SCD-2016-0190-ver9-DAlimonte_1P
Type: research-article

ORIGINAL RESEARCH REPORT

Osteogenic Differentiation of Mesenchymal Stromal Cells: A Comparative Analysis Between Human Subcutaneous Adipose Tissue and Dental Pulp

Iolanda D'Alimonte,[1–3,*] Filiberto Mastrangelo,[4,*] Patricia Giuliani,[1] Laura Pierdomenico,[2,3,5]
Marco Marchisio,[2,3,5] Mariachiara Zuccarini,[1,5] Patrizia Di Iorio,[1] Raimondo Quaresima,[6]
Francesco Caciagli,[1] and Renata Ciccarelli[1–3]

Abstract

White adipose tissue is a source of mesenchymal stromal/stem cells (MSCs) that are actively studied for their possible therapeutic use in bone tissue repair/remodeling. To better appreciate the osteogenic potential of these cells, we compared some properties of MSCs from human subcutaneous adipose tissue [subcutaneous-adipose stromal cells (S-ASCs)] and dental pulp stem cell (DPSCs) of third-impacted molars, the latter representing a well-established MSC source. Both undifferentiated cell types showed similar fibroblast-like morphology and mesenchymal marker expression. However, undifferentiated S-ASCs displayed a faster doubling time coupled to greater proliferation and colony-forming ability than DPSCs. Also, the osteogenic differentiation of S-ASCs was greater than that of DPSCs, as evaluated by the higher levels of expression of early osteogenic markers Runt-related transcription factor-2 (RUNX2) and alkaline phosphatase at days 3–14 and of extracellular matrix mineralization at days 14–21. Moreover, S-ASCs showed a better colonization of the titanium scaffold. In addition, we investigated whether S-ASC osteogenic commitment was enhanced by adenosine A1 receptor (A1R) stimulation, as previously shown for DPSCs. Although A1R expression was constant during DPSC differentiation, it increased in S-ASC at day 21 from osteogenesis induction. Accordingly, A1R stimulation by the agonist 2-chloro-N^6-cyclopentyl-adenosine, added to the cultures at each medium change, stimulated proliferation only in differentiating DPSC and enhanced the osteogenic differentiation earlier in DPSCs than in S-ASCs. These effects were counteracted by cell pretreatment with a selective A1R antagonist. Thus, our findings suggest that S-ASCs could be advantageously used in regenerative orthopedics/dentistry, and locally released or exogenously added purines may play a role in bone repair/remodeling, even though this aspect should be more thoroughly evaluated.

Keywords: regenerative medicine, mesenchymal stromal cells from human subcutaneous adipose tissue (S-ASCs), characteristics of the osteogenic differentiation of S-ASCs

Introduction

MESENCHYMAL STEM CELLS (MSCs) are spindle-shaped plastic-adherent cells that can be isolated from fetuses and extra embryonic tissues; in addition, the existence of MSCs-like cells has been reported in specialized niches of many adult tissues [1], where they are responsible for normal tissue structure homeostasis and cellular regeneration after injury [2]. Whether the latter are true stem cells still remains controversial [3]. Thus,

the International Society for Cellular Therapy (ISCT) has recommended that cells from adult tissues be termed "mesenchymal stromal cells" (coincidentally preserving the same acronym MSCs), even though the two names are interchangeably used in literature (reviewed in Bhartiya [4]).

MSCs are today actively studied for their capability of self-renewal and multi-lineage differentiation [5] that seem to offer advantages in regenerative medicine in comparison not only to traditional therapies but also to the potential use

Departments of [1]Medical, Oral and Biotechnological Sciences and [2]Medicine and Aging Science, and [3]Aging Research Center and Translational Medicine (CeSI-MeT), University of Chieti-Pescara, Chieti, Italy.
[4]StemTeCh Group, Chieti, Italy.
[5]Unit of Dentistry, IRCCS San Raffaele Scientific Institute, Vita e Salute University, Milano, Italy.
[6]Department of Civil Engineering, Architecture and Environment, University of L'Aquila, L'Aquila, Italy.
*These authors equally contributed to the study.

of embryonic or induced pluripotent stem cells. Indeed, MSCs do not raise ethical issues; they show no evident antigenic capacity when used for allogeneic transplantation and have a very low tumorigenic potential, even though some reports indicate that a risk exists [6,7].

MSCs were first isolated from bone marrow [8], and they are currently considered as the reference cell type with which those deriving from other sources such as dental tissues (ie, pulp, apical papilla, periodontal ligament) or embryonic annexes (ie, amniotic fluid, umbilical cord) are compared [2]. We and other researchers have demonstrated that cells isolated from the tissues or fluids mentioned earlier are multi-potent [9–13], as well as preclinical and clinical studies have pointed out the possibility to use these cells in tissue engineering, especially for bone repair/remodeling [14–19]. However, the actual availability of MSCs from the currently analyzed sources is doubtful. Indeed, bone marrow shows a low rate of MSCs, which further decreases with age [20]. The same is true for dental tissues. Moreover, dentists are more oriented toward conservative or restorative dental practices today [18]. Again, harvesting bone marrow from patients or amniotic fluid from pregnant women requires invasive procedures associated with pain and morbidity that discourage their routine employment; on the other hand, MSCs from umbilical cords, which are usually discarded after childbirth, seem to be useful until the fifth passage in vitro [21]. Therefore, the isolation of MSCs from more accessible sources that can be expanded to large cell numbers, while carrying a good multi-lineage differentiation potential, remains the ideal goal. A good perspective is offered by the white adipose tissue (WAT), which is widely distributed throughout the body. WAT is constituted by two main depots, namely subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) adipose tissue; the former represents >80% of the total body fat, and the latter is associated with internal organs. In SAT and VAT cells, different biological properties are present, including a population of adipose stromal cells (ASCs) [22]. However, those isolated from VAT are fewer and exhibit lower proliferation and differentiation capacity than ASCs from SAT, mostly because they are adipogenic precursors [23]. In contrast, SAT, which can be obtained from multiple body sites, offers the possibility of harvesting large ASC numbers from a single and less technically demanding procedure as compared with other tissues [18,24]. Moreover, cells retain their multi-lineage differentiation potential [25].

Based on this evidence, we started our study on MSCs isolated from human SAT [subcutaneous-adipose stromal cells (S-ASCs)] to examine whether they could represent an effective source of stem cells for mineralized tissue engineering in comparison to human dental pulp stem cells (DPSCs), which we previously studied [12]. The comparison included the evaluation of some distinctive features for MSCs in relation to their expansion and differentiation ability. Moreover, we investigated whether exogenous compounds were able to enhance S-ASC differentiation toward an osteogenic phenotype. Among the possible candidates, we chose to examine the effects of purines, as in previous years several reports showed that extracellular purines, usually released from all cell types, promote the differentiation of MSCs toward the osteogenic lineage. Since, in particular, (i) the stimulation of metabotropic receptors for the purine nucleoside adenosine are mainly involved in controlling osteogenesis [26–28]; and (ii) previously we pointed out that the stimulation of the adenosine A1 re-

AU8 ▶

ceptors (A1R) subtype increased the osteogenic differentiation of DPSCs, [12], in the second part of this article, we examined the effects of 2-chloro-N^6-cyclopentyladenosine (CCPA), a selective A1R agonist, on the osteogenic differentiation ability of S-ASCs, previously ascertaining the expression of this receptor in these cells along their differentiation process.

Methods

Materials

Disposables for tissue culture were from Falcon (Steroglass, Perugia, Italy). L-Glutamine for culture medium was purchased from EuroClone S.p.A. (Milan, Italy). Minimum essential medium (MEM) Alpha Medium, penicillin/streptomicin (P/S), amphotericin B, ascorbic acid, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), β-glycerophosphate disodium salt, and all the other chemicals, unless differently indicated, were from Sigma-Aldrich (Milan, Italy).

Cell culture

Pulp samples ($n = 10$) were obtained from the third molars, scheduled to be removed for orthodontic purposes at our Department from six patients (two women and four men), with a mean age of 18.00 ± 1.34 years. Informed consent for clinical research and for the processing of personal data was obtained from each of them. All teeth samples were de-identified. Dental pulps were isolated after tooth extraction as previously reported [12,29]; they were digested (1 h; 37°C) in MEM Alpha Medium containing L-Glutamine, P/S $100 \times$, $500 \mu g/mL$ clarithromycin (Abbott, Italy), 0.1 U/mL collagenase, and 0.8 U/mL dispase (Sigma-Aldrich). Cells were separated by filtering through a 70-μm strainer (Falcon, Becton Dickinson, Franklin Lakes, NJ); then resuspended in growth medium consisting of MEM Alpha Medium, 15% fetal bovine serum (FBS; Gibco by Life Technologies Italia, Monza, Italy), 1% P/S, and amphotericin B $1,000 \times$; and centrifuged (10 min; 1,200 rpm). The cell pellet was resuspended in the same medium and plated in $25 cm^2$ flasks. Cultures were incubated at 37°C in 5% CO_2, and the medium was changed twice a week.

The human S-ASCs were purchased from the Zen-Bio Company (Research Triangle Park, NC) and cultured by using a growth medium consisting of MEM Alpha Medium, 20% FBS (Gibco), 1% P/S, and 1% Amphotericin B. The mean age of six human subjects (women) was 28 ± 3 years. Cultures were incubated at 37°C and 5% CO_2, and the medium was changed twice a week. Experiments were performed only in the first six–eight cell passages.

Detection of surface and intracellular markers by flow cytometry

Samples of 5×10^5 cells were treated and then stained for surface or intracellular antigens, as previously described [30], using the following fluoresceinisothiocyanate (FITC)-, orphycoerythrin (PE)-, or allophycocyanin (APC)-conjugated antibodies: Anti-CD13 (CD13-FITC), anti-CD45 (CD45-FITC), anti-CD29 (CD29-PE), anti-CD105 (CD105-FITC), and anti-CD166 (CD166-FITC) were obtained from Ancell (MN); anti-CD14 (CD14-FITC) was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany); anti-CD90 (CD90-FITC),

anti-CD73 (CD73-PE), and anti-CD146 (CD146-PE) were obtained from Becton Dickinson; anti-CD144 (CD144-FITC) was obtained from Acris Antibodies (Herford, Germany); and anti-CD34 (CD34-PE) was purchased from Beckman Coulter (Fullerton, CA). Cells were incubated with these antibodies for 15 min at room temperature (RT). Then, tubes with cells were washed (3 mL of washing buffer) and centrifuged (4°C, 400 g, 8 min); cells were resuspended with 1 mL 0.5% paraformaldehyde, incubated for 5 min at RT, washed, centrifuged (4°C, 400 g, 8 min), and stored at 4°C in the dark until the acquisition. Finally, the antibody-bound cells were analyzed by using a flow cytometer (FACS Calibur, BD) and CellQuest™ software (BD). Debris was excluded from the analysis by gating on morphological parameters; 20,000 nondebris events in the morphological gate were recorded for each sample. All antibodies were titrated under assay conditions, and optimal photomultiplier gains were established for each channel. Cells incubated with phosphate-buffered saline (PBS) served as a control, and all experiments were performed in triplicate. The data from flow cytometry were then analyzed by using FlowJo™ software (TreeStar, Ashland, OR) and are presented as mean fluorescence intensity ratio, which was calculated by dividing the MFI of positive events by the MFI of negative events.

Cell proliferation

Counting of live cells. It was performed by using the trypan blue exclusion method. Briefly, cells were harvested after different culture periods (from 0 up to 10 days), incubated with trypan blue, and counted with an hemocytometer (three different fields for each sample evaluated in triplicate). Results are expressed as number of live cells/mL.

Doubling time. Cells at the passage 5 (P5) were seeded at 1×10^4 cells/cm^2 and cultured for different periods. They were detached by trysin-EDTA (Gibco) at 48, 72, and 96 h after seeding, and alive cells were counted by the trypan blue exclusion assay. The doubling time (D$_t$) can be computed as follows: $D_t = \frac{duration * \log(2)}{\log(\text{final concentration}) - \log(\text{initial concentration})}$ (Website doubling time computing, Available from: www.doubling-time.com/compute.php)

Colony-forming unit-fibroblast. Cells at P5 were plated in six-well plates at different densities (100, 250, and 500 cells/cm^2) and cultured in humidified 5% CO2 at 37°C. Culture medium was usually changed every 3 days. After 2 weeks, the dishes were washed twice with PBS, fixed with cold 100% methanol, and stained with 3% Crystal violet (Sigma). Colonies were counted under an optical microscope (Leica Microsystems, Germany) that was equipped with a digital camera (Canon, Tokyo, Japan), and the aggregates formed by 16 to 20 or more cells were scored as a 1 colony-forming unit-fibroblast.

In vitro osteogenic differentiation assay

The osteogenic differentiation was induced by culturing the human S-ASCs or DPSCs with MEM Alpha medium that was supplemented with 10% FBS, 0.05 mM ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone for as many as 28 days, with changes of medium every 3 days. To visualize calcium phosphate depositions, cultures were stained at different time points with Alizarin Red S (ARS; Sigma-Aldrich). Calcium depots were either visualized by a phase-contrast microscope (Eclipse TS100; Ni-

kon), equipped with a D200 digital camera (Nikon), or measured by the following spectrophotometric measurements. Briefly, 800 μL of 10% (v/v) acetic acid was added to each well; cells were incubated for 30 min with shaking, then removed by scraping, transferred into a 1.5-mL vial, and vortexed for 30 s. The obtained suspension was overlaid with 500 μL mineral oil (Sigma–Aldrich), heated to 85°C for 10 min, transferred to ice for 5 min, carefully avoiding the opening of the tubes until fully cooled, and centrifuged at 20,000 g for 15 min. The samples were acidified (pH between 4.1 and 4.5) with 200 μL of 10% (v/v) ammonium hydroxide. Aliquots (150 μL) were read in triplicate at 405 nm by a spectrophotometer (Spectramax SM190; Molecular Devices, Sunnyvale, CA).

Evaluation of early osteogenic markers

Alkaline phosphatase activity assay. Tissue-nonspecific alkaline phosphatase (ALP) activity was measured after 1 week of cell culture. Cell monolayers were washed with PBS, lysed with 1 mL of Tris buffer (10 mM, pH 7.5, 0.1% Triton X-100), and centrifuged (2,000 rpm; 1 min). Then, 20 μL of supernatant from each sample was combined with 20 μL of 1 mM p-nitrophenyl phosphate (p-NPP, Sigma; solution at pH 10.3 with MgCl$_2$-diethanolamine buffer) substrate and dispensed into 96-well plates. The samples were incubated in the dark at RT for 30 min. The reaction was stopped with 10 μL of 2 N NaOH. The amount of p-NPP released was measured as absorbance at 405 nm on a microplate spectrophotometer (Spectramax SM190). The protein content of each sample was determined by the BioRad protein assay (Bio-Rad Laboratories, Milan, Italy). The enzyme activity was expressed as nmoles of p-NPP released per mg of protein per 30 min.

RNA isolation and real-time PCR analysis of osteogenic markers. Total RNA was isolated from adipose and DPSCs by using the RNase Mini Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol. In detail, cells were lysed with 350 μL of RLT lysis buffer. After the addition of an equal volume of 70% ethanol, a total of 700 μL of each sample was transferred to the RNase spin column. After several washes with elution buffers, 30 μL of water was added to each column to elute the RNA. Eluted RNA concentration (OD at 260 nm) and purity (OD$_{260/280}$) were quantified by the use of a NanoDrop (Agilent). Afterward, RNA was reverse transcribed into cDNA by using the Omniscript Reverse Transcriptase Kit (Qiagen). In brief, 7 μL of RT mix solution containing 2 μL of oligo (dt) was added to 2 μg of total RNA solution. The reaction mixture was loaded to the Gene Amp PCR system 9700 (Applied Biosystem, Foster City, CA) undergoing the cycle at 37°C for 60 min.

Real-Time RT-PCR was carried out with the ABI Prism 7900 Sequence Detection System (Applied Biosystems). The expression of ALP and RUNX2 was evaluated at 0, 3, 7, and 14 days; cells were cultured in osteogenic medium. Commercially available TaqMan Gene Expression Assays (RUNX2, Hs00231692_m1, ALP, Hs01029144_m1) and the TaqMan Universal PCR Master Mix (Applied Biosystems) were used according to standard protocols. Samples were normalized (ΔCt) against the housekeeping gene beta-2 microglobulin (B2M, Hs99999907_m1, Applied Biosystems), and the -ΔΔCt value of ALP and RunX2 relative to undifferentiating medium was calculated by using the ΔΔCt method.

Scanning electron microscopy and electron-dispersive X-ray analysis

Sandblasted and etching (SLA) Ti6Al4 V disks [diameter 12 mm; thickness 3 mm, SLA (grade 5 Titanium Alloy); Strumann, Switzerland] were placed into 24-well plates and on each one 5×10^5 cells were plated in MEM alpha medium that was supplemented with 10% FBS. After 3 days, the culture medium was replaced with the usual medium (described earlier for the induction of the osteogenic differentiation) that was renewed every 3 days till 6 weeks. After this period, samples were fixed in 2% of glutaraldehyde in 0.1 M cacodylate buffer at pH 7 for 16 h at RT. To remove the fixative, the samples were washed three times with ice-cold PBS and then, they were first dehydrated by immersion in solutions of crescent concentration of ethanol/ water (20%, 50%, 90%, 100% v/v) and subsequently immersed in hexamethyldisilazane for 15 min. Afterward, the samples were dried for 1 day and sputter-coated with gold for 40 s before a scanning electron microscopy examination (Philips XL30CP) was conducted at an accelerating voltage of 20 kV. Electron-dispersive X-ray microanalysis (EDAX) of calcium, phosphorus, carbon, and titanium was performed (EDS, Oxford, Inca Energy 250) at a voltage of 20 kV. Spectra were collected at a magnification of $1,000 \times$ and a collection time of 1 min.

Expression of A1R

At the indicated time points, total protein extracts were prepared to perform western blot analysis as previously described [12]. Protein concentration was determined by the BioRad protein assay. The antibodies used were a rabbit polyclonal anti-A1 adenosine receptor primary antibody (final dilution 1:1,000; overnight incubation at 4°C; Alomone Labs, Israel) and a donkey anti-rabbit IgG HPR-conjugated secondary antibody (final dilution 1:5,000; 1 h incubation at RT; GE Healthcare Life Sciences, Milan, Italy). To determine the equal sample loading, the blots were stripped and reprobed with an anti-ß-actin antibody (1:100 dilution; Santa Cruz Biotechnologies, DBA Italia, Segrate, Milan, Italy). Immunocomplexes were visualized by using the enhancing chemiluminescence detection system (GE Healthcare Life Sciences) and quantified by densitometric analysis using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, https://imagej.nih.gov/ij).

Statistical analysis

The Graphpad Prism 6 Software was used for statistical analysis. The data are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance was used when three or more groups within one variable were compared. To analyze two groups within each cell type, the unpaired Student's t-test was used; to compare data from the two cell types, the multiple t-test was used; and the statistical significance was determined by using the Holm–Sidak method. Values of $P < 0.05$ were considered significant.

Results

Morphological, phenotypical, and proliferative characteristics of undifferentiated MSCs from human dental pulp and WAT

We started our study with the comparison of some characteristics in both kinds of undifferentiated MSCs. We first considered their morphology in vitro as well as the expression of some markers selected according to the criteria of ISCT [31]. At passage 5, S-ASCs exhibited a spindle-shaped (fibroblast-like) morphology comparable to that of DPSCs (Fig. 1a). Flow cytometry analysis revealed that both kinds ◄F1 of cells were also phenotypically similar (a representative scheme of marker expression is reported in Fig. 1b). Undifferentiated human S-ASCs and DPSCs were negative for hematopoietic markers (CD13, CD14, CD34, CD45) and for the surface vascular endothelial-cadherin adhesion molecule CD144, whereas they positively stained for the typical MSC markers CD29, CD73, and CD90 and to a lesser extent for CD105, 166, and 146.

We then evaluated further characteristics of the cells under study that were useful for their potential utilization in bone regenerative medicine. The doubling time (DT) was ◄AU9 significantly lower for S-ASCs than for DPSCs (Fig. 1d). Accordingly, cell proliferation, evaluated by counting live cells by the classic trypan blue exclusion method, was greater in S-ASCs than in DPSCs, leading to a significantly higher number of cells on the eighth day in vitro (DIV) in the former than in the latter cultures (Fig. 1c). On the 10th DIV, proliferation plateaued in both types of cells, but their number remained significantly different.

When cells were examined for clonogenicity, the increase in colony formation ability was parallel to that of the seeding density in both cell types (Fig. 1e, f). However, S-ASCs formed a greater number of colonies than DPSCs that was statistically significant when cells were seeded at 500 cells/cm^2.

Characterization of the osteogenic differentiation of S-ASCs and DPSCs

We then characterized the osteogenic differentiation of both types of cells by measuring the extracellular matrix mineralization and the expression of early osteogenic markers during the differentiation process. In Fig. 2a and b, ◄F2 besides the visualization by microscopy (Fig. 2c, d), we quantified ARS staining by spectrophotometry. S-ASCs in comparison to DPSCs displayed an enhanced extracellular calcium deposition, with a maximal increase on day 21 ($P < 0.0001$ measured by multiple t-test and Holm–Sidak method). Data from real-time PCR performed to evaluate the expression of early osteogenic markers along the differentiation process indicated that ALP was massively expressed in S-ASCs over a period of 14 days, during which RUNX2 expression was also significantly increased (Fig. 2e, g). The increase in ALP and RUNX2 expression was smaller in DPSCs ($P < 0.0001$, multiple t-test) and limited to the first 7 days of differentiation (Fig. 2f, h). In agreement with these findings, the ALP activity, which was significantly enhanced on the seventh DIV in both cell types, was more remarkably increased in S-ASC than in DPSC cultures (Fig. 2i; $P < 0.01$, multiple t-test). Finally, morphological analysis by scanning electron microscopy (Fig. 3a, b, d, e) showed that after ◄AU10 ◄F3 40 days, S-ASCs, but not DPSCs, covered most part of the standard titanium scaffold, demonstrating a good ability of these cells to adhere and colonize the scaffold surface. The presence of a fiber network is relevant, presumably due to the deposition of extracellular matrix proteins and minerals (Fig. 3b), as impressive is the EDAX-identified mineralized depots (Fig. 3c). The spectrum related to DPSCs showed
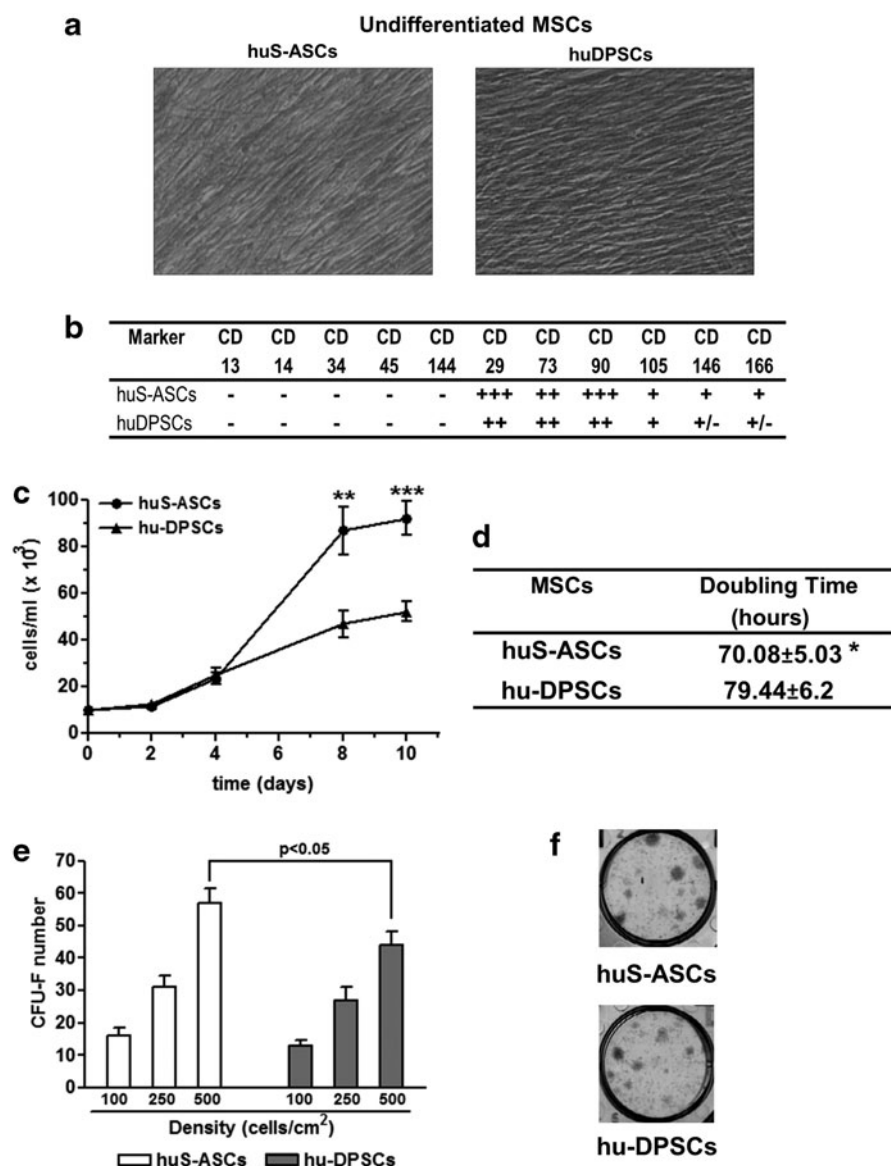
FIG. 1. Characterization of undifferentiated S-ASCs and DPSCs. **(a)** Similar fibroblast-like morphology visualized with an inverse microscope in confluent cell cultures at passage 5. **(b)** Identification of the principal mesenchymal/stemness and hematopoietic markers by flow cytometry analysis. Cells obtained from adult subcutaneous adipose tissue and dental pulp of third molars are a homogeneous population that stains positively, even though to a different extent, for mesenchymal (CD29, CD73, CD90, CD105, CD146, CD166) markers and negatively for hematopoietic (CD13, CD14, CD35, CD45) or epithelial (CD144) markers. Their expression is indicated as follows: −, negative expression; +/−, low expression; +, moderate expression; ++, positive; +++, high expression. Cut-off mean fluorescence intensity ratio positivity >2. **(c)** Cell proliferation measured as count of live cells at different time points by the trypan blue exclusion method. **(d)** Doubling time evaluated on S-ASCs and DPSCs at passage 5 by counting the cells every day for 4 days. **(e)** Colony-forming unit-fibroblast (CFU-F) assay. Graphs show the number of CFU-Fs counted when cells were seeded at different densities. **(f)** Representative images showing colonies formed by DPSCs and S-ASCs and positively stained (magnification: $10\times$). In all panels, values are the mean ± SEM of 12 values. A significant increase in the cell count (**$P < 0.01$ and ***$P < 0.001$) and the number of colonies (*$P < 0.05$) in S-ASC versus DPSC cultures was evaluated by one-way ANOVA plus Bonferroni post hoc test, whereas the greater increase in the doubling time in S-ASC than in DPSC cultures (*$P < 0.05$) was estimated by Student's *t*-test. ANOVA, analysis of variance; DPSCs, dental pulp stem cells; S-ASCs, subcutaneous-adipose stromal cells; SEM, standard error of the mean.

AU13 ▶

only the presence of titanium (Fig. 3f), whereas that of S-ASCs elicited minerals such as calcium, phosphorus, as well as carbon and oxygen (Fig. 3c). Particles analyzed at 4 weeks of cell cultures, indicated by the white circle in the figure, displayed a mean Ca: P ratio of 1.23 (Fig. 3b, c).

Expression of A1R in S-ASCs and DPSCs during their osteogenic commitment

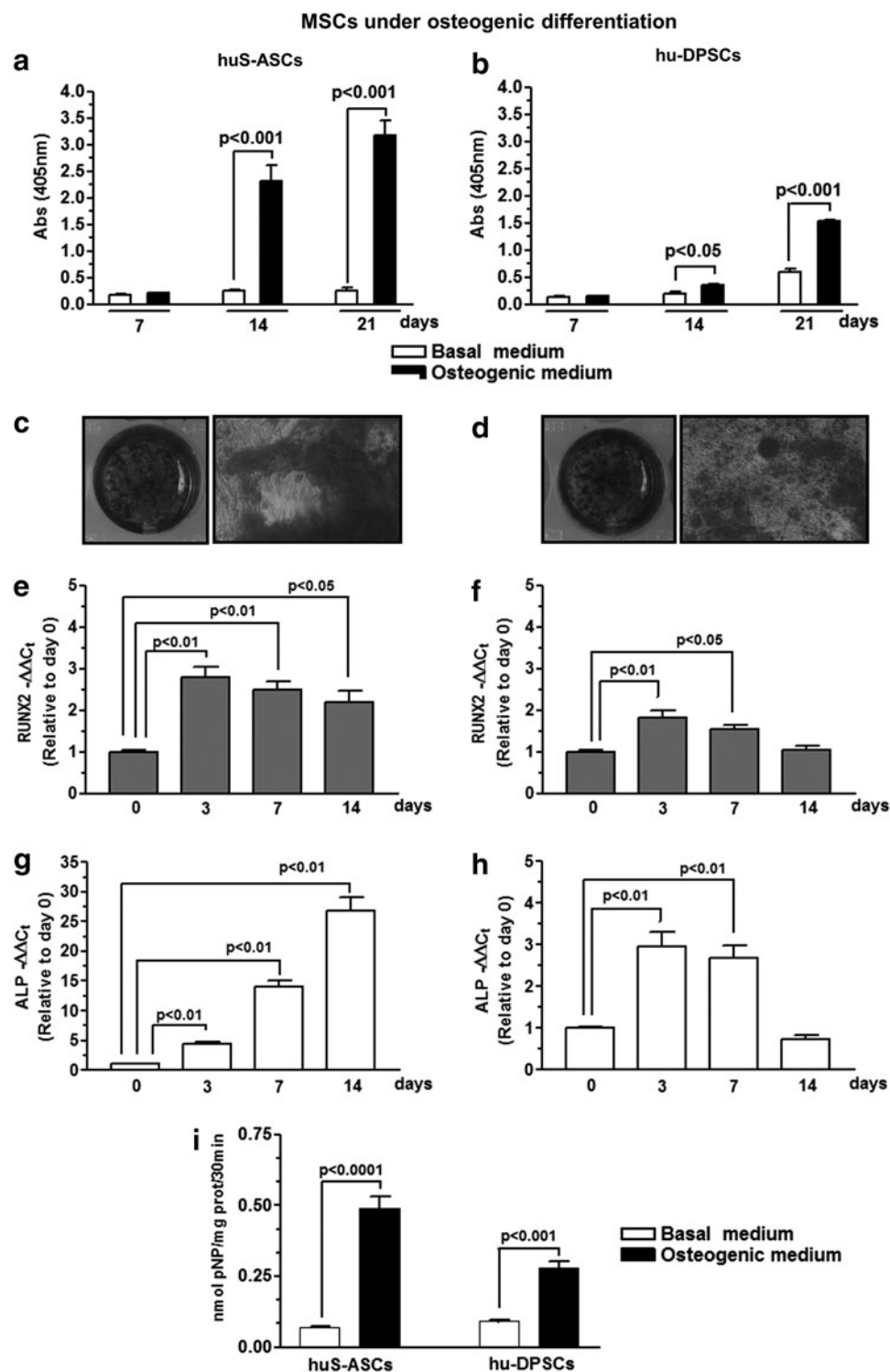We previously demonstrated that purines interacting with A1R can stimulate the proliferation and the osteogenic

FIG. 2. Evaluation of the osteogenic differentiation of S-ASCs and DPSCs. Panels **(a, b)** Extracellular matrix mineralization evaluated by ARS staining and its spectrophotometric evaluation (absorbance read at 405 nm). Values are the mean ± SEM of 12 values. Statistical significance of differentiated versus undifferentiated cells by one-way ANOVA plus Bonferroni post hoc test. **(c, d)** Representative microscope images of 12 independent experiments, carried out on cells derived from samples of different human subjects, showing cell cultures at 21 days after the osteogenic commitment, positively stained for calcium depots by ARS (original magnification: 10×). **(e–h)** ALP and RUNX2 expression evaluated as real-time PCR. This analysis was performed on total RNA extracted from cultured cells after different periods (3, 7, and 14 DIV) from the osteogenic commitment. Data are the mean ± SEM of six separate experiments. Statistical significance of differentiated versus undifferentiated cells (time 0) by one-way ANOVA plus Bonferroni post hoc test. **(i)** Evaluation of ALP activity by a spectrophotometric assay. Cells were grown for 7 days. ALP activity was assayed as described in the Materials and Methods section and expressed as nmol of p-nitrophenol produced per mg of cell protein within a period of 30 min (nmol/mg protein/30 min). Values are the mean ± SEM of six separate experiments. Statistical significance of differentiated versus undifferentiated cells (time 0) was estimated by Student's *t*-test. ALP, alkaline phosphatase; ARS, Alizarin Red S; DIV, days in vitro; RUNX2, Runt-related transcription factor-2; SEM, standard error of the mean.
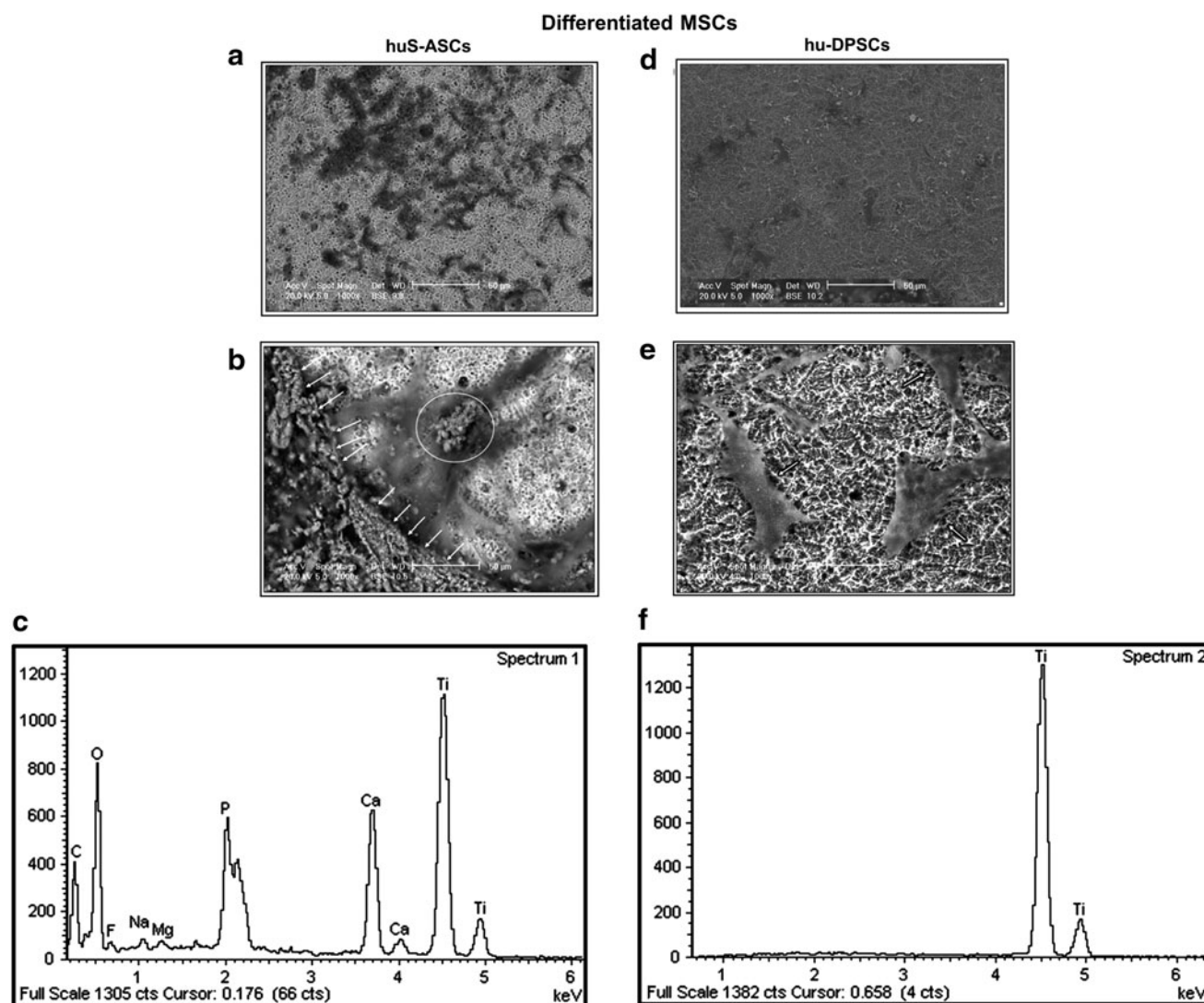
FIG. 3. Analysis by scanning electron microscopy and EDAX assay. Cells were seeded on titanium disks (diameter: 12 mm; thickness: 3 mm); after 3 days, basal growth medium was replaced with osteogenic medium, in which cells were cultured for a further 40 days. **(a, d)** Representative images of scanning electron microscopy analysis of S-ASCs and DPSCs grown on titanium disks (magnification bar = 50 μm). **(b, e)** The *white arrows* indicate the front of the inorganic matrix deposition and the contact network of cells with the titanium surface **(b)** or the initial interconnection between cells without any presence of inorganic matrix **(e)**. Moreover, the *white circle* indicates the area in which EDAX was performed (see next panels). **(c, f)** Electron-dispersive X-ray microanalysis (EDAX) of some minerals such as calcium, phosphorus, carbon, and titanium was performed (EDS, Oxford, Inca Energy 250) at a voltage of 20 kV. Spectra were collected at a magnification of 1,000× and a collection time of 1 min.

differentiation of DPSCs [12]. Here, we wanted to verify whether the A1R activation may influence the same parameters as in S-ASCs. First of all, S-ASCs and DPSCs were grown under osteogenic conditions and were analyzed at different time points for the expression of A1R by western blotting (Fig. 4a, b). Noteworthy, the A1R protein content, which was low in undifferentiated cells (corresponding to the lane at time 0), significantly increased along the S-ASC differentiation process, becoming maximal at 21 DIV after the differentiation induction (Fig. 4a), whereas it remained constant during the osteogenic differentiation of DPSCs and was similar to the A1R expression in undifferentiated cells (lane at time 0) (Fig. 4b).

F4 ▶

S-ASC and DPSC exposure to CCPA, an A1R agonist, differently affected cell proliferation and osteogenic differentiation

Based on our previous experience, we exposed both cell types undergoing osteogenic differentiation to the known A1R agonist, CCPA, that was added to the cultures at each medium change at the concentration of 30 nM. CCPA was able to stimulate DPSC proliferation and osteogenesis [12]. In comparison to untreated (control) cells, CCPA did not modify the S-ASC proliferation (Fig. 4c), whereas it increased the number of DPSCs on the eighth day from their commitment toward osteogenesis (Fig. 4d), as previously
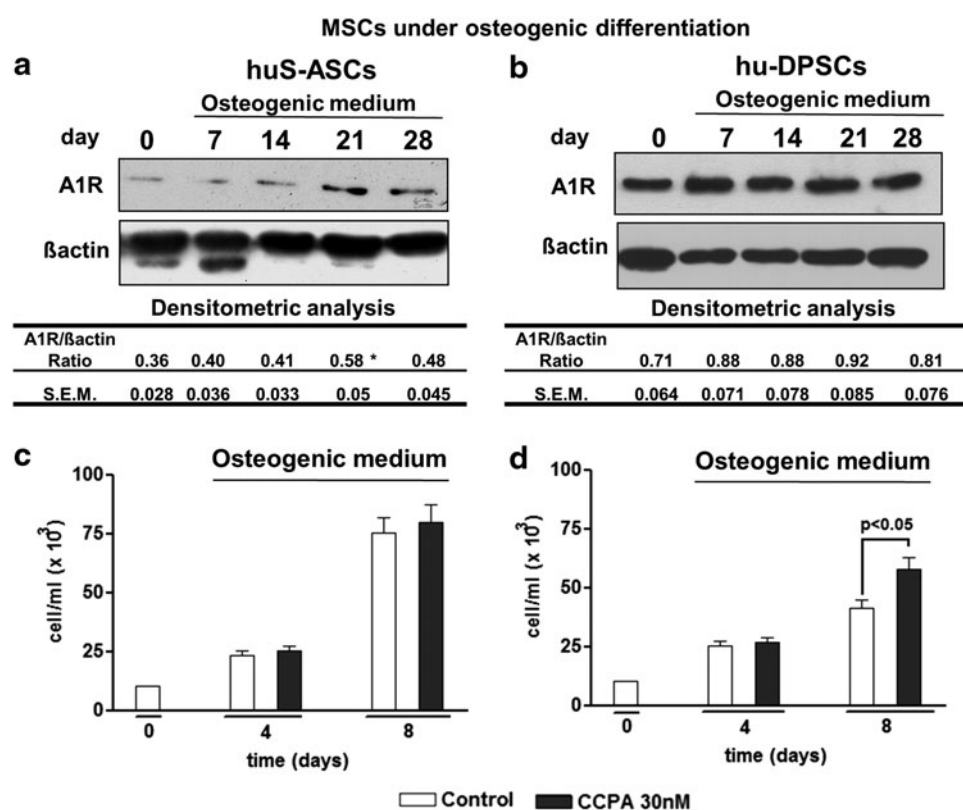
FIG. 4. Expression of adenosine A1 receptor (A1R) and effect of its stimulation on S-ASC and DPSC proliferation. **(a, b)** Immune-detected bands by western blot analysis have been obtained from cells grown as undifferentiated cells (lane at time 0) or in an osteogenic conditioning medium for different days (7–14–21–28). Immunoblots, reprobed with an antibody against β actin to ensure equal sample loading, were quantified by densitometric analysis, the values of which, normalized to β actin, are reported under the blots. Images are representative of six independent experiments, with very similar results. Densitometric values reported under the images are the mean ± SEM of these experiments. **(c, d)** Effect of the culture exposure to CCPA for different times (0, 4, and 8 days) on cell proliferation measured as a count of live cells by the trypan blue exclusion method. Values are the mean ± SEM of six separate experiments. *$P < 0.05$: significantly different from undifferentiated cells (control) (one-way ANOVA plus Bonferroni post hoc test). CCPA, 2-chloro-N^6-cyclopentyladenosine.

observed [12]. In contrast, CCPA significantly enhanced the differentiation process in both kinds of cells, even though with different timing. Indeed, in comparison to untreated S-ASCs undergoing differentiation, CCPA significantly increased the expression of the osteogenic markers RUNX2 and ALP in the period between 14 and 21 days as well as the accumulation of extracellular calcium depots at 21–28 DIV (Fig. 5a–c) after the differentiation induction. In contrast, in DPSCs, CCPA induced an earlier increase of osteogenesis, as demonstrated by the greater expression of RUNX2 and ALP, which was significant at 3–7 days and at 7–14 days, respectively, in comparison to the control, and by the evaluation of the extracellular matrix mineralization, which was enhanced from the 14th day onward (Fig. 5d–f). The CCPA stimulatory effect induced in differentiated S-ASCs or DPSCs was abolished by cell pretreatment with the selective A1R antagonist 1-Butyl-8-(hexahydro-2,5-methanopentalen-3a(1*H*)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1*H*-purine-2,6-dione (PSB36, 10 nM) (Fig. 5), which when administered alone did not affect the differentiation process (data not shown).

F5▶

Discussion

Regenerative medicine based on the experimental use of MSCs is a science in rapid development, especially in the fields of Orthopedics and Dentistry, where MSCs have a great therapeutic potential in bone repair/remodeling (ie, see Refs. [18,32,33]). A great body of evidence has demonstrated that MSCs, isolated from embryonic as well as adult tissues, are able to differentiate into osteogenic or chondrogenic precursors, interestingly lacking histocompatibility antigens, and can be implanted into allogeneic tissues [33]. However, MSCs show a different ability to proliferate and/or to respond to external or microenvironmental influences depending on their tissue origin [34–36]. Since this behavior could lead to different in vivo results after transplantation, it seems crucial to investigate the characteristics of selected MSCs to choose the most appropriate MSC type for healing injured bone tissue. Other important features to consider are the ease of removing tissues containing MSCs with abundant cells inside the tissue and the possibility to expand these cells to obtain a great number of MSCs. With this in mind, our study aimed at investigating the properties of cells
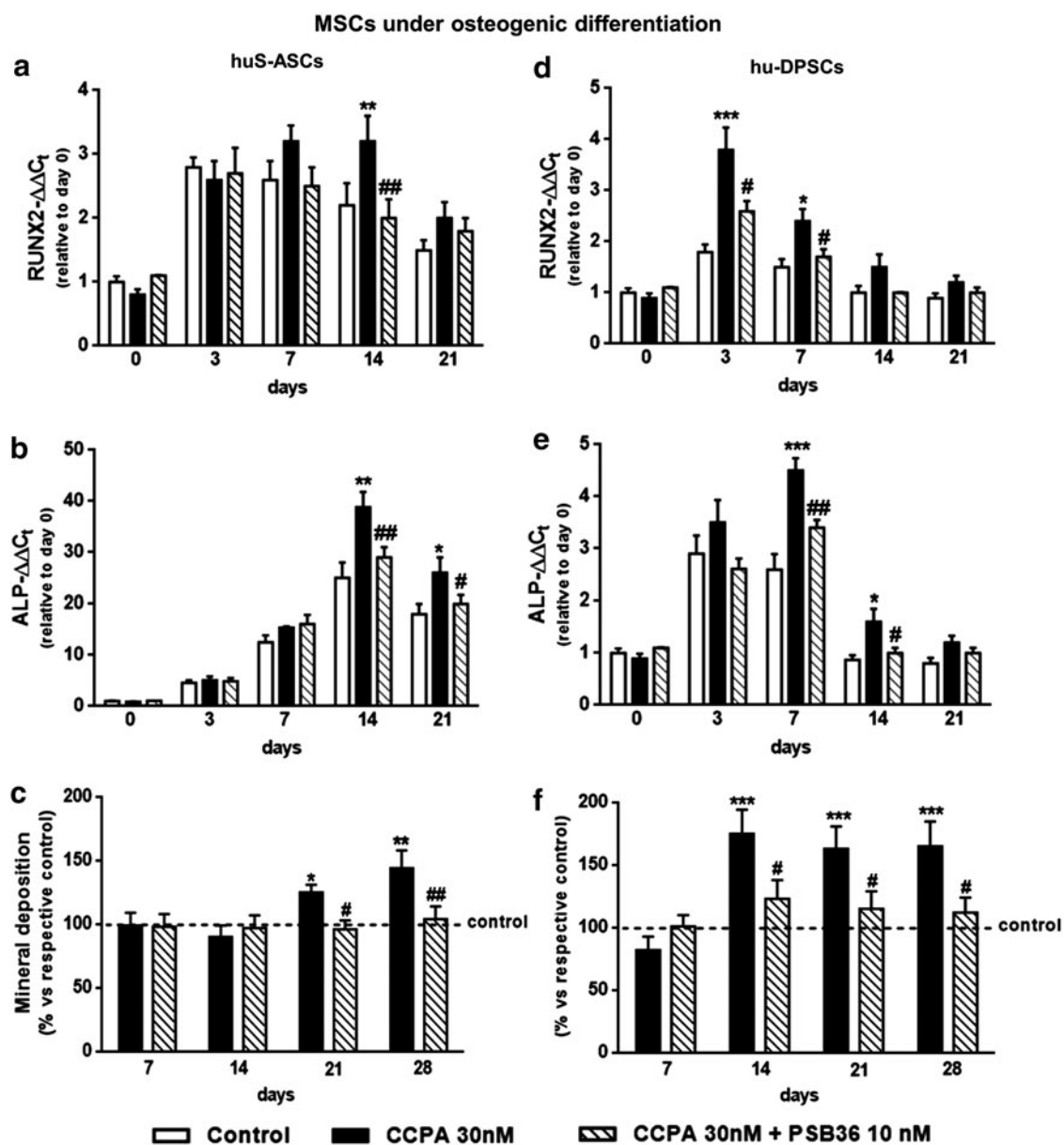
FIG. 5. Effect of the stimulation f A1R on S-ASC and DPSC osteogenic differentiation. The cell treatment with CCPA was performed in DPSCs and S-ASCs for different periods from the cell induction till the osteogenic differentiation. When present, the A1R antagonist PSB36 was added to the culture medium 1 h before the agonist CCPA. The effects on the expression of osteogenic markers (RUNX2 and ALP) were evaluated by real-time PCR **(a, d** and **b, e)**, and the effects on the extracellular matrix mineralization were evaluated by ARS staining and its spectrophotometrical analysis **(c, f)**. The values are the mean ± SEM of six independent experiments, in which different cell samples were used. For all experiments, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$: significantly different from undifferentiated cells (control); #$P < 0.05$, ##$P < 0.01$: significantly different from CCPA-treated cells (Student's *t*-test). PSB36, 1-Butyl-8-(hexahydro-2,5-methanopentalen-3a(1*H*)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1*H*-purine-2,6-dione; RUNX2, Runt-related transcription factor 2.

AU14 ▶

isolated from the SAT, which may correspond to the requirements reported earlier. Previous studies have pointed out that those deriving from the subcutaneous component of WAT have the properties of MSCs [37,38]. A number of papers as well have compared the differentiation properties of human S-ASCs with those of cells derived from other human sources such as bone marrow, umbilical cord, or placental and dental tissues (periodontal ligament, deciduous teeth, or maxillary periosteal tissue) other than adult dental pulp [39–41]. However, the results so far obtained are

conflicting, as they point out a greater osteogenic differentiation potential of MSCs from one or another source, with this also depending on the tissue toward which the regeneration was estimated [42–44]. Eager to contribute toward shedding light on this intricate aspect and based on our previous experience with DPSCs [12,29], here we report findings obtained by comparing some characteristics of S-ASCs with those of MSCs derived from adult dental pulp, which represents another consolidated paradigm for studies in regenerative bone medicine [43]. To our knowledge, only

one paper has reported a similar comparison, using, however, cells from rat incisors and inguinal adipose tissue, and the authors showed a relatively limited potential for mineralized tissue engineering of adipose-derived MSCs, in comparison to DPSCs [44]. In contrast, it was demonstrated that S-ASCs from rats are more resistant to cryo-preservation so that they constitute a robust source of MSCs in comparison to bone marrow cells and DPSCs, which are also derived from rats [45].

Our study started with the characterization of some aspects of human adult S-ASCs and DPSCs, the evaluation of which may contribute toward expanding knowledge of the basic biology of these stem cells, and which may constitute the natural premise for the clinical translation of cell-based therapies. Here, we show that the two cell types share similar morphology and expression of mesenchymal markers. For the latter, we refer to a seminal paper by Dominici et al. [31], which has been cited in the majority of articles related to stem cells, even though there is still a certain debate on the list of the most appropriate markers to define the mesenchymal nature/behavior of cells [46]. However, despite some similarities, when we better analyzed other features that were indicative of cell expansion ability and osteogenic differentiation, we found that S-ASCs exhibited a greater proliferation and osteogenic differentiation than DPSCs. Both these properties may be of value for the use of these cells in bone regeneration medicine. Indeed, the lower duplication time and the higher proliferation shown by S-ASCs in comparison to DPSCs would allow us to obtain a greater number of the former cells in a shorter time during their expansion. Apart from this, the greater differentiation potential of S-ASCs than DPSCs toward an osteogenic phenotype, also evident when cells were cultured on a standard titanium surface, could be very useful for bone repair that was performed with the aid of standardized scaffolds. Thus, our results confirm what is found at present in the literature, which reports the ability of S-ASCs to colonize biotic and abiotic materials, also in in vivo studies [47–51]. Moreover, the same results would make the use of S-ASCs very suitable in Dentistry, since MSCs obtained from dental tissues are few, being small in size compared with the original source. This represents a limit, mainly in relation to the entity of bone reconstruction. Conversely, it is possible to select a large number of stromal cells from SAT with minimal discomfort for patients, given the abundance of this tissue in the human body.

Therefore, we hope that our findings may further stimulate the research on the bone regenerative properties of S-ASCs. Given the high variability reported in different studies, in our opinion it would be important that an international Committee establish precise guidelines that researchers should follow, thus rendering the data analysis more homogenous and therefore the targeted use of suitable cells in regenerative medicine.

Of course, in vitro data should be corroborated by further analysis of cell behavior in vivo, where cells are induced to differentiate not by means of a conditioned medium but by local factors, which are released from the cells themselves. Therefore, it is also crucial to investigate the nature of external signals inducing the activation of these cells and the best way(s) to increase the number and the efficiency of their differentiation. Numerous papers

investigated the influence of specific growth factors and/or selected scaffolds on the osteogenic differentiation of MSCs [47–53]. Here, we focused on purines, ubiquitous molecules that are present inside cells, where they perform many vital functions (ie, as fundamental components of nucleic acids or as a source of energy supply). However, it is also recognized that these molecules, in particular adenine-based nucleotides, are released from every type of cell in the pericellular fluid, where they are rapidly transformed by specific ecto-enzymes into the corresponding nucleoside adenosine [26–28]. Both ATP and adenosine behave as signaling molecules, which are able to interact with specific receptors [26–28]. In relation to bone homeostasis, a recent review emphasized the useful role played by adenosine and related analogs acting on metabotropic receptors (namely, A1, A2A, A2B, and A3) that are specific for adenosine [28]. Here, we investigated the effects consequent to the activation of A1R subtype, based on our experience on the protective effects exerted by the stimulation of these receptors on neural cells [54] and also on the favorable results obtained with the use of an A1R agonist in DPSCs osteogenic differentiation [12]. Our results confirmed that A1R is expressed at a constant level throughout the osteogenic differentiation of these cells. Likely, this feature is important to elicit an enhanced DPSC proliferation that is induced by CCPA. In contrast, probably due to the late increased expression of A1R in differentiating S-ASCs, and also due to the higher proliferative rate of these cells in comparison to DPSCs during the differentiation phase, the stimulation of these receptors by CCPA was ineffective in S-ASC duplication. Apart from this, the A1R activation was able to enhance the S-ASC osteogenic process, but only belatedly. Thus, the different expression of A1R between the two cell types influenced the achievable effects by the stimulation of the same receptor, indicating that the tissue of origin can condition the potential results. Indeed, in MSCs derived from other tissues, the A1R-induced effects on cell proliferation and/or osteogenic differentiation were not so evident or prevailing [55,56]. As previously reported [12], the discrepancy might likely be due to differences in the species (animals or humans) or in the health state or age of subjects used to obtain MSCs (ie, aged women with osteoarthrosis versus young healthy subjects, as in our case). Lastly, in the field of purines, it is also important to underline that other receptors for these substances belonging to the family of P1 receptors for adenosine (A2A, A2B, and A3 receptor subtypes) may control cell differentiation, in particular osteogenesis [26–28,55,56]. Thus, further experiments are needed to evaluate the involvement of other adenosine receptors in the same cells. On the other hand, it has been reported that A1R activation in osteoclast precursors leads to stimulation of the receptor activator NFkB Ligand (RANK), which, in turn, causes the activation of NFkB, a requirement for osteoclastogenesis [57]. Therefore, in vivo, the effects consequent to the stimulation of a single (purine) receptor are the sum of the effects resulting from the simultaneous activation of that receptor subtype, in our case the A1R, on different cells such as osteoblasts and osteoclasts, which ultimately concurs to bone plasticity and homeostasis.

In conclusion, with our study, we provide new insights on the differentiation of S-ASCs that could represent a useful

model to better appreciate biological mechanisms underlying bone homeostasis/repair, as well as a manageable tool to be used in bone recovery/remodeling, thanks to the remarkable cell proliferation and propensity to differentiate in osteoblasts, even on a classical titanium scaffold. The role played by purines in the osteogenic differentiation of S-ASCs deserves to be further evaluated, as these compounds, released from all kinds of cells, especially during tissue injury, may differently contribute to bone repair. Only more extensive studies performed not only on isolated cells but also on more integrated experimental models (ie, co-cultures of different cell types, isolated organs, animals) can elicit the complex effects consequent to the stimulation of selected receptors by exogenous purinergic compounds and could show the real therapeutic potential of these compounds in bone regeneration.

Acknowledgments

Author Disclosure Statement

No competing financial interests exist.

References

1. Augello A, TB Kurth and C De Bari. (2010). Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches. Eur Cell Mater 20:121–133.
2. Baksh D, L Song and RS Tuan. (2004). Adult mesenchymal stem cells: characterization, differentiation and application in cell therapy. Mol Med 8:301–316.
3. Bianco P, X Cao, PS Frenette, JJ Mao, PG Robey, PJ Simmons and CY Wang. (2013). The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat Med 19:35–42.
4. Bhartiya D. (2013). Are mesenchymal cells indeed pluripotent stem cells or just stromal cells? Oct-4 and VSELs biology has led to better understanding. Stem Cells Int 2013:547501.
5. Lenoir N. (2000). Europe confronts the embryonic stem cell research challenge. Science 287:1425–1427.
6. Kang NH, KA Hwang, SU Kim, YB Kim, SH Hyun, EB Jeung and KC Choi. (2012). Potential antitumor therapeutic strategies of human amniotic membrane and amniotic fluid-derived stem cells. Cancer Gene Ther 19:517–522.
7. Prockop DJ, M Brenner, WE Fibbe, E Horwitz, K Le Blanc, DG Phinney, PJ Simmons, L Sensebe and A Keating. (2010). Defining the risks of mesenchymal stromal cell therapy. Cytotherapy 12:576–578.
8. Friedenstein AJ, RK Chailakhyan, NV Latsinik, AF Panasyuk and IV Keiliss-Borok. (1974). Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplant 17: 331–340.
9. D'Alimonte I, A Lannutti, C Pipino, P Di Tomo, L Pierdomenico, E Cianci, I Antonucci, M Marchisio, M Romano, et al. (2013). Wnt signaling behaves as a ''master regulator'' in the osteogenic and adipogenic commitment of human amniotic fluid mesenchymal stem cells. Stem Cell Rev 9:642–654.
10. Pipino C, L Pierdomenico, P Di Tomo, F Di Giuseppe, E Cianci, I D'Alimonte, C Morabito, L Centurione, I Antonucci, et al. (2015). Molecular and phenotypic characterization of human amniotic fluid-derived cells: a morphological and proteomic approach. Stem Cells Dev 24:1415–1428.
11. Trohatou O, NP Anagnou and MG Roubelakis. (2013). Human amniotic fluid stem cells as an attractive tool for clinical applications. Curr Stem Cell Res Ther 8:125–132.
12. D'Alimonte I, E Nargi, A Lannutti, M Marchisio, L Pierdomenico, G Costanzo, P Di Iorio, P Ballerini, P Giuliani, F Caciagli and R Ciccarelli. (2013). Adenosine A_1 receptor stimulation enhances osteogenic differentiation of human dental pulp-derived mesenchymal stem cells via WNT signaling. Stem Cell Res 11:611–624.
13. Hakki SS, SA Kayis, EE Hakki, SB Bozkurt, G Duruksu, ZS Unal, G Turaç and E Karaoz. (2015). Comparison of mesenchymal stem cells isolated from pulp and periodontal ligament. J Periodontol 86:283–291.
14. Crovace A, A Favia, L Lacitignola, MS Di Comite, F Staffieri and E Francioso. (2008). Use of autologous bone marrow mononuclear cells and cultured bone marrow stromal cells in dogs with orthopaedic lesions. Vet Res Commun 32 (Suppl. 1):39–44.
15. Ishihara A and AL Bertone. (2012). Cell-mediated and direct gene therapy for bone regeneration. Expert Opin Biol Ther 12:411–423.
16. Evans NR, EM Davies, CJ Dare and ROC Oreffo. (2013). Tissue engineering strategies in spinal arthrodesis: the clinical imperative and challenges to clinical translation. Regen Med 8:49–64.
17. Karamouzian S, SN Nematollahi-Mahani, N Nakhaee and H Eskandary. (2012). Clinical safety and primary efficacy of bone marrow mesenchymal cell transplantation in subacute spinal cord injured patients. Clin Neurol Neurosurg 114:935–939.
18. Egusa H, W Sonoyama, M Nishimura, I Atsuta and K Akiyama. (2012). Stem cells in dentistry–part I: clinical applications. J Prosthodont Res 56:229–248.
19. Norambuena GA, M Khoury and C Jorgensen. (2012). Mesenchymal stem cells in osteoarticular pediatric diseases: an update. Pediatr Res 71:452–458.
20. Wagner W, P Horn, M Castoldi, A Diehlmann, S Bork, R Saffrich, V Benes, J Blake, S Pfister, V Eckstein and AD Ho. (2008). Replicative senescence of mesenchymal stem cells: a continuous and organized process. PLoS One 3: e2213.
21. Li S, Y Wang, L Guan and M Ji. (2015). Characteristics of human umbilical cord mesenchymal stem cells during ex vivo expansion. Mol Med Rep 12:4320–43255.
22. Choudhery MS, M Badowski, A Muise, J Pierce and DT Harris. (2015). Subcutaneous adipose tissue-derived stem cell utility is independent of anatomical harvest site. Biores Open Access 4:131–145.
23. Lee Mi-J, Y Wu and SK Fried. (2013). Adipose tissue heterogeneity: implication of depot differences in adipose tissue for obesity complications. Mol Aspects Med 34: 1–11.
24. Crespo-Diaz R, A Behfar, GW Butler, DJ Padley, MG Sarr, J Bartunek, AB Dietz and A Terzic. (2011). Platelet lysate consisting of a natural repair proteome supports human mesenchymal stem cell proliferation and chromosomal stability. Cell Transplant 20:797–811.
25. Zuk PA, PA Zuk, M Zhu, P Ashjian, DA De Ugarte, JI Huang, H Mizuno, ZC Alfonso, JK Fraser, P Benhaim and

MH Hedrick. (2012). Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295.

26. Ciciarello M, R Zini, L Rossi, V Salvestrini, D Ferrari, R Manfredini and RM Lemoli. (2013). Extracellular purines promote the differentiation of human bone marrow-derived mesenchymal stem cells to the osteogenic and adipogenic lineages. Stem Cells Dev 22:1097–1111.

27. Kaebisch C, D Schipper, P Babczyk and E Tobiasch. (2015). The role of purinergic receptors in stem cell differentiation. Comput Struct Biotechnol J 13:75–84.

28. Strazzulla LC, Cronstein BN. (2016). Regulation of bone and cartilage by adenosine signaling. Purinergic Signal 12:583–593.

29. D'Alimonte I, E Nargi, F Mastrangelo, G Falco, P Lanuti, M Marchisio, S Miscia, I Robuffo, M Capogreco, et al. (2011). Vascular endothelial growth factor enhances in vitro proliferation and osteogenic differentiation of human dental pulp stem cells. J Biol Regul Homeost Agents 25: 57–69.

30. Lanuti P, V Bertagnolo, L Pierdomenico, A Bascelli, E Santavenere, L Alinari, S Capitani, S Miscia and M Marchisio. (2009). Enhancement of TRAIL cytotoxicity by AG-490 in human ALL cells is characterized by downregulation of cIAP-1 and cIAP-2 through inhibition of Jak2/Stat3. Cell Res 19:1079–1089.

31. Dominici M, K Le Blanc, I Mueller, I Slaper-Cortenbach, F Marini, D Krause, R Deans, A Keating, DJ Prockop and E Horwitz. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317.

32. Wang X, Y Wang, W Gou, Q Lu, J Peng and S Lu. (2013). Role of mesenchymal stem cells in bone regeneration and fracture repair: a review. Int Orthop 37:2491–2498.

33. Ryan JM, FP Barry, JM Murphy and BP Mahon. (2005). Mesenchymal stem cells avoid allogeneic rejection. J Inflamm (Lond) 2:8.

34. Panepucci RA, JL Siufi, WA Silva Jr., R Proto-Siquiera, L Neder, M Orellana, V Rocha, DT Covas and MA Zago. (2004). Comparison of gene expression of umbilical cord vein and bone marrow-derived mesenchymal stem cells. Stem Cells 22:1263–1278.

35. Elahi KC, G Klein, M Avci-Adali, KD Sievert, S MacNeil and WK Aicher. (2016). Human mesencymal stromal cells from different sources diverge in their expression of cell surface proteins and display distinct differentiation patterns. Stem Cells Int 2016:5646384.

36. Mattioli-Belmonte M, G Teti, V Salvatore, S Focaroli, M Orciani, M Dicarlo, M Fini, G Orsini, R Di Primio and M Falconi. (2016). Stem cell origin differently affects bone tissue engineering strategies. Front Physiol 6:266.

37. Baglioni S, G Cantini, G Poli, M Francalanci, R Squecco, A Di Franco, E Borgogni, S Frontera, G Nesi, et al. (2012). Functional differences in visceral and subcutaneous fat pads originate from differences in the adipose stem cell. PLoS One 7:e36569.

38. Kornicka K, K Marycz, KA Tomaszewski, M Marędziak and A Smieszek. (2015). The effect of age on osteogenic and adipogenic differentiation potential of human adipose derived stromal stem cells (hASCs) and the impact of stress factors in the course of the differentiation process. Oxid Med Cell Longev 2015:309169.

39. Jin HJ, YK Bae, M Kim, S-J Kwon, HB Jeon, SJ Choi, SW Kim, YS Yang, W Oh and JW Chang. (2013).

Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. Int J Mol Sci 14:17986–18001.

40. Heo JS, Y Choi, H-S Kim and HO Kim. (2016). Comparison of molecular profiles of human mesenchymal stem cells derived from bone marrow, umbilical cord blood, placenta and adipose tissue. Int J Mol Med 37: 115–125.

41. Trivanović D, A Jauković, B Popović, J Krstić, S Mojsilović, I Okić-Djordević, T Kukolj, H Obradović, JF Santibanez and D Bugarski. (2015). Mesencymal stem cells of different origin: comparative evaluation of proliferative capacity, telomere length and pluripotency marker expression. Life Sci 141:61–73.

42. Jeon J-Y, J Kim, JH Cho, H-M Chung and J-I Chae. (2016). Comparative analysis of human mesenchymal stem cells derived from bone marrow, placenta, and adipose tissue as sources of cell therapy. J Cell Biochem 117:1112–1125.

43. Gronthos S, M Mankani, J Brahim, PG Robey and S Shi. (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A 97:13625–13630.

44. Davies OG. PR Cooper, RM Shelton, AJ Smith and BA Scheven. (2015). A comparison on the in vitro mineralization and dentinogenic potential of mesenchymal stem cells derived from adipose tissue, one marrow and dental pulp. Bone Miner Metab 33:371–382.

45. Davies OG, AJ Smith, PR Cooper, RM Shelton and BA Scheven. (2014). The effects of cryopreservation on cells isolated from adipose, bone marrow and dental pulp tissues. Cryobiology 69:342–347.

46. Lv FJ, RS Tuan, KM Cheung and VY Leung. (2014). Concise review: the surface markers and identity of human mesenchymal stem cells. Stem Cells 32:1408–1419.

47. Detsch R, S Alles, J Hum, P Westenberger, F Sieker, D Heusinger, C Kasper and AR Boccaccini. (2015). Osteogenic differentiation of umbilical cord and adipose derived stem cells onto highly porous 45S5 Bioglass¬-based scaffolds. J Biomed Mater Res 103:1029–1037.

48. Pullisaar H, JE Reseland, HJ Haugen, JE Brinchmann and E Ostrup. (2014). Simvastatin coating of TiO_2 scaffold induces osteogenic differentiation of human adiposetissue-derived mesenchymal stem cells. Biochem Biophys Res Commun 447:139–144.

49. Wittenburg G, V Flade, AI Garbe, G Lauer and D Labudde. (2014). Scaffold preferences of mesenchymal stromal cells and adipose-derived stem cells from green fluorescent protein transgenic mice influence the tissue engineering of bone. Br J Oral Maxillofac Surg 52:409–414.

50. Liang H, X Li, AL Shimer, G Balian and FH Shen. (2014). A novel strategy of spine defect repair with a degradable bioactive scaffold preloaded with adipose-derived stromal cells. Spine J 14:445–454.

51. Semyari H, M Rajipour, S Sabetkish, N Sabetkish, FM Abbas and A-M Kajbafzadeh. (2016). Evaluating the bone regeneration in calvarial defect using osteoblasts differentiated from adipose-derived mesenchymal stem cells on three different scaffolds: an animal study. Cell Tissue Bank 17:69–83.

52. Almalki SG and DK Agrawal. (2016). Key transcription factors in the differentiation of mesenchymal stem cells. Differentiation 92:41–51.

53. Chen Q, P Shou, C Zheng, M Jiang, G Cao, Q Yang, J Cao, N Xie, T Velletri, et al. (2016). Fate decision of mesenchymal stem cells: adipocytes or osteoblasts? Cell Death Differ 23:1128–1139.

54. Rathbone MP, PJ Middlemiss, JW Gysbers, C Andrew, MA Herman, JK Reed, R Ciccarelli, P Di Iorio and F Caciagli. (1999). Trophic effects of purines in neurons and glial cells. Prog Neurobiol 59:663–690.

55. Costa MA, Barbosa A, Neto E, Sá-e-Sousa A, Freitas R, Neves JM, Magalhães-Cardoso T, Ferreirinha F and Correiade-Sá P. (2011). On the role of subtype selective adenosine receptor agonists during proliferation and osteogenic differentiation of human primary bone marrow stromal cells. J Cell Physiol 226:1353–1366.

56. Gharibi B, Abraham AA, Ham J and Evans BAJ. (2011). Adenosine receptor subtype expression and activation influence the differentiation of mesenchymal stem cells to osteoblasts and adipocytes. J Bone Miner Res 26:2112–2124.

57. He W and Cronstein BN. (2012). Adenosine A1 receptor regulates osteoclast formation by altering TRAF6/TAK1 signaling. Purinergic Signal 8:327–337.

Address correspondence to: ◄AU11
Prof. Renata Ciccarelli
Section of Pharmacology ◄AU12
Department of Medical, Oral and Biotechnological Sciences
University of Chieti-Pescara
Via dei Vestini 29
Chieti 66100
Italy

E-mail: renata.ciccarelli@unich.it

AUTHOR QUERY FOR SCD-2016-0190-VER9-DALIMONTE_1P

AU1: Please note that gene symbols in any article should be formatted per the gene nomenclature. Thus, please make sure that gene symbols, if any in this article, are italicized.

AU2: Please review all authors' surnames for accurate indexing citations.

AU3: Please confirm the correctness of authors' affiliations.

AU4: Please fix the expansion of the acronym S-ASCs: ''subcutaneous-adipose stromal cells'' or ''subcutaneous adipose-derived stromal cells''?

AU5: Keywords have been taken from the given PDF file. Please check.

AU6: Please check the hierarchy of heading levels in the article.

AU7: The Publisher requests for readability that no paragraph exceeds 15 typeset lines. Please check for long paragraphs and divide where needed.

AU8: Please fix the expansion of the acronym S-ASCs: ''subcutaneous-adipose stromal cells'' or ''subcutaneous adipose-derived stromal cells''?

AU9: Both ''DT'' and ''D_t'' have been used. Please fix for consistency.

AU10: ''Fig. 3a-b-d-e'' has been changed to ''Fig. 3a, b, d, e.'' Please confirm.

AU11: Please confirm the corresponding author's address.

AU12: ''Section of Pharmacology'' is mentioned in the corresponding author's address, but it is not mentioned in the author's affiliation. Please check.

AU13: Please fix the expansion of the acronym S-ASCs: ''subcutaneous-adipose stromal cells'' or ''subcutaneous adipose-derived stromal cells''?

AU14: ''a-d and b-e'' has been changed to ''a, d and b, e.'' Please confirm.