figure 3.6: Decreased aspect ratio stimulates adipogenesis. ASCs cultured in cytochalasin-D for 6 days exhibited a more rounded morphology and as a result, stained positively for lipid accumulation via Oil Red O (arrows) regardless of the underlying substrate stiffness of 2 kPa (A), 20 kPa (B), 40 kPa (C), or standard tissue culture plastic (D). Investigating the gene expression also revealed their adipogenic nature, as these cells exhibited significantly higher levels of all three adipogenic markers PPARγ, CEBPα, and aP2 compared to control cells cultured on tissue culture plastic in growth media. Scale bar = 5 µm. * indicates p < 0.05 compared to tissue culture plastic control group; # indicates p < 0.01 compared to all groups.

3.4. Discussion

The field of tissue engineering has recently emphasized designing materials that closely mimic the specific microenvironment of a damaged tissue in order to stimulate natural regeneration, a trend that has carried over into adipose tissue engineering. Within just the past 4 years, several groups have developed novel methods to extract ECM components from human adipose tissue. Presenting this complex combination of adipose-specific proteins has already been seen to foster the development of ASCs toward an adipogenic lineage [103, 140, 150]. We hoped to expand on these results by not only recreating the biochemical cues of the adipose microenvironment, but also simulate the biomechanical properties of the tissue as well. Using a polyacrylamide gel system we were able to produce substrates that were both at and above the native
stiffness of adipose tissue. We kept the acrylamide monomer concentration constant at 8% to minimize the potential variance of available protein binding sites between the different stiffness gels. As a result, we investigated the influence of three distinct substrate stiffnesses, 2 kPa, 20 kPa, and 40 kPa, on ASC adipogenesis and compared those results to cells cultured on tissue culture plastic, a substantially stiffer substrate and the standard platform for *in vitro* cell culture.

The substrates used in this study were functionalized with components isolated from decellularized human lipoaspirate to allow the cells to directly interact with adipose ECM components while still sensing the stiffness of the underlying substrate. After just 6 days in this recreated adipose microenvironment, we were able to induce significant differentiation of ASCs, without the need for any chemical additives in the media. When cultured on adipose-relevant 2 kPa substrates, ASCs showed elevated expression of early, mid, and late adipogenic transcription factors, and also began accumulating intracellular lipid vacuoles. It is possible that proteins adsorbed to tissue culture plastic are presented to the cells in a different conformation than those that were chemically attached to the polyacrylamide gels. However, the adipogenic gene expression of cells on the 40 kPa substrates was not significantly different than that of cells cultured on tissue culture plastic, suggesting that the polyacrylamide gel system itself was not responsible for changes in adipogenesis. As seen here, by mimicking the biochemical and biomechanical cues associated with adipose tissue, we were able to provide cues for adipogenic differentiation.

We began to elucidate how ASCs were induced toward an adipogenic lineage by quantifying the morphological changes associated with their differentiation. The
immediate consequence of culturing ASCs on softer substrates was a noticeable reduction in cellular area. ASCs adopted a more rounded morphology on soft substrates compared to their characteristic elongated, fibroblast-like shape that was noted when cultured on tissue culture plastic. A recent study by Guvendiren and Burdick detailed a similar observation, where soft substrates facilitated the adoption of a smaller cell area for bone marrow-derived stem cells (MSCs). When cultured with a chemically-modified differentiation media, the MSCs also expressed a higher percentage of adipogenesis than those on stiffer substrates [145]. However, as seen in our studies, simply reducing the cellular area alone did not appear to be the controlling mechanism for inducing adipogenesis. When using adipogenic media, ASCs undergoing adipogenesis maintained a similar cell area to undifferentiated cells, but showed a significantly reduced cellular aspect ratio, indicating that the aspect ratio may be a more significant morphological change associated with adipogenic differentiation. This was further explored in our polyacrylamide gel studies, where the ASCs cultured on 20 kPa and 40 kPa substrates, despite having significantly reduced average cellular area compared to cells on tissue culture plastic, did not express lipid accumulation when cultured in growth media. Cells on 40 kPa substrates also showed negligible adipogenic gene expression. Accordingly, cells that were on the 20 kPa and 40 kPa substrates had either similar or slightly larger cellular aspect ratios, respectively, compared to cells cultured on tissue culture plastic. Only ASCs that were cultured on the 2 kPa substrates, which demonstrated both significantly reduced cellular area and aspect ratio, exhibited lipid accumulation and upregulated gene expression of all three adipogenic markers. Based on these results, the ASCs either needed to be below a certain threshold cellular area or have a significantly
reduced aspect ratio, or both, in order to spontaneously differentiate down an adipogenic lineage.

We further investigated these controlling mechanisms by using an inhibitor of actin polymerization to decouple the mechanical influence on the cells. Adding cytochalasin-D to the growth media encouraged all cells to adopt a more rounded morphology, regardless of the underlying substrate stiffness, and begin accumulating lipids. The ASCs on 2 kPa substrates maintained their small cell area and aspect ratio, and those on 20 kPa and 40 kPa gels significantly reduced their cellular area and aspect ratio compared to when they were cultured in normal growth media. Strikingly, ASCs on tissue culture plastic only saw a reduction in aspect ratio as a result of the cytochalasin, a similar result to what was seen when adding adipogenic media. These ASCs on tissue culture plastic showed positive lipid staining and upregulated expression of all three adipogenic genes despite having the same cellular area as ASCs that did not receive cytochalasin-D, highlighting that the difference in aspect ratio could be the stimulus for adipogenic differentiation. Recently, a study by Yao et al. also suggested that a reduced aspect ratio could encourage adipogenesis of neonatal rat MSCs on micropatterned surfaces [151]. This adds further support to the assertion that reduced aspect ratio appears to be the driving force for mechanically stimulating adipogenesis of ASCs. McBeath et al. proposed a possible pathway for adipogenic differentiation of MSCs beginning with the influence of soluble differentiation factors, followed by an inhibition of the RhoA-ROCK pathway. They determined the influence of mechanical forces worked downstream of soluble media additives but could still be overridden through inhibition of RhoA-Kinase (ROCK) and thus decreasing intracellular tension [152]. Our
research adds to this model by suggesting that these mechanical forces consist of both a reduction in cellular area and, more importantly, a reduction in cellular aspect ratio in order to minimize cellular tension and drive adipogenic differentiation.

Several studies have indicated that pre-committing ASCs toward an adipogenic lineage may improve function once implanted \textit{in vivo} [49, 153]. With the increasing use of ASCs within the clinic to augment autologous lipotransfer procedures, the ability to encourage adipogenesis of ASCs prior to implantation without having to use soluble chemical factors could be an advantage. Pre-treating ASCs on an adipose-mimicking substrate could prove to be a translationally-relevant method for priming the cells for adipogenic function once injected into the patient. We have demonstrated here a method for inducing adipogenesis of human-derived ASCs, a potentially autologous cell source, without the use of any chemical additives or steroids. Overall, this study offers insight into the influence of both ECM composition and mechanical properties on adipogenic differentiation of ASCs, as well as suggests that novel materials for adipose tissue engineering should emulate adipose biomechanical properties to encourage \textit{de novo} adipogenesis of ASCs.
Chapter 3, in part, has been submitted for publication as: D. Adam Young, Yu Suk Choi, Adam J. Engler, and Karen L. Christman. Stimulation of adipogenesis of adult adipose-derived stem cells using substrates that mimic the stiffness of adipose tissue. 2013.

The author of this dissertation is the primary author of this manuscript.
CHAPTER 4:

Encouraging *in vivo* adipogenesis with a decellularized adipose matrix hydrogel
4.1. Introduction

Researchers have begun to recognize the need of certain patients to actually gain or regenerate lost adipose tissue and, therefore, have started to apply the principles of tissue engineering to achieve this goal. In addition to the more common elective cosmetic procedures, there is a population of patients that would benefit from adipose regeneration due to congenital defects, trauma, or surgical resections. These patients highlight the growing need for biomaterials that can not only replace lost or damaged adipose tissue, but also encourage its natural regeneration and continual integration with surrounding tissue throughout the lifetime of the patient.

Recall from Chapter 1 that several injectable materials are currently being marketed for filling subcutaneous voids, but often face limited longevity due to rapid resorption [37, 128]. Their inability to encourage natural adipose formation or ingrowth necessitates repeated injections for a prolonged effect, and thus classifies them as temporary fillers. Furthermore, materials that are specifically designed to resist in vivo degradation are associated with chronic inflammatory responses and fibrous encapsulation [37, 67]. As a result, a significant demand for injectable materials that not only act as fillers, but also promote in vivo adipogenesis, is beginning to be realized.

As discussed earlier in this dissertation, decellularization is process by which tissue-specific extracellular matrix (ECM) components can be isolated [85]. These ECM components can provide an instructive environment for stem cell differentiation, and are also associated with positive remodeling responses in vivo, including neovascularization and host tissue regeneration based upon on the source material [96, 97, 154, 155]. Adipose is one of the few tissues that can be readily obtained in a minimally-invasive
manner, from either an autologous or allogeneic source, to be used for decellularization processes. We have previously published a method for decellularizing adipose tissue obtained from lipoplasty procedures. This adipose ECM can then be reduced to a liquid formulation that will self-assemble into a hydrogel when it is brought to physiologic temperature and pH [139]. This self-assembly characteristic of the adipose matrix hydrogel provides a cohesive, three-dimensional network of fibrous proteins that can fill the patient’s specific adipose void and potentially aid in cellular infiltration. The cohesive, gel-like nature of the self-assembled material could also prevent migration of the material after injection. In this study, our objective was to evaluate the performance of this adipose matrix hydrogel in vivo compared to Juvederm, a clinical standard composed of crosslinked hyaluronic acid gel that is widely used in the filling of adipose deficits.

Several studies have also implicated the vasculogenic benefits of incorporating adipose-derived adult stem cells (ASCs) into an adipose tissue engineering strategy. We have already demonstrated the ability of the adipose matrix to support ASC viability [139]. Based on our results in Chapter 3 suggesting the adipogenic influence of the adipose matrix on ASCs, we also hoped to investigate its potential to be utilized as a delivery vehicle for these cells in vivo. Furthermore, previous studies have shown that the body gradually breaks down decellularized materials over the course of a couple weeks. Adipogenesis usually takes 3-5 weeks to occur in vivo, therefore we also investigated the use of an in situ crosslinking enzyme, transglutaminase, within our adipose matrix hydrogel. Transglutaminase (TG) is a clinically-relevant crosslinker that promotes the formation of covalent bonds between free amines and gamma-carboxamide
groups. The many free amines present in our digested adipose matrix will provide multiple active sites for TG crosslinking to strengthen the material. We hypothesize that adipose matrix hydrogels will not only show better host integration than Juvederm, but will also support the formation of new adipose tissue, which can be further amplified through the addition of either ASCs or a biocompatible crosslinker.

4.2. Methods

4.2.1. Production of adipose matrix hydrogel

Human adipose tissue was obtained and decellularized according to published protocols, as described in Chapters 2 and 3 [139]. For this study, lipoaspirate from three donors, collected from Ranch & Coast Plastic Surgery Clinic, was combined in equal amounts and decellularized to create a batched source of material, following the protocol established in Chapter 2. The donors ranged in age from 48-60 years old, with an average age of 53. Briefly, the cellular content of the tissue was removed by spinning in SDS, followed by sodium deoxycholate supplemented with 500 U lipase and colipase (Sigma-Aldrich). The resulting decellularized tissue was then rinsed in DI water with 5000 I.U. penicillin and 5 mg/mL streptomycin and then briefly rinsed in isopropanol to ensure lipid removal. Finally, the tissue was washed in DI water, lyophilized, and milled with a Wiley Mini-mill to produce a fine white powder. This decellularized adipose ECM was then digested at 15 mg/mL in 0.1 M HCl with 2 mg/mL pepsin (Sigma-Aldrich). The resulting liquid adipose matrix was then neutralized with 1 M NaOH and diluted to 12 mg/mL in 1x PBS. Control aliquots of adipose matrix were brought to body
temperature in an incubator to confirm self-assembly and gelation. The remaining liquid adipose matrix was then lyophilized and stored at -80°C until use.

4.2.2. Isolation and culture of stem cells

Prior to decellularization, human adult adipose-derived stem cells (ASCs) were isolated from the lipoaspirate according to previously published protocols, as described in Chapters 2 and 3 [104]. Briefly, aliquots of lipoaspirate were digested at 37°C for 20 minutes in 0.075% collagenase I (Worthington Biochemical). The resulting suspension was centrifuged at 5000 x g to obtain an ASC-rich pellet. The cell pellet was resuspended in 160 mM ammonium chloride buffer to lyse red blood cells and again centrifuged at 5000 x g. The new cell pellet was resuspended in growth media (DMEM/F12 plus 10% Fetal Bovine Serum, 100 I.U. penicillin, and 100 µg/mL streptomycin) and passed through a 40 µm cell strainer. The remaining cells were plated on standard tissue culture plastic overnight at 37°C and 5% CO₂. After 24 hours, the non-adherent cells were removed with two rinses with 1x PBS, and then serially passaged at 70% confluence. Growth media was changed every 3-4 days. At Passage 2, cells from 3 donors, ranging in age from 34-48 years old with an average age of 41, were combined together and cultured as a pooled cell line. ASCs used in this study were from this pooled cell line at either Passage 4 or 5.

4.2.3. In vivo injections

All experiments in this study were performed in accordance with the guidelines published by the Institutional Animal Care and Use Committee at the University of
California, San Diego and the American Association for Accreditation of Laboratory Animal Care. Female athymic mice, aged 3-4 weeks, were obtained from Harlan Laboratories and used for this study.

On injection days, digested adipose matrix was rehydrated with sterile water at 12 mg/mL. Mice were briefly anesthetized with isoflurane and received 150-250 µL subcutaneous injections of one of the following materials: 1) adipose matrix hydrogel, 2) adipose matrix hydrogel with ASCs, 3) adipose matrix hydrogel with transglutaminase (TG, from Sigma-Aldrich), 4) adipose matrix hydrogel with TG and ASCs, or 5) Juvederm (Allergan). Adipose matrix hydrogel was delivered at 12 mg/mL, ASCs at 1.5 x 10^5 cells/mL, and TG at 0.1 U/mL. Each mouse received bilateral, subcutaneous injections in the dorsal region using a 25G needle and was monitored for any pain or discomfort during recovery. After 1, 2, and 4 weeks, the mice were sacrificed and the injection regions excised. Adipose matrix hydrogel samples were fresh frozen in OCT compound and Juvederm samples were fixed for 24 hours in 10% formalin and paraffin embedded (n=4 per group, per time point).

4.2.4. Histological staining and analysis

Tissue samples were cryosectioned into 20 µm slices and mounted onto charged microscope slides. Sections from the middle of the injection bolus were briefly fixed with acetone and then stained with hematoxylin and eosin (H&E). These sections were given to an experienced histopathologist for qualitative assessment of the inflammatory response. Separate sections were fixed for one hour in 4% paraformaldehyde and stained for one hour in Oil Red O working solution, following a previously published protocol.
These sections were counterstained with hematoxylin solution for 2.5 minutes and mounted with 10% glycerol in PBS. Images of both stains were acquired using a Zeiss Imager A1 microscope.

### 4.2.5. Immunohistological staining and analysis

Sections from each injection bolus that were at least 200 µm apart were fixed in acetone and then blocked with staining buffer (PBS plus 1% bovine serum albumin and 3% Triton X-100) for 20 minutes. For vessel analysis, sections were co-stained with primary antibodies against CD31 (rat host, 1:200 dilution) and alpha smooth muscle actin (rabbit host, 1:100 dilution) for 1 hour, then with secondary antibodies AlexaFluor 488 (anti-rat, 1:400 dilution) and AlexaFluor 568 (anti-rabbit, 1:200 dilution) for 30 minutes. For macrophage analysis, sections were stained with a primary antibody against pan-macrophage (1:200 dilution) for 1 hour, then with a secondary antibody AlexaFluor 488 (1:200 dilution) for 30 minutes. All primary antibodies were obtained from Abcam. All sections were then counterstained with Hoechst 33342 to label nuclei. Images of the antibody staining were acquired using a Zeiss Observer D1.

All images were analyzed using Zeiss AxioVision software, with 4-6 randomly selected images for each sample. For macrophage analysis, 40x images were taken along the edges of the injection bolus. The area of the injection region visible in the field of view was outlined and a blinded technician counted the number of macrophages and total nuclei in the injection region. For vessel analysis, multiple 20x images across the entire injection bolus were taken. Four images from each sample were randomly selected and the area of the injection bolus within the field of view was outlined. The total area of the
visible vessels and capillaries within this region, as identified by positive CD31 staining, was determined and divided by the area of the injection region to determine the percentage of the injection bolus that was occupied by new vasculature.

4.2.6. Statistical analysis

All data are presented as mean ± standard error of the mean. Statistical significance was determined using a one-way analysis of variance (ANOVA) with a Tukey post hoc test.

4.3. Results

4.3.1. Adipose matrix hydrogels were well tolerated in vivo

Staining of control adipose matrix hydrogels that were gelled in vitro confirmed decellularization and delipidization of the material, as evidenced by the lack of nuclei in H&E staining and lipids in Oil Red O staining, respectively (data not shown). All material injections were well tolerated by the mice, showing no signs of erythema, pain, or dermatitis throughout the study (Fig 4.1). Within 2-3 days, the Juvederm injection sites had noticeably swollen and increased in volume. Adipose matrix hydrogel groups lost volume over the first week but appeared to stabilize over the 2 to 4 week time points, with all groups still visually identifiable through the skin at week 4. Histological analysis of the adipose matrix hydrogel revealed acute inflammation at week 1 with substantial cellular infiltrate, predominantly composed of neutrophils (Fig 4.2). By week 2, macrophages were present throughout the injection bolus that persisted through week 4 with a sporadic neutrophil abscess noted. Incorporation of ASCs into the adipose matrix
hydrogel had a negligible influence on the immune response to the hydrogel. Addition of transglutaminase or transglutaminase with ASCs showed similar acute infiltration of neutrophils but the cells were more evenly distributed throughout the injection bolus, as opposed to concentrated along the boundaries as was seen in the adipose matrix hydrogel alone group (Fig 4.2). Moderate levels of neutrophils and macrophages were noted throughout the bolus at both week 2 and week 4 time points as well, with no indication of abscess formation or foreign body giant cells. This was highlighted by the immunohistochemical staining for macrophages (Fig 4.3A). Several macrophages were identified within the injection region. The addition of TG significantly lowered the macrophage density at week 1, but there was no significant difference in macrophages per area or percent macrophages per total infiltrating cells between the adipose matrix hydrogel groups at weeks 2 and 4 (Fig 4.3B). Furthermore, all adipose matrix hydrogel groups appeared to integrate well with the surrounding tissue with no evidence of capsule formation. This is in contrast to the Juvederm injections, which showed negligible cellular infiltration at week 1 and possible edema in the surrounding tissue. A thin to moderately thin fibrous capsule was noted surrounding all of the Juvederm injections as early as 2 weeks and persisting throughout the study (Fig 4.2).
Figure 4.1: Injectable functionality of adipose ECM. Liquid formulations of adipose ECM could easily be loaded into a syringe and injected through a 25G needle (A). After 1 week in vivo, the ECM bolus was easily identified through the skin and had not initiated any irritation or pain (B). The injection appeared to be vascularized and well integrated with the surrounding tissue (C).
Figure 4.2: ECM hydrogels show improved biocompatibility. ECM hydrogels, with or without transglutaminase or ASCs, integrate well with surrounding tissue. In contrast, Juvederm showed limited cellular infiltration and slight capsule formation. ECM hydrogels with TG or ASCs also induced new adipocytes to form after 4 weeks (indicated by arrows). Scale bar = 100 µm.
4.3.2. Addition of ASCs or transglutaminase improved neovascularization

Adipose matrix hydrogels experienced significant neovascularization after just 2 weeks in vivo (Fig 4.3C). Several capillaries were identified at the boundaries of the injection region. However, this vascularization was not sustained, as evidenced by the negligible capillary or arteriole presence within the injection bolus after 4 weeks. Alternatively, addition of ASCs to the adipose matrix hydrogel sustained this early neovascularization and promoted continual vascular maturation over the course of the study (Fig 4.3D). After 4 weeks, several mature arterioles, surrounded by vascular smooth muscle, were easily identified within the injection bolus. Interestingly, simply adding transglutaminase to the adipose matrix hydrogel also produced a similar sustained vascularization effect regardless of the presence of ASCs. Several mature arterioles were identified both at the boundaries and within the center the injection bolus of both transglutaminase groups at week 4. These results are again in contrast with the Juvederm injections, which showed no evidence of any vascularization within the injection bolus at any time point and thus were not included in analysis.
Figure 4.3: Macrophage infiltration and neovascularization of ECM hydrogels. All ECM hydrogels were infiltrated by macrophages, which were indicated by fluorescent staining (green = pan macrophage, blue = nuclei). Quantification of macrophage density revealed that addition of transglutaminase significantly lowered macrophage density at week 1, but all groups showed similar levels at weeks 2 and 4 (B). ECM hydrogels stimulated significant neovascularization, with arterioles present by 2 weeks (C, red = smooth muscle actin, green = CD31, blue = nuclei). Quantification revealed that the ECM hydrogels alone were substantially more vascularized with the addition of ASCs or TG at weeks 4 (D). Scale = 50 µm. * p < 0.05.

4.3.3. Sustaining neovascularization stimulated adipogenesis in ECM hydrogels

Adipose matrix hydrogels displayed minimal adipogenesis on their own over the course of the one-month study (Fig 4.4A). However, the addition of the ASCs resulted in several adipocytes forming within the interior of the injection bolus of half of the animals, notably in the vicinity of newly developed blood vessels. These cells were not present at 1 week, but were easily identifiable within the injection region by 4 weeks, as confirmed by Oil Red O staining (Fig 4.4B). Furthermore, adipocytes were also found in the injection bolus of all animals receiving transglutaminase groups at week 4, both with and without ASCs added. These adipocytes were further confirmed with Oil Red O
staining at week 4 (Fig 4.4C,D). This is compared to the negligible staining at week 1 for any of the adipose matrix hydrogel groups, indicating these adipocytes were indeed newly formed in the injection region and not simply displaced from the native subcutaneous fat as a result of the initial injection surgery. Finally, no evidence of adipocytes was identified for any of the Juvederm injection regions.

Figure 4.4: ECM hydrogels stimulated adipogenesis. After 4 weeks in vivo, no appreciable adipocytes were noted within the interior of the adipose ECM hydrogel alone injections (A). However, Oil Red O staining confirmed several new adipocytes had developed within the interior of the injection bolus of adipose ECM hydrogel + ASCs (B), hydrogel + TG (C), and hydrogel + TG + ASC (D) samples (arrows indicate positive staining). Scale = 200 µm.

4.4. Discussion

Decellularization of tissues offers the ability to produce tissue-specific scaffolds that recreate many of the biochemical aspects of the tissue of interest. We have previously reported the decellularization of human lipoaspirate tissue to obtain an adipose-specific extracellular matrix material. We then processed this adipose ECM into
a liquid formulation that self-assembles at physiologic temperatures [139]. Unlike other derivations of adipose ECM, this formulation offers the ability to deliver the adipose matrix through a small gauge needle, thus facilitating clinical translation. In this study, we investigated the biocompatibility and in vivo function of this adipose matrix hydrogel compared to the clinical soft tissue filler standard, Juvederm.

From the results of this study, we have shown that adipose matrix hydrogels do not initiate any negative reactions in a mouse model of subcutaneous soft tissue filling. This is similar to what was seen and expected from the clinical standard Juvederm. All of the materials were easily injected subcutaneously with a 25G needle and did not appear to elicit any pain, redness, or irritation in the animals at the injection sites, even in the presence of the in situ crosslinker, transglutaminase. Of note, the Juvederm injections did experience post-injection swelling. This was observed both on a macroscopic level and identified during histological analysis as edema surrounding the injection site. This phenomenon is well documented in the clinic, as clinicians are advised to underfill adipose voids to account for this variable swelling [40]. In contrast, the adipose matrix hydrogels maintained their shape and volume post-injection, although did lose volume over the course of the study. Furthermore, all of the forms of adipose matrix hydrogels experienced persisting cellular infiltration and were well integrated with the surrounding tissue by 4 weeks. This is in contrast to Juvederm injections, which were surrounded by a thin fibrous capsule. While Juvederm did not initiate a negative immune response, it also was not capable of integrating with the surrounding tissue and therefore limited to a role of temporarily filling deficits until it is eventually broken down in vivo.
In addition to positive biocompatibility profiles, adipose matrix hydrogels were also capable of supporting new adipose formation. On its own, the adipose matrix hydrogel did not encourage significant host adipogenesis. However, the addition of ASCs resulted in new adipocytes developing within the injection regions of several samples. It has not yet been elucidated whether these adipocytes were differentiated ASCs or whether they were host cells able to develop as a result of the cytokines and vascular promotion of the delivered ASCs. More importantly, however, the addition of transglutaminase to the adipose matrix hydrogel was also capable of stimulating new adipogenesis in every injection region, regardless of the presence of ASCs. In a study by Cho *et al*., a polymer cage was placed beneath the skin of a mouse and then filled with a fibrin gel. The cage would prevent contraction of the skin on underlying implants and therefore support adipogenesis within the cage [62]. It is possible that the transglutaminase in our studies is providing a similar role by establishing a more structured and supportive environment within the adipose matrix that shields the developing adipocytes from excessive external forces and thus allows for their maturation. Regardless, adipogenesis in this acellular injection suggests that the newly developed adipocytes in each of the groups were indeed host-derived. These results correlated with the vessel analysis, with increased vascularization seen in ASC or TG-containing adipose matrix hydrogels. In comparison, the Juvederm injections showed no formation of new adipose tissue or vascularization. This further highlights the benefits of adipose-derived materials for clinical applications; they are capable of improved biocompatibility, vascularization, and even adipose regeneration compared to current clinical standards.
Recent studies support this concept that adipose ECM-derived materials are capable of stimulating some degree of subcutaneous *in vivo* adipogenesis on their own [129, 141, 156]. These studies presumed that the adipose ECM simply provided a template for adipogenesis of the infiltrating cells. Our results expand on these studies by emphasizing the correlation between neovascularization and adipogenesis. In our study, new adipose formation was associated with increased levels of vascularization in the injection region. This vascularization was provided by both the ASCs and the degradation products of the adipose matrix hydrogel, but needed to be sustained in order for adipose development to occur. This is supported by the many studies that utilize vascular pedicle chambers to assess tissue engineering materials [157]. This model is uniquely pro-angiogenic and accordingly has been shown to support adipogenesis within a variety of materials [100, 158]. Co-delivery of basic fibroblast growth factor (bFGF) has also been associated with increased adipogenic differentiation of implanted ASCs in other animal models [65, 83]. In all studies, adipogenesis was always noted in materials that also displayed neovascularization, collectively highlighting that materials designed for adipose tissue engineering must also possess angiogenic features as well.

In addition to potentially providing a native template for adipose formation and stimulating neovascularization *in vivo*, the adipose matrix hydrogels used in this study were also associated with significant cellular infiltration. In fact, this infiltration was more uniformly distributed in the gels that included either transglutaminase or ASCs, as opposed to being localized to the boundaries in the adipose matrix hydrogel alone after 1 week. This is consistent with what has been seen with other forms of decellularized materials that are available in liquid formulation [97, 138, 154]. From the available
literature, it is becoming apparent that these decellularized materials stimulate more of a positive remodeling response from the infiltrating macrophages as opposed to a negative chronic inflammatory response [86, 159, 160]. Recent studies have also begun to suggest a potential link between macrophage inflammatory responses and increased adipogenesis, particularly in obese patients [161-163]. It is possible that the increased macrophage recruitment in the transglutaminase-crosslinked adipose matrix hydrogels was contributing to the increased adipogenesis seen in those groups. However, a more detailed analysis of the macrophage types and ratios is needed before any definitive conclusions can be drawn. Furthermore, a closer examination of the microscopic differences between crosslinked and non-crosslinked adipose ECM hydrogels is also necessary to identify other features aiding this adipoinductive quality.

The author of this dissertation is the primary author of these manuscripts.
CHAPTER 5:

Development of injectable human ECM particles

without using decellularization agents
5.1. Introduction

The previous chapters have described the development of a novel injectable hydrogel meant to treat adipose deficits by stimulating natural adipose regeneration. However, in certain cosmetic situations, it is more advantageous to provide immediate structural support and then have adipose development as an auxiliary benefit. This might be the case for treating certain deep wrinkles, such as the nasolabial fold, which extends between the crease of the nose to the corners of the mouth. These wrinkles are common in older age and are caused by a gradual degradation of collagen and elastin fibers in the dermal and subcutaneous layers beneath the skin. As a result, deep subcutaneous ridges develop into which the overlaying skin falls. In order to treat these wrinkles, a crosslinked biomaterial or synthetic material is typically placed deep into the subcutaneous layer and must replace the structural support role that used to be provided by the now-degraded collagen and elastin fibers. Unfortunately the soft, compliant nature of the adipose matrix hydrogel that was developed in the previous chapters may not be ideal for this situation, as it was designed primarily to stimulate adipose regeneration, instead of providing structural support.

Current materials designed for treating wrinkles like the nasolabial fold are not ideal solutions either. These materials are either highly crosslinked versions of biopolymers like hyaluronic acid (HA), or more permanent synthetic materials composed of calcium hydroxylapatite or poly-L-lactic acid (PLLA) [39]. While these materials do successfully provide immediate structural support beneath the wrinkle, they are often broken down too quickly or stimulate a negative immune response [37]. Some groups have also begun turning to materials that could possibly replace the degraded collagen...
and elastin fibers. A process known as decellularization offers the ability to remove immunogenic cellular content from an organ and leave behind the extracellular matrix (ECM) proteins of that organ to be used as a tissue repair scaffold. One form of decellularized cadaveric dermis, known as Alloderm, has been investigated in a couple clinical trials for subcutaneous filling. These trials showed that the decellularized dermis material was well tolerated by the body and was able to maintain volume over 3 months [164, 165]. However, these materials showed no development of new adipose tissue and were gradually resorbed by the body. Decellularization processes are often criticized for their use of harsh chemicals. Several reviews have suggested these chemicals have deleterious effects on the ECM proteins, stripping them of their normal form and function [84, 166]. It is possible that the decellularization process of Alloderm disrupts the native conformation of the proteins, preventing them from stimulating a remodeling and regeneration response. Currently, there is no acellular material available for subcutaneous injection that can provide a complex mixture of dermal proteins in an unaltered state.

Recently, a process known as Tissue Engineering by Self-Assembly (TESA) has been developed to produce sheets of human extracellular matrix (ECM) in vitro. In this strategy, human fibroblasts are isolated from a skin biopsy and encouraged to produce ECM. This ECM is then harvested as an intact sheet that can be used as a wound dressing or rolled into a tube for cardiovascular applications [167-169]. Our objective for this study was to utilize the TESA methodology to develop an injectable version of acellular human ECM without utilizing any of the chemicals normally employed during decellularization processing. Furthermore, reducing this TESA-produced ECM into a
particulate formulation would facilitate injectable delivery, potentially providing a novel autologous soft tissue filler that can be delivered subcutaneously to provide structural support to the subcutaneous tissue and replace the degraded proteins that cause deep wrinkles. In this study, we describe the development of TESA-produced ECM particles and compare their in vivo biocompatibility and function against two clinical standard fillers commonly used for treating deep wrinkles.

5.2. Methods

5.2.1. Production of human ECM particles

Human fibroblasts were isolated from skin biopsies of consenting patients as previously described [169]. Cells at passage 6-7 were plated in tissue culture flasks and cultured to confluence in growth medium (DMEM with 20% FBS, 2 mM glutamine, 100U penicillin, 100 µg/mL streptomycin, and 500 µM sodium ascorbate). Medium was changed three times a week and the cells were allowed to produce extensive extracellular matrix (ECM) within the flask, a process referred to as Tissue Engineering by Self Assembly (TESA). After culturing for 4 or 8 weeks, the flasks were rinsed with PBS, aspirated, then stored at -80°C until use.

To generate the particles, flasks with ECM sheets were allowed to gradually thaw at room temperature and rehydrated with PBS. The thickness of ECM sheets was determined by measuring the distance traveled by the objective when focusing on the bottom of the sheet and gradually raising the objective in known increments until the top of the sheet came into focus. The ECM was then lifted off the bottom of the flask as an intact sheet. Sheets of intact ECM were allowed to dry in a laminar flow hood. The
sheets were then milled in a Wiley Mini-mill fitted with a #60 sieve to produce a fine powder of ECM particles. Particles were then sent to Sterigenics Inc. for sterilization by gamma irradiation. Upon return, the particles were stored at -80°C until use. Prior to experiments, particles were warmed to room temperature and rehydrated in 0.9% saline for at least 20 minutes.

5.2.2. Characterization of ECM particles

For light microscopy, rehydrated particles were mounted on microscope slides with 10% glycerol in PBS solution. Brightfield images were obtained using a Nikon Eclipse TS100 microscope fitted with an Infinity 2 camera. Size distribution of the particles was assessed using a Beckman-Coulter Multisizer 4 fitted with 500 µm and 1000 µm apertures. Data was averaged over 4 total runs. To determine DNA content of the particles, a Machery-Nagel NucleoSpin Tissue kit was first used to purify total nucleic acid following the manufacturer’s protocol. An Invitrogen PicoGreen kit was then used to quantify total nucleic acid per weight of particles following the manufacturer’s protocol. A BioTek Synergy 4 plate reader was used to quantify fluorescence with an excitation filter at 480 nm and emission filter at 520 nm. For scanning electron microscopy (SEM), particles were rinsed and diluted in DI water and pipetted onto a silicon wafer. The particles were then dehydrated in a vacuum chamber for 45 minutes. The dry particles were then sputter coated with gold using a Cressington 108A sputter coater. Images were then captured using an Agilent 8500 field-emission scanning electron microscope.
5.2.3. Mass spectroscopy

Tandem mass spectroscopy (MS/MS) was used to identify protein fragments and peptides contained in the ECM particles as described previously [138]. Particle samples were digested with trypsin and then analyzed with liquid chromatography (LC)-MS/MS with electrospray ionization, using an Applied Biosciences QSTAR-Elite hybrid mass spectrometer interfaced to a Tempo nanoscale reversed-phase high pressure liquid chromatographer (Applied Biosciences) using a 10 cm 180 ID glass capillary packed with 5 µm C-18 Zorbax beads (Agilent Technologies). The following buffer compositions were used during the analysis: buffer A was composed of 98% H$_2$O, 2% acetonitrile (ACN), 0.2% formic acid, and 0.005% trifluoroacetic acid (TFA); buffer B was composed of 100% ACN, 0.2% formic acid, and 0.005% TFA. Peptides were eluted from the C-18 column into the mass spectrometer using a linear gradient of 5-60% buffer B over 60 minutes at 400 µL/min. LC-MS/MS data were acquired in a data-dependent fashion by selecting the four most intense peaks with charge state of two to four that exceeds 20 counts, with exclusion of former target ions set to 360 seconds and the mass tolerance for exclusion set to 100 ppm. MS/MS data were acquired from m/z 50 to 2000 Da by using “enhance all” and 24 time bins to sum, dynamic background subtract, automatic collision energy, and automatic MS/MS accumulation with the fragment intensity multiplier set to 6 and maximum accumulation set to 2 seconds before returning to survey scan. Peptides were identified using paragon algorithm executed in Protein Pilot 2.0 (LifeTech). Detected peptide sequences were run against the Swiss Prot databank for protein identification. Proteins were identified based on at least one peptide detected with a confidence of above 99% for that peptide identification. All mass
spectroscopy work was performed at the Biomolecular Mass Spectroscopy Facility at UCSD.

5.2.4. Long-term in vivo injections

All experiments in this study were performed in accordance with the guidelines published by the Institutional Animal Care and Use Committee at the University of California, San Diego and the American Association for Accreditation of Laboratory Animal Care. Female athymic (Foxn1nu) mice, aged 3-4 weeks, were obtained from Harlan Laboratories and used for this study.

On injection days, ECM particles, from ECM sheets that had cultured for 8 weeks, were suspended in 0.9% saline at 175 mg/mL. Mice were briefly anesthetized with isoflurane and received 150 µL subcutaneous injections of one of the following materials: 1) ECM particles, 2) Juvederm (Allergan), or 3) Radiesse (Merz). Each mouse received bilateral, subcutaneous injections in the dorsal region using a 25 G needle and was monitored for any pain or discomfort during recovery. After 2, 4, 8, and 12 weeks, the mice were sacrificed and the injection regions excised. Biopsy samples were fixed for 24 hours in 10% formalin and paraffin embedded (n=4 per group, per time point). At the 3 month time point, one sample from each group was cut in half, and half of the sample was fixed in formalin and paraffin-embedded and the other half was fresh-frozen in OCT for Oil Red O staining.

5.2.5. Immunocompetent animal model
A brief animal study was conducted at Pacific BioLabs to examine the initial biocompatibility of various particle formulations in an immunocompetent animal model. This animal study was reviewed and approved by the Pacific BioLabs animal review board. Two variations of ECM particles were used for this study, one set was produced from ECM sheets that had been culturing for 4 weeks, and one set from ECM sheets that had been culturing for 8 weeks. On the injection day, particles were rehydrated at 60 mg/mL in 0.9% saline. Male Sprague-Dawley rats (Charles River) were used for this pilot study and received an injection of the following groups: 1) ECM particles from 4-week-old sheets, 2) ECM particles from 8-week-old sheets, 3) 0.9% saline, or 4) Juvederm (Allergan). Each rat received bilateral, subcutaneous injections in the dorsal region using a 25 G needle and were monitored for any pain or discomfort during recovery. After 1 and 5 days, the rats were sacrificed and the injection regions excised. Biopsy samples were fixed for 24 hours in 10% formalin and paraffin embedded (n=4 per group, per time point).

5.2.6. Histological staining and analysis

Tissue samples were sectioned into 10 μm slices and stained with hematoxylin and eosin (H&E) and Trichrome stains. These sections were given to an experienced pathologist for qualitative assessment of the inflammatory response. Fresh-frozen samples, from the 3 month time point, were cryosectioned into 20 μm slices, fixed for one hour in 4% paraformaldehyde and stained for one hour in Oil Red O working solution, following a previously published protocol [104]. These sections were
counterstained with hematoxylin solution for 2.5 minutes and mounted with 10% glycerol in PBS. Images of all stains were acquired using a Zeiss Imager A1 microscope.

5.2.7. Statistical analysis

All data are presented as mean ± standard error of the mean. Statistical significance was determined using a one-way analysis of variance (ANOVA) with a Tukey post hoc test.

5.3. Results

5.3.1. Production of human ECM particles

Sheets of human ECM were readily produced by fibroblasts when cultured at confluence for extended periods of time. By 4 weeks, these sheets could be easily lifted off of the surface of the flask and displayed substantial strength, able to withstand a moderate amount of manual handling without tearing. After 8 weeks of culture, these ECM sheets were substantially stronger, reaching approximately 79 ± 2.3 μm in thickness (Fig 5.1A). After drying, the sheets were easily milled into particles that were separated by a #40 and then a #60 sieve (Fig 5.1B). These particles could be concentrated up to 200 mg/mL in 0.9% saline. No appreciable swelling was noted in rehydrated particles over the course of 24 hours. At 200 mg/mL, the particles could be pushed through a 25G needle and at 175 mg/mL could pass through a 27G needle.

The ECM particles ranged in size from 50-250 μm. Examining the particles from the 8-week-old ECM sheets under the microscope revealed a variety of tetrahedral shapes among the relatively planar particles (Fig 5.1C,D). Mass spectroscopy revealed the
particles were composed of a variety of ECM proteins including structural and FACIT collagens, as well as elastin and fibronectin. Several proteoglycans were also identified such as decorin, tenascin, fibrillin, and lumican.

**Figure 5.1: Production of human ECM particles:** Sheets of human ECM (A) were milled into small particles (B). These particles were composed of polygonal shaped planar pieces of assembled ECM, as seen here under a bright field microscope (C, scale = 50 µm) and scanning electron microscope (D, scale = 10 µm).

**5.3.2. ECM particles stimulate adipogenesis and positive integration in vivo**

We utilized a three month study in nude mice to examine the *in vivo* function of these ECM particles against two clinical standards. Juvederm is a highly crosslinked version of hyaluronic acid and Radiesse is a soft tissue filler composed of calcium hydroxylapatite particles. All three materials (ECM particles, Juvederm, and Radiesse) were delivered subcutaneously into the dorsal region of female mice through a 25G needle (**Fig 5.2A**). All of the injections were well tolerated by the animals without any signs of erythema or pain (**Fig 5.2B-D**). Juvederm injections appeared to swell in volume over the first few days, then gradually reduced in volume over the course of the study.
Radiesse injections were delivered as a tight bolus, but gradually flattened into a more amorphous shape. ECM particles lost some volume initially as the saline dispersed but appeared to maintain their shape and volume from the 1 to 3 month time points, with no evidence of migration.

Histologically, all three materials produced distinct morphological profiles and apparent tissue reactions (Fig 5.3). Juvederm injections were generally surrounded by a thin to moderately thin fibrous capsule, and the adjacent tissue characterized by moderate to marked intracellular edema. Other than the fibrous capsule, there was no appreciable cellular infiltrate or neovascularization. By 3 months, some injection sites suggested the Juvederm was beginning to break down, as evidenced by infiltrating fibrous tissue. In contrast, the Radiesse injection sites required decalcification for histological analysis and thus produced characteristic spherical voids throughout the injection region where the calcium hydroxylapatite particles were located. Radiesse sites displayed varying levels of multinucleated cells, characteristic of foreign body syncytial cells. The periphery of the bolus was generally surrounded by a thin to moderate fibrous capsule. Within this bolus existed a mild to marked large mononuclear cell infiltrate and, at times, with admixed neutrophils. Some injections also gave the appearance of mineralizing osteoid. This response to Juvederm and Radiesse was in stark contrast to what was seen for the ECM particles. The ECM particle nodules expressed little to no surrounding fibrous tissue and, other than a few instances of mononuclear cell infiltrate at the borders, no cellular reaction in most sites. The particle injections were also characterized by nearby patent arterioles and limited evidence of particle degradation. Interestingly, beginning at week 4 and expanding in number through week 12, several cells developed within the
ECM particle bolus that showed a clear, expanding cytoplasn, suggestive of lipid in adipocytes (Fig 5.4). These cells were confirmed as adipocytes by Oil Red O staining. This response was seen in every injection site at 3 months, and to a greater degree in scapular injection sites compared to more caudal sites.

**Figure 5.2: Injectable functionality of human ECM particles.** Particles of human ECM were suspended in saline and could be injected through a 27G needle (A). Two weeks after being injected subcutaneously, there were no signs of any pain, redness, or irritation at injection sites of ECM particles (B), Juvederm (C), or Radiesse (D), indicating initial biocompatibility.
Figure 5.3: Biocompatibility of human ECM particles. Human ECM particles initiated only a mild response from the host throughout the 3 months *in vivo*. There is negligible accumulation of inflammatory cells and little fibrous deposition. At 3 months, the injection site is beginning to be filled with adipocytes. In contrast, the Juvederm injections were surrounded by fibrous tissue at 2 weeks and were beginning to break down at 3 months, as indicated by the ingrowth of fibrous tissue. Radiesse injections triggered significant infiltration of immune cells, and were characterized by extensive fibrous tissue deposition and dispersed foreign body giant cells. Scale = 200 µm.
Figure 5.4: Human ECM particles stimulate adipogenesis. At 2 weeks, the ECM particle bolus was mildly infiltrated by fibroblast-like cells, and at 4 weeks pockets of adipocytes could be identified. These areas of adipocytes grew in number over the course of the study until significant portions of the injection bolus contained adipocytes. Several patent capillaries could be identified in the injection regions at weeks 8 and 12 as well. In contrast, Juvederm and Radiesse experienced no adipogenesis. A thickening fibrous capsule can be seen around the Juvederm injections, as well as several multinucleated cells within the Radiesse injections. Scale = 50 µm.

5.3.3. Biocompatibility in an immunocompetent animal model

A short *in vivo* trial was conducted to examine biocompatibility of the ECM particles in an immunocompetent animal and to determine whether the extended 8 week culture time was necessary for particle production. Particles from 4-week-old ECM sheets were produced in the same manner as before with the 8-week-old ECM sheets. Although the two sets of ECM particles behaved similarly *in vitro*, the ECM sheets that had been cultured for 8 weeks produced roughly 10 times the amount of particles
compared to sheets that had only been cultured for 4 weeks. Examining the DNA content of the particles revealed a nearly 4-fold difference, with particles from 4-week-old ECM sheets having $3.96 \pm 0.09 \mu g$ of DNA per mg of ECM compared to only $0.96 \pm 0.04 \mu g$ of DNA per mg of ECM for the 8-week-old sheets.

Both sets of particles were suspended in saline and injected subcutaneously into the backs of Sprague-Dawley rats, again being compared to the clinical standard Juvederm. In this pilot study, all ECM particle injections were well tolerated by the rats and caused no signs of redness, pain or irritation, further highlighting the injectable functionality of these ECM particles. At day 5, the particles from the 4-week-old sheets showed significant cellular infiltration (Fig 5.5A). However, in all injection sites there remained an area in the center of the bolus that had not yet been infiltrated by the immune cells. The 8-week sheet particles and Juvederm injections showed comparable immune responses at day 5, both of which were significantly less severe than what was seen with the 4-week sheet particles. There was a mild influx of cells around the periphery of the injection site but limited accumulation. The beginnings of a fibrous capsule were noted around the Juvederm implant and some fibrous tissue deposition could be seen in the boundary areas of the 8-week sheet particle injection (Fig 5.5B,C). Of particular interest, in all of the 8-week sheet particle injections some of the infiltrating cells appeared to be adopting a more elongated, fibroblast-like morphology in the interior of the implant region, characteristic of a more positive remodeling response than a negative inflammatory response.
5.4. Discussion

In this study we have shown the development of a novel soft tissue filler composed of human extracellular matrix particles. Using only a small skin biopsy, these ECM particles can be produced from an autologous source or potentially, from an allogeneic source if an off-the-shelf version is desired. Through the Tissue Engineering by Self-Assembly (TESA) approach, we were able to produce a complex composition of human ECM components without the use of the harsh processing methods associated with decellularization techniques that may disrupt the native conformation of the proteins \[84, 166\]. The ECM particles we developed were composed of a variety of proteins and proteoglycans characteristic of basement membrane tissues, including collagen and elastin. The degradation of these two proteins is typically responsible for the formation of wrinkles. Therefore, delivery of unmodified collagen and elastin by our ECM particles could potentially facilitate a more natural healing response.

We conducted a three month animal study to compare the function of our ECM particles compared to two clinical standards, Juvederm and Radiesse. Since the ECM
particles will be delivered as an autologous or allogenic material in the clinic and are not specifically decellularized, we chose to use athymic mice for our long-term animal studies to eliminate the confounding variable of an innate immune response to a xenograft. These studies demonstrated the injectable delivery and excellent biocompatibility profile of the ECM particles. No fibrous capsule was noted around the particles and a minimal inflammatory response, highlighting the positive integration of the particles with the surrounding tissue. These results are supported by the positive clinical outcomes seen with blood vessels that were produced using the TESA approach [167, 170]. In contrast, Juvederm and Radiesse, which are used extensively in the clinic, were associated with fibrous encapsulation and chronic inflammation, respectively. Also of note, the Juvederm injections experienced post-injection swelling. This was observed both on a macroscopic level and identified during histological analysis as edema surrounding the injection site. This phenomenon is well documented in the clinic, as clinicians are advised to underfill adipose voids to account for this variable swelling [40]. Comparatively, the ECM particles displayed no inflammation or migration from the injection site for the length of the study, providing a more accurate compensation for adipose voids in the clinic.

In addition to the improved biocompatibility, the ECM particles were also associated with *de novo* adipogenesis in the injection region. Sparse adipocytes were first noted in the injection region, in close association with the particles, as early as 4 weeks in some animals. The number and density of these adipocytes continued to increase with each time point until significant portions of the injection region were filled with adipocytes after 3 months, suggesting that the adipocytes were not merely displaced from
pre-existing tissue as a result of the injection procedure, but were truly developing within the injection region over time. Several patent arterioles were also present near the particles providing a blood supply to these adipocytes for continued development. This is in contrast to both the Juvederm and Radiesse injections, which displayed no signs of adipogenesis at any time point. The exact mechanism stimulating this adipogenesis has yet to be elucidated. However, several previous studies have suggested that biomaterials composed of basement membrane proteins are capable of stimulating adipose formation \textit{in vivo} [81, 83, 100]. Our ECM particles are composed of several components characteristic of basement membrane that were not exposed to harsh chemicals or other denaturing agents. It is possible that these unmodified proteins and proteoglycans, as assembled by human fibroblasts, are positively interacting with the surrounding tissue to stimulate adipose formation.

Finally, initial results from our Sprague-Dawley rat studies further indicated a biocompatible response for the ECM particles that was comparable to a clinical-standard soft tissue filler in Juvederm. This is especially significant considering that these human-produced particles were injected into an immuno-competent animal (xenograft) without a specific decellularization step, yet did not initiate a severe reaction. The increased immune response to particles from younger ECM sheets could possibly be attributed to their 4-fold increase in DNA content. Furthermore, the mild response to the older ECM sheet particles compared to younger ones suggested that there exists a threshold level of cellular material to trigger an inflammatory response. However, the actual component causing the increased cellular infiltration and its threshold level remain to be elucidated. Due to the popularity of decellularization techniques, several groups have offered
guidelines for the amount of remaining DNA that is acceptable for a decellularized graft [120, 166]. While it is well-established that excessive cellular remnants can trigger inflammatory responses, the results from this pilot study suggest that a higher threshold may exist than previously considered.

The author of this dissertation is the primary author of this manuscript.
CHAPTER 6:

Conclusions and future directions for adipose tissue engineering
6.1. Summary of work

As has been seen throughout this dissertation, a variety of materials have been examined for adipose tissue engineering. However, it has not been until just recently that these materials were designed specifically to regenerate adipose tissue. In 2008, we set out to decellularize human adipose tissue in order to develop a novel biomaterial that closely recapitulated the biochemical microenvironment of adipose tissue: a concept that had not yet been previously reported. While we were not the only group that conceived this idea, we were the first to publish a method for decellularizing human lipoaspirate and to reduce it to a liquid formulation capable of injectable delivery. We were then able to show that this adipose matrix hydrogel was capable of not only delivering ASCs into an animal but also stimulating natural adipogenesis \textit{in vivo}, either with or without the exogenous ASCs. These advancements provided a significant benefit to the field, representing one of only a couple clinically translatable materials capable of stimulating \textit{de novo} adipogenesis in an animal.

We also sought to examine the biochemical and biomechanical mechanisms responsible for driving adipogenesis of ASCs. From our work and the work of others in the field, we have concluded that the extracellular matrix of adipose tissue is capable of stimulating differentiation of ASCs toward an adipogenic lineage. Therefore, these decellularized adipose materials likely provide a template for natural adipogenesis of both endogenous and exogenously supplied ASCs. We expanded on these results by showing that the mechanical environment surrounding ASCs also plays a role on adipogenesis. By keeping human ASCs on a substrate that resembles the stiffness of adipose tissue, and therefore reduces intracellular tension, we were able to drive
adipogenesis of the cells without using stimulatory chemicals, a feature that had not previously been shown. With the increasing use of ASCs within the clinic to augment autologous lipotransfer procedures, the ability to encourage adipogenesis of ASCs prior to implantation without having to use soluble chemical factors could offer a significant improvement. These results also suggest that novel materials for adipose tissue engineering should emulate adipose biochemical and biomechanical properties in order to encourage \textit{de novo} adipogenesis of ASCs.

Lastly, we described the development of a second novel biomaterial for adipose tissue engineering using a particle-based approach instead of a hydrogel. By developing these ECM particles, we were still able to offer the benefit of encouraging adipogenesis with basement membrane components but also could take advantage of the increased stability of the particles and avoided the use of potentially damaging decellularization chemicals. This provided a material better suited for the treatment of deep wrinkles that also need to provide immediate structural support, as well as an adipogenic response and neovascularization.

6.2. Future directions for adipose tissue engineering

The field of adipose tissue engineering has experienced substantial growth over just the past five years. Part of this rapid progression can be attributed to the growing demand for soft tissue filler products and part is simply the extension of established tissue engineering principles into a field that was lacking adequate solutions for adipose deficits. The emergence of decellularized adipose tissue provides a promising, versatile biomaterial to be investigated for adipose regeneration (Fig 6.1). It has already been
shown to be a biocompatible scaffold capable of stimulating adipose differentiation of ASCs and *de novo* adipogenesis in animal models. It can also be fabricated into various forms, including self-assembling gels, powders, beads, and foams [139, 141, 142, 156]. However, as with all biologically-derived materials, it does breakdown over time in the body if it is not modified to some degree. Because a majority of the clinical applications for these materials will involve sustained fulfillment of an adipose deficit, it will be imperative to tightly control the degradation of these adipose ECM-derived biomaterials. Future research will be needed to minimize the degradation of the scaffold or to ensure that the degradation is matched to the in-growth of regenerating tissue. Alternatively, decellularized adipose ECM could be combined with other biomaterials in order to match its biocompatibility and adipo-inductive properties with other desirable degradation or mechanical properties.

Alongside material development, there is also a need to design better animal models that better reflect the clinical setting. As seen previously, the diversity of species and strains currently being used in different laboratories precludes direct comparisons of the various materials and results being published. Furthermore, most laboratory rodents possess limited innate adipose tissue. Because several studies have demonstrated the influence of mature adipose tissue within the proximity of an experimental material, a standardized injection site should be established for mouse and rat models in order to better control confounding variables. Subcutaneously implanted materials will likely experience drastically different mechanical loading, cytokine stimulation, and degradation rates in caudal vs cranial and ventral vs dorsal locations. Additionally, the development of a breast tumor resection model would also be beneficial for assessing the
performance and behavior of biomaterials within an implant site that has already experienced a traumatic injury. These improvements to adipose tissue engineering models will help narrow the gap between laboratory experiments and clinical translation.

Incorporation of ASCs into various adipose tissue engineering strategies has also become a popular trend recently. These cells, being derived from adipose tissue, are an ideal candidate for clinical adipose tissue engineering strategies. In fact, anecdotald clinical evidence has surfaced suggesting their ability to improve fat graft retention, stimulate subcutaneous vascularization, and even improve the appearance of skin. However, there are several ongoing regulatory debates that could dramatically affect the fate of ASC clinical usage. In terms of allogeneic usage, several issues must be addressed in terms of reproducible and sterile harvesting techniques, cell storage, and donor variability. In fact, the Supreme Court case of United States v Regenerative Sciences is currently debating the role of the FDA in regulating the use of autologous stem cells in a clinical setting [171]. If this case rules that ASCs should indeed be classified as a “drug” by the FDA, then any procedure involving ASCs will necessitate clinical trials and pre-market approval by the FDA. While these measures will help ensure the safety of the procedures for patients, they will also significantly lengthen the timeline for establishing these procedures in the clinic. Therefore, while ASCs offer significant promise for adipose tissue engineering strategies, there is still much work to be done before they can become a clinically viable resource

Based on the current research, several design criteria have begun to emerge that can guide future adipose tissue engineering strategies. First of all, the material should be biocompatible, integrating well with the surrounding tissue without triggering a chronic
inflammatory response. It should also be durable and provide a long-term solution to adipose deficits without the need for repeated treatment. Part of this solution could be to stimulate natural adipose regeneration at the site of implantation. Our work and the work of others in the field suggest that ECM-based materials that recapitulate the microenvironment of adipose tissue offer the natural encouragement for this de novo adipogenesis. In this case, it is also imperative for the strategy to facilitate neovascularization while promoting the formation of new adipose tissue. This last scenario would represent the pinnacle result for adipose tissue engineering, by providing the patient with a natural means to replace lost adipose tissue with healthy, mature, and functional fat tissue. With the development of decellularized adipose ECM materials and a better understanding of adipogenesis, this goal could become an achievable milestone for tissue engineering within the near future.
Figure 6.1: Various forms of decellularized adipose tissue. Within just the past 5 years, the process of decellularization of human adipose tissues has become an important topic within the field of adipose tissue engineering, offering the ability to generate scaffolds from adipose ECM. Several different forms of this adipose ECM have been developed, ranging from injectable gels and powders (A), to beads (B), to microporous foams (C), and even solid three-dimensional structures in a variety of shapes and sizes (D). Figure adapted from Turner et al, Yu et al, and Choi et al with permission.[139, 142, 156, 172]
Chapter 6, in part, is in preparation for submission for publication as: D. Adam Young, Brian Mailey, Jennifer Brown, Anne M. Wallace and Karen L. Christman. Adipose tissue engineering and stem cells. 2013.

The author of this dissertation is the primary author of this manuscript.
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