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Proliferation and Differentiation of Human Adipocyte Precursor Cells: Differences Between the Preperitoneal and Subcutaneous Compartments

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ABSTRACT

Human adipocyte precursor cells (APC) have been characterized in their proliferation and differentiation potential from subcutaneous, omental, and mesenteric depots, mostly from morbidly obese patients. Cells from the preperitoneal adipose compartment have not been characterized yet, least of all when obtained from normal weight subjects. The aim was to compare proliferation and differentiation of subcutaneous (SC) and preperitoneal (PP) APC derived from adipose tissue in healthy subjects with different body mass. SC and PP adipose tissue was obtained during surgery of inguinal hernias in five healthy non-obese subjects and three obese otherwise healthy men. APC, obtained by collagenase digestion, were cultured. Proliferation was assayed by cell counting and differentiation by oil red O staining and flow cytometry using Nile Red staining. Proliferation of SC was higher than PP APC. Such differences between both compartments were even higher in APC obtained from obese patients. Conversely PP APC differentiated earlier in vitro compared with SC cells. These results agree with published data on fat cell proliferation. However regarding differentiation, our data show that APC from deeper depots (in this case PP) differentiate earlier than subcutaneous APC. This is different to previous studies performed in mesenteric or omental adipose tissue. J. Cell. Biochem. 111: 659–664, 2010.

KEY WORDS: ADIPOSE; PROLIFERATION; DIFFERENTIATION; FAT; HUMAN; PRECURSOR; PREADIPOCYTE

One of the main roles of adipose tissue is storage of triglycerides, according to energy balance. This is accomplished by hypertrophy of mature adipocytes as well as proliferation and differentiation of adipocyte precursor cells (APC) towards lipid-laden adipocytes. However, there is scarce information on human adipocyte expansion, and most studies have been performed in morbidly obese subjects. Limited information derived from normal or slightly overweight subjects is available; furthermore, tissue has been obtained from cancer patients requiring laparotomy. Obviously, adipose tissue samples obtained during hernia surgery and from non-obese subjects are smaller and thus, it is harder to accomplish primary cultures of APC.

The main cellular components of adipose tissue are mature adipocytes of different sizes and stromal-vascular cells, which include macrophages and preadipocytes or APC. Adipose tissue mass is determined by both adipocyte number and size. Therefore, obesity is caused both by hyperplastic growth through mitotic activity in precursor cells and further differentiation, and by hypertrophic growth, this is an increase in the size of adipocytes, due to lipid accumulation within the cell. Hyperplastic growth predominates during the third trimester of gestation and during prepuberty and puberty, but it also occurs in adulthood; long-term overfeeding and weight regain could induce adipocyte hypertrophy and hyperplasia [Löfgren et al., 2005; Jackman et al., 2008; Spalding et al., 2008]. It has been shown that adipose cells are able to re-enter the cell cycle during early stages of adipogenesis, resulting in increase of cell number [Fajas et al., 2001].

In adult human beings, adipose tissue depots show specific body distribution, in relation to degree of overweight, gender and genetic background, among other factors. Abundant research data relate abdominal fat with cardiovascular risk and metabolic diseases. Abdominal fat depots vary in size, function, and potential contribution to disease. Most studies focus on two main compartments: subcutaneous (SC) and visceral or intraperitoneal. However, recent studies have even detected differing capacities for replication, adipogenesis, and apoptosis between APC obtained from two distinct intraperitoneal visceral fat depots, mesenteric, and omental [Tchkonia et al., 2007]. Another less studied abdominal fat depot is the preperitoneal (PP), which widens in obese patients [Bortolotto et al., 2005], can be estimated adequately by ultrasonography [Liu

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et al., 2003], and has been reported to be less related with cardiovascular risk, compared to visceral fat [Liu et al., 2006]. Samples from this compartment can be obtained during dissection of inguinal hernias [Read and Schaefer, 2000; Carilli et al., 2004]. Proliferation and differentiation of APC from this compartment has not been studied up to date.

Published information on proliferation and differentiation capacity of adipose tissue, according to the compartment from which the samples were obtained (SC vs. visceral), is controversial. No information exists about PP fat. SC APC from obese subjects exhibit a higher proliferative capacity compared with deep abdominal (mesenteric or omental) APC [Read and Schaefer, 2000; Zuk et al., 2002]. However, regarding differentiation of the former versus the latter APC in obese subjects, some authors report differences (SC higher than omental and mesenteric) [Tchkonia et al., 2002], while others do not [Van Harmelen et al., 2004; Shahparaki et al., 2002]. Discrepancies are probably explained by methodological differences, or to different types of APC predominating in each fat compartment [Van Harmelen et al., 2004].

Since APC capacity for replication is probably increased in massively obese subjects compared with lean controls, it is also possible that regional differences in adipogenesis could also be influenced by obesity [Carilli et al., 2004]. Therefore, our main objective was to compare proliferation and differentiation of APC obtained from SC and PP adipose tissue, extracted during hernia surgery in healthy non-obese subjects, where regional differences could not be influenced by obesity. Data on proliferation was also compared with a few samples obtained from obese patients.

METHODS

SUBJECTS AND ADIPOSE TISSUE

SC and PP adipose tissue was obtained from eight healthy adult men (three obese), aged 45 ± 12 years, who underwent hernia surgery, after signing a written informed consent. All the procedures were approved by INTA's ethics committee.

ISOLATION OF ADIPOSE PRECURSOR CELLS (APC)

Under sterile conditions, fat tissue samples were washed with HBSS ($CaCl \cdot 2H_2O$ 1.2 M; KCl 5.4 mM; KH_2PO_4 44 mM; $MgCl_2 \cdot 6H_2O$ 40 mM; NaCl 0.1 M; $NaHCO_3$ 4.2 mM; $Na_2HPO_4 \cdot 7H_2O$ 22 mM; D-glucose 5.5 mM; pH 7.2), blood vessels were dissected and discarded. The remaining tissue was cut in 1 mm pieces, and incubated with 1 mg/ml solution of type I collagenase (Worthington Biochemical Corporation, NJ) in HBSS for 1 h at 37°C, then filtered through a sterile gauze. The cell suspension was centrifuged at 480*g* for 7 min; the supernatant (containing mature adipocytes) was discarded. The cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 0.1 mg/ml streptomycin (growth medium). Cells were seeded in culture dishes and kept at 37°C in a controlled atmosphere with 5% CO_2 until confluence was reached, after which cells were detached by incubation with trypsin/EDTA (0.05/0.02%, w/v) and replated. Culture medium was changed every 2 days until confluence was reached, and at third passage, cells were used for proliferation or differentiation assays [Zuk et al., 2002].

PROLIFERATION ASSAYS

5×10^4 APC were seeded in triplicate in 35 mm culture dishes and kept in growth medium. Proliferation was assessed by counting cell number after 1, 3, 6, 9, 13, and 17 days of culture. Proliferation was expressed as cell number at the different periods of time mentioned above, compared with cell number 24 h after seeding, as a ratio. Non-viable cells, identified trough trypan blue staining, were not included for counting; they did not increase throughout the culture period, never exceeded 10% and were similar between groups.

DIFFERENTIATION ASSAYS

After seeding, APC were maintained in control medium (DMEM:F12/10% FBS) until reaching confluence and then replaced by differentiation medium (control medium supplemented with 1 μM dexamethasone, 0.1 mg/ml isobutylmethylxanthine (IBMX), 0.1 mM indomethacin, and 10.5 μg/ml insulin) [Zuk et al., 2002]. Cells were kept in differentiation medium for 4, 7, and 11 days, replacing it every 2 days [Shahparaki et al., 2004; Pino et al., 2006].

ASSESSMENT OF DIFFERENTIATION BY OIL RED O STAINING

2×10^4 cells were seeded on sterile glass slides in 4-well culture dishes. After 7 or 11 days in differentiation medium cells were washed with HBSS and stained with a saturated Oil Red O solution in 60% isopropanol for 30 min at room temperature. Then the cells were washed with PBS (0.1 M NaCl; 3 mM KCl; 10 mM $Na_2HPO_4 \cdot 7H_2O$; 2 mM KH_2PO_4; pH 7.2) and stained with 20% hematoxylin solution for 5 min at room temperature. The stained cells were photographed with a digital camera attached to an inverted microscope at 40×. Stained areas were analyzed through Image J (two to four photographs for each condition) and corrected by nuclei number (in average 40 ± 15 cells per picture). The lipid accumulation using Oil red O staining can be assessed after 5 days of the differentiation stimuli [Ramírez-Zacarías et al., 1992].

FLOW CYTOMETRY ASSAYS

3×10^4 cells were seeded in 24-well culture plates and cultured under the same conditions described above, then detached with trypsin/EDTA solution (0.05/0.02%, w/v) and washed with PBS solution. Cells were stained with 1 μg/ml of Nile Red solution for 15 min at room temperature, and then washed twice with PBS. Fluorescence was measured in a flow cytometer (FACSCalibur; Becton Dickinson, Franklin, NJ), and expressed as mean fluorescent intensity (MFI) relative to that in undifferentiated cells (day 0).

STATISTICAL ANALYSIS

For proliferation studies and quantitative differentiation data, analysis were performed by four-way ANOVA for repeated measures and post hoc Tukey test to compare within and between times and study groups, respectively. All analysis were performed in Stata for Windows v 9.0 and presented as mean ± standard deviation (SD).

RESULTS

After obtaining adipose tissue from eight patients, the available APC for the assays was limited, because most subjects were not obese, and

the existence of the so-called "pre-hernial lipoma," which renders a higher amount of PP fat, is unpredictable. For these reasons we retained enough APC for both proliferation and differentiation studies in the two depots, in a smaller number of cases.

Proliferation assays were performed both in SC and PP cells obtained from four non-obese subjects (BMI 26–28 k/m^2) and three obese patients (BMI 32–44 k/m^2). All volunteers were healthy except for the hernia, were not diabetics, did not take any medications and did not smoke. In the non-obese subjects the rate of proliferation was higher for APC derived from SC compartments compared with those extracted from the PP areas (ratio of cell number at day 17th in relation to day $1 = 3.9 \pm 1.0$ vs. 2.4 ± 0.7 cells). In APC obtained from obese men a similar pattern was observed but with an even higher proliferation rate in the SC compartment (5.8 ± 3.4 in the SC, vs. 2.4 ± 0.8 in the PP), P within and between groups <0.001 (Fig. 1).

Differentiation was analyzed qualitatively by oil red O staining of APC obtained from the same four non-obese adult men, plus one more comparable subject. After 7 days of differentiation stimuli, PP APC accumulated more intracellular lipids compared with SC cells. A similar pattern was observed at 11 days ($P = 0.04$ between groups, $P = 0.8$ within groups; Fig. 2A and B). These data were confirmed by flow cytometry in APC from two subjects. As seen in Figure 3A and B, significant intracellular lipid accumulation started after the fourth day of treatment with differentiation media, and was significantly higher in PP APC compared with SC cells, at days 7 and 11.

DISCUSSION

Most studies regarding adipose tissue regional differences focus on their metabolic and endocrine characteristics. However, there is scarce information about the contribution of fat location on the proliferation and differentiation capacity of fat cells in humans. Moreover, for the most part, data derive from severely obese patients, where obtaining adipose tissue during bariatric surgery is effortless, but there is seldom information about behavior of adipose cells in normal or moderately overweight men. This can be attributed mainly to the smaller sample size (3–5 g adipose tissue per patient). In fact, the present report includes only four subjects, in which, enough tissue was obtained for both proliferation analysis, and these plus one more subject for differentiation assays, using staining and flow cytometry. In the other cases, we obtained less adipose tissue and APC were not sufficient for these assays. This was similar in the three obese patients studied, because the surgery of inguinal hernias does not offer the access to massive amounts of fat tissue.

Previous observations in APC obtained from obese subjects report that cells derived from SC compartments have a higher proliferation capacity compared with those obtained from intra-abdominal visceral areas [Tchkonia et al., 2005; Ohnishi et al., 2009]. In non-obese subjects we found a similar proliferation pattern (higher in SC compared with PP depots), and also detected that proliferation of SC APC was even higher in cells obtained from obese patients compared to non-obese patients (Fig. 1). The PP compartment has not been adequately studied, but, when measured by ultrasonography, has been found to be less related to the metabolic syndrome compared to mesenteric fat [Liu et al., 2003]. Regarding differentiation capacity we found that lipid accumulation in PP APC preceded that of SC cells, starting from day 7. Compared to visceral depots, no conclusions can be drawn. Tchkonia et al., reported the opposite behavior in adipose tissue obtained from obese patients, that is earlier differentiation in SC versus omental adipocytes at days 10 and 15. However, he employed another methodology, including a chemically defined adipogenic media enriched with rosiglitazone but without fetal calf serum (FCS) and differentiation was assayed by glycerol-3-phosphate dehydrogenase activity [Tchkonia et al., 2002,

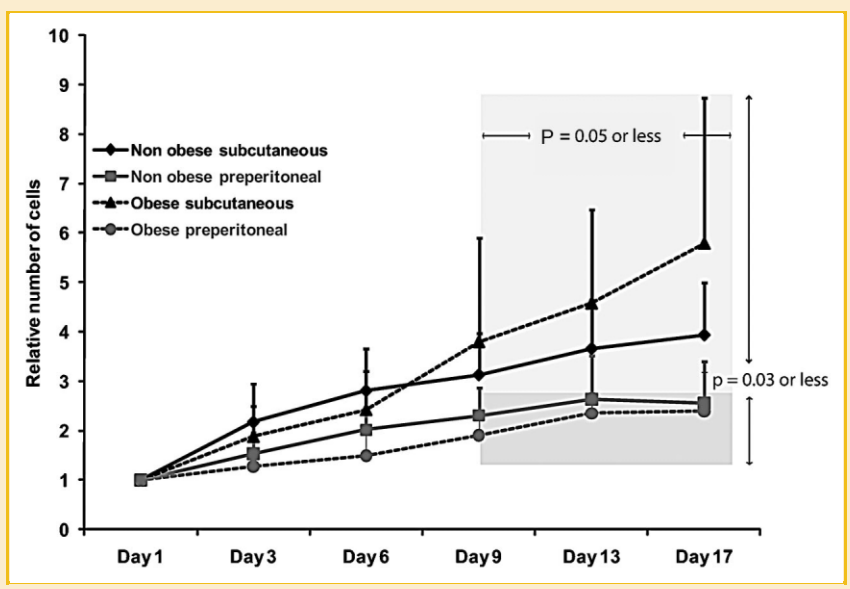

Fig. 1. Proliferation capacity (expressed as ratio of cell number at day 1/cell number at day 3, 6, 9, 13, and 17) was significantly higher in APC obtained from subcutaneous fat (SC–) compared with preperitoneal fat (PP–), in obese and non-obese subjects. Repeated measures ANOVA between and within groups $P < 0.001$.

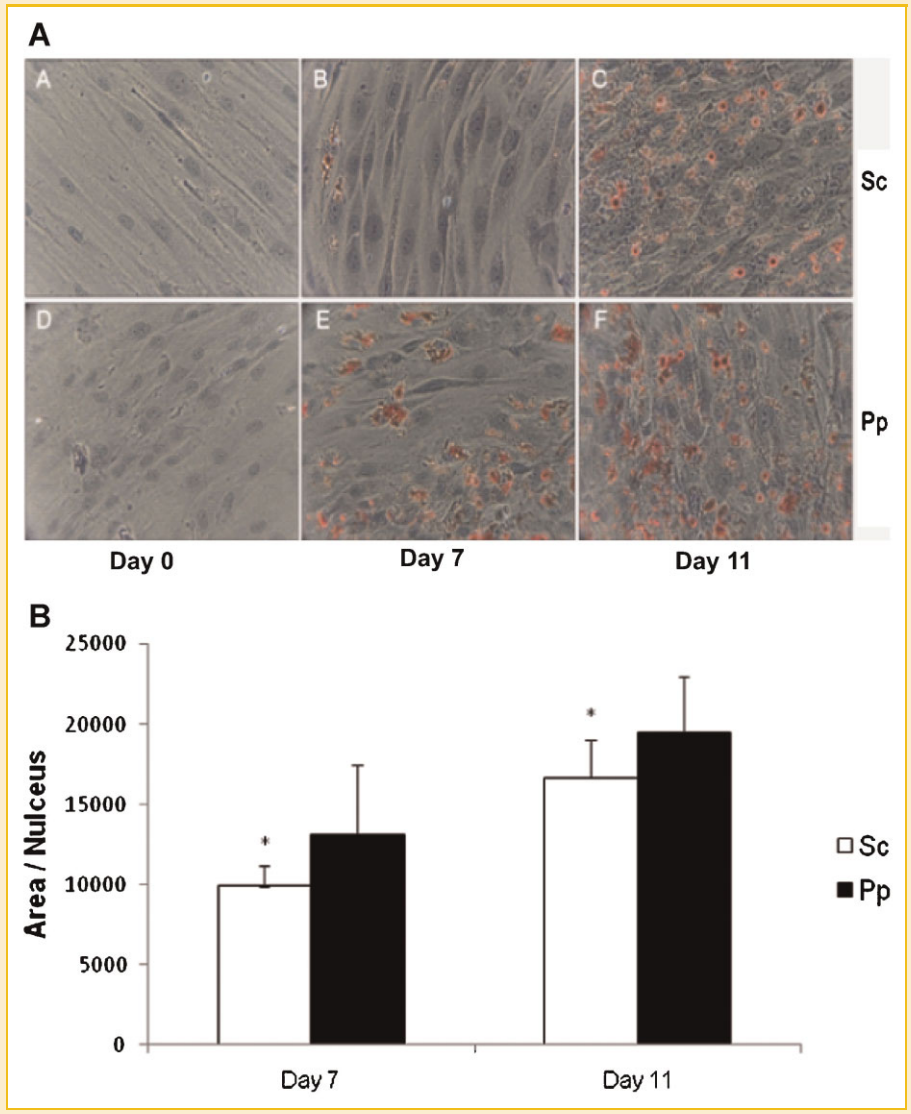

Fig. 2. A: Representative microphotographs showing fat accumulation by oil red O staining in subcutaneous (SC) and preperitoneal (PP) APC, treated for the indicated periods with differentiation medium (40×). B: Quantification of oil red O staining in subcutaneous and preperitoneal APC, expressed as stained area per nucleus (image J). At days 7 and 11 preperitoneal APC had significantly higher lipid accumulation compared with subcutaneous APC (repeated measures ANOVA between groups $P = 0.04$), while differences within groups were not significant. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

2005]. In other studies performed in massively obese subjects, differentiation kinetics was similar among SC and intra-abdominal APC [Van Harmelen et al., 2002, 2004]. Shahparaki et al. [2002] obtained comparable results in overweight subjects. Discrepancies between our results and those showing no differences in adipocyte differentiation between fat compartments [Shahparaki et al., 2002; Van Harmelen et al., 2004] could depend upon methodological issues. The latter studies assessed fat accumulation at 16 and 21 days, while we studied it starting from day 7 (oil red O) and day 4 (flow cytometry). Other factors that could influence our results could be gender and nutritional issues (we included only normal or slightly overweight men) and the attainment of cells from PP instead of omental or mesenteric depots.

Since mature adipocytes are unable to proliferate, an increase in adipocyte number within adipose tissue must originate from differentiation of APC. These cells, also called preadipocytes are committed cells originated from mesenchymal stem cells [Sengenes et al., 2005]. In addition to mature adipocytes, adipose tissue contains stromal-vascular cells that can remain undifferentiated or committed towards adipocytes, at several degrees. Preadipocyte dynamics could be explained by cell composition, which differs between compartments, and nutritional status of individuals. By immunofluorescent staining for the intracellular adipocyte fatty acid binding protein (aP2), Tchoukalova et al. [2007] found a decrease in the proportion of committed SC APC, among obese women, compared with lean, which was related to less differentiation and higher apoptosis. However, these authors did not obtain deeper abdominal cells, only SC. Our results show that PP cells differentiate before SC cells, which could suggest that committed APC predominate in the PP compartment.

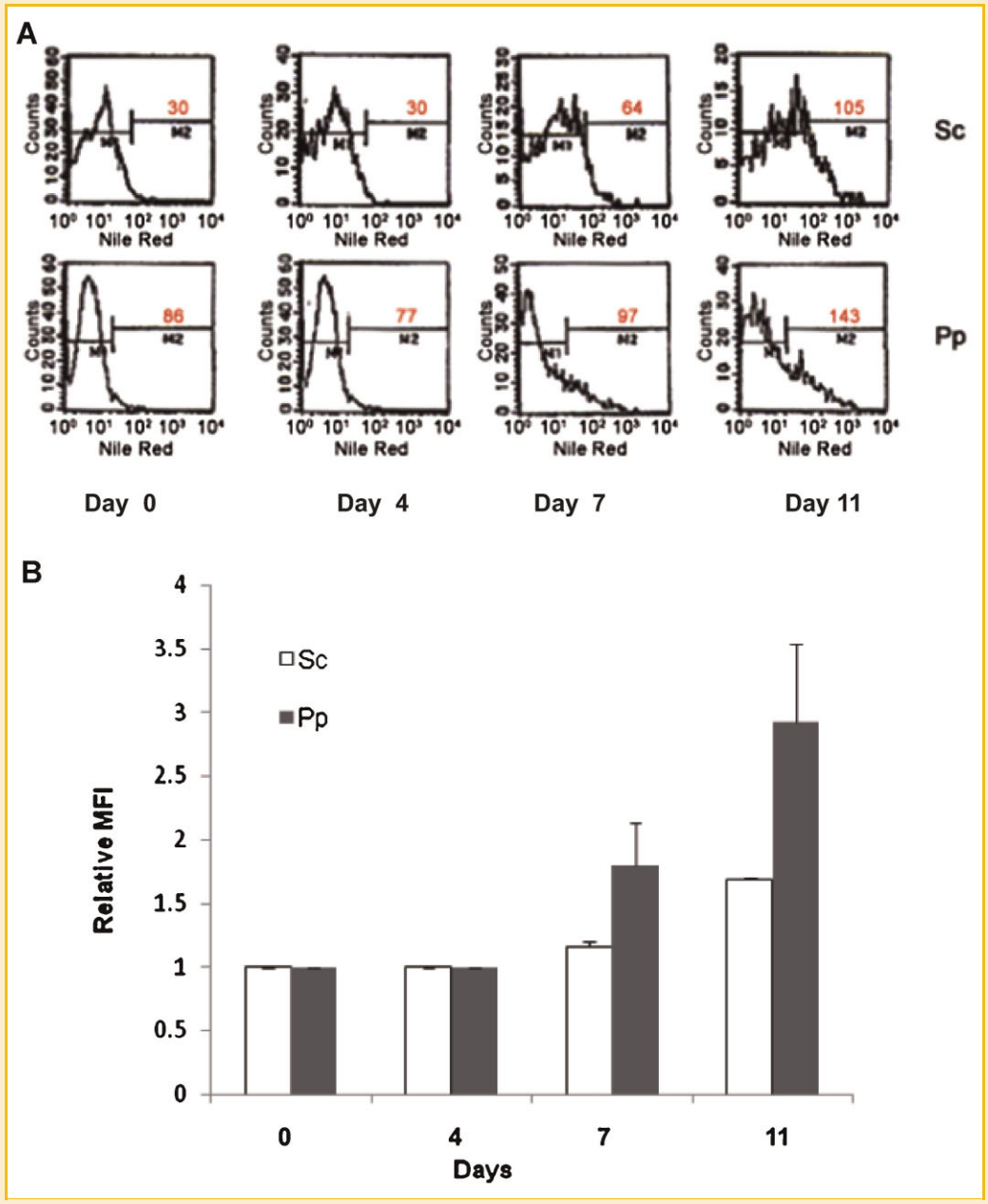

Fig. 3. A: representative histogram of flow cytometry assays, showing fat accumulation by Nile red staining, after treatment with differentiation media and (B) relative to day 0, mean fluorescence intensity (MFI) was higher in preperitoneal (PP) compared with subcutaneous (SC) cells, at days 7 and 11 (day 0 or Control refers to undifferentiated cells). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In adipose tissue, the role of cellularity in relation to its behavior, is not completely clear. On one hand, it has been shown that a long-term positive energy balance engenders more available adipose cells, which would allow a better metabolic performance [Heilbronn et al., 2004; Kim et al., 2007]; therefore, a greater APC proliferation and differentiation rate would be desirable. But on the other hand, it is obvious that enhanced proliferation of APC and further differentiation to mature cells leads to adipose tissue expansion and progressive obesity (further maintained by a greater proliferation capacity of APC in obese subjects as shown in Fig. 1). However, the in vitro evidence not necessarily represents the in vivo situation, where APC responds to a certain microenvironment. Only assuming that these in vitro observations can be extrapolated to human physiology, we would be able to state that SC adipose tissue has a greater ability to expand the number of cells that are able to become preadipocytes, compared to the PP compartment. Therefore, since small but numerous adipocytes represent a metabolic advantage over less but more hypertrophic cells, one can assume that the higher proliferation capacity of the SC compartment, and the opposite earlier differentiation but slower proliferation of the PP APC, would be equivalent to the harmful pattern of deep abdominal fat enlargement. Thus, PP adipose tissue should represent a more visceral-like fat depot in terms of insulin sensitivity and cardiovascular risk.

Depending on the localization, adipose tissue differs in several characteristics. SC and visceral adipose tissue have been well studied in the physiological characteristics that distinguish them. However, the PP depot has been described mostly in radiological and clinical terms. Our results suggest that PP adipose tissue has differences in terms of both proliferation and differentiation compared with SC depot. It remains unclear whether this is translated clinically in a visceral-like behavior or not.

In conclusion, we describe the cellular proliferation and differentiation kinetics of preadipocytes obtained from the less characterized, PP fat compartment and compare it with SC APC. These preliminary results agree with published data on fat cell proliferation; however, respect differentiation, further studies using comparable methodologies are required to answer whether behavior of PP is similar to deeper intra-abdominal depots.

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