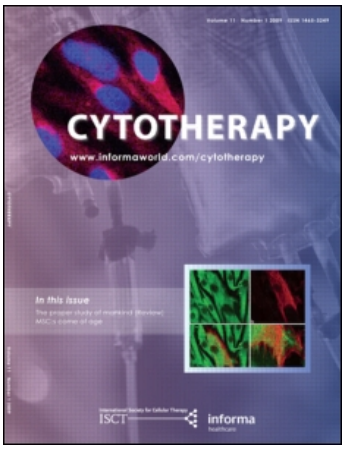

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Human adipose-derived stem cells isolated from young and elderly women: their differentiation potential and scaffold interaction during *in vitro* osteoblastic differentiation

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Background aims

Several authors have demonstrated that adipose tissue contains multipotent cells capable of differentiation into several lineages, including bone, cartilage and fat.

Methods

This study compared human adipose-derived stem cells (hASC) isolated from 26 female donors, under 35 and over 45 years old, showing differences in their cell numbers and proliferation, and evaluated their in vitro *adipocytic and osteoblastic differentiation potential.*

Results

The cellular yield of hASC from older donors was significantly greater than that from younger donors, whereas their clonogenic potential appeared slightly reduced. There were no significant discrepancies between hASC isolated from young and elderly women regarding their in vitro *adipocytic differentiation, whereas the osteoblastic potential was significantly reduced by aging. We also assessed the influence of hydroxyapatite (HAP) and silicon carbide (SiC–PECVD) on hASC. Even when cultured on scaffolds, hASC from younger donors had better differentiation into osteoblast-like cells than hASC from older donors; their differentiation ability was up-regulated by the presence of HAP, whereas SiC–PECVD produced no significant effect on hASC osteoblastic differentiation.*

Conclusions

The large numbers of hASC resident in adipose tissue and their differentiation features suggest that they could be used for a successful bone regeneration process in vivo. *We have shown that age does not seem to affect cell viability and* in vitro *adipocytic differentiation significantly, whereas it does affects osteoblastic differentiation, in the absence and presence of two-dimensional and three-dimensional scaffolds.*

Keywords

Aging, ex vivo *expansion, human adipose-derived stem cells, multipotential differentiation, osteoprogenitor cells, tissue regeneration.*

Introduction

Because of a low self-regenerative faculty, bone and cartilage defects caused by trauma, tumors and congenital deficiency never restore spontaneously. Innovative musculoskeletal regeneration approaches include tissue-engineering techniques using adult mesenchymal stromal cells (MSC) [1–10]. Bone marrow (BM), muscle, synovial membrane and adipose tissue are sources of MSC [11–14]. It is known that adipose tissue changes during life, together with a coordinated remodeling of the adipose vascularization [15];

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these modifications are mediated by resident stem cells [15]. Many authors have shown that these multipotent cells share quite similar features with BM stromal cells (BMSC) [13,16,17]; indeed, human adipose-derived stem cells (hASC) are clonogenic, proliferate easily and their progenies show a broad multilineage differentiation potential [18–21]. Furthermore, hASC proliferation and differentiation do not appear to be negatively affected by donor age, in contrast to reports on BMSC [22,23]. Compared with other adult tissues, adipose tissue has several advantages for both research purposes and clinical applications: a minimally invasive procedure is required for tissue withdrawal and a large number of multipotent cells can be isolated rapidly.

Musculoskeletal tissue regeneration often requires the combination of cells and scaffolds. The ideal material should be bio-degradable or bio-absorbable, suitable for cellular adhesion, and not induce inflammatory and immunologic responses [2]. In particular, in bone tissue engineering, the biomaterial should provide biomechanical support until tissue regeneration is completed and, during this process, the scaffold needs to disappear progressively to allow new tissue formation and cell colonization. Loading stem cells on scaffolds *in vitro* before implantation may lead to a faster bone formation and osteointegration *in vivo* [24,25].

We compared hASC isolated from female donors who were under 35 and over 45 years old, and examined their *in vitro* adipocytic and osteoblastic differentiation potential. We also investigated the influence of natural and synthetic scaffolds on hASC osteoblastic differentiation.

Methods

Isolation and culture expansion of hASC

The study was carried out after institutional review board approval. Subcutaneous fat was obtained from 26 healthy female donors [age range 21–68 years, body mass index (BMI) <30, without any pathologic obesity] undergoing plastic surgery by elective lipoaspiration, after written consent. Primary cultures of the stromal vascular fraction (SVF) were established as described previously [13]. Briefly, the raw lipoaspirates (50–100 mL) were washed at least three times with phosphate-buffered saline (PBS); the matrix was then enzymatically digested with 0.075% type I collagenase (Worthington, Lakewood, NJ, USA) at 37°C with continuous agitation for 30 min. The SVF was then centrifuged (1200 g, 10 min) and filtrated through a sterile medication lint. The collected SVF cells were plated in control medium [Dulbecco's modified Eagle medium (DMEM) +10% fetal bovine serum (FBS) supplemented with 50 U/mL penicillin, 50 µg/mL streptomycin and 2 mM L-glutamine] at approximately 10^5 cells/cm^2. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. After 48 h non-adherent cells were removed and the medium was changed every other day. Adherent cells were grown until 80% confluence, detached with 0.5% trypsin/0.2% EDTA (Ethylene Diamine Tetraacetic acid), and replated at a density of 10^4 cells/cm^2 for further analyzes.

For the raw fat manipulation study, lipoaspirates were separated into two experimental groups, 'filtered' and 'centrifuged', according to the intra-operative manipulation procedure, as described previously [26–28]. The hASC isolation was performed as described above.

Fibroblast and osteoblast colony-forming unit assays

A colony-forming unit–fibroblast (CFU-F) assay was performed as described previously [29] with minor modifications. hASC were plated in six-well plates at low density by limiting dilution (starting dilution 48 cell/cm^2, ending dilution 1 cell/cm^2) and cultured at 37°C in a humidified atmosphere with 5% CO_2 in DMEM/20%FBS. After 6 days the medium was replaced, and 10 days after cells were fixed with methanol and stained with Gram's crystal violet. The frequency of CFU-F was established by scoring the individual colonies and expressed as a percentage relative to the seeded cells.

A colony-forming unit–osteoblast (CFU-O) assay was performed by plating cells in six-well plates by limiting dilution and culturing at 37°C in osteogenic medium (as described below) for 14 days. Colonies were then stained with Alizarin Red S (pH 4.1).

Cell lineage differentiation and evaluation of differentiation markers

Osteoblastic differentiation

10^4 hASC/cm^2 were induced to differentiate on monolayer in osteogenic medium consisting of control medium supplemented with 10 mM glicerol-2-phosphate, 10 nM dexamethasone, 150 µM L-ascorbic acid-2-phosphate and 10 nM cholecalciferol. After 14 days of differentiation, alkaline phosphatase activity (ALP) was determined (Triton X-100 0.1% in ddH_2O as lysis buffer) using 1 mM p-nitrophenylphosphate in alkaline buffer (100 mM diethanolamine and 0.5 mM $MgCl_2$, pH 10.5) as substrate [30] and normalized

for protein content, determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The ALP assay was performed at 37°C and absorbance was read at 405 nm (Wallac Victor II, Perkin Elmer Western Europe, Monza, Italy). To evaluate calcium deposition, 10^4 cells/cm^2 were cultured for 21 days without any cell detachment then samples rinsed with PBS and fixed with ice-cold 70% ethanol for 1 h. They were stained with 40 mM Alizarin Red S (pH 4.1) for 15 min and finally rinsed with ddH_2O. Each sample was destained for 30 min with 10% cetylpyridinium chloride monohydrate (CPC) in 0.1 M phosphate buffer (pH 7.0) and the absorbance read at 550 nm (Wallac Victor II) [31]. All steps were performed at room temperature (RT).

Adipocytic differentiation

10^4 hASC/cm^2 were induced to differentiate into the adipogenic lineage using a pulsed induction comprising 48 h in control medium supplemented with 1 μM dexamethasone, 10 μg/mL insulin, 500 μM 3-isobutyl-1-methylxanthine (IBMX) and 200 μM indomethacin, followed by 48 h maintenance in control medium supplemented only with 10 μg/mL insulin. After 14 days, samples were rinsed and fixed in 10% neutral buffered formalin for 1 h and stained with fresh Oil Red O solution (2% w/v Oil Red O in 60% isopropanol) for 15 min. To quantify the lipid vacuole content, the dye was extracted with 100% isopropanol and absorbance was read at 490 nm.

PPAR-γ (Peroxisome Proliferator-Activated Receptor) expression of 14-day adipogenic differentiated hASC was evaluated by Western blot. Cells were collected by centrifugation, and the cell pellet resuspended in lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P40, 0.1% SDS) containing proteinase inhibitors and incubated at 4°C for 30 min. Following centrifugation at 14,000 *g* for 10 min, the supernatant containing total cell extract was collected and kept at –20°C. The protein concentration of cell lysates was measured using a Pierce protein assay (Pierce). After boiling for 5 min, 15 μg protein were loaded on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel (PAGE) and electrotransferred onto Hybond™–ECL™ extra nitrocellulose membrane (Amersham Bioscience, Pittsburgh, PA). After blocking in 5% non-fat dry milk in TBS (Tris Buffer Solution) and 0.1% Tween-20 (TBST) for 1 h at RT, the membrane was incubated with rabbit anti-PPAR-γ (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and mouse anti-β-actin (Sigma Aldrich, Milan, Italy), diluted at 1:500 and 1:3000, respectively, in blocking buffer at 4°C overnight. This was followed by incubation with horseradish peroxidise (HRP)-conjugated secondary antibodies (GE Healthcare, Pittsburgh, PA) for 1 h at RT. Protein visualization was performed using the ECL™ Western blotting analysis system kit (GE Healthcare, Pittsburgh, PA) according to the manufacturer's protocol. The images were then analyzed with ImageJ.

hASC–scaffold constructs

10^5 undifferentiated hASC were seeded on porous (60%) hydroxyapatite blocks (HAP) (Finceramica, Faenza, Italy) and silicon carbide–plasma-enhanced chemical vapor deposition (SiC–PECVD) fragments (kindly provided by Galileo Avionica, Carsoli, Italy). Cells were allowed to adhere overnight to scaffolds in a polypropylene vial, then transferred to a 24-well plate and cultured in static conditions for 14–21 days. The SiC–PECVD samples were assessed for ALP activity after 14 days of differentiation and for calcium deposition after 21 days; ALP activity at 14 and 21 days was evaluated for HAP samples.

Statistical analysis

All results are expressed as mean ± SD. Student's *t*-test and two-way ANOVA (with Bonferroni's post-test) were performed to analyze data; *P*-values to be considered were $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

Results

Features of hASC derived from under 35- and over 45-year-old female donors

hASC were isolated from liposuction of abdominal subcutaneous adipose tissue aspirates from 26 female donors. We analyzed the progenitor cell numbers and clonogenic potential of hASC derived from females under 35 years old ($n=12$, mean age 31 ± 4 years) and over 45 years old ($n=14$, mean age 56 ± 7 years). We isolated an average of $2.7\pm1.3\times10^5$ hASC/mL raw adipose tissue from the younger donors, in contrast to $6.1\pm4\times10^5$ from the older donors, as shown in Figure 1A. Despite the large fluctuations among samples derived from the over 45-year-old group, as shown in the scatter dot-plot in Figure 1B, the cellular yield turned out to be significantly different between the two groups ($P=0.007$). However, this broad variation did not correlate with donor age within the group (data not shown). CFU-F assays were also performed (Figure 1A). At earlier passages, hASC from both groups were able to produce colonies, although cells from younger donors showed a two-fold increase in clonogenic

A

	Under 35y/o	Over 45y/o
Yield	$n = 12$	$n = 14$
(hASCs/ml of tissue)	$2.7\times10^5 \pm 1.3\times10^{5**}$	$6.1\times10^5 \pm 4\times10^5$
CFU-F (%)		
Passage in culture		
I ($n = 4$)	15 ± 5	7 ± 3
II ($n = 3$)	12 ± 3	8 ± 2
CFU-O (%)		
Passage in culture		
II ($n = 3$)	4 ± 2	3 ± 1

B

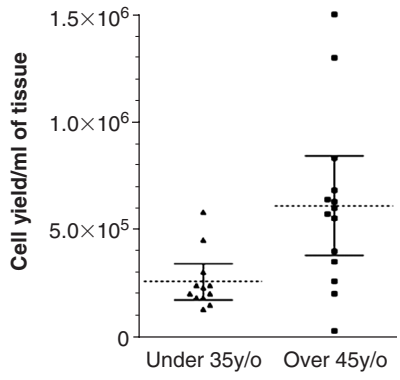

Figure 1. *Comparison of cell number and clonogenicity of hASC derived from two groups of females (under 35 and over 45 years old). (A) Cellular yield is expressed as the average of hASC/mL of raw lipoaspirates ± SD. CFU-F and CFU-O frequencies are expressed as a percentage (number of colonies/number of plated cells) ± SD. A two-tailed Student's t-test was performed: **P < 0.01. (B) The cellular yield variability between the two experimental groups is represented by a scatter dot-plot; each donor's yield is represented as a dot, with the mean (dotted line) and 95% confidence interval shown.*

activity. This trend was maintained during passages, whereas hASC from older donors lost their clonogenic ability sooner (data not shown) [32]. We then assessed the presence of osteoblast-forming units in hASC derived from the two age groups at the second passage. About 30% of cells able to form fibroblast colonies generated CFU-O. However, we did not observe any significant differences between CFU-O produced by hASC from younger and older donors.

We assayed specific MSC markers (CD13, CD14, CD29, CD34, CD44, CD45, CD49d, CD54, CD71, CD90 and CD105) by flow cytometric analysis. Comparing these data with previously published data [33,34], no significant variations related to donor age were observed (data not shown).

hASC differentiation potential

hASC were induced to differentiate towards adipocyte- and osteoblast-like cells. Two weeks in differentiating culture conditions showed significant morphologic changes (Figure 2B, C, E, F) compared with undifferentiated hASC (Figure 2A, D). The classic fibroblastoid-like shape of undifferentiated hASC was progressively lost when cells were maintained in adipogenic and osteogenic media; hASC became either larger, with a cytoplasm full of lipid vacuoles, or less outstretched, with an indented cellular membrane, respectively. We did not observe any relevant morphologic variations among all the older and younger derived hASC.

hASC from younger and older donors show no significant differences in their *in vitro* adipocytic differentiation potential

We evaluated the hASC adipocytic differentiation potential by comparing the amount of lipid vacuoles produced by several samples of adipogenic-differentiated hASC (Figure 3A, B). As shown in Figure 3A, significant Oil Red O staining was observed in adipogenic-differentiated cells. The *in vitro* adipocytic differentiation potential of younger and older donors was not statistically different, although the increase in lipid content of differentiated hASC was 140% and 360% for cells derived from under 35- and over 45-year-old donors, respectively ($n = 5$, $P < 0.05$). This apparent discrepancy was because of a lower level of undifferentiated cells derived from older donors (Figure 3B).

PPAR-γ expression in undifferentiated and adipogenic-differentiated hASC was analyzed (Figure 3C). PPAR-γ was expressed both in undifferentiated and adipogenic-differentiated cells, with an up-regulation of about 35% in the differentiated samples from both age groups ($n = 4$, $P < 0.05$). However, no significant difference in PPAR-γ expression of differentiated hASC between the two groups was detected.

Comparison of hASC isolated from differently manipulated adipose tissue

We also compared hASC isolated from subcutaneous adipose tissue manipulated by either Coleman's method

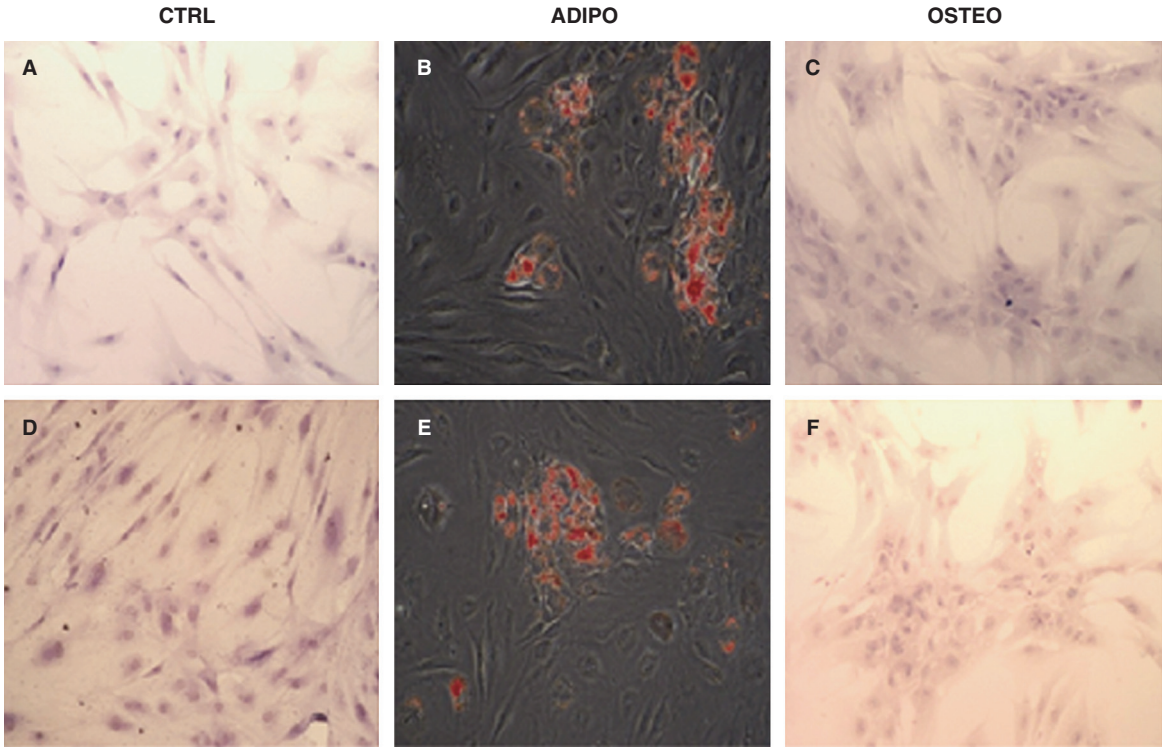

Figure 2. *Morphology of cultured hASC. Hematoxylin-stained hASC from donors under 35 years old (upper line) and over 45 years old (lower line) were cultured in control (A, D), adipogenic (B, E) and osteogenic (C, F) differentiation media (100×).*

[28,35] or filtration, as described by others [26,27]. Abdominal subcutaneous adipose tissue from four women belonging to the over 45-year-old group (mean age 57 ± 11) was both filtered and centrifuged directly in the surgery room and then hASC isolated. Immediately after isolation, $5.73 \pm 1.51 \times 10^5$ hASC/mL from filtered tissues (FILT-hASC) and $6.1 \pm 2.41 \times 10^5$ hASC/mL from centrifuged tissues (CENT-hASC) were obtained, indicating that different tissue manipulations did not affect the yield of progenitor cells. At passage 1, we counted $2.36 \pm 1.1 \times 10^5$ FILT-hASC/mL of digested tissue, and $4.6 \pm 3.3 \times 10^5$ CENT-hASC, suggesting that centrifugation may be less traumatic and cells may recover faster, although the difference was not statistically significant. In addition, no difference in cellular proliferation was observed, and both FILT-hASC and CENT-hASC showed similar clonogenic activities, of 8 ± 2 and $6 \pm 2\%$, respectively. As shown in Figure 4, different cell populations (hASC-1 and hASC-2) showed great variability in their *in vitro* adipocytic differentiation; however, within each population no significant differences were observed between FILT-hASC and CENT-hASC, suggesting that the intrasurgery room manipulation of adipose tissue does not influence progenitor cell number and their adipocytic differentiation ability.

Donor age mildly affects hASC in vitro osteoblastic differentiation potential

hASC from younger donors cultured in osteogenic differentiating medium for 14 days showed a higher ALP activity compared with cells from older donors ($n = 5$, $P < 0.001$) (Figure 5A). Indeed, differentiated cells from the younger group showed an increase in ALP activity of 280% with respect to undifferentiated ones, whereas the increase was just 40% for hASC derived from the over 45-year-old group. Moreover, the ALP basal level in undifferentiated hASC derived from younger donors was three-fold higher than from older donors.

These observations were partially confirmed by quantification of calcium deposition: cells from the under 35-year-old group produced a greater amount of calcium deposits compared with cells from the over 45-year-old group (Figure 5B). However, the great variability among donors meant no significant difference between the two groups ($n = 5$). The increase in extracellular calcium formation by differentiated hASC was 220 and 73% from younger and older donors, respectively.

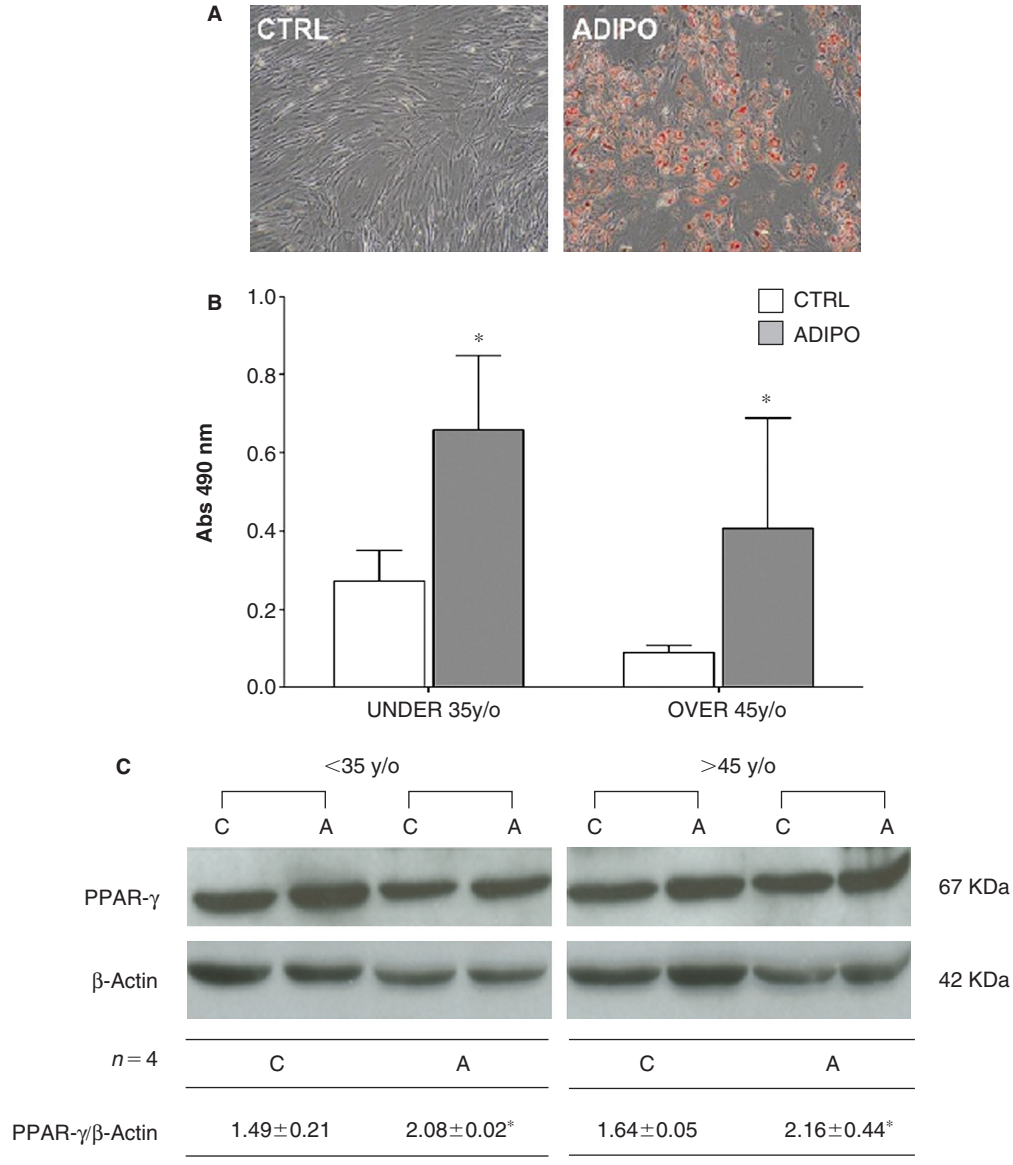

Figure 3. *Adipocytic differentiation of hASC derived from donors under 35 and over 45 years old. (A) Microphotographs (40×) of undifferentiated (left) and adipogenic-differentiated (right) hASC stained with Oil Red O after 14 days of culture. (B) Quantification of lipid deposition in undifferentiated (CTRL, white bars) and adipogenic-differentiated hASC for 14 days (ADIPO, filled bars) in both age groups; data are expressed as mean ± SD* (n = *5). (C) PPAR-γ expression in younger and older donors. Western blot analysis of two populations for each group (upper line) and quantification of PPAR-γ expression standardized on* β*-actin content; data are expressed as mean ± SD* (n = *4). A two-way ANOVA (with Bonferroni's post-test) was performed:* *P < *0.05, ADIPO versus CTRL.*

Scaffolds may affect hASC *in vitro* osteoblastic differentiation

hASC cultured on scaffolds were analyzed (Figures 6 and 7). Their osteoblastic differentiation ability in the presence of HAP scaffolds was monitored by ALP assay only, as the amount of calcium produced by cells could be masked by the calcium released by HAP.

As shown in Figure 5A, even when cultured on HAP, the ALP activity of hASC from younger donors, differentiated for either 14 and 21 days, was significantly increased compared with the one determined in cells from older donors, with an increase of about 10-fold (Figure 6A, B) ($P < 0.001$). Comparing the ALP activity on the plastic (polystyrene; PS) and scaffold, we observed osteoinductive properties of

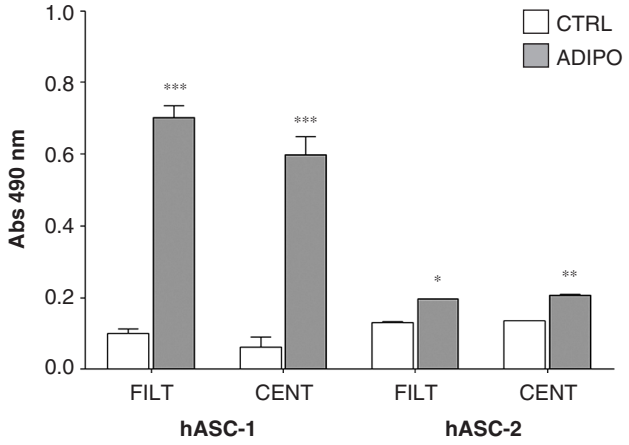

Figure 4. *Comparative study of adipocytic-differentiation potential of hASC isolated from two donors (from the over 45-year-old group) differently manipulated in the surgery room (FILT, filtered; CENT, centrifuged). A two-way ANOVA (with Bonferroni's post test) was performed:* $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, *ADIPO versus CTRL.*

HAP on hASC; indeed, ALP levels of hASC from both younger and older donors cultured on HAP were significantly higher compared with those maintained on PS (Figure 6A, B).

The influence of another scaffold on the osteoblastic differentiation ability of hASC was assessed by culture on SiC–PECVD, a two-dimensional (2-D) metallic scaffold (Figure 7). SiC–PECVD did not show any particular osteoinductive property: no significant difference in ALP activity was detected from cells derived from both groups cultured on PS and SiC–PECVD (Figure 7A). These data were confirmed by quantification of calcium deposition (Figure 7B): there was no significant difference in extracellular calcified matrix production between cells cultured on PS and SiC–PECVD derived from both age groups. Also, donor age negatively influenced the osteoblastic marker expression analyzed ($P<0.001$) (Figure 7A, B).

Discussion

Although BM provides an important source of MSC, adipose tissue also possesses abundant and easily accessible progenitor cells. The harvesting procedure for adipose tissue is less invasive and discomfort for a patient is minimal. Moreover, fat may have a further advantage when the morbidity associated with large volumes of BM harvest is taken into consideration [36]. In this study, we analyzed and compared hASC harvested from female donors aged under 35 and over 45 years. The significantly more abundant hASC yield per milliliter of raw lipoaspirate from the over 45-year-old donors may be related to the hormonal disequilibrium in this period of a woman's life, which may enhance the number of progenitor mesenchymal cells in adipose tissue, known to increase with age. On the other hand, the number of CFU-F was just slightly reduced from older donors. No significant differences were detected in terms of the *in vitro* adipocytic differentiation potential of hASC from younger or older donors; indeed, in both cases cells were able to differentiate into adipocyte-like cells (Figure 3). On the other hand, age influenced the hASC *in vitro* osteoblastic differentiation

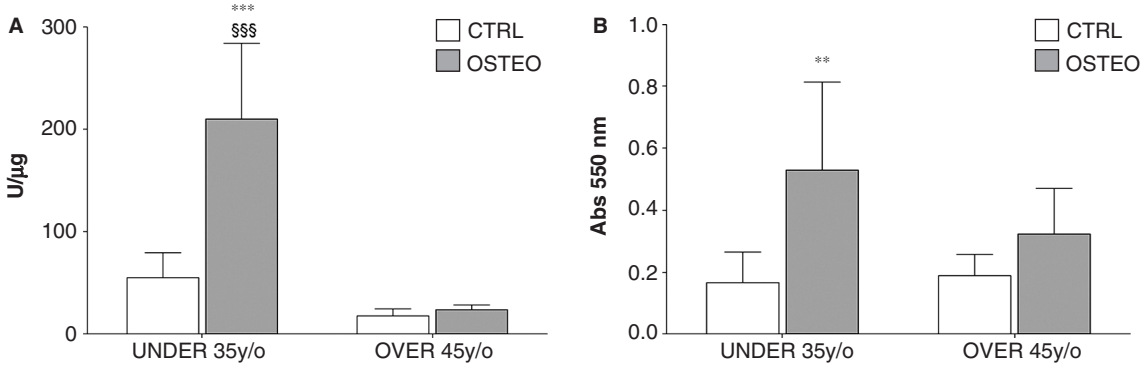

Figure 5. *Osteoblastic differentiation of hASC derived from donors under 35 and over 45 years old. (A) ALP activity, in undifferentiated hASC and osteogenic-differentiated hASC for 14 days. Data are normalized with respect to protein content and expressed as mean ± SD (n = 5). (B) Quantification of calcium deposits in undifferentiated and osteogenic-differentiated hASC for 21 days. Alizarin Red S-stained samples were extracted with CPC; data are expressed as mean ± SD (n = 5) (CTRL, white bars; OSTEO, filled bars). A two-way ANOVA (with Bonferroni's post-test) was performed:* $^{**}P<0.01$, $^{***}P<0.001$, *OSTEO versus CTRL;* §§§$P<0.001$, *UNDER 35 years old versus OVER 45 years old.*

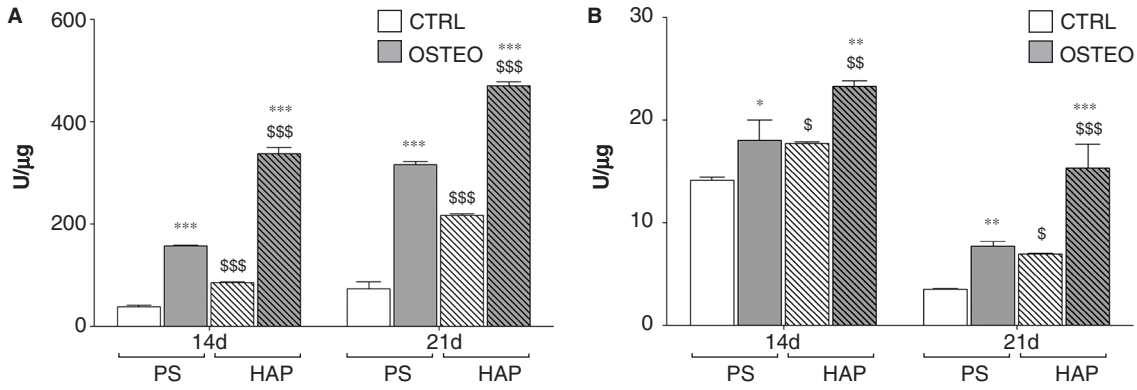

Figure 6. *Influence of HAP on* in vitro *osteoblastic differentiation of hASC belonging to the two age groups. ALP activity, in undifferentiated (CTRL, white bars) and osteogenic-differentiated (OSTEO, filled bars) hASC, from (A) the under 35-year-old and (B) the over 45-year-old groups cultured for 14 and 21 days on PS and HAP (patterned bars). Data were standardized with respect to protein content and expressed as mean ± SD* (n = *3). A two-way ANOVA (with Bonferroni's post-test) was performed:* *P < *0.05;* **P < *0.01;* ***P < *0.001, OSTEO versus CTRL;* $P < *0.05*, $$P < *0.01*, $$$P < *0.001, HAP versus PS.*

potential; a reduced differentiation capacity of cells derived from female donors over 45 years of age was observed, as assessed by ALP activity and calcium deposition.

Despite the observed differences in terms of osteoblastic marker expression between cells from younger and older donors, the osteoprogenitor density was comparable between the two age groups, as assessed by CFU-O assay performed on hASC at the second passage, when we usually start to differentiate them into the osteoblastic lineage. This result is interesting, although apparently contradictory; from our data, we could hypothesize that the observed difference in terms of osteoblastic differentiation potential between younger and older donors is related to the intrinsic osteoblastic capacity of each single hASC or its differentiation rapidity, as we based our evaluation on early osteogenic markers. Certainly, this aspect needs further investigation to understand better the relationship between osteoprogenitor cell density and their effective osteoblastic potential.

Hence our data suggest the potential use of autologous hASC, although interindividual differences related to sex, age and tissue inflammatory state [37] need to be taken into account for regenerative medicine application, together with

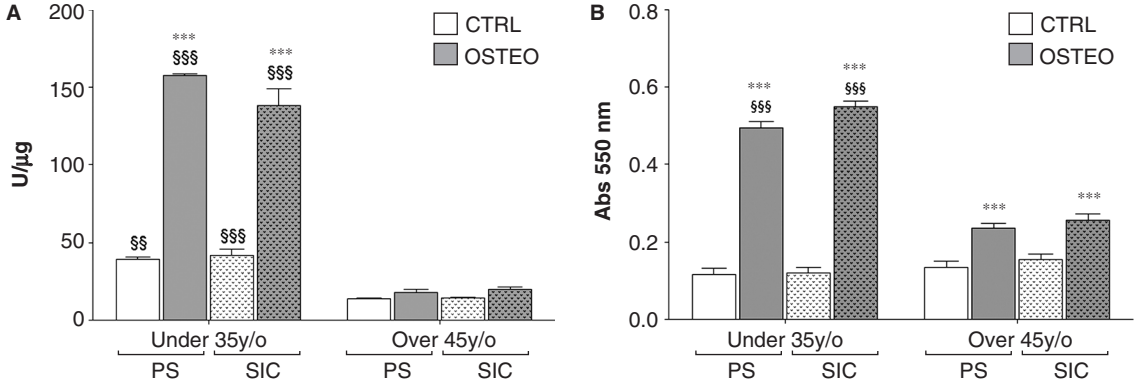

Figure 7. *Influence of SiC–PECVD on* in vitro *osteoblastic differentiation of hASC belonging to the two age groups. (A) ALP activity, in undifferentiated (CTRL, white bars) and osteogenic differentiated (OSTEO, filled bars) hASC cultured for 14 days on PS and SiC–PECVD (patterned bars). Data were standardized with respect to protein content and expressed as mean ± SD* (n = *3). (B) Quantification of calcium deposits in undifferentiated and osteogenic-differentiated hASC cultured for 21 days on PS and SiC–PECVD. Alizarin Red S-stained samples were extracted with CPC; data are expressed as mean ± SD* (n = *3). A two-way ANOVA (with Bonferroni's post-test) was performed:* ***P < *0.001, OSTEO versus CTRL;* §§P < *0.01*, §§§P < *0.001, UNDER 35 years old versus OVER 45 years old.*

the opportune indications for this kind of treatment. Aspirated fat is usually used as a filler material and autologous fat transfer is the most used method for soft-tissue augmentation, performed without detectable scarring on either the donor or recipient site and with very rewarding clinical results [38,39]. Fat tissue has been used as filler for more than 100 years [40], and different purification techniques have been developed. Currently the most common fat-processing methods are filtering [26,27] and centrifuging [28,35]. We compared the adipose-derived progenitor cells isolated from four middle-aged women who had undergone aesthetic surgical intervention and processed by both centrifuging and filtering. We observed no significant differences in terms of hASC viability, even though we noticed a slightly broader cell number variability for CENT-hASC. The centrifugation process may be less traumatic. At early passages, the CENT-hASC proliferated faster than the FILT-hASC; however, this difference was not statistically significant, because of the previously described variability.

Our results indicate that fat progenitor cell commitment is maintained in both types of manipulated cells, as they are able to differentiate efficiently into adipocytes, confirming clinical reports where both isolation procedures provide progenitor cells able to contribute to defect remodeling [26,27,28,35].

As the use of MSC in regenerative medicine applications requires tissue engineering skills [41,42], and the selection of a suitable scaffold is fundamental in order to set up hybrid constructs [43], we have shown that hASC may be used conveniently to screen several biomaterials, natural and synthetic, with potential clinical application. We chose a well-known osteoinductive material, HAP, and a less characterized material, SiC–PECVD. Recently, many manufacturers have developed a coating system of metal components, with different types of materials, for example for prosthetic use, to improve the osteointegration of the devices with the surrounding tissue. So we focused our interest on SiC–PECVD, a suitable ameliorative coating layer, which can be layered on a metal implant surface and is known to be biocompatible [44].

In a previous study, we compared the differentiation efficiency of hASC either pre-differentiated and then loaded on biomaterials, or directly differentiated on biomaterials; no significant difference was observed, suggesting that hASC do not need a pre-differentiating period to allow them to attach to biomaterials and to generate further osteoblast-like cells [34]. This observation allowed us to reduce the time period spanning the withdrawal of adipose tissue progenitor cell isolation and its application for the generation of constructs available for tissue engineering. With this assumption, we seeded undifferentiated hASC on the two different types of scaffold and then compared their ability to differentiate into osteoblast-like cells with cells cultured on PS. Osteogenic induction was assessed by comparing ALP activity and calcium deposit formation of hASC differentiated on biomaterials and PS. hASC from younger donors, when cultured on both HAP and SiC–PECVD, produced higher ALP activity and a greater amount of calcium deposition compared with cells derived from older donors (Figures 6 and 7).

As expected, HAP showed a strong and direct osteoinductive effect on hASC; indeed, cells derived from both young and old donors, when cultured on HAP, showed greater ALP activity compared with cells grown on PS, even if maintained in control medium. This result is quite interesting as it shows that an enhanced osteogenic effect may also be observed *in vitro* on hASC, and it may be because of the combined effects of the biomaterials and some biochemical signals, provided by the medium, and the cells themselves.

In conclusion, we have shown that hASC isolated from donors under 35 years old and over 45 years old efficiently differentiate towards adipocyte-like cells *in vitro*, whereas aging seems to affect their osteoblastic differentiation potential as assessed *in vitro* during a short differentiation period. These results were confirmed by experiments performed on scaffolds in static conditions.

Because of the high frequency of progenitor cells in subcutaneous adipose tissue, which may also be withdrawn easily, we believe that ASC are a suitable tool for musculoskeletal tissue regeneration. Hence liposuction, now just an aesthetic surgery procedure, may become an additional method for autologous progenitor cell collection.

Further analyzes, such as dynamic culture conditions and *in vivo* pre-clinical models, need to be tested to probe these new observations. In addition, as it is well known that hASC may generate endothelial cells [45,46], they theoretically may be induced to differentiate towards both endothelial and osteoblastic lineages, promoting a simultaneous neovascularization of regenerating bone.

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