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A novel efficient method to isolate human adipose-derived stromal cells from periumbilical biopsies without enzymatic digestion

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Abstract

Background: **Among all the possible sources of mesenchymal stem cells, adipose tissue has raised great interest and has become one of the most investigated sources. Several protocols are available for isolating adipose-derived stromal cells (ASCs) and, recently, the International Fat Applied Technology Society (IFATS) highlighted the importance of standardization and harmonization of these procedures. The enzymatic digestion is among the most frequently used and it could represent a problematic issue for transfer to clinical applications.**

AIM: **Set up a novel manual non-enzymatic method combined with the use of stem cells medium (SCM) for isolating ASCs from human periumbilical biopsies with a high yield and rapidity.**

Materials and Method: **ASCs isolated without enzymatic step and using SCM were cultured in both SCM and α-MEM, and characterized for plastic adherence, growth capability, expression of both mesenchymal markers and multipotency genes, and differentiation features into mesenchymal lineage.**

Results: **By following our method, cells can be easily maintained in α-MEM after their isolation in SCM, and they fully adhere to the requirements defined by both IFATS and International Society for Cellular Therapy (ISCT) guidelines. Moreover, we also report that isolated ASCs express neural markers such as β-tubulin III, Nestin and glial fibrillary acidic protein (GFAP).**

Conclusions: **Our method allows the quickly and easily ASCs isolation from human periumbilical biopsies. The obtained cells can be cultured in a commercially available medium without altering their mesenchymal properties. Moreover, the lack of the enzymatic step is a great advantage for all those translational preclinical studies that require a great amount of cells, without affecting their biological features.**

Introduction

Mesenchymal stem cells (MSC) are multipotent fibroblast-like cells firstly isolated in 1970 by Friedenstein et al[1] and now considered a validated tool for therapy and research in various diseases. MSCs are characterized by self-renewal capability, multilineage differentiation and represent an attractive source for tissue engineering[2]. Among all the possible sources of

mesenchymal stem cells, adipose tissue has raised great interest and nowadays has become one of the most used sources for the isolation of MSC. Multipotent stromal cells can be obtained from subcutaneous fat, lipoaspirate samples or abdominal biopsies[3].

According to the International Fat Applied Technology Society (IFATS), a consensus has been reached over the use of the term "adipose-derived stromal/stem cells" (ASC) to name the plastic adherent, cultured and serially passaged multipotent population obtained from adipose tissue[4,5]. It has been reported that stem and precursor cells in the freshly isolated stromal vascular fraction (SVF) usually account for up to 3% of the total cell population, meaning a 2500-fold more than the frequency of stem cells in bone-marrow[6]. In 2006 a standard set of rules was proposed by the International Society for Cellular Therapy (ISCT) to define the identity of these cells. Accordingly, MSCs, in standard culture conditions, must be plastic adherent, express surface molecules such as CD105, CD73, CD90 and CD44, and lack both hematopoietic and endothelial markers such as CD45, CD14 or CD11b, CD79a or CD19, nor MHC II (HLA-DR). They also should be able to differentiate *in vitro* into osteoblasts, adipocytes and chondroblasts[7].

There are several isolation protocols for ASCs and there is a need for standardization and harmonization[5,8]. The enzymatic digestion methods is among the most frequently used[9,10]. Enzymatic digestion is a validated alternative method for those experimental approaches in which a large number of cells cannot be obtained in sufficient quantity and quality through mechanical dissociation alone. Despite the advantage, enzymatic procedures may profoundly affect the expression of a variety of cell-surface molecules and may be the causative origin of discrepancies observed among data obtained by immunohistochemical staining of intact tissues, and gene expression or flow cytometry techniques[11].

In this study we describe the isolation of human ASCs from periumbilical biopsies with a manual method that avoids enzymatic digestion and employs a stem cell medium (SCM), commonly used in our laboratories for the isolation of human embryonic neural stem cells[12] and multilineage differentiation of human muscle-derived stem cells[13]. Particularly, here we propose the novel use of SCM as the most effective medium for obtaining ASCs, with a yield quantitatively and qualitatively comparable to what obtained with the classical standard enzymatic approach, in association with the Minimum Essential Medium Alpha Medium (α-MEM). We isolated human ASCs from n. 10 periumbilical biopsies with SCM and cultivated the derived cells from passage 1 in both SCM and α-MEM media. Cells were characterized by following recently published guidelines[14], and growth capabilities, immunophenotype, ability of *in vitro* differentiation in the three main mesenchymal lineages (osteoblasts, adipocytes and chondroblasts), karyotype stability and expression of the typical multipotency markers (Oct4, Nanog and Sox2) were evaluated.

Matherials and methods

Human periumbilical biopsies

Periumbilical adipose tissue was obtained from 10 healthy male volunteers, undergoing surgery for ventriculo-peritoneal shunt. The mean age was 55 ± 14 years (40 min - 74 max), mean weight was 82.9 ± 22.8 kg (70 min - 146 max), and mean body max index was 26.7 ± 8.5 (21.1 min-50.5 max) (*see Table 1*). All patients were HIV and HCV negative.

All volunteers signed an informed written consent, and the study was approved by local Fondazione I.R.C.C.S. Istituto Neurologico "Carlo Besta" (Milan, Italy) Institutional Review Board. The study also conformed to the 2013 WMA Declaration of Helsinki.

Adipose-derived stromal cells (ASCs) isolation

Procedure for isolation and expansion of ASCs were conducted in a Biological Safety Cabinets (HERA Safe, HERAEUS, USA) and cells were cultured at 37°C in a humidified, 5% CO_2 incubator (Series II Water Jacket-Thermo Scientific, USA). As schemat-

Table 1. Patients demographic and clinical data.

ID sample	Age (yrs)	Gender	Weight (kg)	BMI
ASC pt1	71	M	75	24,5
ASC pt2	74	M	80	23,6
ASC pt3	61	M	70	25,3
ASC pt4	56	M	76	24,8
ASC pt5	40	M	82	23,4
ASC pt6	53	M	146	50,5
ASC pt7	50	M	70	24,2
ASC pt8	31	M	67	21,1
ASC pt9	69	M	83	26,8
ASC pt10	46	M	80	22,9

Legend: BMI body max index

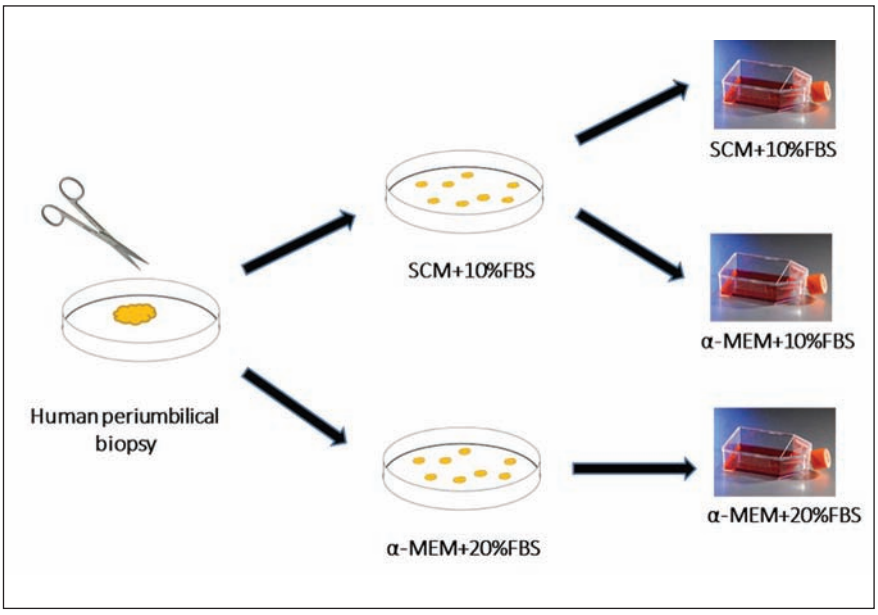

Figure 1. Schematic representation of isolation method adopted in this paper.

ically described in Figure 1, cells isolation was performed starting from the same adipose tissue preparation. About 3 grams of adipose tissue were collected from each male healthy volunteer, and tissues were minced either by sterile scalpel or with surgical scissors in a 100 mm^2 petri dish (CORNING, Tewksbury, MA, USA). The adipose pieces were then harvested with sterile PBS 1X (EuroClone, Pero, MI, Italy) using a 10 ml sterile pipette (CORNING) in a 50 ml Falcon tubes (SARSTEDT AG & Co, Nümbrecht, Germany), and centrifuged at 2000 rpm for 10 minutes. The adipose upper phase resulted by centrifugation was plated into T75-cm^2 flasks (CORNING) and, after adhesion of the pieces, commercially available α-MEM (EuroClone) or SCM medium, consisting of DMEM/F12 (Euroclone, Pero, MI, Italy), 20 μg/L human recombinant epidermal growth factor (EGF, Life Technologies Carlsbad, California, USA) and 10 μg/L basic fibroblast growth factor (bFGF; Life Technologies Carlsbad, California, USA), 2 mmol/L L-glutamine, 33 mmol/L glucose, 9.26 g/mL putrescine, 6.3 μg/L progesterone, 5.3 μg/L sodium selenite, 0.0025 g/L insulin, and 0.1 g/L grade II transferring sodium salt (Sigma, St Louis, MO, USA)[12], was added (Figure 1). α-MEM and SCM media were supplemented with 20% or 10% FBS (USA Origin, Gibco®Life Technologies, Italy), respectively, and with 1% penicillin/streptomycin solution (EuroClone). The same serum batch was used for all isolation experiments and cell cultures. When cells reached 50% of confluence, fresh media was replaced, whereas at 70-80% confluence, cells were harvested by trypsinization (TRYPSIN 1X, EuroClone) and reseeded.

Maintenance of ASC cultures

When cells at passage 1 reached the 80% confluence, ASC cultures were harvested by trypsin, counted, washed in 1 ml of PBS 1X (2000 rpm for 10 min) and re-seeded at $1x10^4$ cells/cm^2 in T75-cm^2 flasks in SCM +10% FBS+1% pen/strep[13] or α-MEM (EuroClone) + 20% FBS + 1% pen/strep + 1% L-Glutamine (EuroClone). To compare the amount of cells obtained by plating adipose biopsies in the two different media (α-MEM or SCM), cells were maintained in culture as follows: cells isolated with α-MEM were maintained in α-MEM + 20% FBS; cells isolated in SCM + 10% FBS were maintained either in this medium or, at passage 1, cultured in α-MEM + 10% FBS (Figure 1). Demographic and clinical data of patients included in the study are reported in Table 1.

For all the experiments, cells were used before passage 9.

Adipose-derived stromal cells (ASCs) were seeded at $1x10^4$ cells/cm^2 in T75-cm^2 flasks in the presence of SCM and passed weekly for expansion or freezing procedures. Expansion was done by trypsinization and by assessing viability, with Trypan Blue dye exclusion assay (EuroClone), counted in triplicates. Freezing was performed in FBS with 10% dimethyl sulfoxide (DMSO, WAK-Chemie Medical GmbH, Steinbach/Ts, Germany) (-80°C

freezer for 24 hours, then stored in liquid nitrogen), while de-freezing by quickly thawing at 37°C, washed in PBS and assessed for viability by Trypan Blue dye exclusion assay.

Growth capability: cumulative population doublings

ASCs obtained and grown in α-MEM at passage 1 were seeded for growth capability by determining cumulative population doublings[15]. At passage 4 these cultures stopped to grow, therefore we could not perform all further investigations. Indeed, ASCs isolated from the other piece of the same specimen biopsy, plated in SMC, were maintained in either SCM and α-MEM and were evaluated for their growth capability at 80-85% of confluence. Briefly, from passage 4 to 9, $1x10^5$ cells, cultured in SMC, or $2x10^5$ cells cultured in α-MEM, were seeded in T25-cm^2 flasks with 3.5 ml of respective medium. After 4 (SCM) or 6 (α-MEM) days of culture, cells were harvested, counted by Trypan Blue dye exclusion and re-seeded for the next passage growth at the same concentration. The number of population cell doublings was calculated using the formula:

Cell doublings = $Log_{(10)}$ N / $Log_{(10)}$ 2

where N = cells harvested / cells seeded.

The total number of cell doublings was calculated by the sum of doublings for each passage.

Flow cytometric immunophenotypic analysis (FACS)

Each ASCs culture, in SCM and α-MEM, was phenotypically characterized by Fluorescence-Activated Cell Sorting (FACS) at different passages (p3 and p8). Briefly, $1x10^5$ cells re-suspended in 250 μl of PBS 1X were incubated for 30 minutes at 4°C in the dark with 10 μl of the following antibody: anti-CD44 (BD Biosciences Italy), anti-CD144 (R&D System USA), anti-CD166 (BD Biosciences, Italy), CD105 (AbDSerotec, Raleigh, NC, USA), CD90 (Millipore Temecula, CA, USA), CD73, CD19, CD31, CD34, CD45, HLA-DR (BD Pharmingen, San Jose, CA, USA). For negative controls, cells were stained with respective isotype Ab. After centrifugation with 1 ml of PBS 1X at 2000 rpm for 10 minutes, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and about $5x10^3$ events were acquired for each sample. Non-viable cells were excluded by physical gating. The results of the analysis were expressed as mean ± SD.

G-banding karyotype analyses

Cytogenetic analyses were performed on "in situ" cultures obtained by seeding human ASCs cultures directly onto a coverslip inside Petri dishes containing 2 ml of media (SCM or α-MEM). Cells were treated with Colcemid (0.02 μl/ml) (Life Technologies Carlsbad, California, USA) for 90 minutes, hypotonic solution (1:1 Na citrate 1%: NaCl 0.3%) (Sigma-Aldrich St. Louis, MO, USA) and fixative solution of 3:1 methanol: acetic acid (VWR International Radnor, Pennsylvania, USA), replaced twice. At least twenty-five QFQ banding metaphases were observed for each sample. The images were acquired using a fluorescence microscope (BX 60 Olympus) and analyzed with Powergene PSI system (Orchard systems/PSI, Newcastle upon Tyne, UK).

RNA extraction and qRT-PCR analyses

Total cellular RNAs were extracted using TRI Reagent® (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instruction. RNA purity and quantity were assessed by Nanodrop (Fisher Scientific) (A260/A280 1.8-2 was considered suitable for further analysis), possible contaminating DNA was removed, and cDNA was prepared from 1 μg of RNA using MBI-Fermentas kit (Fermentas, St. Leon-Rot Germany). SYBG master mix (Biorad 172-5124) was used in StepOne™ Real-Time PCR technology (Applied Biosystems) to perform Quantitative real-time PCR. Total cDNA from foetal brain stem cells and total cDNA samples obtained from human oral mucosa were used as positive and negative control of gene expression, respectively. Melting curve analysis was always performed at the end of each PCR assay to control specificity. Data were expressed as Ct values and used for relative quantification of targets with ΔΔCt calculation. Potential bias, due to averaging data transformed through the equation 2-ΔΔCt to give N-fold changes in gene expression, were excluded performing all statistics with ΔCt values, using 18S rRNA as housekeeping gene The data were only used if the calculated PCR efficiency ranged between 1.85 and 2.0. Template and reverse transcription negative controls were also included in all amplification experiments.

PRIMERS used: Homo_SOX2 NM_003106.3

F: aatcatcggcggcggcaggatcggccagag

R: GCCGGCCGCCGCCGCGGTGGAGTTGCCGCC

Homo_NANOG AY230262.1

F: TGATTTGTGGGCCTGAAGAAAACTATCCATC

R: AGGCTGGGGTAGGTAGGTGCTGAGGCCTT

Homo_OCT-4 DQ486513

F: ccggaggagtcccaggacatcaaagctctg

R: CCCCAGGGTGAGCCCCACATCGGCCTGTGT

In vitro differentiation potential

ASCs at passage 4, either in SCM and α- MEM (n = 3 for each medium), were tested for their ability to differentiate into adipocytes, chondrocytes and osteocytes using Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, Minneapolis MN, USA), according to the manufacture instructions.

For adipogenic differentiation $2.1x10^4$ cells/cm^2 were plated. When cells reached 100% of confluence, growth medium was replaced with Adipogenic Differentiation Medium. After 21 days in Adipogenic Differentiation Medium (changed every 3-4 days), cells were fixed with 4% paraformaldehyde for 1 hour and visualized by Oil Red O Staining (Sigma Aldrich).

For osteogenic differentiation, $4.2x10^4$ cells/cm^2 were plated. When cells reached 70-80% of confluence, growth medium was replaced with Osteogenic Differentiation Medium (changed every 3-4 days). Cells were maintained in culture for 3 weeks, fixed in 70% ethanol and stained with 1 mg/ml Alizarin Red S dye (Sigma-Aldrich St. Louis, MO, USA) to detect mineralized matrix[16].

Images from adipogenic and osteogenic differentiations were obtained at 20X magnification, using Nikon Eclipse TE300 equipped with the Axiovision device camera (Zeiss Instr., Oberkochen, Germany). Images were processed using Axiovision release 4.6.3 (Zeiss Instr., Oberkochen, Germany).

Chondrogenic differentiation was performed with $2.5x10^5$ cells seeded in 1.5 ml eppendorf tubes (Eppendorf) directly in Chondrogenic Differentiation Medium (replaced every 2-3 days). After 21 days, chondrocyte pellet was fixed with 0.3 ml of 4% paraformaldehyde for 20 minutes, washed with PBS 1X and cryopreserved with 15% and 30% Sucrose (o/n 4°C each). Pellet were included and frozen in Killik (Bio-optica, Milano, Italy) and 12μm slices were prepared through cryostat cutting. Immunostaining for human aggrecan was performed, according to the manufacture instructions Kit (R&D System). Briefly, each slice was first defrosted for 10 min at 4°C, then at RT. Permeabilization of tissue was performed with permeabilization solution (PBS 1X+1% BSA (Gibco®Life Technologies, Italy) +10% NGS [Euroclone, Pero (MI) Italy) +0.3% TRITONX100 (Sigma-Aldrich)] for 45 min at RT. Subsequently, human aggrecan antibody was diluted in the permeabilization solution at a final concentration of 10 μg/ml and incubated on slices o/n at 4°C in humidified chamber. Slices were then washed for three times with PBS 1X+1%BSA and incubated for 1h with secondary antibody Alexa594 donkey anti-goat (1:200) in PBS 1X+1% BSA. After three washes with PBS 1X+1% BSA, slices were mounted with Fluorsave™ Reagent (Millipore- Billerica, MA, USA) and analyzed with Nikon Eclipse TE300 equipped with the Axiovision device camera (Zeiss Instr., Oberkochen, Germany). Images were processed using Axiovision release 4.6.3 (Zeiss Instr., Oberkochen, Germany).

Immunofluorescence of ASCs cultured in SCM and α-MEM

$5x10^4$ cells/cm^2 were seeded onto glass slides and fixed with 4% paraformaldehyde after 24 hours of culture. After saturation (4% BSA, 0.3% Triton X-100) (BSA Sigma-Aldrich St. Louis, MO, USA, Triton X-100 VWR International Radnor, Pennsylvania, USA) and permeabilization cells were incubated overnight at 4 °C with primary antibodies against human Nestin (Clone #196908, R&D Systems), Vimentin (Polyclonal, Santa Cruz, CA, USA), GFAP (Polyclonal, Covance) and βIII-tubulin (Millipore). Cells were rinsed and then probed 45 minutes with secondary antibodies Alexa Fluor 594 anti-mouse, rabbit or goat (Invitrogen, Carlsbad, CA, USA). Nuclei were counterstained with DAPI (2 μg/ml in PBS) (Sigma-Aldrich St. Louis, MO, USA). Glasses were mounted with Fluor-SaveTM (Millipore, Billerica, MA, USA). Images were taken using Leica SP2 confocal microscope with He/Kr and Ar lasers (Heidelberg, Germany). In negative control experiments, primary antibodies were replaced with equivalent concentrations of unrelated IgG of the same subclass.

Cell Protein Extraction and Western Blotting

Cells were seeded onto 6-well culture plates and maintained in culture in growth medium (SCM and α-MEM). At 85% confluence the medium was removed, and cell protein extracts were obtained in SDS-containing buffer as previously described[17]. Equal amounts (75 μg) of solubilized proteins were separated by SDS-PAGE gel under reducing conditions, and electro-blotted onto nitrocellulose membrane. Primary antibodies used were: anti-β-actin, (Santa Cruz Biotechnology, clone K3004), anti-GFAP (Polyclonal, Covance).

Statistical Analysis

Data are expressed as means ± SD. The two-way analysis of variance (Anova) and Bonferroni's post-test were applied using Prism 5 software (GraphPad Software Inc, La Jolla, CA, USA) assuming a p value less than 0.05 as the limit of significance.

Results

ASCs isolation and culture

ASCs isolation from the same human periumbilical biopsy was performed in SCM + 10% FBS and α-MEM + 20% FBS by plating small pieces (2 ± 0.5 mm) of tissue at the bottom of the 100 mm^2 petri dish, without enzymatic treatment (see *Materials and Methods* for details). Routinely, starting from a 3 ± 0.5 grams biopsy specimen, we were able to obtain at least 120 ± 20 fragments and to seed 20 ± 5 pieces/100 mm^2 petri dish. Figure 2A shows ASCs from biopsies after 5 days in culture in SCM. From the earliest days of seeding, we observed that cells with fibroblastic morphology adhere to plastic and then spread out from the adipose pieces. Cells were elongated with a classical fibroblast-like morphology and, when split at passage 1 in the two different media (SCM and α-MEM), at passage 3 showed the same morphological properties described above (Figure 2A). Figure 2B shows ASCs after 10 days of biopsies plating in α-MEM + 20% FBS. In this condition, the number of cells diffusing out of the adipose tissue pieces were fewer than in SCM + 10% FBS condition. The ASCs morphology, at passage 3 in α-MEM + 20% FBS, was still fibroblastic-like but with a larger cytoplasm (Figure 2B). With this isolation method, combined with SCM supplementation, we obtained, from the first seeding, a confluent T75-cm^2 flask in 14 days, whereas cells cultured with α-MEM reached the confluence only after 4 weeks.

ASCs growth capability

To evaluate the amount of cells obtained from biopsies plating without enzymatic digestion, using the two different media, growth curves were set up. ASCs growth was evaluated in terms of cumulative population doublings[15], from passage 1 to passage 4. In particular, cumulative population doublings of α-MEM + 20% FBS cells were significantly lower, from passage 2 to passage 4, compared to the same passages of cells cultured in SCM (p2: 1.25 ± 0.35

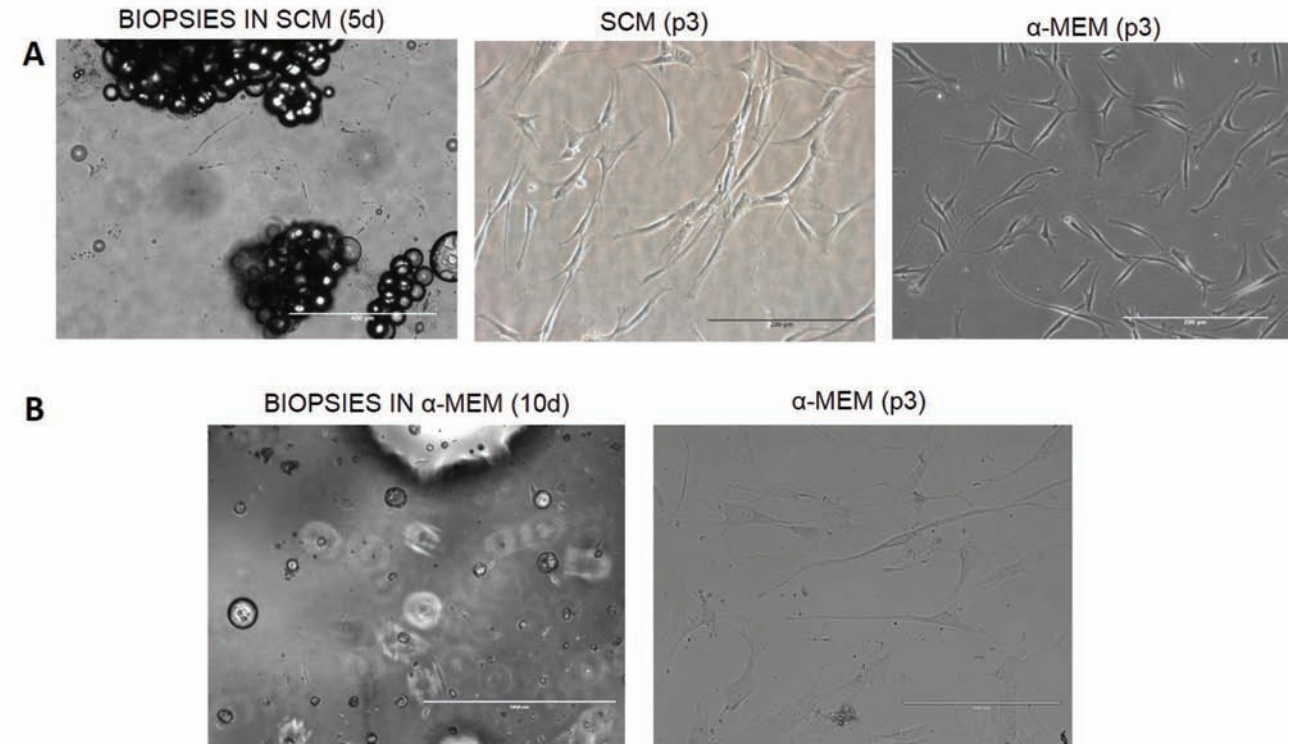

Figure 2. Inverted light microscopy images of ASC primary cultures. *A,* Magnification of adipose pieces plated in SCM after 5 days and ASCs cultured with SCM and α-MEM at passage 3. *B,* Magnification of adipose pieces plated in α-MEM and ASCs cultured with α-MEM at passage 3 (Original magnification: x20).

α-MEM vs 3.47 ± 0.85 SCM; p3: 2.27 ± 0.58 α-MEM vs 6.26 ± 0.81 SCM; p4: 2.67 ± 0.89 α-MEM vs 8.62 ± 0.37) (Figure 3A). Moreover, after passage 4, ASCs obtained in α-MEM stopped to growth, therefore these cells could not be further investigated.

For cells isolated in SCM and grown in either media, instead, cumulative population doublings were calculated from passage 4 to passage 9, as shown in Figure 3B. The median time to reach 80% confluence for all passages was 4 days for ASCs isolated and expanded in SCM and 6 days for ASCs isolated in SCM and expanded in α-MEM. In particular, from passage 7 to passage 9 we found a mean value of cumulative population doublings statistically higher in SCM cultures, compared to α-MEM cultures (p7: 6.07 ± 1.70 α-MEM vs 10.96 ± 2.07 SCM; p8: 7.04 ± 1.60 α-MEM vs 13.67 ± 2.31 SCM; p9: 7.86 ± 2.70 α-MEM vs 16.25 ± 2.68 SCM) (Figure 3B).

Figure 3C shows the percentage of viable cells after cryopreservation procedures for all ASC cultures maintained both in SCM and α-MEM media. We found high percentage of viable cells in both culture conditions: 86.58 ± 9.89% and 88.3 ± 10.48% of viable cells for SCM and α-MEM, respectively.

Flow cytometric immunophenotypic analysis

ASCs were analyzed through flow cytometry for mesenchymal markers expression (Figure 4). Our data highlight that the expression of surface markers molecules was very similar in ASCs obtained from different individuals. ASC cultured in SCM adhered to the criteria defined by ISCT and IFATS[9,5], showing a high percentage of typical mesenchymal markers expression, as CD90, CD73, CD105,CD44, CD144, CD166 (>99 ± 0.3% cells), both at low (passage 3) and high (passage 8) passages, and at the same time low levels of endothelial and hematopoietic markers, as CD31, CD34, CD45, CD19 and HLA-DR (<20 ± 2.5% cells), at either conditions (Figure 4A). A comparable expression profile can be appreciated when ASCs were isolated in SCM and, at passage 1, were transferred to α-MEM medium for maintenance (Figure 4B). A similar expression of mesenchymal markers (>99 ± 0.6% cells) and a lower expression of endothelial and hematopoietic markers (<10 ± 1.5% cells) were found at passage 3 and passage 8, compared to SCM cultured cells. ASCs cultured in α-MEM medium, in fact, appear to express a lower phenotypic panel of endothelial and hematopoietic markers at both passages, even if they were isolated in SCM medium.

Karyotype analysis

The presence of chromosomal rearrangement was studied by QFQ-banding, performed at both earlier and later growth passages. Figure 5 shows a representative result of karyotype stability of late passage ASCs cultured in both media (Figure 5). ASCs did not present any chromosomal rearrangement and chromosome number was normal in five analyzed samples at any passages.

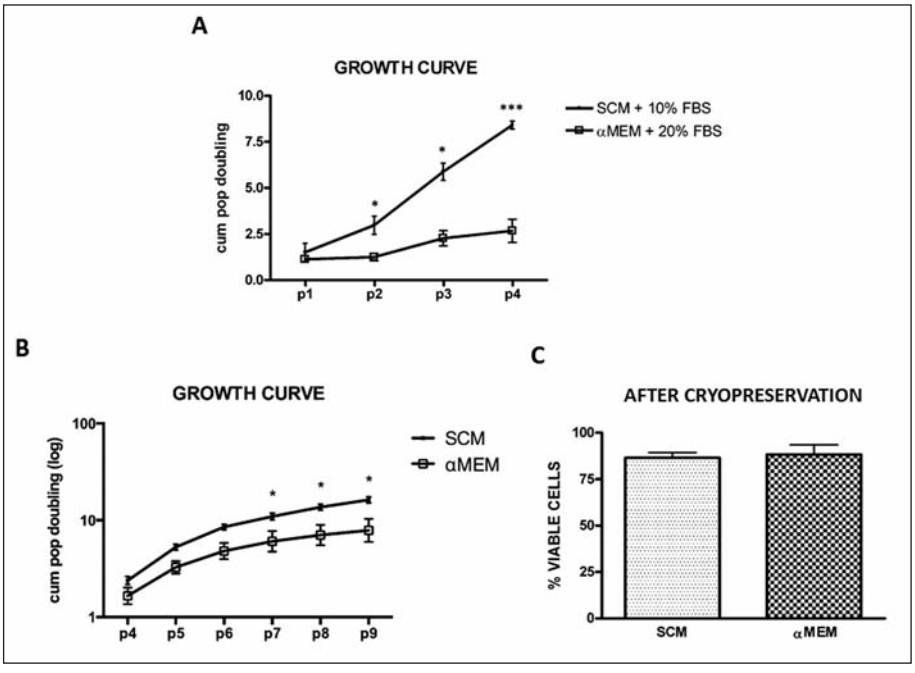

Figure 3. *In vitro* characterization of ASC cell cultures. *A,* Growth curve of ASC primary cultures isolated from the same biopsy specimen with two different media, SCM and α-MEM (*=p<0.05; ***=p<0.001; n=3). *B,* Growth curve of ASC primary cultures isolated with SCM and split for culture in either SCM and α-MEM (*=p<0.05; n=4). Values are expressed as cumulative population doublings calculated with the formula reported by Avanzini et al.[15]. *C,* Cell viability after freezing and thawing evaluated by trypan blue dye exclusion assay, comparing the number of viable cells after thawing to the number of cells previously frozen (n=5). All data are expressed as mean ± SEM.

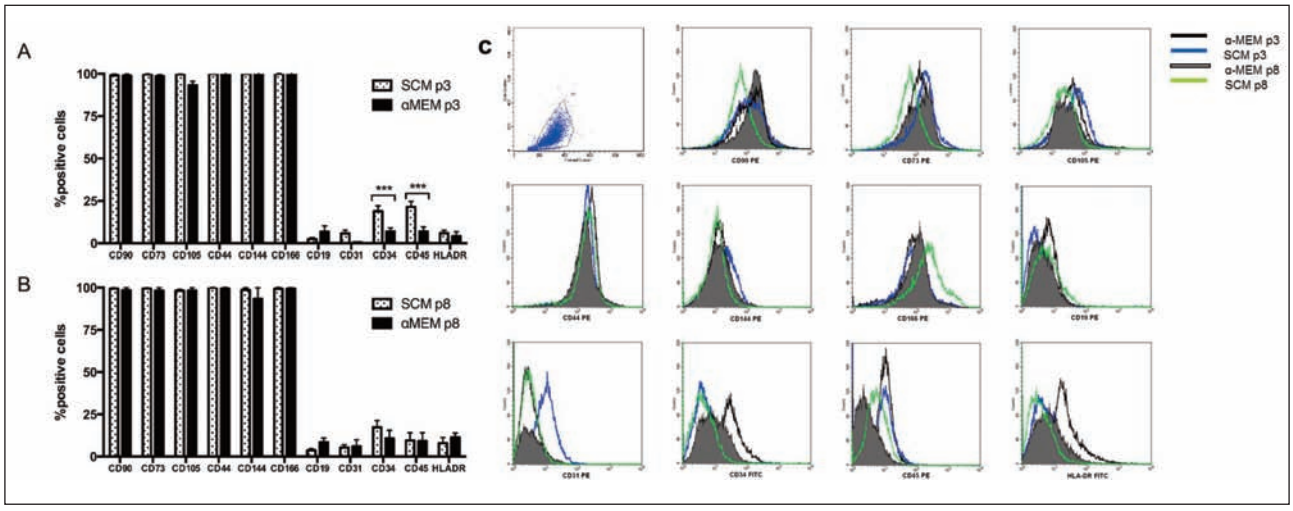

Figure 4. FACs analysis from adherent cells. Analysis were performed at 80% of confluent monolayer at the end of passage 3 and passage 8. *A,* SCM ASC cultures and *B,* α-MEM ASC cultures. Data are expressed as mean ± SEM (n = 5 per group). (**C**) Representative expression of mesenchymal markers in one ASC cultured in SCM (blue line) and α-MEM (black line) at passage 3, and at passage 8 in SCM (green line) and α-MEM (grey fill). Statistical analysis performed in order to compare MFI of α-MEM *vs* SCM cultures, at both passages, revealed that no statistically significant differences in MFI of all considered markers are present (p> 0.05, t test). Moreover no statistically significant differences were observed in MFI between p3 and p8.

Expression of self- renewal genes

To investigate the expression of molecular indicators of self-renewal and plasticity, real time RT-PCR assays were set up. The results reported in Figure 6 show that ASCs grown in either growth media (α-MEM and SCM) expressed spontaneously embryonic stem cell genes, namely Sox2, Nanog, and Oct4 at early growth passage (p3). In particular, Sox2 and Oct4 mRNA levels were significantly higher in cells maintained in SCM, while Nanog mRNA levels were significantly higher in cells maintained in α-MEM (*= $p < 0.001$). The expression of stemness and self-renewal genes was decreased with the maintenance in culture and the corresponding mRNA levels fell down significantly as observed at passage 8 in both culture media (*= $p < 0.001$) (see Figure 6).

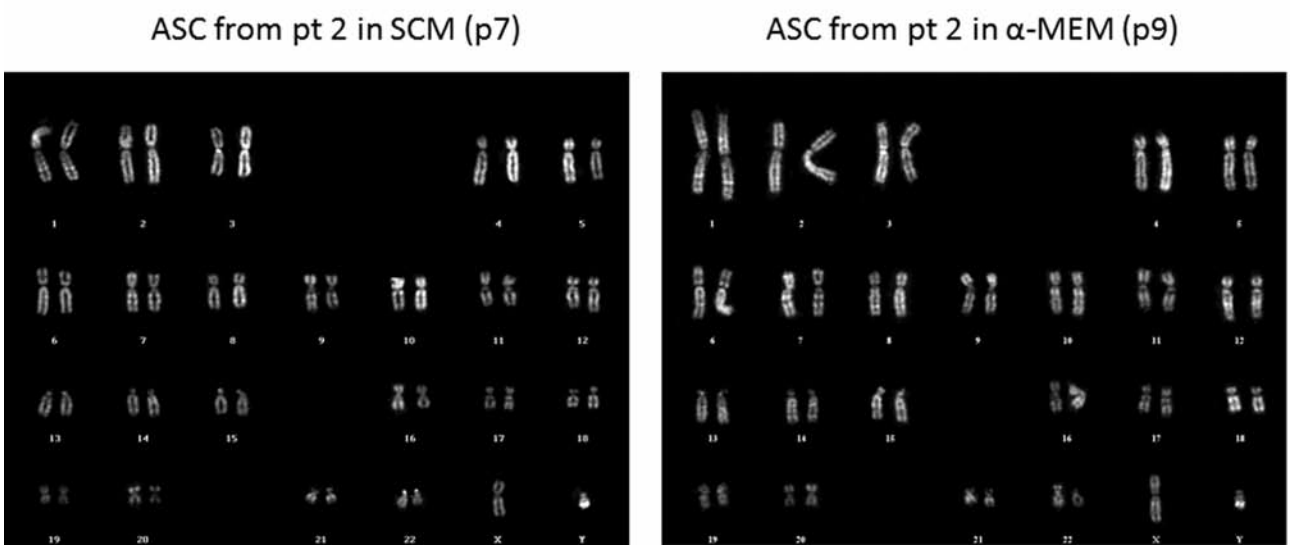

Figure 5. Karyotype analysis. Representative results of QFQ-banding analyses on ASC cultures maintained in both growth media at high passages, passage 7 and passage 9.

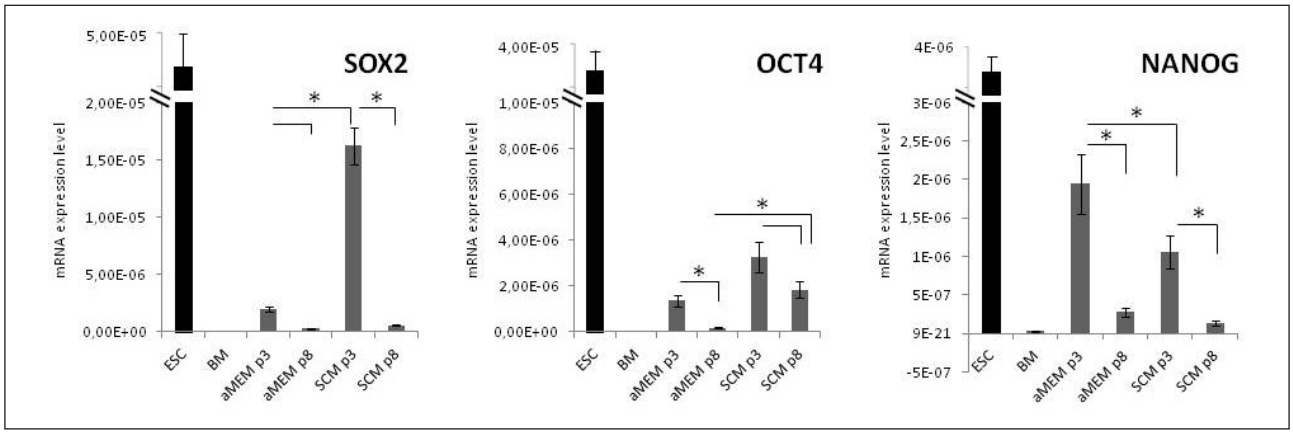

Figure 6. Pluripotency associated endogenous gene expression. Realtime-PCR expression of self-renewal genes Sox2, Oct4 and Nanog in ASC cultures, at early and late passages, and maintained in both growth media, SCM and α-MEM. Values are presented as mRNA expression level. Total cDNA of foetal brain stem cells and samples obtained from human oral mucosa were used as positive and negative control of gene expression, respectively. Data are expressed as the mean of five independent experiments ± SD (*=p<0.001; n=5).

Differentiation potential of ASCs from biopsies

ASCs cultured in SCM and α-MEM media at passage 3 were tested for *in vitro* differentiation potential in adipogenic, osteogenic and chondrogenic phenotypes. Figure 7 shows ASCs positivity for all of the three investigated phenotypes after 21 days of dedicated cultures (see *Materials and Methods* for details). Particularly, panels a, b and c report ASCs maintained in SCM after adipogenic, osteogenic and chondrogenic differentiation, respectively, showing lipid droplets, calcium aggregates and aggrecan positive staining. Panels *d, e* and *f* show differentiation capabilities of ASCs isolated in SCM and maintained in cultures in α-MEM medium (Figure 7). For negative controls, cells were cultured in their classic medium, SCM or α-MEM for the same time period, and negative results are shown in panels g, h and i (Figure 7). The differentiation capabilities towards mesenchymal lineages were comparable between the cells maintained in the two culture media.

Markers expression of ASCs obtained from biopsies

To consolidate the characterization of ASCs derived through biopsies plating, the expression of cellular markers was evaluated by immunofluorescence at low and high growth passages. As shown in Figure 8, cells expressed the mesenchymal marker vimentin and the neuronal marker Nestin and βIII-tubulin at approximately 100%, both in α-MEM and SCM. A very high level of positivity was also detected when cells were maintained in culture up to passage 8 (Figure 8). Moreover, cells cultured with either media showed a low positive expression of GFAP, both at passage 3 and 8 (Figure 9A). Western Blotting (Figure 9B) also confirmed this expression. No significant difference between the two growth media was observed.

Discussion

In the last decade, great interest has arisen around MSCs, as they may become a powerful tool for cell therapy applications in a wide range of diseases, including myocardial infarction, multiple sclerosis and diabetes[18,19]. Adipose tissue represents one of the most important reservoir of MSCs, considering the great accessibility and availability of fat tissue in the organism. In spite of the extensive literature on this topic, a commonly accepted procedure for the isolation of ASCs, from different human fat tissue sources, has not been defined yet. The most frequently used method for ASCs isolation includes enzymatic digestion of fat samples[10], a step that allows a good yield of cells for culture, but at the expense of possible alterations of cell surface molecules. Here we propose the isolation of human ASCs from periumbilical biopsies without enzymatic digestion and with the use of a stem cell medium (SCM), largely used in our laboratories for the multilineage differentiation of human stem cells[13]. In this study we did not made an internal comparison with the enzymatic digestion method, in terms of cells isolation efficiency, and this could

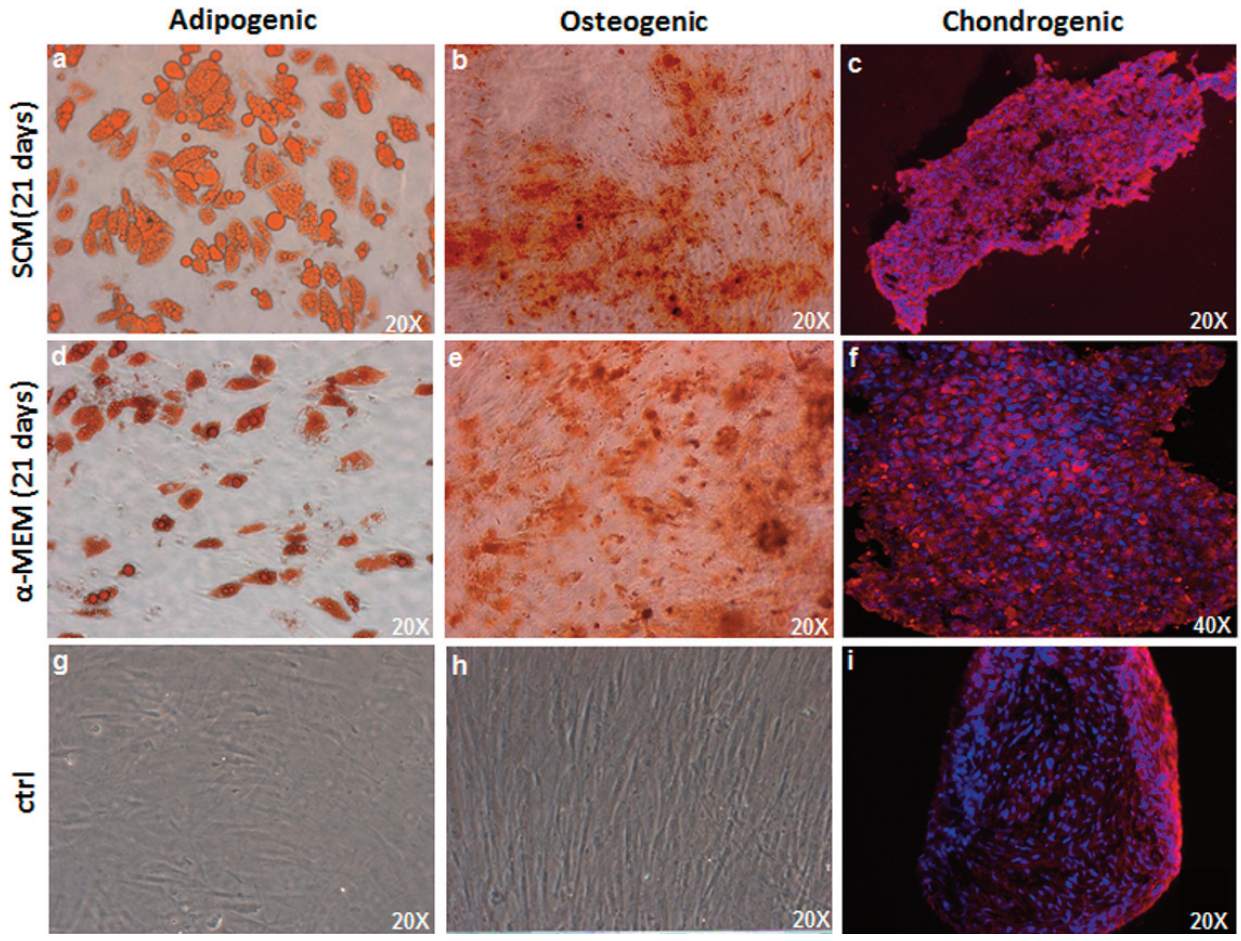

Figure 7. ASC *in vitro* differentiation potential toward the three different lineages. *A-C,* Adipogenic, osteogenic and chondrogenic differentiation of SMC cell cultures. *D-F,* Adipogenic, osteogenic and chondrogenic differentiation of α-MEM cell cultures. *G-I,* Representative images of normal morphology in absence of differentiation stimuli. Adipogenic differentiation was confirmed by Oil Red O Staining and osteogenic by Alizarin Red staining (original magnifications x20). Chondrogenic differentiation was visualized by Aggrecan immunofluorescence (red); nuclei were stained with DAPI (blue). (Original magnification: x20 and x40). Pictures are representative of three independent experiments with similar results.

be considered as a limitation. However, with our isolation method we were able to isolate ASCs from all 10 human fat biopsy samples of the study in a reproducible way and with a good yield of cells obtained. The first cells were detected after 5 days of biopsies plating at the periphery of fat pieces. In this condition their growth was optimal and cells reached 80% of confluence after 14 days in culture.

The comparison with α-MEM, as one of the classical mesenchymal medium accepted worldwide, was performed to verify the mesenchymal characteristics and differentiation potential of ASCs isolated and cultured in SCM. Our ASC cultures showed a good proliferation ability, in both media conditions, and nearly a 90% of viability after cryopreservation procedures. Moreover, the proper expression of phenotypical markers and the karyotype analyses both confirmed the mesenchymal characteristics of healthy ASCs, in terms of surface markers and the absence of cytogenetic alterations. This was observed also at higher passages of culture in both media condition (Table 2). These data support our isolation method as reproducible and rapid, in terms of cell viability and yield, and safety. Moreover, we can confirm that our ASCs, cultured in either SCM or α-MEM, do express the mesenchymal phenotype required by the International Society for Cellular Therapy (ISCT)[7,14] and can differentiate in adipocytes, osteocytes and chondrocytes, completing cell identification and potency assessment required for mesenchymal cells definition[14] (Table 2). Considering the age variability of the selected 10 donors we were not able to find any differences in terms of both yield and phenotypic features of isolated cells.

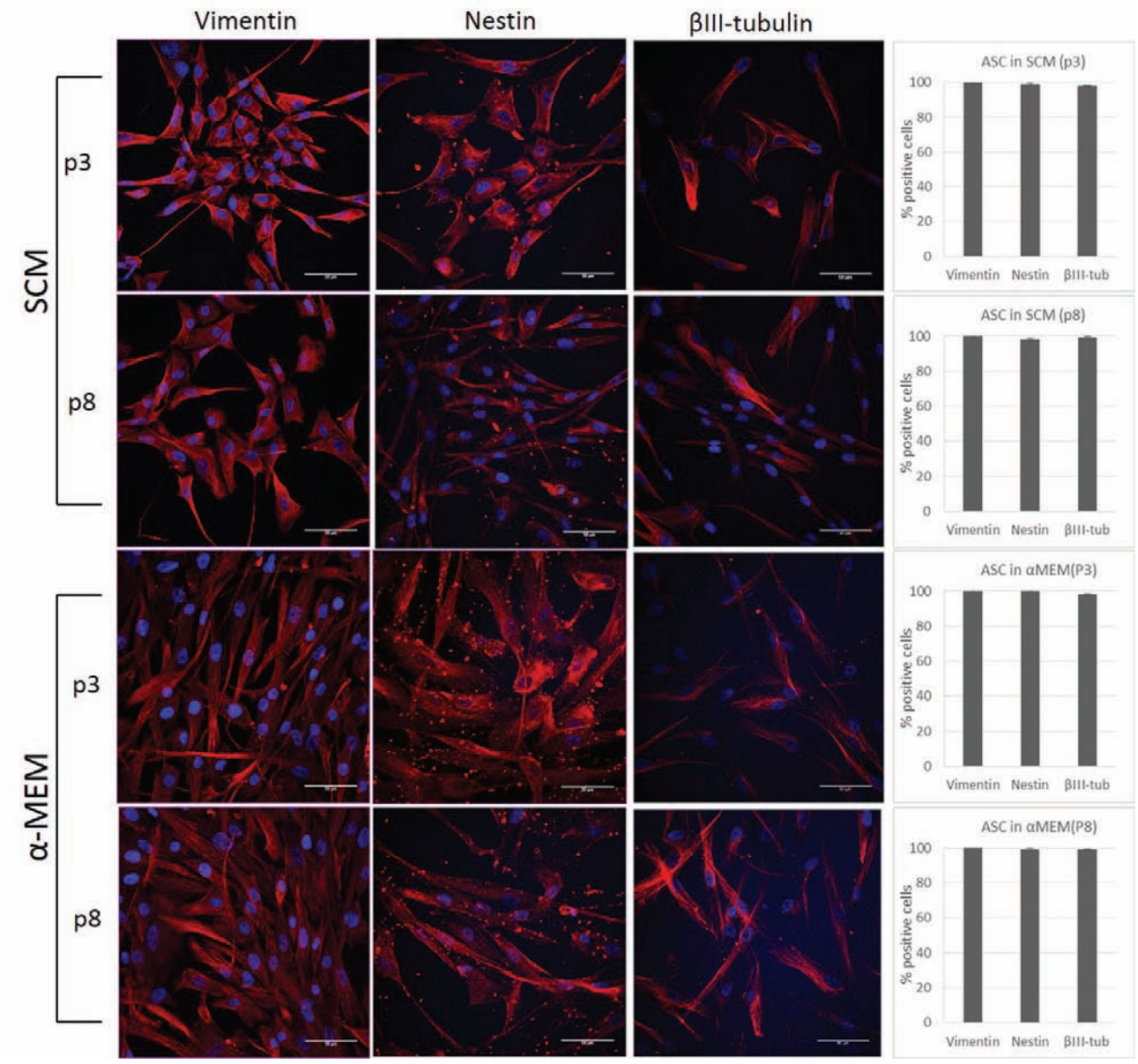

Figure 8. Markers expression. ASCs expression of Vimentin, Nestin, βIII-tubulin was investigated with immunofluorescence analysis. Cells at both early (passage 3) and high (passage 8) passages were maintained in SCM or α-MEM medium and stained for mesenchymal and stemness markers expression. Pictures are representative of five independent experiments with similar results. Quantifications of positive cells were performed by counting at least 5 fields. Results report the number of positive cells divided by the total number of cells (nuclei) and are reported in the graphs as percentage. Data are expressed as the mean of five independent experiments ± SD.

In order to analyze pluripotency of our cultures, we evaluated three pluripotent cell-specific factors expression, Sox2, Oct4 and Nanog in both SCM and α-MEM conditions. Among these, Sox2 and Oct4 are known as essential transcription factors for mesenchymal self-renewing phenotypes[20] and they are described as usually being expressed at low levels in early passages, then decreasing at higher passages[21-23]. Our results confirm this tendency, as we found the mRNA of all three transcription factors in our cultures, although mRNA levels at higher culture passages were lower, in either medium conditions (Table 2). Moreover, their altered expression, found between ASCs cultured with the two different media, could be explained by the presence of growth factors in the SCM, such as bFGF, which is in accordance with what already reported by other authors[23].

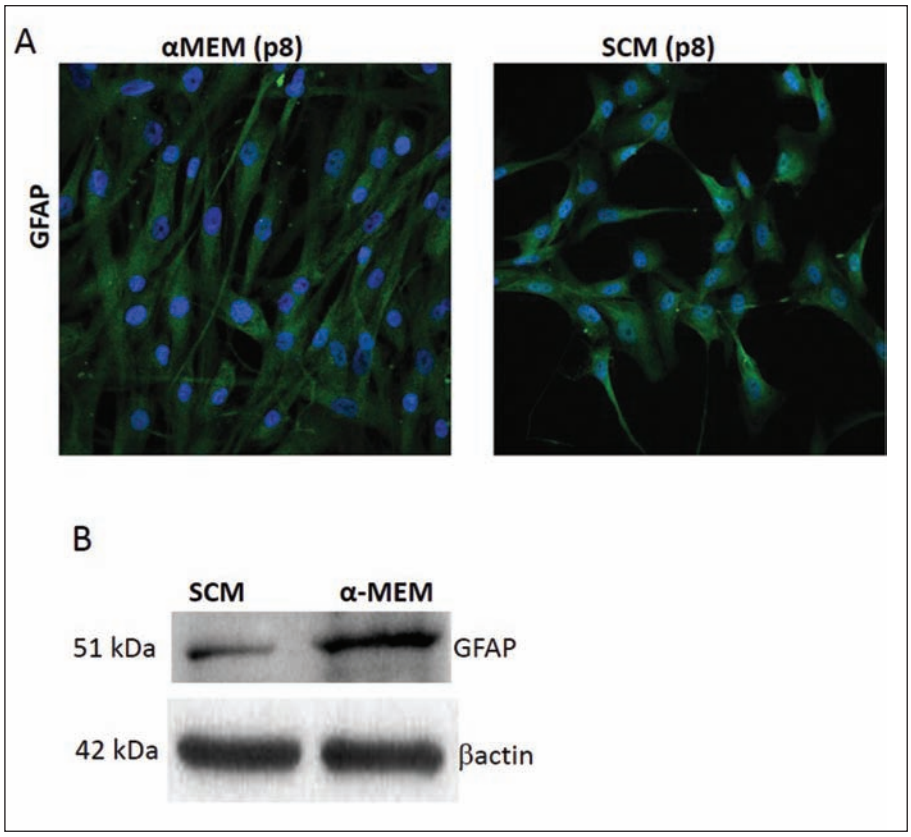

Figure 9. Evaluation of GFAP expression at passage 8**.** *A,* GFAP immunofluorescence staining in ASC cultures maintained in SCM and α-MEM. *B,* Western blotting of GFAP in protein extracts of ASCs maintained in SCM and α-MEM. Results are representative of two different experiments with similar results.

Regarding the expression of neuronal markers, it has been shown that MSCs from different origin can express different ones, such as Nestin, βIII-tubulin and GFAP, already in their undifferentiated state[24,25]. Neurodegenerative disease and other neurological disorders, for which many clinical trials have already been undertaken with promising evidence[2], may also be their target, due to this phenotypical peculiarity. The expression of Nestin, βIII-tubulin and GFAP, at low and high passages, independently of the medium used, support the suggestion of the neuronal differentiation predisposition of ASCs, derived from biopsies, described also by other authors[25].

Table 2. Summary of key differences between ASCs maintained in α-MEM and SCM .

	a-MEM	SCM
% FBS	10%	10%
Morphology	Fibroblastic-like, but larger cytoplasm	Fibroblastic-like
Days to reach confluence	6 days	4 days (p<0.05 vs α-MEM)
Cum Pop Doubling (p8)	7.04 ± 1.60	13.67 ± 2.31 (p<0.05 vs α-MEM)
% Viability after freezing	88.3 ± 10.48%	86.58 ± 9.89%
Mesenchymal markers (p3)	$CD44^+/CD73^+/CD90^+/CD105^+/CD144^+/CD166^+$ $CD19^{low}/CD31^{low}/CD34^{low}/CD45^{low}/HLADR^{low}$	$CD44^+/CD73^+/CD90^+/CD105^+/CD144^+/CD166^+$ $CD19^{low}/CD31^{low}/CD34^{low}/CD45^{low}/HLADR^{low}$
Mesenchymal markers (p8)	$CD44^+/CD73^+/CD90^+/CD105^+/CD144^+/CD166^+$ $CD19^{low}/CD31^{low}/CD34^{low}/CD45^{low}/HLADR^{low}$	$CD44^+/CD73^+/CD90^+/CD105^+/CD144^+/CD166^+$ $CD19^{low}/CD31^{low}/CD34^{low}/CD45^{low}/HLADR^{low}$
Karyotype	No aberration	No aberration
Self-renewal gene express (p3)	Higher	Higher (p<0.001 vs α-MEM)
Self-renewal gene express (p8)	Lower (p<0.001 vs p3)	Lower (p<0.001 vs p3)
Neural markers (p3)	$Nestin^+/ßTubIII^+/GFAP^{low}$	$Nestin^+/ßTubIII^+/GFAP^{low}$
Neural markers (p8)	$Nestin^+/ßTubIII^+/GFAP^{low}$	$Nestin^+/ßTubIII^+/GFAP^{low}$

Legend: (+) means >90% of positivity; (low) means < 25% of positivity.

In conclusion, we propose a novel, easy, rapid and reproducible isolation method of human adipose-derived stem cells, from periumbilical biopsies, which avoid the enzymatic digestion step. Using our stem cell medium we obtained a good yield of starting cells and we did not found any alteration of mesenchymal features, or aberrant chromosome alteration, even at higher passages. By using the same isolation protocol, with α-MEM medium, the amount of cells obtained was very low and insufficient to perform any required characterization step. Indeed, with the SCM isolation method, after passage 1 ASCs can also be cultured in other commercial medium, such as the aforementioned α-MEM, due to the good yield of cells obtained with SCM isolation. This will avoid the risk relative to the use of a growth factor enriched medium, such as SCM, even though we demonstrated that in our culturing conditions cells maintained in SCM remain very stable and still express mesenchymal features. As a matter of fact, our results demonstrate that cells shifted in α-MEM after passage 1 retain comparable mesenchymal phenotype and characteristics of the cells cultured in SCM. Moreover, in both conditions, ASC cultures express neuronal markers, identifying them as good candidates for cell therapy of neurodegenerative disorders. Furthermore, the lack of enzymatic digestion could represent great advantage for future pre-clinical optimization of ASCs isolation protocols in Good Manufacturing Practice conditions[7,14].

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Author Disclosure Statement

The authors declare no competing financial interest.

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