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Research Article

Human adipose derived stroma/stem cells grow in serum-free medium as floating spheres

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ABSTRACT

With the goal of obtaining clinically safe human adipose-derived stroma/stem cells (ASC) and eliminating the use of serum, we have developed a new culture system that allows the expansion of ASC as spheres in a defined medium. These spheres can be passaged several times. They are not only aggregated cells but rather originate from single cells as clonal spheres can be obtained after seeding at very low density and reform clonal spheres after dissociation. These spheres can also revert to monolayer growth when plated in medium containing human plasma and even generate fibroblast-like colonies (CFU-f). Under several differentiation-specific media, spheres-derived ASC maintain their capacity to differentiate into osteoblasts, endothelial cells and adipocytes. These results indicate that human ASC can be maintained in a serum-free 3D culture system, which is of great interest for the expansion in bioreactors of autologous ASC and their use in clinical trials.

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Introduction

Adipose tissue has long been known as the main energy source of the organism. The discovery of its ability to produce and secrete adipokines such as leptin and adiponectin has revealed its endocrine function and its pivotal role in metabolism regulation and physiological homeostasis [1,2]. More recently, certain cells have been isolated from the stromal vascular fraction of adipose tissue that are capable of multilineage differentiation even at the clonal level [3–10]. These cells are termed ASC for adipose derived stroma/stem cells. Although different, they share numerous features with mesenchymal stem cells isolated from bone marrow (MSC) and umbilical blood cord [11–14] and represent an alternative source of

adult stem cells. Indeed, access to subcutaneous deposit is a routine surgical procedure that can be performed under local anaesthesia with minimum discomfort for the patient; moreover, ASC isolation is a simple enzyme-based protocol, making this tissue a more attractive source of stem cells. ASC isolated from human adipose tissue have the potential to differentiate into bone, cartilage, fat and endothelial cells when cultivated under lineage specific conditions [4,9,15,16]. Engineering of these mesenchymal tissues is of major interest in human diseases such as inherited, traumatic or degenerative tissue defects or after tumour surgery [17]. The critical point related to clinical use is that *in vitro* culture of ASC is still dependent of fetal calf serum or human plasma [8,18]. Plasma and serum contain undefined factors, which vary in composition from

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individual to individual and may activate or inhibit cell proliferation and differentiation. Moreover, their stock depends on donors and each batch must be tested for innocuousness (infectious pathogens such as prions). The above combined with the fact that differential gene expression was revealed between ASC cultures in human or fetal bovine serum [18], highlight the need for a substitutive defined cell medium formulation and independent of interest in a serum free medium, to understand the specific effect of any compound.

Multiple approaches employ sphere cluster technology to study stem cells [19]. In contrast to conventional monolayer cell culture, in which cells grow only in two dimensions on a flat surface of a plastic dish, suspension cultures allow cell growth in all three dimensions. Stem cells have been grown as spheres in numerous studies using different protocols (non-adherent plates, methylcellulose semi-solid media, hanging drops and round-bottom 96-well plates) [19,20]. The most studied of these models are embryoid bodies, defined as spherical clusters of both pluripotent and committed stem cells that can organize in a developmental-specific manner and give rise to mature cells from any differentiation lineage. The primary goal of growing cells as spheres has been to reveal the basic principles of a normal tissue organization that gives cells the opportunity to arrange in a three dimensional context similar to tissues [19,21]. This sphere technology is now largely used in cancer research to isolate cancer initiating cells, as tumour spheres are very similar to solid tumours *in situ* [22]. They are also used as molecular assay systems in toxicology and pharmacology [23]. Additionally, embryonic stem cells can be driven to generate insulin-producing cell clusters, that undergo rapid vascularization when injected into diabetic mice and display an organization and functionality similar to pancreatic islets [24]. This has led us to investigate a 3D culture system that enables maintenance of ASC in serum-free conditions.

Materials and methods

Adipose tissue cell isolation

Subcutaneous adipose tissue was obtained from patients undergoing elective abdominal dermolipectomy. No objection certificates were obtained according to bioethics law no. 2004-800 of August 6, 2004. Cells were isolated as previously described [25]. Briefly, adipose tissue (AT) was digested in DMEM-F12 medium (Invitrogen, Carlsbad, USA) supplemented with 2 mg/ml collagenase A (Roche Diagnostic, Indianapolis, IN) and bovine serum albumin (BSA) (2%) for 45 min at 37 °C under agitation. The suspension was filtered through 100 µm and 25 µm nylon membrane and centrifuged at 600 g for 10 min to separate floating mature adipocytes from the stromal-vascular fraction (SVF). SVF was incubated in erythrocyte lysis buffer (ammonium chloride solution, StemCell Technologies, Vancouver, Canada) for 5 min at 4 °C and washed in PBS (phosphate-buffered saline, Sigma, St Quentin Fallavier, France). SVF cells were counted and either cultured *in vitro* or used for flow cytometry analysis.

Cell culture

For ASC monolayer culture, SVF cells were seeded at 4000 cell/cm² in flasks treated for cell culture (TPP, Dominique Dutscher, Brumath, France) in ASC expansion medium which consisted of

alpha-MEM (Invitrogen) supplemented with 2% human plasma enriched with human platelet growth factors (PGFEP), heparin Choay (1 U/ml, Sigma), amphotericin (0.25 µg/ml), streptomycin (100 µg/ml) and penicillin (100 U/ml) (Invitrogen). Cells were incubated at 37 °C under 5% CO₂ and the medium was changed twice a week. For sphere culture, SVF cells were seeded at 60,000 cell/cm² in ultra low adherent flasks (Corning, Avon, France) in defined culture medium which consisted of GMEM supplemented with L-glutamine (2 mM), non essential amino acids (1×), B27 (1×) (Invitrogen), glucose (0.6%, Sigma), human bFGF (10 ng/ml), human EGF (20 ng/ml), human thrombin (1 U/ml) (PeproTech, Rocky Hill, NJ) and ciprofloxacin (2 µg/ml, Bayer, France). Cells were incubated at 37 °C under 5% CO₂ and half of the medium was changed once a week.

To assess the presence of proliferating cells in defined culture medium, BrdU (10 µM) was added for 3 days on the third day after seeding. To test if sphere containing cells can revert to monolayer growth, spheres were dissociated with trypsin-EDTA (0.05%, Invitrogen) and plated in flasks treated for cell culture (TPP) in ASC expansion medium.

Flow cytometry

Dissociated spheres were incubated with PBS (phosphate-buffered saline) supplemented with 0.5% new calf serum and FcR Block reagent (StemCell Technologies, Vancouver). Sextuplet stainings were performed by incubating cells for 30 min at 4 °C with the following conjugated primary antibodies or appropriate IgG isotype controls: CD45-APC-Cy7, CD90-FITC, CD73-PE, CD34-PerCP, CD44-FITC, CD29-APC, CMH1-APC, CD117-PE-Cy7, CD15-FITC, CD56-PE, CXCR4-PE, Lin-FITC, CD38-PE-Cy7, CMH2-FITC, CD3-PE-Cy7, CD11b-APC, CD19-PerCP, CD14-PE-Cy7 (BD Biosciences, Le Pont de Claix, France), CD105-APC, CD146-FITC, CD31-APC (eBiosciences, Paris, France), VEGFR2-PE (R&D Systems, Lille, France), and CD133-APC (Miltenyi, Paris, France). Cells were analyzed on a FACS Canto II (BD Biosciences). Data acquisition and analysis were performed with FACS Diva software (BD Biosciences). Only viable cells that excluded the LIVE/DEAD dye (Molecular Probes, Interchim, Montluçon, France) were considered.

Cell differentiation

Spheres cultured for 7 days were dissociated with trypsin/EDTA (0.05%, Invitrogen) and seeded on gelatin-coated plates (0.1% in PBS, Sigma) for differentiation. The day of plating was considered as day 0. Differentiation was assessed after 24 h (noted as day 1) and after 10 days or 21 days depending on differentiation lineage.

Adipogenic differentiation

On day 0, cells from dissociated spheres were plated at 65,800 cell/cm² in 12-well tissue-culture plates (Falcon, Dominique Dutscher, Brumath, France) and cultured for 3 days in adipogenic differentiation medium which consisted in ASC expansion medium supplemented with dexamethasone (1 µM), IBMX (450 µM), and indomethacin (60 µM) (Sigma). Subsequently, IBMX was removed from the medium and differentiation was extended during 19 days. The medium was changed every 3 days. The extent of differentiation was noted by observation of multilocular refringent droplets in the induced cells and by staining of neutral lipids by Oil red-O as previously described [26]. Cellular triglyceride

(TG) content was measured with a commercial test (Triglycerides Enzymatique PAP 150, Biomerieux). The protein content was determined using the DC Protein Assay Kit (BioRad, Marne la Coquette, France). Differentiation quantification was evaluated by calculating the ratio of TG content per μg of protein.

Osteogenic differentiation

On day 0, cells from dissociated spheres were plated at 15,200 cell/cm² in 12-well tissue-culture plates (Falcon) and cultured for 21 days in osteogenic differentiation medium which consisted in ASC expansion medium supplemented with dexamethasone (0.1 μM), ascorbic acid (250 μM), and NaH₂PO₄ (3 mM). The medium was changed every 4 days. Mineralization was revealed by staining calcium-rich deposits with Alizarin red [6]. Alizarin red quantity (μg) was assessed at day 1 and day 21 by addition of 10% acetic acid to the stained culture dishes and measurement of the optical density at 405 nm with a spectrophotometer.

Angiogenic differentiation

On day 0, cells from dissociated spheres were plated at 100,000 cell/cm² in 48-well tissue-culture plates (Falcon) and cultured for ten days in angiogenic differentiation medium which consisted in ASC expansion medium supplemented with VEGF (10 ng/ml, Sigma). The medium was replaced every 3 days. Angiogenic differentiation was assessed by CD31 immunostaining. CD31-positive extension lengths were measured using the Elements AR 3.0 image analyzer software (Nikon, Champigny sur Marne, France).

Colony forming unit-fibroblast assay

To evaluate the frequency of mesenchymal-like progenitors among cells growing in defined medium, spheres were dissociated and cells seeded in 25 cm² flasks (TPP) at a final concentration of 8 cells/cm² in ASC expansion medium as previously described. The medium was renewed every 2 or 3 days. After 12 days, cells were washed with PBS and fixed with methanol for 15 min. The colony forming unit-fibroblasts (CFU-f) were stained with Giemsa (6%) for 30 min and scored under an optical microscope. Colonies were considered as clusters of more than 50 cells.

Immunostaining

Adherent cells were fixed for 15 min with 4% paraformaldehyde. To avoid non specific binding, cells were first incubated for 1 h in PBS and BSA (2%), then for 2 h with the mouse anti human CD31 antibody (1/10, Dako, Trappes, France) or mouse IgG1 control isotype (Dako). After washing, the secondary antibody goat anti-mouse conjugated to Alexa 488 was added for 1 h (1/200, Invitrogen). Nuclei were stained with DAPI (1/10,000). Cell staining was observed with a fluorescence microscope (DMRB Leica, Gennevilliers, France) and analyzed with the image analyzer software.

For BrdU detection, whole spheres and freshly dissociated spheres were plated by centrifugation (10 min, 1000 rpm) on poly-L-ornithine-coated coverslips before fixation. They were treated with 2 N HCl for 30 min, washed with 0.1 M borate buffer, and incubated in TBS (Tris buffer saline) triton (0.1%) and goat serum (5%) for 1 h and stained with the mouse anti human BrdU antibody conjugated to FITC (1/20, e-biosciences) or mouse IgG1 control isotype; nuclei were counterstained with Hoechst 33242 (5 $\mu\text{g}/\text{ml}$, Sigma).

For paraffin sections, spheres were successively fixed with paraformaldehyde (4%, 1 h), ethanol (70% 15 min–95% 15 min–100% 3 × 1 h), toluene substitute (Microclearing, 1 h–30 min) and embedded in paraffin. Sections of 4 μm thickness were deparaffined and stained with hematoxylin/eosin solution.

Real time PCR

Cell total RNA was isolated using RNeasy minikit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations. RNA (1 μg) was reverse-transcribed using the High-Capacity cDNA Reverse Transcription kit. All amplification reactions were performed in duplicate from 25 ng cDNA in 50 μl Power SYBR® Green PCR Master Mix containing 0.2 μM of each specific primers (for control, Platinum® Taq DNA polymerase was omitted):

hPUM1 Fp: GCCATGTTGTCGGAGTGAAA/Rp: ACACATGCAACGCT-CATTC,
 hOCT4 Fp: CCCCTGGTGCCGTGAAG/Rp: GCAAATTGCTC-GAGTTCITTTCTG,
 hNANOG Fp: AGAACTCTCCAACATCCTGAACCT/Rp: ATTCTTCGGC-CAGTTGTTTTTC.
 hPPAR γ Fp: GATACACTGTCTGCAAACATATCAC/RP: CCACGGAGCT-GATCCCAA
 hLPL Fp: GGTGAAGCATTGGAATCCAG/RP: TAGGGCATCTGA-GAACGAGTC
 hRUNX 2 Fp: ATTCTGTAGATCCGAGCACC/Rp: GCTCACGTCGCT-CATTTTTGC
 hOsteoprotegerin Fp: AGCACCTGTAGAAAACACAC/Rp: ACAC-TAAGCCAGTTAGGCGTAA.

Amplification and detection were performed with the ABI PRISM 7500 Sequence Detection System as follows: initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. To evaluate the specificity of the amplifications a dissociation curve was analyzed (95 °C (15 s), 60 °C (20 s), and 95 °C (15 s)). All PCR amplifications were analyzed in duplicates and included positive and negative (H₂O) control samples. Relative quantification of specific genes mRNA levels was determined using the 2^{- $\Delta\Delta\text{CT}$} method. Results were analyzed with the GeneAmp 7500 software, and genes mRNA expression level was first normalized by PUM taken as reference gene and then to a calibrator consisting of human ASC

Table 1 – Serum-free culture conditions tested for sphere formation. Serum free supplements B27 and N2 as well as glucose and the mitotic cell toxin ARA-C were tested in several combinations with the following basic medium: GMEM medium supplemented with L-glutamine, non essential amino acids, human bFGF, human EGF and human thrombin. All these media formulations were tested on cells plated in culture dishes treated for adherent cell culture or in ultra low adherent supports.

	A	B	C	D	I	J	K	L
B27 (1×)	+	+	+	+	–	–	–	–
N2 (1×)	–	–	–	–	+	+	+	+
Glucose (0.6%)	–	+	–	+	–	+	–	+
ARAC (5–100 μM)	–	–	+	+	–	–	+	+

control. All PCR products and analyzer were purchased from Applied Biosystems, Villebon, France.

Data analysis

Results were expressed as means \pm SEM. Data from at least three independent preparations of ASC grown as spheroids or as a monolayer were statistically processed with the Prism 4 software (GraphPad, San Diego, CA). Significance was defined as * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Results

Serum-free culture conditions allowing for sphere formation

We have evaluated the capacity of several enzymatic digestion conditions, medium formulations and culture dishes to support the growth of human ASC as spheres without the use of serum. Two collagenases were tested for digestion of adipose tissue: collagenase A type II or VIII, during 45, 60 or 90 min. These

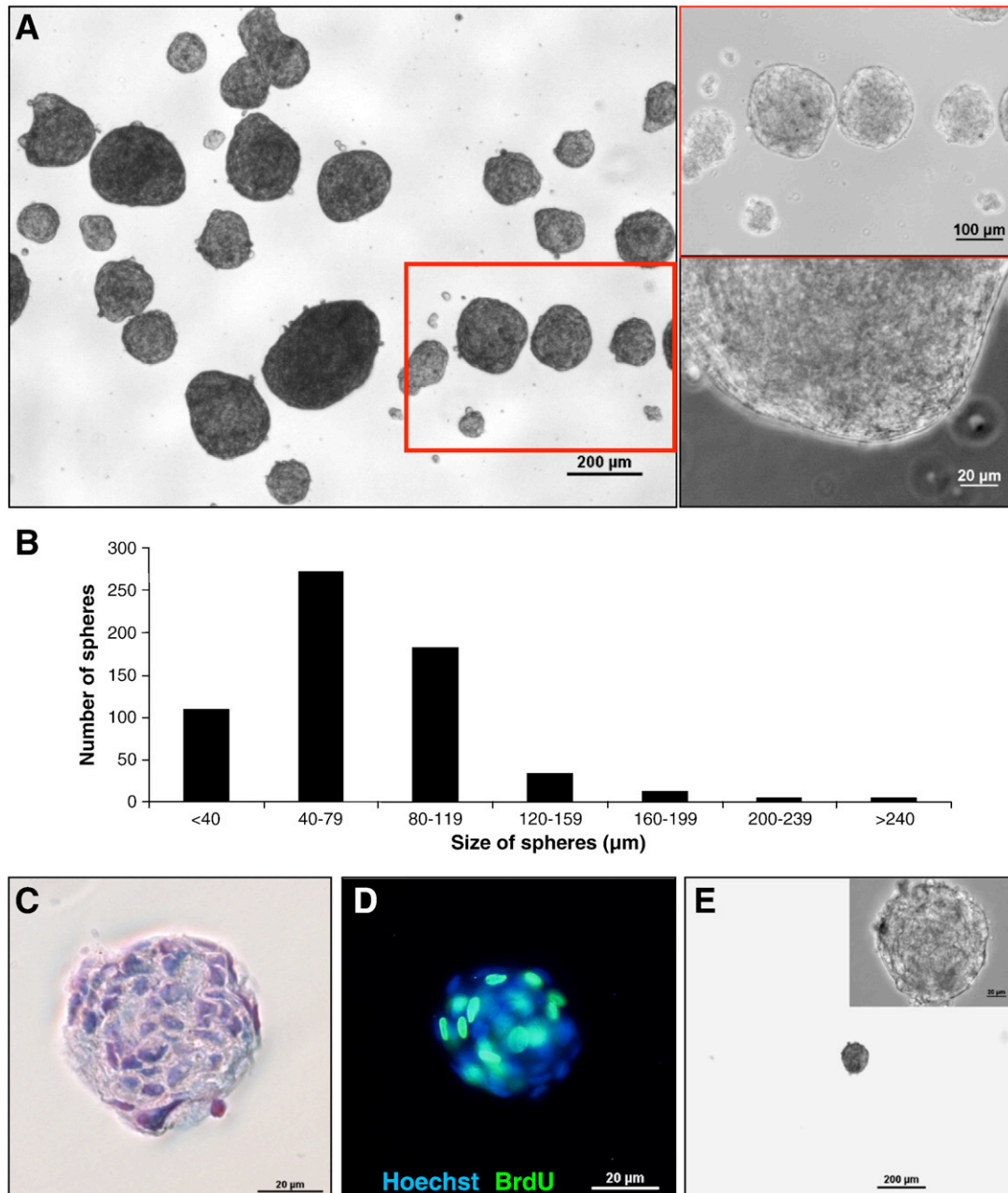


Fig. 1 – Characterization of spheres. (A) Morphology of spheres 7 days after seeding at 190 cells/ μ l (scale bars 200 μ m, 100 μ m and 20 μ m). (B) Spheres display heterogeneous sizes after 7 days of culture. (C) Hematoxylin and eosin staining on 4 μ m-thick paraffin sections of spheres (scale bar 20 μ m). (D) BrdU immunodetection (green) on spheres grown for 7 days in presence of BrdU during 3 days. Nuclei are stained with Hoechst (blue) (scale bar 20 μ m). (E) Clonal spheres obtained after 20 days of culture from primary sphere cells dissociated and seeded at 0.5 cell/ μ l (scale bars 200 μ m and 20 μ m).

conditions were associated with several products combinations listed in Table 1, in culture dishes treated for adherent cell culture or in ultra low adherent supports and with the following medium formulation: GMEM medium supplemented with L-glutamine (2 mM), non essential amino acids (1×), human bFGF (10 ng/ml),

human EGF (20 ng/ml), human thrombin (1 U/ml) and ciprofloxacin (2 μg/ml).

Only collagenase A type II allowed the formation of regular spheres and 45 min of digestion appeared to be sufficient. Using this collagenase, clonal spheres were only obtained in conditions

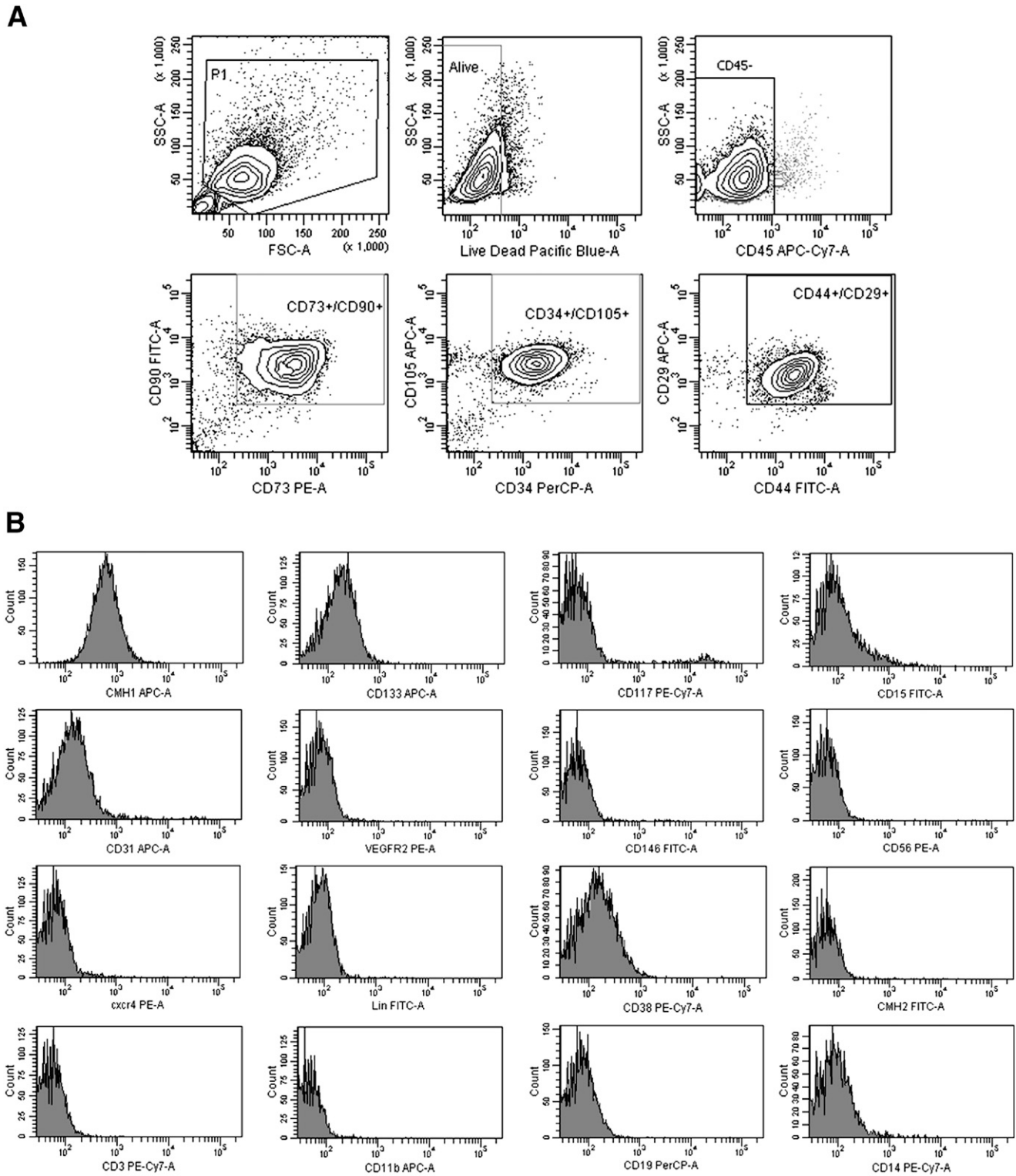


Fig. 2 – Flow cytometry analysis of cells composing the spheres. The vast majority of cells composing the spheres are CD34⁺ and display a mesenchymal phenotype CD105⁺, CD90⁺, CD73⁺, and CD45⁻. They are CD29⁺, CD44⁺, and CMH1⁺ but CD133⁻, CD117⁻, CD15⁻, CD31⁻, VEGFR2⁻, CD146⁻, CD56⁻, CXCR4⁻, Lin⁻, CD38⁻, CMH2⁻, CD3⁻, CD11b⁻, CD19⁻, and CD14⁻.

A, B and J and among them, sphere structure appeared to be most regular under condition B. In flasks treated for cell culture, some cells adhered to the bottom of the flask while spheres formed in the supernatant. In ultra low adherent flasks, no cell adhered to the bottom and only spheres were obtained in the supernatant. Thus, in order to better evaluate the potential of the cells that composed the spheres, subsequent studies were performed using collagenase A type II for 45 min for tissue digestion, and culture in medium formulation B in ultra low adherent flasks. These optimum conditions allowing sphere formation in defined medium are gathered in the [Materials and methods](#) section.

Characterization of spheres

Spheres were round with defined borders and varied in size 7 days after their first emergence (Figs. 1A and B). As shown in Fig. 1B, most spheres (80%) were small in diameter ($<100\ \mu\text{m}$), 18% were medium ($100\text{--}200\ \mu\text{m}$) and the remaining spheres were large ($>200\ \mu\text{m}$).

To determine their structure, spheres were sectioned and stained with hematoxylin and eosin. The cells that composed the spheres appeared homogeneous in size and morphology. No cavity was observed (Fig. 1C). Secretion of extracellular matrix proteins was evaluated on spheres sections by immunocytochemistry or dye staining. Neither collagen, fibronectin, hyaluronic acid nor elastic fibers were detected (data not shown).

Evidence of a proliferation rate was observed as few cells were KI67 positive (data not shown) and 3 days after addition of BrdU in the medium, around 15% of the cells that composed the spheres appeared to have incorporated BrdU (Fig. 1D). A very

limited amount of dead cells was assessed by TUNEL assay (data not shown). Spheres could be passaged at least three times, as enzymatically dissociated spheres could reform new spheres at clonal densities as low as 0.5 cells per μl (Fig. 1E). These data suggest that a sphere can be formed at clonal level through proliferation. However, a very small amount of cells can be considered as sphere initiating cells, as upon dissociation, only 0.8% of the cells that compose a sphere could form secondary spheres.

FACS analysis revealed that, similar to ASC cultured directly from SVF in ASC expansion medium, most of the cells that composed the spheres expressed simultaneously CD34 and classical mesenchymal cell-associated markers such as CD105, CD90, and CD73, and were negative for CD45 (Fig. 2A). Evaluation of the expression of other markers revealed that the majority of cells contained in the spheres expressed CD29, CD44, and CMH1 but not CD133, CD117, CD15, CD31, VEGFR2, CD146, CD56, CXCR4, Lin, CD38, CMH2, CD3, CD11b, CD19 nor CD14 (Fig. 2B). No difference in marker expression profiles was observed between primary and secondary spheres (spheres formed initially and passaged once respectively) except for CD34 positive cells which represent 80% (± 10) and 43% (± 11) of the cells in primary and secondary spheres respectively.

After enzymatic dissociation and culture in ASC expansion medium containing fetal calf serum, spheres could revert to monolayers in flasks treated for adherent cell culture. They re-adhered and morphologically resembled primary cultured ASC obtained from SVF plating and continued to proliferate.

Taken together, these results suggest that ASC can grow in serum-free medium in suspension as clonal spheres, maintain their mesenchymal phenotype and revert to monolayer growth.

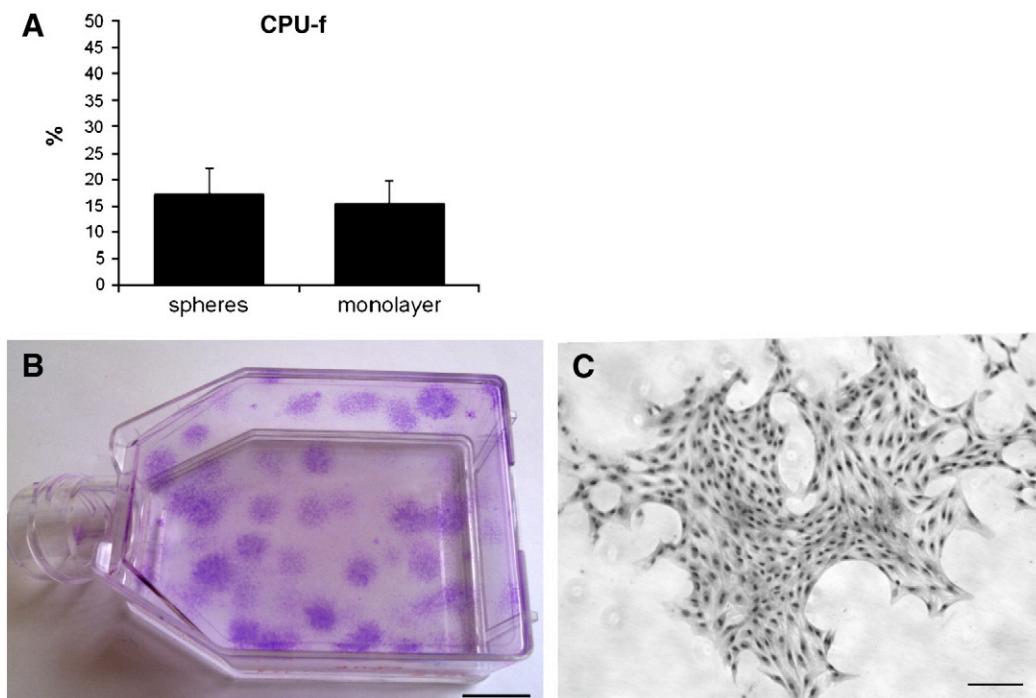


Fig. 3 – Colony forming unit fibroblast assay (CFU-f). Spheres were dissociated and plated at 8 cells per cm^2 for 12 days in ADSC expansion medium. (A) Sphere-derived CFU-f score is similar to ADSC-derived CFU-F. (B) CFU-f stained with Giemsa. (C) High magnification of a colony stained with Giemsa. Scale bars 1 cm and 200 μm .

ASC maintain their multipotentiality when cultured as spheres in defined medium

In order to determine whether cells that composed the spheres expressed pluripotency-associated markers, the expression of Oct4 and Nanog was evaluated by RT-PCR. Both markers were expressed but no significant difference was observed compared to

the expression of these markers in ASC cultured directly from SVF in ASC expansion medium (data not shown).

The progenitor content of spheres was evaluated by the CFU-f test. This revealed that the proportion of true progenitors contained in spheres ($17 \pm 5\%$) was similar to that in ASC cell population cultured under conventional conditions ($15 \pm 4\%$) (Fig. 3).

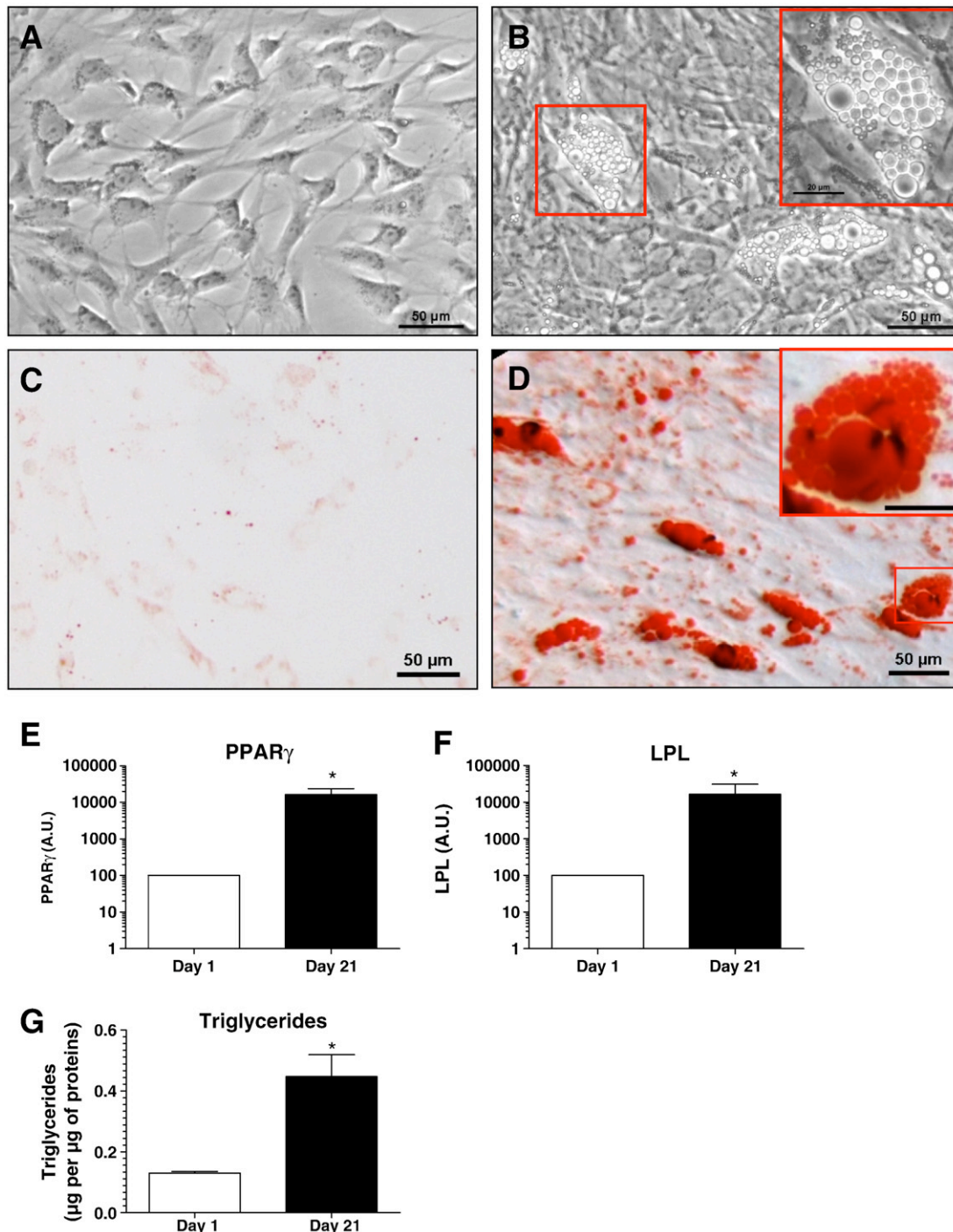


Fig. 4 – Adipogenic differentiation. SVF-derived cells were cultured for 7 days as spheres in defined medium before adipogenic differentiation. After 21 days in differentiation medium, cells display numerous lipidic droplets (B) stained by Oil red-O (D) which were rare at day 1 (A and C) (scale bars 50 μm and 20 μm). (E) PPAR γ gene expression is upregulated after 21 days of differentiation. (F) LPL gene expression is upregulated after 21 days of differentiation. (G) Triglyceride content is significantly increased on 21 days relative to day 1 of differentiation. * $p \leq 0.05$.

To confirm that the differentiation capacity of sphere-derived ASC was maintained 7 days, spheres were dissociated and incubated in lineage specific differentiation media. Early differentiation (noted day 1) was assessed 24 h after plating, whereas late differentiation was assessed 10 days after plating for angiogenic differentiation or 21 days after plating for adipogenic and osteogenic differentiation. Following 21 days in adipogenic differentiation medium, sphere-derived ASC displayed intracellular accumulation of lipid droplets detected by Oil red-O staining (Figs. 4A–D). The peroxisome proliferator-activated receptor gamma (PPAR γ) and the lipoprotein lipase (LPL) genes, which play a key role in lipid metabolism, were upregulated (Figs. 4E and F) and the triglyceride content was 3 fold higher after 21 days of adipogenic differentiation relative to day 1 (Fig. 4G). It is noteworthy that lipid accumulation is an early and spontaneous event as sphere-derived ASC displayed some Oil red-O positive droplets as soon as day 1 in differentiating medium. Sphere-derived ASC cultured for 10 days under angiogenic conditions showed a typical endothelial-like organization in culture as they expressed CD31 (PECAM-1) and formed highly branched networks (Figs. 5A and B). Process length of cells differentiated for 10 days were $625 \pm 22 \mu\text{m}$ whereas they were only $109 \pm 6 \mu\text{m}$ at day 1 (Fig. 5C). When induced towards osteogenic lineage, dissociated spheres were capable of forming mineralized nodules visualized by Von Kossa (Figs. 6A and B) and Alizarin red staining (Figs. 6C and D). The Runx-related transcription factor 2 (Runx 2) and osteoprotegerin genes, which play a central role in bone metabolism, were upregulated (Figs. 6E and F) and Alizarin red accumulation was highly increased

after 21 days of differentiation relative to day 1 (Fig. 6G). Thus, our results show that sphere-derived ASC display *in vitro* multipotency.

Discussion

Our data provide the first evidence that mesenchymal cells derived from human adipose tissue can be grown in serum-free medium in suspension as floating spheres through several passages and maintain their multilineage differentiation capacities.

Similarly, it has been reported that mesenchymal cells derived from other tissues like umbilical cord [27] or skeletal muscles [28] can grow in serum free medium as spheres and maintain multipotency. Studies that aimed to use serum-free media to culture mesenchymal cells from adipose tissue were all restricted to monolayer cultures [29–31] and the ones that derived aggregates from ASC all required a first step of monolayer culture in 10% fetal bovine serum containing media [32–34]. In the present work, we plated cells from freshly dissociated tissue directly in non-adherent conditions without any preliminary monolayer culture and only in serum-free media. Our culture process induced the loss of the vast majority of the harvested primary SVF cells, while some of the growth factor-responsive cells survived, divided, and generated spheres. The amount of cells initiating the spheres was reflected by the number of secondary spheres formed after the dissociation of primary spheres and reseeded at clonal density. It represented 0.8%, revealing that only rare cells owned the property to develop as spheres. In comparison, 17% of

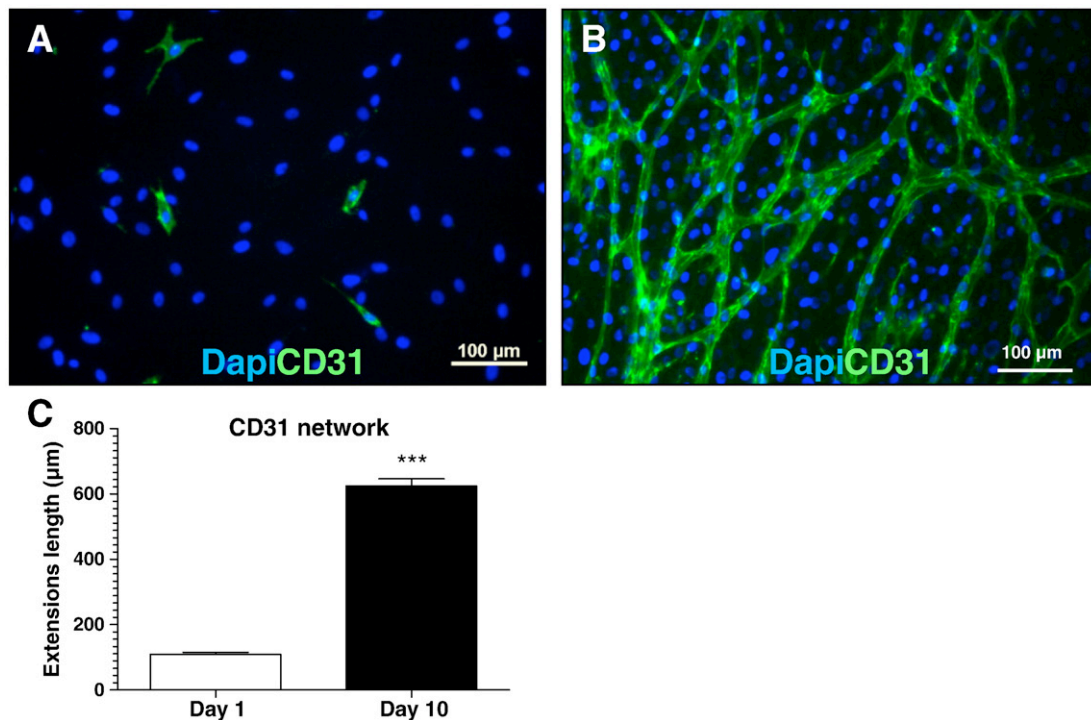


Fig. 5 – Angiogenic differentiation. SVF-derived cells were cultured for 7 days as spheres in defined medium before angiogenic differentiation. (A–B) After 10 days of differentiation, cells expressed CD31 (green) and formed highly branched networks. Nuclei were stained with DAPI (blue). Scale bars 50 μm . (C) Process length (μm). *** $p \leq 0.0001$.

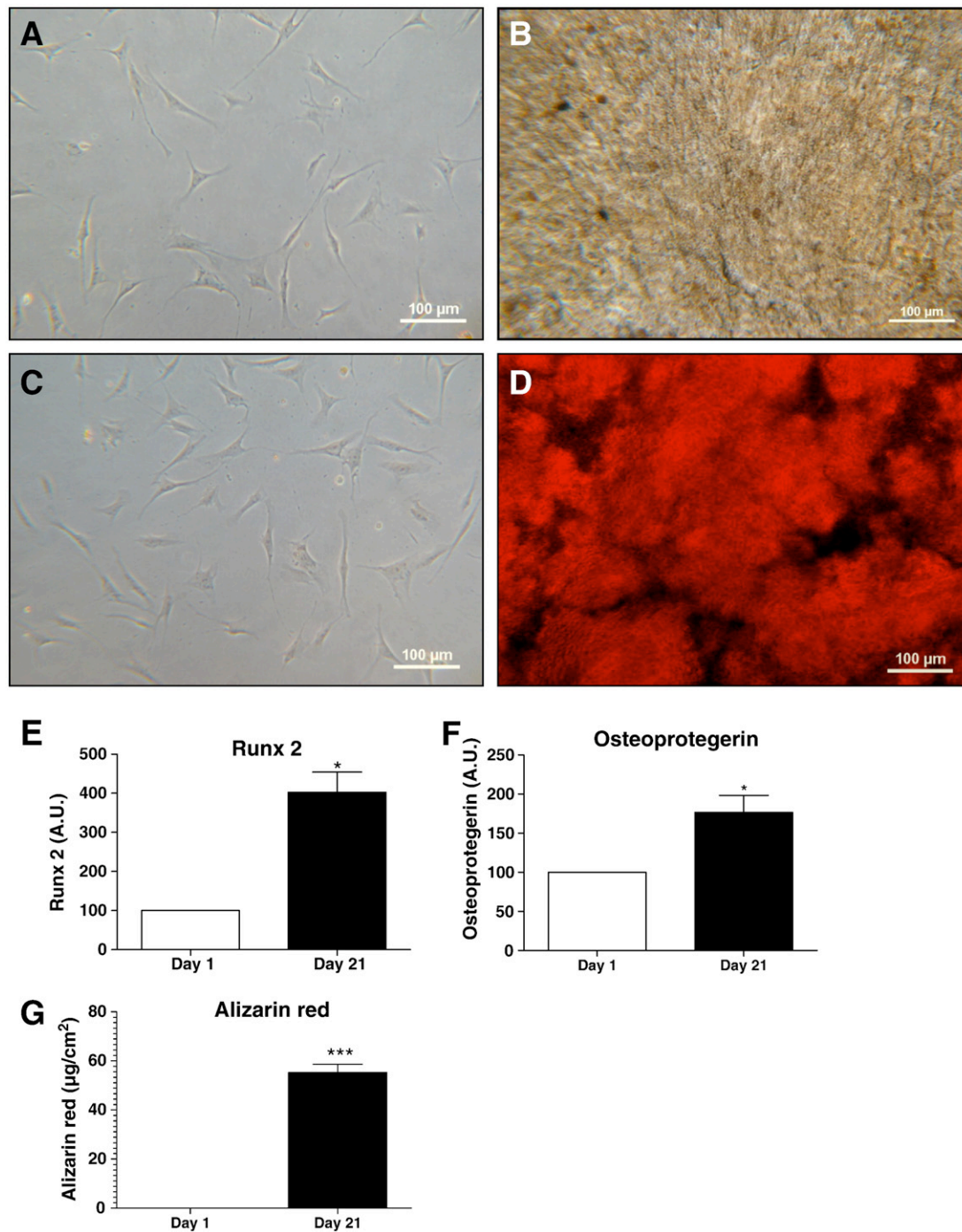


Fig. 6 – Osteogenic differentiation. SVF-derived cells were cultured for 7 days as spheres in defined medium before osteogenic differentiation. After 21 days, differentiated cells formed calcic deposits. (A–B) Von Kossa staining. (C–D) Alizarin red staining (scale bars 50 µm). (E) Runx2 gene expression is upregulated after 21 days of differentiation. (F) Osteoprotegerin gene expression is upregulated after 21 days of differentiation. (G) Quantification of Alizarin red staining (µg/cm²). * $p \leq 0.05$ and *** $p \leq 0.0001$.

cells obtained from dissociated spheres could form CFU-f, suggesting that cells forming CFU-f are not necessarily able to initiate a new sphere or at least have distinct requirements in culture. Therefore, cells initiating spheres could be considered as a non-adherent stem cell population. Indeed, they behold capacities to proliferate in non-adherent condition, to renew medium size spheres (100 µm diameter) at clonal level, to maintain *in vitro*

through several passages (i.e. to be dissociated and re-plated to form secondary then tertiary spheres) and to differentiate into the three primary mesenchymal phenotypes – adipocytes, osteoblasts and endothelial cells, demonstrating the key *in vitro* properties of a progenitor/stem cell – self-renewal at clonal level in culture and multilineage differentiation. In the same way, Westerman et al. who derived myspheres from skeletal muscles in serum-free

media, demonstrated the pre-myogenic nature of the mysphere initiating cells [28]. However, they were not able to generate clonal myspheres. To demonstrate the clonal nature of spheres, individual cells can be plated in single well of 96-well plates. However, their propagation under these conditions is difficult because it prevents paracrine communications between the cells. For these reasons we have chosen to derive clonal spheres through plating at very low cell density (0.5 cell/ μ l). Indeed, Coles-Takabe et al. demonstrated the clonal nature of spheres through mixing experiments, in which cells were labeled with two different markers (yellow and red fluorescent proteins) and then mixed together [35]. These experiments showed that from plating density of 1 cell/ μ l, almost all the spheres generated after mixing contained only one of the two fluorescent labels. Moreover, we left the cells undisturbed during sphere formation to prevent any fluid motion that could increase the probability of aggregation and thus chimeric sphere formation.

In addition, the sphere culture of ASC may enhance their biological activity as the administration of human ASC increased the rate of diabetic wound healing when ASC were derived from aggregates, but not when delivered ASC derived from monolayer culture [34]. Indeed, in this study, ASC formulated as three-dimensional aggregates by the hanging droplet method produced significantly more extracellular matrix proteins and secreted soluble factors compared to monolayer culture. In our sphere culture process that differs by the absence of serum we could not find the presence of the extracellular matrix proteins collagen, fibronectin, hyaluronic acid and elastin (data not shown).

This new non-adherent and serum-free method presented here is a first step towards developing improved protocols for stem cell culture. First, it appears necessary to extend the analyze of gene expression central for cell cycle progression and cell differentiation in sphere-derived ASC, as differential gene expression was established between human serum and fetal bovine serum cultured ASC [18]. The next steps will involve the optimization of medium formulation for higher expansion rate, followed by the development of serum free differentiation media as described for bone-marrow mesenchymal stem cells [36]. The differentiation potential of sphere-derived ASC needs to be further analyzed as we have only searched for the differentiation potential of the mesenchymal lineage. For example, ASC derived from rhesus adipose tissue cultured as monolayer in proliferation medium with 10% fetal calf serum could be committed to neuronal differentiation after a step of sphere culture [37]. Finally, there is a growing interest in designing bioreactor systems, that provide safe and controlled high-density cell culture in production.

In this context, our results represent an important step in our ability to isolate and maintain ASC in culture and extend the possibilities of cell-based therapies.

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Histopathology of the IFR-BMT/150 (Toulouse - Midi-Pyrénées génopole), Toulouse, France.

REFERENCES

- [1] P. Trayhurn, J.H. Beattie, Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ, *Proc. Nutr. Soc.* 60 (2001) 329–339.
- [2] M.H. Fonseca-Alaniz, J. Takada, M.I. Alonso-Vale, F.B. Lima, Adipose tissue as an endocrine organ: from theory to practice, *J. Pediatr. (Rio J)* 83 (2007) S192–S203.
- [3] J. Gimble, F. Guilak, Adipose-derived adult stem cells: isolation, characterization, and differentiation potential, *Cytotherapy* 5 (2003) 362–369.
- [4] V. Planat-Benard, J.S. Silvestre, B. Cousin, M. Andre, M. Nibbelink, R. Tamarat, M. Clergue, C. Manneville, C. Saillan-Barreau, M. Duriez, A. Tedgui, B. Levy, L. Penicaud, L. Casteilla, Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives, *Circulation* 109 (2004) 656–663.
- [5] G.R. Erickson, J.M. Gimble, D.M. Franklin, H.E. Rice, H. Awad, F. Guilak, Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo, *Biochem. Biophys. Res. Commun.* 290 (2002) 763–769.
- [6] Y.D. Halvorsen, D. Franklin, A.L. Bond, D.C. Hitt, C. Auchter, A.L. Boskey, E.P. Paschalis, W.O. Wilkison, J.M. Gimble, Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells, *Tissue Eng.* 7 (2001) 729–741.
- [7] A. Miranville, C. Heeschen, C. Sengenès, C.A. Curat, R. Busse, A. Bouloumie, Improvement of postnatal neovascularization by human adipose tissue-derived stem cells, *Circulation* 110 (2004) 349–355.
- [8] A. Schaffler, C. Buchler, Concise review: adipose tissue-derived stromal cells—basic and clinical implications for novel cell-based therapies, *Stem Cells* 25 (2007) 818–827.
- [9] P.A. Zuk, M. Zhu, H. Mizuno, J. Huang, J.W. Futrell, A.J. Katz, P. Benhaim, H.P. Lorenz, M.H. Hedrick, Multilineage cells from human adipose tissue: implications for cell-based therapies, *Tissue Eng.* 7 (2001) 211–228.
- [10] F. Guilak, K.E. Lott, H.A. Awad, Q. Cao, K.C. Hicok, B. Fermor, J.M. Gimble, Clonal analysis of the differentiation potential of human adipose-derived adult stem cells, *J. Cell. Physiol.* 206 (2006) 229–237.
- [11] T.M. Liu, M. Martina, D.W. Hutmacher, J.H. Hui, E.H. Lee, B. Lim, Identification of common pathways mediating differentiation of bone marrow- and adipose tissue-derived human mesenchymal stem cells into three mesenchymal lineages, *Stem Cells* 25 (2007) 750–760.
- [12] S. Kern, H. Eichler, J. Stoeve, H. Kluter, K. Bieback, Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue, *Stem Cells* 24 (2006) 1294–1301.
- [13] R. Izadpanah, C. Trygg, B. Patel, C. Kriedt, J. Dufour, J.M. Gimble, B.A. Bunnell, Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue, *J. Cell. Biochem.* 99 (2006) 1285–1297.
- [14] S. Roche, B. Delorme, R.A. Oostendorp, R. Barbet, D. Caton, D. Noel, K. Boumediene, H.A. Papadaki, B. Cousin, C. Crozet, O. Milhavel, L. Casteilla, J. Hatzfeld, C. Jorgensen, P. Charbord, S. Lehmann, Comparative proteomic analysis of human mesenchymal and embryonic stem cells: towards the definition of a mesenchymal stem cell proteomic signature, *Proteomics* 9 (2009) 223–232.
- [15] P.A. Zuk, M. Zhu, P. Ashjian, D.A. De Ugarte, J.I. Huang, H. Mizuno, Z.C. Alfonso, J.K. Fraser, P. Benhaim, M.H. Hedrick, Human adipose tissue is a source of multipotent stem cells, *Mol. Biol. Cell* 13 (2002) 4279–4295.

- [16] A. Dicker, K. Le Blanc, G. Astrom, V. van Harmelen, C. Gotherstrom, L. Blomqvist, P. Arner, M. Ryden, Functional studies of mesenchymal stem cells derived from adult human adipose tissue, *Exp. Cell Res.* 308 (2005) 283–290.
- [17] J.M. Gimble, A.J. Katz, B.A. Bunnell, Adipose-derived stem cells for regenerative medicine, *Circ. Res.* 100 (2007) 1249–1260.
- [18] B. Lindroos, K.L. Aho, H. Kuokkanen, S. Raty, H. Huhtala, R. Lemponen, O. Yli-Harja, R. Suuronen, S. Miettinen, Differential gene expression in adipose stem cells cultured in allogeneic human serum versus fetal bovine serum, *Tissue Eng. A* 16 (2010) 2281–2294.
- [19] P.G. Layer, A. Robitzki, A. Rothermel, E. Willbold, Of layers and spheres: the reaggregate approach in tissue engineering, *Trends Neurosci.* 25 (2002) 131–134.
- [20] H. Kurosawa, Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells, *J. Biosci. Bioeng.* 103 (2007) 389–398.
- [21] W. Mueller-Klieser, Three-dimensional cell cultures: from molecular mechanisms to clinical applications, *Am. J. Physiol.* 273 (1997) C1109–C1123.
- [22] M.T. Santini, G. Rainaldi, Three-dimensional spheroid model in tumor biology, *Pathobiology* 67 (1999) 148–157.
- [23] P.G. Layer, T. Weikert, E. Willbold, Chicken retinospheroids as developmental and pharmacological in vitro models: acetylcholinesterase is regulated by its own and by butyrylcholinesterase activity, *Cell Tissue Res.* 268 (1992) 409–418.
- [24] N. Lumelsky, O. Blondel, P. Laeng, I. Velasco, R. Ravin, R. McKay, Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets, *Science* 292 (2001) 1389–1394.
- [25] C. Saillan-Barreau, B. Cousin, M. Andre, P. Villena, L. Casteilla, L. Penicaud, Human adipose cells as candidates in defense and tissue remodeling phenomena, *Biochem. Biophys. Res. Commun.* 309 (2003) 502–505.
- [26] P. Laharrague, D. Larrouy, A.M. Fontanilles, N. Truel, A. Campfield, R. Tenenbaum, J. Galitzky, J.X. Corberand, L. Penicaud, L. Casteilla, High expression of leptin by human bone marrow adipocytes in primary culture, *FASEB J.* 12 (1998) 747–752.
- [27] F. Zaibak, P. Bello, J. Kozlovski, D. Crombie, H. Ang, M. Dottori, R. Williamson, Unrestricted somatic stem cells from human umbilical cord blood grow in serum-free medium as spheres, *BMC Biotechnol.* 9 (2009) 101.
- [28] K.A. Westerman, A. Penrose, Z. Yang, P.D. Allen, C.A. Vacanti, Adult muscle 'stem' cells can be sustained in culture as free-floating myospheres, *Exp. Cell Res.* 316 (2010) 1966–1976.
- [29] P. Lund, L. Pilgaard, M. Duroux, T. Fink, V. Zachar, Effect of growth media and serum replacements on the proliferation and differentiation of adipose-derived stem cells, *Cytotherapy* 11 (2009) 189–197.
- [30] K. Rajala, B. Lindroos, S.M. Hussein, R.S. Lappalainen, M. Pekkanen-Mattila, J. Inzunza, B. Rozell, S. Miettinen, S. Narkilahti, E. Kerkela, K. Aalto-Setälä, T. Otonkoski, R. Suuronen, O. Hovatta, H. Skottman, A defined and xeno-free culture method enabling the establishment of clinical-grade human embryonic, induced pluripotent and adipose stem cells, *PLoS ONE* 5 (2010) e10246.
- [31] A.M. Parker, H. Shang, M. Khurgel, A.J. Katz, Low serum and serum-free culture of multipotential human adipose stem cells, *Cytotherapy* 9 (2007) 637–646.
- [32] F. De Francesco, V. Tirino, V. Desiderio, G. Ferraro, F. D'Andrea, M. Giuliano, G. Libondi, G. Pirozzi, A. De Rosa, G. Papaccio, Human CD34/CD90 ASCs are capable of growing as sphere clusters, producing high levels of VEGF and forming capillaries, *PLoS ONE* 4 (2009) e6537.
- [33] O.M. Martinez-Estrada, Y. Munoz-Santos, J. Julve, M. Reina, S. Vilaro, Human adipose tissue as a source of Flk-1+ cells: new method of differentiation and expansion, *Cardiovasc. Res.* 65 (2005) 328–333.
- [34] P.J. Amos, S.K. Kapur, P.C. Stapor, H. Shang, S. Bekiranov, M. Khurgel, G.T. Rodeheaver, S.M. Peirce, A.J. Katz, Human adipose-derived stromal cells accelerate diabetic wound healing: impact of cell formulation and delivery, *Tissue Eng. A* 16 (2010) 1595–1606.
- [35] B.L. Coles-Takabe, I. Brain, K.A. Purpura, P. Karpowicz, P.W. Zandstra, C.M. Morshead, D. van der Kooy, Don't look: growing clonal versus nonclonal neural stem cell colonies, *Stem Cells* 26 (2008) 2938–2944.
- [36] T. Felka, R. Schafer, P. De Zwart, W.K. Aicher, Animal serum-free expansion and differentiation of human mesenchymal stromal cells, *Cytotherapy* 12 (2010) 143–153.
- [37] S.K. Kang, L.A. Putnam, J. Ylostalo, I.R. Popescu, J. Dufour, A. Belousov, B.A. Bunnell, Neurogenesis of Rhesus adipose stromal cells, *J. Cell Sci.* 117 (2004) 4289–4299.