Using a 3D Culture System to Differentiate Visceral Adipocytes In Vitro

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It has long been recognized that body fat distribution and regional adiposity play a major role in the control of metabolic homeostasis. However, the ability to study and compare the cell autonomous regulation and response of adipocytes from different fat depots has been hampered by the difficulty of inducing preadipocytes isolated from the visceral depot to differentiate into mature adipocytes in culture. Here, we present an easily created 3-dimensional (3D) culture system that can be used to differentiate preadipocytes from the visceral depot as robustly as those from the subcutaneous (sc) depot. The cells differentiated in these 3D collagen gels are mature adipocytes that retain depot-specific characteristics, as determined by imaging, gene expression, and functional assays. This 3D culture system therefore allows for study of the development and function of adipocytes from both depots in vitro and may ultimately lead to a greater understanding of site-specific functional differences of adipose tissues to metabolic dysregulation. (Endocrinology 156: 4761–4768, 2015)
To compare these 2 fat depots, most studies have taken the form of in vivo experiments of genetically modified animals and mechanisms of cell autonomous regulation were often elucidated through in vitro studies using primary adipocytes. Although methods have been developed to directly culture mature adipocytes (12), most in vitro studies use adipocytes converted from preadipocytes that were isolated from the stromal vascular fraction (SVF) (13, 14). Although this method has been used to successfully study sc fat, it is less effective for visceral fat; visceral cells differentiate poorly, only 30%–40%, compared with the robust differentiation seen in sc cells (15). As a result, many in vitro studies have been done on only sc fat, potentially missing visceral-specific regulatory mechanisms and differential physiological functions of these 2 depots.

Previous work has used 3-dimensional (3D) culture in collagen matrices to study the interaction of adipocytes with the extracellular matrix and manipulate adipocytes for bioengineering applications (16–18). In this study, we designed and optimized a novel protocol to differentiate visceral adipocytes in a 3D collagen hydrogel system and demonstrate that visceral cells grown in these hydrogels differentiate as robustly as sc preadipocytes. We additionally show that these visceral adipocytes are functional fat cells that retain characteristics specific to the visceral depot. This user-friendly 3D culture protocol will enable in vitro studies of primary visceral preadipocytes, which may lead to new mechanistic insights into the development and function of visceral fat.

Materials and Methods

Animals

All animal experiments were carried out following protocols approved by the University Committee on Use and Care of Animals at the University of Michigan and conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Similar results were observed with mice of both genders. Individual experiments were performed on cells that were isolated from the sc and visceral tissues of the same animals and processed side by side.

Preadipocyte isolation and culture

Mice were killed, fat tissue was dissected from the inguinal (sc) and perigonadal (visceral) depots, and SVF was isolated using a protocol adapted from Soukas et al (19). Briefly, tissue was minced and digested, then washed and filtered twice before being plated on 10-cm collagen-coated plates. After 1–2 rounds of culture, adherent preadipocytes were trypsinized and plated for differentiation at a concentration of 300 000 cells per well of a collagen-coated 12-well plate (2-dimensional [2D]) or seeded at a concentration of 300 000 cells/500-μL gel into a collagen hydrogel (3D). To create the gel, preadipocytes were resuspended in DMEM/F12 to 10 × the final seeding density. Each gel consists of 50-μL cell suspension, 50-μL Fetal Bovine Serum, 100-μL 5 × DMEM, 50-μL 0.1N NaOH, and 250-μL 8- mg/mL collagen. 2D and 3D cultures were induced to differentiate the day after plating or gel creation. Cells were stimulated in media supplemented with dexamethasone (5 μM; Sigma), insulin (0.5 μg/mL; Sigma), isobutylmethyloxanthine (0.5 mM; Sigma), and rosiglitazone (1 μM; Cayman). After 2 days, the cells were maintained in growth media + insulin (0.5 μg/mL). Experiments were performed 6–7 days after stimulation. For a more detailed protocol, see Supplemental Materials and Methods and Supplemental Figure 1.

Imaging

Oil Red O staining was performed as previously described (20). For fluorescence, cells grown and differentiated in a gel (3D) or on a collagen-coated glass bottom culture dish (2D) (MatTek) were fixed in 10% neutral-buffered formalin, washed twice with PBS, and incubated rocking at 4°C in the dark in PBS supplemented with 0.01-mg/mL boron dipyrromethene (BODIPY; a green fluorescent dye that is often used for staining lipids) 493/503 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diazas-indacene; Life Technologies) and 1-μg/mL propidium iodide (Life Technologies). Samples were then washed twice with PBS and imaged using a confocal microscope. Gels were transferred to a glass bottom dish before imaging.

Gene expression analysis

To isolate RNA from 3D samples or adipose tissue, gels, or fat tissues were homogenized in TRIZol reagent (Life Technologies) with a handheld homogenizer. RNA from cells grown in 2D was also isolated with TRIZol reagent, and total RNA from 2D and 3D samples was isolated according to the protocol provided by the manufacturer. Total RNA was reverse transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen) and analyzed using SYBR green (Fisher Scientific). All primer sequences are listed in Supplemental Table 1.

Enzyme-linked immunosorbent assays

Gels were incubated in 900-μL media supplemented with 1-μg/mL lipopolysaccharide (LPS) for 4 hours. ELISAs were performed using R&D DuoSet kits specific to mouse IL-6 or mouse TNF-α according to the instructions provided by the manufacturer.

Western blotting

Gels were serum starved for 4 hours and treated with or without 10 μM isoproterenol for 15 minutes. Samples were placed in 150-μL ice-cold Radioimmunoprecipitation assay buffer supplemented with a Complete protease inhibitor cocktail tablet (Roche), homogenized using a handheld homogenizer, incubated, rocking, at 4°C for 1 hour, and centrifuged before being analyzed using SDS-PAGE. Antibodies used are listed in Table 1.

Lipolysis

Gels were incubated with and without 10 μM isoproterenol (Sigma) at 37°C for 1 hour. The glycerol content of the supernatant was determined using free glycerol reagent (Sigma) according to the protocol provided by the manufacturer.
cumulation in many visceral cells, as visualized by the absence of Oil Red O staining, demonstrated that these cells differentiated much more poorly than the sc cells in 2D cultures (Figure 1A, upper panels). Fluorescent imaging of 2D cultures stained with BODIPY and propidium iodide (a red fluorescent dye that stains the nucleus) further showed a near complete differentiation in sc cells and poor differentiation of visceral cells (Figure 1A, lower panels). When cultured in collagen hydrogels, the cells from both depots demonstrated robust differentiation (Figure 1B and Supplemental Figure 2A). Additionally, the visceral cells contain larger and fewer lipid droplets than the sc cells, consistent with morphology found in vivo and with previous reports of adipocytes grown in collagen gels (Supplemental Figure 2, B–D) (24–26).

To evaluate adipogenesis at the molecular level, we tested the expression levels of mature adipocyte markers in sc and visceral fat cells in both 2D and 3D cultures. We found that Adipoq, Fabp4 (also called aP2), and Pparg are significantly higher in sc 2D culture than in visceral 2D culture but are expressed at similar levels in 3D cultures (Figure 1C). We also tested a number of genes involved in lipid and glucose metabolism Atgl, Dgat1, Glut4, Hsl, Plin1, and Scd1. Expression of these genes was low in visceral 2D culture and greatly increased in both sc 2D and sc and visceral 3D cultures, showing that these cells have robust expression of metabolic genes that are necessary for mature adipocyte function (Figure 1D). We also tested our 3D gel culture system with precursors isolated from mesenteric fat, another visceral depot. We found that, similar to those from the perigonadal depot, preadipocytes isolated from the mesenteric depot also undergo robust differentiation in the 3D culture system upon adipogenic stimulation (Supplemental Figure 3). Overall, these data

### Table 1. Antibody Table

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<th>Antigen Sequence (if Known)</th>
<th>Name of Antibody</th>
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### Statistics

Data are expressed as mean ± SE and significance was determined using a Student’s t test. The reported n refers to the number of biological replicates of 2D wells or 3D gels tested in each experiment. *P < 0.05 was considered significant, **P < 0.01 is represented as ***, and ***P < 0.001 is represented as ****.

### Results

**Visceral preadipocytes differentiate as robustly as sc preadipocytes in 3D collagen hydrogels**

It has been previously reported that visceral preadipocytes do not differentiate to the same extent as sc cells in monolayer culture conditions (15, 21). One possible reason for this is that they are intrinsically different than sc cells; visceral cells tend to form larger and fewer lipid droplets than sc cells in vivo (8) and thus may need more structural support and the ability to assume more natural shapes in order to differentiate into structurally fragile mature adipocytes with large lipid droplets. Collagen is a major structural protein in many tissues and has been used as a scaffolding biomaterial for a variety of cell culture and tissue engineering applications (22, 23). We therefore tested collagen hydrogels to evaluate their utility in improving the differentiation of visceral preadipocytes. Various concentrations of collagen and different cell densities were tested and compared before we chose 4 mg/mL of collagen and 3 x 10^5 cells/500-μL gel as conditions that were ideal for promoting optimal visceral cell differentiation.

To test whether the collagen hydrogel system allows visceral preadipocytes to differentiate into mature fat cells to the same extent as sc preadipocytes, we grew and differentiated preadipocytes obtained from the SVF of sc and visceral depots side by side. The lack of lipid droplet ac-
suggest that our collagen 3D gels allow for vigorous differentiation of visceral preadipocytes in vitro.

Adipocytes differentiated in the collagen hydrogels retain depot-specific characteristics

We next tested the expression levels of previously identified depot-specific gene signatures to investigate whether cells cultured in collagen gels maintain their original depot identity. We assayed Sbox2 and Tbx15, both of which are enriched in the sc depots of mice and humans (11, 27) as well as Agt and Wt1, which are enriched in the visceral depots of both mice (9, 24) and humans (27). We first verified that these genes are enriched in their respective depots by assaying expression levels in tissue, then tested whether they were similarly enriched in 2D and 3D culture. The inter depot expression differences are more pronounced in 3D culture compared with 2D culture in all cases, and depot-specific enrichment of Agt is even reversed in 2D culture (Figure 2, A and B). These data suggest that tissue-specific gene expression patterns can be obscured in subphysiological 2D culture conditions and that the 3D culture system reliably replicates the magnitude of the differential gene expression and restores the dysregulated gene expression that sometimes occurs in 2D culture.

We next tested the expression of genes related to functional characteristics of both depots. Thermogenic genes have been shown to be expressed at higher levels in the sc depot (10), whereas the visceral depot has been shown to produce greater amounts of some cytokines (6). We differentiated visceral and sc preadipocytes in gels and tested the expression levels of Cidea, Cox7a1, Dio2, Ppargc1a, and Prdm16, which are genes associated with thermogenesis (9, 28), as well as Ccl2 (also called Mcp1), Ccl5 (also called Rantes), Il6, Il10, and Tnfa, which are cytokines known to be secreted from fat (6). Thermogenic gene expression was higher in the sc cells (Figure 2C), whereas cytokine gene expression was higher in the visceral cells (Figure 2D). LPS is an endotoxin found in the cell wall of gram-negative bacteria that produces a strong immune response in many tissues, including adipose tissue. We treated cells from both depots cultured in 3D gels with LPS and observed clear inter depot difference in both cytokine gene expression (Figure 2E) and cytokine secretion, as measured by ELISA (Figure 2F). Taken together, these data show that the collagen 3D culture system enables visceral and sc adipocytes to retain their depot-specific characteristics and can thus be used to study the intrinsic differences between adipocytes from the 2 depots.
Cells differentiated in collagen hydrogels are functional adipocytes

Cells grown and differentiated in 3D culture were further tested to ensure that they retain the functional- ities of mature adipocytes. It has been shown that upon cold exposure, the adaptive thermogenic response is activated in white fat via adrenergic signaling (29). We treated visceral and sc cells grown in 3D gels with isoproterenol, a pan β-adrenergic agonist, significantly increasing thermogenic gene expression in 3D cultures of both visceral and sc cells (Figure 3A). Phosphorylation of p38 MAPK (p38), a key regulator of β-adrenergic-stimulated thermogenesis, was also increased in the isoproterenol treated samples (Figure 3B). Hydrolysis of triglycerides to fatty acids and glycerol (lipolysis) is an important function of mature adipocytes and helps to maintain nutritional and energy homeostasis. We treated the gels with isoproterenol to mimic in vivo catecholamine-stimulated lipolysis and saw significantly increased glycerol content in the supernatant of stimu-

Figure 2. Cells grown in the collagen hydrogels retain depot-specific gene expression and function. A and B, Expressions of genes preferentially expressed in either sc (Shox2 and Tbx15) or visceral (Agt and Wt1) adipose depots were assayed using RT-qPCR with RNA extracted from sc/visceral adipose tissue or sc/visceral cells differentiated in 2D or 3D culture. Relative enrichment of depot-specific expression is shown as mean ± SEM (n = 3); **, P ≤ .01 and ***, P ≤ .001. C, Expression of thermogenic genes (Cidea, Cox7a1, Dio2, Ppargc1a, and Prdm16) was measured in 3D cultures of differentiated visceral and sc cells by RT-qPCR. D and E, Expression of cytokine genes (Ccl2, Ccl5, Il6, Il10, and Tnfa) was measured in basal (D) or LPS-stimulated (1-µg/mL LPS for 4 h) (E) sc or visceral cells differentiated in 3D gels. Values are mean ± SEM (n = 5); *, P ≤ .05; **, P ≤ .01; ***, P ≤ .001. F, Secretion of IL-6 and TNF-α by visceral or sc cells grown in 3D gels was measured in the supernatant of gels stimulated with 1-µg/mL LPS for 4 hours. Values are mean ± SEM (n = 3); **, P ≤ .01; *** P ≤ .001.
lated visceral and sc gels (Figure 3C), as well as an increase in the phosphorylation of hormone-sensitive lipase (HSL) (Figure 3D), an important step in the initiation of lipolysis (30). We were also able to measure oxygen consumption of adipocytes differentiated in 3D gels (Supplemental Figure 4) and assess their ability to respond to hormonal stimulation (Supplemental Figure 5). These results suggest that cells grown in the hydrogels not only exhibit the morphological characteristics and gene expression profiles of mature adipocytes but also can fulfill their many functions.

Discussion

In an attempt to find a culture condition that would allow visceral preadipocytes to differentiate as robustly as sc preadipocytes in vitro, we optimized a collagen hydrogel
system to determine the ideal growth and differentiation conditions for visceral cells. Compared with many commercially available 3D culture systems, our collagen gels are easy to create and do not require specialized equipment or technical skills. Culture and differentiation using this protocol does not demand more cells than what is normally used in the 2D culture system, and downstream functional analyses can be carried out using the same techniques commonly used on whole tissues or cells grown in 2D. Using this system, we are able to differentiate visceral cells as robustly as sc cells grown in 2D and 3D culture, thus providing a system where cells from both depots can be studied side by side.

The reason why visceral cells do not differentiate as well as sc cells in 2D culture is not yet well understood. In this study, we compared the differentiation potential of preadipocytes from these 2 depots in 2D and 3D cultures. The fact that the adipogenic potential of visceral cells can be restored in our collagen gel system suggest that the compromised adipogenesis seen in 2D is at least in part caused by some intrinsic properties of these precursors that can be ameliorated by the enhanced structural support of the 3D culture or other external factors. In visceral cells differentiated in gels, we observed robust expression of genes associated with metabolism as well as previously identified depot markers and functional markers, including cytokines. This reliable replication of in vivo depot-specific characteristics underlines the utility of the 3D hydrogel system in studying the intrinsic differences of adipocytes from different depots.

In summary, we show that 3D collagen hydrogels are a valuable system for culturing and differentiating visceral adipocytes. These gels are easy to create, can be used for a variety of molecular and functional studies, and do not require specialized equipment beyond those used for 2D cell culture. With this system, we can study the cell autonomous regulation of visceral and sc adipocytes side by side and gain a better understanding of their individual contributions to the pathogenesis of metabolic disease.

Acknowledgments

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References