Abundance of two human preadipocyte subtypes with distinct capacities for replication, adipogenesis, and apoptosis varies among fat depots

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methods

human subjects. Fat tissue was resected during gastric bypass surgery for management of obesity from 18 subjects who had given informed consent. The protocol was approved by the Boston University Medical Center and Mayo Clinic Institutional Review Boards for Human Research. All subjects had fasted at least 10 h. Four of the subjects were men and 14 were women. Subjects were 43 ± 3 yr of age (means ± SE; range 29–57 yr). The subjects’ mean body mass index (BMI) was 52 ± 2 kg/m². Subjects with malignancies were excluded. No subjects were taking TZDs or steroids. None had fasting plasma glucose concentrations over 120 mg%. One to 10 g of abdominal subcutaneous (external to the fascia superficialis), mesenteric (colonic appendicies epiploices), and greater omental fat were obtained from each subject.

Preadipocyte culture. Fat tissue was minced and then digested in Hank’s balanced salt solution (HBSS) containing 1 mg/ml collagenase and 0.05% EDTA in a 37°C shaking water bath until fragments were no longer visible and the digest had a milky appearance. Digests were filtered and centrifuged at 800 g for 10 min. The digests were treated with an erythrocyte lysis buffer (37, 81). The cells were then plated using a low-serum plating medium [1:1 Dulbecco’s modified Eagle’s medium (DMEM)-Ham’s F-12 that contained 0.5% bovine serum and antibiotics]. After 12 h, a period during which no replication occurs (21), the adherent preadipocytes were washed extensively, trypsinized, and replated at a density of 4 × 10⁴ cells/cm² in plating medium. Replating helps to eliminate mesothelial cell and macrophage contamination. Linearity of preadipocyte recovery has been shown previously using this approach (51). Macrophages were rare (<5 × 10⁶ cells as assessed by phase contrast microscopy) in the replated cultures, irrespective of depot origin. In previous studies (79), we cloned cells from similarly prepared replated primary cultures and treated the resulting colonies with differentiation-inducing medium. The proportion of clones that contained cells capable of more extensive lipid accumulation than lung fibroblasts or other nonpreadipocyte cell types was determined. Essentially 100% of the clones prepared from the replated primary cultures accumulated lipid more extensively than nonpreadipocyte cell types, confirming the purity of our preadipocyte preparations. Plating medium was changed every 2 days until confluence.

Measurement of replication. To measure the replicative potential of preadipocyte populations, crystal violet and bromodeoxyuridine (BrdU) methods were used. For both methods, preadipocytes that had been subcultured three times at a 1:2 split ratio were plated at a density of 1.5 × 10⁵ cells/cm². For crystal violet staining, after 24 h, the nuclei of cells in triplicate wells were fixed in 1% glutaraldehyde, stained with crystal violet, dissolved in Triton X-100, and measured spectrophotometrically (87). After a further 48 h, crystal violet staining intensity was measured in a parallel set of triplicate wells and the fold increase in crystal violet staining intensity calculated. In yet another parallel triplicate set of wells, cells were labeled with BrdU 24 h after initial plating (BrdU Cell Proliferation ELISA kit; Roche Molecular Biochemicals, Pleasanton, CA). The extent of BrdU labeling was determined after a further 24 h. Both methods yielded similar results and were validated beforehand by comparison with cell numbers determined with a Coulter counter.

To determine the proportion of clones preadipocytes, cells in 96-well plates were maintained in plating medium without phenol. Medium was aspirated, and 100 μl of a lysis buffer (0.5 N NH₄OH, 0.1% Triton X-100) were added to each well. The plates were shaken and frozen at −80°C for ≥16 h to ensure complete lysis, which was confirmed by microscopic evaluation of cells using Trypan blue dye exclusion (26). The plates were thawed at room temperature, and 50 μl of lysis buffer and 50 μl of a 2× stock of CyQuant dye (Molecular Probes, Eugene, OR) were added to each well. Contents of the wells were mixed and incubated for 10 min at room temperature in the dark. Flocculence measurements at excitation and emission wavelengths of 485 and 535 nm, respectively, were compared with standard curves using known concentrations of DNA. In preliminary studies, the validity of this method was confirmed by direct cell counting (as in Refs. 48 and 50). CyQuant staining intensity varied linearly with cell number across a range of 50 to >50,000 cells.

coculture. Abdominal subcutaneous preadipocytes (test cells) were seeded in 24-well plates at 10⁵ cells/cm² (10% of confluent density after correction for plating efficiency). Abdominal subcutaneous, mesenteric, and omental preadipocytes isolated in parallel from three subjects were seeded in transwell inserts on semipermeable membranes at 6 × 10⁴ cells/cm². The inserts were placed in the wells containing the test cells. After 3 days, the number of test cells in the wells was determined with a Coulter counter.

Preadipocyte cloning. Preadipocytes were isolated as described above and were plated at a density of 50 cells/96-well plate in plating medium containing 10% FBS and a total of 4 mM glutamine. At this density, the probability of any one well’s being seeded by more than one cell is <2% (48, 86). Indeed, no more than one cell was found in any of the experiments 48 h after preadipocytes were seeded. After 2 wk, colonies were evident, and by 3 wk some were near confluence. In some experiments, after 14 days the clones were exposed to the differentiation-inducing medium described below. The percentage of clones that contained at least one differentiated cell was determined by observers who did not know the fat depot origin of the cultures. A differentiated cell was considered to be one that contained doubly refractile inclusions visible by low-power phase contrast microscopy, as previously described (48, 79, 86). The lipid nature of these inclusions was confirmed by staining with Oil red O. Human lung fibroblasts cultured under identical conditions did not develop such large lipid inclusions after ≤60 days of treatment with differentiation-inducing medium.

Preadipocyte differentiation. For differentiation, a previously published method (38) was used with modifications that included the following. Preadipocytes were treated for 2–3 wk, as indicated in RESULTS and the figure and table legends, with plating medium (without serum) enriched with 100 nM dexamethasone, 500 nM human insulin, 200 pM triiodothyronine, 0.5 μM rosiglitazone, anti-insulin, and 540 μM isobutylmethylxanthine (removed after 2 days). In preliminary studies, higher rosiglitazone and insulin concentrations did not further enhance differentiation of subcutaneous, mesenteric, or omental preadipocytes. Medium was changed weekly.

telomerase transfection. Preadipocytes were isolated from a 44-yr-old female subject (BMI 77, fasting glucose 100). After cells had undergone seven population doublings, they were transfected with the plasmid pBABE-hTERT-Hygro (13), as previously reported (78). This plasmid expresses the human telomerase catalytic component driven by the Moloney murine leukemia virus long terminal repeat promoter and a hygromycin resistance sequence driven by the SV40 promoter. hTERT expression was confirmed in hygromycin-resistant clones by RT-PCR analysis and activity using a PCR-based TRAP protocol (46) with a kit (TRAPeze; Intergen, Purchase, NY).

karyotyping. Twenty microliters of colcemide were added to preadipocytes in T-75 flasks at 60–70% confluence for 2 h at 37°C. Cells were washed with HBSS solution, trypsinized, and washed again with plating medium. Plating medium was removed, and 20 ml of 0.075 M KCl were added for 20 min at 37°C. The hypotonic KCl solution was removed, and cells were fixed in methanol and acetic acid (66).

apoptotic index. Cells were stained with bisbenzamide and examined using flocculence microscopy by observers unaware of the depot origin or treatment of the cells. Cells were classified as apoptotic if they exhibited irregular nuclear condensation (15). The apoptotic index was the percentage of such nuclei as a function of all of the nuclei in a field.

confocal immunofluorescence studies. Preadipocyte clones were exposed to differentiation medium for 3 wk. The clones were washed twice with phosphate-buffered saline (PBS), fixed in 1% paraformaldehyde for 20 min, and washed again with water. The clones were
incubated for 10 min in DAKO serum-free protein blocking buffer (cat. no. X0909; DAKO, Carpinteria, CA) and 0.1% saponin (cat. no. S-7900; Sigma Chemical, St. Louis, MO). Next, the clones were incubated for 1 h in 50 µl of a 1:37.5 dilution of rabbit polyclonal anti-C/EBPα antibody (cat. no. sc-61; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature, washed in PBS, and labeled using a 1:200 dilution of a chicken anti-rabbit antibody conjugated with Alexa Fluor 488 fluorochrome (cat. no. A-21441; Molecular Probes). After a final wash with PBS, images of the labeled cells were immediately collected on an LSM510 confocal system (Carl Zeiss, Oberkochen, Germany) equipped with an Axiovert 100M inverted microscope and an LD-Acrophan 40×/0.6NA objective lens. Excitation was by the 488 nm line of an argon/krypton laser. Emission was detected above 505 nm. Clones were then counterstained with bisbenzamide, and images of the stained nuclei were acquired using a laser for UV-excited dyes. The sizes of 150 abdominal subcutaneous clones were determined by counting stained nuclei with KS400 imaging software (Carl Zeiss). C/EBPα expression was analyzed in the 40 largest and 40 smallest colonies. The proportion of C/EBPα-positive cells within colonies was determined, as were cell nuclear areas, mean fluorescence intensities, and integral nuclear fluorescence.

Statistical analysis. Results are means ± SE and significance determination was by t-tests or ANOVA with appropriate post hoc comparisons (43, 45). Two-tailed P < 0.05 was considered significant. Statistical analyses of digestion time, preadipocyte recovery, and proportions of cells that accumulated lipid were done using applied regression analysis (56). We estimated the likelihood that there is more than one preadipocyte subtype, each differing in replicative capacity (number of population doublings undergone by a colony arising from a single cell), within cell populations. To do so, we used the Hormone Pulsatility Analysis Software from the University of Virginia Center for Biomathematical Technology (http://www.biomath.virginia.edu/Biomath_DS.html), since correctly identifying "peaks" of cell doublings on histograms was a similar statistical challenge to identifying pulses of hormone secretion. The program discriminates between random variations in values across intervals (in this case bin size) and true peaks. We used the program to identify the number of peaks to infer the number of discrete populations of cells. The standard parameters for the program were employed. For the analysis of the number of doublings the bin size was set to "1" for this program, and the replicates were "1". We used "3" as a minimum for a peak. Presence of the subtypes was also confirmed by using a dip test of unimodality (33, 34), with P < 0.05 that the population is unimodal being considered significant.

RESULTS

Fat depot origin affects preadipocyte replicative potential. Cells were plated at 1.5 × 10^4 cells/cm^2 or 15% of confluent density after allowing for plating efficiency (Fig. 1). After 24 h, a period following plating during which little or no replication occurs (86), cell numbers were estimated by crystal violet nuclear staining. After a further 48 h in growth-promoting medium, cell numbers were again determined. The fold increase in cell number was greater in subcutaneous preadipocyte cultures than in those from the other depots (n = 4; P < 0.005, Duncan’s multiple range test). The increase in mesenteric cell number was intermediate and omental cell number the lowest (difference between mesenteric and subcutaneous, P < 0.05; difference between mesenteric and omental, P < 0.05). Results were confirmed using BrdU (data not shown).

To test whether autocrine factors (e.g., cytokines, growth factors, Wnt pathway components) are responsible for these regional differences in replicative potential, we cocultured abdominal subcutaneous, mesenteric, and omental preadipo-cytes plated at high density with preconfluent cells (10% of confluent density), replicating subcutaneous test preadipocytes. After 3 days, the number of subcutaneous test cells was determined. The presence of mesenteric or omental preadipocytes actually enhanced replication of the preconfluent test cells. Coculture with mesenteric cells resulted in an increase in subcutaneous test cell number that was 129 ± 8% of that resulting from coculture with confluent abdominal subcutaneous cells (means ± SE, n = 3 experiments; P < 0.01, Duncan’s multiple range test) and with omental cells, 134 ± 2% compared with subcutaneous cells (P < 0.01). This suggests that reduced replication in visceral compared with subcutaneous preadipocytes is not a result of production of soluble antireplicative factors by visceral cells, presence of other cell types in visceral cultures that release such factors, or lower production of soluble mitogenic factors by visceral than subcutaneous cultures.

Proportion of preadipocyte subtypes varies among fat depots. Two preadipocyte subtypes with respect to replicative potential were found in primary cultures of human subcutaneous, mesenteric, and omental preadipocyte populations (Fig. 2). The presence of both subtypes was confirmed in each of the depots from three subjects by use of pulsatility analysis software and a test for unimodality (see METHODS). More of the cells in omental preadipocyte populations were slowly replicating subtype cells than in subcutaneous or mesenteric populations (Table 1), consistent with the lower replicative capacity of omental preadipocytes. The modal number of population doublings each of the two subtypes could achieve was similar across all three depots.

A potential explanation for the presence of preadipocyte replicative subtypes is that effects of position within the 96-well culture plates, related to variations in humidity or gas exchange, could affect replicative potential, resulting in a predominance of either small or large colonies near the edges.
of the plates. However, no statistically significant differences between the subtypes could be found in the proportions of colonies of each subtype in the outside compared with inside rows of the plates in any of six experiments involving 10 plates each. Also, because cloning efficiency was similar among depots, it is unlikely that failure of the rapidly replicating subtype to attach accounted for the much lower abundance of this subtype in the omental than abdominal subcutaneous or omental preadipocyte populations.

To test whether proliferation had stopped in the slowly replicating subtype explaining small colony size, 49 colonies that contained fewer than 500 cells by 21 days after plating were followed for a further 11 wk. Thirty five (71%) achieved confluence (≥14 population doublings) by 35 days after plating, 42 (86%) by 41 days, and 44 (90%) by 79 days. Two of the remaining colonies reached >30% confluence (≥13 population doublings) and three lifted off. Thus slowly replicating subtype cells were capable of continued proliferation, confirming their viability. However, they took longer to achieve confluence than cells of the rapidly replicating subtype.

Two preadipocyte subtypes are evident in cloned telomerase-expressing human preadipocyte cell strains. By transfecting human preadipocytes with a telomerase expression vector, we generated stable strains, each originating from single preadipocytes (78). When clones were derived from these strains, the two preadipocyte subtypes were still present (confirmed by analysis using the pulsatility analysis software described in METHODS) despite their identical ancestry (Fig. 3). Thus daughter cells of both subtypes can arise from a single cell, suggesting that contamination with a nonadipose cell type does not need to be invoked as an explanation for the presence of the two subtypes. Furthermore, the telomerase-expressing clones, all derived from single subcutaneous preadipocytes from the same subject, varied in their tendencies to give rise to either slowly or rapidly replicating subtype daughter cells. Thus the propensity of cells to acquire the characteristics of one or the other subtype may be a trait passed on to daughter cells.

The two preadipocyte subtypes can arise from each other. Rapidly replicating subtype colonies were prepared by cloning abdominal subcutaneous preadipocytes from the same subject. Three of these clones were then cloned again (Fig. 4A). After 4 wk, cell numbers in the resulting subclones were determined. Two preadipocyte subtypes were evident in frequency distributions of the numbers of population doublings achieved by the resulting subclones (confirmed in the pooled data with pulsatility analysis software). Of the subclones, 39 ± 19% were of the rapidly replicating subtype. Thus rapidly replicating subtype cells can give rise to preadipocytes of the slowly replicating subtype.

Slowly replicating cells can also give rise to cells of the rapidly replicating subtype. Six slowly replicating subtype colonies that contained fewer than 500 cells after culturing individual abdominal subcutaneous preadipocytes from the same subject for 21 days were subcloned (Fig. 4B). After 5 wk, both preadipocyte subtypes were found, with 22 ± 11% of the subclones becoming the rapidly replicating subtype. Transformation is unlikely to have accounted for switching from the slowly to rapidly replicating phenotype, as the number of population doublings such cells could achieve was limited (to 12 population doublings after subcloning). There was no morphological evidence of transformation (abnormal nuclei, loss of contact inhibition, or formation of cell islands). Furthermore, cytogenetic analyses demonstrated that these cells had a normal complement of chromosomes (normal karyotype was found in 10 of 10 metaphase spreads). Thus the two preadipocyte subtypes can switch into each other.

Apoptosis does not account for the slowly replicating subtype; however, slowly replicating subtype cells are more susceptible to TNF-α-induced apoptosis. TNF-α causes more apoptosis in omental than in abdominal subcutaneous preadipocytes.

Table 1. Cloning efficiency and replicative potential of human preadipocyte subtypes

<table>
<thead>
<tr>
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<th>Abdominal</th>
<th>Mesenteric</th>
<th>Omental</th>
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<tbody>
<tr>
<td>Cloning efficiency, %</td>
<td>63±6</td>
<td>66±6</td>
<td>52±7</td>
</tr>
<tr>
<td>Rapidly replicating subtype cells, %</td>
<td>47±4</td>
<td>51±4</td>
<td>8±1*</td>
</tr>
<tr>
<td>Slowly replicating subtype cells, %</td>
<td>53±4</td>
<td>49±4</td>
<td>92±1*</td>
</tr>
<tr>
<td>Modal number of doublings achieved by rapidly replicating subtype cells</td>
<td>12.7±0.9</td>
<td>12.0±1.5</td>
<td>12.3±0.9</td>
</tr>
<tr>
<td>Modal number of doublings achieved by slowly replicating subtype cells</td>
<td>4.3±0.9</td>
<td>4.7±1.2</td>
<td>4.3±0.9</td>
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Abdominal subcutaneous, mesenteric, and omental preadipocytes from 3 subjects were cloned by plate dilution. Fifty cells from each depot were plated into each of 10 96-well plates. Twenty-one days after cloning, cell numbers were determined. Cloning efficiency is expressed as the percentage of cells plated that had formed colonies within 21 days after plating (means ± SE). Significance was determined by ANOVA with post hoc determinations by Duncan’s multiple range test. *Significant difference from other values in the same row (P < 0.01; n = 3). Cloning efficiencies did not differ significantly (P = 0.31, ANOVA).
pocytes (69, 70). This prompted us to ask whether apoptosis could account for the limited replication and adipogenesis in omental cells and the greater abundance of the slowly replicating subtype in omental preadipocyte populations under the conditions used in our studies. The slowly replicating subtype preadipocytes did not exhibit morphological features of apoptosis, such as an increased proportion of nonadherent cells, membrane retraction, bleb formation, or nuclear condensation. The proportion of apoptotic nuclei was similar in the slowly and rapidly replicating subtype colonies: in comparing 17 slowly with 17 rapidly replicating clones, apoptotic nuclei evident by bisbenzamide staining were rare (<0.1%) in colonies of both subtypes (Fig. 5). These results are inconsistent with extensive apoptosis under basal conditions.

Preadipocytes of both subtypes were treated with 10 nM TNF-α for 12 h. Three times the proportion of nuclei in the slowly replicating subtype colonies were apoptotic as in rapidly replicating colonies (75 ± 4 vs. 25 ± 9% of cells were apoptotic in slowly and rapidly replicating subtype clones, respectively; n = 4 subjects (100 cells examined in each of 10 colonies/subject); P < 0.01, t-test). Thus, under the basal conditions in our studies, apoptosis is unlikely to cause appearance of the slowly replicating subtype, but once developed, these cells are more susceptible to induction of apoptosis by TNF-α.

Potential for adipogenesis is greater in the rapidly than in slowly replicating preadipocyte subtype and depends on depot origin. Colonies of undifferentiated rapidly and slowly replicating subtype cells were prepared from individual abdominal subcutaneous, mesenteric, and omental cells from three subjects. The cells were plated 14 days before the resulting colonies were exposed to differentiation-inducing medium for a further 14 days. Extent of lipid accumulation by individual cells was estimated in each colony by observers who were not aware of the depot from which cells were isolated. The proportion of cells that contained doubly refractile lipid inclusions visible by low-power phase contrast microscopy was determined. In other experiments, we demonstrated that such inclusions develop only in differentiating preadipocytes and not in other cell types and that the proportion of such cells is highly correlated with glyceraldehyde-3-phosphate dehydrogenase activity (48, 79, 86). All of the 2,260 colonies examined contained at least one such cell, indicating that each colony arose from a preadipocyte. The proportion of cells that accumulated lipid was higher within rapidly than within slowly replicating colonies (72 ± 11% of abdominal subcutaneous rapidly replicating clones vs. 50 ± 2% of slowly replicating clones; 48 ± 9 vs. 28 ± 6%, respectively, for mesenteric clones; and 29 ± 7 vs. 15 ± 6% for omental clones; P < 0.01, logistic regression analysis). A lower proportion of mesenteric than subcutaneous cells of each subtype accumulated lipid (P < 0.01) as did omental than subcutaneous cells of each

![Telomerase-Expressing Clone 22](image1.png)

![Telomerase-Expressing Clone 17](image2.png)

Fig. 3. Two preadipocyte subtypes are evident in cloned telomerase-expressing human preadipocyte cell strains. Cells of two different strains, each derived from a single preadipocyte from the same subject by stable hTERT transfection, were cloned by plate dilution and grown in a medium that promotes replication for 3 wk. Colony sizes were determined by CyQuant staining with a plate reader. Frequencies of clones that achieved various numbers of population doublings are shown. Two preadipocyte subtypes were evident. Clone 22 was capable of more rapid replication and gave rise to a higher proportion of cells of the rapidly replicating subtype than clone 17.

![Fig. 4. A](image3.png)

![Fig. 4. B](image4.png)

Fig. 4. A: cells of each subtype can develop into the other. Abdominal subcutaneous cells were cloned by plate dilution. Clones that had reached confluence 21 days after plating were subcloned. Cell numbers of the resulting subclones were determined after a further 28 days. The percentage of subclones that achieved different numbers of population doublings is indicated. Both slowly and rapidly replicating preadipocyte subtypes arose from rapidly replicating subtype cells. Pooled results from subcloning 3 rapidly replicating clones are shown (n = 550/1,410 subclones). B: slowly replicating subcutaneous clones (containing <500 cells after 21 days in culture) were subcloned. After being maintained in culture for 30 days, cell numbers were determined in resulting subclones. Frequencies of clones that had achieved different numbers of population doublings are indicated. Pooled results from subcloning 6 slowly replicating clones are shown; 409/1,877 subclones had become the rapidly replicating subtype.
subtype \( (P < 0.0001) \). Thus characteristics of both preadipocyte subtypes are regionally distinct.

**Cell-cell contact is not required for adipogenesis to proceed in human preadipocytes.** Unlike rodent preadipocyte cell lines, we found that primary human preadipocytes did not need to be confluent to undergo adipogenesis, as reported by others in the case of abdominal subcutaneous preadipocytes (24). We found this to be true in omental and mesenteric as well as in abdominal subcutaneous preadipocytes (Fig. 6). Furthermore, despite being confluent, omental cultures comprising principally the slowly replicating subtype (Table 1) are more resistant to adipogenesis than confluent subcutaneous or mesenteric cultures (79), in which approximately one-half of cells are the rapidly replicating subtype. Thus reduced cell-cell contact alone is unlikely to account for the lower capacity for lipid accumulation in slowly replicating subtype cells.

\textit{C/EBP\alpha} protein abundance is greater in colonies of the rapidly than slowly replicating subtype. \textit{C/EBP\alpha}, a transcription factor whose expression is necessary for adipogenesis to proceed (14, 30, 60–62, 77, 88), is expressed much more extensively in differentiating preadipocytes and fat cells than in undifferentiated preadipocytes. To determine whether processes that contribute to more extensive lipid accumulation by clones of the rapidly than of slowly replicating subtype are upstream or downstream of adipogenic transcription factor expression, cellular \textit{C/EBP\alpha} protein abundance was measured immunohistochemically. After 2 wk of growth and a further 3 wk of treatment with differentiation-inducing medium, 40 large and 40 small colonies, each arising from single abdominal subcutaneous cells, representing the highest and lowest quartiles of cell numbers achieved, were examined. Some cells in all of the colonies examined, of both the slowly and the rapidly repli-

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**Fig. 5.** The slowly replicating subtype does not appear to arise because of apoptosis. Rapidly (A) and slowly replicating subtype (B) abdominal subcutaneous clones grown in the absence of TNF-\( \alpha \) were stained with bisbenzamide and examined by fluorescence microscopy. Cells with irregular nuclear condensation, characteristic of apoptotic nuclei (C; arrow), were no more abundant in the slowly than in the rapidly replicating clones \( (n \approx 17 \text{ slowly and rapidly replicating clones}) \). Magnification, \( \times 200 \).

**Fig. 6.** Cell-cell contact is not required for adipogenesis to proceed in human preadipocytes. Preconfluent 5th-passage abdominal subcutaneous, mesenteric, and omental preadipocytes were plated at densities from 1,000 to 20,000 cells/cm\(^2\), treated with differentiation-inducing medium for 15 days, and then stained with Oil red O. Cells from each depot were capable of accumulating lipid, despite lack of contact with other cells. At all densities, subcutaneous preadipocytes were capable of more extensive lipid accumulation than omental cells, with mesenteric cells being intermediate. Magnification, \( \times 100 \).
cating subtypes, expressed C/EBPα, indicating that the colonies arose from preadipocytes. Within the rapidly replicating colonies, C/EBPα was detected in 81 ± 2% of the cells, whereas it was detected in only 56 ± 3% of cells in the slowly replicating colonies \( (n = 40 \) slowly and 40 rapidly replicating clones; \( P < 0.0001 \), \( t \)-test; Fig. 7). C/EBPα abundance was 19.1 ± 0.9 units/cell in the rapidly replicating clones and 14.7 ± 1 in slowly replicating clones \( (P < 0.005) \). Thus events preceding the increases in C/EBPα expression that occur during adipogenesis may contribute to differences in the capacities of the two preadipocyte subtypes to accumulate lipid.

When C/EBPα was measured using single-cell analysis only in those cells that expressed any detectable C/EBPα, C/EBPα-expressing cells in slowly replicating subtype clones did not express less C/EBPα than similar cells in rapidly replicating subtype clones. Indeed, in the cells that expressed any detectable C/EBPα, C/EBPα per cell tended to be higher in individual cells within the smaller clones \( (27.0 \pm 1.5 \) and 23.6 ± 0.9 arbitrary units/cell in small and large clones, respectively, \( P = 0.06) \). Particularly in the slowly replicating subtype clones, single cells expressing C/EBPα were observed that were distant from other cells. Coupled with the data in Fig. 6, this experiment further demonstrates that human preadipocytes do not need to be confluent in order to differentiate and that absence of cell-cell contact alone may not explain reduced adipogenesis in cells of the slowly replicating subtype. Furthermore, we observed that all of the cells expressing C/EBPα had accumulated lipid, consistent with the contention that mechanisms responsible for preadipocyte subtype differences in adipogenesis may operate upstream of C/EBPα induction. If downstream events were principally responsible, cells expressing C/EBPα, but which had not accumulated lipid, would have been seen.

**DISCUSSION**

Regional differences in characteristics of preadipocytes are reflected in the fat cells that develop from them \( (12, 80, 86) \). For example, preadipocytes from rat perirenal depots are capable of more extensive replication than preadipocytes from epididymal depots \( (20, 21, 48, 63, 86) \). This interdepot variation in rat preadipocyte replicative capacity is reflected in the extent of subsequent increases in fat cell number during depot growth in vivo \( (86) \). Similarly, interdepot variation in cultured rat preadipocyte differentiation-dependent gene expression is reflected in patterns of fat cell expression of the same genes among depots \( (49) \).

Human preadipocyte capacity for adipogenesis varies among fat depots, as demonstrated in several studies \( (1, 19, 36, 39, 42, 65, 79) \). In other studies, these differences were not found \( (75, 82, 83) \), perhaps because of variability among subjects, protocols for inducing differentiation, or the time point at which extent of differentiation was assessed. We found that, if left long enough in differentiation-promoting medium, omental preadipocytes can eventually catch up to subcutaneous cells, potentially obscuring regional differences evident earlier during adipogenesis \( (79) \). In the studies in which regional differences were evident, human abdominal subcutaneous preadipocytes had a greater capacity for adipogenesis than omental cells. TZDs promote more extensive differentiation of human subcutaneous than omental preadipocytes \( (1, 19) \). Consistent with this, TZDs also promote accumulation of fat in subcutaneous but not visceral depots \( (44, 67) \). Despite exposure to hormonal manipulations in vivo, such as estrogen treatment, hypophysectomy, or castration, preadipocytes cultured from various depots retain distinct cell-dynamic and biochemical responses relative to other depots, further suggesting that there are site-dependent, inherent differences in preadipocyte hormone responsiveness \( (47, 57) \). Thus cultured preadipocyte characteristics vary depending on fat depot origin in both rats and humans, and inherent differences in fundamental characteristics of fat cells and their precursors could contribute to regional variation in fat tissue function.

Part of the basis for these regional differences in cellular characteristics of preadipocyte populations is variation in abundance of preadipocyte subtypes. The two subtypes identified, both of which can arise from single preadipocytes, have distinct capacities for replication, differentiation, and adipogenic transcription factor expression and susceptibility to apoptosis. The subtype with the lower capacities for replication, lipid accumulation, and C/EBPα expression and greatest susceptibility to TNF-α-induced apoptosis is most abundant in omental preadipocyte populations, which exhibit the same characteristics compared with subcutaneous populations. Although differences in preadipocyte subtype abundance may contribute to regional variation in preadipocyte function, the characteristics of the subtypes also vary among depots. The omental rapidly and slowly replicating subtypes both accumulated less lipid than their subcutaneous counterparts. This implies that the subtypes from different depots are themselves...
distinct, that developmental imprinting of the cells occurs related to their environment in vivo, or a combination of these mechanisms.

Rapidly replicating subtype cells can produce slowly replicating subtype daughter cells, even after many cell generations. Slowly replicating cells also give rise to euploid rapidly replicating cells. This switching may account for the heterogeneity in cell-dynamic properties within preadipocyte lineages that was evident in the subcloning, lipid accumulation, and C/EBPα expression studies. Heterogeneity is evident even among individual preadipocytes arising within a few generations from a single cell. Analogous variability is also found in capacity for adipogenesis among cells within colonies derived by subcloning from 3T3-L1 clones (29). The interconversion that can occur between the preadipocyte subtypes could contribute to this heterogeneity in cell dynamic characteristics.

Although the probability that daughter cells will belong to one or the other subtype is depot dependent, interconversion ensures the presence of at least some cells of each subtype in all fat depots. The presence of both subtypes could serve to permit plasticity of the progenitor pool over time through selection for clones with particular properties. For example, inflammatory cytokine exposure could favor selection of the rapidly over the slowly replicating preadipocyte subtype, potentially with long-term consequences for fat depot cellular composition and function. Additionally, the preadipocyte subtypes may develop into differentiated cells with distinct properties. Of note, two populations of fat cells, one large and the other small, have been found in human fat and in fat-specific insulin receptor knockout mice (9, 10, 16–18).

We considered the possibilities that the slowly replicating subtype cells appear because of cellular stress, senescence, presence of “postadipocytes” or other cell types, dysdifferentiation, cell isolation or culture artifacts, or apoptosis. However, none of these potential mechanisms could adequately explain the appearance of these cells. Declining replication and adipogenesis occur with aging in rodent preadipocytes and with serial passage in human preadipocytes, related to activation of cellular stress responses or cellular senescence (53). These processes could conceivably explain the existence of the slowly replicating subtype or, by implication, the cell dynamic features of omental cells. The following observations suggest that this is unlikely. We found no difference in the frequency of cells with morphological features of stress or cellular senescence (poor attachment, nucleolar abnormalities, toxic granulations, cytoplasmic budding) among depots or subtypes. Events leading to generation of the subtypes are independent of telomerase activity or, by implication, replicative history and cellular senescence, as both subtypes were present despite telomerase expression. Also, slowly replicating preadipocyte subtype cells can give rise to euploid rapidly replicating cells. Thus replicative senescence may not account for regional or subtype differences. Furthermore, the two subtypes are unlikely to result from the presence of one population that had never differentiated and another arising from dedifferentiated fat cells [postadipocytes (89)], for the following reasons. If dedifferentiated fat cells had a different replicative potential than preadipocytes, one or the other population should become dominant with serial subculturing, but both subtypes remained evident after many population doublings. Both subtypes were found in populations derived from single cells in both the telomerase expression and subcloning experiments. Furthermore, neither of the subtypes is likely to have originated from brown adipocytes or a nonadipocyte cell type, as both subtypes could arise from single rapidly or slowly replicating subtype preadipocytes. Thus contaminating cell types or the presence of postadipocytes do not explain the origin of the two preadipocyte subtypes.

The subtypes do not appear to be artifacts of the methods used to isolate and culture preadipocytes, as both subtypes were found in primary cultures as well as after serial subculturing. Cells of both subtypes were evenly distributed within multiwell plates, indicating that plate position effects are unlikely to account for the presence of the two subtypes. The two subtypes were also found by us (50) in rat fat, implying conservation of the mechanisms giving rise to the preadipocyte subtypes across mammals. This also indicates that the presence of the two subtypes is unlikely to be a result of the particular characteristics of the subjects selected for the current study, although it is possible that relative abundance of the subtypes may be different in lean subjects than in the obese subjects we studied. Thus the presence of the two subtypes does not appear to be an artifact of culture conditions or subject selection, although it will be interesting to test effects of BMI, regional fat distribution, hormonal status (e.g., glucocorticoid excess), sex, and age on subtype abundance and characteristics.

The proportion of differentiated cells in clones of the rapidly replicating subtype was higher than in slowly replicating subtype clones. In 3T3-L1 cells, cell-cell contact appears to be important in promoting adipogenesis. Indeed, confluence, followed by a round of proliferation, precedes onset of adipogenesis in 3T3-L1 cells (25, 68). Thus reduced capacity for differentiation in the slowly replicating subtype cells could conceivably result from decreased cell-cell contact. However, this does not completely explain the low adipogenic capacity of the slowly replicating subtype for the following reasons. Human preadipocytes do not require cell-cell contact, confluence, or a round of replication to undergo adipogenesis (Figs. 6 and 7; Ref. 24). Confluent cultures of omental preadipocytes, in which slowly replicating subtype cells are abundant and in close contact with one another, are more resistant to induction of adipogenesis than confluent subcutaneous cultures, in which the rapidly replicating subtype cells are more abundant (1, 65, 79). Thus, although a causal link between capacities for replication and differentiation is very possible, failure to achieve confluence or lack of cell-cell contact does not fully explain reduced adipogenesis in omental preadipocytes or colonies of slowly replicating subtype cells.

Apoptosis is also unlikely to account for the limited replication and adipogenesis in omental or slowly replicating subtype cells for the following reasons. Under basal conditions, 86% of colonies derived from single slowly replicating subtype cells were able to achieve confluence 42 days after plating, and <10% of these colonies had detached after 79 days. Slowly replicating subtype cells were able to differentiate and express adipogenic transcription factors. These cells were capable of switching into cells of the rapidly replicating subtype. Colonies of slowly replicating subtype cells did not exhibit morphological features of apoptosis (increased nonadherent cells, mem-
brane retraction, bleb formation, or nuclear condensation). The proportion of apoptotic nuclei was similar in slowly and rapidly replicating subtype colonies (Fig. 5). Thus, under basal conditions, regional differences in apoptosis or viability did not appear to contribute to reduced capacities for replication and adipogenesis in slowly replicating subtype cells. However, these cells were more susceptible to apoptosis induced by TNF-α than cells of the rapidly replicating subtype. This may partly explain the greater susceptibility of omental than subcutaneous preadipocyte populations to TNFα-induced apoptosis (69, 70).

We noted that mesenteric preadipocyte populations are very different from omental cells with respect to replicative potential and subtype abundance. In a previous study (79), we found that the capacity of mesenteric preadipocytes for differentiation was higher than that of omental cells. Thus visceral fat does not appear to be homogeneous from a cell dynamic perspective. Some subjects with visceral obesity have been noted anecdotally by surgeons to have more extensive enlargement of their omental than mesenteric depots, whereas in other subjects mesenteric enlargement predominates. Because mesenteric and omental preadipocytes appear to be distinct, it would be very interesting to determine health consequences of predominantly omental compared with mesenteric enlargement.

The presence of two preadipocyte subpopulations with distinct characteristics, both capable of differentiation, may constitute a mechanism that allows plasticity of fat tissue development through regulation of subtype abundance. This regulation may occur both by subtype selection, for example by inflammatory cytokines or their differences in responses to inducers of adipogenesis, and by variation in the propensity of cells to give rise to daughter cells that have the characteristics of a particular subtype. Overlaid upon this, the preadipocyte subtypes from different fat depots have distinct characteristics. Thus, in addition to processes that regulate replication, adipogenesis, dedifferentiation, and apoptosis, preadipocyte subtype selection appears to be a mechanism through which fat tissue development can be shaped.

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