Development of a 3-Dimensional Fat Model Using Alginate Hydrogels

An Honors Thesis for the Dapartment of Chemical and Biological Engineering

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Abstract

In 2010, more than 35.7% of U.S. adults were considered obese [24]. With obesity comes increased risk for conditions such as hypertension and type 2 diabetes [23]. To combat obesity, more needs to be known about the process of adiposity, the excessive expansion of adipose tissue.

As of now, adiposity has been studied through both *in vivo* and *in vitro studies*. *In vivo* studies however, are challenged by numerous uncontrolled factors, which has lead to inconsistent results [8, 12, 20, 41]. Traditional tissue culture plastic, however, is a vastly different system than found *in vivo* which has shown to cause discrepancies in morphological features and gene expression when compared to adipocytes *in vivo* [33].

To address these limitations of current *in vitro* models, we developed a 3-dimensional culture system that allows for study of adipogenesis in more physiological conditions. Alginate hydrogels are promising as the cells are easily incorporated into the gels, and allow for control of the mechanical properties by altering crosslinking parameters [18]. Our studies have shown some affect of scaffold modulus on cell differentiation, as well as a strong effect of cells binding to the scaffold through RGD functionalization. Our gels also showed an ability to function as an adipogenesis model without the need for pre-differentiation. This alginate hydrogel model was also used to begin studying the effects of obesogens, a group of chemicals thought to promote obesity, through studies involving the introduction of TBT into the medium. Overall, alginate hydrogels have proven to be very valuable as a 3-dimensional model, our current studies having possibly discovered new directions for the study and understanding of adipogenesis.

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List of Abbreviations and Acronyms

AIM	Adipogenic Induction Medium
AFM	Atomic Force Microscopy
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ECM	Extra cellular matrix
GDL	D-(+)- Gluconic acid Delta-Lactone
FBS	Fetal Bovine Serum
HBSS	Hank's Balanced Salt Solution
IBMX	Isobutyl methylxanthine
MPB	Methylparaben
Pen Strep	Penicillin Streptomycin
RGD	Peptide sequence Arginine, Glycine, Aspartic Acid
TBT	Tributyl Tin Chloride

1 Introduction

In the United States the threat of obesity is rapidly increasing. In 2010, 35.7% of U.S. adults were considered obese as compared to the year 2000 when none of the 50 states had obesity levels of over 24% [10, 24]. This sharp rise of obesity brings with it many concerns as obesity increases the risk of many other life threatening conditions such as hypertension and type 2 diabetes [23]. Due to a long history of huge health risks associated with obesity drugs, the search for new treatments has been very slow [2]. To better and more safely attack the problem of obesity more must be learned about the process of adiposity.

As of now, it is known that obesity is caused not only by the enlargement of lipids in the adipocytes (hypertrophy) by also by the increase in number of adipocytes (hyperplasia) [34]. Pre-adipocytes are actually posses a fibroblastic nature, and it is not until they differentiate that they round up and accumulate lipids. [25] Before differentiation, pre-adipocytes can undergo proliferation, however, once differentiated they loose this capability. Thus, adipogenesis is a normal part of the cycle of the body, as it is needed for a natural turnover of cells [4] though the body also can induce increased proliferation and then adipogenesis as a response to needs of increased fat storage [28]. The sudden increase in obesity, though often attributed to increased caloric intake and lack of exercise of the current generation, is also thought to be connected to changes in our environment. One area of particular interest is a group of chemicals called obesogens which, as the name suggests, are thought to promote obesity. Among these chemicals is tributyl tin, an obesogen which has previously been studied and been shown to increase obesity in a 2-Dimensional model [16], but has proven to be more difficult to study *in vivo* [41]. Tributyl tin is also of particular interest since it and other organotins have been used as fungicides on food crops, and have been found to contaminate seafood and shell fish [13]. The culture system presented in this paper would provide an effective system in which to study the effects of tributyl tin while cells are in an environment that mimics their native tissue.

Studies of adipogenesis has been stunted by two large problems with the current method of studies. First, in vivo studies being preformed can present widely different results [12, 20], since

in vivo studies have a lot of variables that are difficult to control. Regulation of the studies makes it near impossible to be certain whether variables are causing the effect or if some unaccounted for variable is occurring in some of the trials. As a response to this, many people perform in vitro studies, but this also presents another major problem; growing the cells on hard, flat tissue culture plastic is not reminiscent of the conditions that would be found in the body. To combat these two problems, we have come up with a novel model for adipogenesis studies using alginate hydrogels.

Alginate hydrogels show much promise for 3-dimensional in vitro studies for many reasons. Alginate hydrogels do not gel before being crosslinked, thus cells are easily incorporated into the gel. Also, the gel's mechanical properties are easy to control by varying calcium crosslinking densities [18]. This is very important fact since numerous studies have shown that cells are very sensitive to their environment. As small as 3 fold-changes in scaffold modulus have been shown to have noticeable effects on cell lineage [9]. Tissue culture plastic, however, has a modulus of around 1000kPa [36] while studies have shown white adipose tissue (WAT) in breast tissue to have a modulus around 1.9 if measuring through indentation methods with preconditioning [31] or 24 kPa if using magnetic resonance elastography [38]. This means that tissue culture plastic is at least 50 fold increase in modulus as compared to *in vivo* conditions. Not only that, but the cells are forced into a flat conformation very unlike tissue. With alginate gels, the modulus can be comfortably brought down to about 3kPa and can be increased easily, making the alginate gels perfect for the range of adipose tissue. In addition, by changing percentage of alginate in the system and using 4x calcium the gels can have a modulus of up to 150 kPa. This versatility in the system could be potentially used for study of diseased adipose environments such as atherosclerotic lesions, which, by using AFM (atomic force microscopy), has been found to have an average young's modulus ranging from 5.5+/-3.5kPa up to 59.4+/-47.4 kPa depending on the region of the fibrotic tissue [37].

Though not many studies have been done on the preferred modulus for the differentiation of adipose progenitor cells, many studies have been looking at mesenchymal stem cells and the substrate stiffnesses needed to promote differentiation towards different lineages. Engler showed that

by changing the scaffold modulus of a 2D collagen coated gel system to meet *in vivo* conditions, it was possible to make MSCs tend towards neurons, muscle cells, and osteoblasts, however, adipose was not investigated [9]. McBeath investigated a similar idea looking at the cytoskeletal tension effects on stem cell lineage, however, these studies did not use changes in substrate modulus and instead induced cell shape effects through methods such as the addition of cytochalasin D [22]. Other studies grew off of these ideas and attempted to help dictate differentiation of MSCs through modulus, in combination with molecules from the ECM. Once again though, these stem cell studies focus on bone and muscle and do not attempt studies on adipogenesis [29]

The study of adipogenesis in a 3-D model is still a very new concept. A few groups have attempted other variations on alginate hydrogels. Galateanu made layer-shapped alginate hydrogels using calcium gluconate and calcium chloride for crosslinking for differentiation of ADSCs (adipose-derived stem cells). The calcium in this more soluble form, however, was suspected to have slowed and reduced adipogenesis in the model thus presenting a hurdle for representative *in vitro* analysis. The study also, lacked a focus on the ability to manipulate the mechanical properties the gels could provide, as well as the potential need for cell binding to the gel for differentiation, which leaves the model a less adaptable environment [11]. Another study by Chandler attempted the use of Alginate gels with controllable properties through UV exposure. The UV exposure, however, leads to a harsh encapsulation of the cells with around 50% cell death by day 7 in the softest gel [5]. Collagen gels have also been used for adipogenesis studies [6] though the properties of collagen gels are not as easily controlled and have their own potential for biological signaling. Very recently, some work has also been done using hydrogels from actual adipose tissue as well. It is a very intensive process and thus these studies have focused on their ability to be used as a method of implanting stem cells for tissue repair, rather than a simple model for study [27, 40].

As mentioned, one problem people have faced with alginate gels, is that to increase the modulus, an increase in calcium crosslinking must occur. Extracellular calcium, however, has been shown to reduce the ability of 3T3-L1s (mouse adipocyte progenitor cells) to differentiate [17]. To account for this, some studies have attempted to use UV crosslinking as another method. This,

however, subjects the cells to harmful rays that have even been used in other studies as a means of causing mutations [5,39]. This study, however, overcomes this challenge by using calcium carbonate as its source of calcium. Calcium carbonate is highly insoluble at neutral pHs as found in cell culture conditions. For a 4x Ca gel, 51mM of calcium carbonate is added, which if it were very soluble could cause attenuation of adipogenesis [**?**, jensen04] At 25 C^{o}the solubility of calcium carbonate is only 0.0013g/100mL [1], which gives a soluble concentration of 0.013 mM. Studies have shown that more than 2.5mM of soluble calcium is need to affect adipogenesis [17], which is order of magnitude larger than the calcium dissolved into solution from calcium carbonate. At higher temperatures, the solubility of calcium carbonate actually decreases due to a decrease in the solubility of atmospheric CO_2 thus meaning that even less calcium would be soluble in the ECM (extra cellular matrix) [3, 7]. Since the majority of calcium is either used up in crosslinking with the alginate or in an insoluble form, the calcium cannot enter the cell as a secondary messenger to trigger adipogenesis inhibiting pathways.

Alginate gels have another valuable property, they can be functionalized. Without peptides, the cells cannot bind to the alginate and are merely suspended [18]. The gels, however, can easily be functionalized with numerous different peptides at different concentrations, giving even more control over the environment of the cells [30].

Though the initial inspiration for this project was to create a controllable model for adipogenesis studies, these studies also lend themselves well towards engineering fat for tissue regeneration such as breast reconstruction. Pre-adipocytes are being investigated for breast reconstruction after tumor removal [26]. It has also been seen that adipose progenitor cells have the ability to produce nearly all of the growth factors known to be a part of normal wound healing as well as being able to promote vessel development through many mechanisms including secretion of VEGF [5]. Thus, with a suitable model for the culture of adipose progenitor cells, breast reconstruction efforts, as well as obesity, could benefit.

In this paper, the effect of mechanical and chemical changes in the gels is explored as a means to form a better model for future adipogenesis studies. The aims include: exploring the effect of

different seeding densities, analyzing the effect of change in scaffold modulus, as well as investigating the effect of functionalizing the gel with RGD on adipogenesis. As more data was gathered, the aims were expanded to include looking at the effect of pre-differentiation and examining the effects of specific obesogens on adipogenesis in a 3-D model.

2 Aims

2.1 Aim 1: Cell Density

The first aim was to determine the effects of cell density on adipogenesis in our 3-dimensional culture system. In a 2-dimensional culture system cells are often grown to confluency before induction because close contact of cells seems to be necessary for differentiation [22]. When the cells are suspended in a 3-dimensional system, however, the same idea of confluency cannot be applied. Studies have shown though, that the close contact found necessary for differentiation may actually be due to a need to halt the cell cycle between G_0 and G_1 since cells suspended after halting the cell cycle were able to differentiate [14]. This however, does not mean that cell-cell communication is not important in differentiation. Thus, it is important to determine suitable concentrations inside the gels. The experiment was designed to look at 10 million cells per mL and 2 million cells per mL to see which would produce a higher percentage of adipogenesis. The hypothesis was that the 10 million cells per mL would have an increased amount of adipogenesis due to the fact that the differentiating cells could better communicate, and previous studies have shown high densities to be beneficial [22].

2.2 Aim 2: Morphology

Adhesion was another major area that needed characterization. Up till now, it has been uncertain whether or not adipocytes are rounded due to their environment and thus can differentiate, or if they, as pre-adipocytes, spread until they round later due to the differentiation process. To look

at this, cells suspended in alginate with nothing to grab on to were compared with cells suspended in gels functionalized with RGD allowing the cells to attach to the scaffold. It was hypothesized that the presence of RGD would lower the amount of differentiation due to an increase of tension in the actin cytoskeleton which was seen to reduce differentiation [22].

2.3 Aim 3: Scaffold Modulus

The effect of substrate stiffness has been a major center of study in recent years. Many studies have shown that substrate stiffness can have a drastic effect on differentiation of stem cells with changes as small as 3 fold [9, 22]. One of the major drives for development of this model was to reduce the uncharacteristic modulus of tissue culture plastic. As such, the effects of stiffness changes on adipogenesis were studied. This study tested a 3 fold change, testing a modulus of 3 kPa as well as a modulus of 10 kPa to determine the difference modulus could make on the differentiation process. Both of these values are within the lower range of *in vivo* adipose tissue. The lower range of normal fat tissue moduli was used due to studies that showed that a high substrate modulus inhibits adipogenesis. In this same vein, it was hypothesized that the lower scaffold modulus, 3 kPa, would have a high percentage of adipogenesis.

2.4 Aim 4: Obesogens

After the characterization of how gel properties effect adipogenesis, it was desirable to begin assessing the effects of a component thought to promote adipogenesis, obesogens. In this case, TBT was selected since it is both thought to promote adipogenesis [16] as well as being known to be in considerable contact with humans through its use as a fungicide for plants, as well as in plastics that come in contact with peoples daily food and water [13, 41]. It was hypothesized that the presence of TBT would cause an increase of both number of differentiated cells as well as the lipid content in each of the cells.

2.5 Aim 5: Pre-Differentiation

To study the full process of adipogenesis, it is obviously optimal to have the entire process shown within the gel. Other environments, such as collagen gels, and associated lab saw a need for pre-differentiation to prevent gel contraction. Pre-differentiation, however, could potentially be difficult on the cells and cause unexpected side effects due to the disruption of the process. As such, it was desirable to test to see if this alginate hydrogel system was capable of studying the process of differentiation from the beginning. Two sets of gels were looked at, those that had received one cycle of predifferentiation, and one cycle that had be induced a day after encapsulation though after cell density inhibition had been established in 2-dimensions. It was hypothesized that the gels would not only be able to produce differentiated adipocytes, but would in fact produce more differentiation and earlier differentiation due to the lack of disruption of the process.

2.6 Aim 6: Need for Chemical Induction

Studies on adhesion as well as scaffold modulus brought up the idea that just as chemical cues can be used to promote adipogenesis even in uncharacteristic environments, perhaps the environment can also have a strong enough effect on cells to promote differentiation despite a lack of additional chemical signaling. To test this, one group of gels were given only growth medium, while another group of cells was given the normal two adipogenic medium cycles. The hypothesis was, that even without chemical signaling, some differentiation would occur in the gels with a proper environment (adhesion capabilities and a soft modulus of either 3 kPa or 10 kPa).

3 Materials and Methods

3.1 List of Solvents and Chemicals

Chemical	Supplier
Calcium Carbonate	Sigma Aldrich
Dex	Sigma Aldrich
DMEM	Gibco
FBS	Atlanta Biological
10% Formalin GDL	Sigma Aldrich
HBSS	Gibco
Human Insulin 10mg/mL	Sigma Aldrich
IBMX	Sigma Aldrich
Isopropanol	Sigma Aldrich
MPB	Fisher Scientific
Oil Red O	Sigma Aldrich
Penstrep	Sigma Aldrich
Pronova Alginate	NovaMatrix
RGD	Sigma Aldrich
TBT	Sigma Aldrich
0.025% Trypsin-EDTA	Gibco

Table 1: A list of all chemicals used in the methods described later in this paper

3.2 Materials

Product	Supplier
CL2 Centrifuge	Thermo Scientific
Microscope	Nikon Eclipse TE300
Microscope Camera	Hamamatsu Camera C4742-95
Nalgene bottle-top Sterile filter unit, pore size 0.2μm	Sigma Aldrich
24-well Non-Tissue Culture Plates	BD-Falcon
T-175 Tissue Culture Flasks	BD-Falcon

Table 2: A list of all the materials used in the methods described later in this paper

3.3 Medium

There were 3 different types of medium used during these experiments a standard growth medium/maintenance medium, as well as two adipogenic inducing cocktails used in another paper [19] based on the findings that adding combination of Dex, IBMX, and insulin to a standard culture mixture makes the best adipogenic induction medium [35]. All three mediums were mixed in the same fashion of adding materials in order of increasing volume together and then filtering. The compositions of the medium follow.

3.3.1 Growth Medium/ Maintenance Medium

Component	% Volume
DMEM	89%
FBS	10%
Penstrep	1%

Table 3: The composition of the standard growth and maintenance medium used based on percent of each component in terms of total volume

3.3.2 Adipogenic Medium A

Component	% Volume
DMEM	88.88%
FBS	10%
Penstrep	1%
0.5M IBMX	0.1%
Insulin 10mg/mL	0.01%
10 mM Dex	0.01%

Table 4: The composition of the AIM A based on percent of each component in terms of total volume

3.3.3 Adipogenic Medium B

Component	% Volume
DMEM	88.99%
FBS	10%
Penstrep	1%
0.5M IBMX	0%
Insulin 10 mg/mL	0.01%
10 mM Dex	0%

Table 5: The composition of AIM B based on percent of each component in terms of total volume. Note the composition of AIM B is the same as AIM A except for the removal of dex and IBMX in AIM A

3.4 Cell Culture

All the experiments in this paper used 3T3-L1s, a mouse adipocyte progenitor cell line, as they are one of the best characterized preadipocyte cell lines [25]. In preparation for experiments cells were plated in T-175 flasks from BD-falcon at 5,000 cells/cm^2. Cells were then grown at 37^o C in atmospheric air with 10% CO_2 added. At 75% confluency the cells were passaged. To passage the cells, they were washed in 10mL of HBSS per flask which was then aspirated out followed by treating the cells with 5 ml per T-175 flask of 0.05% trypsin for 5 minutes. The trypsin had been diluted from the original 0.25% stock from gibco by adding HBSS. After the cells had sat in 0.05% trypsin for 5 minutes an equal part of growth medium was then added to neutralize the trypsin and the cell liquid solution was carefully collected and spun down in a centrifuge for 8 minutes at 1,200 rpm. After being spun down, the medium was aspirated, leaving only the cells which were resuspended in HBSS and counted using a hemocytometer. Cells were then replated at 5,000 cells/cm^2 and allowed to grow.

3.5 Gel Fabrication

Based on a paper by Kuo and Ma, gels were considered to have 1x calcium if there was 0.18 mol Ca/mol COOH in alginate [18]. This paper looks exclusively at 2x calcium and 4x calcium (0.36 mol Ca/mol COOH in alginate and 0.72 mol Ca/mol COOH in alginate respectively). The functionalization of alginate with RGD was based on a protocol by Rowley in 2002. As such, 30xRGD is based off of their protocol as 316 μM with a 55-60% efficiency [30].

Gels were fabricated based on the protocol developed by Kuo and Ma in 2001 [18]. A calcium carbonate in HBSS (6% g/ml) slurry was added to 5% alginate stock and mixed thoroughly. Cells suspended in HBSS were then added and mixed well. Powdered GDL was added to HBSS making a 21% solution and then immediately added to the alginate, calcium carbonate, cell mixture. Everything was mixed well and then pipetted into a 96 well teflon mold. After 2 hours, the gels were pushed out of the mold using a teflon rod into individual wells in a 24 well non tissue culture plate. The gels were then each covered with 2 ml of medium.

3.6 3T3-L1 Differentiation in Gels

3.6.1 With Predifferentiation

For all of the trials using predifferentiation, the cells were grown to 100% confluency in tissue culture flasks. After 2 days of being at 100% confluency the induction process was started by giving the cells AIM A instead of standard growth medium. After another 2 days, the cells were given AIM B, which was followed by seeding another 2 days later. On the day of seeding, AIM A was given to the cells. After the cells had been seeded for 2 days the last round of induction medium, AIM B was given. After the cells and gels had been in AIM B for 2 days, the gels were switched to standard maintenance medium. All future medium changes were 1/2 medium changes every other day.

3.6.2 Without Predifferentiation

For all of the trials that did not pre-differentiate, the cells were grown to 100% confluency in tissue culture flasks. After 2 days of being at 100% confluency the cells were seeded. The day after seeding the gels, the induction process was started by giving the cells AIM A instead of standard growth medium. After another 2 days, the cells were given AIM B, which was followed by seeding another round of AIM A 2 days later. After the cells had been in AIM A again for 2 days the last round of induction medium, AIM B was given. After the cells and gels had been in AIM B for 2 days, the gels were switched to standard maintenance medium. All future medium changes were 1/2 medium changes given every other day.

3.7 2-Dimensional Controls

3.7.1 Without Predifferentiation

For the 2-Dimensional controls done without predifferentiation, cells were plated at 5,000 cells/cm^2 in 6 well tissue culture plates. Each control was done in a replicate of 3 and each well was given 2 ml of growth medium. Cells were then grown to 100% confluency and given 2 days

at confluency while still in growth medium. Then, the cells were given 2 cycles of adipogenic induction medium where AIM A for 2 days then AIM B for 2 days is 1 cycle. After the end of the second cycle, the cells were given maintenance medium. Following this change in medium, 1/2 medium changes were given every other day until the end of the experiments.

3.7.2 With Predifferentiation

For the 2-dimensional controls done with predifferentiation, the cells were again plated at 5,000 cells/cm^2 in 6 well tissue culture plates. Each control was done in a replicate of 3 and each well was given 2 ml of growth medium. Cells were then grown to 100% confluency and given 2 days at confluency while still in growth medium. Then, the cells were given 1 cycles of adipogenic induction medium where AIM A for 2 days then AIM B for 2 days is 1 cycle. After the end of the first cycle, the cells were treated with 0.5 ml per well of 0.05% trypsin in HBSS for 5 minutes. An equal part of growth medium was then added to neutralize the trypsin and the cells suspended in liquid were spun down in a centrifuge for 8 minutes at 1,200 rpm. After being spun down, the medium was aspirated, leaving only the cells which were resuspended in HBSS and counted using a hemocytometer. The cells were then replated in 6 well plates at 1 M cells /well. Following the replating, the cells were then given another cycle of adipogenic induction medium then followed by maintenance medium. Once the cells had been given maintenance medium, all subsequent medium changes were 1/2 medium changes every other day until the end of the experiments.

3.8 Obesogen Treatment

All gels treated with TBT were given TBT starting with the first cycle of AIM A and B. A 100μM solution was created by added HBSS as a vehicle to the TBT. This solution was then added to the medium at 1μL of solution for every 1mL of medium. This gave a final concentration of 100nM TBT, which was a value determined based off of previous studies by a partnering group that looked at what levels did TBT become toxic. TBT treatment was added to all medium for the entirety of the experiment. As a control, a set of gels was also given medium with an equal volume

of pure HBSS as a vehicle control to ensure that it was the presence of the TBT, not the HBSS that caused any effects seen.

3.9 Lipid Staining

The method of oil red o staining used was adapted from a paper by Mauney [21]. Gels were fixed in a 10% neutral buffered formalin at 4^{o}C for 30 minute followed by a 30 minute was with 60% isopropanol in HBSS. A solution of Oil Red O in isopropanol was made at 2.5mg/mL and was filtered and then diluted down to 60% solution in HBSS to make the working Oil Red O solution. After the wash was removed, the cells were put in the working solution at 4SPoC for 30 minutes. Afterwards, the Oil Red O solution was removed and the gels were put in HBSS. The gels were then washed twice quickly with HBSS followed by two 45 minutes washes in HBSS.

3.10 Imaging

Images were taken on a Nikon Eclipse TE300 with a 20x optical lens and a Hamamatsu Camera C4742-95 unless otherwise specified. Images looked for signs of spreading or lipid development. Examples of each can be found in the figure below. All, images were plain brightfield images. For each gel 3 images were taken and there images of 3 gels per condition were taken. Representative images were chosen for use in this report. Scale for the images was recorded and then scale bars were added to the images later using Image J.

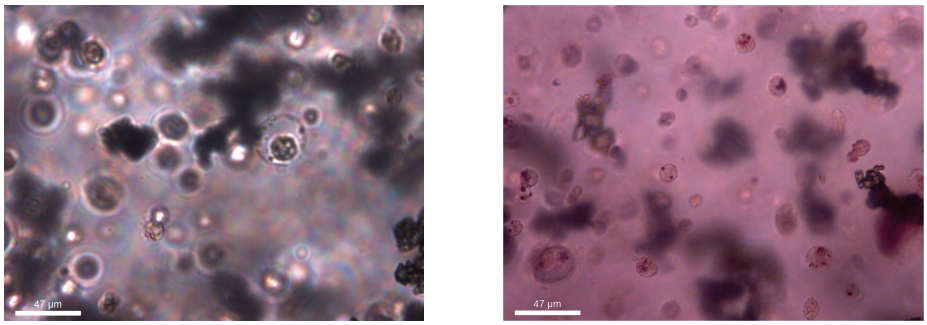
4 Results

4.1 The Effect of Cell Density

To create a useful 3-Dimensional model, it was imperative to determine a suitable cell density for adipogenesis to occur. In 2-Dimensional models, cells are grown to confluency and then left for two days before treatment with adipogenic medium [19]. Though some studies have shown

that as long as 3T3-L1s are halted at the G0/G1 cell cycle boundary, that cells can be suspended without contact and still differentiate [14], studies have shown that high seeding density is still a very important factor in the cells' ability to become adipose [22]. Thus, it was hypothesized that in an increase in cell density there would be a greater amount of differentiation in the 3T3-L1s.

To look at the effects of cell density, two conditions were tested: 2 million cells per mL and 10 millions cells per mL. Both sets of gels had 1.5% alginate with a modulus of 3 kPa and no RGD present and used predifferentiated cells. It was expected, that due to an apparent need for a high seeding density in 2 dimensional culture [22], that the larger cell density would induce differentiation in a larger number of cells. As can be seen in 1, this appears to be true.



(a) 2 million cells/mL (b) 10 million cells/mL

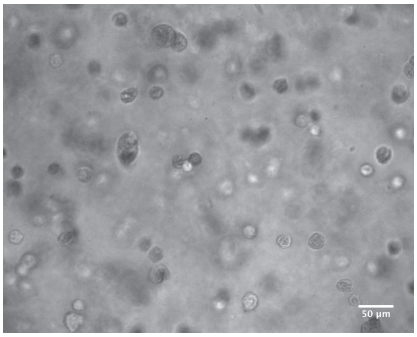
Figure 1: Representative images from day 7 gels. The gels were composed of 1.5% alginate with a modulus of 3 kPa and 3 replicates. These images were taken with the 40x optical lens. It was noticed that the gels with the higher cell density have slightly more lipids developing.

After 7 days, though the cells at 2 million cells per mL do show some signs of small lipid development the 10 million cells per mL gels did appear to have slightly larger lipid droplets on average, and more of the cels seemed to have lipid droplets. The difference between the two however, did seem very slight.

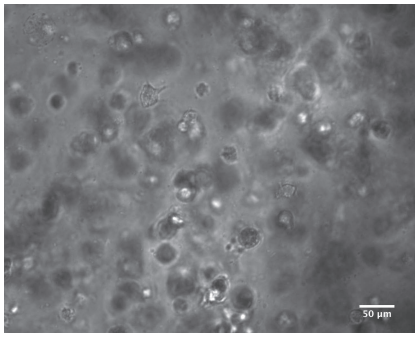
4.2 The Effect of Morphology

Though cell attachments could cause strain on the cells which has been shown to inhibit adipogenesis [22], cell attachment still could be necessary to the differentiation of pre-adipocytes. As such, an investigation of whether or not the presence of RGD promoted or inhibited the process of adipogenesis was conducted. It was hypothesized, that because of an increase in cytoskeleton tension, cells grown in gels where they can adhere to the scaffold (gels with RGD) would have less prevalent differentiation.

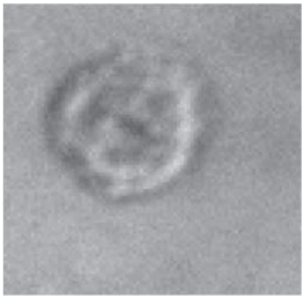
RGD had an effect on the cells as early as 2 days after encapsulation. Figure 2 shows that cell spreading can be seen in the gels functionalized by RGD, the cells being able to pull on the alginate now, while the cells without RGD to grab on to stay rounded.



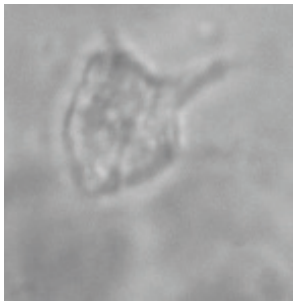
(a) Without RGD



(b) With RGD



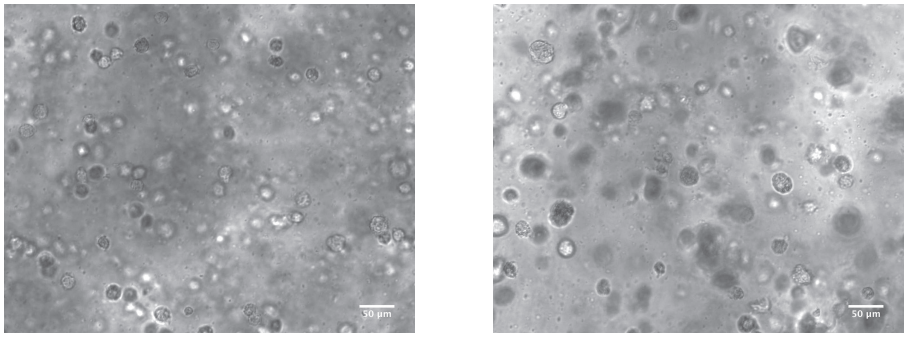
(c) Without RGD



(d) With RGD

Figure 2: Representative images from day 2 gels. Three replicate gels were composed of 1.5% alginate with a final modulus of 3 kPa and three images of each gel were taken. In the gels containing RGD some spreading of cells can be seen.

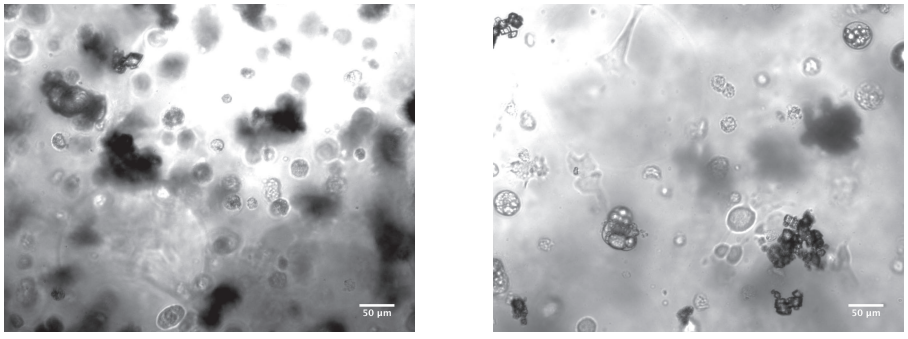
The introduction of differentiation medium, however, seemed to cause the cells to round back up, as by day 7, no cell spreading was seen even in the presence of RGD.



(a) Without RGD (b) With RGD

Figure 3: Representative images from day 7 gels. The gels were composed of 1.5% alginate with a modulus of 3 kPa and 3 replicates. Lipids can be seen in the gels with RGD though scarce to no large lipids can be found in the gels without RGD.

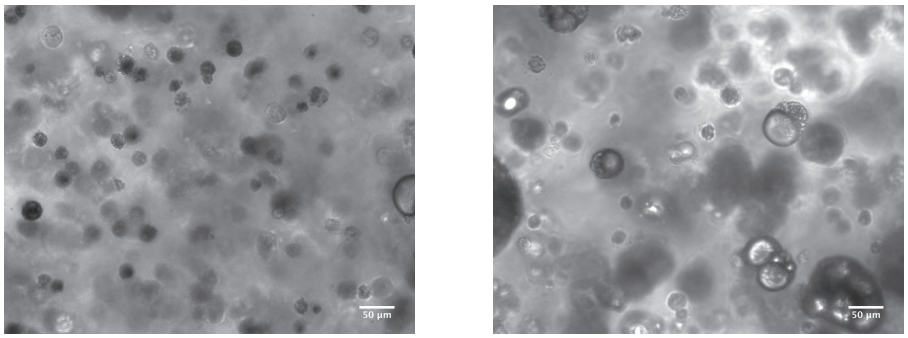
By day 31 there became a very strong correlation between the ability for cells to bind to RGD and their ability to differentiate, as can be seen in figure 4. At day 31, the gels with no RGD have almost exclusively tiny lipids, or no lipids at all. In contrast, though the gels with RGD had many cells that only contained small lipids, and there was a noticeable number of cells with large lipid droplets accumulating.



(a) Without RGD (b) With RGD

Figure 4: Representative images from day 31 gels. The gels were composed of 1.5% alginate with a 3 kPa modulus and 3 replicates. Lipids can be seen in the gels with RGD though scarce to no large lipids can be found in the gels without RGD.

By day 64 (see figure 5) the cells without RGD still show almost no signs of development except for perhaps a lone cell. The gels with RGD however show not only large, but unilocular lipid droplets consistent with mature adipocytes [15] The presence of unilocular adipocytes in the 30x gels show these gels to have true potential as a model for 3-dimensional *in vitro* adipogenesis.



(a) Without RGD (b) With RGD

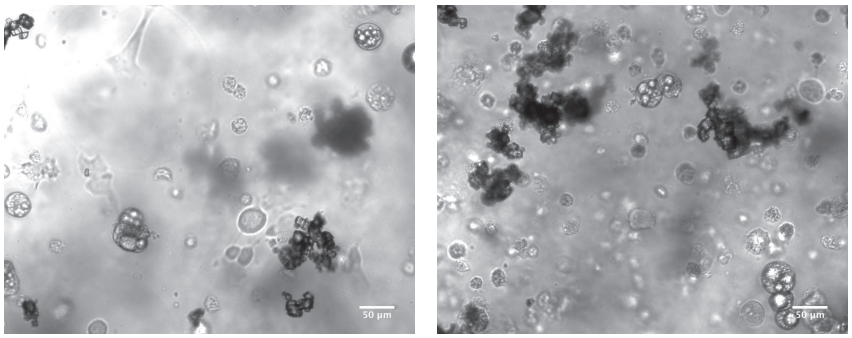
Figure 5: Representative images from day 64 gels. The gels were composed of 1.5% alginate with a modulus of 3 kPa and 3 replicates. Some of the lipids in the gels with RGD have become unilocular while lipids in the gels without RGD are still very rare.

4.3 The Effect of Changes in Scaffold Modulus

As papers have shown that modulus changes in the scaffold by as little as 3 fold can have drastic effects on a cells ability to differentiate [9], two crosslinking densities 2x Ca and 4x Ca were used the gels having a young's modulus of 3kPa and 10 kPa respectively based on previously conducted tests using atomic force microscopy. These two values are both at the lower end of the ranges for the modulus of fat, and it was hypothesized that the 3 kPa gels would produce a higher percentage of differentiation due to the fact that stiffer moduli have been shown to negatively affect the ability of cells to differentiate due to the formation of stress fibers in the actin cytoskeleton [22].

When looking at the images taken on day 31, it is difficult to see any difference between 3 kPa and the 10 kPa conditions. So, even with a 3 fold change in modulus, there seems to be little noticeable change in amount of cells accumulating lipids or in size of the lipids. More quantifiable

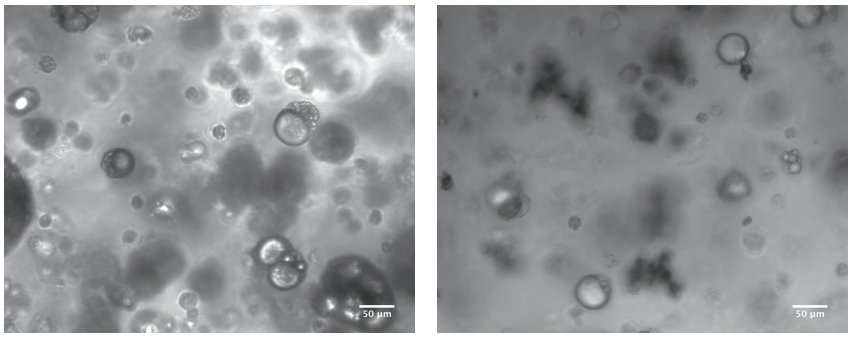
results however could still prove to show slight variations between the two levels.



(a) 3 kPa (b) 10 kPa

Figure 6: Representative images from day 31 gels. The gels were composed of 1.5% alginate with RGD for cell attachment and 3 replicates. Lipids can be seen in both sets of gels.

As seen before, by day 64 the cells were able to develop large unilocular cells as long as RGD was present. Once again though, it was difficult to see any difference in the number and size of the lipids being produced in the 3 kPa gels verses the 10 kPa gels.



(a) 3 kPa (b) 10 kPa

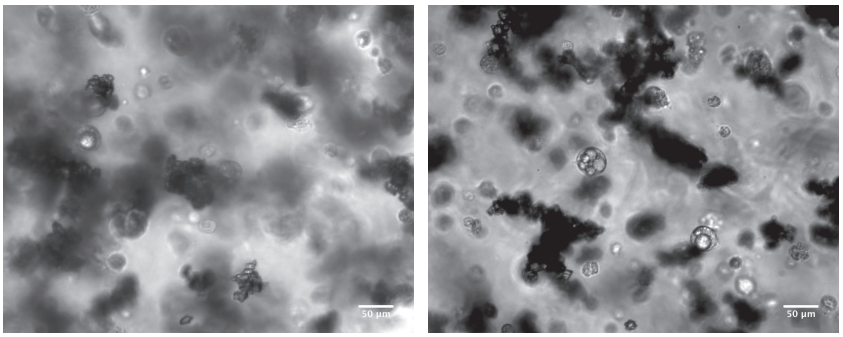
Figure 7: Representative images from day 64 gels. The gels were composed of 1.5% alginate with a 3 kPa modulus and 3 replicates. Unilocular lipids can be seen in both sets of gels.

4.4 The Effect of Obesogens: TBT

With a characterization of gel the changes in gel properties affects on the gels, studies began to move towards changing the environment of the gels through chemicals added. The obesogen

TBT was added to the gels to see if our model could perhaps be affected by a small dose of this chemical thought to promote obesity. It was hypothesized that the gels with TBT would show a significant increase in number of cells differentiating as well as the size of the lipids of the mature adipocytes.

The images did indeed show there to be a difference with the presence of TBT. By day 31 noticeably more large lipids were seen in the gels treated with TBT than those without treatment as can be see in figure 8.

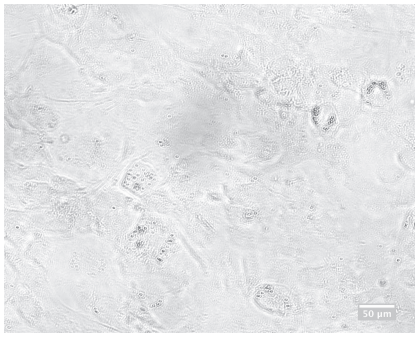


(a) Without TBT (b) With TBT

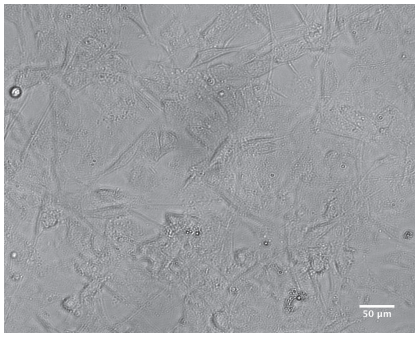
Figure 8: Representative images of gels with and without TBT treatment. The gels were made with 1.5% alginate with RGD, and were made to have a modulus of 3 kPa. The gels with TBT had more prevalent large lipids

4.5 The Effect of Predifferentiation

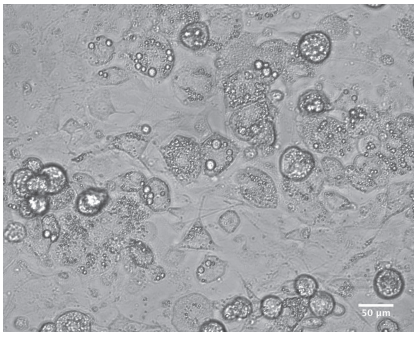
To reconcile the trypsinization of cells in the middle of their differentiation treatment, 2-dimensional controls were run both with replating halfway through treatment as well as without replating through the treatment. As can be seen in 9 there was a drastic difference the number of cells that differentiated in each condition. The cells that were predifferentiated and then replated showed a drastic decrease in the number of lipids found and almost no small lipids found after 7 or 14 days. On the other hand, without replating cells by day 7 started developing larger lipids and by day 14 there were significant colonies of cells filled with large lipid droplets



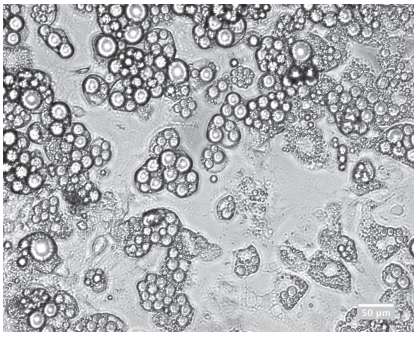
(a) Day 7, With Replating



(b) Day 15, With Replating



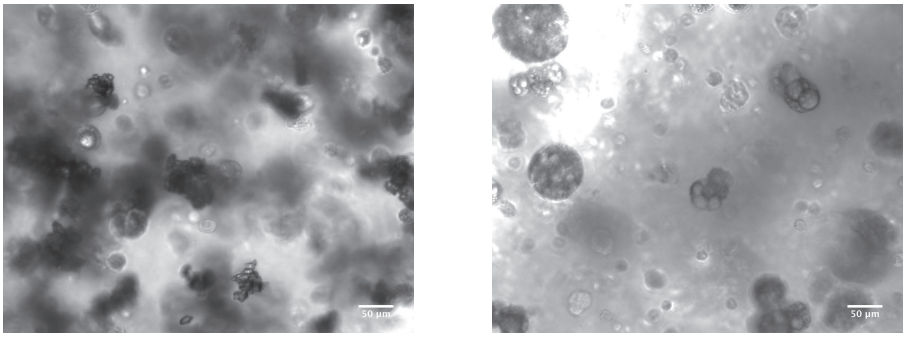
(c) Day 7, No Replating



(d) Day 15, No Replating

Figure 9: Representative images of 2D plates with and without replating the cells during differentiation. The cells that have been replated show no large lipids at all, while the non replated cells have a large number of lipids.

Since the cells showed adverse affects to being replated, the effect of predifferentiating the cells before putting them into the gels was investigated. The images show, that after day 31 the gels with RGD that were not predifferentiated showed similar if not higher levels of differentiation by day 27 than the predifferentiated gels had at day 31. The non predifferentiated gels did also show more signs of clumping in the later stages though, which could be due to proliferation.



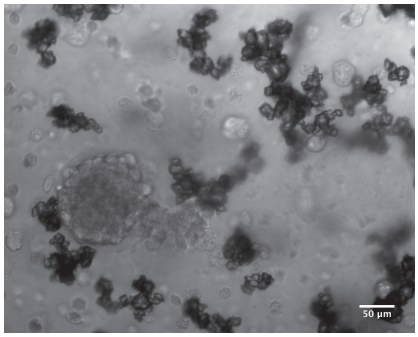
(a) Without TBT (b) With TBT

Figure 10: Representative images of gels with and without TBT treatment. Gels with TBT treatment show a greater number of differentiated cells

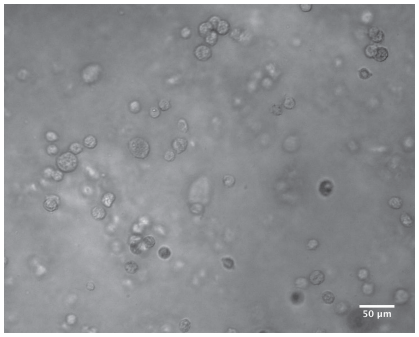
4.5.1 The Effect of Chemical Induction

With there being drastic changes in the ability of cells to change based on the composition of the gel, particularly the presence of RGD, a study was done to see what effect the mechanical properties of the gels had on the cells without induction. It was hypothesized, that even without induction medium, the gels would still have a low level of differentiation in the gels with RGD due to mechanical cues.

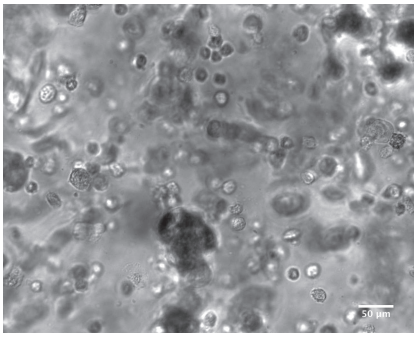
It can be seen in figure 11 that the gels without differentiation medium were not able to differentiate at noticeable levels. In the gels without RGD or growth medium, it was noticed that the cells formed large clumps over time. This is perhaps due to cell proliferation inside the gels.



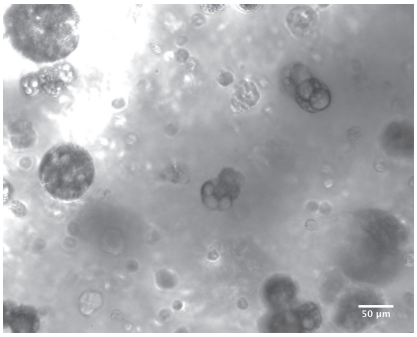
(a) Growth Medium with No RGD



(b) Adipogenic Medium with No RGD



(c) Growth Medium and RGD



(d) Adipogenic Medium and RGD

Figure 11: Representative images of gels with and without adipogenic induction, in 1.5% alginate gels with a scaffold modulus of 3 kPa with and without RGD. Clumping can be seen in gels without RGD or adipogenic medium. Adipogenesis is only noticeable in the gels with both RGD and induction medium.

4.6 Discussion

4.6.1 The Effect of Cell Density

In 2-dimensional experiments, cells are often grown to confluency and left for 2 days, in order to cause cell halt at the cell cycle boundary between G0 and G1. It has been shown that the cells can then be suspended and maintain the ability to differentiate despite the lack of cell contact. [14] This begs the question as to what the correct cell density is for the cells suspended in the gels. Images showed that 10 million cells per mL had a slight increase in number of cells with lipids. This

indicates that the higher cell density might indeed increase the amount of adipogenesis, perhaps due paracrine signaling. The difference between the two groups though was small and it is difficult to say with utmost certainty that there was a significant difference, since no quantitative image analysis or other assays were preformed during this experiment. For future experiments, however, we continued using 10 million cells per mL since it better mimics the close environment shown in other studies to promote adipogenesis.

4.6.2 The Effect of Morphology

The effect of adhesion to RGD on the cells was very large and noticeable. In all of the experiments without RGD, little lipid formation was ever seen. With the presence of RGD, however, a noticeable amount of cells were able to develop large lipid droplets, even achieving unilocular lipid droplets by 64 days. It is very clear that the presence of RGD plays an important role in differentiation though whether it works as a chemical or mechanical cue is unknown. Binding of the cells to the RGD may cause a chemical signal to be sent through the cell telling of its environment and triggering differentiation pathways. Also, the cells may need to have something to attach to providing a mechanical stimulus, before the cells are able to differentiate. This would make sense since previous studies have shown various peptides cause changes in morphology of preadipocytes [32].

4.6.3 The Effect of Changes in Scaffold Modulus

Many studies have shown that the changes in scaffold modulus can have drastic effects on cell differentiation with even as small as a 3 fold change changing the fate of the cell [9]. In this experiment, there was also a 3 fold change, 3kPa (2x Ca) to 10 kPa (4x Ca) though there was not a very clear difference between the two sets of gels in terms of differentiation. This could be because both sets of gels were within the range of normal adipose tissue 2-24kPa, and being progenitor cells instead of stem cells as seen in other modulus based experiments, they could be less susceptible to such small changes in modulus. Though they may be less susceptible to small

modulus changes than stem cells are, 500 fold change of adipose tissue to tissue culture plastic, is almost certainly significant to the way the cells will react and having a controllable, within *in vivo* modulus range model is most certainly important to the development of the cells. This is especially true since many studies have shown that too much cytoskeletal tension, often caused by hard tissue culture plastic, inhibits cell abilities to become adipocytes [22].

4.6.4 The Effect of Obesogens: TBT

With a 3-Dimensional scaffold that does not inhibit adipogenesis, the next step was to look at what effect chemical under the group obesogens have on adipogenesis. TBT was chosen as it is has been labeled as an obesogen, however, *in vivo* studies have given somewhat ambiguous results. Also, as a suspected obesogen, TBT has a huge ability to impact society as people come in contact with it through its used as a fungicide for plants as well as in some plastics used to store food and water [13, 41]. As obesogens are thought to promote obesity, it was hypothesized that with the addition of TBT to the medium, more cells would undergo adipogenesis, and those that had become mature adipocytes, would contain larger lipids. This hypothesis is supported by the images taken which can be seen in 8. On day 31, both sets of gels with RGD for the cells to attach to had some large lipids. Those with TBT though did have a larger number of cells with large lipids. The overall size of the lipids was, however, very similar and it was difficult to fully discern which sets of gels and the largest lipids if looking at the largest adipocytes in each condition. The greater amount of adipogenesis in the TBT group still strongly signifies that TBT promotes adipogenesis. It is also interesting to note however, that without the cells being able to adhere to RGD, even the presence of TBT was not able to compensate for the lack of that particular signaling, and none of the cells that were suspended in alginate hydrogels without RGD formed noticeable lipids.

4.6.5 The Effect of Predifferentiation

When doing the initial studies on the effect of cell density, scaffold modulus, attachment to RGD, and the effect of obesogens a process of predifferentiation, the same as what had been seen

in studies using collagen gels in a partnering lab, was used since there was a basis to compare with for possible troubleshooting. In a true model of adipogenesis however, it is invaluable that the entire process of differentiation is preformed inside the 3-Dimensional culture model. If the process is preformed entirely inside the culture system, then early stage cues and morphological changes during the initial stages of differentiation can better be analyzed. These initial steps are very important to study and understand, since increased proliferation of and differentiation of pre-adipocytes, not just increased lipid size, are a major cause of obesity [34]. There is also the possibility that trypsonizing and seeding the gels in the middle of their differentiation process could be harmful to their development, which removing predifferentiation could avoid. This second claim seems supported by the 2-Dimensional controls that were preformed, one set of cells having been pre-differentiated with the gels, and then trypsonized and replated and the other half, merely being given the entire differentiation process. In the control group given predifferentiation, very few small lipids were seen after both 7 and 15 days, and no large lipids could be seen at all. In the gels that were not replated, many small lipids, and even some large lipids were seen as early as day 7, with a large population of large lipids seen by day 15. This strongly suggests that something in the replating process disrupts the cells ability to differentiate. This effect could also be at least partially elicited from the fact that for differentiation in 2-dimensional culture it has been found imperative that the cells are grown to confluency. Upon replating, even when trying to plate the cells at 100% confluency levels or higher, not all of the cells reattach often leaving gaps on the plate and thus removing that contact environment.

Inside the gels, it was also seen that predifferentiation seemed to make an impact on development, though not nearly as strong. In the gels, as long as there was RGD for the cells to adhere to, the some of the cells were able to form large lipids. In the gels that were not predifferentiated, there seemed to be more signs of clumping on later days, which could be a sign that those not pre differentiated were proliferating slightly. Also in the gels that were not predifferentiated however, were increased signs of differentiation. It was also noticed that the cells with large lipids tended to be clumped in the non predifferentiated gels, possibly suggesting that there is indeed communication

between cells which, at the least, promotes differentiation.

4.6.6 Need for Chemical Induction

The previous experiments had shown different ranges of effect on the cells based on changes in gel properties given the addition of induction medium. It was however hypothesized, that due to the more *in vivo* like nature of the gels, mechanical cues such as rounding of the cells, could potentially drive small amounts of adipogenesis even without induction medium. To test this gels with either RGD or no RGD was made each group having gels given only growth medium and gels which were given induction medium in the beginning. It could be seen, that without RGD or induction medium, large masses of cells formed over time. This is potentially due to proliferation inside the gels. Since there is no RGD for the cells to pull onto, when they split, the child cells stay close to the parents. In the cels with RGD but only growth medium, cell spreading could be seen, but no noticeable differentiation occurs. This could imply that though a mechanical signal is needed, it still cannot compensate for the lack of a chemical signal to induce the differentiation pathway. As expected, the cells in the gels with RGD and adipogenic medium produced a noticeable amount of differentiated cells while the gels without RGD still struggled to differentiate.

4.6.7 Future Directions

These gels have shown much promise for a 3-dimensional fat model however more repetitions need to be run to confirm the results seen. For these trials more quantitative and exhaustive protocols need to be developed to assess the changes in the gels and gain a more concrete understanding of the amounts of change actually occurring. Also, the effect of RGD on the cells was very large, however, from the experiments preformed, it is uncertain whether the RGD is effecting the cells through a chemical signal, or if it is the mechanical signal of the cells stretching that is actually allowing for adipogenesis. As such, future experiments plan to look at the difference of effect between soluble RGD within the gels and functionalized RGD as seen in the experiments here, to differentiate the effect of RGD as a chemical stimulus and a mechanical stimulus. In a similar vein,

determining what type of peptide would work best for the gels is important, since peptides such as IKVAV shown promise in 2-D studies for future adipogenesis models. [32] This culture system will also be used to study interactions of 3T3-L1s with HUVEC cells, since angiogenesis is a key part of being able to eventually engineer fat tissue for application such as breast reconstruction surgery for cancer patients.

4.7 Conclusion

Obesity is a growing problem in the United States and the world. In order to combat it, more needs to be learned about exactly what it is that causes obesity so as to know how best to prevent it. Many studies have been done on obesity but those done *in vivo* have proven difficult to control and reproduce, while 2-Dimensional *in vivo* experiments are preformed under completely different conditions than would be found in the body. To address this problem, a 3-dimensional hydrogel fat model was designed. Experiments were run to determine suitable conditions for the gels, including looking at cell density, ability of cells to adhere to the gel, and the scaffold modulus. These experiments explored some of the major benefits of the alginate hydrogels, which include the ability to functionalize the gels with different peptides, as well as easily control the mechanical properties of the gel. Imaging showed little effect of cell density or scaffold modulus on the cells ability to differentiate, while showing a huge impact of cell adhesion to RGD. It is now thought that mechanical cues of cells being able to stretch slightly and latch onto the ECM (extra cellular matrix) may be a necessary step in cell differentiation. More work needs to be done however, to explore the possibility of RGD working as a necessary chemical signal as well. As for changes in scaffold modulus, is could be that little change was seen due to sensitivity in progenitor cells to changes in modulus, particularly given that both of the tested moduli (3 kPa and 10 kPa) were within the range of normal adipose tissue found in the body.

After looking at the effects of changing the physical environment of the cells, the experiments were expanded to include controlling the chemical environment through the introduction of the obesogen TBT. It was found that TBT does indeed seem to have an effect on differentiation of pre-

adipocytes to adipocytes, which correlates with previous 2-Dimensional studies preformed [16]. Studies were also preformed looking at the effect of pre-differentiation vs. differentiation which showed, that pre-differentiation was not necessary in the alginate hydrogel model, and in fact could have mild inhibitory effects on differentiation. Finally, with seeing some effects due to presence of RGD, and some small effects with scaffold modulus, the ability of the cells to differentiate without an induction cocktail was analyzed to see if mechanical cues, could potentially be strong enough to change the gels on their own. Though random cells were found to have been able to differentiate within the gels, without induction medium, the induction medium clearly had an important role in differentiation, irreplaceable with changes in the environment to better produce adipocytes.

5 References

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