Published in final edited form as:

Process Biochem. 2017 August ; 59: 312–320. doi:10.1016/j.procbio.2017.02.003.

Adipogenic Differentiation of Human Adipose-Derived Stem Cells Grown as Spheroids

Paul A. Turner¹, Bhuvaneswari Gurumurthy¹, Jennifer L. Bailey², Carrie M. Elks², and Amol V. Janorkar^{1,*}

¹Department of Biomedical Materials Science, School of Dentistry, University of Mississippi Medical Center, 2500 N State Street, Jackson, MS

²Matrix Biology Laboratory, Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, LA

Abstract

Understanding the process of adipogenesis is critical if suitable therapeutics for obesity and related metabolic diseases are to be found. The current study presents proof of feasibility of creating a 3-D spheroid model using human adipose-derived stem cells (hASCs) and their subsequent adipogenic differentiation. hASC spheroids were formed atop an elastin-like polypeptide-polyethyleneimine (ELP-PEI) surface and differentiated using an adipogenic cocktail. Spheroids were matured in the presence of dietary fatty acids (linoleic or oleic acid) and evaluated based on functional markers including intracellular protein, CD36 expression, triglyceride accumulation, and PPAR- γ gene expression. Spheroid size was found to increase as the hASCs matured in the adipocyte maintenance medium, though the fatty acid treatment generally resulted in smaller spheroids compared to control. A stable protein content over the 10-day maturation period indicated contact-inhibited proliferation as well as minimal loss of spheroids during culture. Spheroids treated with fatty acids showed greater amounts of intracellular triglyceride content and greater expression of the key adipogenic gene, PPAR- γ . We also demonstrated that 3-D spheroids outperformed 2-D monolayer cultures in adipogenesis. We then compared the adipogenesis of hASC spheroids to that in 3T3-L1 spheroids and found that the triglyceride accumulation was less profound in hASC spheroids than that in 3T3-L1 adipocytes, correlated with smaller average spheroids, suggesting a relatively slower differentiation process. Taken together, we have shown the feasibility of adipogenic differentiation of patient-derived hASC spheroids, which with further development, may help elucidate key features in the adipogenesis process.

Keywords

3-D cell culture; spheroids; adipose tissue model; elastin-like polypeptide; cell morphology; stem cells

*Corresponding author (ajanorkar@umc.edu; Phone: 1-601-984-6170; Fax: 1-601-984-6087).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Adipose tissue is no longer considered merely a passive reservoir of energy, but an important endocrine organ with systemic homeostatic responsibilities. One of the major stumbling blocks in obesity research continues to be the lack of appropriate *in vitro* multicellular architectures that mimic adipose tissue structure and function found *in vivo*. Planar 2-D monolayer adipocyte cultures do not represent the complex architecture of adipose tissue *in vivo*, while the optimum scaffold material to achieve engineered 3-D adipose tissue remains elusive [1,2]. The creation of *in vitro* models of adipose tissue will allow researchers to test various hypotheses, study altered metabolic pathways due to the disease state, and therefore, facilitate the efforts in understanding adipose pathophysiology.

The International Federation for Adipose Therapeutics and Science (IFATS) has applied the term “adipose-derived stem cells,” or ASCs, to pluripotent mesenchymal cells as well as more differentiated adipose progenitor cells found amongst the fibroblastic undifferentiated population of the adipose tissue [3]. A variety of substrate types and materials have recently been investigated for engineering functional adipose tissue by encapsulating ASCs, including decellularized tissue [4], naturally-derived biopolymers [4-7], and degradable synthetic polyesters [6,8,9]. However, due to the restriction placed on the size of the maturing cells embedded directly in such scaffolds, the resulting adipocytes typically remain functionally impaired. 3-D aggregates or “spheroids” of adipocytes display morphology similar to the one found in native adipose tissue (minimum extracellular matrix and closely-packed cells) [10-15]. Benefits of 3-D spheroids over 2-D monolayer cultures include increased adipogenic markers such as triglyceride accumulation as well as expression of adipose-specific genes C/EBP- α , PPAR- γ , and adiponectin [12]. Spheroid organization *in vitro* prior to implantation has been shown to improve *in vivo* angiogenesis [13] as well as survivability under hypoxic physiological conditions [10]. 3-D spheroid configuration has also been found to enhance pluripotent potential and differential efficacy into multiple mesenchymal cell lines when exposed to appropriate differentiation media *in vitro* [14]. We believe that 3-D spheroids may be advantageous over other encapsulation-based 3-D adipose tissue engineering methods as the growth potential of the 3-D spheroids is not restricted by the exogenous encapsulating substrate material. 3-D ASC spheroids have been fabricated by mechanically-induced suspension [10] and hanging drop [11] techniques. Unfortunately, the suspension culture techniques suffer from declining cellular function over a longer culture period possibly due to the increased shear stress experienced by the cells and the hanging drop culture techniques suffer from logistical drawbacks such as difficulty in providing fresh medium and maintaining the stability of cultures over a longer culture period. To minimize these disadvantages, substrate features such as surface morphology [12], porosity [15], and non-adhesive chemistry including immobilized fibroblast growth factor 2 [13] and chitosan films [14] have been exploited to induce spheroid formation *in vitro*. These approaches, while successfully achieving spheroid formation due to the non-adhesive nature of the substrates, suffer from detachment of the spheroids as the formed spheroids fail to tightly adhere to the substrates.

Our method of 3-D spheroid culture used a surface modification approach and creatively avoided using the size-limiting porous or hydrogel scaffolds, unstable suspension or hanging

drop cultures, as well as quick detachment of spheroids from the non-adhesive substrates. Specifically, our research utilized the hASCs from the stromal-vascular fraction from human breast adipose tissue from a donor undergoing elective liposuction or abdominoplasty. hASCs were cultured atop a coating of elastin-like polypeptide-polyethyleneimine (ELP-PEI) copolymer, where the charged PEI segment encouraged spheroid formation, while the biocompatible ELP segment allowed surface attachment of the formed spheroids. To achieve adiposity, we exposed our adipose cultures to a physiologically relevant fatty acid level (500 μ M) [16]. We have screened key fatty acids representing clinically and nutritionally relevant classes implicated in influencing adipogenesis; namely, monounsaturated oleic acid (C18:1, OA) and polyunsaturated linoleic acid (C18:2, LA). These differentially treated adipose cultures were then assessed for their growth and adipogenic potential over a 10-day maturation period. 3T3-L1 mouse preadipocytes are unipotent cells committed to adipocyte lineage and have been widely used in adipose functionality studies [17-19]. Therefore, finally, we compared the adipogenic differentiation of hASCs to that of the 3T3-L1 cells.

2. Experimental Methods

2.1 Synthesis and Characterization of ELP-PEI

Conjugate of ELP with backbone structure Valine-Proline-Glycine-Valine-Glycine (molecular weight = 17,000 Da) and PEI (molecular weight = 800 Da, Sigma-Aldrich, St. Louis, MO) was prepared using carbodiimide chemistry as described elsewhere [20,21]. The phase transition temperatures of ELP and ELP-PEI solutions (5 mg/mL) were determined on 3 replicates ($n = 3$) using a Cary 100 spectrophotometer equipped with a temperature controller by measuring the absorbance at 350 nm as the solutions were heated from 20 to 80 °C at the rate of 1 °C/min. The primary amine content of 5 independently synthesized ELP-PEI conjugates ($n = 5$) was measured using the O-phthalaldehyde (OPA) assay (Thermo Scientific, Pittsburgh, PA) according to the manufacturer's protocol.

2.2 Modification of Tissue Culture Polystyrene (TCPS)

ELP-PEI conjugate was adsorbed to 24-well TCPS plates by placing 200 μ L of a 5 mg/mL, 5 mol% ELP-PEI solution in deionized water in each well. The plates were incubated at 37 °C for 48 hours in a dry incubator to remove all solvent.

2.3 hASC Isolation and Maintenance

Our experiments utilized normally discarded breast whole adipose tissue provided during elective procedure from a single unidentified adult female patient in accordance with the protocol approved by the University of Mississippi Medical Center Institutional Review Board (Approval # 2012-0004). Donated adipose tissue was dissected and digested to harvest undifferentiated ASCs for subsequent culturing and differentiation using techniques demonstrated by Flynn [4,22], adapted from methods developed by Hauner et al. [23]. Whole breast adipose tissue was collected and maintained in sterile $\text{Na}^+/\text{Ca}^{++}$ free PBS, arriving in the lab immediately after extraction. Working under a sterile laminar-flow hood, the tissue was placed in a tissue culture dish, minced into small ($\sim 1 \text{ mm}^3$) sections, and washed with PBS to help remove blood, oil, serum, other vascular components, and lymph vessels. The tissue fragments were then digested in Liberase TM collagenase (0.12 AU/mL)

in PBS supplemented with 3 mM glucose, 25 mM HEPES, and 20 mg/mL BSA for 20 minutes at 37 °C in a shaker bath. The tissue was then filtered through sterile layered cotton mesh to remove undigested fragments. Cell media (DMEM, 10% FBS) was then added to the filtrate and the cells allowed to gravity separate. The supernatant was aspirated to remove mature adipocytes and the remaining filtrate centrifuged at $1,200 \times g$ for 5 minutes. The cell pellet was suspended in preadipocyte media (DMEM + 10% calf serum). The remaining cells were pelleted again, resuspended in whole media, and filtered through a 60- μ m mesh nylon net to remove mature adipocytes, clusters of erythrocytes and endothelial cells, and any remaining tissue and cell aggregates. The remaining stromal-vascular fraction including ASCs was cultured on TCPS dishes in 50:50 DMEM:Ham's F12 with 10% calf serum at 37 °C and 5% CO₂, with regular media changes every 2-3 days. Cells were used for experiments between their 6th and 9th passage.

2.4 hASC Culture and Adipogenic Differentiation

50,000 hASCs were seeded per well of a 24-well TCPS plate (26,000 cells/cm²) coated with the ELP-PEI conjugate and allowed to form 3-D spheroids over a 72 hour period. To prepare 2-D monolayer culture, 50,000 hASCs were seeded per well of an uncoated 24-well TCPS plate and cultured to confluence over the same 72 hour period. hASC differentiation was then carried out for 72 hours in media containing 50:50 DMEM:Ham's F12 media supplemented with 1 μ M dexamethasone, 0.5 mM IBMX, 0.1 U/mL insulin, 1 μ M indomethacin, and 100 units/mL penicillin -100 μ g/mL streptomycin, a similar hormone cocktail demonstrated to induce adipose differentiation [14,24-26].

2.5 Induction of Adiposity

Fatty maturation medium was prepared by supplementing the maintenance medium with 2% bovine serum albumin (BSA) acting as the carrier agent and 500 μ M of linoleic acid (LA) or oleic acid (OA). Control maturation medium was prepared by supplementing the maintenance medium with 2% BSA and no additional fatty acid. Media were sonicated at 40°C for 45 minutes, cooled to 4 °C, and supplemented with 0.2 U/mL insulin, 100 units/mL penicillin, and 100 μ g/mL streptomycin. Fatty acid loading efficacy was verified by gas chromatography analysis [20]. hASCs were exposed to control or fatty-acid containing maturation medium for up to 10 days after differentiation.

2.6 Measurement of Spheroid Size

Spheroid size was assessed using an Olympus IX 81 optical microscope (Olympus, Center Valley, PA) with a total 100 $\times$ magnification. Images were captured at 3 locations per well on 3 replicate wells per condition (total n = 9 images per condition) using a digital camera (Hamamatsu, Bridgewater, NJ) with Slidebook image acquisition and analysis software (Intelligent Imaging, Denver, CO). Spheroid dimensions on a minimum of 90 spheroids (minimum 10 spheroids per image) were tracked (n > 90) throughout the experiment by time-lapse imaging and measured with ImageJ digital analysis software.

2.7 Functional Biochemical Evaluation

All functional biochemical evaluation assays were performed on 3 replicate wells per condition. hASC spheroids were collected after 5 and 10 days in maturation media by aspiration. hASC cells in 2-D monolayer were collected by trypsinization. All aliquots were centrifuged for 2 minutes at 1,000 rpm, resuspended in PBS, and sonicated for 30 seconds at 10% amplitude using a Branson Digital Sonifier 450 (Danbury, CT). Protein and intracellular triglyceride content were analyzed using assays performed per manufacturers' protocols (Thermo Scientific and Sigma-Aldrich, respectively) and measured on an absorbance plate reader (Biotek ELx800, Winooski, VT) with a 540 nm filter. Measurements were taken in triplicate from each of three replicate wells ($n = 9$) exposed to unique maturation media. CD36, a cell surface marker related to fatty acid transporters, was measured to determine cell competency to consume exogenous fatty acids. An ELISA assay specific to human CD36 (Abcam, Cambridge, MA) was performed in accordance with manufacturer's protocol in triplicate for each culture condition, one sample per experimental well, yielding a total of three measurements per condition ($n = 3$).

2.8 Gene Expression Analyses

200,000 hASCs per well of 6-well TCPS plate were cultured as 2-D monolayer and 3-D spheroids. Total RNA was isolated from three replicate wells ($n = 3$) using the RNeasy Mini Kit (Qiagen) and concentrations were quantified fluorometrically (Qubit Fluorometer, ThermoFisher Scientific, Waltham, MA). cDNA was synthesized and gene expression analyzed as previously described [27]. Sequences of human primers for cyclophilin A/PPIA (Integrated DNA Technologies, Coralville, IA) and PPAR- γ (Sigma) were as follows:

PPIA forward: CGTTTTGCAGACGCCACCGC;

PPIA reverse: TGCAAACAGCTCAAAGGAGACGC;

PPAR- γ forward, CATAAAGTCCTTCCCCGCTGA; and

PPAR- γ reverse: GGGGGTGATGTGTTTGAAC.

2.9 Statistical Analysis

Quantitative results reported as mean $\pm$ 95% confidence intervals. Data were analyzed with SPSS Statistics package (IBM, Armonk, NY) using a one-way ANOVA with Games-Howell post-hoc test for unequal variances. Statistical significance was determined using an alpha value of 0.05.

3. Results and Discussion

Amphiphilic conjugates containing the biocompatible, hydrophobic ELP segment and a hydrophilic, charged segment have shown potential for cell culture [28,29] as well as drug delivery [30]. For this work, we prepared an ELP-PEI conjugate by reacting the C-terminus carboxylic acid group of the ELP with one of the primary amine groups present on the PEI using carbodiimide activation chemistry. The increase in the primary amine content of the ELP-PEI compared to neat ELP attributes a net positive charge to the conjugate. The primary amine content was assessed using the OPA assay, wherein the increased

fluorescence (360 nm excitation / 460 nm emission) after the OPA reaction corresponded with the increased primary amine content. Figure 1a shows that the fluorescence of the OPA treated ELP and ELP-PEI (30 μ M solution) were 53 ± 14 and 121 ± 26 , respectively ($p = 0.0004$). The fluorescence of the OPA treated PEI (30 μ M solution) was 453 ± 42 (data not shown). Attributing 0% conversion to the neat ELP fluorescence and 100% conversion to the neat PEI fluorescence, we calculated the conversion for the ELP-PEI conjugation reaction to be 15 mol%, with the remaining 85 mol% material being the unreacted ELP. Based on our previous research which showed a 5 mol% ELP-PEI solution induced uniform spheroid formation [20,21], we further diluted the reaction product solution with more ELP to obtain a 5 mol% ELP-PEI solution with the remaining 95% material being neat ELP. As shown in Figure 1b, the turbidity of the ELP solution increased due to coacervation as the solution was heated and exhibited an inverse phase transition temperature (T_i) at 37.6 ± 0.9 °C (Fig. 1b). Similarly, the 5 mol% ELP-PEI solution exhibited the T_i at 36.6 ± 0.1 °C, which was statistically indistinct ($p = 0.17$) compared to that of the ELP. The similar T_i values for ELP and ELP-PEI solutions are not unexpected as the ELP-PEI solution largely (about 95%) contained unreacted ELP. More importantly, the T_i values were close to the cell culture temperature (37 °C) and ensured that the ELP-PEI remained stably coated atop the TCPS surface and did not dissolve back in the culture medium during the cell culture period.

Adipose tissue possesses superior stem cell content compared to other tissues; as much as 2% of the cellular content of adipose may be stem cells (ASCs), compared to 0.002% of bone marrow cells. Adipose cells may be harvested by liposuction, biopsy/lipectomy, or abdominoplasty [3]. Plentiful availability of adipose tissue and its inherent regenerative potential allow patients to donate a sufficient quantity for cell isolation without encountering adverse effects [31]. For this research, we used discarded adipose tissue from a patient undergoing elective abdominoplasty or liposuction to study the impact of 3-D spheroid culturing technique on primary stromal vascular preadipocyte (hASC) differentiation and overall functionality. hASCs were cultured using similar methods pioneered with H35 rat hepatoma and 3T3-L1 preadipocyte model cells [20,21], though using maintenance media optimized for hASCs [26,32]. The experimental timeline is shown in Figure 2.

Micrographs of 2-D monolayer cultures (not shown) captured after differentiation and 10 days of fatty acid treatment showed an increased surface coverage, loss of fibroblastic preadipocyte morphology, and modest lipid accumulation predominantly exhibited in the form of multilocular microspheres within the cell cytoplasm, which are morphological features associated with immature adipocytes [44,45]. This result contrasts with our previous studies using mouse 3T3-L1 adipocytes, which showed greater lipid accumulation and, in many cases, achieving unilocular morphology after only 5 days of treatment with the same dosage of fatty acid media [20]. From this perspective, we conclude that our primary adipocytes cultured as 2-D monolayer achieved a relatively less complete differentiation state than the 3T3-L1 model cell counterparts.

Micrograph of the cells cultured atop the ELP-PEI coated surface for 3 days in the differentiation medium showed small 3-D spheroid aggregates (Figure 3a). During the maturation phase, these aggregates started to increase in size as soon as day 2 (Figures 3b,d,f) and continued to increase in size throughout the 10 day maturation period (Figures

3c,e,g). Measurements taken from hASC micrographs (Figure 4) tracked spheroid organization and growth throughout the maturation phase and showed that the initially formed small spheroids gradually increased in size during maturation, achieving a maximum aggregate size within 10 days (Figure 4). At the start of the maturation phase, hASC spheroids had an average diameter of $21.9 \pm 0.8 \mu\text{m}$, which gradually increased to $45.2 \pm 5.0 \mu\text{m}$ by the end of the 10-day culture in the control maturation medium. This spheroid diameter increase translates to ~10-fold volume increase over this culture period (Figure 4).

The metabolism of a particular fatty acid may depend on its structure (e.g., chain-length, position and number of double bonds) [35]. Therefore, we sought to investigate if there was any differential effect of LA, which is an essential polyunsaturated ω -6 fatty acid that may influence inflammatory processes through its conversion to arachidonic acid [36], and OA, which is the most abundant monounsaturated ω -9 fatty acid in human adipose tissue [37], on hASC spheroid culture. Importantly, LA and OA have similar molecular weight and chain-length, except the number of double bonds. As seen in Figure 4, the average diameter was generally found to be lower for the spheroids treated with LA or OA over the entire maturation phase. For instance, the average diameters for spheroids cultured for 10 days in 500 μm LA or OA containing maturation media were $36.2 \pm 2.4 \mu\text{m}$ and $38.6 \pm 3.3 \mu\text{m}$, respectively, compared to $45.2 \pm 5.0 \mu\text{m}$ in the control maturation medium ($p < 0.05$). However, there was no significant difference between the diameters for spheroids treated with the two fatty acids. This spheroid diameter increase translates to ~5-fold volume increase over this culture period (Figure 4).

Human stromal cells from either lipoaspirated or resected fat have been shown to produce a 3-D adipose tissue using a “self-assembly” culture methodology, where adipocyte differentiation was induced with ascorbic acid [38]. Kaplan and co-workers used porous silk-based fibroin, collagen, and poly-lactic acid (PLA)-based scaffolds *in vitro* and *in vivo* to support the attachment and growth of human bone marrow and adipose-derived mesenchymal stem cells (MSCs) [6]. Mao and co-workers prepared 3-D adipose-like tissue structures by seeding human MSCs on a gelatin sponge and subsequent exposure to adipogenic differentiation medium [39]. Our system distinguishes itself from these techniques, whereby the PEI segment of the ELP-PEI surface coating discourages individual cell attachment to substrate surface leading to spheroid formation, while the ELP segment of the ELP-PEI surface coating encourages attachment of the formed spheroids (Figure 3). The result is a culture where the individual cells maintain minimum fidelity with the surface, while surface-tethering of the formed spheroids is achieved [20]. Overall, our 3-D spheroid system promotes cell-cell interaction compared to cell-material interaction, which is commonly observed in endocrine tissues. More importantly, the surface-tethering of spheroids may minimize any spheroid loss due to media changes during a long-term culture. This hypothesis was tested by comparing the total intracellular protein content of the cultures after 5 days and 10 days in the maturation media. Protein content was statistically indistinct between the two time points and between the two fatty acid (OA and LA) treatments (Figure 5), indicating no significant spheroid loss over the 10-day culture period. This stable protein content over the 10-day maturation period also indicated contact-inhibited proliferation within the spheroid culture, which would likely support superior differentiation under the adipogenic culture conditions. The total intracellular protein

measured showed ~5 fold higher protein content in the 2-D monolayer cultures (793 ± 63 μg) compared to 3-D spheroids (142 ± 54 μg). This increase was due to the extensive proliferation that occurred during the first 3 days after cell seeding to achieve a confluent monolayer. Interestingly, the protein content across each of the fatty acid treated 2-D monolayer cultures was significantly (~25%) lower than untreated 2-D controls (Figure 5).

hASCs and mature adipocytes may receive exogenous fatty acids either as transported by plasma albumin or as triacylglycerides packaged into chylomicrons or lipoproteins. Fatty acid uptake is believed to be due to a combination of diffusion and active transport by membrane proteins such as CD36 in humans [40]. CD36 is an important marker for physiological homeostasis and differentiated function and has previously been found to be associated with both long-chain fatty acid transport [41] and advanced glycation end products [41] in both 3T3-L1 mouse [41,42] and human adipocytes [42]. An ELISA assay specific to human CD36, for the first time to our knowledge, measured the expression of the CD36 fatty acid transporter by differentiated hASCs in 3-D spheroid cultures. Our data revealed that the CD36 expression was statistically indistinct between the two time points (day 5 and day 10) and between the two fatty acid (OA and LA) treatments (Figure 6). CD36 ELISA assay also indicated general upregulation of the fatty acid transporter in 3-D spheroid cultures versus all 2-D monolayer cultures (Figure 6).

hASCs and preadipocytes are primed for intracellular triglycerides accumulation during the differentiation phase and then typically start accumulating intracellular triglycerides during the maturation phase. Immediately after the differentiation phase (3 days in the adipogenic differentiation medium and 0 days in the maturation medium), the hASCs did not show any triglyceride accumulation in 2-D monolayer as well as 3-D spheroid cultures (data not shown). Triglyceride accumulation was evident in all hASC cultures by day 5 in the maturation media (Figure 7). As shown in Figure 7a, hASC spheroids cultured in the control maturation medium showed an average intracellular triglyceride content of 0.09 ± 0.03 $\mu\text{g}/\mu\text{g}$ protein by day 5, which remained statistically unchanged at 0.10 ± 0.04 $\mu\text{g}/\mu\text{g}$ protein by the end of the 10-day culture period ($p > 0.05$). hASC spheroids cultured for 5 days in 500 μM LA or OA containing maturation media showed an average intracellular triglyceride content of 0.12 ± 0.01 and 0.13 ± 0.06 $\mu\text{g}/\mu\text{g}$ protein, respectively. Intracellular triglyceride content was statistically indistinct between the spheroids cultured for 5 days in the control maturation medium and the two fatty acid (OA and LA) treatments ($p > 0.05$). However, the intracellular triglyceride content of the hASC spheroids cultured in 500 μM LA or OA containing maturation media increased by approximately 2-fold to 0.21 ± 0.05 and 0.28 ± 0.05 $\mu\text{g}/\mu\text{g}$ protein, respectively, by the end of the 10-day culture period ($p < 0.05$). On the other hand, as shown in Figure 7b, hASCs cultured as 2-D monolayer in the control maturation medium showed an average intracellular triglyceride content of 0.01 ± 0.009 $\mu\text{g}/\mu\text{g}$ protein by day 5, which remained statistically unchanged at 0.03 ± 0.006 $\mu\text{g}/\mu\text{g}$ protein by the end of the 10-day culture period ($p > 0.05$). This level of the intracellular triglyceride content was significantly ($p < 0.05$) lower than that observed in the hASC spheroids cultured in the control maturation medium (Figure 7a). Interestingly, the 2-D monolayer cultured for 5 days in 500 μM LA or OA containing maturation media showed a significantly ($p < 0.05$) higher average intracellular triglyceride content of 0.25 ± 0.01 and 0.21 ± 0.04 $\mu\text{g}/\mu\text{g}$ protein, respectively. However, the intracellular triglyceride content of the 2-D monolayers

cultured in 500 μ M LA or OA containing maturation media did not show any further increase by the end of the 10-day culture period ($p > 0.05$). This is an important finding that shows that while the intracellular triglyceride accumulation in hASC spheroids as well as the 2-D monolayers was stimulated by exogenous fatty acid supplementation, the 2-D monolayer converted the available fatty acids into triglycerides at an earlier time point, while the 3-D spheroids processed the available fatty acids at a more moderate pace. Such head-to-head comparison between the 3-D spheroid model against the conventional 2-D monolayer model may lead to a better *in vitro* model to uncover important biological mechanisms involved in diet-induced obesity.

Next, to demonstrate the successful differentiation and long-term maintenance of the 3-D human adipose stem cell spheroids as functional adipocytes, we analyzed expression of PPAR- γ , a key gene involved in adipogenesis, using qRT-PCR. In 3-D hASC spheroids, the PPAR- γ gene expression was enhanced 2-5 fold (Figure 8) when exposed to LA or OA compared to those cultured in the control medium. By contrast, in 2-D monolayer, the PPAR- γ gene expression was enhanced $\sim$ 2 fold when exposed OA, but not LA (Figure 8). Increased expression of PPAR- γ gene expression in response to LA or OA is expected, as fatty acids are known PPAR- γ ligands [43,44]. Collectively, these results underscore the suitability of the 3-D spheroid adipogenesis model in primary human cells. During the maturation phase, the differentiated hASCs lose their proliferative capability and gradually exhibit the phenotypic markers and behaviors associated with the adipose tissue – including triglyceride accumulation and the expression of adipogenic genes and transcription factors such as adipocyte lipid-binding protein/Fatty acid binding protein (aP2/FABP4), PPAR- γ , lipoprotein lipase (LPL), hormone-sensitive lipase (HSL), glycerol-3-phosphate dehydrogenase (GPDH), and the synthesis and secretion of adiponectin, and leptin as well as matrix proteins such as type VI collagen [33,45]. Here, we have shown that the hASC spheroids accumulated triglycerides during the maturation phase (Figure 7) with a concomitant increase in the expression of a key adipogenic gene, PPAR- γ (Figure 8).

We have previously shown that the ELP-PEI conjugate induced 3-D spheroid formation in primary rat hepatocytes [46], H35 rat hepatoma cells [21] as well as 3T3-L1 mouse preadipocytes [20]. Here we have successfully demonstrated that hASCs formed spheroid cultures and were differentiated along the adipogenic lineage. While animal-derived preadipocyte cell lines (e.g., mouse 3T3-L1) are popular subjects for tissue engineering feasibility studies, there is much contention in the literature as to the relevancy of these analogues to human lipid metabolism. Therefore, we compared the performance of the hASC spheroids with the 3T3-L1 spheroids. 50,000 hASCs or 3T3-L1 preadipocytes formed spheroids atop the ELP-PEI surface coating and were differentiated in the adipogenic media for 3 days and subsequently matured in the control maturation media for 5 days. The hASCs readily formed spheroids on the ELP-PEI coated surfaces on the same time scale (72 hours) as the 3T3-L1 cells. In fact, many other types of cells (liver cells, adipose cells, bone cells, cancer cells from animal and human origin as well as various cell lines) also readily form spheroids on the ELP-PEI coated surfaces over a 72-hour period [20,21,46]. As shown in Figure 9, hASC spheroids do not achieve similar levels of adipose-specific functionality as 3T3-L1 mouse cell analogues. As such, the hASC spheroids (diameter = $42.1 \pm 3.8 \mu$ m) were significantly smaller than the 3T3-L1 spheroids (diameter = $132.5 \pm 9.6 \mu$ m). The total

protein content, CD36 expression, and intracellular triglyceride accumulation were also 2–4 fold smaller for hASC spheroids compared to the 3T3-L1 spheroids (Figures 9b,c,d). We attribute these observed differences to significant differences in the *in vitro* behavior of human versus mouse cells, resulting in incomplete or divergent differentiation. Particularly, the slower hASC differentiation compared to the 3T3-L1 cells is likely due to fact that hASC need to first go through a lineage commitment phase and then the adipogenic maturation phase. Nevertheless, for both cells, increased spheroid sizes correlated with increased intracellular triglyceride and the triglyceride accumulation in differentiated hASC spheroids was further upregulated by exogenous fatty acid treatments (Figure 7).

4. Conclusions

Our work describes construction of a 3-D spheroid culture system atop positively charged ELP-PEI coating to study the differentiation and maintenance process of hASC adipocyte spheroids when subjected to fatty acid treatment. Measurements taken from spheroid micrographs indicated spheroid size increased during the maintenance phase, though the spheroids treated with LA or OA had smaller size compared to those cultured in the control maintenance medium. LA and OA treatments stimulated triglyceride accumulation in hASC cultures. The CD36 expression, representing cell competency for consuming extracellular fatty acids, was consistently found to be higher in 3-D hASC spheroids compared to 2-D monolayers. Expression of a key adipogenic gene, PPAR- γ was found to be elevated after the fatty acid treatment. Overall, the hASCs cultured in our 3-D spheroid model vastly outperformed the 2-D monolayer with respect to CD36 and PPAR- γ expression, likely due to contact-inhibited proliferation serving as a cue for enhanced adipogenic differentiation. We observed that the hASC spheroids undergo slower differentiation along the adipogenic lineage and attainment of physiological functionality compared to the 3T3-L1 mouse spheroids.

Acknowledgments

Support from the School of Dentistry and the University of Mississippi Medical Center intramural research support programs, the National Science Foundation (Award No: 1033525), and the National Institutes of Health (Award No: R01EB020006) is gratefully acknowledged. C.M.E. is supported by K01DK106307 from the National Institutes of Health. This work was supported in part by U54GM104940 from the National Institute of General Medical Sciences of the National Institutes of Health, which funds the Louisiana Clinical and Translational Science Center. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Science Foundation or the National Institutes of Health. We thank Dr. Peter Arnold for acquiring the donated whole adipose tissue. This work made use of instruments in the Department of Biomedical Materials Science User Facility. Authors have no conflicts of interest to disclose.

References

1. Gomillion CT, Burg KJ. Stem cells and adipose tissue engineering. *Biomaterials*. 2006; 27:6052–6063. [PubMed: 16973213]
2. Patrick CW. Tissue engineering strategies for adipose tissue repair. *Anatom Rec*. 2001; 263:361–366.
3. Tanzi MC, Farè S. Adipose tissue engineering: state of the art, recent advances and innovative approaches. *Expert Rev Med Devices*. 2009; 6:533–551. [PubMed: 19751125]
4. Flynn L, Semple JL, Woodhouse KA. Decellularized placental matrices for adipose tissue engineering. *J Biomed Mater Res A*. 2006; 79:359–369. [PubMed: 16883587]

5. Halbleib M, Skurk T, de Luca C, von Heimburg D, Hauner H. Tissue engineering of white adipose tissue using hyaluronic acid-based scaffolds. I: in vitro differentiation of human adipocyte precursor cells on scaffolds *Biomaterials*. 2003; 24:3125–3132. [PubMed: 12895585]
6. Mauney JR, Nguyen T, Gillen K, Kirker-Head C, Gimble JM, Kaplan DL. Engineering adipose-like tissue in vitro and in vivo utilizing human bone marrow and adipose-derived mesenchymal stem cells with silk fibroin 3D scaffolds. *Biomaterials*. 2007; 28:5280–5290. [PubMed: 17765303]
7. von Heimburg D, Kuberka M, Rendchen R, Hemmrich K, Rau G, Pallua N. Preadipocyte-loaded collagen scaffolds with enlarged pore size for improved soft tissue engineering. *Int J Artif Organs*. 2003; 26:1064–1076. [PubMed: 14738190]
8. Neubauer M, Hacker M, Bauer-Kreisel P, Weiser B, Fischbach C, Schulz MB, Goepferich A, Blunk T. Adipose tissue engineering based on mesenchymal stem cells and basic fibroblast growth factor in vitro. *Tissue Eng*. 2005; 11:1840–1851. [PubMed: 16411830]
9. Marra KG, Defail AJ, Clavijo-Alvarez JA, Badylak SF, Taieb A, Schipper B, Bennett J, Rubin JP. FGF-2 enhances vascularization for adipose tissue engineering. *Plast Reconstr Surg*. 2008; 121:1153–1164. [PubMed: 18349632]
10. Bhang SH, Cho SW, La WG, Lee TJ, Yang HS, Sun AY, Baek SH, Rhie JW, Kim BS. Angiogenesis in ischemic tissue produced by spheroid grafting of human adipose-derived stromal cells. *Biomaterials*. 2011; 32:2734–2747. [PubMed: 21262528]
11. Bhang SH, Lee S, Shin JY, Lee TJ, Kim BS. Transplantation of cord blood mesenchymal stem cells as spheroids enhances vascularization. *Tissue Eng Part A*. 2012; 18:2138–2147. [PubMed: 22559333]
12. Miyagawa Y, Okita H, Hiroyama M, Sakamoto R, Kobayashi M, Nakajima H, Katagiri YU, Fujimoto J, Hata J, Umezawa A, et al. A microfabricated scaffold induces the spheroid formation of human bone marrow-derived mesenchymal progenitor cells and promotes efficient adipogenic differentiation. *Tissue Eng Part A*. 2011; 17:513–521. [PubMed: 20818998]
13. Kim JH, Park IS, Park Y, Jung Y, Kim SH, Kim SH. Therapeutic angiogenesis of three-dimensionally cultured adipose-derived stem cells in rat infarcted hearts. *Cytotherapy*. 2013; 15:542–556. [PubMed: 23352461]
14. Cheng NC, Wang S, Young TH. The influence of spheroid formation of human adipose-derived stem cells on chitosan films on stemness and differentiation capabilities. *Biomaterials*. 2012; 33:1748–1758. [PubMed: 22153870]
15. Laschke MW, Schank TE, Scheuer C, Kleer S, Schuler S, Metzger W, Eglin D, Alini M, Menger MD. Three-dimensional spheroids of adipose-derived mesenchymal stem cells are potent initiators of blood vessel formation in porous polyurethane scaffolds. *Acta Biomater*. 2013; 9:6876–6884. [PubMed: 23415749]
16. Kotani A, Fuse T, Kusu F. Determination of plasma free fatty acids by high-performance liquid chromatography with electrochemical detection. *Anal Biochem*. 2000; 284:65–69. [PubMed: 10933857]
17. Huypens P, Quartier E, Pipeleersand D, Van de Casteele M. Metformin reduces adiponectin protein expression and release in 3T3-L1 adipocytes involving activation of AMP activated protein kinase. *Eur J Pharmacol*. 2005; 518:90–95. [PubMed: 16039647]
18. Kang X, Xie Y, Kniss D. Adipose tissue engineering with cells in engineered matrices. *Tissue Eng*. 2005; 11:458–468. [PubMed: 15869424]
19. Bradley RL, Fisher FM, Maratos-Flier E. Dietary Fatty Acids Differentially Regulate Production of TNF- α and IL-10 by Murine 3T3-L1 Adipocytes. *Obesity*. 2008; 16:938–944. [PubMed: 18356844]
20. Turner PA, Harris LM, Purser CA, Baker RC, Janorkar AV. A surface-tethered spheroid model for functional evaluation of 3T3-L1 adipocytes. *Biotechnol Bioeng*. 2014; 111:174–183. [PubMed: 24038000]
21. Turner PA, Weeks CA, McMurphy AJ, Janorkar AV. Spheroid organization kinetics of H35 rat hepatoma model cell system on elastin-like polypeptide-polyethyleneimine copolymer substrates. *J Biomed Mater Res A*. 2014; 102:852–861. [PubMed: 23564487]

22. Flynn LE. The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells. *Biomaterials*. 2010; 31:4715–4724. [PubMed: 20304481]
23. Hauner, H., Skurk, T., Wabitsch, M. Cultures of Human Adipose Precursor Cells. In: Ailhaud, G., editor. *Methods in Molecular Biology*. Totawa, NJ: Humana Press Inc.; 2001. p. 239-247. *Adipose Tissue Protocols*
24. Stillaert FB, Abberton KM, Keramidaris E, Thompson EW, Blondeel PN, Morrison WA. Intrinsic and dynamics of fat grafts: an in vitro study. *Plast Reconstr Surg*. 2010; 126:1155–1162. [PubMed: 20885240]
25. Daquinag AC, Souza GR, Kolonin MG. Adipose tissue engineering in three-dimensional levitation tissue culture system based on magnetic nanoparticles. *Tissue Eng Part C Methods*. 2013; 19:336–344. [PubMed: 23017116]
26. Bunnell BA, Flaatt M, Gagliardi C, Patel B, Ripoll C. Adipose-derived stem cells: isolation, expansion and differentiation. *Methods*. 2008; 45:115–120. [PubMed: 18593609]
27. Sanchez-Infantes D, White UA, Elks CM, Morrison RF, Gimble JM, Considine RV, Ferrante AW, Ravussin E, Stephens JM. Oncostatin M is produced in adipose tissue and is regulated in conditions of obesity and type 2 diabetes. *J Clin Endocrinol Metab*. 2014; 99:E217–25. [PubMed: 24297795]
28. Janorkar AV, Rajagopalan P, Yarmush ML, Megeed Z. The use of elastin-like polypeptide–polyelectrolyte complexes to control hepatocyte morphology and function in vitro. *Biomaterials*. 2008; 29:625–632. [PubMed: 18006054]
29. Swierczewska M, Hajicharalambous CS, Janorkar AV, Megeed Z, Yarmush ML, Rajagopalan P. Cellular response to nanoscale elastin-like polypeptide polyelectrolyte multilayers. *Acta Biomater*. 2008; 4:827–837. [PubMed: 18178532]
30. Chen TH, Bae Y, Furgeson DY. Intelligent biosynthetic nanobiomaterials (IBNs) for hyperthermic gene delivery. *Pharm Res*. 2008; 25:683–691. [PubMed: 17762916]
31. Flynn L, Woodhouse KA. Adipose tissue engineering with cells in engineered matrices. *Organogenesis*. 2008; 4:228–235. [PubMed: 19337402]
32. O'Connor KC, Song H, Rosenzweig N, Jansen DA. Extracellular matrix substrata alter adipocyte yield and lipogenesis in primary cultures of stromal-vascular cells from human adipose. *Biotech Lett*. 2003; 25:1967–1972.
33. Ailhaud, G. Development of white adipose tissue and adipocyte differentiation. In: Klaus, S., editor. *Adipose Tissues*. Eureka.com Inc; Austin, TX: 2001. p. 27-48.
34. Klaus, S. Significance of Fat and Adipose Tissues. In: Klaus, S., editor. *Adipose Tissues*. Austin, TX: Eureka.com Inc; 2001. p. 1-9.
35. Madsen L, Petersen RK, Kristiansen K. Regulation of adipocyte differentiation and function by polyunsaturated fatty acids. *Biochim Biophys Acta*. 2005; 1740:266–286. [PubMed: 15949694]
36. Calder PC. n–3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am J Clin Nutr*. 2006; 83:S1505–S1519.
37. Kokatnur MG, Oalman MC, Johnson WD, Malcom GT, Strong JP. Fatty acid composition of human adipose tissue from two anatomical sites in a biracial community. *Am J Clin Nutr*. 1979; 32:2198–2205. [PubMed: 495536]
38. Vermette M, Trottier V, Ménard V, Saint-Pierre L, Roy A, Fradette J. Production of a new tissue-engineered adipose substitute from human adipose-derived stromal cells. *Biomaterials*. 2007; 28:2850–2860. [PubMed: 17374391]
39. Alhadlaq A, Tang M, Mao JJ. Engineered adipose tissue from human mesenchymal stem cells maintains predefined shape and dimension: implications in soft tissue augmentation and reconstruction. *Tissue Eng*. 2005; 11:556–566. [PubMed: 15869434]
40. Large V, Peroni O, Letexier D, Ray H, Beylot M. Metabolism of lipids in human white adipocyte. *Diabetes & Metabolism*. 2004; 30:294–309. [PubMed: 15525872]
41. ohl J, Ring A, Korkmaz U, Ehehalt R, Stremmel W. FAT/CD36-mediated long-chain fatty acid uptake in adipocytes requires plasma membrane rafts. *Mol Biol Cell*. 2005; 16:24–31. [PubMed: 15496455]

42. Kuniyasu A, Ohgami N, Hayashi S, Miyazaki A, Horiuchi S, Nakayama H. CD36-mediated endocytic uptake of advanced glycation end products (AGE) in mouse 3T3-L1 and human subcutaneous adipocytes. *FEBS Lett.* 2003; 537:85–90. [PubMed: 12606036]
43. Schoonjans K, Staels B, Auwerx J. Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. *J Lipid Res.* 1996; 37:907–925. [PubMed: 8725145]
44. Furuhashi M, Hotamisligil GS. Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. *Nat Rev Drug Discov.* 2008; 7:489–503. [PubMed: 18511927]
45. Choi JH, Gimble JM, Lee K, Marra KG, Rubin JP, Yoo JJ, Vunjak-Novakovic G, Kaplan DL. Adipose tissue engineering for soft tissue regeneration. *Tissue Eng Part B.* 2010; 16:413–426.
46. Weeks CA, Aden B, Zhang J, Singh A, Hickey RD, Kilbey SM II, Nyberg SL, Janorkar AV. Effect of Amine Content and Chemistry on Long-Term, Three-Dimensional Hepatocyte Spheroid Culture atop Aminated Elastin-Like Polypeptide Coatings. *J Biomed Mater Res Part A.* (Accepted for Publication 09/16/2016).

Highlights

- 3-D spheroid model using human adipose-derived stem cells (hASCs)
- Differentiated 3-D stem cell spheroids display functional markers of adipogenesis
- Stem cell spheroids follow slower differentiation compared to 3T3-L1 adipocytes

Figure 1.

(a) Fluorescence (360 excitation / 460 emission) of OPA-treated solutions (5 mg/mL) of ELP and ELP-PEI ($n = 5$). (b) Representative turbidimetry result showing increase in turbidity of the solutions (5 mg/mL) of ELP and ELP-PEI as they are heated. Solution turbidity is indicated by absorbance at 350 nm. Inset shows the transition temperatures of ELP and ELP-PEI solutions defined as the temperature at which the solution turbidity was half of the maximum turbidity ($n = 3$).

Figure 2.
Experimental timeline for 3-D spheroid culture and analyses.

Figure 3.

Spheroid formation of hASCs atop ELP-PEI coated surface. Images were captured at 3 locations per well on 3 replicate wells per condition (total $n = 9$ images per condition). (a) hASC cultures formed small spheroids at the end of the differentiation phase. Subsequent 10-day culture in the maturation media increased spheroid size. (b,d,f) 5 day culture in maturation media. (c,e,g) 10 day culture in maturation media. (b,c) control maturation medium, (d,e) maturation medium containing 500 μM LA, (f,g) maturation medium containing 500 μM OA.

Figure 4. Quantitative measurement of the hASC spheroid sizes ($n > 90$) during the maturation phase. Spheroids treated with 500 μM LA (■) or 500 μM OA (▲) were smaller than those cultured in the control maturation medium (◆). * $p < 0.05$ vs. Control.

Figure 5.

Total Protein content of hASCs cultured as (a) 3-D spheroids and (b) 2-D monolayer for 5 days (purple bars) and 10 days (blue bars) in maturation media containing no additional fatty acid (control), 500 µM LA, or 500 µM OA. * $p < 0.05$ vs. Control on same day; # $p < 0.05$ vs. 2-D monolayer. Measurements were taken in triplicate from each of three replicate wells ($n = 9$) exposed to unique maturation media.

Figure 6.

CD36 expression of hASCs cultured as (a) 3-D spheroids and (b) 2-D monolayer for 5 days (purple bars) and 10 days (blue bars) in maturation media containing no additional fatty acid (control), 500 μ M LA, or 500 μ M OA. * $p < 0.05$ vs. 2-D monolayer. Measurements were taken in triplicate for each culture condition, one sample per experimental well, yielding a total of three measurements per condition ($n = 3$).

Figure 7.

Triglyceride content of hASCs cultured as (a) 3-D spheroids and (b) 2-D monolayer for 5 days (purple bars) and 10 days (blue bars) in maturation media containing no additional fatty acid (control), 500 µM LA, or 500 µM OA. Triglyceride content was increased by fatty acid treatment. * $p < 0.05$ vs. Control on same day; # $p < 0.05$ vs. 2-D monolayer; \$ $p < 0.05$ vs. day 5 in the same maturation medium. Measurements were taken in triplicate from each of three replicate wells ($n = 9$) exposed to unique maturation media.

Figure 8. PPAR- γ gene expression of hASCs cultured as (a) 3-D spheroids and (b) 2-D monolayer for 5 days (purple bars) and 10 days (blue bars) containing no additional fatty acid (control), 500 μ M LA, or 500 μ M OA. Measurements were taken in triplicate for each culture condition, one sample per experimental well, yielding a total of three measurements per condition ($n = 3$).

Figure 9.

Comparison of hASC and 3T3-L1 spheroids cultured in the control maturation medium for 5 days. (a) spheroid diameter ($n > 90$), (b) total protein content ($n = 9$), (c) CD36 expression ($n = 3$), and (d) triglyceride content ($n = 9$). * $p < 0.05$ vs. hASC spheroids. Measurements were taken using the same sample sizes as described before in Figures 4-7.