

Human pericytes isolated from adipose tissue have better differentiation abilities than their mesenchymal stem cell counterparts

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Abstract Multi-potent mesenchymal stem/progenitor cells are present in almost all organs and tissues, although their identity remains elusive. Several isolation strategies have been pursued to identify these cells prospectively, leading to the isolation of various cell populations endowed with multi-lineage mesodermal potential. Historically, mesenchymal stem cells (MSCs) were the first cell population to be isolated from the stromal fraction of most connective tissues. These cells are able to differentiate towards various mesodermal lineages and are currently the most studied adult mesodermal progenitors. Recently, the isolation of a subpopulation of microvascular pericytes (PCs) endowed with multi-lineage mesodermal potential has led to the identification of mesenchymal progenitors that reside in a defined anatomical location, namely the wall of small blood vessels. To gain insight into these two related cell populations, we performed a detailed analysis of the mesodermal potential of isogenic human MSCs and PCs isolated from white adipose tissue. Although both cell populations expressed known mesodermal markers at similar levels and displayed a comparable growth rate, PCs differentiated towards osteocytes, adipocytes and myocytes more efficiently than their MSC counterparts, as revealed by both histological and molecular assays. Our results show that microvascular PCs are more prone to mesenchymal differentiation than MSCs and therefore represent a preferable source of human adult mesenchymal progenitors when adipose tissue is used as a cell source.

Keywords Pericytes · Mesenchymal stem cells · Adipose tissue · Differentiation · Mesodermal lineage · Human

Introduction

Connective tissues maintain their homeostasis because of the presence of resident undifferentiated progenitor cells that guarantee cellular turnover throughout life. In humans, these progenitors were isolated for the first time from bone marrow (Pittenger et al. 1999) and later, following similar isolation procedures, from connective tissues and organs such as adipose tissue, dermis, cord blood, liver, or pancreas (Zuk et al. 2001, 2002; Toma et al. 2001; In't Anker et al. 2004; Beltrami et al. 2007; Gallo et al. 2007). Based on their differentiation abilities, these cells are usually referred to as mesenchymal stem cells (MSCs). Although several alternative protocols have been further developed to obtain multi-potent progenitors from various tissues, “standard” MSCs are currently defined as adult multi-potent progenitors that are isolated following the enzymatic digestion of the stromal fraction of organs and further selected as plastic-adherent cells (Baksh et al. 2004; Da Silva Meirelles et al. 2008). More recently, a distinct human adult stem cell population endowed with multi-mesodermal potential has been identified in the wall of small blood vessels and isolated following explant cultures and/or fluorescence-activated cell-sorting (FACS) purification of the vascular portion of various organs and tissues (Dellavalle et al. 2007; Crisan et al. 2008). These progenitors have been further identified both by in vivo and in vitro approaches as microvascular pericytes (PCs). Although MSCs have been proposed to represent a subpopulation of pericytes (Caplan 2008), lineage-tracing experiments that definitively support this hypothesis have not as yet been performed, because of the absence of highly selective markers (Bianco 2011; Feng et al. 2011). Accordingly, MSCs and PCs

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are currently considered as two closely related multi-potent cell populations that are present in several adult tissues and that can be isolated by using various experimental protocols and expanded under various culture conditions.

Both MSCs and PCs are able to differentiate towards mature cells of mesodermal origin such as adipocytes, osteocytes, chondrocytes and muscle cells (Pittenger et al. 1999; Crisan et al. 2008). Several studies indicate that the differentiation abilities of MSCs result from the presence of a heterogeneous mixture of cells with null-, single-, or multi-lineage potential (Muraglia et al. 2000; Guilak et al. 2006; Russell et al. 2010). This implies that the overall differentiation potential of MSCs reflects the effective frequency of these progenitors in a given MSC population (Phinney 2012). In contrast, PCs are a more homogeneous cell population consisting in a pool of progenitors with equal or comparable multi-lineage differentiation abilities. However, this latter aspect has been formally demonstrated only in PCs from human skeletal muscle (Crisan et al. 2008). Regardless of the relative frequency of clonal multi-potent progenitors, whose number and distribution can vary across different adult tissues (Manini et al. 2011), preliminary evidence that MSCs and PCs differ in their overall differentiation abilities has been obtained in a study in which MSCs isolated from bone fragments and PCs isolated from skeletal muscle have been compared (Roobrouck et al. 2011). Actually, the direct quantitative analysis of the differentiation abilities of MSCs and PCs isolated from the same tissue might improve our current knowledge of the differentiation properties of these cell populations. A major goal of the current research on human adult stem cells is to provide multi-potent cells that can be isolated with minimal invasive procedures and that retain robust differentiation abilities. Among adult tissues, adipose tissue is widely available, easy to access and loaded with a conspicuous number of multi-potent cells, thus representing a good source from which progenitor cells can be isolated and used for experimental and clinical applications (Orbay et al. 2012). Here, we report the detailed analysis of the mesodermal differentiation properties of isogenic MSCs and PCs prepared in parallel from biopsies of human white adipose tissue. Our results indicate that PCs have better differentiation abilities than their MSC counterparts, thus suggesting that, when adipose tissue is used as a source of multi-potent mesodermal progenitors, the isolation of microvascular PCs should be preferred to that of standard MSCs.

Materials and methods

Cell isolation and culture

All biopsies were taken following informed consent obtained from patients who underwent diagnostic surgery (later classified as “non-pathologic”) at the Division of Obstetrics and

Gynecology and at the Molecular Medicine Section of the University of Siena. All reagents were purchased from Sigma-Aldrich if not otherwise specified. To reduce genetic and sampling variability, MSCs and PCs were derived from the same subcutaneous white adipose tissue biopsy. MSCs were isolated as previously described (Pierantozzi et al. 2011). Briefly, a portion of each biopsy was digested by collagenase type IA and filtered through 100- μ m and 70- μ m cell strainers. The resultant single cell suspension was plated in proliferation medium consisting of alpha-minimum essential medium (α -MEM; Lonza), supplemented with heat-inactivated 10 % fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin (Lonza). PCs were isolated from the remaining part of each adipose tissue biopsy (Fig. 1a) as previously described, with minor modifications (Tonlorenzi et al. 2007). Briefly, adipose tissues were mechanically dissected into small pieces that were transferred to collagen-coated Petri dishes. After at least 4 h of adherence, growing medium consisting of Dulbecco’s modified Eagle’s medium (DMEM)-mega-cell supplemented with 5 % FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1 % non-essential amino acids, 0.1 mM β -mercaptoethanol and 5 ng/ml basic fibroblast growth factor (Peprotech) was added. At 8–12 days after plating, both floating and weakly adherent cells that emerged from the explants were transferred to a new non-coated dish. This step was considered as passage 1. All cell populations were trypsinized and transferred to a new dish when they reached 70 %–80 % confluence. Population doublings (PD) were calculated by using the following formula: $PD = \ln 2 / (\ln C2) / (\ln C1)$, where C1 represents the number of cells seeded at a given passage and C2 the number of cells harvested at the next passage, as previously described by Manini et al. (2011). As a control for some experiments, PCs from the perimysium of the *vastus lateralis* muscle were isolated and cultured by using the above-described procedures.

In vitro myogenic, osteogenic and adipogenic differentiation

Skeletal muscle differentiation was induced by seeding 1×10^4 cells/cm² onto dishes coated with Matrigel (growth-factor-reduced; BD), referred to in the text as “spontaneous”-12 days of differentiation, or by co-culturing MSCs and PCs with C2C12 mouse myoblasts or L6 rat myoblasts at a ratio of 1:4, referred to in the text as “co-culture”-10 days of differentiation. In both protocols, differentiation medium consisted of DMEM supplemented with heat-inactivated 2 % horse serum (Euroclone), 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μ g/ml streptomycin (D2 medium).

Smooth muscle differentiation (8 days of differentiation) was induced by seeding 1×10^4 cells/cm² onto Matrigel-coated dishes in D2 medium supplemented with transforming

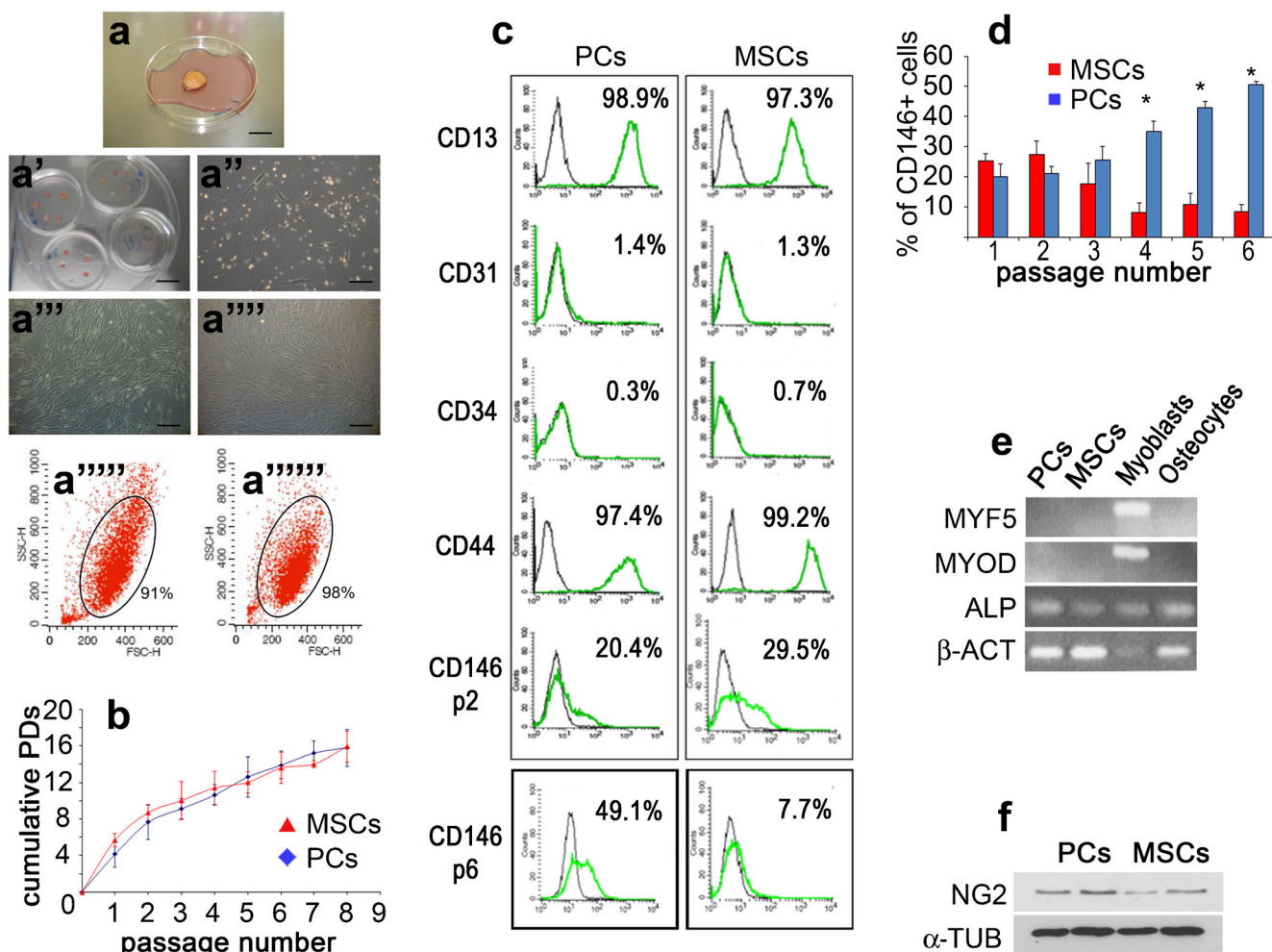


Fig. 1 Adipose-tissue-derived pericytes (PCs) and mesenchymal stem cells (MSCs) share common mesenchymal and pericytic markers. **a–a''''''** Each biopsy (**a**) was divided in two pieces and PCs and MSCs were isolated by explant-cultures and enzymatic digestion protocols, respectively (**a'**, **a''**). Representative micrographs (**a'''**, **a''''**) and FSC-H/SSC-H plots (**a'''''**, **a''''''**) of PCs and MSCs are shown. Bars 21 mm (**a**, **a'**), 100 μm (**a'''–a''''**). **b** Growth curves of PCs and MSCs (PD population doubling; $n=4$ for both cell populations). **c** Representative plots of flow cytometry analysis of PCs and MSCs at passage two ($p2$) for CD13, CD31, CD34, CD44 and CD146 surface antigens. CD146 expression was also evaluated at passage six ($p6$; bottom panels). The percentage of positive cells (green peak) for each marker is

shown. Ig isotype-matched antibodies (black peak) were used to set basal fluorescence. **d** Analysis of CD146+ cells from $p1$ to $p6$ in MSCs and PCs ($n=3$ for both cell populations). Values are expressed as means of positive cells \pm SEM; * $P<0.05$, Two-way analysis of variance. **e** Reverse transcription plus polymerase chain reaction (RT-PCR) analysis of MYF5, MYOD and alkaline phosphatase (ALP) expression. Human myoblasts and terminally differentiated osteocytes were used as positive and negative controls for MYF5 and MYOD, respectively. β -Actin (β -ACT) was used as normalization internal standard. **f** Western blot analysis of expression of NG2 (a proteoglycan) in two PC populations and two MSC populations. α -Tubulin (α -TUB) was used as a loading control

growth factor- β (5 ng/ml; R&D) that was freshly added every other day. To visualize differentiated skeletal myotubes or smooth-muscle-differentiated cells, cells grown on Matrigel-coated glass slides were fixed with 3 % (v/v) paraformaldehyde, permeabilized with HEPES-Triton buffer (HEPES 20 mM, sucrose 300 mM, $MgCl_2$ 3 mM, Triton X-100 0.5 %) saturated with 10 % FBS or normal goat serum and incubated with the following primary antibodies: monoclonal anti- α -actinin (30 μg/ml), polyclonal anti-SM22- α (Abcam, 0.5 μg/ml), monoclonal anti-MyoD (Abcam, 20 μg/ml). In some experiments, anti- α -actinin and anti-MyoD were directly

conjugated with Alexa Fluor 647 and Alexa Fluor 488, respectively, following the manufacturer's instructions (Life Technologies). Nuclei were visualized by 6-diamidino-2-phenylindole (DAPI; Calbiochem) or propidium iodide (PI) staining. In co-culture experiments, human nuclei were counter-stained with monoclonal anti-human lamin A/C (Novocastra, 1:50). Specific secondary antibodies (anti-mouse IgG conjugated to cyanine2, 1:5000; anti-goat IgG conjugated to cyanine3, 1:20,000; both purchased from Jackson Immuno-Research Laboratories) were used to reveal bound primary antibodies. Slides were analyzed with a LSM-510 META

confocal microscope (Zeiss, Jena, Germany). In co-culture experiments, the percentage of myogenic efficiency was calculated as the number of human nuclei inside α -actinin-positive cells and myotubes, divided by the total number of nuclei inside the myotubes. In each experiment, more than two hundred nuclei inside myotubes were scored out of seven to fifteen microscopic fields randomly chosen among three different glass-slides. Microscopic fields without α -actinin-positive myotubes were not scored. Each experiment was performed twice with three different cell populations of PCs and of MSCs.

Osteogenic differentiation (21 days of differentiation) was induced by plating 2×10^4 cells/cm² in the presence of osteogenic medium consisting of α -MEM supplemented with 10 % FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.1 μ M dexamethasone, 50 μ M ascorbate-2-phosphate and 10 mM β -glycerophosphate. The production of the extracellular calcified mineral matrix was visualized by Alizarin-Red staining. Quantification of osteogenic differentiation was performed by Alizarin-Red extraction as previously described (Manini et al. 2011).

Adipogenic differentiation (14 days of differentiation) was induced by plating 2×10^4 cells/cm² in the presence of adipogenic medium consisting of α -MEM supplemented with 10 % FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.5 mM isobutyl-methylxanthine, 1 μ M dexamethasone, 10 μ M insulin and 200 μ M indomethacin. The presence of lipid droplets was visualized by Oil-red O staining. Quantification of adipogenic differentiation was performed by Oil-Red-O extraction as previously described (Manini et al. 2011).

RNA extraction, reverse transcription and polymerase chain reaction amplification

Total RNA was extracted from proliferating and differentiating cell populations by using the RNeasy kit (Qiagen) following the manufacturer's instructions. From each RNA sample, 200 ng were reverse-transcribed by using the High Capacity cDNA reverse transcription kit following the manufacturer's instructions (Applied Biosystems). Target cDNAs were amplified for 30 cycles at pair-specific annealing temperatures by using the following primers: MYF5 (Fw 5'-CTATAGCCTGCCGGACA-3'; Rev 5'-TGGACCAGACAGGACTGTTCAT-3'); MYOD (Fw 5'-GAAGCTAGGGGTGAGGAAGC-3'; Rev 5'-CCCGGCTGTAGATAGCAAAG-3'); ALP (Fw 5'-CCTCCTCGGAAGACACTCTG-3'; Rev 5'-GCAGTGAA GGGCTTCTTGTC-3'); β -ACTIN (Fw 5'-CAACTCCATCATGAAGTGTGAC-3'; Rev 5'-GCCATGCCAATCTCATCTTG-3'); peroxisome proliferator-activated receptor- γ (PPAR- γ ; Fw 5'-TTCTCCTATTGACCCAGAAAGC-3'; Rev 5'-TCCACTTTGATTGCACTTTGG-3'); runt-related transcription factor (RUNX2; Fw 5'-GCAGCACGCTATTA

AATCCAA-3'; Rev 5'-ACAGATTCATCCATTCTGCCA-3'); Osterix (OSX; Fw 5'-GCCAGAAGCTGTGAAACCTC-3'; Rev 5'-GCAACAGGGGATTAACCTGA-3'); LincMD1 (Fw 5'-CACTGCCAGCTCTGGAAAAT-3'; Rev 5'-ACTTGGTTCCGTTTGACCAG-3'). MicroRNAs were extracted and purified from proliferating cell populations by using the RNeasy mini kit (Qiagen), following the manufacturer's instructions. The concentration of the microRNA-enriched fraction was evaluated by NanoDrop ND-1000. MicroRNAs were converted into single-strand cDNA with the miScript II RT kit (Qiagen), following the manufacturer's instructions. cDNA samples were amplified in triplicate by using the miScript SYBR Green PCR kit (Qiagen). Real-time quantitative PCR analysis was performed on a StepONE Plus detection system (Applied Biosystems) and the relative microRNA expression was calculated by using the Pfaffl quantification method (Pfaffl 2001). Primers used in this set of experiments were the following: hsa-miR-1: 5'-UGGAAUGUAAAGAAGU AUGUAU-3'; hsa-miR-133b: 5'-UUUGGUCCCCUACA ACCAGCUA-3'; hsa-miR-206: 5'-UGGAAUGUAAGGAA GUGUGUGG-3' (Qiagen, miScript Pimer Assay); with hsa-RNU6-2 (Qiagen) as an internal control.

Protein extraction and Western blotting

Pellets of undifferentiated or differentiated cells were lysed in RIPA buffer (TRIS 50 mM, NaCl 150 mM, SDS 0.1 %, sodium deoxycholate 0.5 %, Triton X-100 or NP40 1 %) containing a protease inhibitor cocktail for 30 min at 4 °C and further disrupted through sonication. Insoluble materials were removed by centrifugation at 18,000 relative centrifugal force for 10 min at 4 °C and protein concentration was measured by Bradford's protein assay (Bio-Rad). Proteins (50 μ g) were loaded onto 10 or 12 % SDS-polyacrylamide gel for electrophoresis and then transferred onto polyvinylidene difluoride membrane. The membranes were blocked in 5 % no-fat milk in TRIS-buffered saline and Tween 20 for 1 h at room temperature and incubated overnight with the following primary antibodies: polyclonal anti-NG2 (Chemicon, 2 μ g/ml), polyclonal anti-SM22- α (Abcam, 0.1 μ g/ml), monoclonal anti- α -tubulin (ascite fluid, 1:5000). Anti-rabbit, anti-goat and anti-mouse IgG antibodies conjugated to horseradish peroxidase (Ge-Healthcare, 1:3000) were used to detect immunoreactive bands by SuperSignal West Pico chemiluminescent reagent (Pierce). Protein expression levels of SM22- α were quantified by optical densitometry by using ImageJ software.

Flow cytometry

Proliferating cells were analyzed by fluorescence-activated cell-sorting (FACS-Aria, BD) at early (p1–p3) and late passages (p4–p8). Cells were washed and re-suspended in ice-cold PBS

containing 0.5 % bovine serum albumin (FACS buffer). Cells (1×10^5) were incubated for 30 min at 4 °C with the following monoclonal antibodies: phycoerythrin (PE)-conjugated CD13 (DAKO, 1:50); PE-conjugated CD31 (Santa Cruz, 1:50); allophycocyanin (APC)-conjugated CD34 (BD, 1:50); APC-conjugated CD44 (e-Bioscience, 1:50); fluorescein isothiocyanate (FITC)-conjugated CD45 (BD, 1:50); PE-conjugated CD146 (BD, 1:50). Identical Ig isotypes conjugated to PE, FITC, or APC were used to set the fluorescence background.

Statistics

Statistical analysis was performed by using the Student's *t*-test or the two-way analysis of variance by using GraphPad Prism6 software. Results are presented as means \pm SEM; a *P*-value less than 0.05 was considered statistically significant.

Results

Characterization of MSCs and PCs from white adipose tissue

To avoid individual variability, MSCs and PCs were isolated in parallel from the same biopsy of human white adipose tissue ($n=4$) as detailed in [Materials and methods](#) (Fig. 1a–a’). All cell populations were grown in culture for at least eight passages and displayed a similar growth rate (Fig. 1b). They also had comparable cell morphology when observed under a light microscope and were found in the same gated-region when analyzed by FACS (Fig. 1a’’–a’’’’’). MSCs and PCs were analyzed for the expression of known mesenchymal and pericytic markers. As reported in Fig. 1c, FACS analysis showed that both cell populations uniformly expressed CD13 and CD44 antigens. MSCs and PCs did not express endothelial markers such as CD31 and CD34. The percentage of cells expressing the CD146 antigen was similar in both cell populations at early passages (p1–p3). However, starting from passage four, the percentage of CD146-positive cells was markedly decreased in MSCs, whereas it was maintained or even increased in PCs (Fig. 1c [bottom panels], d). RT-PCR and Western blot experiments indicated that both cell populations expressed alkaline phosphatase (ALP), a classic marker of mesodermal precursors (Fig. 1e) and NG2, a proteoglycan known to be expressed by perivascular mural cells (Crisan et al. 2009; Fig. 1f). Finally, no analyzed MSCs and PCs expressed the muscle-specific transcription factors MYOD and MYF5 (Fig. 1e).

Analysis of mesodermal differentiation potential of MSCs and PCs

To analyze the mesodermal differentiation potential of MSCs and PCs, cell populations were induced to differentiate towards adipogenic, osteogenic and myogenic lineages. Adipogenic and osteogenic differentiation were first assessed by histological staining after 14 and 21 days of differentiation, respectively. Indeed, lipid droplets were visualized by Oil-Red-O staining, whereas the apposition of the extracellular calcified mineral matrix was detected by Alizarin-Red staining. As shown in Fig. 2a, b, MSCs and PCs were both able to differentiate into adipocytes and osteocytes. However, quantitative analysis of cell differentiation performed by means of dye-extraction indicated that PCs were able to accumulate a total amount of lipids that was about 15-fold higher than that accumulated by MSCs (Fig. 2a’’). Similarly, the amount of extracellular mineral matrix produced by PCs was about 30-fold higher than that measured with MSCs (Fig. 2b’’), thus suggesting that PCs can differentiate towards adipogenic and osteogenic lineages more efficiently than MSCs. We previously observed that MSCs but not PCs, showed a decrease in CD146 expression after 4–6 passages in culture (Fig. 1d). We thus wondered whether the reduction in CD146-positive cells correlated with differences in the adipogenic and osteogenic abilities of MSCs and PCs at later passages. However, quantitative analysis of both adipogenic and osteogenic differentiation demonstrated no significant differences between early and late passages of both cell populations (data not shown). The myogenic potential of MSCs and PCs was analyzed by using standard protocols for smooth and skeletal muscle differentiation (see [Materials and methods](#) for details). MSCs and PCs readily differentiated into smooth muscle cells as revealed by both immunostaining and immunoblotting with an antibody against SM22- α (Fig. 2c–c’’). On the contrary, following the induction of skeletal muscle differentiation, neither MSCs nor PCs were able to fuse and form α -actinin-positive myotubes (Fig. 2d–d’’, e–e’’). However, when co-cultured with L6 rat myoblasts or C2C12 mouse myoblasts, PCs but not MSCs, were recruited to form myotubes with a recorded myogenic efficiency of 7 % and 5 %, respectively (Fig. 2g–g’’, h–h’’, j; data not shown). To verify the myogenic conversion of PCs but not of MSCs, MYOD expression in human cells co-cultured with either L6 or C2C12 was analyzed following the induction of myogenic differentiation. MyoD was detected by immunofluorescence in the nuclei of PCs, as identified with an antibody against human lamin A/C (Fig. 2k–k’’, l–l’’) but not in those of MSCs co-cultured with either L6 or C2C12 cells (Fig. 2m–m’’, n–n’’), further supporting that only PCs are endowed with myogenic potential that can be unmasked under appropriate conditions. As a control for in vitro myogenic differentiation, we used pericytes isolated from skeletal muscle (SK) tissue. Notably, SK-PCs were more

efficient than MSCs and PCs prepared from adipose tissue in fusing and forming α -actinin-positive myotubes in both spontaneous and co-culture differentiation conditions (Fig. 2f–f”, i–i”).

Molecular analysis of adipogenic, osteogenic and myogenic differentiation of MSCs and PCs

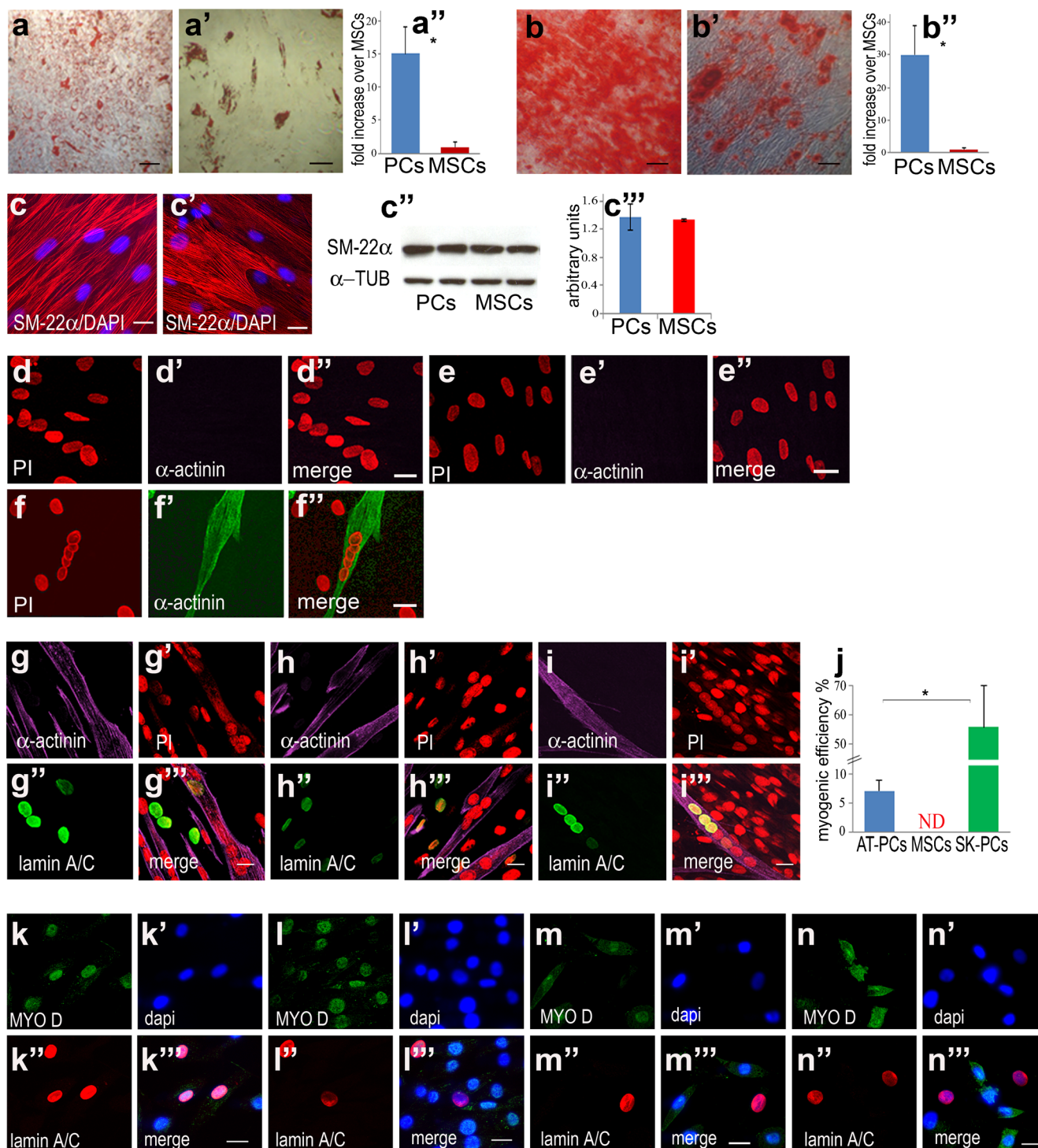
Next, the adipogenic, osteogenic and myogenic differentiation of MSCs and PCs was analyzed at the molecular level. Accordingly, the expression of early genes involved in the onset of adipogenesis and osteogenesis was analyzed by quantitative RT-PCR in PCs (Fig. 3a–c) and MSCs (Fig. 3d–f) at various time points: (1) before the induction of differentiation; (2) 2 days after the induction of adipogenesis; (3) 12 days after the induction of osteogenesis.

Expression of PPAR γ , one of the main adipogenic transcription factors, did not significantly differ between undifferentiated PCs and MSCs (data not shown). After 2 days of adipogenic induction, we observed a 22-fold increase of PPAR γ expression in PCs compared with a 10-fold increase in MSCs (Fig. 3a, d). The early expressed osteogenic transcription factor RUNX2 was expressed at similar basal levels in both undifferentiated MSCs and PCs and the presence of OSX was detectable in undifferentiated MSCs, whereas it was nearly negligible in undifferentiated PCs (data not shown). After 12 days of osteogenic differentiation, the expression of RUNX2 and OSX in PCs increased by 13-fold and 5-fold, respectively, with respect to undifferentiated cells (Fig. 3b, c). On the other hand, in MSCs, we observed a 7-fold increase of only RUNX2 expression, whereas OSX expression decreased (Fig. 3e, f). According to the results obtained on spontaneous skeletal muscle differentiation, the expression of the early muscle-specific genes MYOD and MYF5 was undetectable in both MSCs and PCs following 3 days of myogenic differentiation (data not shown), further validating that neither MSCs nor PCs can efficiently support myogenic differentiation. To understand this point, we analyzed the expression of three major pro-myogenic microRNAs, namely mir-1, mir-133 and mir-206 (Townley-Tilson et al. 2010) and the expression of a recently discovered pro-myogenic long non-coding RNA, namely linc-MD1 (Cesana et al. 2011), in undifferentiated MSCs and PCs. Indeed, quantitative RT-PCR experiments showed that both cell populations expressed extremely low levels of mir-1, mir-133, mir-206 and linc-MD1 (Fig. 3g, h) with respect to those measured in undifferentiated pericytes from skeletal muscle that were used herein as references.

Fig. 2 Qualitative and quantitative analysis of adipogenic, osteogenic and myogenic differentiation abilities of PCs and MSCs. **a–a”** Representative micrographs of Oil-Red-O staining of lipid droplets in differentiated PCs (**a**) and MSCs (**a”**), with quantification of bound Oil-Red-O being reported in the *histogram right* (**a”**). **b–b”** Representative micrographs of Alizarin-Red staining of extracellular calcified mineral matrix in differentiated PCs (**b**) and MSCs (**b”**), with quantification of bound Alizarin-Red being reported in the *histogram right* (**b”**). Normalized dye content in MSCs was arbitrarily set as “1”. Values are expressed as the fold increase of PCs over MSCs (\pm SEM). Biological replicates: 3; experimental replicates: 3. * $P < 0.05$, Student’s *t*-test. **c–c”** Representative micrographs of SM22- α immunostaining in PCs (**c**) and MSCs (**c’**). Nuclei were counter-stained with DAPI (*blue*). SM22- α protein expression was determined by Western blot analysis (**c”**) and quantified by densitometric analysis (**c”**). α -Tubulin (α -*TUB*) was used as a loading control. **d–f”** Spontaneous skeletal myogenic differentiation of adipose tissue PCs (**d–d”**), adipose tissue MSCs (**e–e”**) and skeletal muscle PCs (**f–f”**) was visualized by α -actinin staining (*green*) of differentiated cells and/or myotubes (**d’**, **e’**, **f’**). All nuclei were counter-stained with propidium iodide (*PI*; **d**, **e**, **f**). Merged images are shown in **d”**, **e”**, **f”**. **g–j** Myogenic differentiation was also induced by co-culturing adipose tissue PCs (**g–g”**), adipose tissue MSCs (**h–h”**) and skeletal muscle PCs (**i–i”**) with L6 rat myoblasts. Differentiating cells or myotubes were visualized by α -actinin staining (*violet*; **g**, **h**, **i**). Human nuclei were visualized by counter-staining with human lamin A/C (**g”**, **h”**, **i”**). Both rat and human nuclei were stained with PI (**g’**, **h’**, **i’**). Merged images are shown in **g”**, **h”**, **i”**. **j** Myogenic differentiation efficiency in each co-culture experiment was calculated as described in [Materials and methods](#). * $P < 0.05$, Student’s *t*-test (*ND* not detected, *AT-PCs* adipose tissue PCs, *MSCs* adipose tissue MSCs, *SK-PCs* skeletal muscle PCs). **k–n”** MyoD immunostaining of PCs co-cultured with either L6 (**k–k”**) or C2C12 (**l–l”**) and MSCs co-cultured with either L6 (**m–m”**) or C2C12 (**n–n”**). Anti-MyoD antibody recognized both human and rodent protein (**k**, **l**, **m**, **n**). Human nuclei were visualized by counter-staining with human lamin A/C (**k”**, **l”**, **m”**, **n”**). All nuclei were stained with DAPI (**k’**, **l’**, **m’**, **n’**). Merged images are shown in **k”**, **l”**, **m”**, **n”**. *Bars* 100 μ m (**a**, **a’**, **b**, **b’**), 20 μ m (**c–n”**)

Discussion

Although mesenchymal stem/progenitor cells are present in the stromal fraction of all human organs, their identity remains poorly defined. Bone marrow was the first tissue from which mesenchymal progenitors were isolated and it is currently the elective tissue for obtaining multi-potent cells used for clinical applications (Trounson et al. 2011). By adapting the original isolation procedure developed in bone marrow, various types of stroma-residing progenitors have been isolated from virtually all organs (Da Silva Meirelles et al. 2006). At present, however, MSCs prepared following the enzymatic digestion of tissue and selection of plastic-adherent cells remain the most characterized adult mesodermal progenitors. More recently, multi-potent perivascular-residing cells, namely PCs, have been identified by exploiting an alternative isolation procedure (Dellavalle et al. 2007; Crisan et al. 2008). Nevertheless, although both cell populations are well known as being able to differentiate towards distinct mesodermal lineages, whether MSCs and PCs have distinct or equivalent differentiation potential is still an open question (Bianco and Cossu 1999; Da Silva Meirelles et al. 2008; Caplan 2008;



Roobrouck et al. 2011). In this study, we analyzed and compared isogenic MSCs and PCs isolated in parallel from the same biopsy of human white adipose tissue. Because of its wide availability and its less invasive harvesting procedure compared with that of bone marrow, adipose tissue has gradually emerged as a valid alternative source of undifferentiated mesodermal progenitors that can also be used in clinical

applications. We isolated MSCs from the stromal fraction of the tissue according to the original isolation procedure developed by Zuk and colleagues (2002), whereas PCs were obtained following culture of explants of the vascularized portion of the tissue, as previously described (Tonlorenzi et al. 2007). We found that the two isogenic cells populations are similar in terms of growth rate and the expression pattern of

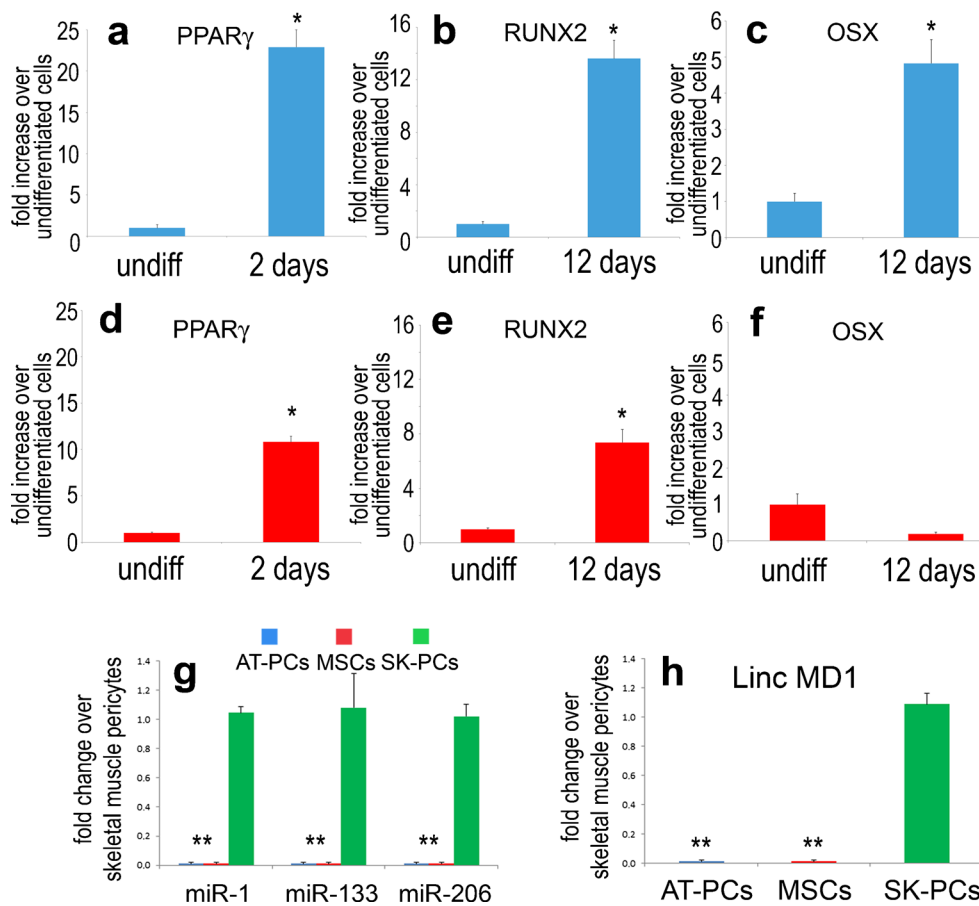


Fig. 3 Quantitative RT-PCR analysis of adipogenic, osteogenic and myogenic factors. The expression of peroxisome proliferator-activated receptor- γ (*PPAR* γ ; **a**, **d**), runt-related transcription factor (*RUNX2*; **b**, **e**) and Osterix (*OSX*; **c**, **f**) was analyzed in both adipose tissue PCs (**a–c**) and MSCs (**d–f**) in: (1) undifferentiated cells (*undiff*), (2) differentiating cells 2 days after the induction of adipogenesis (2 days) and (3) differentiating cells 12 days after the induction of osteogenesis (12 days). The expression of target genes in undifferentiated cells was arbitrarily set as “1”. Data are reported as the fold increase of target transcription in differentiating cells over undifferentiated cells, as calculated by using the Pfaffl quantification method (Pfaffl 2001). * $P < 0.05$, Student’s *t*-test. **g**

Expression of microRNAs miR1, miR133 and miR206 was analyzed in undifferentiated PCs from adipose tissue (*AT-PCs*) and mesenchymal stem cells from adipose tissue (*MSCs*). MicroRNA expression in undifferentiated pericytes from skeletal muscle (*SK-PCs*), herein used as a reference control, was arbitrarily set as “1”. **h** Expression of linc-MD1 was analyzed in undifferentiated PCs from adipose tissue (*AT-PCs*) and mesenchymal stem cells from adipose tissue (*MSCs*). Linc-MD1 expression in undifferentiated pericytes from skeletal muscle (*SK-PCs*) was arbitrarily set as “1”. In **g**, **h**, data are reported as fold changes of the expression of the targets in *AT-PCs* and *MSCs* with respect to *SK-PCs*. ** $P < 0.01$, two-way analysis of variance

mesenchymal markers. These observations are consistent with previous studies of human MSCs and PCs in which cells isolated from other tissues and/or selected by CD146 expression were compared (Covas et al. 2008; Roobrouck et al. 2011). In agreement with previous studies that revealed that the expression of CD146, both in vivo and in vitro, is dynamic and dependent on cell type and culture conditions (Roobrouck et al. 2011; Lv et al. 2014; own unpublished results), we noted that the presence of CD146⁺ cells varies during the in vitro growth of isogenic MSCs and PCs from adipose tissue. CD146 has been used to identify and purify adult progenitors from bone marrow stroma and from the vascular niche of various tissues (Sacchetti et al. 2007; Crisan et al. 2009). However, we found that the percentage of CD146⁺ cells does not correlate with either the proliferation or differentiation

abilities of adipose-derived isogenic MSCs and PCs. The analysis of the differentiation abilities of MSCs and PCs indicates that they are both able to differentiate towards adipogenic and osteogenic lineages. Interestingly, we demonstrated that PCs activate the adipogenic and the osteogenic molecular programs more efficiently than their MSC counterparts, leading to a more robust terminal differentiation. To our knowledge, this is the first time that this evidence has been reported for isogenic adipose-tissue-derived MSCs and PCs. In addition, the analysis of the myogenic potential of MSCs and PCs revealed that both cell populations are able to differentiate in smooth muscle cells, whereas none of the analyzed cell populations are able to proceed towards skeletal muscle differentiation. Human adipose pericytes transplanted into mouse skeletal muscle can be recruited into the fibers of recipient

mice, whereas MSCs cannot (Crisan et al 2008; Valadares et al. 2014). Accordingly, we found that the myogenic potential of PCs but not of MSCs, can be partially rescued when induced to differentiate in co-culture with murine or rat myoblasts, further confirming that, in adipose tissue, PCs are also endowed with a “latent” myogenicity that is not displayed by their MSC counterparts. The inability of MSCs to undergo skeletal myogenic differentiation was also reported by Roobrouk and colleagues (2011) in a set of experiments performed on MSCs prepared from bone fragments. Of note, the inability of adipose tissue PCs to undergo spontaneous skeletal muscle differentiation is in contrast with that of PCs prepared from skeletal muscle that, at variance, readily fuse and form myotubes when cultured in myogenic differentiation medium. Interestingly, we found that, before the induction of differentiation, skeletal-muscle-derived PCs but not adipose-tissue-derived PCs, express detectable basal levels of known pro-myogenic factors (i.e., mir-1, mir-133, mir-206 and lincMD1), indicating that skeletal-muscle-derived PCs are more prone to myogenic differentiation than PCs derived from adipose tissue. This evidence indicates that PCs from different tissues are not equivalent in terms of their differentiation abilities.

Taken together, our results indicate that isogenic MSCs and PCs differ in terms of their adipogenic, osteogenic and myogenic differentiation abilities. If adipose tissue is used as a source of adult multi-potent progenitors, the isolation of microvascular PCs is therefore to be preferred, instead of standard MSCs. MSCs and PCs, including MSCs from adipose tissue, are known to comprise clonal progenitors that are not equivalent in terms of multi-lineage potential (Muraglia et al. 2000; Guilak et al. 2006; Crisan et al. 2008). Whether this reflects the differences observed between the MSCs and PCs in our analysis needs to be further investigated.

References

- Baksh D, Song L, Tuan RS (2004) Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. *J Cell Mol Med* 8:301–316
- Beltrami AP, Cesselli D, Bergamin N, Marcon P, Rigo S, Puppato E, D’Aurizio F, Verardo R, Piazza S, Pignatelli A, Poz A, Baccarani U, Damiani D, Fanin R, Mariuzzi L, Finato N, Masolini P, Burelli S, Belluzzi O, Schneider C, Beltrami CA (2007) Multipotent cells can be generated in vitro from several adult human organs (heart, liver, and bone marrow). *Blood* 110:3438–3446
- Bianco P (2011) Back to the future: moving beyond “mesenchymal stem cells”. *J Cell Biochem* 112:1713–1721
- Bianco P, Cossu G (1999) Uno, nessuno e centomila: searching for the identity of mesodermal progenitors. *Exp Cell Res* 251:257–263
- Caplan AL (2008) All MSCs are pericytes? *Cell Stem Cell* 3:229–230
- Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A, Bozzoni I (2011) A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Cell* 147:358–369
- Covas DT, Panepucci RA, Fontes AM, Silva WA Jr, Orellana MD, Freitas MC, Neder L, Santos AR, Peres LC, Jamur MC, Zago MA (2008) Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts. *Exp Hematol* 36:642–654
- Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Bhuring HJ, Giacobino JP, Lazzari L, Huard J, Péault B (2008) A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 3:301–313
- Crisan M, Chen CW, Corselli M, Andriolo G, Lazzari L, Péault B (2009) Perivascular multipotent progenitor cells in human organs. *Ann N Y Acad Sci* 1176:118–123
- Da Silva Meirelles L, Chagastelles PC, Nardi NB (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* 119:2204–2213
- Da Silva Meirelles L, Caplan AI, Nardi NB (2008) In search of the in vivo identity of mesenchymal stem cells. *Stem Cells* 26:2287–2299
- Dellavalle A, Sampaolesi M, Tonlorenzi R, Tagliafico E, Sacchetti B, Perani L, Innocenzi A, Galvez BG, Messina G, Morosetti R, Li S, Belicchi M, Peretti G, Chamberlain JS, Wright WE, Torrente Y, Ferrari S, Bianco P, Cossu G (2007) Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. *Nat Cell Biol* 9:255–267
- Feng J, Mantesso A, De Bari C, Nishiyama A, Sharpe PT (2011) Dual origin of mesenchymal stem cells contributing to organ growth and repair. *Proc Natl Acad Sci U S A* 108:6503–6508
- Gallo R, Gambelli F, Gava B, Sasdelli F, Tellone V, Masini M, Marchetti P, Dotta F, Sorrentino V (2007) Generation and expansion of multipotent mesenchymal progenitor cells from cultured human pancreatic islets. *Cell Death Differ* 14:1860–1871
- Guilak F, Lott KE, Awad HA, Cao Q, Hicok KC, Fermor B, Gimple JM (2006) Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. *J Cell Physiol* 206:229–237
- In’t Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, Kanhai HH (2004) Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. *Stem Cells* 22:1338–1345
- Lv FJ, Tuan RS, Cheung KM, Leung VY (2014) Concise review: the surface markers and identity of human mesenchymal stem cells. *Stem Cells* 32:1408–1419
- Manini I, Gulino L, Gava B, Pierantozzi E, Curina C, Rossi D, Brafa A, D’Aniello C, Sorrentino V (2011) Multi-potent progenitors in freshly isolated and cultured human mesenchymal stem cells: a comparison between adipose and dermal tissue. *Cell Tissue Res* 344:85–95
- Muraglia A, Cancedda R, Quarto R (2000) Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. *J Cell Sci* 113:1161–1166
- Orbay H, Tobita M, Mizuno H (2012) Mesenchymal stem cells isolated from adipose and other tissues: basic biological properties and clinical applications. *Stem Cells Int* 2012:461718
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45
- Phinney DG (2012) Functional heterogeneity of mesenchymal stem cells: implications for cell therapy. *J Cell Biochem* 113:2806–2812
- Pierantozzi E, Gava B, Manini I, Roviello F, Marotta G, Chiavarelli M, Sorrentino V (2011) Pluripotency regulators in human mesenchymal stem cells: expression of NANOG but not of OCT-4 and SOX-2. *Stem Cells Dev* 20:915–923
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* 284:143–147

- Roobrouck VD, Clavel C, Jacobs SA, Ulloa-Montoya F, Crippa S, Sohni A, Roberts SJ, Luyten FP, Van Gool SW, Sampaolesi M, Delforge M, Luttun A, Verfaillie CM (2011) Differentiation potential of human postnatal mesenchymal stem cells, mesoangioblasts, and multipotent adult progenitor cells reflected in their transcriptome and partially influenced by the culture conditions. *Stem Cells* 29: 871–882
- Russell KC, Phinney DG, Lacey MR, Barrilleaux BL, Meyertholen KE, O'Connor KC (2010) In vitro high-capacity assay to quantify the clonal heterogeneity in trilineage potential of mesenchymal stem cells reveals a complex hierarchy of lineage commitment. *Stem Cells* 28:788–798
- Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, Bianco P (2007) Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 131:324–336
- Toma JG, Akhavan M, Fernandes KJL, Barnabé-Heider F, Sadikot A, Kaplan DR, Miller FD (2001) Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 3:778–784
- Tonlorenzi R, Dellavalle A, Schnapp E, Cossu G, Sampaolesi M (2007) Isolation and characterization of mesoangioblasts from mouse, dog, and human tissues. *Curr Protoc Stem Cell Biol* Chapter 2:Unit 2B.1
- Townley-Tilson WH, Callis TE, Wang D (2010) MicroRNAs 1, 133, and 206: critical factors of skeletal and cardiac muscle development, function, and disease. *Int J Biochem Cell Biol* 42:1252–1255
- Trounson A, Thakar RG, Lomax G, Gibbons D (2011) Clinical trials for stem cell therapies. *BMC Med* 9:52
- Valadares MC, Gomes JP, Castello G, Assoni A, Pellati M, Bueno C, Corselli M, Silva H, Bartolini P, Vainzof M, Margarido PF, Baracat E, Péault B, Zatz M (2014) Human adipose tissue derived pericytes increase life span in *Utrn^{tm1Ked} Dmd^{mdx}/J* mice. *Stem Cell Rev* 10:830–840
- Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 7: 211–218
- Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 13:4279–4295