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ADIPOSE DERIVED CELLS

Comparison between pediatric and adult adipose mesenchymal stromal cells

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Abstract

Background. Adipose-derived mesenchymalstromal cells (ASC) are currently tested in regenerative medicine to promote tissue reconstruction after injury. Regardingautologous purpose, the possible loss of therapeutic function and cell properties during aging have been questioned in adults. To date no reliable information is available concerning ASC from pediatric patients and a better knowledge is required for clinical applications. *Methods.* Subcutaneous adipose tissue was collected from 27 donors (0–1 years old) and 50 donors (1–12 years old) and compared with adult ASC for *in vitro* characteristics. ASC were then tested in a mouse model of limb ischemia. *Results.* Cells from the stromal vascular fraction (SVF) and subsequent cultured ASC were prepared. Only a greater amount in SVF cell number and ASC proliferative rate were found. Cell phenotype, colony formingunit-fibroblast (CFU-F) content, immunomodulation effect and adipogenic, osteoblastic and angiogenic potentials were not significantly different. *In vivo*, pediatric ASC induced an increase in microangiographic score in a mouse model of limb ischemia, even though improvement in vascular density was not significantly correlated to limb rescue. Finally messengerRNA (mRNA) analysis using a microarray approach identified that only 305 genes were differentially expressed (217 down- and 88 up-regulated) in pediatric versus adult ASC, confirming that ASC from both age groups shared very close intrinsic properties. *Conclusion.* This is the first study reporting a comparative analysis of ASC from a large number of donors and showing that their *in vitro* and *in vivo* properties were similar and maintained during aging.

Key Words: *adipose mesenchymalstromal cells, cell therapy, pediatric cells*

Introduction

Reconstructive surgery is a key feature of pediatric surgery, whatever the organ involved [1]. During the last century, the management of neonates and infants with congenital malformations or acquired disabilities has considerably improved, thanks to the development of both anesthetic and surgical techniques. However, multiple procedures are sometimes required during the growth of the child to achieve an acceptable, stable and functional outcome. As an example, the management of labio-alveolar cleft requires a long-term follow-up after the initial surgical procedure. Healing complications such as cutaneous scar retraction, secondary acquired palatal fistulae and other orthodontic abnormalities have to be detected rapidly to ensure their early correction. To overcome these limits, alternative therapies have been proposed to better restore initial anatomical and functional properties of the wounded tissue and then avoid subsequent procedures. Stem cell–based therapy has been pointed out as a good candidate to reach this purpose [1].

Adipose mesenchymal stromal cells (ASC) are well known within the field of reconstructive surgery, with lots of pre-clinical and now clinical applications [2,3]. Due to specific properties, such as differentiation

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potential into different tissue lineages, secretion and immunomodulation characteristics [4], their abundance, with little invasive access, and the absence of ethical issue, they have been widely studied in adults [5]. However, to date, very little information concerning pediatric ASC properties has been reported and it is insufficient to consider their clinical use in children. Maiorana et al. first reported the isolation and characterization of ASC from omental adipose tissue in 13 children (6.5 ± 4.3 years old) and 2 newborns describing their adipogenic differentiation potential and immunophenotype [6]. Guasti et al. focused on the plasticity of pediatric ASC that express pluripotency markers such as c-Myc, OCT4, Nanog, KLF4 and DNMT3B but not Sox2 and their multipotency, as pediatric ASC also express many lineage markers and differentiate into adipose, chondrogenic, osteoblastic and neuronal cell types [7]. Due to such skeletogenic differentiation potential of ASC from the 16 children tested, they concluded that adipose tissue could be a potential source of stem cells for cartilaginous or bone replacement. Wu et al. performed the only comparative study in infants (age < 1 year), adults and elderly ASC and concluded that infant ASC have long telomeres and exhibit enhanced angiogenic and osteogenic capabilities, based on four samples [8]. Moreover, the *in vivo* therapeutic potential of children ASC has never been evaluated. If the goal of an ideal therapy is not to repair but to regenerate a limb or organ, a more extent characterization of cells from younger donors is required as well as efficacy data in a model.

The aim of our study was, therefore, to assess the potentiality of pediatric ASC obtained from 27 donors from Group 0–1 years old and 50 donors from Group 1–12 years old to study sampling and phenotyping of the crude stromal vascular fraction (SVF) of adipose tissue, culture, differentiation, immunomodulation, gene expression profile and *in vivo* beneficial effect in ischemic tissue, depending on the age of the donor.

Methods

Adipose tissue sample collection

Pediatric subcutaneous adipose tissue (1 g) was obtained from donors aged from 0 to 1 year (Group 0–1; 0.3 ± 0.5 year old; n = 27; male = 23; female = 4) and from 1 to 12 years (Group 1–12; 6.6 ± 6.1 years old; n = 50; male = 47; female = 3) undergoing elective inguinal surgeries (inguinal hernia repair and orchidopexy). The investigation was approved by the local ethics committee (AS Child 1 no. 08-082-03 and AS Child 2 no. 11-228-02), national agency (CPP-ID-RCB no. 2008-A01469-46 and no. 2011-A01469-32) and written informed consent from both parents. Adult subcutaneous adipose tissue (10 g) was obtained from donors undergoing elective abdominal dermolipectomy (age 20–35 years, body mass index [BMI] < 28; n = 85; male = 4; female = 81). No objection certificate was obtained according to bioethics law number 2004-800 of August 6, 2004.

ASC isolation and culture

Adipose tissue samples were digested in alpha - Minimum Essential Medium (α-MEM, Invitrogen) supplemented with 0.4 U/mL NB4 collagenase (Serva electrophoresis) for 45 min at 37°C under agitation. Cellular suspension was filtrated through 25 µm nylon membrane and centrifuged at 600*g* for 8 min to separate floating mature adipocytes from SVF. SVF was incubated in erythrocyte lysis buffer (ammonium chloride solution, StemCell Technologies) for 5 min at 4°C and washed in phosphate-buffered saline (PBS). SVF cells were re-suspended in culture medium α-MEM for viability and cell numeration (Thoma hemocytometer).

SVF cells were then seeded at 4000 cells/cm^2 in flasks treated for cell culture (TPP, D. Dutscher) in ASC expansion medium that consisted of α-MEM supplemented with 2% human plasma enriched with human platelet growth factors (EFS-PM Toulouse), 1 U/mL heparin Choay (Sanofi Aventis), 0.25 µg/mL amphotericin, 100 µg/mL streptomycin and 100 U/mL penicillin (Invitrogen). Cells were incubated at 37°C under 5% CO_2 and the medium was changed twice a week. After 7 days, ASC were harvested with trypsin-ethylenediaminetetraacetic acid (EDTA; LifeTechnologies). The number of viable cells was determined using Trypan blue exclusion on a Countess cell counter. Cells were then used (ASC P0) or plated at a density of 2000 cells/cm^2 and cultured until use at passage P1 (ASC P1). For long-term culture ASC were successively passaged at 80% of confluency until passage 10 (ASC P10).

Colony forming unit-fibroblasts assay

Freshly prepared SVF cells or ASC P0 were seeded in 25 cm^2 flasks at 16 cells/cm^2 in ASC expansion medium. The medium was renewed every 2 or 3 days. The cultures were ended at day 14 for ASC P0 and day 10 for ASC P1. The flasks were stained with the kit RAL stainer MCDh (RAL Diagnostics) to score the fibroblast colonies using an optical microscope.

Cell phenotyping

SVF cells were incubated with PBS supplemented with FcR Block reagent (Miltenyi Biotec). Sextuplet staining were performed by incubating cells for 30 min at 4°C with the following conjugated primary antibodies or appropriate immunoglobulin (Ig) G isotype

controls: Lin-FITC, CD166-PE, CD34-PerCP, CD38-PE-Cy7, CD117-APC, CD31-FITC, CD73-PE, CD117-PE-Cy7, CD36-APC, HLA-DR-FITC, CD14-PerCP, CD11b-PE-Cy7, CD15-APC, CD56-PE, CD19-PerCP, CD3-PE-Cy7, CD11b-APC, TCRγδ, CD45-PerCP, CD4-PE-Cy7, CD8-APC (FITC, fluorescein isothiocyanate, PE, phycoerythrin, PerCP, peridinin chlorphyll protein, PE-Cy7, phycoerythrin-cyanin 7, APC, allophycocyanin, BD Biosciences), CD20-FITC (BioLegend), CD1a-PE (Beckman), CD45-APC-vio770 and CD-3 APC-vio770 (Miltenyi Biotec). DAPI was used for viability control. Cells were analysed on LSRFortessa™ X20 (BD Bioscience). Data acquisition was performed with FACS Diva software and analysis was performed with Kaluza software (Beckman-Coulter).

Adipogenic differentiation

Cells were plated at 4000 cells/cm^2 in 12-well tissue culture plates (Falcon). Subconfluent ASC were induced for 3 days in adipogenic differentiation medium, which consisted in ASC expansion medium supplemented with 1 µmol/L dexamethasone, 5 µg/mL insulin, 1 µmol/L rosiglitazone, 450 µmol/L IBMX and 60 µmol/L indomethacin (all Sigma-Aldrich). Subsequently, IBMX was removed from the medium and differentiation was extended during 11 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 (Sigma-Aldrich) and was compared with control cells (no differentiation induction). 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). Differentiation quantification was evaluated by calculating the ratio of TG per total protein content.

Osteogenic differentiation

Cells were plated at 4000 cells/cm^2 in 12-well tissue culture plates (Falcon) and cultured until 70%–80% of confluence. ASC were then cultured for 21 days in osteogenic medium, which consisted in ASC expansion medium first supplemented with 0.1 µmol/L dexamethasone, 50 µmol/L ascorbic acid and 3 mmol/L NaH_2PO_4 (all Sigma-Aldrich) for 14 days and then with 0.1 µmol/L dexamethasone, 50 µmol/L ascorbic acid and 10 mmol/L β-glycerophosphate (all Sigma-Aldrich) until day 21. The medium was changed every 3 days. Mineralization was revealed by staining calcium-rich deposits with alizarin red (Sigma-Aldrich) and compared with control cell (no differentiation induction). Alizarin red quantity (µg) was assessed at day 0 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. For messenger RNA (mRNA) study, the differentiation medium consisted in ASC expansion medium supplemented with 10 mmol/L β-glycerophosphate, 50 µmol/L ascorbic acid and 50 ng/mL BMP4 (R&D Systems).

Endothelial differentiation

SVF cells were plated at 100,000 cells/cm^2 in gelatin-coated 48-well tissue culture plates (Costar) and cultured for 10 days in ASC expansion medium supplemented or not with 10 ng/mL VEGF (Sigma-Aldrich). The medium was replaced every 3 days. Vascular tube formation was assessed using CD31 (Dako) immunostaining. CD31-positive extension lengths were measured using the Elements AR 3.0 image analyzer software (Nikon).

Immunosuppression assay

Five × 10^4 ASC were co-cultured with 10^5 CD3$^+$ T cells isolated from peripheral blood monnuclear cells (PBMCs) (T-cell purification kit, Miltenyi Biotec), in RPMI medium supplemented with 10% fetal bovine serum (FBS; Hyclone, Thermo Scientific), 0.25 µg/mL amphotericin, 100 µg/mL streptomycin and 100 U/mL penicillin (Invitrogen). Before the co-cultures, CD3$^+$ T cells were labeled with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE; Life Technologies) as fluorescent cell-tracing reagent. T cells were then activated with CD3/CD28 coated beads (Life Technologies). After 5 days, all cells were recovered, and T cells were stained with fluorochrome coupled anti-CD3 and anti-CD45 antibodies (Miltenyi Biotec). The proportion of cycling CD3$^+$CD45$^+$ T cells was quantified by fluorescence decrease of CFSE in comparison with both uncycling cells (non-stimulated T cells) and stimulated T cells cultured without ASC. Analysis involved use of the Cyan™ flow cytometer (Beckman Coulter) and Kaluza™ software. The percentages of CD3$^+$ T lymphocytes proliferating during the co-cultures were calculated in comparison with stimulated T lymphocytes without ASC as control to set the 100% of cycling cells.

Microarray transcriptome hybridization

After extraction, the RNAs were controlled using Nanodrop ND-1000 and Bioanalyzer 2100 Expert from Agilent. The resulting RNA was then subjected to reverse transcription using random hexamers tagged with a T7 promoter sequence followed by second-strand complementary DNA (cDNA) synthesis using a DNA polymerase (GeneChip WT cDNA Synthesis Kit; Affymetrix). The resulting double-stranded cDNA was then used for amplification of antisense

cRNA and cleaned (Gene Chip Sample Cleanup Module; Affymetrix). A second cycle of cDNA synthesis was performed using random primers to reverse transcribe the cRNA into sense single-stranded DNA. This DNA was fragmented, labeled and hybridized to a human gene chip (Human GeneChip Gene 1.0 ST Arrays; Affymetrix). Target labeling, array hybridization, washing and staining were performed upon manufacturer recommendation (GeneChip Whole Transcript [WT] Sense Target Labeling; Affymetrix). Arrays were hybridized, washed and stained (GeneChip Hybridization, Wash & Stain Kit in a GeneChip Hybridization Oven 645 and GeneChip Fluidics Station 450; Affymetrix), then scanned (GeneChip Scanner GCS3000 7G; Affymetrix) and analyzed using Command Console software.

Microarray data analysis

Microarray data were analyzed using tools furnished by the Bioconductor 3.1 project and R software 3.2.2 (R project). Background correcting, normalizing and calculating expressions were achieved by the Robust Multi-Array Average (RMA) expression measure (RMA method from the affy package, 1.46.1). The limma package 3.24.14 was used to determine, using Bayesian analysis, the differently expressed genes from five pediatric (P1–P5) and five adult (A1–A5) ASCs. Only significant genes (*P* value level set at 0.05) with more than log2 1-fold up- or down-regulated were kept [9–11]. Such a set of genes was annotated using oligo package 1.8.0 (pd.hugene.1.0.st.v1). Hierarchical cluster analysis was performed using Euclidean distance (average linkage clustering) and represented as a heatmap with dendrograms (gplots package 2.17.0).

All differentially expressed genes were annotated using the Gene Ontology (GO) term biological processes database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database from Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 [12,13]. Terms were considered significantly enriched at a 0.05 EASE score level, corrected by Benjamini–Hochberg procedure. Reduce and Visualize Gene Ontology (REViGO) [14] was then used to summarize GO terms, removing redundancy (medium similarity [0.7]).

Eighteen genes from Affymetrix and quantitative–polymerase chain reaction (q-PCR) were respectively transformed using the $2^{\Delta\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ method, normalized to adults, using PUM as reference gene. Analysis of variance model with a random effect on patient was used to detect and interpret any significant difference between the two obtained levels of expression (Supplementary Figure S1).

Real time–q-PCR

Total RNA was isolated using RNAeasy minikit (Qiagen) according to the manufacturer's recommendations. RNA was quantified using Nanodrop (ThermoScientific, NanoDrop Products) and 500 ng RNA was reverse-transcribed using random hexamers and Multiscribe reverse transcriptase (High Capacity cDNA Reverse Transcription kit, Thermo Fisher Scientific/Applied Biosystems).

For differentiation marker expression, cDNA quantification was performed using the StepOnePlus technology and the Syber green Fast Master Mix according to manufacturer instructions (Life Technologies/Applied Biosystems). Primers peroxisome proliferator-activated receptor gamma (PPARγ), adipose fatty acid-binding protein (aP2), lipoprotein lipase (LPL), run-related transcription factor 2 (Runx2), Osterix, Osteocalcin, parathyroid hormone 1 receptor (PTHR1), distal-less homeobox 5 (DLX5), zinc finger transcription regulator SNAIL, Fms Related Tyrosine Kinase 1 (FLT1), Kinase insert Domain Receptor (KDR), inter cellular adhesion molecule (ICAM), von Willebrand factor (vWF), CD31 (PECAM-1) and endothelial NO synthase (eNOS) were used at 300 nmol/L and PUM at 100 nmol/L.

For senescence marker expression (except for p16), q-PCR was performed with Sso Fast EvaGreen Supermix (Bio-Rad) and CFX96 thermal cycler (Bio-Rad). Taqman Gene expression Master kit (Life Technologies/Applied Biosystems) was used for p16 amplification. All primers PPIA, YWHAZ, p21, p53, Myc, Rb, MDM2, Oct-4, Sox2 and hTERT were used at 500 nmol/L, except P16-FAM and pri Nanog (300 nmol/L). The geometric mean of YWHAZ and PPIA was used to normalize gene expression values.

For microarray validation, 20 ng of cDNA was analyzed by real time PCR in a final volume of 20 µl using Power SYBRgreen master mix (Life Technologies/Applied Biosystem) with the primers for Vascular Endothelial Growth Factor, Hepatocyte Growth Factor, transforming growth factor (TGF)beta2, interleukin (IL)-8, IL-6, tumor necrosis factor (TNF) alpha and glucose transporter 1 (GLUT1) (0.3 µmol/L final). Real-time PCR assays were run on StepOne detection system instrument (Life Technologies/Applied Biosystem). Relative gene expression was calculated by the delta-Cycle Threshold (dCT) method and normalized to PUM.

All primers sequences are detailed in Supplementary Table S1.

Mouse model of hindlimb ischemia

Six-week-old adult athymic nude mice (Harlan) were housed in pathogen-free animal facilities (Anexplo/

GenoToul). All experimental procedures were done in compliance with the French Ministry of Agriculture regulation (animal facility registration no. MP/01/14/03/11) for animal experimentation. After anesthesia by isoflurane inhalation, a ligature was placed on the left femoral pedicle by a short inguinal incision as previously described [8]. Two h later, 1×10^6 ASC were administrated by intramuscular injection in three different sites (gastrocnemius, gracilis and quadriceps muscles, 20 µL per injection) of the ischemic leg. For each of the six pediatric ASC samples, 4–6 mice were injected with cells (treated group; n = 29) and compared with 5–6 mice (control group; n = 31) undergoing a similar procedure with injections of 20 µL NaCl 0.9%. Independent experiments were repeated with distinct ASC samples.

Vascular function was evaluated as the percentage of conserved ischemic limb and by the measurement of the length of the wounded leg in comparison with the non-ischemic leg of the same animal. Leg perfusion was assessed using laser Doppler imaging and limb perfusion expressed as a ratio of right ischemic to left non-ischemic leg during a 14-day follow-up. At the end point, a microangiography analysis was performed under anesthesia after intracardiac injection of 1 mg/mL barium sulfate for limb vascular network imaging (Faxitron MX-20). The vessel density was expressed as a percentage of pixels per image occupied by vessels in the quantification area and compared with the non-ischemic limb of the same animal (Osirix Imaging Software, Pixmeo). Quantification zone was delineated by the ligature site on the femoral artery, the knee, the edge of the femur and the external limit of the leg.

Statistical analysis

Quantitative results were expressed as the mean ± standard error of the mean (SEM) from independent experiments. Comparisons between groups or conditions were made with the unpaired *t* test using Prism 5 software (GraphPad). Significance was defined as $^{\star}P \leq 0.05$, $^{\star\star}P \leq 0.01$ and $^{\star\star\star}P \leq 0.001$.

Results

Pediatric adipose tissue cell properties

The efficiency of adipose tissue cell isolation from pediatric samples was compared with the conventional procedure validated for adult ASC [2]. SVF cell number obtained from children of Group 0–1 was significantly higher than Group 1–12 or adults ($1.59 \pm 0.15 \times 10^6$ compared with $0.66 \pm 0.06 \times 10^6$ and $0.69 \pm 0.04 \times 10^6$, respectively). Similar amounts of SVF cells were obtained with Group 1–12 and adults samples (Figure 1a). The amount of progenitor cells in SVF cells was assessed by the number of colony forming unit-fibroblasts (CFU-F). No significant difference was noticed between groups (Figure 1b). The phenotype of SVF cells was homogeneous between pediatric donors from 0 to 12 years of age. When compared with adults, no major difference was observed (Figure 1c). Surface markers analysis just pointed out that the amount of endothelial cells ($CD34^+$ $CD31^+$ $CD45^-$) was higher ($P = 0.038$) in pediatric SVF cells, whereas lymphocyte T tented to slightly more important in adult samples ($P = 0.307$ for LT $CD3^+$ and $P = 0.290$ for LT $CD4^+$). Considering more specifically the ASC subpopulation, the yield of ASC obtained in primary culture (ASC P0) was similar between Group 0–1 and Group 1–12 but Group 0–1 was significantly higher compared with adults with an expansion factor of 4.77 (Group 0–1), 4.69 (Group1-12) and 3.87 (Adult; Figure 1d). The amount of CFU-F obtained from ASC was very close in the three groups (13.6% ± 3, 12.1% ± 4.5 and 12.9 % ± 3.8, respectively; Figure 1e).

Pediatric ASC differentiation potentials

Adult ASC are multipotent and known to be able to differentiate into adipogenic, osteogenic and chondrogenic lineages and to support vascular network formation *in vitro* [5]. In the present study, the adipogenic and osteogenic potentials of pediatric ASC from both groups were found to be similar as adult ASC (Figure 2a and 2b). TG accumulation under adipogenic induction appeared to be two times higher in the ASC Group 0–1 (Figure 2a) and mineralization under osteogenic induction was higher in ASC 1–12 (Figure 2b), however, these differences were not statistically significant from adult ASC. When assessing specific differentiation marker expression no difference was observed. Concerning the level of expression of endothelial markers under VEGF stimulation, there was no significant difference between the three groups of ASC, as well as for their capacity to support CD31vascular network formation *in vitro* (Figure 2c).

Immunosuppressive properties

Immunomodulatory properties of adult ASC have been previously described *in vitro* [15] and were challenged *in vivo* in graft-versus-host disease [16]. The immunosuppressive activity of ASC towards T-lymphocyte proliferation was estimated *in vitro* and results highlighted that pediatric ASC displayed similar inhibitory activity to adult ASC (Figure 3).

Effect of in vitro *replicative stress on pediatric ASC*

In vitro cell expansion might be associated with replicative senescence and stress that may generate

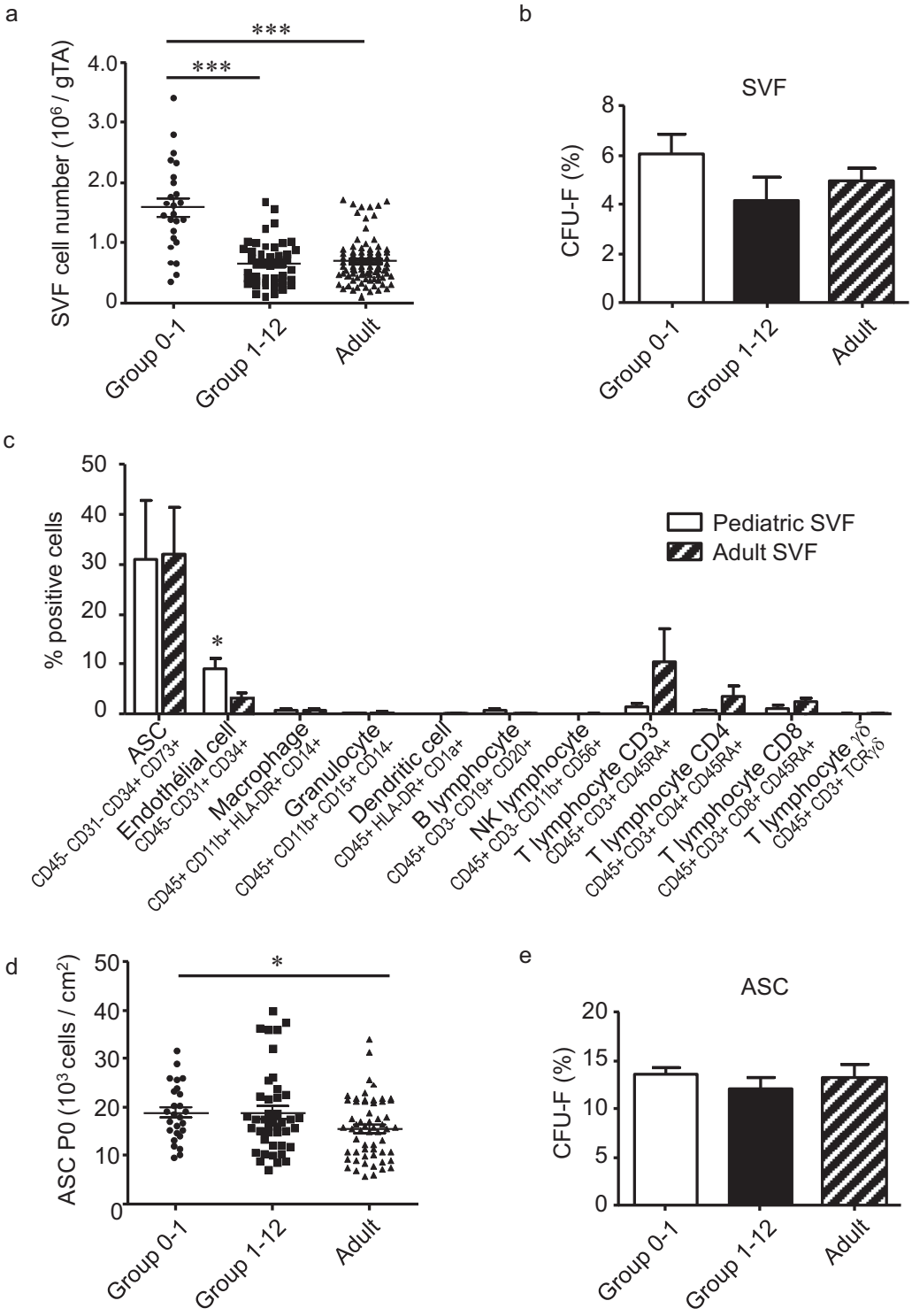

Figure 1. *In vitro* properties of pediatric ASC. The amount of freshly prepared SVF cells from adipose tissue (a), n = 25 Group 1–0 (22 male and 3 female samples), n = 45 Group 1–12 (42 male, 3 female samples), n = 85 Adult group (4 male, 81 female samples), as well as the number of CFU-F formed (b), n = 17 of each group, were compared between children and adults (Group 0–1: 14 male and 3 female samples, Group 1–12: 16 male and 1 female samples). SVF cells from pediatric and adult donors were analyzed for the indicated phenotypes of cell subpopulations (c), n = 3 pediatric SVF, n = 4 adult SVF. The amount of ASC obtained after 8 days in primary culture (ASC-P0) (d), n = 26 Group 0–1 (22 male and 4 female samples), n = 42 Group 1–12 (39 male and 3 female samples), n = 57 Adult group (1 male and 56 female samples), and the number of CFU-F formed (e), n = 17 Group 0–1 (14 male and 3 female samples), n = 17 Group 1–12 (16 male and 1 female samples), n = 4 Adult group, were compared between children and adults. *$P \leq 0.05$ and ***$P \leq 0.001$ between indicated groups.

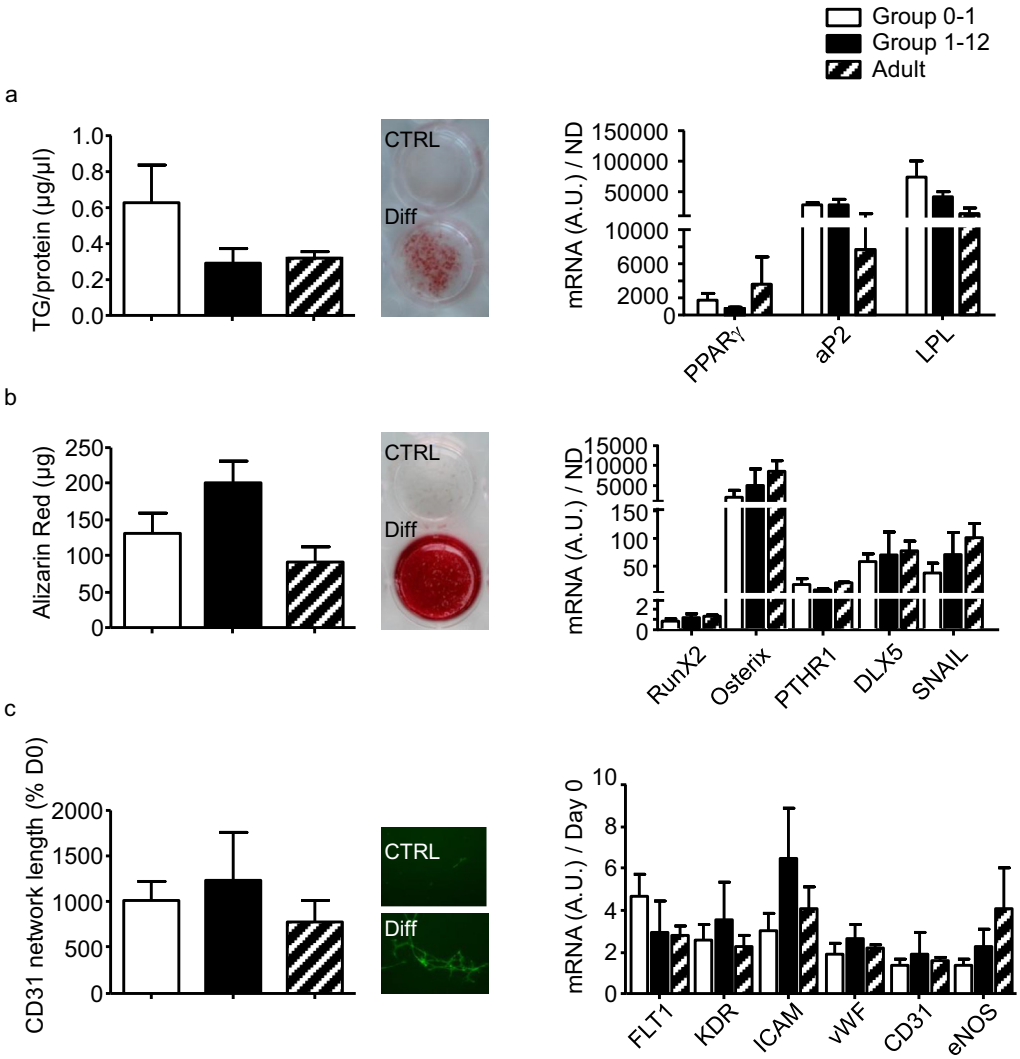

Figure 2. Differentiation (Diff) potentials of pediatric ASC. ASC from the three different groups of donors were cultured in control (CTRL) or Diff medium as shown in representative images. Adipose differentiation was estimated by measuring TG content and the mRNA expression level of the Diff factors: peroxisome proliferator-activated receptor gamma (PPARγ), adipose fatty acid-binding protein (aP2) and the lipoprotein lipase (LPL) (a) n = 6 (5 pediatric male, 1 pediatric female and 6 adult female samples). Osteoblasic Diff was estimated by measuring mineralization after alizarin red staining and the mRNA expression level of the transcription factors: run-related transcription factor 2 (Runx2), Osterix, parathyroid hormone 1 receptor (PTHR1), distal-less homeobox 5 (DLX5) and the zinc finger transcription regulator SNAIL (b) n = 6 (5 pediatric male, 1 pediatric female and 6 adult female samples). Angiogenic effect was estimated by measuring the branched networks after CD31immunostaining and by mRNA expression level ofVEGF receptor 1 (FLT1) and 2 (KDR), intercellular adhesion molecule (ICAM), von Willebrand factor (vWF), CD31 (PECAM-1) and endothelial NO synthase (eNOS) (c), n = 3–5.

mutations, chromosomal abnormalities and many other cell defects not suitable with the perspective of cell administration for therapeutic purpose. Some data are available concerning adult ASC [17] but this point has never been addressed with pediatric ASC. Because cell transformation has been associated with an increase in telomerase activity that shortens telomere, and a loss in proliferative activity control, the risks induced by the cell culture process were evaluated. We first calculated the doubling time at each passage and observed that ASC from Group 0–1 only tended to cycle more rapidly than ASC from Group 1–12 (data not shown). A significant difference was obtained when expressing the cumulated doubling time over 10 passages showing that ASC from Group 0–1 divided faster than ASC from Group 1–12 ($P = 0.0025$) and adult ($P = 0.036$). Doubling time corresponded to 26.5 ± 1.2 days for Group 0–1, 37.8 ± 2.3 days for Group 1–12 and 33.4 ± 0.9 days for the adult group (Figure 4, first panel). We also assessed the level of expression of the main molecules of the replicative senescence checkpoint (p53, p21, p16, MDM2 and pRB), hTERT, Myc for proliferative activity and Oct4, Sox2 for cell stemness status during 10 passages, each performed at the subconfluence cell density. The result showed that, except for p16, whose expression was

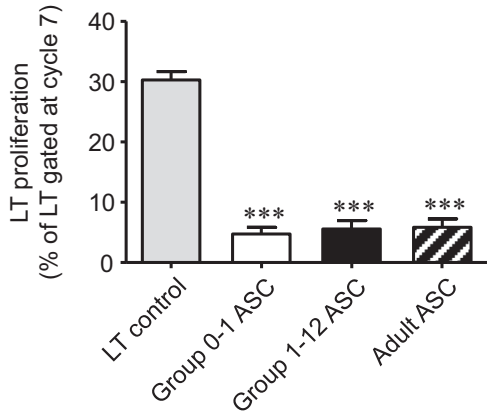

Figure 3. Immonumodulation activity of pediatric ASC. CSFE-labeled purified CD4 + T cells were stimulated with CD3/CD8 beads and ASC from the different groups were added or not (control) to quantify the rate of proliferating, in comparison with non-stimulated T lymphocytes, n = 8 (pediatric male versus adult female samples). ***$P \leq 0.001$.

increasing with passages, the expression of all the other factors stayed stable or even tented to decrease in long-term culture (Figure 4).

In vivo *angiogenic potential of pediatric ASC in a mouse model of limb ischemia*

To investigate the *in vivo* potential of pediatric ASC, we used the previously described mouse model of limb ischemia where pediatric ASC P1 were injected [11,14]. First, we assessed the percentage of hind limb conservation with or without *in situ* injection of pediatric ASC (Figure 5a). No significant difference was found between the two groups with 66.6% and 73.3%, respectively, of limb conservation in animals treated with ASC from Group 0–1 and 1–12 versus 70.1% in untreated animals. When ischemic lesions were observed in the conserved limb (from distal finger necrosis to partial limb amputation), we compared the length of the remaining hind limb (expressed as the ratio of the full non-ischemic length) (Figure 5b). The remaining ischemic limb was always shorter in the control group (0.63 ± 0.05) compared with the ASC-injected groups (0.83 ± 0.08 and 0.68 ± 0.07 with ASC from Group 1–0 and 1–12, respectively) without reaching significant difference. When no macroscopic lesion occurred, we compared the superficial blood flow recovery. No difference was observed comparing the injected and non-injected groups, whatever the day tested after femoral artery ligation (Figure 5c). In both groups, the blood flow similarly increased from day 0 to day 14. Finally, we assessed the microvascularization development in response to ischemia in both groups by measuring the pixel density on microangiography imaging (Figure 5d). The vascular density was significantly improved when pediatric ASC were injected; 0.87 ± 0.02 for Group 0–1 (n = 8) and 0.91 ± 0.03 for Group 1–12 (n = 15) compared with the control. Taken together pediatric ASC from both groups significantly improved neovascularization in ischemic tissue reaching 0.9 ± 0.02 (n = 23) versus 0.84 ± 0.01 in the control (n = 23; $P = 0.0184$). To sum up, no strong benefit was associated with pediatric ASC administration at the macroscopic level. Only an improvement in mircrovessel density was observed in the ischemic muscle, suggesting that the angiogenic potential is somehow sustained from pediatric to adult ASC.

Global gene expression profile between pediatric versus adult ASC

Due to the modest changes noticed in ASC during aging we developed a global approach to focus our attention on the possible significant variations in the gene expression program. Gene expression pattern for a total of 33297 genes was then assessed in microarray and only 217 and 88 were significantly down- and up-regulated in pediatric compared with adult ASC ($P < 0.05$ with fold change > log2; Figure 6a). The complete lists of genes sorted by log fold change are detailed in Supplementary Table S2 and Supplementary Table S3. The clustering of pediatric and adult ASC into two separated groups was obtained as expected, even though the P1 donor was closer to adults according to the gene expression profile (Figure 6a). Within each group a quite similar profile was obtained. Two KEGG pathways mapping molecular interaction and reaction between genes down-regulated in children were identified (Supplementary Table S4): the nucleotide oligomerization domain (NOD)-like receptor pathway ($P = 0.04$) and genes about circadian rhythm ($P = 0.05$). One pathway was found up-regulated and was related to the aminoacyl-tRNA biosynthesis pathway ($P = 0.008$). Differentially expressed gene sets could also be classified into biological processes (Figure 6b) such as biosynthesis (clustered in GO terms “tRNA aminoacylation for protein translation”, “amino acid activation” and “L-serine metabolic process”) that were up-regulated in pediatric versus adult ASC. On the contrary, several genes about immune response and stress cellular response were down-regulated (e.g., clustered in GO terms “positive regulation of response to external stimulus”, “response to lipopolysaccharide”, “inflammatory response” or “response to wounding”) in children. Up- and down-regulated genes involved in each identified GO term are detailed in Figure 6b. Taken together this analysis confirmed that only minor differences could be highlighted between pediatric and adult ASC according to their gene expression profile. These differences may be attributed to the age of the donor even though we may not exclude a role for gender, as due

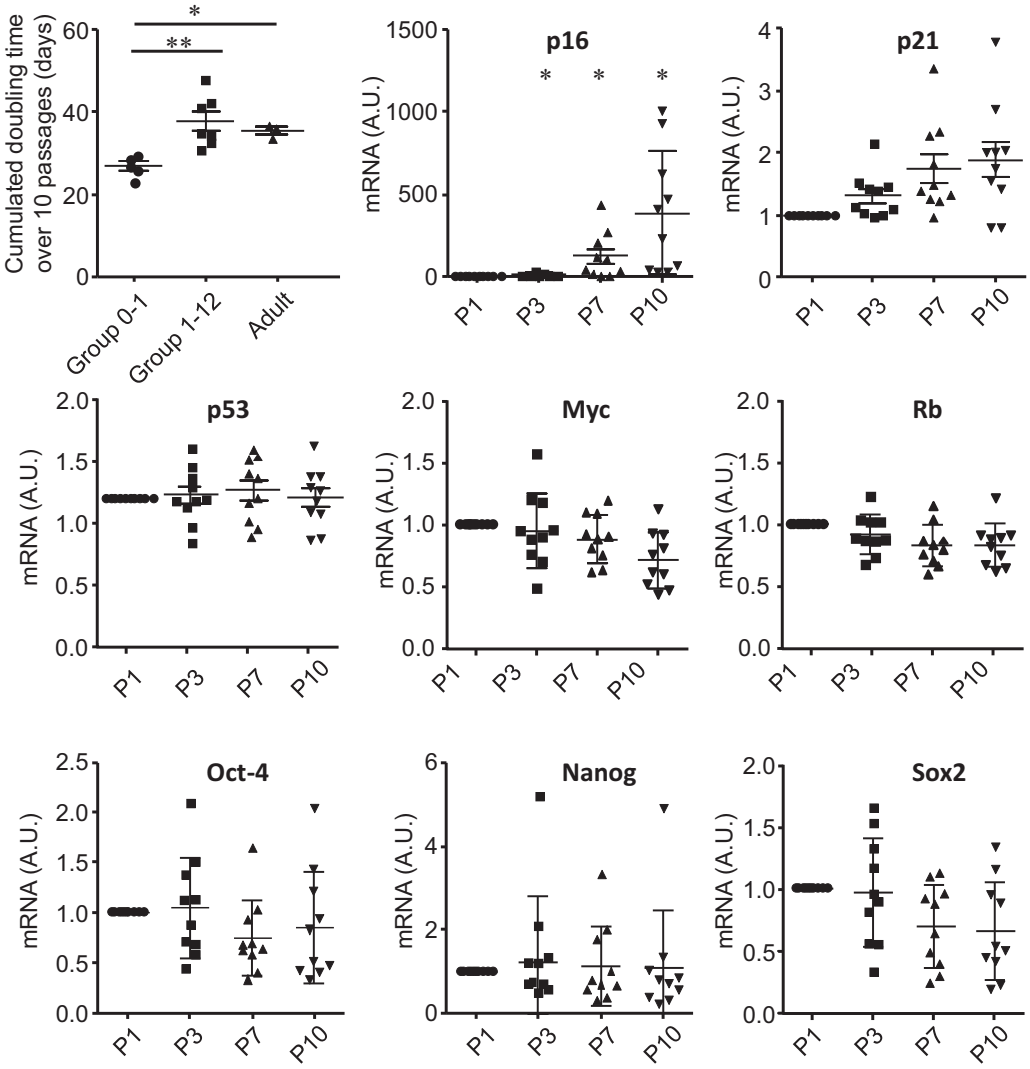

Figure 4. Effect of long-term culture on pediatric ASC. Doubling time was calculated every 2 to 4 days and the sum over the 10 passages corresponded with cumulative doubling time (first panel) n = 5 Group 0–1 (3 male and 2 female samples), n = 7 Group 1–12 (6 male and 1 female samples), n = 3 Adult group (female samples). Quantitative analyses of the expression of p16, p21, p53, Myc, Rb cell-cycle regulators and Oct-4, Nanaog and Sox2 stemness markers along passages, n = 10. *$P \leq 0.05$.

to genomic material availability, gene expression analyses were performed with ASC from pediatric males versus adult women.

Discussion

Studies reporting the potential of human ASC in various experimental models mainly focused on the use of ASC from adult donors (even elderly) [18] but information about pediatric cells are still lacking with only three previous studies [6–8]. In accordance with the unique comparative study [7] our data led to the conclusion that pediatric ASC have similar *in vitro* properties as adult ASC concerning extraction, *in vitro* expansion and immunosuppressive effect, based on 77 samples tested. Regarding functional assays and gene expression we also found that ASC from each group of donors displayed similar differentiation potentials into bone and adipose cells. This result is in agreement with the study by Guasti et al. [7] that has been enthusiastic concerning the possible use of pediatric ASC to reconstruct bone and cartilage defect or malformations. The authors also pointed out that differentiation should be strictly directed by defined medium as cells from young donors may easily engage into multiple lineages simultaneously.

In our hands pediatric ASC can express endothelial cells markers and can also support vascular network formation *in vitro* and *in vivo*. The *in vitro* angiogenic properties of pediatric ASC were similar to adult cells. This result correlates with the mRNA expression level of the angiogenic factor VEGF-A comparable

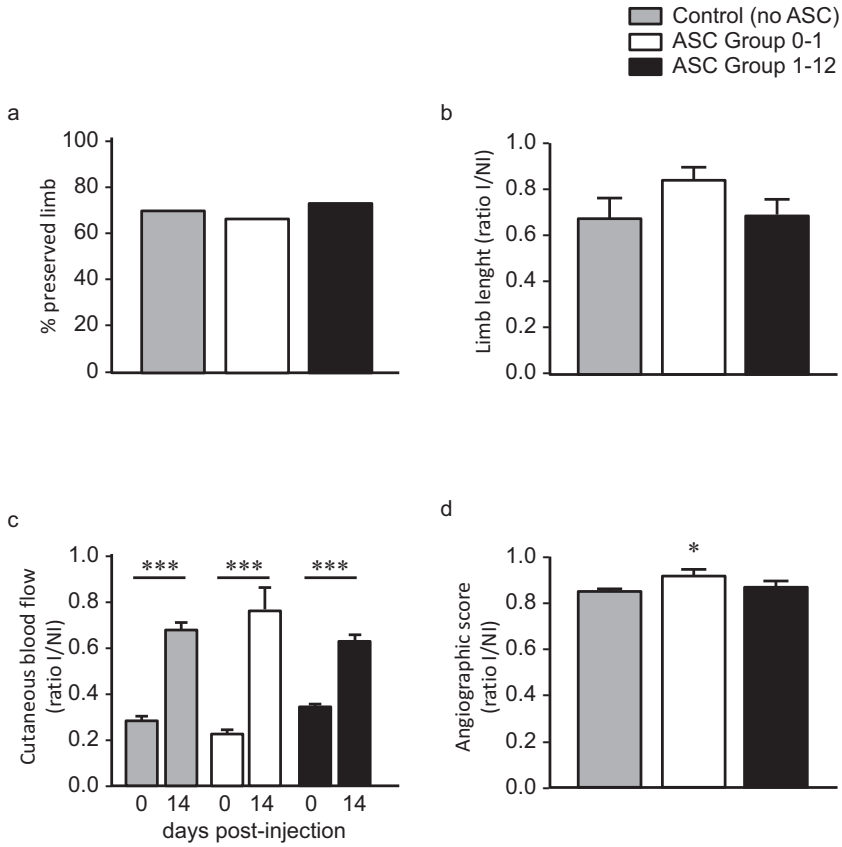

Figure 5. *In vivo* angiogenic properties of pediatric ASC. Injection of 1 x 10^6 pediatric ASC (n = 6 male samples) was performed in the ischemic mouse hind limb (n = 4–6 per ASC sample) in comparison with non-injected control animals (n = 31). Vascular function was analyzed by the percent of limb preservation (a), the length of ischemic limb (b), the quantitative evaluation of cutaneous blood flow measured by laser Doppler (c) and the angiographic score representative of the vessel density (d) expressed as the ratio of the ischemic (I)/the non-ischemic (NI) leg of each animal. *$P \leq 0.05$.

in children and adults, according to Affymetrix or reverse transcriptase (RT)-qPCR analyses. However, this observation differs from the findings by Wu et al. They reported that the expression level of VEGF and FGF-2 were higher in children than in adult and elderly samples and concluded that infant ASC represent a crucial source for angiogenesis and vasculogenesis [8]. The ASC culture conditions or individual donor status should be closely compared because they may generate the observed differences.

In the mouse model of limb ischemia the beneficial effect of ASC in neo-vascularization seemed to be weaker than the effect previously reported with adult ASC [19,20]. To clearly and definitively conclude on the *in vivo* effect, additional samples from children should be tested as due to the small amount of fat obtained it was not possible in the present study to systematically perform *in vivo* experiments with pediatric samples (n = 6 distinct samples tested). Nevertheless, among the parameters tested, the angiographic score was improved with pediatric ASC, which is encouraging. When considering the effect of aging, we reported that ASC from senior donors (>50 years old) showed a marked decrease in angiogenic potentials *in vitro* and *in vivo* as well compared with ASC from 20- to 35-year-old adults [13]. Taken together, this may suggest that pediatric and adult ASC display angiogenic properties that seem to be more efficient in adult cells but are severely impaired during aging.

To identify a possible specific signature in gene expression or representative markers of pediatric ASC compared with adult ASC, microarray experiments were performed. Minor changes **in genome**-wide **expression were highlighted because genes differentially expressed in children represented less than 1%**. Among the 88 genes up-regulated in children, some are related to the Y chromosome due to the fact that the pediatric cells analyzed came from boys whereas most of the adults were women. Thus, we have to consider that the differences observed in gene expression profile may be attributed either to the age and/or to the gender of the donors. The aminoacyl tRNA synthesis pathway up-regulation probably re-

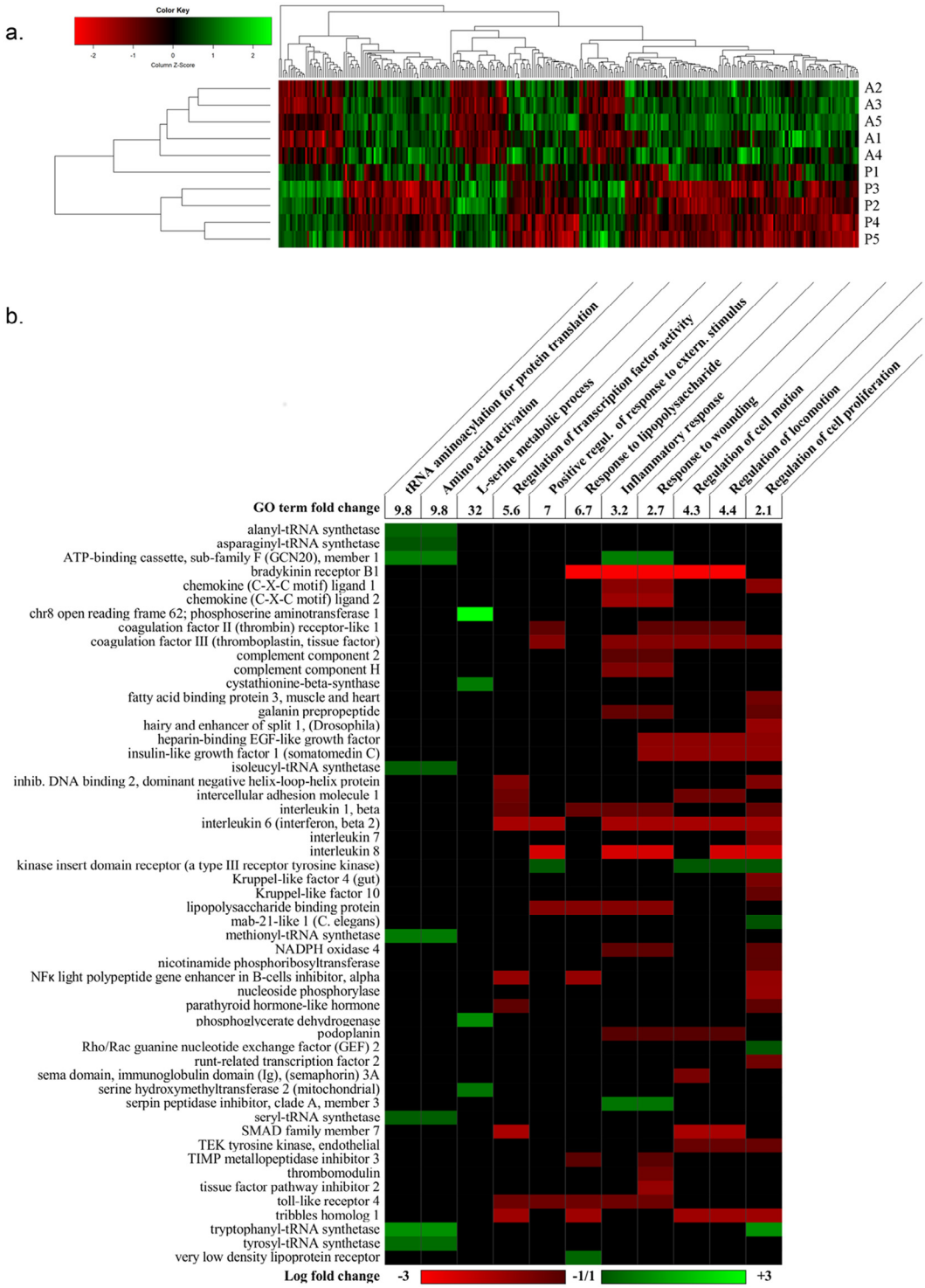

Figure 6. Gene expression profiling of ASC from pediatric and adult donors. Microarray data from 5 pediatric male (P1-5) and 5 adult female (A1-5) ASC samples were analyzed considering that genes were significantly ($P < 0.05$) differentially expressed when more than log2 1-fold was obtained and were represented in hierarchical cluster (a). The 53 genes involved in the significantly enriched GO terms about biological processes were detailed (b).

flects an elevated rate of protein synthesis in children. To the opposite genes related to inflammatory process including cytokines and chemokine expression, defense and wounding responses are down-regulated in children. This lower basal expression may reflect the fact that children probably faced less episodes of inflammation or wounding compared with adults. This hypothesis is at least supported and documented in

adults where the aging process is associated with an increase in the expression of inflammatory molecules and pathways in adipose tissue [21]. One major difference that distinguishes adult from pediatric donors is that adult adipose tissue is obtained from formerly obese donors who may have developed a sustained local inflammation signal in their adipose tissue. It is described that the obese environment alters *in vitro* properties of ASC [22]. The loss of tissue homeostasis during obesity can then affect ASC biology. These findings in addition to our present results suggest that this *in situ* conditioning seems to be maintained beyond the culture process. Accordingly, ASC from morbid obesity patients are demonstrated to secrete higher concentrations of IL-8 and IL-6 in the culture medium compared with the control [23]. Both cytokine expressions are found significantly down-regulated in children (–2.49 and –1.95 log fold change, respectively), suggesting that their high level in adults may be linked to a disorder in their metabolic status. To confirm this hypothesis, adult lean donors could be compared with our adult group.

To extend our comparison to other sources of mesenchymal stromal cells (MSC), such as bone marrow, various fetal tissues, dermis and dental pulp, among many others [24], studies of age-related MSC properties mostly refer to the aging process in adult cells. It is widely reported that MSC decrease in their immunomodulatory and wound-healing abilities in the elderly, together with reduction of proliferative and differentiation capacities and an increase in oxidative stress and in senescence markers [25,26]. Very few data are available on pediatric MSC properties *in vitro*. However, the use of MSC for clinical applications in children is already reported, showing promising results for further developments in the treatment of graft-versus-host disease for example [27], in osteoarticular diseases [28] and in tissue engineering [29,30].

The question of gender has to be closely stated because pediatric samples collected were from males and adult samples were from a group of almost all females. A possible gender bias has to be considered. Except for the higher SVF cell number obtained in Group 0–1 (Figure 1a) where significant differences were still maintained when separating male and female data within the three groups of age (data not shown), all the other conclusions have to be cautious as we did not have enough samples to systematically separate male and female in our statistical analyses. Thus, we may not completely exclude that some significant differences observed might be due to the gender instead/or in addition to the age. However, any absence of difference might also be due to the gender. Additional female pediatric and male adult samples are necessary to validate or invalidate such possibility. Unfortunately adult male samples represented less than 10% and it was not possible to enroll more girls with appropriate surgical indications during the 2-year period of inclusion in our studies.

In conclusion, our study reports that pediatric ASC share similar *in vitro* properties as adult ASC. Samples collected from the youngest donors just have a slightly higher proliferation rate than older ones. Angiogenic properties are maintained in both groups of donors although pediatric ASC appeared to be less effective *in vivo* than previously reported with adult ASC in ischemic limb. Further investigations are required to better explore the functional properties of pediatric ASC to confirm this difference in efficacy and to determine if a pre-conditioning of pediatric ASC before administration may be of interest to enhance their therapeutic benefit. Complementary studies to increase the number of female pediatric and male adult samples would be necessary to firmly state the effect of the gender comparison with the effect of the age of the donors.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jcyt.2016.11.012.