COMPOSITE ADIPOSE DERIVED DELIVERY SYSTEMS CONTAINING ADIPOGENIC FACTORS FOR SOFT TISSUE RESTORATION

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Musculoskeletal injuries inflicted by wars, congenital deformities, tumor resection, and general traumatic injuries often require soft tissue reconstruction. Damages to soft tissue not only affect cosmetic appearance, but also hinder function and emotional well-being. Autologous adipose grafting using processed lipoaspirate is a safe, resourceful, and minimally invasive option gaining tremendous momentum in clinical practice due to potential applications in trauma and reconstructive surgery, especially for breast cancer reconstruction. However, results can be unpredictable due to graft resorption rates reaching as high as 90%. These limitations serve as motivation for the development of new therapies to regenerate adipose tissue. We examined the use of an injectable adipose derived extracellular matrix (AdECM) combined with dexamethasone encapsulated polymer microspheres (Dex MS) as a scaffold for soft tissue restoration. First, we implemented a decellularization process to remove lipids and cellular content from discarded adipose tissue. The remaining material, known as AdECM, was thoroughly characterized. Triglycerides and residual DNA content were significantly reduced to ensure biomaterial safety. Adipose matrix proteins and glycosaminoglycans were evident and measurable following the decellularization process. Additionally, polymer microsphere encapsulation techniques were used to release dexamethasone in a controlled fashion.

Hydrogels derived from AdECM have shown potential in the ability to generate new adipose tissue in vivo. To further enhance adipogenesis, a composite adipose derived delivery system (CADDS) containing single- and double-walled dexamethasone encapsulated microspheres (SW and DW Dex MS) was developed. Previously, our laboratory has published the use of Dex MS as an additive to enhance adipogenesis and angiogenesis in adipose tissue grafts. In the current work, AdECM and CADDS are extensively characterized, in addition to conducting in vitro cell culture analysis. Characterization studies indicate the AdECM used for the CADDS has minimal cellular and lipid content allowing for gelation of its collagen structure under physiological conditions. Human adipose-derived stem cell (hASC) culture studies confirmed viability with the CADDS, and adipogenesis was increased in experimental groups containing the hydrogel scaffold. In vitro studies of AdECM hydrogel containing microspheres demonstrated a controlled release of dexamethasone from SW and DW formulations. In vivo studies indicated increased volume retention from CADDS material implants with Dex MS. Additionally, in vitro studies comparing hASCs to rat ASCs (rASCs) showed that hASCs differentiate and accumulate lipids on CADDS material at a higher capacity than rASCs. The comparison offers an explanation to the low retention rates seen from the animal studies. The delivery of Dex MS via an injectable hydrogel scaffold combines two biologically responsive components to develop a minimally invasive, off-the-shelf biomaterial for adipose tissue engineering.

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PREFACE

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1.0 INTRODUCTION

Adipose tissue plays an essential regulatory role in normal substrate metabolism by storing and releasing high-energy compounds when the needed by the body. Adipose tissue is found throughout the body to protect underlying structures, provide insulation, and impart a typical human appearance (Choi et al. 2010; Philips et al. 2012a). Human appearance can become compromised by disease, trauma, tumor resection, or congenital defects. Soft adipose tissue engineering has recently generated great interest to surgeons treating patients with disfiguring contour imperfections, particularly when using autologous fat grafting as a treatment (Philips et al. 2012a). Autologous fat grafting, using a patient's own processed lipoaspirate, has surged in recent years as a minimally-invasive solution. Although long-term outcomes of fat graft survival and durability still remain highly unpredictable due to post-graft resorption with the possibility of as little as ten percent of the original fat volume retained (Choi et al. 2010; Minteer et al. 2015; Philips et al. 2012a). The main requirements of adipose tissue engineering can be summarized into three main categories: host compatibility, bioactivity, and sustainability. Host compatibility is imperative so that the implanted construct does not elicit host immunological rejection. Moreover, tissue-specific soft tissue reconstruction would optimize host compatibility (Choi et al. 2010). We utilized the tissue-specific approach to design a composite adipose derived delivery system from decellularized adipose tissue and dexamethasone encapsulated polymer microspheres for adipose tissue defect restoration.

1.1 THE CLINICAL NEED FOR ADIPOSE TISSUE RECONSTRUCTION

Adipose tissue is distributed throughout the body and is categorized by location such as subcutaneous (arm, abdominal, and gluteal), intraabdominal (omental, retroperitoneal, and visceral) and other sites (retroorbital, periarticular regions, bone marrow, intramuscular, and pericardial). Adipose tissue performs an essential role in the protection of underlying structures, providing insulation, contributing function, and imparting a normal human appearance. This appearance, and even function, can be compromised by disease, trauma, tumor resection or congenital defects of the subcutaneous adipose tissue. These pathologies and defects of the adipose tissue remain a reconstructive challenge for plastic surgeons and clinicians. According to the American Society of Plastic Surgeons, the most common reconstructive procedures are breast reconstruction, burn care, cleft lip/palate repair, tumor removal, and treatment from dog bites (ASPS 2017). In particular, from 2009 to 2014, the Agency for Healthcare Research and Quality reported an increase in breast reconstruction after mastectomy by 62 percent (Miller et al. 2017).

Two major reconstruction options for breast adipose tissue deficits in the clinic include implant-based procedures or flap surgery using the patient's own skin, fat, and/or muscle. Currently, flap surgeries are not without morbidity. Patients receiving autologous reconstruction are at high risk for specific complications including wound infection, flap/prosthesis failure, and reoperation. Additionally, general complications of major surgery such as deep vein thrombosis, blood transfusions, pneumonia, and pulmonary embolism can lead to a prolonged length of stay in the hospital (Agha, Goodacre, and Orgill 2013b; Tachi and Yamada 2005). Implant-based breast reconstruction is the most frequently used technique globally (Schmauss et al. 2016). The first documented implant-based breast reconstruction was performed by Vincent Czerny in 1895 using the patient's lipoma to augment a post-surgical asymmetry after tumor resection (Champaneria et

al. 2012). Implant-based procedures require adequate skin envelope that allows covering the implant that is normally introduced in a sub-muscular plane detaching the medial insertions of the pectoralis major muscle from the ribs. The most common implants are silicone gel-filled breast implants that contain a highly viscous and more or less form-stable gel. Implants are available in a variety of shapes and sizes (Schmauss et al. 2016). Patients undergoing implant-based reconstruction may encounter complications such as rupture, migration, discontent, implant exposure/extrusion, rippling, and deformation/distortion (Rocco et al. 2016). These complications can occur during the subsequent 10 years following the procedure with a risk of reoperation of 70% (Schmauss et al. 2016). With the volume of complications to consider using invasive procedures such as flap and implant-based surgeries, clinicians and patients are looking for less or minimally invasive options.

In effort to reduce the risk of complications, clinicians have increased the practice of a widely-used procedure in plastic surgery known as autologous fat grafting (AFG). Assuming the patient has adipose tissue to spare, which is not always the case, AFG harvests the patient's own fat, typically from the abdomen or thighs, via liposuction and deposits the tissue into the defect site with syringe injections. AFG is minimally invasive and carries several advantages in comparison to implant- or flap-based soft tissue reconstruction. With minimal scarring from the defect site and no foreign body reaction, the patient recovery time is typically less than 48 hours.

Depending on the clinical indication, the standard of care for adipose tissue repair and regeneration may vary as well as their outcomes. For cases such as breast reconstruction, lipoatrophy, scleroderma, face rejuvenation, gluteal augmentation, cranio-maxilla-facial deformities, etc., the gold standard of care is an AFG (Monfort and Izeta 2012). AFG is a safe, resourceful, and minimally invasive option which allows the patient's body to provide its own

non-immunogenic, compatible biomaterial (Agha, Goodacre, and Orgill 2013a, Hong et al. 2010a, Gir et al. 2012a). The liposuction technique and fat processing of AFG has significantly evolved over the past century with the advancement of technologies used to perform the procedure and examination of studies to optimize graft survival. As shown in Figure 1, many plastic and reconstructive surgeons have adopted the "lipo-structure technique" or Coleman technique for the microinjection of fat particles to minimize fat graft loss (Mahoney et al. 2018). Using a Coleman microcannula, light negative pressure is created by drawing the plunger of a 10-ml syringe connected to a 3-mm cannula when introduced in the subcutaneous cavity per a small incision. The cannula is moved through the adipose section manually, loosening the tissue, and collecting the fat into the syringe. For processing, the collected fat tissue is transferred to 10 mL tubes for centrifugation to separate into four layers: (a) oily fraction; (b) aqueous fraction; (c) a cell pellet; and (d) the purified fat flanked by the oily and aqueous fractions (Simonacci et al.). Centrifugation at 3000 rpm for 3 min is the standard for separation of fractions, but there is literature that suggests slower speeds may reduce adipocyte disruption (Gir et al. 2012a). Following centrifugation, the middle layer of viable adipocytes is transferred to 1 cc syringes. Successful graft implantation is executed using blunt tip cannulas to create tunnels at insertion, with small aliquots of fat injected at different depths of the defect. Multiple passes are utilized to reach throughout the site of interest. Due to the unpredictable volume results from AFG, the majority of clinicians overcorrect volume deficiencies from 20-30% (Kaufman et al. 2007, Tabit et al. 2012).

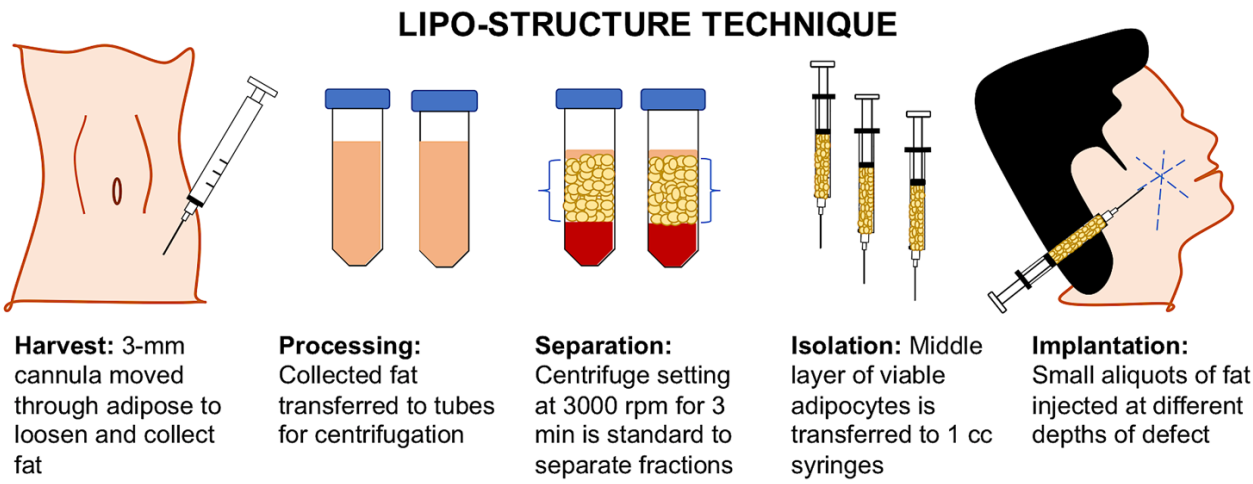

Figure 1. Lipo-structure technique for autologous fat grafting (Mahoney et al. 2018)

Adipose grafts contain adipose derived stem cells (ASCs) with the capability of differentiating into various cell lineages in the correct environment (Maione et al. 2015). Other advantages of ASCs include the expression/secretion of growth factors such as IGF (insulin-like growth factor), HGF (hepatocyte growth factor), TGF-β1 (transforming growth factor beta 1) and VEGF (vascular endothelial growth factor). Several studies have shown that the addition of ASCs to increase stem cell content of the fat graft lead to healthier engraftment (Philips, Marra, and Rubin 2012, Choi et al. 2014a). Protocols involving the injection of ASCs from the stromal vascular fraction (SVF), seen in Figure 2, of processed adipose tissue require an additional operation. However, while the general outcomes for these procedures are successful in the short-term, the long-term results can be unpredictable due to post-graft resorption rates reaching as high as 90% (Monfort and Izeta 2012, Hong et al. 2010a). These studies demonstrated no evidence to support an improved protocol (Gir et al. 2012b, Yu et al. 2015). Although fat transplantation techniques have been refined and enhanced in recent years, further improvement is required due to the continued lack of predictability in volume restoration. Several techniques have been evaluated for their differences in fat harvesting, fat processing, fat reinjection, and fat storage.

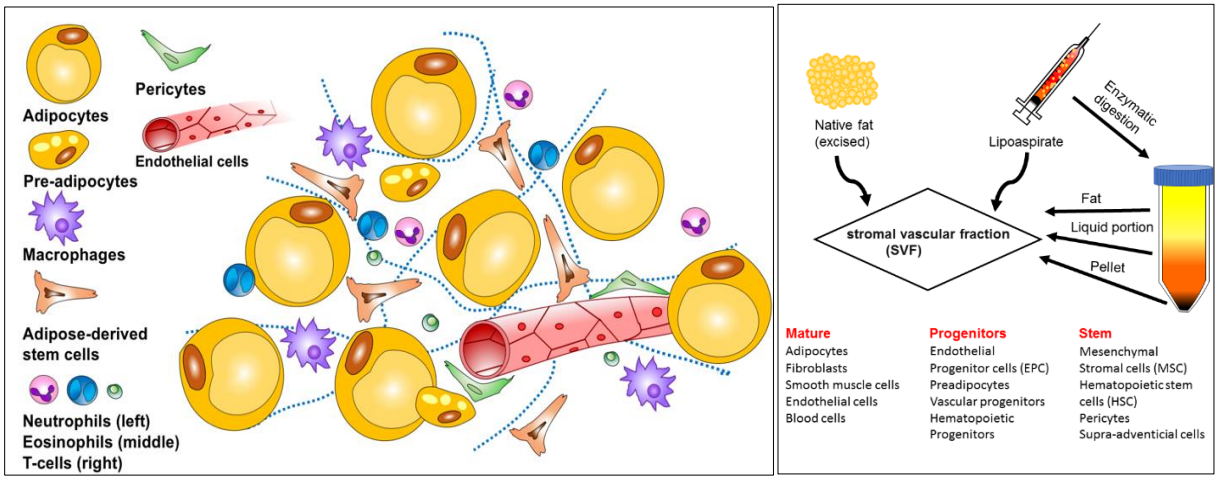

Figure 2. Cellular and Extracellular Constituents of Adipose Tissue and SVF

While AFG is a widely utilized treatment, there can be complications. Experienced physicians using the preferred technique may encounter unsatisfactory results such as fat necrosis, calcification, oil cyst formation, and hypertrophic scarring (Simonacci et al. , Gir et al. 2012a, Nahabedian 2015, Philips, Marra, and Rubin 2014). Largo et al. reviewed several publications following the results of over 1400 patients (Largo et al. 2014). The studies containing volume retention data documented values varying from 55 to 82 percent retention. Other studies have reported resorption rates from 30 to 70 percent (Kaufman et al. 2007, Kurita et al. 2008). There are several mechanisms that contribute to the variability of outcomes from AFG. The trauma caused by liposuction can lead to apoptosis of adipocytes and cyst formation. The different degrees of blood supply and fluctuations in oxygen delivery of the recipient wound may range from adequate revascularization to insufficient revascularization and ischemia, apoptosis, and dedifferentiation of essential adipocytes. An accepted principle of fat grafting is that adipocytes within 2 mm of an arterial blood supply have greatest chance of survival (Tabit et al. 2012, Bourne et al. 2016). Overall, autologous fat grafting is limited by graft absorption and necrosis due to lack of vascularization after fat transfer.

1.2 ADIPOSE TISSUE ENGINEERING

Tissue engineers have begun to address the challenges of variable resorption of autologous fat grafting with strategies to engineer tissue tailored to a specific site of injury (Keck et al. 2011a, Jennifer H. Choi 2010). Synthetic polymer biomaterials for adipose tissue engineering have the advantage of being able to tailor mechanical properties, chemical properties, and degradability. In addition, a number of biologically derived materials, such as collagen, hyaluronic acid, fibrin, and alginate, have been utilized as injectable scaffolds for soft tissue repair and shown successful adipose tissue growth (Hemmrich and von Heimburg 2006). However, significant work *in vivo* remains in order to confirm and optimize treatments for clinical use, including investigating the role of macrophage in the remodeling process following implantation. Since the extracellular matrix of each tissue and organ consists of specialized secreted products for its resident cell population, it would be appropriate to develop an injectable hydrogel from adipose tissue as replacement material that contains the constitutive peptides, cytokines, and complement factors which regulate cell growth, vascularization and homeostasis (Choi et al. 2014b, Cheung et al. 2014b, Sano et al. 2014, Choi et al. 2011).

Adipose tissue engineering requires the essential permutation of bioactivity, sustainability, and host compatibility. Several studies have shown that tissue site influences both amount of adipose tissue regeneration and metabolic function of regenerated tissue. Schipper et al. demonstrated that human adipose-derived stem cells harvested from various subcutaneous depots exhibited significant differences in adipogenic differentiation, sensitivity to apoptosis, and lipolytic function (Schipper et al. 2008). A review by Giorgino et al. describes the metabolic and hormonal differences between fat depots, as well as the different signaling properties of distinct fat depots (Giorgino, Laviola, and Eriksson 2005). Hence, there needs to be a thorough

understanding in the design of an engineered adipose tissue construct. Similarly, it is necessary to thoroughly characterize the biological and mechanical properties of an implanted biomaterial, as the construct is typically degraded as new adipose tissue and extracellular matrix is remolded (Sano et al. 2014).

Bioactivity of the regenerated adipose tissue needs to mimic that of native adipose tissue, not only in function, but also in structure, which includes the incorporation of a functional vasculature consisting of blood vessels, nerves, and lymphatic vessels. Incorporation of the construct upon transplantation is also critical, as the ability of the tissue to completely regenerate over the long term without associated donor-site morbidity or loss of function with time is essential for full restoration of native tissue function. There are several assays that are commonly used to evaluate the biochemical functions of adipose tissue resulting from an engineered construct. Mature adipocytes, the predominant cell type in the adipose tissue, contain microscopic intracellular lipid droplets, which are the first visible signs of adipose tissue formation. Nile red, Oil Red O staining and enzymatic assays are conducted to observe and quantify the intracellular lipid, respectively (Gerlach et al. 2012, Minteer, Marra, and Rubin 2015). Real-time reverse transcription polymer chain reaction (RT-PCR) is also implemented in analysis to detect specific transcripts related to adipogenic differentiation and lipogenic activity. Key transcription factors that are measured most frequently include peroxisome proliferator-activated receptor-gamma (PPAR-γ), glucose transporter-4 (Glut4), fatty acid binding protein-4 (FABP4), and acyl-CoA synthetase (ACS) (Sano et al. 2014, Choi et al. 2011, Poon et al. 2013, Young et al. 2011). Glycerol-3-phosphate dehydrogenase (GDPH), a key enzyme in triacylglycerol biosynthesis, is frequently detected at the levels of both transcript and activity (Rubin et al. 2009b, Giorgino, Laviola, and Eriksson 2005). Another key measure of adipose function is its sensitivity to hormone

stimulation. Standard assays involve measurements on glucose uptake or glycerol and fatty acid release after stimulation with lipolytic (catecholamine) or lipogenic (insulin) hormones.

As discussed earlier, adipose tissue is recognized as a vital endocrine organ. Therefore, the ability to engineered adipose to secrete appropriate adipokines, such as leptin and adiponectin, should be and has been thoroughly investigated. The adipose tissue environment, comprising of adipogenic stimulants, inflammatory cytokines, thrombosis-associated cytokines, and growth factors, is a complex niche that stimulates and maintain native adipose tissue (Peckett, Wright, and Riddell 2011, Rubin et al. 2009b, Choi et al. 2014b). There are several known mediators of adipose tissue formation that are commonly exploited. These include the phosphodiesterase inhibitor 3-isobutyl-1-nethyl-xanthine; the glucocorticoid dexamethasone; indomethacin, an inhibitor of cyclooxygenase; and insulin, which is active in glucose uptake and triglyceride formation (Minteer, Marra, and Rubin 2015). Basic fibroblast growth factor (bFGF) is an essential growth factor present in native adipose tissue that has been widely utilized in adipose tissue engineering strategies. In addition, the microenvironment consists of constitutive cell populations, specific pH and oxygen levels, and appropriate ECM. Collectively, these key components should be considered as the design and analysis of engineered biomaterials should reflect closely the native tissue environment.

In the effort to achieve a sustainable adipose environment, the ability of the new tissue to incorporate blood vasculature is important. Vasculature has been incorporated either in vitro through the addition of vascular cells or in vivo through the recruitment of host cells and is often evaluated through histological analysis for specific endothelial markers such as CD31 and von Willebrand factor (Chung et al. 2012b, Schipper et al. 2008, Choi et al. 2014b). Complete regeneration of functional adipose tissue and incorporation of functional vasculature will only be

attained through long-term analyses of both in vivo and in vitro constructs. This includes measurement of viability and function of the engineered/regenerated tissue, as well as ensuring minimal donor-site morbidity over the years. Additionally, new ECM production is another indicator of mature adipogenesis. Specifically, new ECM deposition, composed of collagen type IV, laminin, and fibronectin, plays a role in the maintenance and remodeling of adipose tissue (Uriel et al. 2008). Studies have also confirmed the secretion of ECM from human adipose stem cells (hASCs) which can be used as a biological scaffold for adipose formation.

1.3 BIOMATERIALS FOR ADIPOSE TISSUE ENGINEERING

1.3.1 Synthetic Biomaterials

Synthetic biomaterials have the advantage of being tailored and specifically designed for adjustment of mechanical/chemical properties as well as degradation (Place et al. 2009). Synthetic materials such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and poly(lactic-co-glycolic acid) (PLGA) have been extensively investigated and used for adipose tissue regeneration (Sharma et al. 2015, Patrick et al. 2002, Patrick et al. 1999, Place et al. 2009, Dhandayuthapani et al. 2011, Elamparithi et al. 2016, Itoi et al. 2010, Patrick 2001). These polymers degrade via hydrolysis and their degradability can be controlled by altering the molecular weight, crystallinity, and ratio of lactic to glycolic acid subunits (Place et al. 2009).

As ASCs and SVF are used in AFG retention, these biologics have also been seeded within scaffold biomaterials as a therapy for adipose tissue engineering. Patrick et al., demonstrated in vivo tissue formation compared ASC-seeded PLGA scaffolds to acellular PLGA scaffolds

implanted subcutaneously on the dorsal aspect of Lewis rats for 12 months (Patrick et al. 2002). Histological assessment of explanted constructs showed maximum adipose tissue formation at 2 months for both groups. However, as PLGA scaffolds degraded, adipose tissue volume also decreased with time. Long term maintenance of viable adipose tissue was not obtained using the preadipocyte-seeded PLGA scaffolds (Patrick et al. 1999, Patrick et al. 2002). Patrick et al., continued to analyze synthetic biomaterials to engineer adipose tissue using PLA reinforced PGA (Cho et al. 2005). The biodegradable PGA/PLA support scaffold was supplemented with fibrin matrix and seeded with human ASCs prior to implantation into subcutaneous pockets of athymic mice. After six weeks, adipose tissue formation with scaffold-fibrin matrix containing ASCs indicated differentiation in vivo. Implanted scaffolds without ASCs in fibrin gel demonstrated limited adipocyte formation (Cho et al. 2005). The survival of adipose tissue beyond six weeks was not reported. It is possible that an immunocompetent animal model with autologous cells may provide additional information into the mechanism. These preliminary studies introduced the use of ASCs for adipose tissue formation. Several limitations such as time, feasibility, and reproducibility have been addressed by investigators. With the maximum adipose tissue formation being shown at 2 months, additional therapeutics are needed to provide stronger outcomes in vivo with synthetically-based scaffolds. Synthetic materials possess several drawbacks when compared to their natural counterparts, particularly the absence of intrinsic surface ligands for cell attachment and a potential impact of their degradation products on cell function (Dhandayuthapani et al. 2011, Place et al. 2009). These limitations render the use of solely biomaterials for soft tissue repair quite challenging.

1.3.2 Extracellular Matrix-Derived Materials

The use of ECM-derived matrices has gained significant interest in tissue engineering due its potential to be easily recognized by the host environment. Constitutive ECM proteins retained after decellularization are the ideological scaffold material for tissue restoration. An ECM scaffold-based approach to regeneration will elicit an immediate host response distinctly different from other scaffold materials after implantation due to their surface topologies and ligand landscapes (Brown, Ratner, et al. 2012). Cellular infiltrate consisting of polymorphonuclear leukocytes and mononuclear cells occurs within minutes. After 72 hours, most infiltrating cells are entirely mononuclear, specifically of the pro-inflammatory macrophage phenotype (M1), with early signs of neovascularization. For two weeks, the mononuclear cell population continues to infiltrate the ECM scaffold, including macrophages for apoptotic neutrophil removal, angiogenesis dramatically increases, and degradation of scaffold is advanced as newly formed host ECM is deposited. By day 14, a reduced mononuclear cell population consisting mostly of immunoregulatory macrophage phenotype (M2) resides and site-specific parenchymal cells appear to begin the final phase of constructive remodeling along with circulating, marrow derived progenitor cells. By day 35, there should be minimal to no scaffold present and a dense, highly organized connective tissue present (Brown, Ratner, et al. 2012, Badylak 2002, 2004, Brown, Londono, et al. 2012).

Despite a multitude of publications investigating the use of synthetic and natural materials for adipose tissue engineering, there remains a clinical need to have an off-the-shelf biomaterial for adipose restoration. Recent advances in decellularization have provided researchers an alternative material to employ in all areas of tissue engineering. Ideally, decellularization is designed to remove the immunogenic material from donated biological sources while retaining as

many constitutive components as possible. ECM can be derived from a specific tissue source to exploit its unique assortment of endogenous factors to restore its respective milieu. Many endogenous factors such as vascular endothelial growth factor, transforming growth factor (TGF) beta 1, basic fibroblast growth factor, placenta growth factor and insulin-like growth factor 1 affecting angiogenesis have been identified in adipose tissues (Choi et al. 2014a). Decellularized adipose tissue (DAT) has gained significance in the tissue regeneration field as scaffold material in the form of hydrogels, sponges, injectable hydrated material, and fibers constructed from adipose ECM.

Several groups have validated DAT hydrogel from human and porcine as a support structure for cellular activity in vitro (Sano et al. 2014, Choi et al. 2014b, Choi et al. 2011, Young et al. 2011, Uriel et al. 2008, Poon et al. 2013). The investigation of porcine adipose ECM utilized freeze-thaw cycles along with trypsin/EDTA to decellularize porcine tissue. Resultant porcine derived biomaterial demonstrated DNA content 99.5% less than native tissue and successful cellular infiltration in vitro over 8 days. However, the absence of animal studies undermines its actual potential (Roehm, Hornberger, and Madihally 2016). Pepsin was utilized for digestion to create an injectable hydrogel from dry milled human DAT; endogenous proteins like collagen I, III, and IV were present after processing (Young et al. 2011). Gelation of the hydrogel occurred with subcutaneous bolus injections in athymic mice. However, implants were excised 15 minutes later and further points in time were not investigated. In a later publication, this research was updated to include ASC-seeded scaffolds with or without transglutaminase (TG), an angiogenic cross-linker (Adam Young, Bajaj, and Christman 2014). It was discovered that the addition of TG and ASCs significantly improved vascularization in vivo compared to the human DAT hydrogel. Oil red O staining of lipids within the scaffolds after 4 weeks in vivo also indicated superior

adipogenesis in human DAT hydrogel containing TG and ASCs (Adam Young, Bajaj, and Christman 2014).

Several groups have investigated seeding ASCs with DAT as a therapy for stimulating natural adipose tissue restoration including the fabrication of an injectable microparticle formulation in saline for engineered fat graft evaluation. Human ASCs were combined with human DAT to study adipose tissue engineering implanted subcutaneously in nude rats for up to 8 weeks compared to human fat graft and hASC injection. Vessel density confirmed by IHC staining showed that human DAT-ASCs grafts at 4 and 8 weeks contained less blood vessels compared to the fresh human fat grafts. H&E staining also confirmed remodeling of fresh graft and surrounding tissue resembling new adipose formation (Wang, Johnson, et al. 2013). In a cell culture comparison study, ASCs were induced in adipogenic media for two weeks and seeded onto various scaffolds (type I collagen sponge, PGA, and hyaluronic acid) prior to implantation into athymic mice. Compared to ASCs seeded in normal culture media in various scaffolds, the area of newly generated adipose tissue was significantly increased in the induced ASCs group. However, within the induced ASC group, only the type I collagen sponge displayed noticeable amounts of adipogenesis after 8 weeks. Induced ASCs in PGA and hyaluronic acid gel displayed minimal adipocyte growth (Itoi et al. 2010). In summary, the results confirm that current investigations of composite scaffolds are not inductive towards adipose tissue formation with deficient angiogenic penetration (Itoi et al. 2010).

As a strategy to increase clinical relevance, Han et al. assessed allogenic rat ASC-seeded DAT scaffolds implanted subcutaneously in an immunocompetent Wistar rat model. Cells were seeded 72 hours prior to implantation (Han et al. 2015). Compared to non-seeded scaffolds, significant remodeling into mature adipocytes was observed in the ASC-seeded groups at 8 and 12

weeks. Adipogenic marker expression in surrounding macrophages confirmed these results as well as correlated increases blood vessel diameter at 12 weeks. The scaffold platform investigated in this study provides invaluable preclinical data towards clinical trials (Han et al. 2015).

Specific ECM and cellular parameters can be adjusted to influence cellular behavior. For example, the effect of DAT particle size and cell density on ASC proliferation and differentiation was assessed in a composite methacrylate chondroitin sulfate (MSC)-DAT hydrogel. At a cellular density of 2500 cells/μl of MSC-DAT hydrogel, proliferation of human ASCs was significantly higher than using DAT of large particle size (~280 μm) compared to the small particle size group (~40 μm). However, at an ASC concentration of 5000 cells/μl of hydrogel, the small particle size group displayed a significantly outperformed all groups with the greatest amount of adipogenesis, identified by glycerol-3-phosphate dehydrogenase activity and laminin production (Brown et al. 2015).

In an attempt to enhance fat graft volume retention, FGF-2 was loaded into an injectable decellularized adipose matrix (mouse-derived tissue) to be investigated as a potential soft-tissue replacement for reconstructive surgery (Zhang et al. 2016). A 12-week animal study compared to performance of FGF-2-Loaded DAT to PBS hydrated DAT in the form of subcutaneous injections. Results indicated significantly more adipose neotissue formation and higher volume retention at 6 weeks in the FGF-2 group. Adipocyte quantifications at 12 weeks for the FGF-2-loaded group were also comparable to endogenous mouse adipose (Zhang et al. 2016).

The use of decellularized ECM from tissues other than adipose have also been examined for soft tissue repair. For example, PLGA microspheres containing FGF-2 were combined with decellularized small intestinal submucosa (SIS) particulates and ASCs in an athymic mouse subcutaneous model for adipose tissue growth (Marra et al. 2008). Initially, ASCs were cultured

and allowed to proliferate on SIS particulate before injection. Following the SIS injection, mice received either 1 ng of free FGF-2, 1 ng dose of FGF-2 in PLGA microspheres, or no FGF-2. Blood vessel quantification was significantly higher in mice receiving FGF-2 microspheres (Marra et al. 2008).

1.3.3 Naturally-Derived Materials

Collagen and hyaluronic acid (HA) are two of the most common naturally-derived biomaterials for scaffolds examined in tissue engineering. The enzymatically degradable glycosaminoglycan, hyaluronic acid, consists of multiple repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid. HA is a major component of the extracellular matrix due to its prominent role in cellular behavior, mechanical support, nutrient diffusion, high water retention and intrinsic swelling property. HA-based scaffolds are currently available on the market for various surgical procedures (Itoi et al. 2010, Stillaert et al. 2008, Cohen et al. 2013).

The potential of HA as an off-the shelf biomaterial for adipose tissue growth has been studied by several research groups. Tan et al. developed an injectable thermosensitive HA gel that confirmed *in situ* gel formation after subcutaneous injection into athymic mice up to 5 days (Tan et al. 2009). However, HA undergoes rapid absorption in vivo (Hemmrich et al. , Okabe et al. 2009). A composite device involving an HA-based (HYAFF®11) pre-adipocyte seeded scaffold implanted in subcutaneous pockets of human volunteers, using a 1 cm median sub-umbilical skin incision, up to 16 weeks, has been reported (Stillaert et al. 2008). Histological analysis results indicated that the HA-based scaffold with and without preadipocyte seeding lacked evidence of any mature adipocytes at 16 weeks. In summary, the results confirm the current proposed

composite scaffolds are not inductive towards adipose tissue formation with deficient angiogenic infiltration (Itoi et al. 2010).

Enhancing the adipogenic properties of hydrogels may be achieved by combining drugs or cytokines. For example, Fan et al. attempted to improve adipose tissue formation in HA hydrogels through aqueous Diels-Alder chemistry (Fan et al. 2015). The HA hydrogel was functionalized for a sustained release of dexamethasone over a two-week time period. In vitro studies conveyed significant increases of the ASC population in groups containing the functionalized Dex-HA hydrogel compared HA gels after 14 days. The same mechanism of drug release was used in magnetic HA nanospheres capable of delivering dexamethasone controlled by an external magnetic field (Jia et al. 2015). In vitro ASC culture results confirmed an increase in cell viability and activity with magnetic HA nanospheres with Dex under a magnetic field. Controlled drug/biomolecule delivery continues to be an evolving asset in tissue restorative therapies.

Collagen is a naturally-derived ECM protein that provides biodegradability, biocompatibility, and weak antigenicity. Several collagenous based biomaterials have been studied due to their favorable characteristics, long history of safety, and FDA-approval. One of the earliest studies of collagen and FGF-2 therapy reported the use of reconstituted basement membrane extracellular matrix, Matrigel™, to deliver FGF-2 subcutaneously in BALB/c nude mice at several concentrations (Kawaguchi et al. 1998). Experimental groups receiving Matrigel™ supplemented with FGF-2 at 10 ng/ml, 100 ng/ml, and 1000 ng/ml, observed neovascularization within 7 days, followed by migration of endogenous ASCs. A visible fat pad was observed between 1-2 weeks and was maintained up to 10 weeks (Kawaguchi et al. 1998). An alternative to free FGF-2 delivery has been investigated in gelatin microspheres containing 1 μg incorporated in the Matrigel™ scaffold along with ASCs (Kimura et al. 2003). Using a BALB/c nude mouse model, a significant

difference in adipose tissue formation was discovered using gelatin microsphere encapsulated FGF-2 compared to free FGF-2 in the collagen sponge subcutaneous implant. Additionally, the highest degree of adipose tissue was formed using 2.5 x 10^4 ASCs/cm^2 of the implant site compared to 1.25 x 10^4 cell/cm^2 and 5 x 10^3 cells/cm^2 (Kimura et al. 2003).

Matrigel™ was investigated with alginate microbeads containing fibroblast growth factor (FGF-1) to induce neovascularization and adipogenesis in a rat vascular pedicle model of adipose tissue engineering (material of interest deposited in silicone tube with vasculature wrapped in local fat pad) (Moya et al. 2010). Vessel density and area of adipose tissue was measured after 6 weeks. Compared to a bolus injection of FGF-1 and empty alginate microbeads, Matrigel™ containing 2.5 µg/ml of FGF-1 in microbeads experienced higher rates of vessel density. However, no difference in adipose tissue formation was observed between the bolus injection group and FGF-1 microbeads group at 2.5 µg/ml dose (Moya et al. 2010).

Porous collagenous microbeads (CultiSphers; Sigma.) have been evaluated as an injectable cell delivery scaffold for ASCs (Rubin et al. 2007). In vitro studies using a spinner flask showed ASCs in culture attach and seed into microbeads at a high capacity. The ability to proliferate and differentiate was also confirmed with clear potential in culture up to 49 and 21 days, respectively (Rubin et al. 2007). An assessment of cell viability post-injection and tissue growth in vivo are the logical next steps to advance this technology for adipose tissue regeneration. Since these early studies, there have been multiple in vivo studies examining collagen in adipose tissue engineering (Cherubino et al. 2016, Itoi et al. 2010, von Heimburg et al. 2001, Kimura et al. 2003, Vashi et al. 2006). Overall, collagen-based scaffolds require ASC cell seeding or controlled release FGF-2 to promote new adipose tissue formation and vascularization.

While the majority of studies with collagen consisted of bovine collagen, salmon collagen has also been evaluated as a scaffold for its ligand protein interactions. This protein biomaterial was prepared with and without pioglitazone, a known inducer of adipogenesis via peroxisome proliferator-activated receptor-γ (PPARγ) (Yazawa et al. 2015). Using a C3H/He/N mouse model, biomaterials were injected subcutaneously and analyzed at 1 week and 4 weeks. Histological results showed mature adipocyte growth substituting the pioglitazone collagen after 4 weeks compared to minimal tissue growth in the control group without pioglitazone.

While scaffold performance of collagen and HA has been analyzed individually, the combined form of these natural biomaterials have implications on the future use of multi-component scaffold. Collagen (type I, derived from bovine Achilles) and HA (derived from bovine vitreous humor) has been crosslinked to develop a three-dimensional scaffold that encourages adipose tissue development (Davidenko et al. 2010). Collagen-HA scaffolds were characterized, seeded with ASCs, and compared to collagen scaffolds. The results indicated that collagen-HA significantly increased gene expression of adipsin, an enzyme involved in lipid metabolism predominately in mature adipocytes (Lu et al. 2014), and noticeably higher levels of PPARγ. The inclusion of HA in collagen scaffolds may aid adipogenesis by hastening cell-contacted growth arrest prior to adipogenic conversion (Davidenko et al. 2010). Preadipocytes seeded on collagen coated with elastin has also been recognized as a potential scaffold with the ability to encourage cell proliferation, infiltration, and adhesion (Keck et al. 2011b).

Gelatin, a partially degraded product of collagen, contains amino acid sequences which can enhance cell attachment (Chang, Liao, and Chen 2013a). Gelatin has also been combined with hyaluronic acid as a complementary ingredient for cryogel scaffolds. Gelatin-HA cryogel scaffolds were seeded with porcine ASCs and implanted into the subcutaneous pocket of two separate

animal models: murine and porcine. Acellular cryogels were also implanted for comparison. The relative gene expression of adipocyte-specific genes (PPARγ, LPL, aP2, and leptin) were significantly greater in the seeded gelatin-HA scaffolds than acellular scaffolds at weeks 2, 4, and 8 in both animal models. It is important to note that both seeded and acellular scaffolds exhibited positive CD31 staining at 8 weeks showing how adequate porosity sufficiently provided space for vascularization in the acellular scaffold implants, a characteristic many other acellular scaffolds failed to demonstrate (Chang, Liao, and Chen 2013b).

Chitosan (CS) is another biodegradable natural material currently being used in tissue engineering applications (Wang, Cao, et al. 2013b, Tan, Rubin, and Marra 2010, Wang, Cao, et al. 2013a). The biocompatible characteristics of cell adhesion and growth were determined using crosslinked CS with poly(L-glutamic acid) to mimic natural ECM (Wang, Cao, et al. 2013a). A porous scaffold was developed via lyophilization for evaluation of engineered adipose tissue in severe combined immunodeficiency (SCID) mice. Acellular and ASC-seeded scaffolds were implanted. After 6 weeks, scaffolds remained intact with vascularization reported in the cell-seeded scaffolds. Cell-seeded poly(L-glutamic acid)/CS scaffolds retained 90% volume and Oil Red O staining indicated adipose tissue formation within the implant (Wang, Cao, et al. 2013a). Minimal adipose tissue was formed within acellular implants. CS has also been crosslinked with HA to produce an injectable hydrogel scaffold capable of delivering dexamethasone for adipose tissue growth (Sun et al. 2013). Results in ASC culture revealed dexamethasone as an important factor in hydrogel performance with increased proliferation and cell adhesion.

Silk is another widely examined biomaterial for soft tissue repair produced by members of the arachnid animal class and cultured silkworms (Vepari & Kaplan 2007). Silk is a naturally occurring biocompatible protein with tunable mechanical strength, adjustable degradation rate, and

low inflammatory and immunogenic responses (Abbott et al. 2016, Bellas et al. 2015). In reference to the application of silk to soft tissue regeneration, Bellas et al. evaluated silk at three concentrations in the form of an injectable foam scaffold. Silk foams supported in vitro ASC survival and migration over a 10-day period. Subcutaneous injections of silk scaffolds in Sprague-Dawley rats demonstrated significant degradation after 90 days with new tissue formation, and cell-seeding ASCs into the silk scaffold may have contributed to new adipose tissue growth in vivo.

Overall, the use of natural-biomaterial based therapies has led to considerably positive results both in vitro and in vivo. The use of ASCs for in vivo injection studies showed significance for several research groups. Additional comparison studies of natural biomaterials containing ASCs with various therapeutic agents in vivo will identify the ideal combination of components.

1.4 DRUG DELIVERY SYSTEMS FOR ADIPOSE TISSUE ENGINEERING

An increasingly widespread trend for adipose restoration has been to incorporate additional biomolecules or biological factors with scaffold material to facilitate new adipose tissue formation, thus creating composite biomaterials. Basic fibroblast growth factor (FGF-2) (Zhang et al. 2016, Kimura et al. 2003, Kawaguchi et al. 1998, Vashi et al. 2006), fibroblast growth factor (FGF-1) (Moya et al. 2010), dexamethasone (Fan et al. 2015, Kelmendi-Doko et al. 2017, Sun et al. 2013, Rubin et al. 2009a, Jia et al. 2015), adipose-derived stem cells (ASCs) (Wang, Johnson, et al. 2013, Cheung et al. 2014a, Brown et al. 2015), pioglitazone (Yazawa et al. 2015), insulin (Masuda, Furue, and Matsuda 2004, Hong et al. 2010b, Rubin et al. 2009a), and insulin-like growth factor I

(IGF-1) (Masuda, Furue, and Matsuda 2004) are among several additive components that have been evaluated in vitro/in vivo. Several methods of encapsulation have been used to localize the delivery of the therapeutic agents. Table 1 contains a comprehensive list of therapeutic agents, encapsulation strategies used, and the expected biological responses from their use. The effects of these therapeutic agents on preadipocyte differentiation and adipose tissue have been described further in illustration (Figure 3). FGF-2 is a member of fibroblast growth factors and most widely used angiogenic factor to stimulate blood vessel infiltration and subsequent de novo adipogenesis (Lu et al. 2014, Zhang et al. 2016, Jiang et al. 2015). Insulin binds to IGF-1 receptors to mimic most of the biological effects of IGF-1 (Lu et al. 2014). IGF-1 and insulin activates phosphorylation of cAMP response element binding protein (CREB) in ASCs to induce differentiation while also regulating triglyceride synthesis through sterol regulatory element-binding protein-1c (SREBP1c) (Lu et al. 2014, Widberg et al. 2009). Vascular endothelial growth factor (VEGF) is a major regulator of vasculogenesis and angiogenesis, serving as a mitogen for vascular endothelial cells (EC) along with several other roles (Chung et al. 2012a). Pioglitazone hydrochloride (P-zone) promotes peroxisome proliferator receptor-γ (PPAR-γ) mRNA expression in ASCs, a critical step in adipogenesis, as well as increases mRNA levels of insulin responsive glucose transporter (GLUT4) (Yazawa et al. 2015, Lu et al. 2014). It is believed that FGF-1 increases expression of PPAR-γ and members of the CCAAT enhancer binding protein (C/EBP) family of transcription factors to prime ASCs for proliferation and differentiation (Hutley et al. 2004, Widberg et al. 2009). Dexamethasone (Dex) targets activating transcription factor 4 (ATF4) to initiate adipocyte differentiation in ASCs (Widberg et al. 2009). Direct comparisons of these additives in the appropriate animal model as well as their feasibility for commercial production are imperative for developing a clinically relevant adipogenic biomaterial.

Table 1. Therapeutic Agents for Adipose Tissue Defects

Drugs/Biomolecules	Methods of Delivery	Cellular Response	References
Dexamethasone	double-emulsion/solvent extraction (PLGA microspheres)	Down-regulate expression of preadipocyte factor-1 (pref-1)	(Fan et al. 2015, Kelmendi-Doko et al. 2017, Sun et al. 2013, Jia et al. 2015, Rubin et al. 2009a)
Insulin	double-emulsion/solvent extraction (microspheres); photocured to gelatin microspheres	Stimulates glucose uptake, lipogenesis, and inhibits lipolysis through IGF-1	(Rubin et al. 2009a, Masuda, Furue, and Matsuda 2004, Tan, Rubin, and Marra 2010, Kim et al. 2016)
Insulin-like growth factor-1 (IGF-1)	photocured to gelatin microspheres	Activates AKT and MAPK signaling pathways	(Masuda, Furue, and Matsuda 2004, Kim et al. 2016)
Basic fibroblast growth factor (FGF-2)	mixed heparinized protein; photocured to gelatin microspheres; hydrogel supplement; double emulsion (PLGA microspheres)	Upregulates mitotic genes	(Masuda, Furue, and Matsuda 2004, Zhang et al. 2016, Kimura et al. 2003, Kawaguchi et al. 1998)
Pioglitazone hydrochloride (P-zone)	saline gel mixture	Ligand for PPAR-γ; increase GLUT4 expression	(Yazawa et al. 2015)
Vascular endothelial growth factor (VEGF)	double-emulsion/solvent extraction (PLGA microspheres)	Mitogen for vascular EC; increase EC migration, regulate microvascular permeability and vasodilation	(Chung et al. 2012a, Zhu et al. 2010, Topcu et al. 2012)
Fibroblast growth factor (FGF-1)	heparin incubation	Increase PPAR-γ expression	(Moya et al. 2010, Widberg et al. 2009)

The effects of therapeutic agents on ASCs, vascular cells, and adipocytes have been exploited in numerous studies to enhance the functional behavior of biomaterials and autologous fat grafts.

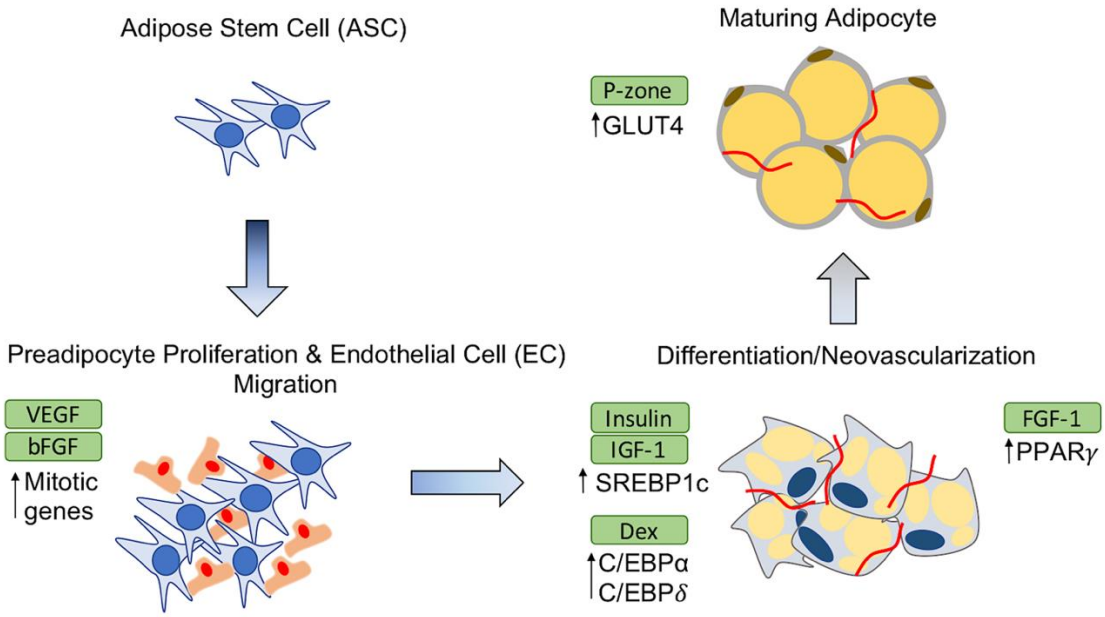

Figure 3. Effects of therapeutic agents on ASC differentiation

1.4.1 Polymeric Microspheres

Polymers such as poly (lactic-co-glycolic acid) (PLGA) can be used to encapsulate drugs or proteins using an oil-in-water technique in which the polymer is dissolved into an organic phase (oil) that is emulsified with a surfactant or stabilizer (water). Hydrophobic drugs are added directly to the oil phase, whereas hydrophilic drugs are first emulsified with the polymer solution prior to formation of polymer droplets. High intensity sonication bursts facilitate the formation of small polymer droplets. The resulting emulsion is added to an aqueous phase and stirred for several hours, which allows the solvent to evaporate. The double emulsion technique results in microspheres with a more controlled release and reduces the burst drug release effect observed in

the studies of single walled microspheres. Double walled microspheres can be prepared by incorporating a previously sonicated PLGA/drug mixture in solvent into another dissolved polymer solution such as PLA in solvent and administering high intensity sonication to create droplets around the previously formed polymer droplets (McCall and Sirianni 2013, Pekarek, Jacob, and Mathiowitz 1994). The diameter of drug encapsulated microspheres generally range from ~1-1000 μm which can be suspended in a gel or solution for a localized controlled delivery of the drug directly to the intended targeted tissue while avoiding systemic side effects caused by oral administration and possibilities of user error which include as under- and overdosing.

Researchers have utilized microspheres to encapsulate growth factors and pharmaceuticals aiming to develop clinically relevant tissue engineering therapies (Rubin et al. 2009a, Topcu et al. 2012, Zhang et al. 2016, Chung et al. 2012a). VEGF was encapsulated in calcium alginate microspheres to create a controlled release system and was evaluated in a Wistar rat model receiving fat grafts (Topcu et al. 2012). Rats in groups 1 and 2 received microspheres (MS) containing 2 μg/0.1 mL VEGF subdermally 21 days prior to fat grafting and mixed within fat graft, respectively. After 90 days, the fat graft control group lost over 50% of mass while groups receiving VEGF calcium alginate MS showed an increase in weight over 25%. Microvascular density results were also significantly higher in the VEGF microsphere pre-injection group and VEGF MS mixed with fat graft group. VEGF has also been encapsulated in poly(lactic-co-glycolic) (PLGA) MS as a potential therapy for transferred human lipoaspirate (Chung et al. 2012a). Human lipoaspirate with and without PLGA VEGF MS were injected into the flanks of athymic mice and evaluated up to 6 weeks for vascularization and tissue survival. Significantly higher mean masses and volumes in the VEGF-loaded microsphere group were seen at 6 weeks.

Additionally, there was IHC staining qualitatively displayed increased vascularization in the VEGF MS group at 6 weeks compared to 3 weeks results.

Rubin et al. utilized PLGA MS to encapsulate two adipogenic factors, dexamethasone (Dex) and insulin, as a method to stimulate adipogenesis in ASCs. Results showed that ASCs treated with dexamethasone and insulin microspheres contained a significantly higher number of adipocytes compared to control groups (Rubin et al. 2009a). More recently, Doko et. al utilized this same microsphere delivery system with encapsulated adipogenic factors to increase retention of transplanted adipose tissue (Kelmendi-Doko et al. 2014). Dex and insulin were loaded into PLGA MS and mixed at various doses with human fat grafts to be injected subcutaneously into the dorsal aspect of athymic mice. After 5 weeks, volume analysis showed increased graft retention with use of Dex-loaded PLGA MS. Blood vessel density was also increased with the use of the adipogenic encapsulated MS. Doko et al. extended the investigation of Dex MS in a double-walled (DW) formulation mixed into human fat grafts (Kelmendi-Doko et al. 2017). Control human fat grafts explanted from athymic mice after 6 months demonstrated significant volume loss (i.e. ~90%) while groups containing the DW Dex MS resulted in a mean volume loss of ~ 33%, thus demonstrating the beneficial effects of a slow, controlled release delivery system.

In another study, Masuda, et al. proposed the co-release of insulin with additional encapsulated drugs from styrenated, gelatin-based microspheres (SGM) (Masuda, Furue, and Matsuda 2004). Insulin, IGF-1, and FGF-2 were encapsulated to develop a drug delivery system that induces neovascularization and migration of endogenous ASCs, followed by proliferation and differentiation into adipocytes. Subcutaneous lesion injections of SGMs in Wistar rats were evaluated after 6 weeks. The total lipid content extracted from the experimental group receiving FGF-2, insulin, and IGF-1 SGMs were significantly higher than groups receiving insulin SMGs

alone, IGF-1 SGMs alone, and insulin/IGF-1 combined SGMs (Masuda, Furue, and Matsuda 2004).

1.4.2 Hydrogels

Hydrogels are three-dimensional networks of hydrophilic polymers crosslinked by covalent bonds, physical intramolecular, and/or intermolecular attractions (El-Sherbiny & Yacoub 2013; Van Tomme et al. 2008). Hydrogels can absorb large amounts of water or biological fluids without dissolving while resembling the characteristic of soft living tissue. The soft and hydrophilic nature of hydrogels make them particularly suitable as a drug delivery system or as a cell-entrapping scaffold for tissue engineering (Toh & Loh 2014). Hydrogels designed for drug delivery typically do not contain chemical crosslinking agents due to incompatibility with fragile molecules such as pharmaceutical proteins and living cells (Van Tomme et al. 2008). Injectable hydrogel drug delivery systems are of particular interest as a non-invasive therapy for their ability to form *in situ*, at the site of injection, without the aid of potentially toxic or denaturing crosslinking agents (Van Tomme et al. 2008). Hydrogels comprised of ECM proteins have the ability to gel spontaneously under physiological conditions while delivering pharmaceutical agents for tissue regeneration.

1.4.3 Dexamethasone

Dexamethasone is an FDA approved synthetic adrenocortical steroid in the form of a white, odorless, crystalline powder that is stable at room temperature. Glucocorticoids (GCs), naturally occurring and synthetic, are readily absorbed from the gastrointestinal tract. The synthetic analog, Dex, is primarily used for their anti-inflammatory effects in disorders of many organ systems. As

an adipogenic factor, Dex and other GCs are required for the full differentiation of adipose precursors and for the maintenance of key genes in glucose and lipid metabolism in cultured adipocytes and adipose tissue. As a result of its anti-inflammatory nature, Dex decreases the expression of inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor alpha that are mainly expressed in non-adipocyte fraction in human adipose tissue (Lee and Fried 2014). Side effects of administering Dex orally include anxiety, irritability, mental depression, mood changes, blurred vision, decrease in urine volume, dizziness, shortness of breath, and weight gain. In order to avoid the side effects of oral administration of drugs such as Dex, localized drug delivery and polymer microsphere encapsulation has allowed for a more advanced, controlled administration of therapies.

1.5 PROJECT OBJECTIVES

1.5.1 Design, develop, and characterize a composite adipose derived delivery system (CADDS)

The first objective was to confirm suitability of the adECM to be used as a biomaterial as well as the dexamethasone microsphere delivery system. Specifically, it was critical to ensure the removal of immunogenic components such as residual lipids/DNA while preserving the fundamental ECM structure. Characterization, including cumulative dexamethasone release and homogeneous distribution of the microspheres, was a vital step in the development of a unique and reproducible drug delivery system. We hypothesized that if ECM from human adipose tissue can aid in adipose tissue restoration, the ECM must be decellularized to prevent adverse reactions as a biomaterial.

1.5.2 Determine the bioactivity and adipogenic potential of CADDS

The objective of this aim was to evaluate the biological potential of the hydrogel with cell culture studies. The *in vitro* assessment of biocompatibility, adipogenesis, and bioactivity was a comprehensive step to determine efficacy of the composite adipose-derived delivery system (CADDS) prior to examination in an animal model. We hypothesized that human adipose derived stem cells (hASCs) cultured with CADDS will demonstrate bioactivity in vitro and respond with an increased degree of adipogenesis.

1.5.3 Evaluate the in vivo response and volume retention of CADDS implanted in an immunocompetent animal model

The objective of this aim was to compare the administration of hydrated adipose derived scaffolds to CADDS by tracking volume retention and examining histological sections at the end of the study. Explant tissue sections were compared and evaluated for structure and integration with the surrounding tissue. We hypothesized that if CADDS is implanted in an immunocompetent small animal model, CADDS will result in higher volume retention in groups containing dexamethasone encapsulated microspheres with a healthy adipose architecture.

2.0 DESIGN, DEVELOP, AND CHARACTERIZE A COMPOSITE ADIPOSE DERIVED DELIVERY SYSTEM (CADDS)

2.1 INTRODUCTION

The current limitations surrounding adipose tissue restoration involve rapid resorption of natural biopolymers, lack of adipose stimulation by synthetic biomaterials, and volume loss of autologous adipose grafts (Nahabedian 2015; Hong et al. 2016; Patrick 2001; Boccaccini et al. 2005; Davidenko et al. 2010; Hemmrich & von Heimburg 2006). When normal appearance is compromised by chronic disease, trauma, or tumor resection, the options for adipose tissue restoration either have a vascularized or non-vascularized approach. The field of tissue engineering has begun to address challenges within the category of non-vascularized approaches using strategies that engineer tissue tailored to a specific site of injury (Keck et al. 2011; Choi et al. 2010). Additionally, researchers have also been incorporating biological and growth factors to enhance these strategies. The objective of this study was to characterize the components of an adipose-derived hydrogel containing adipogenic factors as a potential biomaterial to restore volume in adipose defects.

Scaffolds and hydrogels comprised of extracellular matrices have been used clinically for over a decade and have shown moderate success in adipose tissue engineering (Hemmrich & von Heimburg 2006; Choi et al. 2011; Yu et al. 2013; Cheung et al. 2014; Choi et al. 2014). Adipose extracellular matrix isolated through decellularization can contain specific components such as collagen, elastin, sGAGs, and laminin that provide specific instructive biological cues for growth, migration, and differentiation to residing cells *in situ* (Yu et al. 2013; Choi et al. 2014). The

following study combines the latest advancements in adipose tissue engineering to develop an injectable hydrogel scaffold derived from discarded adipose tissue enhanced with dexamethasone-encapsulated microspheres for the controlled delivery. Discarded adipose tissue was decellularized and evaluated for suitability as a biomaterial. Dexamethasone encapsulated microspheres were fabricated in single- and double-walled formulations and fully characterized as well. This knowledge was critical for the development of further studies in vitro involving adipose derived stem cells.

2.2 METHODS

2.2.1 Adipose Tissue Processing and Decellularization

Adipose tissue samples were collected as discarded biological material from female patients (non-diabetic, age 21 to 60) undergoing elective abdominoplasty within the University of Pittsburgh Medical Center system as approved by the institution review board. The decellularization process for adipose tissue has been established in the laboratory and optimized for reproducibility (Brown et al. 2011). The process reduces human adipose tissue matrix to decellularized extracellular in approximately seven days (Figure 4). Briefly, mechanical processing using a commercial meat grinder breaks the fibrous tissue apart and creates a homogeneous mixture. Alcohol and salt solution rinses were used to extract triglycerides and cellular material from the matrix. After disinfection via low concentrate peracetic acid, 10x PBS rinsing (phosphate buffer solution) balances the pH before flash freezing to prepare the matrix for lyophilization. The final step results in a dry decellularized adipose ECM, seen in Figure 5a.

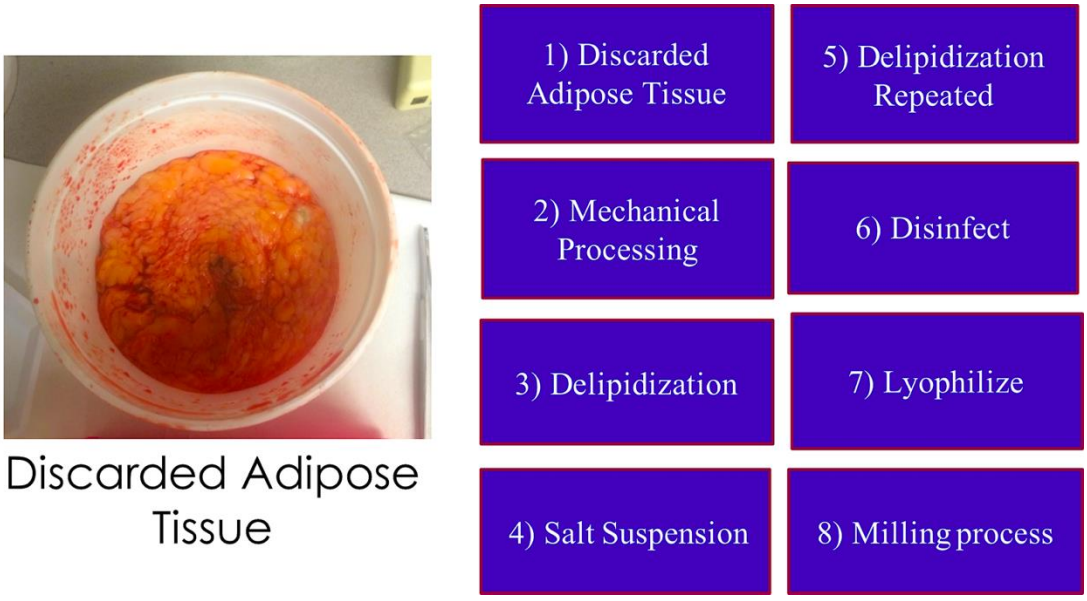

Figure 4. Decellularization Process of Discarded Adipose Tissue

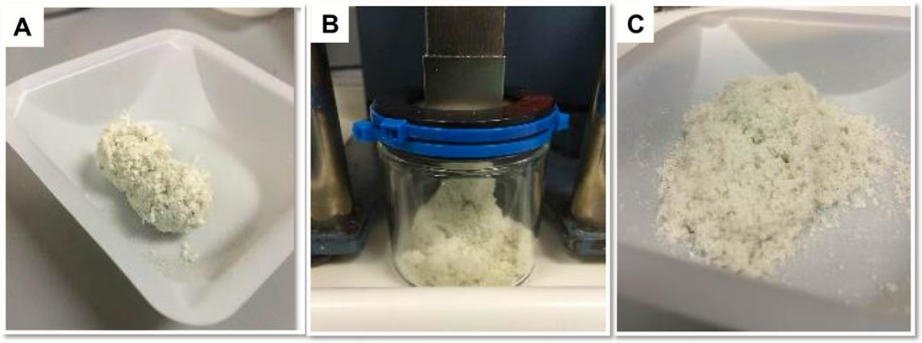

Figure 5. A) Dry AdECM after lyophilization, B) Milling process of AdECM, C) Powder-form AdECM

2.2.2 Decellularized matrix characterization

Decellularized matrix were fixed and embedded in paraffin and/or optimal cutting temperature compound to be prepared for tissue sections. Tissue sections were stained for collagens, (types 1, 3, 4, and 6), heparan sulfate proteoglycan (HSPG), and nidogen along with hematoxylin and eosin (H&E) and Oil Red O. Polyclonal anti-mammal collagen (types 1, 3, 4, and 6), nidogen, and HSPG

antibodies were purchased from abcam (Cambridge, MA, USA) and Life Technologies (Grand Island, NY). These specific proteins were chosen for staining due to their influence on cell guidance and migration, support, binding growth factors, and attachment within the ECM.

Total DNA content was also assessed using a Quant-IT PicoGreen double-stranded DNA (dsDNA) assay kit (Invitrogen, Carlsbad, CA). Samples will be proteinase K-digested for up to 1 h (60 mAU/ml, 50 °C), then heated at 95° for 5 min. Samples were centrifuged and the supernatant was added to 1X TE buffer for incubated with fluorescent PicoGreen reagent for 30 minutes. Fluorescence was measured using an Infinite® 200 Pro microplate reader (TECAN, Inc.) with excitation wavelength of 480 nm and emission wavelength of 520 nm. dsDNA was quantified by relating sample absorbance to the absorbance measured for standards of known DNA concentration.

Residual lipid content of the decellularized ECM was assessed using an AdipoRed Reagent Assay (Lonza, Walkersville, MD). According to manufacturer's instructions, in a 24-well plate, 10 mg of decellularized ECM (n=6) per were compared to the same amount of adipose tissue (n=6) per well by mixing the samples with 1 ml of phosphate buffer solution and 30 µl AdipoRed™.

2.2.3 Hydrogel formation and characterization

A Wiley Mill was used to break down the lyophilized adipose ECM into a powder like substance as seen in Figure 5b and Figure 5c. Next, the ECM was digested by porcine solution at 0.1 M hydrochloric acid (HCl). After two days of digestion, 1 M sodium hydroxide (NaOH) was added dropwise to slowly raise the pH to about 7.4. The adipose hydrogel formed after the solution was raised to physiological temperature of 37 °C. Scanning electron microscopy (SEM) was used to visualize the microstructure of the hydrogel. The hydrogels were fixed in an aqueous solution of

2% glutaraldehyde and 4% paraformaldehyde followed by dehydration with graded series of ethanol (30-100%). Dehydrated gels were critical point dried using CO_2 and gold coat sputtered to prepare for clear imaging under SEM. Hydrogel degradation was observed under physiological conditions in 1X PBS (ThermoFisher Scientific, Waltham, MA) and collagenase/dispase buffer solution (1 mg/ml, Millipore Sigma, St. Louis, MO).

2.2.4 Dexamethasone microsphere encapsulation

Single emulsion/solvent extraction protocols established by the laboratory was used to fabricate dexamethasone encapsulated in single walled microspheres (SW Dex MS) (Rubin et al. 2009; Chung et al. 2012). Poly (lactic-co-glycolic acid) (PLGA) (75:25) was dissolved in methylene chloride (MC). Dexamethasone disodium phosphate (Dex) was dissolved in methanol and added to the polymer solution. A vortex was used to mix the solution for at least 60 seconds. The mixed solution was added dropwise to a 2% poly (vinyl alcohol) (PVA) solution to extract solvent under magnetic stirring. After 3 hours, the MC solvent evaporates leaving behind microspheres to be collected by centrifugation. Lastly, the microspheres were frozen in deionized water (DI) with liquid nitrogen and lyophilized to remove all water content. Double-walled dexamethasone microspheres (DW Dex MS) were fabricated using the following protocol. PLGA (75:25) was dissolved in MC to produce a 10% polymer solution. Dex dissolved methanol was added to the polymer in a glass vial and placed under vortex for about a minute for the first emulsion. In a separate vial, polylactic acid (PLA) (50:50) was dissolved in MC to produce a 10% PLA solution. The PLA solution was then added to the PLGA-Dex emulsion to be placed under vortex for the second emulsion. Drops of the final solution were added to a 1% PVA solution under magnetic stirring which continues for about 3 hours to allow the MC solvent to evaporate. Morphology and

size of the Dex MS were determined along with the adipose ECM hydrogel using a scanning electron microscope (JSM-6335F).

2.2.5 In vitro release studies

The encapsulation efficiency was determined using a UV-Vis Spectrometer. Dimethyl sulfoxide was used to quickly dissolve the PLGA causing the coating to release the encapsulated Dex from the MS. After balancing the pH, small diluted portions were measured with the spectrometer set at 242 nm absorbance wavelength. After comparing the absorbance to a standard curve, the correlated amount of Dex was divided by the known amount of Dex used in the emulsion protocol to calculate the encapsulation efficiency.

Spectrophotometry was also used to determine the release of Dex from single and double-walled MS. An *in vitro* drug release study was conducted by placing 5 mg of Dex MS (DW or SW) into a 1.5 ml Eppendorf tube containing 1 ml of 1X PBS. Tubes (n=5) were kept in an incubator at 37 °C between readings by the spectrometer. Readings were taken every 2-3 days and a fresh ml of 1X PBS will be replaced each time.

2.2.6 Statistical analysis

All quantitative data is presented as a mean ± standard error of the mean. Differences between the AdECM, human adipose tissue, and porcine dermis ECM were calculated for statistical significance by one-way analysis of variance (ANOVA) using GraphPad Prism 6. (GraphPad Software, Inc.). Differences are considered significant if $p < 0.05$.

2.3 RESULTS

2.3.1 Decellularized matrix and CADDS microstructure

AdECM microstructure and composition were evaluated in multiple ways. In Figures 6a and 6b, the scanning electron microscopic images show the morphological and structural properties of AdECM (100X magnification) and the CADDS with SW Dex MS (170X magnification), respectively. SW Dex MS examined by SEM range from 70-125 μm in diameter. Both matrices share the characteristics of porosity, microstructural build, and fiber organization.

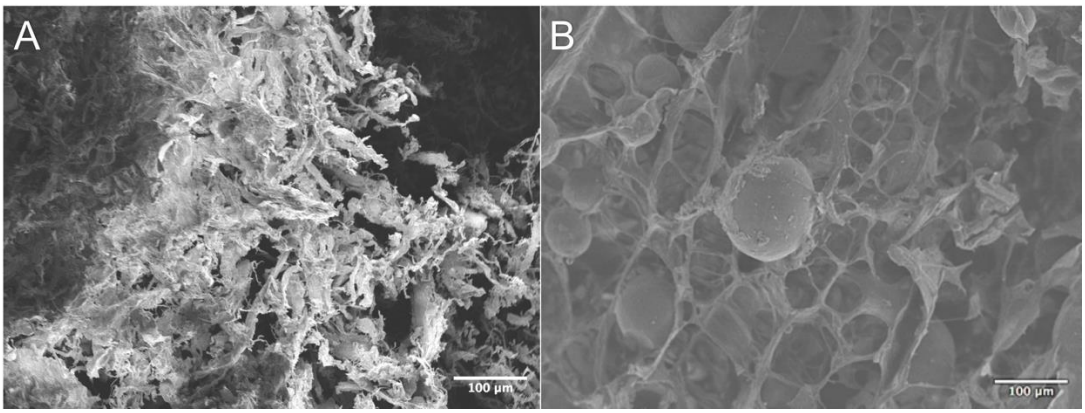

Figure 6. SEM imaging of A) AdECM, B) Composite adipose derived delivery system (CADDS)

2.3.2 AdECM histology characterization

H&E staining of AdECM histological sections display a lack of nuclei stains (Figure 7) and DNA quantification analysis resulted in less than 4 ng of DNA per mg of AdECM (Figure 8). These characteristics are indicative of a properly decellularized matrix. Immunohistochemistry staining of AdECM sections show that constitutive proteins, laminin, nidogen, HSPG, Figure 9A-F, and Collagen (I, III, IV, and VI), Figure 10 and 11, were retained post decellularization.

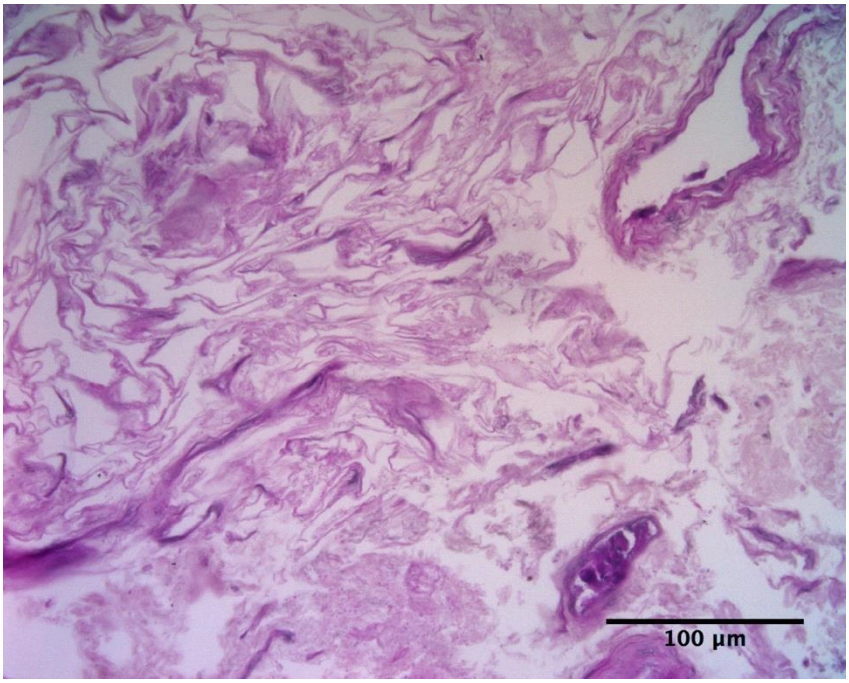

Figure 7. H&E stain of AdECM

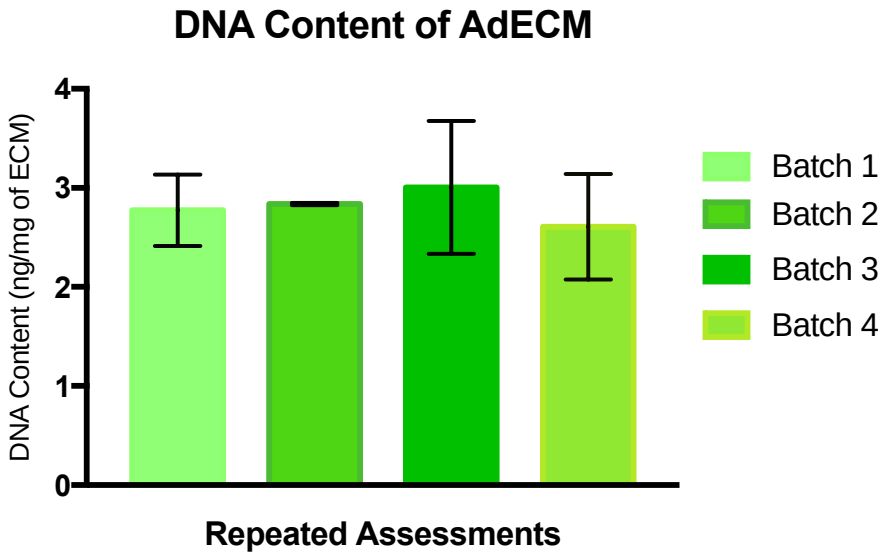

Figure 8. Quantification of DNA content in four batches of AdECM

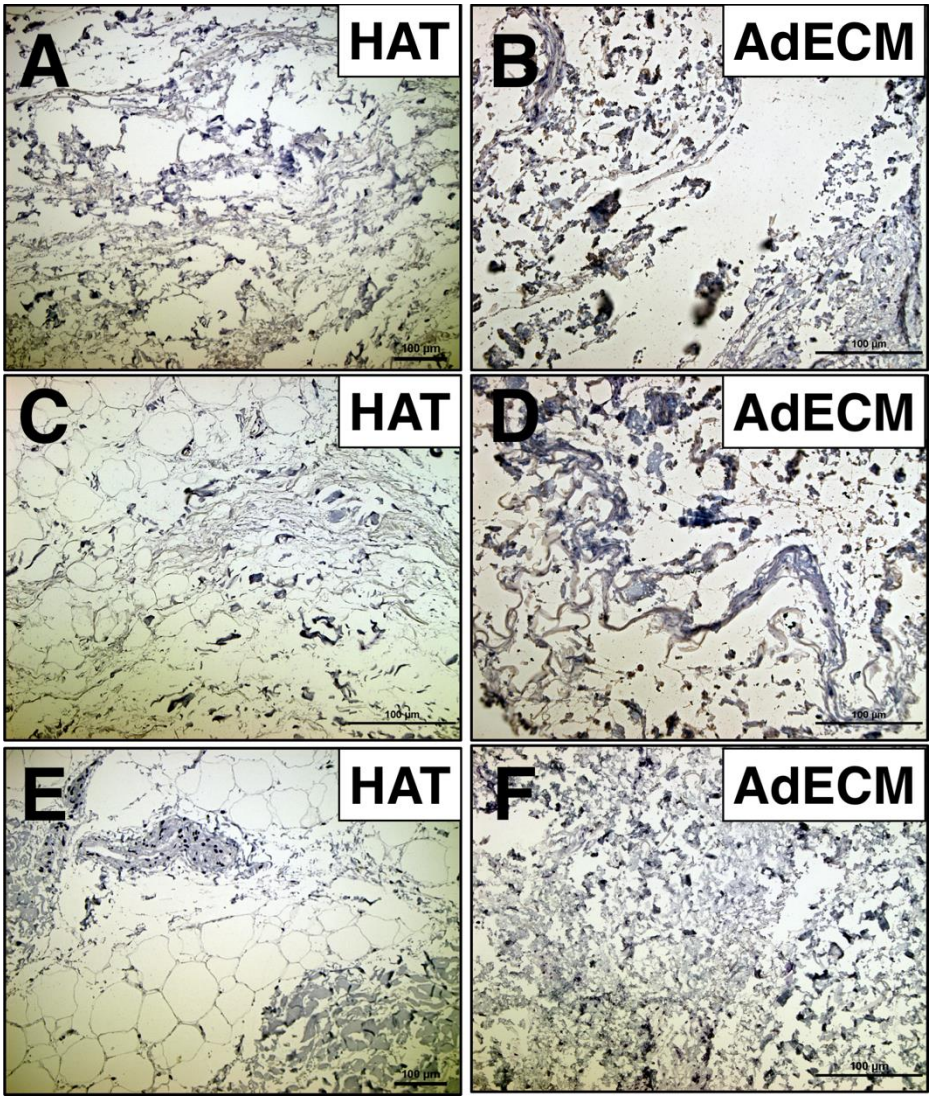

Figure 9. IHC staining of tissue sections for: Heparin sulfate proteoglycan (HSPG), (A) HAT, (B) AdECM; Laminin, (C) HAT, (D) AdECM; Nidogen, (E) HAT, (F) AdECM (100 µm scale bar).

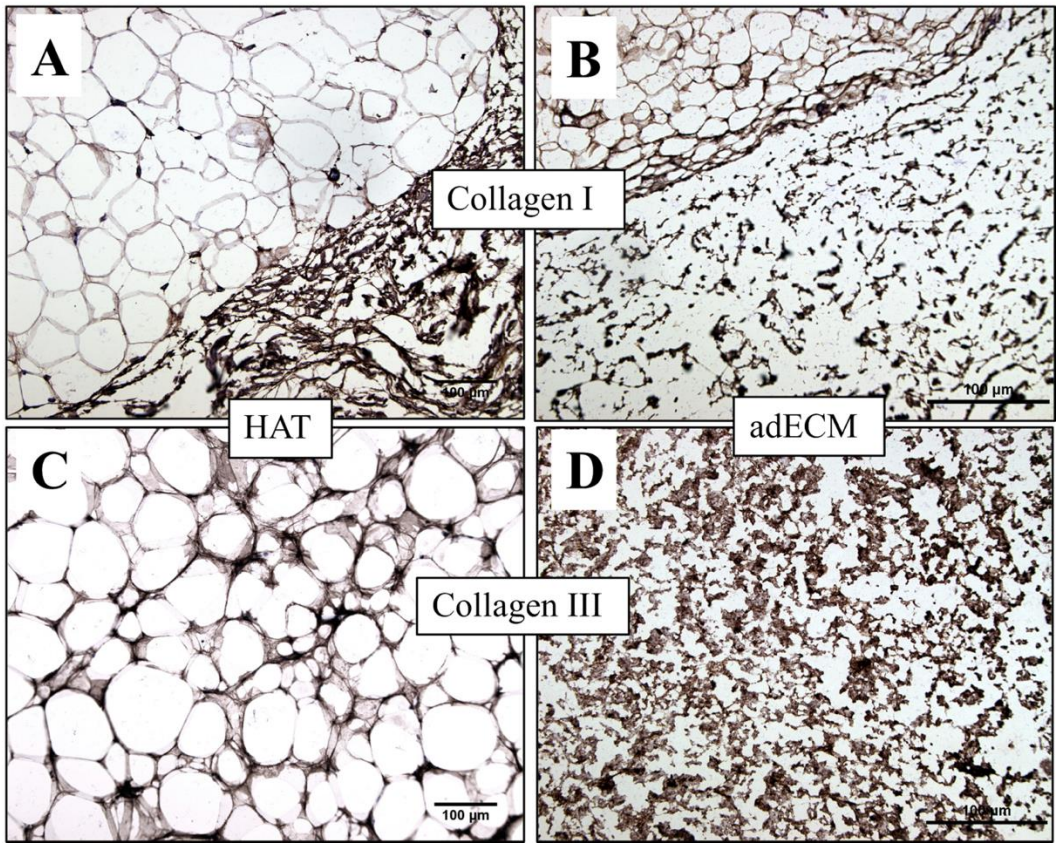

Figure 10. IHC stains of tissue sections (A) Collagen 1, Human Adipose Tissue (HAT), (B) Collagen 3, Human Adipose Tissue, (C) Collagen 1, AdECM, (D) Collagen 3, AdECM

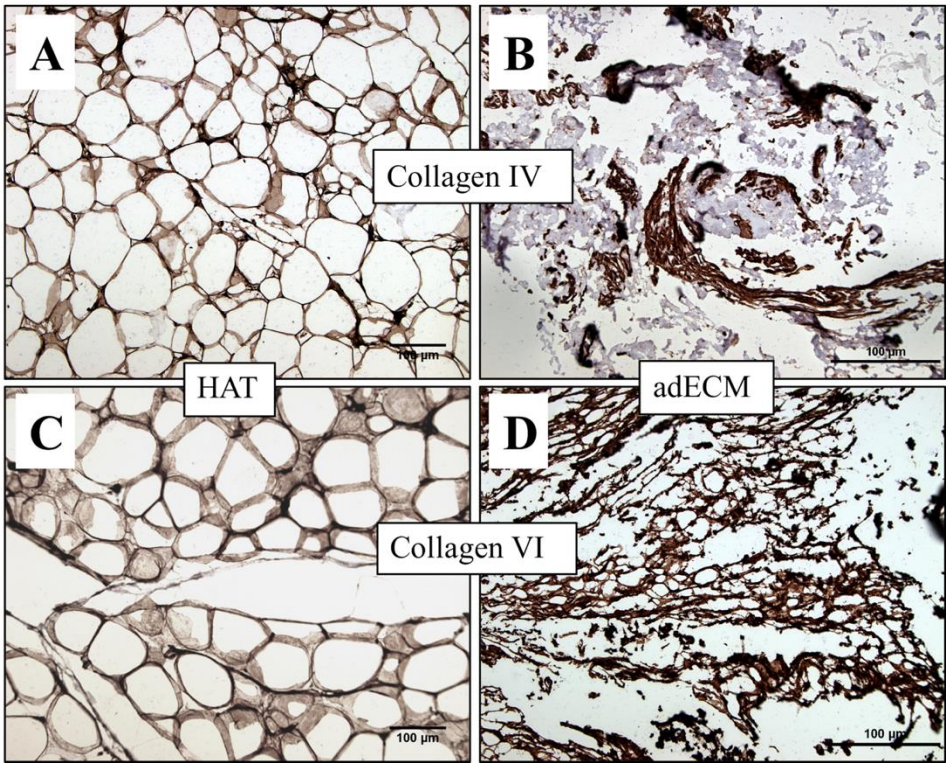

Figure 11. IHC stains of tissue sections (A) Collagen 4, Human Adipose Tissue (HAT), (B) Collagen 4, Human Adipose Tissue, (C) Collagen 6, AdECM, (D) Collagen 6, AdECM

2.3.3 AdECM composition analysis

The decellularizaton process removed a significant amount of cellular and lipid content reducing the human adipose tissue to a protein-enriched extracellular matrix. AdECM lipid content was comparable to commercial dermis ECM, significantly lower than human adipose (Figure 12). DMMB assay measured the remaining sGAGs to be approximately 100 ug/ml in the AdECM sample, compared to 50 ug/ml in the dermis ECM, and 9 ug/ml in the HAT sample (Figure 13). Total protein content measured by Pierce BCA assay reported significantly higher amounts of protein in the AdECM group compared to dermis material (Figure 14).

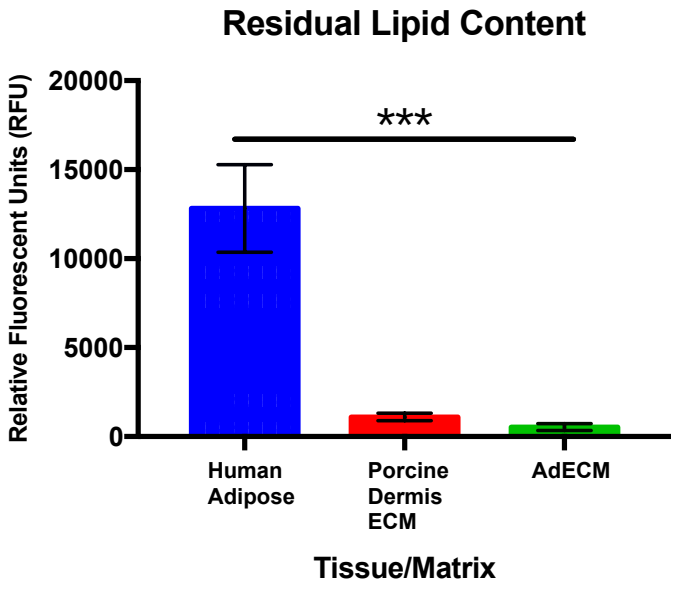

Figure 12. Quantification of lipid content in human adipose tissue, commercial dermis ECM, and AdECM

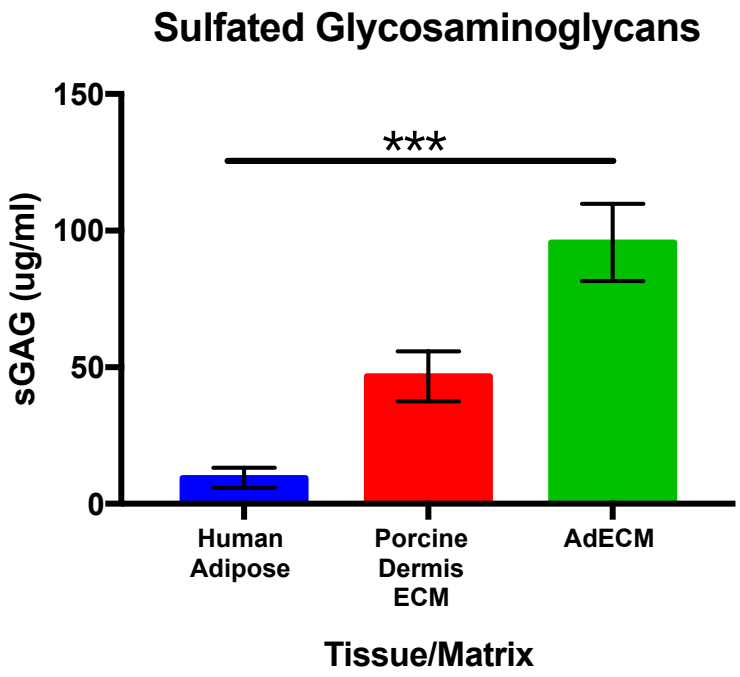

Figure 13. Quantification of sGAG content in human adipose tissue, commercial dermis, and AdECM

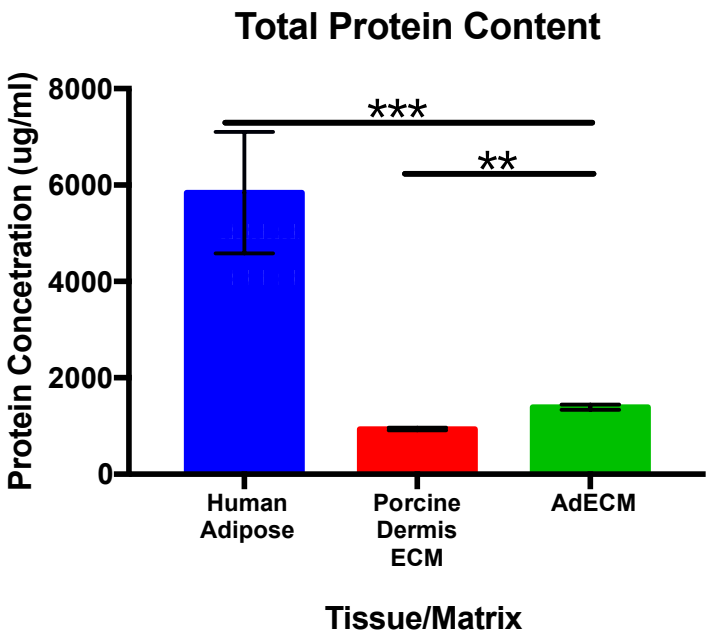

Figure 14. Quantification of protein in 5 mg sample of human adipose, commercial dermis matrix, and AdECM

2.3.4 SW and DW Dex MS characterization

In vitro dexamethasone release from DW and SW MS were monitored for at least 50 days. Figure 15 shows that within the first week of Dex release from SW MS, a large burst effect caused approximately 50% of the encapsulated drug to be released compared to a more controlled 50% release of drug after about 120 days from the DW MS containing the additional coating of PLA. Average size of the microspheres were determined by SEM with SW average diameter to be ~50 µm (Figure 16A) and DW average diameter at ~90 µm (Figure 16B).

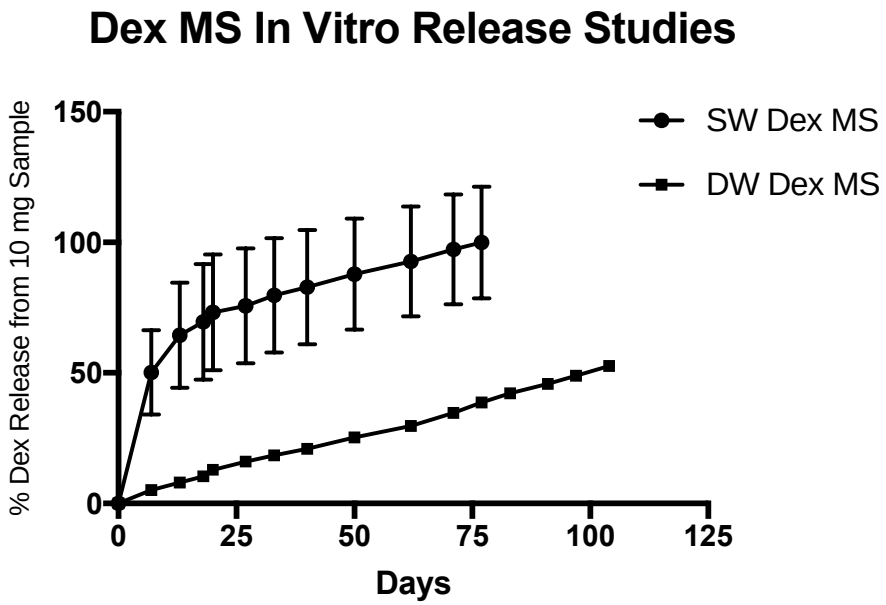

Figure 15. Cumulative in vitro release of dexamethasone SW and DW Dex MS

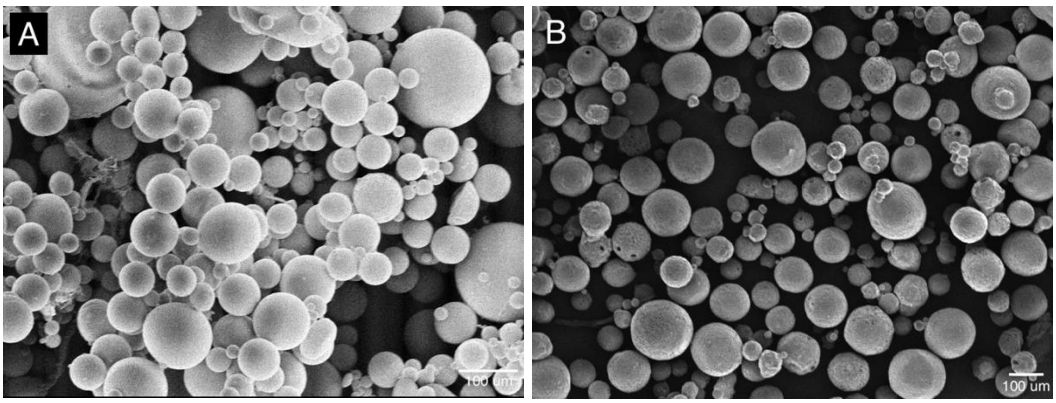

Figure 16. Scanning electron microscopy images of (A) SW Dex MS and (B) DW Dex MS

2.4 DISCUSSION

Ideally, biologic scaffolds derived from ECM are free of cell associated antigens while the microstructure and composition remain intact. Once complete decellularization is reached, an

acellular adipose matrix can provide constitutive cues, biochemical milieu, and sufficient structural scaffolding to promote precursor adipocyte infiltration while influencing adipose tissue restoration and remodeling *in situ* (Brown et al. 2011; Brown et al. 2010). With the addition of biocompatible polymers, cells, or growth factors, matrices can be enhanced to form a composite for further stimulation towards tissue restoration. An established adipose ECM extraction method was implemented to develop an acellular, protein-enriched, biologically active matrix and hydrogel as a delivery vehicle of stable, adipogenic factors as an off-the-shelf therapy for adipose tissue engineering.

Several important characteristics need to be established when developing an adipogenic biomaterial from biological tissue. Initially, decellularization can be seen as a deciding factor with recent findings demonstrating that host response to cell remnants and amount of DNA in scaffold material can significantly alter tissue remodeling (Keane et al. 2012). More specifically, researchers have established a criterion for complete decelluarization such as lack of visible nuclei per H&E/DAPI stain, less than 50 ng of DNA per mg of dry weight matrix, and lengths of DNA have less than 200 base pairs (Londono & Badylak 2015). Consequences of ineffective decellularization ultimately can cause an immunological host response that hinders recruitment of cells and a cascade of biological signals that facilitate functional tissue repair and constructive remodeling. Therefore, the adECM obtained for the study was thoroughly evaluated for cellular and DNA content.

As a hydrogel delivery system, the adECM is easy to manipulate and administer as a minimally invasive, off-the-shelf, injectable scaffold for adipose tissue engineering. Post-implantation the collagen-based hydrogel forms into a solid gel with similar composition as adipose tissue at physiological conditions. Some advantages of this three-dimensional scaffold

include network of connecting pores, mass transport properties, and mechanical integrity for tissue formation. With the addition of Dex MS to the scaffold, the composite becomes a highly adipogenic specifically targeting precursor adipocytes to form new adipose tissue as demonstrated by the in vitro testing.

Specific ECM elements with its own complex composition and concentration of chemical constituents known to regulate numerous cell processes, including attachment, survival, migration, proliferation, and differentiation (Young et al. 2011). The adipose derived matrix from the study retained its fundamental essential proteins namely collagen I, collagen III, collagen IV, and collagen VI in addition to nidogen, HSPG, and laminin. Each component plays an essential role in adipose development and homeostasis. Specifically, type I and type VI collagen provide structural support. Extracellular laminin form networks with collagen fibers and provide attachment points for integrins anchored in the adipocyte membrane. Similar to paracrine signals, integrins transduce cues from the extracellular matrix to regulate gene expression and function (Pope et al. 2016). Basal laminae of the adipose matrix consists of laminin (principal structural protein), HSPG, and type IV collagen. The basal laminae provides high affinity binding sites for other components of the ECM and for cell surface receptors (Teti 1992). By securing the presence of physiologically relevant matrix proteins in the composite delivery system, the host's implant site receives the fundamental cues familiar with adipose tissue regeneration and homeostasis.

2.5 CONCLUSION

The research in this investigation has led to the development of a composite adipose derived delivery system for dexamethasone as an adipogenic factor in tissue engineering. Decellularization

of human adipose tissue was effective to produce an acellular biocompatible scaffold system while retaining essential proteins. Overall, the investigation introduces a unique combinations of tissue engineering strategies as a potential therapy to encourage adipogenesis and restore tissue function.

3.0 BIOACTIVITY AND ADIPOGENIC POTENTIAL OF THE COMPOSITE ADIPOSE DERIVED DELIVERY SYSTEM

3.1 INTRODUCTION

Human preadipocytes were first isolated over forty years ago and it was only ten years ago that their multi-lineage potential was discovered (Minteer et al. 2015). Since then, many biological properties of ASCs have been characterized utilizing in vitro and vivo studies (Patrick et al. 2002; Philips et al. 2012b; Otto & Lane 2005; Philips et al. 2014). As a result of their exquisite plasticity, human ASCs are known to have the ability to differentiate into numerous cell lineages including adipogenic, osteogenic, cardiomyogenic, neurogenic, myogenic, and chrondrogenic cell types. Additionally, numerous studies have demonstrated that ASCs express/secrete multiple growth factors including IGF, HCF, VEGF, and TGF-β1 (Widberg et al. 2009). In consideration of these biological characteristics, adipose tissue has recently proven to be an invaluable stem cell source for clinical application in tissue engineering instead of its previous role as a discarded and unwanted tissue. ASCs have also been established as a valuable tool to evaluate the adipogenicity of biomaterials and drug delivery techniques. As previously mentioned, glucocorticoids such as Dex are required for the full differentiation of adipose precursors and for the maintenance of key genes in glucose and lipid metabolism in cultured adipocytes and adipose tissue.

We hypothesized that hASCs in contact with CADDS differentiate at a higher rate due to the constitutive protein interaction and Dex release from polymer microspheres. In order to assess the adipogenic potential of the CADDS material, in vitro experiments were conducted to evaluate the interaction of differentiating hASCs on CADDS and bioactivity of Dex MS.

3.2 METHODS

3.2.1 Isolation of human ASCs (hASCs)

Human ASC isolation was conducted as previously reported. This study was conducted in accordance with the regulations of the Human Studies Committee of the University of Pittsburgh. Tissue collection was performed according to a protocol approved by the University of Pittsburgh Institutional Review Board. ASCs were isolated from fresh human abdominal adipose tissue harvested during elective surgery. Human adipose tissue was minced in Hank's Balanced Salt Solution containing 1 mg/ml collagenase and 3.5% fatty acid free BSA. Tubes containing minced tissue shaken and submerged in 37° C water bath. Once the solution appears homogenous, it is filtered with double layer gauze into a sterile tube. Following centrifugation at 1000 rpm for 10 minutes, the supernatant and fatty layer is aspirated leaving behind a pellet of cells. Pellet is resuspended in erythrocyte lysis buffer and centrifuged again. Next, supernatant of lysis buffer is aspirated leaving behind a pellet to resuspend in ASC expansion/growth media and plate in T-75 flask. After 4-6 hours in incubator (37° C and 5% CO_2), cells have attached to culture plate. Finally, cells are washed 3 times with sterile 1X PBS and fresh ASC growth media is added to begin feeding. Rat ASCs were expanded on T-175 cm^2 tissue culture flasks (Fisher Scientific, Waltham, MA) in expansion media until passage 3 to obtain a homogenous population of precursor cells for *in vitro* studies.

3.2.2 Live/dead stain of hASCs on CADDS

Cytocompatibility assessment was conducted in a 24-well tissue culture plate along with transwell tissue culture inserts to suspend the composite hydrogel above the cells in culture medium. Control wells were left as a normal tissue culture plastic. Human ASCs were seeded at approximately 1 x 10^5 per well in maintenance medium and medium is changed every 2-3 days. On days 5, cells were stained with fluorescent Live/Dead viability/cytoxicity kit (Life Technologies, Grand Island, NY). A solution of 4 µM calcein and 2 µM ethidium homodimer (EthD-1) was prepared in PBS. The solution was added to cells and incubated for 30-45 min at room temperature. Stained cells were subsequently rinsed twice with PBS and then observed under fluorescent microscopy to determine the viability of the cells. Four treatment conditions were used: Hydrogel adipose ECM, hydrogel and single-walled microspheres (SW MS), hydrogel and double-walled microspheres (DW MS), and positive control ASCs in maintenance media.

3.2.3 Alkaline phosphatase (ALP) activity of hASCs with Dex MS

To access bioactivity of Dex MS, ALP activity was quantified in differentiating cultures of hASC containing varied doses of Dex. Concentrations of Dex in differentiation media, from 0 to 800 nM, were prepared using Dex MS, according to loading capacity. Human ASC cultures were seeded at 85,0000 cells/cm^2 in 48-well tissue culture polystyrene plate with adipogenic media (StemPro Adipogenesis Differentiation Kit, Invitrogen, Inc.) replaced every 2-3 day. Cells were maintained and incubated at 37 °C and 5% CO_2 for 2 weeks. Alkaline phosphatase activity (ALP) was measured using assay kit (BioVision, Milpitas, CA) and plate reader absorbance at 405 nm.

3.2.4 Differentiation of hASCs on CADDS

In vitro differentiation of hASCs with CADDS was conducted to biomaterial interaction with cells, specifically adipogenesis. Transwell baskets were used to suspend CADDS (SW), CADDS (DW), CADDS (Empty), and Hydrated ECM in culture with hASCs seeded at 85,000 cells/cm^2 in 24 well plate. Cultures were grown on tissue culture plastic with adipogenic media (StemPro Adipogenesis Differentiation Kit, Invitrogen, Inc.) and basal media (DMEM media, 3% FBS, pen/strep, fungizone) for positive and negative control, respectively. Cells were maintained and incubated at 37 °C and 5% CO_2 for 2 weeks.

3.2.5 Oil Red O stain of hASCs on CADDS

After 2 weeks, hydrogels were removed, and cultures were rinsed with PBS and fixed for an hour in 4% formaldehyde. After fixing, cultures were rinsed with sterile water and 60% Isopropanol was added to sit for 2 min. Isopropanol solution was moved and replace by Oil Red O working solution to sit for 5 minutes. Oil Red O was removed by tap water rinsing and a hematoxylin counter stain was used to stain nuclei before the final rinse. Oil red O stock solution was prepared by combining 300 mg of Oil Red O powder (Sigma-Aldrich, St. Louis, MO, USA) to 100 ml of 99% isopropanol. The working solution was developed by using stock dilution of 3:2 (v:v) with deionized water.

3.2.6 Image J Quantification of Adipogenesis

Brightfield light microscopy was used to visualize and obtain an objective overview the differentiating cultures at 20X magnification. 15 Images (15) were taken at 20X magnification. ImageJ was used to compile 15 images of each replicate in experimental group and quantify the area of red stained lipids. First, the scale within each image was converted from pixels to micrometers. Next, images were converted to grayscale within the RGB stack function. Once in grayscale, stained lipids were isolated using the threshold function. Lastly, the threshold area was measured as collected for data analysis.

3.2.7 Lipid Extraction from differentiated hASCs on CADDS

An additional method was used to quantify the amount of lipids stain by Oil Red O. After Brightfield imaging each well, culture plates were placed in 37° C to evaporate any remaining water content. Next, 200 μl of 100% isopropanol was added to well to wash Oil Red O stain off the cells. Lastly, samples were collected and transferred to 96 well plates for measurement by plate reader at 510 nm.

3.2.8 Statistical Analysis

All quantitative data is presented as a mean ± standard error of the mean. For cell cultures, differences between the experimental conditions were calculated for statistical significance by one-way analysis of variance (ANOVA) using GraphPad Prism 6 (GraphPad Software, Inc.). Differences are considered significant if $P < 0.05$.

3.3 RESULTS

3.3.1 ASC viability on AdECM and CADDS biomaterial

Cellular biocompatibility was assessed by staining of adipose stem cell cultures in contact with AdECM hydrogel and CADDS, compared to a positive control. Overall, quantitative data analysis in Figure 17 showed that experimental groups containing hydrogel systems maintained a high percentage of cell viability, totaling approximately 80-90% living cells in culture, comparable to adipose-derived stems cells alone which had around 92% viable cells after 5 days in culture. Representative images of these cultures, Figure 18A-D, consistently reflect a high number of viable cells (green fluorescence) compared dead cells (red fluorescence), a similar outcome for all groups in the assay.

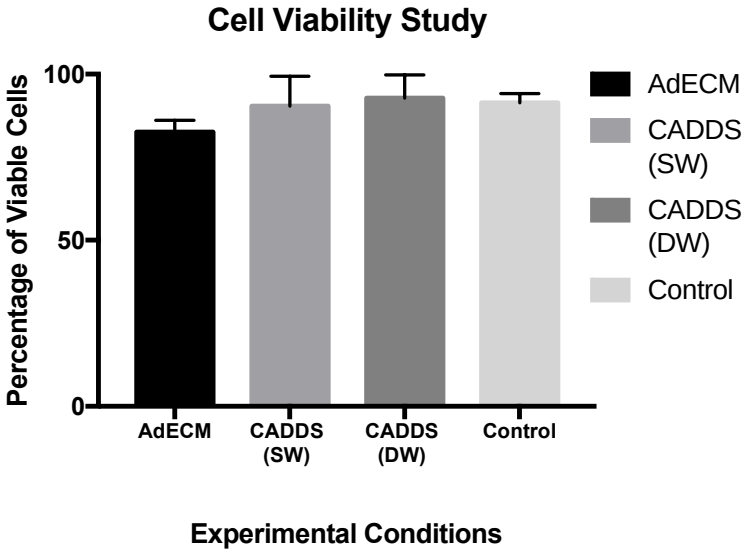

Figure 17. ASC viability on CADDS biomaterial

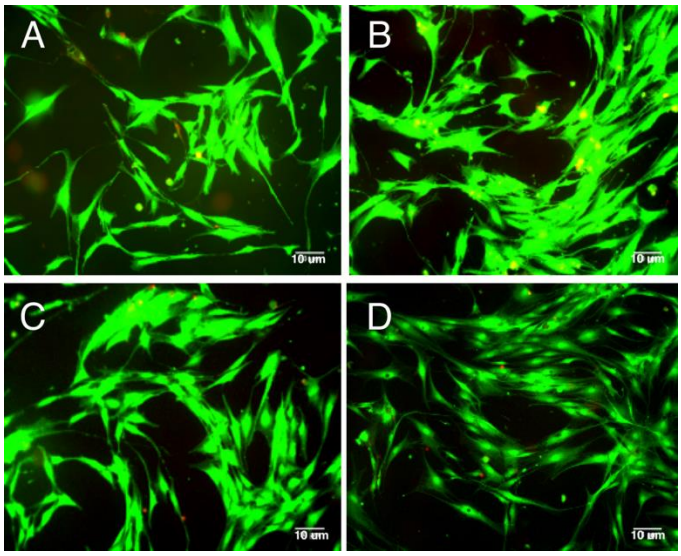

Figure 18. Live/dead stain of ASCs on (A) AdECM hydrogel, (B) CADDS (SW), (C) CADDS (DW) and (D) media (10 um scale bar)

3.3.2 Bioactivity of Dex MS in ASC culture

In vitro study results indicate that increased amounts of Dex correlated with ALP intracellular activity in groups with higher Dex MS concentration. The trend of ALP intracellular activity in ASCs decreased as Dex MS media concentration decreased across the six groups of 800, 400, 200, 100, 50, and 0 nM Dex, seen in Figure 19.

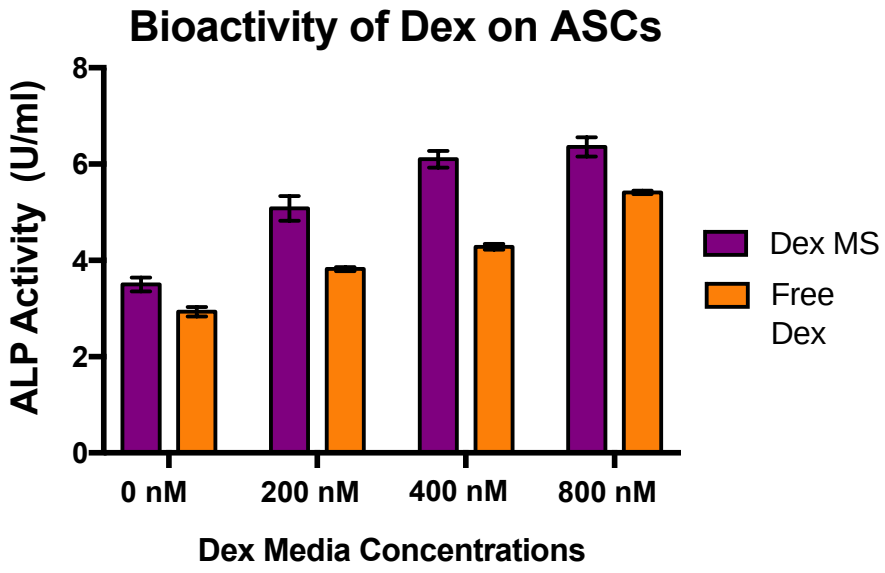

Figure 19. Alkaline phosphatase activity in Dex MS-ASC cultures

3.3.3 Quantification of adipogenesis

A 14-day adipogenesis study was conducted to determine the effect of the CADDS on the formation of adipocytes in a cell culture. Representative brightfield images in Figure 20 displays the differentiation of adipose derived stem cells with clear observational differences in lipid accumulation between the positive control and the cells in culture with the CADDS.

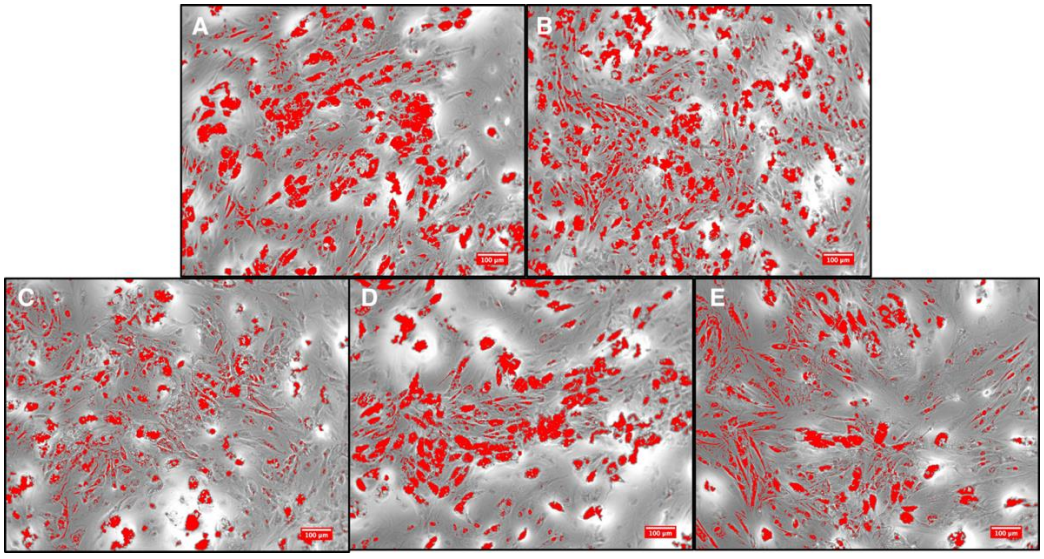

Figure 20. ImageJ modified images of differentiated hASCs cultured with (A) CADDS (SW), (B) CADDS (DW), (C) tissue culture plastic, (D) CADDS (Empty), and (E) Hydrated AdECM

ImageJ was used to capture, measure, and quantify the amount of lipid accumulation. Quantitative results in Figure 21 showed that ASC differentiated samples with CADDS resulted in higher amounts of lipid droplets. Additionally, the extracted lipid stains of Oil Red O produced similar results with high absorbance measurements from samples with CADDS scaffold in Figure 22. In both cases of analysis, CADDS DW groups containing the highest degree of lipid content and Oil Red O staining.

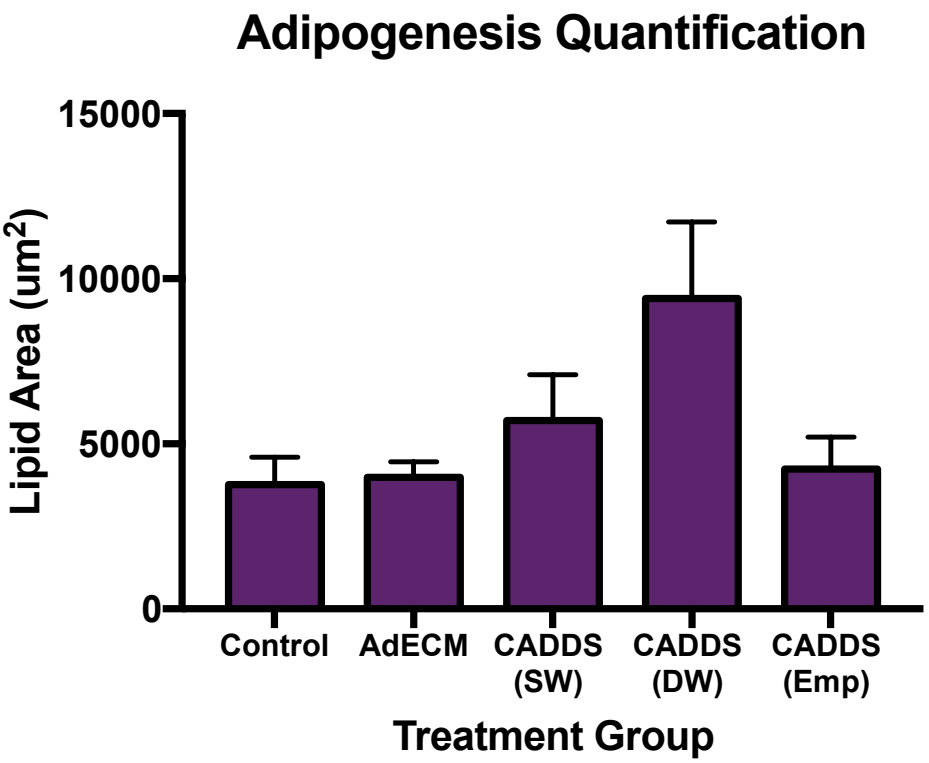

Figure 21. ImageJ quantification of lipid area in differentiated hASC culture

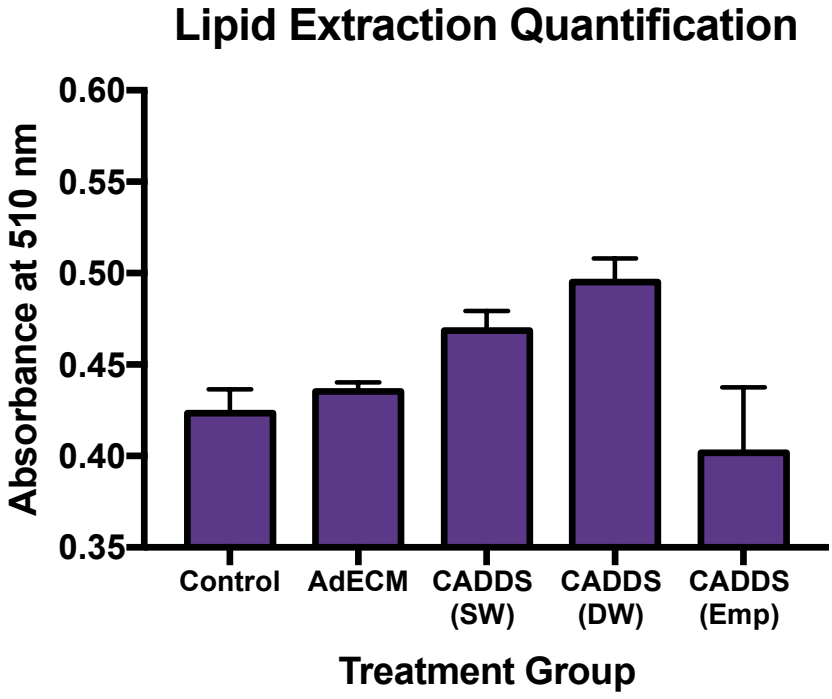

Figure 22. Absorbance readings of Oil Red O extract from differentiated ASCs

3.4 DISCUSSION

By encapsulating the dexamethasone in polymer microspheres, biomaterial scientists are able to harness the adipogenic function of Dex for local delivery to the targeted defect site while also avoiding the systemic side effects associated with oral administration. Results from the *in vitro* adipogenesis assay demonstrate the ability of dexamethasone released from microspheres to increase adipogenesis in adipose derived stem cells compared to cell cultures alone in adipogenic media. The dexamethasone and other glucocorticoids are required for the full differentiation of adipose precursors and for the maintenance of key genes in glucose and lipid metabolism in cultured adipocytes and adipose tissue. As a key component of the composite adipose derived delivery system, dexamethasone microspheres can release Dex for several weeks in its single and double emulsion form. The SW Dex MS releases a significant portion of the adipogenic drug within the first 10 days to initiate adipogenesis in the desired region. This initial burst release caused the CADDS SW group to lose the majority of its drug during the first week of the study while the CADDS DW group was able to release Dex consistently throughout the study which may have led to the higher degree of lipid formation in the ASC differentiation cultures. The single layer of PLGA that encapsulates the Dex is hydrolyzed and degrades quickly compared to the DW Dex MS formulation which traps the Dex within the inner PLGA core. The PLLA layer surrounding the PLGA core degrades much slower due to its methyl side groups causing the sustained release of Dex from the DW formulation.

It was also important to analyze the role of AdECM within the CADDS construct in regard to the ASC differentiation studies. The CADDS (Empty MS) and AdECM groups conditions were essential to assess the cellular response without Dex MS. Results from the in vitro studies indicate

that AdECM is unable to induce a higher degree of lipid formation. Dex MS were required in order to influence the adipogenic potential of AdECM in vitro.

3.5 CONCLUSION

ASCs are the precursor cells from which adipocytes are formed. In vitro bioactivity studies of ASCs with Dex MS demonstrated that increases Dex MS dosage led to a higher degree of cellular ALP activity. ASCs were also cultured with CADDS (SW and DW MS) to evaluate viability and adipogenic response. ASCs remained highly viable after 5 days in culture with the biomaterial, similar to the TCP control, demonstrating a moderate degree a suitability and safety. ASCs differentiation on CADDS was also compared to TCP positive control. CADDS containing SW and DW microspheres induced ASCs to differentiate to a higher degree and produce a great amount of lipids. ASC groups differentiating on CADDS DW were found to have the greatest amount of lipid occurrence. Lipid occurrence was analyzed using two different methods which resulted in similar outcomes.

CADDS (SW/DW MS) construct for the purpose of delivering adipogenic bioactive molecules represents potential off-the-shelf biomaterial for adipose tissue repair. In this chapter, it was demonstrated that CADDS containing SW or DW microspheres positively affects ASC differentiation via the release of dexamethasone in cell culture.

4.0 IN VIVO RESPONSE AND VOLUME RETENTION OF THE COMPOSITE ADIPOSE DERIVED DELIVERY SYSTEM IN SMALL ANIMAL MODEL

4.1 INTRODUCTION

A variety of animals has been used in adipose tissue research, each serving a specific purpose. Athymic mice are frequently used to assess volume retention of human fat grafts under various experimental conditions (microspheres) as well as the growth/differentiation of human ASCs in scaffolds within a living system. Athymic mice provide the advantage of physiological conditions for a multi-week study without having to match cell-to-animal species. Substantial information can be obtained from these studies, but it is distant from clinical application in terms of size, function, and immune health.

The use of an immunocompetent animal such as the Wistar rat is also common. The use of allogenic cells, autologous fat, and multi-component scaffolds have been explored and evaluated in the Wistar rat model. Materials are normally injected as a bolus subcutaneously with studies lasting up to 12 weeks. An animal model with a healthy immune system allows investigators to fully consider material degradation and immune responses related to adipose tissue formation. Adipose volume retention has been reported in this animal model at 12 weeks (Topcu et al. 2012), providing significant data with some clinical relevance.

4.2 METHODS

4.2.1 Pilot In Vivo Volume Retention Study

Fisher 344 immunocompetent rats were chosen to appropriately assess adipogenic potential of CADDS biomaterial in vivo. Rats (n = 16) were divided into 4 groups (n = 4) receiving subcutaneous bilateral 1 ml volume injections of hydrated AdECM containing either (1) 300 µg dose of SW Dex MS, (2) 300 µg dose of DW Dex MS, (3) Empty MS, or (4) saline only. The photograph in Figure 23 shows the implant sites. A 6-week time point was chosen for evaluation of biomaterials in regard to explant structure, tissue integration, and volume retention. Photographs of the rat were taken each week to observe animal health and outer appearance of implant until the end of the study.

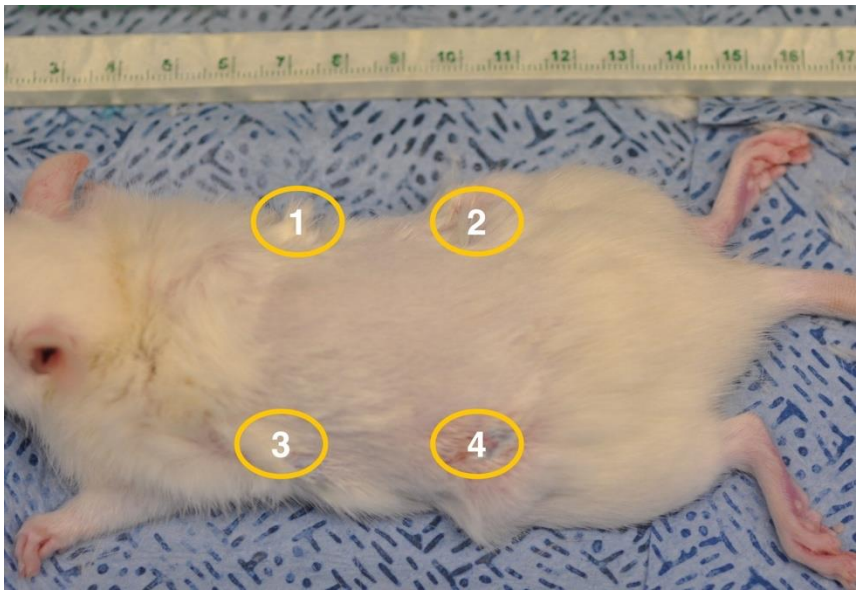

Figure 23. Implant sites for volume retention study

4.2.2 Surgical Procedure Validation Study

After high mortality rates occurred in the pilot study, an additional in vivo animal study was conducted to validate the safety of surgical procedure for the rats. Rats (6) were divided into 3 groups (n = 2) receiving either (1) CADDS (SW), (2) Renuva (Musculoskeletal Transplant Foundation, Edison, NJ), or (3) sham procedure. The Renuva product was added to compare the biological safety of the CADDS material to an industry developed AdECM. A 6-week time point was chosen for evaluation of biomaterials in regard to explant structure, tissue integration, and volume retention. Photographs of the rat were taken each week to observe animal health and outer appearance of implant until the end of the study.

4.2.3 Implant Surgical Procedure

All experiments with animals in this study were performed in accordance with the guidelines set by the Institutional Animal Care and Use Committee at the University of Pittsburgh in Pittsburgh, PA. AdECM and Renvua were reconstituted in sterile saline at a protein concentration of 22.5% and implanted subcutaneously on the bilateral dorsal flanks of female Fisher 344 rats using a diffuse injection technique. Animals received isoflurane gas during the procedure for immobilization and ketoprofen after the procedure for pain management. Over the next 72 hours, animals were monitored daily to address any health concerns.

4.2.4 Post Implantation Evaluation

Photos were taken every week for 6 weeks to monitor size of implant over the course of time. Additionally, rat weight was recorded to ensure a healthy eating habits resumed after the surgical procedure.

4.2.5 Implant Harvest Procedure and Volume Measurement

Animals were euthanized by CO_2 asphyxiation and cervical dislocation at 6 weeks prior to removing the implant. An incision was made above the implantation site to harvest implants from underlying subcutaneous tissue. Photos of animals were used to document findings. Half of the harvested implants were used for volume measurement via gas pycnometer (AccuPyc II 1340 Pycnometer, Micrometrics, Norcross, GA). The other half of harvested implants were used for histology.

4.2.6 Histological Analysis

Immediately after harvesting, implants were placed and submerged in 10% formalin solution as a method of fixation. After 8 days, implant tissues were removed and processed for paraffin embedding. Once embedded in paraffin blocks, a microtome was used to section samples at 5 μm for tissue staining. Hematoxylin and Eosin (H&E) staining was conducted to view the overall structure of the implant and surrounding tissue. Masson's Trichrome (M&T) (American MasterTech, Lodi, CA) staining was performed according to manufacturer's protocol. MT staining

distinguished the implant from the surrounding animal tissues in the sections. Sections were imaged using brightfield microscopy (BZ-X700 Fluorescence microscope, Keyence, Itasca, IL).

4.2.7 Statistical Analysis

All quantitative data is presented as a mean ± standard error of the mean. For cell cultures, differences between the experimental conditions were calculated for statistical significance by one-way analysis of variance (ANOVA) using GraphPad Prism 6. (GraphPad Software, Inc.). Differences are considered significant if $P < 0.05$.

4.3 RESULTS

4.3.1 Pilot Study Animal Weight and Health

6 out of the initial 12 animals survived to the end of the pilot study. The remaining 6 animals remained healthy and increased their weight according to the range provided by the supplier. In Figure 24, animals weighed between 59 to 75 grams at week 0 and increased over time to as high as 130 grams. Overall, the animals starting at a lower weight remained smaller than the rest of the animals in the study.

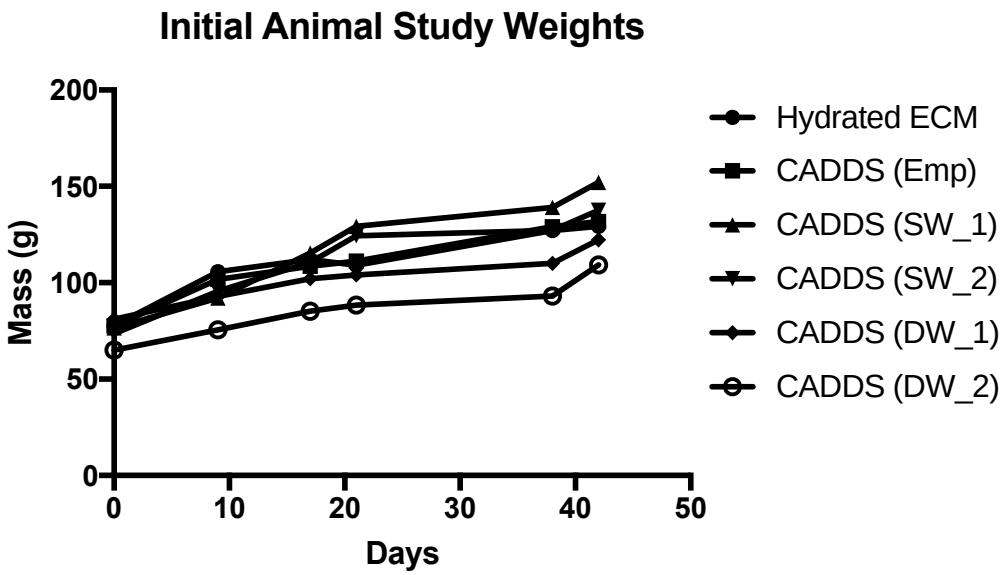

Figure 24. Volume Retention Study Animal Weight over 6 weeks

4.3.2 Pilot Study Pycnometer Data

Half of the harvested implants were used to measure the remaining volume and calculate retention (added explant pictures). The hydrated AdECM and CADDS (empty MS) implants had the smallest volume with less than 20% of the 1 ml injection remaining as seen in Figure 25. The average volume retention rate for CADD (SW) implants was approximately 38%. The harvested CADDS (DW) implants were found to have the largest volume remaining after 6 weeks in vivo. Retention rates for CADDS (DW) were approximately 50%.

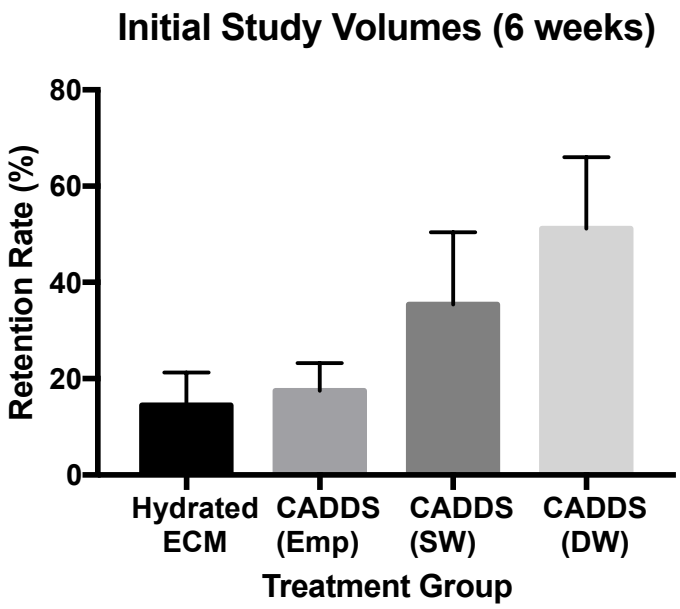

Figure 25. Volume Retention of Implants in Pilot Study

4.3.3 Pilot study explant histology

Half of the excised implants were fixed, sectioned, and stained with H&E and Masson's Trichrome to assess general structure and morphology of the implant and surrounding tissue. Typically, H&E stains nuclei dark blue while cytoplasm and extracellular matrix are stained varying degrees of pink. Masson's trichrome stains the nuclei purple/black, cytoplasm, muscle, erythrocytes and keratin bright-red, and blue. Figure 26 and 27 shows the hydrated ECM implant (dark pink/dark blue) surrounded by a fibrous capsule (bright pink/bright blue). After 6 weeks, a substantial number of mononuclear cells infiltrated the scaffold. Figure 28 shows a noticeably darker CADDS SW implant that seemed to degrade at a high degree in the insert microscopy image. The CADDS empty implant seemed to have similar results in morphology and degradation as the CADDS SW, seen in Figure 30. The CADDS DW implant in Figures 32 and 33 shows a scaffold with darker

areas (black/purple) and brighter areas surrounding pockets of adipose-like structure similar to adipose tissue found under the skin.

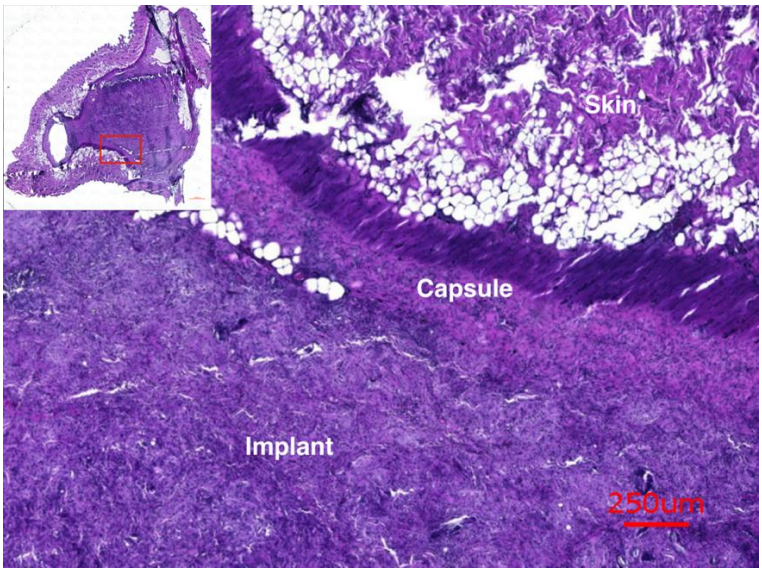

Figure 26. Representative image of H&E stained Hydrated AdECM implant section

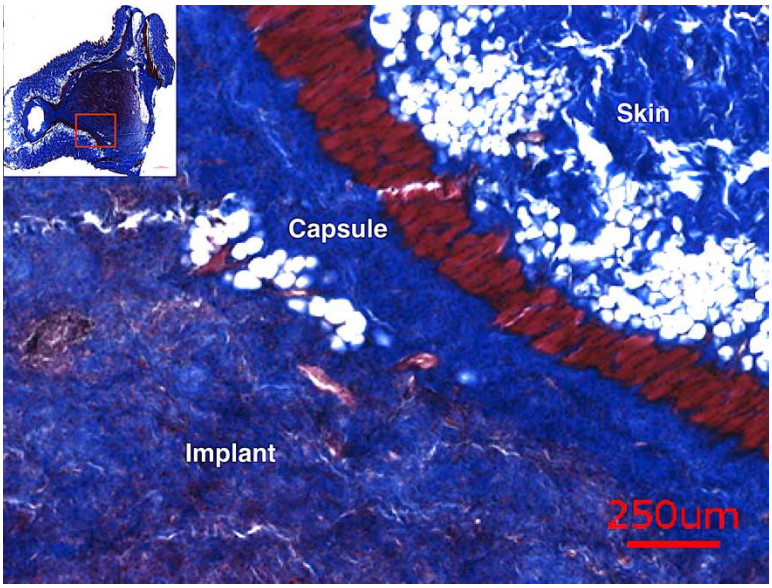

Figure 27. Representative image of Masson's stained Hydrated AdECM implant section

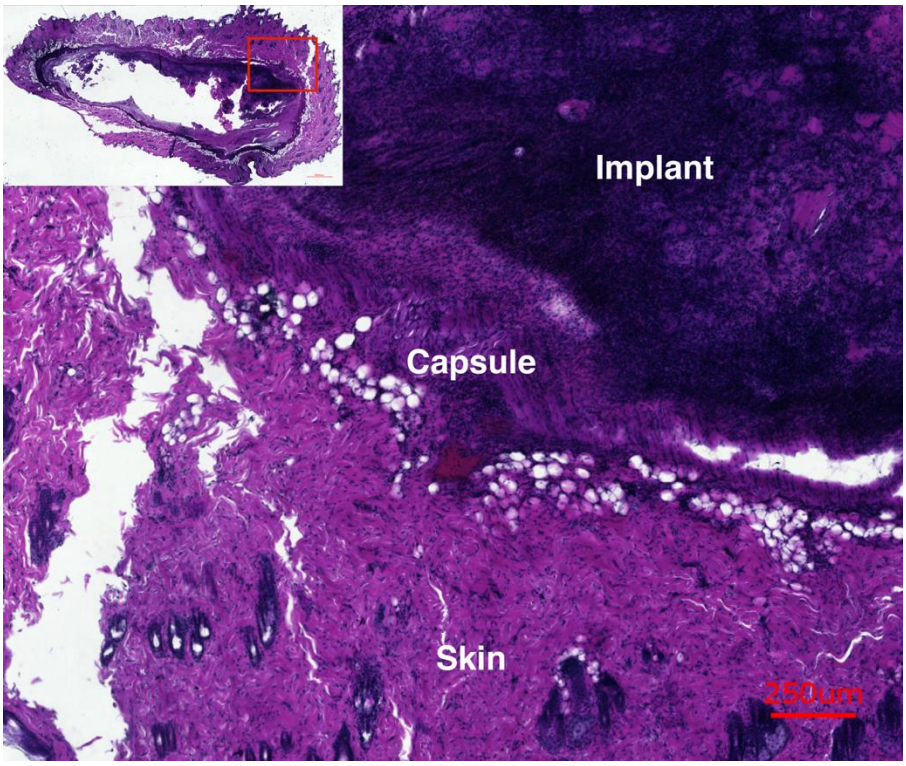

Figure 28. Representative image of H&E stained CADDS (SW) implant section

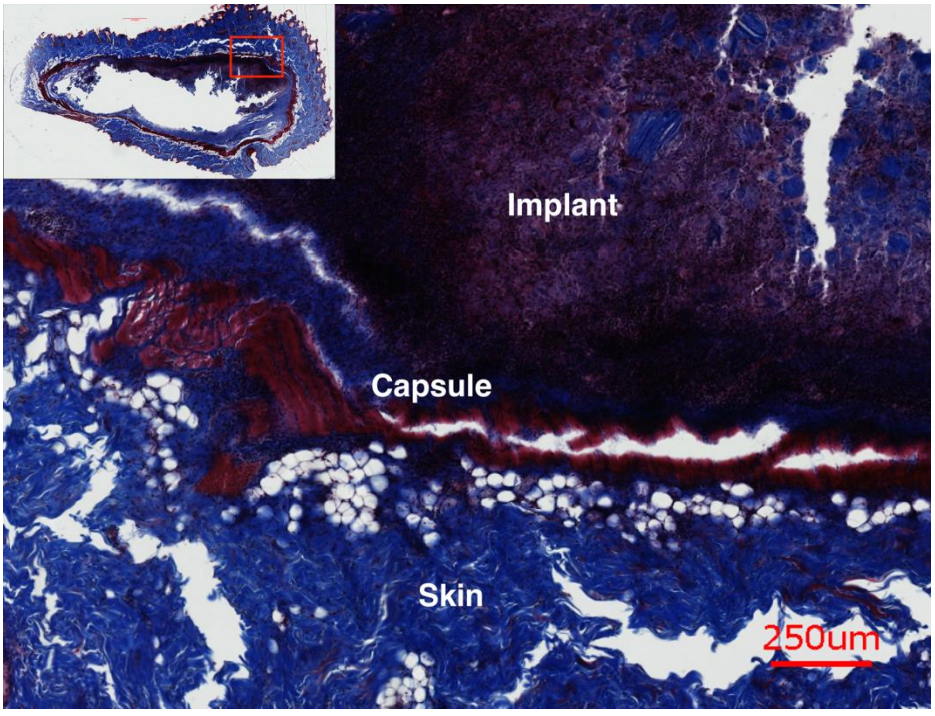

Figure 29. Representative image of Masson's Trichrome stained CADDS (SW) implant section

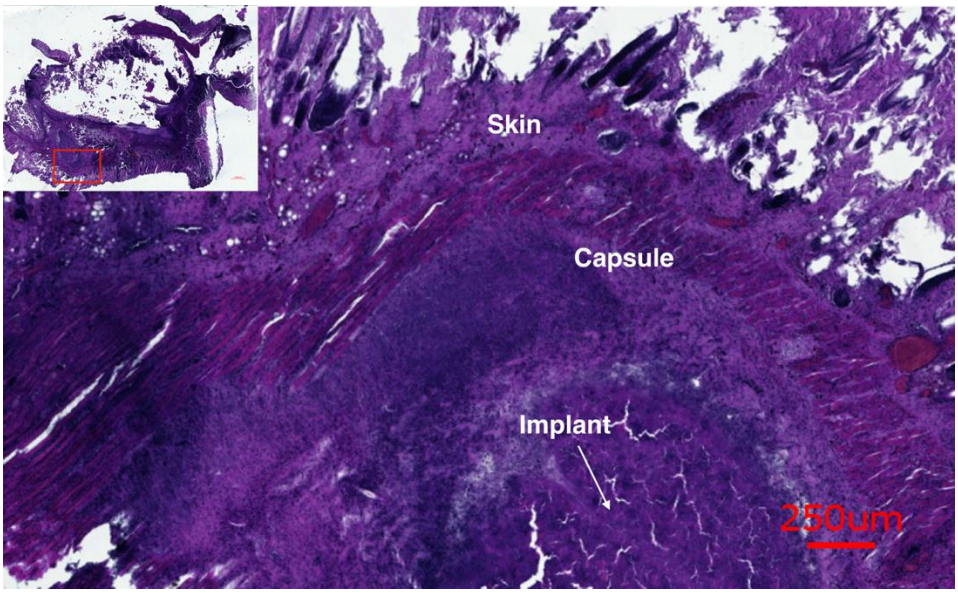

Figure 30. Representative image of H&E stained CADDS (Empty) implant section

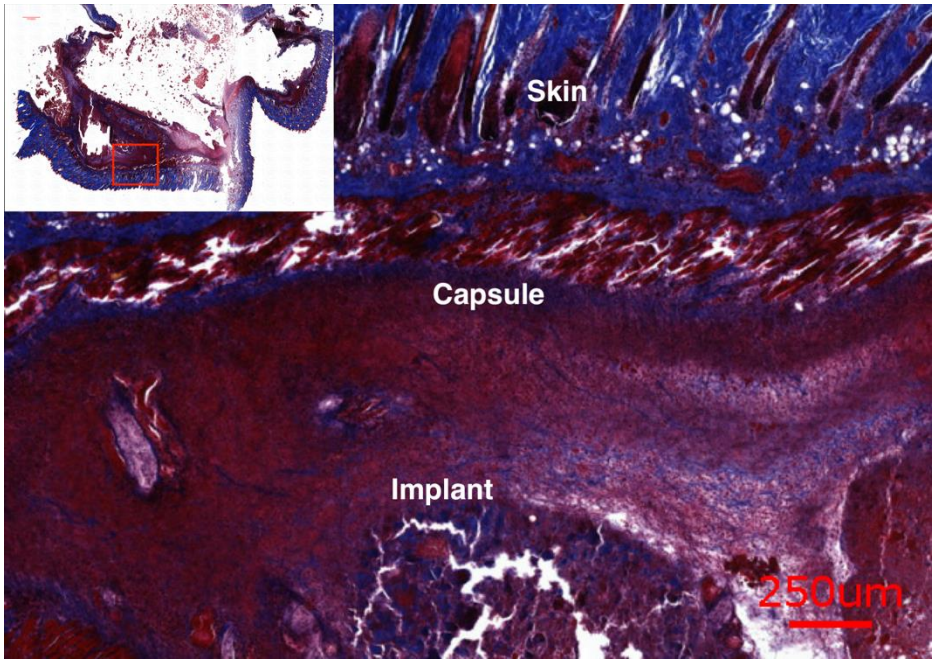

Figure 31. Representative image of Masson's trichrome stained CADDS (Empty) implant section

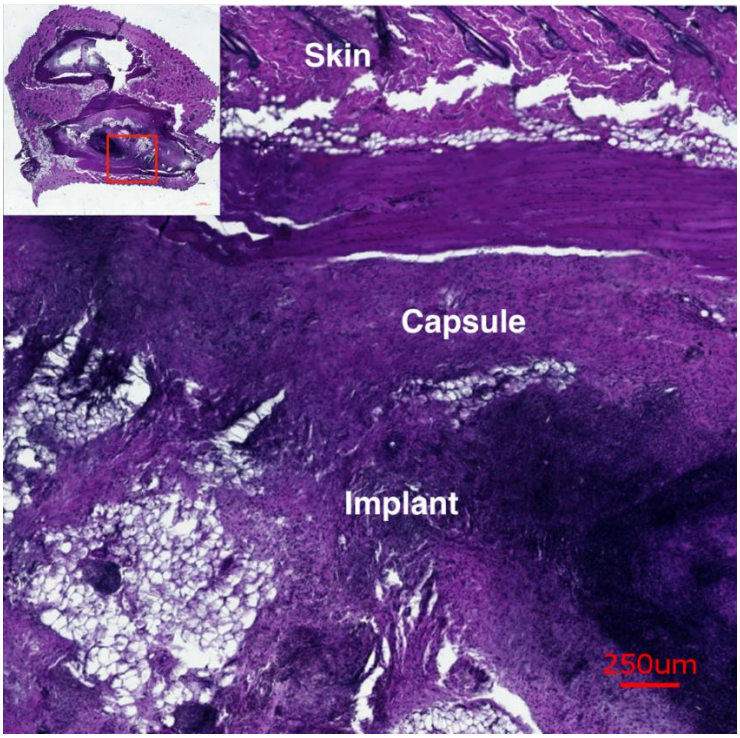

Figure 32. Representative image of H&E stained CADDS (DW) implant section

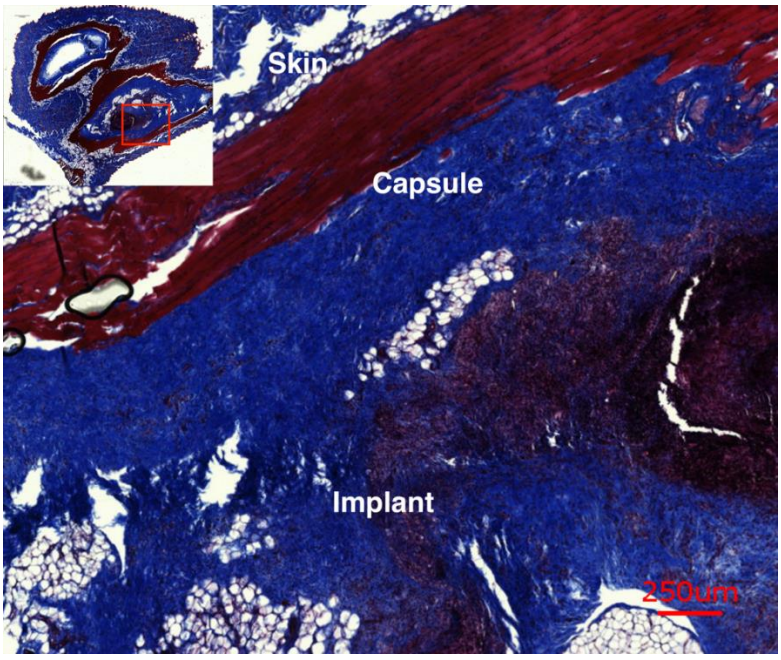

Figure 33. Representative image of Masson's trichrome stained CADDS (DW) implant section

4.3.4 Validation Study Animal Weight and Health

All of the animals in the validation study survived to the 6-week surgical procedure validation study. All 6 animals remained healthy and increased their weight according to the range provided by the supplier. In Figure 34, animals weighed between 98 to 112 grams at week 0 and increased with time to as high as 150 grams. By the end of the study, all animals were within a close range of 7 grams or less.

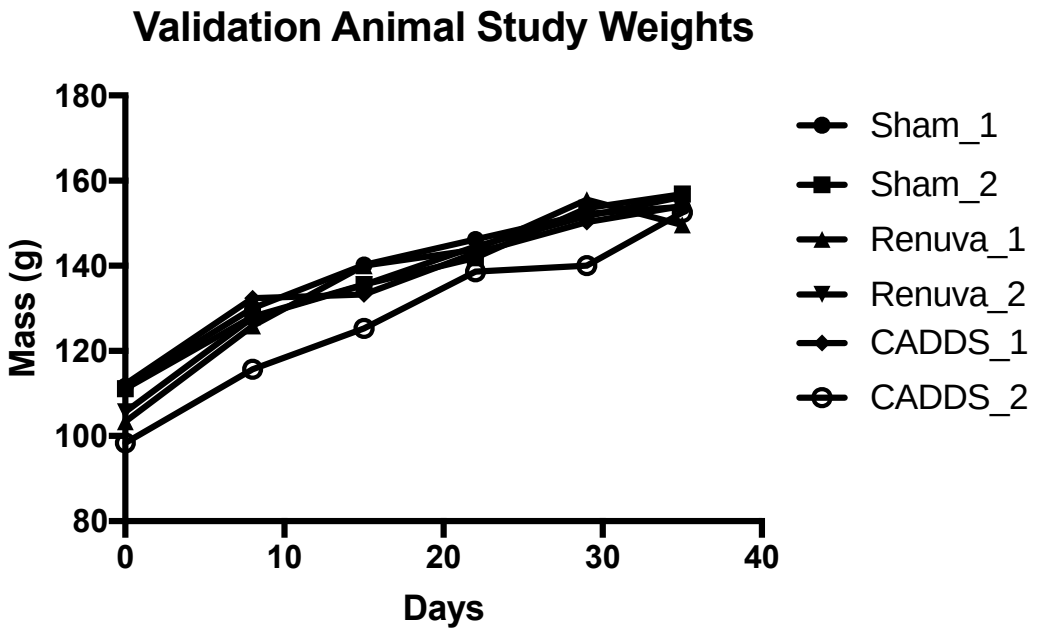

Figure 34. Surgical Procedure Validation Study Animal Weight over 6 weeks

4.3.5 Validation Study Pycnometer Data

Half of the harvested implants were used to measure the remaining volume and calculate retention (add explant pictures). The hydrated AdECM and CADDS (empty MS) implants had the smallest volume with less than 20% of the 1 ml injection remaining as seen in Figure 35. The average

volume retention rate for CADD (SW) implants was approximately 38%. The harvested CADDS (DW) implants were found to have the largest volume remaining after 6 weeks in vivo. Retention rates for CADDS (DW) were approximately 50%.

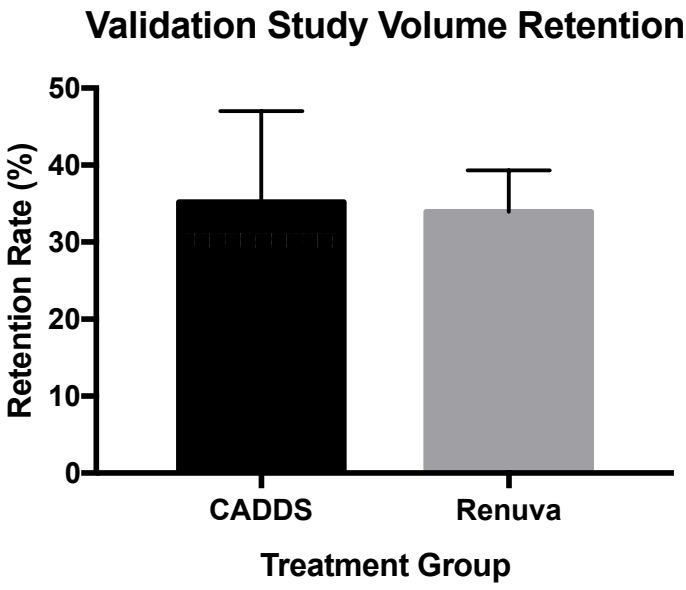

Figure 35. Volume Retention of Implants in Procedure Validation Study

4.3.6 Validation study explant histology

Half of the excised implants from the validation study were fixed, sectioned, and stained with H&E and Masson's Trichrome. Figure 36 and 37 shows a CADDS (empty) implant infiltrated with cellular bodies throughout. Histological sections also seem to show a presence of adipose-like structures throughout the capsule and implant interface. The Renuva implant in Figure 38 and 39 exhibit a very dark shade of pink and blue, respectively. This is most likely cause by a very high occurrence of cell infiltration in the implant. Additionally, the implant seems to be detached from the capsule interface.

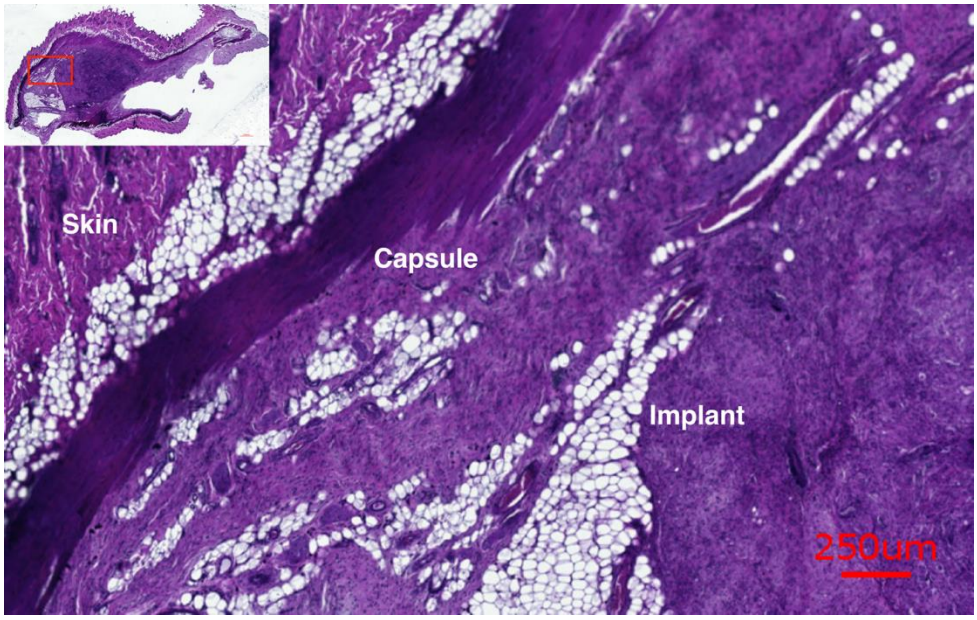

Figure 36. Representative image of H&E stained CADDS (Empty) implant section

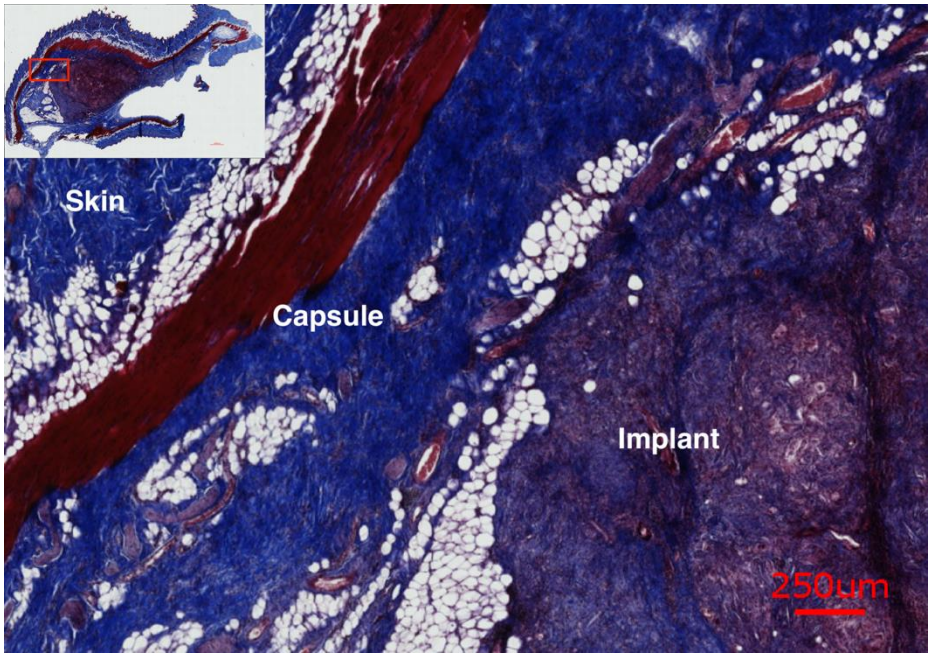

Figure 37. Representative image of Masson's trichrome stained CADDS (Empty) implant section

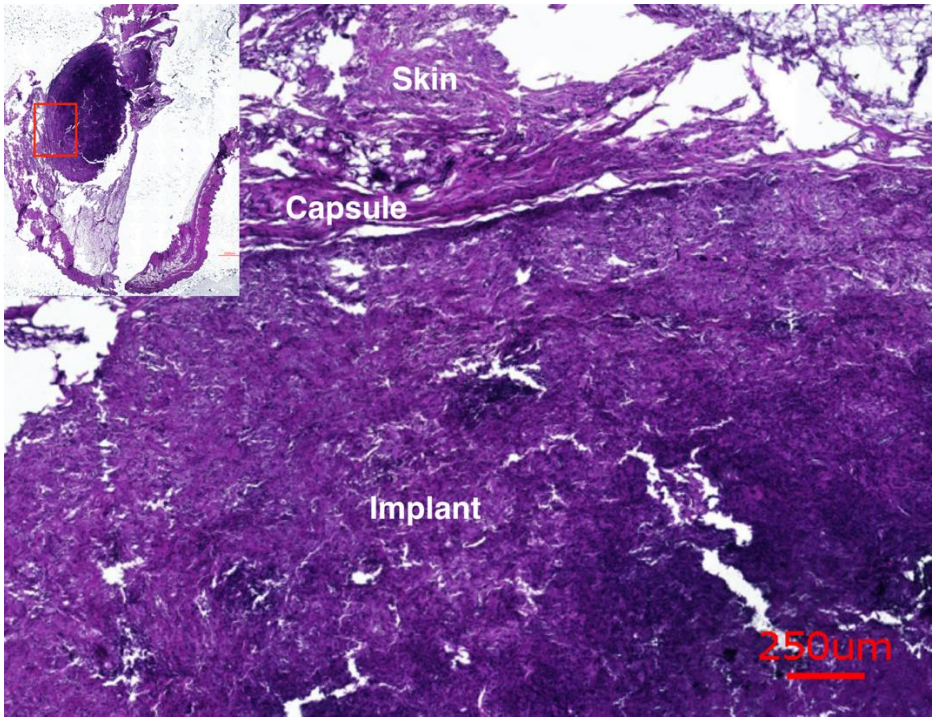

Figure 38. Representative image of H&E stained Renuva implant section

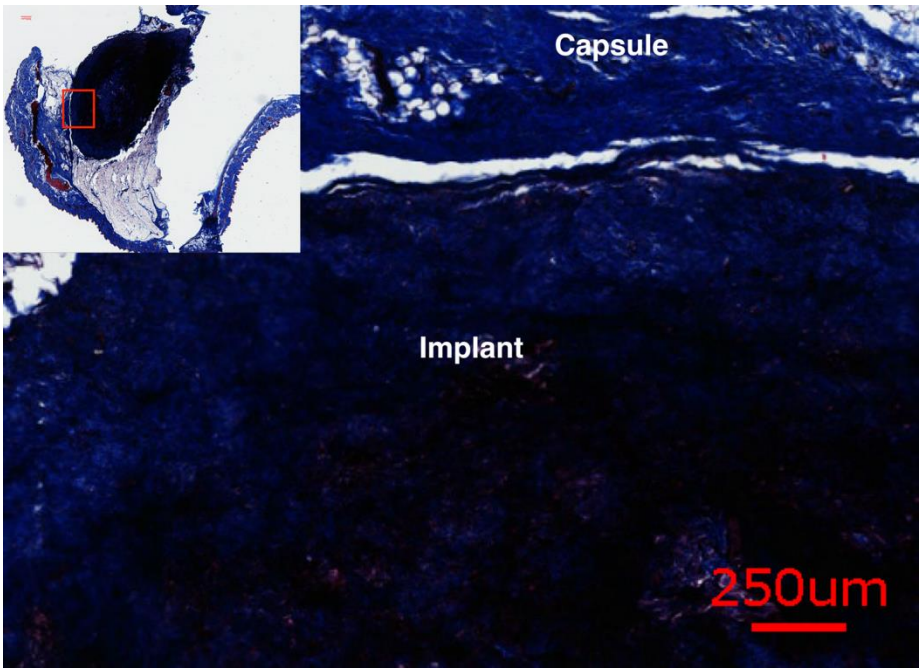

Figure 39. Representative image of Masson's trichrome stained Renuva implant section

4.4 DISCUSSION

The immunocompetent animal model is essential for the evaluation of implant biomaterials for tissue engineering. The response of the functioning immune system to the implanted biomaterials provides vital information regarding host response, tissue reconstruction, and remodeling/degradation. The majority of the adipose tissue engineering in vivo studies insert the respective scaffold subcutaneously similar to the location of an adipose defect area in the clinic.

The short-term pilot study was designed to observe the initial changes in CADDS scaffold structures with and without the release of Dex MS. Unfortunately, only half of the animals survived the surgical procedure. However, the remaining animals were able to provide valuable information given the multiple inject sites per subject. The volumes of implant scaffolds containing Dex MS were higher than scaffolds without Dex. These results suggest that Dex drug delivery may support retention in vivo. Similar results were reported in animal studies investigating retention in autologous fat grafts (Kelmendi-Doko et al. 2017).

Histological sections of the pilot study implants demonstrated various degrees of cell infiltration, capsule integration, and adipose-tissue like structures. It is worth noting that the CADDS DW implant sections contained a noticeable amount of adipose-like structures within the construct. Comparatively, the other groups were not about to produce these structures including the CADDS SW implants. These results suggest that a slow release of Dex may also support positive tissue remodeling in the short term.

An additional animal study was performed to validate the safety of the surgical procedure and compare CADDS (Empty) to an industry manufactured material. All of the animals remained viable through the end of the short-term study. The surgical procedure appeared to be safe for the animals and resulted in similar retention rates found in Dex-free samples of the initial study.

Additionally, CADDS (empty) appeared to promote adipose-like structures formation in histological sections without Dex. Longer studies containing a higher population of animals may be helpful to clearly defining the role of Dex MS in the CADDS implanted in vivo. However, the low retention rates overall from both studies suggest two theories: (1) the immunocompetent rat model may be insufficient in assessing CADDS material or (2) the CADDS material promotes an insufficient amount of new volume stable adipose tissue. Additional studies were conducted to investigate the first theory. It was hypothesized that rat precursor cells (rASCs) from the immunocompetent rat will not differentiate on CADDS to the same degree as hASCs in adipogenic media.

4.4.1 Additional Experimentation

ASCs are an essential population of cells required for new adipose tissue formation. Therefore, rASCs should be able to differentiate on scaffold material in an adipogenic environment. In Chapter 3, we demonstrated the ability of hASCs to differentiate on CADDS. An in vitro differentiation study was designed to identify any differences in adipogenic potential of rASCs compared to hASCs on CADDS.

4.4.2 Methods

4.4.2.1 Isolation of human ASCs

Human ASC isolation was conducted as previously reported (Minteer et al. 2015).

4.4.2.2 Isolation of rat ASCs

Subcutaneous adipose depots, particularly the inguinal, dorsolumbar, and axillary, were used to isolated adipose tissue of Fischer 344 female rats (Charles River Laboratories, Wilmington, MA). Rat adipose tissue was minced in Hank's Balanced Salt Solution containing 1 mg/ml collagenase and 3.5% fatty acid free BSA. Tubes containing minced tissue shaken and submerged in 37° C water bath. Once the solution appears homogenous, it is filtered with double layer gauze into a sterile tube. Following centrifugation at 1000 rpm for 10 minutes, the supernatant and fatty layer is aspirated leaving behind a pellet of cells. Pellet is resuspended in erythrocyte lysis buffer and centrifuged again. Next, supernatant of lysis buffer is aspirated leaving behind a pellet to resuspend in ASC expansion/growth media and plate in T-75 flask. After 4-6 hours in incubator (37° C and 5% CO_2), cells have attached to culture plate. Finally, cells are washed 3 times with sterile 1X PBS and fresh ASC growth media is added to begin feeding. Rat ASCs were expanded on T-175 cm^2 tissue culture flasks (Fisher Scientific, Waltham, MA) in expansion media until passage 3 to obtain a homogenous population of precursor cells for *in vitro* studies.

4.4.2.3 Flow cytometry of ASCs

Cultured human or rat ASC cells (passage 3) were analyzed for the expression of various cell surface markers using an LSR-II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, OR).

Antibodies used are listed as following: Fluorochrome-conjugated mouse anti-human CD45 (clone: 2D1, BD Bioscience), mouse anti-rat CD90 (clone: 5E10, BD Bioscience), mouse anti-rat CD235a (clone: GA-R2, BD Biosciences), mouse anti-rat CD34 (clone: 581, BD Biosciences), mouse anti-rat CD73 (clone: AD2, BD Biosciences), mouse anti-rat CD146 (clone:

541-10B2, Miltenyi Biotec), mouse anti-rat CD14(clone: RMO52, Beckman Coulter), mouse anti-rat CD33 (clone: D3HL60.251, Beckman Coulter), mouse anti-rat CD31 (clone: WM59, BioLegend), mouse anti-rat CD105 (clone: N1-3A1/SN6, Fitzgerald). Fluorochrome-conjugated mouse anti-rat CD45 (clone: OX-1, BD Biosciences), mouse anti-rat CD90 (clone: OX-7, BD Biosciences), mouse anti-rat CD44 (clone: OX-50, BIO-RAD) and hamster anti-rat CD29 (clone: Ha2/5, BD Biosciences).

4.4.2.4 ASC seeding and differentiation on CADDS material

In vitro studies were conducted on polystyrene 96-well cell culture microplates (Falcon™, Fisher Scientific, Hampton, NH) for glycerol quantification and black 96 well μ-plates (ibidi®, Fitchburg, Wisconsin) for fluorescent staining followed by microscopy. ASCs isolated from human and rat adipose were seeded at 30,000 cells per well under the following experimental groups (n=5): tissue culture plastic (TCP) with adipogenic media, TCP with basal media, hydrated AdECM with adipogenic media, CADDS (SW Dex) with adipogenic media, CADDS (DW Dex) with adipogenic media, and CADDS (Emp. MS) with adipogenic media. CADDS and hydrated ECM was prepared as previously stated. On day 0 of the 15-day study, 100 μl of CADDS or hydrated ECM was deposited onto the well plate using 1 ml sterile syringe. Plates were placed in incubator for 3 hours to allow scaffolds to stabilize before cell seeding. Expansion media was removed from flasks containing ASCs and then rinsed in PBS-EDTA solution to initiate lifting protocol. Cells were collected after trypsinization and resuspended in fresh media following centrifugation. Using pipette, 30,000 cells were seeded in 50 μl of expansion media and allowed to attach to scaffolds in the incubator for 1 hour. Lastly, 200 μl of expansion media was added each well. On day 1,

media was changed to either basal or adipogenic media according to experimental conditions. Media changes occurred every 2-3 days until the end of the study.

4.4.2.5 Nile Red/DAPI stain of differentiated ASCs

At the last day of the study, media was removed, and cell cultures were gently washed with 1X PBS (twice). CADDS/AdECM culture samples were fixed in 4% paraformaldehyde for 30 minutes at 4° C. TCP samples were fixed for 10 minutes. After fixation, paraformaldehyde was removed, and all samples were rinsed 3 times with 1X PBS. Next, CADDS/AdECM samples were stained for 60 minutes with 5 μg/ml Nile Red solution (Sigma-Aldrich). TCP samples were stained with Nile Red solution for 30 minutes. After rinsing 3 times with 1X PBS, CADDS/AdECM samples were stained with DAPI for 15 min. TCP samples were stained with DAPI for 5 min. Once staining was complete, all samples were rinsed with 1X PBS 3 times prior to imaging under a fluorescent microscope.

4.4.2.6 Fluorescent imaging of differentiated ASCs on CADDS

The BZ-X700 (Keyence) Fluorescence microscope was used to obtain fluorescence images of differentiated human and rat ASCs. After fixation and staining, cell culture plates were placed into the microscope. The Texas Red channel was used to identify lipids stained with Nile Red. The DAPI channel was used to identify the stained nuclei. Ten images were taken from each well of all experimental conditions at 20X magnification.

4.4.2.7 Glycerol Assessment of differentiated ASCs on CADDS

Glycerol release from lipids in differentiated cultures was stimulated by isoproterenol for 1 hour. Media was collected after 1 hour in fresh media and saved for measurements. Glycerol content in

culture medium across experimental conditions served as an index of lipid accumulation and was determined at the absorption of 570 nm by use of a colorimetric assay kit (Glycerol Assay Kit, Sigma-Aldrich, St. Louis, MO). Glycerol data was expressed in micromoles of glycerol.

4.4.3 Results

4.4.3.1 ASC characterization

Flow cytometry using antibodies against specific antigens of stem cells was performed. The immunotypic profile of hASCs was $CD45^-$, $CD146^-$, $CD14^-$, $CD90^+$, $CD73^+$, $CD33^+$, $CD105^+$, $CD31^+$, $CD34^+$, and $CD235^-$, seen in Figure 40. The immunotypic profile of rASCs was $CD45^-$, $CD29^+$, $CD90^+$, and $CD44^+$, seen in Figure 41.

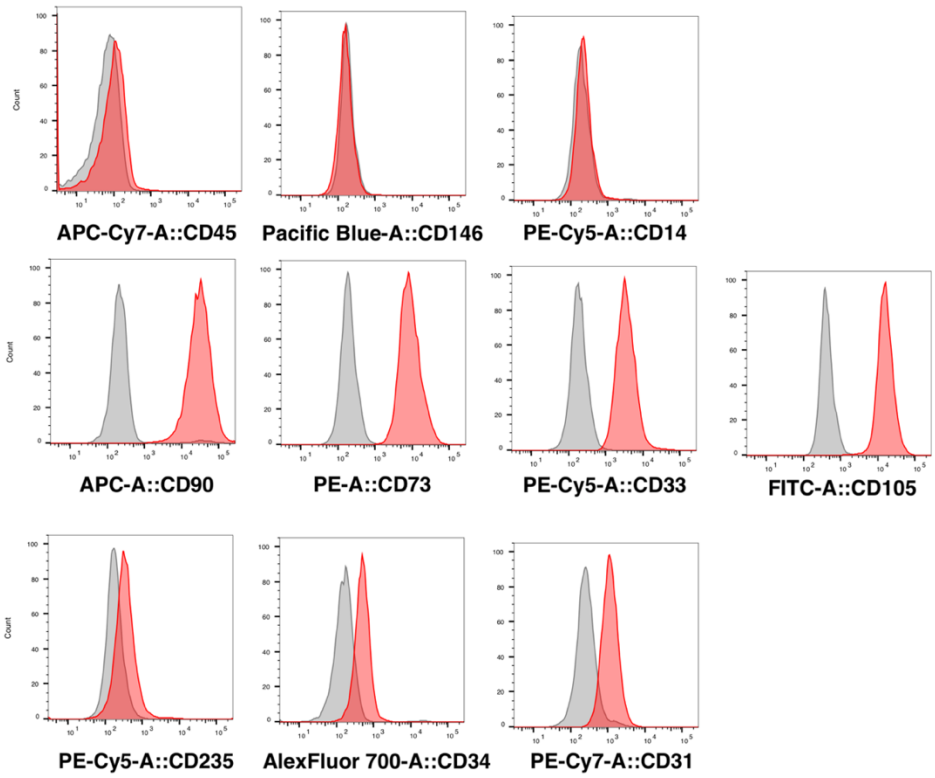

Figure 40. Flow Cytometry characterization of hASCs

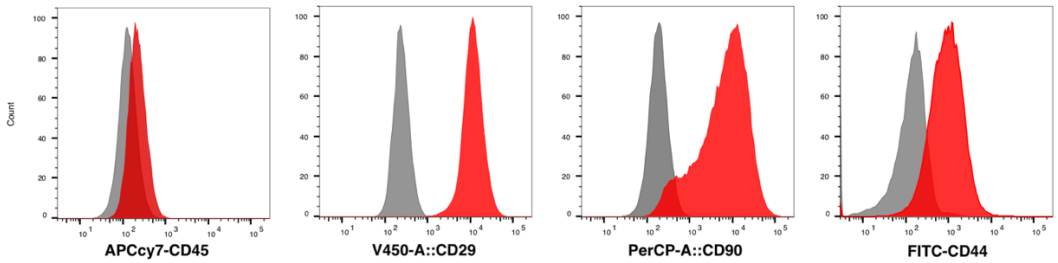

Figure 41. Flow cytometry characterization of rASCs

4.4.3.2 Microscopy images of hASCs compared to rASCs

There were observational differences in the cellular behavior between the rASCs and hASCs. In Figure 42, a greater degree on hASC differentiation is seen on Hydrated ECM compared to rASC differentiation. Differentiation on TCP was comparable between the cell groups containing adipogenic media and both ASC groups did not differentiate on TCP in basal media as expected. Differences in differentiation is also seen in Figure 43 with microscopic images of hASCs shower a higher degree of Nile Red staining on CADDS (empty), CADDS (SW), and CADDS (DW). Microscopic images of differentiated rASCs seem to show a trend of cluster cellular structures on the CADDS structures as opposed to the spreading seen in differentiated hASC groups. Additionally, the hASCs differentiating on CADDS (DW) seemed to result in the highest occurrence of Nile Red staining despite the slower release of Dex from the MS.

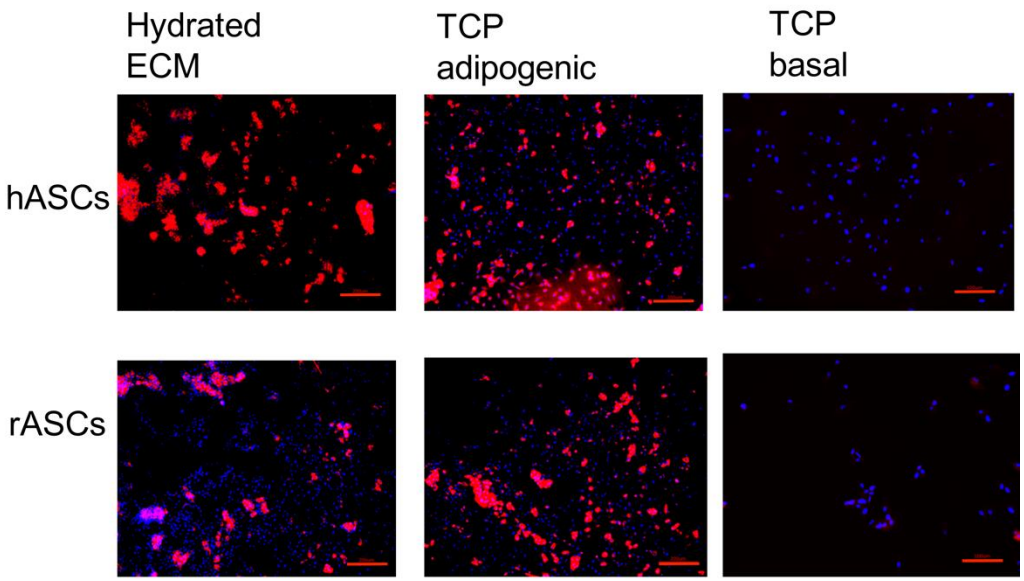

Figure 42. Cultured rASCs and hASCs stained with DAPI/Nile Red

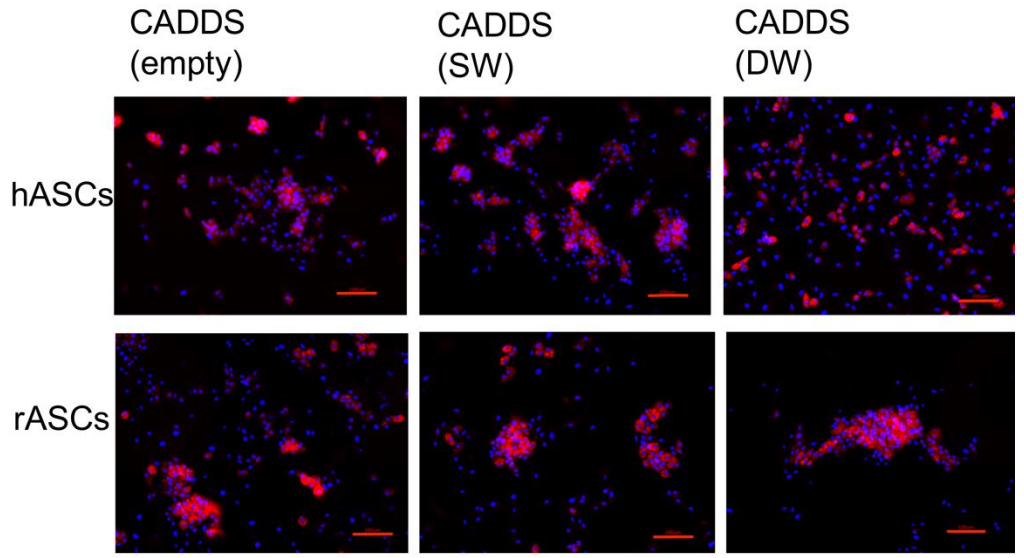

Figure 43. Cultured rASCs and hASCs stained with DAPI/Nile Red

4.4.3.3 Free Glycerol Quantification

Differences in glycerol content between differentiated rASCs and hASCs on TCP, hydrated AdECM, and CADDS can be seen in Figure 44. Glycerol concentrations were highest for both rat and human ASCs on tissue culture plastic compared to other experimental conditions.

Comparisons of glycerol content between two species indicate a significantly higher occurrence in the hASC cultures on all scaffold material. Additionally, glycerol content was higher for hASCs differentiating on CADDS material contain SW and DW MS compared to hydrated ECM and CADDS containing empty microspheres.

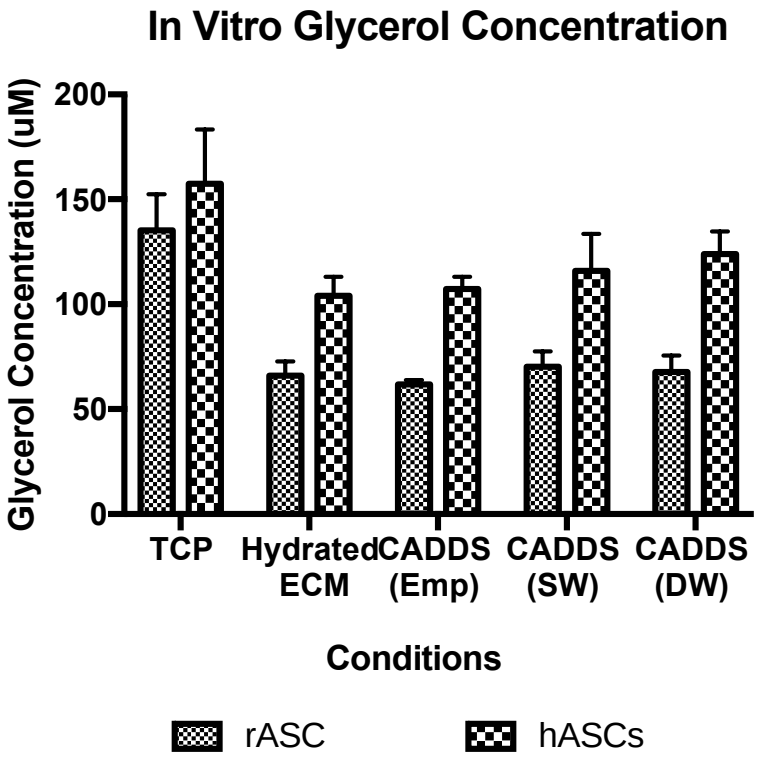

Figure 44. Glycerol content of differentiated rASCs and hASCs

4.4.4 Discussion

ASCs from the human and rat subjects were characterized in regard to the stem phenotype. Researchers have confirmed that hASCs with high expressions of CD90, CD 105, CD73, and CD44 exhibit the stem phenotype with the ability to differentiate into at least 3 different cell lineages (Gir et al. 2012; Minteer et al. 2015; De Francesco et al. 2015). The hASCs used in this study also positively expressed these markers. Rat ASCs also expressed progenitor markers with

the majority of cells being $CD45^-$, $CD29^+$, $CD90^+$, and $CD44^+$ (Amerion et al. 2018; Ying et al. 2012). Both species of species possessed the inherent potential to differentiate into multiple cell types.

The differences between differentiated rASC and hASCs on CADDS were evident. Observationally, microscope images displayed how rASCs differentiated at a lesser degree and clustered on scaffold material. In contrast, hASCs spread across the scaffold and generated more lipids indicated by the Nile Red stain. The glycerol content provided a qualitative measurement to support the initial findings. Significant differences in glycerol content between rASCs and hASCs was found under each condition of scaffold material. The results suggest that rASCs do not possess the ability to differentiate on ECM-based scaffolds as well as hASCs. This difference in differentiation capacity for rASCs may have contributed to the underwhelming volume retention results from the animal studies.

4.5 CONCLUSION

Hydrated AdECM and CADDS were implanted in immunocompetent rats to investigate volume retention and host tissue response. Overall, the scaffolds were found to be suitable biomaterial after the completion of validation study. Although volume retention was unexpectedly low, new adipose like-structures were seen in CADDS (DW) implants. Results from in vitro studies showed ASCs derived from the immunocompetent rat exhibit limited adipogenic capacity on CADDS. Moreover, the immunocompetent rat appears to be an insufficient animal model for assessing the volume retention and adipose structure formation from CADDS.

5.0 CONCLUSIONS

A minimally invasive treatment that results in volume-stable, healthy adipose tissue would be an ideal therapy for patients with soft tissue defects. A variety of biomaterials, cells, growth factors, delivery methods, and culture strategies have been explored resulting in exciting and intriguing results that warrant further investigation if their potential. However, it is important to focus on the most appropriate avenue to advance clinical applications. Clinical goals for adipose tissue replacement include regenerated tissue that both looks and feels similarly to that of native tissue. This includes mechanical integrity as well as sustainability and viability of the tissue over time. Additionally, adipose tissue that possesses active metabolic function would serve as a significant advancement over current adipose tissue engineering strategies.

Currently, some clinical trials that have been conducted for soft tissue contouring applications include a cell-assisted lipotransfer technique developed for cosmetic breast augmentation, hyaluronic acid injected into the lips and hands, and face injections containing synthetic calcium hydroxylapatite microspheres. Overall, these clinical trials show positive results but require bigger population studies to begin establishing a new standard of care. Additionally, the current defect requirements for these studies are typically on the order of cubic centimeters. Therefore, engineered adipose constructs of significant dimension are necessary. To address this particular clinical need, several researchers recommend that significant emphasis on vascularizing 3D adipose tissue and dynamic pre-cultivation would allow for larger constructs and enhance functional adipose tissue formation and maintenance. The ability to deliver appropriate nutrients and oxygen to an adipose that is not limited by diffusion, as well as improvements in long-term

sustainability of the engineered tissue made through appropriate biomaterial selection, would significantly enhance the potential of engineered adipose constructs.

Naturally derived biomaterials and decellularized proteins are currently dominating the research area as the base component of the scaffold. The current project was a reflection this peak in interest. We have presented the use of an extensively characterized composite adipose derived material containing adipogenic factors as an off the shelf biomaterial. The scaffold was designed to address adipose defects especially in cases where autologous fat grafting is not an option. These types of scaffolds are still needed in the clinic.

AdECM and Dex MS are the fundamental components of the CADDS biomaterial. The AdECM exhibited favorable characteristics including residual ECM proteins and the removal of immunogenic substances such as lipids. Dex MS showed the ability to release Dex quickly and in a slower controlled profile over several months. Dex MS also showed bioactivity with hASCs in vitro. Additional, in vitro experiments showed that CADDS was adipogenic and increased lipid accumulation in hASCs.

CADDS were implanted in immunocompetent rats as an animal model in the short-term evaluation of volume retention, host tissue interaction, and adipose tissue formation. The role of Dex MS remains unclear from host tissue interactions. However, Dex MS in CADDS appeared to promote moderately higher volume retention. Overall, the low retention rates and lack of adipose tissue formation called into question the use of the immunocompetent rat and the adipogenic capacity of its ASCs. Additional in vitro studies suggested that rASCs are unable effectively differentiate on CADDS which may have played an essential role in the undesirable behavior of CADDS in vivo.

Next steps include identification of animal models containing ASCs with comparable adipogenic potential to hASCs. Once this comparison has been established, the animal model should be able to sufficiently assess whether CADDS has the ability to promote adipose formation at a high rate of volume retention. Moreover, model should also be used to create a true adipose tissue defect that reflects some of the major challenges seen in the clinic.

It is anticipated that biodegradable biomaterials will continue to dominate the future of tissue engineering, specifically adipose tissue. These complex, multi-component scaffolds require several fabrication steps to develop each component. With this degree of complexity involved in fabricating each scaffold, consistency and standardized batch testing will be of immense importance. Although these factors are in the preclinical stage, the addition of personnel with controlled drug delivery as well as manufacturing experience in the research lab can give investigators a clear advantage for the implementation of their complex biomaterial technology.

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