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# Evidence of *in Situ* Proliferation of Adult Adipose Tissue-Derived Progenitor Cells: Influence of Fat Mass Microenvironment and Growth

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**Context:** Adipocyte formation in human adult adipose tissue (hAT) originates from resident progenitor cell differentiation in the stroma vascular fraction of the AT. The processes involved in the self-renewal of this cell population remain to be defined.

**Objective:** The objective was to study *in situ* and *in vitro* hAT progenitor cell (defined as $CD34^+$/$CD31^-$ cells) proliferation.

**Design and Participants:** *In situ* progenitor cell proliferation was assessed by immunohistochemistry and flow cytometry analyses on hAT from lean to obese subjects using the proliferation marker Ki-67. The effects of adipokines, hypoxia, and conditioned media (CM) from adipocytes, capillary endothelial cells, and macrophages isolated by an immunoselection approach were studied on hAT progenitor cell growth. Cell death in hAT was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein end labeling method.

**Results:** Ki-67-positive staining was observed in AT progenitor cells. Fat mass enlargement in obese patients was associated with an increased $Ki\text{-}67^+$ progenitor cell population together with a new fraction of small adipocytes and increased cell death. HIF-1$\alpha$ mRNA expression in freshly harvested progenitor cells was positively correlated with body mass index. Adipocyte- and capillary endothelial cell-CM, hypoxia, leptin, IL-6, lysophosphatidic acid, and vascular endothelial growth factor, all increased hAT progenitor cell proliferation *in vitro*. Macrophage-CM had an antiproliferative effect that was suppressed by an antioxidant.

**Conclusions:** The fraction of proliferative progenitor cells in adult hAT is modulated by the degree of adiposity. Changes in the progenitor cell microenvironment involving adipokines, hypoxia, and oxidative stress might play a key role in the control of the self-renewal of the local pool of AT progenitor cells. **(*J Clin Endocrinol Metab* 93: 4098–4106, 2008)**

The excessive development of human adipose tissue (AT) results from both hypertrophy of preexisting adipocytes and hyperplasia due to the formation of new adipocytes (1, 2). Hypertrophy which consists of excessive triglyceride accumulation in mature adipocytes, is due to both positive energy balance and reduced lipolysis. It is the initial event occurring during the establishment of obesity (3). In severe forms of obesity, an apparent increase in number of adipocytes follows the mature adipocyte hypertrophy (4, 5). Interestingly, Spalding *et al.* (6) recently showed that the number of mature fat cells is constant in adulthood in lean and obese individuals. Furthermore, the authors suggest that mature adipocytes exhibit a high turnover rate, indicating that fat cell number is tightly regulated during adulthood. Because adipocytes are terminally differentiated cells and, as such, are considered incapable of division (2, 7), the apparent increase in adipocyte

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Abbreviations: AT, Adipose tissue; BMI, body mass index; BrdU, bromo-2′-deoxyuridine; CEC, capillary endothelial cell; CM, conditioned media; ECBM, endothelial cell basal medium; FCS, fetal calf serum; HIF-1$\alpha$, hypoxia-inducible factor-1$\alpha$; LPA, lysophosphatidic acid; NAC, *N*-acetyl cysteine; SVF, stroma-vascular fraction; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; VEGF-A, vascular endothelial growth factor A.

number is thought to originate from adipogenesis, the proliferation/differentiation of adipocyte progenitor cells (preadipocytes). Adipogenesis has been extensively studied *in vitro* on several cell models such as established murine preadipocyte cell lines and primary cultures of stroma-vascular fraction (SVF) cells in adipogenic culture conditions (8, 9). It was reported in mice that adipocytes may derive from circulating bone marrow cells (10), whereas a recent study found the opposite and suggests that bone-marrow-derived cells do not contribute in any way to AT development (11). Whatever the final issue of this debated question in rodents, clinical studies are requested to investigate adipogenesis in human AT. The exact nature of human preadipocytes still remains elusive. Because of the difficulty in studying cell turnover in humans, few data are available concerning human adipocyte precursor renewal within AT, although such a process is necessary to maintain a pool of preadipocytes to be recruited during AT enlargement. Our previous studies showed that human preadipocytes are present in the AT-derived SVF. Using a combination of cell surface markers, CD34 (mucosialin) expressed on hematopoietic stem cells and capillary endothelial cells (CECs), CD31 (platelet endothelial cell adhesion molecule-1), expressed on leukocytes and endothelial cells and CD14 (lipopolysaccharide receptor), expressed on myeloid cells, the main cell types composing the AT-SVF were defined as AT CECs ($CD34^+/CD31^+$), AT macrophages ($CD34^-/CD14^+$), and AT progenitor cells ($CD34^+/CD31^-$) (12). The $CD34^+/CD31^-$ cell fraction was the only one, among the SVF-derived cells, to differentiate into adipocytes (13). Additionally, the $CD34^+/CD31^-$ cells exhibited angiogenic potential (14) and were therefore defined as progenitor cells.

The present study was undertaken to evaluate the *in situ* proliferative status of the $CD34^+/CD31^-$ progenitor cells in human sc AT and to determine whether self-renewal of a local pool of human progenitor cells could be modulated by adipocyte hypertrophy and/or hyperplasia. *In vitro* studies were performed on freshly isolated $CD34^+/CD31^-$ cells to analyze the effects of local changes in the microenvironment in which the progenitor cells reside, notably the effects on the proliferative responsiveness of $CD34^+/CD31^-$ cells of secreted factors originating from adipocytes, AT-CECs, and AT-macrophages as well as low oxygen tension.

## Materials and Methods

### Materials

Chemicals were from Sigma (Saint-Quentin Fallavier, France). Collagenase NB4 was from Serva (Coger, Paris, France). $CD34^+$ and $CD14^+$ cell selection kits were from StemCell Technologies (Grenoble, France), and the $CD31^+$ cell selection kit was from Dynal (Invitrogen, Cergy-Pontoise, France). Culture media were from Promocell (Heidelberg, Germany). Flow cytometry antibodies were from BD Biosciences (Le-Pont-de-Claix, France). Adiponectin, leptin, and vascular endothelial growth factor A (VEGF-A) were from Peprotech (Levallois-Perret, France) and IL-6 from R&D Systems (Lille, France). Lysophosphatidic acid (LPA) was a gift from Dr. Saulnier-Blache [Institut National de la Santé et de la Recherche Médicale (INSERM) Unit 858, France].

### Patients

Human abdominal sc AT was obtained from a group of normal to class I obese women undergoing plastic surgery (dermolipectomy and/or liposuction) [n = 61, body mass index (BMI) ranging from 20.8–34 $kg/m^2$; mean age = 42 ± 1 yr] (15) and from a group of class II to class III obese women (n = 25, BMI ranging from 35–55 $kg/m^2$; mean age = 40 ± 3 yr) undergoing vertical banded Mason gastroplasty, with a stable weight for at least 3 months before surgery (supplemental data 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The AT samples were immediately processed after removal. The protocol has been conducted in accordance with the Declaration of Helsinki guidelines and was approved by the Institutional Research Board of INSERM and the Toulouse University Hospital.

### Isolation of the cell types from human AT

Mature adipocytes and SVF were harvested as previously described (12, 16). The SVF cells were isolated using an immunoselection/depletion approach (14, 17). Freshly isolated $CD34^+/CD31^-$ cells (progenitor cells), $CD34^+/CD31^+$ cells (AT-CECs) and $CD34^-/CD14^+$ cells (AT-macrophages) were cultured in appropriate media.

### Isolation of human foreskin fibroblasts

Human foreskin fibroblasts were prepared according to a modified version of Rheinwald and Green protocol (18). The cells were cultured in DMEM/10% fetal calf serum (FCS) (passages 3–14 were used).

### Adipocyte diameter determination

After isolation from dermolipectomies (normal to class I obese individuals) or sc fat biopsies (class II and III obese patients) (n = 51, BMI ranging from 20.8–55 $kg/m^2$), mature adipocytes were suspended in endothelial cell basal medium (ECBM, containing no growth factors)/0.5% BSA (1/10, vol/vol), and 5 $\mu$l cell suspension was placed onto plastic slides. Three distinct calibrated fields were taken to measure manually adipocyte diameters with NIS software (Nikon, Champigny-sur-Marne, France). Fat cell-like shapes were labeled with the nucleus dye 4′,6-diamidino-2-phenylindole (DAPI). Among them, 94 ± 1% were nucleated and identified as adipocytes (85 ± 7 and 99 ± 1%, for small and large size adipocytes, respectively).

### Preparation of conditioned media (CM)

To prepare CM from the different cell fractions composing AT, cells were isolated from 21 abdominal liposuctions (BMI = 30.2 ± 0.9 $kg/m^2$). Adipocytes were plated in fibrin gels (12), and basal medium (*i.e.* ECBM/0.1% BSA) was added. After 24 h, the adipocyte-CM was collected, centrifuged (20,000 × g for 3 min at room temperature), and stored at −20 C until further use. The AT-CECs were plated on fibronectin-coated plates (5 $\mu g/cm^2$) in endothelial cell growth medium-microvascular. At confluence, AT-CECs were rinsed, and basal medium was added. Twenty-four hours later, CM was collected, centrifuged, and stored at −20 C. AT-macrophages were plated in basal medium supplemented or not with *N*-acetyl cysteine (NAC) for 24 h. The CM was collected, centrifuged and stored at −20C until further use.

### Immunohistochemistry and cell death measurement

Immunohistochemistry analyses were performed on freshly harvested human sc AT cut into small pieces and fixed in neutral buffered 4% (wt/vol) paraformaldehyde (24 h at room temperature). The microwave antigen retrieval was performed in citrate buffer (10 mmol/liter, pH 6) three times for 7 min each. The AT was permeabilized in PBS/0.1% Triton (20 min) followed by an overnight incubation in PBS/2% BSA with mouse monoclonal CD34 antibody (Santacruz, Le Perray-en-Yvelines, France) (1/50), rabbit polyclonal Ki-67 antibody (Dako, Trappes, France) (1/50), or mouse polyclonal hypoxia-inducible factor-1$\alpha$ (HIF-

1α) antibody (R&D Systems) (1/20). AT was then incubated for 1 h with the corresponding fluorescent-labeled second antibodies (goat anti-mouse or goat antirabbit coupled to AlexaFluor 488 or 546) (Invitrogen) (1/200). Cell death was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) assays according to the manufacturer's instructions (Roche, Meylan, France). Briefly, after fixation and permeabilization (PBS/0.5% Triton/0.1% sodium citrate for 20 min at room temperature) AT pieces were incubated in the dark with the TUNEL reaction mixture or with the control solution (for negative controls) for 1 h at 37 C in a humidified atmosphere. Nuclei were stained with Hoescht 33258 (Invitrogen). After washes, AT pieces were mounted between two slides, and positive TUNEL cells (green nuclei) were determined under fluorescent microscope analyses (Nikon) on three fields for each AT sample.

## Cell proliferation assay

The $CD34^+/CD31^-$ progenitor cells (or foreskin-derived fibroblasts) (n = 14; BMI = 28.5 ± 1.6 kg/m$^2$) were plated at a density of $1.10^4$ cells/cm$^2$ in ECBM/10% FCS. After 24 h, cells were rinsed and treated with ECBM/0.1% BSA (control), 5% FCS (positive control), adipocyte-, CECs-, or macrophage-CM in normoxia or with ECBM/0.1% BSA in hypoxia chamber (1% $O_2$; Sanyo, Avon, France). Progenitor cells were also treated with adiponectin (1, 10, and 100 ng/ml), IL-6 (1, 10, and 100 ng/ml), leptin (1, 10, and 50 ng/ml), LPA (0.1, 1, and 10 μmol/l), or VEGF-A (0.1, 1 and 10 ng/ml). After 48 h, bromo-2′-deoxyuridine (BrdU) was added to the medium (20 μl at 100 μmol/liter) for 6 h. The cell proliferation index was evaluated according to the manufacturer's instructions (Roche).

## Flow cytometry analysis

AT-fixed SVF (CellFix; BD Bioscience; vol/vol, 4 C, 30 min) were permeabilized (20 h at −20C in 70% ethanol). At least $1 \times 10^5$ cells in PBS/0.5% BSA/2 mmol/liter EDTA were incubated with fluorescein isothiocyanate (FITC)-conjugated Ki-67 antibody, peridinin chlorophyll protein (PerCP)-conjugated CD34 antibody and phycoerythrin (PE)-conjugated CD14 antibody or the respective isotype controls. The labeled cells were analyzed by multiparameter flow cytometry using a FACSCalibur flow cytometer and the CellQuest Pro software (BD Bioscience).

## RNA isolation and real-time PCR

Total RNA was extracted from $CD34^+/CD31^-$ cells or human foreskin fibroblasts using the RNeasy kit (QIAGEN, Courtaboeuf, France) and the RNA concentrations determined using a fluorometric assay (Ribogreen; Invitrogen). RNA (0.5 μg) was reverse-transcribed using the Superscript II (Invitrogen) (Random Hexamers and dNTPs were from Invitrogen). Reverse transcription was also carried out without the superscript enzyme on RNA samples. Primers for the adiponectin receptors (Adipo R1, Adipo R2), the leptin receptor, the VEGF receptors (VEGF-R1, VEGF-R2, and VEGF-R3), the IL-6 receptor, and HIF-1α were provided by Applied Biosystems (Courtaboeuf, France) (assay on demand: hs00360422_m1, hs00226105_m1, hs00174497_m1, hs00176573_m1, hs00176676_m1, hs00176607_m1, hs00169842_m1, and hs00153153_m1, respectively). The primers for the LPA receptor (LPA1R) were forward, 5′-TGGGCCATTTTCAACTTGGT-3′, and reverse, 5′-TCTGGCGAACATAGCCAAAGA-3′. The amplification reaction was carried out on 15 ng of cDNA samples in 96-well plates (Applied Biosystems) in a GeneAmp 7000 sequence detection system. The PCR mixture contained either 5 μl TaqMan primers (5× prediluted in water) or 900 nmol/liter for LPA1R and 10 μl 2× TaqMan PCR Master Mix or 10 μl 2× SybrGreen PCR Master Mix (Applied Biosystems). All reactions were performed

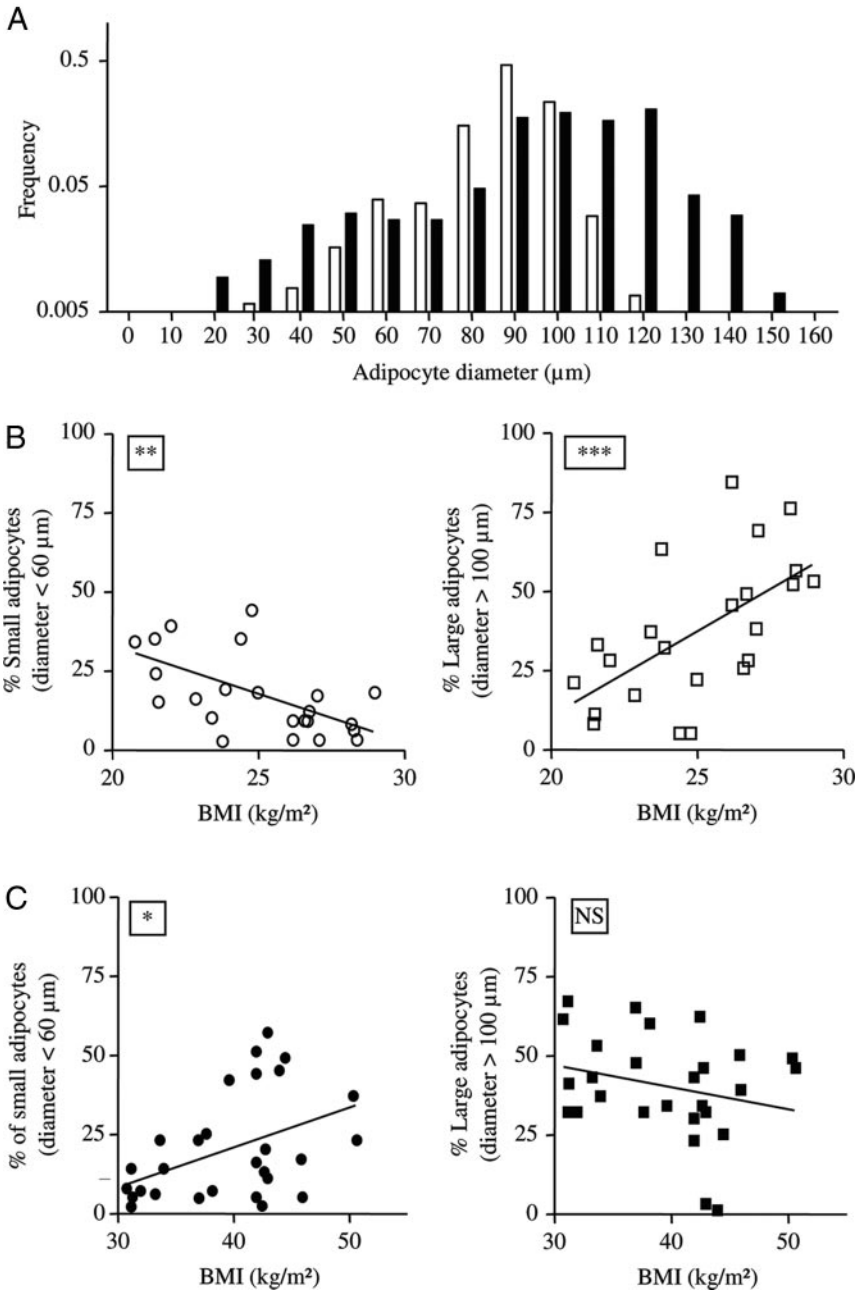


**FIG. 1.** Evolution of adipocyte size and number with the growth of human sc AT. A, Adipocytes from human sc AT of lean/overweight (BMI <30 kg/m$^2$; n = 23; *white bars*) and obese (BMI ≥30 kg/m$^2$; n = 28; *black bars*) patients were isolated for counting and diameter determination. Results are expressed as frequency and presented in log10 scale. B and C, Correlations between the percentage of small (diameter <60 μm, ○ and ●) and large (diameter >100 μm, □ and ■) adipocytes according to the BMI in lean/overweight patients (B, *white symbols*; ***, $P < 0.01$, Spearman r = −0.55, n = 23; and ***, $P < 0.001$, Spearman r = 0.62, n = 23, respectively) and in obese patients (C, *black symbols*; *, $P < 0.05$, Spearman r = −0.40, n = 28; and $P$ = nonsignificant, Spearman r = −0.23, n = 28, respectively).

under the same conditions: 50 C for 2 min, 95 C for 10 min, followed by 40 cycles of 95 C for 15 sec and 60 C for 1 min. The results were analyzed with the GeneAmp 7500 software, and all the values were normalized to 18S rRNA levels.

### Statistical analysis

The statistical analysis was performed with GraphPad Software (San Diego, CA). Correlations were analyzed with a Spearman test. Values are given as mean ± SEM of (n) separate experiments. The comparisons between different groups were analyzed by one-way ANOVA followed by Dunnett *post hoc* test or with a Student's *t* test for two groups. Differences were considered significant when $P < 0.05$.

## Results

### Changes in adipocyte size distribution with the growth of human sc AT

Fat cell size distribution was determined in AT from lean to overweight patients ($20 < BMI < 30$ kg/m$^2$; n = 23) and was compared with the obese patients (BMI ≥30 kg/m$^2$; n = 28). As shown in Fig. 1A, the most representative population of adipocytes, with a mean diameter of 90 $\mu$m in lean/overweight AT, shifted to 100–120 $\mu$m in obese AT. Moreover, the frequency of very small adipocytes (diameter 20–40 $\mu$m) increased from 0.015 in lean/overweight AT to 0.045 in obese AT. To further analyze the influence of the degree of adiposity on adipocyte size, adipocytes were classified according to their diameter (*i.e.* large with a diameter more than 100 $\mu$m and small with a diameter less than 60 $\mu$m), and their percentage within the whole adipocyte population was determined. As shown in Fig. 1B, increased adiposity in lean to overweight patients was negatively correlated with the percentage of small adipocytes but positively with that of large adipocytes. The further increase in fat mass seen in obese patients was characterized by stabilization or a statistically nonsignificant decrease in the percentage of large adipocytes together with an increase in the percentage of smaller adipocytes (Fig. 1C).

### Changes in cell death with the growth of human sc AT

Cell death in human sc AT was assessed by TUNEL in lean/overweight (n = 9) and in obese patients (n = 9). As shown in Fig. 2A, occasional TUNEL-positive cells were observed in lean/overweight AT (mean value of 5 ± 2% of the total nuclei), whereas a clear increase was seen in obese AT (Fig. 2B) (mean value of 20 ± 6% of total nuclei, $P < 0.05$; Fig. 2C).

### Changes in the proliferation rate of the CD34$^+$/CD31$^-$ progenitor cells with the growth of human sc AT

The expression of the Ki-67 protein that is expressed during all active phases of the cell cycle ($G_1$, S, $G_2$, and mitosis) but absent from resting cells ($G_0$) was studied in the sc AT of lean/overweight and obese patients. As shown in a representative histological assay (Fig. 3A), immunohistochemistry revealed the presence of cells positive for CD34 in the stromal compartment surrounding mature adipocytes and defined as progenitor cells. Among the progenitor cells, Ki-67-positive cells were identified (Fig. 3A). To quantify the number of proliferative progenitor cells, flow cytometry analyses were performed on the SVF from the sc AT of lean to obese patients using the peridinin chlorophyll protein (PerCP)-conjugated CD34 antibody together with the fluorescein isothiocyanate-conjugated Ki-67 antibody. The per-

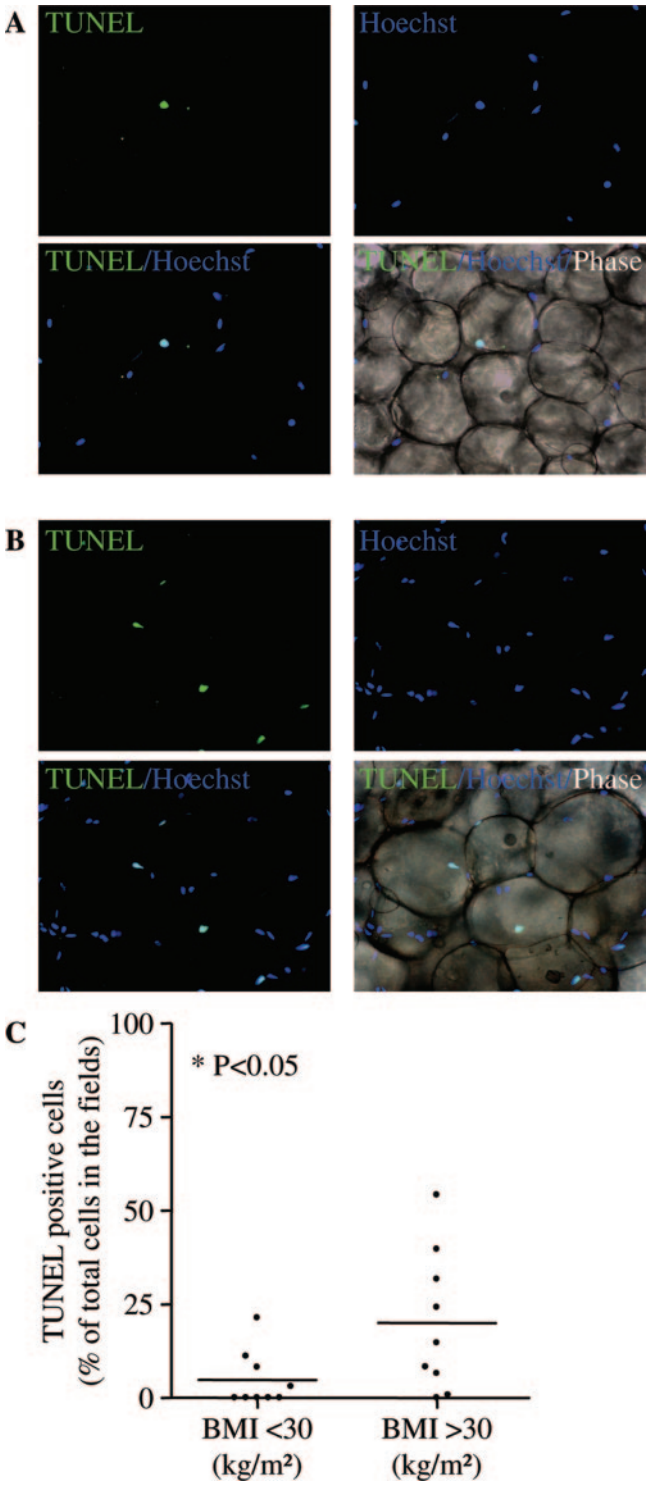


**FIG. 2.** Effect of human sc AT growth on cell death. A and B, Representative photomicrographs of TUNEL staining (*green*) and nuclei (*blue*) in fixed and permeabilized human sc AT of lean/overweight patients (BMI <30 kg/m$^2$) (A) and obese patients (BMI ≥30 kg/m$^2$) (B) (original magnification, ×200); C, mean values of TUNEL-positive cells expressed as percentage of the total cell number in nine lean/overweight patients and nine obese patients determined on three independent microscopic fields for each adipose tissue sample. *, $P < 0.05$, lean/overweight *vs.* obese.

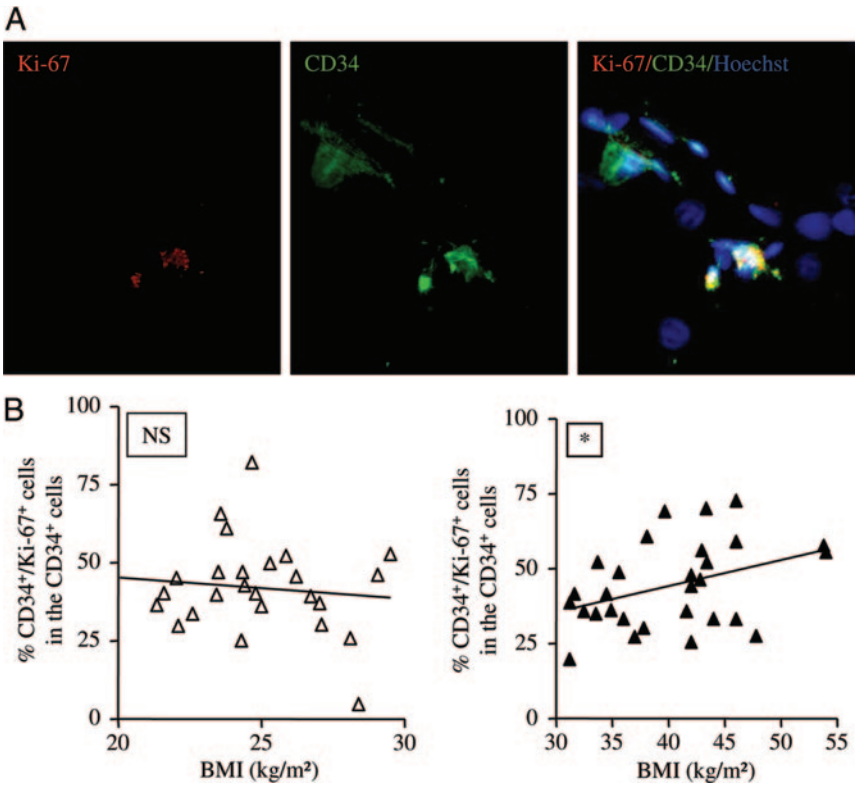


**FIG. 3.** Effect of human sc AT growth on the expression of Ki-67 in AT-progenitor cells. A, Representative photomicrographs of immunohistochemical analyses in fixed and permeabilized human sc AT stained with Ki-67-antibody (*red*), CD34-antibody (*green*), and Hoescht 33258 (original magnification, ×200). B, Two-color flow cytometry analyses were performed on freshly harvested SVF using fluorescent-labeled antibodies directed against CD34 and Ki-67. The percentage of $CD34^+/Ki\text{-}67^+$ cells within the $CD34^+$ cell population according to BMI is shown in lean/overweight patients (BMI <30 kg/m$^2$, △; $P$ = nonsignificant; Spearman r = 0.008; n = 26) and in obese patients (BMI ≥30 kg/m$^2$, ▲; *, $P < 0.05$; Spearman r = 0.34; n = 29).

centage of progenitor cells positive for Ki-67 remained constant with increasing BMI in the SVF of lean/overweight patients (Fig. 3B; n = 26). Interestingly, positive Ki-67 progenitor cell percentage was positively correlated with the BMI in obese patients (Fig. 3B; n = 29; $P < 0.05$). As shown in Fig. 3B, large inter-obese individual differences exist; one cannot rule out that they might be the consequence of each patient's weight history (weight cycling, pregnancy, and number of diet restriction periods). Even though the present work was not designed as a follow-up study, prebariatric data such as obesity duration and waist circumference were retrieved from 16 class II to class III obese patients of a group of 25 (supplemental data 1). Obesity duration was not correlated with the BMI or with the percentage of progenitor cells positive for Ki-67 (not shown). Finally, the age of the patients included in the present work was not correlated to the percentage of progenitor cells positive for Ki-67 (not shown).

### Local control of $CD34^+/CD31^-$ progenitor cell proliferation by adipokines

Freshly harvested AT progenitor cells were treated for 48 h with CM originating from mature adipocytes, AT-CECs, and AT-macrophages. Their proliferation was determined by BrdU incorporation assays as well as by nuclei counting after Hoescht staining. As shown in Fig. 4A, CM originating from mature adipocytes and from AT-CECs increased the proliferation of the AT progenitor cells as shown by the enhanced BrdU incorporation (Fig. 4A) and nuclei number (data not shown). The stimulatory effects of the adipocyte- and CEC-derived CM on the progenitor cell proliferation was cell type specific because they did not affect the proliferation activity of human foreskin-derived fibroblasts treated under the same conditions (Fig. 4B). Finally, the growth responsiveness of the AT progenitor cells to adipokines known to be derived from adipocytes and CECs and whose production is known to be related to fat mass growth, such as leptin, adiponectin, LPA, IL-6, and VEGF-A, was studied. First, the expression of their corresponding receptors in the AT progenitor cells was determined by real-time PCR analysis and compared with the one of human foreskin-derived fibroblasts. As described in Table 1, the AT progenitor cells specifically expressed the mRNA transcripts encoding for the receptor for leptin (LEPR), adiponectin (Adipo R1 and R2), LPA, IL-6, the VEGF-R2 for the VEGF-A receptor as well as VEGF-R1 and -R3. Leptin triggered a concentration-dependent increase in BrdU incorporation (Fig. 4C) whereas adiponectin was without effect (data not shown). In parallel, VEGF-A, LPA, and IL-6 increased the proliferation of the progenitor cells (Fig. 4D).

AT macrophage-derived CM led to a reduction in the proliferation rate of the progenitor cells (Fig. 5). To investigate whether reactive oxygen species could be responsible for the antiproliferative effect of macrophage-CM, an antioxidant, NAC, was also added. As shown in Fig. 5, the presence of NAC abolished the antiproliferative effect of macrophage-CM.

### Local control of progenitor cell proliferation by oxygen tension

The potential presence of a hypoxic area within human sc AT was investigated by immunohistochemistry. As shown in Fig. 6A, the hypoxia-sensitive transcription factor HIF-1$\alpha$ could be identified in human sc AT. Moreover, the expression of HIF-1$\alpha$ was determined by real-time PCR analysis in the progenitor cells isolated from sc AT from patients with increasing BMI. As shown in Fig. 6B, the expression of HIF-1$\alpha$ in $CD34^+/CD31^-$ progenitor cells was positively correlated with the BMI of the patients. Finally, the proliferation rate of the $CD34^+/CD31^-$ progenitor cells was studied using BrdU incorporation assays under 20 and 1% $O_2$ in a hypoxia chamber for 48 h. As shown in Fig. 6C, hypoxia increased the BrdU incorporation by the $CD34^+/CD31^-$ progenitor cells (1.7 ± 0.3-fold compared with normoxic conditions; n = 6; $P < 0.05$).

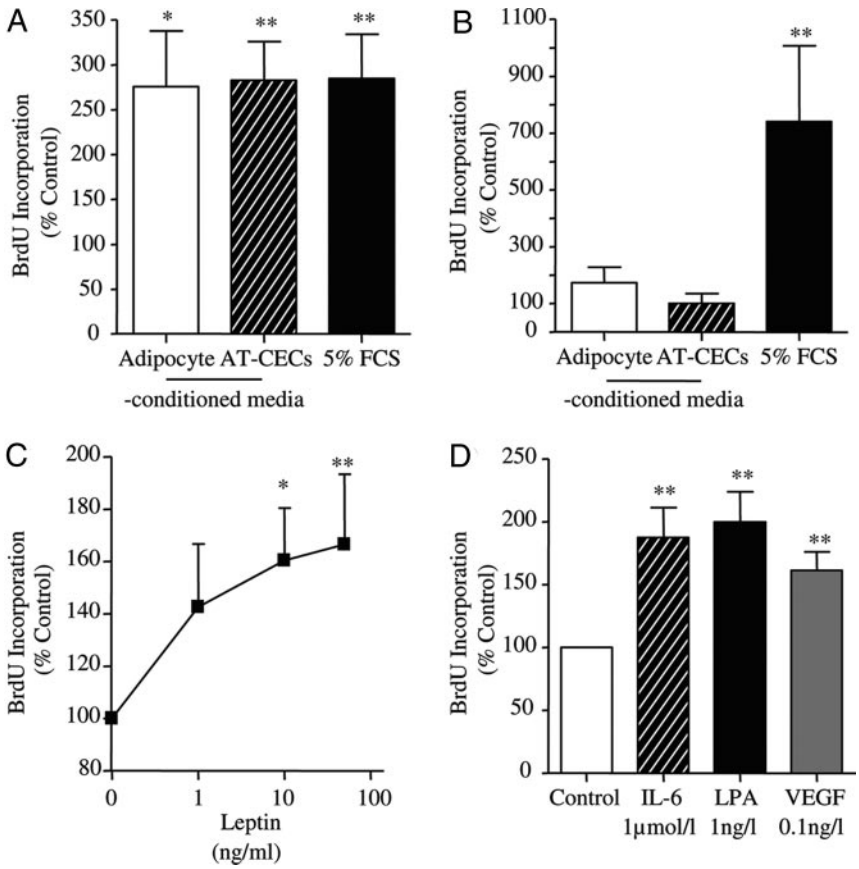


**FIG. 4.** Effects of adipokines on CD34$^+$/CD31$^-$ progenitor cell proliferation. The effects of CM from adipocytes and CECs were evaluated on the proliferation of CD34$^+$/CD31$^-$ progenitor cells (A) and compared with human foreskin-derived fibroblasts (B). Concentration response curves of leptin (C) and maximal concentration effects of LPA, IL-6, and VEGF-A (D) on the proliferation of the CD34$^+$/CD31$^-$ progenitor cells are presented. Proliferation was evaluated by BrdU incorporation assays. Results are expressed as the percentage of control (basal medium) and are presented as means ± SEM from eight to 10 separate experiments. *, $P < 0.05$; **, $P < 0.01$.

## Discussion

The present study demonstrates that human AT progenitor cells characterized by the expression of the mucosialin CD34 and the lack of platelet endothelial cell adhesion molecule-1 (CD31) exhibit *in situ* expression of markers of proliferative activity, the proportion of which increases in obese patients. Moreover, *in vitro* assays showed that the growth of AT progenitor cells is modulated by the AT microenvironment and more particularly by soluble factors originating from adjacent cells (adipocytes, AT-CECs, and AT-macrophages) and by hypoxia.

The enlargement of the human fat mass is associated with two major cell changes in adipocytes: size enlargement and increased number (3). In the present study covering a BMI range from 20–55 kg/m$^2$, the progressive development of adipocyte hypertrophy was characterized by the concomitant increase in large adipocytes and reduction in small adipocytes in lean to overweight patients. Such a phenomenon may be explained by an increase in lipogenic activity due to positive energy balance leading to higher BMI. Further increases in the degree of adiposity in obese patients were associated with the gradual replenishment of a pool of small adipocytes, whereas the fraction of large adipocytes remained constant or tended to decrease. This observation fits with previous reports showing that hyperplasia follows hypertrophy in patients during fat mass increment (1, 3). Moreover, and in accordance with Spalding *et al.* (6), cell death in human AT was found. Altogether, the present results suggest that cell death occurring in AT might be, in addition to adipocyte hypertrophy and hyperplasia, a contributor to the remodeling events that take place in the growing fat mass. The appearance of small adipocytes in the AT of obese patients was more likely related to the formation of new adipo-

**TABLE 1.** mRNA receptor expression of adipokines receptors by freshly harvested CD34$^+$/CD31$^-$ progenitor cells and human foreskin fibroblasts

| Receptor | mRNA expression (AU) in CD34$^+$/CD31$^-$ progenitor cells | mRNA expression (AU) in human foreskin fibroblasts |
|---|---|---|
| LEPR | 1.17 ± 0.34 | ND[a] |
| Adipo R1 | 0.84 ± 0.24 | 0.05 ± 0.04[a] |
| Adipo R2 | 0.48 ± 0.14 | ND[a] |
| LPA R | 2.03 ± 0.62 | 0.60 ± 0.11[a] |
| IL-6 R | 1.15 ± 0.42 | ND |
| VEGF-R1 | 0.07 ± 0.04 | ND |
| VEGF-R2 | 0.42 ± 0.31 | ND |
| VEGF-R3 | 0.05 ± 0.03 | ND |

The mRNA expression of the receptors was determined in CD34$^+$/CD31$^-$ progenitor cells and human foreskin fibroblasts. Total RNAs were extracted from cells and reverse transcribed, and real-time RT-PCR analysis was performed using specific cDNA primers. The results obtained were normalized to the levels of 18S rRNA and expressed in arbitrary unit (AU). The presented data are mean ± SEM for four to eight independent experiments. ND, Not detectable.

[a] $P < 0.05$ progenitor cells *vs.* fibroblasts.

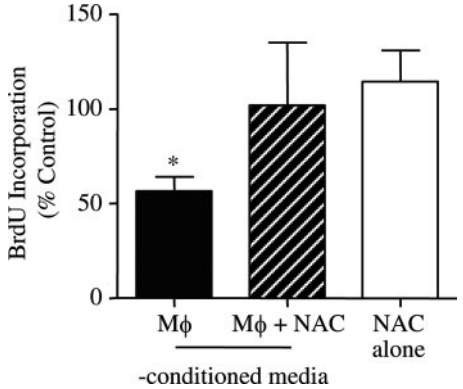


**FIG. 5.** Effects of macrophage-secreted factors on CD34$^+$/CD31$^-$ progenitor cell proliferation. CD34$^+$/CD31$^-$ progenitor cells were treated for 48 h with macrophage (M$\phi$)-CM, in the presence or absence of the antioxidant NAC (0.5 mmol/liter). Proliferation was evaluated by BrdU incorporation assays. Results are expressed as the percentage of control (basal medium) and are presented as means ± SEM from five to seven separate experiments. *, $P < 0.05$.

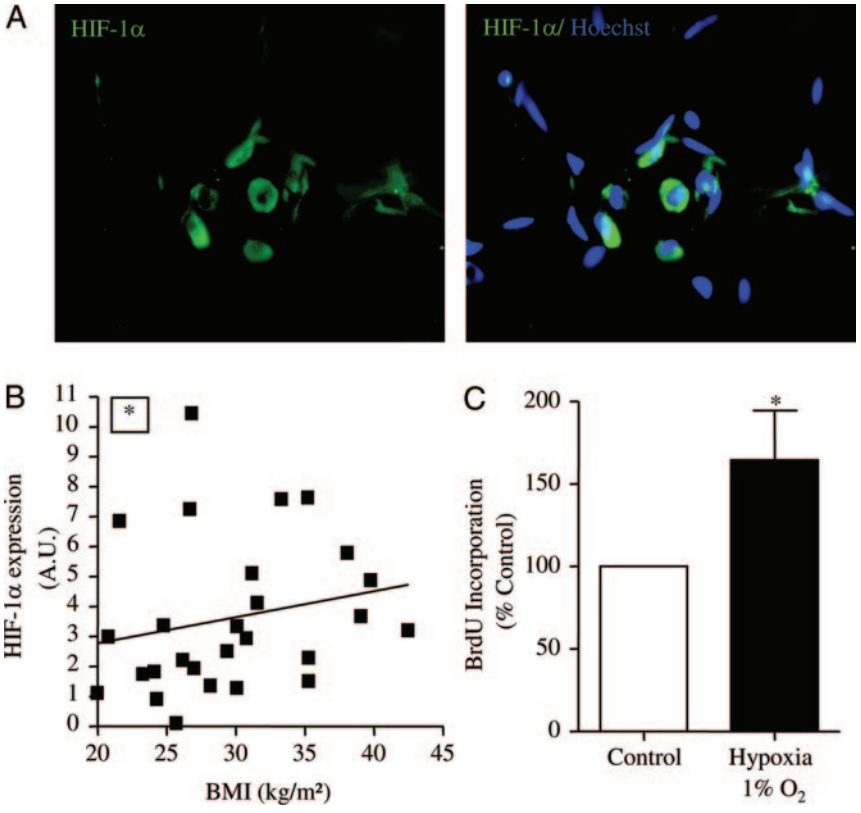


**FIG. 6.** Control of progenitor cell proliferation by low oxygen tension. A, Representative photomicrographs of immunohistochemical analyses of fixed and permeabilized human sc AT stained with HIF-1$\alpha$ antibody (*green*) and Hoescht 33258 (original magnification, ×200); B, correlation between HIF-1$\alpha$ mRNA levels in freshly harvested progenitor cells according to BMI ($P < 0.05$; Spearman r = 0.36; n = 27); C, $CD34^+/CD31^-$ progenitor cells were incubated for 48 h in basal medium in normoxia (20% $O_2$) (control) or in hypoxia (1% $O_2$). Proliferation was evaluated by BrdU incorporation assays. Results are expressed as a percentage of control (basal medium) and are presented as mean ± SEM from six separate experiments. *, $P < 0.05$.

cytes rather than an increased lipolysis because the proportion of hypertrophic adipocytes was not statistically altered. The formation of new adipocytes is thought to occur via the differentiation of the preadipocytes. Although several lines of evidence show that human preadipocytes are present in the AT-SVF, the exact nature of this cell population needs to be clearly defined (19). Moreover, whether this cell population is resident within the fat mass and/or originates from the recruitment of bone marrow-derived circulating progenitor cells, as recently suggested in mice (10), remains to be determined, because conflicting results have been recently published (11). The study of Nishimura *et al.* (20) in db/db obese mice has shown that newly formed adipocytes originated from AT stromal cells that had recently undergone cell division. Moreover, postconfluent mitosis and clonal expansion have been described as being required for the final expression of the adipogenic differentiation program in established preadipocyte cell lines (21). Finally, the study of Strawford *et al.* (22) demonstrated, using heavy water labeling, the presence of proliferating cells within the human sc AT. The present study demonstrates stromal cells that coexpress CD34 and the proliferation marker Ki-67 (23, 24). Because our previous reports defined the stromal $CD34^+$ cells as endothelial and adipocyte progenitor cells (13, 14), this result indicates that human AT contains proliferating progenitor cells located in the stromal fraction. Moreover, although a basal level of proliferative cells in lean/overweight patients with large interindividual differences was observed, adipocyte hyperplasia in obese patients was associated with an increase in the proportion of progenitor cells expressing the proliferative marker. Even though a potential effect of weight cycling on the control of progenitor cell proliferation cannot be ruled out, our results are in agreement with the study of Neese *et al.* (25) showing, by the use of heavy water labeling, an increased proliferation rate within the AT of obese *vs.* lean mice. In addition, obesity duration correlated neither with the percentage of progenitor cells expressing Ki-67 molecular marker of proliferation nor with the age of the class II to class III obese patients. Our results are in agreement with the study of Spalding *et al.* (6) because they propose, on one hand, that adipocyte number stays constant during adulthood, the number of which is the balance between adipocyte death and generation, and, on the other hand, that adipocyte cell number in adult obese subjects is not caused by a prolonged expansion period in adulthood. Whether proliferation could be considered as an index of progenitor cell entry into adipogenesis and/or a mechanism to replenish the local pool of immature progenitor cells of the expanding AT remains to be determined. The observation that the increased progenitor cell proliferation was associated with adipocyte hypertrophy suggests that hypertrophied adipocytes could control the growth of AT-derived progenitor cells. Changes in the production of adipokines such as leptin, IL-6, and LPA, described as being increased with the adipocyte size (26, 27), may be key players in such a process. In addition, hypoxia, recently reported to occur in the AT of obese mice (28), might also be considered as a consequence of an increased adipocyte diameter (29, 30). To note, there is no direct evidence of hypoxia in human AT. Freshly harvested AT progenitor cells expressed receptors for leptin, IL-6, and LPA and thus could be targets for such factors released in their neighborhood. Moreover, immunohistochemistry showed stromal cells positive for HIF-1$\alpha$ within AT, the expression of which was positively correlated with BMI. HIF-1$\alpha$ expression has been described to be increased in the entire AT of obese *vs.* lean subjects (31). To note, in addition to its major role in low oxygen sensing, studies performed on various preadipocyte models showed an increased expression of HIF-1$\alpha$ during adipogenesis and under normoxic culture conditions (32–34). Moreover, the pharmacological inhibition of HIF-1$\alpha$ activity led to the abrogation of adipocyte formation (34), strongly suggesting that HIF-1$\alpha$ could control adipogenesis. Thus, it is tempting to speculate that the HIF-1$\alpha$ transcript level increase with BMI in the progenitor cells might be related

to adipogenic events. CM originating from mature adipocytes, as well as leptin, IL-6, and LPA, stimulated the AT progenitor cell proliferation. Such an effect of leptin, IL-6, and LPA has already been described in established rodent preadipocyte cell lines or primary cultures of AT-derived SVF (35, 36). In addition, hypoxic culture conditions and CM from AT-CECs as well as the proangiogenic and hypoxia-regulated VEGF enhanced the growth of progenitor cells, suggesting that in addition to the direct effects of adipokines and hypoxia, the proliferation of the AT progenitor cells might be modulated by indirect effects through endothelial-derived hypoxia-sensitive factors. Such an observation is in agreement with Hutley *et al.* showing that factors secreted by human AT-derived endothelial cells promoted the human preadipocytes' proliferation (37). AT progenitor cells are considered to be mesenchymal stem cells (38). Interestingly recent investigations suggest that oxygen tension affects the physiology of mesenchymal stem cells including growth and *in vitro* development (39). Finally, the effects of macrophage-derived secretions were also studied. Macrophage accumulation within AT is associated with fat mass enlargement (12). Histological analyses have shown that macrophages localize to crown-like structures at sites of adipocyte death, the frequency of which is positively correlated with adipocyte size or inflammation (40, 41). In the same way, we found enhanced cell death in the AT from obese patients using the TUNEL method. Conflicting results were recently published by Spalding *et al.* (6) reporting no adipocyte death rate increase in obese patients, which might be explained by the use of a different approach. It is anyway tempting to speculate that adipocyte hypertrophy may be a triggering signal for macrophage accumulation. In the present study, macrophage-CM reduced the proliferation rate of the AT progenitor cells. This effect was suppressed in the presence of the antioxidant NAC, suggesting that reactive oxygen species produced by AT macrophages are able to inhibit the AT progenitor growth. Such an antiproliferative effect mediated by oxidative stress has been reported in the 3T3-L1 preadipocyte cell line (42).

Taken together, the present results demonstrate that human AT progenitor cells *in situ* exhibit a proliferative potential that is modulated by fat mass enlargement. The microenvironment in which the AT progenitor cells reside plays a key role in the regulation of the progenitor growth via the balance between proliferative signals originating from paracrine soluble factors released by the cellular partners of the AT-progenitor cells, adipocytes, endothelial cells, and oxygen tension. The antiproliferative reactive oxygen species derived from macrophages also contribute to the control of these progenitor cells. Adipocyte hypertrophy and/or death might also trigger the initial signals leading to increased AT-progenitor cell proliferation. Such an hypothesis is in agreement with the early observations of Hirsch and Batchelor (1) showing that new adipocyte formation occurs in AT once mature adipocytes have reached a critical size. Considering recent results in rodents demonstrating that blood-bone contribution to adipogenesis does not happen with detectable frequency, the study of fat cell progenitor proliferation/differentiation determinants *in vivo* remains a challenge.

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