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Development of a three-dimensional adipose tissue model for studying embryonic exposures to obesogenic chemicals

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Abstract

Obesity is a rising issue especially in the United States that can lead to heart problems, type II diabetes, and respiratory problems. Since the 1970s, obesity rates in the United States have more than doubled in adults and children. Recent evidence suggests that exposure to certain chemicals, termed “obesogens,” *in utero* may alter metabolic processes, predisposing individuals to weight gain. There is a need to develop a three-dimensional human tissue system that is able to model the effects of obesogens *in vitro* in order to better understand the impact of obesogens on early development. Human embryonic-derived stem cells in three-dimensional collagen embedded silk scaffolds were exposed to three different obesogens, Bisphenol A (BPA), Bisphenol S (BPS), and Tributyltin (TBT). The exposed tissues accumulated triglycerides and increased expression of adipogenic genes (PLIN1, PPAR γ , FABP4) compared to equivalent control cultures with no obesogen exposure. These cultures were also compared to human adult stem cell cultures, which did not respond the same upon addition of obesogens. These results demonstrate the successful development of a representative tissue model of *in utero* obesogen exposures. This tissue system could be used to determine mechanisms of action of current obesogens and to screen other potential obesogens.

Keywords

Obesogens; Obesity; Adipocytes; Silk; Scaffold; Bisphenol-A; Bisphenol-S; Tributyltin Chloride

3. Introduction

Since the 1980s, obesity rates in the United States have more than doubled in adults and children.[1] Today, in the United States, more than two-thirds of adults and one-third of children are overweight or obese.[2] Obesity is a risk factor for many diseases, including type II diabetes, heart attack, heart failure, and respiratory problems. Previously, lack of exercise and excess consumption of unhealthy foods were believed to be the sole cause of

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Conflict of Interest

The authors have no conflicts of interest to declare.

obesity. However, recent evidence suggests that poor diet and lack of exercise are not the only causes, but instead exposure to certain environmental chemicals, termed “obesogens”, *in utero* may alter metabolic processes and leave individuals more prone to weight gain.[3] Blumberg first termed the word obesogens in 2006 when he discovered that certain organotins predisposed laboratory mice to gain weight.[3] Since then, more obesogenic compounds have been proposed, each acting through different mechanisms.[3]

Obesogens are prevalent in society and include dietary, pharmaceutical, and industrial compounds.[3] Based on prior studies, three obesogens were chosen for study, tributyltin (TBT), bisphenol-A (BPA), and bisphenol-S (BPS). TBT is an industrial compound found in pesticides, and BPA and BPS are found in many plastic products. TBT is one of the most well studied obesogens[3–7] and was chosen for this study as a positive control to verify the 3D model. Although there was a TBT ban recently in antifouling paints, it was found that TBT still presents a danger to humans and the environment since toxic effects can occur at just 1ng.[8] TBT is also capable of bonding to suspended materials and sediments, where it can remain as a reservoir and subsequently be released for up to 30 years.[8] TBT is known to predispose mesenchymal stem cells (MSCs) to differentiate into adipocytes at the expense of osteocytes by controlling Peroxisome proliferator-activated receptor gamma (PPAR γ).[9] Furthermore, TBT increases the number of, and the size of adipocyte cells.[3, 7]

BPA is another well-studied obesogen and was also included for tissue model verification. BPA enhanced preadipocyte adipogenic differentiation and lipid accumulation.[10, 11] At nanomolar concentrations, BPA increased lipid accumulation, PPAR γ expression, and lipoprotein lipase activity in children.[10] In 2008, the FDA withdrew many BPA-based products due to the effects that BPA had on humans. Although this ban was enforced in 2008, in 2010 multiple studies showed that about 90% of the United States population had a detectable amount of BPA in urine, blood, or breast milk, showing that the potential effects of BPA are still prevalent and persistent.[12]

Many plastics now use BPS instead of BPA as a result of the FDA ban. In 2014, a study showed that one of the risks of BPA exposure, neurological disorders, is also a risk of BPS exposure in embryonic zebrafish.[13] Since BPS was shown to have similar neurological embryonic effects as BPA, we hypothesized that BPS would have similar adipogenic embryonic effects as BPA. Additional research showed that BPS, like BPA, increased the amount of triglycerides stored intracellularly.[14]

Since obesogen research is a relatively new field of study, many of the previous studies have either focused on exposing pregnant mice to these chemicals or using twodimensional *in vitro* models featuring non-human cells[3–5, 9, 11, 14–16]. However, since animal models are not always indicative of human responses,[17] in the present study we focused on creating a three-dimensional *in vitro* human tissue model. The three-dimensional structure was formed using a collagen I gel supported by a porous silk protein scaffold as a superstructure. Silk is a biocompatible and stable material that has been used in other *in vitro* adipose applications previously.[18–29] Silk also has adjustable mechanical strength and the rate of degradation can be tuned for long-term culture[20, 30, 31]. Alternative methods such as cell electrospinning were considered for scaffold formation, but cell death

during cell electrospinning can be significant [32, 33]. Since it was unknown if the hES-MSCs would survive electrospinning, the scaffolds were fabricated and seeded as discussed in the methods section.

Since obesogens are known to affect fetal development, human embryonic derived mesenchymal stem cells (hES-MSCs) were chosen as an appropriate cell type to use in the tissue model because they are more primitive than ASCs and are an intermediate phenotype between ESCs and ASCs [34]. ASC responses to obesogens were compared to that of the ES-MSCs because obesogens have little effect on adult cells [3].

The goal of the present research was to generate a tissue system for evaluating human responses to obesogenic chemicals. It has been postulated that the biological effects resulting from early-life exposure to obesogenic chemicals are irreversible, but if exposure is limited, the risk of the long-term effects decreases.[3] If a 3D *in vitro* tissue model is able to successfully recapitulate the effects of obesogen exposure on humans, this can improve predictable outcomes and in turn, raise awareness of problematic chemicals before they enter mainstream use. Future studies can also be considered with the systems described here to reduce or prevent the obesity epidemic.

4. Materials and Methods

Abcam (Cambridge, UK): Lactate Dehydrogenase (LDH) Assay kit (#ab102526)
Advanced BioMatrix (Carlsbad, CA): Bovine collagen solution, type I (#5005-100ML)
Applied Biosystems (Foster City, CA): Power SYBR[®] Green PCR Master Mix (#4368706)
BD (Franklin Lakes, NJ): Alexa Fluor[®] 488 Mouse Anti-Human Leptin Receptor (CD295) (#564376), Alexa Fluor[®] 488 Mouse Anti-Oct3/4 (#560217), Anti-Mouse IgG (FITC) (#555988), BV Mouse Anti-Human CD73 (#562430), Purified Mouse anti-Mouse Nanog (#560259) **Bioassay Systems (Hayward, CA):** EnzyChrom Adipolysis assay kit (#EAPL-200), EnzyChrom Triglyceride assay kit (#ETGA-200) **Biorad (Hercules, CA):** Reverse Transcription Supermix for RT-qPCR (#170-8841) **Life Technologies (Carlsbad, CA):** Alexa Fluor[®] 488 Phalloidin (#A12379), DAPI (#D1306), Live/Dead Viability/Cytotoxicity Kit (#L-3224), Picogreen kit (#P11496), Primers (See Table 7 for sequences)
Lonza: AdipoRed Assay Reagent (#PT-7009) **Millipore (Billerica, MA):** Human mesenchymal stem cells (derived from hES cells) (#SCC036), FibroGRO LS complete media kit for culture of human fibroblasts (#SCMF002), Ultrapure water with 0.1% gelatin (#ES-006-B) **Qiagen (Hilden, Germany):** QIAshredder (#79656), RNeasy Mini Kit (#74104) **Sigma Aldrich (St. Louis, MO):** Anti-Rabbit IgG (FITC) (#F0382), DMEM/F12 (#D2429), Absorbic acid (#1043003), Bisphenol A (#239658), 4,4'-Sulfonyldiphenol (Bisphenol S) (#103039), Dexamethasone (#D4902), Indomethacin (#I7378), Insulin, recombinant human (#91077C), Tributyltin chloride (#T50202), 1,1,1,3,3,3-Hexafluoro-2-Propanol (HFIP) (#105228), Sodium Chloride (NaCl, ASC reagent) (#S9888)
ThermoFisher Scientific (Waltham, MA): 4 mm biopsy punches (#NC9840633), Accutase (#A1110501), Antibiotic-antimycotic (#15240-062), Basic fibroblast growth factor (#13256-029), DMEM/F12 (#11320-033), DMEM/F12 Phenol Free (#21041-025), Fetal bovine serum (#10437-028), Charcoal stripped fetal bovine serum (#12676029), L-glutamine (#25030-081), Rabbit serum (#16120107), Trypsin (#25300062).

4.1. Silk Processing and Scaffold Formation

Silk and silk scaffolds were prepared as described previously.[35] Frozen silk was placed in the lyophilizer and dissolved in hexafluoro-2-propanol (HFIP) at a 1:9 ratio. Canisters were filled with sodium chloride with grain sizes of 500–600 μ m and covered with the silk/HFIP solution. Dried scaffolds were placed in methanol, before transferring to ultrapure milliQ water for 3–5 days with daily water exchanges. Scaffolds were cut to 2mm (height) $\times$ 4mm (diameter).

4.2. Isolation of human adipose derived stem cells (hASCs)

To isolate hASCs, human adipose tissue samples were acquired from abdominoplasty procedures with institutional review board approval (Protocol #0906007). Two different patient samples were used: a female patient age 50 (BMI: 27) and a female patient age 54 (BMI: 35). The adipose tissue was liquefied with a blender, mixed with an equal volume of a 0.1% collagenase type I solution, and incubated at 37°C for 30 minutes. The tubes were centrifuged at 300g for 5 minutes and the pellet was resuspended in Dulbecco's phosphate-buffered saline (DPBS) and centrifuged again at the same settings. The pellet was then resuspended in hASC maintenance media (DMEM/F12 with 10% fetal bovine serum (FBS) and 1 $\times$ Antibiotic-Antimycotic), centrifuged again, and seeded into flasks with fresh maintenance media.

4.3. Cell Culture

hES-MSCs were seeded and maintained in T75 flasks coated with 0.1% ultrapure gelatin water. The hES-MSC maintenance media was comprised of DMEM/F12 without phenol red, 2% FBS, 5ng/mL basic fibroblast growth factor, 50 μ g/mL ascorbic acid, 100 $\times$ glutamax, 5 μ g/mL insulin, and 1 $\times$ Antibiotic-Antimycotic. The hASCs were maintained in T75 flasks with hASC maintenance media. For both cell types, media was changed twice a week. When cells reached 70–90% confluency, which was determined through observation on a microscope, they were passaged using accutase (hES-MSCs) or trypsin (hASCs).

For the 2D studies, the hES-MSCs were seeded on 24 well plates coated with 0.1% ultrapure gelatin water at a cell density of 9000 cells/cm². The cells were treated with hES-MSC maintenance media with media changes twice a week until they reached 90% confluency when were treated with the appropriate media for differentiation (see below for different conditions).

For the 3D studies, the silk scaffolds were autoclaved in water and soaked in media prior to seeding the cells. To better promote cell adhesion, the media soaked scaffolds were dried using aspiration before seeding. Seeding densities of 50,000 cells/scaffold, 100,000 cells/scaffold, and 500,000 cells/scaffold were tested both with and without collagen to determine the optimal density. For the scaffolds with collagen, hES-MSCs were suspended on a collagen gel mixture before being pipetted into the silk scaffold. The collagen gel mixture was made from 1 part 10 $\times$ DMEM, 8 parts type I collagen, and neutralized to a pH of 7.4 with 0.1M NaOH. The cells were resuspended in the collagen mixture at the desired concentration in 20 μ L (e.g. 500,000 cells/20 μ L). 10 μ L of the cell and collagen solution was

pipetted into both the top and bottom of the scaffold. The scaffolds were then placed in an incubator for 2 hours before adding hES-MSC maintenance media.

Cells were maintained in maintenance media until they reached ~80% confluency before obesogen treatment for 14 days (2D cultures) or 21 days (3D cultures). The 3D cultures were treated for 21 days because the cells needed more time to attach and differentiate in the 3D matrix. Obesogens were added to the media in the following concentrations: 1nM TBT, 10 μ M BPA, and 10 μ M BPS. The rationale for these concentrations is provided in Results based on initial screening. Three different media conditions were tested in combination with these obesogens, “Main,” in which the cells were maintained in maintenance media (no differentiation components), “Prime,” in which the cells were maintained in media containing 10% of the normal amount of differentiation components, and “WAT,” in which cells were maintained in white adipose differentiation media.

To perform qRT-PCR, lysates were stored in RLT buffer (containing 2-mercaptoethanol and an RNA carrier) at -80°C . Supernatant samples were collected throughout culture for determining LDH and glycerol levels and stored at -20°C . Cell lysates were also stored at -20°C in a Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) to determine DNA and Triglyceride content. In addition, some samples were used for immunohistochemistry and live/dead assays.

4.4. qRT-PCR

For qRT-PCR, samples were thawed and homogenized with QIAshredders. RNA was prepped according to the manufacturer's protocol with the RNeasy[®] Mini Kit. To synthesize cDNA, an iScript Reverse Transcription Supermix kit was used according to the manufacturer's protocol. The cDNA was stored at -20°C until use. 2 μ L of each sample was pipetted into a well on a 96 well PCR plate with 7.2 μ L of DNase/RNase free water, 0.4 μ L of the appropriate forward primer and 0.4 μ L of the appropriate reverse primer (Supplemental Table 1), and 10 μ L of Sybrgreen master mix. The thermal profile setup was as follows: 10 minutes at 95°C for one cycle, 30 seconds at 95°C , 1 minute at 58°C , and 1 minute at 72°C for 40 cycles, and 30 seconds at 95°C , 30 seconds at 58°C , and 30 seconds at 95°C for 1 cycle. Relative gene expression was calculated using the comparative Ct method normalized to cells maintained with no obesogens in the respective media condition (i.e. BPA stimulation in WAT media was normalized to cells cultured in WAT media without the obesogen to account for differences caused by the different media groups) and GAPDH.

4.5. LDH Secretion

To measure LDH secretion, an Abcam LDH assay was used on supernatant samples according to the manufacturer's instructions. Briefly, 50 μ L of either the cell culture supernatant sample or the positive control (provided in the kit) was pipetted into a well in a 96 well plate. In addition, a 1.25mM NADH standard was made using the NADH standard solution provided and distilled water. 50 μ L of the reaction mix was added to each well and the optical density at 450nm was read once at time zero, and once after the plate was incubated at 37°C for 30 minutes. If LDH activity was low, the plate was read again at 60 minutes.

4.6. DNA Content

To determine DNA content, an Invitrogen Picogreen Assay Kit was used according to the manufacturer's protocol. Briefly, 25 μ L of each lysate sample and 75 μ L of 1 $\times$ TE buffer were added to a well on a 96 well plate. Standards were made using the standard supplied in the kit and 1 $\times$ TE buffer (standard DNA concentrations ranged from 0 to 250 ng/mL). 100 μ L of each standard was also pipetted into each well. 100 μ L of 2 μ g/mL dye (diluted with 1 $\times$ TE buffer) was added to each well and the fluorescence was read in a plate reader at an excitation of 480nm and an emission of 520nm.

4.7. Glycerol Secretion

To determine the amount of glycerol secretion, supernatant samples were assayed with the Bioassay Systems EnzyChrom Adipolysis assay kit according to the manufacturer's instructions. Briefly, 10 μ L of each supernatant sample was pipetted into a well on a 96 well plate. A standard curve was made using cell culture medium and the 100mM glycerol standard supplied with concentrations ranging from 0 to 100 μ g/mL. 100 μ L of the working reagent was pipetted into each well and the plate was incubated for 20 minutes at room temperature before reading the optical density at 570nm.

4.8. Intracellular Triglyceride

To quantify triglyceride accumulation within the cells, a Bioassay Systems EnzyChrom Triglyceride assay kit was used according to the manufacturer's instructions. Briefly, 10 μ L of each sample was pipetted into a well on a 96 well plate. A standard curve was made using 1 $\times$ TE buffer and the standard supplied with concentrations ranging from 0 to 1.0mmol/L. 100 μ L of the working reagent was pipetted into each well and the plate was incubated for 30 minutes at room temperature before reading the optical density at 570nm.

4.9. AdipoRed Fluorescence Assay

To assess lipid content, cells were rinsed with DPBS and 30 μ L of AdipoRed was added to 1mL of DPBS in each well of a 24 well plate. The plate was incubated at room temperature for 10 minutes and the fluorescence was measured with an excitation of 485 nm and an emission of 572 nm.

4.10. Immunohistochemistry

2D and 3D cultures were fixed in 10% formalin for 20 minutes. The samples were washed twice with DPBS and permeabilized for one hour in 0.01% Triton-X. The samples were stained with a 1:1000 DAPI, 1:40 Phalloidin, and 1:35 AdipoRed solution (diluted in DPBS) for one hour. All of these steps were performed at room temperature. The solution was removed and replaced with DPBS. The samples were protected from light and stored at 4°C before imaging with a BZX-700 microscope (2D) or confocal microscope (3D).

4.11. Live/Dead Staining

To visualize the distribution of live and dead cells in the scaffolds, the cells were stained with a 4 μ M Ethidium homodimer-1 and 2 μ M calcein AM solution. The cells were incubated

for 30–45 minutes at room temperature and then imaged on an inverted Leica DMIRE2 confocal microscope.

4.12. Statistics

Sample sizes are indicated in the figure legends, with all samples run in either duplicate or triplicate. Error bars were used when applicable to represent the standard deviation. Graphpad Prism was used with a one way Analysis of variance (ANOVA) test to determine statistically significant differences between groups. Groups on graphs were labeled with letters, where groups sharing the same letter are not significantly different from each other. A one way t-test was used with a hypothetical mean of 1 to analyze significant differences between the obesogen groups and the no obesogen control groups (indicated by a * on graphs). Significance was defined as $p < 0.05$.

5. Results

5.1 Determination of the optimal concentration of obesogens to elicit the greatest accumulation of lipids

2D cultures were used to screen the concentration of obesogens that yielded the optimal accumulation of lipids within adipocytes. AdipoRed, a lipophilic dye, was used to quantify lipid accumulation. Treatment with 1nM of TBT resulted in the highest AdipoRed fluorescence, compared to 5nM and 10nM of TBT (Figure 1(i)). The 5nM and 10nM TBT levels were toxic to the cells, causing cell detachment. Therefore, 1nM of TBT was chosen as an appropriate concentration to use in further studies. Since the amount of lipids accumulated for various concentrations of BPA and BPS were not significantly different from each other (Figure 1(ii)), 10 μ M was chosen to use in further studies to optimize lipid accumulation and prevent other sources of variability that may occur from higher concentrations. The amount of LDH secreted by cells cultured in these different concentrations of BPA and BPS were also not significantly different, suggesting that none of these concentrations were toxic to the cells (Supplemental Figure 2).

Figure 1(ii) shows images of the cells with the optimal concentration for TBT (1nM) and 10, 20, and 40 μ M of BPA and BPS, respectively, stained with AdipoRed (lipids = red), phalloidin (actin cytoskeleton = green), and DAPI (nuclei = blue). Images at 5nM and 10nM TBT are not shown since the cells detached from the bottom of the plate; likely due to toxicity from the obesogen. Consistent with the AdipoRed fluorescence quantification data, there were no observable differences in lipid accumulation between 10 μ M, 20 μ M, and 40 μ M concentrations of the BPA and BPS obesogens. Importantly, there was a clear increase in the presence of intracellular lipids in groups treated with obesogens compared to groups without.

5.2 The effects of obesogens on lipid metabolism in 2D

Next, we wanted to test whether obesogens would alter the metabolic activity of the cells. Adipocyte lipid metabolism involves the storage of triglycerides (lipogenesis) as well as the breakdown of triglycerides into glycerol and free fatty acids (lipolysis). To determine the effect of the compounds on lipolysis (glycerol secretion) and lipogenesis (triglyceride

accumulation) the obesogens were supplemented in three different media groups with varying degrees of adipogenic factors (*Maintenance* = no adipogenic differentiation factors, *Prime* = 10% adipogenic differentiation factors, *WAT* = 100% adipogenic differentiation factors). WAT and Prime media components were chosen based on screening studies shown in Supplemental Figure 1.

The maintenance groups shown in Figure 2(i) were not stimulated with adipogenic factors, therefore, it was not expected that there would be significant lipolysis or lipogenesis without obesogen treatment. For both the hASC and hES-MSC groups, there was no significant difference in glycerol secretion between the different groups with and without obesogen treatment. Although there was a slight difference in some of the groups for triglyceride content, the magnitude of the values obtained for triglyceride content were all too low to conclude that obesogens had an effect on undifferentiated cells.

The prime groups in Figure 2(ii) were treated with media composed of 10% of the normal amount of differentiation components to see if the combination of the obesogens and low levels of differentiation components would result in altered lipid metabolism. Figure 2(ii) shows that the values for glycerol secretion were higher than the glycerol secretion values for the maintenance groups for the hES-MSC groups, indicating the low levels of differentiation components initiated hES-MSCs to secrete glycerol. However, there were no significant differences with and without the addition of obesogens, demonstrating that the addition of the obesogen alone did not affect lipolysis. For the adult hASCs, there was no increase in glycerol secretion for the priming groups compared to the maintenance groups, with no differences in response to obesogens, as well. Figure 2(ii) also shows that the triglyceride content only increased in the TBT groups for both hES-MSC and hASC groups. Since glycerol secretion was lowest for the hES-MSC prime TBT group, this suggests that TBT promoted an increase in lipogenesis rather than lipolysis in embryonic cells. A similar trend was also shown in Figure 3(iii) for the groups with full differentiation potential (WAT, where the cells were treated with 100% differentiation media). Again, the TBT group secreted less glycerol compared to the group without obesogen supplementation, but had a higher degree of triglyceride content. These results support the hypothesis that TBT promoted an increase in lipogenesis over lipolysis.

5.3 2D changes in gene expression in response to obesogens

Four markers were tested in qPCR: PLIN1, TGF β 1, FABP4, and PPAR γ . All were normalized with GAPDH as a housekeeping gene and the no obesogen control. Perilipin (PLIN1), fatty acid binding protein 4 (FABP4), and PPAR γ are all adipocyte specific markers, while TGF β 1 is not.

Consistent with the 2D results on lipid metabolism, hES-MSCs and hASCs cultured in maintenance media and exposed to obesogens (Figure 4(i)) did not show increased expression for any of the adipogenic genes compared to the control with no obesogen addition. Therefore, for 3D studies obesogens were paired with differentiating factors to see differences and the maintenance group was discontinued.

When obesogens were added to priming media, there was an upregulation of FABP4 and PPAR γ expression (Figure 3(ii)). There was also a significant decrease in TGF β 1 expression, indicating the cells were not proliferating, and were likely differentiating. Similarly, when obesogens were added to the hASCs in the priming media (Figure 3(v)), the adipose tissue markers FABP4 and PPAR γ , were upregulated. Again, TGF β 1 expression was not upregulated, suggesting the cells were differentiating rather than proliferating.

Lastly, the hES-MSC differentiation groups (Figure 3(iii)) showed a similar trend as the priming groups. There was an upregulation of FABP4 and PPAR γ expression and no increase in TGF β 1 expression for all groups. Interestingly, the hASC differentiation groups (Figure 3(vi)) showed that the addition of obesogens did not have a significant impact on changes in adipogenic gene expression. Collectively, these results supported the hypothesis that obesogens had little effect on adult stem cell differentiation.

5.4 Optimization of 3D culture conditions

For 3D culturing of hES-MSCs, three seeding densities were tested with and without the addition of a collagen gel, and stained with calcein and ethidium to determine viability. Based on the results from the live/dead assay, 500,000 cells/scaffold with collagen was chosen to be the most effective seeding method (Figure 4).

5.5 The effects of obesogens on lipid metabolism in 3D

Since hASCs did not respond as effectively to obesogen stimulation, only hES-MSCs were used in 3D studies. Similar to the 2D models, there were no significant differences in glycerol secretion in response to BPA and BPS, and TBT was lowest when added to the priming media (Figure 5 (a,b)). For triglyceride content, the 2D data showed that there was not a significant increase for the BPA and BPS groups, however the 3D data showed that there was a significant increase in triglyceride content for all three obesogens compared to the no obesogen control. This result suggests that differences in responsiveness to obesogens exist in 2D and 3D. This is consistent with the literature finding that BPA, BPS, and TBT promoted higher intracellular storage of triglycerides in mice *in vivo* and in non-human *in vitro* models.[3, 5–7, 11, 14] The difference between 2D and 3D could be due to the fact that the 3D platform provided a better model for the cells and they were able to grow spherically (as they are *in vivo*).

5.6 3D changes in gene expression in response to obesogens

When supplemented in priming media, TBT was the only obesogen to promote a significant increase in adipogenic markers - PLIN1 and FABP4 (Figure 6(i)). However, when the obesogens were supplemented in differentiation media in 3D (Figure 6(ii)), all three obesogens produced robust adipose marker expression (PLIN1, FABP4, and PPAR γ expression). In particular, PLIN1 expression was higher for all of the obesogen groups, showing that the obesogens promoted lipid storage in the 3D models. Interestingly, TGF β 1 expression was also higher than the control in the differentiation groups showing that the 3D model provided a platform for the cells to not only differentiate, but also to further proliferate (likely with different subpopulations of cells).

6. Discussion

To test the obesogenic potential of different compounds *in utero*, 2D and 3D human embryonic adipose tissue models were developed. In response to known obesogenic compounds, BPA and TBT, both models upregulated adipogenic genes. In addition, the 2D and 3D models both responded to a hypothesized obesogen, BPS, in a similar manner to the known obesogens, indicating it likely has obesogenic effects as well. In particular, our results indicated that when hES-MSCs were seeded into a 3D collagen embedded silk scaffold system differences could be detected in lipogenesis that were not observed in the 2D models in response to obesogens.

The amount of media supplementation with differentiation factors was an important indicator of responsiveness to obesogens in the model systems (2D and 3D). The groups treated with maintenance media did not show any significant differentiation into adipose tissue, with or without an obesogen stimulus. This result indicates that obesogens do not have the capability to differentiate undifferentiated stem cells without the presence of differentiation components. Furthermore, supplementing the groups with priming media (which contained 10% of the differentiation components as normal differentiation media) with an obesogen also did not have the capability to push the cells to further differentiate in both 2D and 3D cultures. However, looking at the 3D qRT-PCR data in Figure 6, it is clear that the groups treated with obesogens in differentiation media showed more robust differentiation into adipose tissue than the groups treated with priming media, as PLIN1, FABP4, and PPAR γ expression are all higher than the differentiation control. Therefore, in the future differentiation media would be optimal for screening obesogens in this type of tissue system.

It was also discovered that culturing the cells in 2D and 3D had significant effects on the responsiveness of cells to obesogens. While the 2D intracellular triglyceride quantification data (Figure 3) suggested that BPA and BPS promote secretion rather than storage of lipids and TBT promotes increased lipid storage rather than secretion, a finding inconsistent with the literature where BPA increased the size of adipocytes in mice *in vivo* [3, 11]. The 3D data presented in Figure 5, on the other hand, was consistent with the *in vivo* literature results. The 3D data showed that the addition of obesogens increased lipogenesis. This is likely due to differences between the environment provided by the 2D environment compared to the 3D scaffolds; where the three dimensional scaffold was able to provide an environment that was more similar to an *in vivo* environment, allowing the adipocytes to form more spherically accommodating the lipids.

The 2D and 3D differences were also seen in qRT-PCR data. The 2D hES-MSC qRT-PCR data (Figure 3), indicated that obesogens upregulated FABP4 and PPAR γ expression in both the priming and differentiation media conditions. However, PLIN1, the gene that codes for a protein that coats lipid storage droplets, expression showed inconsistent trends. This is again hypothesized to be due to the fact that the two dimensional surface did not allow the adipocytes to fully develop and become large enough for intracellular triglyceride storage. For the 3D qRT-PCR data (Figure 6), however, all three obesogens, BPA, BPS, and TBT, promoted significantly higher PLIN1 expression than the differentiation control. Many

studies have claimed that TBT increased the size of adipocyte cells, which has been shown in this study using many different methods.[3, 5, 6, 9] It was also reported in literature that BPS and BPA increased the amount of triglycerides stored intracellularly both *in vivo* and *in vitro*. [10, 11, 14] Since these results were found in 3D, but not in the 2D models, this suggests that the 3D scaffold tissue systems are a better representation of how obesogens affect adipose tissue compared to 2D models.

For the hASCs, the difference between the cell groups is less clear. There is not a clear trend shown in Figures 2 or 3. This was concluded to be because obesogens do not have a significant effect on adult cells. This finding is consistent with the obesogen hypothesis, which states that obesogens have the greatest effect on cells that are still developing *in utero*. Overall, since the hASC trends were not as conclusive as the hES-MSC trends, only the hES-MSCs were brought into 3D scaffolds.

In conclusion, a successful tissue model was developed to test obesogenic effects on cell cultures using hES-MSCs in a three dimensional collagen embedded silk scaffold system. Glycerol secretion, intracellular triglyceride content, and qRT-PCR data demonstrated that hES-MSCs had distinct differences in responses to obesogen exposures compared to adult hASCs. The 3D tissue models upregulated important adipogenic markers in response to known obesogens, TBT and BPA. In addition, this tissue model was able to confirm the hypothesis that BPS has detrimental effects similar to the more widely known obesogen BPA.[14] In the future this tissue system could be useful in the screening of other potential unknown obesogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. 2D cultures were used to screen what concentration of obesogens would cause the optimal accumulation of lipids within adipocytes

(i) Quantification of lipids using AdipoRed fluorescence after 14 days of culture. Treatment with 1nM of TBT resulted in the highest AdipoRed fluorescence, compared to 5nM and 10nM of TBT. In contrast, 10, 20, and 40 μ M of BPA and BPS, respectively, resulted in an increase in lipid accumulation that was independent of concentration. Groups with the same letter are not significantly different (ANOVA, significance was defined as $p < 0.05$, $n = 3$ samples run in duplicate). (ii) Staining with AdipoRed (lipids = red), phalloidin (actin cytoskeleton = green), and DAPI (nuclei = blue) indicated there was an observable increase in the presence of intracellular lipids in groups treated with obesogens compared to groups without. Error bars are 100 μ m.

Figure 2. The effect of obesogens on lipogenesis and lipolysis in 2D hES-MSC and hASC cultures Glycerol secretion (top) and triglyceride content (bottom) were normalized per microgram of DNA for groups treated with and without obesogens in (i) maintenance media, (ii) priming media, and (iii) differentiation media. Groups with the same letter are not significantly different (significance was defined as $p < 0.05$, $n = 3$ samples run in duplicate). Error bars represent standard deviation.

Figure 3. PLIN1, TGF 1, FABP4, and PPAR expression of 2D cultures
qRT-PCR data after 14 days for (i) hES-MSC maintenance, (ii) hES-MSC priming, (iii) hES-MSC differentiation groups, (iv) hASC maintenance, (v) hASC priming, and (vi) hASC differentiation groups. Delta Delta CT was calculated from the GAPDH housekeeping gene and the no obesogen control in the same media. Groups with the same letter within a gene group are not significantly different (significance was defined as $p < 0.05$, $n = 3$ samples run in duplicate). Error bars represent standard error.

Figure 4. Calcein and ethidium staining were used to determine the optimal seeding conditions of the 3D scaffolds

Live/Dead (calcein and ethidium, respectively) staining on hES-MSCs with and without collagen in silk scaffolds at different seeding densities. For all seeding densities, the cells had enhanced cellular infiltration and adherence to the scaffold with the addition of the collagen gel. The 500,000 cells/scaffold with the addition of collagen had the highest number of live (green) compared to dead (red) cells. Scale bars are 300 μm.

Figure 5. The effect of obesogens on lipogenesis and lipolysis in 3D hES-MSC cultures
Glycerol secretion (top) and triglyceride content (bottom) were normalized per microgram of DNA for the 3D models, with or without obesogens in (i) priming media or (ii) differentiation media. Groups with the same letter are not significantly different (ANOVA, significance was defined as $p < 0.05$, $n = 3$ samples run in duplicate). Error bars represent standard deviation.

Figure 6. PLIN1, TGF 1, FABP4, and PPAR expression of 3D cultures
 qRT-PCR data for (i) priming groups and (ii) differentiation groups. Delta Delta CT was calculated from a GAPDH housekeeping gene and the no obesogen control in the same media. Groups with the same letter within a gene group are not significantly different (significance was defined as $p < 0.05$, $n = 3$ samples run in duplicate). Error bars represent standard error.