Effect of Engineered Matrix Environment on Adipogenesis of Adult Adipose-Derived Stem Cells

A thesis submitted by

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Abstract

Obesity has become a major concern for today's society over the last decades without any foreseeable solution to the problem. However, it is known that adipose tissue is not just fat storage, and is found to secrete several proteins that regulate every day processes in the body. Studying the process of adipogenesis will help with understanding diseases that stem from obesity. Here, we use a 3D alginate hydrogel system to help understand how cell differentiation is specifically affected by the matrix environment, for a broader application for development of an *in vitro* model of adipose tissue. Using varying amounts of RGD adhesion peptide within the gels, we aim to change cell morphology of ASCs, and understand it this will have an effect on adipogenesis. Results indicate that the amount of RGD within the gel does affect morphology. Gene expression is higher in gels with RGD than without. Also using this gel system, we aim to show that substrate elastic modulus has an effect on adipogenesis of 3T3-L1 preadipocytes, that is, the higher the elastic modulus, the less adipogenesis will occur. Results show that adipogenesis does appear to be inhibited when cells are in gels with an elastic modulus outside the range of normal adipose tissue; however, more investigation is needed. Overall, results indicate that cell differentiation is influenced by the matrix environment, and shows potential for future studies for adipogenesis, leading to an *in vitro* model of adipose tissue.

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1 Introduction

The goal of this research focuses on studying how microenvironment factors of the extracellular matrix for cells affect adipogenesis within a scaffold, and leading to trying to develop an *in vitro* model of adipose tissue. To do this, we focus on understanding different environmental factors that may influence a cell's ability to differentiate. In this thesis, these factors are scaffold elastic modulus and cell adhesion peptide changes to induce cell morphological changes.

Chapter 2 discusses the motivation for studying adipogenesis, and related background information. It will examine related studies on adipogenesis previously performed by other labs, and will highlight the 3D alginate system that will be used for studying adipogenesis in this thesis.

Chapter 3 focuses on understanding how cell morphology can influence cell behavior towards an adipogenic lineage. It will show the effects of encapsulating cells in gels with different amounts of adhesion peptides and how this can affect the ability of ASCs to undergo adipogenesis.

Chapter 4 outlines the work done to understand how scaffold elastic modulus has an effect on cell differentiation. It explains the effects seen on adipocyte progenitor cells when encapsulated in gels with different elastic moduli, and how the differentiation of the cells is affected.

Chapter 5 summarizes the conclusions drawn from this work, as well as the future directions to which this work might lead.

2 Background

2.1 Obesity and adipose tissue

Obesity is a condition that is a result of a person having too much body fat, and occurs over time when a person consumes more calories than used, resulting in accumulation and storage of fat (NIH). It has become a major concern for today's society over the last decades without any foreseeable solution to the problem. In the 2012, almost 35% of the adult population in the United States were considered obese, and even more disturbing, 17% of youths were considered obese (Ogden, et al., 2014). This problem has resulted in increases in medical costs due to obesity; in 1998, it is estimated that direct medical costs related to obesity totaled $78.5 million and, in 2008, almost doubled to $147.5 million (Finkelstein, et al., 2009). Being obese is known to increase a person's risk of diabetes, heart disease, stroke, arthritis, high blood pressure and some cancers (NIH), all which contribute to the growing medical costs related to obesity. Over the last 20 years, obesity in the United States has increase rapidly. In 1994, no state had a population with an obesity percentage over 19%. In 2012, the majority of states had a population that ranged between 25-30% being obese, with some states having a population that ranged between 30-35% obese. All states had at a population that was at least 20% obese, a percentage that did not exist for any state 20 years ago (Figure 1, CDC).

Despite the current obesity problem in the United States, adipose tissue can be beneficial in healthy people. It is known that adipose (fat) tissue is not just

fat storage. It has been found to secrete several proteins that regulate processes like haemostasis, blood pressure, immune function, angiogenesis and energy balance, and healthy levels of adipose tissue are necessary for normal body functions (Rosen, et al, 2006).

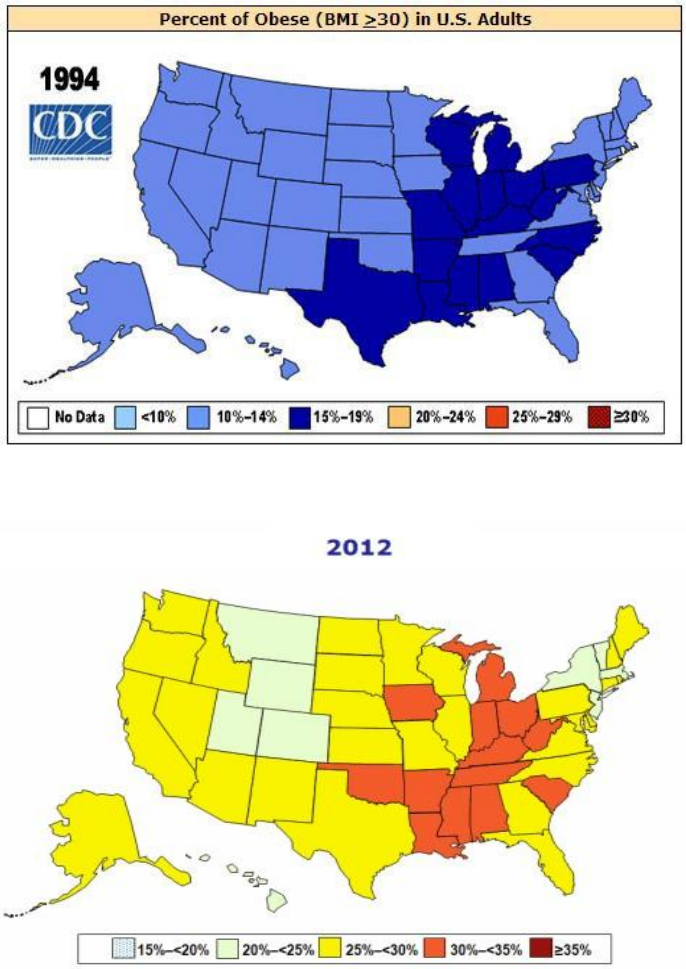


Figure 1. **Maps of the US showing obesity prevalence for the years 1994 and 2012, demonstrating huge increases over the last 20 years (CDC)**

It is known that an increased production of adipose tissue results from both an increased number of adipocytes, which are mature fat cells (hyperplasia),

and enlargement of these cells (hypertrophy), both of which are causes for health problems related to obesity (Spaulding, et al., 2008). Understanding how normal adipose tissue develops can lead to normal adipose tissue models *in vitro*, and lead to disease models for conditions that are known to be related to adipose tissue development, such as obesity and diabetes.

Adipocytes are derived from multipotent mesenchymal stem cells, which can be isolated from either bone marrow or adipose tissue itself. Figure 2 depicts the phases of differentiation. The first phase of differentiation is known as determination. This involves the commitment of a pluripotent stem cell to the adipocyte lineage. This results in the stem cell being converted to a preadipocyte, which is morphologically undistinguishable from its precursor cell, but has lost the potential to differentiate into other cells types. The second phase is known as terminal differentiation. In this phase, the preadipocytes take on the characteristics of the mature adipocyte. It is where it acquires the machinery necessary for lipid transport and synthesis, insulin sensitivity, and the secretion to adipocyte-specific proteins (Rosen and MacDougald, 2006).

2.2 Understanding adipogenesis *in vitro*

The most traditional way to study cell behavior *in vitro* is to culture them in a 2D monolayer on tissue culture plastic (TCP). There are, however, two major problems with this method. First, TCP has an elastic modulus of about 1000 megaPascals (MPa) (Sun 2012; Gilbert, et al., 2010). In contrast, adipose tissue

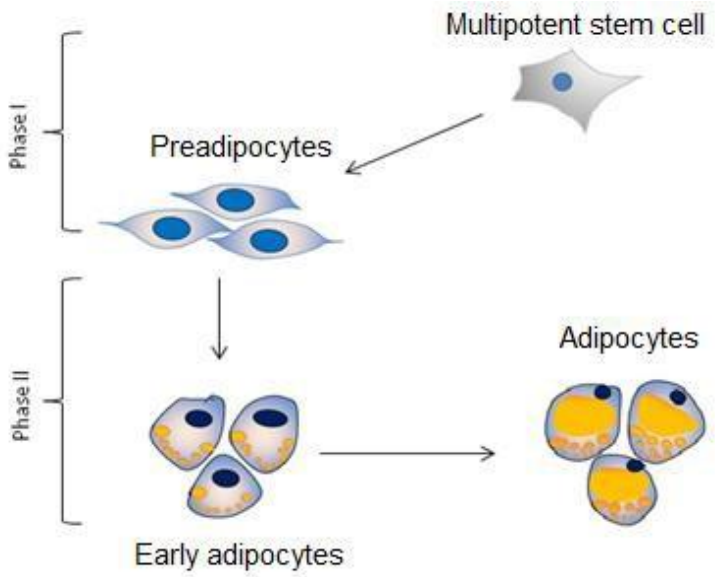


Figure 2. **Phases of adipocyte differentiation**. Stem cells undergo determination to a preadipocyte, where it still maintains a fibroblastic morphology. Next, preadipocytes terminally differentiates into an adipocyte, where it has the ability to store lipids and secrete proteins

has an elastic modulus measuring anywhere from 2 to 24 kPa. This range results from different techniques of measuring elastic moduli of the tissue: Magnetic Resonance Elastography (MRE), indentation measurement techniques, and micromechanical model (Van Houten et al., 2003; Samani, et al., 2003; Comley and Fleck, 2010). Regardless of the way the tissue is measured, the elastic modulus of adipose tissue is at least 50,000 times less than that of TCP. This is significant, when it is known that stem cells are perceptive to single digit fold changes of elastic modulus during differentiation (Engler, 2006). Secondly, culturing cells in a 2D environment is not what they experience *in vivo*. Cells in the body are fully surrounded by the extracellular matrix (ECM) of their native tissue, and can bind via integrins on their entire surface, unlike 2D, where

integrins distribution is concentrated to the bottom of the cell. To combat these two problems, we will use a 3D alginate hydrogel system in this thesis. Culturing cells in this 3D system allows cells to be fully surrounded by the matrix environment. The interaction of cells with the ECM may play an important role in determining cell differentiation. It is known that the fate of embryonic stem cells is determined not only by soluble factors but also by physical interactions with the surrounding matrix and molecules embedded within this EMC (Czyz and Wobus, 2001). In adipose tissue, the ECM organizes fat cells into discrete cell communities and gives rise to fat clusters *in vitro*, and fat lobules *in vivo* (Kubo, et al., 2000). This brings to light the importance of understanding how physical and biochemical environment influences adipogenesis, in order to develop a 3D model of adipose tissue.

2.3 Potential cell sources for in vitro adipogenesis

For the study of adipogenesis, there are several cell types that have the potential to be used, and are described below.

Preadipocyte cell lines: Mouse preadipocyte cells lines are widely used in research (Kang, et al., 2005; Fischbach, et al., 2004, Chandler, et al., 2011), and have the advantage that they are well characterized, easy to expand, and uniform in their differentiation potential. There cell lines are immortalized, and have been used in identifying key molecular markers, transcription factors and other interactions that are required for preadipocyte differentiation. They are frequently

used to rapidly screen and assess the adipogenic potential of various reagent and physical cellular cues (Poulos, et al., 2010).

Embryonic stem (ES) cells: These are pluripotent stem cells derived from the inner cell mass of a blastocyst, which is an early-stage pre-implantation embryo (Thomson, et al., 1998). Ethical concerns and legal constraints limit the use and availability of human ES cells (Baldwin 2009); however, they have been used in limited studies on adipogenesis (Hillel, et al., 2009). As a result, ES cells from other species, such as mouse, are available and used for research on adipogenesis (Kang, et al., 2007).

Mesenchymal stem cells (MSCs): MSCs are multipotent cells that can be isolated from bone marrow (Minguell, et al., 2001). They have the ability to differentiate into adipocytes, chondrocytes, and osteoblasts, as well as other types of cells (Gregory, et al., 2005). However, there is a limitation of use due to lower yields of stem cells present in the bone marrow, when comparing to other tissues (Fraser, et al., 2006) and the invasive isolation process. Still, they have been used in studying adipogenesis (Sekiya, et al., 2004; Khetan, et al., 2013)

Adipose-derived stem cells (ASCs): ASCs, like MSCs, are multipotent cells that have the potential of differentiate into many different cell lineages, including adipocytes, osteoblasts, chondrocytes, neurons, endothelial cells, as well as others (Fraser, et al., 2006, Locke, et al., 2011). ASCs can be isolated from adipose tissue and by harvested by direct excision or from the discarded tissue following liposuction, which is most common (Fraser, et al., 2006). Use of

these cells has been shown to be beneficial for understanding adipogenesis (Schiller, et al., 2013; Gerlach, et al., 2012; Mauney, et al., 2007)

2.4 Adipogenesis and tissue engineering

There have been previous studies that focused on adipogenesis in 3D culture using various scaffold materials. Studies of adipogenesis use two main cell types, adipocyte progenitor cell line or stem cells. Two studies from the same group showed the importance of 3D culture for adipogenesis for both adipocyte progenitor cells and embryonic stem (ES) cells. Both cell types were seeded onto a fibrous matrix in 3D (polyethylene terephthalate for the progenitor cells or polycaprolactone for ES cells) and showed that cells express higher adipogenic markers than 2D cultures, and correct cell morphology was adapted by the cells. They even showed the chemical induction for progenitor cells was not necessary for these results (Kang, et al., 2005; Kang, et al., 2007).

Silk is another material that has been used in the development of a 3D scaffold for adipogenesis. A study out of the Kaplan lab showed that silk, as a biomaterial, has the potential to be used for developing a 3D model of adipogenesis *in vitro*. Additionally, due to its biodegradable nature, the silk scaffold may also have the ability to be used for i*n vivo* implantation, as compared with other commonly used scaffold materials (Mauney, et al., 2007).

Other materials used for a scaffold in adipose tissue engineering include gelatin sponges (Hong, et al., 2005), poly(lactic-co-glycolic) acid (PLGA; Patrick et al., 1999; Patrick, et al., 2002; Kang, et al., 2008), collagen scaffolds (Hiraoka

et al, 2006; Vashi, et al., 2006; von Heimburg, et al, 2003) hyaluronic acid (HA; Flynn, et al., 2007; Halbleib, et al., 2003; 27-31) fibrin matrices (Torio-Padron, et al.,2007) and various hydrogels (Stacey, et al, 2009; Chandler, et al, 2011; Galateanu, et al, 2012; Handel et al, 2012).

Some studies were performed with using methods beyond just differentiation by induction media. One study done by Stacy, et al. (2009) showed that when preadipocyte-seeded scaffolds were cultured in the presence of mature adipocytes, via transwell co-culture plates produced more glycerol than those cells cultured in differentiation media. The overall observation of the study was that in 2D, cells with differentiation media did not achieve the same level of adipogenesis as compared to the positive control; however, in 3D, the same levels of adipogenesis were seen the differentiation media conditions and transwell co-culture with mature adipocytes. This highlights evidence that the 3D culture itself provides an environment that is more conducive to adipogenesis.

Beyond the use of just 3D scaffolds, other studies have been conducted on the potential use of bioreactors in the development of adipose tissue. Gerlach, et al. (2012) investigated the potential of a 3D hollow fiber-based perfusion bioreactor for adipogenesis. The bioreactor allowed for medium and air perfusion in and out of the system, across the fibers where the cells were growing. Result show that tissue formation by ASCs could be seen in and around hollow fiber membranes by the end of the differentiation period, which resembled native adipose tissue. Cells also were shown to have more unilocular lipid-filled vacuoles compare with multilocular lipid droplets of cells in 2D cultures.

Previous studies certainly indicated that 3D has more potential than 2D for studying adipogenesis. Taking from this, out study will use a 3D system described below.

2.5 Alginate and alginate hydrogel system

Alginate is an anionic polysaccharide derived from brown algae. It has been used for cell encapsulation and transplantation, because of its biocompatible nature (Ghidoni, 2008; Kuo and Ma, 2001; Kuo and Ma, 2008). The biocompatibility of a scaffold or matrix for tissue engineering refers to its ability to perform as a substrate that will support cell growth and not negatively interfere with normal cellular activity, including of molecular and mechanical signaling systems (Williams, 2008). Alginate has many qualities that make it attractive as a cell scaffold. It is inert to cells, but it can be functionalized with peptides to allow cells to bind to alginate (Rowley, et al., 1999). Alginate hydrogels are useful for 3D studies beyond their biocompatibility. Alginate has the ability to have a slow gelation time to allow for a uniform mixture of cells and homogeneous mechanical properties when ionically crosslinked (Kuo and Ma, 2001; Kuo and Ma, 2008). Many hydrogel materials, including alginate, can be photo-crosslinked with the addition of a photo initiator by UV light to cause gelation. However, this can have deleterious effects on encapsulated cells. To form a gel, alginate can be crosslinked with calcium ions.

Different forms of calcium ions have been used to crosslink the alginate and cause gelation. Some studies have used calcium chloride ($CaCl_2$) as the crosslinker (Galateanu, et al, 2012; Handel et al, 2012), but using this form of

calcium ions does not allow for slow gelation, and it is hard to achieve homogeneous mechanical properties. Previous work from our lab has developed a slow ionic crosslinking system using calcium carbonate ($CaCO_3$) as the source of divalent calcium ions (Ca^{2+}) used for crosslinking the alginate. Calcium is presented in the alginate mixture, along with d-gluconic acid lactone (GDL). GDL is dissolved in HBSS and is added to the alginate/calcium carbonate mixture. This causes a reaction that results in Ca^{2+} ions and $CO_2 + H_2O$. The calcium ions are then able to bind to the carboxyl groups on the alginate chains. Alginate contains "G" and "M" blocks, and binding of carboxyl groups on the G blocks results in an "egg box" structure. The formation of the egg box structure contributes to the mechanical properties of the gel (Kuo and Ma, 2001). Results showed that using calcium as an ionic crosslinker allowed for formulation of a gel with uniform structure and consistent material properties. Increasing both the amount of calcium and alginate polymer concentration will increase the elastic modulus of the gel (Kuo and Ma, 2001).

Alginate can also be functionalized with binding peptides to provide sites for cell attachment (Rowley, 1999). One of the most commonly used peptides for functionalization is Arginine-Glycine-Aspartic Acid (RGD), a cell binding site found within many EMC proteins (Ruoslahti, 1996). The RGD can be covalently attached to the alginate, allowing cells to bind to the RGD, and exert tension on the alginate. This functionalization process is critical because it has been shown that any soluble RGD that is present for the cells actually causes cell death. Cells internalize the peptide and it directly induces apoptotic proteins (Buckley, et al., 1999).

RGD concentration used in this thesis was the same as in previous work done in our lab (Marturano in prep). We used a mass ratio of RGD to alginate, of 0.03, previously selected by our lab as a concentration in which tendon cell spreading was observed. In this current study, the concentration was given the designation of "RGD". Kang, et al. (2013) show adipogenesis of ASCs is affected by using RGD within alginate gels. They compared using RGD within the gels to their control of no RGD and concluded that the presence of RGD enhances adipogenesis. However, they define the amount of RGD within the gel as a degree of substitution (DS) as the number of peptides per 100 uronic acid residues in the alginate chain. The DS of the modified alginate used in their study was 0.15. Because of this method used to determine the amount of RGD is different from other studies, it is difficult to compare to among the similar studies. In addition to adipogenesis, other studies have looked at how RGD affect differentiation of other cell types. Connelly, et al. (2007) showed that the presence of RGD inhibited chondrogenesis of mesenchymal stem cells. This study also used the same RGD amount as presented in Rowley, et al. In 2D, is has been shown that nanospacing (the distance from one peptide to the next) has an effect on cell differentiation (Wang, et al., 2013). This is important to note because it could indicate that there is an optimal amount (and thus spacing) of the functionalized peptide within the alginate gels that could be influencing the ability of the encapsulated cells to differentiate.

Alginate gel mechanical properties are easily controlled by changing the compositional parameters. For example, elastic modulus depends on both the alginate polymer concentration and calcium crosslinking densities. (Kuo and Ma,

2001). Work from our lab has shown that alginate hydrogels can have an elastic modulus ranging from 2.5kPa to almost 150kPa, as tested by atomic force microscopy (AFM) (Marturano, in prep). Thus, gels can be made with a similar modulus to that of native adipose tissue, which has been reported to range between 2-24kPa (Van Houten et al., 2003; Samani, et al., 2003; Comley and Fleck, 2010).

Previous works have attempted to use varying types of hydrogels for tissue engineering purposes, for adipose tissue, as well as other tissues. Chandler, et al, (2011) used alginate hydrogels to establish a biomaterial-based 3D system in order to evaluate the effect of matrix elastic modulus on adipose-progenitor cells. Unlike the alginate system describe above, they used UV-photocrosslinking to induce gelation. The paper states that this does not negatively affect the cells; however, they show cell viability was reduced to 50% in some of the conditions.

Other studies focus on adipose tissue for the goal of soft tissue regeneration. There have been attempts at this using alginate hydrogels, but instead of using calcium ions for slow crosslinking, others have used $CaCl_2$, where the alginate bead must be placed in the calcium solution and gels within minutes (Galateanu, et al, 2012; Handel et al, 2012). This leads to lack of uniform gelation, and makes it harder to finely control the mechanical properties.

2. 6 Thesis Aims

Using our system of alginate hydrogels, with slow crosslinking, we intend to show that it is a controllable (i.e., control of mechanical properties) 3D *in vitro*

system for studying adipogenesis, and can aid in guiding cells towards an adipogenic lineage. Not only can the mechanical properties be finely tuned, but binding peptides can be covalently bonded to the alginate to allow for cell adhesion. With this, our aim is to use this system to understand how both stem cells and adipocyte progenitor cells perceive the matrix environment around them, and whether this can affect differentiation toward the adipose lineage, leading to an overall goal of developing an in vitro model of adipose tissue. Two characteristics we will focus on are how changes in cell morphology can be induced by adhesion peptides and elastic modulus of surrounding environment. Both have been shown to have a great deal of influence on cellular behavior. Cell morphology has been shown to affect differentiation (McBeath et al., 2004; Lee et al., 2013), while matrix elastic modulus has been shown to effect cells in many ways, including proliferation (Subramanian and Lin, 2005), differentiation (Engler, et al, 2006), and angiogenesis (Mammoto et al., 2009).

Using the alginate gels to understand cell morphology, we varied the amount of binding peptide present, and looked to see if 1) cell morphology would be affected, and 2) if a change in cell morphology would affect the adipogenic potential of the encapsulated cells. To look at how elastic modulus affects adipogenesis, the amount of alginate polymer concentration and calcium amount within the gels was varied, causing a change in elastic modulus in the gels. This allowed us to understand if the cells were able to sense the elastic modulus of the gels, and if this had an effect on cell differentiation. The following chapters will describe the work done on each of these.

3 Effect of cell morphology on adipogenesis

3.1 Introduction

Obesity is a major health issue in our society, with no solution to the problem in the near future. People who are obese are at higher risk to develop type II diabetes, heart disease, and even certain cancers (NIH). In order the combat this growing problem, the basic methods of adipogenesis must be understood.

In order to develop an *in vitro* model of adipogenesis, we must understand how cells within the tissue are affected by their surrounding environment. The makeup of the matrix environment in the tissue allows the cells to perceive their surroundings. A specific area of interest for how cells perceive the matrix environment of the tissue is whether changes in adhesion peptides within a 3D environment influence cell morphology and affects differentiation towards an adipogenic lineage.

Studies have shown that differences in cell shape can lead to differences in potential differentiation of cells. McBeath, et al. (2004) examined whether changes in cell shape were able to regulate the commitment of human mesenchymal stem cells (hMSCs). They demonstrated that cells grown individually on small islands of fibronectin, which restricted their ability to spread, resulted in adipogenesis, and cells grown on larger islands results in cells undergoing osteogenesis. This study focuses only on changing the area of the island to increase or decrease spreading. Others have used this technique to show

opposite effects, that is, changing cell morphology will cause cells to dedifferentiate. A recent study done by Ding and colleagues (2014) cultured individual rat chondrocytes on PEG hydrogels, using RGD to create different shape islands on which the cells could grow. They grew cells on round islands with varying diameters and areas, and also islands with the same area but varying aspect ratios. Results indicated that as spreading area and diameter increased, the proportion of dedifferentiated chondrocytes increased and the proportion of normal chondrocytes decreased. In addition, for cells grown on islands with the same area but different aspect ratios, the proportion of de-differentiated chondrocytes increased with increasing aspect ratio, indicating that cell morphology has the ability to cause differentiation.

Another technique used to change morphology on a 2D surface is changing the distance between the cell adhesion molecules, such as RGD, which is known as 'nanospacing.' RGD is an amino acid sequence that was found to be part of many ECM molecules, and known to participate in cell integrin binding. It is used most commonly in *in vitro* studies due to its presence in so many ECM molecules. Wang and colleagues conducted a study that focused on the possibility of nanospacing having a direct effect on differentiation of stem cells. They cultured MSCs on PEG surfaces that had RGD ligands printed at specific distances from each other, and these distances were varied to change cell shape. The idea was that the further the RGD spacing was to each other, the more rounded the cells would be, because neighboring RGD would be too far to reach. Their results illustrated that as the spacing of RGD nanopatterns increased, cell

spreading decreased. Results also showed that adipogenesis of the cells increased as the nanospacing increased (Wang, et al, 2013).

In a study by Lee, et al. (2013), they showed that cell morphology could have an effect on differentiation of cells grown on a substrate with a matched elastic modulus of two different tissues. When cells were allowed to spread on a soft substrate, they showed higher levels neurogenic markers, whereas cells that were confined to a small area showed higher levels of adipogenic markers. Because it is known that elastic modulus can have an effect on cell behavior, and even influence cell morphology, it is important to eliminate that as a potential factor. Therefore, our study will look at cell morphology independently of elastic modulus, doing so by using a model with a similar elastic modulus to that of normal adipose tissue.

All of these studies showed that cell shape has the potential to regulate differentiation of cells, but one major downfall of each study is that they were conducted in 2D, unlike the 3D *in vivo* environment. There are very few studies that look at cell morphology in a 3D environment independently from elastic modulus. A study from the Burdick lab indicates that cell morphology can in fact regulate cell differentiation regardless of initial elastic modulus of the 3D hydrogel. Comparing a degradable and non-degradable hydrogel system, they demonstrated that cells show favor towards osteogenesis when presented with degradable hydrogels and allowed to spread, and favored adipogenesis when cells remained rounded and could not displace the matrix, regardless of initial elastic modulus of the gel (Khetan, et al, 2013).

For this experiment, Adipose-derived Stem Cells (ASCs) will be used. ASCs are multipotent cells that have been shown to have great potential in the field of tissue engineering. The discovery of these cells resulted in an increased appreciation for white adipose tissue (WAT) as a valuable source of adult stem cells with the same potency as mesenchymal stem cells for use in regenerative medicine. These cells are advantageous because they are abundantly present in WAT, where they make up as much as 1% of human adipose cells, as compared to only 0.001-0.002% of MSCs in bone marrow (Fraser, et al, 2006). ASCs can also be harvested with a minimally invasive process by liposuction from subcutaneous fat deposits (Ong and Sugii, 2013).

Because these cells offer advantages over other cell types, they are a good candidate to use to the development of an in vitro model of adipose tissue. These cells have the ability to be differentiated towards an adipogenic lineage. Mature adipocytes are terminally differentiated cells, and are considered incapable of division (Hausman, et al., 2001). It is suggested that ASCs participate in adipogenesis during excessive adipose tissue development, and are responsible for the increase in cell number; the number of ASCs in subcutaneous adipose tissue has been shown to be lower in people with certain types of obesity, while the number of mature adipocytes and progenitor cells are increased. (Maumus, et al., 2013).

The focus of the study that follows is to understand how adipogenesis can be influenced by cell morphology when all other conditions are kept constant. The aim of the study is to change cell morphology by using different amounts of RGD

peptide within the alginate gels, and cause a difference in cell differentiation by changing cell morphology. It is hypothesized that changing the amount of peptide within the 3D alginate gels with change cell morphology. Cells must have the ability to sense their surrounding environments (i.e. bind to peptide), but adipogenesis may be affected by the amount of peptide present. Therefore, the amount of peptide that leads to rounder cell morphology will enhance adipogenesis

To do this, we are using the alginate hydrogel system that was previously mentioned. Using this system, different amounts of RGD binding peptide were incorporated into the gels. We then investigated the influence of RGD concentration on cell morphology and adipogenic potential of the encapsulated cells. Using the alginate system, the cells will be encapsulated in gels with RGD, an amount that was previously described by our lab (Marturano, in prep), 50% of the original RGD, and CTRL gels with no RGD. We aim to show the encapsulated ASCs must be able to sense their surrounding environment, but are also sensitive to the amount of binding peptide present

3.2 Materials and Methods

3.2.1 List of Solvents and Chemicals

Table 1: A list of all chemicals used for experiment with ASCs

Chemical Name	Supplier
Alexa Fluor 488 dye	Life Technologies
Antibiotic-Antimycotic 100x (AA)	Invitrogen
Biotin	Sigma Aldrich
Brilliant II SYBR Green qPCR Master Mix	Aligent Technologies
Calcium Carbonate ($CaCO_3$)	Sigma Aldrich
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Life technologies
Dexamethasone (Dex)	Sigma Aldrich
DMEM/Ham's F12K (DMEM/F12K)	Gibco
Fetal Bovine Serum (FBS)	Gibco
D-(+)- Gluconic acid Delta-Lactone (GDL)	Sigma Aldrich
Hank's Balanced Salt Solution (HBSS)	Gibco
Human Insulin 10 mg/mL	Sigma Aldrich
Hoechst 33258 dye	Invitrogen
Isobutyl methylxanthine (IBMX)	Sigma Aldrich
RGD Peptide sequence Arginine, Glycine, Aspartic Acid (RGD)	Peptides international
Panthothenate	Sigma Aldrich
Pronova Alginate	NovaMatrix

Thiazolidinedione	Sigma Aldrich
Triglyceride determination kit	Sigma Aldrich
Tris-HCl	Sigma Aldrich
TRIzol LS	Invitrogen
0.025% Trypsin-EDTA	Gibco

3.2.2 Materials

Table 2: A list of all the materials used for experiment with ASCs

Material	Supplier
CL2 Centrifuge	Thermo Scientific
Confocal Microscope	Leica Microsystems
Microcentrifuge	Thermo Scientific
Microscope	Nikon Eclipse TE300
Microscope Camera	Hamamatsu Camera C4742-95
MX3000p qPCR System	Aligent Technologies
SpectraMax Gemini EM, fluorescence microplate reader	Molecular Devices
Thermocyler	Eppendorf
24-well Non-Tissue Culture Plates	BD-Falcon

3.2.3 Medium

3.2.3.1 Growth Medium

Growth Medium	% Volume
DMEM/F12K	89%
FBS	10%

AA	1%

3.2.3.2 Adipogenic Medium

Adipogenic Medium	% Volume
DMEM/F12K	92.887%
FBS	3%
AA	1%
0.5M IBMX	1.099%
Insulin 10 mg/mL	0.059 %
10nM Dex	0.1%
TZD	0.1%
Biotin	0.055%
Pantho	1.7%

3.2.3.3 Maintenance Medium

Maintenance Medium	% Volume
DMEM/F12K	94.087%
FBS	3%
AA	1%
Insulin 10 mg/mL	0.059 %
10nM Dex	0.1%
Biotin	0.055%
Pantho	1.7%

3.2.4 ASC culture

Primary human ASCs used in this study were isolated from subcutaneous adipose tissue samples harvested from the abdomen of three healthy adult female donors (body mass index (BMI) range: 21 to 27; age range: 40 to 59). ASCs were obtained from existing stores and were de-identified and, therefore, were not considered human research subjects and did not require ethics approval; donors provided written informed consent for the collection of the adipose tissue. Cells

were plated at about 5000 cells/cm^2 and cultured in ASC growth medium. Cells were passaged at 90% confluent. Cells were not used beyond passage number 4 (Handel, et al., 2012).

3.2.5 Alginate preparation

Alginate was prepared and functionalized as previously described by work in our lab (Marturano, et al., *in prep.* 2014). Alginate was suspended at 1% (w/v) in 0.3 M 4-morpholineethanesulfonic acid (MES) and 0.1 M NaCl, adjusted to pH 6.5 with NaOH. Alginate is functionalized with GGGGRGDSP peptides (RGD) using the carbodiimde coupling method of Rowley et al. (1999). *N*-hydroxysulfosuccinimide (NHS), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and RGD peptides were dissolved sequentially in mass ratios of 0.88, 1.64, and 0.03 relative to alginate, respectively. This reaction proceeded for 20h at 22°C with stirring until termination by addition of hydroxylamine HCL (HA) at a 0.043 mass ratio relative to alginate. Alginate solutions were dialyzed (3500 Da cut-off) for 72 h in distilled water. After treatment with activated charcoal, the alginate was passed through a 0.2 μm filter, lyophilized, and re-suspended at 5% (w/v) in HBSS.

There are three different conditions of alginate in this study. Alginate with RGD is at the same ratio described by Marturano, et al., and given the designation of "RGD." Non-functionalized alginate was designated as "CTRL." A third alginate condition was formed by mixing 1:1 of RGD and CTRL alginates, and designated as "RGD/2," and has 50% less RGD than the "RGD" condition.

3.2.6 Gel fabrication

Cells were grown to confluence and cultured for 2 days. On the second day, cells were encapsulated in alginate hydrogels, according to the protocol described by Kuo and Ma (2001). First, a $CaCO_3$ in HBSS suspension was mixed into the alginate to yield a 2X Ca^{2+} content, where "X Ca^{2+} Content" represents the molar ratio between added $CaCO_3$ and COOH groups on the alginate polymer. Next, ASCs were trypsinized and re-suspended in HBSS for either a final cell seeding density of $1.5x10^6$cells/mL or $5x10^6$ cells/mL. A fresh 21.4% (w/v) GDL solution in HBSS was then added in a 1:2 molar ratio of $CaCO_3$ to GDL. The final alginate polymer concentration was 1.5% (w/v). A 55 μl volume of alginate-cell solution was pipetted into custom PDMS molds and allowed to cross-link for 2 hours at 37° C. All three condition alginate gels (RGD, RGD/2, and CTRL) were transferred into 24-well plates and cultured in growth medium overnight in a standard cell incubator at 37° C. The percentage of alginate polymer concentration and amount of calcium used was determined by previous work that determined the elastic moduli of the alginate gels (Marturano, in prep). The amount of each used in this experiment was measured to be around 3 kPa, which falls within the normal range of adipose tissue.

3.2.7 Differentiation of ASCs in alginate gels

After gel fabrication, gels were placed in wells with growth medium overnight. The following day, medium on gels was either replaced with adipogenic induction medium (AIM), or new growth medium for a total of 2 mL.

All gels were given half medium changes (1 mL was removed and 1 mL of fresh medium was added) every other day. On day 9, the induction medium was removed from the gels, and adipogenic maintenance medium was added. Growth medium was also replaced. All gels received half medium changes every other day for the remainder of the experiment.

3.2.8 Actin cytoskeletal imaging

To visualize the morphology of cells, gels were fixed with 4% paraformaldehyde, and stained with both AlexaFluor 488-phalloidin and DAPI for actin visualization and cell nuclei, respectively. Gels were mounted in glass-bottomed petri dishes and imaged on a Leica TCS SP2 inverted confocal microscope (Leica Microsystems, Buffalo Grove, IL) with a 63X objective.

Circularity of the cells was quantitatively analyzed using the circularity function in ImageJ (NIH, Bethesda, MD) to measure the area-to-perimeter ratio of the actin cytoskeleton. Circularity was defined by equation 1:

$$C = 4\pi\left(\frac{A}{P^2}\right)$$

where C is circularity, A is the area, and P is the perimeter of the cell. A perfectly smooth circle has a circularity measurement of "1". Circularity was calculated from 9 different gels per condition, and 3 different images from each gel.

In addition, each individual data point for each replicate was plotted on a scatter plot to compare across replicates to investigate if differences in circularity were a result of the different conditions, and not a result of differences in

biological replicates.

3.2.9 Quantitative-PCR

Gels were harvested on days 1, 7 and 14. They were homogenized in TRIzol LS reagent and total RNA was extracted according to chloroform extraction protocol. RNA concentration was quantified by spectrophotometry using a Nanodrop ND 2000. Reverse transcription was performed using Superscript III First-Strand Synthesis System, with 2 μg total RNA reverse transcribed with oligo(dT) primers. qPCR was performed with Brilliant II SYBR Green qPCR Master Mix and the MX3000p qPCR System Primer pairs were designed and optimized for qPCR analysis of expression levels of human 18S (forward 5′-gactcaacacgggaaacctcacc-3′; reverse 5′-accagacaaatcgctccaccaact-3′), PPARγ (forward 5′-aatgccatcaggtttgggcgga-3′; reverse 5′-cgccctcgcctttgctttgg-3′) and FABP4 (forward 5′ -tgataaactggtggtggaatgcgtc-3′; reverse 5′-ctctctcataaactctcgtggaagtg-3′) . Expression levels relative to 18S gene were calculated using the delta-delta cycle threshold method.

3.2.10 Triglyceride analysis

Metabolic analysis of triglyceride content was conducted on the cell lysates. On days 1 and 14, gels were weighed and stored in 400 μL of SDS solution (0.1% SDS, 1mM EDTA, 100mM Tris-HCl, pH 7.4) and frozen at -80°C until analysis. Previously frozen samples were thawed, homogenized and sonicated using 60% amplification for ten seconds. Triglyceride content of the supernatant was measured using a triglyceride determination kit, which analyzed the release of glycerol from triglycerides by lipoprotein lipase. Triglyceride values were normalized to total DNA content using Hoechst 33258 dye.

3.2.11 DNA content

DNA content of the samples was conducted by staining an aliquot of the cell lysates from days 1 and 14 with Hoechst dye. Fluorescence values for these samples were acquired on a fluorescence microplate reader using an excitation wavelength of 365 nm and an emission wavelength of 458 nm. These values were then converted to total DNA content by comparing the fluorescence values to a standard curve of fluorescence readings generated from solutions with known DNA concentrations.

3.2.12 Statistical Analysis

A one- or two-way ANOVA with Tukey's post hoc test or a t-test, and $p < 0.05$ was used to evaluate statistical significance. All statistical calculations were performed with Graphpad (GraphPad Software Inc, San Diego, CA).

Additional statistical analysis was performed on the circularity data. The data was subjected to a cross-validation "leave-one-out" analysis. A data point from each experiment replicate was randomly excluded from each N (each biological replicate; a total of 3 excluded data points). This was done 20 different times, each time at random, producing a different combination of excluded points. The data points for each N were then averaged, producing a new N=3 for the measurements. All 20 of the new N=3 points were plotted, and then compared to the original values for the circularity measurements. An ANOVA was run to compare all the new points to the original values, as well as to every other new data set. This was done for every set of measurements on days 1, 7, and 14, for each gel condition and medium condition.

3.3 Results

3.3.1 Preliminary ASC density test

ASCs were encapsulated in alginate gels at two different cell densities, to compare how that would affect adipogenesis. Table 3 lists experimental conditions. Gels were harvested on days 1, 7 and 10 for PCR gene expression analysis.

Table 3. Experiment conditions for preliminary density experiment with ASCs.

Gel Elastic Modulus (kPa)	Gel Condition	Cell density (M/mL)	Medium
3	CTRL	1.5	Growth
3	CTRL	5	Growth
3	CTRL	1.5	Adipo
3	CTRL	5	Adipo
3	RGD	1.5	Growth
3	RGD	5	Growth
3	RGD	1.5	Adipo
3	RGD	5	Adipo

Figure 3A shows PPARγ gene expression for ASCs cultured in alginate gels for 10 days. On day 10, cells cultured at a seeding density of 1.5M/mL with RGD show the highest expression of PPARγ. All gene expression is normalized to the condition of 1.5M/mL, cultured in growth medium without RGD. The same condition shows highest PPARγ gene expression when compared to only conditions grown in adipogenic medium, normalized to the condition of 1.5M/mL, grown in adipogenic medium without RGD (Figure 3B). As a result of this experiment, further experiments were conducted with a seeding density of 1.5M/mL.

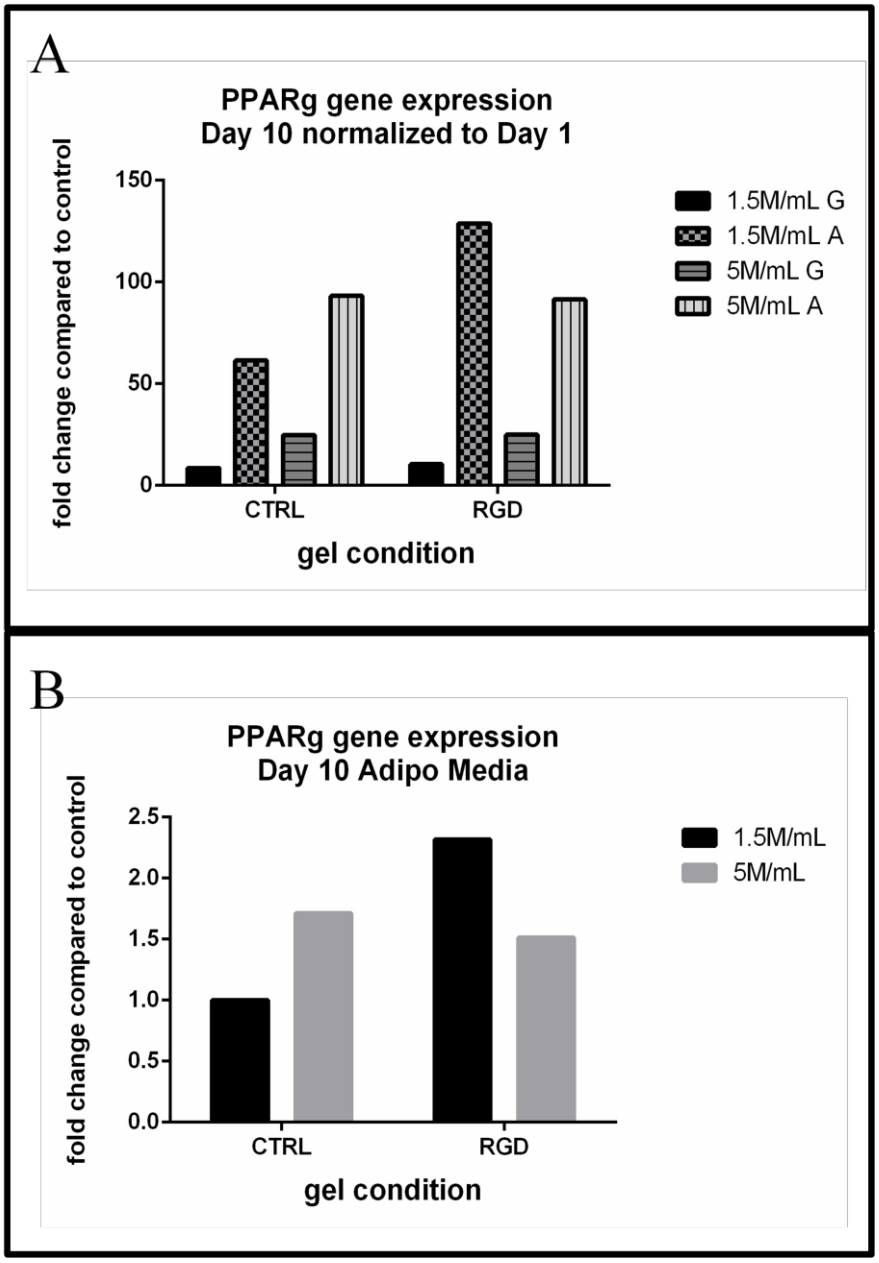


Figure 3. **Gene expression of ASC density experiment.** A) Graph shows PPARγ gene expression comparing ASCs seeded in gels at 1.5 M/mL and 5 M/mL, with and without RGD, and with growth and adipogenic medium. They are normalized to day 1 CTRL gels, seeded at 1.5 M/mL. The cells seeded at 1.5 M/mL with RGD and adipogenic medium have the highest gene expression. B) Gene expression of both seeding densities comparing RGD and CTRL conditions, all grown in adipogenic medium, normalized to day 10, 1.5M/mL CTRL group. Cells seeded at 1.5 M/mL with RGD have the highest PPARγ gene expression. G = Growth Medium; A = Adipogenic Medium

3.3.2 The effect of cell morphology on adipogenesis

ASCs were encapsulated in alginate gels with all the same elastic modulus of 3 kPa, with a seeding density of 1.5M/mL. The elastic modulus of the gels is representative of the elastic modulus of normal adipose tissue. Table 4 lists experimental conditions. Cells were cultured for 14 days. Samples were for harvested for PCR gene expression and actin cytoskeleton imaging on days 1, 7 and 14, and harvested on days 1 and 14 for TG and DNA content analysis

Table 4. Experiment conditions for ASC morphology experiment

Gel elastic modulus (kPa)	**Gel condition**	**Cell density (M/mL)**	**Medium**
3	CTRL	1.5	Growth
3	CTRL	1.5	Adipo
3	RGD/2	1.5	Growth
3	RGD/2	1.5	Adipo
3	RGD	1.5	Growth
3	RGD	1.5	Adipo

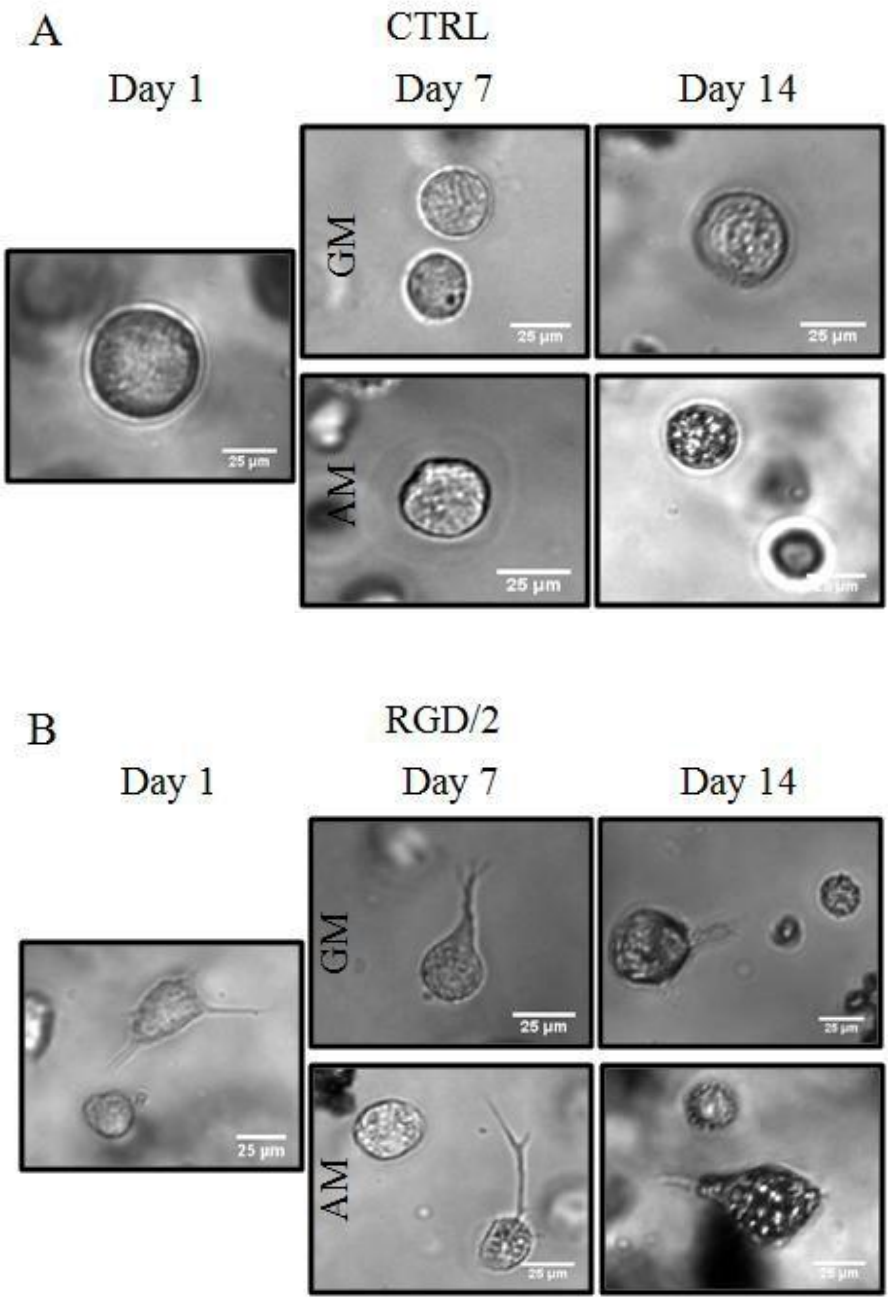
3.3.2.1 Image analysis for morphology and circularity

Cells in the gels were imaged with bright-field microscopy to visualize cell morphology. Figure 4 shows representative images for each condition. For the CTRL gels, cells are rounded throughout the 14 days due to the lack of RGD within the gel. These cells do not have the ability to bind to the gels. At the end of the 14 days, cells in the growth medium condition do not appear to have changed since the start of the experiment. Cells in the adipogenic medium condition have the same morphology throughout the 2 week period, but some of the cells have

visible lipids, as shown by the images. Lipids start to become visible by day 9 (Figure 4A).

Day 1 images were taken the following day after seeding the gels. Even after less than 24 hours, many cells in RGD/2 gels are spread. Many cells continue to be spread throughout the 14 days, but some are rounded. At day 7 in the adipogenic medium condition, what look like the beginning of lipids start to appear in some of the cells, and by day 9, visible lipids are present in some cells. By the end, many of the cells have larger lipids, even though most cells have not rounded back up. Cells with lipids seem to be less spread than those without lipids (Figure 4B)

Cells in the RGD gels are extremely spread, even by day 1 and look more spread than those cells is the RGD/2 gels. Most cells continue to be spread for the duration of the experiment. As with the other groups, lipids start to be visible in cells around day 9, and increase until the last day, even though cells were not rounded. Cells with lipids tended to be less spread than those without lipids (Figure 4C)


A
CTRL
Day 1
Day 7
Day 14
GM
AM
25 µm
B
RGD/2
Day 1
Day 7
Day 14
GM
AM
25 µm

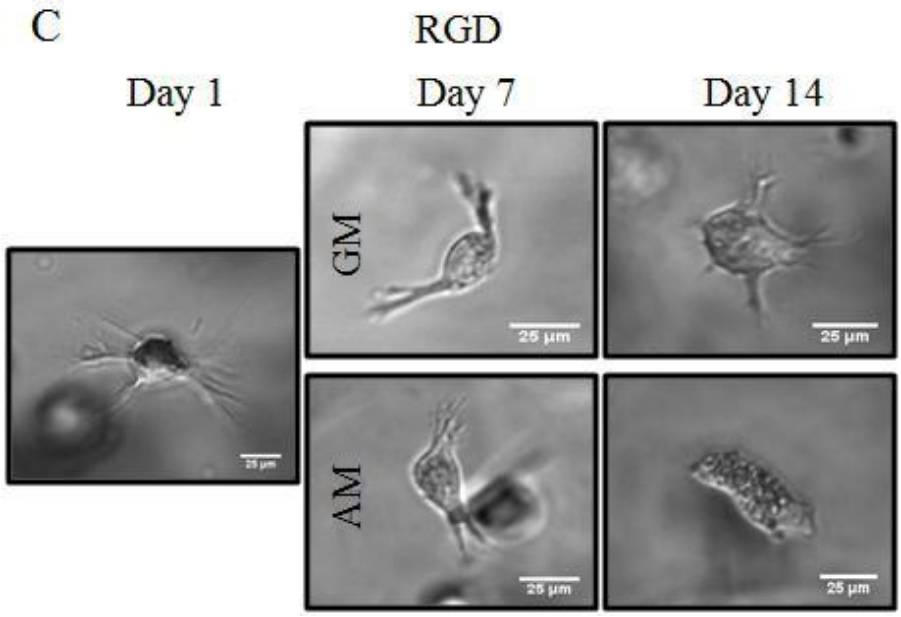


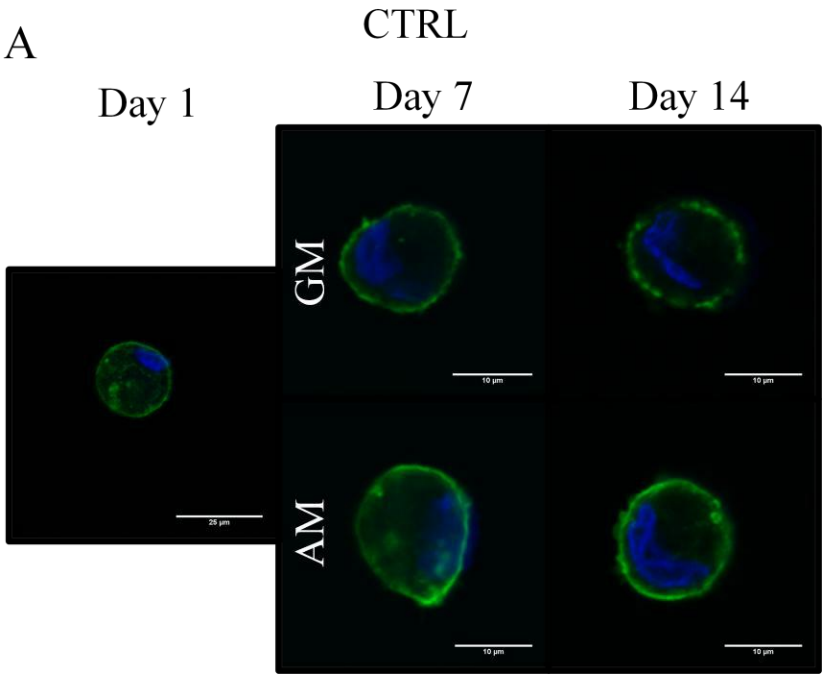
Figure 4. **Bright field images of ASCs in each gel condition.** A) Cells in CTRL condition are rounded due to lack of binding peptide. Cells continued to be rounded after 14 days. Lipids appear around day 9 in adipogenic medium. B) Cells in RGD/2 condition are spread, even after just 1 day. For the duration of the experiment, some cells are spread, and some are rounded. Lipids appear around day 9 in adipogenic medium. Cells with lipids tended to be less spread than those without lipids. C) Cells in RGD condition are very spread after 1 day. Majority of cells are spread, and continue this way over 14 days. Lipids appear around day 9 in adipogenic medium. Cells with lipids tended to be less spread than those without lipids.

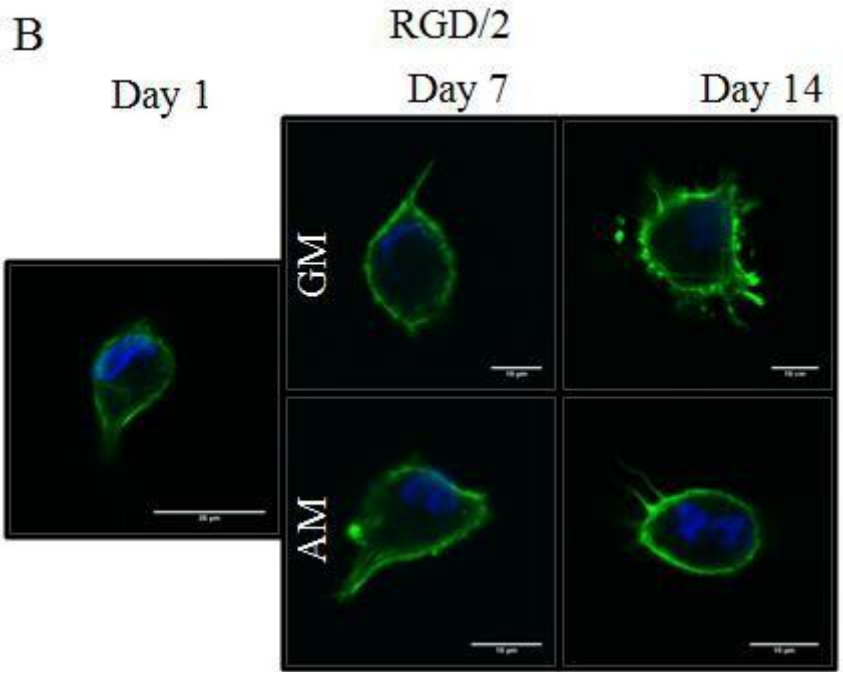
On days 1, 7, and 14, ASCs were stained to visualize the actin cytoskeleton and using those images, circularity of the cells was measured. Figure 5 is representative images for each condition and each time point for actin cytoskeleton imaging. In order to measure circularity using these images, they were processed into binary images. Figure 6 is a representation of how cell images look as a fluorescent image and then as a binary image in order to measure the circularity. Results of the circularity measurements can be seen in Figure 8. On day 1, the day after cell seeding, cells in CTRL gels are significantly more circular than those cultured in either of the RGD conditions. Cells cultured in the RGD/2 gels are significantly more rounded than those cells culture in the RGD gels (Figure 7A). On both days 7 and 14, cells cultured in CTRL condition are still significantly rounder than those cells cultured in either RGD condition, regardless of medium type. Cells are slightly rounder when cultured in the RGD/2 condition than those cultured in the RGD condition, but results are not significant (Figures 7B & C). There are no differences in circularity across the same condition (i.e. CTRL, RGD/2 or RGD) over 14 days, regardless of medium (Figure 8).

Each individual circularity data point was graphed on a scatter plot to ensure that there was similar distribution among biological replicates, and to show differences in circularity were due to the different gel conditions and not an effect of the different biological replicates. Figure 9 shows each repeat of the experiment (N=3; Exp 1, 2, and 3 represents each biological replicate) graphed on the scatter plot. Day 1 distribution shows circularity points are influence by gel conditions, and are not replicate dependent (Figure 9A). On days 7 and 14,

distribution among replicates shows dependence on gel condition, and not on the different replicates. As noted in a previous figure, circularity is more dependent on gel condition than medium type (Figure 9 B&C).

Additional statistical testing was performed on the circularity data to support validity of these data. A "leave-one-out" cross validation analysis was complete for all circularity measurements on days 1, 7, and 14, for both growth and adipogenic medium. This analysis is done by randomly removing a data point from each N, and then averaging the data points to obtain a new averaged value for circularity (a new N=3). This was done 20 different times. Figure 10 through 14 show the results. On day 1, for all three gel conditions, there are not significant differences between the original (when all data points from all biological replicates are included) circularity value and any of the new values (Figure 10). On day 7, there are no significant differences between the original circularity value and any of the new values, for both growth and adipogenic media (Figure 11&12). On day 14, the same results hold true. There are no significant differences between the original value and any of the new circularity values that were obtained, for both growth and adipogenic media (Figure 13&14).


A
CTRL
Day 1
Day 7
Day 14
GM
AM


B
RGD/2
Day 1
Day 7
Day 14
GM
AM

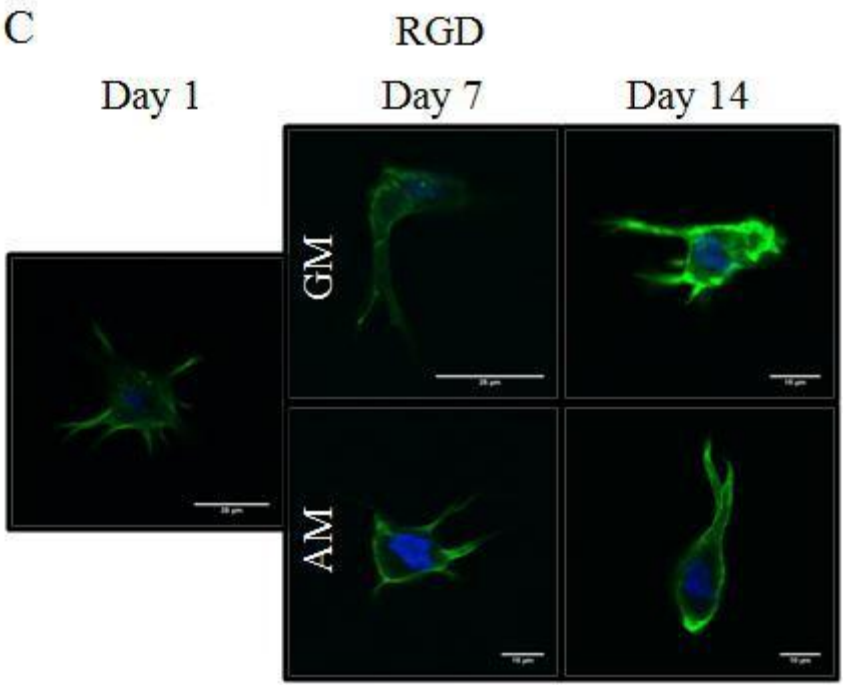


Figure 5. **Representative confocal images of ASCs in each gel condition.**

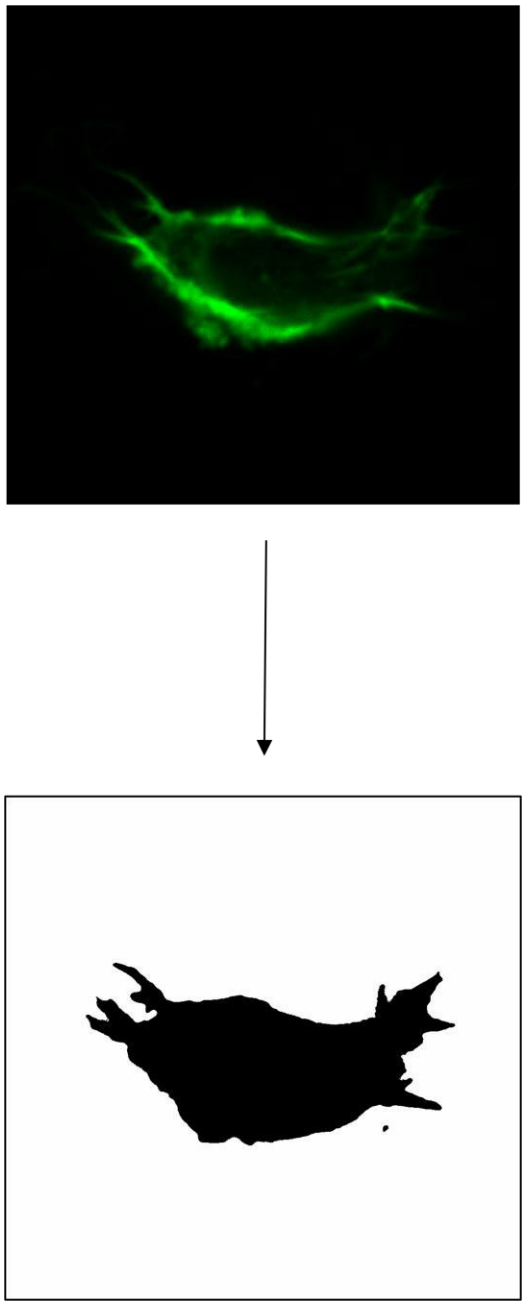


Figure 6. **Representative image of ASC showing how it is changed from confocal image to binary image in order to measure circularity**

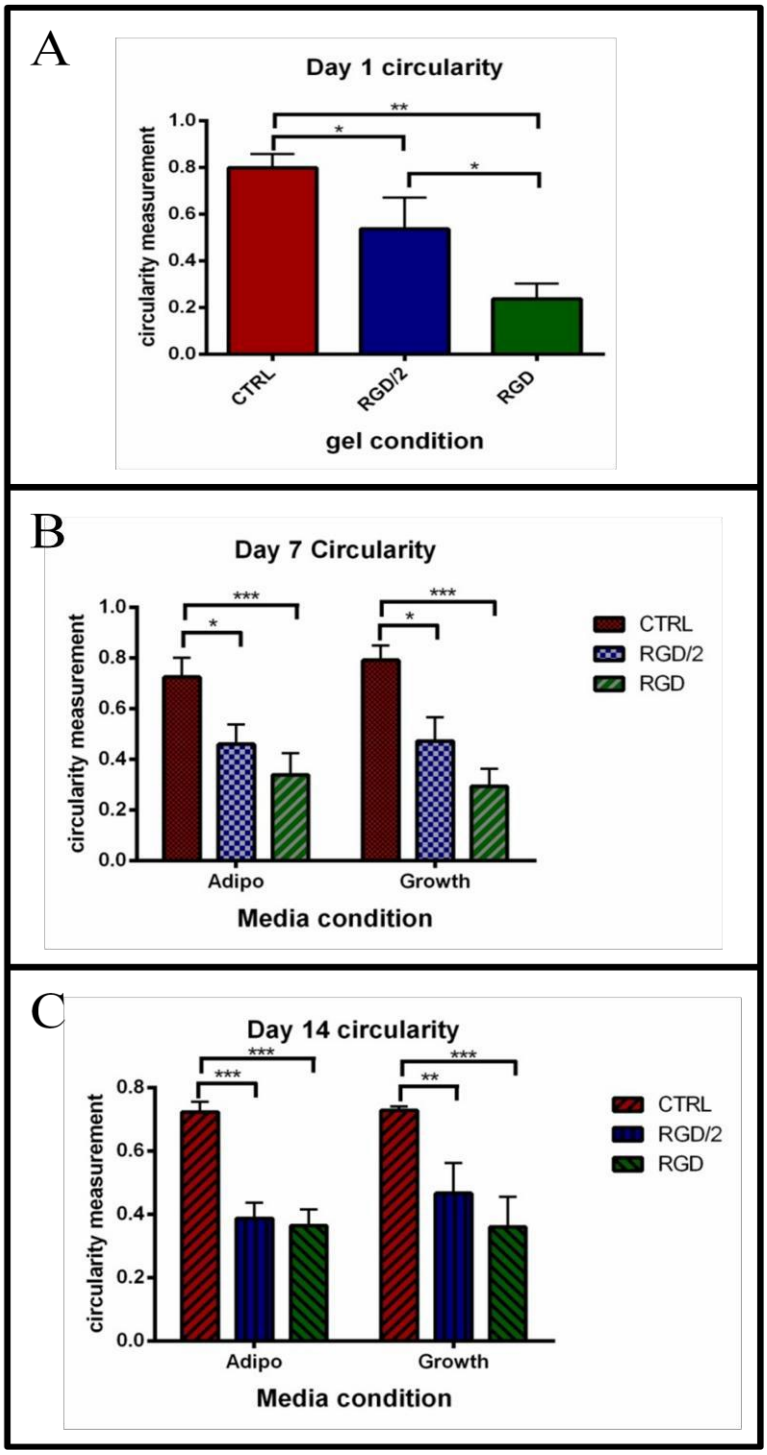


Figure 7. **Comparing circularity measurements for ASCs among different RGD concentrations and medium types at days 1, 7, and 14 (N=3, and each replicate represents an average of 5-10 individual cells). Circularity was determined by using ImageJ program, where 1.0 was assessed to be a perfect circle.** A) At day 1, the circularity of cells in each type of gel is significantly different from those is all other types. B) At day 7, cells in CTRL gels are significantly rounder than cells in both RGD/2and RGD gels. C) At day 14, cells in CTRL gels are significantly rounder than cells in both RGD/2 and RGD gels. * = $p<0.05$; ** = $p<.01$; *** = $p<0.001$

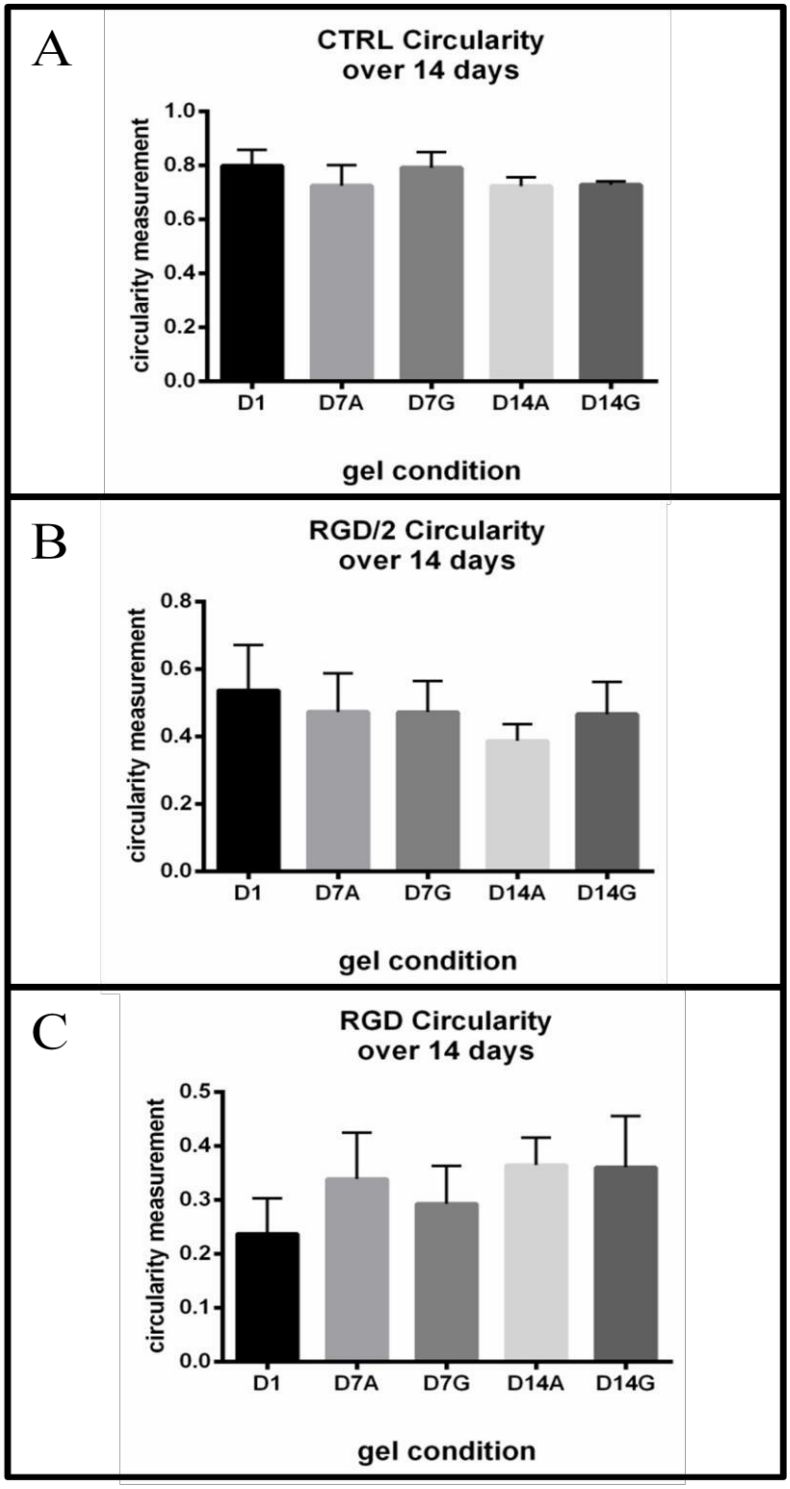


Figure 8. **Comparing circularity measurements for ASCs over time for each gel condition (N=3, and each replicate represents an average of 8-10 individual cells).** A) Circularity does not change over time for cells in CTRL gels. B) Circularity does not significantly change over time for cells in RGD/2 gel condition C) Circularity appears to increase slightly over time, but no significant changes. D = day; A = adipogenic medium; G = growth medium

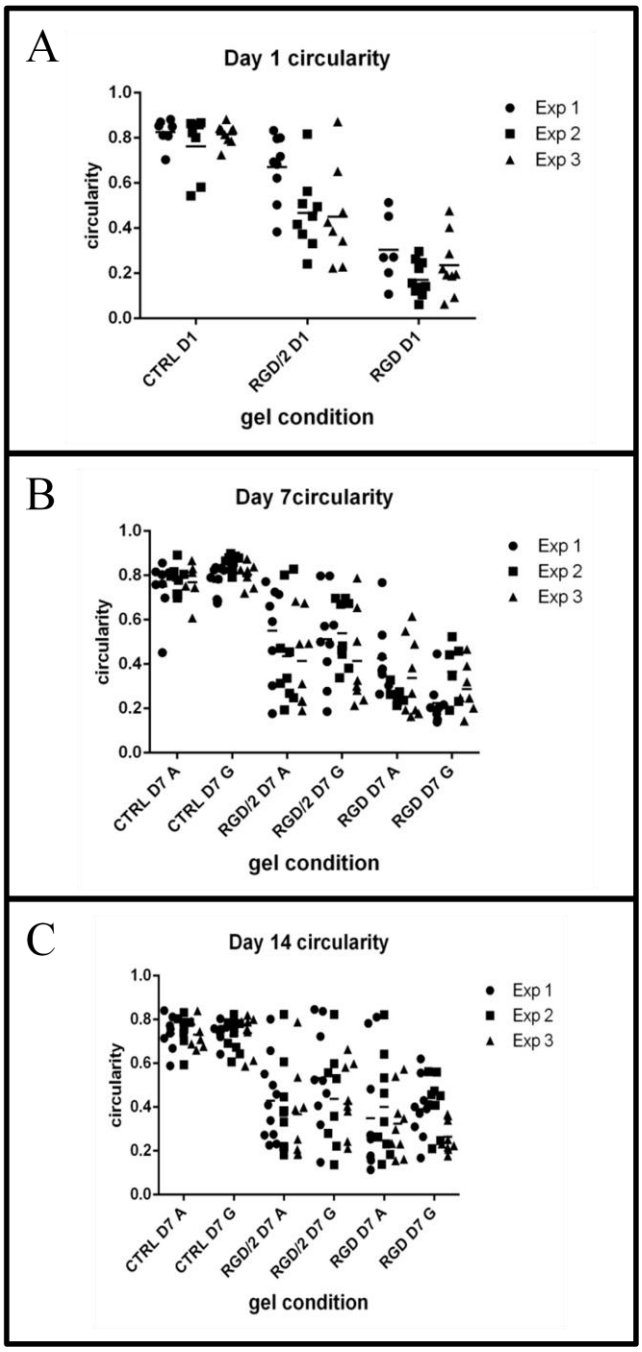


Figure 9. **Scatter plots showing each individual data points for N=3 replicates for days 1, 7, and 14, for each gel condition. Exp 1, 2, and 3 represents each of the biological replicates conducted.** A) Scatter plot for day 1 shows individual data point distribution. Random distribution is seen among the different biological replicates. B) Scatter plot for day 7 shows individual data point distribution. Random distribution is seen among the different biological replicates. C) Scatter plot for day 14 shows individual data point distribution. Random distribution is seen among the different biological replicates. D = day; A = adipogenic medium; G = growth medium

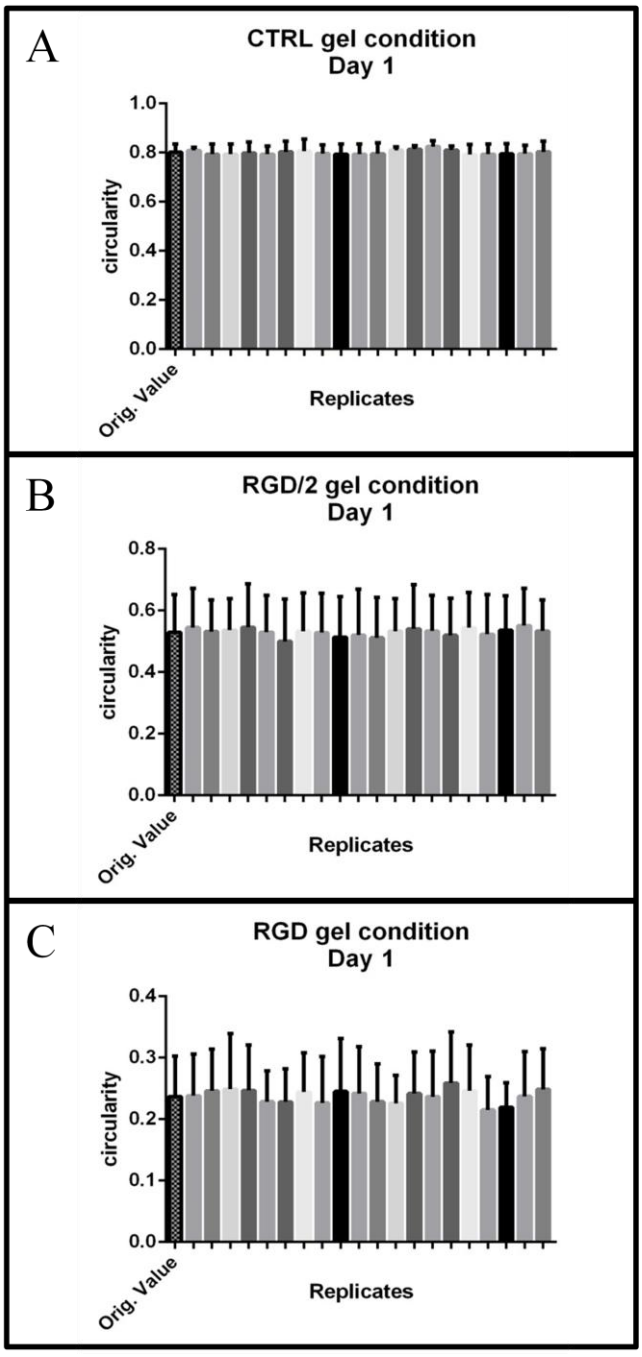


Figure10. **"Cross validation" analysis for each gel condition on day 1.** A) Circularity for CTRL condition on day 1. There are no significant differences among the new values compared to the original value. B) Circularity for RGD/2 condition on day 1. There are no significant differences among the new values compared to the original value. C) Circularity for RGD condition on day 1. There are no significant differences among the new values compared to the original value.

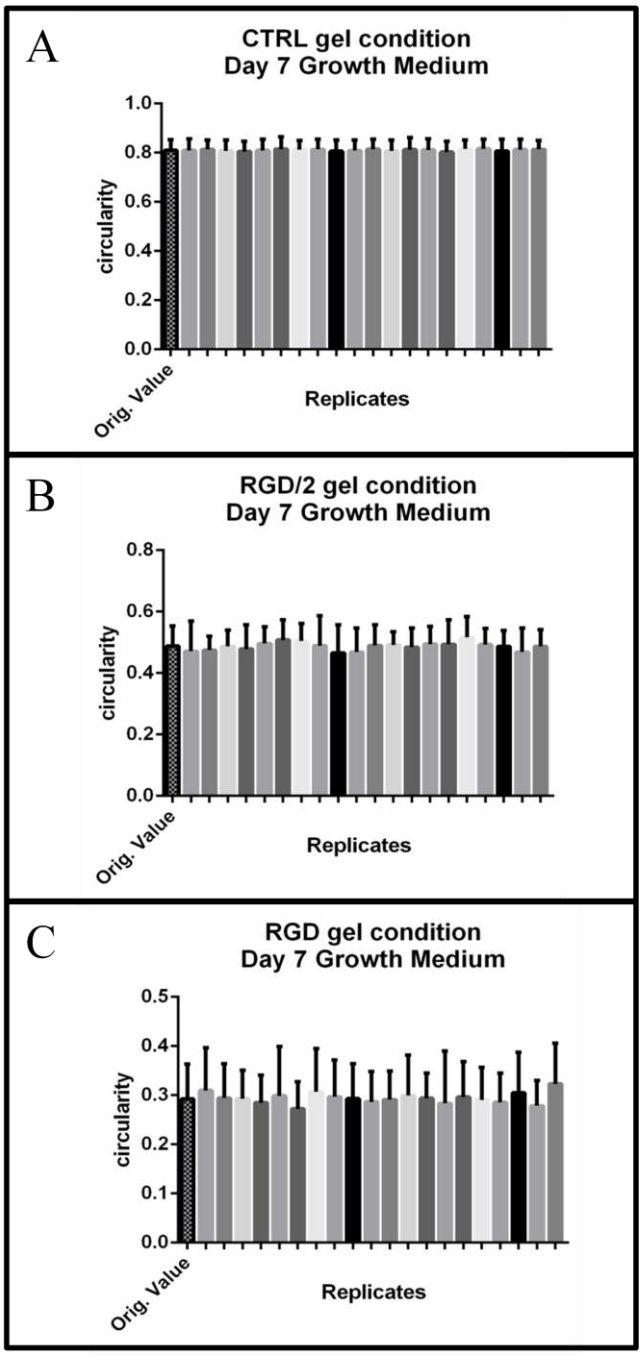


Figure 11. **"Cross validation" analysis for each gel condition on day 7 with growth medium.** A) Circularity replicates for CTRL condition with growth medium on day 7. There are no significant differences among the new values compared to the original value. B) Circularity replicates for RGD/2 condition with growth medium on day 7. There are no significant differences among the new values compared to the original value. C) Circularity replicates for RGD condition with growth medium on day 7. There are no significant differences among the new values compared to the original value.

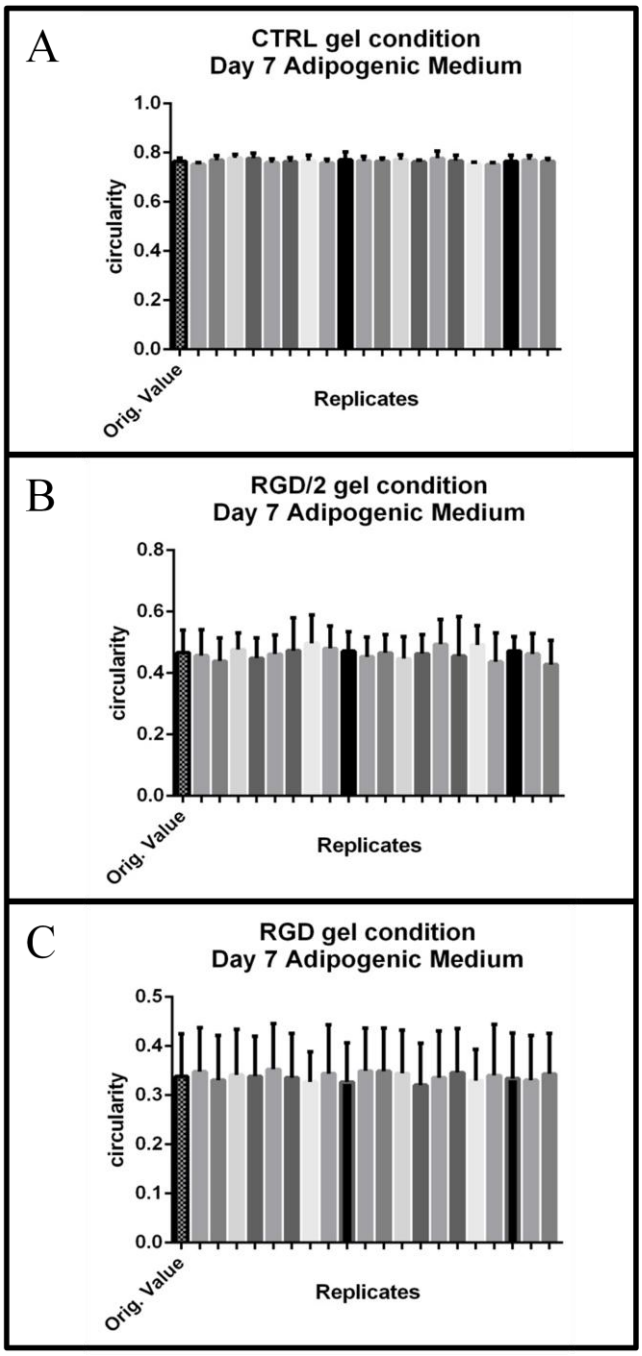


Figure 12. **"Cross validation" analysis for each gel condition on day 7 with adipogenic medium.** A) Circularity replicates for CTRL condition with adipogenic medium on day 7. There are no significant differences among the new values compared to the original value. B) Circularity replicates for RGD/2 condition with adipogenic medium on day 7. There are no significant differences among the new values compared to the original value. C) Circularity replicates for RGD condition with adipogenic medium on day 7. There are no significant differences among the new values compared to the original value.

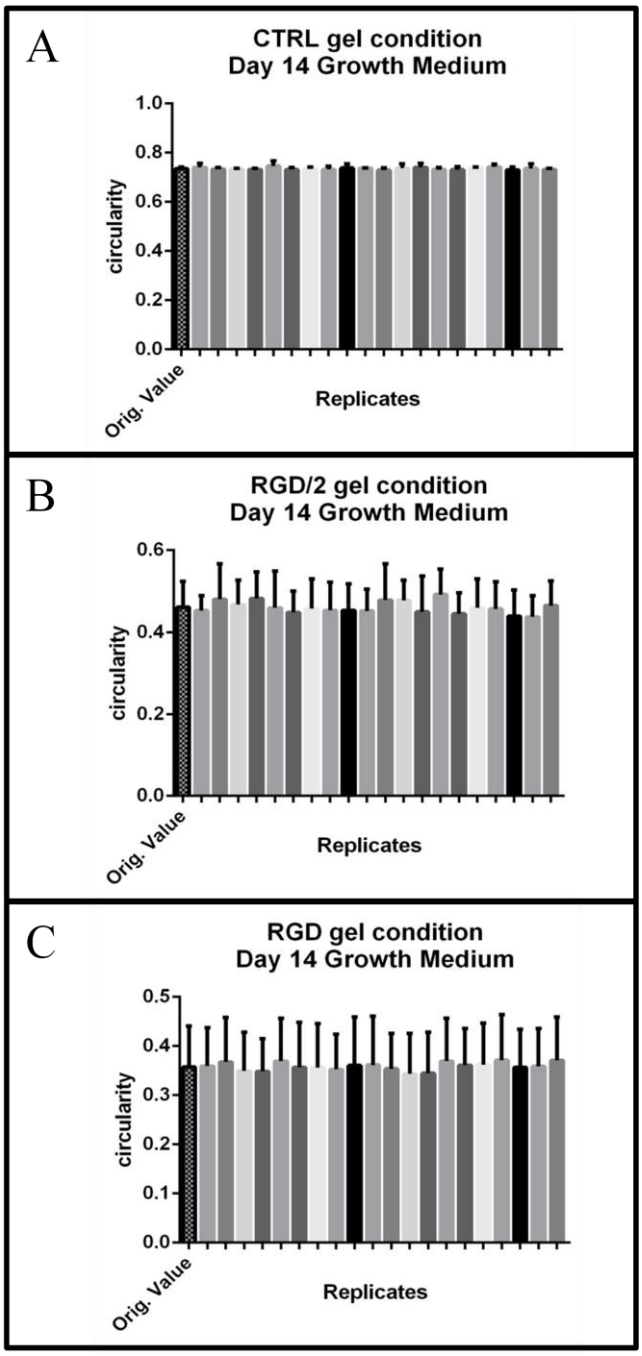


Figure 13. **"Cross validation" analysis for each gel condition on day 14 with growth medium.** A) Circularity replicates for CTRL condition with growth medium on day 14. There are no significant differences among the new values compared to the original value. B) Circularity replicates for RGD/2 condition with growth medium on day 14. There are no significant differences among the new values compared to the original value. C) Circularity replicates for RGD condition with growth medium on day 14. There are no significant differences among the new values compared to the original value.

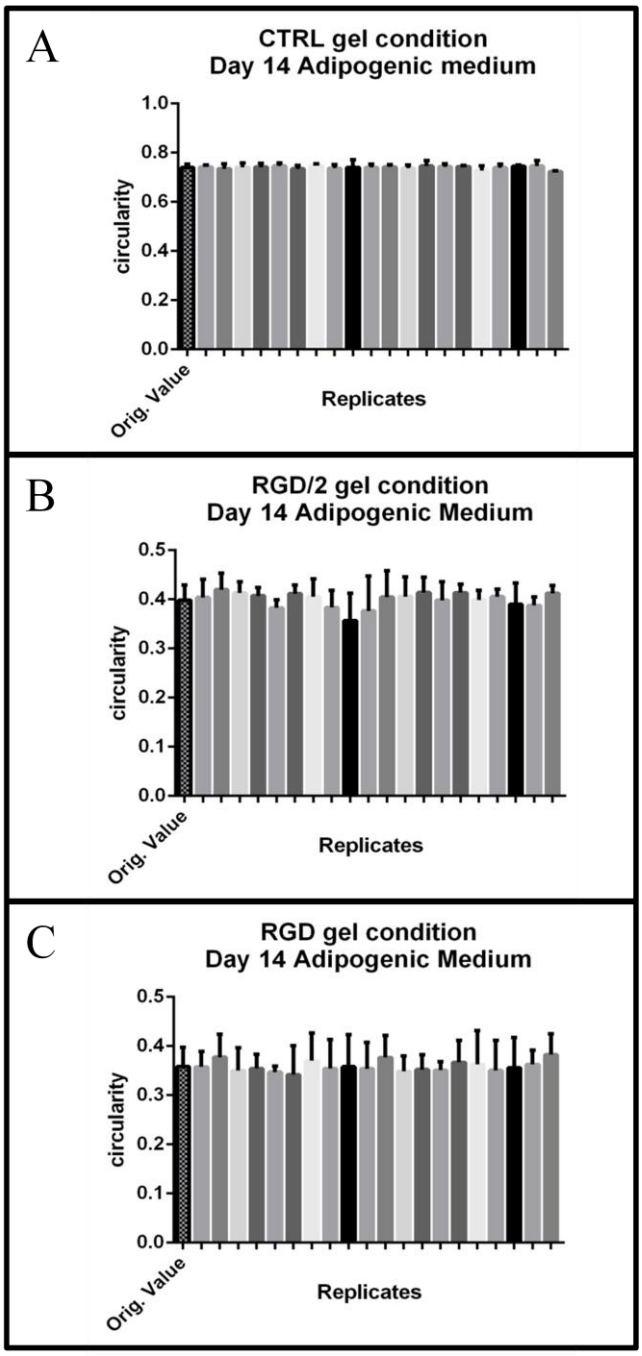


Figure 14. **"Cross validation" analysis for each gel condition on day 14 with adipogenic medium.** A) Circularity replicates for CTRL condition with adipogenic medium on day 14. There are no significant differences among the new values compared to the original value. B) Circularity replicates for RGD/2 condition with adipogenic medium on day 14. There are no significant differences among the new values compared to the original value. C) Circularity replicates for RGD condition with adipogenic medium on day 14. There are no significant differences among the new values compared to the original value.

3.3.2.2 DNA content

DNA content was measure by using Hoechst's fluorescent dye on days 1 and 14. Hoechst's dye preferably binds to the AT region of double stranded DNA. The amount of DNA was measured in micrograms of DNA and normalized per milligram of alginate gel. This is to account for any variation in gel size. When gels were grown in growth medium for the entire 2 week period, there was no significant difference in DNA amount between days 1 and 14, for all gel conditions (Figure 15A). However, when gels were cultured in adipogenic medium for 14 days, there is a slight decrease in DNA content on day 14 when compared to day 1 for all conditions, with RGD gels having a significant decrease in DNA (Figure 15B). On Day 14, gels cultured in growth medium had significantly more DNA than that those gels cultured in adipogenic medium, across all three gel conditions (Figure 15C).

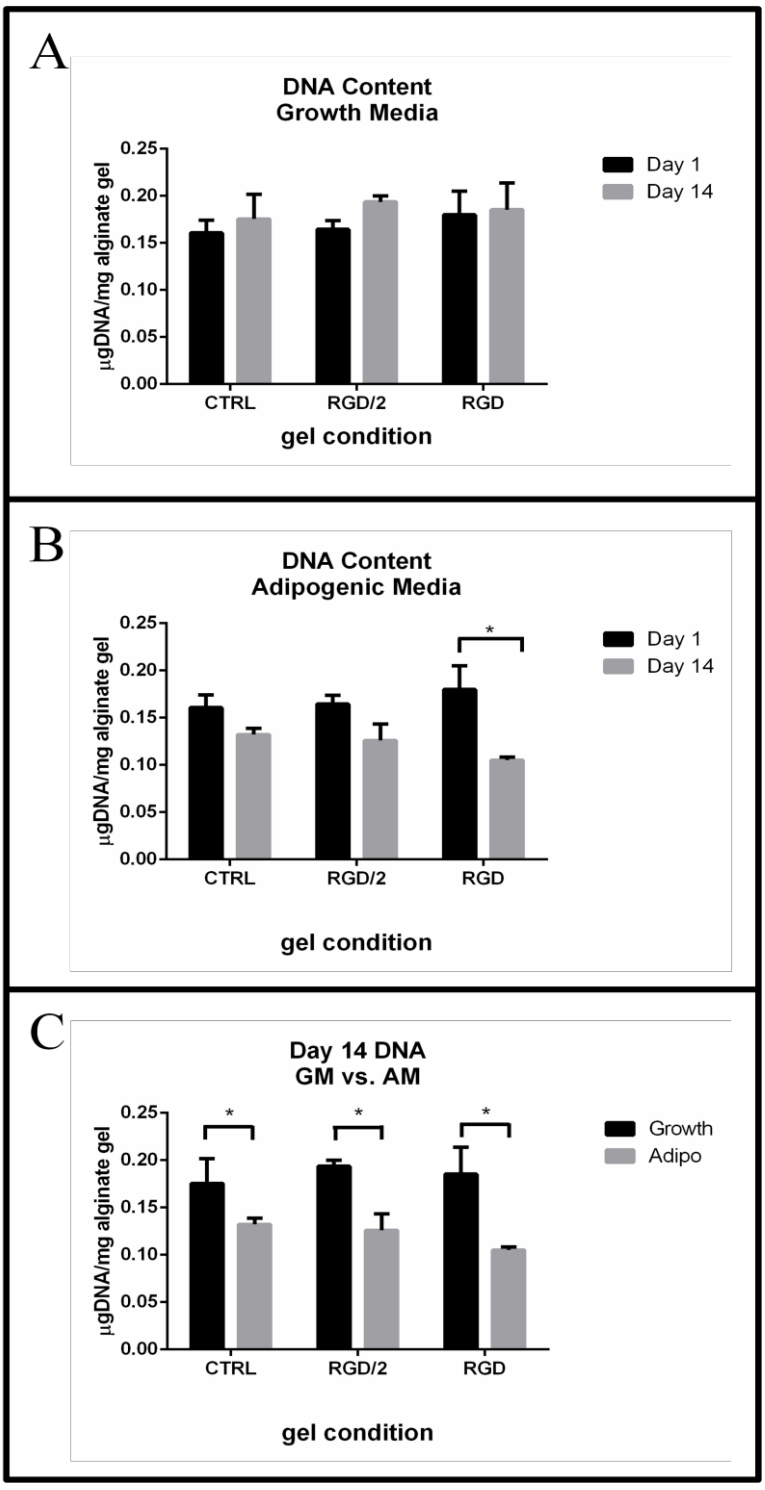


Figure 15. **Changes in DNA for ASCs as a result of medium type over 14 days using Hoechst's dye (N=3)**. A) Amount of DNA does not change after 14 when gels cultured in growth medium. B) After 14 days, cells in RGD condition gels, cultured in adipogenic medium, saw significant decrease of DNA, as compared to day 1. C) On day 14, gels is all three conditions showed significant decrease of DNA when grown in adipogenic medium as compared to the same gel type grown in adipogenic medium; * = $p<0.05$

3.3.2.3 Gene expression

On days 1, 7, and 14, gels were harvested for Quantitative-PCR. Results were graphed and normalized several different ways in order to understand every aspect that may be affected during the experiment. First, we were interested in how gene expression was influenced over the length of the experiment within each gel condition and medium type. Figures 16 through 21 look at how gene expression is influenced over the duration of the experiment when cells are grown in either growth or adipogenic medium. For CTRL gels, there is an increase in both PPARγ and FABP4 gene expression over 14 days when cultured in growth medium, and FABP4 gene expression is significantly higher than day 1 at both days 7 and 14 (Figure 16 A&B). Gene expression is increased for both PPARγ and FABP4 for RGD/2 gels cultured in growth medium. FABP4 gene expression is significantly higher than day 1 on both days 7 and 14, and gene expression for PPARγ on day 14 is significantly higher than that of both days 1 and 7. Day 7 is also significantly higher than day 1 (Figure 17 A&B). RGD gels also show an increase in PPARγ and FABP4 gene expression over 14 days, but only FABP4 expression on day 14 shows significant increase over RGD condition day 1 control (Figure 18 A&B).

For CTRL gels grown in adipogenic medium, there is an increase in both PPARγ and FABP4 gene expression over 14 days, however, only days 7 and 14 for FABP4 gene is this significantly increased, as compared to CTRL condition day 1 control (Figure 19 A&B). Cells grown in RGD/2 gels with adipogenic medium show significantly increased gene expression on day 14 for both PPARγ

and FABP4. Both have significantly more gene expression than that of RGD/2 day 1 control and day 7. Both genes have significantly more expression on day 7 than RGD/2 day 1 control (Figure 20 A&B). For cells in RGD gels, there is a significant increase in gene expression on days 7 and 14 for both PPARγ and FABP4, as compared to day 1. FABP4 gene expression on day 14 is also significantly higher than day 7 (Figure 21 A&B).

After looking at how time influences gene expression, we next were interested in how medium influenced adipogenesis on days 7 and 14 comparing growth and adipogenic medium. Conditions were normalized to their respective day 1 controls. Figure 22 looks at gene expression for just the CTRL gels. On day 7, both PPARγ and FABP4 show higher gene expression when cells are grown in adipogenic medium as compared to growth medium, with significant increase for FABP4 (Figure 22 A&C). The same trend is seen on day 14; there is higher gene expression when gels are cultured in adipogenic medium, but neither are significant (Figure 22 B&D).

For RGD/2 gels, day 7 shows significantly higher gene expression for both PPARγ and FABP4 when gels are cultured in adipogenic medium (Figure 23 A&C). On day 14, gene expression is also significantly higher for those gels cultured in adipogenic medium, as compared to growth medium, for both PPARγ and FABP4 genes (Figure 23 B&D). For RGD gels, there is significantly higher gene expression for PPARγ and FABP4 on day 7 for gels grown in adipogenic medium, as compared to gels cultured in growth medium (Figure 24 A&C). On day 14, both PPARγ and FABP4 have significantly higher gene expressions for

gels grown in adipogenic medium, as compared to growth medium (Figure 24 B&D).

Figure 25 shows how gel condition (i.e. CTRL, RGD/2, and RGD) influences gene expression at day 14 of the experiment, for both medium conditions. On day 14, when cells are cultured in growth medium, there is slightly more gene expression for both PPARγ and FABP4 for the RGD/2 gel condition, as compared to CTRL condition at day 14 with growth medium. There is, however, slightly less gene expression in the RGD condition, as compared to CTRL condition, for both genes. None of the results are significant (Figure 25 A&B). When cells are cultured in adipogenic medium, there is somewhat more gene expression in both RGD/2 and RGD gel conditions, as compared to CTRL condition with adipogenic medium, for both PPARγ and FABP4 (Figure 25 C&D). However, results are not significant.

3.3.2.4 TG Analysis

Samples were harvested on days 1 and 14 for triglyceride analysis assay. After doing the assay on each replicate, values for the samples were below the detectable range, and therefore, a quantitative lipid measurement was unable to be provided. Due to the nature of the cells in 3D, a lipid count was also unable to be performed.

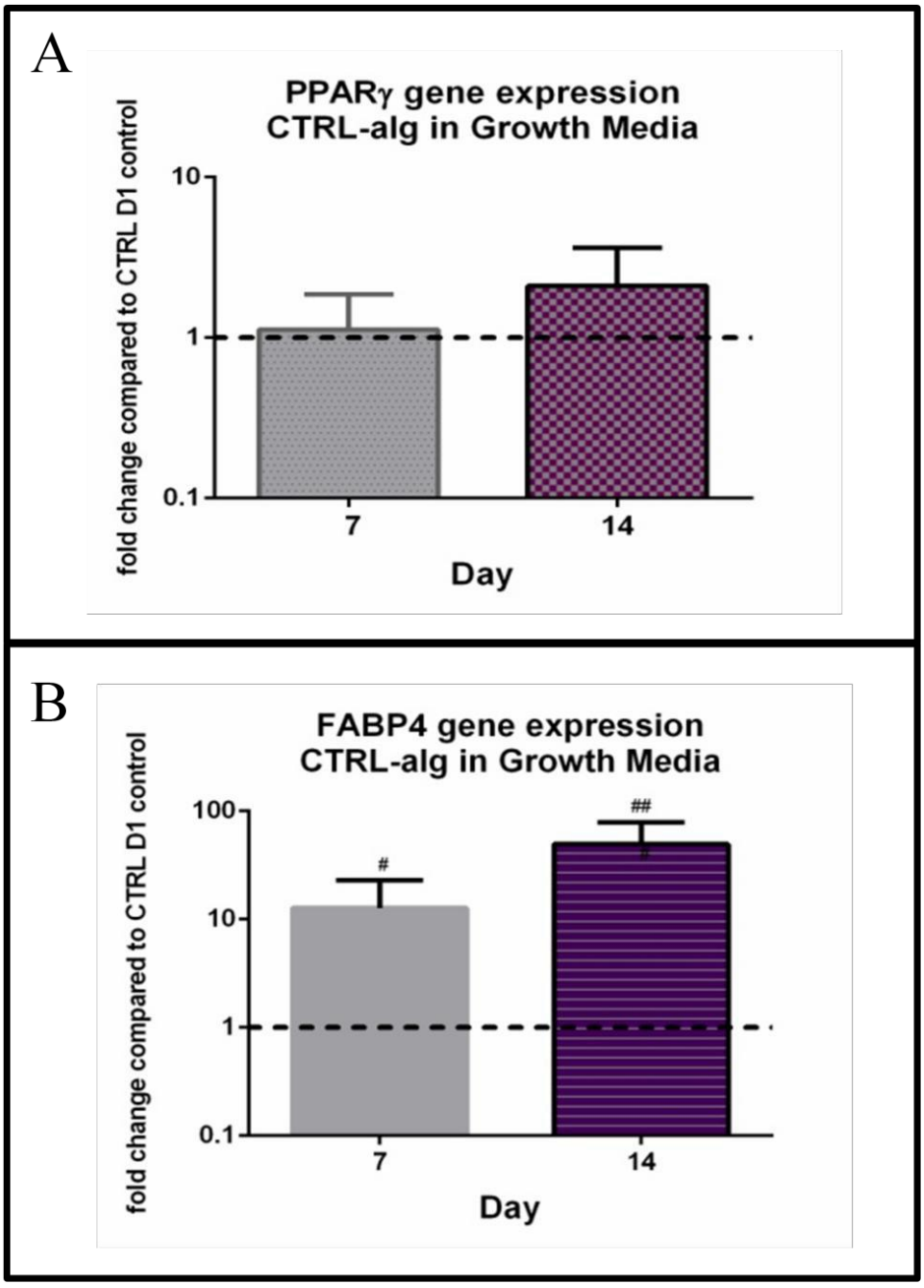


Figure 16. **Changes over time of gene expression for CTRL gel condition on days 7 and 14, when cultured in growth medium; dashed line represents respective day 1 control to which gene expression is normalized (N=3).** A) PPARγ gene expression in CTRL gels with growth medium did not significantly change on days 7 or 14, as compared to day 1. B) FABP4 gene expression in CTRL gels with growth medium was significantly higher on days 7 and 14, as compared to day 1. # = significance to day 1 control.

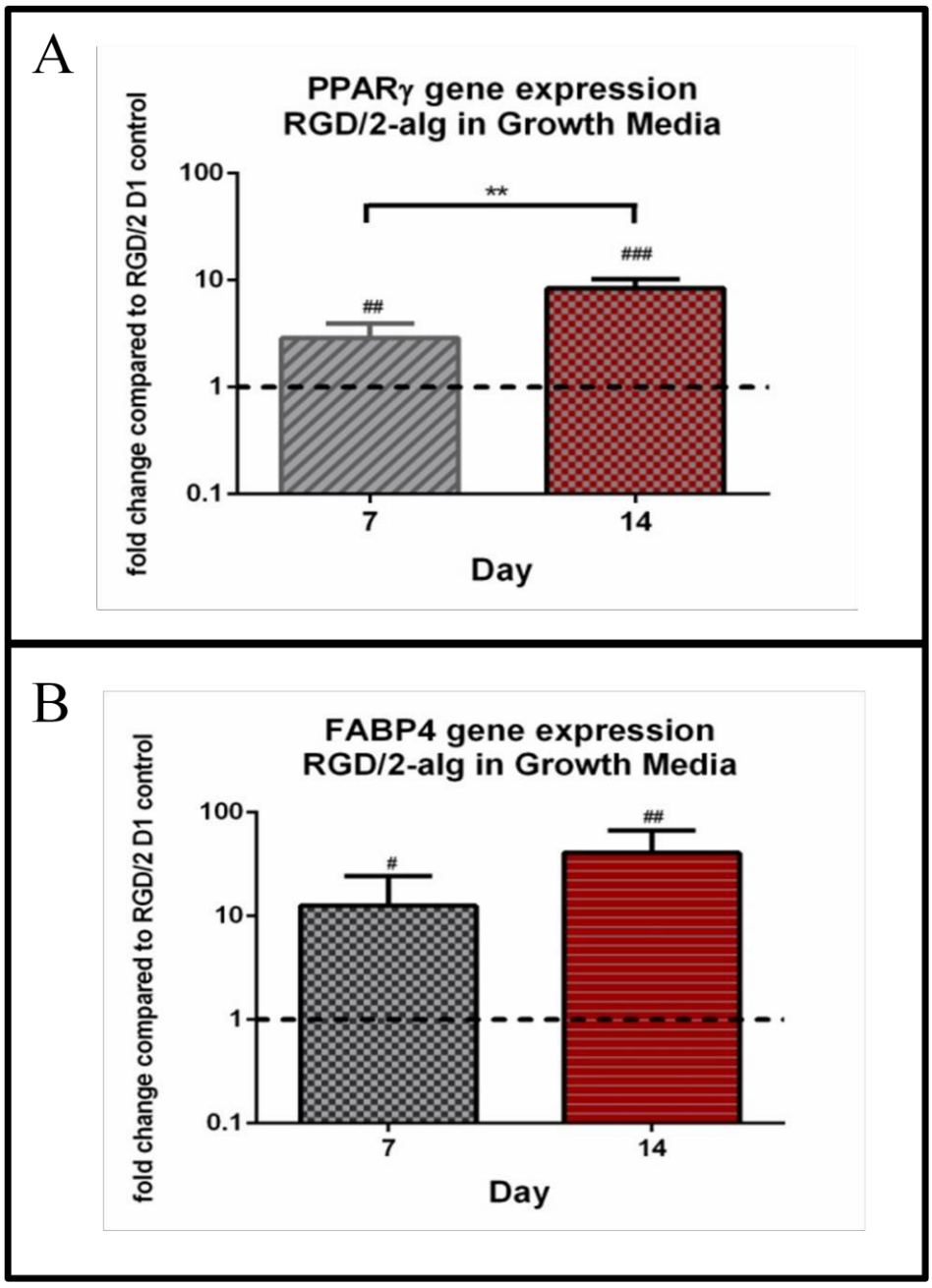


Figure 17. **Changes over time of gene expression for RGD/2 gel condition on days 7 and 14, when cultured in growth medium; dashed line represents respective day 1 control to which gene expression is normalized (N=3).** A) PPARγ gene expression in RGD/2 gels with growth medium on day 7 is significantly higher than day 1. Day 14 is significantly higher than both days 1 and day 7. B) FABP4 gene expression in RGD/2 gels with growth medium is significantly higher on days 7 and 14, as compared to day 1. ** = $p<0.01$; # = significance to day 1 control.

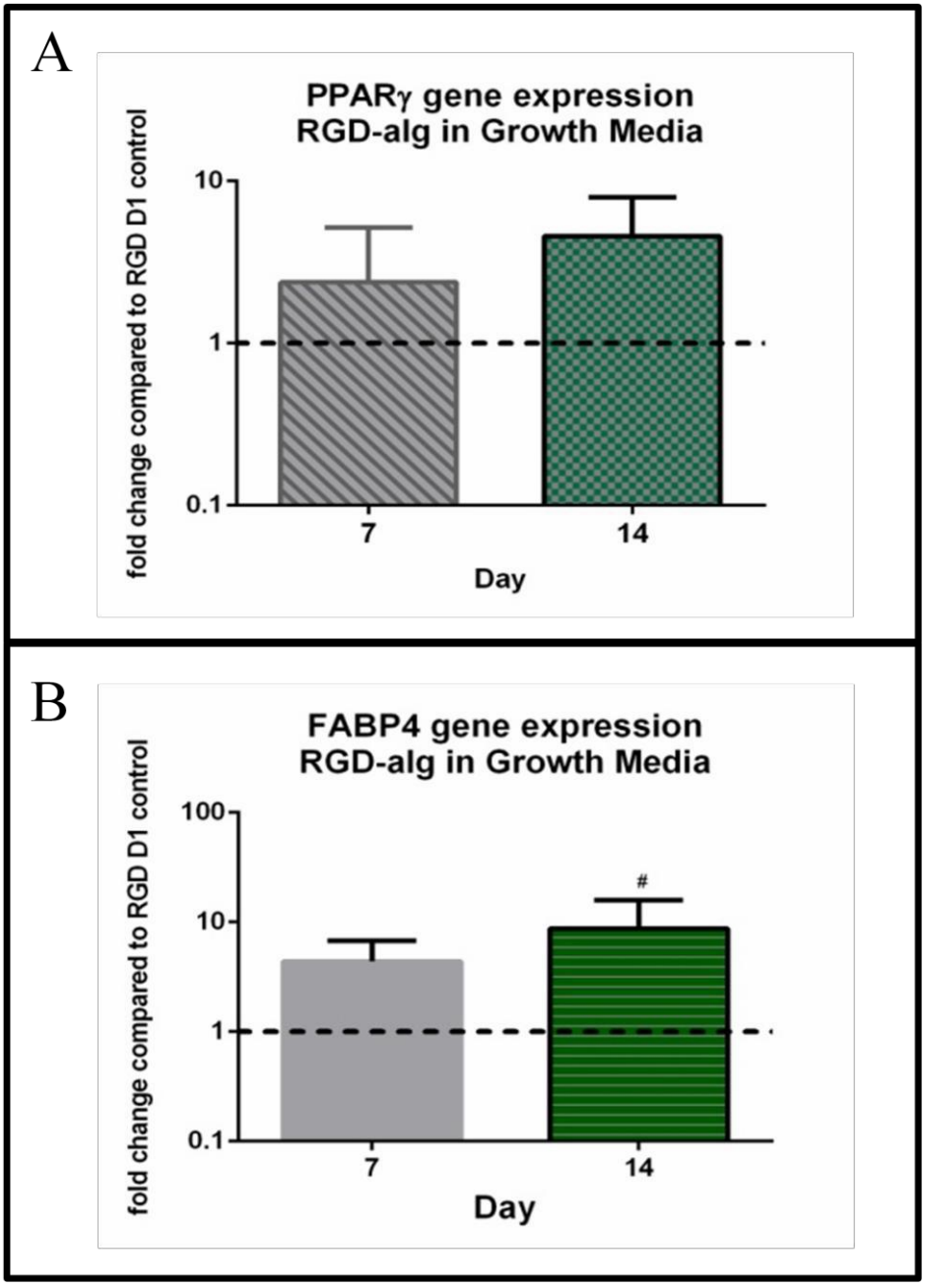


Figure 18. **Changes over time of gene expression for RGD gel condition on days 7 and 14, when cultured in growth medium; dashed line represents respective day 1 control to which gene expression is normalized (N=3).** A) PPARγ gene expression in RGD, growth medium gels did not significantly change on days 7 or 14,
as compared to day 1. B) FABP4 gene expression in RGD gels with growth medium on day 14 is significantly higher day 1. # = significance to day 1 control.

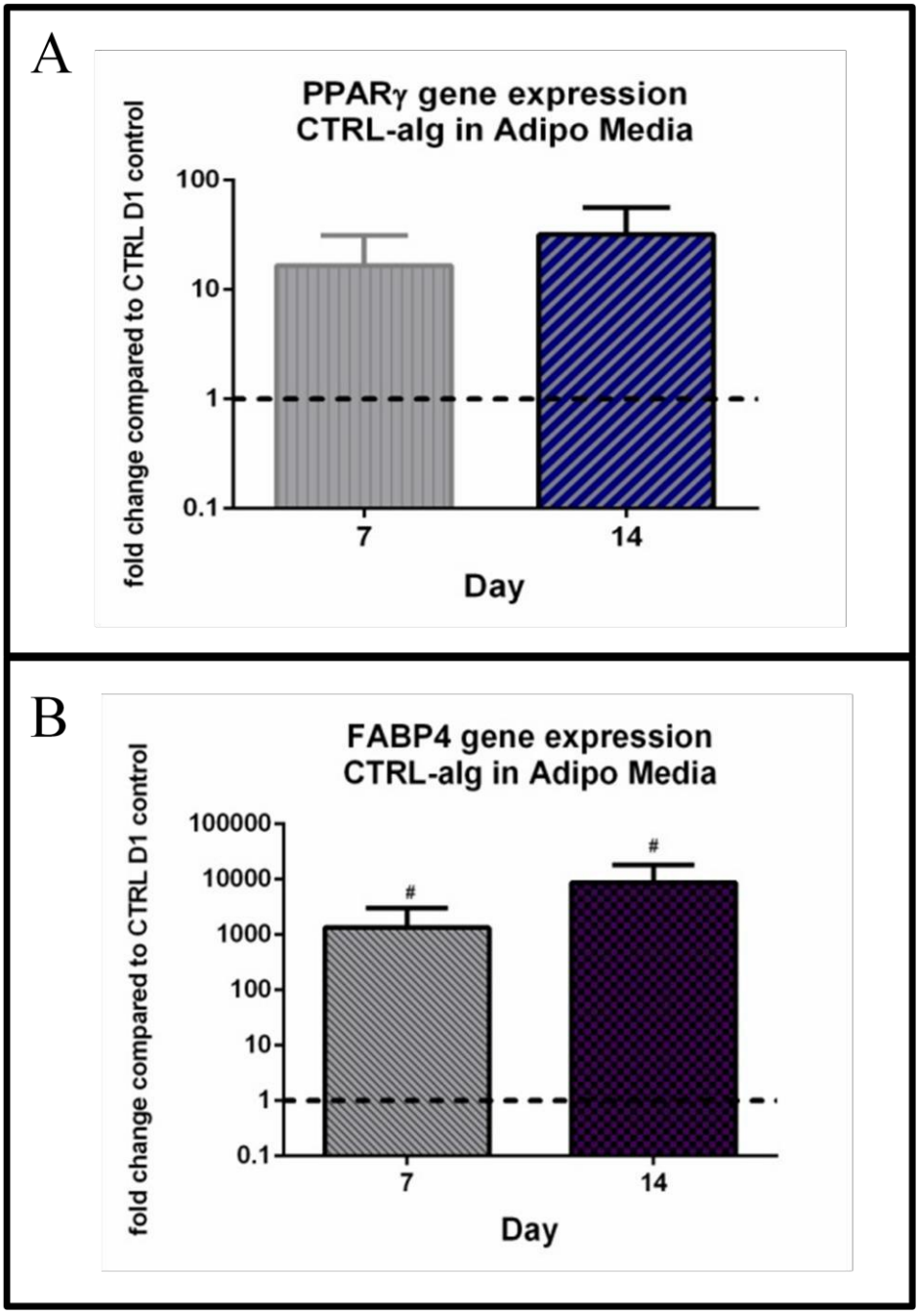


Figure 19. **Changes over time of gene expression for CTRL gel condition on days 7 and 14 with adipogenic medium; dashed line represents respective day 1 control to which gene expression is normalized (N=3).** A) PPARγ gene expression in CTRL gels with adipogenic medium is not significantly increased on days 7 or 14, as compared to day 1. B) FABP4 gene expression in CTRL gels with adipogenic medium is significantly increased on both days 7 and 14, as compared to day 1. # = significance to day 1 control

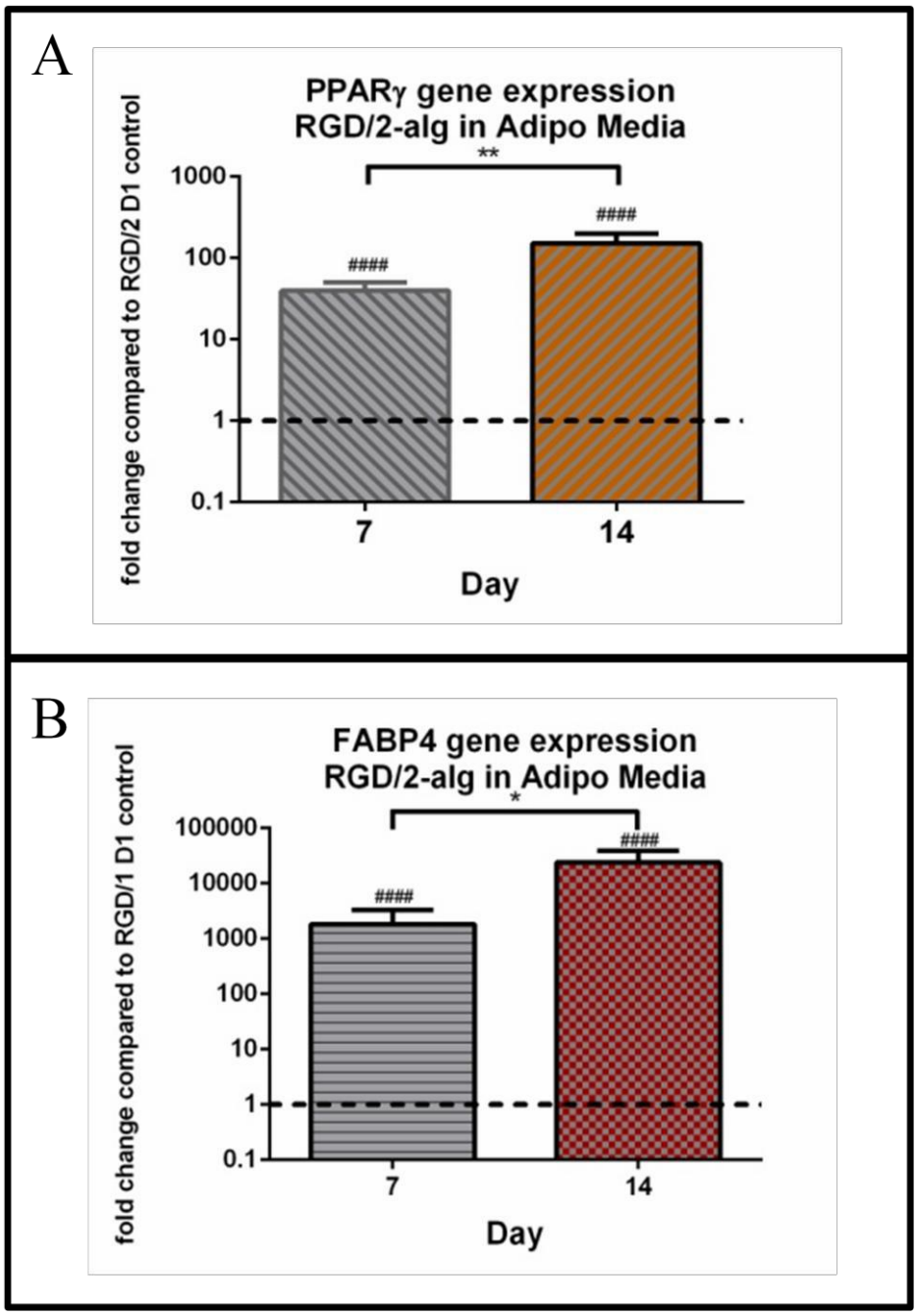


Figure 20. **Changes over time of gene expression for RGD/2 gel condition on days 7 and 14 with adipogenic medium; dashed line represents respective day 1 control to which gene expression is normalized (N=3).** A) PPARγ gene expression in RGD/2 gels with adipogenic medium is significantly higher on day 7 compared to day 1 control. Day 14 gene expression is also significantly higher, as compared to both days 1 and 7. B) FABP4 gene expression in RGD/2, adipogenic medium gels is significantly higher on day 14 than either day 1 or 7 gene expression. Day 7 is also significantly higher than day 1. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; # = significance to day 1 control

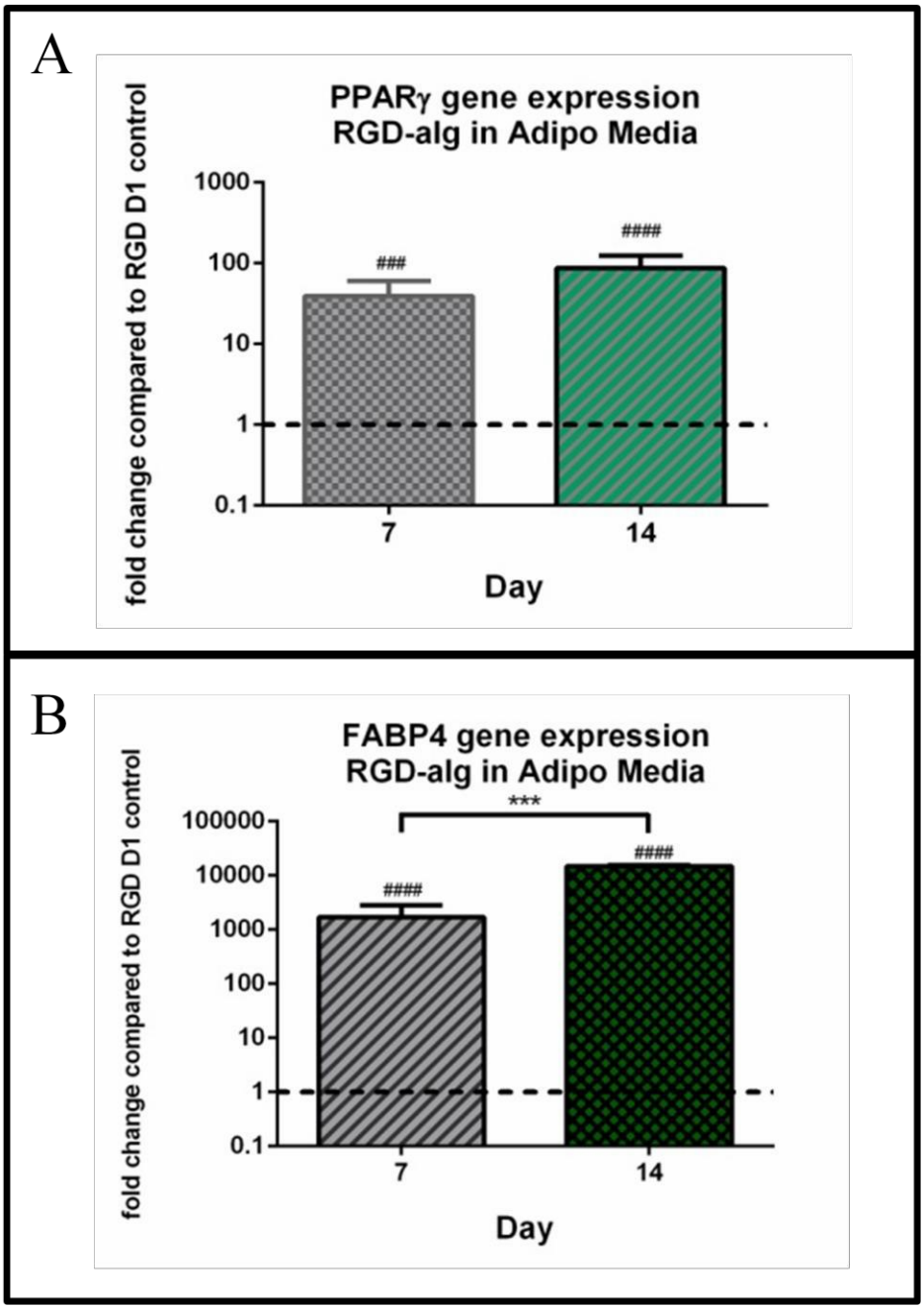


Figure 21. **Changes over time of gene expression for each RGD concentration on days 7 and 14 with adipogenic medium; dashed line represents respective day 1 control to which gene expression is normalized (N=3).** A) PPARγ gene expression in RGD gels with adipogenic medium is significantly increased on days 7 and 14, as compared to day 1. B) FABP4 gene expression in RGD, adipogenic medium gels is significantly increased on days 7 and 14, compared to day 1 control. Day 14 expression is also significantly higher than day 7. *** = $p<0.001$; # = significance to day 1 control

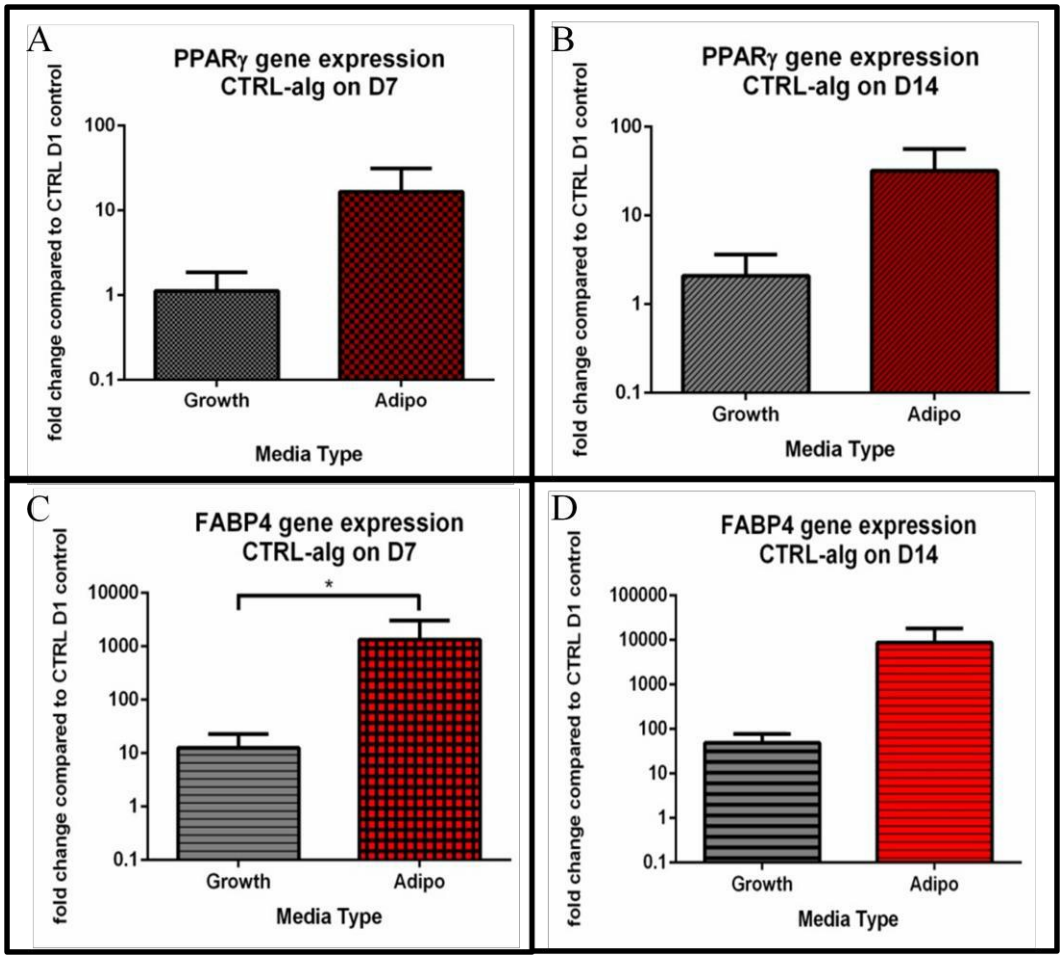


Figure 22. **Influence of medium on gene expression for days 7 and 14 in CTRL-alginate gels (N=3).** A) PPARγ gene expression in CTRL gels on day 7 is higher with adipogenic medium. B) PPARγ gene expression on day 14 is higher is gels with adipogenic medium. C) FABP4 gene expression is significantly higher on day 7 in gels with adipogenic medium, as compared to growth medium. D) FABP4 gene expression is higher on day 14 in gels with adipogenic medium. * = $p<0.05$

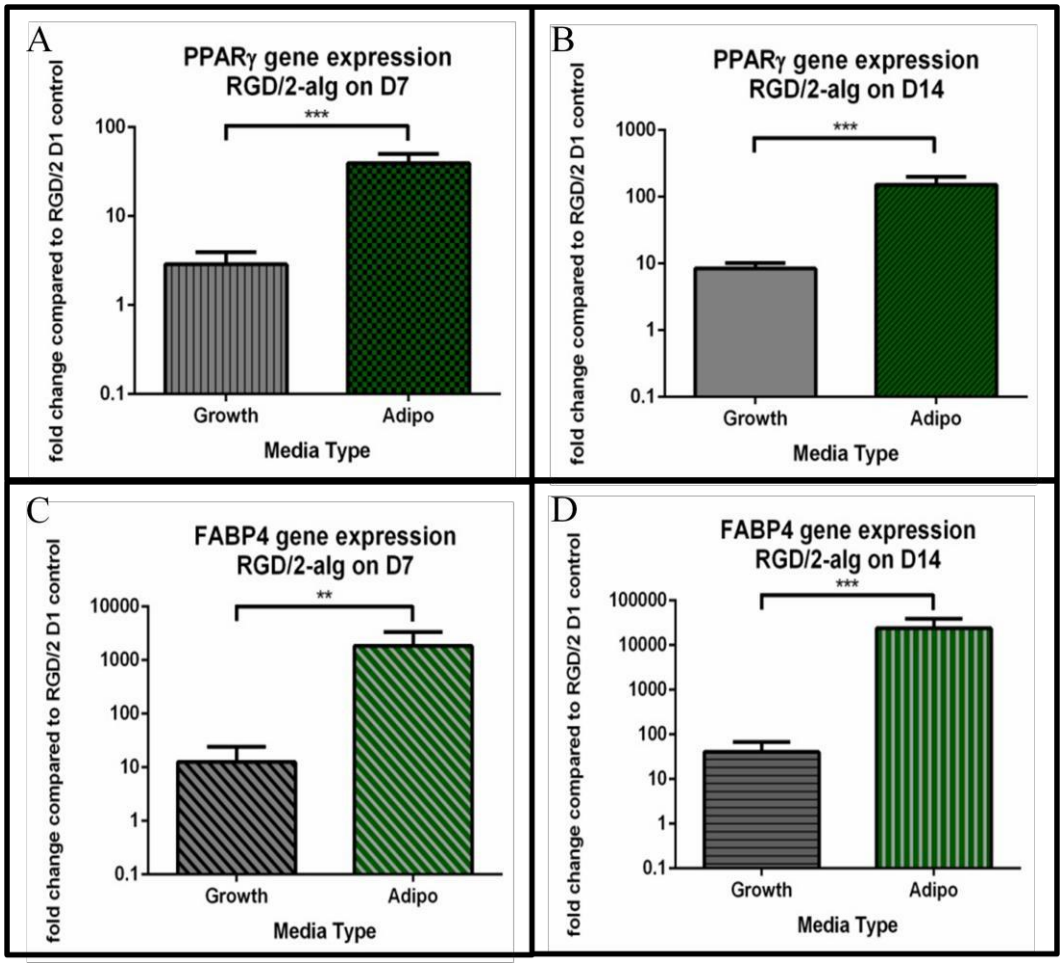


Figure 23. **Influence of medium on gene expression for days 7 and 14 in RGD/2-alginate gels (N=3).** A) PPARγ gene expression in RGD/2-alg. gels on day 7 is significantly higher with adipogenic medium, as compared to growth medium. B) PPARγ gene expression on day 14 is significantly higher in gels with adipogenic medium. C) FABP4 gene expression is significantly higher on day 7 in gels with adipogenic medium. D) FABP4 gene expression is significantly higher on day 14 in gels with adipogenic medium. ** = $p < 0.01$; *** = $p < 0.001$

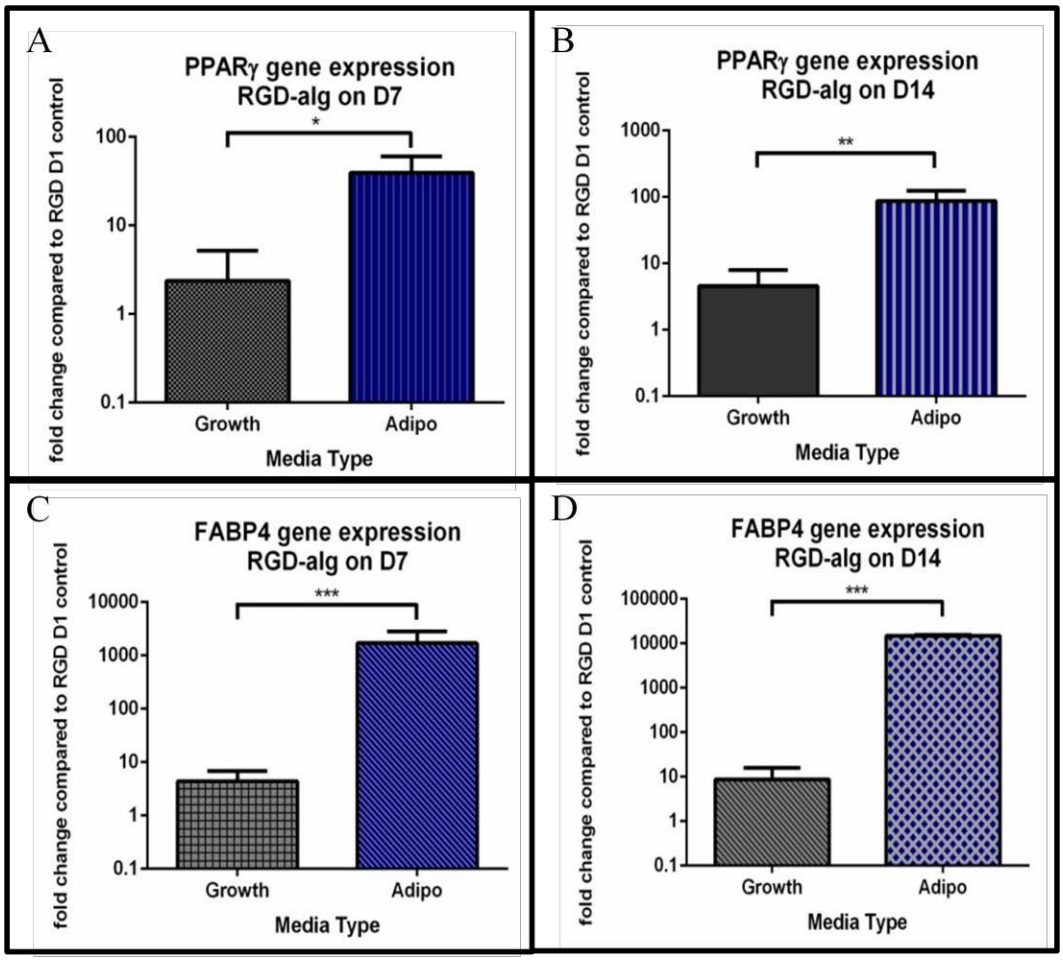


Figure 24. **Influence of medium on gene expression for days 7 and 14 in RGD-alginate gels (N=3).** A) PPARγ gene expression in RGD-alg. gels on day 7 is significantly higher with adipogenic medium, as compared to growth medium. B) PPARγ gene expression on day 14 is significantly higher in gels with adipogenic medium, as compared to growth medium. C) FABP4 gene expression is significantly higher on day 7 in gels with adipogenic medium. D) FABP4 gene expression is significantly higher on day 14 in gels with adipogenic medium, as compared to growth medium. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$

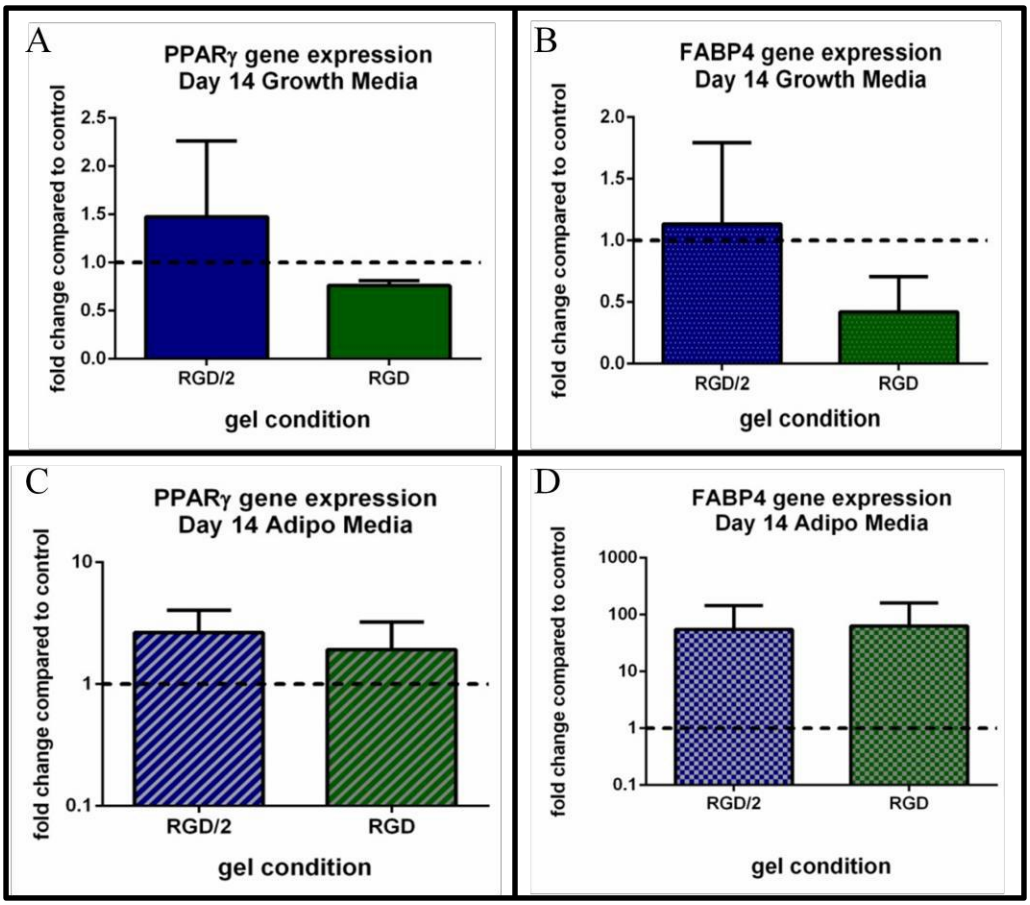


Figure 25. **Gene expression on day14, comparing gel conditions in both growth and adipogenic medium; dashed line represents respective day 14 CTRL gels to which gene expression is normalized (N=3)** A) PPARγ gene expression is increased in gels with RGD/2 when cultured in growth medium, compared to CTRL and RGD conditions, but results are not significant. B) FABP4 gene expression is increased in RGD/2 gels and growth medium compared to CTRL and RGD conditions, but results are not significant. C) PPARγ gene expression is increased in RGD/2 gels with adipogenic medium compared to CTRL and RGD conditions, but results are not significant. D) FABP4 gene expression is increased in RGD/2 and RGD gels and adipogenic medium compared to CTRL condition, but results are not significant.

3.4 Discussion

Many studies have shown that cell morphology has the ability to affect cell behavior (McBeath, et al., 2004; Lee, et al., 2013). This occurs when traction forces exerted by the cell through integrins binding interactions influence cytoskeleton tension and lead to changes in cell morphology and associated signaling cascades that ultimately regulate gene expression (Lee, et al., 2013). Here, we show that differences in cell morphology of ASCs have the ability to affect differentiation of the cells. In intact adipose tissue, ASCs were found to exhibit a spread morphology with very long protrusions allowing them to form networks surrounding mature adipocytes (Maumus, et al., 2013). Because mature adipocytes cannot proliferate, production of new adipocytes is thought to be a result of stem cell differentiation. In both *in vivo* and *in vitro*, preadipocytes are still fibroblastic in nature, whereas mature adipocytes are rounded, but the mechanism behind the changes in morphology is still unclear. In gels with different amounts of RGD binding peptide, we aim to see differences in cell morphologies of encapsulated cells, and understand if these morphological changes induced by differences in peptide amount result in variations of adipogenic marker gene expression. Previous work from our lab showed that by changing cell morphology, and the associated actin cytoskeletal tension, adipogenic differentiation of ASCs was altered. It was demonstrated that treating ASCs in 2D cultured with cytochalasin D and blebbistatin inhibited cells from spreading and increase adipogenic gene expression (Schiller, et al., 2013). Here

we aim to alter cell morphology of ASCs in 3D alginate gels using varying amounts on RGD.

Before any experiments were done on cell morphology, a cell density test was done to understand if that would influence differentiation. Gene expression showed that a cell seeding density of 1.5M/mL resulted in more PPARγ expression. Although more replicates could have been done, or more seeding densities tested, we chose to continue on with a 1.5M cells/mL seeding density for the subsequent experiments.

To understand if cell morphology influences differentiation of ASCs in 2D alginate gels, cells were seeded into gels at 1.5M/mL, with CTRL, RGD/2, and RGD conditions. As previously described, RGD is the designated amount given to the alginate when it is functionalized, done by previous work from our lab (Marturano, in prep). CTRL gels have been subjected to the same functionalization process, but without RGD. RGD/2 gels are a 1:1 mix of CTRL and RGD alginate. Gels were cultured for 14 days in either adipogenic medium or growth medium.

Our interest was to understand how cell morphology influenced adipogenic marker gene expression of ASCs between gel conditions, but also within each condition. Over 14 days, none of the gel conditions (CTRL, RGD/2, or RGD) had any change in circularity (Figure 8).

Additional statistical analysis was done on the circularity data points. All the individual points were graphed on a scatter plot to show that our different gel conditions were truly influencing the circularity of the cells, and not caused by

differences in biological replicates used in this experiment (Figure 9). The results indicate that we can conclude that the different gel conditions are affecting circularity and are independent of the particular biological replicate. A "leave-one-out" cross validation was then performed on the data for days 1, 7, and 14, for both growth and adipogenic media. The original value was compared to the 20 new averaged circularity values. For all conditions, regardless of media type and day, there were no significant differences between the original circularity values, and any of the new values. These results support that the circularity measurements are indicative of a random distribution within the gels, and can be considering representative images for our circularity measurements.

DNA content also did not significantly change from day 1 to 14 in any of the groups, except RGD when is cultured in adipogenic medium. This change could indicate that cells are not proliferating, but instead, are differentiating. However, when looking at how gels in growth medium compare to day 1, there is no significant increase in DNA content. This could be the result of both cell death and proliferation within the gels; but a live/dead assay was not conducted and, therefore no definitive reasons can be concluded as to why there is no increase in DNA. DNA content of the gels is normalized to the amount of alginate in each gel by weighing them, but this unfortunately does not take into account if the gels are swelling, and any additional weight that the medium may add. Other assays, such as live/dead staining and metabolism assay, would give insight to how the cells are functioning in the gels.

Gene expression changes were analyzed for PPARγ and FABP4 genes.

PPARγ is a central regulator during adipogenesis. It is induced during differentiation, and is responsible for activating a number of other genes involved in fatty acid binding, uptake and storage (Otto and Lane, 2005), and lipid and glucose homeostasis (Damcott, et al., 2004). FABP4 (fatty acid binding protein 4) gene encodes for an intracellular fatty acid binding protein that is found in differentiated adipocytes (Bernlohr, et al., 1985), and is a mediator of intracellular transport and metabolism of fatty acids (Damcott, et al., 2004), and during adipogenesis, PPARγ, along with other transcription factors, induces FABP4 mRNA expression, resulting in an increase of FABP4 protein expression (Tang, et al., 2004). Over the duration of the experiment, even when cultured in growth medium, there is an increase in both PPARγ and FABP4 gene expression for all gel conditions. Interestingly, RGD/2 condition shows a significantly higher PPARγ gene expression than both days 1 and day 7 at day 14. FABP4 gene expression is also significantly higher than day 1 at day 14 in all gel conditions. This increase in gene expression is not caused by any chemical induction and therefore, may be attributed to the ECM environment in which the cells are encapsulated.

Not unexpectedly, gene expression also increases for all gel conditions when grown in adipogenic medium. On days 7 and 14, FABP4 gene expression is significantly higher that day 1 for CTRL gels. Both RGD/2 and RGD show significantly higher gene expression on days 7 and 14 over day 1 for both PPARγ and FABP4. RGD/2 gene expression on day 14 is also significantly higher than that of day 7 for both genes. Although medium is causing cells to undergo

differentiation, the right matrix environment seems to be important, and therefore are contributing to why some gene expression is significantly higher and some is not. For both medium conditions, FABP4 is significantly increased in all conditions over the duration of the experiment, but not PPARγ. This is most likely due to the interactions of these two transcription factors. PPARγ is located in the nucleus, and activation depends on the transport of fatty acids to the nucleus (Damcott, et al., 2004). Studies have found that FABP4 localizes to the nucleus of adipocytes (Helledie, et al., 2000), and that FABP4 activates PPARγ In turn, PPARγ regulates transcription of FABP4 through a positive feedback mechanism (Tan et al., 2002).

For the experiment, differences in medium caused differences in DNA content for the conditions. Even thought circularity of the cells was not influenced by medium, for all conditions, DNA content was significantly less on day 14 when cultured in adipogenic medium, as compared to growth medium. As mentioned earlier, this could indicate that cells are committing to differentiation, and are no longer proliferating. On days 7 and 14, gene expression is higher in gels with adipogenic medium for both PPARγ and FABP4 genes. This was seen in all gel conditions. Both RGD/2 and RGD-alg. gels showed significant differences in PPARγ and FABP4 gene expression with adipogenic medium on both days 7 and 14. Since all parameters are the same for the experiment for all gel conditions, apart from the amount of RGD within the gels, differences in gene expression could be attributed to the presence of the binding peptide. One study suggested that the presence of RGD in alginate gels does indeed enhance

adipogenesis of ASCs, but show lack of quantitative measurements, and how much peptide needed was unclear (Kang et al., 2011).

When comparing across gel conditions with both growth and adipogenic medium, cells in CTRL gels are still significantly rounder than cells both RGD/2 and RGD gels, regardless of medium, throughout the experiment. Gene expression shows that RGD/2 gels have the highest levels of both PPARγ and FABP4 on day 14, but the difference among the groups is small and results are not significant.

The function of RGD within the gel may cause a chemical signal via integrin binding, as well as a physical attachment to the gels, causing mechanical force and signaling via the actin cytoskeleton. Soluble RGD is known to cause cell death by cells internalizing the peptide and inducing apoptosis (Buckley, et al., 1999). Therefore, to understand if RGD is producing chemical signaling for adipogenesis, functionalizing gels with other peptides may elucidate the direct effects of RGD. Another peptide that is used for cell adhesion, IKVAV, which is a laminin-derived peptide, has shown promise in 2D studies towards the goal of adipogenesis (Santiago, et al., 2006).

Triglyceride analysis was performed on samples harvested at days 1 and 7. These samples, however, were below a detectable range of the assay, and no quantitative lipid measurement is available. Because the seeding density is much lower compared to other cell types for adipogenesis (See chapter 4), it could be that there is just not enough sample. Future studies would benefit from harvesting more gels per sample to increase the cell number. It could also be beneficial to extend the experiment for another 1 to 2 weeks to increase lipid production.

3.5 Conclusions

ASCs are human cells, and have a lot more variation than other mammalian cells because they are primary cells taken from a human donor. As mentioned before, obesity development is influenced by a variety of factors, including diet and genetics. The differences in these two factors from donor to donor are certainly large, and most likely can cause differences in cell behavior in *in vitro* culture. It may be that more biological replicates would show greater differences in gene expression, and more statistical significance.

Here, it is shown that the amount of RGD peptide as the ability to cause differences in cells morphology. Differences in gene expression of adipogenic markers were also seen among the different gel conditions (CTRL, RGD/2, and RGD). Further studies could include the use of other amounts of RGD, such as RGD/3 or RGD/4 in this same experiment setup. This could cause more detectable differences in cell circularity, and more definitive differences in cell differentiation. It may also be beneficial to use other adhesion peptides, to understand if the presence of RGD is affecting differentiation.

4 Effect of scaffold elastic modulus of adipogenesis

4.1 Introduction

In addition to understanding how cell morphology is influenced by the matrix environment, we are looking to understand how cells respond to substrate elastic modulus. The range of ECM elastic modulus in the body is huge, from soft, pliable brain tissue with an elastic modulus of tenths of a kilopascal (kPa) to hard, calcified bone with a modulus of hundreds of megaPascals (MPa). With many orders of magnitude separating the softest and stiffest tissue in the body, these tissues contain cells that are tuned to the specific mechanical environments in which they reside (Park, et al., 2011)

The elastic modulus of a material is defined by Young's Modulus, which is the slope of a classical linear stress-strain curve, which stress is defined as the force per unit area and the strain as the normalized deformation induced (Dado et al. 2009). Cells can exhibit mechanotransduction by probing the rigidity of their substrate, along with other external mechanical signals, and reacting by way of biochemical mediators such as the cytoskeleton (Ingber, 2006)

There are several 2D studies that look at effect of elastic modulus of substrate. In 2D, however, it is difficult to show how cells are influenced by elastic modulus without having an effect on cell morphology. In 2006, Engler, et al. showed that MSCs grown on substrates of varying elastic moduli resulted in cell morphologies, gene expressions, and protein expressions that mimicked native cells in tissues with the same elastic modulus of that substrate. Another

study cultured ASCs on polyacrylamide gels with varying elastic moduli and showed that cells on the softest substrate had a significant increase of PPARγ gene expression as compared to the control (Young, et al., 2013). Saha et al. (2008) looked at the effect of substrate stiffness on neural stem cells, and grew cells on substrates with varying moduli between 10 and 10,000 Pascals (Pa). Cell spreading, self renewal and differentiation were inhibited on soft substrates (10 Pa), and cells proliferated on substrates with a modulus of 100 Pa or greater, exhibiting maximum levels of beta-tubulin III, a neuronal marker, on substrates that was approximately the same elastic modulus of brain tissue. A study out of the Blau lab shows that when muscle stem cells are cultured on a substrate that mimics the rigidity of muscle tissue, they are able to self renew to generate stem cell progeny that can effectively repair damaged muscle when subsequently transplanted into mice. This regenerative capacity of the cells is lost when these cells are plated for expansion in culture on tissue culture plastic (Gilbert, et al., 2010). These studies highlight the degree to which cells have the ability to perceive the elastic modulus of the substrate they are grown on, and why it is an important aspect to understand in order to develop tissue models.

Although a 2D environment is the easiest way to observe cell behavior, it has been shown that there is a mode of matrix adhesion that is unique to cells in 3D culture (Cukierman, et al., 2001), suggesting that the effect of biological signaling due to cell-matrix interactions should not be taken from 2D studies, but needs to reexamined for the case of 3D microenvironment. Pek, et al. (2010) encapsulated MSC in thixotropic gels, meaning shear stress could liquefy them,

with varying liquefaction stresses (the minimum shear stress to liquefy the gel). This was used to characterize matrix stiffness; the higher the shear stress required to liquefy the gel, the higher the matrix stiffness. Results showed that the highest expression of neural, myogenic and osteogenic transcription factors were obtained from low, intermediate, and higher liquefaction stresses, respectively. They also showed that immobilized RGD within the gel had a greater effect on matrices of higher stiffness, which they suggest is because a higher degree of cytoskeletal tension could be generated for the stiffer matrix, and it is this tension that transduces the signals for differentiation. Taking from this, cells grown in 3D are more *in vivo*-like, but also, the presence of a binding peptide is necessary for cells to detect the elastic modulus of a substrate.

3T3-L1 cells are a cell line that was developed through clonal expansion of mouse-derived cells and only contain a single cell type (Fernyhough, et al, 2005). Even though it is thought that these cells are immortalized, it is known that the capacity of the cells to differentiation into adipocytes declines with increased passaging. They have been used in identifying key molecular markers, transcription factors and other interactions that are required for preadipocyte differentiation, and are frequently used to rapidly screen and assess the adipogenic potential of various reagent and physical cellular cues (Poulos, et al., 2010).

Using our alginate hydrogel system, we can control the elastic modulus of the gels by varying the calcium content and/or the alginate polymer percentage within the gel. With this system, we aim to show that cells are influenced by the elastic modulus of their surrounding matrix environment, and this affects the

adipogenic potential of the cells. It is hypothesized that adipogenesis of the cells will decrease as the elastic modulus increases, as cells can sense the surrounding elastic modulus of the matrix.

4.2 Methods

4.2.1 List of solvents and chemicals

Table 5. List of chemicals and reagents used for experiments with 3T3-L1 cells

Chemical Name	Supplier
Calcium Carbonate (CaCO3)	Sigma Aldrich
Calf Serum (CS)	Gibco
Dexamethasone (Dex)	Sigma Aldrich
Dulbecco's Modified Eagle Medium (DMEM)	Gibco
Fetal Bovine Serum (FBS)	Gibco
D-(+)- Gluconic acid Delta-Lactone (GDL)	Sigma Aldrich
Hank's Balanced Salt Solution (HBSS)	Gibco
Human Insulin 10 mg/mL	Sigma Aldrich
Isobutyl methylxanthine (IBMX)	Sigma Aldrich
RGD Peptide sequence Arginine, Glycine, Aspartic Acid (RGD)	Peptides international
Penicillin/streptomycin (Pen/Strep)	Invitrogen
Pronova Alginate	NovaMatrix
Triglyceride determination kit	Sigma Aldrich
Tris-HCl	Sigma Aldrich
TRIzol LS	Invitrogen
0.025% Trypsin-EDTA	Gibco

4.2.2 Materials

Table 6. List of materials used for experiments with 3T3-L1 cells

Material	Supplier
CL2 Centrifuge	Thermo Scientific
Microscope	Nikon Eclipse TE300
Microscope Camera	Hamamatsu Camera C4742- 95
SpectraMax Gemini EM, fluorescence microplate reader	Molecular Devices
24-well Non-Tissue Culture Plates	BD-Falcon

4.2.3 Medium

4.2.3.1 Growth Medium and Maintenance Medium

	Growth Medium		Maintenance Media
DMEM	89%	DMEM	89%
CS	10%	FBS	10%
Pen/Strep	1%	Pen/Step	1%

4.2.3.2 Adipogenic induction media

	Adipogenic Medium A		Adipogenic Medium B
DMEM	88.88%	DMEM	88.99%
FBS	10%	FBS	10%
Pen/Strep	1%	Pen/Strep	1%
0.5 M IBMX	0.1%	Insulin 10 mg/mL	0.01%
Insulin 10 mg/mL	0.01%		
10 nM Dex	0.01%		

4.2.4 Cell Culture

3T3-L1 cells, a mouse adipocyte progenitor cell line, were plated in T-175 flasks at 5000 cells/cm^2. Cells were grown at 37° C in atmospheric air with 10% CO2. Cells were passaged when they reached 75% confluency. This was done by washing cells with HBSS, and then adding 5 mL of 0.05% trypsin for 5 minutes. Trypsin was neutralized by adding 5 mL of growth medium. Cells were spun down, counted and then replated at 5000 cells/cm^2, and allowed to grow.

4.2.5 Alginate Preparation and Gel fabrication

Alginate was prepared and functionalized as mention in the previous chapter. Alginate is functionalized with GGGGRGDSP peptides at the same ratio described by Marturano et al. (*in prep*. 2014), and given the designation of "RGD." Non-functionalized alginate, and designated as "CTRL."

Gel fabrication was like that of gels in the previous chapter. Cells were grown to confluence and cultured for 2 days. On the second day, cells were encapsulated in alginate hydrogels. Seeding density was either $10x10^6$ cells/mL or $50\ x10^6$cells/mL. A volume of 200 μL of alginate-cell solution was pipette into custom Teflon molds and allowed to cross-link for 2 hours at 37° C

4.2.6 Differentiation

After gel fabrication, gels were placed in 24-well non-tissue culture plate with growth medium overnight. The following day, medium on gels was either replaced with adipogenic medium A (AM A), or maintenance medium for a total of 2 mL. Gels that were given AM A received a medium change to AM B 2 days later. Medium changes continued for 9 days, so that gels received both AM A and

B two times throughout the differentiation process. Gels with maintenance medium also received medium changes through this time. After 9 days, all gels were given new maintenance medium regardless of the medium they originally started with, and received half medium changes (1 mL was removed and 1 mL of fresh medium was added) every other day for the remainder of the experiment.

4.2.7 Imaging

Gels were imaged using the Nikon Eclipse TE3000 microscope and Hamamatsu Camera every day for the first week, and then imaged every other day for the remainder of the experiment. Three gels per condition were imaged and a representative of those images is shown here.

4.2.8 Triglyceride analysis

Metabolic analysis of triglyceride content was conducted on the cell lysates. On days 9 and 28, gels were weighed and stored in 900 μL of SDS solution (0.1% SDS, 1mM EDTA, 100mM Tris-HCl, pH 7.4) and frozen at -20°C until analysis. Previously frozen samples were thawed, homogenized and sonicated using 60% amplification for ten seconds. Triglyceride content of the supernatant was measured using a triglyceride determination kit, which analyzed the release of glycerol from triglycerides by lipoprotein lipase. Triglyceride values were normalized to total DNA content using Hoechst 33258 dye.

4.2.9 DNA content

DNA content of the samples was conducted by staining an aliquot of the cell lysates from days 1 and 14 with Hoechst dye. Fluorescence values for these

samples were acquired on a fluorescence microplate reader using an excitation wavelength of 365 nm and an emission wavelength of 458 nm. These values were then converted to total DNA content by comparing the fluorescence values to a standard curve of fluorescence readings generated from solutions with known DNA concentrations.

4.3 Results

4.3.1 Cells in gels with elastic moduli of 3 and 10 kPa

3T3-L1 cells were encapsulated in alginate gels with two different elastic moduli, each with CTRL and RGD conditions. Each different elastic modulus and gel condition received both adipogenic induction medium and maintenance medium. Table 7 lists experimental conditions.

Table 7. Experimental conditions for 3T3-L1 gel seeding with elastic moduli of 3 and 10 kPa

Elastic modulus (kPa)	**Gel Condition**	**Medium**
3	CTRL	Maintenance
3	CTRL	Adipo
3	RGD	Maintenance
3	RGD	Adipo
10	CTRL	Maintenance
10	CTRL	Adipo
10	RGD	Maintenance
10	RGD	Adipo

All gels were seeded at a cell density of $10x10^6$ cells/mL. Gels were in culture for 49 days, images were taken every day for the first 7 days, and every other day for the remainder of the experiment.

One day after seeding, cells were spread in the RGD groups, and gels with an elastic modulus of 3 kPa seemed to have more spreading. No spreading was seen in CTRL gels (Figure 26). Cells continued to spread in RGD groups, regardless of elastic modulus or medium, until about day 4. After this point, cells looked like they were becoming less spread, and started to round up. By day 7,

most cells were round; this process happened faster for cells in adipogenic medium. By day 11, lipids were present in RGD condition with adipogenic medium. By day 14, lipids could be seen in both RGD and CTRL gels with adipogenic medium. At this point, cells in gels with RGD and maintenance medium were also starting to produce visible lipids. By day 21, lipid formation had increased slightly. Cells in CTRL gels with maintenance medium still did not have lipids (Figure 27). After day 30, some cells had larger lipids that filled the entire cell, although most were still small. After this point, gels got increasingly more difficult to image, for unknown reasons. Cells were cultured out to day 49 (Figure 28). Larger lipids were more noticeable at this time.

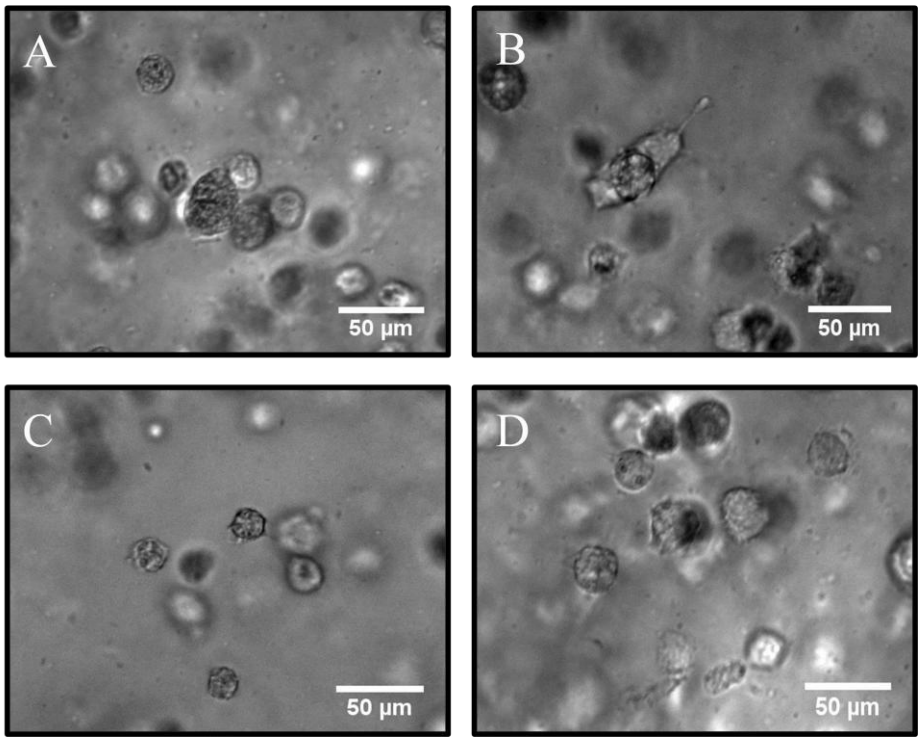


Figure 26. **Representative images for day 1 of 3T3-L1 cells.** A) Cells in 3 kPa, CTRL gels; cells are rounded after day 1. B) Cells in 3 kPa RGD gels; cell spreading can be seen on day 1 after seeding. C) Cells in 10 kPa CTRL gels; cells are rounded after day 1. D) Cells in 10 kPa, RGD gels; some cells are spread after day 1, but not as many as 3 kPa, RGD gels.

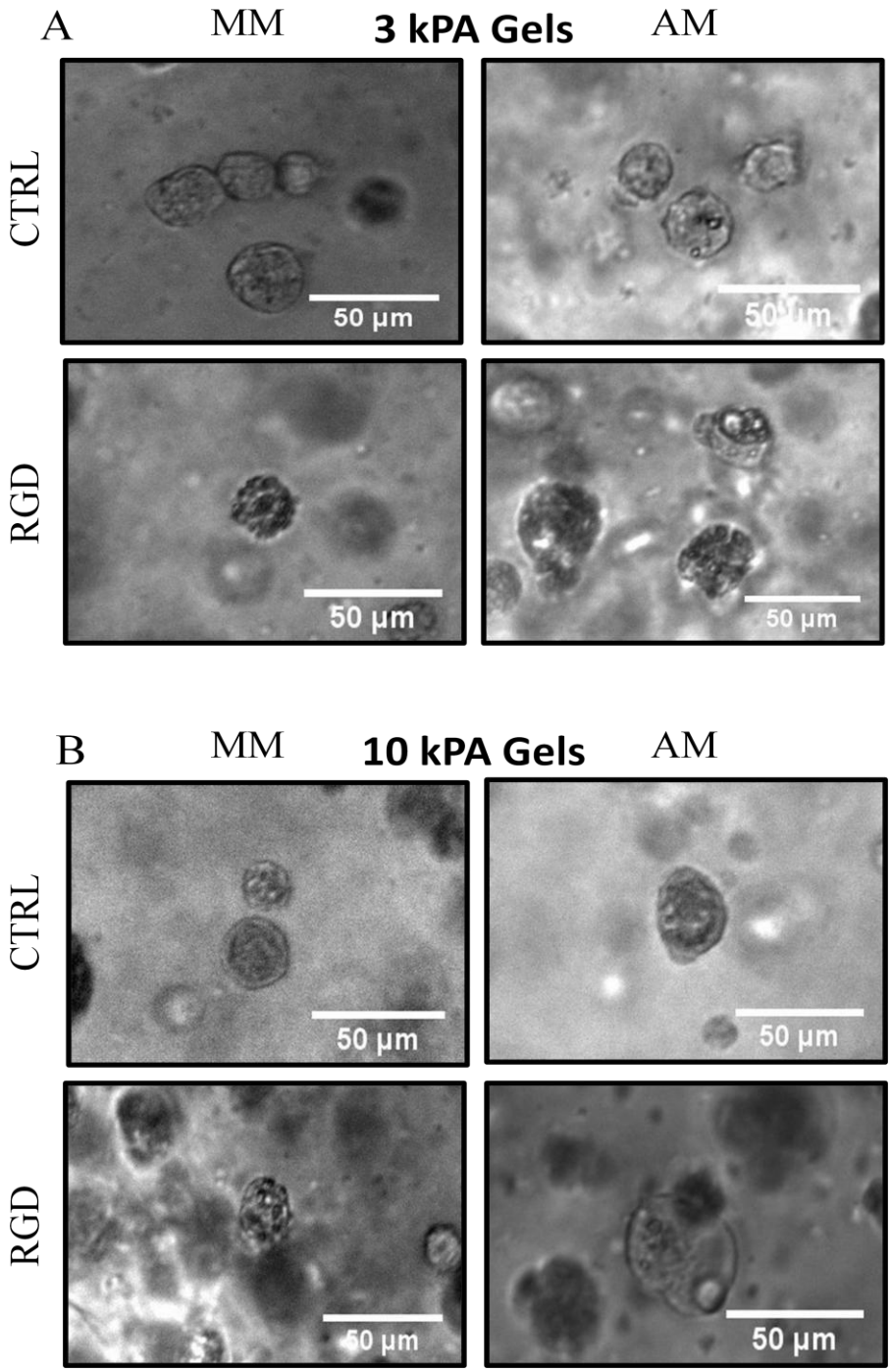


Figure 27. **Representative images for day 21 of 3T3-L1 cells.** A) By day 21, lipids could be seen in cells in 3 kPa, RGD gels, with both adipogenic and maintenance medium. Small lipids could also be seen in CTRL gels with adipogenic medium. B) Lipids were present in 10 kPa, RGD gels, regardless of medium, at day 21. Cells in CTRL, adipogenic medium also look to have very small lipids. MM = maintenance medium; AM = adipogenic medium

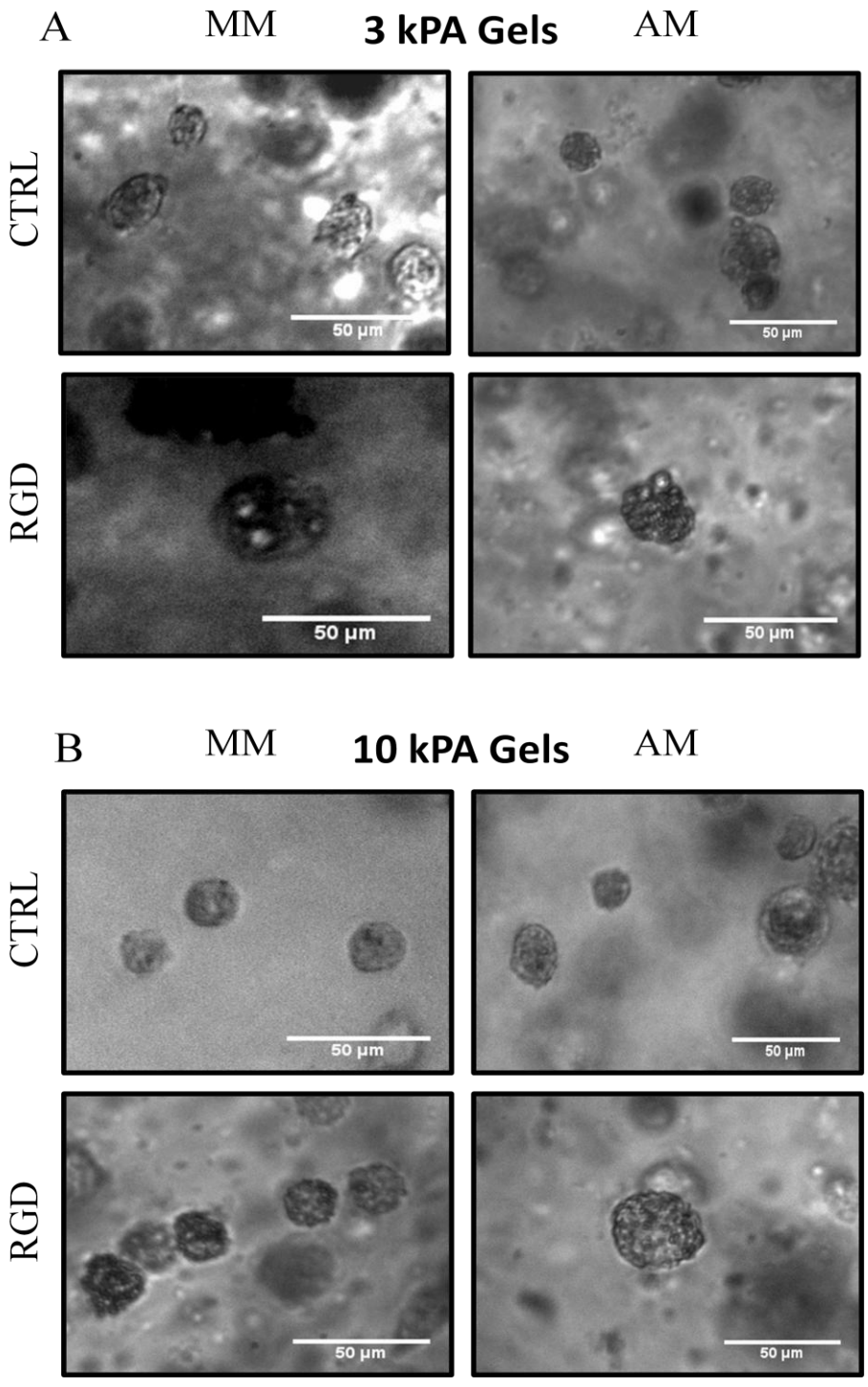


Figure 28. **Representative images for day 49 of 3T3-L1 cells.** A) At day 49, larger lipids could be seen in cells in 3 kPa, RGD gels, with both adipogenic and maintenance medium. Small lipids continued to be seen in CTRL gels with adipogenic medium, but did not progress to larger lipids B) Lipids continued to be seen in 10 kPa, RGD gels, regardless of medium, at day 49. Cells in CTRL gels did not noticeably change. MM = maintenance medium; AM = adipogenic medium

4.3.2 Repeat of cells in gels with elastic moduli of 3 and 10 kPa

3T3-L1 cells were encapsulated in alginate gels with elastic moduli of 3 and 10 kPa, with CTRL and RGD gel conditions. Table 5 lists experimental conditions. All gels were seeded at a cell density of 10M/mL. Gels were in culture for 33 days, and images were taken every day for the first 7 days, and every other day for the remainder of the experiment. Samples were harvested for TG analysis on the last day.

Like the previous experiment, one day after seeding cell spreading could be seen in RGD gels. Cells in 3 kPa gels looked slightly more spread than those is 10 kPa gels. Cells in CTRL gels never spread (Figure 29). By day 7 most cells in RGD gels rounded back up, including cells with maintenance medium. By day 10, lipids could be seen in RGD gels with adipogenic medium. By day 13, small lipids could be seen in all RGD gels, regardless of medium. Larger lipids started to form by day 23 in RGD gels with adipogenic medium (Figure 30), and by day 30, all RGD gels, regardless of medium, started to look similar. There were no noticeable changes after this point. Gels were cultured until day 33 (Figure 31).

On the last day, samples were taken for DNA content and TG analysis. Results show that on day 33, TG amount is highest in the 10 kPa CTRL gels conditions. Within this condition, adipogenic medium causes slightly more adipogenesis than maintenance medium (Figure 32).

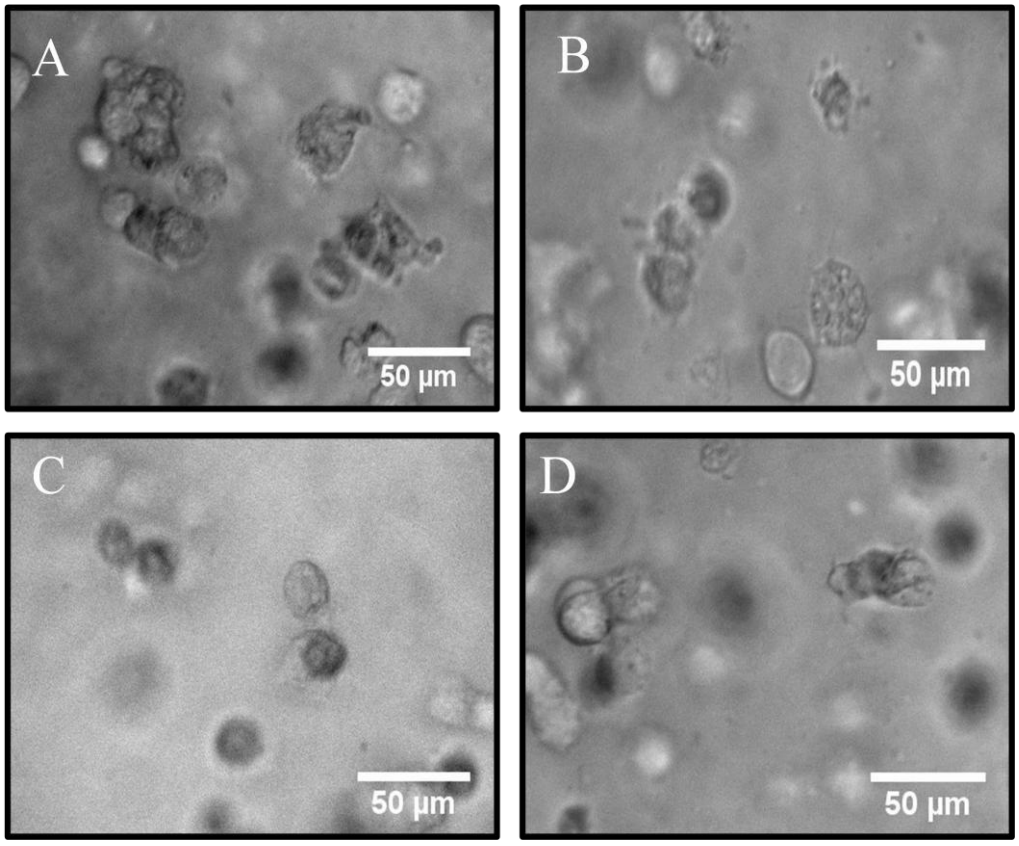


Figure 29. **Representative images for day 1 of 3T3-L1 cells.** A) Cells in 3 kPa, CTRL gels; cells are rounded after day 1. B) Cells in 3 kPa RGD gels; cell spreading can be seen on day 1 after seeding. C) Cells in 10 kPa CTRL gels; cells are rounded after day 1. D) Cells in 10 kPa, RGD gels; some cells are spread after day 1, but not as many as 3 kPa, RGD gels.

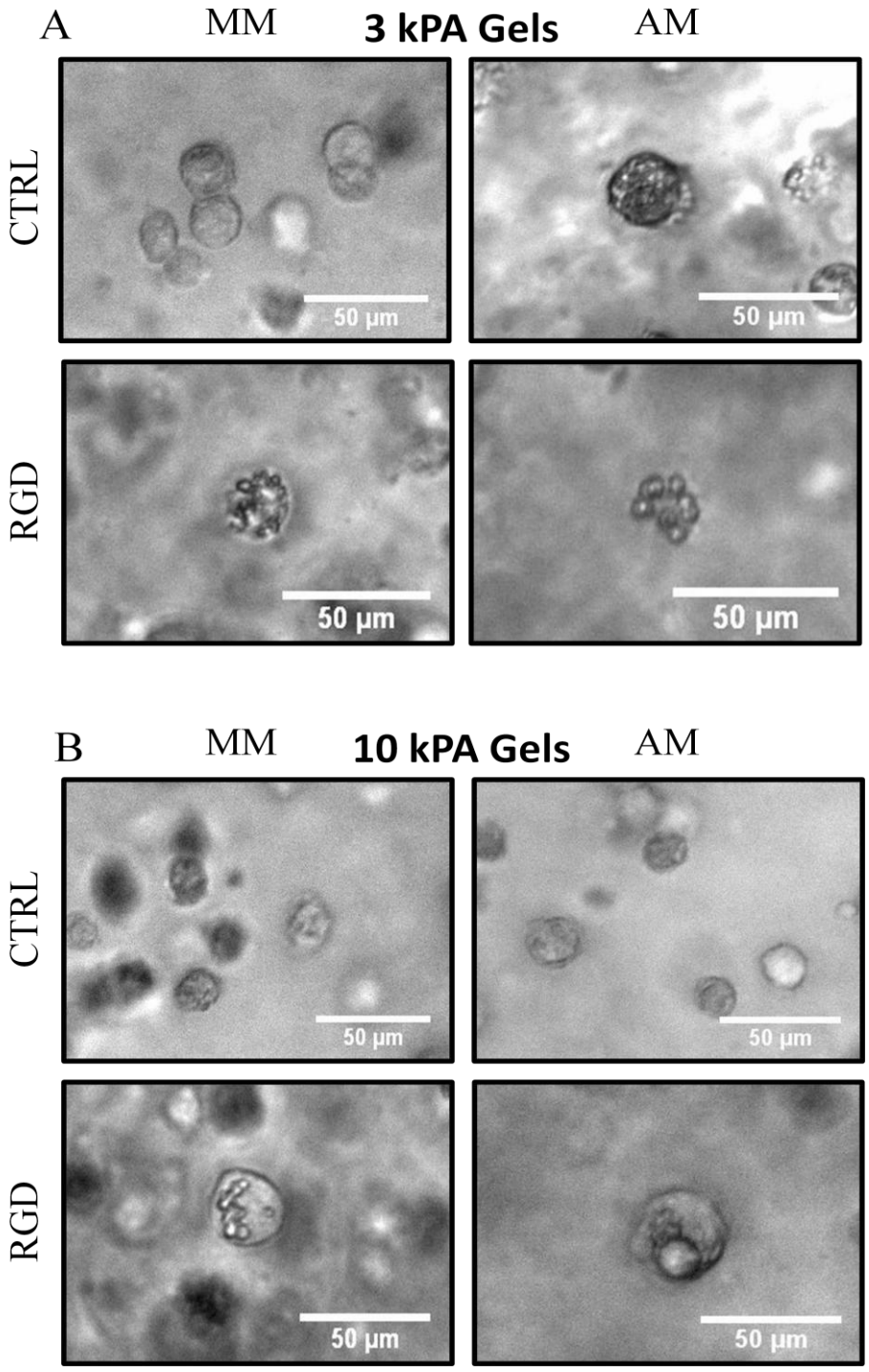


Figure 30. **Representative images for day 23 of 3T3-L1 cells.** A) At day 23, 3 kPa, RGD gels have larger lipids, regardless of medium. Some cells in CTRL gels with adipogenic medium had very small lipids. B) Larger lipids could also be seen in the 10 kPa, RGD gels, regardless of medium. CTRL gels did not have many cells with lipids. MM = maintenance medium; AM = adipogenic medium

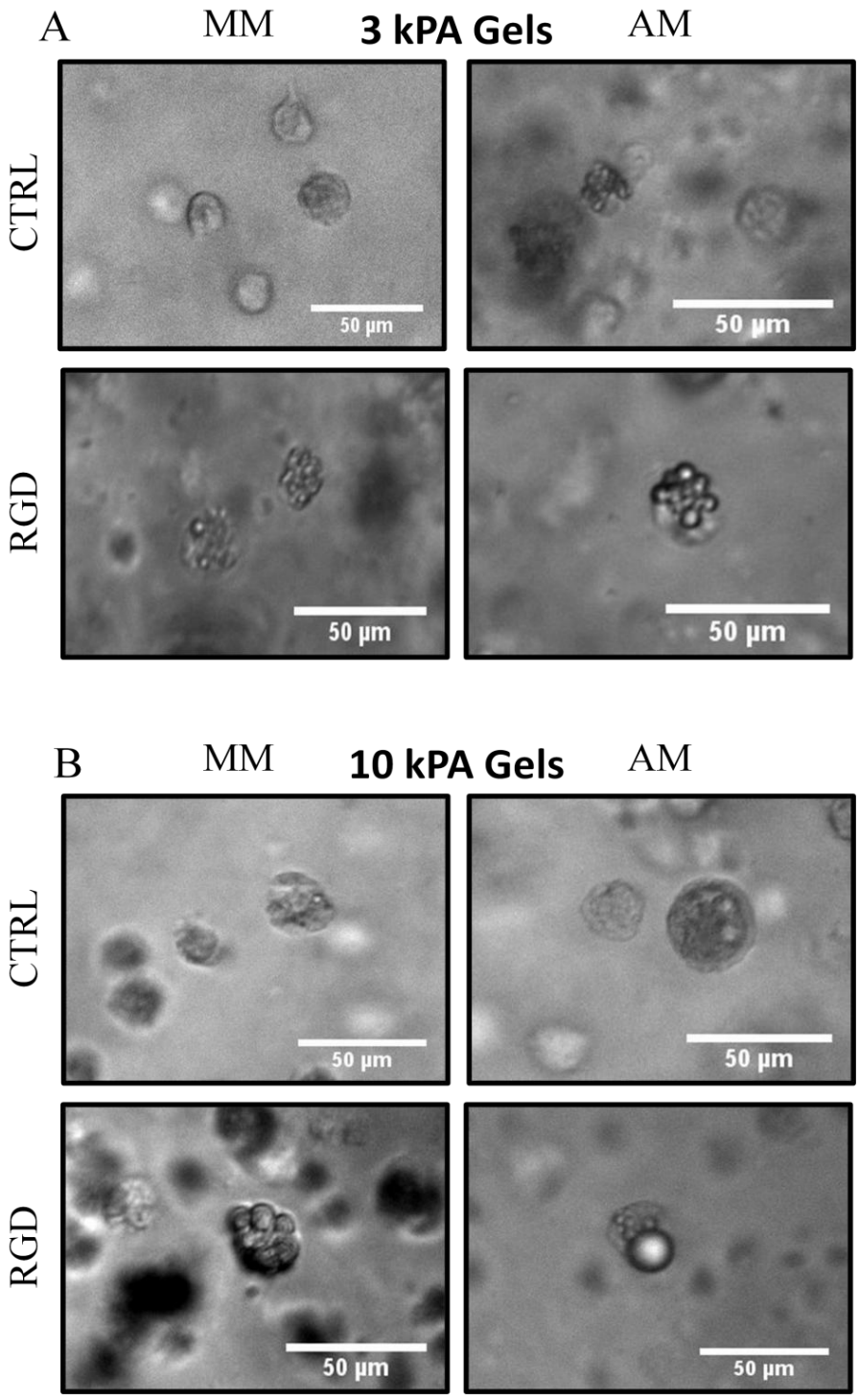


Figure 31. **Representative images for day 33 of 3T3-L1 cells.** A) On day 33, 3 kPa, RGD gels continued to have visible lipids, regardless of medium. Some cells in CTRL gels with adipogenic medium still had very small lipids, but never developed into larger lipids. B) Larger lipids persisted in the 10 kPa, RGD gels, regardless of medium. CTRL gels with adipogenic medium had small lipids. MM = maintenance medium; AM = adipogenic medium

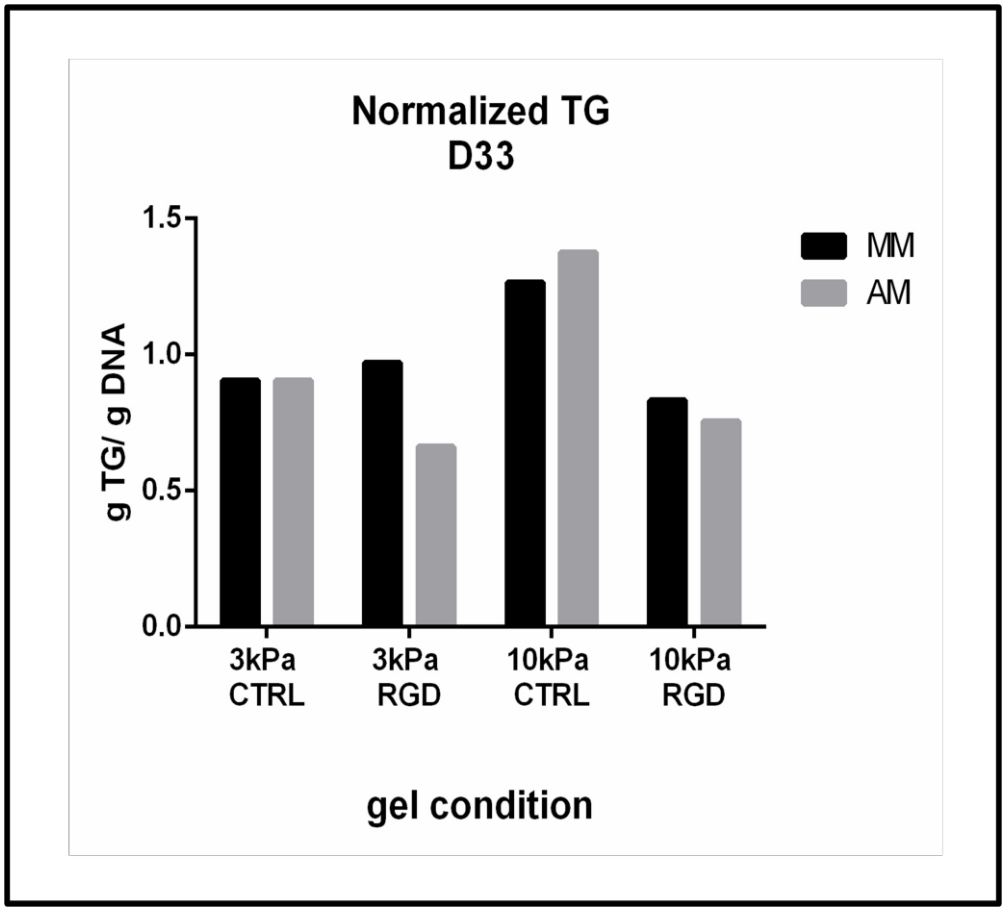


Figure 32. **TG amount in 3T3-L1 cells on day 33 of experiment.** Results show that 10 kPa CTRL gels have more TG at day 33 than the other conditions. Adipogenic medium causes slightly more TG than maintenance medium. MM = maintenance medium; AM = adipogenic medium

4.3.3 Cells into gels with elastic moduli of 10 and 55 kPa

3T3-L1 cells were encapsulated in alginate gels with two different elastic moduli, 10 kPa and 55 kPa, to achieve a greater difference in elastic modulus between the gels. There were both CTRL and RGD gels. Table 8 lists experimental conditions. For the stiffer gels, double the amount of alginate was used for both the CTRL gels and RGD gels. Because double the volume of RGD gel was used, there is twice as much RGD in the gels with the higher elastic modulus than the softer gels, even though the ratio of alginate: RGD is the same. Alginate was functionalized all the same way.

Table 8. Experimental conditions for 3T3 gels seeding with elastic moduli of 10 and 55 kPa

Elastic Modulus (kPa)	**Gel condition**	**Medium**
10	CTRL	Maintenance
10	CTRL	Adipo
10	RGD	Maintenance
10	RGD	Adipo
55	CTRL	Maintenance
55	CTRL	Adipo
55	RGD*2	Maintenance
55	RGD*2	Adipo

All gels had a seeding density of $10x10^6$cells/mL. Images were taken for the first 7 days, and every other day for another 28 days. Samples were harvested on days 9 and 28 for TG analysis.

Cells in both RGD groups were starting to spread one day after seeding. Cells without RGD were rounded (Figure 33). Cells continued to spread for the

first 5 days, and by day 7, many cells were rounded back up. Very small lipids could be seen by day 10, and by day 13, cells in the 10 kPa gels with RGD have defined lipids. Cells in the 55 kPa gels look like they may have very small lipids, but they are not as visible and defined as the 10 kPa gels (Figure 34). By the end of the experiment, lipids are still present in the 10 kPa gels with RGD, regardless of medium. Small lipids look to be present in the 55 kPa gels, but are poorly defined (Figure 35). An observation that was made was that the cells in the 55 kPa gels tended to be larger, and contain what look like to be very small lipids throughout the experiment, but never progress into anything more.

At days 9 and 28, samples were harvested for DNA and TG analysis. On day 9, medium type does not cause a difference in the 10 kPa gels, for both CTRL and RGD conditions, and RGD gels have more DNA than CTRL gels. Interestingly, the same trend is not seen in the 55 kPa gels. For these gels, medium causes a larger difference in DNA, where gels with adipogenic medium have much more DNA than maintenance medium, for both CTRL and RGD*2 gels. The adipogenic medium gels have the same amount of DNA in CTRL and RGD gels, where RGD*2 gels have more DNA than CTRL gels when culture in maintenance medium (Figure 36A). At day 28, 10 kPa gels, CTRL and RGD conditions, all have similar DNA content, regardless of medium. For the 55 kPa gels, there is more DNA in the maintenance medium gels than in the adipogenic medium gels, and CTRL conditions having more than those in RGD*2 conditions. Adipogenic medium produces similar amounts of DNA for both CTRL and RGD*2 conditions (Figure 36B). For TG, similar trends can be seen on both days

9 and 28. On day 9 10 kPa CTRL gels have the most TG as compared to all other groups. Interestingly, medium does not cause a huge difference for both 10 kPa conditions. For 55 kPa gels, maintenance medium shows more TG than adipogenic medium for both CTRL and RGD conditions. Overall, CTRL conditions seem to have more TG than RGD conditions (Figure 37A). On day 28, 10 kPa RGD gels have had the most increase in TG, reaching similar levels to that of the CTRL groups. Medium still does not seem to produce a difference in TG. 55 kPa gels still have less TG than 10 kPa gels, but there is an increase from day 9. Adipogenic medium seems to produce slightly more TG in both CTRL and RGD*2 gel conditions (Figure 37B).

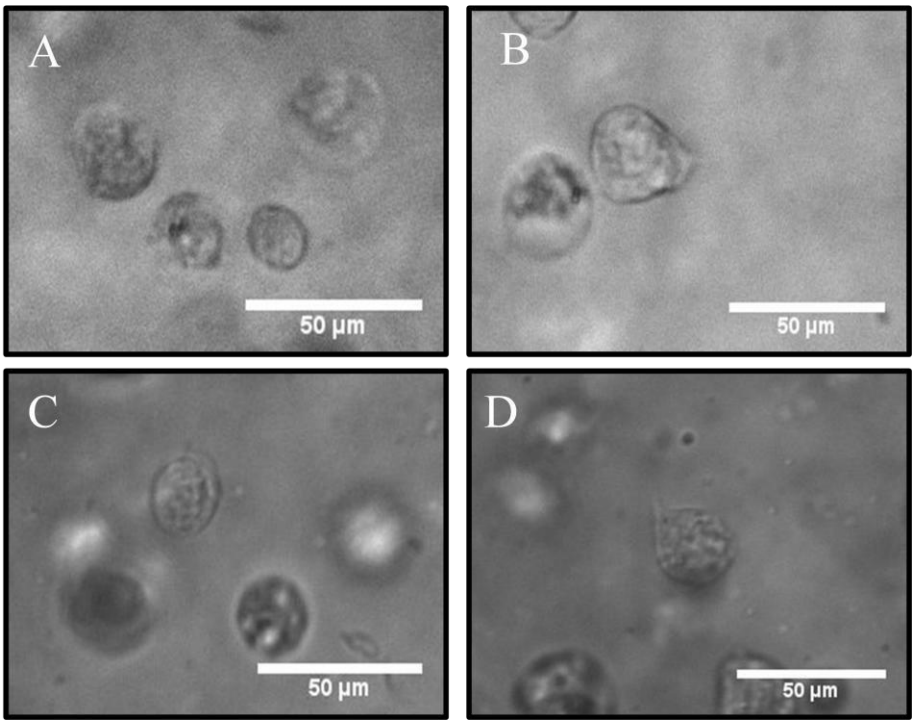


Figure 33. **Representative images for day 1 of 3T3-L1 cells.** A) Cells in 10 kPa, CTRL gels; cells are rounded after day 1. B) Cells in 10 kPa RGD gels; cell spreading can be seen on day 1 after seeding. C) Cells in 55 kPa CTRL gels; cells are rounded after day 1. D) Cells in 55 kPa, RGD*2 gels; some cells are spread after day 1, but not as many as 10 kPa, RGD gels.

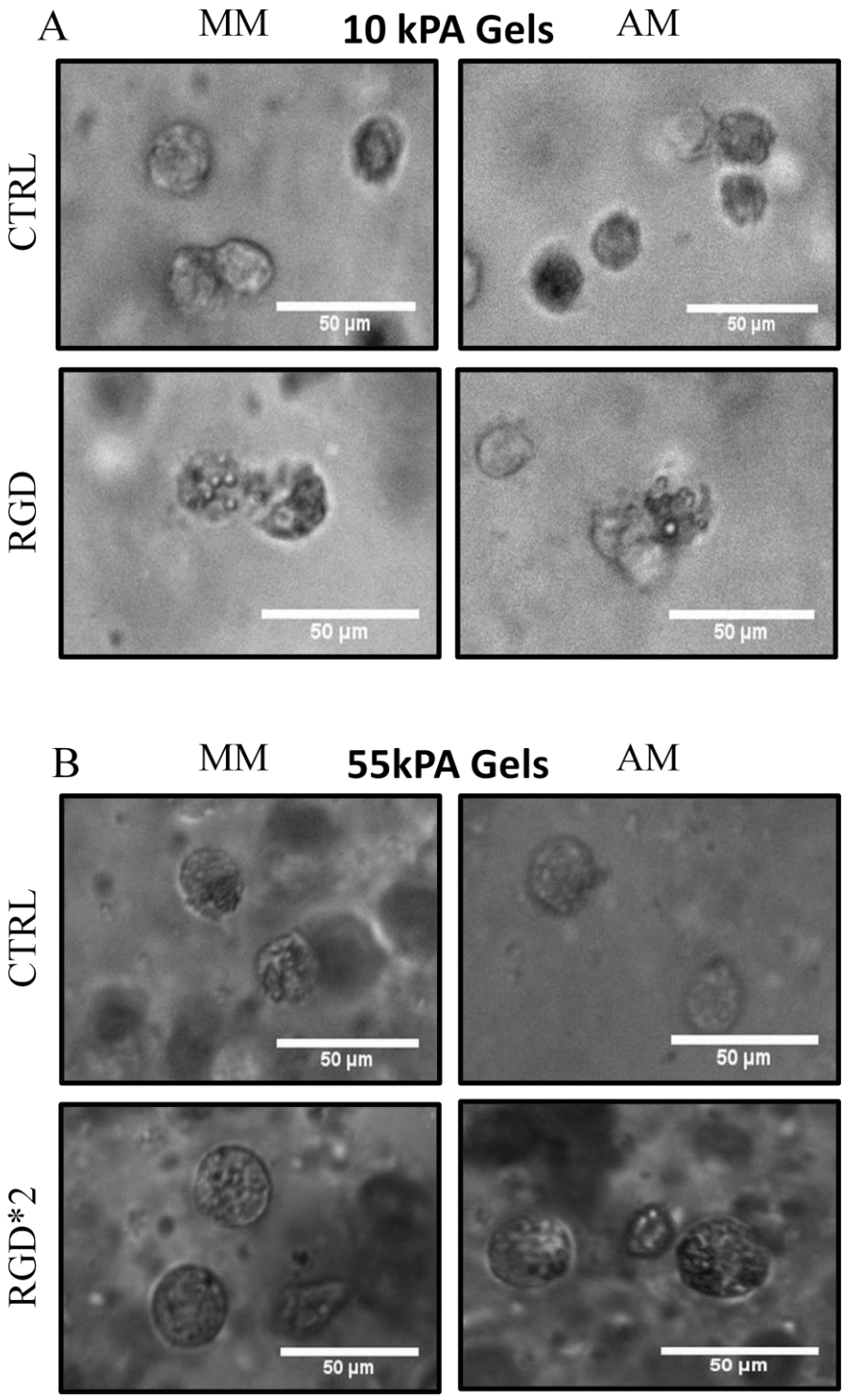


Figure 34. **Representative images for day 13 of 3T3-L1 cells.** A) Cells in 10 kPa gels with RGD have visible lipids, regardless of the medium. CTRL gels do not have any visible lipids. B) Cells in 55 kPa gels with RGD*2 and adipogenic medium look like they may have small lipids, but are not well defined. Other conditions do not have lipid formation. MM = Maintenance medium; AM = Adipogenic Medium

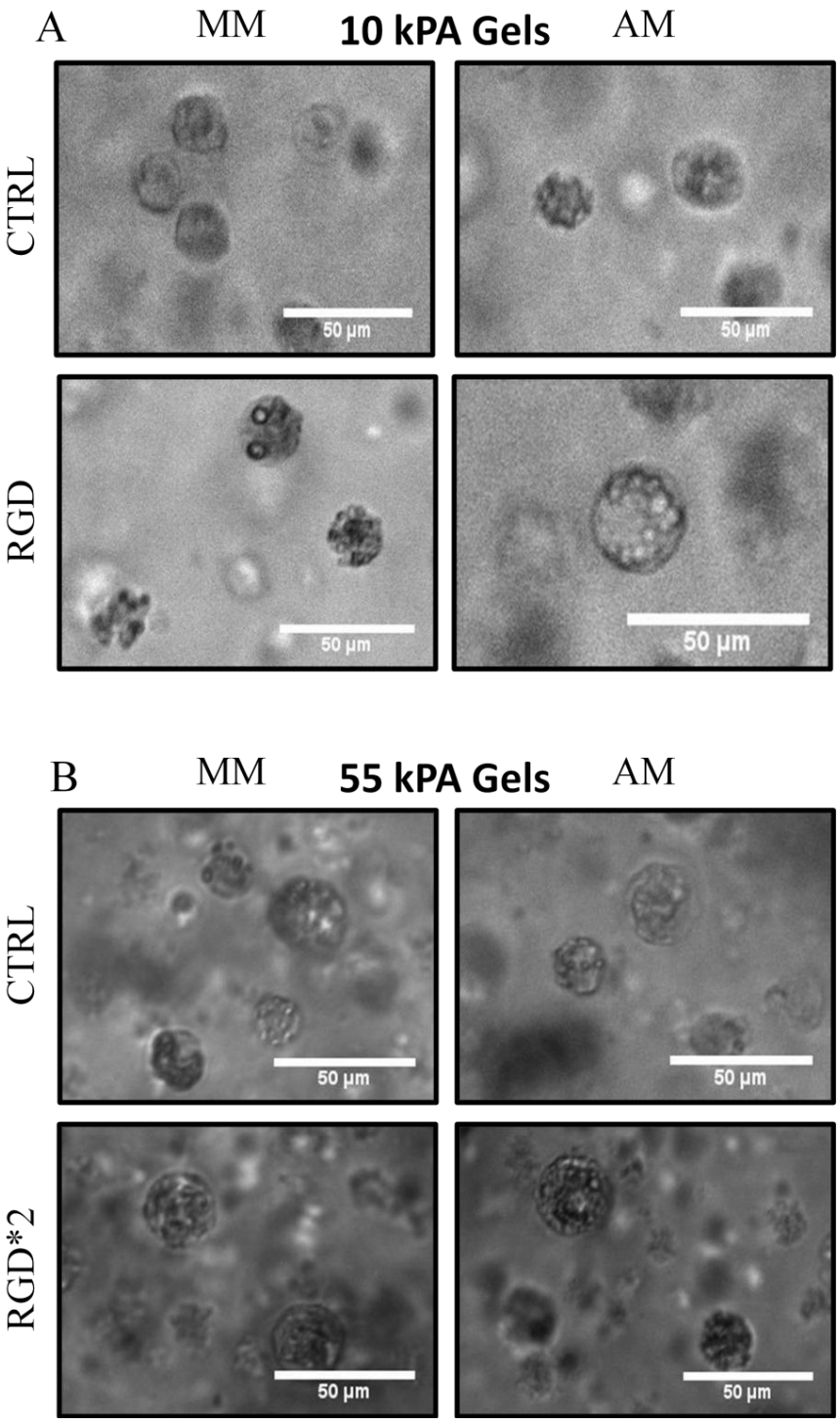


Figure 35. **Representative images for day 28 of 3T3-L1 cells.** A) Cells in 10 kPa gels with RGD have larger lipids by day 28, regardless of medium. Cells in CTRL gels with adipogenic medium look like there could be small lipids. B) Cells in 55 kPa gels with RGD, both maintenance and adipogenic medium, look like they have small lipids, but none have progressed into larger lipids. MM = Maintenance medium; AM = Adipogenic medium

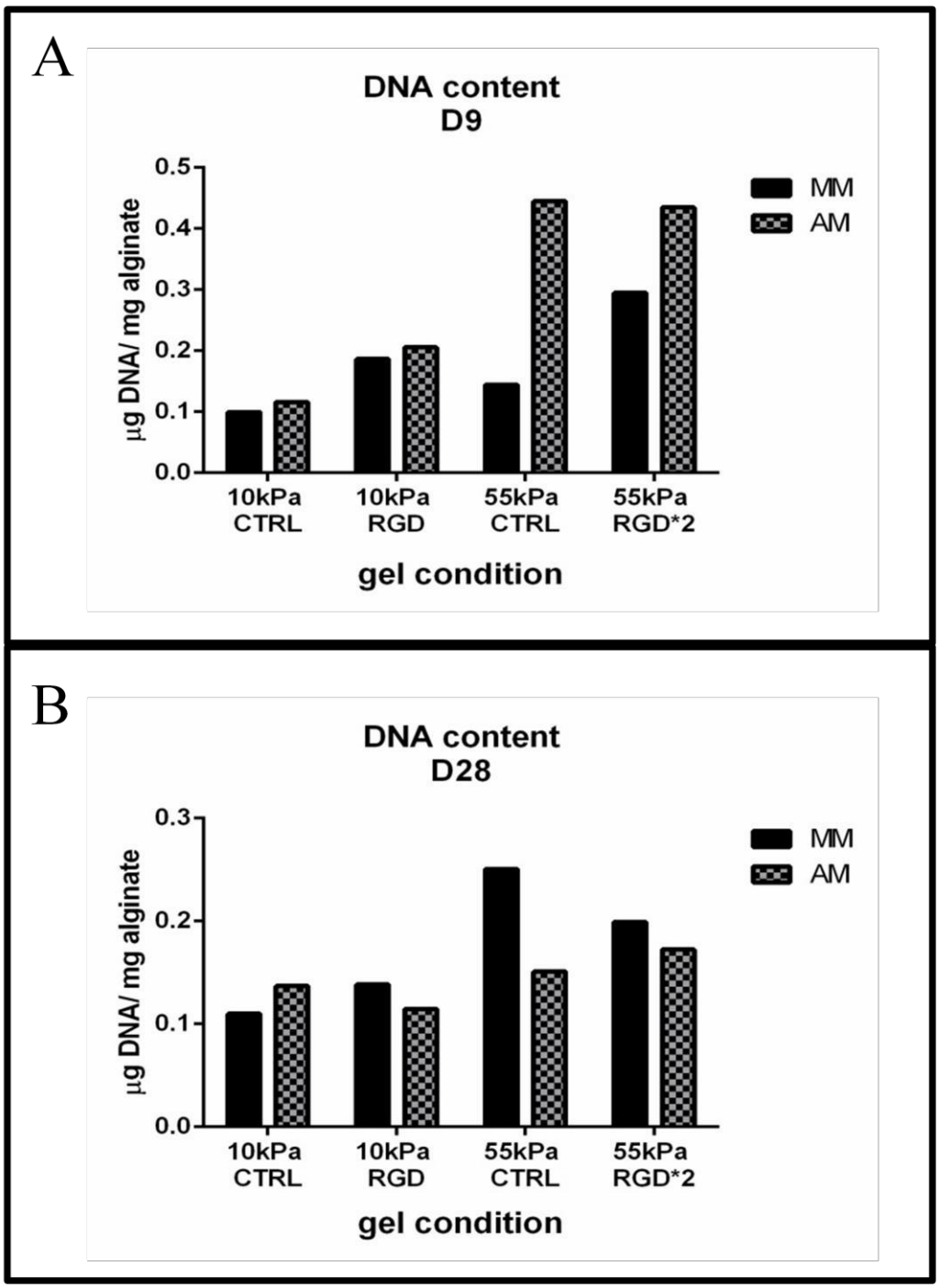


Figure 36. **DNA content for 3T3-L1 cells on days 9 and 28.** A) On day 9, 55 kPa gels have more DNA than 10 kPa gels, with adipogenic medium, for both CTRL and RGD gels. B) On day 28, DNA in 10 kPa, CTRL and RGD gels, have not changed, whereas 55 kPa gels have decreased in all conditions but CTRL gels with maintenance medium.

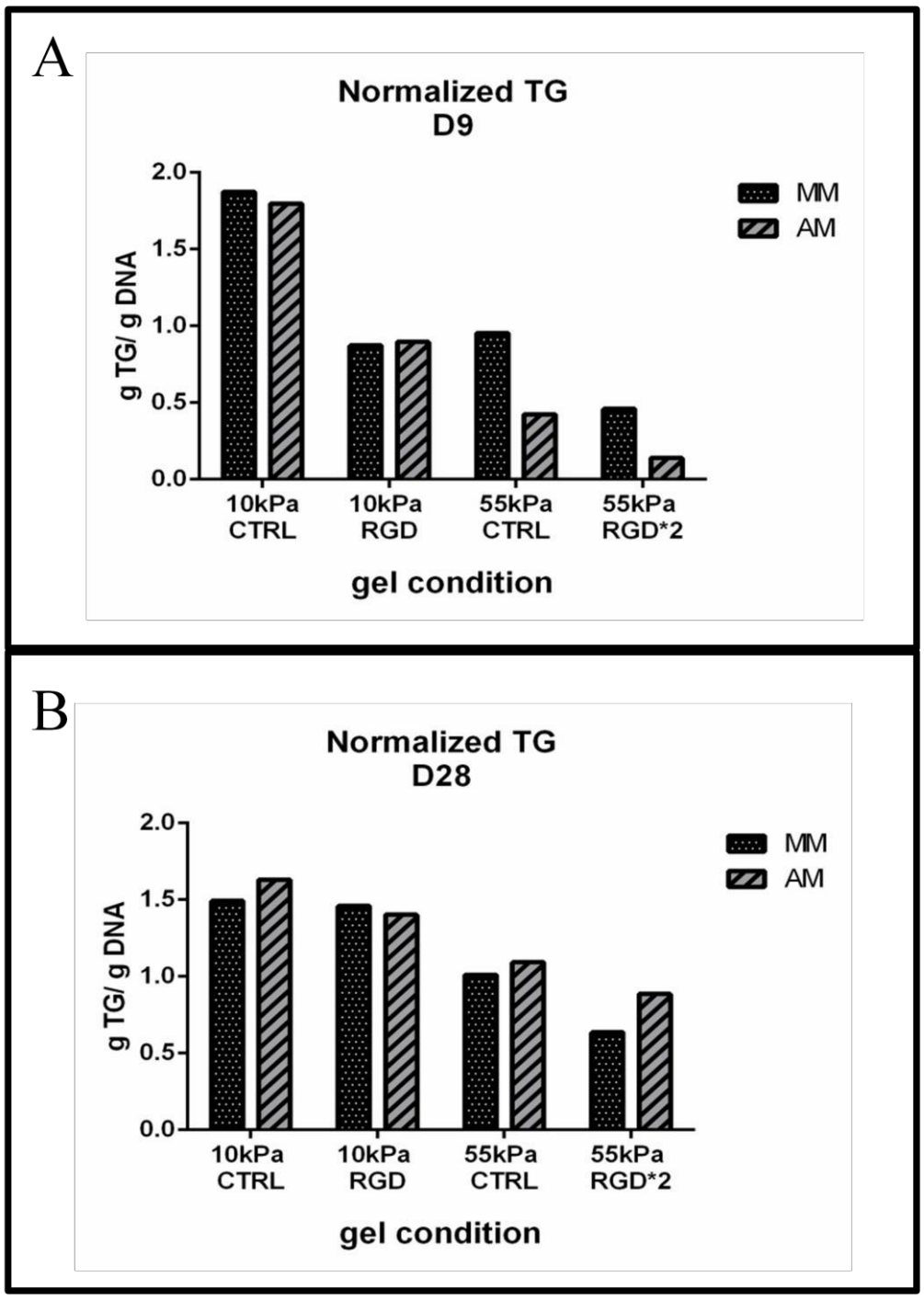


Figure 37. **TG amount for 3T3-L1 cells on days 9 and 28.** A) On day 9, results show there is more TG in the 10 kPa CTRL gels, with medium causing very little difference in TG. B) On day 28, 10 kPa gels, CTRL and RGD conditions, show similar amounts of DNA, with medium causing little difference. MM = maintenance; AM = adipogenic medium

4.3.4 Repeat cells in gels with elastic moduli of 10 and 55 kPa

3T3-L1 cells were encapsulated in alginate gels with different elastic moduli, 10 kPa and 55 kPa, to achieve a greater difference in elastic modulus between the gels. There were CTRL and RGD gels. Table 9 lists experimental conditions. For 55 kPa gels, double the alginate polymer percentage was needed, as compared to the 10 kPa gels. Previous experiment used alginate that was functionalized with RGD for the entire volume of alginate for 55 kPa gels, so keeping the alginate: RGD ratio the same between the different conditions, the amount of RGD was twice as much in the 55 kPa RGD condition as the 10 kPa RGD condition. For this experiment, the same volume of functionalized alginate was used for each gel condition, and non-functionalized alginate was used for the remainder of the volume for the 55 kPa gels. Therefore, there is the same amount of RGD in both the 10 kPa and 55 kPa gels.

Table 9. Experimental conditions of 3T3-L1 gel seeding with elastic moduli of 10 and 55 kPa

Elastic Modulus (kPa)	**Gel condition**	**Medium**
10	CTRL	Maintenance
10	CTRL	Adipo
10	RGD	Maintenance
10	RGD	Adipo
55	CTRL	Maintenance
55	CTRL	Adipo
55	RGD	Maintenance
55	RGD	Adipo

Similar trends could be seen as the last experiment. On the day 1 after seeding, cells both RGD groups have begun to spread, and cells in CTRL gels are rounded. Cells in RGD gels continue to spread for the first 4 or 5 days, where at this point they begin to round back up. By day 7, most cells have rounded back up, regardless of medium type. By day 9, small lipids could be seen in the 10 kPa RGD gels and by day 12, cells have more defined lipids. By the end of the experiment, well defined larger lipids could be seeing in 10 kPa gels with RGD, regardless of medium and smaller lipids in the 10 kPa CTRL gels with adipogenic medium. Smaller lipids would also be seen in the 55 kPa RGD gels, with both medium types, and CTRL gels with adipogenic medium.

Gels were harvested on days 9 and 28 for DNA content and TG analysis. On day 9, generally, there is similar DNA content in 10 kPa and 55 kPa gels, for both CTRL and RGD conditions, regardless of medium. 55 kPa, CTRL gels with adipogenic medium shows slightly more DNA content than any other condition (Figure 38A). On day 28, 10 kPa, CTRL gels have the most DNA, regardless of medium. Medium type does not seem to result in differences of DNA for any of the conditions (Figure 38B). On day 9, 10 kPa gels, both CTRL and RGD conditions, have similar amounts of TG, and both more than the 55 kPa gels (Figure 39A). On day 28, both 10 kPa and 55 kPa, CTRL gels, with adipogenic medium have similar amounts of TG, and more than other conditions. Overall, 55 kPa gels seem to have more TG than 10 kPa gels. For the 10 kPa gels, adipogenic medium seems to have a greater affect than on the 55 kPa gels (Figure 39B)

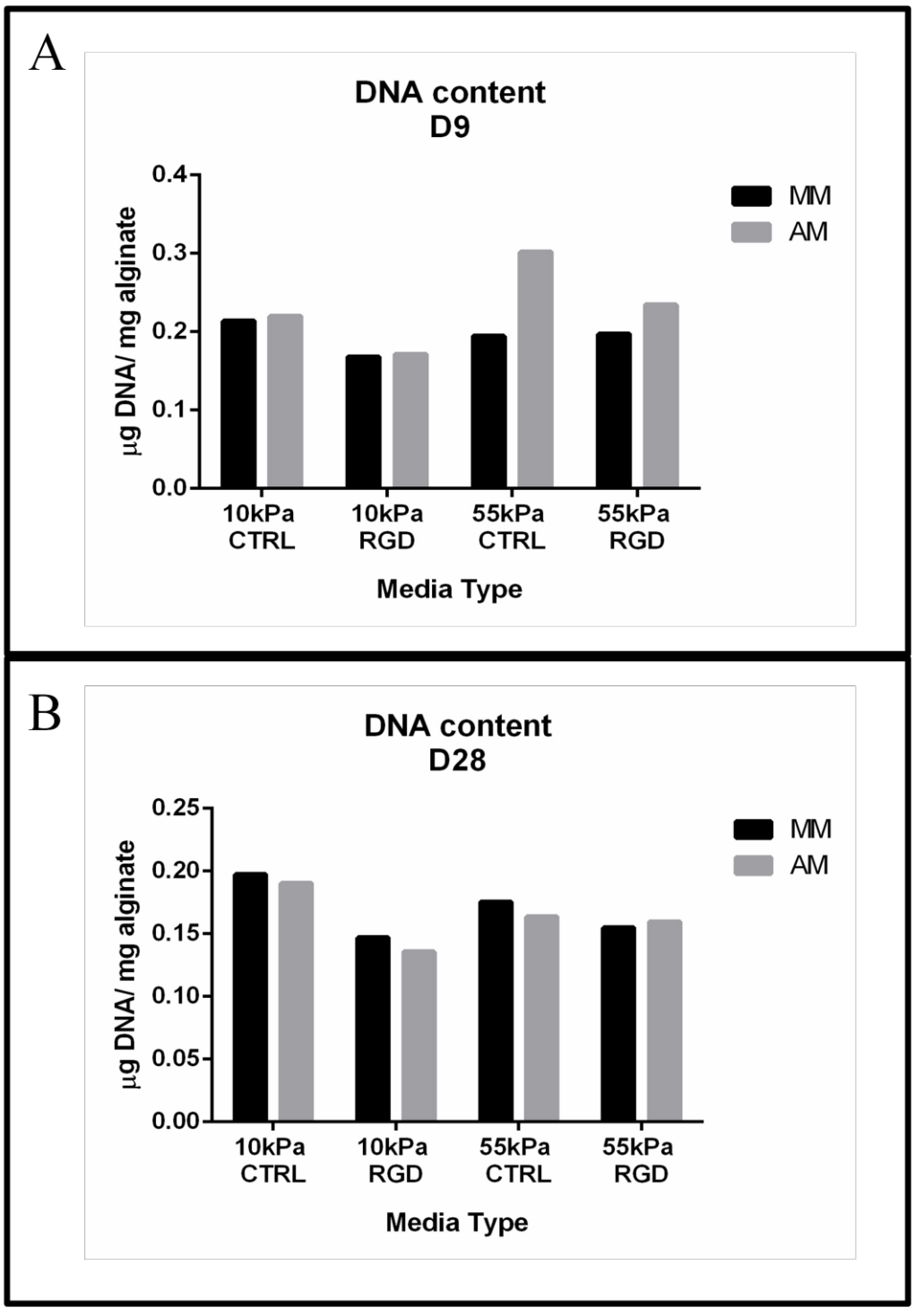


Figure 38. **DNA content for 3T3-L1 cells on days 9 and 28.** A) On day 9, DNA content is similar in all conditions except 55 kPa CTRL gels with adipogenic medium, which has more than all other conditions. B) On day 28, 10 kPa CTRL gels have slightly more DNA than all other condition, with medium type causing little difference within a condition. MM = maintenance medium; AM = adipogenic medium

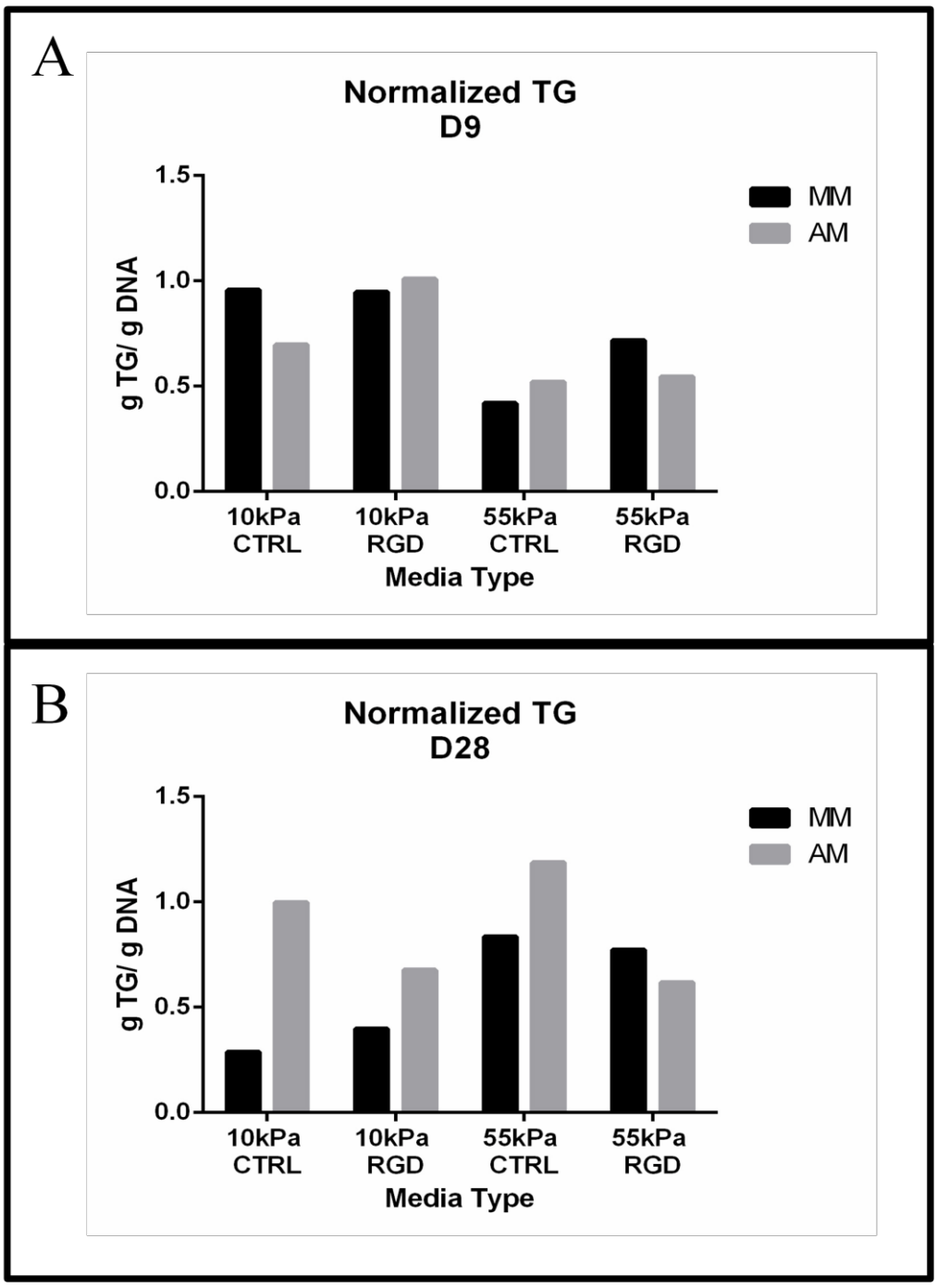


Figure 39. **TG amount for 3T3-L1 cells on days 9 and 28.** A) On day 9, 10 kPa gel, both CTRL and RGD conditions, have similar amounts of TG, and more than both 55 kPa CTRL and RGD gels. Medium type did not cause drastic differences in TG amounts. B) On day 28, both 55 kPa and10 kPa CTRL gels with adipogenic medium have the most TG

4.3.5 Seeding cells in gels at low and high cell density

3T3-L1 cells were encapsulated into alginate gels at two different seeding densities, 10M/mL and 50M/mL, both with CTRL and RGD conditions. All gels had the same elastic modulus and received adipogenic medium. Table 10 lists experimental conditions. This was to understand if different seeding densities influenced adipogenesis.

Table 10. Experimental conditions for density test

Elastic Modulus (kPa)	**Gel condition**	**Cell density (M/mL)**
10	CTRL	10
10	CTRL	50
10	RGD	10
10	RGD	50

One day after seeding, many cells in the 10 M/mL gels with RGD were spread, and all cells in the CTRL gels were rounded. The high density of 50M/mL gels made it difficult to see cells in the gels, but there were a few cells that looked spread. By day 7, many cells had rounded back up in both seeding densities and by day 9, there were very few cells that were not rounded. By day 18, many cells in the 10 M/mL gels have lipids, with RGD gels having more lipids. The 50 M/mL gels were too dense to be able to focus on individual cells, therefore, extremely difficult to see if lipids were present. Cells in 10 M/mL gels progressed like previously experiment; however, gels with the high seeding density still made it difficult to see cells.

Gels were harvested on day 28 for DNA content and TG analysis. Not surprisingly, the high cell density (50 M/mL) gels had more DNA, than the low cell density (10 M/mL) gels. There is marginally more DNA in the CTRL gels than the RGD gels for both cell densities (Figure 40A). Triglyceride analysis shows that there is more TG in the low cell density gel than the high density gels, and CTRL and RGD gel conditions have similar amounts. (Figure 40B)

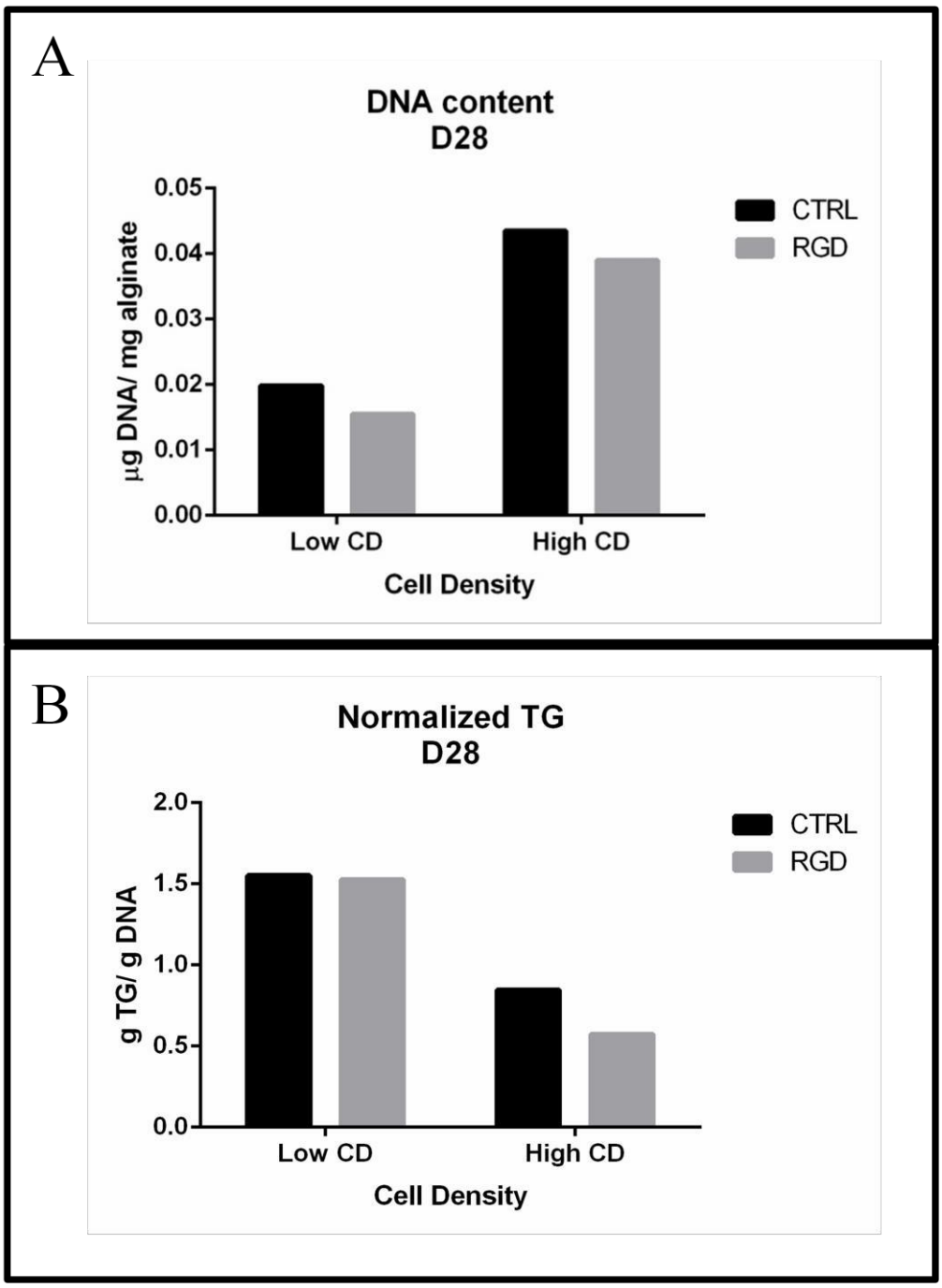


Figure 40. **DNA content and TG amount for cell density experiment.** A) Results show more DNA in gels seeded at the high cell density at day 28 B) Results show more TG is gels seeded at the low cell density

4.4 Discussion

Elastic modulus of a substrate is known to have an effect on cell behavior. Studies have shown that as little as a 3-fold change in elastic modulus of a scaffold can have drastic effects on cell differentiation potential (Engler, et al., 2006). Here we look at adipose progenitor cells, and their ability to perceive the elastic modulus of the scaffold they have been encapsulated in and differentiate into mature adipocytes.

3T3-L1 cells, a mouse adipose progenitor line, were encapsulated in alginate gels with elastic moduli of 3 and 10 kPa. Although this experiment has a 3-fold change in elastic modulus, differences in differentiation of cells are hard to detect. Both elastic moduli with RGD seem to produce large lipids, regardless of whether they received induction medium or not. Images seem to indicate that RGD is beneficial for cell differentiation and lipid formation. Although the 3 kPa gels look to produce more large lipids by the end, a quantitative measurement is needed to detect any real differences. A repeat of this experiment was set up in order to test a quantitative measurement for differentiation differences between the two scaffolds.

3T3-L1 cells were encapsulated in alginate gels with elastic moduli of 3 and 10 kPa. This was to understand any differences in differentiation by using a quantitative assay to detect lipid formation. Again, by looking at images, it is hard to detect any differences in differentiation. By the end of the experiment, both elastic moduli gels show larger lipids in the RGD conditions, regardless of

medium type they received. Images seem consistent with the last experiment in that the presence of RGD seemed more beneficial for lipid formation. When looking at TG amount, 10kP gels actually show more than 3 kPa gels. This was unexpected, but could be explained by the fact that both elastic moduli are in the range of what is reported for normal adipose tissue. 3T3-L1 cells, because they are an adipocyte progenitor line, and can only differentiate into adipocytes, may not be sensitive to such a small fold change in elastic modulus of the scaffold. Our measurements of elastic moduli of the alginate gels are taken by a different method than others have done, so our 10 kPa gels may actually be a better match to the elastic modulus of normal adipose tissue.

3T3-L1 cells were encapsulated into alginate gels with elastic moduli of 10 and 55 kPa. Here, our aim is to see a difference in differentiation of cells when seeded into gels that have elastic moduli within and out of the normal range of adipose tissue, as was reported to range 2-24kPa. Unlike the last experiment, images show a difference in lipid formation, favoring the 10 kPa gels with RGD, regardless of medium. First, we looked at DNA content for days 9 and 28. On day 9, 55 kPa cells have more DNA than 10 kPa gels, which would indicate that they may be proliferating, rather than differentiating. It is, however, interesting that adipogenic medium produces more DNA content on all gel types, which does not support the idea that adipogenic medium causes proliferation to stop and differentiation to occur. It is also possible that by day 9, medium is not causing an effect on the 55 kPa gels. On the last day of the experiment, day 28, results still show that 55 kPa gels, both CTRL and RGD conditions have more DNA content

than the 10 kPa gels, regardless of medium. However, there is less DNA at day 28 for the 55 kPa gels than there was at day 9. 10 kPa gels show similar DNA amounts for the two days, regardless of medium. This would indicate that 10 kPa have not proliferated during the experiment. A loss in DNA for the 55 kPa gels could indicate that the stiffness of the gels is inducing apoptosis, or programmed cell death, due to the abnormal stiffness that these cells would not normally be subjected to. TG analysis, for both days 9 and 28, shows that 10 kPa gels have more TG than the 55 kPa gels. Interestingly, medium does not seem to have an effect of TG amount. At day 9, 10 kPa CTRL gels have more TG than RGD gels, but at day 28, they have the same amount. The amount of TG in the CTRL gels goes down, whereas the TG in the RGD gels goes up, by day 28. This could mean that because cells are rounded in the CTRL gels, initial TG is higher, but as the cells in the RGD gels round up, they produce more TG. Taking the experiment beyond 28 days could show that this is the case, and there may be a greater difference in TG over time, favoring the RGD gels. This experiment was also done with different amounts of RGD for the 10 and 55 kPa gels. Because these amounts were different, a repeat of the experiment was done to keep RGD amount constant.

3T3-L1 cells were encapsulated in gels with elastic moduli of 10 and 55 kPa, with keeping the amount of RGD within the gels consistent. Similar trends were seen in the images as with the previous experiment. Visually, it looked like 10 kPa gels with RGD, regardless of medium, produce the most lipids. The 55 kPa gels with RGD look like they may have small lipids, but none produced

larger, more defined lipids. On day 9, results show that there are similar amounts of DNA in all the gels, regardless of medium or stiffness. By day 28, the 10 kPa CTRL gels have slightly more DNA than all the other conditions. This trend is opposite of what was seen in the previous experiment, where this particular condition had the least. Medium also does not seem to have an effect, where as the last experiment, medium caused differences in DNA. On day 9, 10 kPa gels, for both CTRL and RGD conditions, have more TG than either CTRL or RGD gels with an elastic modulus of 55 kPa. On day 28, however, 55 kPa CTRL gels with adipogenic medium have the most TG. 10 kPa CTRL gels with adipogenic medium also have a similar amount. It is expected that adipogenic medium produces more TG; however, it is unexpected that 55 kPa gels have the most TG. It is also unexpected that CTRL gels seemed to produce more TG than RGD gels, because there were more large visible lipids in the RGD conditions. This is a trend that is seen throughout many of the experiments. One explanation could be that many small lipids, although not as visible when looking that the cell, actually results in more over all TG. More investigation is needed to understand if this is the case. Because they are a progenitor cell line, causing them to stay round in the CTRL gels may be alone causing adipogenesis, and 3T3-L1 cells may not be the most ideal cells to use for this stiffness experiments.

After looking at how differences in elastic moduli of scaffold have an effect on cell differentiation, we questioned whether seeding density of the gels would have an effect on adipogenesis. Gels were seeded with a low cell density of 10 M/mL, which is the density for other previous experiment, and high cell

density of 50 M/mL. Only adipogenic medium was used. The cells in the low cell density gels behaved as previously described. Cells in the high density gels were very hard to visualize and focus on under the microscope. On the last day of the experiment, day 28, the gels were harvested for DNA and TG analysis. There was more DNA in the high cell density gels, although not 5 times as much, which would indicate that cell death was occurring. TG analysis showed that there was more TG in the low cell density gels, but equal amounts in the CTRL and RGD gels. Because samples were not taken on any other day, it is hard to say how both DNA and TG changed over the duration of the experiment. However, results support the use of the low cell density for the previous experiments. It may be beneficial to do further density studies with cell seeding densities even lower than our low value.

4.5 Conclusions and future directions

The results from all the studies conducted indicate that adipogenic potential of 3T3-L1 cells could be affected by different elastic moduli, but more studies needed to be done. The initial studies with elastic moduli of 3 and 10 kPa show that this may not be enough of a difference in fold change to induce any differences in adipogenesis of the cells. This could be a result of the two elastic moduli being within the reported range of adipose tissue. The subsequent experiments where the difference in elastic moduli is greater show that cells may be affected by the differences. More studies and replicates are needed to confirm this. Other quantitative measurements, such as Real-Time PCR, and a live/dead assay would be beneficial to help understand how cells are behaving. It may also be necessary to increase the difference in elastic moduli, to show more drastic changes in differentiation. These cells are a progenitor cell line, and only have the ability to differentiate into mature adipocytes. Because most stiffness studies are done using stem cells, it may be that 3T3-L1 cells are not sensitive to smaller fold changes in elastic modulus.

5 Thesis conclusions and future directions

This thesis focuses on the ability for the ECM of cells to determine cell differentiation. We looked at how the presence of a binding peptide within a 3D alginate gel can influence cell morphology and thus affect the ability of ASCs to undergo adipogenesis. We also looked at how the elastic modulus of a scaffold influences 3T3-L1 cells to differentiate into mature adipocytes.

In our alginate system, we used calcium ions from calcium carbonate (CaCO3) to crosslink the gels. Free calcium ions in the alginate gel system may influence cell behavior, and may be a potential confounding factor. For this reason, it is important that total calcium content is kept constant for all conditions being investigated. For the alginate gels, a calcium ion to carboxyl molar ratio was used, where 0.18 is designated as 1X (Kuo and Ma, 2001). In this thesis, experiments with ASCs used a crosslinking density of 2X for every condition. Therefore, in experiments with ASCs, calcium content was always constant. Whereas, a crosslink density of 2X and 4X was used in experiments with 3T3-L1 and may be potential confounding factor. Therefore, it is important to be aware of the effects that calcium can have on cells. Previous studies have shown excess extracellular calcium can reduce adipogenesis of 3T3-L1 mouse preadipocytes without causing apoptosis or affecting proliferation. At concentrations greater or equal or 5mM of soluble calcium, differentiation of 3T3-L1 preadipocytes was altered through inhibition of the expression of key adipogenic transcription factors and differentiation marker genes (Jensen, et al., 2004). Other studies have shown that high concentrations of intracellular calcium ions have a biphasic

affect. The results indicate that increasing the concentration of intracellular calcium in the early stages of differentiation inhibits human adipocyte differentiation, whereas increasing the concentration of intracellular calcium in late stage promotes human adipocyte differentiation (Shi, et al., 2000). However, our alginate system is presumably overcoming the problem of calcium available to the cells because the calcium carbonate used in our experiments is highly insoluble at neutral pHs (only 0.014g/L; Klieger and Hooton, 1990). While, it is likely that the majority of calcium is either consumed in crosslinking with the alginate or in an insoluble form, it is currently unknown if the calcium in the alginate gels influences adipogenesis by the ASCs or 3T3-L1s. Future studies are needed to investigate if calcium is available to the cells and how it may influence cell behavior.

Using either ASCs or 3T3-L1 cells for developing a model of adipose tissue has both advantages and disadvantages. 3T3-L1 cells are a cell line, which are easier to grow and work with. There is most likely less variability among replicates, which makes for more consistent results. However, these are mouse cells, and may not be as relevant for human models. Because they are an adipocyte progenitor cell line, there differentiation ability is limited to that of only mature adipocytes. They are much more prone to differentiation regardless of conditions, and therefore it is harder to elucidate how different external factors are affecting differentiation (Smas, et al., 1995). ASCs are human cells, with a fairly easy isolation process from donors. However, being primary cells from human donor can cause variation among different lots, which may cause result variation

in experiments. Because they are stem cells, they have the ability or differentiate down several lineages. This means they are more likely affected by small changes in their matrix environment and may be better at elucidating how these changes affect differentiation.

Future directions for understanding how cell morphology affects cell differentiations include more repeats of the same experiment with ACS. Because there is variation between the replicates, it may be beneficial to have more repeats, which could help show more significant differences among the gel conditions (CTRL, RGD/2 and RGD. It may also be beneficial to use other amounts of RGD, such as RGD/3 or RGD/4 in this same experiment. This could cause more detectable differences in cell circularity, and more definitive differences in cell differentiation.

To better understand how scaffold elastic modulus affects adipogenesis, it is beneficial to understand the mechanical properties of the materials used in the experiments, and is needed to be able to compare to other studies. Elastic modulus of a material can be determined in different ways. A commonly used method is compression testing, which tests the elastic modulus at a bulk level. A sample is compressed under a certain amount of load. Another way of testing bulk elastic modulus is with tensile testing, where a material is stretched. In these methods of materials characterization, elastic modulus can be measured by calculating the slope of the stress-strain curve.

Elastic modulus can also be measured by atomic force microscopy (AFM) at nano- and microscales by using nano- or microscale AFM tip probes,

respectively. Nano- or microscale indentation by AFM measures material properties at the cellular level, which may be particularly relevant as mechanical cues experienced by cells during tissue development (Marturano, et al., 2013). For measurements at the nanoscale, AFM uses a cantilever with a sharp tip (radius apex curvature 1-10 nm) that is scanned over the surface and the interaction forces between tip and sample lead to a deflection of the cantilever (in contact mode) or a reduced vibrational amplitude (in dynamic mode). As a result the topography of a sample's surface is recorded with lateral resolution of 1–10 nm and height resolution down to 0.1 nm (Bald and Kellar, 2014).

It can be difficult to compare elastic modulus values if testing conditions and modes of testing are different (i.e., bulk vs. AFM). Work done previously in our lab shows that when measuring the elastic modulus of embryonic chick tendon using AFM, the resulting elastic moduli ranges from 5-108 kPa, depending on what stage of development and which size tip (micro- or nanoscale) is used (Marturano, et al., 2013). In comparison, other works have shown that these embryonic chick tendons can have an elastic modulus of 20.5 MPa when determined by bulk tensile testing (Kalson, et al., 2011).

The elastic moduli of the alginate gels used in this thesis project were measured by AFM, by previous work done in our lab (Marturano, et al., *in prep*). The work showed that the calcium content (i.e., crosslinking density) and alginate polymer concentration used in these gels had an elastic modulus of around 3.4 kPa. One limitation is that the reported values for adipose tissue have not been determined by AFM, which makes it difficult to directly compare these gels to adipose tissue, and even other *in vitro* studies, that do not use AFM. The value of

1.9 kPa for normal adipose tissue from breast tissue was reported by Samani, et al., who determined the value by unconstrained indentation compression testing (2003). Another study reported that at low strain rates of compression testing, the effective elastic modulus of adipose tissue is 1 kPa (Comley and Fleck, 2009). To date, the elastic modulus of adipose tissue as measured by AFM has not been reported.

One study by Lee, et al. (2013), used 2D substrates with an elastic modulus of 0.6 kPa to represent adipose tissue, a value that was determined by AFM, which is over five times less than the value of 3.4 kPa that we use for our alginate gels. Because the elastic modulus of adipose tissue determined by AFM has not been reported, it is difficult to say which of these values more closely represents native adipose tissue. Another study used alginate gels that had an elastic modulus of about 3 kPa to represent normal adipose tissue; however, this value was determined by aggregate compression testing (Chandler, et al., 2011). The different mechanical testing methods used (i.e., bulk vs. AFM) are a challenge for comparing the results of different studies.

To better mimic the mechanical properties of native adipose tissue with engineered tissue constructs *in vitro*, it may be beneficial in future studies to do additional mechanical testing of adipose tissue and our engineered tissues. To better inform alginate gel composition (e.g., polymer concentration, crosslink density) and the resulting mechanical properties, both alginate gels and adipose tissue should be tested using the same mechanical testing method, whether that is by AFM, or compression testing.

In addition, it may be beneficial to understand whether matching bulk mechanical properties or matching cellular mechanical properties has more of an impact on cell differentiation. After having a better understanding of the mechanical properties of our materials, we want to further investigate scaffold elastic modulus affects cell differentiation using 3T3-L1 cells. In doing so, it may be more beneficial to have a greater difference between the low and high elastic moduli within the experiments. Because these are a progenitor line, they may be less sensitive to small changes in substrate elastic modulus. It could also be more helpful to use ASCs for this study. After understanding if there is an optimal amount of RGD needed, we could use this amount in gels with different scaffold elastic moduli, and see if changes in differentiation can be detected. It has been shown that stem cells have greater responses to small changes in matrix environment, and therefore ASCs may be better in the goal of developing a model of adipose tissue. Also, having more than 2 different elastic modulus can better show how they are affected by differences, as seen in the cell morphology experiment that had 3 different gel conditions. Overall, these experiments can elucidate how cell differentiation is affected by matrix environment factors. Combined with future experiments, they will be helpful towards the goal of developing an *in vitro* model of adipose tissue to help study obesity and other related diseases.

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