AN ADIPOSE EXTRACELLULAR MATRIX-DERIVED BIOMATERIAL FOR SOFT TISSUE RECONSTRUCTION

by

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ABSTRACT

Soft tissue defects can result from traumatic injury, congenital defects, and medical procedures such as tumor resection. Patients who require soft tissue reconstruction have limited options and often must use autologous tissue for reconstruction, which has the adverse effects of donor site morbidity. This study explores the development of an adipose extracellular matrix – based biomaterial for the repair of soft tissue defects. The adipose extracellular matrix (ECM) is obtained through mechanical and chemical treatments to remove all the cells and lipids from intact human adipose tissue. The degradation properties of the ECM-based biomaterial can be modulated with chemical crosslinking, allowing for control over the degradation rate of the biomaterial. Material characterization of the adipose ECM was carried out through proteomics, mechanical testing, and thermal analysis. The adipose matrix supported adipose stem cell growth and differentiation in vitro, providing tissue-specific bioactive cues to improve adipogenic differentiation. In vivo biocompatibility studies showed minimal inflammatory response to the biomaterial and stable volume retention through 12 week studies. When compared with the clinical standard of fat grafting, the acellular adipose matrix demonstrated the advantages of facilitating new adipose tissue regeneration rather than relying on transplantation of autologous fat which has the risk of necrosis, cyst formation and calcification due to the limited viability of transplanted adipocytes. The adipose ECM can also be used with adipose stem cell delivery, providing a protective environment for stem cell engraftment, which in turn facilitates new adipose tissue formation. The adipose matrix provides a versatile biomaterial for soft tissue regeneration hat can be used for allogenic applications, circumventing the need for autologous tissue for reconstruction.

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1. Introduction

Adipose tissue occupies a unique role in providing structural support, establishing contour, and serving as a key energy reservoir for the body. As one of the most dynamic tissues of the body, the size of the adipose organ can vary considerably both between individuals and within a single individual's lifetime. To accommodate the drastic changes in adipocyte size based on an individual's metabolic state, the extracellular matrix of adipose tissue is highly active and must undergo constant remodeling to support the parenchymal cells of adipose tissue. These reasons, along with the availability of adipose tissues from common plastic surgery procedures such as abdominoplasty and brachioplasty, make the adipose extracellular matrix an intriguing source for developing a biomaterial for soft tissue regeneration.

The field of regenerative medicine develops biomaterials and cell-based approaches to regenerate damaged or diseased tissues. Current clinical strategies for soft tissue reconstruction highlight the need for regenerative medicine-based therapies. Patients who have significant soft tissue loss often require autologous flap reconstructions whereby tissue is removed from a donor site to repair the defect, resulting in donor site morbidity. Improved aesthetic outcomes have important effects on quality of life and physical and psychological aspects of a patient's well-being. Development of an off-the-shelf biomaterial for soft tissue regeneration could provide an easily available source for replacing soft tissue in cases of trauma or tumor resection.

In the following dissertation, the author investigated the use of an adipose-derived biomaterial for application in soft tissue regeneration. The extracellular matrix provides a promising scaffold for adipogenesis as it carries out many of the supportive functions for adipocytes in vivo, providing not only a biocompatible surface for cell adhesion and growth, but also retaining bioactive cues for maintaining adipose tissue function. The extracellular matrix is isolated through a series of mechanical and chemical treatments to remove all antigenic components including cells and lipids, leaving an acellular biomaterial that can be used allogenically without eliciting a severe inflammatory response.

Due the shortcomings of current reconstructive options, many researchers have tackled the challenge of engineering adipose tissues. Chapter 2 of this thesis reviews the current literature and state of the art in tissue engineering approaches to adipose tissue regeneration. In Chapter 3, we discuss the development of an adipose extracellular matrix-based biomaterial and the design considerations framing the process. Additionally, we characterize the biomaterial through proteomic, mechanical and thermal analysis. Chapter 4 evaluates the in vitro bioactivity of the matrix in supporting cell migration, growth, and differentiation on the scaffold and explores tissue specificity of the adipose matrix through comparison with dermal extracellular matrix. In Chapter 5, we investigate the in vivo biocompatibility of the biomaterial and its long term efficacy in retaining a stable volume. To further evaluate the adipogenic inductive capacity of the matrix, we delivered it in conjunction with adipose-derived stem cells to evaluate neoadipogenesis in vivo.

The extracellular matrix of tissues has been found to perform an increasing number of roles in supporting tissue function. Through our studies, we demonstrate a way to utilize it as a blueprint for tissue regeneration by taking advantage of its innate bioactivity. As a natural scaffold for soft tissue reconstruction, the adipose extracellular matrix is an easily accessible biomaterial with the potential to facilitate soft tissue repair.

2. Adipose Tissue Engineering

2.1 Introduction

Soft connective tissues function to provide structural support and establish contour. Soft tissue defects present a challenging problem as the aesthetic function is intricately linked with the patient's psychological well-being. As such, the benefits of improved aesthetic outcomes must be weighed against the risks from the complicated reconstructive surgeries often required. Soft tissue defects can arise from trauma, medical conditions, or after ablative procedures such as tumor resection. In addition to the aesthetic function, there are also many negative biological sequelae of scar formation involved in soft tissue loss and reconstruction.[1]

Traumatic injuries such as those sustained from explosive devices and automotive accidents often result in extensive soft tissue loss. Laceration and dog bite repair account for over 320,000 reconstructive cases performed in 2012, making it the second most common condition requiring reconstructive surgery.[2] Despite advances in reconstructive procedures, regeneration of damaged soft connective tissues has yet to be achieved and autologous flap reconstructions are often the only option for replacing areas of soft tissue loss. Significant contour defects and scarring often persist, resulting in poor aesthetic outcomes for individuals who have sustained soft tissue trauma.

In addition to traumatic injury, soft tissue loss can also arise secondary to medical conditions or as a result of surgical management of cancer. Lipodystrophy is a condition characterized by dysfunction or degenerative loss of adipose tissue and can lead to severe insulin resistance and dyslipidaemia.[3] There are both acquired and congenital forms and it is often associated with antiretroviral therapy for HIV patients. Patients with this condition have a unique need for soft tissue replacements as they typically do not have adequate autologous adipose tissue available for grafting. Tumor resection is the most common reconstructive surgery carried out, with over 4.2 million operations performed annually for tumor removal.[2] Without a suitable soft tissue replacement, patients typically opt for contralateral reduction, prosthetic implants or autologous flap reconstructions to restore symmetry after lumpectomy or mastectomy procedures.[4] The limited options available highlight the need for an alternative that avoids the risk of complications for prosthetic implants and negates the prerequisite of donor site tissue for flap reconstruction.

2.1.1 Current clinical strategies

For small volume corrections, dermal fillers or fat grafting can be used to fill the defect space and restore contour. Dermal fillers range from naturally occurring components of the extracellular matrix such as collagen, cross-linked forms of hyaluronic acid, and calcium hydroxyapatite, to synthetic polymers including poly-lactic acid and polymethylmethacrylate.[5] However, most dermal fillers are not permanent and require patients to return regularly for repeat injections to maintain a proper volume correction and there are a number of known complications that can occur with application of specific fillers.[6]

Autologous fat grafting is another option and involves harvesting tissue by liposuction at one site of the body and reinjecting the lipoaspirate to fill a soft tissue defect. The procedure suffers from inconsistent outcomes due to highly variable rates of resorption. To rectify the unpredictability of graft take, several groups have established protocols to standardize and

improve outcomes through harvest, preparation, and grafting techniques.[7,8] However, there are still complications that can arise from central necrosis of the transplanted lipoaspirate as poor viability results from rupture of adipocytes and lack of oxygen and vascular supply post transplantation.[9] Cyst formation and calcification often occur secondary to the necrosis and can interfere with radiological detection of breast cancer.[10]

One of the most common reconstructive procedures to correct loss of soft tissue volume is autologous flap reconstruction, where a tissue flap from a donor site in the body is used for the reconstruction. Advances in reconstructive surgery have decreased the associated morbidity by attempting to keep the donor site muscle intact when possible.[11] However, the basic requirement of a donor site to repair a soft tissue defect at another location in the body inevitably results in donor site morbidity.[12,13] Availability of donor tissue can also be a limiting factor for patients who are thin or those who have multiple injury sites. Patients who undergo mastectomy have the additional option of prosthetic implants. However, complications can arise including capsular contracture, implant rupture, and infection, often requiring a second surgery to resolve the problem.[14]

2.2 Biomaterials

2.2.1 Synthetic polymers

Synthetic polymers commonly used in numerous biomedical devices offer the distinct advantage of high level of control over the chemical properties of the polymer. As a scaffold for adipose tissue engineering, polylactic acid, polyglycolic acid and copolymers incorporating both have been widely investigated due to their ability to degrade over time. These polymers degrade by bulk hydrolytic degradation of the ester bonds and the rate of degradation can be modulated by changing the molar mass and molar ratios of the monomers.

Cells can be combined with synthetic polymers to create a cell-scaffold construct for adipose tissue engineering. Several groups have seeded 3T3-L1 preadipocytes[15-17] or adipose-derived stem cells[18,19] on polyglycolic acid meshes and observed adipogenesis over time both in vitro and in vivo. Electrospun polylactic acid nanofibers were shown to support adipogenic differentiation of bone marrow stem cells in vitro. The copolymer of poly(lactic-co-glycolic acid) (PLGA) is more commonly used since the degradation rate of the polymer can be modulated by altering the molar ratios of the two monomers. Injectable PLGA spheres were used to deliver stem cells and it was found that pre-differentiating stem cells attached to the PLGA spheres improved the adipose tissue formation in vivo.[20-23] In a different model of adipose tissue formation, PLGA was loaded in closed chambers incorporating a vascular pedicle for angiogenesis but researchers mainly observed granulation and fibrous tissue formation.[24,25] PLGA hollow fibers were also used for formation of cell aggregates and adipogenic differentiation of stem cells showing cells could persist in the hollow fibers in vivo.[26] Other groups have also used solid forms of PLGA scaffolds where cells are seeded directly on the scaffold and differentiated towards adipogenesis before in vivo application.[27-31]

In addition to the solid polymer scaffolds previously described, polymers can also take the form of a hydrogel. Hydrogels are characterized as water-soluble polymers that can be crosslinked to form a hydrogel scaffold that shows high swelling properties in aqueous solutions. Polyethylene glycol is a commonly used hydrogel in which cells are typically encapsulated within the hydrogel prior to crosslinking. Photopolymerized polyethylene glycol hydrogels supported adipogenic differentiation of embryonic[32] and adult mesenchymal stem cells[33] although it was found that the addition of bFGF to hydrogel scaffolds greatly improved vascularization and cell infiltration in vivo.[34]

2.2.2 Naturally derived scaffolds

Scaffolds for tissue engineering can also be derived from natural sources and generally have high biocompatibility. Some examples of naturally derived biomaterials are alginate, silk and chitosan. Alginate is a natural polysaccharide derived from seaweed. Stem cells differentiated along the Adipogenic lineage were encapsulated in alginate and implanted subcutaneously.[35] To render alginate susceptible to degradation, an oxidized form of alginate was also investigated[36] as well as combined collagen alginate microspheres to better mimic the native extracellular matrix environment.[37] Similar to alginate, chitosan is a natural polysaccharide that is isolated from chitin. So far researchers have used it in combination with hyaluronic acid to form an injectable hydrogel[38] or with PLGA to form a porous scaffold[31] for adipose tissue engineering. Silk is another naturally derived scaffold that is fibrous in nature and has high mechanical strength. In adipose tissue engineering, it has been applied as a scaffold for adipogenic differentiation of stem cells and subsequently implanted in rodent models.[39-42] The silk scaffolds alone produced a fibrous tissue, but when seeded with adipogenic differentiated cells, maintained their adipogenic state with lipid-laden cells observed over time.

2.2.3 Extracellular matrix - based materials

A subset of naturally derived scaffolds are extracellular matrix-based biomaterials. This includes extracted components of the extracellular matrix such as collagen, dextran, fibrin, matrigel and hyaluronic acid, as well as bulk extracellular matrix obtained through decellularization procedures. These natural ECM-based scaffolds typically have high biocompatibility, and since they originate from the matrix, they contain the cell-binding domains required for the survival and growth of anchorage-dependent cells.

Collagen

Collagen is perhaps the most studied, as it forms the structural basis for much of the extracellular matrix of our tissues. Collagen can be used as a scaffold in which cells can be seeded directly on

the collagen matrix and differentiated with adipogenic media[43,44] Collagen sheets with an elastin component have also been tested for adipose engineering, based on a scaffold designed to be a skin substitute[45] Collagen sponges are a porous form of the collagen based scaffold and provide increased surface area and permeability.[46,47] A few groups have also incorporated gelatin microspheres in the collagen scaffolds for controlled release of bFGF in a collagen sponge[48] and collagen gel[49] to improve adipogenesis. Collagen microparticles or beads have been fabricated and used as microcarriers for cell delivery.[50,51] In a modified design based on the collagen gel, short embedded collagen fibers were incorporated in a collagen gel to improve structural support and limit cell-mediated contraction of the constructs.[52]

Gelatin

Gelatin is derived from collagen, typically from bovine sources, and can be used in a number of forms similar to collagen. For application in adipose tissue engineering, it has primarily been used as a gelatin sponge onto which cells are seeded and differentiated before in vivo implantation.[19,43,53,54] Gelatin has also been used as microparticles for controlled release of bFGF in several systems to aid adipogenesis.[48,49,55,56]

Fibrin

Fibrin is a blood component involved in the clotting process and can be used as a matrix for tissue engineering applications. Polymerization of fibrin is initiated with thrombin, and the fibrin gel can be naturally degraded in vivo. Fibrin matrices have been used for culture of cells in vitro as well as in vivo delivery for stem cells and in conjunction with endothelial cells.[50,57-63] One of the groups using fibrin matrices has also observed that providing a mechanical support for the developing construct greatly enhances generation of new adipose tissues.[59,60]

Hyaluronic acid

Hyaluronic acid (HA) is a linear polysaccharide and is a natural component of the extracellular matrix. Hyaluronic acid can be chemically modified to modulate its material and biological properties, making it a popular choice as a biomaterial for tissue engineering. A hyaluronic acid sponge fabricated from an ester derivative of hyaluronic acid allowed evaluation of the adipogenic potential for sponges with varying pore sizes and degrees of esterification.[64-66] In another formulation, a hyaluronic acid gel was formed with varying degrees of amidation of carboxyl groups and studied for application in adipose tissue engineering.[67] The two were compared in a study that investigated the in vivo response to the scaffolds alone and found less inflammation in the HA gels with dodecyl amidation and improved adipogenesis compared to the esterified HA sponges.[68] Additionally, cross-linked HA can be used as a hydrogel for adipogenesis[69] and can be combined with extracts from adipose tissue to improve bioactivity.[70] A polyethylene glycol- HA composite with encapsulated VEGF in heparin nanoparticles was investigated by one group to see if the added growth factor would enhance adipogenesis.[71] In a different approach to develop a material that can be modulated by physiological signals, a chitosan-HA hydrogel was developed for insulin delivery in a glucose responsive hydrogel upon swelling.[38] Through this design, the scaffold could deliver insulin, an adipo-inductive factor, locally after swelling.

Matrigel

Some of the most successful cell-free approaches to adipose tissue regeneration use Matrigel, a basement membrane extract derived from the EHS sarcoma in a mouse. Researchers found that subcutaneous injections of Matrigel with bFGF resulted in de novo adipose tissue formation, regardless of the site of injection.[72,73] Gelatin microspheres have also been used for controlled release of bFGF in Matrigel and were found to be more effective at lower concentrations of bFGF compared to doping the bFGF directly in Matrigel.[55,56] Other groups have also established

a model in which Matrigel and bFGF are enclosed in a chamber in which a vascular pedicle is inserted to observe adipogenesis in a closed environment isolated from the surrounding tissue such that only circulating cells from the vasculature would contribute to new tissue formation.[24,74,75]

Tissue based protein extracts

Extracellular matrix proteins are a promising class of biocompatible scaffold materials since they comprise the natural environment for cells in the body. Similar to the approach of extracting basement membrane proteins from the EHS sarcoma to produce Matrigel, investigators have also explored using different tissues as the starting material to form tissue extracts composed of extracellular matrix proteins. Groups have isolated proteins from the adipose matrix in a similar preparation to Matrigel, producing a protein extract enriched for adipose matrix components that can form a hydrogel at physiological temperature.[76,77] Additional tissue-derived protein extracts have been produced using dermis,[78] skeletal muscle,[79] and cardiac muscle.[80] Adipose stem cells grown on these tissue-derived hydrogels show spontaneous adipogenic differentiation, suggesting that bioactive factors from the tissues partition with these extracted components. Another group has also incorporated adipose tissue extracts into electrospun nanofibers, showing that the nanofibers support stem cell attachment and survival.[81]

Bulk decellularized extracellular matrices

Another class of extracellular matrix-based biomaterials is derived through decellularization of bulk tissues to obtain an acellular matrix. Typically tissues are processed with various solvents such as detergents, acids, and enzymes to remove the cellular component and prevent antigenicity. Lipoaspirate taken from patients undergoing liposuction procedures can be decellularized and milled to a powder to produce an injectable adipose ECM powder.[82-85] Adipose ECM powders can be formed into a thermoresponsive hydrogel by acid solubilization so that it undergoes gelation upon injection.[86] Intact adipose tissue from patients who undergo

procedures where bulk tissue is excised, such as abdominoplasty or brachioplasty procedures, can also be decellularized to produce an extracellular matrix that remains intact.[87,88] This adipose extracellular matrix can also be solubilized using pepsin and formulated into microcarrier beads[89] or foams[90] for adipose tissue engineering. Placental tissue has also been decellularized and explored as a tissue source for use as a scaffold for adipose tissue regeneration.[69,91,92]

2.3 Cell sources

2.3.1 Cells of adipose tissue

The parenchymal cells of adipose tissue are adipocytes, terminally differentiated lipid-filled cells providing a key energy reserve for the rest of the body. Although they are the primary cells transplanted in fat grafting procedures, their inability to replicate, high metabolic demands, and fragile nature limit their utility in adipose tissue regeneration. As an alternative, many researchers have looked to progenitor cells that can be readily expanded and are capable of differentiating into adipocytes. Another prominent cell type in adipose tissue is endothelial cells due to the extensive vascularization of adipose tissue. Each adipocyte in the body has immediate access to at least one blood vessel, and the processes of adipogenesis and vascularization are tightly coupled during development. Endothelial cells have often been used in conjunction with adipose stem cells to encourage vascularization of constructs for nutrient diffusion to cells and to encourage adipogenesis.[37,41,57,62,93]

2.3.2 Adult stem cells

One of the early sources of adult stem cells to be investigated was bone marrow. A subset of multipotent progenitor cells was found to reside in the bone marrow niche and could be induced to differentiate down specific mesenchymal lineages.[94] The bone marrow aspirate is collected from the iliac crest, followed by selection for plastic-adherent cells.

Adipose-derived stem cells present an intriguing cell population for adipose tissue engineering due to the relative ease of access for cell harvesting by liposuction. A resident

population of multipotent cells has been identified in the stromal vascular fraction of adipose tissue and is likely to be the compartment in which adipocyte precursor cells reside. These adipose-derived cells have also been shown to differentiate down multiple mesenchymal lineages including adipose, bone, cartilage, neurons, and myocytes. In comparison to mature adipocytes, ASCs are easily expanded in culture and have a fibroblastic morphology. When exposed to adipogenic induction media, the cells begin to undergo adipogenic differentiation and start accumulating lipid.

The adipose-derived stem cells are typically isolated from adipose tissue by enzymatic digestion, generating a heterogeneous cell population of stromal vascular cells. Adipose-derived stem cells express many of the mesenchymal stem cell surface markers, including CD 13, CD 29, CD 44, CD 71, CD 73, CD 90, CD 105, and STRO-1 while being negative for the hematopoietic lineage markers CD14, CD16, CD45, CD56, CD61, CD62E, CD104, CD106, and endothelial markers CD31 and von Willebrand factor.[95] Initially a heterogeneous population of stromal vascular cells, selection of the plastic adherent population depletes many cells of hematopoietic origin. After in vitro expansion over several passages, cells tend to show decreased expression of CD11, CD14, CD34, CD45, CD86, and HLA-DR while becoming uniformly positive for the other stromal cell surface markers with successive passages, suggesting that a more homogeneous population of cells is established over time.[96,97] Continuing development of point of care systems to automate stromal vascular cell isolation can potentially allow the use of autologous cells for treatment to be widely adopted in the clinic.

2.3.3 Embryonic stem cells

Embryonic stem cells are pluripotent cells that have high proliferative capacity in culture and can be expanded through far more passages compared to adult stem cells without reaching senescence. The cells are isolated from the inner cell mass of blastocysts and can be differentiated towards numerous somatic cells with varying phenotypes. The use of embryonic

stem cells in research has been fraught with controversy and is subject to political policies that can limit access to their use.

2.3.4 Induced pluripotent stem cells

In 2006 researchers discovered that it was possible to induce adult somatic cells to revert to an embryonic-like state by genetically reprogramming the cells to express the transcription factors Oct3/4, Sox 2, and Klf4.[98-100] Cells expressing these factors exhibited key characteristics of embryonic stem cell pluripotency, including embryoid body and teratoma formation, and proved capable of differentiating into cells of all three germ layers. As a result, any adult somatic cell may be used as a cell source, thereby circumventing the ethical issues and political influences controlling the use of embryonic stem cells in research.

IPS cells have been successfully differentiated down the adipogenic lineage showing similar efficacy as embryonic stem cells for adipogenic differentiation.[101] Thus far, the use of iPS cells in adipose tissue engineering has been limited, and consistent directed differentiation of cell populations still remains a challenge.

3. Development of an Adipose Matrix – Based Biomaterial

3.1 Introduction

There is a significant need for soft tissue replacements in the field of reconstructive surgery. Autologous tissue is currently the treatment of choice for repairing soft tissue defects due to trauma, tumor resection, and congenital defects.[4,102,103] However, current autologous fat transfer techniques have a number of limitations. For many HIV patients suffering from lipodystrophy and patients undergoing chemotherapy for cancer, the associated cachexia leaves them without the adipose volume required for autologous tissue transfer. Additionally, the persistence of fat transfer varies widely and is often surgeon, technique, and patient dependent. With resorption causing up to 40-55% reduction of total graft volume over six months, multiple procedures are often required in order to maintain the desired correction and volume limitations need to be taken into account to prevent necrosis.[104] Finally, implanted adipose tissue can lead to post-operative calcifications which is detrimental to patients with a history of breast cancer as this may interfere with early radiologic detection of cancer.[105]

Developments in plastic surgery and microsurgical techniques have established autologous tissue flaps as excellent options for post-mastectomy breast reconstruction, offering patients outstanding aesthetic outcomes without the concern for long-term alloplastic implant complications such as capsular contracture and rupture.[4,13] However, a complex procedure like

autologous flap transplantation carries its own set of risks including necrosis of transplanted tissue[106] and donor site morbidity, such as abdominal wall weakness, scarring, and hernia.[4,107-109] Perforator flap techniques and new imaging protocols have been developed to help leave the musculature intact in order to decrease donor site morbidity; nevertheless, they remain complex and lengthy undertakings.[11,110-113]

Regenerative medicine approaches to soft tissue reconstruction provide a potential alternative in which biomaterials facilitate tissue regeneration in the defect area, thus avoiding the need for tissue transplantation from a donor site. Several studies have investigated the use of various synthetic polymers in conjunction with a cell delivery using polyethylene glycol, poly L-lactic acid, polyglycolic acid, and poly(lactic-co-glycolic) acid copolymers.[15,21,22,26,27,29,32,33,59,114] Naturally-derived polymer scaffolds such as collagen, hyaluronic acid, gelatin, silk fibroin, alginate, and fibrin have also been applied to adipose tissue engineering.[35,42,48,61,65-67,115] Additionally, researchers have tried to harness extracellular matrix (ECM) components for use in adipose tissue engineering using ECM derived from EHS sarcomas (Matrigel), adipose tissue, dermis, muscle, and placenta. [55,72,75,76,79,83,91,92,116,117] However, a recent review highlights the lack of three-dimensional scaffolds derived from adipose tissue.[118]

Here we show the development of a novel adipose-derived extracellular matrix biomaterial that serves as a three-dimensional scaffold for tissue regeneration. Intact ECM-based biomaterials have not been previously studied *in vivo* for use as a volume-stable soft tissue implant that can be crosslinked to control degradation properties.[118,119] We hypothesized that intact human adipose tissue could be decellularized to obtain a matrix that would provide an instructive environment for the repair of soft tissue defects. To control the rate of degradation, chemical crosslinkers can be used to reinforce the collagen and other proteins in matrix-based scaffolds. Thus, the aims of this study are to develop a biocompatible adipose-derived biomaterial by removing lipids and resident cells from adipose tissue, use crosslinkers to

modulate degradation properties of the extracellular matrix, and evaluate the biocompatibility of the matrix in vivo.

3.2 Materials and Methods

Adipose extracellular matrix was produced by decellularizing adipose tissue using a combination of mechanical and chemical processing steps. Upon obtaining the acellular adipose tissue, the material was characterized, before being crosslinked to modulate degradation profiles. In vitro and in vivo biocompatibility studies were performed to investigate the cell and tissue response to the adipose-derived biomaterial. Materials were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

3.2.1 Adipose extracellular matrix preparation

Subcutaneous adipose tissue was subjected to mechanical processing and extensive rinsing which was followed by incubation with 0.1%, 1%, 3%, or 5% peracetic acid for 3 hours to remove cells. Samples were brought back to physiological pH using PBS and incubated overnight with 1% Triton X-100 in 2 mM EDTA to remove residual lipids. Finally, samples were treated with 600 U/mL DNase (Roche, Indianapolis, IN) in 10 mM $MgCl_2$ overnight at 37°C.

3.2.2 Material characterization

Biochemical assays

Adipose ECM scaffolds were lyophilized and digested in a 125 ug/mL papainase solution (Worthington) for 16 hours at 60°C. DNA content was determined by fluorescence intensity using 1 ug/mL Hoechst dye (Molecular Probes, Eugene, OR) in TNE buffer.[120] Total collagen content was determined by a hydroxyproline assay with acid hydrolysis in 6N hydrochloric acid at 115°C, followed by reaction with *p*-dimetylaminobenzaldehyde and chloramine-T hydrate to obtain an absorbance reading at 525 nm.[121]

Scanning electron microscopy (SEM)

Samples were prepared for SEM by fixing in 3.0% formaldehyde/1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer with 2.5% sucrose for 1 hour. Samples were then post-fixed with 1% osmium tetroxide for 30 minutes before dehydration with graded ethanol solutions. Samples were dried using CO_2 critical point drying, which was followed by sputter-coating with platinum and imaging with a FEI Quanta 200 SEM (Hillsboro, OR).

Proteomic analysis

Adipose extracellular matrix samples were prepared for mass spectrometry analysis by cryomilling (SPEX Sample Prep, Metuchen, NJ) and solubilizing in 4 M guanidine HCl and 50 mM sodium acetate at pH 5.8 with vortexing for 48 hours. 100 ug of protein quantified with the BCA assay was moved to a new eppendorf tube and diluted down to 0.5 M guanidine HCl using distilled water. Samples were reduced by adding 2 ul of 50 mM TCEP, incubated at 37°C with vortexing for one hour, alkylated by adding 1 ul of 0.1 M MMTS, and incubated at room temperature for 15 minutes. Deglycosylation was carried out using 5 ul of deglycosylation enzyme mix (New England Biolabs, Ipswich, MA), which includes 500,000 U/ml PNGaseF, 40,000,000 U/ml O-Glycosidase, 50,000 U/ml Neuraminidase, 8,000 U/ml β1-4 Galactosidase, and 4,000 U/ml β-N-acetylglucosaminidase. Samples were incubated at 37°C for 4 hours to complete deglycosylation. Samples were digested overnight with trypsin (Promega) at a 1:50 ratio and maintained at 37°C with vortexing. In the morning a second aliquot of trypsin at a 1:50 ratio was added and samples were incubated at 37°C for an additional 4 hours.

For 1D SDS-PAGE gels, protein samples were boiled with Laemmli sample buffer (Bio-Rad) containing β-mercaptoethanol for 10 minutes at 100°C. Samples were loaded on Criterion 4-20% Tris-HCl gels (Biorad) and run for 50 minutes at 200 volts on a Criterion Cell (Bio-Rad). Protein bands were visualized with silver staining (Thermo Scientific Pierce).

Protein identification by liquid chromatography tandem mass spectrometry (LCMS/MS) analysis of peptides was performed using an LTQ Orbitrap Velos MS (Thermo Scientific) interfaced with a 2D nanoLC system (Eksigent). Peptides were fractionated by reverse-phase HPLC on a 75 um x 10 cm PicoFrit column with a 15 um emitter (PF3360-75-15-N-5, New Objective) in-house packed with Magic C18AQ (5 μm, 120Å, Michrom) using 1-45% acetonitrile/0.1% formic acid gradient over 90 min at 300 nl/min. Eluting peptides were sprayed directly into an LTQ Orbitrap Velos at 2.0 kV. Survey scans (full ms) were acquired from 350-1800 m/z with up to 10 peptide masses (precursor ions) individually isolated with a 1.2 Da window and fragmented (MS/MS) using a collision energy of HCD35, 30s dynamic exclusion. Precursor and the fragment ions were analyzed at 30,000 and 15,000 resolution, respectively. Peptide sequences were identified from isotopically resolved masses in MS and MS/MS spectra and searched against all human entries in RefSeq 2012, with oxidation on M, carbamidomethylation on C and Mascot Daemon (Matrix Science) software. Mass tolerances on precursor and fragment masses were 10 ppm and 0.03 Da, respectively. Mascot search result files were processed in Scaffold (Proteome Software) or Proteome Discoverer to validate protein and peptide identifications.

Differential Scanning Calorimetry

Differential scanning calorimetry was carried out using a DSC 8500 (Perkin Elmer) to determine the thermal properties of the adipose matrix through the different processing steps and determine whether the terminal sterilization process of gamma irradiation has a detrimental impact on the collagen structure. Hydrated samples between 3-10 mg were loaded onto sample pans and hermetically sealed. The endotherm was measured against an empty reference pan from -20°C to 95°C at a rate of 5° per minute with two heating cycles per sample.

Rheology

The rheological properties were tested using an ARES-G2 Rheometer (TA Instruments). 1 ml samples were loaded onto 25 mm parallel plate fixtures with the gap set to 1.0 mm for each run. A strain sweep was run at frequencies of 0.1, 1, 10, 100 rad/s and the strain percent for subsequent runs were set based on the linear viscoelastic region. Dynamic frequency oscillation measurements were taken over an angular frequency range of 0.1 to 100 rad/s to determine the storage modulus and complex viscosity.

3.2.3 Crosslinking

Crosslinking of adipose extracellular matrix

Adipose ECM was crosslinked using 1-Ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) in 50 mM 2-(*N*-morpholino) ethanesulfonic acid buffer at pH 5.5.[122] Samples were incubated with crosslinking solutions for 4 hours at concentrations of 5, 10, 50, and 100 mM EDC with an EDC:NHS molar ratio of 2:1. Residual crosslinkers were removed by rinsing with 0.1M Na_2HPO_4 for 2 hours followed by four additional 30 minute incubations with distilled water.

EDC crosslinking was compared with hexamethylene diisocyanate (HMDC). Due to its instability in aqueous solutions, previous studies have used secondary alcohols as the solvent or used surfactants to preserve HMDC reactivity.[123,124] For the secondary alcohol suspension, 1% and 5% HMDC solutions were dissolved in 2-propanol and samples crosslinked for 4 hours. Alternatively, samples were crosslinked with 1% and 5% HMDC in a surfactant solution containing 1% Tween 20 in a phosphate buffer (0.054 M Na_2HPO_3, 0.013 M NaH_2PO_4) at pH 7.4 for 4 hours. Residual crosslinkers and surfactant were removed with distilled water rinses.

Resistance to enzymatic degradation

The crosslinking conditions were characterized by comparing susceptibility to enzymatic degradation. Crosslinked samples and uncrosslinked controls (n=3) were lyophilized and

incubated with 200 U/mL collagenase I in 0.05 M Tris-HCl (pH 7.5) at 37°C.[123,125] At timepoints of 1, 3, 8, 16, and 24 hours, the samples were spun down and supernatants collected and stored at -20°C until completion of the experiment and fresh collagenase added to samples. At 24 hours, any remaining ECM was digested with papainase. Finally, the supernatants and papain digested samples were analyzed via the collagen assay to quantify solubilized collagen. The percentage of total collagen degraded was calculated at the different timepoints.

3.3 Results

3.3.1 Adipose extracellular matrix

We obtained an acellular adipose-derived matrix that retains properties from adipose tissue for soft tissue repair. The processing methods developed for decellularizing adipose tissue resulted in significant macroscopic changes as lipids and other cell components were removed. Intracellular lipids contribute to the characteristic yellow color of intact adipose tissue and as the lipids are removed, the ECM remains as a white fibrous tissue scaffold (**Fig 3.1**). Histological analysis revealed the complete removal of nuclei and the absence of intracellular lipid vacuoles characteristic of adipose tissue. Immunostaining for collagen I confirms it as the predominant component of adipose ECM.

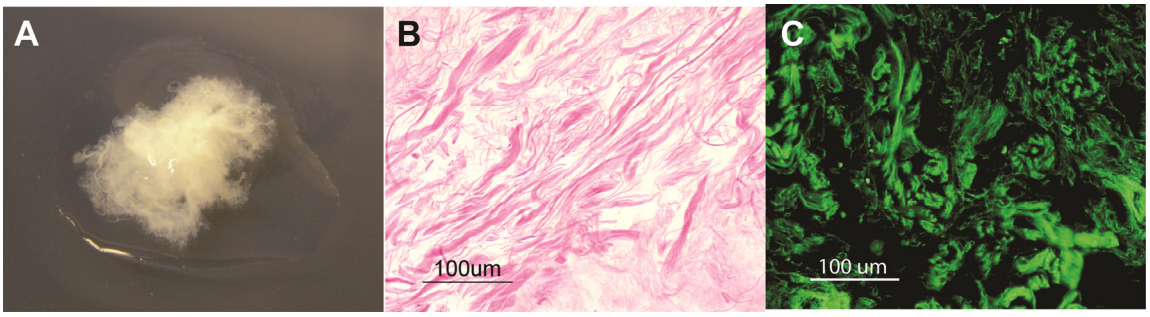


Figure 3.1 Processed adipose tissue matrix. Processed adipose tissue matrix (A) and histological image of decellularized adipose tissue showing no remnants of cell nuclei (B). Immunostaining for type I collagen confirms it as the predominant component of adipose extracellular matrix (C).

3.3.2 Material characterization

Various processing conditions were compared to determine the optimal processing scheme to decellularize adipose tissue. Biochemical analysis of the composition of adipose scaffolds showed little donor variability across the three different donors. Samples treated with 0.1% peracetic acid had the highest DNA content in comparison with samples from higher acid concentrations and also showed incomplete lipid removal as evidenced by residual yellow lipids in the tissue.

Collagen content in the ECM decreased at higher peracetic acid concentrations, most notably with 5% peracetic acid. The optimal processing condition was determined by the ability to best satisfy the two goals of completely removing cellular material while preserving extracellular matrix structure. Ultimately, 3% peracetic acid was determined to give optimal results and was used for subsequent experiments.

SEM images of the decellularized adipose matrix show the fibrillar structure of the ECM with bundles of varying thickness (**Fig 3.2)**. The ECM is porous in nature, facilitating cell migration and nutrient diffusion. In vitro experiments with ASCs reveal extensive cell adhesion and spreading on the ECM of the decellularized tissue. Multiple cell-ECM contacts can be seen for each cell by day 7 in culture.

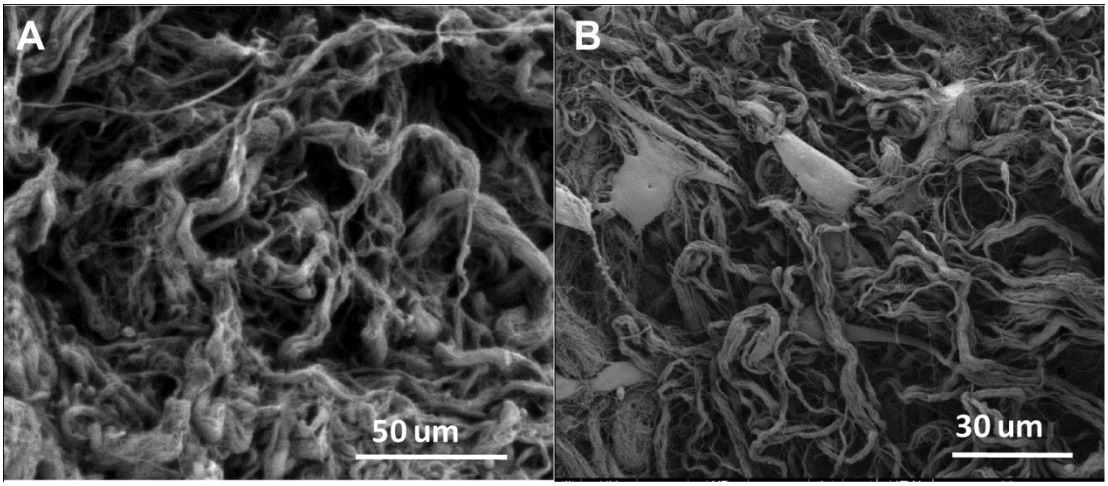


Figure 3.2 Scanning electron microscopy images of adipose ECM without cells (A) and 48 hours after seeding with cells (B).

Proteomic analysis

To identify the protein composition of the adipose extracellular matrix and determine tissue-specific differences between adipose and dermal extracellular matrices, samples were solubilized with an organic solvent prior to reduction and alkylation. Deglycosylation was also adapted to improve enzyme access for the digestion and samples were digested overnight in trypsin to obtain peptides for identification with mass spectrometry. After carrying out liquid chromatography tandem mass spectrometry (LC-MS/MS), the resulting spectra were searched against a database of the human proteome to identify the proteins they originate from, and were classified based on the matrisome proteins as defined by Naba et al.[126] The 1D SDS-PAGE gel of the solubilized proteins show different bands when comparing the adipose and dermis samples, and deglycosylation changes the migration of proteins as the sugars are cleaved (**Fig 3.3A**). After the overnight trypsin digestion, we were able to obtain a complete digestion with most of the bands no longer visible as the tryptic peptides run off the gel (**Fig 3.3B**)

Proteomic analysis with LC-MS/MS identified a total of 25 proteins corresponding to the matrisome or matrisome-associated proteins (**Fig 3.4**). There was some contamination from residual intracellular proteins such as cytoskeletal proteins. There were 11 proteins shared in common between the adipose and dermal matrix, 1 protein unique to the dermis, and 13 unique to adipose tissue. When observing the breakdown by the number of peptides contributing to each category of proteins, peptides belonging to the collagen family dominate the spectra for both dermal and adipose tissues since they are a primary structural component of the ECM. Proteoglycans also had good coverage for both tissues. There were very few ECM glycoproteins identified in dermis compared to adipose and ECM-affiliated proteins were identified only in adipose tissues.

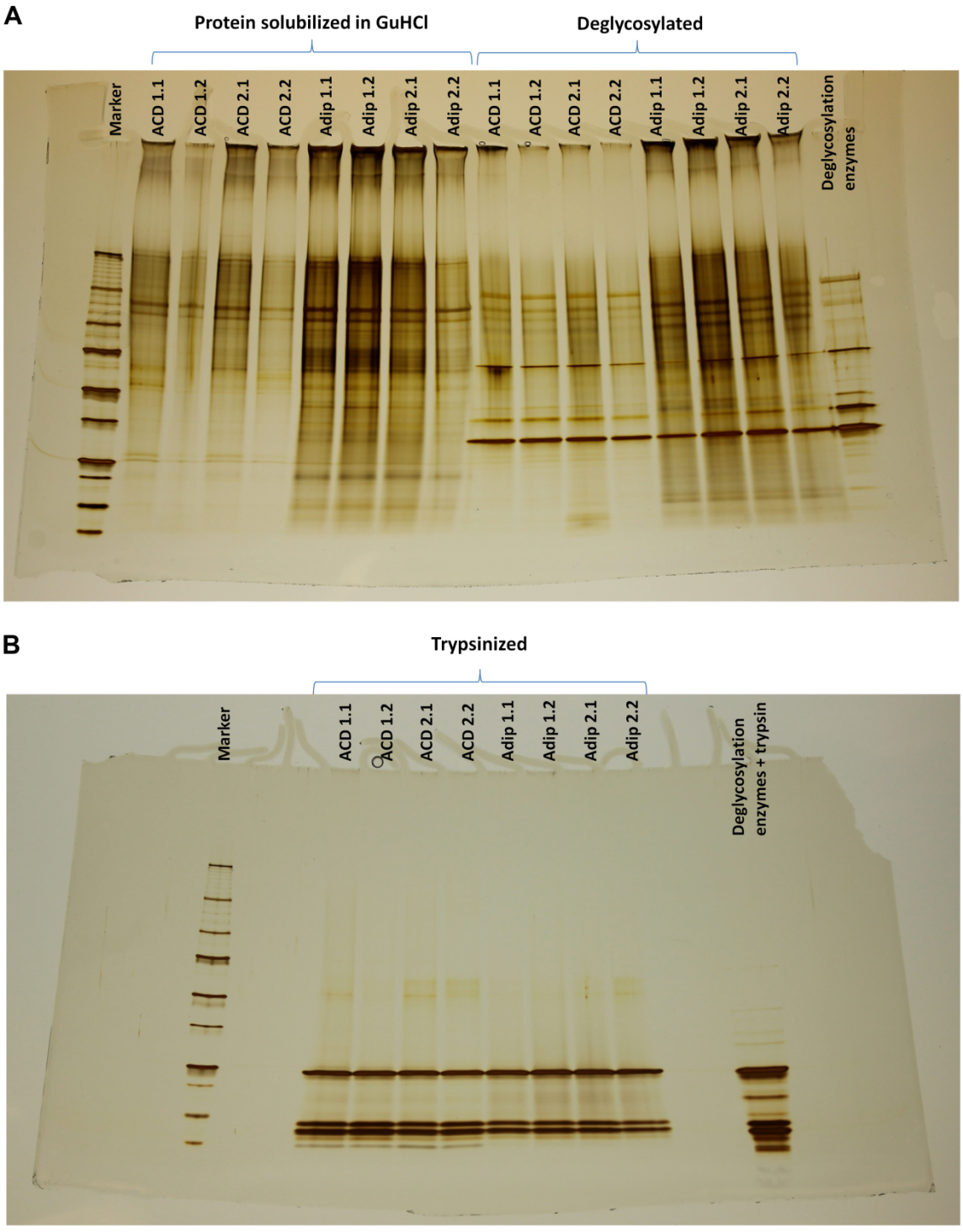
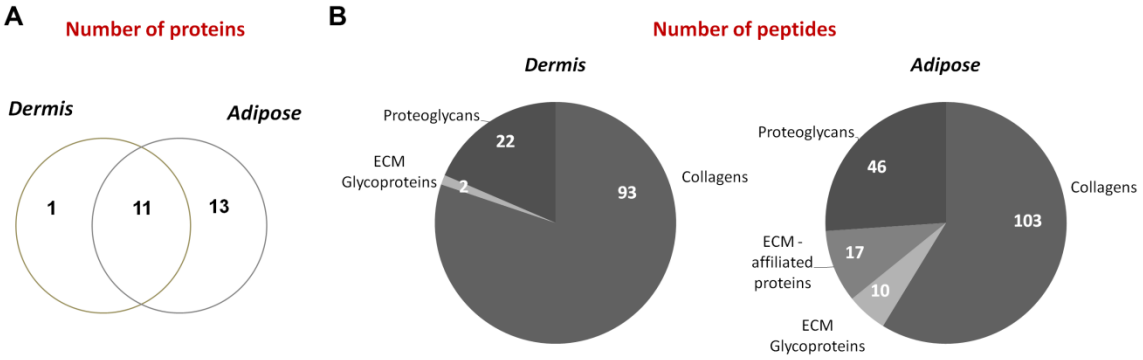


Figure 3.3 1D SDS-PAGE gels of the adipose extracellular matrix and acellular dermis. Samples from two separate donors (Adip 1, Adip 2) with technical replicates (Adip 1.1, Adip 1.2) solubilized with guanidine HCl and deglycosylated (A). After trypsin digestion the bands are no longer visible (B).



C

DIVISION*	CATEGORY	PROTEIN	DERMIS 1	DERMIS 2	ADIPOSE 1	ADIPOSE 2
Core matrisome	Proteoglycan	asporin	0	0	4	4
Core matrisome	Proteoglycan	heparan sulfate proteoglycan 2	0	0	2	4
Core matrisome	Proteoglycan	biglycan	1	3	7	10
Core matrisome	ECM Glycoprotein	cartilage intermediate layer protein	0	0	1	2
Core matrisome	Collagen	collagen, type I, alpha 1	29	36	17	21
Core matrisome	Collagen	collagen, type I, alpha 2	21	16	8	9
Core matrisome	Collagen	collagen, type III, alpha 1	5	9	3	3
Core matrisome	Collagen	collagen, type VI, alpha 1	6	6	9	14
Core matrisome	Collagen	collagen, type VI, alpha 2	3	8	7	9
Core matrisome	Collagen	collagen, type VI, alpha 3	19	28	36	66
Core matrisome	Collagen	collagen, type XIV, alpha 1	0	0	0	3
Core matrisome	Proteoglycan	decorin	8	9	6	5
Core matrisome	ECM Glycoprotein	dermatopontin	0	0	2	2
Core matrisome	ECM Glycoprotein	fibrillin 1	0	0	5	0
Core matrisome	ECM Glycoprotein	laminin, alpha 4	0	0	1	2
Core matrisome	ECM Glycoprotein	laminin, beta 2 (laminin S)	0	0	2	2
Core matrisome	Proteoglycan	lumican	0	2	5	12
Core matrisome	Proteoglycan	osteoglycin	7	6	10	13
Core matrisome	ECM Glycoprotein	periostin, osteoblast specific factor	1	2	0	0
Core matrisome	Proteoglycan	proline/arginine-rich end leucine-rich repeat protein	3	4	3	6
Matrisome-associated	ECM- affiliated	annexin A1	0	0	4	4
Matrisome-associated	ECM- affiliated	annexin A2	0	0	6	6
Matrisome-associated	ECM- affiliated	annexin A5	0	0	1	5
Matrisome-associated	ECM- affiliated	annexin A6	0	0	3	3
Matrisome-associated	ECM- affilitated	coagulation factor XIII, A1 polypeptide	0	0	0	2

Probability Legend
Over 95%
80% – 94%
50% – 79%

Figure 3.4 Proteomic characterization of the adipose and dermis ECM by LC-MS/MS. Shared and unique proteins for adipose and dermis ECM (A) Distribution by number of peptides corresponding to the matrix protein categories (B). List of the proteins and their classifications and number of peptides identifying them (C).

Most of the collagens were shared between the two tissues (Collagen types I, III, VI) with type XIV found only in adipose tissue. Most proteoglycans, including biglycan, decorin, lumican osteoglycin and prolargin, were also common to both tissues. The two proteoglycans detected only in adipose tissues were aspirin and heparin sulfate. ECM glycoproteins and ECM-affiliated proteins were differentially expressed in the two tissues; this is where most differences were

observed. Most ECM glycoproteins, including cartilage intermediate layer protein, dermatopontin, fibrillin, and laminin, were found only in adipose tissues, while the single protein unique to dermis was the ECM glycoprotein periostin. ECM-affiliated proteins that were observed in adipose tissues were annexins A1, A2, and A6 and coagulation factor XIII, while no ECM-affiliated proteins were detected in dermis.

Differential Scanning Calorimetry

The thermal stability of the adipose matrix through the different stages of processing for lipid removal and decellularization were determined using DSC (**Fig 3.5**). The endotherm for intact adipose tissue shows two characteristic peaks at around 7°C and 32°C, most likely attributed to phase changes of the lipids. Adipose samples immediately after pressing for lipid removal show the characteristic peak at around 66°C, typical of collagen based materials where the peak corresponds to the denaturation temperature of the collagen. It is an irreversible process and so the peak is no longer observed during the second heating cycle. With peracetic acid treatment, there is a slight negative shift of a few degrees to around 64°C. The denaturation temperature does not change significantly with incubation using the detergent Triton X-100, but with gamma irradiation there is a larger negative shift down to 57°C, indicating slight weakening of the collagen structure with irradiation for terminal sterilization (**Table 3.1**).

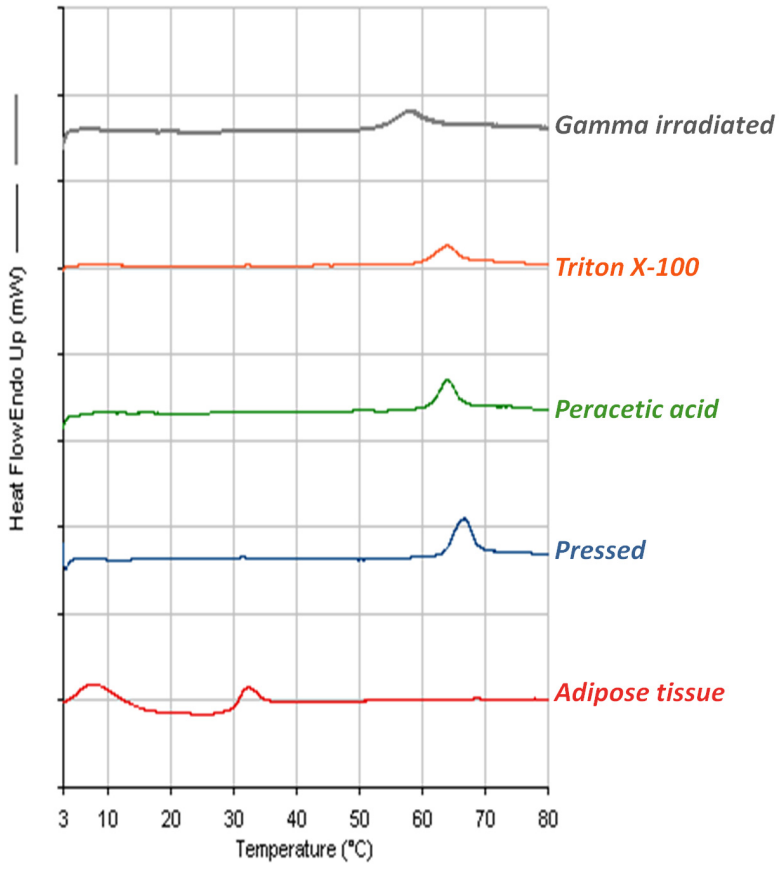


Figure 3.5 DSC thermogram of the adipose ECM through the processing stages.

Table 3.1Denaturation peak temperature

Sample	Peaks (°C)
Gamma irradiated	57.23 ± 0.48
Triton X-100	63.53 ± 0.48
Peracetic Acid	63.99 ± 0.21
Pressed	66.37 ± 0.48
Adipose Tissue	7.35 ± 0.35, 32.29 ± 0.05

Rheology

Rheological testing was carried out to compare the viscoelastic properties of the adipose matrix with current clinical options of lipoaspirate and the dermal fillers calcium hydroxyapatite (Radiesse) and crosslinked hyaluronic acid (Juvederm). The storage modulus of Juvederm was the lowest over all the frequencies tested. Lipoaspirate had a slightly lower storage modulus than adipose ECM but both materials stayed fairly constant over the frequency range tested and were nearly parallel throughout. Radiesse started with a lower storage modulus than lipoaspirate, but quickly increased with increasing angular frequency. The sharper rise in the storage modulus resulted in Radiesse having the highest storage modulus at frequencies above 2 rad/s (**Fig 3.6A**).

Juvederm also had the lowest complex viscosity of all the materials tested, making it easiest to undergo deformation. Adipose ECM again had a slightly higher complex viscosity compared to lipoaspirate, but both had similar rates of decrease in complex viscosity with increasing angular frequency. Radiesse started with a similar complex viscosity modulus as lipoaspirate at the lowest frequencies, but showed a slower rate of decrease in complex viscosity with increasing frequencies and ended up surpassing both lipoaspirate and adipose ECM at around 1 rad/s to end up with the highest viscosity modulus at higher frequencies (**Fig 3.6B**).

3.3.3 Crosslinking

To control the degradation of the ECM, different chemical crosslinking methods were compared. EDC-crosslinked samples showed greater resistance to enzymatic degradation in comparison to uncrosslinked controls when incubated with collagenase (**Fig 3.7)**. Resistance to degradation increased with higher concentrations of the chemical crosslinker. HMDC crosslinking resulted in different degradation profiles depending on the solvent used. For samples reacted in Tween 20, very little degradation was observed with no differences between 1%-5% HMDC concentrations, whereas HMDC in 2-propanol showed greater resistance to enzymatic degradation for 5% HMDC samples compared to 1% HMDC.

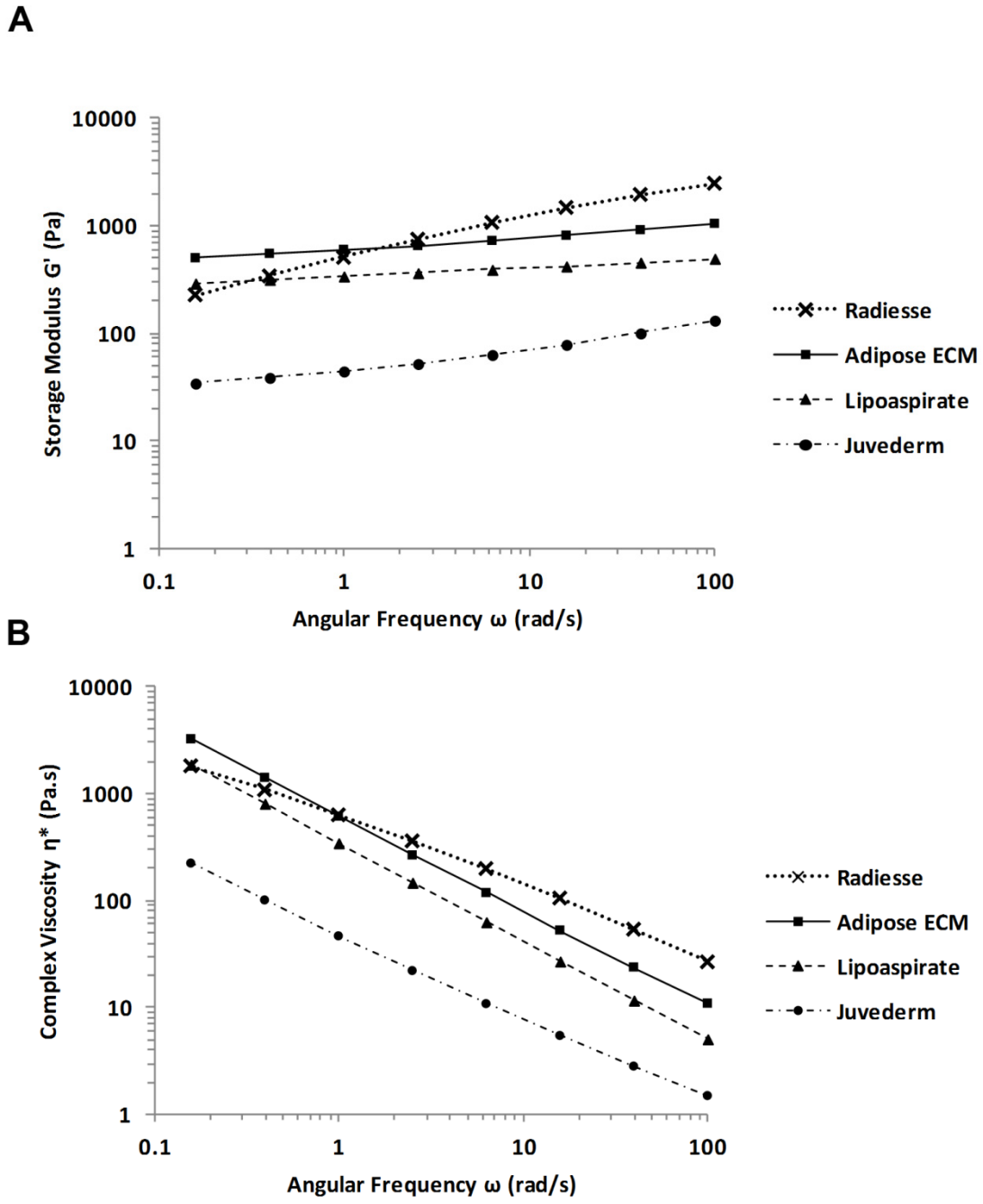


Figure 3.6 Mechanical properties of the adipose ECM and commercial fillers. Rheological testing of the adipose ECM, lipoaspirate, and commercial dermal fillers Radiesse (calcium hydroxyapatite) and Juvederm (crosslinked hyaluronic acid) gives measures of the storage modulus (A) and complex viscosity (B).

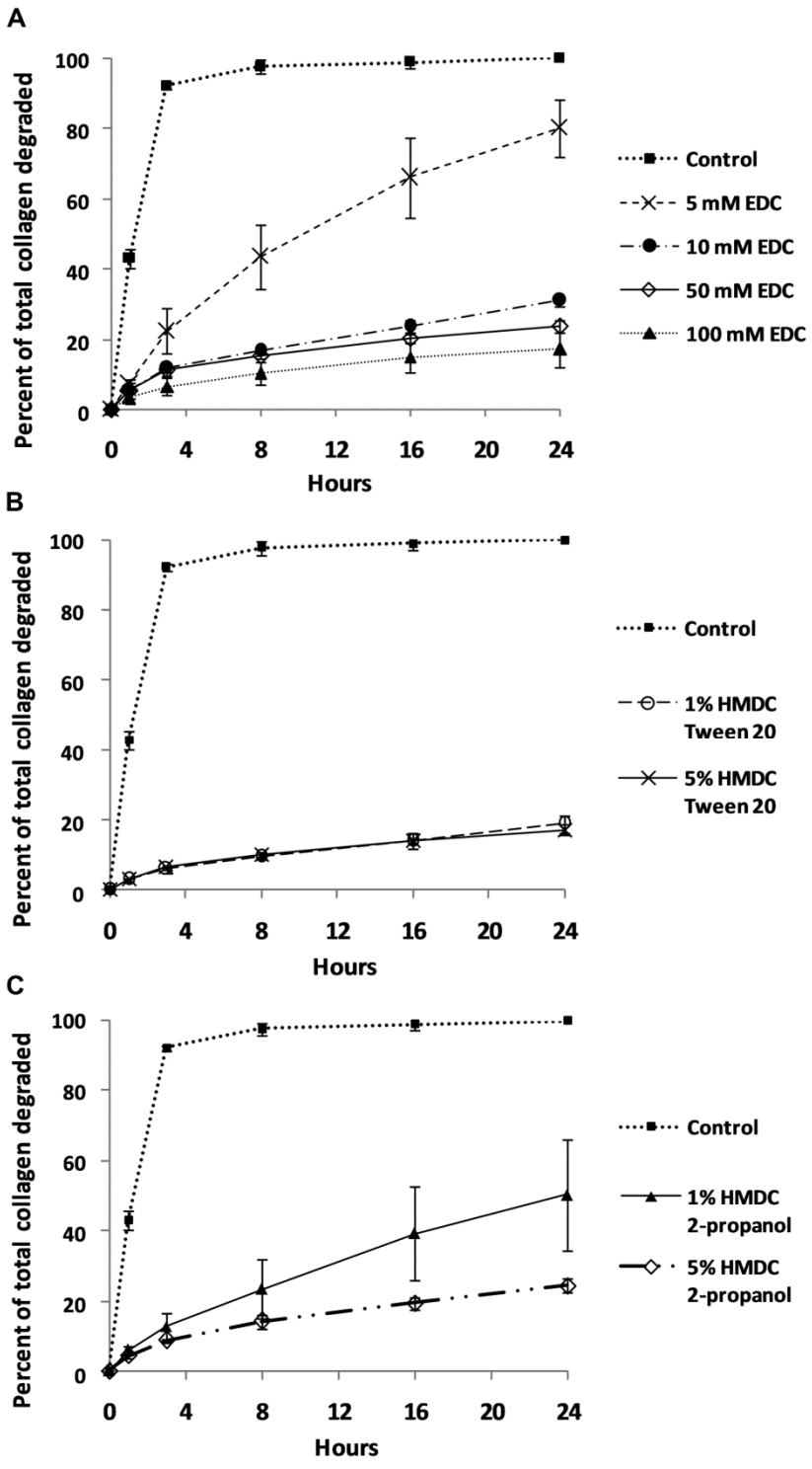


Figure 3.7 Crosslinked matrices show increased resistance to enzymatic degradation. Percent of total collagen degraded over 24 hours when incubated with collagenase for uncrosslinked control tissue and matrices crosslinked with 5-100 mM EDC (A), 1% and 5% HMDC in Tween 20 (B), 1% and 5% HMDC in 2-propanol (C). Error bars denote standard deviation.

3.4 Discussion

Decellularization of tissues has become increasingly popular as a method to obtain a matrix of proteins and sugars ideally suited to direct the migration of cells into the matrix and provide the structural cues to maintain cell survival.[127] A range of applications has been explored for decellularized ECM derived from dermal, cardiovascular, musculoskeletal, hepatic, and gastrointestinal tissues, many of which are already in clinical use and have been shown to facilitate tissue integration and restoration.[128]

Our studies showed that a decellularized ECM biomaterial can be derived from adipose tissue to obtain a biocompatible scaffold for the repair of soft tissue defects. Proteomic analysis of the adipose matrix and acellular dermis identified a number of glycoproteins and ECM affiliated proteins that were unique to the adipose ECM. The annexins are a class of proteins found only in the adipose matrix. Annexin A1 is a mediator of the inflammatory response with anti-inflammatory properties that prevent recruitment and migration of leukocytes while Annexin A2 has been found to show antithrombogenic activity.[129] For the glycoproteins, dermatopontin is a widely studied protein with known functions in ECM assembly, cell adhesion, and modulation of TGF-β1 activity.[130] Fibrillin is a glycoprotein important in the formation of microfibrils which form elastin fibers and store TGF-β1.[131,132] Laminin is a critical component of the basement membrane, affecting cell movement, differentiation, shape, and survival.[133] The proteomic analysis provided a comprehensive protein profile for the extracellular matrices of the different tissues. Tissue specific differences were prevalent, even between two closely related connective tissues, adipose and dermis.

Thermal analysis of the adipose matrix showed slight changes in the structure through the different processing stages using differential scanning calorimetry, a method sensitive enough to capture changes in the molecular stability of collagen-based materials. Collagen undergoes irreversible denaturation with heating, undergoing a conformational change from the triple helical structure to a random coil state due to disruption of the intramolecular crosslinks. For

both the peracetic acid and gamma irradiation steps, a negative shift of the collagen denaturation peak was observed. This suggests that the structure is slightly weakened by these processes. Acid treatment likely solubilizes a number of proteins and proteoglycans from the matrix, reducing its structural stability as well as altering its hydration state. Gamma irradiation has been found to cause oxidative damage to collagen structure by disrupting crosslinks and causing fragmentation by peptide scission.[134-137] For products intended for clinical use, terminal sterilization methods are one of the precautionary steps often taken to ensure minimal bioburden, but must be balanced against the possible structural damage from the irradiation.

Rheological analysis of the adipose matrix, lipoaspirate, and commercial dermal fillers demonstrate the range of mechanical properties for the different products. For the commercial fillers, crosslinked hyaluronic acid (Juvederm) had the weakest viscoelastic properties while calcium hydroxyapatite (Radiesse) had the highest storage modulus and complex viscosity for the majority of frequencies tested. Human adipose ECM and lipoaspirate were positioned between the two, with adipose ECM having a slightly higher storage modulus and complex viscosity, and having similar rates of change in their respective moduli with change in angular frequencies. This suggests that many of the mechanical properties of the original tissue are retained in the adipose ECM and the viscoelastic behavior of the matrix was most similar to lipoaspirate across the range of frequencies tested. Calcium hydroxyapatite proved to be most resistant to deformation, with the greatest increase in storage modulus and most gradual decrease in complex viscosity with increasing frequencies. Alternatively, crosslinked hyaluronic acid was the most susceptible to deformation. Based on the mechanical properties, the adipose matrix is a suitable filler for soft tissue defects due to the similarity in viscoelastic properties with the surrounding tissue.

Crosslinking the decellularized scaffold provided an additional element of control over the degradation properties of the tissue and allows the material to be tailored to the target site of implantation, which may be subject to different mechanical forces. Additionally, the degradation

rate can be tuned to the rate of host tissue regeneration to ensure that the biomaterial provides sufficient structural support until newly developed tissues repopulate the scaffold. Resistance to enzymatic degradation is critical when using natural ECM-based scaffolds since host cells can easily degrade the proteins with various endogenous matrix metalloproteinases.

As our understanding of the role of the extracellular matrix in tissue and organ development expands, it is clear that matrix components do not just serve a structural role in tissue, but also actively participate in the instructional aspect of cell development. Much has recently been elucidated about the role of the various ECM degradation components in influencing cell migration and proliferation, particularly in aiding tissue regeneration.[138-140] Studies of adipose tissue ECM proteins have also shown that they facilitate in vivo adipogenesis.[76] In view of this instructional role for the ECM, we demonstrated that adipose tissue can be successfully processed to remove inflammatory lipids and cells to produce a biomaterial for soft tissue reconstruction.

The components of the adipose-derived scaffold are all naturally occurring ECM proteins and proteoglycans that are highly conserved between different species, such that cells are not exposed to any materials or synthetic polymers that can induce a foreign body response. Host tissue integration is also enhanced since matrix components can be readily degraded by cell-secreted enzymes as tissue remodeling takes place. Mechanisms for matrix turnover are already established in host cells, avoiding any concerns over proper clearance of scaffold materials from the body. These factors contribute to the biocompatibility of ECM-based scaffolds and highlight their utility in regenerative medicine.

4. In Vitro Bioactivity of the Adipose Extracellular Matrix

4.1 Introduction

Soft tissue repair remains a challenge for surgeons who have limited options for reconstructing areas of soft tissue loss or disfigurement. Ablative surgeries for tumor removal, traumatic injuries, and congenital defects all require reconstructive procedures to restore contour and symmetry. Current clinical strategies often require autologous tissue for flap reconstructions or fat grafting to fill defects. Surgeons are making strides in developing new reconstructive procedures to minimize donor site morbidity, but these approaches still suffer from the key drawback that they inherently require donor site tissue, which is limited by availability. Since the requirement of autologous tissue results in donor site morbidities, the procedure essentially creates a second defect site to repair the first.[141] Additionally, transplanted tissue can have complications including necrosis, infection, thrombosis, and hematoma formation.[12] These complications can lead to total flap loss or unpredictable rates of resorption for fat grafts. This predicament highlights the utility and advantages of an off-the-shelf alternative that can fill soft tissue defects while promoting soft tissue regeneration.

Approaches to engineer soft connective tissues traditionally involve choosing a scaffold, seeding it with cells, differentiating those cells towards adipogenesis, and finally using the construct as a soft tissue replacement. Adipose tissue constructs can be formed by seeding cells

on synthetic scaffolds like poly glycolic acid[15,16] or encapsulating cells in polyethylene glycol hydrogels.[32,33] Naturally derived scaffolds can also be used for adipose tissue formation such as silk,[39,41,42] alginate,[35,37] collagen,[43,52] and hyaluronic acid.[65,67] Adipose extracellular matrix based approaches to replacing damaged soft connective tissue have also been explored. Decellularized lipoaspirate has been reformed into microcarriers and sponges to provide a substrate for cell growth,[84,85] and adipose tissue has also been decellularized and fabricated into porous foams for adipogenesis.[90]

The use of the extracellular matrix as the basis of a biomaterial scaffold is promising due to the bioactive properties of the extracellular matrix and its critical role in maintaining cell functions in vivo. For the following studies, we wanted to investigate the bioactive properties of the adipose matrix for both cell migration and differentiation, as well as the tissue specificity of the extracellular matrices when comparing scaffolds derived from different tissue sources. We decided to compare the adipose matrix with acellular dermis since it is also a connective tissue and is currently available for clinical use.

4.2 Materials and Methods

Materials were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

4.2.1 Adipose extracellular matrix preparation

Subcutaneous adipose tissue was subjected to mechanical processing and extensive rinsing followed by incubation with 0.1%, 1%, 3%, or 5% peracetic acid for 3 hours to remove cells. Samples were brought back to physiological pH using PBS and incubated overnight with 1% Triton X-100 in 2 mM EDTA to remove residual lipids. Finally, samples were treated with 600 U/mL DNase (Roche, Indianapolis, IN) in 10 mM $MgCl_2$ overnight at 37°C.

4.2.2 Adipose-derived stem cell isolation

Subcutaneous adipose tissue was obtained from patients undergoing abdominoplasty procedures with approval from Johns Hopkins Medicine Institutional Review Board. Adipose-derived stem cells (ASCs) were isolated by digestion with 1 mg/mL collagenase I (Worthington) in DMEM F-12 (Gibco) for 1.5 hours on an orbital shaker at 37°C. The resulting cell suspension was centrifuged and filtered through 70 uM and 40 uM cell strainers. ASCs were seeded at 5,000 cells/cm^2 and cultured in DMEM-F12 supplemented with 10% fetal bovine serum (Thermo Scientific HyClone). Cells were used between passages 3-5 for all studies.

4.2.3 In vitro cell studies

Cell viability on crosslinked matrices

To evaluate cell viability on the crosslinked matrices, cells were seeded on the scaffolds and cultured for 5 days in ASC maintenance media (DMEM-F12, 10% FBS, 100 U/mL penicillin, 10 ug/mL streptomycin). Cell viability was assessed using a LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, Carlsbad, CA). Cell-ECM scaffolds were incubated with 1 uM calcein AM and 4 uM ethidium homodimer-1 in DMEM-F12 for 30 minutes at 37°C to visualize live and dead cells.

Migration assay

Adipose-derived stem cells were serum starved for 24 hours. Cells were trypsinized and seeded in the top chamber of a 6.5mm 7.0 um pore size polystyrene transwell (Corning) at 30,000 cells per transwell. The adipose extracellular matrix was added to the media in the bottom chamber at 0, 1, and 10 ug/ml. A positive control of 10% FBS in media was used. Migrated cells were quantified after 6 hours using ImageJ (NIH).

Adipogenic differentiation of cells in 2D culture on the adipose matrices

Adipose extracellular matrix was embedded in OCT and cryosectioned at 200 um sections and collected on a plus charge coated glass slide. OCT was removed by incubating slides in PBS on an orbital shaker with three changes each at 1 hour intervals. Adipose-derived stem cells were seeded directly on the adipose matrix on slides or on empty glass slides and cultured in ASC expansion media or adipogenic media.

Adipogenic differentiation was carried out for ASCs seeded on the matrix with adipogenic induction media (1 μM dexamethasone, 200 μM indomethacin, 500 μM methylisobutylxanthine, 10 μg/ml insulin, 1% penicillin/streptomycin, and 10% FBS in High Glucose DMEM). Cells were differentiated for 7 days in culture before fixation for histological analysis. Slides were fixed for 10 minutes in 10% formalin and stained with Texas Red-phalloidin (Invitrogen) for actin, Nile Red for lipids, and rabbit anti-collagen type I antibodies (Fitzgerald) followed by FITC- AffiniPure goat anti rabbit IgG secondary antibodies (Jackson) for visualization of the ECM scaffold and coverslipped with Vectashield plus DAPI (Vector Labs).

Adipogenic differentiation of cells in 3D constructs comparing the adipose and dermis ECM

To evaluate adipogenesis of the cells in a 3D environment and to determine if there is any tissue specificity of the extracellular matrices, adipose-derived stem cells were resuspended in 50 ul of cryomilled adipose ECM or reconstituted micronized acellular dermis (Cymetra) at 2 million cells per construct and seeded in the top chamber of a 6.5 mm transwell (Corning). After culturing in ASC expansion media for 48 hours, the constructs were removed from the transwells and moved to a 24 well plate. The constructs were cultured on an orbital shaker with adipogenic induction media for an additional 15 days.

For histological analysis, constructs were fixed in 10% formalin overnight, infiltrated with sucrose, and embedded in OCT. Samples were cryosectioned at 10 um sections and stained with hematoxylin and eosin or Oil Red O for lipid accumulation.

RT-PCR

Samples for determining gene expression levels were snap frozen in liquid nitrogen and crushed with a mortar and pestle under liquid nitrogen. Homogenized samples were immediately placed in Trizol Reagent (Invitrogen) and total RNA extraction was carried out according to manufacturer instructions with the addition of 1 ug of glycogen added to each sample to aid in RNA precipitation and phase separation carried out by centrifugation in phase lock gel tubes (heavy, 5 PRIME). RNA was resuspended in DEPC water and quantified using a Nanodrop (Thermo Scientific).

cDNA was synthesized using the SuperScript RT III system (Invitrogen) according to manufacturer instructions and RT-PCR was carried out with Power SYBR Green (Applied Biosystems) on an Applied Biosystems 7500 Real-Time PCR System. Each PCR reaction was carried out in triplicate with three biological replicates. Relative quantitation was done using the ΔΔCt method[142] with beta actin as the housekeeping gene with normalization to day 3 acellular dermis data. Primer sequences for adipogenic genes are listed in Table 1.1.

Table 4.1 RT-PCR Primer sequences

Gene	Forward Sequence 5' – 3'	Reverse Sequence 5' – 3'
PPARG	AGGAGAAGCTGTTGGCGGAGA	TGCTTTGGTCAGCGGGAAGG
LPL	GTCAGAGCCAAAAGAAGCAGCAA	GGGTTTCACTCTCAGTCCCAGAA
LEP	TGACACCAAAACCCTCATCAAGACAA	GGAGCCCAGGAATGAAGTCCAA
CEBPA	TCACCGCTCCAATGCCTACTG	CCTGCTCCCCTCCTTCTCTCAT
FABP4 (ap2)	ACAGGAAAGTCAAGAGCACCATAACC	TGACGCATTCCACCACCAGTTT
BACT	GGCACCCAGCACAATGAA	GCTAACAGTCCGCCTAGAAGC

HUVEC tube formation assay

Human endothelial cells (HUVECs) were used to evaluate network tube formation on a basement membrane extract.[143] HUVECs (Lonza) were cultured in EGM-2 media (Lonza) and passaged before reaching confluence. HUVECs were used between passages 2-4 for all studies. To analyze HUVEC tube formation in basal media, 30,000 cells were seeded on growth factor

Reduced Matrigel (BD) in a 96 well plate and cultured in EBM (Lonza) with adipose ECM at concentrations of 0, 0.01, 0.1, 1 mg/ml. For HUVEC tube formation in growth media, 10,000 cells were seeded on growth factor reduced Matrigel per well of a 96 well plate and cultured in EGM-2 with adipose ECM added at concentrations of 0, 0.01, 0.1, 1 mg/ml. After 6 hours in culture, cells were imaged with brightfield microscopy and branching was quantified with the Angiogenesis Analyzer (written by Gilles Carpentier, 2012. The macro is available at http://imagej.nih.gov/ij/macros/toolsets/Angiogenesis%20Analyzer.txt) for ImageJ (NIH).

4.3 Results

4.3.1 Cell viability on cross-linked matrices

Adipose-derived stem cells cultured on the crosslinked adipose extracellular matrices adhered and spread on the scaffold and showed good viability when stained with calcein AM and ethidium homodimer-1 (**Fig 4.1**). The adipose matrix supported cell survival with few cells staining red to indicate non viable cells.

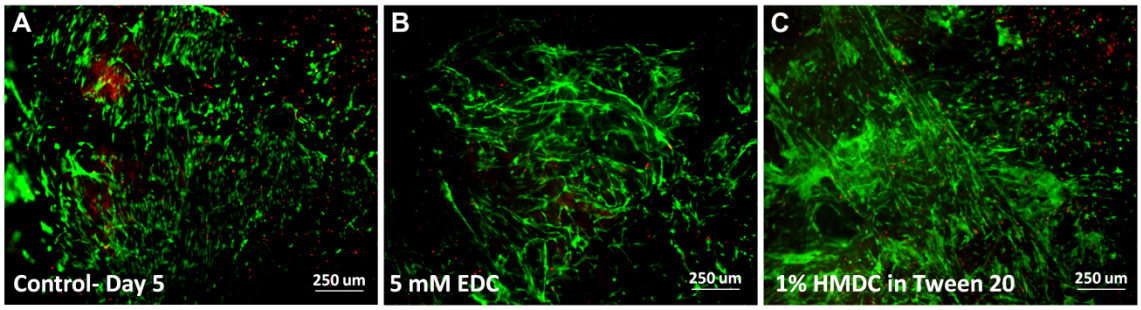


Figure 4.1 LIVE/DEAD staining of cells on crosslinked matrices. Cells are stained with calcein AM for live cells (green) and ethidium homodimer-1 for dead cells (red). Cells show high viability on uncrosslinked adipose extracellular matrix (A), 5 mM EDC crosslinked (B), and 1% HMDC crosslinked in Tween 20 matrices (C).

4.3.2 Migration assay

Serum starved ASCs were screened using a Boyden chamber assay to determine whether they would migrate towards adipose ECM proteins. A dose-dependent increase in the number of cells migrating across the membrane was observed with the addition of adipose ECM to the media, similar to that observed with the positive control of 10% FBS (**Fig 4.3A**).

4.3.3 Adipogenic differentiation of cells in 2D culture on the adipose matrices

ASCs adhered and spread on positively-charged, coated glass slides. When cultured on 200 um thick sections of adipose matrix collected on the glass slides, cells preferentially adhered to the adipose matrix over areas of exposed glass and adapted a spindle-shaped morphology that was less spread than cells cultured directly on the glass slides. With the addition of adipogenic induction media, cells underwent adipogenic differentiation and began accumulating lipid droplets that stained positively with Nile Red (**Fig 4.2**).

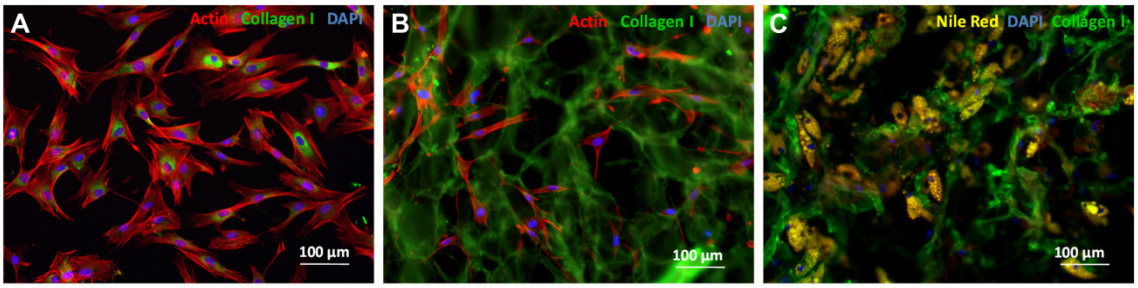


Figure 4.2 In vitro adipogenesis of ASCs seeded on adipose matrix in 2D culture. ASCs seeded on glass slides (A), 200 um sections of adipose ECM (B), and on adipose ECM sections with adipogenic differentiation media (C).

4.3.4 Adipogenic differentiation of cells in 3D constructs comparing the adipose and dermis ECM

Efficiency of adipogenic differentiation of ASCs was compared on adipose and dermal extracellular matrices in a 3D culture environment more representative of the native adipocyte environment. The ASCs were resuspended in either adipose ECM or acellular dermis and at the end of 15 days in culture, looked indistinguishable upon gross examination (Fig 4.3B). After cryosectioning, histological sections of the constructs were stained with Oil Red O to stain for lipid accumulation, which is one of the hallmarks of adipogenic differentiation (**Fig 4.3C**). Hematoxylin and eosin staining showed similar cell distribution and morphologies between cells grown on the two scaffolds. Gene expression supported the results from the Oil Red O staining as cells grown on the adipose matrix had higher expression of adipogenic genes for both early and late markers of adipogenesis compared to cells grown on the dermal matrix (**Fig 4.3 D**)

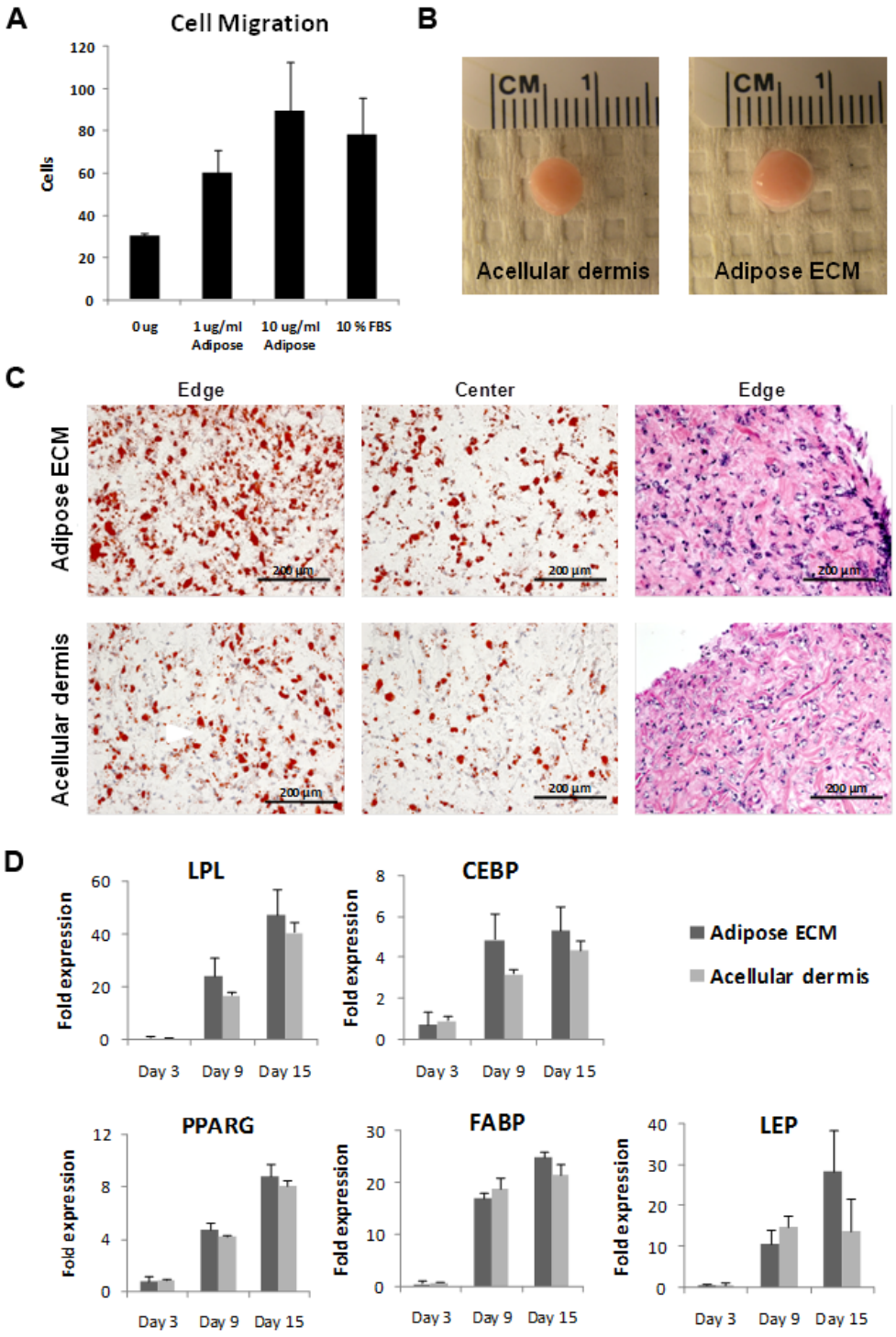


Figure 4.3 In vitro chemoattractive properties and adipo-inductive capacity of the adipose matrix. Migration of ASCs in response to an adipose ECM gradient using a Boyden chamber assay (A). 3D construct culture of ASCs in acellular dermis and adipose ECM (B). Oil red O staining for adipogenesis from both the edge and center of the constructs and hematoxylin and eosin staining of cells cultured in adipose and dermis ECM (C). Gene expression of adipogenic markers for cells cultured in the two matrices (D).

4.3.5 HUVEC tube formation assay

HUVECs seeded on Matrigel form capillary-like structures within several hours of seeding. The tube formation can be enhanced with the addition of angiogenic factors in the media such as the growth factors commonly used in endothelial growth media. Since growth factors are naturally found in the basic Matrigel preparation, the use of growth factor-reduced Matrigel in the tube formation assay allows us to evaluate this angiogenic potential against a less potent background. HUVECs begin to branch out and form immature networks when cultured in EBM but are unstable over long periods in culture. The addition of adipose ECM enhances network formation in EBM, resulting in more branching and contacts between neighboring cell aggregates. In EGM, HUVECs develop more mature vascular networks with extensive branching. The addition of adipose matrix resulted in a slight increase in the branching and network formation (**Fig 4.4**).

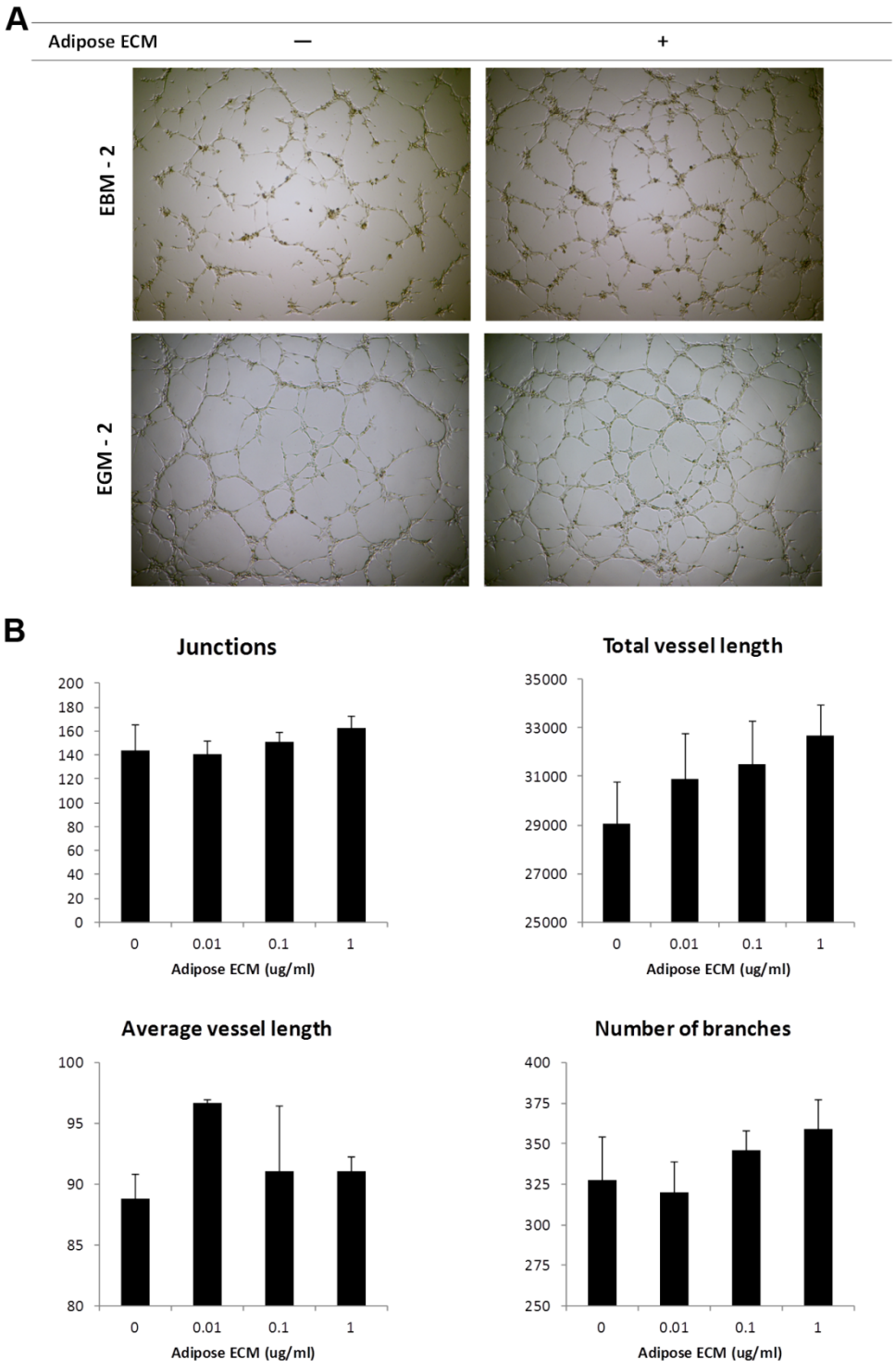


Figure 4.4 HUVEC tube formation assay with adipose ECM. Soluble adipose ECM was added to either endothelial basal media or endothelial growth media to evaluate vascular network formation of HUVEC cells seeded on Matrigel after 6 hours (A). Parameters of branching networks were quantified using the Angiogenesis Analyzer for ImageJ (B).

4.4 Discussion

In these studies, we were able to investigate the ability of the adipose matrix to support cell adhesion, growth, and differentiation. Even with chemical crosslinking of the adipose ECM, cells showed high viability and adhered to the matrix. Chemical crosslinkers often are very toxic to cells and any uncrosslinked molecules must be completely removed through the rinsing steps. There is also the risk that crosslinking will alter the protein structures to an extent that cells will no longer recognize them as the original matrix, thus losing any bioactivity through the crosslinking process. These concerns did not end up materializing and the matrices were capable of supporting cell survival in vitro.

In the culture of ASCs on sections of adipose ECM attached to glass slides, the ASCs preferentially bound to the adipose matrix even when they had equal access to the glass surface and adopted a spindle-like morphology. The cells appeared to have a natural affinity for the ECM scaffold and it is likely that it provided a more natural surface for their growth compared to the glass slides. The cells also differentiated and initiated formation of lipid droplets with the addition of adipogenic induction media, demonstrating that the adipose matrix could indeed support adipogenic differentiation of the cells. However, the adipose matrix was not sufficient alone, and required the addition of the adipogenic factors to induce differentiation.

We also investigated the bioactive properties of the adipose extracellular matrix in the in vitro environment. The adipose matrix had chemoattractive properties and guided stem cell migration in similar fashion to FBS in a migration assay, suggesting that there is likely an optimal gradient to which stem cells are inclined to migrate towards ECM-based scaffolds. This is supported by studies that have found that mesenchymal stem cells home to areas of tissue injury in vivo and contribute to the wound healing response.[144,145] In sites of tissue injury, fragments of ECM can potentially provide the local trophic factors to induce stem cell migration.[139] More importantly, this highlights the potential of utilizing the acellular scaffolds to

facilitate cell migration and tissue regeneration, and bypassing the need to deliver exogenous cells for tissue repair.

Despite the increased use of extracellular matrix-based approaches to tissue regeneration, tissue specificity of the extracellular matrix remains an enigma as it remains unclear which tissues are optimal for specific applications. In this study we compared the efficacy of adipogenic differentiation on adipose and dermal ECM to determine whether the tissue specificity of the matrices is retained through all the decellularization procedures. We found that ASCs cultured on the adipose matrix showed improved adipogenic differentiation by gene expression and histological markers, suggesting that tissue-specific factors may render particular tissues more appropriate for certain applications. Thus, care must be taken in deciding the starting material as the tissue of origin does impart different bioactive cues that control the cellular microenvironment. This is further supported by studies that have identified differences in the ECM composition of distinct tissues and organs.[126] Additionally, other groups have found that extracting protein components from the extracellular matrix of adipose tissue has adipo-inductive effects and can lead to adipogenic differentiation of stem cells.[76,77]

To date, attempts to generate adipose tissue constructs in vitro have been met with mixed success. One of the major challenges is to get adipogenic differentiated cells to adopt the unilocular morphology characteristic of adipocytes in the native environment. Although it has been well established that multipotent stem cells can be differentiated toward the adipogenic lineage with a defined set of media components in 2D culture, differentiated cells tend to accumulate multiple small lipid droplets as opposed to a single large lipid droplet similar to their native morphology in adipose tissue. Fischbach et al were able to do this successfully with poly glycolic acid scaffolds seeded with 3T3-L1 preadipocytes, which produced constructs that looked morphologically similar to native adipose tissue after long term culture in vitro.[15,16]

Difficulties in histological sectioning of some of these materials are also likely to be a factor in the mixed outcomes observed in the literature. Oil Red O staining requires cryosectioning so

that the lipids are not processed away, and this can contribute to histological artifacts in sections. In most cases, cells are sparsely distributed in the matrix and are not in close approximation with each other, as found in native adipose tissue. Additionally lipid accumulation is not as drastic as in native adipose tissue where lipids disproportionately take up the majority of the cell volume. Specific in vitro culture conditions can improve outcomes for generating adipose tissue constructs, such as the use of dynamic culture either with a stirred bioreactor or on an orbital shaker, which improves tissue production and maintenance of larger tissue constructs by enhancing nutrient diffusion.[15,39]

Nonetheless, these engineered constructs are intended for in vivo use and as a result, the tissues need not be fully formed at the time of implantation. Instead many researchers try to capitalize on the host in vivo regenerative processes to complete tissue maturation in the constructs. Our studies demonstrated that the adipose ECM can serve as a biological scaffold for adipose tissue engineering. It provides a supportive environment for stem cell adhesion and differentiation and its biological properties make it advantageous for application in soft tissue regeneration. These aspects of the ECM, along with its availability and potential for allogenic use make it uniquely suited for soft tissue repair.

5. The Adipose Extracellular Matrix as a Scaffold for In Vivo Adipose Tissue Regeneration

5.1 Introduction

Soft tissue damage can occur as a result of traumatic injury, congenital and acquired medical conditions, infection, or ablative surgical procedures like tumor resection. Intensive reconstructive surgeries are often required to repair these defects to improve aesthetic outcomes and often rely on the use of autologous tissue flaps for the reconstruction. The major drawback of this approach is the requirement of a donor site to provide tissue for the reconstruction, which can result in significant donor site morbidities.[107] In cases of extensive soft tissue loss such as injuries sustained from blast trauma, availability of donor sites can become limited as multiple flap reconstructions are required. Additional complications can include infection, necrosis, thrombosis, hematoma, and flap loss.[12,141] Another approach for repairing smaller volume defects is to perform fat grafting. In this procedure, patients undergo liposuction and the collected lipoaspirate is injected at the defect site. This technique suffers from high variability in the rates of resorption and poor viability of transplanted adipose tissue, which leads to necrosis and cyst formation.[8,10]

Current engineered approaches to regenerate adipose tissues have used both synthetic and naturally-derived biomaterials.[118] The extracellular matrix of tissues (ECM) is composed of a network of structural proteins and proteoglycans that provide both mechanical and biochemical

support for the parenchymal cells of the tissue. The ECM is an intriguing prospect as a biomaterial for tissue regeneration since it is the native environment that cells are found in and can potentially retain biological signals from its tissue of origin that may aid in regeneration.

Decellularized lipoaspirate has been processed into various formulations including injectable powders, sheets, and pepsin digested to form a hydrogel for adipose regeneration.[82-86] Others have extracted proteins from the extracellular matrix in a preparation similar to Matrigel, forming a temperature hydrogel that forms a gel at physiological temperatures.[76,77] Intact decellularized adipose matrices have been used as an acellular scaffold and formulated into microcarriers and foams for use as a scaffold in vivo.[87,89,90] However, many of the approaches lose structural integrity of the ECM with enzyme digestion and acid solubilization, or extract only specific fractions of the adipose matrix.

In this study, we introduce a method of decellularizing intact adipose tissue to obtain a bulk extracellular matrix scaffold that is minimally processed to retain as much of the original structure as possible. The use of a bulk extracellular matrix would be advantageous since it would more effectively fill a volume defect and provide the structural support required while encouraging host tissue regeneration to take place. Additionally, we monitor the long term volume retention of the scaffold in vivo and compare it with the current clinical standard of fat grafting.

5.2 Materials and Methods

Materials were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

5.2.1 Adipose extracellular matrix preparation

Subcutaneous adipose tissue was subjected to mechanical processing and extensive rinsing, followed by incubation with 3% peracetic acid for 3 hours to remove cells. Samples were brought back to physiological pH using PBS and incubated overnight with 1% Triton X-100 in 2 mM EDTA to remove residual lipids, followed by detergent removal with rinsing the next day.

5.2.2 In vivo studies

All animal studies were conducted with approval by the Johns Hopkins Animal Care and Use Committee.

In vivo biocompatibility of crosslinked adipose ECM

A total of 18 subcutaneous injections (6 per crosslinking condition) were carried out in two male and two female Sprague-Dawley rats (n=4) between 4-13 weeks old. The following conditions were used: uncrosslinked control, 5 mM EDC-crosslinked, and 1% HMDC-crosslinked in Tween 20, with 300 ul injections of each condition along the dorsum. Implants were removed after 2 and 3 weeks and fixed for histology.

Long term biocompatibility

Upon completion of the initial study, a longer term study was carried out with 6-week old female Sprague Dawley rats (n=12) over the course of 12 weeks. Animals were grouped by study endpoints of 1, 4, and 12 weeks with 4 animals per timepoint. 200 ul injections of uncrosslinked adipose-derived matrix were performed subcutaneously in the rats to characterize the immune response and tissue remodeling without introducing additional crosslinkers, which can potentially elicit a more severe inflammatory response.

In vivo volume retention compared to fat grafting

Athymic mice (n=8) were used to evaluate the volume retention of the adipose ECM in comparison to the clinical standard of fat grafting. Human lipoaspirate was obtained from patients undergoing liposuction with prior approval by the Institutional Review Board. A solution containing normal saline with 1:500,000 epinephrine was infiltrated into the area at a ratio of 1 ml of solution per ml of fat and adipose tissue was aspirated using a 2.5 mm diameter blunt tip cannula attached to a 10-ml Luer-lock syringe, creating light negative pressure by slowly withdrawing the plunger in a gradual manner. Lipoaspirate was washed three times with

normal saline added at a 1:1 ratio and allowed to decant at room temperature for five minutes between each wash to aid in removal of blood and infiltration fluids. Excess saline was removed and 200 ul of lipoaspirate was loaded in syringes for injection.

Subcutaneous injections of lipoaspirate and adipose ECM were injected along the dorsum of the athymic mice. Each mouse received a 200 ul injection of each condition and volume measurements were taken using digital calipers. Volume persistence was monitored with regular measurements starting at day 0 immediately after injection and continued every two weeks until the study endpoint of 12 weeks. At the conclusion of the study, implants were removed and fixed for histology.

In vivo adipogenesis of adipose ECM with ASCs

Subcutaneous adipose tissue was obtained from patients undergoing abdominoplasty procedures with approval from Johns Hopkins Medicine Institutional Review Board. Adipose-derived stem cells (ASCs) were isolated by digestion with 1 mg/mL collagenase I (Worthington) in DMEM F-12 (Gibco) for 1.5 hours on an orbital shaker at 37°C. The resulting cell suspension was centrifuged and filtered through 70 uM and 40 uM cell strainers. ASCs were seeded at 5,000 cells/cm^2 and cultured in DMEM-F12 supplemented with 10% fetal bovine serum (Thermo Scientific HyClone). Cells were used between passages 3-5 for all studies.

To evaluate whether in vivo adipogenesis could be enhanced by the addition of ASCs, a combined approach with adipose ECM and stem cell delivery was used. ASCs were expanded and resuspended with 200 ul of adipose ECM right before injection. Athymic mice (n=12) received injections of adipose ECM with and without 2 million ASCs. Volume persistence was monitored with digital calipers starting 24 hours after initial injection at day 1 to prevent over manipulation of the implants and measured at regular intervals every 2 weeks. Implants were removed from 4 mice at each of the study timepoints of 1, 4, and 12 weeks for histological analysis.

5.2.3 Histology

Specimens for paraffin embedding were fixed with 10% formalin, dehydrated through graded ethanol solutions, cleared in xylene, and embedded in paraffin. Samples were sectioned at 5 um thickness and stained with hematoxylin and eosin or Masson's trichrome. For Oil Red O (0.75% (w/v) in 36% triethyl phosphate) staining of lipids, samples were fixed and infiltrated with graded sucrose solutions, followed by OCT embedding and cryosectioned in order to visualize new adipose tissue formation.

5.2.4 Immunostaining

Antigen retrieval was performed using heat-induced epitope retrieval with sodium citrate buffer. Deparaffinized and rehydrated slides were boiled in 10 mM sodium citrate, 0.05% Tween-20, pH 6 for 30 minutes at 100°C. Sections were blocked with 10% normal goat serum or 1% BSA for 2 hours at room temperature and incubated with primary antibody overnight at 4°C. Secondary antibodies were applied the next day and incubated for 2 hours at room temperature. Slides were coverslipped with Vectashield + DAPI and sealed. The following primary antibodies were used: mouse anti-human nuclei (Millipore), and mouse anti-rat CD44 (BD Pharmingen, San Diego, CA), and mouse anti-CD31 (Abcam). The secondary antibodies for fluorescent detection were Alexafluor 488 goat anti-mouse IgG1 (Invitrogen) and Alexafluor goat anti-mouse IgG2a. The SuperPicture Mouse DAB Kit (Invitrogen) was used for chromogen detection.

5.3 Results

5.3.1 In vivo biocompatibility of crosslinked adipose ECM

The in vivo study was carried out with control, 5 mM EDC, and 1% HMDC in Tween 20 crosslinked ECM. After two weeks of implantation, adipose ECM implants were opaque and vascularization could be observed at the surface (**Fig 5.1**). Adipose tissue development was observed at the periphery of the implant. No sign of a severe immune response was evident, but a focal acute inflammatory response was observed at the periphery, extending inwards. The

inflammatory reaction was primarily comprised of lymphocytes and neutrophils and the most prominent fibrous capsule formed with the 5 mM EDC crosslinked ECM.

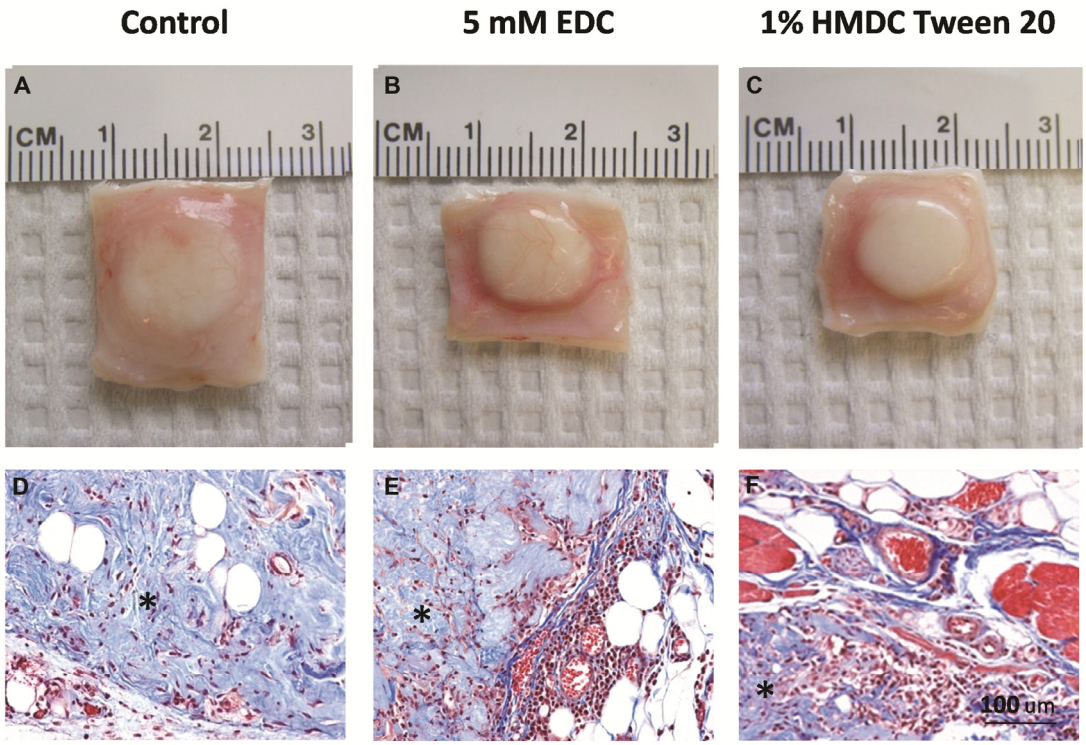


Figure 5.1 In vivo biocompatibility of crosslinked matrices. Gross (A-C) and histological images (E-F) of adipose matrix from the in vivo rat study for uncrosslinked control (A, D), 5 mM EDC crosslinked (B, E), and 1% HMDC crosslinked ECM (C, F). Specimens are stained with Masson's trichrome and the adipose-derived matrix is denoted by an asterisk.

5.3.2 Long term biocompatibility

In the long term study, the adipose-derived matrix developed into newly formed adipose tissue at the edges near the scaffold interface with host subcutaneous tissue. Oil Red O staining accentuated the band of adipose tissue forming from the edges of the implant at four weeks (**Fig 5.2**). Additionally, cells in the center of the matrix stained positive for CD31, indicating vascular development, which is the necessary prerequisite for formation of new adipose tissue. A number of cells also stained positive for CD44, a cell surface receptor for hyaluronic acid that is expressed on both cells of hematopoietic and non-hematopoietic origin and is involved in

lymphocyte homing. By 12 weeks, the adipose tissue development continued in the implant and areas of collagen remodeling were observed by histology (**Fig 5.3**).

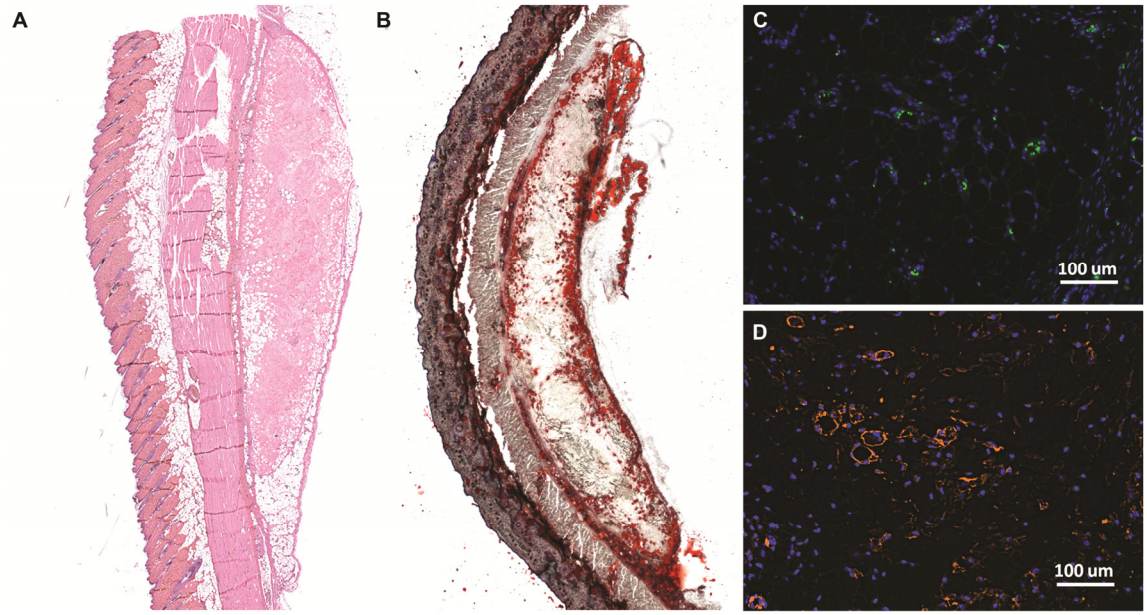


Figure 5.2 In vivo biocompatibility in a rat model. Histology of adipose ECM implants after four weeks in Sprague Dawley rats stained with hematoxylin and eosin (A), Oil red O for lipids (B), CD31 (C), and CD44 (D).

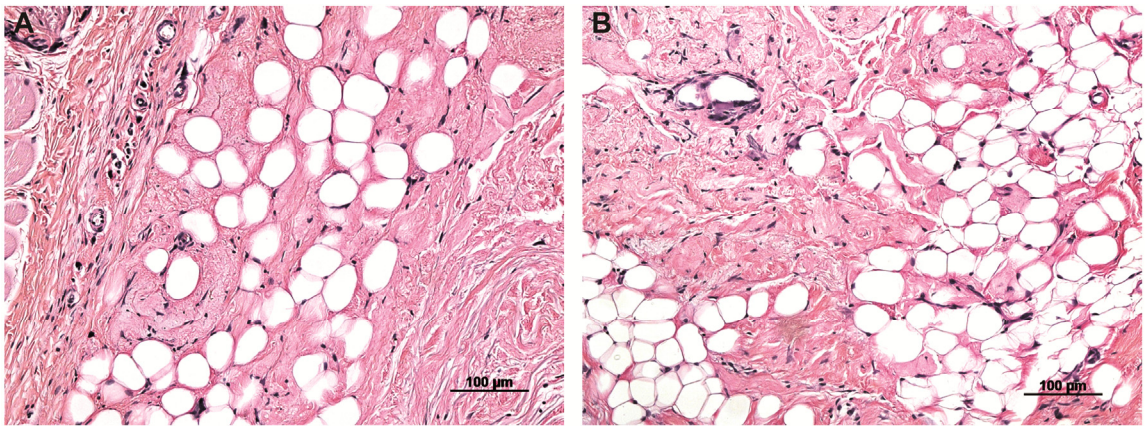


Figure 5.3 De novo adipogenesis at implant periphery. At 12 weeks in the subcutaneous rat study, adipose matrix implants show substantial areas of adipose tissue development and collagen remodeling by host cells.

5.3.3 In vivo volume retention compared to fat grafting

There was no significant difference in the volume persistence between adipose ECM implants and lipoaspirate fat grafts (**Fig 5.4**). At one week post implantation, there was slightly more swelling for the adipose ECM implants compared to fat grafts whereas fat grafts just showed a continual decrease in volume after injection. Once the swelling subsided, the volume retention of the two conditions was nearly indistinguishable. Upon gross examination of the implants at 12 weeks, the adipose ECM implants were white in color in contrast to the characteristic yellow color of the adipose fat grafts from the lipids in human adipose tissue.

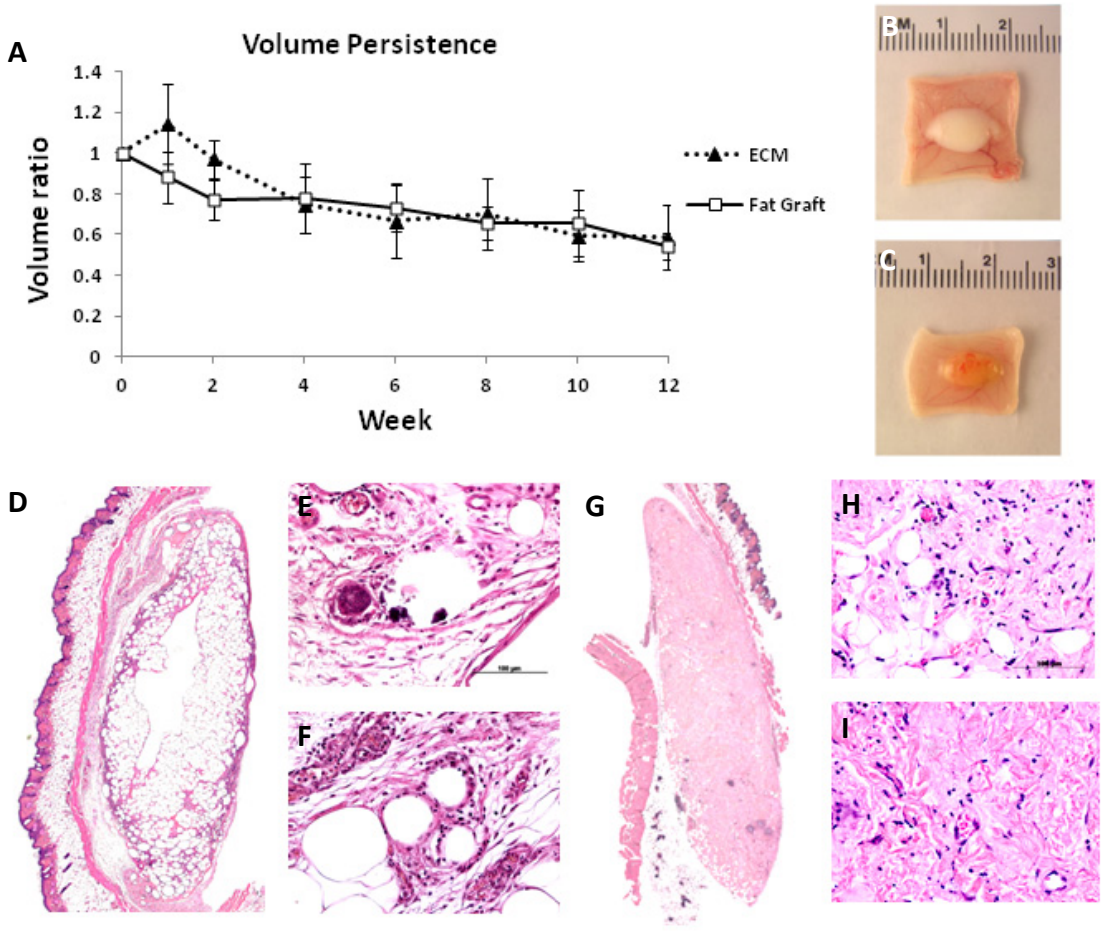


Figure 5.4 Comparison of in vivo fat grafting and adipose ECM. Volume persistence of the fat grafts and adipose ECM measured with digital calipers (A). Gross images of the adipose ECM (B) and human lipoaspirate fat graft (C) at the end of the 12 week study period. Hematoxylin and eosin staining of the fat graft (D-F) with signs of calcification (E) and cyst formation (F). Hematoxylin and eosin staining of human adipose ECM implants (G-I). High magnification images showing adipogenesis (H) and vascularization within the implant (I).

Histology of the adipose fat graft showed a layer of viable adipocytes around the edges, but a central necrotic cyst had formed in the center. Areas of calcification were also observed, which typically occur secondary to necrosis and phagocytes were observed in the implant area, clearing away lipid and cell debris. Hematoxylin and eosin staining of the adipose matrix showed the scaffold largely remained, with few areas of adipose tissue formation along the periphery. There was also some vascularization in the matrix and minimal inflammation.

5.3.4 In vivo adipogenesis of adipose ECM with ASCs

To determine if adipogenesis could be improved with the addition of ASCs to the matrix, ASCs were expanded in culture and resuspended with the adipose matrix just prior to delivery by subcutaneous injection in an athymic mouse model (**Fig 5.5**). The volume persistence showed that the adipose matrix alone maintained a constant volume throughout the study period. The adipose matrix with ASCs showed a slight decrease between weeks 4-10 but stabilized thereafter.

The histology of the acellular adipose matrix scaffold was similar to the results obtained in the previous study, with the adipose matrix maintaining volume stability and the scaffold remaining largely in place. Staining of the adipose matrix with cells at 12 weeks showed extensive de novo adipose tissue formation. Much of the implant was replaced by new adipose tissue with the edges completely populated by new adipocytes and progressing inward to the center of the implant, with small areas in the center where the original scaffold was still visible. The remaining areas of the adipose matrix were sites with high levels of activity and matrix remodeling, as evidenced by the staining pattern of the collagen-based scaffold.

Immunostaining was performed to determine whether the implanted human ASCs still remained in the scaffold and at 12 weeks, ASCs were observed in the implant, staining positively for antibodies against cells of human origin. It is unclear whether the ASCs have differentiated

into adipocytes or whether they remain in the stroma and adopt a supportive role in facilitating new adipose tissue formation.

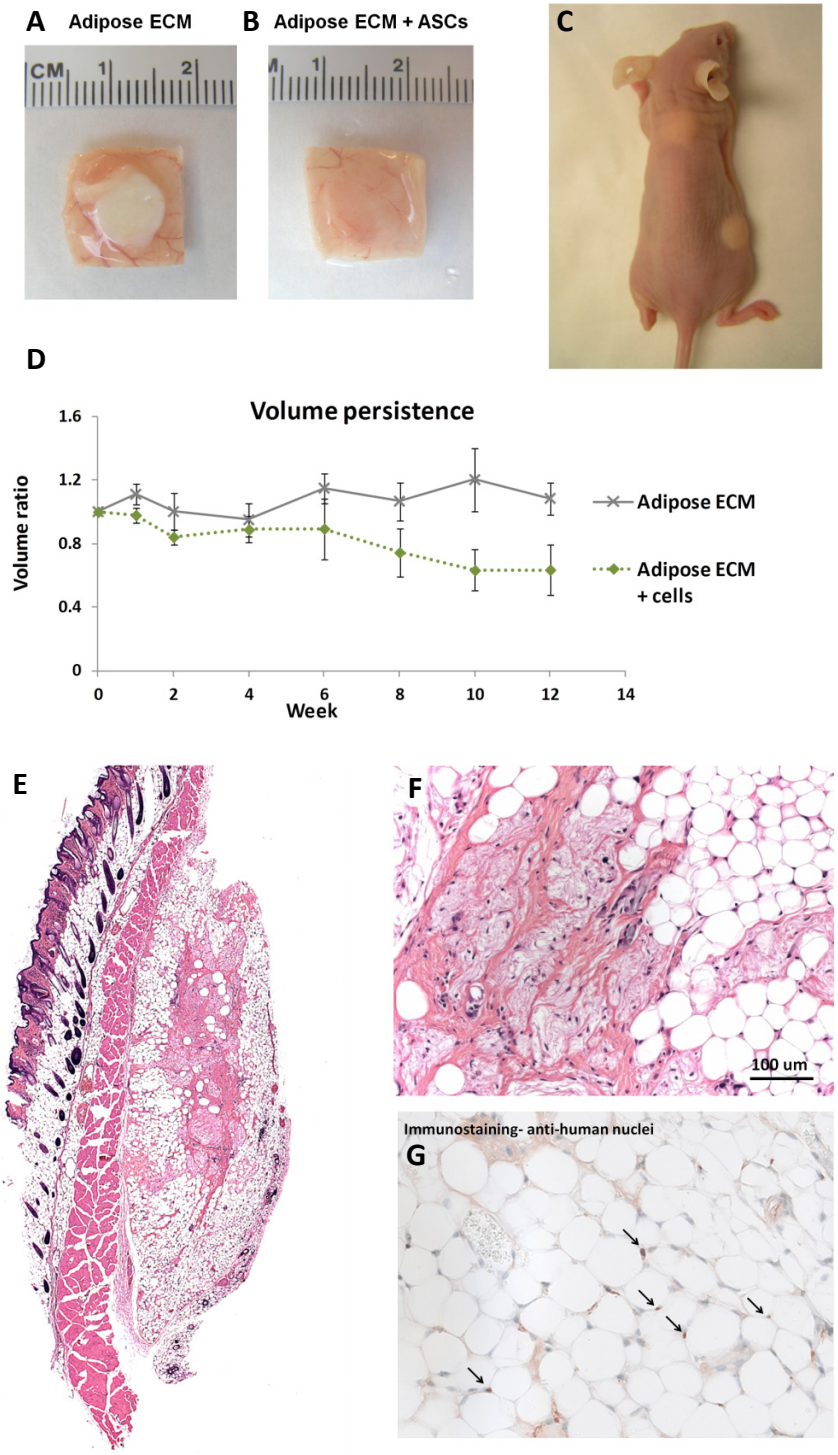


Figure 5.5 In vivo adipogenesis for adipose ECM with ASC delivery. Gross images of an adipose ECM implant (A) and adipose ECM with ASCs (B) at the end of 12 weeks in an athymic mouse model (C). Volume measurements of both conditions using digital calipers (D). Histology with hematoxylin and eosin staining of the adipose ECM with ASCs at 12 weeks (E). High magnification images of areas of new adipose tissue formation (F) and immunostaining for anti-human nuclei to identify implanted human cells at 12 weeks (G).

5.4 Discussion

Our studies showed that a decellularized ECM biomaterial can be derived from adipose tissue to obtain a biocompatible scaffold for the repair of soft tissue defects. Crosslinking the decellularized scaffold provided an additional element of control over the degradation properties of the tissue and allows the material to be tailored to the target site of implantation. In vivo implantation in rats revealed good tissue integration with adipose tissue development starting at the edges of the ECM scaffold.

The adipose-derived matrix supported cell migration and infiltration, with a considerable number of host cells at the center of the implant for the uncrosslinked control and 1% HMDC crosslinked matrices. There was a more prominent fibrous capsule observed for the crosslinked matrices, so while they do not appear to elicit a severe inflammatory reaction, fewer cells migrate into the scaffold. In the long term in vivo study, adipogenesis was observed at the periphery of the implant, suggesting that despite the acellular nature of the implant, the bioactive properties of the matrix render it conducive to host cell migration, which could be sufficient for tissue regeneration. The adipose scaffold was able to maintain a stable volume, proving efficacy in restoring contour and maintaining a volume correction.

When the volume retention of the adipose ECM was compared to the clinical standard of fat grafting, the retention rates were comparable and were not significantly different. Regarding in vivo efficacy, the adipose matrix performed just as well as fat grafting and would thus provide patients with an alternative in which they would no longer need to undergo a surgical procedure of liposuction to collect the required lipoaspirate. Instead, the adipose matrix could be produced as an off-the-shelf product that could be used allogenically. Histology of the fat grafts revealed the troubling disadvantages of fat grafting. At 12 weeks, a defined central necrotic cyst had formed with several smaller cysts throughout the implant and areas of calcification. Higher levels of inflammation were also observed as phagocytic cells migrate into the graft to clear away nonviable adipocytes and residual intracellular lipid.

Adipocytes in their native environment each have access to at least one blood vessel for oxygen and nutrient exchange. During the fat grafting procedure, adipocytes are ripped from their native environment and reinjected at another site of the body. Adipocytes are fragile by nature, due to the oversized lipid droplet they safeguard and are easily ruptured by the negative pressure applied during liposuction. Once injected in their new site, adipocytes in the center no longer have access to a blood supply and these metabolically active cells undergo necrosis. Surgeons have developed numerous protocols to improve viability of the grafted cells by injecting into small channels rather than a bolus, using more involved preparations of lipoaspirate such as centrifugation or washing, and adding stromal vascular cells to aid in graft survival. However the unpredictable rates of resorption and lack of standardization of techniques remain a problem.

Thus, one of the distinct advantages of the adipose ECM compared to fat grafting is that the high metabolic burden of transplanting adipocytes is bypassed. Instead, the adipose ECM is an acellular graft that relies on host cells to migrate into the scaffold and repopulate it to form new tissue. This also ensures that the rate of vascularization is coupled with the rate of new adipose tissue formation to generate sustainable adipose tissue with high cell viability.

In the final in vivo study, the adipose matrix scaffold was investigated as a delivery vehicle for ASCs. The rationale behind the approach was twofold: first, the adipose stem cells could enhance adipogenesis by differentiation into adipocytes and secretion of paracrine factors, and second, the adipose matrix could provide a protective environment for stem cell engraftment and survival. The ASCs were just expanded in culture prior to differentiation and not predifferentiated, so any induction of adipogenesis was not due to addition of exogenous factors. The addition of the ASCs drastically improved de novo adipogenesis compared to delivery of the acellular adipose matrix. At 12 weeks, the adipose scaffold is almost entirely replaced by new adipose tissue whereas scaffolds without the inclusion of cells had adipogenesis only at the periphery of the implant. The mechanisms by which the ASCs speed up this process of

adipogenesis are still unclear. With immunostaining, we found that the ASCs were still present in the implants at week 12, but it was impossible to elucidate whether they had differentiated into adipocytes or if they remained in the stromal compartment.

Promising developments in point-of-care systems for automated isolation of stromal vascular cells from lipoaspirate could make intraoperative isolation of a patient's own cells possible. ASCs do not have the high metabolic demands of the adipocytes from the previous study and have been found to have significant immunomodulatory and paracrine activity.[146] The use of combined cell-scaffold delivery approaches to tissue regeneration may soon be realized with technological advances that will allow for automatic isolation of adipose stromal cells.

To date, intact adipose-derived ECM has not been studied extensively in vivo for use in soft tissue repair, but results from this study suggest that adipose tissue can be effectively decellularized and stripped of its antigenic components to produce a biocompatible matrix for adipose tissue engineering. The adipose matrix can be used as an acellular scaffold for filling soft tissue defects, or it can be used in conjunction with delivery of progenitor cells to enhance new tissue formation. The acellular scaffold is sufficient in restoring volume for most soft tissue defects but there may be cases where the regenerative activity of ASCs may be beneficial. In areas known to have poor regenerative capacity such as irradiated tissue beds or scarring from burn wounds, ASC delivery may be ideal for encouraging tissue regeneration.

6. Discussion and future work

In this dissertation we have demonstrated the development of an adipose extracellular matrix-based biomaterial. By removing the cells and lipids from human adipose tissue, we obtained a biocompatible scaffold for soft tissue repair. The degradation properties of the adipose matrix can be modulated through chemical cross-linking, allowing the user to tune the rate of degradation based on the rate of tissue regeneration. The adipose matrix was further characterized to determine the protein composition of the ECM. Through mass spectrometry, the proteomic profile of the adipose matrix was acquired, allowing us to identify the proteins and proteoglycans that constitute the adipose ECM. The mechanical and thermal properties of the matrix were measured with rheology and differential scanning calorimetry, respectively, providing a complete characterization of the material properties.

We next evaluated the cell response to the adipose-derived biomaterial through in vitro studies. Through migration studies with adipose-derived stem cells, we found that the adipose matrix had chemoattractive properties that guided stem cell migration, which would benefit in vivo tissue regeneration. The adipose matrix supported cell adhesion, providing an environment conducive to cell growth and differentiation. To investigate whether there was tissue specificity in extracellular matrices from different tissues, we compared the adipogenic differentiation of adipose-derived stem cells on adipose and dermal matrices. Results from the study showed that

the adipose matrix did improve adipogenic differentiation, supporting unique tissue-specific bioactivity of the adipose matrix.

Finally, we investigated the in vivo host response to the adipose matrix. The acellular scaffolds were well tolerated in vivo and had minimal inflammatory response, even with chemical cross-linking. Over 12 weeks, the volume persistence was steady, proving efficacy of the adipose matrix in maintaining a volume correction. When compared with the clinical standard of fat grafting, the acellular adipose scaffold had clear advantages since there was no cellular component that needed to be transplanted. Limited cell viability in the fat grafts led to necrosis and inflammation, which in turn caused cyst formation and calcification over the course of the study. Alternatively, the adipose matrix can also be used as a delivery vehicle for adipose-derived stem cells. The addition of the stem cells enhanced adipogenesis in the scaffold, resulting in significant new adipose tissue formation, replacing much of the original scaffold at 12 weeks.

Through this work, we have established a novel adipose-derived biomaterial that facilitates adipose tissue regeneration. The adipose matrix presents an off-the-shelf alternative for repairing soft tissue defects that would circumvent the need for donor site tissue for reconstruction, thereby preventing donor site morbidities for the patient. Future directions for this work include elucidating differences in the adipose extracellular matrix derived from different adipose tissue depots of the body and disease-related changes to the extracellular matrix, such as in a diabetic donor. Additionally, further studies would need to be carried out to reveal the mechanisms by which adipose-derived stem cells enhance adipogenesis in the cell plus scaffold approach. It is still unclear whether the adipose-derived stem cells act by paracrine factors or if they fully differentiate into adipocytes to contribute to the new adipose tissue formation. If the activity is indeed attributed to the secretion of trophic factors, one would ideally be able to identify which factors are responsible and incorporate them into the scaffold design in order to harness the beneficial effects of the ASCs without having to perform cell transplantation. Thus, the adipose

extracellular matrix serves as a foundation for new adipose tissue growth, making it a promising therapeutic tool for soft tissue regeneration.

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145. Karp JM, Leng Teo GS. Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell.* Mar 6 2009;4(3):206-216.
146. Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. *Circ Res.* May 11 2007;100(9):1249-1260.

CURRICULUM VITAE

EDUCATION

Ph.D. Biomedical Engineering, *May 2013*
Johns Hopkins University
Department: Biomedical Engineering

B.S. Bioengineering, *June 2008*
University of California, San Diego
Major: Bioengineering: Biotechnology
Minor: Cognitive Science

TEACHING EXPERIENCE

Freshman Modeling and Design – Johns Hopkins University	2010
Cell and Tissue Engineering – Johns Hopkins University	2011
General Chemistry – University of California, San Diego	2006 – 2008

RESEARCH EXPERIENCE

Translational Tissue Engineering Center, Johns Hopkins University
7/2008– 5/2013
Department of Biomedical Engineering, Advisor: Jennifer Elisseeff, Ph.D.

Graduate student researcher- Developed an extracellular matrix-based scaffold for soft tissue reconstruction and characterized material properties through mechanical testing and proteomic analysis. Investigated the in vitro bioactivity of the extracellular matrix in guiding stem cell differentiation and in vivo biocompatibility including the host immune response to the biomaterial.

Cartilage Tissue Engineering Lab, University of California, San Diego
1/2007- 7/2008
Department of Bioengineering, Advisor: Robert Sah, M.D., Sc.D.

Research Assistant- Optimized the use of human osteoarthritic chondrocytes for cartilage repair through the cell isolation, expansion and redifferentiation process for engineering cartilage

constructs. Improved bioreactor design for whole joint culture incorporating continuous passive motion to apply dynamic mechanical forces on articulating cartilage surfaces.

Pacific Rim Undergraduates Experiences Program Scholar, Monash University, Australia
6/2006-8/2006
Department of Bioengineering, Advisors: Roy Kerckhoffs, Ph.D. (UCSD)
Department of Computer Science, Advisor: David Abramson, Ph.D. (Monash University)

Intern at Monash University, Melbourne, Australia- Carried out computational cardiac modeling of the human heart and stabilized ion concentrations in simulations of different types of myocardium during contraction.

National Center for Microscopy and Imaging Research (NCMIR), UCSD
2/2006- 6/2006
Advisor: Guy Perkins, Ph.D.

Research Assistant- Performed electron microscope tomography of membrane-bound cellular organelles including computer image processing for 3D visualization and reconstruction of cellular proteomes and wet lab specimen preparation for electron microscopy.

Cardiac Mechanics Research Group, UCSD
8/2005-12/2005
Department of Bioengineering, Advisor: Andrew McCulloch, Ph.D.

Research Assistant- Conducted high throughput cardiac phenotyping of *drosophila Melanogaster* and a systematic genetic screen to characterize cardiac adaptation to hypoxic conditions.

JOURNAL PUBLICATIONS

Wu I, Condé-Green A, Graham IB, Berglund T, Chae J, Elisseeff JH. A translational study of the acellular adipose matrix for soft tissue regeneration. (*In preparation)*

Condé-Green A, Wu I, Graham IB, Chae J, Drachenberg C, Singh D, Holton L, Slezak S, Elisseeff J. Comparison of three techniques of fat grafting and cell-supplemented lipotransfer in athymic rats: a pilot study. *Aesthetic Surgery Journal.* (*In press*)

Wu I, Nahas Z, Kimmerling KA, Rosson GD, Elisseeff JH. An Injectable Adipose Matrix for Soft-Tissue Reconstruction. *Plastic and Reconstructive Surgery*. 129(6): 1247-1257, 2012.

Hsieh-Bonassera ND, Wu I, Lin JK, Schumacher BL, Chen AC, Masuda K, Bugbee WD, Sah RL: Expansion and redifferentiation of chondrocytes from osteoarthritic cartilage: cells for human cartilage tissue engineering. *Tissue Engineering Part A*. 15: 3513-3523, 2009.

BOOK CHAPTERS

Wu I, Elisseeff J. Extracellular matrix – based approaches for the engineering of soft connective tissues. Li S, L'Heureux N, Elisseeff JH (Eds.), *Stem Cells and Tissue Engineering*. Hackensack, NJ: World Scientific. (*In preparation*)

CONFERENCE PRESENTATIONS

Wu I, Condé-Green A, Graham I, Chae J, Elisseeff J. Use of an adipose matrix-based scaffold as an alternative to fat grafting. *International Federation for Adipose Therapeutics and Science*. Quebec City, Canada. October 5-7, 2012.

Wu I, Graham I, Condé-Green A, Chae J, Nahas Z, Elisseeff J. Intrinsic bioactive properties and tissue specificity of the adipose extracellular matrix guide soft tissue regeneration. *Tissue Engineering and Regenerative Medicine International Society World Congress*, Vienna, Austria. September 5-8, 2012.

Wu I, Chae J, Berglund T, Graham I, Nahas Z, Elisseeff J. Inductive capacity of adipose extracellular matrix in guiding stem cell differentiation and tissue regeneration. *Tissue Engineering and Regenerative Medicine International Society*, Houston, TX, December 11-14, 2011.

Wu I, Nahas Z, Rosson G, Elisseeff J. Acellular Adipose Tissue Scaffold for Soft Tissue Reconstruction. *American Society of Plastic Surgeons,* Denver, September 23-27, 2011.

Wu I, Nahas Z, Kimmerling K, Elisseeff J. Adipose-Derived Biomaterials for Soft Tissue Reconstruction. *Tissue Engineering and Regenerative Medicine International Society*, Orlando, December 5-8, 2010.