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# Stimulation of adipogenesis of adult adipose-derived stem cells using substrates that mimic the stiffness of adipose tissue

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ABSTRACT

Biochemical and biomechanical extracellular matrix (ECM) cues have recently been shown to play a role in stimulating stem cell differentiation towards several lineages, though how they combine to induce adipogenesis has been less well studied. The objective of this study was to recapitulate both the ECM composition and mechanical properties of adipose tissue *in vitro* to stimulate adipogenesis of human adipose-derived stem cells (ASCs) in the absence of exogenous adipogenic growth factors and small molecules. Adipose specific ECM biochemical cues have been previously shown to influence adipogenic differentiation; however, the ability of biomechanical cues to promote adipogenesis has been less defined. Decellularized human lipoaspirate was used to functionalize polyacrylamide gels of varying stiffness to allow the cells to interact with adipose-specific ECM components. Culturing ASCs on gels that mimicked the native stiffness of adipose tissue (2 kPa) significantly upregulated adipogenic markers, in the absence of exogenous adipogenic growth factors and small molecules. As substrate stiffness increased, the cells became more spread, lost their rounded morphology, and failed to upregulate adipogenic markers. Together these data imply that as with other lineages, mechanical cues are capable of regulating adipogenesis in ASCs.



## 1. Introduction

Adipose tissue engineering has recently emerged as a growing field within regenerative medicine that strives to replace lost adipose tissue due to severe burns or breast tumor resection. Unfortunately, materials currently available for adipose replacement do not mimic native adipose tissue and thus offer few cues for natural regeneration, and as such, many of these synthetically-derived materials are either encapsulated in fibrous tissue or fail to integrate with the surrounding tissue [1]. Biologically-based materials, such as hyaluronan and collagen, typically interact well with the surrounding tissue but offer few cues for adipose-specific regeneration and therefore are gradually broken down and cleared by the body with no positive remodeling [2]. Thus there remains a significant clinical need to develop materials for the treatment of burns or tumor resection that positively interact with the surrounding tissue to not only fill the void left by the damaged tissue, but also facilitate the natural remodeling and regeneration of adipose tissue.

Clinicians have begun to address this issue by offering a procedure termed “lipotransfer”, or the direct implantation of liposuctioned adipose tissue back into the subcutaneous space of a different area of the body [3,4]. The variable outcomes of these procedures have led the field to begin mixing lipotransfer with autologous, adipose-derived adult stem cells (ASCs). These multipotent cells have been shown to possess the ability to differentiate down multiple lineages, including adipogenic, chondrogenic, osteogenic, and myogenic pathways [5]. The success of ASCs within a lipotransfer graft has been partly attributed to their ability to both encourage neovascularization and also produce new adipocytes within the implant [6]. However, adipogenic differentiation of ASCs *in vitro* has occurred predominantly via soluble factors within the culture media, which typically include insulin, dexamethasone and various other steroids [7]. Chemically-based differentiation protocols provide a reductionist approach to pinpoint the effect of specific induction pathways, but they likely stimulate few of the pathways utilized *in vivo* when ASCs differentiate. On the other hand, lipotransfer procedures with ASCs are relatively uncontrolled and outcomes can greatly vary between clinicians. Identification of materials, which could encourage ASC maturation prior to implantation to produce a more uniform population of ‘primed’ cells or which could be implanted with ASCs to guide their maturation,

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would improve retention and standardize outcomes of adipose defect treatments.

Because of the inability of current materials to offer encouragement for natural fat regeneration, many investigators have begun to turn to natural alternatives that better recapitulate the extracellular cues present within adipose tissue. The process of decellularization offers the possibility of isolating tissue specific extracellular matrix (ECM) components by removing the cellular content from whole organs or tissues. This produces a material composed of various proteins and proteoglycans characteristic of the tissue of interest, but devoid of the immunogenic cellular components [8]. These ECM-based materials have already been seen to stimulate stem cell differentiation for a variety of organs and facilitate constructive remodeling *in vivo* [9–15]. In particular, several structural proteins, such as collagen VI, have been shown to have a dramatic impact on adipose tissue metabolic function and adipocyte physiology [16]. Recently, several groups have published methods for decellularizing human adipose tissue. Adipose ECM can be prepared in a variety of forms, from solid scaffolds to injectable gels and powders, and encourages adipogenic differentiation of ASCs [17–22]. While these materials mimic the ECM composition of adipose tissue, ECM mechanical cues are also a major contributor to stem cell differentiation. Biomechanical cues can stimulate mesenchymal stem cell differentiation toward a variety of cell lineages by mimicking the stiffness of the desired tissue [23,24]. For example, a substrate that mimics the stiffness of healthy muscle or bone encourages ASCs to express myogenic or osteogenic transcription factors, respectively, in the absence of specific exogenous growth factors [24]. ECM stiffness can even regulate differentiation in the presence of a mixture of soluble factors for osteogenic and adipogenic differentiation; softer substrates encourage bone marrow-derived mesenchymal stem cells to favor adipogenesis despite the presence of osteogenic inductive factors while stiffer substrates favor osteogenesis despite the presence of adipogenic factors [25].

These data suggest the important regulatory potential for matrix-based cues, which could be used *in vitro* to differentiate ASCs prior to their introduction with other tissue-engineered products in a reconstructive therapy. Thus our goal for this study was to recreate the adipose microenvironment *in vitro* by combining both biochemical and biomechanical adipose-specific cues to stimulate adipogenesis of ASCs. By recapitulating both the stiffness and ECM composition of adipose tissue, we can present natural stimuli to the ASCs to encourage adipogenic differentiation. Furthermore, identification of an "adipogenic stiffness" could provide an important design criteria for the future development of materials meant to encourage adipogenesis of ASCs.

## 2. Materials and methods

### 2.1. Isolation of adipose stem cells

Human adipose tissue was collected from patients undergoing elective liposuction surgery at the Ranch & Coast Plastic Surgery Clinic (Del Mar, CA). All procedures involving tissue from human patients were reviewed and approved by the UCSD Institutional Review Board. Tissue was collected from seven female patients ranging in age from 34 to 52, with an average age of 42 ± 6. Human adipose-derived adult stem cells (ASCs) were isolated from the lipoaspirate following a previously established protocol [26]. Briefly, the lipoaspirate was digested for 20 min in 0.075% collagenase I (Worthington Biochemical) at 37 °C. The resulting suspension was centrifuged at 5000 ×*g* to obtain an ASC-rich pellet. The cell pellet was resuspended in 160 mM ammonium chloride buffer to lyse red blood cells and again centrifuged at 5000 ×*g*. The new cell pellet was resuspended in growth media (DMEM/F12 plus 10% Fetal Bovine Serum, 100 I.U. penicillin, and 100 μg/mL streptomycin) and passed through a 40 μm cell strainer. The remaining cells were plated on standard tissue culture plastic overnight at 37 °C and 5% $CO_2$. After 24 h, the non-adherent cells were removed with two rinses with 1× PBS, and then serially passaged at 70% confluence. Growth media was changed every 3–4 days. The remaining lipoaspirate was frozen at −80 °C until further processing.

### 2.2. Adipose decellularization

Human liposuctioned adipose tissue was decellularized following our previously published protocol to isolate adipose extracellular matrix [17]. Briefly, the tissue was rinsed in 1× phosphate-buffered saline (PBS) to remove blood and free lipids. The cellular content of the tissue was then removed by rinsing with 1% sodium dodecyl sulfate (SDS). Residual SDS was removed by brief washes in DI water and then 0.01% Triton X-100. The tissue was then delipidized using a solution of 2.5 mM sodium deoxycholate with a 1:1 ratio of lipase to colipase enzymes (all chemicals were obtained from Sigma–Aldrich unless otherwise noted). The remaining white tissue was then rinsed overnight in DI water followed by a brief rinse with isopropanol. The resulting decellularized adipose ECM was then milled at room temperature using a Wiley mini-mill to produce a fine powder. To facilitate use in cell culture, the adipose ECM was reduced to a liquid form by digesting in a solution of 0.1 M HCl and 1 mg/mL pepsin as previously described [17]. After digestion, the liquid adipose matrix was brought to physiological pH with sodium hydroxide on ice, then diluted to 100 μg/mL in 50 mM HEPES buffer at pH 8.5.

### 2.3. Fabrication of polyacrylamide gels

Polyacrylamide gels were produced using a constant 8% acrylamide solution. Glass coverslips were first activated by treating them for 10 min in a UV/Ozone ProCleaner (Bioforce Nanosciences) and then functionalized with 3-(Trimethoxysilyl)propyl methacrylate (20.3 mM in ethanol, 3 min). Coverslips were briefly rinsed with 100% ethanol and water and then dried. Solutions of 8% acrylamide and varying percentages of bis-acrylamide (Fisher BioReagents) were diluted in 1× PBS and the reaction was catalyzed by adding 10% ammonium persulfate and 1/1000 volume of N,N,N′,N′-tetramethylethylenediamine. This solution was then sandwiched between the methacrylate-functionalized coverslip and a DCDMS-coated glass slide. After reacting for 30 min, the polyacrylamide gels were rinsed three times with 1× PBS and allowed to hydrate in PBS overnight. Gels were then sterilized in a tissue culture hood by exposure to 254 nm, 30 W UV light for 4 h. For protein attachment, the surface of the polyacrylamide gel was reacted with the photoactivated, bi-functional crosslinker Sulfosuccinimidyl 6-(4′-azido-2′-nitrophenylamino)hexanoate (sulfo-SANPAH. Pierce, 0.2 mg/mL in 50 mM HEPES pH 8.5) under 365 nm UV light with a surface intensity of 0.85 mW/cm$^2$ for 10 min. Solutions of 100 μg/mL adipose matrix, in 50 mM HEPES buffer at pH 8.5, were added on top of the gels and allowed to attach for 1 h at 37 °C. To confirm protein attachment, adipose matrix was first labeled with sulfo-NHS-functionalized biotin (Pierce). After attaching the adipose matrix, the polyacrylamide gels were then reacted with neutravidin-horseradish peroxidase complex and visualized with 3,3′-Diaminobenzidine (DAB) substrate kit (Pierce).

### 2.4. Atomic force microscopy

Polyacrylamide gel stiffness was confirmed by atomic force microscopy (AFM; MFP3D, Asylum Research) as detailed previously [27]. Briefly, a pyramidal probe, 0.08 N/m force constant with a 35° half angle (PNP-TR-SPL, Nanoworld), was used to indent each gel in triplicate over 5 random regions of the gel to assess heterogeneity. Probe indentation velocity was fixed at 2 μm/s with the trigger force of 2 nN. Elastic moduli were determined by the Sneddon cone model with a sample Poisson ratio of 0.5 fit over a range of 10%–90% indentation force. Custom software written in Igor pro 6.22 was applied to analyze elastic modulus via Sneddon's model [28]. Four polyacrylamide gels of each bis-acrylamide percentage were measured to determine the average elastic modulus for that group.

### 2.5. Cell culture

For media differentiation studies, the liquid adipose matrix was added to tissue culture polystyrene wells at a concentration of 100 μg/mL and incubated at 37 °C for 1 h to allow protein adsorption, as previously described [17]. Human ASCs were then seeded in the functionalized wells in growth media at a density of 1 × 10$^4$ cells/mL and allowed to attach overnight. The next day, the media was aspirated and either fresh growth media was added, or adipogenic media (AM: DMEM/F12 plus 10% FBS, 500 μM isobutylmethyl xanthine, 1 μM dexamethasone, 10 μg/mL insulin, 200 μM indomethacin, 100 I.U. penicillin, and 100 μg/mL streptomycin). Each respective media was then changed every 2 days until completion of the study. At 48 h after cell seeding, separate gels were stained with a Live/Dead kit (Life Technologies) to confirm cell viability, following the manufacturer's protocol (see Supplementary Data). For stiffness differentiation studies, human ASCs were seeded in growth media on top of adipose matrix-functionalized polyacrylamide gels in a 24-well plate at a density of 1 × 10$^4$ cells per well and allowed to attach overnight. The next day the media was aspirated and either fresh growth media was added, or growth media supplemented with 0.25 μg/mL cytochalasin-D. Each respective media was then changed every day until completion of the study. All ASCs used in this study were between passages 1 and 3, and cells from at least 3 different patients were used to repeat each study. Brightfield images of the cells were taken every 2 days using a Nikon Eclipse TS100 microscope fitted with an Infinity 2 camera.