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Effect of Ceramide on Mesenchymal Stem Cell Differentiation Toward Adipocytes

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Abstract Proinflammatory cytokines such as tumor necrosis factor (TNF) α are well known to inhibit adipocyte differentiation. TNF-α triggers ceramide synthesis through binding of TNF-α to its p55 receptor. Therefore, ceramide is implicated in many of the multiple signaling pathways initiated by TNF-α. In breast tissue engineering, it is important to know how to modulate adipocyte differentiation of the stem cells with exogenous additives like ceramide *in vitro*. We hypothesized that stem cell adipogenesis could be retained in TNF-α-treated preadipocytes in which ceramide synthesis was blocked and that exogenous ceramide could inhibit adipocyte differentiation. We first studied the effect of ceramide synthase inhibitor, Fumonisin B2, on the adipogenesis of murine mesenchymal stem cells (D1 cells), treated with TNF-α. We then studied the effect of specific exogenous C6-ceramide on D1 cell viability and differentiation. It was found that 1 ng/ml of TNF-α significantly inhibited D1 cell adipogenesis. Cells treated with 5 μM of Fumonisin B2 were able to undergo adipogenesis, even when treated with TNF-α. High concentrations of exogenous C6-ceramide (>50 μM) had an inhibitory effect, not only on the pre-confluent proliferation of the D1 cells but also on the post-confluent cell viability. High concentrations of C6-ceramide (>50 μM) also inhibited mitotic clonal expansion when D1 cell differentiation was induced by the addition of an adipogenic hormonal cocktail. C6-ceramide at low concentrations (10–25 μM) inhibited lipid production in D1 cells, demonstrated by decreased levels of both total triglyceride content and specific fatty acid composition percentages. Genetic expression of peroxisome proliferator-activated receptor (PPAR) γ and aP2 in D1 cells was reduced by C6-ceramide treatment. CCAAT/enhancer-binding protein (C/EBP) β levels in D1 cells were reduced by C6-ceramide treatment during early differentiation; PPARγ and aP2 protein levels were reduced at terminal differentiation. C6-ceramide at lower concentrations also decreased lipid accumulation of differentiating D1 cells. Our results suggest that ceramide synthase inhibitor retains the adipogenic potential of TNF-α-treated mesenchymal stem cells, while exogenous ceramide

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at lower concentrations inhibit the adipogenesis of mesenchymal stem cells. Ceramide, therefore, could be a modulator candidate in breast tissue engineering strategies.

Keywords Adipocyte · Adipogenesis · Ceramide · Differentiation · Fumonisin B2 · Inhibition · Proliferation · Stem cells · TNF-α

Introduction

Tumor necrosis factor (TNF) α is a well-known proinflammatory cytokine [1]. The expression of TNF-α is elevated in the adipose tissue or plasma of obese patients [2–6]. TNF-α inhibits adipocyte differentiation by transcription suppression of peroxisome proliferation-activated receptor (PPAR) γ2 gene expression via an inhibition of CCAAT/enhancer-binding protein (C/EBP) δ during the early stage of adipocyte differentiation [7] without down-regulating the expression of C/EBPβ [8]. A large body of evidence has shown that ceramide has been implicated in many of the multiple signaling pathways initiated by binding of TNF-α to its p55 receptor [9, 10]. Ceramide synthesis is triggered by TNF-α, and intracellular ceramide levels are increased two- to threefold [11]. The effect of TNF-α is mediated through ceramide, atypical PKC and nuclear factor kappa B pathway [12].

Ceramide refers to a family of highly hydrophobic molecules that contain a fatty acid of 2–28 carbons, which is linked to sphingosine or a related long-chain base [13]. Ceramide has a number of important physiologic functions that regulate cellular homeostasis [14]. Intracellular ceramide can be formed either by *de novo* synthesis, or through sphingomyelinase-dependent catabolism, in a range of separate cellular compartments. *De novo* ceramide biosynthesis requires the synergistic actions of serine palmitoyltransferase and ceramide synthase to generate ceramide [15]. The generation of ceramide by both methods is believed to be part of the response to a variety of stress stimuli [16–18], usually resulting in elevated ceramide levels for extended time periods [19, 20]. In general, ceramide is an important intracellular second messenger that plays a key role in the regulation of cell growth, differentiation, and apoptosis [21–24].

For successful breast tissue engineering strategies, the development of methods for generating adipose tissue is essential. Stem cells offer an attractive cell source for breast tissue engineering applications, as adipocyte differentiation of stem cells may be modulated by exogenous additives *in vivo* [25, 26]. Since ceramide is an important downstream effector of TNF-α, it is a possible candidate to serve as an adipocyte differentiation modulator. However, up to now, studies have not shown whether the ceramide synthase inhibitor leads to a recovery of adipogenesis in TNF-α-treated mesenchymal stem cells. Additionally, it is not known what effect exogenous ceramide has on stem cell differentiation to adipocytes. We hypothesized that ceramide synthase inhibitor could recover adipogenesis in TNF-α-treated stem cells and exogenous ceramide could inhibit stem cell adipocyte differentiation. Our hypothesis was tested in an *in vitro* model of D1 cells treated with ceramide synthase inhibitor, Fumonisin B2, and a specific exogenous C6-ceramide.

Materials and Methods

Cell Cultures

D1 cells, bone-marrow-derived mouse pluripotent mesenchymal stem cells, were obtained from American Type Culture Collection (ATCC, Manasssas, VA, USA) and maintained in

Dulbecco's Modified Eagle Medium (DMEM, ATCC) containing 10% fetal bovine serum (Mediatech Inc., Herndon, VA, USA), with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (Gibco/Invitrogen Corporation, Grand Island, NY, USA), in a humidified, 5% CO_2 incubator at 37 °C. Culture media were replaced every 3 days with fresh DMEM with the appropriate supplements in concentrations described above. The passages used in the experiment were from 5 to 15.

Cell Adipogenic Differentiation

The adipogenic induction of confluent D1 cell was accomplished over 12 days by pulsed treatment with an adipogenic hormonal cocktail, which contained 0.5 mM isobutylmethylxanthine (IBMX), 100 μg/ml bovine insulin, and 0.5 μM dexamethasone (Dex) [26]. Briefly, D1 cells were seeded in 12-well plates at 5×10^4 cells/well with 2 mL DMEM per well. After reaching confluence in 4–5 days, the cells were treated with the adipogenic hormonal cocktail for 2 days, followed by 2 days with the insulin additive only (culture medium containing 100 μg/ml bovine insulin). At 8 days, the cells were treated with DMEM culture medium only (Fig. 1). The cells gradually differentiated into mature adipocytes. Triglyceride (TG) and Oil Red O (ORO) assays were used to determine the lipid production at days 3, 6, 9, and 12 following adipogenic induction.

In addition to the conventional 2-D culture system described, a 3-D culture system was also used in this study. The procedure was conducted as follows: first, 26 ml (for one 24-well plate) of collagen gel, comprised of 346 μl 1 N NaOH solution, 5.554 ml culture medium, 2.8 ml 10× phosphate-buffered saline (PBS), and 17.3 ml 3 mg/ml collagen solution (Inamed Biomaterials, Fremont, CA, USA) was prepared. A volume of 0.3 ml of collagen gel was pipetted into each well of a 24-well plate and incubated at 37 °C for at least 1 h to form a base layer. To obtain 2×10^5 cells ml^{-1} $well^{-1}$, 4.8×10^6 cells were collected and pelleted by centrifugation, then suspended in 16.8 ml of collagen gel. A volume of 0.7 ml of cellular collagen gel was pipetted into each well and incubated at 37 °C for at least 1 h to form the top layer. Finally, 1 ml of culture medium was added to each well. After the cells were grown to confluence, the adipogenic induction was conducted over 12 days as previously described. On days 6 and 12, the top medium was gently and thoroughly aspirated from each well. Twenty-four microtubes were weighed. Each gel was transferred into a microtube. A volume of 0.5 ml of 250-unit/ml collagenase solutions was added to each tube. The collagenase solution was comprised of 5 mg 250-unit/mg collagenase, 5 ml 0.05-M Tris buffer (pH 7.4±0.05 at 25 °C) with 1 mM $CaCl_2$. The microtubes were then incubated at 37 °C for more than 2 h. Subsequently, 0.5 ml of 2%

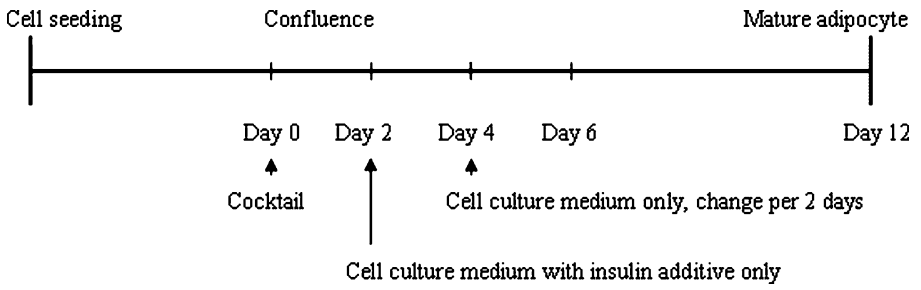

Fig. 1 Experimental flowchart detailing the induction of D1 cells to adipocytes

Triton X-100 solution was added to each tube. The tubes were weighed again, and the weight of cells–gel in each tube was calculated. The samples were frozen and thawed three times and centrifuged at 2,000 rpm for 5 min. The triglyceride content of each sample was assayed.

Effect of TNF-α and Fumonisin B2 on D1 Cell Viability

D1 cells were seeded in 24-well plates at 2.5×10^4 cells/well with 1 mL of DMEM culture medium and then incubated overnight at 37 °C with 5% atmospheric CO_2. Aliquots of TNF-α (Sigma) were added to the wells as appropriate, using a 10-μg/ml distilled water stock solution to achieve final well concentrations of 0, 0.1, 0.5, and 1 ng/ml. The cells were incubated for an additional 4 days. Aliquots of Fumonisin B2 (Sigma) were added to the wells as appropriate, using a 1.4-mM dimethyl sulfoxide stock solution to achieve final well concentrations of 0, 1, 2.5, and 5 μM. The cells were incubated for 4 days. Cell viability was evaluated, using an alamarBlue assay, on day 4, following treatment with TNF-α or Fumonisin B2.

Effect of TNF-α and Fumonisin B2 on D1 Cell Adipogenic Differentiation

D1 cells were seeded as described above in 12-well plates at 5×10^4 cells/well with 2 mL of DMEM culture medium and cultured to confluence. The cells were induced to adipogenic differentiation as shown in Fig. 1. The four groups were as follows: positive control, 1 ng/ml of TNF-α-treated group, 5 μM of Fumonisin B2-treated group, and TNF-α/Fumonisin B2-treated group. On days 6 and 12 following induction, the adipogenesis of D1 cells was evaluated using TG and ORO measurements.

Effect of C6-Ceramide on D1 Cell Viability at Various Stages

D1 cells were seeded in 24-well plates at 2.5×10^4 cells/well with 1 mL of DMEM culture medium and then incubated overnight at 37 °C with 5% atmospheric CO_2. Aliquots of C6-ceramide (Avanti Polar Lipid, Inc., Alabaster, AL, USA) were added to the wells as appropriate, using a 0.5-mg/ml C6-ceramide stock solution to achieve final concentrations of 0, 5, 10, 25, 50, and 100 μM. The cells were incubated for 4 days. The cell viability was evaluated, using an alamarBlue assay, on day 4, following treatment with C6-ceramide.

Next, D1 cells were seeded as described above and cultured to confluence. At confluence, aliquots of C6-ceramide were added to the wells as above, and the cells were continued to incubate for 3 days. On day 3, the cell viability was evaluated using an alamarBlue assay.

Finally, the cells were seeded as described above and cultured to confluence. The cells were then induced into the mitotic clonal expansion (MCE) stage with the adipogenic hormonal cocktail for 2 days. Simultaneously, aliquots of C6-ceramide were added to the wells as described above. The cells were incubated for 4 days. On day 4, the cell viability was evaluated using an alamarBlue assay.

Effect of C6-Ceramide on D1 Cell Adipogenic Differentiation

D1 cells were seeded as above and cultured to confluence. The cells were induced into adipogenic differentiation as shown in Fig. 1. Aliquots of C6-ceramide were added to the

wells as appropriate, using a C6-ceramide stock solution to achieve final well concentrations of 10 and 25 μM, treating the cells for the entire 12 days. Culture medium containing no C6-ceramide was used as a positive control. On days 3, 6, 9, and 12 following induction, the adipogenesis of the D1 cells was evaluated using TG and ORO measurements.

Effect of C6-Ceramide on Differentiating D1 Cells

D1 cells were seeded as above and cultured to confluence. The cells were induced with adipogenic hormonal cocktail as shown in Fig. 1. On day 6 following adipogenic induction, C6-ceramide (10 and 25 μM) was added for an additional 6 days. On day 12, TG and ORO assays were used to evaluate the lipid accumulation of differentiating D1 cells treated with C6-ceramide.

Cell Viability Assay

The cell viability was measured quantitatively using the alamarBlue assay (BioSource International, Inc., Camarillo, CA, USA) [27]. AlamarBlue reagent was added to each well at 10% volume of culture medium and incubated for 2 h on a shaker plate. Then, 150 μl of reaction solution was pipetted into each well of a black flat-bottom 96-well microplate to assay the fluorescence with an excitation wavelength of 544 nm and emission wavelength of 590 nm, using a fluorescence plate reader (Fluoroskan Ascent FL fluorometer; Labsystems Oy; Helsinki, Finland). The effect on cell viability was evaluated using the ratio of the OD values of the treated sample to that of the control sample.

Cell Morphology

Images of the cells were captured using an Axiovert 135 inverted microscope (Zeiss, Germany), Image-Pro 5.0 software (Media Cybernetics; Silver Spring, MD, USA) and a ProgRes^{TM} C10^{Plus} digital camera (Chori Imaging Corporation, Yokohama, Japan). All cellular images were photographed using 320× total magnification.

Triglyceride [28] and Specific Fatty Acid [29] Measurement

The amount of TG produced by the differentiating D1 cells was determined using a Cultured Human Adipocyte Differentiation Assay Kit (Zen-Bio Inc.; Research Triangle Park, NC, USA). Briefly, the cells were rinsed with PBS and subjected to three freeze–thaw cycles and then incubated in 1 ml of 1% Triton®-X-100 solution at room temperature for 30 min. Triplicate mixtures of 100 μl of cellular lysate and 100 μl of Infinity^{TM} triglyceride liquid stable reagent (Thermo Electron Corp., Melbourne, Australia) were mixed in each well of 96-well plate and incubated for 15 min at room temperature. The absorbance of all samples and standards were detected at 490 nm using a plate reader (MRX Tc Revelation; Dynex Technology, Chantilly, VA, USA).

The fatty acid profile for each sample was determined using gas chromatography (GC) as described by Folch et al. [29]. Briefly, the cells on day 12 were rinsed with PBS and scraped into a tube. Total lipid was extracted using chloroform/methanol (2:1 *v*/*v*) plus 0.58% sodium chloride. After evaporation to dryness, methylene chloride and sodium methoxide in methanol was added to the lipid residue for transmethylation. The internal standard (ISTD C23:0, Sigma) was added to each sample tube, and the samples were run on

GC to measure long chain fatty acids. The percentage of fatty acid was calculated according to the following formula:

$$\text{mg fatty acids} = (\text{Total area} - \text{Area}_{\text{ISTD}})/(\text{Area}_{\text{ISTD}}/0.5\text{mg});$$
$$\text{Fatty acid percentage} = \left(\left(\text{Area}_{\text{fatty acid}}/\text{Area}_{\text{ISTD}}/0.5\text{mg}\right)/\text{mg fatty acids}\right)\times 100$$

Oil Red O Stain Measurement [26]

The lipid produced by the differentiating D1 cells was stained with Oil Red O (Matheson Coleman & Bell; Norwood, OH, USA). Briefly, 0.5 g of ORO was dissolved in 100 ml of isopropanol, and the solution was filtered through a 0.22-μm filter membrane to generate a stock solution. To prepare the working solution, ORO stock solution was mixed with distilled water (6:4) and filtered before use. Cells were rinsed with PBS and fixed in 10% neutral buffered formalin for 4 h, washed with distilled water, and stained with 500 μl of ORO working solution for 20 min at room temperature. The wells were rinsed three times with water. The resulting red staining, indicating lipid produced by the cells, was dissolved by adding 500 μl of isopropanol to each well for 15 min. Finally, 200 μl of the destained solution was pipetted into each well of a 96-well plate, and the absorbance at 540 nm was read.

Real-Time Quantitative RT-PCR Analysis

The total RNA in the cells was isolated using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA). The quality and quantity of RNA were evaluated using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano Lab-on-a-Chip kit (Agilent Technologies, Inc., Hewlett-Packard-Strabe, Waldbrone, Germany). One microgram of total RNA was then reversed transcribed and the cDNA sample was further amplified on a Rotor-Gene 3000 thermal cycler (Corbette Life Science, Sydney, Australia) for real time quantitative detection of polymerase chain reaction (PCR) products using QuantiTect SYBR Green reverse transcription polymerase chain reaction (RT-PCR) kit (Qiagen Inc.). Gene expression in each sample was normalized to the expression of a housekeeping gene β-actin and compared with control samples (cells in adipocyte cocktail medium), using the $2^{-\Delta\Delta Ct}$ method [28]. The primers for control and target genes were as follows: β-actin, forward 5′-CTGACAGACTACCTCATGAAGA-3’, reverse 5′-ATGTCAACGTCACACYYCATGA-3′; PPARγ, forward 5′-ACCACTCCCA TGCCTTTGAC-3′, reverse 5′-AACCATCGGGTCAGCTCGTG-3′; aP-2, forward, 5′-ATGTGTGATGCCTTTGTGGGA-3′, reverse, 5′-TGCCCTTTCATAAACTCTTGT-3′.

Western Blotting Analysis

D1 cells were grown to confluence in culture flasks. The cells were induced to adipogenesis as described in Fig. 1. The cells were treated with 25 μM C6-ceramide for 12 days following induction. Cells cultured without C6-ceramide were used as the positive control. On days 3 and 12, the cells were lysed in M-PER mammalian protein extraction reagent (Pierce, Rockford, IL, USA) to extract total protein. The protein concentration was quantified using a bicinchoninic acid protein assay kit (Pierce). Equal amounts of protein extracts from each sample were loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and Western blot onto polyvinylidene difluoride membranes was performed. The various proteins were analyzed using a BM chemiluminescence Western

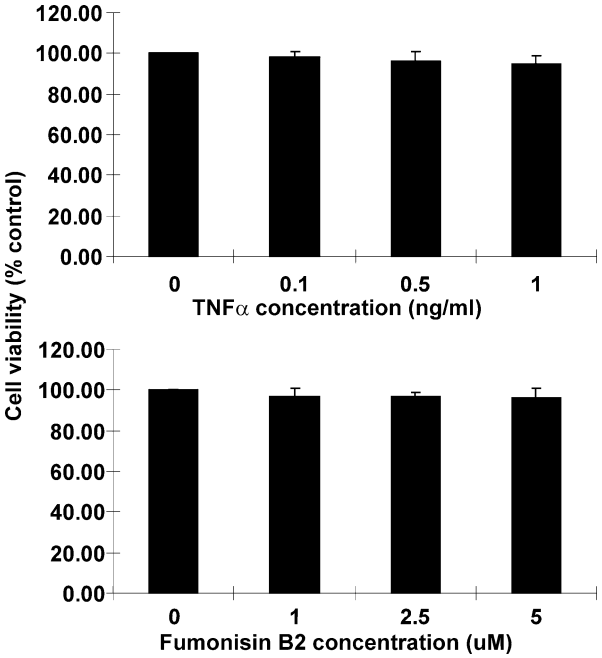

Fig. 2 TNF-α (less than 1 ng/ml) and Fumonisin B2 (less than 5 μM) had no negative effect on cell viability of D1 cells

blotting analysis kit (Roche Diagnostics, Indianapolis, IN, USA). Primary antibodies included: PPARγ, aP-2, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibodies were donkey anti-rabbit immunoglobulin G-horseradish peroxidase (IgG-HRP) and donkey anti-goat IgG-HRP (Santa Cruz).

Statistical Analysis

Statistical significance was assessed using a factorial analysis of variance, and a two-tailed least significant difference test (SAS 9.0 for Window; SAS, Cary, NC, USA). All results were obtained from at least three independent experiments. A p value of less than 0.05 was considered significant.

Table 1 Effect of TNF-α (1 ng/ml) and Fumonisin B2 (5 μM) on D1 cells differentiation.

	Day 6		Day 12	
	TG (μM)	ORO (OD value at 530 nm)	TG (μM)	ORO (OD value at 530 nm)
Control	27.02±1.59	0.69±0.06	44.29±4.78	1.09±0.06
TNF-α	14.22±2.83**·****	0.58±0.06	28.15±2.87**·***	0.77±0.12*
Fumonisin B2	26.54±2.03	0.74±0.06	39.54±5.06	1.10±0.10
TNF-α + Fumonisin B2	25.19±1.83	0.62±0.11	41.29±4.48	1.02±0.16

*P<0.05, **P<0.01, compared to control

***P<0.05, ****P<0.01, compared to Fumonisin B2 and TNF-α+Fumonisin B2

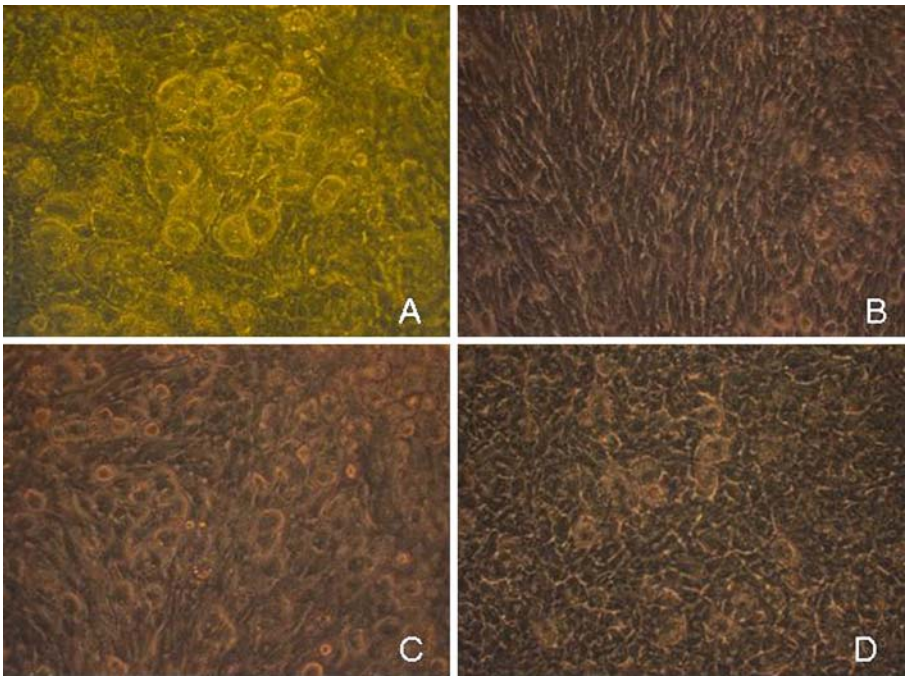

Fig. 3 Cell morphology of D1 cells treated TNF-α, Fumonisin B2, and TNF-α plus Fumonisin B2 on day 12 (×320 total magnification): **a**) D1 cells with adipogenic cocktail, **b**) D1 cells treated with adipogenic cocktail at 1 ng/ml of TNF-α, **c**) D1 cells treated with adipogenic cocktail at 5 μM of Fumonisin B2, **d**) D1 cells cultured with adipogenic cocktail and TNF-α plus Fumonisin B2

Results

Effect of TNF-α and Fumonisin B2 on D1 Cell Viability and Differentiation

After 4 days of treatment with 0.1 to 1 ng/ml of TNF-α and 1 to 5 μM of Fumonisin B2, the viability of the D1 cells was not influenced when compared to the control (Fig. 2). TNF-α at 1 ng/ml, however, did inhibit D1 cell adipogenesis, which was evidenced by the

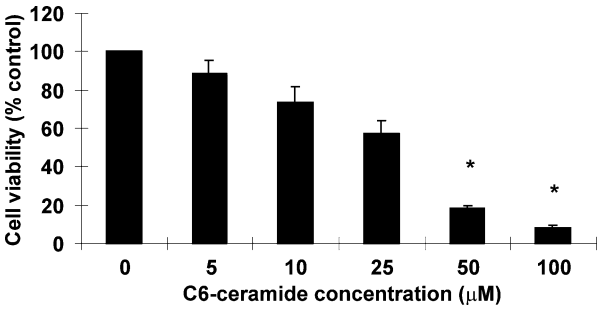

Fig. 4 Effect of C6-ceramide on D1 cell viability on pre-confluent day 4. *$P<0.05$ compared to control; $n=3$

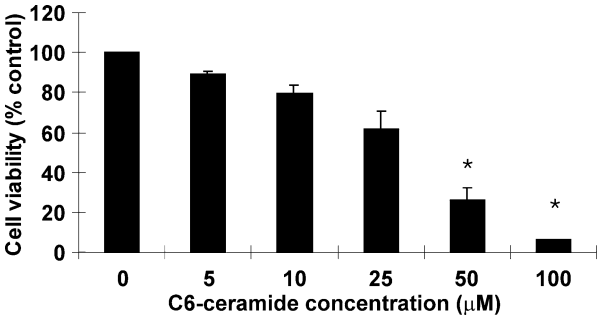

Fig. 5 Effect of C6-ceramide on D1 cells at confluence/growth arrest on day 3. *$P<0.05$ compared to control; $n=3$

significant decrease in TG levels to 47.37% on day 6 and 36.44% on day 12, respectively, and by the decrease in ORO levels to 15.94% on day 6 and 18.08% on day 12, respectively. Fumonisin B2 at 5 μM successfully recovered adipogenesis of TNF-α-treated D1 cells (Table 1). Observations of cell morphology showed that on day 12 following adipogenic hormonal cocktail induction, D1 cells differentiated into mature adipocytes with visible lipid deposition (Fig. 3a). Lipid vesicles were rarely observed in the D1 cells treated with 1 ng/ml of TNF-α (Fig. 3b). D1 cells treated with TNF-α plus Fumonisin B2 and Fumonisin B2 alone resulted mature adipocytes with visible lipid deposition similar to the control samples (Fig. 3c, d).

Inhibitory Effect of C6-Ceramide on Viability of D1 Cells at Various Stages

The alamarBlue assay results showed that C6-ceramide at high concentrations (more than 50 μM) had an inhibitory effect, not only on the pre-confluent cell viability of the D1 cells (Fig. 4) but also on the viability of the D1 cells at the stage of confluence/growth arrest (Fig. 5).

MCE is a synchronous process required for adipogenesis as preadipocytes are induced to differentiate [30]. Our results showed that C6-ceramide at high concentration (more than 50 μM) also inhibited the cell viability of D1 cells at the stage of MCE in the presence of the adipogenic hormonal cocktail (Fig. 6).

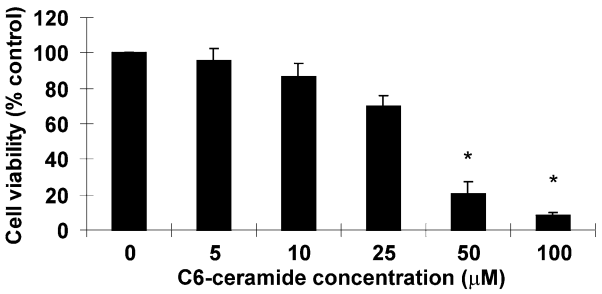

Fig. 6 Effect of C6-ceramide on viability of D1 cells at the stage of mitotic clonal expansion on day 4. *$P<0.05$ compared to control; $n=3$

Table 2 Effect of C6-ceramide on TG levels (μM) of D1 cells during differentiation in 2-D culture system.

C6-ceramide (μM)	Day 3	Day 6	Day 9	Day 12
Control (0)	13.3±5.2	23.8±1.8	25.4±7.7	45.4±4.0
10	6.7±4.3	6.5±3.5**	10.8±4.9*	18.0±5.1**
25	3.6±2.4*	4.2±3.7**	6.8±3.0**	5.0±3.0**

*$P<0.05$, **$P<0.01$, compared to control

Inhibitory Effect of C6-Ceramide on D1 Cell Adipocyte Differentiation

Decreasing TG and ORO values showed that C6-ceramide at low concentrations (10–25 μM) inhibited the differentiation of D1 cells in the 2-D culture system (Tables 2, 3). Since D1 cells cultured in the 3-D culture systems produced more TG than in the 2-D culture systems, the percentage of TG inhibition of D1 cells at day 12, when treated with 25 μM of C6-ceramide, was different in 2-D and 3-D culture systems, specifically 90% and 78%, respectively (Table 4).

On day 12, C6-ceramide treatment changed the fatty acid composition percentage in the samples. Myristic, palmitic, and oleic acids were decreased while stearic acid was increased. The total amount of fatty acids was significantly decreased by 25 μM of C6-ceramide (Table 5).

Observations of cell morphology showed that on day 12 following adipogenic hormonal cocktail induction, the D1 cells differentiated into mature adipocytes with visible lipid deposition (Fig. 7a). Lipid vesicles were rarely observed in the D1 cells treated with 10 and 25 μM of C6-ceramide (Fig. 7b, c). The D1 cells cultured without the adipogenic hormonal cocktail induction maintained an elongated, fusiform appearance (Fig. 7d).

Reduction of Gene and Protein Expression Relative to Adipocyte Differentiation

D1 cells were cultured at 1.25×10^5cells/well in 5 mL of DMEM culture medium in six-well plates and grown to confluence. Then, D1 cells were induced to adipocyte differentiation with a hormonal adipogenic cocktail, as described in Fig. 1, either with or without 25 μM C6-ceramide. On days 3 and 12, the cells were harvested for total RNA isolation and real-time quantitative RT-PCR. The results showed that the gene expression of PPARγ and aP2 was significantly reduced by C6-ceramide treatment ($p<0.05$) at late stage differentiation on day 12 (Fig. 8).

Both PPARγ and aP2 are proteins specific to adipocyte differentiation. As determined by Western blotting analysis, the results showed that the level of PPARγ was reduced significantly on day 3 ($p<0.05$), while the level of aP2 was significantly reduced on day 12 ($p<0.05$) in the D1 cells treated with 25 μM C6-ceramide (Fig. 9).

Table 3 Effect of C6-ceramide on TG levels (μM) of D1 cells during differentiation in 3-D culture system.

C6-ceramide (μM)	Day 6 (μM)	Day 12 (μM)
Control (0)	114.2±85.4	263.9±240.3
10	57.5±38.1*	96.9±38.2*
25	39.6±28.3**	58.6±25.5**

*$P<0.05$, **$P<0.01$, compared to control

Table 4 Effect of C6-ceramide on ORO stain (OD value at 530 nm) of D1 cells during differentiation in 2-D culture system.

C6-ceramide (μM)	Day 3	Day 6	Day 9	Day 12
Control (0)	0.66±0.32	0.82±0.41	0.99±0.43	1.13±0.39
10	0.51±0.24	0.66±0.26	0.74±0.24	0.83±0.33
25	0.45±0.31	0.44±0.15	0.57±0.10	0.57±0.17*

*$P<0.05$, compared to control

C6-Ceramide Reduces Lipid Accumulation in D1 Cells During Adipocyte Differentiation

At confluence, D1 cells were differentiated into adipocytes as induced by the adipogenic hormonal cocktail for 12 days. Thus, on day 6, D1 cells were in the middle of the differentiation process; we investigated the effect of C6-ceramide on differentiating D1 cells at day 6. On day 12, 6 days after C6-ceramide treatment, TG and ORO results indicated that lipid accumulation was decreased (Table 6).

Discussion

Cytokines such as TNF-α are secreted by inflammatory cells and have been shown to impair normal adipocyte differentiation [6–8, 31]. A large body of evidence supports that ceramides are the downstream effectors of TNF-α-mediated inhibition of adipogenesis [9–12]. TNF-α exerts this effect by triggering ceramide synthesis [9–11]. In the present work, we chose TNF-α to inhibit mesenchymal stem cell differentiation to adipocytes with the concentration of 1 ng/ml. High concentrations (more than 1.5 ng/ml) might inhibit the viability of D1 cells. Hammarstedt and co-workers [6] reported that TNF-α completely blocked 3T3-L1 adipose cell differentiation at 1.5 ng/ml. Our results showed that 1 ng/ml of TNF-α inhibited D1 cell adipogenesis by 36% compared to the control. We also chose Fumonisin B2 to study whether it recovers the adipogenesis in TNF-α-treated mesenchymal stem cells at the concentration of 5 μM. Fumonisin B2 is a naturally occurring inhibitor of sphingosine and sphinganine *N*-acyltransferase (ceramide synthase) [32]. Ten to 35 μM of Fumonisin B2 inhibited proliferation of LLC-PK1 cells and higher concentrations of Fumonisin B2 killed the cells [33]. The EC_{50} for alterations in sphingolipid biosynthesis

Table 5 Some specific fatty acid percentage (%) and total fatty acid of D1 cells treated by C6-ceramide.

Group	Control	C6-ceramide (10 uM)	C6-ceramide (25 uM)
Lauric	0.47±0.41	0.60±0.53	0.64±0.55
Myristic	2.04±0.21	1.66±0.15*	1.06±0.92
Palmitic	20.84±0.85	18.07±0.93*	18.52±0.92*
Stearic	15.76±1.08	18.29±1.11*	18.39±0.53*
Oleic	24.11±2.56	25.29±1.03	22.50±5.03
Linoleic	1.82±0.35	2.09±0.32	1.49±1.30
Linolenic	0.42±0.05	0.57±0.12	0.46±0.40
Total fatty acid (mg)	0.12±0.06	0.10±0.03	0.06±0.04*

*$P<0.05$ compared to control

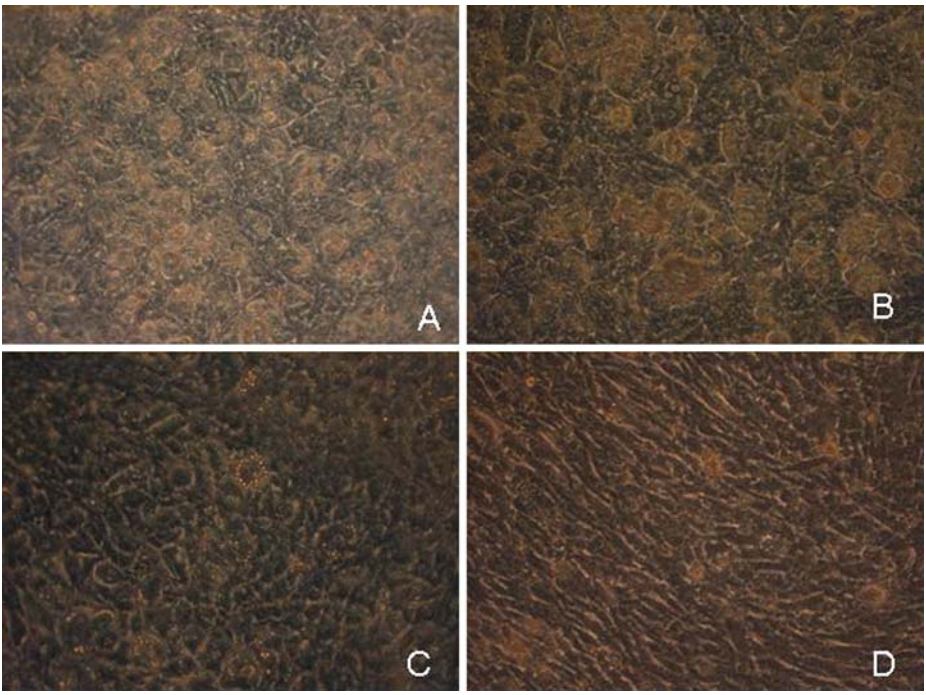

Fig. 7 Cell morphology of D1 cells treated with or without C6-ceramide on day 12 (×320 total magnification): **a**) D1 cells with adipogenic cocktail, **b**) D1 cells treated with adipogenic cocktail at 10 μM C6-ceramide concentration, **c**) D1 cells treated with adipogenic cocktail at 25 μM C6-ceramide concentration, **d**) D1 cells cultured without adipogenic cocktail

was 10–15 μM [33]. The results of the present study showed that treatment with 5 μM of Fumonisin B2 for 4 days had no inhibitory effect on viability of mesenchymal stem cells, and 5 μM of Fumonisin B2 could recover the adipogenesis in the TNF-α-treated mesenchymal stem cells.

There are many kinds of ceramide [34, 35]. C2- and C6-ceramide are synthetic short-chain ceramides, compared to those natural ceramides such as C16, C18, and C20-ceramide [13]. C2- and C6-ceramide are cell-permeable [36] and can decrease HCT116 human colon

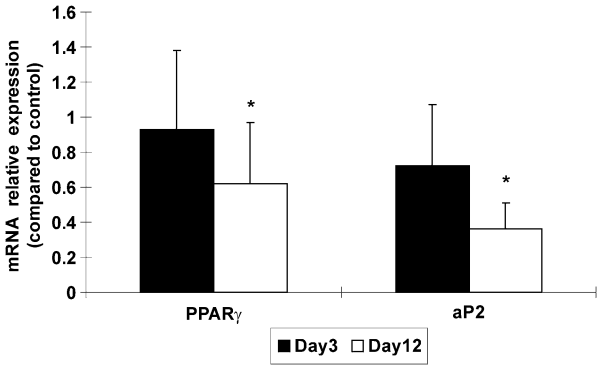

Fig. 8 Real-time quantitative RT-PCR showing inhibitory effect of C6-ceramide on PPARγ and aP2 gene transcription. *$P<$ 0.05 compared to control; $n=3$

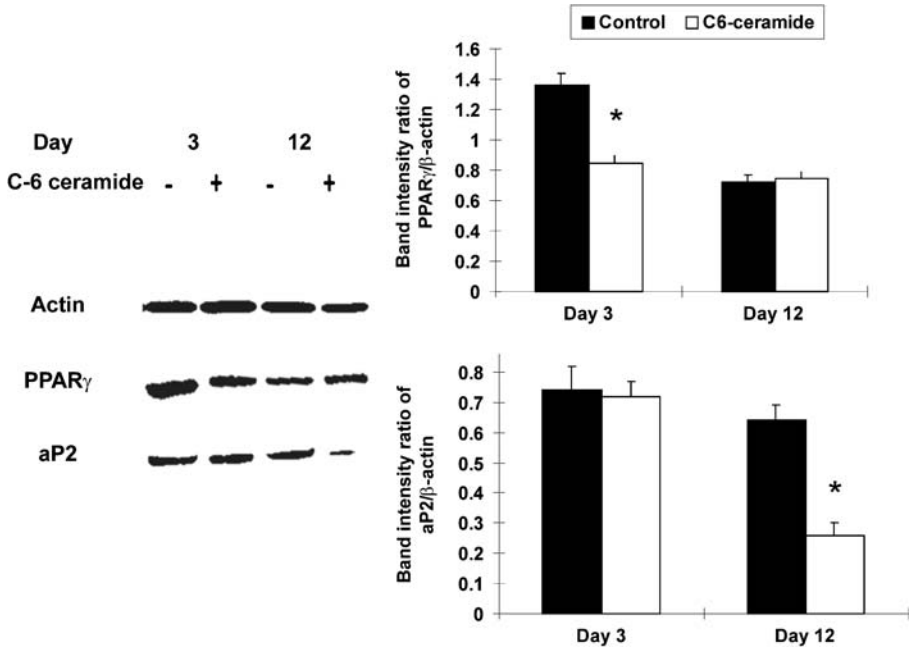

Fig. 9 Western blotting showing inhibitory effect of C6-ceramide on PPARγ and aP2 protein expression. Bands resulting from C-6 ceramide induced cultures are found in columns denoted with *plus symbol*, while bands resulting from control cultures are found in columns denoted by *minus symbol*. PPARγ was significantly reduced on day 3, and aP-2 was significantly reduced on day 12 by C6-ceramide ($P<0.05$), respectively

carcinoma cell and OVCAR-3 ovarian carcinoma cell viability similarly in a concentration- and time-dependent manner, with IC_{50} values around 30 μM [22]. We chose C6-ceramide to test our hypothesis in this study. The D1 murine bone marrow mesenchymal stem cell line has, for many years, provided a useful model to study the differentiation process *in vitro* [37–40]. Induced by an adipogenic hormonal cocktail comprised of insulin, IBMX, and dexamethasone, confluent D1 cells produce lipid and show enhanced expression of a lipid-binding protein (aP2) that is expressed during adipocyte differentiation [41]. Our hypothesis was tested in an *in vitro* model of D1 cells treated by a specific exogenous C6-ceramide. We present evidence for the first time that C6-ceramide has an effect on the proliferation and differentiation of D1 stem cells.

Our data showed that C6-ceramide at high concentrations (more than 50 μM) significantly inhibited D1 cell viability at the pre-confluent stage, the confluence/growth arrest stage, and through the MCE stage, respectively. Some researchers have used the same

Table 6 C6-ceramide reduces lipid accumulation of differentiating D1 cells based on TG levels and ORO stain on day 12 in 2-D culture system.

C6-ceramide(μM)	TG (μM)	ORO (OD value at 530 nm)
Control (0)	39.7±11.1	1.09±0.05
10	33.7±6.7	0.90±0.10**
25	29.7±6.0*	0.73±0.05**

*$P<0.05$, **$P<0.01$ compared to control

levels of other ceramides and obtained similar results [12, 42]. When 50 μM C2-ceramide was added to human abdominal subcutaneous preadipocytes in serum-free medium, cell death was evident at 24 h, increasing from 18% with serum deprivation alone to 40%. An augmentation in cell death also occurred for differentiated adipocytes in serum-free medium treated with C2-ceramide from 32% to 68% [42]. There might be a difference, since various biological effects mediated by ceramide depend on a several factors such as cell type, expression of surface receptors, and the kind, the concentration, and the treatment time of ceramide. Higher levels of ceramide can trigger cell apoptosis, but lower levels of ceramide only inhibit cell function. In primary cultured adipocytes, 1 μM C6-ceramide reduced PPARγ and aP2 messenger RNA levels and protein levels [12]. In this study, low levels of C6-ceramide showed an inhibitory effect on D1 cell differentiation as evidenced by inhibition of both gene and protein expression of PPARγ and aP2, as well as a decrease of TG and ORO levels.

Low levels of exogenous ceramide possibly have a physiological relevance of upregulation of endogenous ceramide because endogenous ceramide in general does not accumulate to a large extent within cells. Both endogenous and exogenous ceramides have been studied widely. Ceramide levels in obese patients or diabetes patients could be high to exert some effects. Shimabukuro and coworkers [43, 44] studied fatty acid-induced β cell apoptosis and confirmed that excess intracellular lipids increase ceramide biosynthesis. Ceramide levels were significantly increased ($p<0.01$) in prediabetic and diabetic islets. Plasma free fatty acids (FFAs) were high (>1 mM) in prediabetic and diabetic Zucker Diabetic Fatty (ZDF) rats. Exogenous C2-ceramides were found to induce apoptosis, while ceramide synthase inhibitor was found to completely block FFA-induced apoptosis in cultured ZDF islets. The researchers concluded that β-cell apoptosis was induced by increased FFAs via *de novo* ceramide formation. At the stage of terminal differentiation of mesenchymal stem cells, long-chain fatty acids are overaccumulated within the cells. The hydrolysis of triglyceride stores will add to the pool of fatty acyl CoA, and the excess of fatty acyl CoA will result in *de novo* synthesis of ceramide [44, 45]. During adipocyte differentiation, trace amounts of ceramide were detected in lipid extractions at the levels of nanomole per milligram protein [46]. Therefore, the overaccumulation of triglyceride could inhibit cellular differentiation via the ceramide pathway.

In conclusion, our experiments showed that C6-ceramide inhibited the differentiation of D1 cells. The inhibitory effect was both time- and dose-dependent. Since PPARγ and aP-2 are adipocyte differentiation-related genes [47–49], the downregulation of both gene transcription and protein expression of PPARγ and aP2 might be related to the mechanism of C6-ceramide. Further study of this mechanism is ongoing.

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References

1. Cawthorn, W. P., & Sethi, J. K. (2008). *FEBS Letters*, *582*, 117–131. doi:10.1016/j.febslet.2007.11.051.
2. Rondinone, C. M. (2006). *Endocrine*, *29*, 81–90. doi:10.1385/ENDO:29:1:81.
3. Petruschke, T., & Hauner, H. (1993). *The Journal of Clinical Endocrinology and Metabolism*, *76*, 742–747. doi:10.1210/jc.76.3.742.
4. Xing, H., Northrop, J. P., Grove, J. R., Kilpatrick, K. E., Su, J. L., & Ringold, G. M. (1997). *Endocrinology*, *138*, 2776–2783. doi:10.1210/en.138.7.2776.
5. Vidal, H. (2003). *Annales d'Endocrinologie*, *64*, S40–S44.

6. Hammarstedt, A., Isakson, P., Gustafson, B., & Smith, U. (2007). *Biochemical and Biophysical Research Communications*, *357*, 700–706. doi:10.1016/j.bbrc.2007.03.202.
7. Kudo, M., Sugawara, A., Uruno, A., Takeuchi, K., & Ito, S. (2004). *Endocrinology*, *145*, 4948–4956. doi:10.1210/en.2004-0180.
8. Kurebayashi, S., Sumitani, S., Kasayama, S., Jetten, A. M., & Hirose, T. (2001). *Endocrine Journal*, *48*, 249–253. doi:10.1507/endocrj.48.249.
9. Ghosh, S., Strum, J. C., & Bell, R. M. (1997). *The FASEB Journal*, *11*, 45–50.
10. Kolesnick, R. N., Haimovitz-Friedman, A., & Fuks, Z. (1994). *Biochemistry and Cell Biology*, *72*, 471–474.
11. Liu, J., Ginis, I., Spatz, M., & Hallenbeck, J. M. (2000). *American Journal of Physiology. Cell Physiology*, *278*, C144–C153.
12. Kajita, K., Mune, T., Kanoh, Y., Natsume, Y., Ishizawa, M., Kawai, Y., et al. (2004). *Diabetes Research and Clinical Practice*, *66*(Suppl 1), S79–S83. doi:10.1016/j.diabres.2003.09.022.
13. Kolesnick, R. N., Goni, F. M., & Alonso, A. (2000). *Journal of Cellular Physiology*, *184*, 285–300. doi:10.1002/1097-4652(200009)184:3<285::AID-JCP2>3.0.CO;2-3.
14. Hannun, Y. A. (1996). *Science*, *274*, 1855–1859. doi:10.1126/science.274.5294.1855.
15. Hannun, Y. A., Luberto, C., & Argraves, K. M. (2001). *Biochemistry*, *40*, 4893–4903. doi:10.1021/bi002836k.
16. Reynolds, C. P., Maurer, B. J., & Kolesnick, R. N. (2004). *Cancer Letters*, *206*, 169–180. doi:10.1016/j.canlet.2003.08.034.
17. Perry, D. K., Bielawska, A., & Hannun, Y. A. (2000). *Methods in Enzymology*, *312*, 22–31. doi:10.1016/S0076-6879(00)12897-6.
18. Hannun, Y. A., & Luberto, C. (2000). *Trends in Cell Biology*, *10*, 73–80. doi:10.1016/S0962-8924(99)01694-3.
19. Liao, W. C., Haimovitz-Friedman, A., Persaud, R. S., McLoughlin, M., Ehleiter, D., Zhang, N., et al. (1999). *The Journal of Biological Chemistry*, *274*, 17908–17917. doi:10.1074/jbc.274.25.17908.
20. Bose, R., Verheij, M., Haimovitz-Friedman, A., Scotto, K., Fuks, Z., & Kolesnick, R. (1995). *Cell*, *82*, 405–414. doi:10.1016/0092-8674(95)90429-8.
21. Chavez, J. A., Knotts, T. A., Wang, L. P., Li, G., Dobrowsky, R. T., Florant, G. L., et al. (2003). *The Journal of Biological Chemistry*, *278*, 10297–10303. doi:10.1074/jbc.M212307200.
22. Fillet, M., Bentires-Alj, M., Deregowski, V., Greimers, R., Gielen, J., Piette, J., et al. (2003). *Biochemical Pharmacology*, *65*, 1633–1642. doi:10.1016/S0006-2952(03)00125-4.
23. Ruvolo, P. P. (2003). *Pharmacological Research*, *47*, 383–392. doi:10.1016/S1043-6618(03)00050-1.
24. Tirosh, O., Aronis, A., & Madar, Z. (2004). *Asia Pacific Journal of Clinical Nutrition*, *13*, S108.
25. Gomillion, C. T., & Burg, K. J. (2006). *Biomaterials*, *27*, 6052–6063. doi:10.1016/j.biomaterials.2006.07.033.
26. Yang, C. C., Ellis, S. E., Xu, F., & Burg, K. J. (2007). *Journal of Tissue Engineering and Regenerative Medicine*, *1*, 146–153. doi:10.1002/term.17.
27. Ahmed, S. A., Gogal Jr, R. M., & Walsh, J. E. (1994). *Journal of Immunological Methods*, *170*, 211–224. doi:10.1016/0022-1759(94)90396-4.
28. Livak, K. J., & Schmittgen, T. D. (2001). *Methods (San Diego, Calif.)*, *25*, 402–408. doi:10.1006/meth.2001.1262.
29. Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). *The Journal of Biological Chemistry*, *226*, 497–509.
30. Tang, Q. Q., Otto, T. C., & Lane, M. D. (2003). *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 44–49. doi:10.1073/pnas.0137044100.
31. Gustafson, B., & Smith, U. (2006). *The Journal of Biological Chemistry*, *281*, 9507–9516. doi:10.1074/jbc.M512077200.
32. Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T., & Merrill Jr., A. H. (1991). *The Journal of Biological Chemistry*, *266*, 14486–14490.
33. Yoo, H. S., Norred, W. P., Wang, E., Merrill Jr, A. H., & Riley, R. T. (1992). *Toxicology and Applied Pharmacology*, *114*, 9–15. doi:10.1016/0041-008X(92)90090-F.
34. Venable, M. E., Lee, J. Y., Smyth, M. J., Bielawska, A., & Obeid, L. M. (1995). *The Journal of Biological Chemistry*, *270*, 30701–30708. doi:10.1074/jbc.270.51.30701.
35. Fishbein, J. D., Dobrowsky, R. T., Bielawska, A., Garrett, S., & Hannun, Y. A. (1993). *The Journal of Biological Chemistry*, *268*, 9255–9261.
36. Kundu, N., Smyth, M. J., Samsel, L., & Fulton, A. M. (2002). *Breast Cancer Research and Treatment*, *76*, 57–64. doi:10.1023/A:1020224503335.
37. Li, X., Cui, Q., Kao, C., Wang, G. J., & Balian, G. (2003). *Bone*, *33*, 652–659. doi:10.1016/S8756-3282(03)00239-4.
38. Diduch, D. R., Coe, M. R., Joyner, C., Owen, M. E., & Balian, G. (1993). *The Journal of Bone and Joint Surgery. American Volume*, *75*, 92–105.

39. Cui, Q., Wang, Y., Saleh, K. J., Wang, G. J., & Balian, G. (2006). *The Journal of Bone and Joint Surgery. American Volume*, *88*(Suppl 3), 148–154. doi:10.2106/JBJS.F.00534.
40. Cui, Q., Wang, G. J., & Balian, G. (1997). *The Journal of Bone and Joint Surgery. American Volume*, *79*, 1054–1063.
41. Hunt, C. R., Ro, J. H., Dobson, D. E., Min, H. Y., & Spiegelman, B. M. (1986). *Proceedings of the National Academy of Sciences of the United States of America*, *83*, 3786–3790. doi:10.1073/pnas.83.11.3786.
42. Papineau, D., Gagnon, A., & Sorisky, A. (2003). *Metabolism: Clinical and Experimental*, *52*, 987–992. doi:10.1016/S0026-0495(03)00165-3.
43. Shimabukuro, M., Zhou, Y. T., Levi, M., & Unger, R. H. (1998). *Proceedings of the National Academy of Sciences of the United States of America*, *95*, 2498–2502. doi:10.1073/pnas.95.5.2498.
44. Shimabukuro, M., Higa, M., Zhou, Y. T., Wang, M. Y., Newgard, C. B., & Unger, R. H. (1998). *The Journal of Biological Chemistry*, *273*, 32487–32490. doi:10.1074/jbc.273.49.32487.
45. Unger, R. H., & Orci, L. (2002). *Biochimica et Biophysica Acta*, *1585*, 202–212.
46. Listenberger, L. L., Han, X., Lewis, S. E., Cases, S., Farese Jr, R. V., Ory, D. S., et al. (2003). *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 3077–3082. doi:10.1073/pnas.0630588100.
47. Ito, S., Suzuki, N., Kato, S., Takahashi, T., & Takagi, M. (2007). *Bone*, *40*, 84–92. doi:10.1016/j.bone.2006.07.012.
48. Gregoire, F. M., Smas, C. M., & Sul, H. S. (1998). *Physiological Reviews*, *78*, 783–809.
49. Cowherd, R. M., Lyle, R. E., & McGehee Jr., R. E. (1999). *Seminars in Cell & Developmental Biology*, *10*, 3–10. doi:10.1006/scdb.1998.0276.