methods

OP9 mouse stromal cells rapidly differentiate into adipocytes: characterization of a useful new model of adipogenesis

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Abstract Much knowledge of adipocyte biology has been learned from cell culture models, most notably 3T3-L1 cells. The 3T3-L1 model has several limitations, including the requirement of 2 weeks to generate adipocytes and the waning of adipogenic potential in culture. We have investigated the capacity of OP9 cells, a line of bone marrow-derived mouse stromal cells, to recapitulate adipogenesis. When OP9 cells are given any one of three adipogenic stimuli, they rapidly accumulate triacylglycerol, assume adipocyte morphology, and express adipocyte late marker proteins, including glucose transporter 4 and adiponectin. OP9 cells can differentiate into adipocytes within 2 days. This rapid rate of differentiation allows for the detection of transiently expressed proteins in mature OP9 adipocytes. Adipogenesis in OP9 cells involves the master transcriptional regulator of adipocyte differentiation, peroxisome proliferator-activated receptor γ (PPARγ). OP9 cells are late preadipocytes in that, before the addition of adipogenic stimuli, they express the adipocyte proteins CCAAT/enhancer binding proteins α and β, PPARγ, sterol-regulatory element binding protein-1, S3-12, and perilipin. OP9 differentiation is not diminished by maintenance in culture at high cell density or by long periods in continuous culture, thereby facilitating the generation of stable cell lines that retain adipogenic potential. Thus, the unique features of OP9 cells will expedite the study of adipocyte biology.—Wolins, N. E., B. K. Quaynor, J. R. Skinner, A. Tzekov, C. Park, K. Choi, and P. E. Bickel. **OP9 mouse stromal cells rapidly differentiate into adipocytes: characterization of a useful new model of adipogenesis. *J. Lipid Res.* 2006.** 47: **450–460.**

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The increase in obesity and the identification of adipocyte-secreted proteins that regulate energy metabolism (1) have generated interest in adipocyte biology. Adipocytes are the primary storage site for energy in vertebrate animals. During fasting, adipocytes release energy-rich molecules that provide metabolic fuels to other tissues. Adipocytes also secrete hormones that orchestrate the storage, release, and oxidation of energy-rich molecules throughout the body and that control behavior, including feeding (2). Primary adipocytes maintain a large dynamic triacylglycerol (TAG) pool and express a specific set of proteins to maintain circulating metabolic fuel levels. Primary adipocytes and adipose tissue have been used to study basic adipocyte biology. However, these systems have several limitations: they do not propagate in culture, they are difficult to transfect with DNA, they have a huge TAG store that interferes with biochemistry and microscopy, they vary as a result of the genetics and conditions of the animals from which they are isolated, and the isolation procedure is tedious and introduces variation. In addition, harvesting primary adipocytes or adipose from animals generally requires the euthanasia of a vertebrate animal and the expense of specialized facilities and protocols. For these reasons and likely others, cell lines have been developed that can be induced to store TAG, to express proteins that are hallmarks of adipocytes, and presumably to recapitulate key events in adipocyte ontogeny.

Three decades ago, Green and colleagues (3–5) reported that a clonal subline of mouse 3T3 cells had a propensity to differentiate into adipocytes when in a

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Abbreviations: ADC, adipogenic cocktail; BMP-4, bone morphogenic protein 4; C/EBP, CCAAT/enhancer binding protein; DN, dominant negative; eGFP-perilipin, perilipin tagged at its N terminus with enhanced green fluorescent protein; GLUT4, glucose transporter 4; IO, insulin oleate; M-CSF, macrophage colony-stimulating factor; PPARγ, peroxisome proliferator-activated receptor γ; SR, serum replacement; SREBP, sterol-regulatory element binding protein; SSC, side scatter; TAG, triacylglycerol.

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"resting state." This 3T3-L1 cell culture model of adipogenesis has been exploited extensively to investigate the mechanisms of adipocyte differentiation, lipid metabolism, insulin signaling, and glucose transport as well as to identify physiologically important adipocyte-secreted proteins, such as adiponectin (6) and resistin (7). Nevertheless, the 3T3-L1 model has significant limitations. First, from the time of initial plating, the generation of 3T3-L1 adipocytes from preadipocytes requires at least 2 weeks (8). Second, if 3T3-L1 cells become confluent and are further propagated or if they are passaged extensively, they no longer differentiate robustly into adipocytes. These issues make culturing of 3T3-L1 cells demanding and limit their utility in the generation of stable cell lines. Third, to our knowledge, it has not been possible to efficiently detect RNAs and proteins from transiently transfected DNA in 3T3-L1 adipocytes. This limitation derives from the facts that most plasmid transfection protocols require subconfluent cells and that levels of RNAs and proteins expressed from such transfections wane before 3T3-L1 cells become adipocytes. Finally, because the 3T3-L1 cell line originated from a single clone and thus has clone-specific traits, it fails to recapitulate the primary cells it models. Thus, an alternative, tractable adipocyte model system is required.

We now report our initial characterization of a new adipocyte cell culture model, OP9 mouse stromal cells, that provides a tractable alternative system for studies of adipocyte biology. The OP9 cell line was established from the calvaria of newborn mice genetically deficient in functional macrophage colony-stimulating factor (M-CSF) (9). OP9 cells are used in coculture to support hematopoietic cell differentiation from embryonic stem cells (9). Specifically, we describe conditions that cause OP9 cells to store TAG, to take up glucose in response to insulin, and to express adipocyte proteins. Unlike 3T3-L1 cells, OP9 cells will robustly differentiate into adipocytes after being confluent and after many passages and long periods in culture. Furthermore, OP9 cells can be differentiated rapidly enough to detect protein expressed from transiently transfected DNA in fully differentiated adipocytes.

EXPERIMENTAL PROCEDURES

Antibodies and antisera

The following rabbit antisera were gifts and have been described previously: anti-adiponectin/Acrp30 (10), provided by Dr. Philipp E. Scherer (Albert Einstein College of Medicine, Bronx, NY); anti-glucose transporter 4 (GLUT4) (11), provided by Dr. Michael Mueckler (Washington University School of Medicine, St. Louis, MO); and anti-perilipin (12), provided by Dr. Constantine Londos (National Institutes of Heath, Bethesda, MD). S3-12 antibody was raised in rabbits to a peptide with the sequence of the first 16 residues of mouse S3-12 and purified against the same peptide (13). The guinea pig adipophilin (14) and perilipin (15) antisera were purchased from Research Diagnostics, Inc. (Flanders, NJ; catalog nos. RDI-PROGP40 and RDI-PROGP29, respectively). The following antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): CCAAT/enhancer binding protein (C/EBP) α (catalog no. sc-61); C/EBP β (catalog no. sc-7962); peroxisome proliferator-activated receptor γ (PPARγ) (catalog no. sc-9000); and sterol-regulatory element binding protein-1 (SREBP-1) (catalog no. sc-367).

Propagation of cells

OP9 cells were generously provided by Dr. Toru Nakano (9). OP9 cells are available from the American Type Culture Collection (Manassas, VA; catalog no. CRL-2749); however, we have not had consistent results differentiating the American Type Culture Collection OP9 cells into adipocytes. OP9 cells were grown in OP9 propagation medium: MEM-α with 20% FBS (Premium Fetal Bovine Serum; BioWhittaker, Walkersville, MD; catalog no. 14-051Q), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. In our hands, the optimal differentiation potential of OP9 cells was achieved when cells were replated every 3 days at a density of at least 7,000 cells/cm^2. OP9 cells grown in this manner are flat with broad cell processes. OP9 cells maintained at low cell density adopt a spindly morphology and differentiate into adipocytes poorly. 3T3-L1 cells were grown in 3T3-L1 propagation medium: DMEM with 10% bovine serum (Invitrogen; catalog no. 16170-078), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Differentiation of adipocytes

Three methods were used to differentiate preadipocytes into adipocytes as described below.

Serum replacement method. OP9 or 3T3-L1 cells were grown to confluence and then cultured for 2 additional days in either OP9 or 3T3-L1 propagation medium as described above. The cells were then cultured up to 4 more days in serum replacement (SR) medium: MEM-α with 15% KnockOut™ SR (Invitrogen; catalog no. 10828-028), 100 U/ml penicillin, and 100 μg/ml streptomycin. For the purpose of studying insulin effects, it is important to change the SR medium to OP9 propagation medium after day 2 of differentiation, because KnockOut™ SR contains very high concentrations of insulin (final concentration of 1.7 μM). In our initial experiments, the SR medium contained 5 ng/ml bone morphogenic protein 4 (BMP-4) (R&D Systems, Inc., Minneapolis, MN). We subsequently found there to be no significant difference in adipocyte differentiation without BMP-4, so all experiments were performed without BMP-4, except for the assays for insulin-stimulated glucose uptake.

Insulin oleate method. OP9 cells were plated at 5,000 cells/cm^2. When cells adhered to the plate, the OP9 propagation medium was replaced with insulin oleate (IO) medium: MEM-α with 0.2% FBS, 175 nM insulin, 900 μM oleate bound to albumin (5.5:1 molar ratio, prepared as described previously) (14), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Adipogenic cocktail method. This method was performed as described previously for 3T3-L1 cells (8). In brief, cells were grown to confluence and then cultured for 2 additional days in 3T3-L1 adipocyte medium: DMEM with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were then cultured for 2 days in DM1: DMEM with 10% FBS, 175 nM insulin, 0.25 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured for an additional 2 days in DM2: DMEM with 10% FBS, 175 nM insulin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. At that point, 3T3-L1 cells were maintained in 3T3-L1

adipocyte medium, and OP9 cells were maintained in OP9 propagation medium.

Labeling lipid droplets with red fatty acid

Adipocytes were incubated for 2 h in IO medium containing 20 μg/ml BODIPY® 12 carbon red fatty acid (Molecular Probes, Invitrogen Detection Technologies; catalog no. D3822, BODIPY®FL C_{12}) delivered from a 20 mg/ml stock dissolved in dimethyl sulfoxide.

Immunofluorescence microscopy

Adipocytes were fixed, stained, and imaged as described previously for 3T3-L1 cells (14).

Live cell imaging

Imaging live OP9 cells on 22 mm coverslips. Thirty-six hours after adding IO medium, the coverslip was washed with PBS, pH 7.4, and the side without cells was dried. A chamber was created by placing two coverslips 18 mm apart and anchoring them to the slide with silicone vacuum grease. Then the coverslip was placed cell-side down spanning the gap between the anchored coverslips. The chamber between the slide and the cell-bearing coverslip was then filled with PBS, and adipocytes were imaged as described previously (14).

Imaging live OP9 cells in culture dishes. Cells were imaged in medium with an Eclipse TS1500 inverted microscope (Nikon, Inc.) using the 20× objective. Images were captured with a Coolpix 5000 digital camera (Nikon, Inc.).

Protein assay

Protein was assayed by the bicinchoninic acid method in a microtiter format using BSA as the standard (Pierce Biotechnology, Inc., Rockford, IL).

TAG measurements

Cells were dispersed in PBS with 10 mM EDTA. Cells or increasing amounts of corn oil dissolved in ethanol (up to 40 μg of corn oil) were extracted from the aqueous phase (16). The organic phases were dried and the colorimetric TAG detection reagent (Thermo Electron Corp., Melbourne, Australia; catalog no. 2780-400H) was added to the lipid residue. Samples and the corn oil standards were transferred to a microtiter plate, and absorbance at 540 nm was measured. The TAG mass extracted from the cells was estimated by interpolation on the line that best fit the corn oil standards.

Immunoblotting

Immunoblotting was done as described previously (14). Cells were lysed in HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.5% cholate with Complete Protease Inhibitor Cocktail (Roche Applied Science; catalog no. 1 836 153), and the supernatants (21,000 *g* for 10 min) were resolved by PAGE and blotted.

Extraction of nuclear proteins

Nuclear protein-enriched supernatants were generated using a commercial kit according to the manufacturer's protocol (Pierce Biotechnology; catalog no. 78833).

Generation of OP9 cell lines stably expressing NLS-LacZ and a PPARγ dominant negative mutant

cDNAs encoding β-galactosidase with a nuclear localization signal (NLS-LacZ) or a FLAG-tagged PPARγ dominant negative (DN) mutant (17) were cloned into the ΔU3 vector, and pseudotyped retroviral particles were produced and used to transduce subconfluent cells, as described (18). To minimize differences in the cell lines not attributable to the virally expressed proteins, we generated each cell line in parallel.

Glucose uptake assays

Specific uptake of 2-deoxy-D-[^{3}H]glucose into adipocytes in response to insulin was measured essentially as described (19). The fold increase in 2-deoxyglucose uptake in response to insulin was calculated as the 2-deoxyglucose uptake of the insulin-treated adipocytes divided by the 2-deoxyglucose uptake of the untreated adipocytes. Nonspecific uptake in the presence of 10 μM cytochalasin B was subtracted from all values. The SR medium used to initiate OP9 cell differentiation in these studies contained 5 ng/ml BMP-4.

Transfection of OP9 cells

A total of 50,000 OP9 cells were plated in a 35 mm dish. After 18 h, cells were transfected with 4 μg of DNA and 12 μl of Optifect reagent, according to the manufacturer's protocol (Invitrogen; catalog no. 12579-017). After 8 h, transfection medium was replaced with either propagation medium or IO medium as indicated.

Flow cytometry

Cells were transfected as described above and treated as indicated in the legend to **Fig. 1**. Immediately before scanning, cells were trypsinized and resuspended in Hanks' balanced salt solution with 0.2% bovine serum albumin. A total of 10,000 events from this cell suspension were analyzed with a Calibur cytometer (Beckman-Coulter, Inc.).

RESULTS

We and others (20) have noted that OP9 cells accumulate lipid droplets when grown to confluence, and we observed that lipid droplet accumulation was increased in serum-free culture conditions. Immunofluorescence studies revealed that the lipid droplets that accumulated in OP9 cells were coated with perilipin, which is expressed almost exclusively in adipocytes or steroidogenic cells. Given the limitations of other available cell culture models of adipocytes, we initiated a characterization of OP9 cells as an adipocyte model. First, we investigated the effects of adipogenic stimuli on OP9 cells. Then, we characterized the expression of adipocyte marker proteins and behaviors.

Adipogenic stimuli cause OP9 cells to acquire adipocyte morphology, accumulate TAG, and express adipocyte marker proteins

We found three treatments that caused OP9 cells to assume the morphology of adipocytes (contain abundant intracellular lipid droplets) and accumulate TAG. The first treatment is the adipogenic cocktail (ADC) method tra-

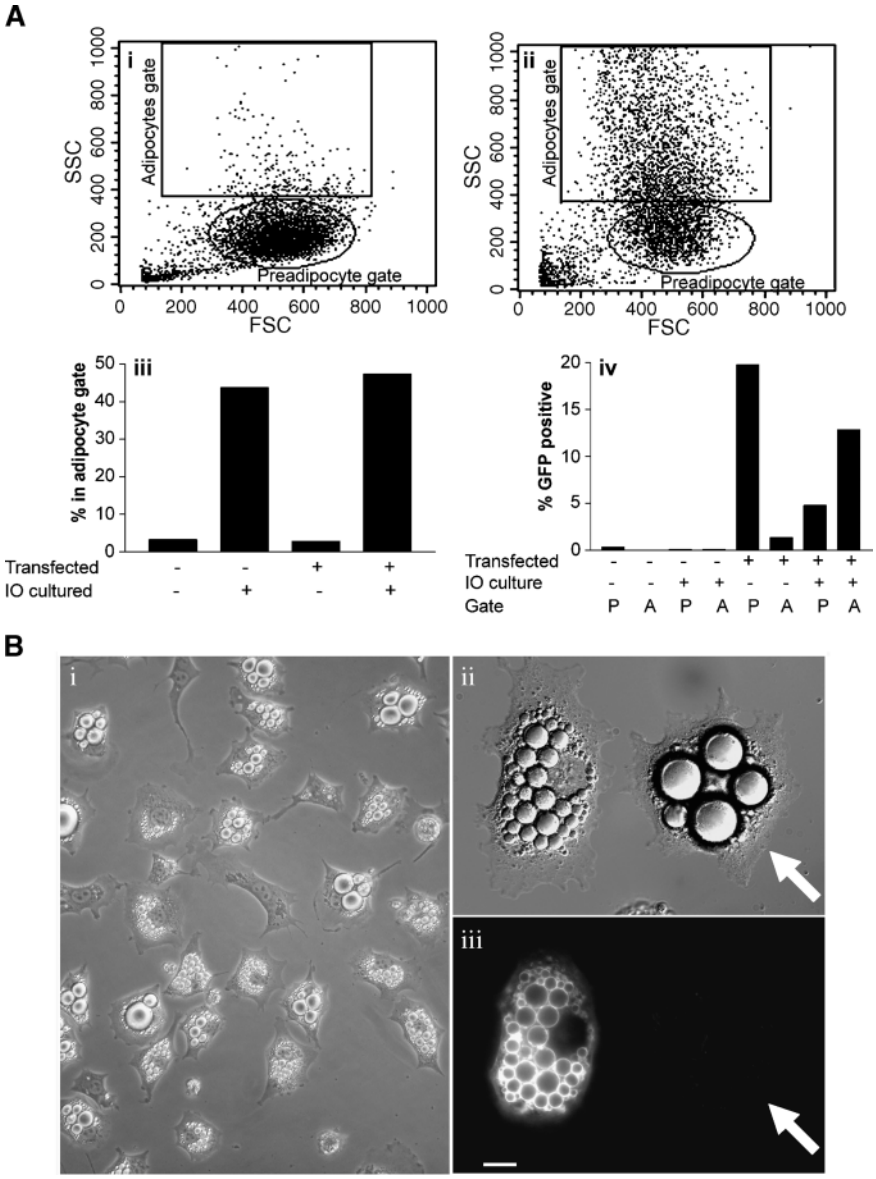

Fig. 1. OP9 adipocytes express transiently transfected perilipin tagged at its N terminus with enhanced green fluorescent protein (eGFP-perilipin). A: OP9 cells were trypsinized from culture dishes and analyzed by flow cytometry. In panels i and ii, side scatter (SSC) is plotted on the y axis and forward scatter (FSC) is plotted on the x axis for preparations of untransfected OP9 cells that had either not received (panel i) or had received (panel ii) adipogenic stimuli with insulin oleate (IO) medium for 36 h. Panel iii shows the number of events that are in the adipocyte gate (rectangle) from cell preparations treated as indicated. Panel iv shows the number of events that fall into either the preadipocyte gate (oval; P) or the adipocyte gate (A) from cell preparations treated as indicated. Approximately 10,000 events were counted, and of those, ~9,000 fell into either the preadipocyte or the adipocyte gate. B: OP9 cells were transiently transfected with an expression plasmid for eGFP-perilipin and differentiated into adipocytes by incubation in IO medium for 36 h, and then live cells were imaged. Panel i shows a low-magnification differential interference contrast image of a field of these cells. Panel ii shows a high-magnification differential interference contrast image of two adipocytes. Panel iii shows the green channel of the same field as panel ii. The arrows point to an untransfected adipocyte. Bar = 10 μm.

ditionally used to differentiate 3T3-L1 cells. ADC treatment causes OP9 cells to accumulate similar amounts of TAG as 3T3-L1 cells (**Fig. 2A**). Like 3T3-L1 cells, OP9 cells must be confluent for ADC culturing to cause robust differentiation; preconfluent ADC-treated OP9 cells fail to accumulate TAG and do not assume the appearance of adipocytes (data not shown). In addition, like 3T3-L1 cells, ADC-differentiated OP9 cells form a thick mat of adipocytes that is not conducive to microscopy. Unlike 3T3-L1 adipocytes, which can be dispersed by trypsinization, replated, and effectively imaged by microscopy (13, 14), we were unable to disperse the mat of OP9 adipocytes by trypsinization. Thus, we were unable to take informative images of OP9 adipocytes that had been differentiated by the ADC method.

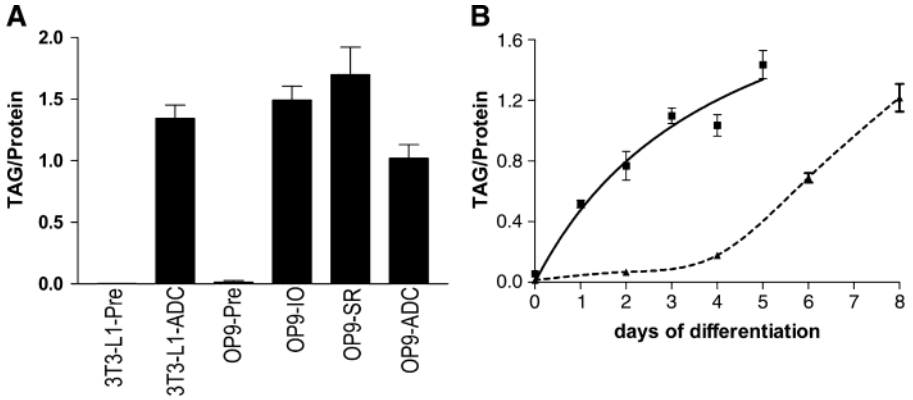

Fig. 2. Culturing of OP9 cells in adipogenic medium causes OP9 cells to accumulate large triacylglycerol (TAG) stores. A: TAG and protein were measured as described in Experimental Procedures, and the TAG/protein ratio was determined in cells treated as follows: 3T3-Ll-Pre, 3T3-L1 cells were harvested before they became confluent (n = 9); 3T3-L1-ADC, 3T3-L1 adipocytes were harvested 8 d after differentiation was initiated with DM1 medium (n = 4); OP9-Pre, OP9 cells were harvested before they became confluent (n = 4); OP9-IO, OP9 adipocytes were harvested 3 d after initiation of differentiation with IO medium (n = 5); OP9-SR, OP9 adipocytes were harvested 5 d after initiation of differentiation with serum replacement (SR) medium (n = 9); OP9-ADC, OP9 adipocytes were harvested 8 d after initiation of differentiation with DM1 medium (n = 6). B: OP9 and 3T3-L1 cells were differentiated for the times indicated by the SR and adipogenic cocktail (ADC) methods, respectively. The TAG/protein ratios of the OP9 cells are shown as a solid line with closed squares, and the TAG/protein ratios of the 3T3-L1 cells are shown as a dashed line with closed triangles. Each symbol represents three dishes for OP9 cells and four dishes for 3T3-L1 cells. Error bars indicate SEM.

The second method of OP9 differentiation, SR, uses a chemically defined, commercially available, insulin-rich SR instead of fetal bovine serum and does not have added dexamethasone or 3-isobutyl-1-methylxanthine. SR-cultured OP9 cells accumulate TAG more rapidly than ADC-treated 3T3-L1 cells (Fig. 2B) and assume the appearance of adipocytes with perilipin-coated lipid droplets (**Fig. 3B, E**). The kinetics of TAG accumulation in

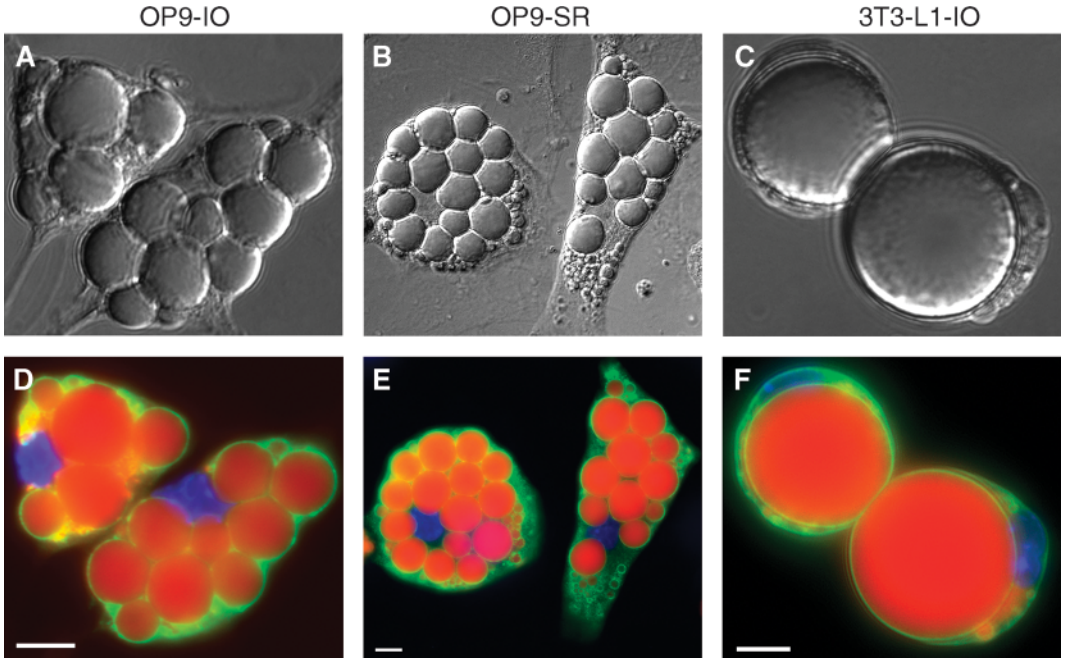

Fig. 3. OP9 cells can be induced to form large perilipin-coated droplets. OP9 cells and 3T3-L1 cells were differentiated, labeled with red fluorescent fatty acids, and stained for immunofluorescence microscopy with guinea pig perilipin antiserum, as described in Experimental Procedures. A–C show differential interference contrast images. D–F show perilipin staining in green and fatty acid labeling in red. OP9 cells were cultured in IO medium for 3 d (OP9-IO) or in SR medium for 4 d (OP9-SR). 3T3-L1 cells were cultured in IO medium for 5 d (3T3-L1-IO). Bars = 10 μm.

SR-treated OP9 cells is remarkable in that within 24 h the TAG/protein ratio increases by >10-fold (Fig. 2B). Moreover, SR-treated OP9 cells accumulate as much TAG per unit of protein within 3 days as 3T3-L1 cells accumulate by 8 days of the standard treatment with ADC. SR culturing of 3T3-L1 cells results in only rare patches of adipocytes.

The third method of OP9 differentiation, IO, uses low serum conditions (0.2%) in MEM-α supplemented with 900 μM oleate and 175 nM insulin. In contrast to ADC differentiation, IO culturing efficiently differentiates preconfluent OP9 cells (Fig. 1B, panel i). A 3 day incubation in IO medium causes OP9 cells to accumulate at least as much TAG as 3T3-L1 cells at 8 d of ADC culturing (Fig. 2A). The IO method also induces adipocyte morphology, including round cell shape and nuclei displaced to the cell periphery by large perilipin-coated lipid droplets (Fig. 3A, D). For comparison, we treated preconfluent 3T3-L1 cells with IO medium. More than 90% of 3T3-L1 cells die when cultured in IO medium for 48 h, but OP9 cell death in IO medium is much less. However, the few surviving 3T3-L1 cells often display morphology similar to that of primary adipocytes, a large unilocular lipid droplet with a peripherally displaced nucleus. Up to 50% of IO-cultured OP9 cells have unilocular droplets, but the most common morphology is one of multiple droplets of >5–10 μm diameter with the nucleus displaced to the cell periphery. Notably, most IO-cultured OP9 cells contain numerous lipid droplets within 48 h of initiating differentiation, and many cells develop lipid droplets within 18 h (data not shown).

Both the IO medium and the SR medium have added insulin and NEFA but little (IO) or no (SR) serum. However, these two methods are qualitatively different. IO medium effectively differentiates subconfluent OP9 cells (Fig. 1B, panel i), whereas SR medium effectively differentiates confluent OP9 cells (**Figs. 4A, 5B**). SR culturing is more effective when cell density is high, whereas IO culturing is less effective as cell density increases (data not shown).

The SR method of OP9 differentiation was chosen for further characterization, because SR differentiation can rapidly produce large numbers of adipocytes, and SR adipocytes can be effectively imaged (Fig. 3B, E). The levels of the following four adipocyte marker proteins were examined because they play distinct roles in the core functions of mature adipocytes: *1*) GLUT4, an insulin-regulated glucose transporter that plays a role in systemic glucose homeostasis and is necessary for effective lipogenesis (21); *2*) adiponectin, an insulin-sensitizing adipokine (22); *3*) perilipin, which constitutively coats adipocyte lipid droplets, helps to conserve the TAG pool in nonlipolytic adipocytes, and organizes lipolysis in lipolytic adipocytes (12, 23–25); and *4*) S3-12, which packages nascent TAG (13, 14). OP9 adipocytes express adipocyte marker proteins at levels similar to 3T3-L1 adipocytes, but at earlier time points during differentiation (Fig. 4). OP9 cells have easily detectable levels of GLUT4, perilipin, and S3-12 proteins 48 h after reaching confluence (day 0), which is before the addition of SR medium.

Adipogenic potential of OP9 cells is persistent and resilient

3T3-L1 cells do not maintain the ability to differentiate into adipocytes robustly during extended times in culture or after extensive passaging. In our hands, the adipogenic potential of 3T3-L1 cells begins to wane between the 20th and 30th passage after initial plating. In contrast, we have maintained OP9 cells in culture for >100 passages with no decrease in the extent of adipocyte differentiation. Furthermore, 3T3-L1 cells do not maintain adipogenic potential if they are replated after having reached confluence. Maintaining OP9 cells at confluence does not limit their adipogenic potential after replating. In fact, we have observed that OP9 adipocyte differentiation is potentiated by maintaining the preadipocytes at high cell density.

OP9 cells have adipogenic transcription factors without exogenous adipogenic stimuli

We investigated by immunoblotting whether the accelerated expression of adipocyte marker proteins in OP9 cells is driven by the early expression of transcription factors known to promote adipogenesis (**Fig. 6**). OP9 cells that have been confluent for 48 h have high levels of PPARγ1, C/EBPα-p42, C/EBPβ-LAP, and SREBP-1, and

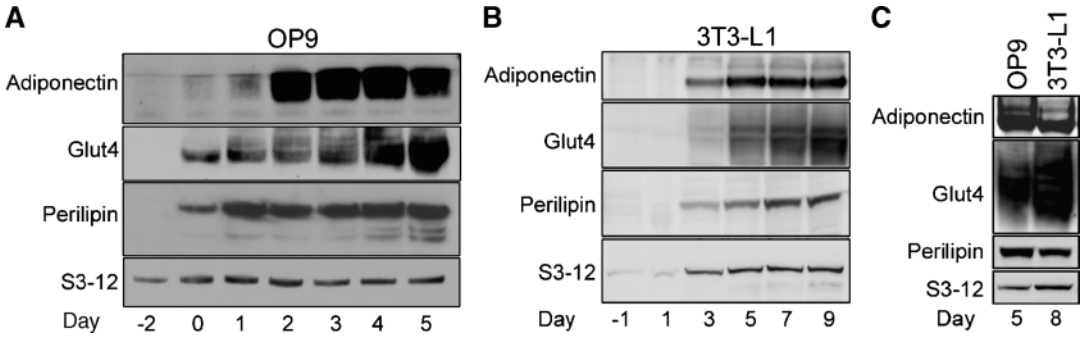

Fig. 4. OP9 cells can be induced to rapidly accumulate adipocyte markers. OP9 and 3T3-L1 cells were differentiated by the SR and ADC methods, respectively, as described in Experimental Procedures. On the days indicated, proteins were extracted and immunoblotted (30 μg/lane). Blots were probed with the rabbit antisera indicated at the following concentrations: adiponectin (1:1,000), GLUT4 (1:500), perilipin (1:1,000), and S3-12 (1:2,000).

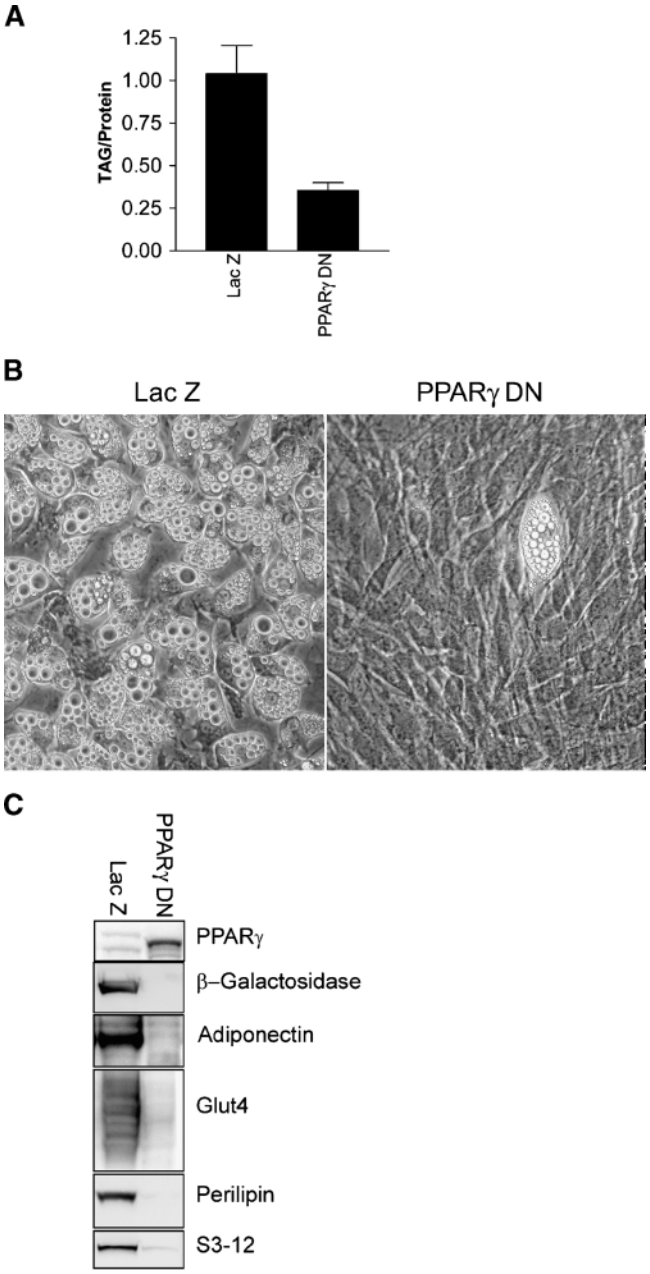

Fig. 5. Dominant negative (DN) peroxisome proliferator-activated receptor γ (PPARγ) mutant blocks OP9 cell adipogenesis. OP9 cells were transduced retrovirally with cDNAs for DN PPARγ or the LacZ gene (LacZ) and were SR cultured for 4 d. A: TAG and protein levels were measured for each stable cell line, and the TAG/protein ratio (mg/mg) was calculated (n = 5). The TAG/protein ratio in DN PPARγ cells was significantly less than that in LacZ cells ($P < 0.002$ by single-tailed Student's *t*-test with the assumption of equal variance). B: Five days after the initiation of differentiation, live LacZ and DN PPARγ OP9 cells were imaged in culture dishes by light microscopy. C: Immunoblots of proteins extracted from either LacZ or DN PPARγ OP9 cells that had been differentiated in SR medium for 5 d were probed with antibodies or antisera against PPARγ (400 ng/ml), β-galactosidase (660 ng/ml), adiponectin (1:1,000), glucose transporter 4 (GLUT4; 1:500), perilipin (1:1,000), and S3-12 (1:2,000).

the expression of PPARγ2 and C/EBPα-p30 increases rapidly during differentiation induced by SR culturing. Moreover, preconfluent OP9 cells express easily detectable levels of PPARγ1 and C/EBPα proteins, as assessed by immunoblotting of nuclear extracts (data not shown). In contrast, PPARγ RNA and C/EBPα protein isoforms are not detectable in confluent but undifferentiated 3T3-L1 cells (26, 27). These data suggest that OP9 cells are at a later stage in adipogenesis than 3T3-L1 cells.

PPARγ is necessary for OP9 differentiation into adipocytes

The early expression of PPARγ in OP9 cells suggests that the adipocyte differentiation program followed by these cells shares common mechanisms with the well-characterized 3T3-L1 model. To test this hypothesis, we generated OP9 cells that stably expressed either β-galactosidase or a DN PPARγ mutant (17). Retrovirus-mediated stable expression of β-galactosidase in OP9 cells has no significant effect on TAG accumulation, adipocyte morphology, or adipocyte marker protein expression (Fig. 5). In contrast, stable expression of the DN PPARγ in SR-treated OP9 cells inhibits the accumulation of TAG (Fig. 5A), stops all but rare cells from acquiring adipocyte morphology (Fig. 5B), and prevents the induction of adipocyte marker proteins (Fig. 5C). These observations suggest that OP9 adipocyte differentiation is through a PPARγ-dependent pathway. Note that there is one well-differentiated adipocyte in the DN PPARγ field (Fig. 5B). Such rare cells likely do not express the DN PPARγ mutant. We were unable to determine the extent of the viral infection of DN PPARγ by microscopy. However, >99% of OP9 cells infected in par-

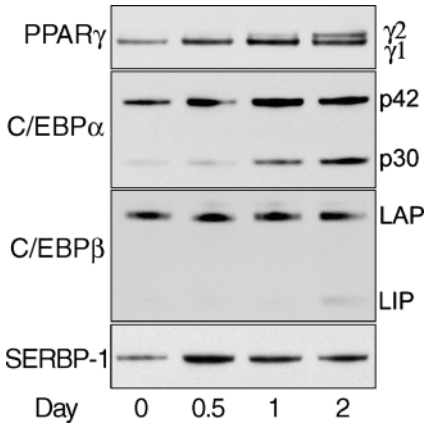

Fig. 6. OP9 cells express adipogenic transcription factors. OP9 cells were differentiated by the SR method. Nuclear and cytoplasmic fraction proteins were extracted at the times indicated after the initiation of differentiation and blotted as described in Experimental Procedures. Nuclear fraction proteins (20 μg/lane) were probed with antibodies to CCAAT/enhancer binding protein (C/EBP) α, C/EBPβ, and PPARγ, and cytoplasmic fraction proteins (35 μg/lane) were probed with an antibody to sterol-regulatory element binding protein-1 (SREBP-1); all antibodies were used at 400 ng/ml. γ1 and γ2 label isoforms of PPARγ. p42 and p30 label the 42 and 30 kDa isoforms, respectively, of C/EBPα. Liver-enriched activating protein (LAP) and liver-enriched inhibitory protein (LIP) label isoforms of C/EBPβ.

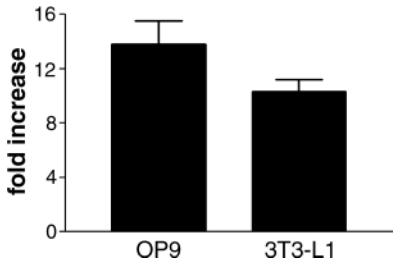

Fig. 7. Insulin stimulates 2-deoxyglucose uptake in OP9 adipocytes. OP9 cells and 3T3-L1 cells were differentiated by the SR and ADC methods, respectively. The resulting adipocytes were either untreated or treated with 100 nM insulin, and then 2-deoxyglucose uptake was measured. The columns show the fold increases in 2-deoxyglucose uptake in response to 100 nM insulin. Each column shows the average and standard error from three independent experiments.

allel with retrovirus particles that encoded β-galactosidase showed β-galactosidase activity. That OP9 cells infected with the control virus differentiated normally suggests that the failure of the DN PPARγ-expressing cells to differentiate is attributable to the expression of the DN PPARγ mutant and is not a general effect of viral infection.

Insulin causes OP9 cells to increase glucose uptake

Adipocytes, like skeletal muscle cells, express the insulin-responsive GLUT4. In mice, adipocyte-specific ablation of GLUT4 leads to insulin resistance in both liver and muscle (28), which demonstrates the importance of insulin-stimulated glucose transport in adipocytes for whole body fuel metabolism. Consistent with the precocious expression of adipocyte transcription factors and marker proteins and the robust accumulation of TAG, day 5 OP9 adipocytes are at least as insulin-responsive as day 8 3T3-L1 adipocytes (**Fig. 7**).

The rapid differentiation of OP9 cells into adipocytes allows proteins expressed from transiently transfected DNA to be detected in adipocytes

The detection of proteins expressed from transiently transfected DNA has not been feasible in 3T3-L1 adipocytes, because there are 10–14 days between when cells are preconfluent and thus transfectable and when the cells become well-differentiated adipocytes (8, 29). During this prolonged period, expression of the transiently expressed RNA or protein wanes. We investigated whether the unique features of OP9 cells would permit the efficient detection of such transiently expressed proteins. To this end, we used a recently described quantitative assay for adipocyte differentiation (30). A conspicuous characteristic of adipocytes is numerous large lipid droplets. Cells with numerous large lipid droplets diffract more light, which is manifested in flow cytometry as increased side scatter (SSC). This lipid droplet-induced shift in SSC can be used to identify adipocytes by flow cytometry (30). As expected, 36 h of IO culturing induced OP9 cells to form large perilipin-coated lipid droplets and to undergo a large increase in SSC compared with preconfluent OP9 cells (Fig. 1A, panels i, ii, and iii). This shift is not appreciably changed by transfection of the cells before differentiation (Fig. 1A, panel iii). When undifferentiated OP9 cells are evaluated by flow cytometry for forward scatter and SSC, most of the events fall into a discreet region labeled the "preadipocyte gate" (Fig. 1A, panel i, oval). IO treatment of OP9 cells shifts the events into a region with increased SSC labeled the "adipocyte gate" (Fig. 1A, panels i and ii, rectangle). That there is very little overlap in SSC between undifferentiated and IO-cultured OP9 cells illustrates the dramatic effect that IO culturing has on OP9 cells (Fig. 1A, panels i and ii). These data are consistent with the morphologic observation that most IO-cultured OP9 cells have numerous large lipid droplets (Fig. 1B, panel i).

3T3-L1 preadipocytes are difficult to transfect with naked DNA (31). Investigators have resorted to more cumbersome and cell-toxic methods, such as electroporation or adenoviral transduction (32). Using flow cytometry, we investigated the efficiency of transfection of OP9 cells with an expression plasmid encoding perilipin tagged at its N terminus with enhanced green fluorescent protein (eGFP-perilipin). Preconfluent OP9 adipocytes were transiently transfected with eGFP-perilipin and then cultured in IO medium for 36 h. Flow cytometry indicated that 19 ± 4% (n = 11) of the transfected OP9 cells had increased fluorescence over untreated cells. Furthermore, in the cell preparation that was both transfected and IO-cultured, ~13% of the total events both were in the adipocyte gate and expressed eGFP-perilipin (Fig. 1A, panel iv). These events represented OP9 adipocytes that expressed the transiently transfected protein. Fluorescence microscopy showed that many IO-cultured OP9 cells that expressed eGFP-perilipin contained numerous lipid droplets that were of uniform size and were surrounded by green fluorescence. A representative cell is shown in Fig. 1B, panel iii.

DISCUSSION

OP9 mouse stromal cells represent a new model of adipocyte differentiation that will be useful for further studies of the mechanisms of differentiation and of mature adipocyte function. We have demonstrated that OP9 cells can be induced to accumulate TAG rapidly, to form numerous, large lipid droplets, to express adipocyte marker proteins (adiponectin, GLUT4, perilipin, and S3-12), and to display robust insulin-stimulated glucose transport. Moreover, by 2 days of confluence, OP9 cells express easily detectable levels of transcription factors that have been implicated in adipocyte differentiation (PPARγ, C/EBPα and C/EBPβ, and SREBP-1). OP9 cells recapitulate common mechanisms of adipocyte differentiation, as suggested by the failure of OP9 cells to differentiate into adipocytes in the context of stable expression of the DN PPARγ mutant.

Lane and Tang (33) have proposed a model of adipogenesis in which multipotent stem cells first commit to the adipocyte lineage to become preadipocytes and then differentiate into "late-stage" preadipocytes before terminal differentiation. In this schema, preconfluent OP9

cells likely represent late-stage preadipocytes, because they already express PPARγ1 and C/EBPα proteins and because they can differentiate into adipocytes within 48 h.

Several practical features of OP9 cells make this cell line a particularly convenient model for adipocyte studies. First, OP9 adipocytes can be rapidly produced from either preconfluent cells (IO method) or confluent cells (SR and ADC methods). Second, in contrast to 3T3-L1 cells, OP9 cells can be maintained at high density without loss of potential to differentiate into adipocytes. Third, OP9 cells continue to differentiate well even at high passage number. These features permit large numbers of OP9 cells to be conveniently maintained in culture and OP9 adipocytes to be produced for experiments within a few days.

3T3-L1 cells are of limited utility for the generation of cell lines for the stable expression of proteins or RNAs. 3T3-L1 cells must be maintained at low density. Therefore, it is cumbersome to subject large numbers of 3T3-L1 cells to the DNA transduction procedure; this limits 3T3-L1 DNA transduction procedures to only the most efficient methods. Because 3T3-L1 cells only differentiate well for a limited number of passages, stable 3T3-L1 cell lines may not maintain the capacity for robust adipocyte differentiation. OP9 cells can be kept in culture at high density and continue to differentiate well at high passage numbers and after months in continuous culture; thus, OP9 cells do not have these limitations. As an illustration, OP9 cells that stably express a scrambled small interfering RNA have been generated, and these cells maintain the ability to differentiate into adipocytes, as shown by TAG storage and gene expression (S. E. Gale, A. Frolov, X. Han, P. Bickel, L. Cao, A. Bowcock, J. E. Schaffer, and D. S. Ory, unpublished data).

Transient transfection is a powerful tool for the initial exploration of the function and intracellular location of proteins (34, 35). This technique is particularly powerful if the effect can be seen morphologically, because the untransfected cells in the same field serve as a negative control. This concept is demonstrated in Fig. 1B, panels ii and iii, in which OP9 adipocytes that express eGFP-perilipin form smaller and more uniform lipid droplets than cells that do not express eGFP-perilipin. Furthermore, transient transfection also can be used to confirm the biochemical effects of a stably expressed protein. This may be important even when dealing with pools of cells that stably express a protein, because the number of copies and the position of integration of the genes vary between the control and experimental pools. These differences are potentially confounding. Finally, overexpression of some proteins interferes with cellular function to such an extent that it is difficult to generate stable cell lines. For these reasons, the ability to express readily detectable levels of exogenous proteins by transient transfection in OP9 adipocytes is potentially valuable.

OP9 cells were derived from the calvaria of a mouse that lacked functional M-CSF (9). Both the site of extraction and the genetics of the mouse of origin have implications for the adipogenic phenotype of these cells. The site of extraction suggests that OP9 cells were destined to be bone marrow adipocytes. M-CSF is a cytokine that promotes the clonal growth and differentiation of cells of the monocyte-macrophage lineage, and M-CSF deficiency leads to bone overgrowth as a result of the failure of osteoclast maturation (36, 37). M-CSF has also been reported to promote adipocyte hyperplasia when locally overexpressed in rabbit subcutaneous adipose tissue (38). Both M-CSF and its receptor are expressed in human subcutaneous adipocytes (38). Obesity is associated with the infiltration of adipose tissue by macrophages, and these infiltrating macrophages release factors that affect adipocytes and adipogenesis (39). It remains to be determined whether the lack of M-CSF affects OP9 adipocyte differentiation positively, negatively, or not at all. There are at least three independent reports of marrow-derived cells isolated from wild-type mice that differentiate into adipocytes upon hormone treatment (40–42).

The standard model of adipocyte differentiation is that preadipocytes must undergo growth arrest followed by mitotic clonal expansion (43), but the necessity of clonal expansion has been questioned (44–46). Mitotic clonal expansion does not appear to occur during IO culture of OP9 or 3T3-L1 cells. Large perilipin-coated lipid droplets that are characteristic of mature adipocytes begin to appear after ~18 h of IO culture, and cell number decreases during IO culture. These observations are consistent with mitotic clonal expansion being dispensable for adipocyte differentiation, but definitive resolution of this important question requires further study.

The rapid and profound effects of IO medium on both OP9 and 3T3-L1 cell morphology suggest an important role for long-chain fatty acids in the promotion of adipocyte differentiation, as has been suggested recently for chicken adipocytes (47). Whether the oleate in the IO medium leads to the generation of ligands for adipocyte transcription factors such as PPARs or acts via other mechanisms remains to be investigated. Given that adipocytes are the primary storage site for long-chain fatty acids and that the storage capacity of each adipocyte is finite, long-chain fatty acids may promote the differentiation of preadipocytes as an adaptive response to increase the overall size of the depot.

The OP9 adipocyte model has great potential to accelerate critically important areas of metabolism research. The robust insulin-stimulated glucose transport displayed by OP9 adipocytes makes this model particularly promising for studies of GLUT4 trafficking and its regulation. We may not have optimized every protocol for OP9 adipocyte differentiation and manipulation. However, the use of OP9 cells by the adipocyte research community should lead to improved methods as each laboratory modifies the procedures we have reported.

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REFERENCES

1. Drevon, C. A. 2005. Fatty acids and expression of adipokines. *Biochim. Biophys. Acta.* **1740:** 287–292.
2. Rajala, M. W., and P. E. Scherer. 2003. Minireview. The adipocyte—at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology.* **144:** 3765–3773.
3. Green, H., and O. Kehinde. 1974. Sublines of mouse 3T3 cells that accumulate lipid. *Cell.* **1:** 113–116.
4. Green, H., and O. Kehinde. 1975. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. *Cell.* **5:** 19–27.
5. Green, H., and M. Meuth. 1974. An established pre-adipose cell line and its differentiation in culture. *Cell.* **3:** 127–133.
6. Scherer, P. E., S. Williams, M. Fogliano, G. Baldini, and H. F. Lodish. 1995. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J. Biol. Chem.* **270:** 26746–26749.
7. Steppan, C. M., S. T. Bailey, S. Bhat, E. J. Brown, R. R. Banerjee, C. M. Wright, H. R. Patel, R. S. Ahima, and M. A. Lazar. 2001. The hormone resistin links obesity to diabetes. *Nature.* **409:** 307–312.
8. Student, A. K., R. Y. Hsu, and M. D. Lane. 1980. Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. *J. Biol. Chem.* **255:** 4745–4750.
9. Nakano, T., H. Kodama, and T. Honjo. 1994. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science.* **265:** 1098–1101.
10. Pajvani, U. B., M. Hawkins, T. P. Combs, M. W. Rajala, T. Doebber, J. P. Berger, J. A. Wagner, M. Wu, A. Knopps, A. H. Xiang, et al. 2004. Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. *J. Biol. Chem.* **279:** 12152–12162.
11. Haney, P. M., J. W. Slot, R. C. Piper, D. E. James, and M. Mueckler. 1991. Intracellular targeting of the insulin-regulatable glucose transporter (GLUT4) is isoform specific and independent of cell type. *J. Cell Biol.* **114:** 689–699.
12. Brasaemle, D. L., B. Rubin, I. A. Harten, J. Gruia-Gray, A. R. Kimmel, and C. Londos. 2000. Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. *J. Biol. Chem.* **275:** 38486–38493.
13. Wolins, N. E., J. R. Skinner, M. J. Schoenfish, A. Tzekov, K. G. Bensch, and P. E. Bickel. 2003. Adipocyte protein S3-12 coats nascent lipid droplets. *J. Biol. Chem.* **278:** 37713–37721.
14. Wolins, N. E., B. K. Quaynor, J. R. Skinner, M. J. Schoenfish, A. Tzekov, and P. E. Bickel. 2005. S3-12, adipophilin, and TIP47 package lipid in adipocytes. *J. Biol. Chem.* **280:** 19146–19155.
15. Brasaemle, D. L., G. Dolios, L. Shapiro, and R. Wang. 2004. Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. *J. Biol. Chem.* **279:** 46835–46842.
16. Coleman, R., and R. M. Bell. 1976. Triacylglycerol synthesis in isolated fat cells. Studies on the microsomal diacylglycerol acyltransferase activity using ethanol-dispersed diacylglycerols. *J. Biol. Chem.* **251:** 4537–4543.
17. Gurnell, M., J. M. Wentworth, M. Agostini, M. Adams, T. N. Collingwood, C. Provenzano, P. O. Browne, O. Rajanayagam, T. P. Burris, J. W. Schwabe, et al. 2000. A dominant-negative peroxisome proliferator-activated receptor gamma (PPARgamma) mutant is a constitutive repressor and inhibits PPARgamma-mediated adipogenesis. *J. Biol. Chem.* **275:** 5754–5759.
18. Ory, D. S., B. A. Neugeboren, and R. C. Mulligan. 1996. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. *Proc. Natl. Acad. Sci. USA.* **93:** 11400–11406.
19. Garcia de Herreros, A., and M. J. Birnbaum. 1989. The regulation by insulin of glucose transporter gene expression in 3T3 adipocytes. *J. Biol. Chem.* **264:** 9885–9890.
20. Yamane, T., T. Kunisada, and S.-I. Hayashi. 2002. Embryonic stem cells as a model for studying osteoclast lineage development. *In* Embryonic Stem Cells: Methods and Protocols. K. Turksen, editor. Humana Press, Totowa, NJ. 97–106.
21. Tozzo, E., P. R. Shepherd, L. Gnudi, and B. B. Kahn. 1995. Transgenic GLUT-4 overexpression in fat enhances glucose metabolism: preferential effect on fatty acid synthesis. *Am. J. Physiol.* **268:** E956–E964.
22. Pajvani, U. B., and P. E. Scherer. 2003. Adiponectin: systemic contributor to insulin sensitivity. *Curr. Diab. Rep.* **3:** 207–213.
23. Sztalryd, C., G. Xu, H. Dorward, J. T. Tansey, J. A. Contreras, A. R. Kimmel, and C. Londos. 2003. Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. *J. Cell Biol.* **161:** 1093–1103.
24. Tansey, J. T., C. Sztalryd, J. Gruia-Gray, D. L. Roush, J. V. Zee, O. Gavrilova, M. L. Reitman, C. X. Deng, C. Li, A. R. Kimmel, et al. 2001. Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. *Proc. Natl. Acad. Sci. USA.* **98:** 6494–6499.
25. Martinez-Botas, J., J. B. Anderson, D. Tessier, A. Lapillonne, B. H. Chang, M. J. Quast, D. Gorenstein, K. H. Chen, and L. Chan. 2000. Absence of perilipin results in leanness and reverses obesity in Lepr(db/db) mice. *Nat. Genet.* **26:** 474–479.
26. Chawla, A., E. J. Schwarz, D. D. Dimaculangan, and M. A. Lazar. 1994. Peroxisome proliferator-activated receptor (PPAR) gamma: adipose-predominant expression and induction early in adipocyte differentiation. *Endocrinology.* **135:** 798–800.
27. Lin, F. T., O. A. MacDougald, A. M. Diehl, and M. D. Lane. 1993. A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity. *Proc. Natl. Acad. Sci. USA.* **90:** 9606–9610.
28. Abel, E. D., O. Peroni, J. K. Kim, Y. B. Kim, O. Boss, E. Hadro, T. Minnemann, G. I. Shulman, and B. B. Kahn. 2001. Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature.* **409:** 729–733.
29. Frost, S. C., and M. D. Lane. 1985. Evidence for the involvement of vicinal sulfhydryl groups in insulin-activated hexose transport by 3T3-L1 adipocytes. *J. Biol. Chem.* **260:** 2646–2652.
30. Lee, Y. H., S. Y. Chen, R. J. Wiesner, and Y. F. Huang. 2004. Simple flow cytometric method used to assess lipid accumulation in fat cells. *J. Lipid Res.* **45:** 1162–1167.
31. Orlicky, D. J., and J. Schaack. 2001. Adenovirus transduction of 3T3-L1 cells. *J. Lipid Res.* **42:** 460–466.
32. Bereziat, V., S. Moritz, B. Klonjkowski, A. Decaudain, M. Auclair, M. Eloit, J. Capeau, and C. Vigouroux. 2005. Efficient adenoviral transduction of 3T3-F442A preadipocytes without affecting adipocyte differentiation. *Biochimie.* **87:** 951–958.
33. Lane, M. D., and Q. Q. Tang. 2005. From multipotent stem cell to adipocyte. *Birth Defects Res. A Clin. Mol. Teratol.* **73:** 476–477.
34. Caplan, S., N. Naslavsky, L. M. Hartnell, R. Lodge, R. S. Polishchuk, J. G. Donaldson, and J. S. Bonifacino. 2002. A tubular EHD1-containing compartment involved in the recycling of major histocompatibility complex class I molecules to the plasma membrane. *EMBO J.* **21:** 2557–2567.
35. Caplan, S., L. M. Hartnell, R. C. Aguilar, N. Naslavsky, and J. S. Bonifacino. 2001. Human Vam6p promotes lysosome clustering and fusion in vivo. *J. Cell Biol.* **154:** 109–122.
36. Dobbins, D. E., R. Sood, A. Hashiramoto, C. T. Hansen, R. L. Wilder, and E. F. Remmers. 2002. Mutation of macrophage colony stimulating factor (Csf1) causes osteopetrosis in the tl rat. *Biochem. Biophys. Res. Commun.* **294:** 1114–1120.
37. Kodama, H., A. Yamasaki, M. Nose, S. Niida, Y. Ohgame, M. Abe, M. Kumegawa, and T. Suda. 1991. Congenital osteoclast deficiency in osteopetrotic (op/op) mice is cured by injections of macrophage colony-stimulating factor. *J. Exp. Med.* **173:** 269–272.
38. Levine, J. A., M. D. Jensen, N. L. Eberhardt, and T. O'Brien. 1998.

Adipocyte macrophage colony-stimulating factor is a mediator of adipose tissue growth. *J. Clin. Invest.* **101:** 1557–1564.
39. MacDougald, O. A., and S. Mandrup. 2002. Adipogenesis: forces that tip the scales. *Trends Endocrinol. Metab.* **13:** 5–11.
40. Gimble, J. M., M. A. Dorheim, Q. Cheng, K. Medina, C. S. Wang, R. Jones, E. Koren, C. Pietrangeli, and P. W. Kincade. 1990. Adipogenesis in a murine bone marrow stromal cell line capable of supporting B lineage lymphocyte growth and proliferation: biochemical and molecular characterization. *Eur. J. Immunol.* **20:** 379–387.
41. Kodama, H. A., Y. Amagai, H. Koyama, and S. Kasai. 1982. Hormonal responsiveness of a preadipose cell line derived from newborn mouse calvaria. *J. Cell. Physiol.* **112:** 83–88.
42. Ding, J., K. Nagai, and J. T. Woo. 2003. Insulin-dependent adipogenesis in stromal ST2 cells derived from murine bone marrow. *Biosci. Biotechnol. Biochem.* **67:** 314–321.
43. MacDougald, O. A., and M. D. Lane. 1995. Transcriptional regulation of gene expression during adipocyte differentiation. *Annu. Rev. Biochem.* **64:** 345–373.
44. Cho, Y. C., and C. R. Jefcoate. 2004. PPARgamma1 synthesis and adipogenesis in C3H10T1/2 cells depends on S-phase progression, but does not require mitotic clonal expansion. *J. Cell. Biochem.* **91:** 336–353.
45. Entenmann, G., and H. Hauner. 1996. Relationship between replication and differentiation in cultured human adipocyte precursor cells. *Am. J. Physiol.* **270:** C1011–C1016.
46. Qiu, Z., Y. Wei, N. Chen, M. Jiang, J. Wu, and K. Liao. 2001. DNA synthesis and mitotic clonal expansion is not a required step for 3T3-L1 preadipocyte differentiation into adipocytes. *J. Biol. Chem.* **276:** 11988–11995.
47. Matsubara, Y., K. Sato, H. Ishii, and Y. Akiba. 2005. Changes in mRNA expression of regulatory factors involved in adipocyte differentiation during fatty acid induced adipogenesis in chicken. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **141:** 108–115.