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Engineering biomimetic scaffolds to encapsulate adipose-derived stem cells: A cell biology approach to regenerative treatments

A dissertation submitted in partial satisfaction
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Doctor of Philosophy
in Molecular, Cellular, and Developmental Biology

by

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September 2016

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ABSTRACT

Engineering biomimetic scaffolds to encapsulate adipose-derived stem cells: A cell biology approach to regenerative treatments

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The last decade has seen tremendous advances in the use of stem cell-based therapies to treat injury and disease, yet there are many hurdles still to overcome. Tissue or organ-specific strategies have begun to emerge as our knowledge of stem cell biology and transplant biology increases. This thesis presents results aimed at understanding and improving cell based therapies for soft tissue repair. Defects in soft-tissue can occur by many means, including traumatic injury, tumor resection, and congenital causes. Current approaches to the treatment of these deficiencies include autologous fat transplantation, which falls short of optimal. There are numerous negative outcomes associated with this procedure, the most common of which is loss of transplanted volume; which occurs in 92% of cases.

This thesis describes efforts to improve autologous treatments by utilizing the stem cell population present in transplanted tissue, adipose-derived stem cells (ASCs), and encapsulating them in an environment that supports survival, improving treatment options. The following chapters provide an in-depth overview of the development of a synthetic scaffolding system that supports the survival

and differentiation of ASCs *in vitro*. By utilizing poly(ethylene) glycol (PEG) we were able to model a biomimetic environment and demonstrate that functionalization of inert PEG with different Arg-Gly-Asp (RGD)-containing peptides resulted in different levels of adhesion.

After the development of the scaffolding system we sought to further engineer capabilities of the system to provide the possibility for numerous applications using the same basic chemistry. Capitalizing on what is known about the remodeling of natural extracellular matrix (ECM) we were able to incorporate peptides that permit the degradation of the scaffolding after the stem cells differentiate. Selecting a cleavage sequence that is sensitive to proteinases that are secreted by mature adipocyte and not by undifferentiated ASCs we succeeded in creating a hydrogel that mimics a natural environment and is degradable upon the differentiation of ASCs to the desired cell types. We have further demonstrated that ASCs encapsulated in this system are viable for 12 weeks, both *in vitro* and *in vivo.* Although we examined just adipogenic differentiation in this work, due to the simple nature of the system it should be possible to systematically alter the incorporated components for applications of other cell types. This affords extensive possible targets for tissue regeneration utilizing a basic scaffolding system. Collectively, the work described here advances the understanding and application of stem cell based therapies for soft tissue repair and regeneration.

TABLE OF CONTENTS

Chapter I **Strategies for bioengineered scaffolds that support adipose stem cells in regenerative therapies**……...…...12
References…….……....32

Chapter II **Vitronectin-based, biomimetic encapsulating hydrogel scaffolds support adipogenesis of adipose stem cells**……...41
References……..69

Chapter III **Cell mediated remodeling of biomimetic encapsulating hydrogels achieved by adipogenic differentiation of adipose stem cells** ……..……...……...……76
References……...……..99

Conclusion ……103

Appendix ….……..……105

LIST OF FIGURES

Figure 1. Analysis of ECM protein and integrin expression in ASCs before and after adipogenic differentiation……54

Figure 2. Effects of full-length ECM proteins on ASCs……56

Figure 3. ASC Adhesion and Spreading on RGD Peptides……58

Figure 4. 3D Hydrogel Proliferation and Adipogenic Differentiation……..60

Figure 5. PEG-Divinyl Sulfone 3-Dimensional Hydrogel Proliferation and Adipogenic Differentiation……62

Figure 6. Remodeling of differentiated MMPc containing hydrogels…….85

Figure 7. Holes are dependent on the differentiation of cells in MMPc containing hydrogels……..87

Figure 8. Average Voronoi areas……88

Figure 9. Cell Morphology in hydrogels +/- MMPc…….90

Figure 10. Expression of PPARγ and MMP3/10 protein in MMPc containing hydrogels……..91

Figure 11. Degradation of gels without MMPc over 12 weeks……..……92

Supplemental Figure 1. Histological stains of MMPc gels…….105

LIST OF TABLES

Table 1. Summary of synthetic matrices used in combination with ASCs……29

Table 2. A List of Primary Antibodies Used in This Study……49

Table 3. TaqMan Probes used for gene expression analysis……82

Chapter I.

Strategies for bioengineered scaffolds that support adipose stem cells in regenerative therapies

Abstract

Regenerative medicine possesses the potential to ameliorate damage to tissue that results from a vast range of conditions, including traumatic injury, tumor resection, and inherited tissue defects. Adult stem cells, while more limited in their potential than pluripotent stem cells, are still capable of differentiating into numerous lineages, and provide feasible allogeneic and autologous treatment options for many conditions. Adipose stem cells (ASCs) are one of the most abundant types of stem cell in the adult human. Here, we review recent advances in the development of synthetic scaffolding systems used in concert with ASCs, and assess their potential use for clinical applications.

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Adipose stem cells in regenerative medicine

Regenerative medicine is an immense field focused on the replacement, and regeneration of human cells and/or tissues to restore normal functions.[1,2] The replacement of damaged or diseased tissue with functioning healthy cells is the

primary goal of this field. The use of stem cells has become fundamental to its rapid expansion and the foundation for developing therapies to treat congenital defects, traumatic injury, and disease in a patient-specific manner through the use of autologous tissue.[3] Since the first documented use of the term “regenerative medicine” and the isolation of human embryonic stem cells, efforts to develop synthetic scaffolds for use in conjunction with stem cells have increased significantly.[4-6] The use of stem cells for regenerative treatments has achieved varying degrees of success with regards to replacing missing or damaged tissue, but progressive improvements have been brought about via recent efforts in tissue engineering.[7]

Indeed, a PubMed search for “regenerative medicine” yields more than 30,000 publications since 1920.[8] When the search is narrowed to include “regenerative medicine and stem cells” a list of 11,770 publications is returned. Refining this search still further by using key words such as “mesenchymal stem cells” yields only 1,148 publications. Finally, using the phrase “regenerative medicine and adipose-derived stem cell” (ASC) produces a total of 156 publications from 2005 to 2016, an indication that research involving ASCs in the field of regenerative medicine remains in its infancy.

Surgeons in the American Society of Plastic Surgeons preformed more than 5.8 million reconstructive surgeries in 2015 alone to repair defects arising from tumor resection, traumatic injury, maxillofacial abnormalities, laceration repair, and scar revision.[9] However, even the most common treatments show a significant and unpredictable loss of transplanted tissue volume over time. Volume loss is a main

reason for treatment failure, and therefore a need exists for a microenvironment that produces repeatable, sustainable results over many years. To this end, a biologically inert scaffolding system that can be tailored to the needs of individual patients and presents a means for maintaining tissue volume may prove to be a significant advancement over current treatments.

Bone marrow-derived mesenchymal stem cells (BM-MSCs) are a type of adult stem cell commonly used in numerous therapies including the treatment of liver failure as a result of hepatitis B.[10] BM-MSCs have been approved for use in humans since 1995, and are currently being used in 272 clinical trials according to clinicaltrials.gov. Mesenchymal stem cells (MSCs) are multipotent cells that possess the ability to differentiate into various cell types, including adipogenic, chondrogenic, osteogenic, muscular, cardiac, and endothelial lineages.[11,12] The International Society for Cellular Therapy (ISCT) uses three criteria to define MSCs regardless of their source: (1) plastic adherence in standard culture conditions; (2) expression of nonspecific surface markers CD105, CD 90, and CD73 and the absence of CD34, CD45, CD14, or CD11b, CD79α and HLA-DR; and (3) differentiation into osteoblasts, adipocytes and chondroblasts under specific stimuli *in vitro*.[13,14] In contrast to pluripotent embryonic stem cells the benefits of using adult mesenchymal stem cells for treatments include the ability for autologous transplants and the absence of ethical controversies surrounding their use. Adipose tissue provides an alternative to bone marrow-derived stem cells; it is a high-yield source of adult stem cells known as ASCs obtained predominately through rudimentary liposuction procedures.[15,16]

Adipose-derived stem cells share many similarities with BM-MSCs including their potential to develop into similar cell lineages, and have no clear distinction between the stem cell populations in terms of surface marker or gene expression.[13,17-20] It has been suggested that ASCs can be distinguished from BM-MSCs by their expression of CD36 (F.A.T. - a protein involved in fatty acid metabolism), or CD49d (integrin α4 – a subunit of the integrin receptor for fibronectin and VCAM-1), as well as the lack of CD106 (VCAM1- a protein involved in the adhesion of vascular cells). However, each of these proteins shows variability in expression patterns between specific ASC populations.[21] Investigations into the gene expression profiles of BM-MSCs and ASCs found that 13.2% of 384 genes examined were differentially expressed between the two populations. Although no identifying markers were specific to each population, genes more highly expressed by ASCs were mainly involved in cellular communication (*FGF9, IL1R2, CCL3* and *KDR*) while those with higher expression in BM-MSCs were involved in WNT signaling and differentiation pathways (*WNT11, WNT7B,* and *SOX6*).[22]

Adipose stem cells are 10 times more abundant than BM-MSCs in the tissues from which they are isolated. Additionally, ASCs demonstrate a higher proliferative potential, show consistent growth rates in culture, and are procured from a minimally invasive procedure by comparison.[23,24] Furthermore, ASCs are robust, capable of self-renewal, can be collected in large quantities and easily expanded in culture. These qualities identify ASCs as a promising source for use in therapeutic regenerative medicine.[25-28]

Over the last 18 years many techniques have been developed to create scaffolding materials that are compatible with stem cells as well as transplantation sites. Scaffolds generated prior to cell seeding allow for the use of reagents typically considered “harsh,” thereby expanding the potential library of materials for therapeutic use. Topical seeding of cells has been one approach used in the development of synthetic scaffolds in regenerative medicine, but often results in a very low penetration throughout the material, leading to a heterogeneous cellular distribution within the scaffold.[29] Another tactic for the development of scaffolds as regenerative therapies is the use of decellularized extracellular matrix. These scaffolds are generated from allogenic or xenogenic tissues and are popular for applications involving heart valves, blood vessels, tendons, and ligaments. Importantly, this technique most closely mirrors the mechanical and biological properties of human tissue.[30] Complications from using this approach may arise if all cellular components of the donor tissue are not thoroughly removed prior to implantation, increasing the likelihood of immunological rejection, thereby requiring the long-term use of immunosuppressant drugs. Cell encapsulation in natural or synthetic hydrogel matrix is yet another method used in scaffold engineering, since these frameworks can be designed to provide biomimetic environments that polymerize from a liquid to a solid polymer network under specific conditions. By using a one-step procedure to encapsulate stem cells instead of topical cell seeding, a more homogenous cell density with exceptional cell viability is achieved. Here, we provide an overview of the field by examining a collection of synthetic scaffolds currently used in conjunction with ASCs to treat defects of various tissue types.

Adipose stem cells in synthetic scaffolds for cartilaginous regeneration

Cartilage is flexible connective tissue located in joints between bones, but regions of cartilaginous tissue also exist in the ear, nose, and rib cage. Unlike bone, cartilage is not rigid; however, it is less flexible than muscle or other types of connective tissues, such as fat. Cartilage is important in providing flexibility to the skeletal system, a critical feature that allows for proper function. Cartilage is primarily composed of chondrocytes, cells responsible for producing the extracellular matrix proteins required for the tissue's unique mechanical characteristics.[31-34] Cartilage is unable to self-repair after blunt-force trauma, athletic injury, disease, or age-related degeneration. The number of knee surgeries to repair articular cartilage damage each year in the United States increases by 5% annually.[35] The natural lack of vascularization in addition to the minimal cell-to-cell contact restricts cartilage to only minimal spontaneous healing because of the slow dissemination of healing factors to distant cells. Because of these characteristics, treatments frequently consist of surgically removing damaged tissue in order to reduce pain and restore function.[32,34,36] A regenerative approach to cartilage replacement therapy involves the restoration of proper cellular morphology, and the prevention of further deterioration. Current treatments, such as allografting, can carry small, but serious risks of infection and disease transmission while treatments such as autologous chondrocytic transplantations may result in degenerative changes accompanied by pain.[33,34,37]

Adipose stem cells can be induced into chondrogenic differentiation *in vitro* by the combinatorial influence of growth factors, such as transforming growth factors β1

and β3, bone morphogenetic protein 4, and basic fibroblast growth factor. Adipose-derived stem cells provide several advantages over autologous chondrocyte treatments because they do not induce an inflammatory response, form new cartilage, and possess the potential for restoring long-term cartilage function. The use of 3-dimensional (3D) scaffolds has gained momentum in the field of cartilage restoration because of their ability to overcome the growth inhibition typically observed in standard *in vitro* cultures.[38,39] Synthetic platforms provide locations for ASCs to adhere, thereby providing an environment conducive for growth and proliferation. Additionally, scaffolds have also been shown to promote differentiation and enable cells to achieve a cartilage-like morphology and express chondro-specific molecules, such as collagen type II alpha1, and aggrecan.[40]

Poly-lactide-co-glycolide (PLGA) is a copolymer approved for numerous therapeutic uses in humans by the Food and Drug Administration (FDA) since 2001. This copolymer may act as a stable or a biodegradable material depending on its formulation, and it possesses a permeable pore network that supports cell adhesion and proliferation. Mehlhorn et al. (2009) showed that these scaffolds were suitable cell carriers for chondrocytes. Furthermore, PLGA networks seeded with ASC-chondrocytes showed excellent volume stability, and sufficient elasticity comparable to natural cartilage.[41] These results suggest that PLGA may serve as an effective scaffolding system for chondrocytes derived from ASCs.

Another avenue employs the use of fibrorous polyglycolic acid (PGA) stabilized by poly lactic acid (PLA). Cui et al. (2009) demonstrated that this combination of polymers produced promising results during the initial attachment of

ASCs, and subsequent proliferation of chondrogenic-induced ASCs. In addition, cells deposited cartilage-specific extra-cellular matrix (ECM) proteins within the polymer. Degradation times of approximately 2 months *in vivo* appeared to match the natural mechanisms of new cartilage formation. Thus, PGA/PLA in combination with ASCs may also serve as a synthetic scaffold for cartilage regeneration.[40]

While PLGA and PGA/PLA comprise the bulk of synthetic polymers used for cartilage regeneration, other synthetic gels incorporate hyaluronic acid (HA), an important component of cartilage, into poly(ethylene) glycol (PEG) polymers. Unterman et al. 2012 showed HA-interacting PEG hydrogels improved cartilage tissue formation *in vitro* and *in vivo* in instances where HA was presented at a later stage of differentiation, subsequently resulting in increased chondrogenic phenotypes.[42] Here, carefully considering properties of the native environment resulted in increased success by incorporating HA components that mimic the desired tissue. This is an important case that demonstrated functionalization of a scaffold to more closely replicate a desired environment had a positive effect on graft viability.[43]

3D cell printing, or bioprinting, has become an increasingly attractive option for the treatment of bone lesions as it provides a means to create scaffold structures that alleviate the limitations of the fields due to the complex 3D geometries associated with defects. The use of cells in prepolymer “bioink” allows a layer by layer deposition in a 3D construct that is analogous to tissues and organs.[44,45] This technique provides unique opportunities to develop complexly shaped scaffolds from synthetic material that encapsulate cells as shown by Lee et al (2014).[46] The

fabrication of a structure with an ear shape with chondrocytes and adipocytes derived from adipose stem cell derived cells in a polycaprolactone (PCL) hydrogel demonstrated a successful composite tissue. The efficient chondrogenesis and adipogenesis of the cell-printed structure resulted in a step forward for the practicality of 3D printing complex organs for tissue regeneration.

Adipose stem cells in synthetic scaffolds for osteogenic therapeutics

In contrast to cartilage, bone has regenerative capacity due to its inherent population of osteoblasts and osteoclasts (bone-forming and bone-resorbing cell-types respectively).[47] However, these processes are frequently perturbed in cases of trauma, disease, or tumor resection. Bone autografts, i.e., harvesting bone from one anatomic site and grafting into another site in the same subject, are one of the primary approaches currently used for bone augmentation in a variety of orthopedic and maxillofacial procedures. Approximately 800,000 patients receive these grafts annually,[48] and while significant skeletal incorporation has been observed in these types of grafts many drawbacks still exist using this approach, such as delayed healing, a complete failure to heal, morbidity at donor sites, quantity restrictions, substantial financial costs due to additional procedures to harvest transplant tissue, and discomfort for the patient.[49-52]

Collectively, focus has begun to shift towards the development of synthetic systems for use in conjunction with adipose stem cells to replace traditional bone grafts. One study determined that PLGA is a viable scaffold for osteogenic differentiation of ASCs. After two weeks of osteogenic induction, mineralized

nodular structures were observed by Alzarian Red and von Kossa staining, indicating successful calcification of the ECM.[53] The use of PLGA scaffolds for osteogenic differentiation provides a viable polymer scaffolding option, however, further investigation is needed to determine what external cues may be necessary prior to graft implantation of this particular material, which has shown promise for applications involving chondrogenic and adipogenic lineages; indicating that the basic polymer supports numerous cell fates and must be modified to help direct differentiation.

While polymers have proven to be useful in a variety of other fields, osteogenesis may require unique materials due to the highly specialized mechanical properties of natural bone. Thus, regenerative osteogenic technology has begun to employ the use of titanium metal to create a space that facilitates the migration of implanted cells and their osteogenic differentiation. Titanium is an inert biomaterial that possesses exceptional mechanical strength, is biocompatible, and therefore, a prime candidate for use in regenerative applications involving bone. Adipose stem cells have shown compatibility with titanium systems, as well as displayed suitable cell adhesion. As a scaffold, titanium enables adhesion and osteoblastic differentiation of ASCs *in vitro*, indicated by an increased deposition of alkaline phosphatase and osteocalcin (ECM proteins necessary for matrix mineralization) as well as calcification, confirmed by von Kossa staining.[54,55] The ability of ASCs to acquire the proper phenotypic differentiation as well as produce an ECM and a mineralized matrix suggest titanium as an attractive material as a filler or support structure for bone ingrowth in regenerative medicine.[54]

Calcium phosphate ceramics (CPC) are another class of scaffolds used for bone regeneration. These are promising synthetic materials due to their resemblance to bone mineral, their malleable bioactive properties, and their surface characteristics which support osteoblast adhesion, proliferation and differentiation *in vivo.*[56,57] Most CPCs examined have been shown to be osteoconductive (growth of bone on a surface) while only certain types exhibit osteoinductive (recruitment and differentiation of immature osteocytes) abilities. There is evidence, however, that increased mircoporosity increases the amount of bone inducing proteins secreted by ASCs *in vitro*.[58] The similarities of CPCs to bone, along with their ability to induce bone growth and promote secretion of important proteins elevate these materials as an intriguing and exciting possibility for osteogenic therapies.

While the similarities of CPC to bone have proven to be beneficial to osteogenic regeneration the use of decellularized bone (DCB) in combination with PCL shows even greater promise. Polycaprolactone is a biodegradable polyester polymer used to circumvent the inability of 3D printers to use decelluarlized bone alone as a printing material. The use of 3D printers to engineer scaffolding systems using PCL has shown enhanced adhesion of ASCs. These cells exhibited significant upregulation of osteogenic genes such as osteocalcin, runx2, and osteonectin. It was also demonstrated by Alzarian Red staining that ASCs on DCB:PCL materials showed increased calcification. When scaffolds were implanted into calvarial defects in mice, DCB:PCL scaffolds invoked nearly twice the volume of regenerated bone in 12 weeks compared to PCL alone.[59]

The use of 3D printed PCL scaffolding without addition of natural components has also shown by varying the internal pore size of the scaffold it is possible to influence cell seeding of ASCs. By manipulating this parameter Temple et al 2014 were able to achieve optimal vascular and osteogenic differentiation in 3D printed scaffolds.[60] This study also showed that maintenance of complex geometrical features such as maxilla and mandible bones maintain this porosity and therefore allow for cell seeding and vascularization similar to previous *in vivo* studies.

Similar to PCL, polymers used in other regenerative studies, such PLGA can be blended with natural components to make them more amenable to 3D printing. Lee et al 2015 determined that by 3D printing PLGA scaffolds impregnated with bone morphogenic protein 2 and ASCs it's possible to achieve mandibular regeneration.[61] The use of a small animal model of mandibular defects allows investigation of the potential for union of transplanted scaffolding with natural bone, within a site of segmental defect. Similarly, Kao et al 2015 demonstrated that coating of 3D printed PLA with bio-inspired synthetic coatings increased the adhesion, proliferation, as well as the osteogenic and endothelial differentiation of ASCs in 3D structures.[62] These simple modifications to synthetic 3D printed scaffolds may serve as the basis for effective delivery carriers in bone tissue engineering.

Adipose stem cells in synthetic scaffolds for soft tissue regeneration

Soft-tissue defects are relatively common, accounting for nearly 10% of all emergency department visits in addition to causes previously examined (i.e. trauma,

tumor resection).[63] More than 100,000 breast reconstructions after mastectomy, and over 200,000 maxillofacial surgeries were performed in 2015 alone and it is predicted that there will be more than 12,000 new cases of soft-tissue sarcomas in the United States in 2016.[9,64] The current treatment for many of these conditions is autologous lipotransfer, a procedure involving collecting fat tissue from a patient, minimally manipulating the resultant lipoaspirate and relocating it to the site of reconstruction.[65-68] Although used widely, reports show that there is extensive variability of long-term lipotransfer graft survival, due to unpredictable degrees of resorption and tissue volume loss that can range from 20-90%.[29,69,70]

Variations in soft-tissue graft survival have been attributed to many causes including a lack of local angiogenesis, sample preparation, as well as innate properties of the transplant site.[67] In a national consensus survey, 92% of physicians stated that their patients experienced some degree of resorption, 52% reported a resorption rate of 50% or greater.[71] Mature adipocytes constitute the majority of the transplant volume, and since these cells are in a terminally differentiated state, they lack the ability for self-renewal and proliferation. The primary cause for transplant death is the lack of re-vascularization of the transplanted tissue. Success rates are often reported to be as low as 20%, while successful transplants are commonly attributed to the relatively small population of ASCs present in the transplanted fat, and can be enhanced by increasing the number of stem cells transplanted.[67,72] Thus, significant volume loss in these types of transplants provides motivation for finding ways to decrease the loss and thereby increase the likelihood of a successful transplant. The recent development

of Cell Assisted Lipotransfer using concentrated ASCs as a lipoaspirate additive before transplantation leads to significantly improved results, specifically in terms of thickness gains observed during the first 6 months, and a reduction in thickness loss at 1 year.[73] However, even cases using stem cell enrichment, marginal volume losses were still documented, and in most cases no gain of regenerated tissue was reported.[67] Therefore, engineering synthetic scaffolds that can support the survival of the transplanted stem cell population while simultaneously promoting adipogenic differentiation has been proposed as a novel avenue for improving the success of these types of transplants.

Various synthetic scaffolding materials have been examined to determine structural viability for stem cell survival and adipose tissue reconstruction. Patrick et al. (2012) demonstrated that ASCs seeded into a PLGA scaffold and implanted subcutaneously into rats showed maximum adipose tissue formation after 2 months, but noted that between 3-12 months, a complete loss of reconstructed adipose tissue and degeneration of the PLGA scaffold occurred.[74] This loss of tissue may arise from the degradation of the scaffolding, especially since signs of PLGA degradation were apparent as early as 1 month post-transplantation. Thus, successful scaffolds for adipose tissue transplantation may require prolonged degradation times in order to allow for the maturation of regenerating tissue.

Cho et al. 2005 demonstrated that implanting a support made of poly(glycolic acid) and poly(L-lactic acid) (PGA/PLA) before injecting pre-adipocytes provided enough support to maintain the volume of the implants and showed regeneration of the adipose tissue after 6 weeks in athymic mice.[75] However, this approach

utilizes an implant primarily acting as structural support for ASCs that are injected in a solution. Although stability of the transplanted volume was reported, there was no systematic method to measure pre-implantation volume, leading to difficulties in determining whether adipose growth was due to the implanted cells. Additionally, no conclusive evidence was shown to indicate that the regenerated cells originated from transplanted ASCs. In clinical applications it will be imperative to determine that the incorporation and differentiation of implanted cells replaces missing tissue.

The use of blended copolymers has recently become increasingly popular for applications in therapeutic treatments. The blending of poly(glycerol sebacate) (PGS), a biodegradable and biocompatible synthetic elastomer specifically designed to imitate the mechanical behavior of soft tissue, with PLA (to overcome the quality and flexibility concerns of using PGS alone) has shown promise. Frydych et al. 2015 showed that ASCs seeded onto the surface of a PGS/PLA scaffold exhibited significant amounts of cellular penetration and substantial collagen accumulation over 21 days.[76] However, *in vitro* degradation assays determined that degradation appeared to progress too rapidly (50% loss after approximately 30 days) for this scaffold to support the growth of target tissue, a phenomenon also observed by Patrick et al.[74]

Blending different scaffolding polymers provides the advantage of utilizing the positive attributes of each material. Lin and colleagues used mixtures of gelatin sponges and polyglycolic meshes encased in microfilament polypropylene mesh to support adipose tissue regeneration using pre-differentiated ASCs.[77] The gelatin-polyglycolic mesh were observed to degrade completely within 60 days, however,

the polypropylene mesh is biostable and remains as a permanent resident of the transplant procedure. In fact, it was demonstrated that after 6 months *in vivo* these scaffolds retained their shape, a trait attributed to the nondegradable mesh, while the newly formed adipose tissue occupied the space within the scaffold.[77] This avenue is an improvement in terms of the longevity of engineered adipose tissue, however, this system is complex and requires lengthy *in vitro* cultures, thus it may prove too difficult to translate to clinical applications.

While each of these synthetic scaffolds possess positive attributes, each neglects to consider important interactions of cells with their surroundings. Immediately following seeding into a synthetic scaffold, cells must be afforded sites of adhesion from which they are able to receive signals for survival, proliferation and differentiation. Since these polymers are biologically inert it is critical to engineer attachment sites that provide favorable interactions between the ASCs and their surroundings. Poly(ethylene) glycol (PEG) is a polymer that was approved for use in humans by the FDA in 1979, and is currently used in a myriad of applications ranging from food additives to pharmaceutical products and drug delivery systems.[78,79] Recently, it was demonstrated that incorporation of Arg-Gly-Asp (RGD) variant peptides (linear RGD, cyclic RGD, and vitronectin derived RGD) into PEG based gels is a feasible approach to functionalizing an inert biomaterial.[80-84] These peptides provide sites for ASC attachment at the time of cell incorporation. It was demonstrated that various adhesion peptides provided transplanted ASCs with enhanced directed adipogenic differentiation by comparison to systems that contained no attachment peptide. Peptides considered to be highly adhesive lead to

smaller lipid vacuoles, and thus immature adipocytes. However, peptides containing RGD derived from vitronectin (less adhesive) allowed ASCs to attach when incorporated into the hydrogel, while remaining rounded morphologically.[85] This approach demonstrates that the initial environment encountered by ASCs may influence their ability to differentiate in 3D scaffolds.

Conclusion

Despite its relative youth, the field of regenerative medicine is expanding quickly, encompassing exciting developments in the area of bioengineering, stem cell biology, and materials research. Adipose stem cells hold enormous potential in this field. The multipotency of ASCs provides the potential building blocks for the treatment and regeneration of damaged tissue. Their relative abundance, and their ease of access, suggests ASCs may provide an improvement over other stem cells used in therapeutic treatments.

The design of complex and smart materials able to interact with cells to direct their biological response and differentiation has been on the rise since the advent of tissue engineering. It has been shown that the interactions of cells with their environment plays a critical role in their health and development.[86] In order to regenerate and restore healthy tissue after an insult, disease, or defect the ability to direct implanted cells along specific pathways may prove to be paramount. There are many avenues currently under investigation to determine the best method of combining ASCs and synthetic scaffolds to obtain an optimal graft or implant for the desired application. Table 1 summarizes various synthetic matrices that have been

Table 1. Summary of synthetic matrices used in combination with ASCs.

	Cartilage	Bone	Adipose
PLGA	Mehlhorn et al. 2009	Lee et al. 2008 Lee et al. 2015	Patrick et al. 2002
PGA/PLA	Cui al. 2009	-	Cho et al. 2005
HA-PEG	Unterman et al. 2012	-	-
PCL	Lee et al. 2014	Temple et al. 2014	-
Titanium	-	Gastaldi et al. 2010 Marycz et al. 2015	-
CPC	-	Samavedi et al. 2013 Barrere et al. 2006 Li et al. 2011	-
DCB/PCL	-	Hung et al. 2016	-
PLA	-	Kao et al. 2015	-
PGS/PLA	-	-	Frydrych et al. 2015
Gelatin/PGA/PP	-	-	Lin et al. 2008
PEG	-	-	Clevenger et al. 2016

evaluated in the last 15 years for treatment of cartilage, bone and adipose tissue defects. One of the most common polymers, PLGA, has been used for all three purposes, showing positive results when used in osteogenic applications, neutral results when used in chongrogenic repair and a notable loss of volume when applied to cases of adipogenic tissue growth. This is a prime example of the diverse capability of synthetic polymers used with stem cell populations. It is critical to consider the downstream consequences for all proposed scaffolding materials; specifically, scaffolding systems successfully used in one tissue type may not be yield similar results in another. Mimicking the native environment of the target tissue is likely to play a significant role for the long-term survival of virtually all transplant scaffolds.

Indeed, it was recently demonstrated that various adhesion peptides provided transplanted ASCs with enhanced directed adipogenic differentiation by comparison to systems that contained no attachment peptide.[85] The use of vitronectin derived attachment peptides promoted the development of larger lipid vacuoles, further suggesting that the interaction of the ASCs with their scaffolding may have a significance impact on the desired differentiation and health of implanted cells.[87,88] Biomimetic PEG hydrogels may prove to be superior synthetic scaffolds for use in tissue reconstruction. [89-91]

Future Perspectives

Applications of adipose-derived stem cell therapies have enormous potential for expanding the field of regenerative medicine. Their ability to differentiate into numerous cell types, as well as their abundance, places ASCs at the forefront in the development of next-generation therapeutic treatments. Vascularization of grafts and implants is another chief concern with respect to cell viability. This is a critical issue in current approaches for regeneration and treatment, and thus an important factor to address when developing new synthetic scaffold systems. The ability of ASCs to differentiate into endothelial cells along with a scaffold that supports a desired differentiation may increase the chances of achieving a scaffold that produces viable, long-lasting, vascularized tissue.[92-95]

Transitioning scaffolding materials from the laboratory to clinical applications poses challenges that require further investigation. For example, additional demonstration of long-term safety in preclinical animal models will be necessary.

This process is timely, labor-intensive, and expensive, however, the treatment benefits will outweigh the initial hurdles encountered in the exploration and development of synthetic scaffold for use as regenerative therapeutics.

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Chapter II.

Vitronectin-based, biomimetic encapsulating hydrogel scaffolds support adipogenesis of adipose stem cells

Abstract

Soft tissue defects are relatively common, yet currently used reconstructive treatments have varying success rates, and serious potential complications such as unpredictable volume loss and reabsorption. Human adipose-derived stem cells (ASCs), isolated from liposuction aspirate have great potential for use in soft tissue regeneration, especially when combined with a supportive scaffold. To design scaffolds that promote differentiation of these cells down an adipogenic lineage, we characterized changes in the surrounding extracellular environment during adipogenic differentiation. We found expression changes in both extra-cellular matrix (ECM) proteins, including increases in expression of collagen-IV and vitronectin, as well as changes in the integrin expression profile, with an increase in expression of integrins such as αVβ5 and α1β1. These integrins are known to specifically interact with vitronectin and collagen-IV, respectively, through binding to an Arg-Gly-Asp (RGD) sequence. When three different short RGD containing peptides were incorporated into 3D hydrogel cultures it was found that an RGD containing peptide derived from vitronectin provided strong initial attachment, maintained the desired morphology and created optimal conditions for *in vitro* 3D adipogenic differentiation of ASCs. These results describe a simple, nontoxic encapsulating scaffold, capable of supporting the survival and desired differentiation of ASCs for the treatment of soft

tissue defects. This chapter was published as Clevenger, T. N., Hinman, C. R., Ashley Rubin, R. K., Smither, K., Burke, D. J., Hawker, C. J., ... & Clegg, D. O. (2016). Vitronectin-based, biomimetic encapsulating hydrogel scaffolds support adipogenesis of adipose stem cells. *Tissue Engineering Part A*, *22*(7-8), 597-609.

Introduction

Soft tissue defects are caused by a number of sources including trauma, deep burns, tumor removal and liposarcomas [1-5]. Current standard reconstruction treatments of these defects include alloplastic implants and autologous fat transplants. While these methods have shown some success, they come with serious potential complication, including foreign body reaction, donor-site morbidity and migration of implants [2,6-8]. The likely occurrence of reabsorption in autologous fat transplants (currently the most commonly used treatment) makes this option less than optimal. It is believed that the benefits demonstrated by fat transplants are attributed primarily to a specific population of stem cells present in the tissue [9-14]. Therefore, a need exists for improved, consistent reconstruction strategies capable of treating these soft-tissue defects.

Adipose tissue, which is a rich source of easily isolated adipose-derived stem cells (ASCs), can be frequently harvested in large quantities utilizing standard liposuction procedures. These procedures have been shown to be effective and safe with low risk of donor-site morbidity [15-19]. Additionally, ASCs are readily obtained in significant quantities from a patient, are robust and capable of self-renewal. ASCs are multipotent, mesenchymal stem cells that have the ability to differentiate down

various lineages, including adipogenic, osteogenic, chondrogenic, muscular, cardiac and endothelial, much like bone-marrow derived mesenchymal stem cells (BM-MSCs) [2,20-22]. While ASCs and BM-MSCs share many similarities beyond their potential lineages, including the majority of their confirmed *in vitro* immunophenotypes [23,24], it has been shown that ASCs have a higher proliferation potential with a more consistent growth rate in culture [2,25-28] and are at least 10 times more abundant than BM-MSCs [2,26,29]. Due to the accessibility of ASCs, the ease of cell culture and the short expansion times after isolation, it is feasible to use these cells to create autologous, patient-specific treatments, avoiding the potential complications of immune rejection. To reduce treatment outcome variability and promote targeted tissue regeneration, implanting only cells that provide the positive benefits (ASCs), in an engineered environment, is thought to be a viable approach. By implanting the desired cell population, volume loss and reabsorption should be kept to a minimum, as all cells present retain the potential to proliferate as well as differentiate. The cells in the scaffold will be influenced by the native tissue and may encourage the cells of the host's tissue to regenerate [10,12,30-32]. A synthetic scaffold that promotes preferential differentiation and supports survival of this population of cells would be critical to the effectiveness of such a treatment. Such a scaffold would need to provide the necessary mechanical support for the graft as well as the long-term survival of the grafted cells.

Poly(ethylene-glycol) (PEG) hydrogels have promising potential to serve as basic scaffolding materials in regenerative medicine [1,3-5,16,18,21,23,26,33]. The inert backbone of PEG hydrogels, and the relative ease to functionalize sites

mimicking extracellular matrix (ECM) proteins to help direct cell fate make this type of hydrogel appealing for soft tissue reconstruction [6-8]. ECM proteins contain sites that cells can bind to and use to interact with their surrounding environment, including neighboring cells. The sites a cell interacts with can affect it in a variety of ways, including inducing proliferation, signaling a specific pathway for differentiation, or initiating apoptosis [9,11,13,14]. A binding site that numerous ECM proteins have been shown to contain is the Arg-Gly-Asp (RGD) sequence. Originally identified as a critical cell-ECM adhesion component in fibronectin (FN) [15,17,19], RGD has since been identified in numerous other ECM proteins, such as vitronectin, collagen I and collagen IV [20,21]. It has been shown that adipocytes *in vivo* are surrounded by an ECM that includes many similar proteins such as multiple collagens (types I and IV included), multiple laminins, fibronectin and others [23]. The expression levels of some of these proteins have previously been shown to change, *in vitro*, during differentiation of ASCs down various lineages [2,26-28]. During osteogenic differentiation increased deposition of collagens I and IV have been observed [2,26], while chondrogenic differentiation shows increases in collagen II [10,12,31]. Changes in ECM expression of adipogenically differentiated mesenchymal cells isolated from bone marrow have been studied previously [16,18,21,23,26,33]. However, little work has been done examining adipogenesis in the more readily abundant cell population from fat lipoaspirate.

Integrin receptors are one of the primary methods used by cells to recognize, and attach to, these RGD sequences and other peptide sequences in ECM proteins [2,19,22,34-36]. These receptors are heterodimeric transmembrane proteins made

up of an alpha and beta subunit. They interact with the surrounding ECM and relay messages from the extracellular environment to the cells, as well as from the cell to the ECM [24,34]. Based on previous work showing BM-MSCs change their integrin expression profile during other types of differentiation [25-28,37], we hypothesized that the integrin profile of ASCs being directed toward an adipogenic lineage will alter in response to a changing ECM environment. We further hypothesize that we can utilize various small integrin interacting peptides, incorporated into a 3-dimensional scaffold, to promote adipogenic differentiation of this stem cell population.

Materials and Methods

Cell lines and culture

Cells were isolated from two donors and a third line was purchased form Life Technologies (San Diego, CA). Donor cells were isolated from lipoaspirate by methods previously established [18,22,29,38-42]. Briefly, 1-4 liters of lipoaspirate were repeatedly washed with an equivalent volume of PBS containing 10 U/mL penicillin-streptomycin (Life Technologies) until the PBS layer was mostly clear. The washed lipoaspirate was then aliquoted into 225 cm^2 flasks containing 0.15% collagenase type I solution (220.00 units/mg) (Life Techonologies) and 20 U/mL pen-strep antibiotics. The flasks were incubated at 37°C on a slow shaker for 2 hours, with additional vigorous shaking every 15 minutes. The collagenase was neutralized by the addition of fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO) to a final concentration of 10%. The cell solution was then centrifuged at 1,000 x g for 10

minutes to pellet the stem cell-dense fraction. The supernatant, was removed and the cell pellet was resuspended in 160 mM ammonium chloride solution to lyse any red blood cells present. Cells were then centrifuged at 1,000 x g for 10 minutes, and the resulting pellets were resuspended in media containing 60% Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) with 10% FBS and 40% MesenPRO medium (Life Technologies). Cells were passed through 50mL sterile vacuum 60μm Nylon cell strainers (Millipore, Billerica, MA), counted via hemocytometer, and then cryopreserved in a 3:2 mixture of DMEM+10% FBS:MesenPRO with 10% dimethyl sulfoxide (DMSO). For culturing and expansion, cells were thawed into 3:2 DMEM+10% FBS:MesenPro media and switched to 100% MesenPRO 24-48 hours later. The cells were characterized using flow cytometery for immunophenotypes in accordance with guidelines set forth by the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT)[43]. The cultures were determined to be CD44, CD90 and CD105 positive as well as CD45 and CD31 negative. All cells were passaged at least one time before use in experiments to ensure a population of only plastic adherent ASCs. Cells were transduced with a constitutive mCherry-luciferase reporter plasmid (provided by Dr. Byron Hann, UCSF) and selected for using neomycin to obtain a population with only fluorescent expressing cells, to facilitate visualization of cells in 3-dimesional (3D) culture. All subsequent experiments were preformed on cells between passage 2 and passage 5.

Adipogenic Differentiation

ASCs were dissociated using TrypLE Select (Life Technologies) at 37 °C for 5-10 min and inactivated by dilution with MesenPRO, per the TrypLE protocol. The cells were spun down at 1,200 rpm and resuspended in MesenPRO then seeded into appropriate vessels at varying densities for 2D or 3D analysis. Cells in 2D cultures were grown in MesenPRO on uncoated tissue culture plastic until confluent and then switched to adipogenic differentiation medium. Cells for 3D cultures were treated in the same manner and then encapsulated in hydrogels at a high confluence, $2 \times 10^6 - 2 \times 10^7$ cells/mL of hydrogel as explained below, and were placed directly into adipogenic differentiation media. Adipogenic differentiation medium consisted of alpha modified Minimum Essential Media (Sigma Aldrich, St. Louis, MO), supplemented with 10% FBS (Atlas Biologicals), 2mM L-Glutamine (Life Technologies), 100μM indomethicin (Sigma Aldrich), 10μg/mL insulin (Sigma Aldirch), 1μM dexamethasone (Sigma), 500μM 3-isobutyl-1-methylxanthine (Sigma Aldrich) and 10 U/mL pen-strep (Life Technologies) [30,32,39,40,44]. 2D and 3D cultures were allowed to differentiate for 21 days prior to analysis. 2D cultures were stained (see below) and imaged on an Olympus IX70 (Olympus, Shinjuku, Tokyo) at 20X magnification. 3D cultures were stained with Hoescht 33342 (2ug/mL; Invitrogen), LipidTOX (1:150; Life Technologies) for detection of lipid vacuoles, and CellMask (1:150; Life Technologies) to enhance mCherry reporter and visualize cell bodies. 3D gels were then imaged on an Olympus Fluoview 1000 Spectral Confocal microscope. Images analyzed using Imaris software (Bitplane, South Windsor, CT). The “surface object” module was utilized to create a 3D representation of the stack

for each channel imaged and generate objects for the nuclei, lipid vacuoles, cellular membranes and the tagged peptides independently. Threshold settings were optimized for each channel to allow for unbiased counting of analyzed surface types. The distance tool in the software was also used to determine which cells contained lipids. The size of the lipid vacuoles was determined using size functions in the Imaris software.

Immunocytochemistry

ASCs at passage 2 were seeded at 2.3 x 10^4 cells/cm^2 on uncoated tissue culture plastic 12 well plates and grown to 100% confluence in MesenPRO. Cells were then either fixed or switched to adipogenic differentiation medium and differentiated for 21 days. Cells were fixed through incubation with 4% paraformaldehyde in 0.2 M sodium cacodylate buffer for 10 minutes and stored in 0.4% paraformaldehyde at 4°C for no more than 6 months. Cells were blocked using 5% BSA and permeabilized using 0.2% TritonX-100 in 1X PBS for 1 hour at 4°C. Cells were stained overnight at 4°C with primary antibodies diluted in blocking buffer. (See Table 2 for a list of primary antibodies, and concentrations used.) Cells were subsequently washed with PBS and stained with AlexaFluor secondary antibodies (20µg/mL) and Hoescht 33342 (2ug/mL; Life Technologies) for 1 hour at room temperature before being imaged.

3D Hydrogels

A solution of 4-arm PEG-Thiol (Creative PEGWorks, Chapel Hill, NC) at 10 wt% was prepared in 37°C cell culture media. A solution of divinyl sulfone (DVS)

Table 2. Primary Antibodies Used in This Study

Antibody	*Species*	*Concentration (µg/mL)*	*Manufacturer*	*Catalog Number*
Integrin α1	Mouse Monoclonal	2	EDM Millipore, Temecula CA	MAB1973Z-20
Integrin α3	Mouse Monoclonal	2	EDM Millipore	MAB1952Z-20
Integrin α5	Mouse Monoclonal	2	EDM Millipore	MAB 1956Z-20
Integrin α6	Rat Monoclonal	2	EDM Millipore	MAB1378-20
Integrin αV	Mouse Monoclonal	2	EDM Millipore	MAB1953Z-20
Integrin β1	Mouse Monoclonal	2	EDM Millipore	MAB1951Z-20
Integrin β4	Mouse Monoclonal	2	EDM Millipore	MAB2060-20
Integrin β5	Rabbit Polyclonal	2	EDM Millipore	AB1926-20
Fibronectin	Rabbit Polyclonal	10	OneWorlLabs, San Diego CA	C0195
Collagen Type IV	Rabbit Polyclonal	4	Abcam, Cambridge MA	ab19808
Laminin- α4	Rabbit Polyclonal	10	OneWorldLab, San Diego CA	bs-11055R
Collagen Type 1	Rabbit Polyclonal	10	Rockland Inc, Pottstown PA	600-401-103-0.1
	Rabbit Polyclonal	5	Abcam, Cambridge MA	ab113700

crosslinker (Sigma Aldrich) was prepared in 4°C cell culture media. Custom ECM-based peptides were synthesized by BioMatik (linearRGD and vitronectin-derived peptides) and received as a kind gift from the laboratory of Erkki Ruoslathi, UC Santa Barbara (cycloRGD) with FITC (liner RGD and vitronectin-derived) and FAM (cylcoRGD) fluorescent markers attached. Gels were functionalized with peptides at 80 µM concentrations before DVS crosslinker addition. Cells were expanded on uncoated tissue culture plastic after transduction of the mCherry plasmid and selection with G418. After dissociation with TrypLE Select and pelleting, cells were resuspended in PEG/peptide solution and the DVS crosslinker was added at a 10:1 ratio (DVS:PEG) immediately prior to plating. 50 µL of gel solution was then plated into each well of a Teflon mold. The polymerizing PEG solution was incubated 30 minutes at 37°C then each gel was placed directly into appropriate culture media (adipogenic differentiation or MesenPRO) in separate wells of a 24-well plate. QGel

without RGD (ref. 1004 QGel, Switzerland) was used following their recommended protocol. Briefly, ASCs were harvested and pelleted in the same manner as in the DVS-PEG gels and resuspended in 125µl of MesenPRO with peptide added at 80 µM. One vial of QGel powder was dissolved in 375µl of Buffer A and quickly vortexed. The hydrogel mixture was then added to the cell suspension and quickly plated into molds in 50 µl aliquots and allowed to polymerize at 37°C for 30 minutes then immediately transferred into culture media.

Adhesion Assay

The ECM-based peptides were covalently conjugated to amine-modified Primaria 96well plates (Corning, 353872) via an *N*-hydroxysuccinimide ester (NHS) NHS-PEG_{12}-Maleimide bi-functional linker (Thermo Scientific Pierce, Carlsbad, Ca; PI22112). NHS groups on the linker were reacted with amines on the plates to form amine bonds, and consequently attach the linker to the plates through incubation with 100 µM NHS-PEG_{12}-Maleimide in PBS for 30 minutes at room temperature. Peptides were diluted to 20 µg/mL in PBS and conjugated overnight at 4°C to react the free cysteine on the peptides with the malemide groups on the linker and create thioester bonds [1,3-5,45]. Plates were then blocked for 2 hours at room temperature with 1%BSA and washed with PBS to remove excess BSA. ASCs were detached from tissue culture plastic after expansion using TrypLE Select (Life Technologies), diluted for inactivation in serum free cell culture media, pelleted, resuspended in serum free media and then stained in suspension with Hoescht 33342 (2 ug/mL; Invitrogen) for 10 minutes, at room temperature for subsequent nuclear visualization. Cells were counted on a hemocytometer then seeded, in triplicate for each peptide,

at 2.9 x 10^4 cells/cm^2 [6-8,15] and allowed to adhere to the modified plates for 3 hours at 37°C. It was determined that a shorter amount of time did not allow for strong enough adhesion to withstand even gentle washing. Non-adherent cells were removed by very gently washing plates three times with room temperature PBS. A negative control of no linker/no peptide and linker/no peptide were included. Each well was imaged immediately after the 3-hour attachment period on an Olympus IX70 fluorescent microscope (Olympus) at 10 X magnification at the center of each well. Fluorescent nuclei were counted and cell area was calculated using ImageJ software (National Institute of Health). Statistical analysis was performed using a two-tailed Student's t-test for this and all subsequent assays.

Culture of ASCs on Full Length ECM Proteins

All proteins were used at a final concentration of 10 µg/mL. Protein dilutions were made fresh and 24-well culture plates were coated overnight at 4°C. Laminin (Sigma Aldrich L4544), fibronectin (R&D systems, Minneapolis, MN; 4305-FN-200), vitronectin (R&D systems; 2308-VN-050), collagen type I (R&D Systems; 6220-CL) and collagen type IV (Millipore; CC076) full length proteins were used. Passage 4 ASCs were thawed from cryopreservation and seeded at a density of 3 x 10^4 cells/cm^2. Cells were harvested at days 1, 3, 5, and 7 using TrypLE Select and counted using the Scepter Handheld Automated Cell Counter (Millipore). Media was harvested from the cultures at each time point before cell harvest and snap frozen in liquid nitrogen, for ELISA testing. Adiponectin, leptin, basic fibroblast growth factor, vascular endothelial growth factor, hepatocyte growth factor (Life Technologies) and stromal cell derived factor-1 (R&D Systems) ELISA kits were used according to the

manufacturer's protocol to assay the amount of each factor present in the spent media at the various time points.

Proliferation Assay

QGel was prepared as described in the 3D hydrogel section above. ASCs were seeded at a density of 10 x 10^6 cells/mL and cultured after polymerization in 24-well plates in MesenPRO media for 7, 14 and 21 days. At each time point, as well as on day 0, QGels were placed in individual 1.5mL Eppendorf tubes and frozen at -80°C. Once all samples had been collected, QGels were digested using Proteinase K (0.5 mg/mL) overnight at 60°C and DNA content was determined following the CyQUANT NF Cell Proliferation Assay protocol for non-adherent cells (Life Technologies). Peptide functionalized PEG-DVS hydrogels were made as described in the 3D hydrogels section above. Cells were seeded at a density of 10 x 10^6 cells/mL and cultured in MesenPRO media for 0, 7, 14 or 21 days. Because the PEG-DVS gels were unable to be digested to quantify DNA content the hydrogels were instead stained with Hoescht, to visualize nuclei, and imaged on an Olympus Fluoview Spectral Confocal microscope. The average number of nuclei for each peptide condition, in three fields, for each of three individual gels was determined at desired time points using Imaris software (Bitplane, CT, USA). The "spots" module was used to create a 3D image of the 150 μm z-stacks and a spot was created based on intensity value of nuclei visualized in the DAPI channel, allowing determination of individual nuclei. Threshold settings were optimized and used for impartial counting of nuclei, independent of peptide condition. Nuclei counts were

averaged for each gel and then for each peptide condition. A Student's t-test was used to determine statistical significance.

Results

As previously described, ASCs isolated from human lipoaspirate can be differentiated down multiple lineages including adipogenic, chondrogenic, osteogenic and endothelial [2,9,11,13,14,22,46]. The differentiation process of many cell types has been shown to be affected by the extra-cellular environment [9,11,13,15,17,19,47,48]. During differentiation, the surrounding extracellular environment must change to properly support the new, maturing cell population. In an effort to understand this process during adipogenic differentiation, we used immunocytochemistry to examine the expression of ECM proteins and integrin subunits in undifferentiated ASCs and in ASCs that had undergone adipogenic differentiation for 21 days. As shown in Figure 1A certain ECM proteins, such as collagen I and vitronectin, were expressed at relatively low levels by undifferentiated ASCs. After 21 days of adipogenic differentiation there was a change in the expression pattern of these proteins as well as an increase in overall expression. Other proteins, such as collagen IV, laminin-α4 and fibronectin were not found to be present in undifferentiated ASC cultures, but could be readily detected in the ASC-derived adipocyte population. Still other ECM proteins such as Elastin (not shown) were detected, but showed no change in immunoreactivity after adipogenic differentiation.

The increase in expression of select ECM components, specifically collagen IV, vitronectin and fibronectin, which were not detected in undifferentiated ASCs,

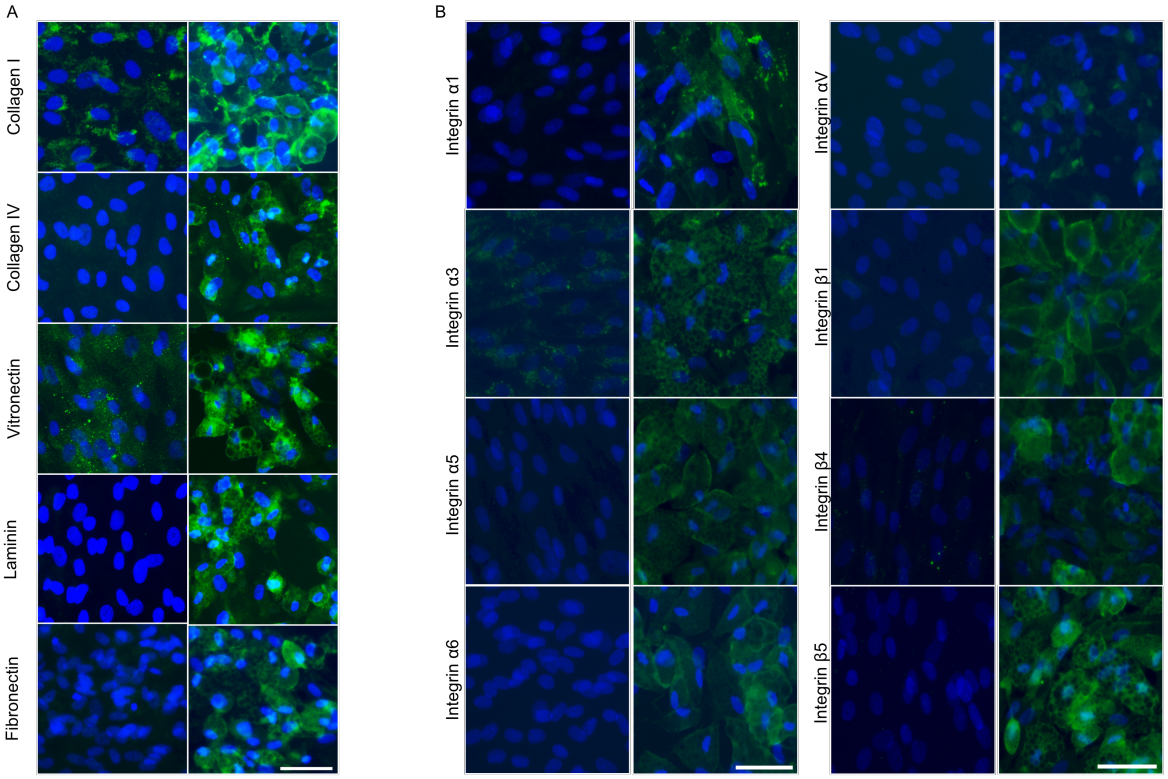

Figure. 1. Analysis of ECM protein and integrin expression in ASCs before and after adipogenic differentiation. (A) ECM proteins indicated (green) were detected by immunocytochemistry in undifferentiated ASCs (left) and ASCs subjected to adipogenic differentiation for 21 days (right). Nuclei (blue) were detected by Hoechst staining. Scale bar=50mm. (B) Integrin subunits indicated (green) were detected by immunocytochemistry in undifferentiated ASCs (left) and ASCs subjected to adipogenic differentiation for 21 days (right). Nuclei (blue) were detected by Hoechst staining. Scale bars = 50 mm. ASCs, adipose-derived stem cells; ECM, extracellular matrix.

supports the idea that the differentiated cells demonstrated increased utilization of integrin receptors that recognize Arg-Gly-Asp (RGD) sequences present in all of these proteins. [20,21,49-53] Laminins containing the α4 subunit are recognized by different integrins not involved in RGD signaling. There are at least eight known integrins that can bind to laminin, [23,54] and adipocytes use a small number of these [33].

While integrin expression of BM-MSC derived adipocytes has been examined previously [26], the expression of integrins in adipocytes derived from lipoaspirate, compared to their undifferentiated counterparts, has yet to be examined fully. Using immunocytochemistry, we characterized the expression of integrin subunits in undifferentiated ASCs and ASC-derived adipocytes that had been differentiated for 21 days (Figure 1B). We detected several integrin subunits in ASC-derived adipocytes but not in undifferentiated ASCs (specifically α1, α5, α6, αV, β4, and β5), as well as some integrin subunits (α3 and β1) that were expressed in both the differentiated and undifferentiated cells, although at higher levels in the ASC-derived adipocytes than the undifferentiated ASCs. In addition, there were subunits that were found to have no discernable change in expression after 21 days of adipogenic differentiation, such as α2, α4 and β3 (not shown). These results indicate that both ECM and ECM receptors are dynamically regulated during adipogenic differentiation.

To examine how the ECM environment might effect the adipogenic differentiation of ASCs, we cultured cells grown on full-length proteins in 2D and assessed their proliferation and expression of adipogenic and endothelial markers over time (Figure 2). These ECM proteins were selected based on changes in expression of ECM proteins and integrins observed during adipogenic differentiation of ASC cultures, with the goal of mimicking the normal adipose ECM environment and promoting this pathway. Proliferation of undifferentiated ASCs cultured in maintenance conditions on selected ECM proteins was assessed over one week. In general, cell proliferation was relatively slow in the low serum media, both on ECM

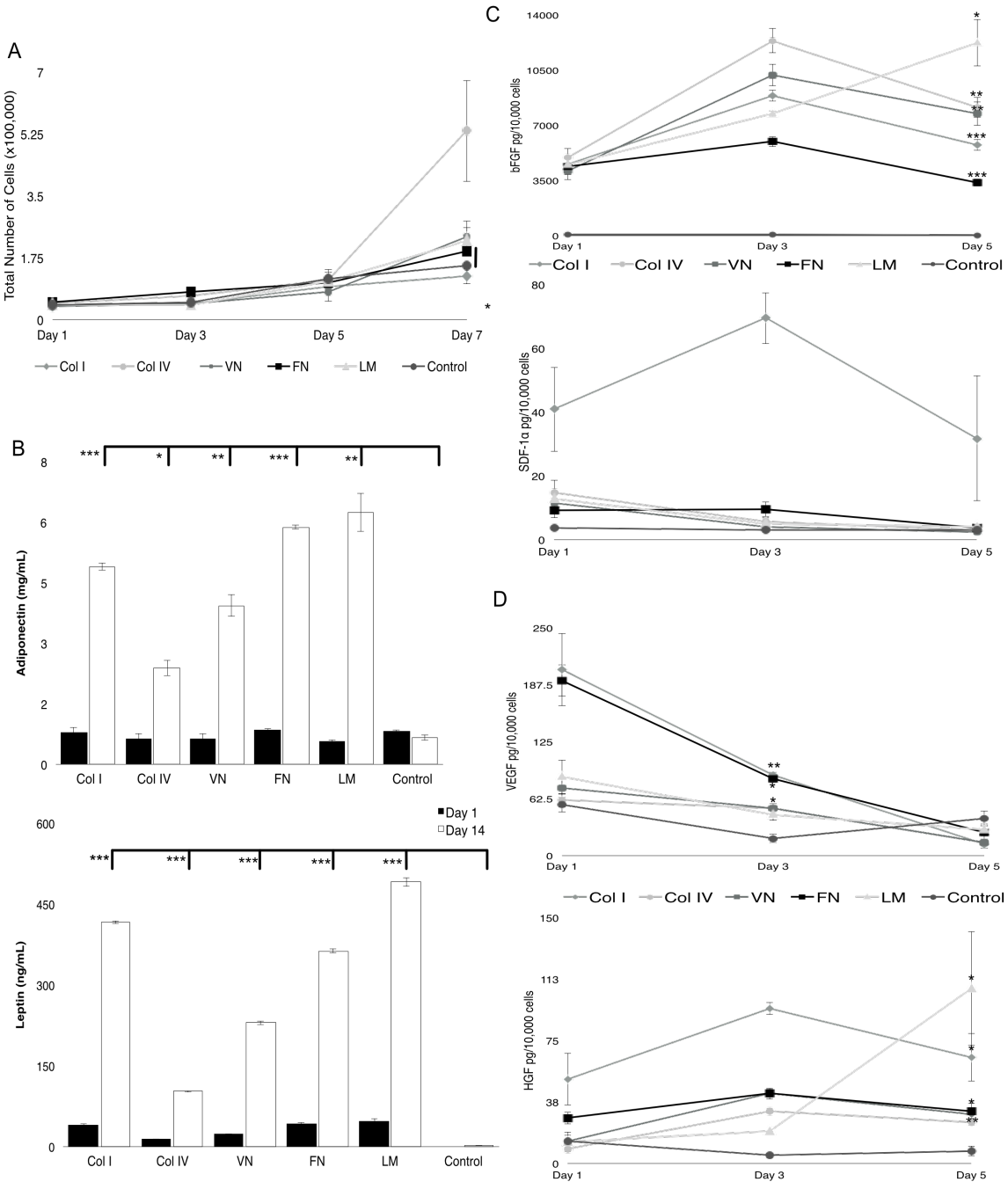

Figure. 2. Effects of full-length ECM proteins on ASCs. (A) Proliferation on different substrates was quantified (*p < 0.05) (B) Secretion of adipogenic factors (adiponectin and leptin) by ASCs cultured on different ECM proteins was quantified by ELISA at day 1 (dark bars) and day 14 (light bars). Secretion of bFGF and SDF1- a (C) and VEGF and HGF (D) by ASCs cultured on different ECM proteins was quantified by ELISA over time (***p<0.001; **p < 0.01; *p < 0.05). bFGF, basic fibroblast growth factor; Col I, collagen I; Col IV, collagen IV; FN, fibronectin; HGF, hepatocyte growth factor; LM, Laminin; SDF-1a, stromal cell-derived factor 1a; VEGF, vascular endothelial growth factor; VN, vitronectin. N=3

proteins as well as tissue culture plastic (control). However, ASCs cultured on collagen IV showed a significant increase in cell number compared to the control (Figure 2A) ($p<0.05$). The only protein that showed a significant, although slight, increase in proliferation at day 7 compared to uncoated tissue culture plastic was fibronectin ($p<0.05$). All other ECM proteins tested supported proliferation rates similar to that of ASCs cultured on untreated tissue culture plastic. Next, we examined secreted factors important in adipogenic differentiation (specifically leptin and adiponectin) using ELISA assays. We found that after 14 days of culture in maintenance conditions on selected ECM proteins ASCs grown on all proteins secreted significantly higher levels of both leptin and adiponectin relative to ASCs cultured on untreated tissue culture plastic (Figure 2B) ($p<0.001$). ASCs cultured on fibronectin or laminin secreted the highest levels of adiponectin and cells on collagen IV secreted the lowest levels. Secretion of four angiogenic factors was also investigated: basic fibroblastic growth factor (bFGF), stromal cell-derived factor 1 (SDF-1α) (Figure 2C), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF)(Figure 2D). Because these factors mediate the formation of new blood vessels, recruit endothelial progenitors, and stimulate vasculogenesis and mitogenesis, their expression during adipogenic differentiation would be useful for eventual clinical applications using ASC-derived adipocytes in tissue grafts and transplants. Results showed that secretion of bFGF and HGF was higher at day 5 on all protein substrates as compared to uncoated control. SDF-1α levels were higher on protein substrates at day one (especially Collagen I), but did not significantly differ from uncoated controls at day 5. VEGF levels decrease over time, but were

higher, compared to uncoated controls, after 3 days on all substrates. Taken together, these results show that ECM protein could be utilized to improve adipogenic differentiation in a 3D culture.

Because four out of the five ECM proteins examined above contain functional RGD peptide sequences, we chose to investigate the ability of different RGD-containing peptides to promote adhesion of ASCs. We investigated peptides with varying adhesivity: a cyclized RGD peptide (cycloRGD) a linear RGD peptide (linearRGD), and a vitronectin-derived RGD peptide (VnRGD) (Figure 3A). It has been previously shown that cells adhere to cycloRGD significantly more strongly (10 times) than to the linearized form [55]. The VnRGD peptide was derived from the full-length vitronectin protein sequence, containing the RGD sequence [56] based on work done previously [57]. To investigate the adhesive properties of each RGD peptide on ASCs, we performed 3

A

Name	Sequence
cycloRGD	FAM-C-X-CRGDC
linearRGD	CRCGG**RGD**SPG-Lys(FITC)
Vitronectin Derived (VnRGD)	CGRCGKGGPQVT**RGD**VFTMPG-Lys(FITC)

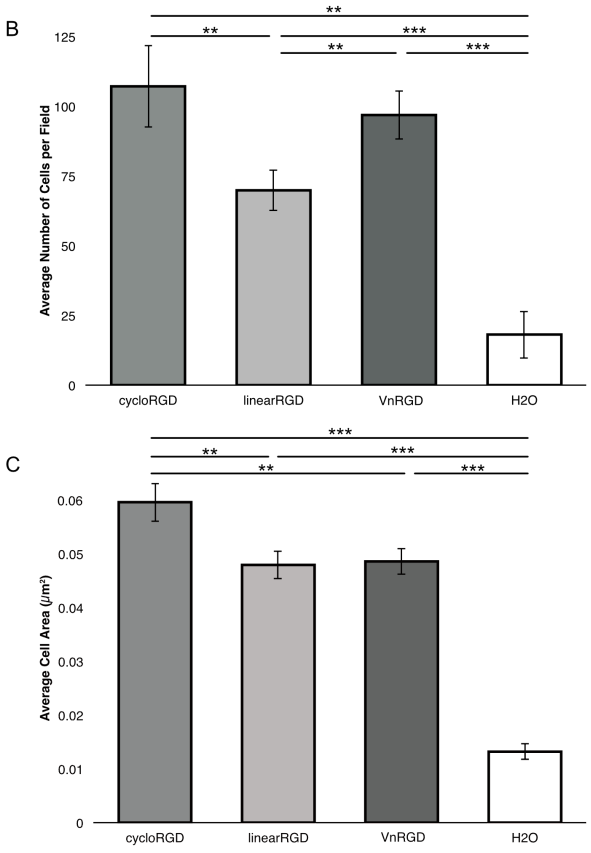

FIG. 3. ASC Adhesion and spreading on RGD peptides. (A) Sequence of peptides. (B) Average number of cells that attach to peptides in two-dimensional culture, after 3h, showing varying adhesion, compared to no-peptide control. (C) Average area of cells after attachment on various pep- tides showing extent of spreading after 3h of attachment compared to no-peptide control (**p < 0.01, ***p < 0.001).

hour-long adhesion assays. We found that significantly greater numbers of ASCs adhered to cycloRGD compared to linearRGD ($p=0.04$), but similar numbers of ASCs adhered to VnRGD and cycloRGD ($p=0.55$) (Figure 3B). ASCs adhered to each RGD peptide in significantly greater number compared to the no peptide control ($p<0.001$). The extent of the ASC spreading on the RGD peptides, which is indicative of the strength of adhesion of the ASCs on the surface, was also quantified (Figure 3C). We found that individual ASCs spread over significantly greater distances when plated on cycloRGD compared to linearRGD, VnRGD and no-peptide controls ($p=0.008$, $p=0.009$, and $p<0.001$ respectively). However, no difference in the amount of spreading was observed when comparing ASCs assayed on linearRGD and VnRGD ($p=0.85$). These results indicate that cycloRGD allows for both strong initial attachment and spreading of ASCs and that the VnRGD peptide provides good initial attachment, but less cell spreading of ASCs. Overall, these three RGD peptides were found to support varying degrees of ASC adhesion and cell spreading, giving us the ability to investigate the effects of varied adhesion on adipogenic differentiation of ASCs in 3D.

We next incorporated the RGD peptides into 3D hydrogels to examine possible effects on proliferation and adipogenic differentiation of the ASCs. We initially used QGel, which is a commercially available gel that has previously been used successfully in *in vivo* studies [58] to examine whether peptides could be incorporated, retained, and whether ASCs could survive and be differentiated down an adipogenic lineage in 3D. Peptides with a free cysteine were added into QGel solution just before cells were encapsulated at a concentration of 80 μM. Using a

CyQUANT assay to determine the DNA content of the QGels after 21 days in maintenance media, it was shown that the undifferentiated ASCs could in fact survive in 3D cultures and that the cycloRGD peptide provided the best condition for the ASCs to attach and proliferate in the Qgels (Figure 4A) with significantly more

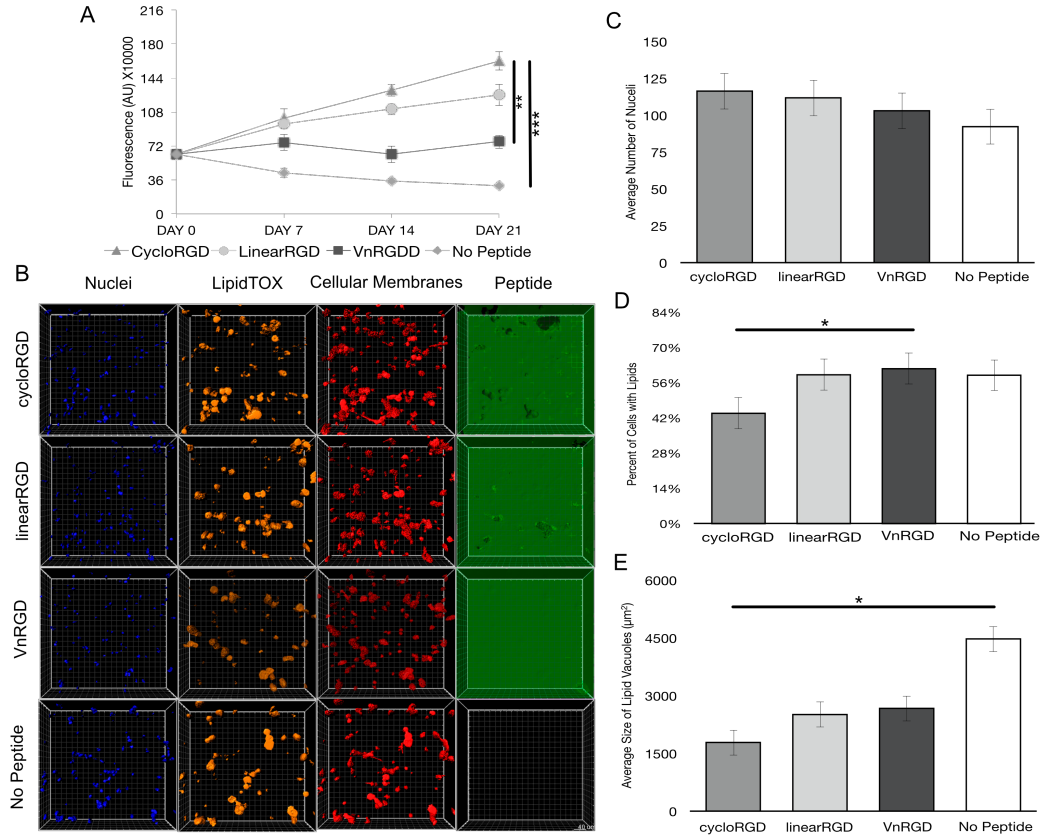

Figure. 4. QGel 3D Hydrogel proliferation and adipogenic differentiation. (A) Proliferation of the ASCs in maintenance conditions in 3D culture as determined by a CyQUANT assay. (B) 3D reconstructions of ASCs adipogenically differentiated for 21 days in Qgel containing various attachment peptides. (C) The average number of nuclei visualized per field showing the overall number of cells present after 21 days of adipogenic culture. (D) The percent of total cells present in each field that are associated with a lipid vacuole. (E) The average size of all the lipid vacuoles present for each condition (*p < 0.05, **p < 0.01, ***p < 0.001). 3D, three-dimensional.

growth at day 21 than VnRGD and no peptide conditions (p=0.0002 and p=0.0018). All three peptides supported proliferation of ASCs in 3D cultures more than gels without attachment sites. After culture for 21 days in QGel with adipogenic

differentiation media, all three peptides were still present, as shown by continued presence of fluorescent signal (Figure 4B; green). Cells were detected under all conditions, including those cultured in QGel without peptides, as shown by staining for nuclei. Likewise, all conditions allowed for adipogenesis, as shown by a LipidTOX stain for neutral lipids in lipid vacuoles (Figure 4B; orange). Cell membranes were visualized using the fluorescent reporter transfected into the cells before encapsulation and enhanced for clarity using CellMask (Figure 4B; red). Quantification of cell numbers showed that all gels contained about the same number of cells, regardless of the peptide (Figure 4C). While all conditions allowed for adipogenic differentiation, it was found that the cycloRGD peptide yielded a significantly lower percentage of cells containing lipid vacuoles ($p=0.04$) when compared to the VnRGD condition (Figure 4D). The percentage of cells containing lipid vacuoles was similar for ASCs differentiated with the linearRGD, VnRGD and QGel without peptides. Using Imaris software we were also able to measure the 3D area of the lipid vacuoles and found that the lipid vacuoles formed by ASCs differentiated with cycloRGD were significantly smaller than those formed in QGel without peptides ($p=0.04$) (Figure 4E). The size of the lipid vacuoles formed by ASCs differentiated with linearRGD, VnRGD and QGel without peptides were not significantly different in size.

We next incorporated RGD peptides in a simple encapsulating, PEG-based hydrogel with a DVS cross-linker (PEG-DVS)[59], and tested proliferation and adipogenic differentiation of ASCs. ASCs were seeded into these PEG-DVS gels at 10×10^6 cells/mL, with consistent peptide concentrations as in the QGels.

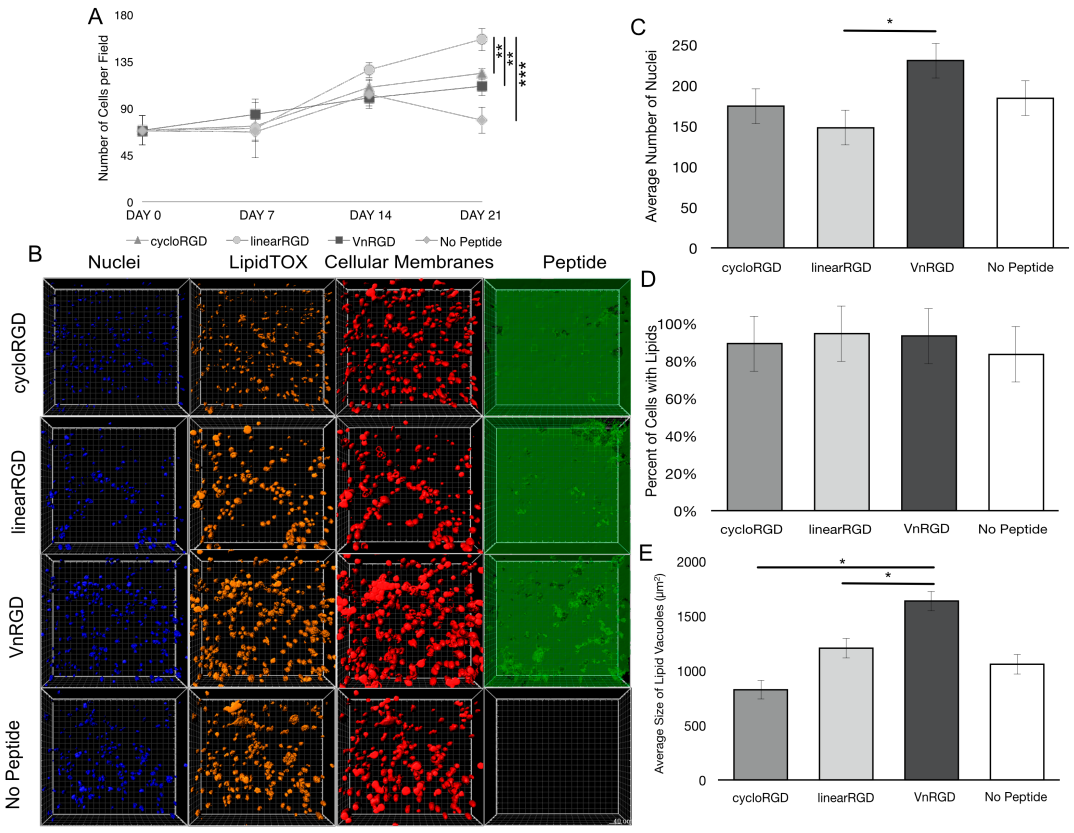

Figure. 5. PEG-DVS 3D hydrogel proliferation and adipogenic differentiation. (A) Proliferation of the ASCs in maintenance conditions in 3D culture over 21 days. (B) 3D reconstructions of ASCs adipogenically differentiated for 21 days in PEG- DVS gels containing various attachment peptides. (C) The average number of nuclei visualized per field showing the overall number of cells present after 21 days of adipogenic culture. (D) The percent of total cells present, in each field that are associated with a lipid vacuole. (E) The average size of all the lipid vacuoles present for each condition (*p<0.05, **p < 0.01, ***p < 0.001). DVS, divinyl sulfone; PEG, poly(ethylene-glycol).

Undifferentiated ASCs were grown in the PEG-DVS gels, in MesenPRO, for up to 21 days and imaged using a confocal microscope to determine the rate of proliferation in each of the peptide conditions. At day 21 it was found that the linearRGD peptide actually supported proliferation significantly better than all other peptide, and no peptide conditions (cycloRGD p=0.009, VnRGD p=0.004, H_2O p=0.0001) (Figure 5A). In this hydrogel system the cycloRGD and vitronectin-derived supported the

same (limited) proliferation and both were more supportive than no peptide. When ASCs were seeded in PEG-DVS gels and grown in adipogenic media for 21 days, all peptide conditions, including the gels without peptide, supported adipogenesis (Figure 5B), as indicated by staining of neutral lipids with LipidTOX (Figure 5B; orange). Additionally, high levels of peptide incorporation were still apparent after 21 days in culture, as assessed by the visualization of the FITC/FAM tag on the peptides (Figure 5B; green). Unlike with the QGel system, we observed a difference in the total number of cells present in the PEG-DVS systems after 21 days of adipogenic differentiation, with a significantly greater number of cells present in the VnRGD system compared to the linearRGD system ($p=0.004$) (Figure 5C). Also differing from the results using QGel, we found that there was no significant difference in the percentage of cells containing lipid vacuoles (Figure 5D). However, the size of the lipid vacuoles in the VnRGD and linearRGD systems were both significantly larger compared to the size of the lipid vacuoles in the cycloRGD system (VnRGD $p=0.001$, linearRGD $p=0.04$) (Figure 5E). Overall, our results with the PEG-DVS system agree with the Qgel data in that the VnRGD peptide was the most supportive of adipogenic

Discussion

Here we have shown that the ECM profile of ASCs changes over 21 days of adipogenic differentiation. Increases in collagens type I and type IV, as well as in vitronectin and fibronectin indicate a likely increase in RGD sites throughout the culture. This change in proteins present outside of the cells correlates to the

changing integrin profile of the cells. Integrin α1β1 has been shown to be involved in attachment to collagen IV and laminins containing the α4 subunit [27,60], while α3β1, α6β1 and α6β4 have all been shown to interact specifically with laminins [60-62]. αVβ5, αVβ1, and α5β1 along with several other integrin heterodimers have been shown to interact with RGD sequences from varying ECM proteins, including collagen I, fibronectin and vitronectin [27,63,64]. The increase of each of these subunits in adipogenically differentiated cultures along with increases in ECM proteins that they are able to interact with supports the hypothesis that these integrins may be important to the differentiation process.

It has been found that BM-MSCs adipogenically differentiated for 21 days only showed a significant increase in α6 integrin subunit and significant decreases most notably in α3 and β4 [26]. While there are a few common changes of integrin subunit expression between BM-MSCs and ASCs (like the increase in α6) there are more notably some potentially important differences. One such difference is the lack of increase in α5 and αV in the BM-MSCs. β1 can dimerize with a large number of α subunits, including α5 and αV, which are both used for binding of RGD sequences in fibronectin. The increase in expression we observed in β1, coupled with the expression of α5 and αV in the derived adipocytes further supports the importance of interaction with RGD containing ECM proteins in adipogenic differentiation of ASCs. Such changes can be potentially utilized in the design of a synthetic 3D environment to enhance adipogenic differentiation.

We found that ECM protein substrates in a 2D environment increases the secretion of factors favorable for adipogenic differentiation (relative to uncoated

controls) such as adiponectin and leptin [65-69]. Other secreted factors important in angiogenesis and vasculogenesis, specifically bFGF, sDF1α, VEGF and HGF [70-73] were also increased. These data are particularly important, as the survival of a 3D tissue graft *in vivo* will most likely require the formation of some vasculature in order to bring blood and essential nutrients to the cells present in the tissue graft. While the expression of these factors is clearly essential for developing tissue grafts, it's also important to keep in mind that ASCs themselves are capable of differentiating into endothelial cells [46,74], which would allow these cells to potentially survive *in vivo* by differentiating into both adipocytes and endothelial cells capable of creating their own vasculature.

By examining the important links between how the changing extracellular environment of differentiating ASCs affects the expression of integrins we were able to determine candidate peptides for incorporation into our 3D structures. Based on the expression of multiple ECM proteins that contain RGD sequences in the adipogenically differentiated cultures we decided to examine how different RGD peptides might affect adipogenic differentiation in a 3D system. Knowing that mature adipocytes are natively found in a relatively soft environment [75], we chose three RGD-based peptides to provide different strengths of adhesion to examine the affect of this initial adhesion strength on the undifferentiated ASCs for their eventual adipogenic differentiation. The initial interaction of these cells with their environment may provide important cues for their eventual differentiation [9,13]. We found that the greatest number of undifferentiated ASCs adhered to cycloRGD although this was not significantly greater than the number of cells adhered to VnRGD. The

difference between the cycloRGD and VnRGD peptides was seen in the extent to which the ASCs spread out during the adhesion period. The size of the undifferentiated ASCs grown on the VnRGD over 3 hours was less than that on the cycloRGD and similar to cell spreading on linearRGD. Overall, while both cycloRGD and VnRGD provide good sites for initial attachment of undifferentiated ASCs, the VnRGD peptide keeps the cells in a more rounded formation, which may be favorable for adipogenic differentiation [76,77].

The strong initial adhesion and reduction in spread morphology of the undifferentiated ASCs on the VnRGD peptide conditions was found to be beneficial to adipogenic differentiation in a 3D environment as well, in both the commercially available QGel as well as a novel formulation of a PEG-DVS hydrogel. We saw that in the QGel the number of cells with lipid vacuoles was significantly lower in the cycloRGD gel, which promoted spreading. While the percentage of lipid vacuole-containing cells did not differ in the PEG-DVS gels between the different peptides used, the size of the lipid vacuoles did vary with cells cultured in the VnRGD-containing gels typically forming significantly larger lipid vacuoles. This production of larger lipid vacuoles is potentially due to the more rounded morphology of the cells differentiated in the VnRGD-containing hydrogels.

When comparing the commercially available QGel and the PEG-DVS gels we found that while both systems incorporate and maintain the presence of the RGD peptides well, the PEG-DVS gels seem to be more supportive of adipogenic growth and differentiation of ASCs compared to QGel. It may be that while the basic thiolene chemistry employed in both gels is acceptable for differentiation of ASCs

into adipocytes, the somewhat different PEG-DVS gel system, provides a slightly more conducive environment for the growth and differentiation of ASCs to adipocytes, possibly due to fewer crosslinking events.

We were able to determine that when ASCs isolated from lipoaspirate were encapsulated into a PEG based hydrogel they were able to not only survive for at least 3 weeks, but also able to proliferate in an undifferentiated state as well as differentiate into adipocytes. The incorporation of RGD-based peptides into these gels affected the efficiency of adipogenic differentiation. Specifically, we found that a strongly adhesive peptide, cycloRGD, may not provide ideal adipogenic inducing interactions for ASCs *in vitro*. VnRGD, however, may create the most inductive environment for adipogenic differentiation; while it supported significant initial adhesion of undifferentiated ASCs, it still allowed the cells to remain more rounded. It also proved to offer the most beneficial environment for adipogenic differentiation in 3D hydrogels, with cells differentiated in its presences showing large lipid vacuoles, indicating more mature adipocytes. These data show the ECM-based attachment sites provided within a 3D environment have a direct effect on the efficiency of adipogenic differentiation of ASCs *in vitro*. While these conditions may not precisely reflect the *in vivo* environment, they represent an important foundation to developing potential treatments. The incorporation of RGD-containing peptides into tissue grafts might later be utilized for improved adipogenic differentiation of ASCs, with the ultimate goal of soft tissue reconstruction *in vivo*.

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Chapter III.

Cell mediated remodeling of biomimetic encapsulating hydrogels achieved by adipogenic differentiation of adipose stem cells

Abstract

One of the most common regenerative cellular therapies is autologous fat grafting, which can suffer from unexpected volume loss and/or reabsorption of the graft. One approach to this problem is to isolate adipose stem cells (ASCs), and deliver them in an engineered, synthetic environment that supports cell survival, differentiation, and integration after transplant. We describe an encapsulating, biomimetic poly(ethylene)-glycol (PEG) hydrogel, with embedded peptides that provide sites for attachment and biodegradation. We sought to develop a long-lasting gel that would remodel upon differentiation of ASCs to adipocytes to provide spaces within the gel supportive of cell survival and differentiation. PEG hydrogels containing an Arg-Gly-Asp (RGD) attachment sequence in addition to a matrix metalloprotease 3 / 10 cleavage site (MMPc) supported ASC survival and showed a regulated remodeling, which is triggered by adipogenic differentiation. RGD-MMPc hydrogels with encapsulated ASCs showed an increased number and area of lacunae or holes, only after ASCs were differentiated to adipocytes. Image analysis of ASCs in RGD-MMPc gels showed larger Voronoi domains, while cell density remained unchanged. Differentiated adipocytes residing within these newly remodeled spaces express proteins and mRNAs indicative of adipocytic differentiation. A biomimetic hydrogel scaffold where remodeling of the gel is

triggered by adipogenic differentiation may be useful in soft tissue regeneration. This work was completed by Tracy N. Clevenger, Gabriel Luna, Daniel Boctor, Steven K. Fisher and Dennis O. Clegg.

Introduction

The development of biocompatible materials has helped advance the field of tissue engineering by providing new cellular platforms for the promotion of tissue regrowth.[1] Many fields in regenerative medicine, including the treatment of chronic wounds, stand to benefit significantly from advances in biomaterial engineering. It is estimated that 1-2% of people in developed countries will experience a chronic wound in their lifetime, and that in the US alone 6.5 million patients suffer from chronic wounds.[2,3] Additionally, soft-tissue injuries constitute a large portion of blast-related wounds in active military personnel, and because of their extensive variability and complexity, they are not amenable to standard treatments.[4,5]

Interest in the use of human adipose stem cells (ASC) as a potential method to treat traumatic or blunt-force injuries has steadily grown over the last decade. Adipose-derived stem cells are a multipotent stem cell population found in abundance in adult humans, and they possess attributes well-suited for regenerative applications. Autologous ASC are easily obtained through standard liposuction procedures, and can be expanded in culture. They have immunomodulatory properties and secrete vital growth factors, such as vascular endothelial growth factor (VEGF), which is an important molecule involved in the wound healing process.[6] Recently, it was demonstrated that the addition of ASCs to a chronic

wound site decreased healing times, and that seeding ASCs on the surface of synthetic membranes showed an even greater benefit, increasing healing by 50%.[7,8]

Biomimetic frameworks that provide structural support and signaling cues derived from the extracellular matrix (ECM) may aid survival of transplanted cells. Poly(ethylene) glycol (PEG), a water soluble polymer, is currently used to extend the duration of interferon-alpha in humans as treatment for Hepatitis C, and can aid in the stimulation of neutrophil production in neutropenia.[9,10] Hydrogels manufactured from this polymer can be functionalized to provide cell attachment and degradation sites, and may also be adapted for use in drug delivery systems and as substrates for cell transplantation.[11-13] The chemical versatility of PEG allows for the incorporation and modification of components that create a synthetic ECM closely mimicking an environment that, at the molecular level, can specifically interact with a given cell type in a directed manner.[14-16]

One approach to encapsulation involves using thiol-functionalized multi-arm PEG groups that use divinyl sulfone as a crosslinker to polymerize the hydrogels through a Michael-type addition chemical reaction. This method avoids the need for a catalyst that has the potentially detrimental effect on encapsulated cells.[17,18] Furthermore, these PEG-based hydrogels possess the ability to incorporate peptides through thiol chemistry using a free cysteine. Peptides containing Arg-Gly-Asp (RGD) sequences can serve as attachment sites for cells, via integrin binding, in the inert PEG gel.[19] Sequences containing RGD have been shown to induce fibroblast spreading,[20] maintain embryonic stem cells[21], influence mineralization

by osteogenic cells,[22] promote the migration of smooth muscle cells through hydrogels[23], and regulate differentiation of various cell lines, such as endothelial cells and myoblasts.[24-27] Modulation of the of RGD-containing sequence can influence survival, proliferation and differentiation, indicating that synthetic environments can be engineered to generate specific cell populations with specific functions.[18]

In this study, we describe efforts to develop an encapsulating hydrogel for ASC that will remodel upon differentiation of ASCs to adipocytes. Because adipocytes produce elevated levels of MMP-3 and MMP-10 (two members of the stromyelsin family of MMPs)[28,29], a scaffolding system was developed that used an MMP peptide cleavage site specific to MMPs 3/10 (GR**C**GRPKPQQ↓FFGLMG; hereafter MMPc) [30,31]. We hypothesize that the MMPc peptide may provide crosslinking abilities via the covalent incorporation at the C-terminus and hydrophobic interactions with PEG at the N-terminal amino acids, here cleavage of MMPc by secreted MMP 3/10 is proposed to alleviate these crosslinks and allow remodeling of the gel. We characterized ASCs encapsulated in this hydrogel *in vitro*, and show accelerated generation of lacunae or holes within the gel after differentiation.

Materials and Methods

Cell Culture

Adipose stem cells were isolated from donated lipoaspirate and purified using methods previously described elsewhere [18,32-34]. Briefly, lipoaspirate was

washed 5-10 times using phosphate buffered saline (PBS; pH 7.4) containing 1X penicillin streptomycin. Next, the lipoaspirate was digested using 0.15% collagenase type I solution (220.00 units/mg; Thermofisher, Carlsbad, CA). The cell solution was then pelleted by centrifugation at 1000 x g for 10 min, and resuspended in a solution containing 160 mM ammonium chloride. Cells were then recentrifuged and resuspended in media containing 60% Dulbecco's modified Eagle's medium (DMEM; Thermofisher, Carlsbad, CA) with 10% FBS and 40% MesenPro medium (Thermofisher, Carlsbad, CA). Finally, the cells were characterized using flow cytometery. Cultures were determined to be CD73, CD90, and CD105 positive, as well as CD45 and CD31 negative, consistent with the ASC immunophenotype defined by the guidelines set forth by the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT).[35]

Cells at passage 2-4 were harvested from tissue culture flasks and encapsulate in a 10kDa PEG hydrogel (PEGworks Chapel Hill, NC) crosslinked using divinyl sulfone (DVS; Sigma Aldrich, St. Louis, MO) (PEG-DVS) as described preveiously [18] A biotin tagged RGD containing peptide (VnRGD) (CGRCGKGGPQVT**RGD**VFTMPG-K(biotin)) derived from the full-length vitronectin amino acid sequence was incorporated to provide attachment sites for undifferentiated ASCs. In some experiments, a peptide was incorporated that contained a site for cleavage by MMP3/10 (ac-GR**C**GRPKPQQ↓FFGLMG-NH_2), derived from the sequence found in Substance P, a known substrate.[30] Cells were

cultured in 3-dimensional (3D) hydrogels in either MesenPro or adipogenic differentiation media for 24 hrs., 4 or 12 weeks.[18]

ELISA

To examine amounts of cleaved biotin, an enzyme-linked immunosorbent assay (ELISA) was performed. Here, ASCs were encapsulated into PEG-DVS hydrogels with VnRGD, in the presence of the MMPc cleavage peptide. Hydrogels were grown in either adipogenic culture media or MesenPro media, media was then harvested 24hrs after polymerization and at 4 weeks. Samples used to analyze 4 week time points were harvested from media that was added 72hrs prior to the time point. Samples were diluted 1:2000, and the amount of biotin released into the media was determined using a Vitamin H ELISA kit (MDBioscience, St. Paul, MD; cat# M046019).

Quantitative Real Time PCR

After 4 weeks of culture, gels containing MMPc peptides were frozen in liquid nitrogen and manually homogenized in 1mL of TRIzol (ThermoFisher; Carlsbad, CA) containing 5μg/mL *S. cerevisiae t*RNA (Sigma Aldrich; Cat# R8508; St. Louis, MO) for 3 mins.[36] Next, 100μl of chloroform was added and samples were centrifuged at 15,000 rpm for 18 min at 4°C. The aqueous phase was removed and recentrifuged at 15,000 rpm for 10 min at 4°C. The aqueous phase was then removed and an equal volume of 100% EtOH was added. The resultant solution was then added to RNeasy spin columns and subsequent steps followed in accordance with the RNeasy plus mini kit protocol (Qiagen; Cat# 74136; Hilden, Germany). RNA from two gels were combined during the elution step and used for cDNA synthesis

using an iScript cDNA synthesis kit (BioRad; Cat#; 1708891; Hercules, CA). Predesigned TaqMan (ThermoFisher; Canoga Park, CA) probes used to examine gene expression are shown in Table 3.

Table 3. TaqMan probes used for gene expression analysis

Gene	Gene Name	Assay ID	Marker cell type
PPARG	Peroxisome proliferator-activated receptor gamma	Hs00234592_m1	Adipocyte
ADIPOQ	Adiponectin	Hs00605917_m1	Adipocyte
ACAN	Aggrecan	Hs00153936_m1	Chondrocyte
ALPL	Alkaline Phosphatase	Hs01029144_m1	Osteocyte
vWF	von Willebrand factor	Hs01109446_m1	Endothelial
MMP3	Matrix metallopeptidase 3 (stromelysin 1)	Hs00968305_m1	-
Gene	Gene Name	Assay ID	Marker cell type
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs02758991_g1	Housekeeper
ACTB	Actin, beta	Hs01060665_g1	Housekeeper
GPI	Glucose-6-phosphate isomerase	Hs00976715_m1	Housekeeper

Preparation and Immunocytochemistry

After 24hr., 4 or 12 weeks in culture samples were washed 3 x 5 min in PBS then immersion fixed in 4% paraformaldehyde in 0.1M sodium cacodylate (Electron Microscopy Sciences, Hatfield, PA) for 30 min at room temperature. Samples were then cryo-protected using a gradient series of 10%, 20%, 30% sucrose in PBS for 2 hr. each and placed in 40% sucrose overnight at 4°C. The following day samples were immersed in optimal cutting temperature (OCT) media (Electron Microscopy Sciences, Hatfield, PA) on a rotator overnight at room temperature. Finally, samples were embedded in OCT media, frozen using liquid nitrogen and sectioned at 20μm.

To identify the hydrogel, slides were immunostained using a streptavidin secondary antibody conjugated to 488 or 568 flurophore (1:100; Jackson

ImmunoResearch, West Grove, PA). In order to further characterize developing ASCs, sections were stained using anti-PPAR gamma (1:50; Santa Cruz Biotechnology; Santa Cruz, CA; Cat: sc-271392), anti-MMP3 (1:50; EMD Millipore; Temecula, CA; MAB3306), anti-MAP2 (1:100; EMD Millipore; Temecula, CA) and Hoescht 33342 (1:5000; Thermofisher; Canoga Park; CA) for 1hr at room temperature in PBS containing 0.5% triton x-100, and 1% BSA (PBT). Samples were then rinsed 2 x 5 mins, in PBT and subsequently mounted using Prolong Gold (Invitrogen, Carlsbad CA) under a glass cover slip.

Mosaic Acquisition and Image Registration

Digital micrographs were captured using an Olympus Fluoview 1000 laser scanning confocal microscope (Olympus America Inc., Center Valley, PA) equipped with a precision automated motorized stage (Applied Scientific Instrumentation Inc., Eugene, OR). Using either an UPlanFLN 40x (N.A. 1.30) or an UPlanSApo 20x (N.A. 0.75) lens. Datasets were collected as individual z-stacks captured at a pixel array of either 1024 x 1024 or 800 x 800. Optical sections were collected at 1-μm intervals with a 5% overlap along the x and y-axes, finally resultant datasets were automatically maximally projected, aligned, and registered using Imago 1.5 (Mayachitra Inc. Santa Barbara, CA) to yield wide-field high-resolution mosaics of the sectioned 3D hydrogels in a manner previously described.[37] All datasets used for spatial analyses have been deposited to Bisque (Bio-Image Semantic Query User Environment) and are publically available for further interrogation with permission

(http://bisque.ece.ucsb.edu/client_service/view?resource=http://bisque.ece.ucsb.edu/data_service/00-BCLMKCcAJrQ2XHUpXx4fnf).[38]

Image Analysis

Images were analyzed using open source ImageJ software (National Institute of Health) to quantify both the number and size of holes present in each mosaic. Each z-stack was first projected as a 2D image, subsequently a threshold for detection of fluorescence was applied across all images analyzed. Images were then converted to a binary format and analyzed for particles using a set size (140-7000) and a circularity of (0.37-1) to ensure that only holes were included while edge artifacts were excluded from quantitative analyses. Here, statistical significance was defined as $p \leq 0.05$ using a one-tailed Student's t-test.

Animal Studies

In accordance with the University of California, Santa Barbara's Institutional Animal Care and Use Committee, 2-month-old athymic mice were anesthetized with a ketamine/xylazine solution (100mg/kg each; Henry Schein; Melville, NY). A small incision was made on the mid-dorsal region of the animal and a pocket was formed using blunt dissection. A premade hydrogel containing undifferentiated ASCs was inserted. The incision was closed using surgical glue and the animals were allowed to recover on a water circulating heating pad. 4 and 12 weeks after the experimental transplantation, animals were euthanized by CO_2 inhalation and the implants as well as surrounding tissue were excised and washed with PBS 3 x 5 min. Tissue samples were then processed for immunocytochemistry as previously described.

Results

In a previous study (Chapter 2), we described a simple, encapsulating RGD peptide hydrogel that supported survival and differentiation of ASCs. In this study, we sought to develop a hydrogel that will be remodeled during differentiation to provide spaces to accommodate adipocytes. Because adipocytes produce elevated levels of MMP-3 and MMP-10 [28,29] , we incorporated peptides containing MMP cleavage sites recognized by these enzymes. The most promising peptide (GR**C**GRPKPQQ↓FFGLMG; hereafter MMPc), was selected for further study [30,31].

After 4 weeks in maintenance media (MesenPro), 3D synthetic scaffolds with and without MMPc peptide showed no structural changes (Figs. 6A, B; green). Similarly, scaffolds that did not contain MMPc and cultured in adipogenic

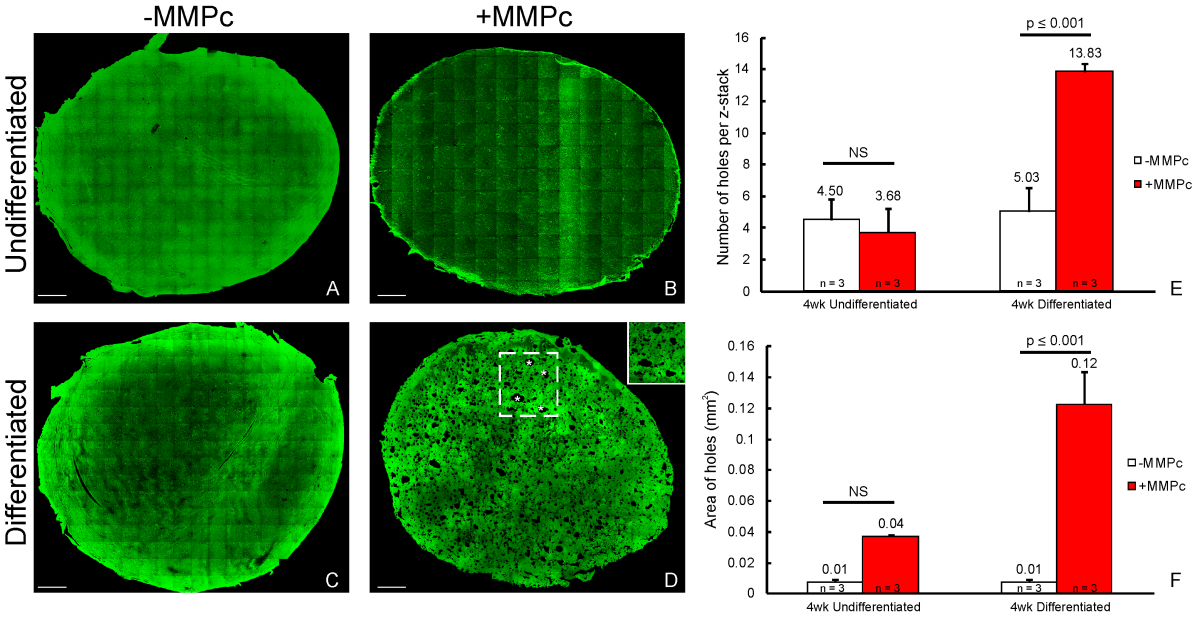

Figure 6. Remodeling of differentiated MMPc containing hydrogels. Gels kept in an undifferentiated state for 4 weeks of culture without (A) and with (B) MMPc peptide show very few holes. The differentiation of hydrogels alone, without the presence of MMPc (C) does not cause remodeling of the gel. Combining differentiation with the incorporation of MMPc peptide causes extensive remodeling of the hydrogel. The number of holes in each condition has been quantified in (E). The size of those holes has been quantified in (F). Scale bars = 500μm.

differentiation media for 4 weeks displayed no structural changes (Fig 6C). However, the inclusion of MMPc resulted in significant structural changes in the gels after 4 weeks of adipogenic differentiation with an increased number of lacunae, or holes observed (Fig. 6D; green; asterisk, inset). After 4 weeks, no statistical significance in the average number of holes was observed between undifferentiated samples with and without MMPc. However, after 4 weeks of differentiation there was a statistical significance in the average number of holes per z-stack between hydrogels that contained the cleavage peptide (13.83 holes/z-stack) and those that did not (5.03 holes/z-stack) (Fig. 6E; $p \leq 0.001$). Additionally, no statistical significance in the average area of holes per z-stack was observed between undifferentiated hydrogel with (0.04 mm^2) and without (0.01 mm^2) the cleavage peptide. The average area of holes in differentiated hydrogels with MMPc was significantly increased compared to differentiated gels without MMPc as well as both undifferentiated conditions (Fig. 6F; $p \leq 0.001$).

To determine whether the ASCs were responsible for the generation of holes in the MMPc scaffolds, hydrogels were cultured for 4 weeks with and without undifferentiated ASCs and no structural changes were observed in number or sizes of holes (Figs. 7A, B; green). Hydrogels containing MMPc that were differentiated for 4 weeks in the absence of ASCs also appeared structurally similar to those in undifferentiated conditions (Fig. 7C; green). However, scaffolds containing differentiated ASCs in the presence of MMPc showed a markedly increased number of holes (Fig. 7D).

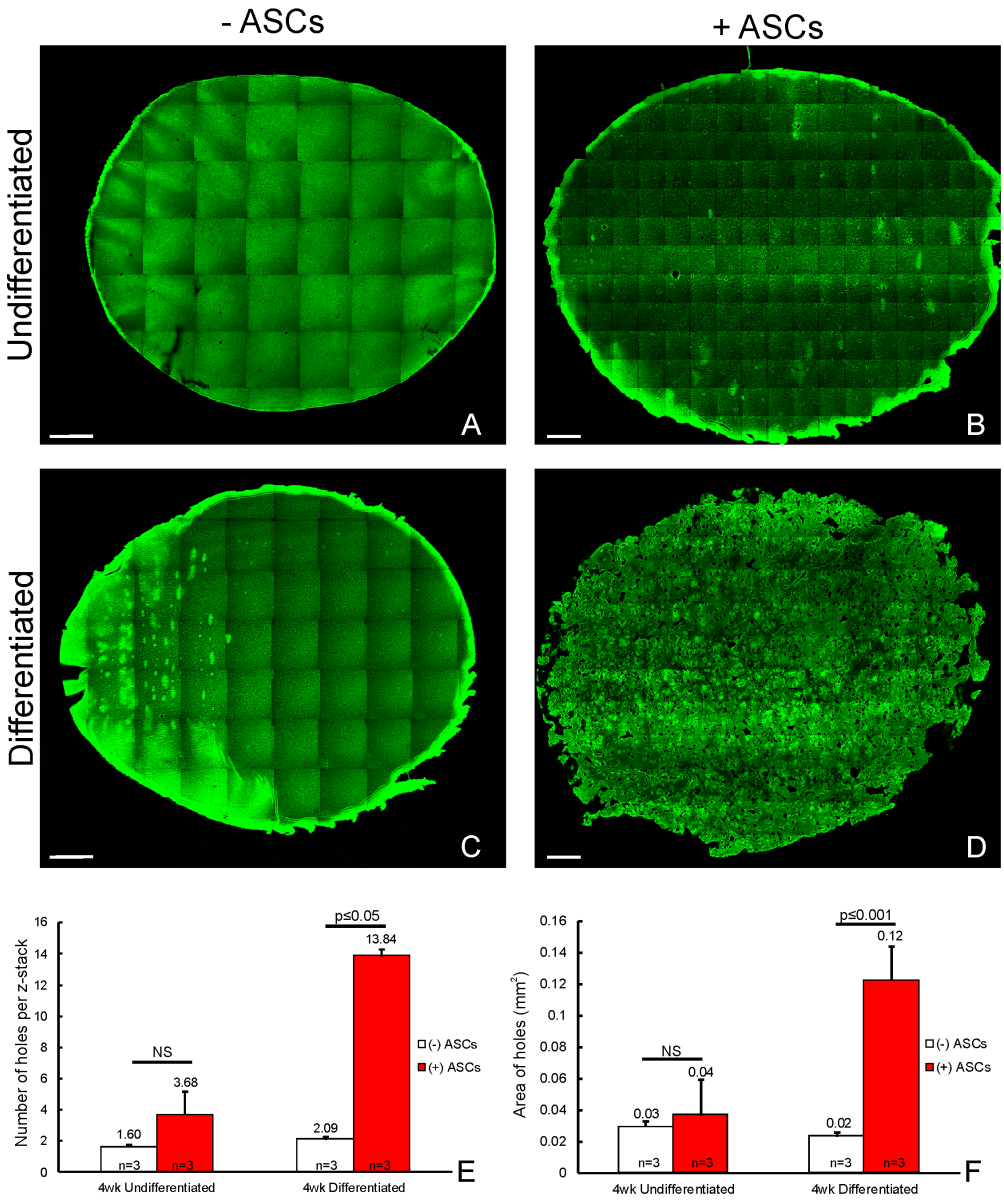

Figure 7. Holes are dependent on the differentiation of cells in MMPc containing hydrogels. Undifferentiated gels containing MMPc, but without cells show no signs of remodeling (A). The addition of cells, in an undifferentiated condition show no signs of remodeling (B). Gels containing MMPc differentiated for 4 weeks in the absence of cells show no signs of remodeling (C). Gels with MMPc, containing cells that have been differentiated for 4 weeks (D) show significant remodeling, quantified in E and F. Scale bars= 500μm

Upon quantification no statistical significant difference was observed in the number of holes per z-stack in hydrogels that were cultured in maintenance media with or without ASCs. In contrast, gels that were differentiated in the presence of MMPc with ASCs showed a greater than 6-fold increase in the number of holes per

z-stack compared to those without ASCs (Fig. 7E; $p \leq 0.001$). Similarly, the area of holes between undifferentiated conditions (i.e. with and without ASCs) showed no statistical significant difference (0.03 mm^2 vs 0.04 mm^2). The area of holes in differentiated gels containing MMPc peptide in the presence of ASCs showed a distinct increase (Fig. 7F; 0.12 mm^2 vs. 0.02 mm^2; $p \leq 0.001$).

Voronoi domains were used to describe the 2-dimensional spatial organization of the ASCs across experimental conditions. Manual annotations of Hoescht-stained nuclei were used and the Cartesian x-y coordinates on these nuclei

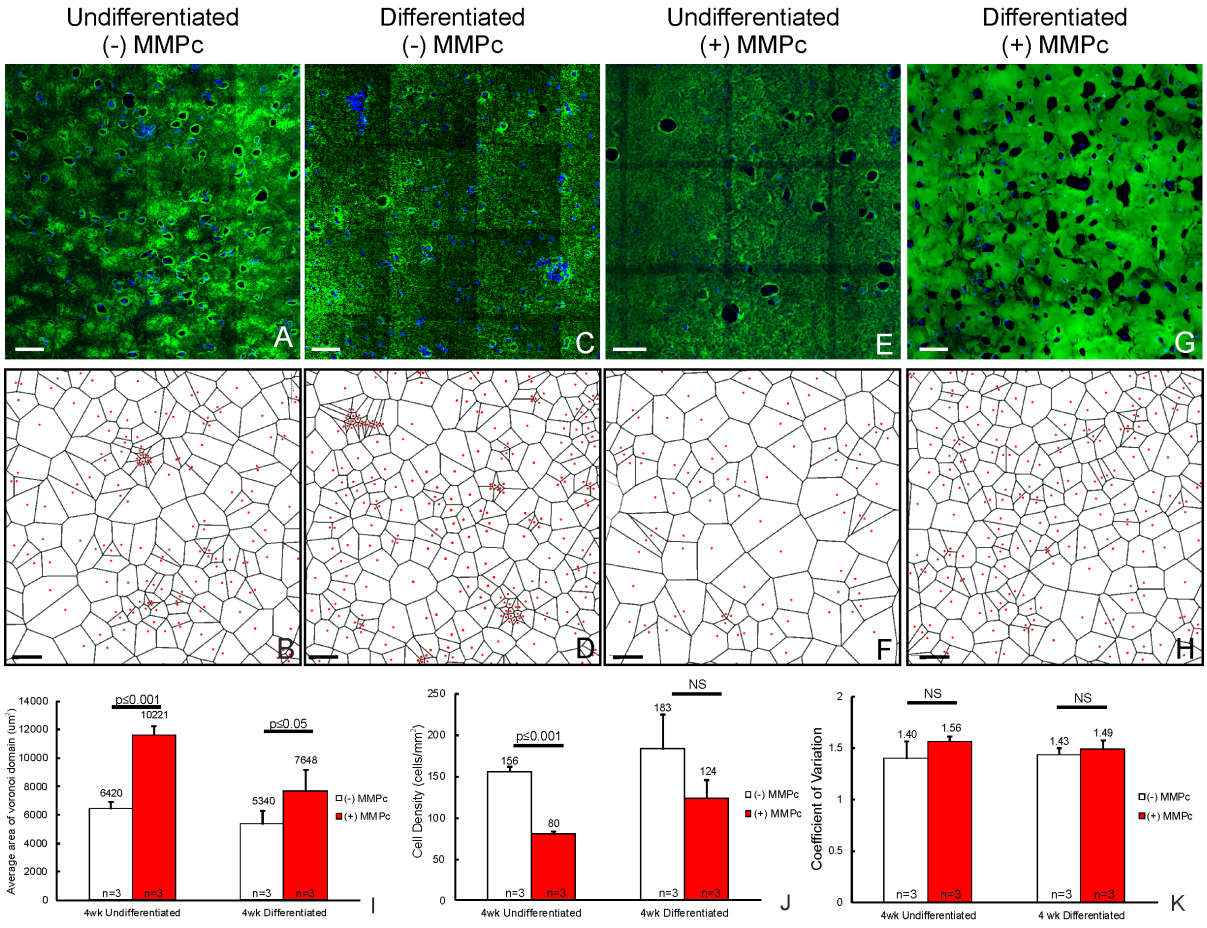

Figure 8. Average Voronoi areas. Micrographs of hydrogels cultured for 4 weeks without MMPc (A and C) and with MMPc (E and G) in undifferentiated and differentiated conditions. Snapshots of Voronoi domain outlines (B, D, F, and H). Quantification of the average Voronoi area (I). Quantification of cell densities in all conditions (J). Scale bars = 100μm

were recorded in hydrogels cultured for 4 weeks (Figs. 8A, C, E, G). Voronoi domain diagrams were generated and their respective areas computed (Figs. 8B, D, F, H). In undifferentiated conditions Voronoi domain area averaged 6,420 um^2. By comparison hydrogels containing the MMPc peptide showed a statistically significant increase in the average area of Voronoi domains (10,221 um^2, $p \leq 0.001$) (Fig. 8I). Likewise, samples cultured in differentiation media also showed a statistically significant increase in the average Voronoi domain area between gels without MMPc (5,340 um^2) and those with MMPc (7,648 um^2; $p \leq 0.05$). To further support the changes observed in the average area of Voronoi domains in the undifferentiated conditions, cell densities were computed and it was determined that in samples not containing MMPc there was a statistically significant increase in the density of cells (156 cells/mm^2) compared to those gels with MMPc (80 cells/mm^2; $p \leq 0.001$). By contrast, samples cultured in differentiation media showed no statistical difference in cell density (124 cells/mm^2; +MMPc) and (183 cells/mm^2; -MMPc) (Fig. 8J). Finally, a coefficient of variance analysis (i.e. standard deviation/mean of the Voronoi domains) was performed to determine whether there was cell clustering observed in any condition. It was determined that no condition demonstrated an increased grouping of cells in 2D (Fig. 8K).

Encapsulated cells were further analyzed using immunocytochemistry. Cells were clustered around the edges of the holes in the hydrogel (Figs. 9A, C, E; arrows). While ASCs that were differentiated for 4 weeks in samples with MMPc remained clustered near the edges of the holes in the remodeled gel, even in spaces that appeared devoid of PEG (Fig. 9G; asterisks). Under these conditions, the gel

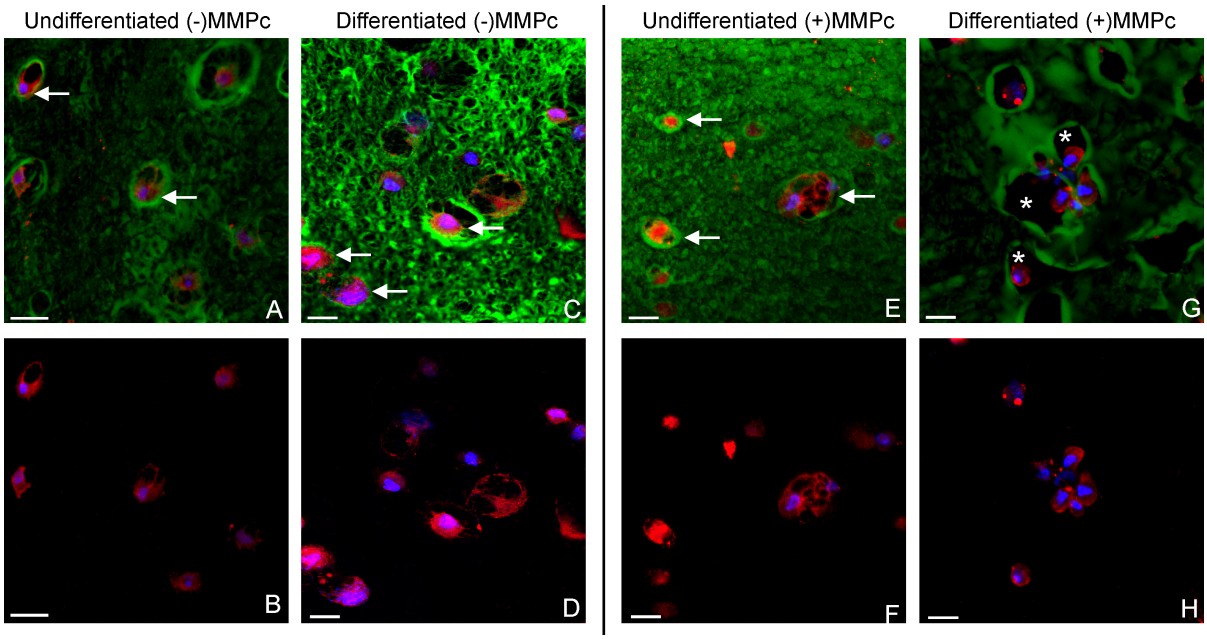

Figure 9. Cell Morphology in hydrogels +/- MMPc. Micrographs of hydrogels cultured for 4 weeks without (A-D) and with (E-H) MMPc, in undifferentiated and differentiated conditions. Arrows indicate protrusions of the cellular membrane. Asterisk indicates holes formed in the +MMPc Differentiated condition. Scale bars = 20μm

appeared to be thickened at the boundary of the holes, as indicated by biotin staining (green). To rule out trapping of streptavidin, sections were stained with hemotoxylin and eosin, which revealed a higher density of gel at the boundary of holes (Supplemental Figure 1). Additionally, cells in each condition had a rounded appearance, indicated by anti-MAP2 staining, a hallmark morphological appearance of adipocytes (Figs. 9B, D, F, H; red).[39]

Adipose-derived stem cells that were undifferentiated in gels with the MMPc cleavage site showed only non-specific anti-PPAR gamma labeling compared to the elevated labeling observed in ASCs that had been differentiated for 4 weeks (Figs. 10A-D). Anti-PPAR gamma labeling appeared to display perinuclear localization in ASCs under differentiated conditions (Fig. 10D; arrows). Additionally, immunolabeling for the MMP3/10 protein was also increased in the differentiated

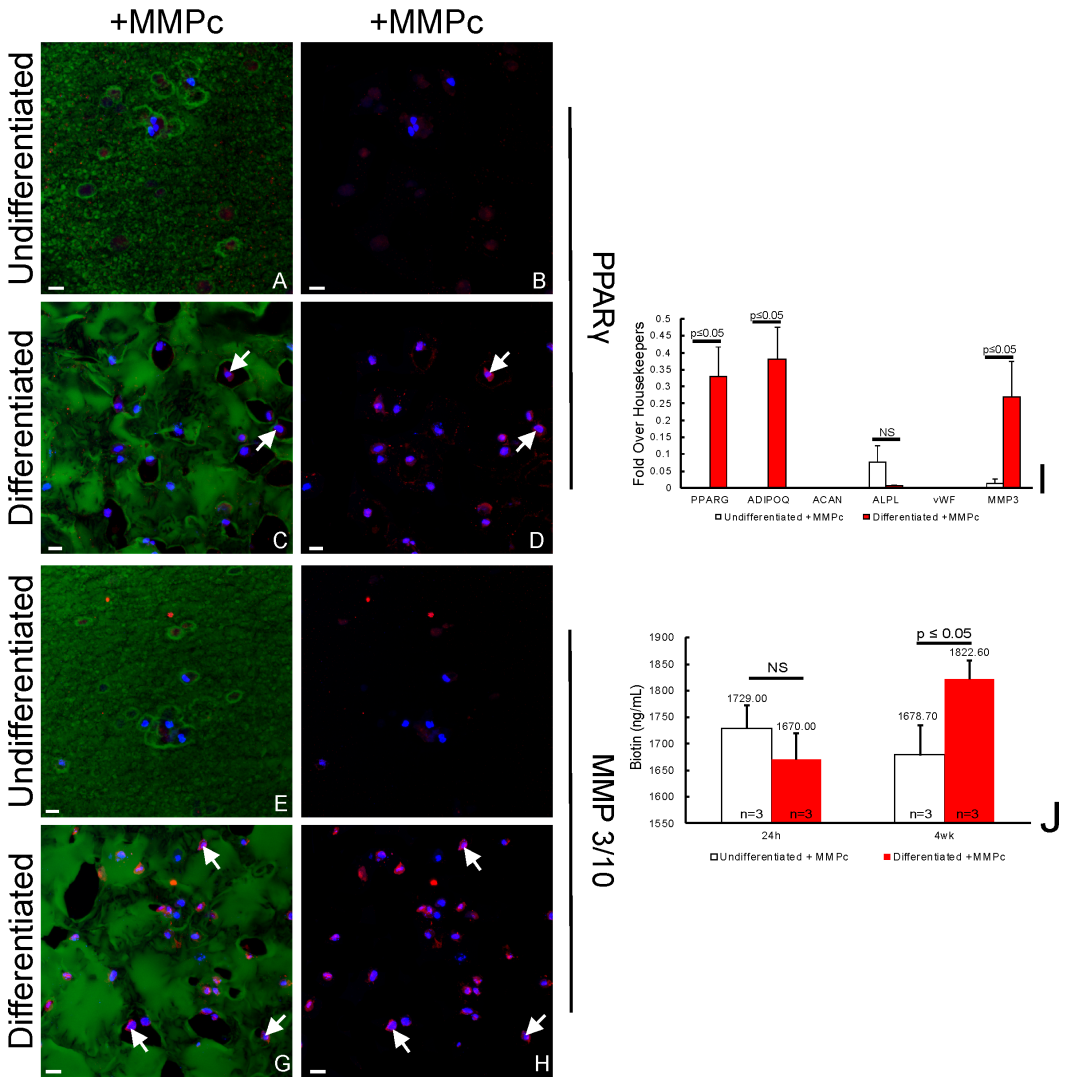

Figure 10. Expression of PPARγ and MMP3/10 protein in MMPc containing hydrogels. There is no expression of PPARγ after 4 weeks of culture in undifferentiated conditions (A and B). Four weeks of adipogenic differentiation elicits expression of PPAR❸ (C and D – arrows). In undifferentiated conditions after 4 weeks of culture there is no expression of MMP3/10 (E and F). Expression in differentiated conditions at 4 weeks is observed (G and H – arrows). Quantification of gene expression shows that differentiated cultures show an increase in adipogenic markers, but not in chondrogenic, osteogenic, or endothelial markers and also show an increase in MMP3 expression (I). Release of biotin from the scaffolding into the media during culture duration shows that gels with MMPc that are differentiated are degrading (J). Scale bars = 20μm

state (Figs. 10E-H). In the differentiated state a majority of the ASCs were MMP 3/10 immunopositive (Fig. 10H; arrows). Gene analysis determined a statistically significant increase in two adipogenic genes (PPARG and *ADIPOQ*) in differentiated samples. Chondrogenic and endothelial genes examined (*ACAN* and *vWF*

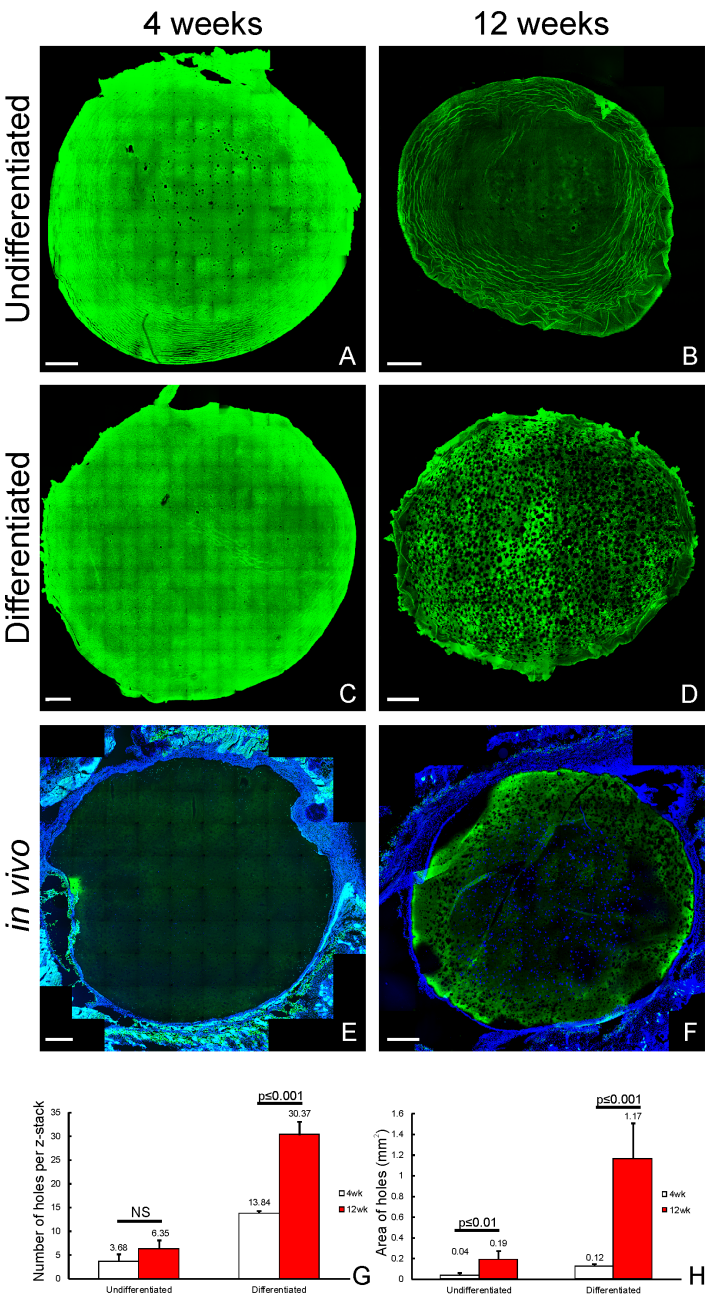

Figure 11. Degradation of gels without MMPc over 12 weeks. After 12 weeks of culture gels maintained in undifferentiated conditions show no increase in the number of holes present compared to 4 weeks (A and D). Gels that are differentiated for 12 weeks show a significant increase in the number (E) compared to 4 weeks (B). After 4 weeks of growth *in vivo* there are no signs of degradation (C), but at 12 weeks holes are apparent (F). Samples that were grown for 4 weeks (G) and 12 weeks (H) in athymic mice. Quantification of the number (I) and size (J) of holes from 4 week *in vitro* samples.

respectively) showed no expression in either differentiated or undifferentiated conditions. Expression of MMP3 showed an increase in samples that were adipogenically differentiated compared to undifferentiated counterparts (Fig 10I).

To investigate degradation of the system, we quantified biotin released into the media after adipocytic differentiation. Biotin is attached to the RGD peptide, which was covalently attached to the PEG backbone. A significant increase in the amount of biotin was detected at 4 weeks in gels with differentiated ASCs (1822.60 ng/ml), compared to the undifferentiated samples (1678.70 ng/ml)(Fig. 10J).

Samples cultured for 4 and 12 weeks were used to examine

the effects of long-term culture on the degradation of gels without the MMPc peptide. Adipose-derived stem cells that were maintained in an undifferentiated state for 12 weeks showed no increase in the number of holes compared to 4 weeks (Figs. 11A, D, G). In contrast, adipogenically differentiated ASCs elicited degradation not seen at 4 weeks (Fig. 11B) in the absence of MMPc, after 12 weeks (Fig. 11E). The appearance of holes in the 12 week differentiated conditions indicates that the differentiation of the cells is a significant contributing factor in the remodeling of the synthetic scaffold. A greater than four fold increase in the number of holes, as well as an increase in size was observed at 12 weeks (Figs. 11G-H).

When hydrogels were subcutaneously implanted into nude mice for 4 and 12 weeks similar trends in remodeling were observed. After 4 weeks there were very few holes apparent in the hydrogel (Fig. 11C) whereas at 12 weeks there was a trend towards an elevated number of holes (Fig. 11F). This increase suggests differentiation of the transplanted cells while *in vivo*.

This time course appears to closely mimic the progression observed *in vitro* and indicates that 3D culture conditions are comparable to *in vivo* models. The consistency between *in vitro* and *in vivo* remodeling times may prove useful for providing insights into scaffold behavior and at the same time reducing the number of animal needed for pre-clinical experiments.

Discussion

Our previous work has demonstrated that the PEG-DVS hydrogel system allows for the survival and differentiation of ASCs into mature adipocytes (Chapter 2).[18] These PEG-based scaffolds have demonstrated robust biostability in culture, showing no significant degradation over a 1-month period. However, the ASCs were constrained in close proximity to the surrounding gel, which may not allow for diffusion of secreted ECM, optimal differentiation, or investiture of the vasculature after transplant. Therefore, we sought to modify the hydrogel to allow remodeling upon differentiation to generate spaces to accommodate adipose cells. Research from a variety of labs have incorporated peptide crosslinks that are susceptible to enzymatic degradation by MMPs.[17,40-44] These previous approaches most often utilize a peptide that includes two cysteine residues flanking an MMP cleavage site for common MMP collagenases, allowing for generic degradation. Collagenases comprise the largest known class of MMPs, and by utilizing these cleavage sites degradation of the hydrogel can be very rapid.

We incorporated a novel MMP peptide containing the cleavage site recognized by MMP-3 and MMP-10, stromelysins which are known to be elevated in adipocytes. Surprisingly, we found that a peptide containing only a single cysteine residue (MMPc) permitted remodeling as encapsulated ASCs differentiate into mature adipocytes. Our data show that these functionalized scaffolds contained an increased number and size of lacunae or holes after differentiating the ASCs for 4 weeks in culture, a phenomenon not seen in samples with undifferentiated ASCs. Furthermore, the remodeling occurred only in the presence of differentiated ASCs,

indicating that it is unlikely a result of a breakdown of the hydrogel over time or an artifact of long-term culture alone.

Voronoi domains quantitatively showed the distribution of ASCs in 2-dimensional space across experimental conditions, and that the incorporation of the MMPc site is correlated with the presence of larger Voronoi areas indicating that the cellular distribution in these degradable scaffolds varied from their counterparts. However, cell density in differentiated conditions was not altered by the inclusion of an MMPc site, indicating that the increase in area of Voronoi domains was not a result of a reduction in the number of ASCs present after 4 weeks of culture. Interestingly, the differentiation of these cells elicited an increase in density not observed in the undifferentiated samples ($p \leq 0.01$). The increase in density may be indicative of increased cell viability due to the deposition of ECM by the differentiating cells, a process that may be absent in undifferentiated cultures. Finally, a coefficient of variation analysis was used to determine the relative distribution of ASCs throughout each condition revealing that no condition exhibited a unique distribution of cells (i.e., clustering). A homogeneous distribution of ASCs in a scaffolding system may be important in improving therapeutic approaches to tissue regeneration.

General cellular morphology was examined by probing for the cytoskeletal marker MAP2. Anti-MAP2 labeling revealed that ASCs in an undifferentiated state in both the presence and absence of MMPc showed protrusions representing potential attachment sites to the hydrogel. These extensions were also noted in the differentiated samples without MMPc although to a lesser extent. However, the

hydrogels containing the MMPc site with differentiated ASCs displayed no visible protrusions emanating from ASCs. Cells in this condition showed a more rounded morphology, congruent with the typical phenotype of adipogenic cell differentiation.[39]

Adipogenic differentiation of the ASCs was confirmed by the immunocytochemical presence of the nuclear receptor PPAR gamma (PPARG). At the transcription level, adipogenic differentiation of ASCs was examined via RT-qPCR. Two genes commonly used to characterized adipose tissue are *PPARG* and *ADIPOQ*, here it was observed both increased in differentiated samples.

Gene expression analysis for contaminating cell-types was also examined in differentiated samples; with no detectable level of chondrogenic (*ACAN*), or endothelial cell (*vWF*) genes observed, and statistically insignificant changes in an osteogenic (*ALPL*) gene. Increased expression of MMP3 was confirmed at both the gene and protein level in the differentiated condition and was not detected in the undifferentiated condition. The increase in MMP3/10 expression (both protein and gene) supports the hypothesis that as ASCs in the hydrogels continue to differentiate into mature adipocytes they increase their expression of MMP3/10.

Investigation into whether this remodeling occurs in the absence of the MMPc peptide revealed evidence of degradation after 12 weeks, indicated by the appearance and size of holes in the sectioned hydrogel. These holes were not found in undifferentiated conditions demonstrating that degradation is dependent on differentiation up to 12 weeks, and that these PEG-based hydrogels do not fully disintegrate over a 12 week period in culture. While the number of holes in the

undifferentiated condition was not increased from 4 to 12 weeks the size of the holes were significantly larger. This may indicate a small amount of natural differentiation of ASCs after long-term culture. Hydrogels lacking the MMPc peptide implanted subcutaneously in athymic mice were still present at 4 and 12 week time points. There was also a clear demarcation between the implanted scaffold and animal tissue surrounding it, suggesting that the scaffolds are able to maintain their structure, thus allowing for systematic identification of implanted components. There was evidence of ASCs present in the gels after 12 weeks showing the ability of these PEG hydrogels to support long-term cell survival. The presence of synthetic gel at 12 weeks indicates a slow degradation process *in vivo*. PEG hydrogels have been reported to degrade in vivo, with varying kinetics.[45] Scaffolds maintained *in vivo* exhibited similar trends to *in vitro* gels, with no significant holes present after 4 weeks. However, after 12 weeks the number of holes was greatly increased. Remodeling appears to be present at 12 weeks, even in the absence of the MMPc peptide. This may signify differentiation of the ASCs *in vivo*, a desirable trait for tissue regeneration in order to reduce the amount of manipulation necessary prior to implantation.

The variation in remodeling times depending on the presence of the cleavage peptide also affords this system versatility. By altering various aspects of the system, such as concentration of MMPc or cell density at time of encapsulation, this scaffold can potentially be modified to remodel and/or degrade in a time or cell dependent manner that is appropriate for the target tissue, ranging from weeks to several months. The generation of holes by the differentiated adipocytes not only provides a

supportive niche, but could aid in vascularization of the gel after implantation. Studies are underway to further characterize these gels after implantation in animal models.

Conclusions

Novel, encapsulating PEG hydrogels containing an Arg-Gly-Asp (RGD) attachment sequence in addition to a matrix metalloprotease 3 / 10 cleavage site (MMPc) supported ASC survival and showed a regulated remodeling triggered by adipogenic differentiation. These RGD-MMPc hydrogels with encapsulated ASCs showed an increase in the number and area of lacunae or holes upon adipogenic differentiation, providing a niche for newly developed adipocytes. Tunable, biomimetic hydrogel scaffolds where remodeling of the gel is triggered by adipogenic differentiation may be useful in soft tissue regeneration.

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Chapter IV

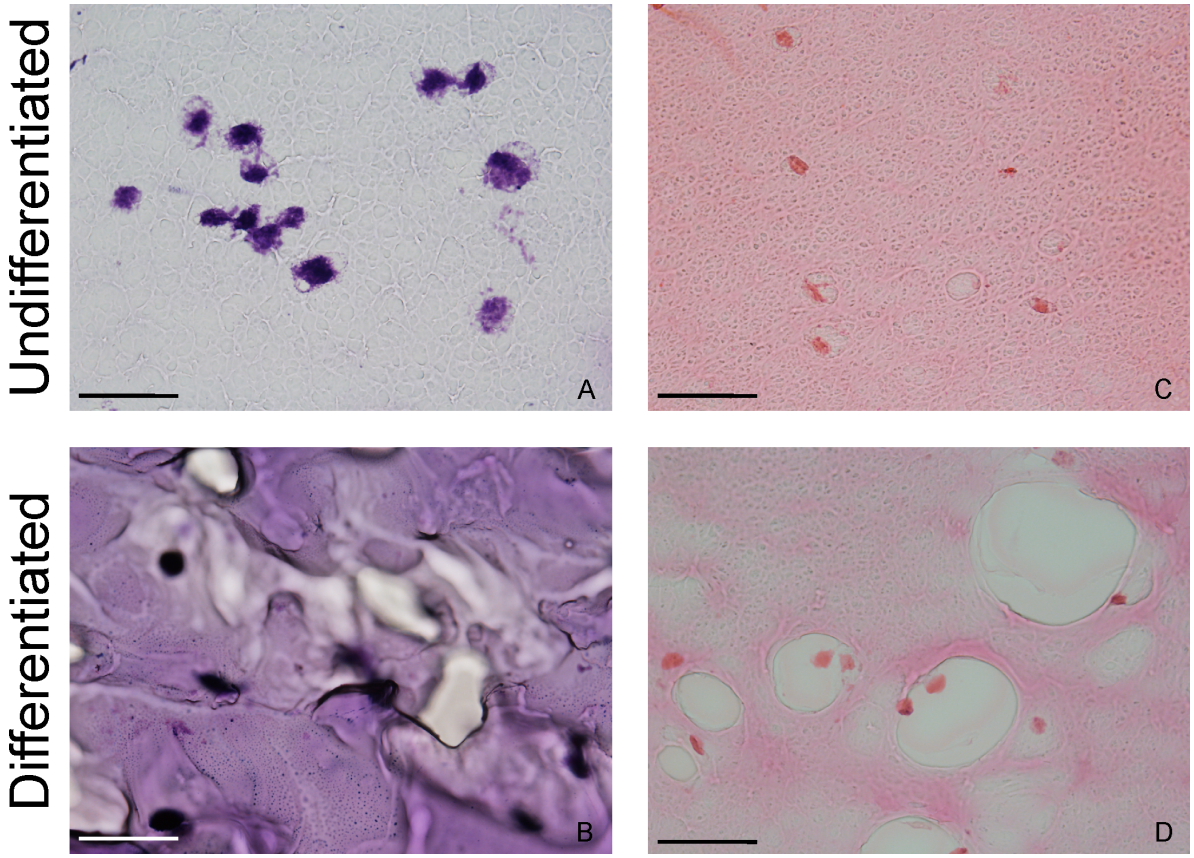
Conclusion

In my opinion it has been an exciting privilege to work in the field of adult mesenchymal stem cells. The boundless opportunities that this cell type provide to the treatment of human disease and injury makes this work all the more exciting. As most of the non-scientific world knows mostly about embryonic stem cells working in this field has enabled me to open the eyes of many of that population to the benefits of potential stem cell therapies that don't involve what they deem to be controversial. Through collaborations and funding from the California Institute for Regenerative Medicine (CIRM), I have been able to spread knowledge to an even broader audience than would have been possible without their support, and for this I feel exceptionally fortunate.

Work on developing synthetic scaffolds has become an area of increasing exploration in the field of tissue engineering. Creating environments that play an active role in the viability and differentiation of cells to be transplanted has become vital to the effectiveness of these regenerative therapies. The system described in this work enables engineering of the basic scaffold for different applications by expanding the possible uses of a simple system to include different target tissues and different treatment options. The use of cleavage sites increases the number of potential applications even further by introducing a mechanism for degradation and remodeling as a function of differentiation. In cases where shorter regeneration times are desirable, such as cases involving wound healing that seek to avoid extensive scaring a scaffolding system that degrades more quickly may be

beneficial, whereas tissues that require more extensive regeneration times, for example, deep ulcers, may require the synthetic environment to remain intact for a longer time. Collectively, the results described here provide a variety of applications for a hydrogel scaffolding system that may be altered to expand the collection of uses this system might benefit.

Appendix



Supplemental Figure 1: Histological stains of MMPc gels. Gels stained with Toluidine Blue show different distributions of nucleic acids present in undifferentiated samples (A) and differentiated samples (B). Hemotoxylin and Eosin staining of undifferentiated (C) and differentiated (D) samples shows altered distribution of hydrogel after adipogenic differentiation.