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Spheroid Culture System Confers Differentiated Transcriptome Profile and Functional Advantage to 3T3-L1 Adipocytes

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Abstract—This study highlights functional differences between 2-D monolayer and 3-D spheroid 3T3-L1 adipocyte culture models and explores the underlying genomic mechanisms responsible for the different phenotypes present. The spheroids showed higher triglyceride accumulation than the monolayer culture and further increase with larger spheroid size. Whole transcriptome analysis indicated significant differential expression of genes related to adipogenesis, including adipocytokine signaling, fatty acid metabolism, and PPAR-γ signaling. Spheroids also showed downregulation of matrix metalloproteinases (MMPs), integrin, actin-cytoskeleton associated genes, and Rho/GTPase3 expression relative to 2-D monolayer, indicating suppression of the Rho-ROCK pathway and thereby promoting adipogenic differentiation. When exposed to linoleic acid (500 μM) and TNF-α (125 ng/mL) to promote chronic adiposity, linoleic acid treatment resulted in increased intracellular triglycerides and subsequent TNF-α treatment resulted in significantly altered adipocytokine signaling, fatty acid metabolism, and PPAR signaling, in addition to upregulation of multiple MMPs in spheroids vs. monolayer. Overall, 3-D spheroids showed enhanced adipogenic phenotype as indicated by triglyceride synthesis and transcriptome changes while retaining sensitivity to a pro-inflammatory stimulus. The 3-D spheroid culture thus may provide a simple, convenient, and sensitive *in vitro* model to study adipocyte response to metabolic stresses relevant to clinical pathologies.

Keywords—Gene expression, Cell culture, Matrix metalloproteinase (MMP), Triglyceride, Adipocytes.

INTRODUCTION

Poor diet, sedentary lifestyle, and genetic susceptibility make obesity the second leading cause of preventable death after smoking. The common mode of treatment for obesity involves low-fat diet and exercise.[25] It is known that excess dietary fat contents present during obesity alter the intracellular triglyceride accumulation as well as the levels of expression of transcription factors and genes involved in adipocytic differentiation.[13,26] Elevated concentrations of inflammatory cytokines in adipose tissue present during obesity, for example, tumor necrosis factor-α (TNF-α), have been associated with type II diabetes and correlated with insulin desensitization and inhibition of metabolic functions including carbohydrate metabolism, lipogenesis, adipogenesis, and thermogenesis, as well as lipolysis (triglyceride breakdown) and impaired endocrine response (leptin feedback). Chronic obesity has been thought to result in such ongoing inflammatory state in adipose tissue due to limited cell expansion, apoptosis of adipocytes due to physical and metabolic stresses, and monocyte recruitment and activation.[4] It follows that optimal treatments for obesity would include intervention at the cellular level, but the complex interactions of the various organs with the adipose tissue occurring *in vivo* makes the exact determination of the effects of dietary fatty acid types and dosages as well as cytokines on cellular metabolic outcomes challenging.

In vitro models that capture *in vivo* cellular morphology and functionality offer a relatively straightforward way to understand such effects. Therefore, directing and controlling such effects *in vitro* has been a burgeoning topic of research.[21,22,31] For instance, ω-3 fatty acids such as eicosapentaenoic acid (20:5 *n*-3) and docosahexaenoic acid (22:6 *n*-3) have been associated with the expression of lipogenic (e.g., fatty acid synthase), lipolytic (e.g., hormone-sensitive lipase), and glyceroneogenic (e.g., phosphoenolpyruvate car-

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boxykinase) enzymes, activation of transcription factors (e.g., CCAAT-enhancer-binding protein α), and expression of genes (e.g., leptin), which all have been correlated with decreased fat cell size.[11] ω-3 fatty acids have also been shown to alleviate inflammatory response in a murine model.[1] In contrast, the dietary intake of ω-6 polyunsaturated fatty acids such as arachidonic or linoleic acid (LA) may directly influence the intensity and duration of inflammatory processes by contributing to the formation of pro-inflammatory cytokines and eicosanoids.[3] To mimic the pathological conditions occurring in progressing obesity, the overall goals of this project are to develop an *in vitro* 3-dimensional (3-D) model of adipose tissue by subjecting preadipocytes to elevated doses of fatty acids to induce triglyceride accumulation and to investigate the responses of such model to a subsequent inflammatory cytokine (TNF-α) exposure.

Recently, we engineered a conjugate of elastin-like polypeptide (ELP) and polyethyleneimine (PEI; a charged polyelectrolyte) and used it as a substrate for inducing 3-D spheroid organization of cultured adipose cells.[29] Once cultured, adipocytes respond to metabolic stresses and adverse conditions relevant to clinical pathologies—more specifically, elevated extracellular LA and subsequent exposure to TNF-α induce oxidative stress, promote inflammation and apoptosis. Previously we have demonstrated: (1) enhanced adipogenic differentiation in 3-D spheroids compared to the traditional two-dimensional (2-D) monolayer cultures based on triglyceride accumulation, CD-36 and CD-40 protein expression, and peroxisome proliferator-activated receptor-γ (PPAR-γ) and adiponectin mRNA expression; and (2) LA-fed cultures have reduced metabolic function and enhanced lipolysis in response to TNF-α.[29] In the current study, we present an in-depth functional (including adipogenic differentiation, viability, and lipolysis) and genomic analysis (whole transcriptome analysis) of the 3-D spheroid culture compared to the 2-D monolayer culture of 3T3-L1 preadipocytes as they are differentiated using adipogenic cocktail, matured in presence of LA, and subsequently exposed to TNF-α. Additionally, expression of key adipogenic genes in 2-D monolayer and 3-D spheroid cultures were compared to the adipose tissue isolated from mouse perirenal fat deposit to identify which culture method more closely resembled the isolated adipose. We also present transcriptome level evidence that the 3-D spheroids, with their reduced cell-substrate interactions, suppress Rho-ROCK signaling pathway to enhance PPAR-γ expression and achieve enhanced adipogenesis.

EXPERIMENTAL

Preparation of ELP-PEI Coated Surface

ELP with structure $(VPGVG)_{40}$ was produced using *E. coli* BLR(DE3) (Novagen (EMD), Madison, WI), purified by inverse phase transition, and chemically conjugated to PEI (M_w = 800 Da, Sigma Aldrich, St. Louis, USA) using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride and *N*-Hydroxysuccinimide (Sigma Aldrich). The conjugation ratio after the amide coupling reaction, that is, the proportion of ELP molecules bound to PEI molecules, was assessed quantitatively using Fluoraldehyde™ o-phthalaldehyde assay (OPA, ThermoFisher Scientific, Pittsburgh, PA). 5 mg/mL solution in deionized water of 5 mol% ELP-PEI conjugate and 95 mol% ELP was adsorbed onto 24-well tissue culture polystyrene (TCPS) plate by placing 200-μL solution in each well. The plates were incubated at 37 °C for 48 h in a dry incubator. The detailed procedures have been described elsewhere.[29]

3T3-L1 Cell Culture

Equal number of 3T3-L1 mouse preadipocytes (passage 7–13, American Type Culture Collection, Manassas, VA) were seeded per well of an uncoated TCPS plate or a TCPS plate coated with ELP-PEI (26,000 cells/cm^2). Cells were cultured for 3 days in high glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% calf serum and 100 U/mL penicillin-100 μg/μL streptomycin at 5% CO_2 and 37 °C. In this period, cells formed a confluent 2-D monolayer on uncoated TCPS or formed 3-D spheroids on TCPS coated with ELP-PEI. Subsequently, cells were differentiated for 3 days using DMEM supplemented with 10% fetal bovine serum (FBS), 1 μM dexamethasone, 0.5 mM IBMX, and 0.1 U/mL insulin.

Induction of Adiposity

Control maintenance medium was prepared by supplementing DMEM with 10% FBS and 2% bovine serum albumin (BSA). Experimental maintenance medium was control maintenance medium plus 0.5 mM exogenous LA. The entire media were sonicated at 40 °C for 45 min. After cooling to 4 °C, the media were supplemented with 0.2 U/mL insulin and 100 U/mL penicillin—100 μg/mL streptomycin. Fatty acid loading efficacy was verified by gas chromatography analysis as described elsewhere.[29] After the differentiation period, cells were fed control or

experimental maintenance medium for up to 5 days. Half of the media volume was changed every 48 h. Cells were subsequently exposed to medium containing 125 ng/mL TNF-α for 24 h.

Biochemical Characterization of Cell Lysate

At several time points over the culture period, 3T3-L1 cells were rinsed with PBS and removed from the culture surface *via* trypsinization. Cell aliquots were centrifuged for 2 min at 1000 rpm, resuspended in PBS, and sonicated for 30 s at 10% amplitude using a Branson Digital Sonifier 450 (Danbury, CT). Total intracellular protein (ThermoFisher), intracellular triglyceride (Sigma Aldrich), and extracellular glycerol (Sigma Aldrich) assays were performed per manufacturers' protocols. The influence of LA and TNF-α treatment on cell viability/metabolism was quantified using MTT assay (Molecular Probes, Eugene, OR) as described elsewhere.[29] Fatty acid uptake upon LA supplementation and fatty acid released into the media after TNF-α treatment were measured by gas chromatography analysis as described elsewhere.[29] All biochemical characterization measurements were taken in triplicate from each of 3 wells, yielding a total of 9 measurements per condition ($n = 9$).

Measurement of Spheroid Diameters

Olympus IX81 optical microscope (Olympus, Center Valley, PA) with a total ×100 magnification was used to capture images at 3 locations per well on 3 replicate wells per condition (total $n = 9$ images per condition) using Slidebook image acquisition and analysis software (Intelligent Imaging, Denver, CO). Spheroid dimensions were measured with ImageJ digital analysis software on a minimum of 50 spheroids ($n > 50$).

Differential Gene Expression of 3T3-L1 Cultures

2-D monolayer and 3-D spheroid cultures were analyzed for differential gene expression after 5 days in control or experimental maintenance media and after a subsequent 24-h treatment with 125 ng/mL TNF-α. First, intracellular RNA was isolated by column affinity using an RNeasy spin-column kit (Qiagen) following manufacturer's protocol. Extracted RNA was suspended in RNase-free deionized water and the isolated RNA ($n = 3$–5 per group/treatment) were evaluated for quality and integrity (Bio-Rad Experion). RNA samples were processed per manufactures directions for specific application [GeneChip® 2.0 ST] using Affymetrix equipment (Scanner 3000 7G System). Hybridized chips were automatically washed, stained, and scanned at the UMMC Institutional Molecular and Genomics Core using Affymetrix equipment as done previously.[30] Data obtained from these gene expression studies are deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) with the GEO accession number GSE00000 (*pending*).

Additionally, the 2-D monolayer and 3-D spheroid cultures were compared to the adipose tissue isolated from perirenal adipose tissue (PRAT) from C567BL/6J mice fed a 45% high fat diet (Diet #D12451, Research Diets, New Brunswick NJ) for 16 weeks as described previously.[14]

Statistical Analysis of Biochemical Assays

All experiments were performed at least in triplicate. Quantitative results reported as mean ± 95% confidence intervals. Assays were measured from three separate wells for a total of nine measurements. Statistical evaluation of the results was performed with ANOVA followed by Games-Howell *posthoc* test for unequal variance. Values with $p \leq 0.05$ were deemed significantly different.

Statistical Analysis and Heat Map Representation of Microarray Data

Microarray analysis was performed using commercially available GeneSifter™ software platform. In brief, Affymetrix imaging files for each array (CEL) were normalized using Robust Multi-array Averaging (RMA) algorithm and differentially expressed genes were evaluated using Benjamani and Hochberg false discovery rate (FDR) method, which corrects for multiple comparisons, with $p \leq 0.05$ and fold-change ≥ 1.2. An unbiased analysis of gene networks/pathways was evaluated using Ingenuity Pathways Analysis. For comparisons within multiple groups, statistical significance ($p < 0.05$) was determined by two-way ANOVA for interaction between the two experimental variables (Table 1). Fold changes were calculated as $2^{\Delta C_T}$, where ΔC_T is the relative change in gene expression. GeneSifter™ software uses the ΔC_T data to create the heat maps.

RESULTS

Comparison of 2-D and 3-D Cultures

Figures 1a and 1b show 3T3-L1 cells cultured as 2-D monolayer and 3-D spheroids, with cells in 2-D monolayer showing a polygonal morphology and cells in 3-D spheroids showing spherical clumps. Total protein content of 3T3-L1 cells grown in monolayer

TABLE 1. Number of statistically significant differentially expressed genes in whole transcriptome comparisons for the selected conditions.

	Selected Conditions for Whole Transcriptome Comparisons									
Models	2-D & 3-D	2-D & 3-D			2-D & 3-D			2-D		3-D	
			LA			TNF-α		LA	TNF-α	LA	TNF-α
	2-D Vs. 3-D	2-D	–	+	2-D	–	+	–	–	–	–
								–	+	–	+
		3-D	–	+	3-D	–	+	+	–	+	–
Pathway								+	+	+	+
Adipocytokine signaling pathway	14	13	**(1)**		43	**(15)**		22	**(2)**	44	**(4)**
Fatty acid metabolism	15	9	**(1)**		29	**(5)**		16	**(0)**	28	**(3)**
PPAR signaling pathway	29	22	**(0)**		49	**(9)**		19	**(1)**	44	**(7)**
ECM-receptor interactions	27	21	**(1)**		47	**(14)**		26	**(0)**	38	**(3)**
Insulin signaling pathway	28	34	**(1)**		91	**(26)**		37	**(1)**	79	**(9)**
JAK-STAT signaling pathway	29	32	**(6)**		81	**(33)**		55	**(3)**	75	**(9)**
MAPK signaling pathway	56	63	**(5)**		138	**(39)**		79	**(5)**	135	**(15)**
Glycolysis/Gluconeogenesis	13	10	**(0)**		31	**(4)**		15	**(0)**	31	**(1)**
Linoleic acid metabolism	4	3	**(0)**		10	**(3)**		8	**(0)**	13	**(1)**
Arachidonic acid metabolism	13	14	**(2)**		29	**(11)**		22	**(1)**	28	**(4)**
Corresponding Figure	**2**	**5**			**7**			**8a**		**8b**	

Bracketed numbers in bold indicate the number of statistically significant differentially expressed genes due to interaction between selected variables (highlighted in red) and show that the 3-D spheroid model is more sensitive in responses to LA and TNF-α treatments compared to the 2-D monolayer culture. Statistical significance was determined by Benjamani and Hochberg false discovery rate (FDR) method with $p \leq 0.05$ and fold-change ≥ 1.2. For comparisons within multiple groups, statistical significance ($p < 0.05$) was determined by two-way ANOVA for interaction between the two experimental variables.

initially increased 5–6 fold before reaching a stable level within 72 h of plating during the pre-adipocyte maintenance period (Fig. 1c, diamonds and triangles). During the same period 3-D spheroid culture was found to maintain the initial (seeding) protein content (Fig. 1c, squares), approximately one-sixth that of the confluent 2-D cultures, while acclimating and organizing into spheroids. This initial increase in protein content in 2-D monolayer has been attributed to the cell proliferation before reaching a confluent state leading to contact inhibited growth phase. In case of the 3-D spheroid culture this contact inhibited growth phase commences soon after seeding and leads to a significantly less cell proliferation (and hence, significantly less protein content).[28,29] The level of total protein content achieved by the end of the acclimation period remained constant over the differentiation and adipocyte maintenance periods in the absence of differentiation factors (Fig. 1c, triangles). However, when treated with differentiation media, protein content of cells grown in monolayer significantly increased during the differentiation period (780 ± 40 μg/mL on day 6 vs. 452 ± 28 μg/mL on day 3, $p < 0.05$), but remained constant thereafter during the adipocyte maintenance period (Fig. 1c, diamonds). Similar to 2-D monolayer culture, the 3-D spheroid culture nearly doubled in protein content following differentiation period (107 ± 10 μg/mL on day 6 vs. 55 ± 6 μg/mL on day 3, $p < 0.05$). However, the differentiated 3-D spheroid culture continued to show increased protein content thereafter during the adipocyte maintenance period (Fig. 1c, squares). Total intracellular protein was used to normalize other functional assays (triglyceride accumulation, fatty acid consumption and release) to changes in cell population.

Intracellular triglyceride data (Fig. 1d) collected from the same cultures as total protein data (Fig. 1c) indicated minimal spontaneous triglyceride accumulation by preadipocytes prior to differentiation. However, following differentiation treatment, both 2-D and 3-D cultures showed steady increase in intracellular triglyceride accumulation, with 3-D spheroid culture outpacing 2-D monolayer (~ 2-fold greater). As these control cultures were provided with no additional fatty acid, the primary mechanism of triglyceride accumulation is presumed to be due to *de novo* lipogenesis. 3T3-L1 cells grown on ELP-PEI surfaces rapidly organize into spheroids whose dimensions were dictated by the cell's differentiated state. When plotted against spheroid size, triglyceride data (Fig. 1e) showed a positive correlation between increased intracellular triglyceride accumulation and larger spheroid size, with the largest average triglyceride (~ 0.25 μg/μg protein) occurring in an average stable spheroid diameter of ~ 125 μm.

Whole transcriptome analysis of RNA between 2-D monolayer and 3-D spheroid cultures (Fig. 2a) demonstrated differential expression of genes related to adipogenesis, including 14 genes associated with adipocytokine signaling, 15 genes with fatty acid me-

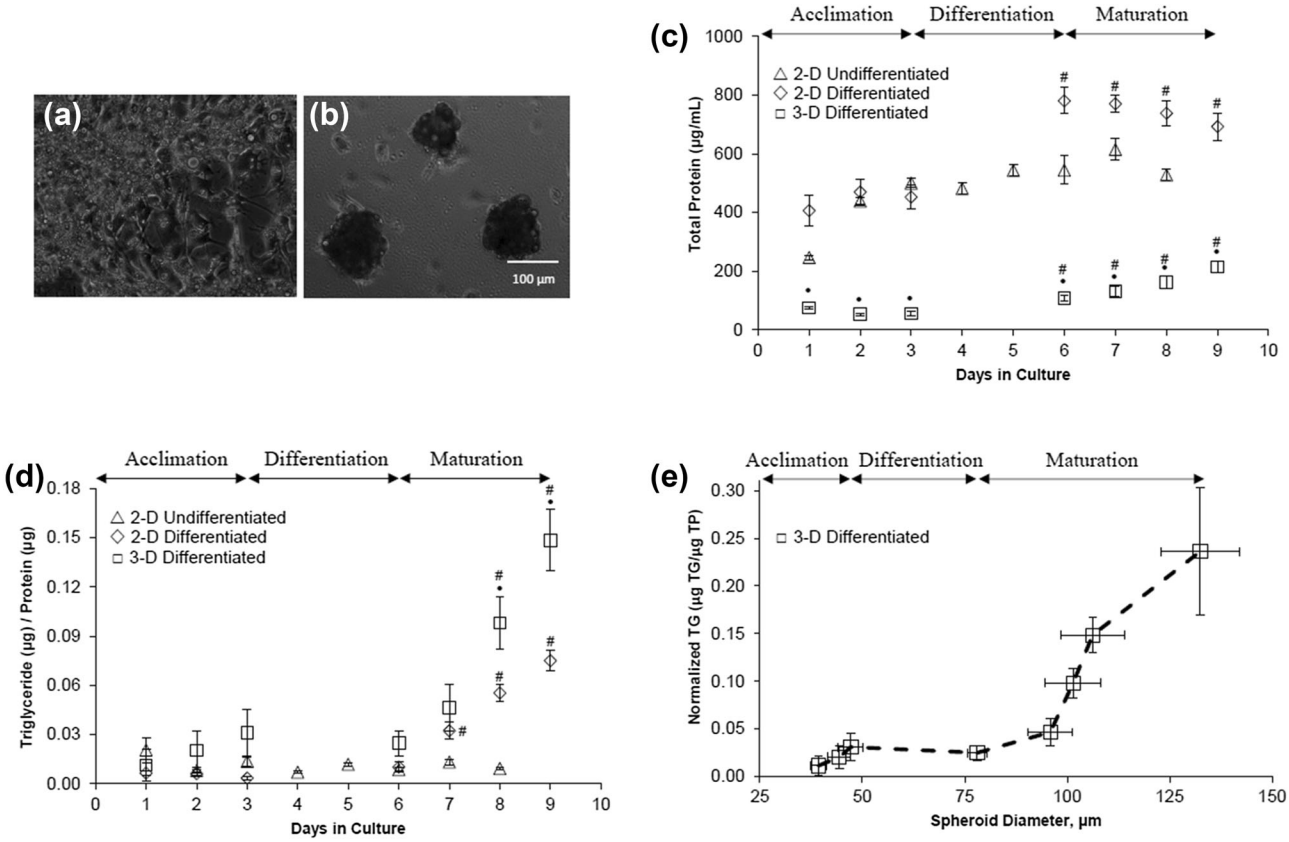
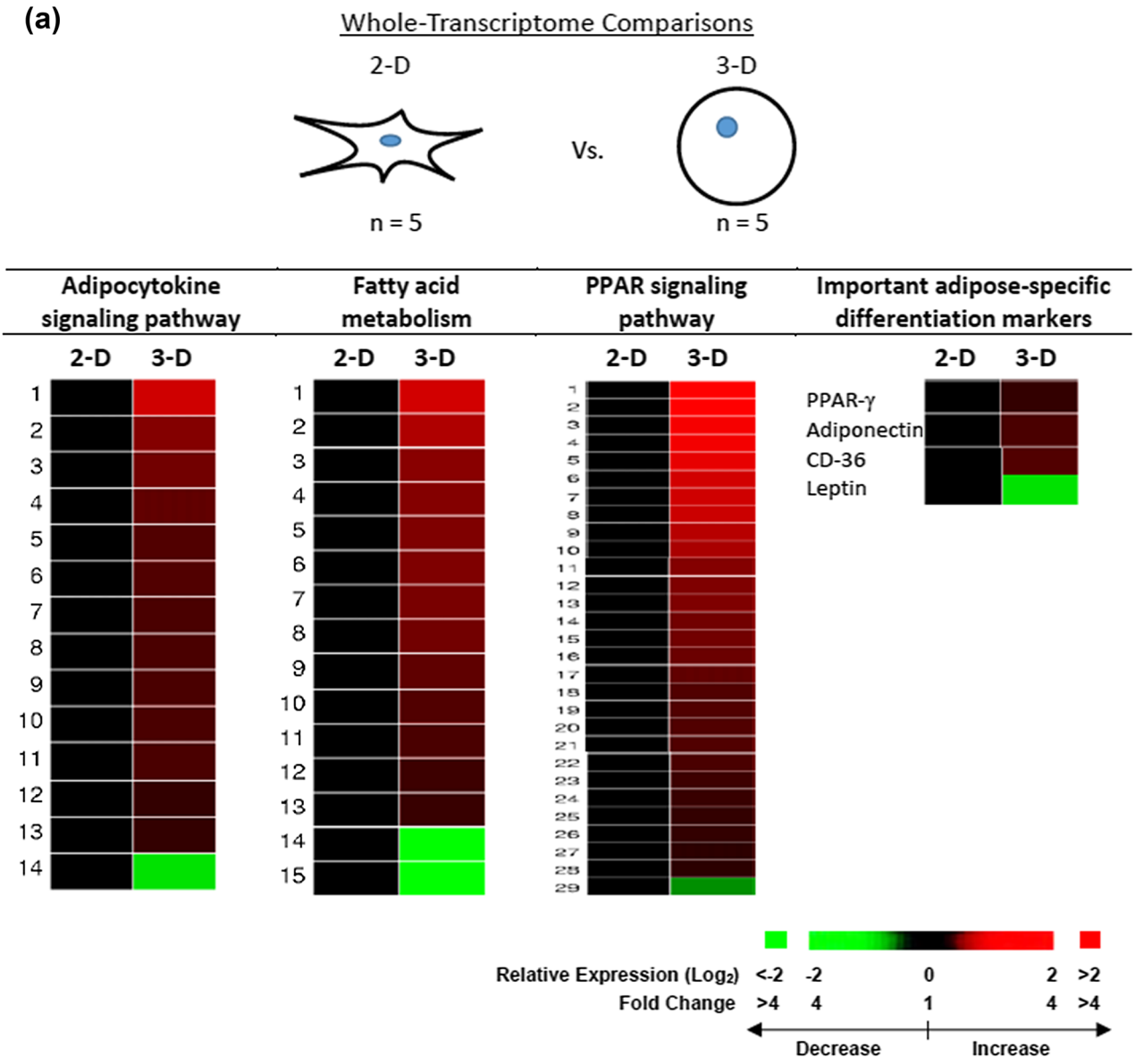
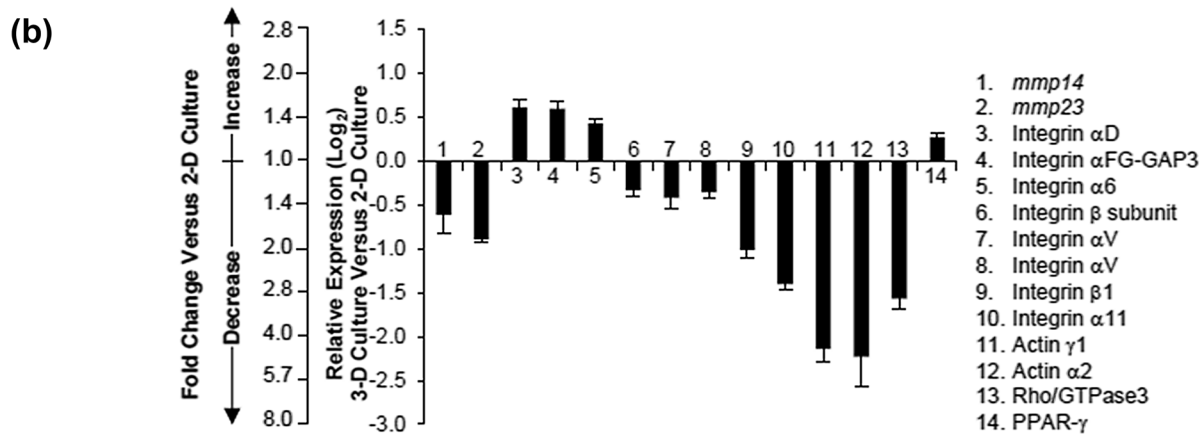


FIGURE 1. Optical microscopy images of 3T3-L1 cells cultured as (a) 2-D monolayer and (b) 3-D spheroids. (c) Total Protein content is shown for 3T3-L1 cultures grown in 2- and 3-D over 3 days of acclimation (Day 0–3), 3 days of differentiation (Day 3–6), and 3 days of maturation (Day 6–9). Stable protein concentrations indicated that confluence in 2-D cultures (open diamond and open triangle) as well as immediate cessation of mitosis in 3-D cultures (open square) was achieved within the acclimation period. Following differentiation, protein concentrations in both 2-D (open diamond) and 3-D (open square) cultures approximately doubled, though 2-D cultures remained steady thereafter whereas 3-D cultures continued to increase. The 2-D culture that was not differentiated (open triangle) did not show this increase in protein concentrations. (d) Normalized triglyceride content is shown for 3T3-L1 adipocytes in differentiated 2-D culture (open diamond), undifferentiated 2-D culture (open triangle), and differentiated 3-D culture (open square). Triglyceride content is negligible during acclimation period, but increases in both 2- and 3-D cultures following treatment with differentiation factors. 3-D culture outpaces 2-D culture in triglyceride accumulation. (e) Triglyceride accumulation is correlated with increased spheroid size. Triglyceride content in 3T3-L1 adipocytes is plotted against spheroid diameter. $^{\bullet}p \leq 0.05$ vs. equivalent 2-D culture; $^{\#}p \leq 0.05$ vs. equivalent day 3 culture. Error bars indicate 95% confidence intervals.

tabolism, 29 genes with PPAR-γ signaling, and 26 genes with extracellular matrix (ECM) interaction (Table 1), with a notable exception of leptin, which was expressed 1.8-fold higher in 2-D monolayer culture. Key adipogenic genes for glucose transporter 4 (Glut4; 1.3-fold), fatty acid transporters (FATs; 1.3–1.7-fold), fatty acid binding proteins (FABP; 1.3–1.6-fold), perilipin (1.5-fold), PPAR-α (1.2-fold), and PPAR-γ (1.2-fold) were all significantly ($p < 0.05$) upregulated in 3-D spheroid culture relative to 2-D monolayer (Fig. 2a). CD-36 gene, responsible for the expression of the surface protein associated with fatty acid uptake, was also upregulated 1.3-fold ($p < 0.05$) in spheroids (Fig. 2a). Our previous studies have noted significantly increased surface protein expression of CD-36 in spheroid cultures relative to 2-D analogues.[29] As shown in Fig. 2b, 3-D spheroid morphology clearly resulted in minimum 1.2-fold upregulation of adipogenic genes and minimum 1.2-fold downregulation of MMP, integrin, and actin-cytoskeleton associated genes as well as Rho/GTPase3 expression relative to cells in 2-D monolayer.

Next, we compared the 2-D monolayer and 3-D spheroid cultures to the adipose tissue isolated from mouse perirenal fat deposit (PRAT). A total of 609 and 672 genes (fold-change > 8; $p < 0.01$) were observed to be differentially expressed between 2-D monolayer and PRAT, and 3-D spheroids and PRAT, respectively. Differential expression of such a large number of genes between the *in vitro* cultures and the PRAT is likely due to the presence of other cell types (e.g., endothelial cells, macrophages) in the PRAT that may confound the observed gene expression. Nevertheless, a large number of genes ($n = 522$) were





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◀**FIGURE 2. (a) Whole transcriptome comparison of 3-D spheroid model vs. 2-D monolayer shows upregulation of several important signaling pathways involved in adipogenesis, fatty acid metabolism, and adipocyte function. n indicates number of replicates tested. All differentially expressed genes in the heat maps show statistically significant differences with $p \leq 0.05$ and fold-change ≥ 1.2 for the 3-D spheroid culture compared to the 2-D monolayer culture. *Key genes referenced in text: Adipocytokine signaling pathway: 1—glucose transporter 4 (Glut4); 9, 12—TNF receptor. PPAR signaling pathway: 8—fatty acid transporter (FATs); 9—fatty acid binding protein (FABP); 10—perilipin; 26—PPAR-α.* (b) Relative expression levels of key genes involved in the Rho/ROCK pathway in 3-D spheroid culture vs. 2-D monolayer culture. Normalized expression of $n = 5$ replicates. Error bars indicate standard errors of mean.**

similarly expressed with respect to PRAT in both 2- and 3-D cultures. Out of those 522 genes, 150 genes (29%) were expressed only in 3-D spheroids (and not 2-D monolayer) similar to PRAT. Top biological functions (GO) of these genes were found to be regulation of protein metabolic processes, response to stress, integrin-mediated signaling, and positive regulation of cell cycle, migration, and motility. Next, we probed the expression levels of key genes involved in adipogenesis (CD-36, adiponectin, PPAR-α, and PPAR-γ). Our results (Fig. 3) show that, while further optimization will be required to achieve responses in *in vitro* models at similar levels to those in the *in vivo* mouse adipose tissue, the CD-36, adiponectin, and PPAR-α levels in 3-D spheroids matched well with those in mouse adipose tissue. Interestingly, PPAR-γ

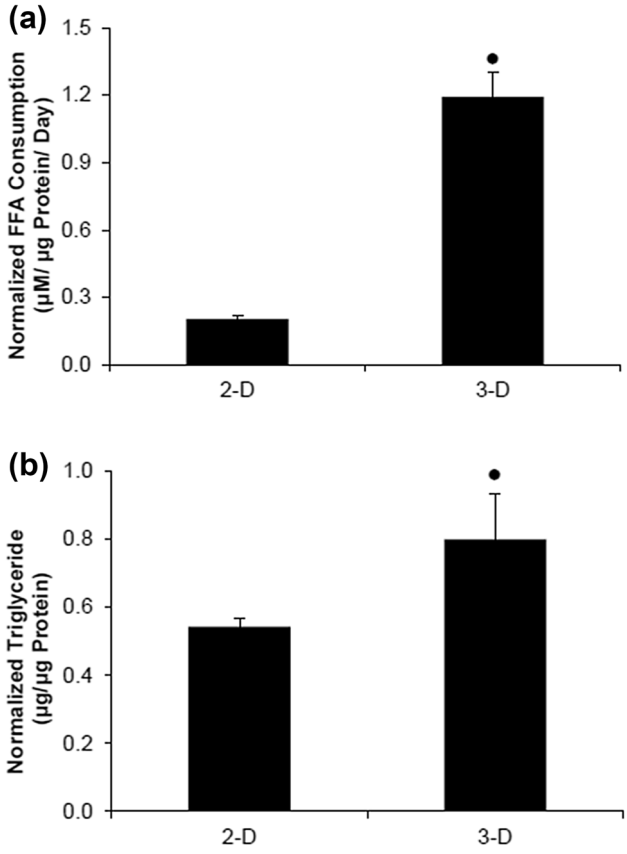


FIGURE 4. (a) LA uptake and (b) Triglyceride content of 3T3-L1 adipocytes treated with 0.5 mM LA. $^{\bullet}p \leq 0.05$ vs. equivalent 2-D culture. Error bars indicate 95% confidence intervals.

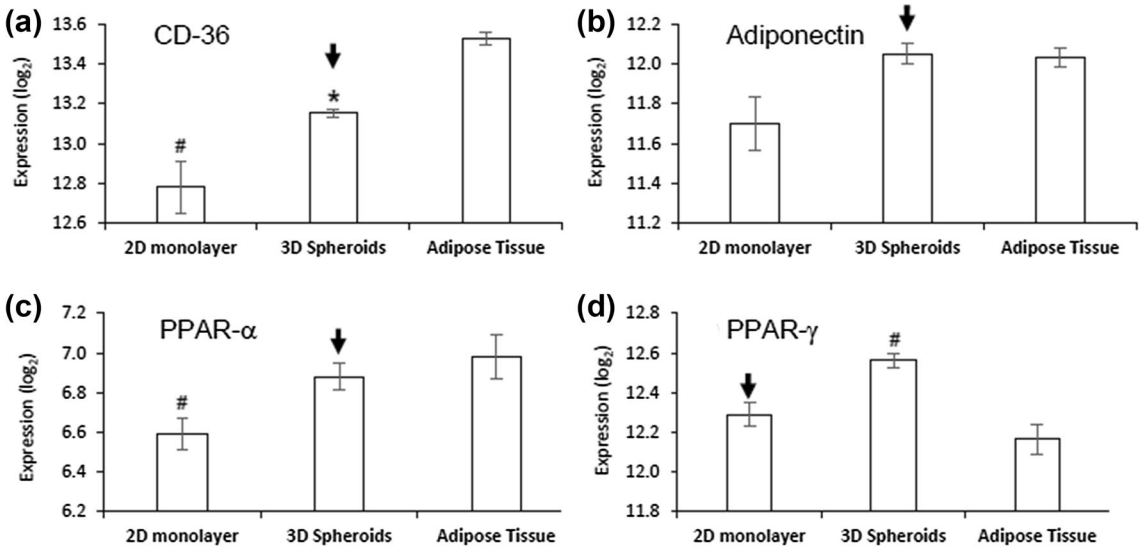


FIGURE 3. Expression levels of key genes in adipose tissue are more similar to 3-D spheroids compared to 2-D monolayer cultures. Normalized expression of $n = 3$ replicates. $^{\#}p \leq 0.05$. Error bars indicate standard errors of mean. Arrows indicate cultures showing the closest response to adipose tissue.

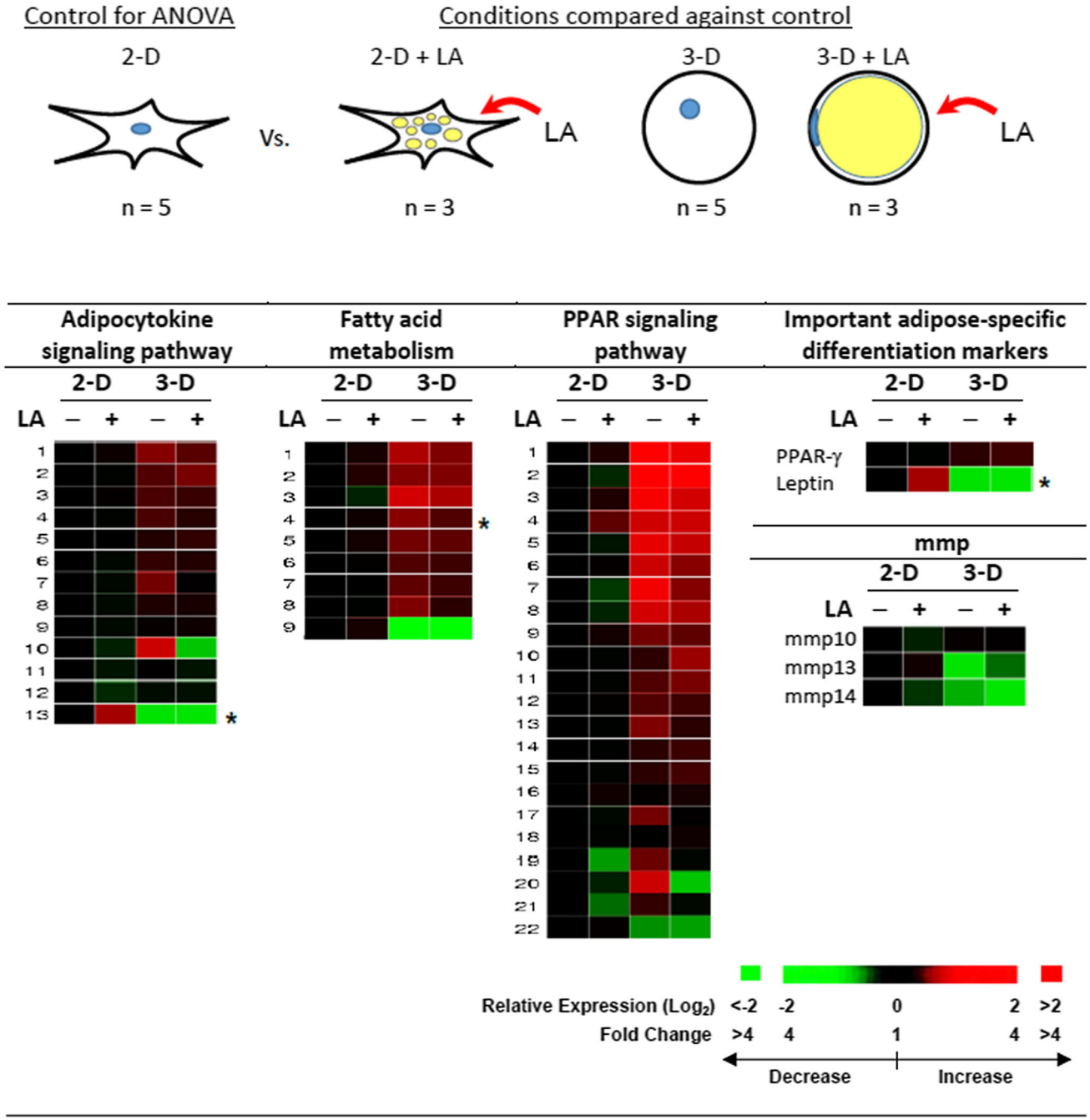


FIGURE 5. Whole transcriptome comparison of 3-D spheroid model vs. 2-D monolayer cultured in media containing no LA or 0.5 mM LA. This data shows slight upregulation of signaling pathways involved in adipogenesis, fatty acid metabolism, and adipocyte function. Downregulation of MT1-MMP (*mmp14*) was observed in the 2-D monolayer as well as the 3-D spheroid model treated with LA. LA treatment appeared to slightly upregulate PPAR-γ. *n* indicates number of replicates tested. *Statistical significance ($p < 0.05$) determined by two-way ANOVA for interaction between the two experimental variables, namely, the 3-D spheroid culturing condition and LA supplementation. *Key genes referenced in text: PPAR signaling pathway: 20—phosphoenolpyruvate carboxykinase (PEPCK).*

expression in 3-D spheroids was superior to that in mouse adipose tissue (Fig. 3d). One possible explanation for this elevated PPAR-γ expression is the absence of relevant chemical cues (e.g., elevated fatty acids, cytokines) that are present *in vivo*, as the elevated levels of inflammatory cytokines such as TNF-α in the obese state act to downregulate PPAR-γ expression.

Effect of LA Supplementation on 2-D and 3-D Cultures

Fatty acid consumption rates (Fig. 4a) indicated nearly 6-fold increased competency of cells in 3-D spheroid culture (1.19 ± 0.11 μM-FFA μg-protein^{-1} day^{-1}) for processing exogenous free fatty acid compared to 2-D monolayer (0.20 ± 0.01 μM-FFA μg-protein^{-1} day^{-1}). Triglyceride assay of cultures treated with fatty media (Fig. 4b) indicated nearly 1.5-fold increased triglyceride accumulation in 3-D spheroid culture (0.80 ± 0.13 μM-TG/μg-protein) vs. 2-D monolayer (0.54 ± 0.02 μM-TG/μg-protein). Whole transcriptome analysis of LA-treated 2-D monolayer and 3-D spheroid cultures indicated minimal changes in signaling pathways involved in adipogenesis, fatty acid metabolism, and adipocyte function as expression of most genes in these pathways remained unchanged (compare colors in heat maps in Fig. 5 for LA supplementation vs. untreated controls). LA treatment appeared to slightly (~ 1.2-fold) upregulate PPAR-γ (Fig. 5) as well as led to differential expression of genes related to glucose metabolism, indicating potentially altered glucose responsiveness. Namely, the expression of phosphoenolpyruvate carboxykinase (PEPCK), associated with gluconeogenesis, and leptin expression were significantly ($p < 0.05$) downregulated in 3-D spheroid culture compared to 2-D monolayer (Fig. 5). Overall, 3-D spheroids showed only modestly altered gene expression to LA supplementation compared to 2-D monolayer. Two-way ANOVA revealed only 1 out of 13 (8%) genes associated with adipocytokine signaling, 1 out of 9 (11%) genes associated with fatty acid metabolism, and none out of 22 (0%) genes associated with PPAR signaling (Table 1) showed a statistical significance ($p < 0.05$) for interaction between the two experimental variables, namely, the 3-D spheroid culturing condition and LA supplementation (* in Fig. 5).

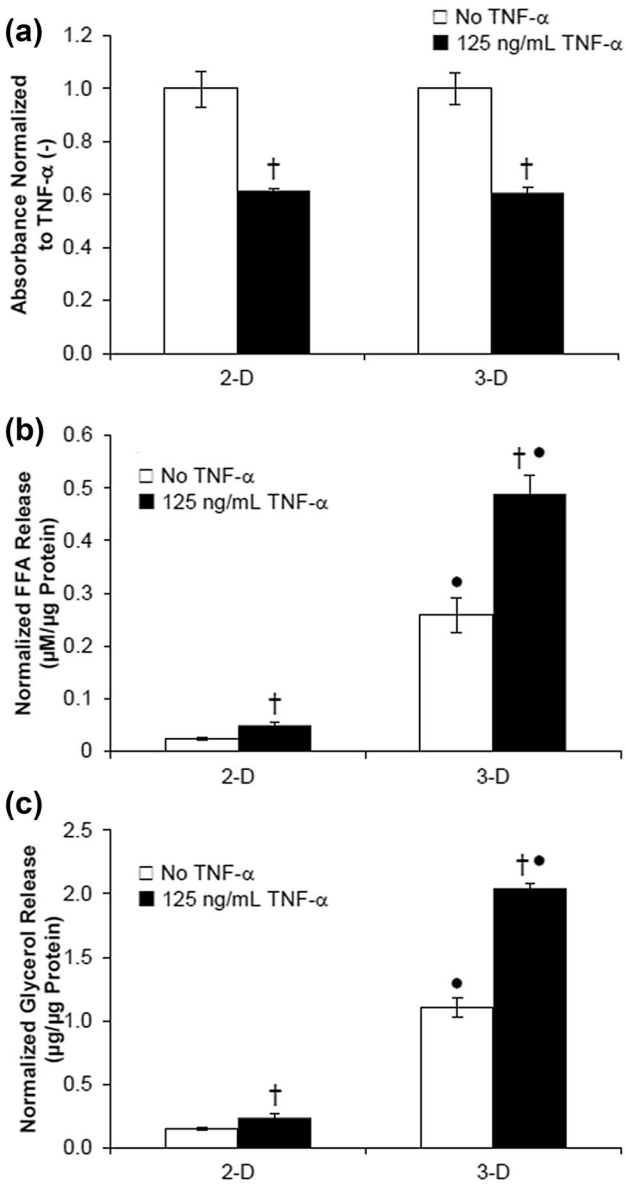
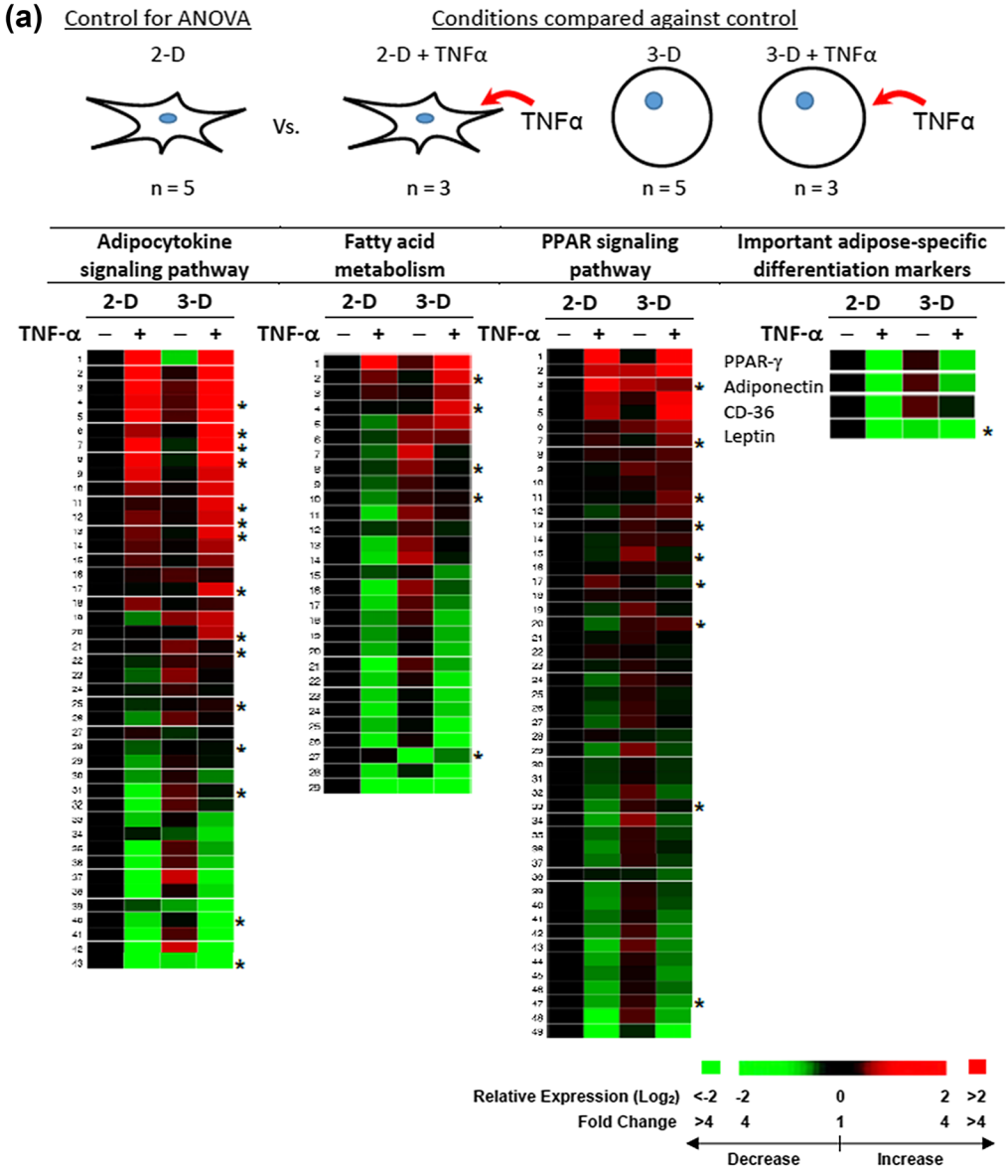


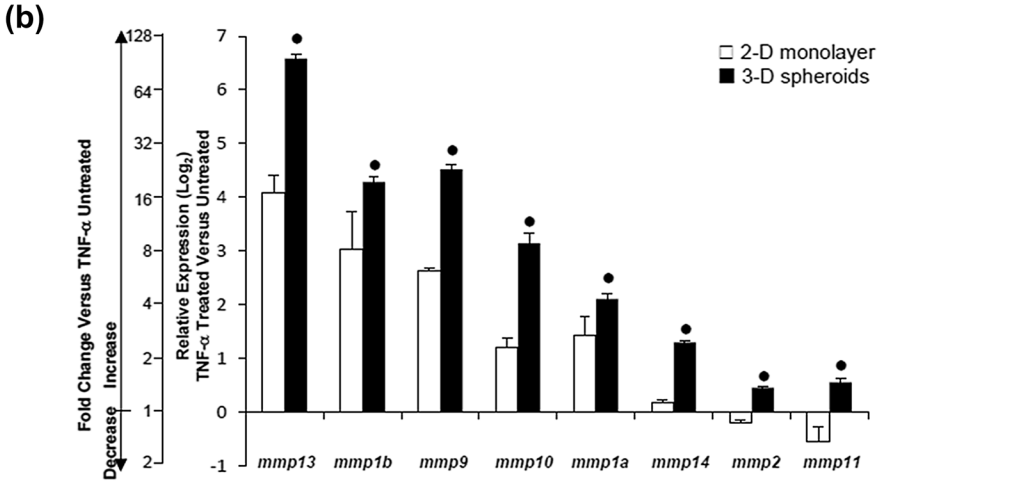
FIGURE 6. (a) TNF-α treatment led to reduction in metabolic function of 3T3-L1 adipocytes fed with 0.5 mM LA. 3T3-L1 adipocytes fed with 0.5 mM LA released (b) free fatty acids and (c) glycerol in response to TNF-α treatment. open square: TNF-α untreated LA-fed culture, filled square: LA-fed culture cultures exposed to 125 ng/mL TNF-α for 24 h. •$p \leq 0.05$ vs. equivalent 2-D culture; †$p \leq 0.05$ vs. equivalent TNF-α untreated culture. Error bars indicate 95% confidence intervals.

Effect of Subsequent TNF-α Treatment on 2- and 3-D Cultures

Cell response to exogenous TNF-α treatment was characterized by metabolic/viability MTT assay (Fig. 6a) and traditional markers for lipolysis: fatty acid release quantified by GC (Fig. 6b) and lipolysis quantified by triglyceride assay (Fig. 6c). Cells in 2-D monolayer and 3-D spheroid cultures fed with 0.5 mM LA showed similar 40% decrease in metabolic potential in response to TNF-α treatment (Fig. 6a). TNF-α treatment stimulated lipolysis in both cultures as indicated by nearly 2-fold increased extracellular free fatty acid (Fig. 6b) and glycerol (Fig. 6c), though the effect was significantly exaggerated in 3-D spheroid culture relative to 2-D monolayer.

Gene expression profiles of 2-D monolayer and 3-D spheroid cultures fed with control maintenance media (no LA) after exposure to TNF-α showed minimum 1.2-fold downregulation of adipogenic genes (PPAR-γ, adiponectin, CD-36, leptin) as well as genes responsible for fatty acid metabolism and PPAR signaling (Fig. 7a), while TNF-α treatment caused an exaggerated upregulation of MMPs in 3-D spheroids (Fig. 7b).





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◀**FIGURE 7. (a) Whole transcriptome comparison of 3-D spheroid model vs. 2-D monolayer cultured in media containing no TNF-α or 125 ng/mL TNF-α. This data shows significant downregulation of fatty acid metabolism and PPAR-γ signaling pathways. In 2-D monolayer treated with TNF-α a slight upregulation of MT1-MMP (*mmp14*) was observed, while in the 3-D spheroid model treated with TNF-α a significant upregulation of MT1-MMP (*mmp14*) was observed. n indicates number of replicates tested. *Statistical significance ($p < 0.05$) determined by two-way ANOVA for interaction between the two experimental variables, namely, the 3-D spheroid culturing condition and TNF-α treatment. *Key genes referenced in text: Adipocytokine signaling pathway: 4—TNF receptor; 8—suppressor of cytokine signaling 3 (SOCS3); 18—inhibitor of kappaB kinase β; 20—inhibitor of kappaB kinase γ; 41—insulin receptor; 42—glucose transporter 4 (Glut4). Fatty acid metabolism: 4, 17—carnitine palmitoyltransferases. PPAR signaling pathway: 32—lipoprotein lipase (LPL); 47—fatty acid binding protein (FABP); 48—perilipin.* (b) Relative expression levels of key matrix metalloproteinases (*mmp*) in 3-D spheroid and 2-D monolayer cultures when treated after exposure to 125 ng/mL TNF-α for 24 h vs. no TNF-α treatment. Normalized expression of $n = 3$ replicates. Error bars indicate standard errors of mean. •$p \leq 0.05$ vs. equivalent 2-D culture.**

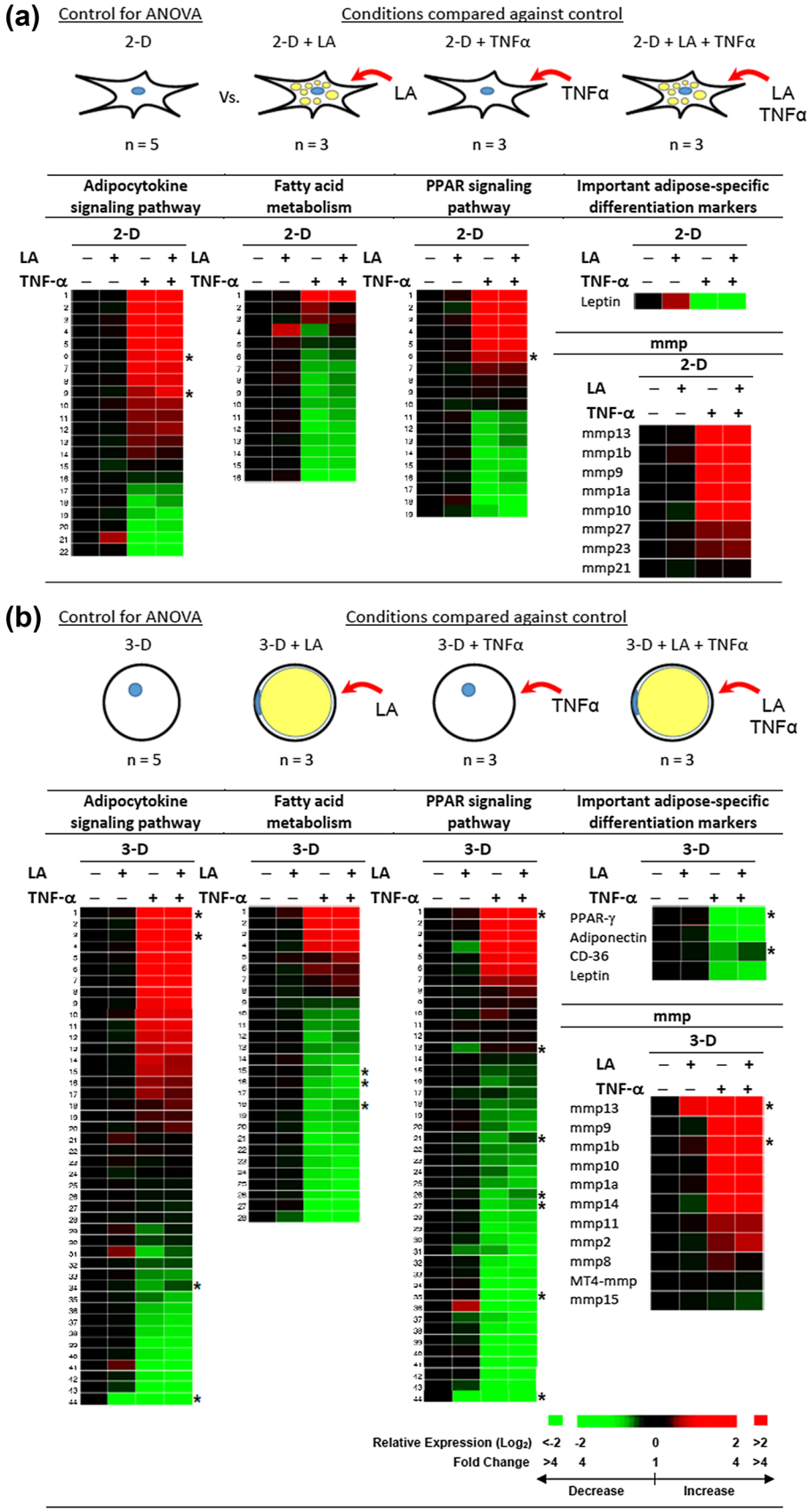
Overall, 3-D spheroids showed enhanced sensitivity to TNF-α treatment, with two-way ANOVA revealing 15 out of 43 (35%) genes associated with adipocytokine signaling, 5 out of 29 (17%) genes associated with fatty acid metabolism, and 9 out of 49 (18%) genes associated with PPAR signaling being differentially expressed (Table 1) with a statistical significance ($p < 0.05$) for interaction between the two experimental variables, namely, the 3-D spheroid culturing condition and TNF-α treatment (* in Fig. 7a).

Gene expression profiles of 2-D monolayer and 3-D spheroid cultures exposed to control maintenance media (no LA), media containing 0.5 mM LA, control media followed by TNF-α, and LA media followed by TNF-α were also compared. From Figs. 8a and 8b, it is evident that 3-D spheroid cultures maintained under identical conditions revealed enhanced sensitivity to LA and TNF-α treatments as shown by the numerous differential gene expressions related to adipogenesis, fatty acid metabolism, and adipocyte function. In addition, several adipogenic genes (PPAR-γ, adiponectin, CD-36) were downregulated by minimum 1.2-fold while MMPs were upregulated minimum 1.2-fold only in the 3-D spheroid culture (Fig. 8b), but not in the 2-D monolayer culture (Fig. 8a). Interestingly, leptin expression (Fig. 8a) was upregulated by LA treatment in the 2-D monolayer culture, but this sensitivity was lost following stimulation by TNF-α. Overall, prior treatment with LA in the 2-D monolayer culture only modestly affected cell response to TNF-α, with two-way ANOVA revealing only 2 out of 22 (9%) genes associated with adipocytokine signaling, none out of 16 (0%) genes associated with fatty acid metabolism, and 1 out of 19 (5%) genes associated with PPAR signaling being differentially expressed (Table 1) with a statistical significance ($p < 0.05$) for interaction between the two experimental variables, namely, the LA supplementation and TNF-α treatment (* in Fig. 8a). However, subjecting 3-D spheroids to LA treatment prior to TNF-α treatment resulted in differential expression of 4 out of 44 (9%) genes associated with adipocytokine signaling, 3 out of 28 (11%) genes associated with fatty acid metabolism, and 7 out of 44 (16%) genes associated with PPAR signaling (Table 1) with a statistical significance ($p < 0.05$) for interaction between the same two experimental variables (* in Fig. 8b).

DISCUSSION

Our previous studies have shown ELP-PEI coated surfaces effectively induced self-aggregating cell spheroids and promoted adipogenic differentiation in 3T3-L1 adipocytes as indicated by free fatty acid uptake, triglyceride synthesis, and expression of adipocytic markers.[28,29] Present study demonstrated functionality, particularly triglyceride synthesis and lipid storage (Figs. 1d and 1e), to be dependent upon the differentiated state of the cells which, in turn, may be influenced by exogenous factors including hormone and fatty acid supplementation as well as 3-D cell organization. It should be noted that cells within a 3-D spheroid inherently display a heterogeneous profile with respect to nutrient exposure, wherein the cells on the outer surface of a spheroid will be more exposed to the hormone and fatty acid supplementation compared to those residing within the spheroid's core. In fact, it is this heterogeneity between cells depending on their location within the spheroid which makes the 3-D spheroid model more physiologically relevant compared to the 2-D monolayer model, where all cells are exposed to same levels of nutrients.

The whole transcriptome comparison of RNA from 3-D spheroid and 2-D monolayer cultures (Figs. 2, 5, 7, 8, and 9) was performed to determine underlying mechanisms responsible for the differences described by functional assays (Figs. 1, 4, and 6). Our study did not use any exogenous ECM (e.g., fibronectin) coating to encourage cell-surface attachment and we observed differential expression of 26 genes related to ECM interaction in the 3-D spheroids. Out of these 26 genes, 17 (65%) genes were downregulated, indicating an overall reduced cell-substrate interaction. The 3-D spheroids showed significantly ($p < 0.05$) downregulated (minimum 1.2-fold) expression of β integrins and α and γ actins (Fig. 2b), owing to the lack of strong cell-substrate attachment by individual cells in the 3-D spheroids. Previous research has also identified the importance of extracellular matrix interactions in



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◀FIGURE 8. Whole transcriptome comparison of (a) 2-D monolayer and (b) 3-D spheroid model cultured in media containing none, either, or both 0.5 mM LA and 125 ng/mL TNF-α. This data shows that TNF-α treatment has a more significant impact on signaling pathways involved in adipogenesis, fatty acid metabolism, and adipocyte function compared to LA treatment. n indicates number of replicates tested. *Statistical significance ($p < 0.05$) determined by two-way ANOVA for interaction between the two experimental variables, namely, the LA supplementation and TNF-α treatment.

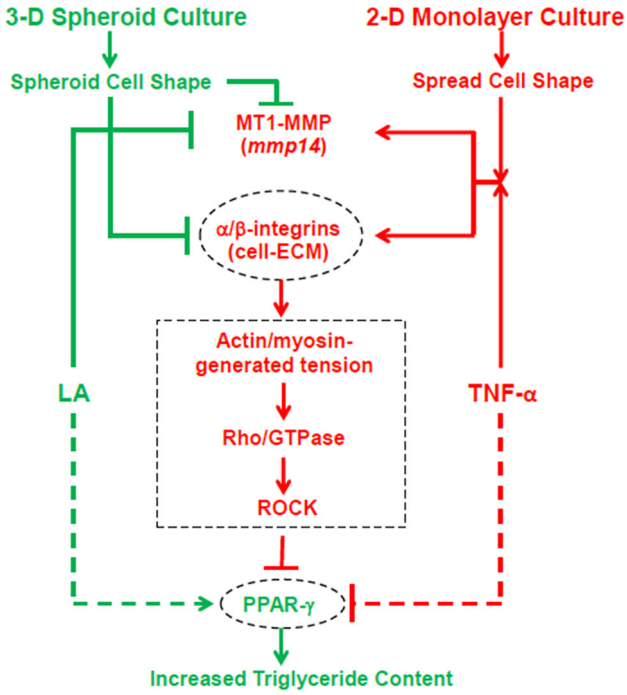

FIGURE 9. Mechanistic description of changes observed in 3-D spheroid model vs. 2-D monolayer during adipogenesis. Factors in green lettering favor adipogenesis, while factors in red lettering downregulate adipogenesis. Dashed lines indicate mechanisms that do not involve MT1-MMP (*mmp14*).

determining the fate of differentiating mesenchymal cells and selecting an appropriate environment for lineage specific differentiation.[8,9,12,17,19,20,23] Adipogenic cells manipulate their extracellular environment through secreted MMPs which soften or solubilize the matrix, allowing the cell to assume a rounded morphology and undergo hypertrophic expansion by lipid accumulation.[2,5] Research by Dr. Weiss's group[6] has highlighted the importance of MT1-MMP (*mmp14*) expression during early *in vivo* adipogenesis. Adipose cells from MT1-MMP knockout mice, unable to remodel their immediate scaffold environment, have been shown to be unable to assume rounded, unilocular morphology and remain immature.[6] Based on these prior findings, we hypothesize that suppression of *mmp14* plays a vital role in the superior adipogenic differentiation observed in our 3-D spheroid cultures (Fig. 9). Specifically, pre-adipose cells grown in 2-D monolayer atop rigid substrates remain bound to the matrix *via* integrins, which may activate the Rho-ROCK pathway through *mmp14* activation. Activation of the Rho-ROCK pathway has been shown to suppress PPAR-γ and thereby may suppress adipogenesis. On the other hand, our 3-D spheroid model denies adipogenic cells stable integrin adhesion sites, which may suppress *mmp14* and Rho-ROCK activation and thereby may promote adipogenic differentiation. Evidence for this hypothesis is present in our transcriptome analysis. For example, Fig. 2b shows the minimum 1.2-fold downregulation of *mmp14*, α and β integrins, α and γ actins, and Rho/GTPase3 as well as 1.2-fold upregulation of PPAR-γ. Downregulation of Rho/GTPase3, an important regulator of mesenchymal stem cell fate,[7,16,18] has been attributed to the lack of organized actin cytoskeleton and plays an important role in adipogenic differentiation. Numerous significant studies have investigated the importance of ECM chemistry and stiffness for directing cell differentiation, with softer, pliable surfaces preferred for adipogenesis.[8,9,12,17,19,20,23] 3T3-L1 adipocytes interact with ECM proteins such as fibronectin *via* α5 integrin, which serve as anchorage points for organized actin stress fibers.[24] Actin acts to propagate signals from the ECM promoting expression of Rho/GTPases, a family of inhibitors of adipogenic genes including PPAR.[7,18] Therefore, due to the lack of integrin interactions with the ECM, cells organized into 3-D spheroids in our study display reduced expression of Rho/GTPases, the PPAR-γ antagonists, promoting adipogenic differentiation. Additionally, we observed that LA supplementation resulted in *mmp14* downregulation and PPAR-γ upregulation (Fig. 5), leading to enhanced adipogenesis (Fig. 4). Conversely, TNF-α treatment resulted in 2.4-fold upregulation of *mmp14* and 2.3-fold downregulation of PPAR-γ (Fig. 7), leading to enhanced lipolysis (Fig. 6).

As such, the gene expression profiles (Fig. 2) of the two culture systems correlate well with functional assays (Figs. 1c, 1d and 1e): inactivation of integrins in 3-D spheroids inhibits proliferation (Fig. 1c) and promotes adipogenic differentiation and triglyceride synthesis (Figs. 1d and 1e). Increased CD-36 gene expression may be responsible for increased fatty acid uptake by 3-D spheroids, likely through the increased CD-36 surface protein expression.[29] Finally, increased glucose uptake (*via* Glut4; Fig. 2a) and fatty acid transport (*via* FATs and FABP; Fig. 2a) may contribute to increased *de novo* triglyceride synthesis by 3-D spheroids. Through limited cell-substrate interaction, the 3-D spheroids also appear to benefit from bypassing early ECM-associated differentiation regulators imposed on 2-D monolayer cultures by the Rho-

ROCK signaling pathway, to enhance PPAR-γ and PPAR-α expression (Fig. 2).

We and others have previously shown significant adipogenic differentiation in 3T3-L1 cells after free fatty acid supplementation.[10,12,15,23,28] In the present study, the analysis of functional markers and gene expression profiles revealed nuanced differences in cell response to exogenous LA. For instance, the 6-fold increased fatty acid uptake by 3-D spheroids (Fig. 4a) compared to the 2-D monolayer culture may be attributed to 1.3–1.7-fold increase in CD-36, FATs, and FABP expression (Fig. 2a). The 2-D culture showed an increased expression of leptin, while the 3-D culture showed a reduced leptin expression (Fig. 5). Similarly, the genes associated with gluconeogenesis (PEPCK) were downregulated by LA treatment in the 3-D culture (Fig. 5), indicating modulation of genes responsible for energy homeostasis.

In our study, the typical functional markers including 40% reduced viability (Fig. 6a) and lipolysis indicated by free fatty acid and glycerol release (Figs. 6b and 6c) were observed in 2- and 3-D cultures following TNF-α treatment, though the effects were more pronounced in 3-D spheroids. These functional markers correlate with 1.3-fold increased expression of TNF-α receptors in 3-D spheroids (Fig. 2a). The TNF-α receptor expression was further increased 2-fold in 2-D and 4.7-fold in 3-D cultures following TNF-α treatment (Fig. 7a). As shown in Fig. 7a, 3-D spheroids also indicated greater expression of lipolytic genes such as carnitine palmitoyltransferases (cpt1c, 1.9-fold) and decreased expression of inhibitors of lipolysis (perilipin, 3.9-fold). Key adipogenic gene PPAR-γ was also significantly ($p < 0.05$) downregulated (2.3-fold) and PPAR-antagonists inhibitor of kappaB kinase β (1.3-fold) and inhibitor of kappaB kinase γ (1.6-fold) were upregulated following TNF-α treatment (Fig. 7a). Fatty acid metabolism was impaired in both 2- and 3-D cultures by downregulation of CD-36 (1.5-fold), lipoprotein lipase (LPL; 2-fold), and FABP (2.9-fold) (Fig. 7a). Insulin sensitivity and glucose metabolism were impaired in both culture conditions, as indicated by 2.3-fold upregulation of SOCS3 genes (suppresses glucose uptake) and downregulation of glucose transporters (Glut4; 4.6-fold) and insulin receptor (1.7-fold), especially in 3-D culture (Fig. 7a). Finally, TNF-α treatment altered cell-ECM interaction (mobility and invasiveness) *via* general upregulation of a suite of MMPs (Fig. 7b), specifically *mmp9* (22.9-fold), *mmp10* (8.8-fold), *mmp13* (96.3-fold), and *mmp14* (2.4-fold).

Overall, the 3-D spheroid model showed enhanced adipocytic differentiation (Figs. 1 and 2) and was found to be more responsive to fatty acid and inflammatory stimuli than cells grown in conventional 2-D monolayer (Fig. 8), as indicated by differential expression of adipose specific genes associated with adipocytokine signaling, fatty acid metabolism, and PPAR signaling (Table 1) as well as ECM interaction, insulin signaling and glucose metabolism, and ERK/MAPK signaling pathways (data not shown). Our recent work has shown that human adipose derived stem cells (hASCs) show similar enhanced differentiation toward the adipogenic lineage when cultured as 3-D spheroids compared to the 2-D monolayer culture.[27] However, pace of such differentiation was significantly slower in hASCs compared to the 3T3-L1 cells. Additionally, adipose tissue is comprised of multiple cell types (endothelial cells, macrophages) and their interactions with the adipocytes is also expected to affect their transcriptome level outcomes. Finally, continuous exchange of nutrients, growth factors, signaling molecules, and waste products will also have a significant impact on adipocyte differentiation. Therefore, while this study has provided important insights into transcriptome level differences in mouse 3T3-L1 adipocyte behavior when cultured as 3-D spheroids vs. 2-D monolayer, it is only a stepping stone to future more complex *in vitro* studies involving multicellular 3-D spheroid models of the adipose tissue.

CONCLUSIONS

The current study highlights functional differences between 2-D monolayer and 3-D spheroid cultures and explores the underlying genomic mechanisms responsible for the different phenotypes. While ECM interaction, modulated by integrin expression and MMP secretion, are important for regulating adipose cell fate and functionality, gene expression profiles of our 3-D spheroid model suggest that differentiated gene expression and functionality may be achieved without ECM anchorage. Indeed, MMP expression is critical for adipogenesis as far as downregulating ECM-associated integrins and actin cytoskeleton organization maintenance of Rho-ROCK genes which otherwise promote chondrogenic or osteogenic cell fates. However, 3-D spheroid cultures appear to bypass these PPAR antagonists, resulting in increased adipogenesis. Thus, we have demonstrated the feasibility of genome-wide analysis of our *in vitro* 3-D spheroid model and its superiority over the conventional 2-D monolayer model.

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CONFLICT OF INTEREST

The authors have no conflict of interests to disclose.

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