The Use of Silk as a Scaffold for Mature, Sustainable Unilocular Adipose 3D Tissue Engineered Systems

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There is a critical need for monitoring physiologically relevant, sustainable, human adipose tissues in vitro to gain new insights into metabolic diseases. To support long-term culture, a 3D silk scaffold assisted culture system is developed that maintains mature unilocular adipocytes ex vivo in coculture with preadipocytes, endothelial cells, and smooth muscle cells obtained from small volumes of liquefied adipose samples. Without the silk scaffold, adipose tissue explants cannot be sustained in long-term culture (3 months) due to their fragility. Adjustments to media components are used to tune lipid metabolism and proliferation, in addition to responsiveness to an inflammatory stimulus. Interestingly, patient specific responses to TNFα stimulation are observed, providing a proof-of-concept translational technique for patient specific disease modeling in the future. In summary, this novel 3D scaffold assisted approach is required for establishing physiologically relevant, sustainable, human adipose tissue systems from small volumes of lipoaspirate, making this methodology of great value to studies of metabolism, adipokine-driven diseases, and other diseases where the roles of adipocytes are only now becoming uncovered.

1. Introduction

White adipose tissues are endocrine organs that secrete adipokines[1] and store excess energy in unilocular lipid-filled vacuoles of adipocytes.[2] Lipid metabolism includes storing triglycerides and lipolysis, where the triglycerides are broken down into glycerol and free fatty acids to meet energy needs.[3] In healthy individuals there is homeostasis between the release of fatty acids from adipose tissues into the circulation and the uptake and oxidation in peripheral tissues. However, in obese individuals energy intake exceeds the storage capacity of adipose tissue, triggering an inflammatory response (as the tissue expands), additional lipolysis, and altered concentrations of fatty acids and glucose in the circulation.[4] Obesity predisposes individuals to complex metabolic disorders including dyslipidemia and type 2 diabetes.[5] As the prevalence of obesity reaches epidemic proportions in many countries, there is a critical need for physiologically relevant, sustainable, human adipose tissue engineered models to generate new insights into disease initiation and progression and to test potential treatment options.

Current in vitro models of white adipose depots fail to recapitulate the complex human tissue. The models utilize animal cells,[6–8] cell suspension cultures,[9] ceiling cultures,[2,10–12] or differentiation of human adipose derived stem cells.[13–15] Animal cell lines (including murine 3T3-L1 and 3T3-F442A) undergo adipogenesis during in vitro culture, however, they lack the single large lipid droplet characteristic of mature in vivo adipocytes.[6] Additionally murine differentiated cells secrete only 1%–2% of the leptin secreted by primary mature adipocytes[7] and often do not translate well to human adipocyte function.[16] Primary cell suspension cultures consist of adipocytes floating in medium, and hence the cells are not equally exposed to treatments of interest. Moreover, this culture method is only a short term solution, as the floating cells are not exposed to adequate nutrition and lyse within 72 h of incubation.[10] Ceiling cultures also take advantage of the buoyancy of adipocytes: a culture flask is filled completely with medium to allow the adipocytes to adhere to the top surface of the flask. Although the adipocytes proliferate and exhibit some adipocyte functions such as lipogenesis (accumulation of multilocular lipid droplets) and lipolysis, they display a fibroblast-like phenotype rather than the round unilocular phenotype typical of adipocytes.[2,12] Finally, the most common method of generating white adipose tissue involves differentiating human adipose-derived stem cells in 2-D or 3D systems. To enhance physiological

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relevance, endothelial cells have been incorporated with differentiated stem cells,[15,17,18] demonstrating improved adipogenic outcomes. However, these methods require lengthy culture times to differentiate the stem cells into adipocytes, and in the absence of perfusion, contain multilocular lipid droplets.[19] Therefore, better in vitro models are required that incorporate the physiologically relevant mature unilocular adipocytes.

Ex vivo culture of unilocular adipocytes is challenging, as lipid laden mature cells are fragile, highly buoyant, and prone to dedifferentiation in culture. Maintenance of cells in their native tissue matrix (explanted tissue) would be ideal to maintain their 3D morphology in vitro; however, explants are delicate and lack the structural integrity required for extended culture periods (>14 d). Culturing tissue fragments in collagen gels has been proposed as a solution to the fragile and buoyant nature of adipocytes/explants[20] for establishing human ex vivo adipose tissue models. However, in collagen gels, preadipocytes only develop actively at the periphery of the adipose tissue fragments. It was hypothesized that a silk scaffold would provide a 3D framework that would capture and maintain mature unilocular adipocytes ex vivo while having the structural integrity required for long-term culture. Silk is a naturally occurring and clinically accepted biocompatible protein material that has tunable mechanical strength, low inflammatory and immunogenic responses, an absence of cell-specific signaling domains, and can be tailored to degrade slowly for long-term culture.[21] Furthermore, silk has demonstrated compatibility with adipose tissue engineering applications.[17,22,23] Liquefied adipose tissue was used in this study in combination with silk scaffolds as this heterogeneous mixture contained the relevant cell types required to create a physiologically relevant adipose system (**Figure 1**) and could penetrate into the scaffold farther than larger tissue fragments used in studies with collagen.[20] In addition, for implanting in vivo, collagen has been shown to rapidly degrade while silk provides longer-term structural integrity to promote the maintenance of soft tissue.[24] Moreover, an important aspect of this method is the applicability to patient-specific in vitro systems. To create a patient specific model of an adipose depot, a small volume of liquefied adipose tissue would be obtained by noninvasive lipoaspirate procedures and cultured within the silk scaffolding material. The adipose depot could then be used to explore treatment options (including high throughput screens of multiple drug targets as well as optimizing the doses), adverse effects to drug treatments (including side effects and toxicity), disease mechanisms (including type II diabetes and cancers that affect the adipose tissue) and other metabolic parameters that vary between patients.

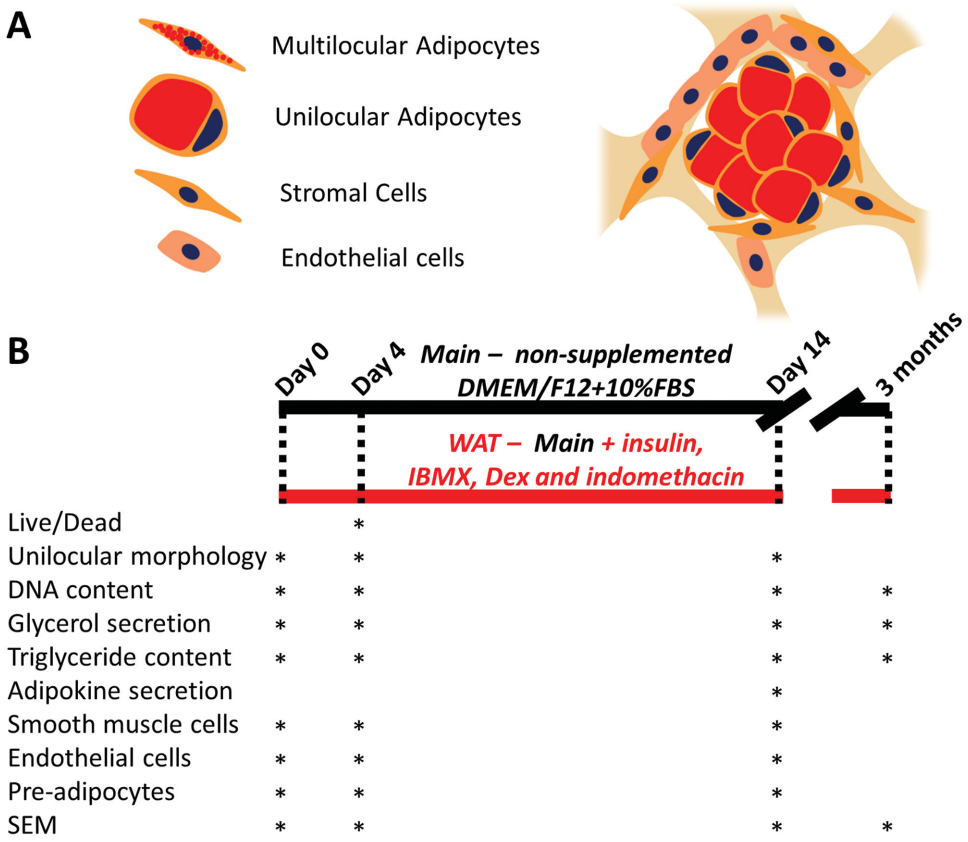

Figure 1. Schematic of cells in the adipose tissue model and culture timeline. Adipose tissue containing unilocular adipocytes (as opposed to multilocular immature adipocytes), stromal cells (preadipocytes, fibroblasts, smooth muscle pericytes), and endothelial cells was liquefied and seeded A) into porous silk scaffolds in B) different media conditions. The maintenance media group, referred throughout as *Main*, was a minimally supplemented media (DMEM/F12 and 10% FBS), while the white adipose tissue stimulation media group, referred throughout as *WAT*, was created by supplementing the maintenance media with insulin, IBMX, dexamethasone, and indomethacin. The different assays that were performed at each time point are shown.

2. Results and Discussion

2.1. Viability and Distribution of Cells in the Scaffolds

Since adipose tissue explants rapidly deteriorate in culture, the goal of this work was to determine whether a white adipose tissue model could be maintained ex vivo in silk scaffold cultures. Liquified adipose tissue was chosen to improve penetration in the scaffolds. In addition, lipoaspirates are an ideal source of obtaining adipose tissue minimally invasively. To validate that cells remained viable through the seeding process, calcein and ethidium staining was used to visualize the distribution of cells throughout the cultures. Live cells were dispersed throughout the explants and scaffolds 4 d after seeding (**Figure 2**) demonstrating a safe and effective isolation, seeding, and culturing process for both culture techniques. Pockets of lipid laden adipocyte cell clusters were also evident in the pores of the scaffolds, demonstrating that the cells were viable, distributed throughout the constructs, and may have maintained their in vivo unilocular morphology. Since current models contain cells with a multilocular morphology and not the characteristic unilocular phenotype observed in vivo,[2,12] maintenance of a unilocular morphology would indicate the model maintained physiologically relevant characteristics of an adipose depot.

2.2. Determination of Cell Types Present

To further test if the unilocular lipid morphology was maintained, lipophilic AdipoRed staining was performed. AdipoRed staining demonstrated that mature adipocytes were maintained in both explant and scaffold cultures under both media conditions tested (**Figure 3**). The two media conditions were maintenance (*Main*) media (DMEM/F12, 10% fetal bovine serum) and white adipose tissue (*WAT*) stimulation media (DMEM/F12, 10% fetal bovine serum, 1X antibiotic-antimycotic, 1 µM insulin, 0.5×10^{-3} M 3-isobutyl-1-methylxanthine (IBMX), 1×10^{-6} M dexamethasone, 0.05×10^{-3} M indomethacin). Non-supplemented maintenance media (*Main*) demonstrated greater numbers of cells while stimulatory supplemented media (*WAT*) showed smaller lipid droplets accumulating in both explant and scaffold cultures. To quantify whether the larger unilocular lipid droplet size was maintained ex vivo, lipid areas were traced and are shown for one patient (Figure 3C). After 2 weeks, there were no differences between scaffold and explant unilocular lipid droplet areas. While there was a significant decrease in unilocular lipid droplet area from day 0 for the explants, scaffold cultures had similar areas as the values measured directly at the time of seeding (day 0). Therefore, this experiment confirmed that the scaffold pores were filled with mature unilocular adipocytes, as well as other populations of cells indicated by phalloidin staining in the absence of AdipoRed staining.

To identify what other cell types remained in the cultures (the phalloidin positive cells), further imaging was performed. Multiple cell types are residents of adipose tissue, including adipocytes, preadipocytes, endothelial cells, fibroblasts, vascular smooth muscle cells and immune cells[25] therefore markers of preadipocytes, smooth muscle cell lineages, and endothelial cells were immunostained with preadipocyte

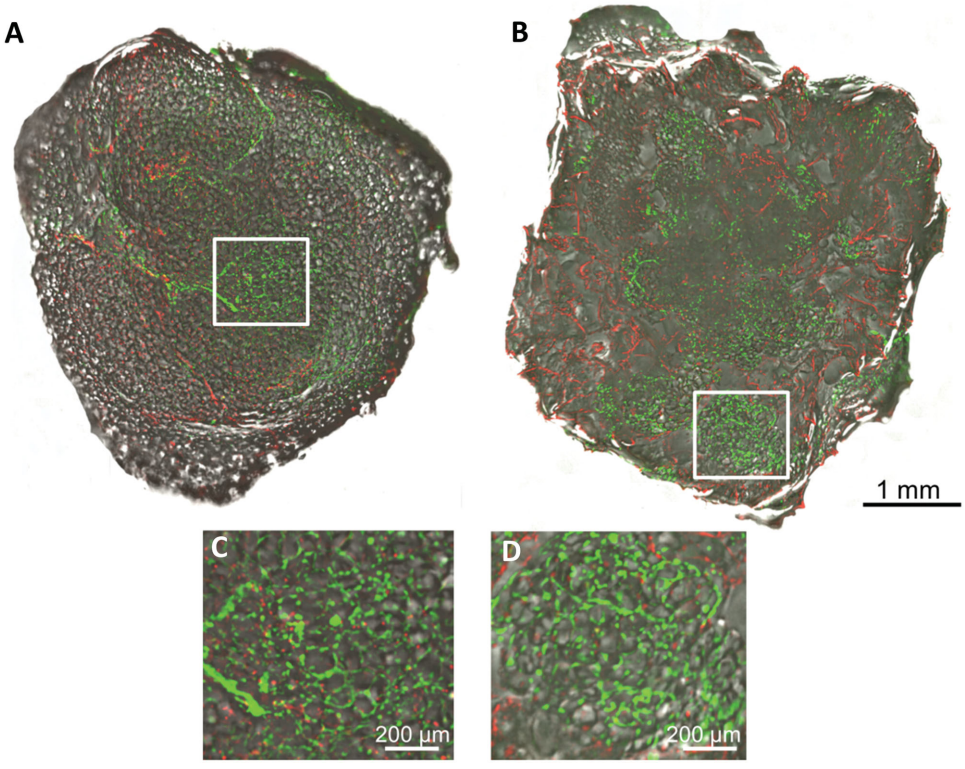

Figure 2. Distribution of cells in explant and scaffold cultures. Calcein and ethidium staining of an A,C) explant and B,D) scaffold demonstrating the distribution of live (green) and dead (red) cells, respectively, throughout the explants and scaffolds after 4 d of culture (silk is also evident in the red channel, but to a lesser degree). C,D) Magnified insets are expanded from the square white region in A,B) the lower magnification images.

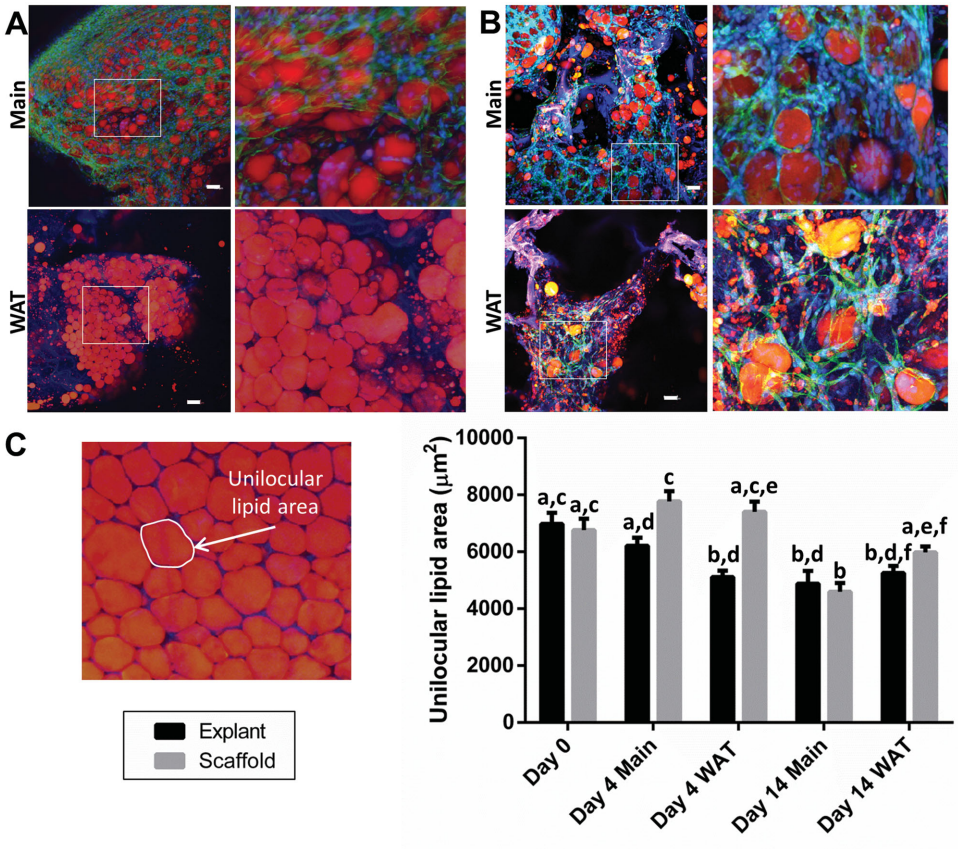

Figure 3. The unilocular morphology is maintained in explant and scaffold cultures. Adipose tissue was either A) cultured directly as explants or B) liquefied and seeded into scaffolds and stained with a lipophilic dye (AdipoRed, red = lipids), DAPI (blue = nuclei), and phalloidin (green = actin cytoskeleton) at 14 d (silk fluorescence is visible in the blue and red wavelengths and therefore is purple). Nonsupplemented media (*Main*) demonstrated greater numbers of cells in both explant and scaffold cultures while white adipose tissue stimulation media (*WAT* = *Main* + insulin, IBMX, dexamethasone and indomethacin) showed smaller lipid droplets accumulating in both culture conditions. Quantification of C) AdipoRed unilocular morphology demonstrated no significant differences in sizes of cells at 14 d of culture for explants (black) and scaffolds (grey). An example unilocular lipid area from an explant culture is shown for reference. Scale bars are 100 μm in length. Groups with different letters are significantly different (two way ANOVA significant effects of culture condition, media supplementation, and the two factors interacting, $p < 0.001$). Error bars represent standard error of the mean. Three different patient samples were stained, imaged, and measured with at least 50 cells measured for each condition; one patient is shown for clarity as each patient had different lipid sizes. See Figure S1 (Supporting Information) for positive staining for smooth muscle cells, endothelial cells, and preadipocytes.

factor 1 (PREF1),[26] alpha smooth muscle actin (αSMA),[27] and cluster of differentiation 31 (CD31),[28] respectively. It was thought that different media conditions might not support all three cell types; however, positive staining was present under all of the conditions for all three cell types (Figure S1, Supporting Information). Since culturing multiple cell types improves the accuracy of tissue engineered systems,[29] this model system will likely have improved physiological relevance over other adipose tissue engineered systems that incorporate only one[13,15] or two[15,17,23] cell types.

2.3. Proliferation and Lipid Metabolism

Adipocytes store excess energy in unilocular lipid-filled vacuoles of adipocytes.[2] Lipid metabolism includes not only storage of triglycerides, but also lipolysis, where the triglycerides are broken down into glycerol and free fatty acids to meet energy needs.[3] Therefore a hallmark of adipocyte functionality in vitro is quantification of lipid accumulation (triglyceride content) and glycerol secretion.[30] Media composition was an important factor in determining the number of cells, lipogenesis and secretion of glycerol and other proteins. The media without supplements; the *Main* group, had increased levels of DNA compared to the initial seeding values, suggesting it encouraged proliferation (**Figure 4**A, consistent with more cells seen in Figure 3). The *WAT* group on the other hand was stimulated by IBMX, indomethacin, dexamethasone, and insulin and stimulated preadipocytes to accumulate lipids, rather than proliferate. Cells cultured in this media demonstrated an increase in triglyceride accumulation (Figure 4C consistent with small lipid droplets observed in Figure 3) and glycerol secretion (Figure 4B).

2.4. Evaluation of the Adipose Secretome

White adipose tissue is actively involved in many physiologic and pathologic processes, including immunity, inflammation,

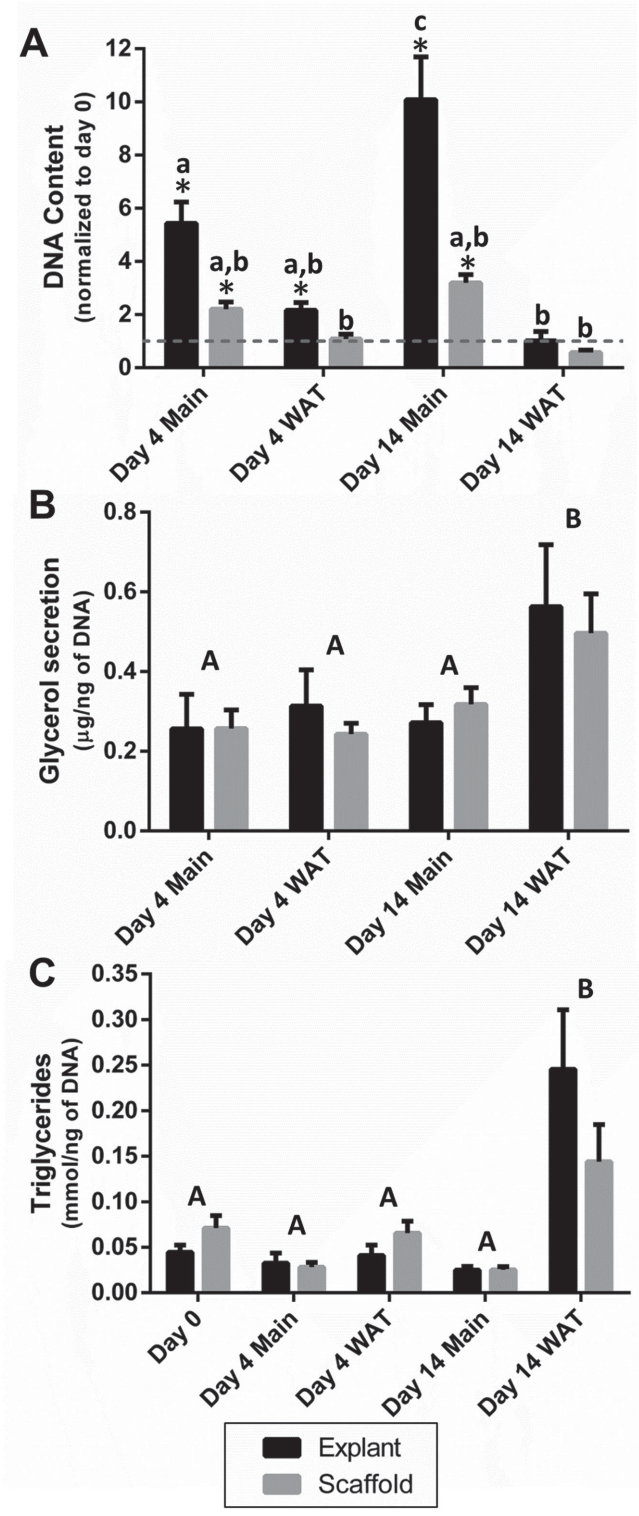

Figure 4. Media supplementation can be used to enhance triglyceride accumulation and glycerol secretion or withheld to enhance proliferation. Nonsupplemented media (*Main*) enhanced A) DNA content, while media supplementation (*WAT*) enhanced B) glycerol secretion and C) triglyceride accumulation. DNA content was also dependent on culture condition ($p < 0.001$) and was higher in explant cultures compared to scaffold cultures ($p < 0.001$, significant interaction between media and culture condition $p < 0.001$). Glycerol secretion was enhanced with media supplementation ($p = 0.002$) without any differences between explant and scaffold culture conditions ($p = 0.667$, no significant interaction between media and culture condition $p = 0.846$). Likewise, after two weeks of cultures, triglyceride content, was not significantly different between explant and scaffold cultures ($p = 0.445$, media supplementation $p < 0.001$, interaction between media and culture condition $p = 0.046$). Groups with different letters are significantly different. A two-way ANOVA was used, where lower case letters indicate significant differences between groups when there was a significant interaction between the two factors and upper case letters indicate significant differences when there was a factor effect. * indicates significant differences from day 0 (one sample *t*-test) for DNA content. Error bars represent standard error of the mean. Assays were performed with two or more patients with five samples from each patient (run in duplicate) for each of the experiments.

and communication with other tissues.[1] Since adipose tissues are endocrine organs that function by secreting cytokines,[1] an adipose specific cytokine array (for a full list of proteins and their abbreviations see Table S1, Supporting Information) was used to screen the in vitro cultures for proteins that are known to be secreted in vivo. For a comparison of scaffold secretion levels to explant secretion levels see Figure S2 (Supporting Information; data repeated from **Figure 5).** In general, explant cultures secreted decreased numbers of adipokines compared to scaffold cultures. This suggests that the scaffold-assisted cultures are a more valid model of endocrine function than explant cultures.

All of the cells seeded in scaffolds secreted important adipokines regardless of media supplementation (Figure 5A) including: leptin R, adiponectin (ACRP30), adipsin (synthesized by adipocytes and circulates in the bloodstream[31]), inflammatory interleukins; including interleukin-6 (IL-6), IL-6 receptor subunit alpha, and interleukin-8 (IL-8), as well as tissue inhibitor of metalloproteinases (TIMP) 1, interferon gamma-induced Protein (IP10, secreted by mature and differentiating adipocytes[32]), angiopoietin (ANGPT) – 2, plasminogen activator inhibitor 1 (PAI-1) and oncostatin-M (OSM). However, some proteins such as resistin and macrophage colony-stimulating factor (M-CSF) varied depending on the patient. While adiponectin and leptin are primarily secreted by adipocytes, the majority of secreted cytokines, including the inflammatory interleukins, are secreted by the other cell types found in adipose tissue.[33,34] Therefore, patient specific variations in resident percentages of different cell types[34,35] could account for differences seen in levels of secreted proteins. In fact, those patient specific differences are likely to play an important role in discovering why some obese patients develop type 2 diabetes and others do not.[36]

2.5. Stimulation with an Acute Inflammatory Mediator

An increasing amount of evidence suggests that low-grade chronic inflammation links excess adipose tissue to metabolic disorders.[37] To test out the applicability of this model to respond to an acute inflammatory stimulus the media was supplemented with tumor necrosis factor alpha (TNFα) and the secretion patterns were compared to values without supplementation (Figure 5B). When scaffold cultures in the *Main*

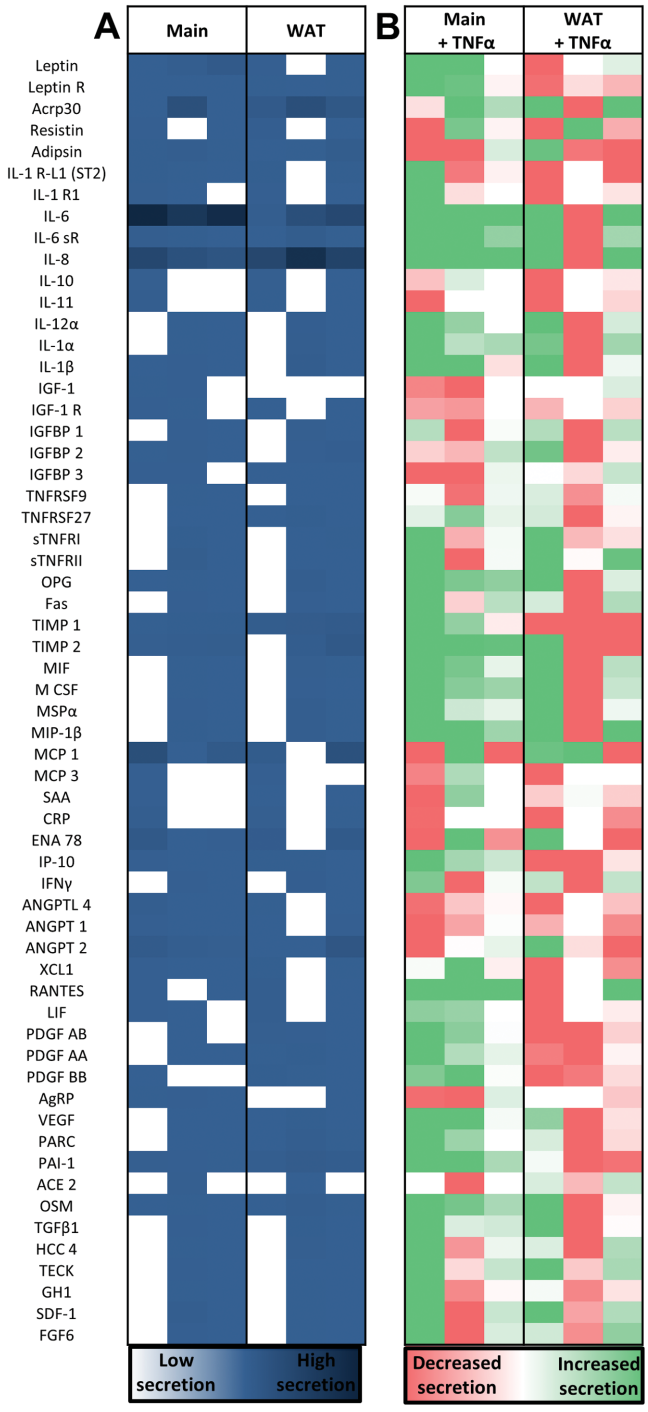

Figure 5. Cells cultured in scaffolds secreted a variety of proteins. Common adipokines were evaluated for the different culture conditions and media supplementations at 14 d of culture (for full protein names see Table S1, Supporting Information). Each column in these heat maps represents the secretion of constructs from three different patients normalized by media only values and negative control intensity values (run in duplicate and averaged). A) The left columns are secretion levels without stimulation (navy blue represents high secretion levels) while B) the right columns represent the change when stimulated with TNFα for 24 h (decreases in secretion levels are red while increases in secretion levels are green). Nonsupplemented media (*Main*) and white adipose tissue stimulation media (*WAT* = *Main* + insulin, IBMX, dexamethasone and indomethacin) showed similar trends in secretion values (A), but showed different responses to TNFα differently (**B**). See Figure S2 (Supporting Information) for a comparison to explant conditions.

group were treated with TNFα there was an increase in the secretion of proteins that are higher in insulin-resistant obese subjects[38] including: proinflammatory IL-1α, angiogenic osteoprotegerin (OPG) and TIMP2, anti-inflammatory macrophage stimulatory protein alpha (MSPα) and chemoattractant macrophage inflammatory protein 1-beta (MIP-1β). Additionally, increased regulated-on-activation-normal-T-Cell-expressed-and-secreted (RANTES), IL-6, and IL-8 levels were elevated with the acute inflammatory stimulus, which is consistent with elevated levels in type 2 diabetic patients.[39] This model also confirmed that PAI-1 production is correlated with TNFα stimulation, reinforcing the idea that there is a possible local contribution of TNFα in the regulation of PAI-1 production by human adipose tissue.[40] Likewise, TGFβ1 levels were increased after TNFα administration, consistent with findings that TGF-β1 induces PAI-1 expression.[41] Finally, there were elevated levels of OSM in the *Main* group treated with TNFα, which is a protein secreted by cells in the stromal vascular fraction and stimulates adipocytes to secrete PAI-1 and IL-6.[42] However, the large variability in responses of the three different samples reinforces the need for patient specific models of disease mechanisms.

2.6. Differences in Secreted Proteins between Media Conditions

The cytokine array demonstrated that scaffolds in the *Main* group consistently secreted a number of proteins that were not secreted in all of the samples cultured in the *WAT* group (Figure 5A). For example, all of the samples in the *Main* group secreted leptin, a hormone that is higher in plasma and serum levels of obese patients and signals the hypothalamus to decrease food intake and increase energy expenditure.[43] Interestingly, all of the samples in the *Main* group also secreted Agouti-related protein (AGRP), which is modulated by leptin,[43] and increases food intake and decreases energy expenditure.[44] Pro-inflammatory cytokines were also secreted consistently in this group and not consistently in the *WAT* group including: interleukin-1 beta (IL-1β), a cytokine that is suspected to mediate the damaging effects of macrophages on insulin signal transduction in adipocytes;[45] monocyte chemoattractant protein 1 (MCP1), a cytokine that regulates macrophage recruitment to sites of inflammation;[46] and the receptor for interleukin-1 receptor-like 1 (IL-1 R-L1, ST2), which is a cytokine mainly expressed by stromal endothelial cells and is associated with the polarization of macrophages in adipose tissues.[47] Angiogenic proteins that are upregulated in obese subjects: OPG, TIMP2 and epithelial-derived neutrophil-activating peptide 78 (ENA78);[38] and insulin-like growth factor-binding protein 2 (IGFBP 2), which is increased in obese children (and associated with increased adiposity and decreasing insulin sensitivity[48]), were also consistently upregulated in this group and not consistently in the *WAT* group.

On the other hand, stimulated cells (*WAT*) consistently secreted many proteins that weren't secreted in all of the samples cultured in nonsupplemented media (*Main*) including (Figure 5A): insulin-like growth factor-binding protein 3 (IGFBP3), pulmonary

and activation-regulated chemokine (PARC), platelet-derived growth factor (PDGF) AB, PDGF AA, PDGF BB, and Vascular endothelial growth factor (VEGF). As opposed to IGFBP-2 (which was always expressed in the *Main* group), IGFBP-3 is involved in glucose homeostasis and is decreased in obesity.[49] PARC protein levels, however, were also secreted and are upregulated in obese patients.[38] The PDGF polypeptides and VEGF belong to a family of structurally and functionally similar growth factors that function in hematopoietic development, neurogenesis, and neuroprotection.[50] The differences in protein secretion levels between media groups is likely related to different populations of cells proliferating and differentiating.

2.7. Evaluation of Long-Term Culture Capability

An important criterion for an adipose tissue model is the ability to maintain the model for extended time frames to enable studies associated with chronic disease signaling. Of significance, explants could not be sustained in long-term culture (3 months) due to their fragility. Explants broke apart at variable points during culture, with some breaking apart before the 14 d time point and others after it. Thus, the last endpoint comparing explant and scaffold cultures was chosen at 14 d (and extra explants were cut to ensure the endpoint had sufficient comparisons to scaffold cultures). Scaffold aided adipose tissues remained intact and were evaluated for DNA content and lipid metabolism at 3 months. Consistent with earlier time points, DNA content varied depending on whether the media was supplemented (**Figure 6**). Triglyceride content was maintained from day 0 to 3 months in the *Main* group and increased in the *WAT* group. Glycerol secretion was also maintained with no significant differences from initial values to values obtained at 3 months. Since DNA content, triglyceride content, and glycerol secretion were all maintained, these results indicate that lipid laden cells can be sustained for long-term culture (>3 months) in the silk scaffolds and maintain lipolytic function. In addition, scanning electron microscopy (SEM) analysis demonstrated that the pores were filled with cells and tissue at 3 months (Figure S3, Supporting Information). Long-term lipid metabolism in vitro makes this technique not only amendable to studying metabolic transitions associated with type II diabetes, but also for studying certain infectious diseases that affect adipose tissue[51] and different cancers affected by obesity;[52] including breast cancer which affects the mammary adipose tissue.[53] All of these diseases are chronic conditions that will likely require long-term culturing to manifest accurately. It also has potential as an approach for autologous soft tissue regeneration, especially where adipose tissue grafts and natural biomaterials resorb too quickly to maintain tissue regeneration.[54]

2.8. Validating the Scaffold-Assisted Method for Use with Lipoaspirate Samples

To validate that this technique could be used with small volumes of lipoaspirate samples, two lipoaspirate patient samples

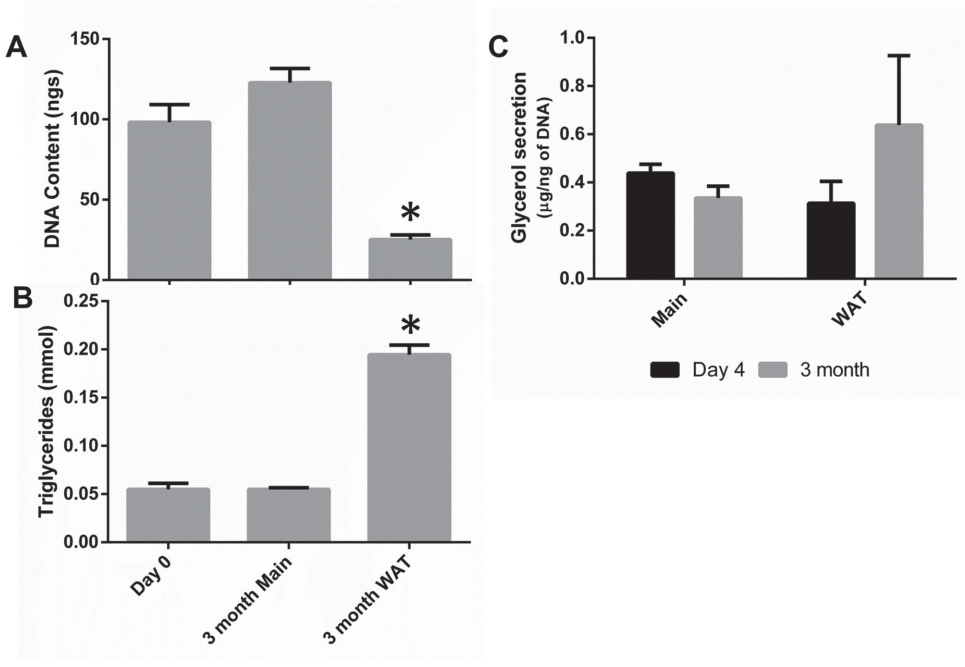

Figure 6. At 3 months glycerol secretion was maintained, while DNA content and triglyceride content depended on media supplementation. A) DNA content varied depending on whether the media was supplemented for 3 months. There was a significant decrease in DNA content in the supplemented group (*WAT*) compared to day 0 ($p < 0.001$). However, there was no significant change in DNA content between day 0 and 3 months for scaffolds cultured in nonsupplemented (*Main*) media ($p = 0.093$). B) Triglyceride content was maintained from day 0 to 3 months in the *Main* group ($p = 0.992$), and increased in the *WAT* group ($p < 0.001$). Additionally, C) glycerol secretion was maintained with no significant differences from initial values to values obtained at 3 months ($p = 0.267$), with no differences between media conditions ($p = 0.370$). * indicates significant difference from day 0 (unpaired sample *t*-test). Error bars represent standard error of the mean. Assays were performed with two patients with five samples from each patient (run in duplicate) for each of the experiments.

were obtained (Figure S4, Supporting Information) and compared to the prior results with blended adipose tissue (results repeated from Figure 4). The lipoaspirate seeded scaffolds showed no differences in the amount of DNA due to the seeding method. Additionally, both seeding methods demonstrated the same trends with media supplementation where supplementing the media (*WAT*) enhanced triglyceride accumulation per cell and glycerol secretion without affecting the DNA content, while media without supplements (*Main*) increased DNA content without affecting the lipid metabolism. Similarity in trends between the two seeding methods indicates that this technique is amendable to a patient specific approach where a small volume of lipoaspirate can be obtained from an individual patient.

2.9. Material Choice

Silk scaffolds were used in this study since they can be extended for months in culture[18] avoiding premature collapse of structures. Silk scaffolds provide biocompatibility, porous features for transport, robust mechanical properties, and retain size with slow proteolytic biodegradation, and require no chemical or photo crosslinking for stability and function.[55] Matrix molecules such as collagen provide structural support and important matrix-mediated cell signaling, however, these systems degrade too rapidly to serve the functional goals of tissue models[24] for long-term models. Crosslinking can be considered, however, cell signaling is impacted and crosslinking agents are often cytotoxic.[56] While silk was an ideal material for providing the structural integrity required to sustain the fragile and buoyant adipose tissue, other materials could be considered that have been used for long-term cultures including: poly (e-caprolactone),[57] poly (ethylene terephthalate),[58] polyglycolic acid scaffolds,[59] and poly (lactide-*co*-glycolide).[60]

3. Conclusion

This study evaluated the potential for maintaining human mature adipocytes in vitro in a nonsignaling, cell compatible, and mechanically tunable 3D silk scaffold system that could support long-term culture. Since liquefied adipose tissue was seeded directly into the scaffolds, it contained the relevant cell types (adipocytes, preadipocytes, endothelial cells, and smooth muscle cells) required to create a physiologically relevant system. Media supplementation was used to tune the lipid metabolism and proliferation of cells in the culture systems. Additionally, the scaffolds remained intact with similar numbers of cells and triglycerides after 3 months of culture, which could not be achieved in explant cultures. Most importantly, soaking silk scaffolds directly from a small volume of liquified adipose tissue makes this technique amendable to the study of patient-specific disease mechanisms and drug responses, where a small volume of lipoaspirate can be obtained from a patient.

4. Experimental Section

Materials: Bombyx mori silkworm cocoons were acquired from Tajima Shoji Co (Yokohama, Japan). All cell culture supplies were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. In addition, calcein, ethidium, phalloidin, 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), and Picogreen kits were obtained from Invitrogen. Human recombinant insulin, IBMX, dexamethasone, indomethacin, bovine serum albumin (BSA), goat serum, and TNFα were obtained from Sigma–Aldrich (St. Louis, MO). AdipoRed was purchased from Lonza (Walkersville, MD). Primary and secondary antibodies were purchased from Sigma-Aldrich and BD Biosciences (San Diego, CA) as noted. Triglyceride and Glycerol quantification kits were obtained from BioAssay Systems (Hayward, CA). The human obesity arrays were obtained from RayBiotech (Norcross, GA). Polyethylene molds (Catalog number: 03-338-1E) were acquired from Fisher Scientific (Waltham, MA).

Silk Scaffold Fabrication: To generate silk scaffolds (see Figure S3A in the Supporting Information for an SEM of a scaffold without cells), silk solution was extracted from *B. mori* silkworm cocoons as described previously.[61] The solution was lyophilized, resolubilized in a 17% w/v hexafluoro-2-propanol (HFIP) solution, and poured over 6.8 g of salt (particles were sieved to only include particles in the range of 500–600 μm therefore creating pores in that range)[62] in a polyethylene vial. The vials were sealed and left in a fume hood for 2 d to ensure even distribution of the HFIP, silk, and salt. The vials were opened for 1 d to allow the HFIP to evaporate and were then immersed in methanol overnight. To allow the methanol to evaporate the vials were removed from the methanol and left open in the hood for 1 d. Finally, the vials were immersed in water to leach out the salt particles. After multiple wash steps, the scaffolds were removed from the containers, cut to size (cylinders, 2 mm height × 4 mm diameter), and autoclaved. The scaffolds were then soaked overnight in media (DMEM/F12, 10% fetal bovine serum, 1X Antibiotic-Antimycotic) prior to cell seeding.

Culturing of Explants and Adipose-Soaked Scaffolds: On the same day of surgery, subcutaneous adipose tissue was acquired from elective abdominoplasty procedures. Five different patient samples were obtained for comparisons between explants and scaffolds (Table S2, Supporting Information). Blunt dissection was used to separate the adipose tissue from the skin and the fascia of Scarpa. Explants were cut to the same size as the scaffolds (2 mm height × 4 mm diameter) and cultured in the same media. The remaining adipose tissue was liquefied in a blender by successive short pulses until the tissue had the viscosity of lipoaspirate. Media was then aspirated from the soaking scaffolds and the scaffolds were added directly to the liquefied adipose tissue in 50 mL falcon tubes. The tubes were placed in an incubator for 1 h (37 °C, 5% CO_2). The scaffolds were then separated from the excess tissue and placed into 24 well plates for 2 h (37 °C, 5% CO_2) without media to allow the cells to attach to the scaffold. Maintenance media or white adipose tissue (WAT) stimulation media (DMEM/F12, 10% fetal bovine serum, 1X antibiotic-antimycotic, 1×10^{-6} M insulin, 0.5×10^{-3} M IBMX, 1×10^{-6} M dexamethasone, 0.05×10^{-3} M indomethacin) was then added and changed twice a week. Scaffolds and explants were cultured in different media conditions (Figure 1) referred throughout as *Main* (maintenance media) or *WAT* (white adipose tissue stimulation media). Endpoint analyses were performed at day 0, day 4, day 14, and in some cases at 3 months. In an additional set of experiments, scaffolds were soaked in lipoaspirate (Table S2 for patient info, Supporting Information) to compare lipoaspirate samples to blended abdominoplasty samples.

Immunostaining: To visualize the distribution of live and dead cells in the explants and scaffolds, brightfield and fluorescent images were taken on a macroscope (Olympus MVX10) after 4 d of culture. The constructs were stained for 30 min at room temperature with 2×10^{-6} M calcein AM and 4×10^{-6} M ethidium homodimer-1 prior to imaging.

Cellular morphology in the explants and scaffolds was evaluated in samples that were fixed with 10% neutral buffered formalin for 30 min. Samples were washed twice with phosphate buffered saline (PBS) containing 0.2% Triton-X100 and stained for 1 h with AdipoRed (1:35), DAPI (1:1000), and Alexa flour 488 phalloidin (1:40). Following the staining process, the samples were rinsed with PBS twice. Samples were imaged with either an inverted Leica DMIRE2 confocal microscope or a Leica SP5X Laser Scanning Confocal Microscope (at the DFCI

Microscopy Core) using Leica LAS acquisition software. Scaffolds were imaged with 10× dry or 20× water-immersion objectives using 488 nm Argon, 405 nm UV diode, or white light lasers (470–670 nm). PMTs (photomultiplier tubes) collected fluorescence signal from DAPI (405 nm/420–440 nm), Phalloidin (488 nm/500–520 nm), AdipoRed (488/564–616 nm), and scaffolds (which are visible in the DAPI and AdipoRed PMTs). Pseudocolors were assigned to each: DAPI/Blue, Phalloidin/Green, AdipoRed/Red, and Scaffold which appeared as violet/purple. Z-stack images were acquired and processed using LeicaLite or LeicaLAS software to create single maximum projection 3D-like images. The final images were analyzed with ImageJ (NIH) to determine unilocular lipid size (three different patient samples were used and at least 50 cells measured for each condition, 1 patient is shown in Figure 3 for clarity as each patient had different lipid sizes, likely related to patient specific differences in BMI, age, and other factors).[63]

Prior to staining for cell type markers, samples were fixed with 10% neutral buffered formalin for 30 min at room temperature and blocked in a buffer for an hour (1% goat serum, 0.2% BSA, and 0.2% Triton-X100 in PBS). The primary antibodies, human cluster of differentiation 31 (CD31, BD 555444, 1:50) produced in mouse, human α-smooth muscle actin (αSMA, Sigma A2547, 1:400) produced in mouse, and human preadipocyte factor 1 (PREF1, Sigma HPA053879, 4 μg mL^{-1}) antibody produced in rabbit, were then applied to the samples in the buffer solution for 1 h, followed by three 10 min washes with the buffer. The secondary antibodies, goat anti-mouse (BD555988, 1:200) and goat anti-rabbit (Sigma F0382, 1:80), were diluted in the buffer and counterstained with DAPI (1:1000) for 1 h, followed by three 10 min washes with the buffer. All staining steps were performed at room temperature. Three different patient samples were imaged for each group, with separate samples used for each marker. The samples were imaged with the macroscope (Olympus MVX10) to determine overall distribution of cell types in the explants and scaffolds.

DNA and Triglyceride Content: Scaffolds and explants were lysed in a Tris-EDTA buffer (10×10^{-3} M Tris-HCl, 1×10^{-3} M EDTA, pH 7.5), shredded using microdissection scissors and stored at −20 °C until the assays were performed. After thawing, the samples were immediately assayed according to the manufacturer's protocol for DNA content and triglyceride content with the Picogreen assay and the EnzyChrom Triglyceride Assay Kit, respectively. Assays were performed with two or more patients with five samples from each patient (run in duplicate) for each of the experiments. Triglyceride content was normalized by DNA content to account for potential variations in cell numbers.

Protein Secretion: To quantify secretion of different proteins, media samples with or without stimulation by 10 ng mL^{-1} TNFα for 24 h were stored at −20 °C until the assays were performed. After thawing, the samples were immediately assayed according to the manufacturer's protocol with either the EnzyChrom Adipolysis Assay Kit or the Human Obesity Array C1. The glycerol assay was performed on two or more patients with five samples from each patient (run in duplicate) for each of the experiments and normalized by DNA content.

Human sandwich-based obesity arrays containing 60 different target proteins (for a full list of proteins and abbreviations see Table S1, Supporting Information) were run according to the manufacturer's protocol. Briefly, the antibody coated arrays were blocked for 30 min, and incubated with 1 mL of the supernatant samples overnight at 4 °C on a shaker. Then, the arrays were washed and incubated with 1 mL of biotin-conjugated antibodies overnight at 4 °C on a shaker. Next, the arrays were washed and incubated with streptavidin-conjugated horseradish peroxidase for 2 h. Finally, the arrays were washed and the chemiluminescence signal was detected with a Syngene G Box imager. Data were extracted using ImageJ software where images were inverted and analyzed with the built in analysis for gels. Lanes were plotted and averages of peaks were determined for each dot. A media control was subtracted from each array value to account for proteins in the media. Additionally, background was subtracted based on negative control intensity values for each array. On each array the proteins were run in duplicate and were averaged. For comparison between patients, media samples from three patient samples were run for each group at the 14 d time point. Technical replicates within the same patient were all done at the same time from the same tissue sample. Glycerol secretion was normalized by DNA content.

Scanning Electron Microscopy: Silk scaffolds with and without cells were cross-linked with a 2.5% glutaraldehyde solution and progressively dehydrated in a graded series of ethanols (30%, 50%, 75%, 95% and twice in 100%, 30 min at each concentration). The samples were critical point dried with a liquid CO_2 dryer (AutoSamdri-815, Tousimis Research Corporation, Rockville, MD). Prior to imaging with a scanning electron microscope (Zeiss UltraPlus SEM or Zeiss Supra 55 VP SEM, Carl Zeiss SMT Incorporation, Peabody, MA), the samples were coated with a thin layer (10 nm thick) of Pt/Pd at a voltage of 2–3 kV using a sputter coater (208HR, Cressington Scientific Instruments Incorporation, Cranberry Township, PA).

Statistics: Statistics were performed with GraphPad Prism software (GraphPad, CA, USA). A two way Analysis of Variance (ANOVA) was performed for unilocular lipid size, DNA content, triglyceride content and glycerol secretion, where the factors were culture condition (explant vs scaffold) and media supplementation at different time points (Day 0, Day 4 Main, Day 4 WAT, Day 14 Main, Day 14 WAT). To test significant differences between day 0 and normalized DNA content, a one sample t test for each group was performed with a theoretical mean of 1. Significant differences from seeding values in DNA content, and triglyceride accumulation at 3 months were determined with an unpaired sample t test. Differences in glycerol secretion from initial values and 3 month values were determined with a two way ANOVA. To determine if there were significant differences between lipoaspirate soaked scaffolds and the scaffolds soaked with liquefied adipose tissue a two way ANOVA was used.

When there was a significant effect of a factor in the two way ANOVA tests, a Tukey post-hoc test was performed between the different groups. When there was a significant interaction between the two factors in the two-way ANOVA all of the groups were compared individually with a Tukey post-hoc test. Significant differences were always defined as $p < 0.05$.

Study Approval: Human adipose tissue samples were obtained with institutional review board approval (Protocol #0906007). Informed signed consent was obtained from either the patient or from the next of kin.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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