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Mesenchymal Stem Cells from Bichat's Fat Pad: *In Vitro* Comparison with Adipose-Derived Stem Cells from Subcutaneous Tissue

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Abstract

Adipose-derived stem/stromal cells (ASCs) are progenitor cells used in bone tissue engineering and regenerative medicine. Since Bichat's fat pad is easily accessible for dentists and maxillo-facial surgeons, we compared the features of ASCs from Bichat's fat pad (BFP-ASCs) with human ASCs from subcutaneous adipose tissue (SC-ASCs). BFP-ASCs isolated from a small amount of tissue were characterized for their stemness and multidifferentiative ability. They showed an important clonogenic ability and the typical mesenchymal stem cell immunophenotype. Moreover, when properly induced, osteogenic and adipogenic differentiation markers, such as alkaline phosphatase activity, collagen deposition and lipid vacuoles formation, were promptly observed. Growth of both BFP-ASCs and SC-ASCs in the presence of human serum and their adhesion to natural and synthetic scaffolds were also assessed. Both types of ASCs adapted rapidly to human autologous or heterologous sera, increasing their proliferation rate compared to standard culture condition, and all the cells adhered finely to bone, periodontal ligament, collagen membrane, and polyglycol acid filaments that are present in the oral cavity or are commonly used in oral surgery. At last, we showed that amelogenin seems to be an early osteoinductive factor for BFP-ASCs, but not SC-ASCs, *in vitro*. We conclude that Bichat's fat pad contains BFP-ASCs with stemness features that are able to differentiate and adhere to biological supports and synthetic materials. They are also able to proliferate in the presence of human serum. For all these reasons we propose BFP-ASCs for future therapies of periodontal defects and bone regeneration.

Key words: amelogenin; biomaterials; buccal fat pad; mesenchymal stem/stromal cells; oral bone regeneration

Introduction

Mesenchymal stem/stromal cells (MSCs) represent important suitable candidates in regenerative medicine applications for the treatment of tissues damaged by trauma or pathological diseases. They have been isolated from bone marrow, adipose tissue, tendon, periodontal ligament, synovial membranes, trabecular bone, skin, periostium, and muscle.[1] Even though bone marrow represents the more used source of stem cells (BMSCs) in the clinical field, adipose tissue is a valid alternative source of MSCs. It is easily accessible in large quantities with a minimal invasive harvesting procedure and allow a high number of adipose-derived mesenchymal stem/stromal cells (ASCs) to be obtained.[2,3] ASCs show a multilineage differentiation capacity similar to that of BMSCs.[4–6] The growth factor secretome of MSCs was characterized by Wang et al.,[7] and the secretory activity of MSCs favors a regenerative microenvironment at sites of tissue injury.[8]

Adipose tissue withdrawn during plastic surgery is a discarded tissue, and the usual anatomical regions from which this tissue is collected are the abdomen, breast, buttock, knee, and thigh. In this study, we have characterized human ASCs isolated from the buccal fat pad, usually called Bichat's fat pad (BFP), one of the encapsulated fat masses in the cheek. It is a deep fat pad located on either side of the face between the buccinator muscle and several more superficial muscles, including the masseter, the zygomaticus major,

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Table 1. Enrollment Criteria for Patients

Clinical inclusion criteria:
• Patients classified as ASA I–II
• Males and females, between 18 and 70 years of age, with compromised wisdom teeth and thin bone at the maxillary sinus
• Possibility of withdrawing subcutaneous adipose tissue from abdominal region at the same time
Clinical exclusion criteria:
• Disorders of the parotid glands
• Disorders affecting fat and carbohydrate metabolism
• Presence of adequate bone support under remaining teeth
• No risk of oro-sinusal communication after tooth extraction
• Medical contraindications to elective surgery

and the zygomaticus minor.[9,10] Several authors described the buccal fat pad functions in relation to mastication and suction.[9] This tissue can usually be easily harvested with minimal discomfort and complications for patients and is used in facial reconstruction.[11–13] Removal of the buccal fat pad is also sometimes done to reduce cheek prominence, although this procedure may carry with it a significant risk of damage to the buccal branch of the facial nerve and the parotid ducts.[14] The buccal fat pad flaps are used in the reconstruction of the periorbital area,[15] for the repair of congenital oro-antral and/or oro-nasal disorders,[16] and for congenital cleft palate repair.[17] More recently, they have also been used for the closure of oro-antral communications[18,19] and treatment of oral submucous fibrosis,[20,21] intraoral malignant defects,[22] and cheek mucosa defects.[23]

The aim of this study was to isolate, characterize, and compare human ASCs isolated from the buccal fat pad and subcutaneous fat regions, and to highlight a new source of more accessible ASCs for use in periodontal, oral implant, and maxillo-facial surgery. Moreover, we evaluated the adhesion of hASCs on resorbable materials and the effect on these progenitor cells by amelogenin (AM)–enamel matrix derivative, a porcine matrix protein used to treat periodontal defects on natural teeth.[24–26]

Materials and Methods

Patient enrollment

Adipose tissues were harvested from dental patients selected by using the clinical criteria reported in Table 1. The enrolled patients (1 man and 1 woman, $n=2$, age 35.5 ± 2.7 years) were in good health without any systemic complications. The informed consent and experimental protocols for this study were reviewed and approved after written consent by the Ethics Committee and Institutional Review Board of University of Milan (authorization #17/12 of April 23, 2012).

Local anesthesia was performed (carbocaine 2% and adrenaline 1:100,000) either in the oral cavity or in the subcutaneous fat tissue; the surgical access for liposuction was performed at the navel using the lipofilling technique: fat was harvested with a sterile 10-mL BD Luer Lock syringe performing a constant manual negative pressure (plunger positioned between 1 and 2 mL).[27,28]

The tooth was extracted and different tissues were collected: alveolar bone, as a result of modeling the residual extraction socket; periodontal ligament; and tooth portions, coronal and root. Finally, BFP was approached, cutting the mucosa in proximity to the second molar teeth, 0.5 cm from the fornix. Blunt dissection was performed to prevent damage, to close the anatomical structures, and to allow the identification and exteriorization of the fat pad. BFP was stretched into the wound to close the residual defect and extra tissue was cut (Fig. 1). This technique is widely used either for aesthetic or reconstructive reasons.[29,30] At the end of the procedure, Ethilon 5.0 sutures were used to close the surgical access.

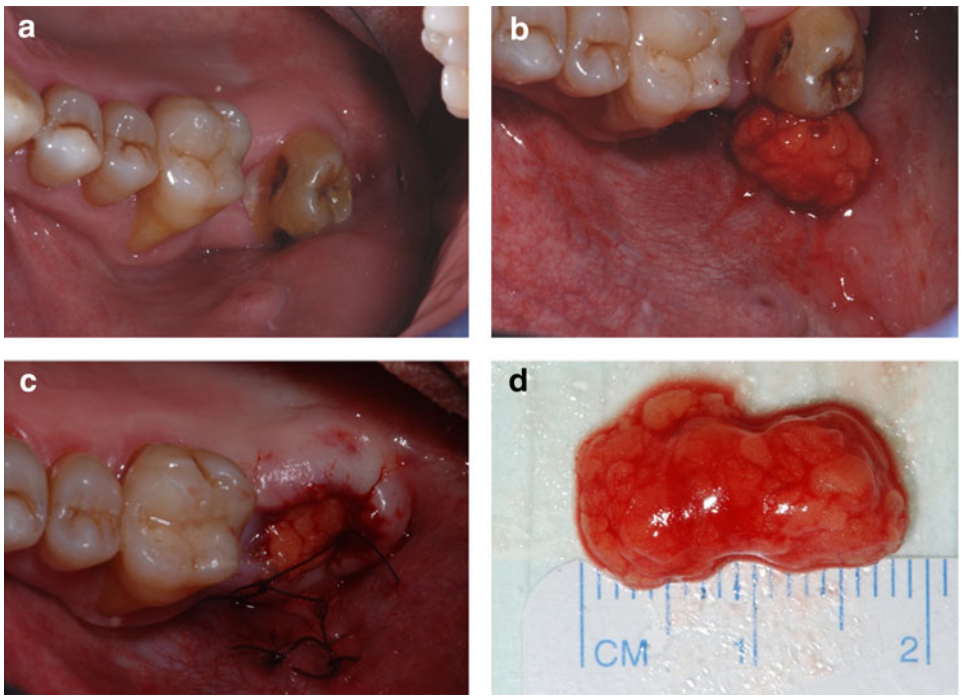

FIG. 1. Extraction of Bichat's fat pad (BFP). **(a)** Preoperative conditions. Poor oral hygiene; 26 compromised by periodontal disease, absence of 27, and caries infiltrating 28. **(b)** Exposure of BFP. **(c)** Tunneling and positioning of the BFP to repair defect. **(d)** Waste material of the BFP at the end of surgery.

Patients' blood was also collected, and all the specimens were sent to the laboratory.

One day before surgery, patients were treated with amoxicillin (1 g twice a day) to prevent bacterial load, and this therapy was maintained for additional 5 days. Sutures were removed after 1 week and the patients were monitored at 2 weeks and at 1 and 6 months.

The pain was controlled for 2 days with naproxen (550 mg twice daily). A mild muscle contraction lasting 4 days was observed in one patient; no other complications were described, and after 2 weeks the tissues were already well-epithelized.

Isolation and characterization of ASCs

Isolation and culture. Subcutaneous adipose tissues were obtained from buccal fat pad (BFP-ASCs) and abdominal subcutaneous tissue (SC-ASCs). ASCs were isolated as previously described.[6] The tissues were enzymatically digested by 0.075% type I collagenase for 30 min at 37°C. The stromal vascular fraction was obtained by centrifugation at 1200 *g* for 10 min, filtered through a sterile medication lint, and plated (10^5 cells/cm^2) in the cDMEM control (CTRL) medium, composed of Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine (Sigma-Aldrich). Cells were then maintained at 10^4 cells/cm^2.

Proliferation. Both cell types were maintained in culture for 21 days and counted every week. The doubling time was calculated as $(\ln [N/N_0])/(\ln 2)$, where N is the number of the counted cells and N_0 represents the number of plated cells.

MTT assay for cell viability. From passage 4 to 7, ASC viability was monitored at day 1 and 7. Cells were incubated for 4 h at 37°C with 0.5 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) in CTRL medium. Viable cells produced formazan precipitate, which was then solubilized in 100% dimethylsulfoxide (DMSO; Sigma-Aldrich). The absorbance was read at 570 nm with the Wallac Victor II plate reader (Perkin Elmer Western Europe, Monza, Italy).

Colony forming unit–fibroblast assay. ASCs were plated in DMEM supplemented with 20% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine in six-well plates by serial dilution starting from 1000 cells/well. Medium was changed after 6 days, and at day 14 cells were fixed in 100% methanol and stained with 0.5% crystal violet (Fluka, Buchs, Switzerland). Individual colonies (consisting of at least 25 cells) were counted, and the colony forming unit–fibroblast (CFU-F) frequency was calculated with respect to the number of seeded cells.

Flow cytometry analysis. ASCs (3×10^5) in PBS with 1% FBS/0.1% NaN_3 per sample were incubated for 30 min on ice with monoclonal antibodies raised against CD14, CD31 CD34, CD73, CD90, and CD105 (Ancell, Bayport, MN). Specific binding was revealed by either streptavidin-PE– or fluorescein isothiocyanate–conjugated sheep anti-mouse antibody. Samples were acquired by FACS Calibur flow cytometer (BD Biosciences Europe, Erembodegem, Belgium) and data were analyzed using CellQuest software (BD Biosciences Europe).

Assessment of multidifferentiative potential of BFP- and SC-ASCs

Adipogenic differentiation. ASCs (10^4/cm^2) were induced to differentiate towards the adipogenic lineage culturing them for 14 days in cDMEM supplemented with 1 μM dexamethasone, 10 μg/mL insulin, 500 μM 3-isobutyl-1-methyl-xanthine, and 200 μM indomethacin (induction medium, Sigma-Aldrich). Then, cells were fixed in 10% neutral buffered formalin for 1 h and stained by fresh Oil Red O solution (20 mg/mL [w/v] Oil Red O in 60% isopropanol) for 15 min. Lipid vacuoles were quantified by extraction with 100% isopropanol for 10 min and reading the absorbance at 490 nm with the Wallac Victor II plate reader.

Osteogenic differentiation. ASCs (10^4/cm^2) at passage 7 were cultured for 14 days in CTRL and osteogenic inductive medium (OSTEO; 10 nM dexamethasone, 10 mM glycerol-2-phosphate, 150 μM L-ascorbic acid-2-phosphate, and 10 nM cholecalciferol [Sigma-Aldrich] added to cDMEM). To evaluate alkaline phosphatase (ALP) enzymatic activity, both undifferentiated and differentiated ASCs were lysed in 0.1% Triton X-100 and incubated at 37°C with 10 mM p-nitrophenylphosphate dissolved in 100 mM diethanolamine and 0.5 mM $MgCl_2$, pH 10.5.[31] Samples were read at 405 nm and ALP activity was calculated with respect to the protein concentration of each sample determined by BCA Protein Assay (Pierce Biotechnology, Rockford, IL).

To determine collagen production, ASCs were fixed for 1 h with Bouin's solution (Sigma-Aldrich) and then stained for 1 h with 1 mg/mL (w/v) Sirius Red F3BA in saturated picric acid (Sigma-Aldrich). Stained samples were extracted with 0.1 M NaOH for 5 min and then read at 550 nm.[32]

Proliferation of ASCs in the presence of human sera

Serum collection. Ten milliliters of blood were collected from each donor, allowed to clot for 30–45 min at 37°C and then transferred at 4°C for 30 min. After centrifugation (1000 *g* for 10 min) the sera were carefully removed and placed in new 15-mL tubes. All sera were collected under sterile conditions.[33]

Cell culture. ASCs (6×10^4/well) were cultured in six-well plates in DMEM supplemented with either 10% or 5% autologous serum (HAS) or 5% heterologous serum (HHS). ASCs were also grown in cDMEM as control medium. Cells were cultured for four passages; they were detached and replated every 5 days and counted each time.

ASCs-scaffold interaction and scanning electron microscopy

ASCs (1×10^5 and 3×10^4) were grown for 4 days on natural supports (alveolar bone and periodontal ligament) and on synthetic scaffolds (collagen membrane; Euroresearch, Milan, Italy and polyglycol acid filaments; Resorb, Sweden and Martina, Padova, Italy), respectively. The constructs were fixed in glutaraldehyde (2% in 0.1 M sodium cacodylate buffer) overnight at 4°C and dehydrated with a series of graded ethanol (from

Table 2. Volume of Adipose Tissue and Cellular Yield of Adipose-Derived Stem/Stromal Cells Derived From Bichat's Fat Pad and Subcutaneous Adipose Tissue

Sample	*Volume (mL)*	*ASCs/mL of adipose tissue*
BFP-ASCs-1	1	1.0×10^5
BFP-ASCs-2	0.5	1.2×10^5
SC-ASCs-1	60	1.2×10^5
SC-ASCs-2	14	1.1×10^5

ASCs, adipose-derived stromal/stem cells; BFP, derived from Bichat's fat pad; SC, derived from subcutaneous adipose tissue.

75% to 100%) for 4–5 h at room temperature and subjected to drying using critical point drying (CPD; Baltec C.P.D. 030). Samples were mounted on aluminum stubs, sputter-coated with gold (Balzers MED 010) and analyzed by scanning electron microscopy (SEM; Sem Quanta Feg 250 esem).

Amelogenin and BFP- and SC-ASCs

Both types of ASCs were cultured for 7 days in the presence of AM (Emdogain, Straumann, Basel, Switzerland). ASCs (1.5×10^4/well) were seeded in a 24-well culture plate, with wells previously spread with a drop of AM, and cultured in either cDMEM or osteogenic medium. ALP activity and collagen deposition were evaluated as previously described. As control, we considered ASCs cultured in the absence of AM.

Statistical analysis

Data are expressed as mean ± standard deviation. Statistical analyses were performed using Student *t*-test. Differences were considered significant at $p<0.05$.

Results

Characterization of human ASCs derived from the BFP

In this preliminary study, we analyzed cell populations derived from two donors who were enrolled for surgery for upper wisdom tooth extraction with sinus communication (Fig. 1). MSCs were isolated from both adipose tissue of BFP (BFP-ASCs, $n=2$) and abdominal subcutaneous tissue (SC-ASCs, $n=2$). We collected 0.8 ± 0.4 mL of BFP and 37.0 ± 32.5 mL of subcutaneous adipose tissue; despite the different amount of raw adipose tissues, we isolated a quite similar amount of cells per milliliter ($1.1\times10^5\pm1.4\times10^4$ BFP-ASCs/mL and $1.15\times10^5\pm7.1\times10^3$ SC-ASCs/mL; Table 2).

The BFP-ASCs were characterized for their immunophenotype; a representative cytofluorimetric analysis of both BFP-ASCs and SC-ASCs is shown in Figure 2. Cells appeared to have similar granularity but were different in size (Fig. 2). Both types of ASCs isolated expressed the characteristic stem cell markers such as CD73, CD90, and CD105, whereas they did not express lymphocyte or leucocyte antigens and hematopoietic markers such as CD14, CD31, and CD34 (Fig. 2).

One week after isolation, ASC populations begin to proliferate and, after 21 days, starting from 6×10^4 ASCs, $5.9\times10^5\pm2.6\times10^5$ BFP-ASCs and $1.3\times10^6\pm3.0\times10^5$ SC-

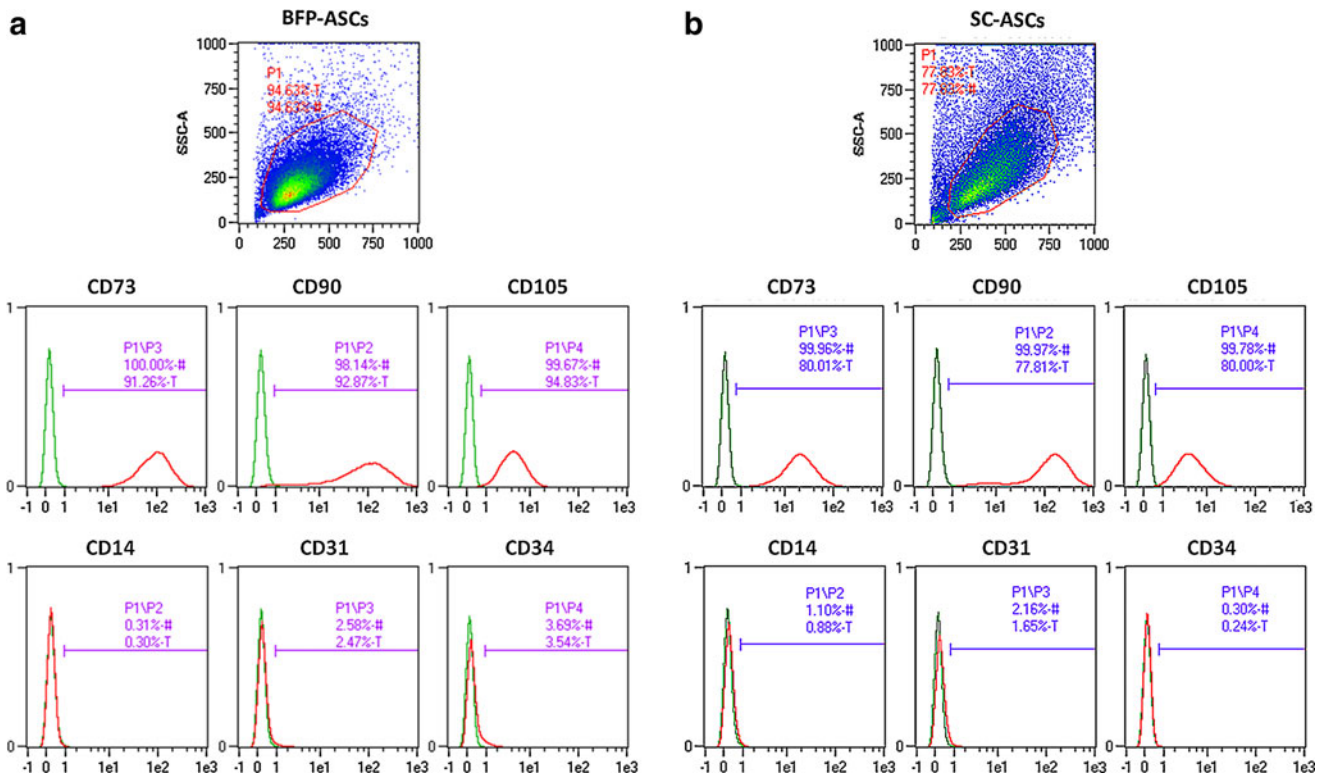

FIG. 2. A representative FACS analysis of BFP-ASCs-1 (**a**) and SC-ASCs-1 (**b**) stained for CD73, CD90, CD105, CD14, CD31, and CD34. Size and granularity are shown (*top*).

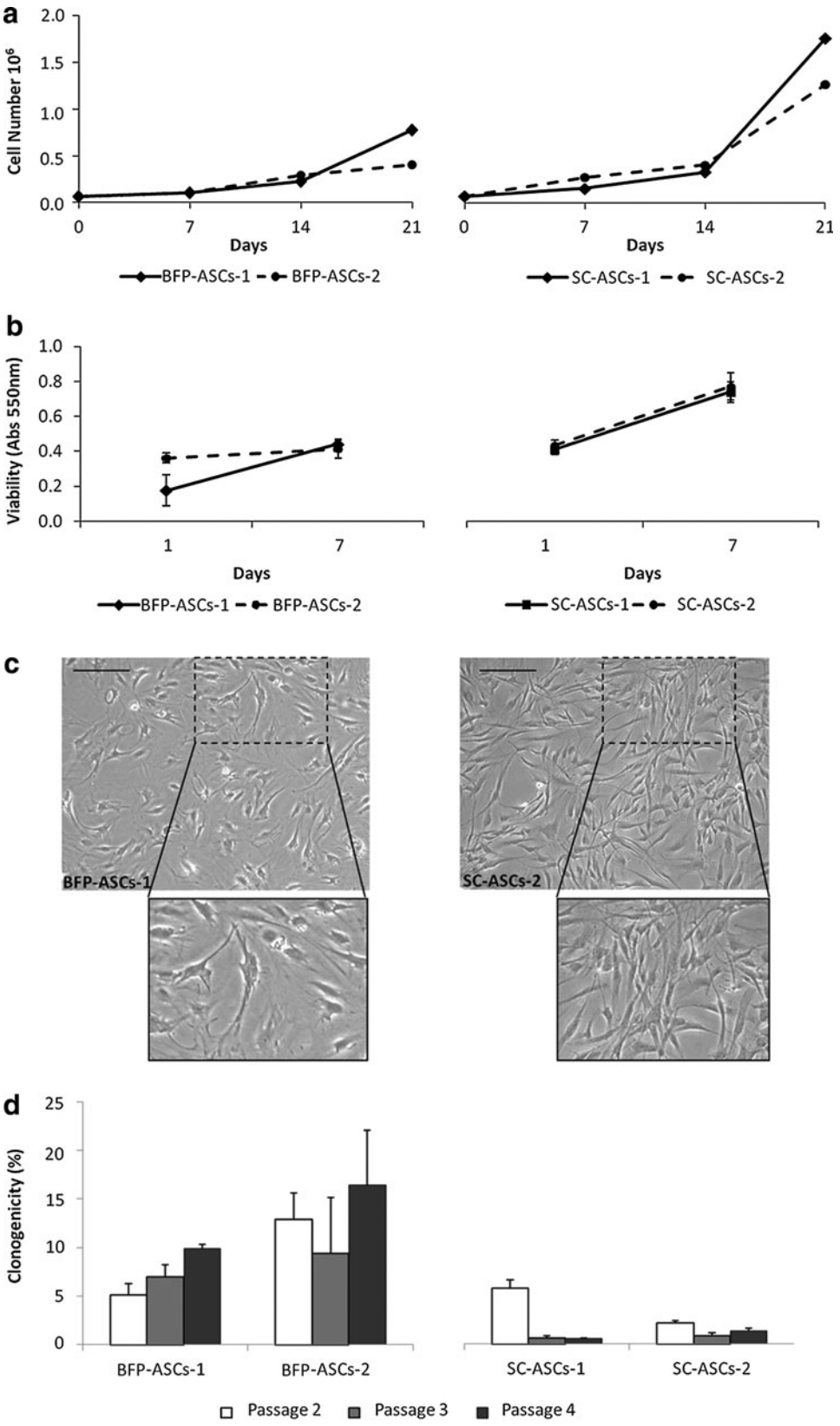

FIG. 3. Comparison of BFP-ASCs-1, -2 (*left*), and SC-ASCs-1, -2 (*right*). **(a)** Cell number of ASCs cultured for 21 days. **(b)** Viability of ASCs monitored for 1 week in cDMEM and tested as MTT metabolic activity. **(c)** Morphology of BFP-ASCs and SC-ASCs (optical microscopy, 100× and 400× magnification, scale bar 100 μm). **(d)** Clonogenicity of both ASCs by CFU-F assay. Percentage of CFU-F was calculated as number of colonies/number of plated cells × 100. ASCs, adipose-derived stem/stromal cells; SC, subcutaneous; cDMEM, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine; CFU-F, colony-forming unit–fibroblast.

ASCs were collected (Fig. 3a); indeed, SC-ASCs proliferated faster than BFP-ASCs, with an average doubling time of 73.5 ± 17.2 h compared to 126.5 ± 33.6 h (data not shown). This was also confirmed through a viability test, in which MTT incorporation by SC-ASCs-1 and -2, was mildly higher than that of BFP-ASCs (Fig. 3b). Another observed small difference, as shown in Figure 3c, concerns cellular morphology: SC-ASCs appeared to be a homogeneous population, with the distinctive fibroblast-like shape maintained for long time in culture (Fig. 3c, *right*), whereas BFP-ASCs, as

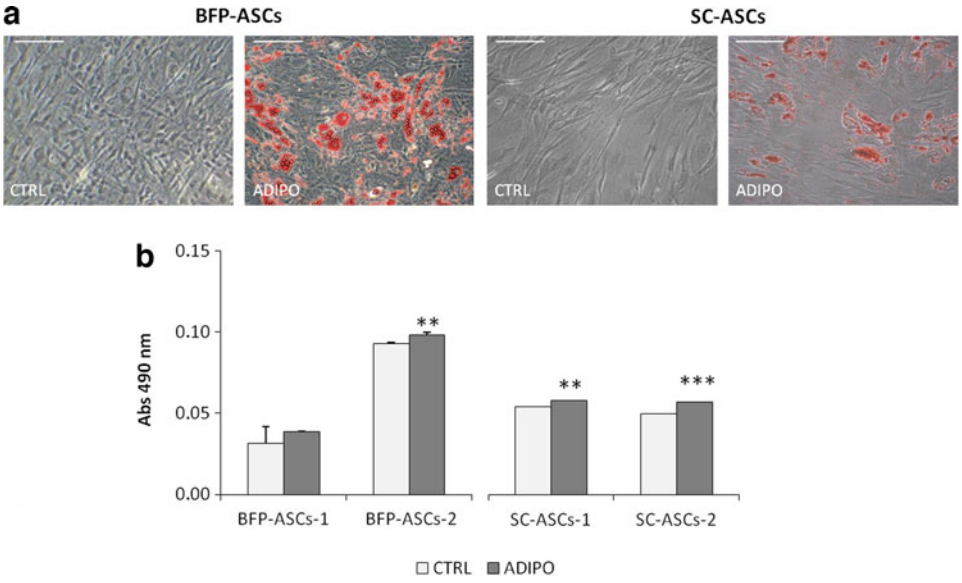

FIG. 4. Adipogenic differentiation. **(a)** Microphotographs of BFP-ASCs and SC-ASCs maintained for 14 days in control (CTRL) and adipogenic medium (ADIPO; 200× magnification, scale bar 50 μm); lipid vacuoles are positively stained by Oil Red O. **(b)** Quantification of lipid vacuole formation in CTRL (white bars) and ADIPO (grey bars) BFP-ASCs-1, -2 and SC-ASCs-1, -2. ADIPO vs. CTRL: $^{**}p < 0.01$, $^{***}p < 0.001$.

previously shown in Figure 2, appeared slightly smaller and rounder compared with SC-ASCs (Fig. 3c, *left*).

Surprisingly, BFP-ASCs, and in particular BFP-ASCs-2, showed a noticeable clonogenic ability respect to the two populations of SC-ASCs (Fig. 3d), with an average of about 9.2%±4.5% BFP-ASCs able to produce CFU-F from passage 2 to 4 (Fig. 3d). Interestingly, BFP-ASCs-1 showed a significant increase in colony formation at late passages (from passage 7 to 9, +19.1%±7.7%; data not shown), suggesting a delayed selection of progenitor cells.

No significant difference between BFP-ASCs and SC-ASCs from the two donors was observed.

Differentiation of human BFP-ASCs

Adipogenic differentiation. After 2 weeks in the presence of adipogenic stimuli, an important morphological change was induced compared to undifferentiated cells (CTRL; Fig. 4a) The classic fibroblast-like shape of human ASCs was lost, and both ASC populations showed lipid vacuoles–enriched cytoplasm (Fig. 4a). A considerable production of lipid vacuoles was observed in differentiated BFP-ASCs (Fig. 4b, *left*) and SC-ASCs (Fig. 4b, *right*); however, no difference was observed in the adipogenic potential of ASCs derived from the two anatomical regions ($p < 0.05$).

Osteogenic differentiation. After 14 days of osteogenic induction (OSTEO), both BFP-ASCs and SC-ASCs showed a significant up-regulation of two specific markers, ALP activity and collagen deposition (Fig. 5). The two differentiated BFP-ASCs populations significantly up-regulated their ALP levels with respect to undifferentiated cells, with an increase of 504.0% and 334.1% for BFP-ASCs-1 and BFP-ASCs-2, respectively (Fig. 5a, *left*). The osteogenic ability of SC-ASCs is also reported in Figure 5a (*right*), which shows that the ALP activity of osteo-differentiated SC-ASCs was significantly augmented, with an increase of about 620% and 486%, for SC-ASCs-1 and SC-ASCs-2, respectively.

OSTEO-BFP-ASCs-1 and -2 produced a considerable amount of collagen, with an average increase of 100% and 49%, respectively, compared to undifferentiated cells (Fig. 5b, *left*). High levels of collagen were also produced by osteo-differentiated SC-ASCs-1 and -2, with an increase of 129% and 146%, respectively, compared to undifferentiated cells (Fig. 5b, *right*). Figure 5c shows both BFP-ASCs and SC-ASCs, maintained for 14 days in both CTRL and OSTEO, fixed and stained by Sirius-Red.

Growth of ASCs with human sera

Considering a possible future clinical application, we studied the ability of BFP-ASCs and SC-ASCs to grow in medium supplemented with human serum. Both populations were cultured in the presence of 10% or 5% HAS or 5% HHS, and their growth was compared to that of cells maintained in standard conditions (10% FBS). No differences in morphology were observed. In all the growth conditions, the ASC populations maintained the fibroblast-like shape (Fig. 6a, b), and their proliferation rate was analyzed at 7, 14 (Fig. 6c, d), and 21 days of culture. The presence of 10% HAS induced a prompt increase (within 7 days) in both BFP-ASC and SC-ASC numbers compared to other sera (Fig. 6c, d). The superior proliferation rate was quite evident for both cell types after 21 days of culture ($8.5 \times 10^6 \pm 6.9 \times 10^6$ BFP-ASCs and $5.4 \times 10^6 \pm 7.6 \times 10^5$ SC-ASCs; data not shown), whereas cells cultured in the presence of 5% HAS and 5% HHS showed a comparable trend of cellular growth with those cultured under standard conditions (Fig. 6c, d).

Human ASCs and scaffolds

The ability of ASCs to adhere to and grow on the types of scaffolds routinely applied in periodontal and oral bone regeneration was tested by seeding them on natural and synthetic biomaterials such as bone, periodontal ligament, collagen membrane, and polyglycol acid filaments. Undifferentiated BFP-ASCs and SC-ASCs were first loaded and then cultured on the scaffolds for 4 days, as shown in Figure 7. Both cell types adhered to the natural supports nicely. The

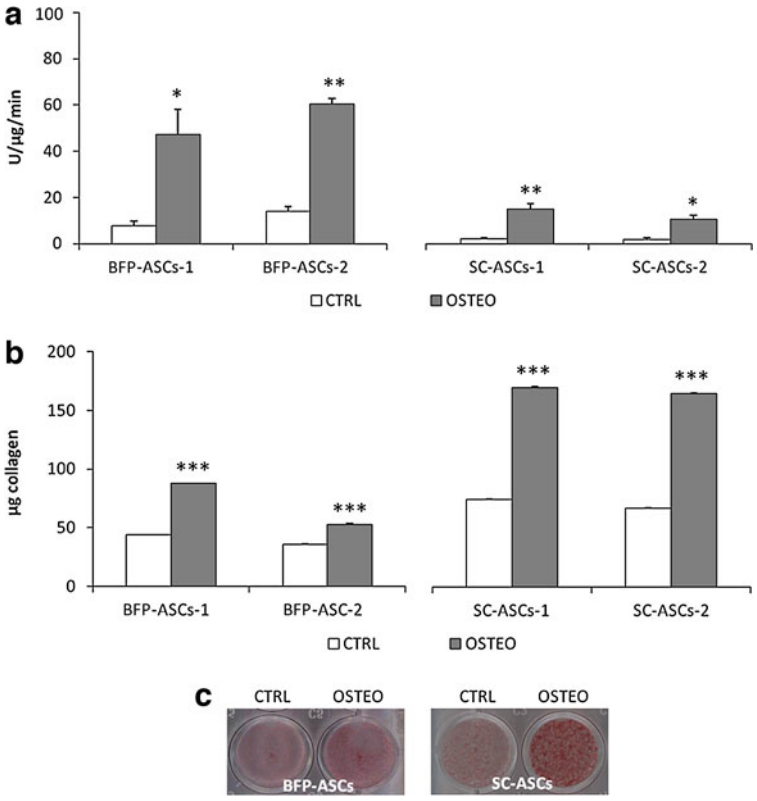

FIG. 5. Osteogenic differentiation. **(a)** Alkaline phosphatase (ALP) activity of undifferentiated (CTRL, white bars) and osteo-differentiated (OSTEO, grey bars) BFP and SC-ASCs-1, -2 at passage 7. Data are normalized vs. protein concentrations of each sample. **(b)** Quantification of collagen deposition in CTRL and OSTEO BFP and SC-ASCs-1, -2 at passage 7. OSTEO vs. CTRL: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. **(c)** Representative images of collagen production by BFP-ASCs and SC-ASCs cultured for 14 days in CTRL and OSTEO media and stained with Sirius Red S.

only slight difference observed was when cells were kept on suture filaments of polyglycolic acid, on which BFP-ASCs did not seem to be tightly bound.

Amelogenin as an osteogenic inductive factor in vitro

In a preliminary experiment BFP-ASCs and SC-ASCs were cultured for 7 days in the presence of 3% AM. This molecule was able to increase ALP activity, both in undifferentiated and OSTEO-differentiated BFP-ASCs. This effect was observed primarily with BFP-ASCs rather than with SC-ASCs, suggesting that the former were more sensitive. In particular, ALP activity was stimulated by AM with an increase of ~220% in undifferentiated cells (−/+), and further up-regulated when BFP-ASCs were OSTEO-differentiated (+/+), suggesting a synergic effect (+950.4; Fig. 8a, b). Collagen deposition was also up-regulated about 32.5% for both OSTEO-differentiated ASCs (Fig. 8c, d).

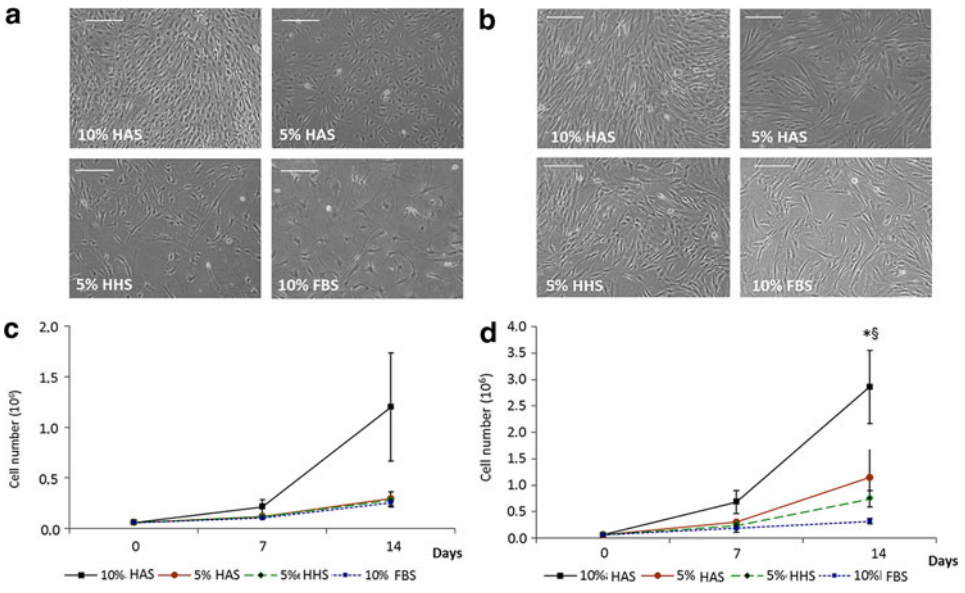

FIG. 6. ASCs cultured in DMEM supplemented with human sera. Microphotographs (100× magnification, scale bar 100 µm) of BFP-ASCs-1 **(a)** and SC-ASCs-1 **(b)** maintained for 14 days in DMEM in the presence of 10% or 5% autologous serum (HAS), 5% heterologous serum (HHS) and 10% FBS. Number of BFP-ASCs **(b;** $n=2$**)** and SC-ASCs **(c;** $n=2$**)** were counted at day 0, 7, and 14 of culture. Data are expressed as mean ± SD. 10% HAS vs. 5% HHS: $^{*}p<0.05$; 10% HAS vs. 10% FBS: $^{\S}p<0.05$.

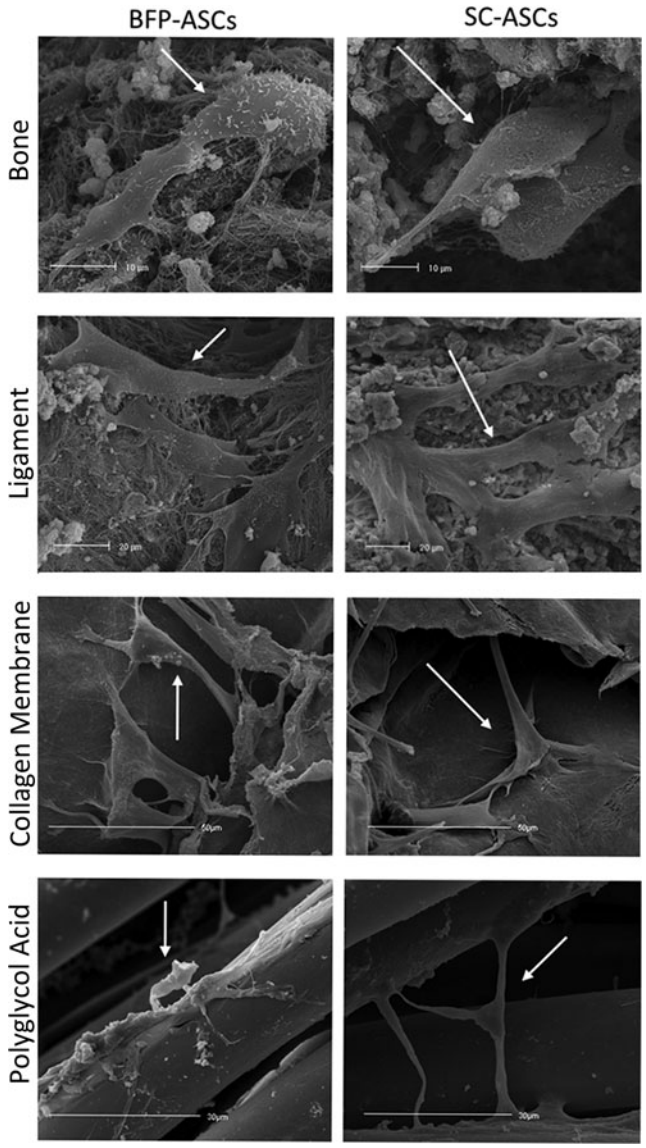

FIG. 7. Undifferentiated BFP-ASCs (*left*) and SC-ASCs (*right*) maintained on natural and synthetic scaffolds for 4 days. Bone fragment (scale bar, 10 μm), periodontal ligament (scale bar, 20 μm), collagen membrane (scale bar, 50 μm), and polyglycol acid (scale bar, 30 μm) are shown.

Discussion

In order to characterize a new source of MSCs, we evaluated human ASCs isolated from the BFP, comparing them with the ones isolated from subcutaneous adipose tissue. Since the final purpose will be the use of these cells for a future therapy of periodontal defects and bone regeneration, we also studied their differentiative ability and their growth on different substrates.

It is feasible to isolate a proper quantity of stem cells even by starting from a small amount of raw adipose tissue (0.5–1 mL) such as BFP. As previously reported for ASCs isolated from subcutaneous tissue,[6,34] we found that BFP-ASCs also share MSC features. In fact, their typical fibroblast-like morphology was present in both populations even though BFP-ASCs appeared to have a smaller and rounder cell body. Moreover, the immunophenotype and the clonogenic activity confirmed their stemness; interestingly, BFP-ASCs were even more prone to produce colonies than SC-ASCs. Both cell types are multipotent and they can be induced to differentiate, as previously described by Farré-Guasch et al.[35] In particular, we observed the production of lipid vacuoles and an up-regulation of ALP activity and collagen deposition already after 14 days of induced differentiation *in vitro*. In our experimental conditions, differentiation was detected in the absence of bone morphogenic proteins, which seemed to be necessary in the study of Shiraishi et al.[36] This could be due either to the different osteogenic media used or to the possible variability among cells derived from healthy donors of our study and the patients with jaw deformity of theirs.

Since our data seem to support future tissue-engineering applications, we investigated the behavior of BFP-ASCs in association either with natural supports, such as bone and periodontal ligament or biocompatible materials that might be in contact with these cells in a clinical setting. It is known that SC-ASCs can be successfully seeded on demineralized and decellularized bone;[37–39] here, we also showed a stable interaction of SC-ASCs and BFP-ASCs with healthy tissues such as autologous alveolar bone and periodontal ligament. Moreover, all the cells also efficiently adhered to collagen membrane and polyglycol acid filaments, two materials chosen for their resorbable property and the widespread use in surgery. This noted adaptability of ASCs, to survive and grow on all supports assayed and present during oral surgery, represents a prominent quality for a future regenerative-medicine approach. Interestingly, one of our experiments indicated that osteo-differentiation of BFP-ASCs is specifically induced and up-regulated by AM, as also previously reported for BMSCs.[40–42] AM is the most abundant enamel matrix protein and dentists favor it for the repair of periodontal defects. The success of this treatment depends on which cell line fills the defect; in particular, it is important to promote the growth of periodontal ligament cells and slow that of gingival and epithelial cells to let the correct cells proliferate close to the dental root. AM enhances the proliferation of periodontal MSCs that secrete cytokines and cause the synthesis of new cementum and periodontal fibers. Subsequently, the regeneration of the periodontium creates the precondition for new formation of functional attachment and for bone regeneration.[24] A recent review by Bosshardt[43] also sheds light on other biological mechanisms of this compound that seems to affect a great variety of cells types included MSCs, pre-osteoblasts, and osteoblasts, influencing their proliferation, expression of transcription factors and cytokines, and their differentiation, showing the direct influence of AM on bone regeneration. Our experimental approach showed the effect of AM on osteogenic differentiation of human ASCs with a synergic effect with others osteo-inductive factors. We may assume that this synergy, observed *in vitro*, could be maintained *in vivo*, where a lot of growth and differentiation factors are released during the fracture healing.[44] Moreover, this effect is more evident for BFP-ASCs than for SC-ASCs, possibly due to the natural localization of BFP-ASC that could make them more prone to respond to stimuli naturally secreted in the same body area.

Since it was already reported that culturing ASCs with autologous serum does not negatively affect them[45,46] and with

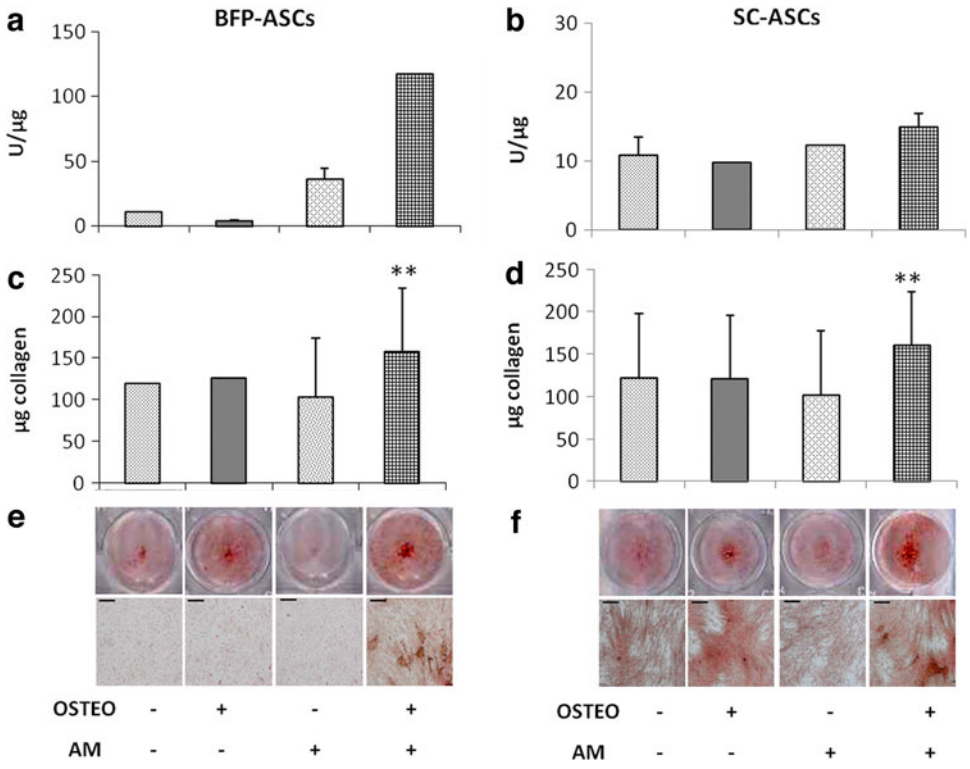

FIG. 8. Effect of amelogenin on BFP-ASCs and SC-ASCs osteogenic differentiation. **(a, b)** ALP activity of undifferentiated and osteodifferentiated BFP and SC-ASCs for 7 days in the presence of amelogenin (+/− and +/+) and in its absence (−/− and +/−). ALP activity is normalized with respect to protein concentration. **(c, d)** Quantification of collagen deposition in the same experimental conditions. +/+ vs. −/−: $^{**}p < 0.001$. **(e, f)** Wells containing stained BFP- and SC-ASCs cultured for 7 days (*top*) and respective microphotographs (*bottom*; magnification 40×, scale bar 300 µm).

prospective clinical applications, we cultured these cells in self and nonself conditions. We noticed that the presence of human serum enhanced the proliferation rate of both cells types. This effect has not been previously observed on ASCs, but similar heterogeneity in the response to autologous sera has been also described for bone marrow stem cells[47–50] and could be explained by the differences of sera from the donors. Growing ASCs in the presence of autologous serum could be a convenient and safe procedure in future cellular therapy, eliminating the concerns about contact with animal proteins.

We conclude that ASCs can be isolated from small specimens such as BFP; their features and behavior are quite similar to the most known SC-ASCs. BFP-ASCs are sensitive to the classical osteo-differentiative stimuli but also, quite interestingly, to AM. We believe that these results are very encouraging, although further experiments are required to confirm the effect of AM on progenitor cells derived from BFP.

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Author Disclosure Statement

The authors state that this manuscript has not been published previously and is not currently being assessed for publication by any journal other than *Bioresearch Open Access*. Each author has contributed substantially to the research, preparation, and production of the paper and approves its submission to the journal. No competing financial interests exist.

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