Tissue-Specific Cultured Human Pericytes: Perivascular Cells from Smooth Muscle Tissue Have Restricted Mesodermal Differentiation Ability

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Microvascular pericytes (PCs) are considered the adult counterpart of the embryonic mesoangioblasts, which represent a multipotent cell population that resides in the dorsal aorta of the developing embryo. Although PCs have been isolated from several adult organs and tissues, it is still controversial whether PCs from different tissues exhibit distinct differentiation potentials. To address this point, we investigated the differentiation potentials of isogenic human cultured PCs isolated from skeletal (sk-hPCs) and smooth muscle tissues (sm-hPCs). We found that both sk-hPCs and sm-hPCs expressed known pericytic markers and did not express endothelial, hematopoietic, and myogenic markers. Both sk-hPCs and sm-hPCs were able to differentiate into smooth muscle cells. In contrast, sk-hPCs, but not sm-hPCs, differentiated in skeletal muscle cells and osteocytes. Given the reported ability of the Notch pathway to regulate skeletal muscle and osteogenic differentiation, sk-hPCs and sm-hPCs were treated with N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a known inhibitor of Notch signaling. DAPT treatment, as assessed by histological and molecular analysis, enhanced myogenic differentiation and abolished osteogenic potential of sk-hPCs. In contrast, DAPT treatment did not affect either myogenic or osteogenic differentiation of sm-hPCs. In summary, these results indicate that, despite being isolated from the same anatomical niche, cultured PCs from skeletal muscle and smooth muscle tissues display distinct differentiation abilities.

Introduction

THE MAINTENANCE OF THE HOMEOSTASIS of human adult tissues is ensured by the presence of undifferentiated precursors that are able to replace those cells that are repeatedly lost following physiological, pathological, or traumatic events. In addition to other tissue-specific stem cells found in tissues like blood, skin, and gut [1], stem cells able to differentiate in the multiple cell types of mesodermal lineages have been identified in the connective tissue that forms the stromal compartment of most organs of the adult organism [2].

Mesenchymal progenitors, originally described in bone marrow [3], have been subsequently isolated from other organs [4–6]. These cells, known as mesenchymal stem cells (MSCs), are capable to differentiate in various types of connective cells such as osteocytes, adipocytes, and chondrocytes [7]. MSCs are currently defined as multipotent cells that are obtained from the stromal portion of the tissues and further selected as plastic-adherent cells [8,9]. However, mutual relations between mesenchymal progenitors of different tissues are still poorly understood and it is still un-

clear if cells with identical differentiation abilities can be isolated from all organs of the adult organism.

In the past years, following an alternative isolation procedure based on the harvesting of weak adhering cells emerging from explant cultures of vascularized connective portions of the tissues, a novel cell population endowed of multilineage mesodermal potential has been isolated from the skeletal muscle of mice, dogs, and humans [10–12] and, subsequently, from the vascularized portion of additional human adult and fetal tissues [13]. These cells were further described, *in vivo*, among the endothelial-associated cells that contribute to the architecture of small blood vessels of organs and tissues and identified as perivascular pericytes (PCs) [14], strongly supporting the notion that the endothelium of the microvessels would represent the *in vivo* stem-niche of mesodermal adult progenitors [15–18].

The perivascular localization of PCs and their multilineage differentiation abilities are in agreement with the hypothesis that these cells are the adult counterpart of the embryonic mesoangioblasts, which were first described as multipotent cells located in the dorsal aorta of the developing embryo

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[19]. Accordingly, it has been postulated that during organogenesis some mesoangioblasts migrate along the outgrowing blood vessels contributing to the formation of the perivascular cell compartment of postnatal tissues [15,20].

Notably, adult PCs still express a subset of integrins and receptors that allows the efficient migration ability of these cells [21,22]. Adult PCs from skeletal muscle have attracted attention because of their ability to differentiate in skeletal muscle cells and even more for their ability to cross the vascular wall, a feature that makes these cells particularly suitable for systemic cell delivery in protocols of cell transplantation [23,24]. PCs from human skeletal muscle are also able to differentiate toward additional lineages of mesodermal origin such as adipocytes, osteocytes, and chondrocytes [12]. However, a comparative analysis of PCs from different human tissues indicated that PCs from skeletal muscle could differentiate toward mesodermal lineages more efficiently than PCs from adipose tissue [25]. In addition, PCs from adult heart are not able to differentiate into skeletal muscle cells, but only into cardiac myocytes, adipocytes, osteocytes, and chondrocytes [26].

These data suggest that, despite the proposed common embryonic origin and the localization at the same anatomical niche, namely the perivascular compartment, PCs from different tissues may show distinct differentiation abilities. Accordingly, a better understanding of the differentiation potentials of PCs from the different adult tissues is still necessary to further improve our current knowledge of the properties of these cells and to assess whether the multilineage potential of PCs may vary among different tissues.

In the present study, we compare the mesodermal differentiation abilities of isogenic human PCs isolated from skeletal (sk-hPCs) and smooth muscle (sm-hPCs). We report here that sm-hPCs differentiated only into smooth muscle cells, in contrast to the multilineage abilities of sk-hPCs. Inhibition of Notch signaling, which is known to regulate both myogenesis and osteogenesis [27–30], had no effect on the differentiation abilities of sm-hPCs. Conversely, inhibition of Notch signaling enhanced myogenic differentiation of sk-hPCs, whereas strongly reduced their osteogenic differentiation.

Materials and Methods

Cells isolation and culture

This study complies with the ethical standards laid down in the 1964 Declaration of Helsinki, and was approved by the local human investigation board.

Skeletal muscle and smooth muscle biopsies were obtained from 34 healthy pregnant women that underwent Cesarean section and 8 women that underwent hysterectomy. Before enrollment, all woman signed a written informed consent at the Division of Obstetrics and Gynecology of “Policlinico Santa Maria alle Scotte” in Siena, Italy. Genetic and sampling variability were reduced by deriving isogenic PCs from skeletal and smooth muscle biopsies from the same patient. All reagents were purchased from Sigma-Aldrich (St. Louis, MO), if not otherwise specified. PCs were isolated as previously described [31].

Briefly, biopsies were cut in small pieces of about 1 mm³ and placed in collagen-coated Petri dishes at 37°C in humidified 5% CO₂ atmosphere. After at least 4 h of adhesion,

growing medium consisting of Dulbecco's modified Eagle's medium (DMEM) MegaCell supplemented with 5% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 1% nonessential amino acids, 0.1 mM β -mercaptoethanol, and 5 ng/mL basic fibroblast growth factor (PeproTech, Rocky Hill, NJ), was added to the explants. After 8 to 12 days from explant plating, floating and weakly adherent cells were poured out from the dishes and transferred to a new noncoated 100-mm Petri dish. This step was considered as passage 1. From this step on, cell populations were maintained in culture, detached by trypsin/EDTA treatment when they reached 70%–80% of confluence, and transferred each time into uncoated 150-mm Petri dishes at a plating density of 3,000 cells/cm². At every passage, 1.5×10^6 cells were harvested from a single 150-mm Petri dish. All the experiments described below were performed by passage 5, on cells that did not exceed 20 population doublings.

Myogenic, osteogenic, and adipogenic differentiation

Skeletal and smooth muscle differentiation were performed seeding 10,000 cells/cm² onto Matrigel (growth factor-reduced; BD, Franklin Lakes, NJ)-coated Petri dishes. After 1 day of adhesion, differentiation was induced by medium change. Skeletal muscle differentiation medium consisted of DMEM supplemented with 2% heat-inactivated horse serum (EuroClone, Picco, Milan, Italy), 2 mM glutamine, 1 mM sodium pyruvate (Lonza, Basel, Switzerland), 100 μ g/mL streptomycin, and 100 U/mL penicillin (D2 medium). Smooth muscle differentiation medium consisted of D2 medium supplemented with transforming growth factor- β (TGF- β) 5 ng/mL (R&D, Minneapolis, MN) freshly added every day. For the assessment of skeletal muscle differentiation, multinucleated myotubes were visualized both by 4',6-diamidino-2-phenylindole (DAPI) and α -actinin staining 10 days after induction of differentiation (see relevant paragraph).

Fusion index of each cell population ($n=6$ for both sk-hPCs and sm-PCs) was calculated as the number of nuclei inside α -Actinin-positive myotubes, divided by the total number of nuclei in the relative microscopic field. Ten to 20 microscopic fields were randomly chosen among two different glass slides. Each experiment was performed twice. For the assessment of smooth muscle differentiation, the presence of smooth muscle cells was verified by both SM22- α staining and smooth muscle actin- α (α -SMA) immunodetection (see relevant paragraphs) in differentiated cells, 8 days after the induction of differentiation.

Osteogenic differentiation was performed by plating 20,000 cells/cm² onto uncoated Petri dishes. Cells were left to adhere for 1 day and then switched to the osteogenic medium consisting of minimum essential medium alpha (α -MEM) supplemented with 10% heat-inactivated 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. Medium was changed every 2 days. Twenty-eight days after induction of differentiation, the production of extracellular calcified mineral matrix was visualized by Alizarin Red staining. Quantification of osteogenic differentiation was performed by the spectrophotometric evaluation of bound Alizarin Red dye as previously described [32].

Adipogenic differentiation was performed by plating 20,000 cells/cm² onto uncoated Petri dishes. The day after plating,

differentiation was induced with adipogenic medium consisting of α -MEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 μ g/mL streptomycin, 100 U/mL penicillin, 0.5 mM isobutyl-methylxanthine, 1 μ M dexamethasone, 10 μ M insulin, and 200 μ M indomethacin. Medium was changed every 2 days. Fourteen days after plating, adipogenic differentiation was visualized by staining lipid droplets with Oil Red O. To quantify adipogenic differentiation, Oil Red O extraction was performed and spectrophotometrically evaluated as previously described [32].

Immunocytochemical analyses

Cells were fixed with 3% (v/v) paraformaldehyde in phosphate-buffered saline (PBS), permeabilized in HEPES/Triton buffer (20 mM HEPES pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, and 0.5% Triton X-100), saturated with 10% FBS or normal goat serum in PBS, and incubated 1 h at room temperature (RT) with the following primary antibodies: monoclonal anti- α -Actinin (1:1,000), polyclonal anti-SM22 α (1:1,000; Abcam, Cambridge, United Kingdom), monoclonal anti- α -SMA (1:400), monoclonal anti-desmin (1:1,000; Millipore, Temecula, CA), monoclonal anti-MYOD (1:50; Abcam), and monoclonal anti-myogenin (1:150; Abcam). Rhodamine-conjugated phalloidin (1:1,000; Thermo Fisher Scientific, Waltham, MA) was used to reveal F-actin cytoskeleton.

To reveal bound primary antibodies, cells were incubated for 1 h at RT with specific secondary antibodies (anti-mouse IgG conjugated to cyanine2, 1:5,000; anti-mouse IgG conjugated to cyanine3, 1:5,000; anti-goat IgG conjugated to cyanine3, 1:2,000; all from Jackson Laboratories, Bar Harbor, ME). Mouse monoclonal anti- α -Actinin and mouse monoclonal anti-myogenin antibodies were directly conjugated to the relevant fluorochrome using the Alexa Fluor 647 Monoclonal Antibody Labeling Kit (Molecular Probes, Life Technologies, Waltham, MA) and the Alexa Fluor 488 Zenon Mouse IgG Labeling Kit (Invitrogen, Life Technologies, Waltham, MA), according to the manufacturer's instruction. Nuclei were visualized by DAPI (1:10,000; Calbiochem, San Diego, CA) or TO-PRO[®]-3 Iodide (1:1,000; Molecular Probes) staining. Slides were analyzed with LSM-510 META confocal microscope (Carl Zeiss, Oberkochen, Germany).

DAPT treatment

The cleavage of Notch intracellular domain was inhibited by adding N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a selective inhibitor of γ -secretase [33], to proliferation and/or differentiation medium, at a final concentration of 20 μ M. DAPT was resuspended in dimethyl sulfoxide (DMSO). Accordingly, all control cell populations used for the comparative analysis were cultured in the presence of equal amount of DMSO. DAPT and DMSO treatment was performed for two consecutive passages (p3 and p4) on undifferentiated growing cells. During myogenic, osteogenic, and adipogenic differentiation, DAPT and DMSO were freshly added at every medium change.

Total RNA and miRNA extraction, cDNA synthesis, and polymerase chain reaction analysis

Total RNA and microRNA were extracted from either undifferentiated or differentiating cells using the miRNeasy

Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. MicroRNA fraction was enriched using RNeasy[®] MinElute[™] Cleanup Kit (Qiagen). RNA and microRNA concentration was evaluated with NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). Two hundred nanograms of total RNA was reverse transcribed using the M-MLV Reverse Transcriptase (Promega, Fitchburg, WI) following the manufacturer's instructions.

MicroRNAs were converted into double-strand cDNA with the miScript II RT Kit (Qiagen), following the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (PCR) analysis was performed on StepOnePlus[™] detection system (Applied Biosystems, Waltham, MA). Power SYBR[®] PCR Master Mix (Applied Biosystems) was used to amplify the cDNA from total RNA and QuantiTect SYBR[®] Green PCR Master Mix was used to amplify microRNAs (Qiagen). All Ct values greater than 35 were not considered. The relative RNA and microRNA expressions were calculated using β -actin and hsa-RNU6-2 (Qiagen) as internal control, respectively, according to the Pfaffl's $2^{-\Delta\Delta Ct}$ quantification method [34].

Primers used were as follows:

Platelet-derived growth factor receptor- β (*PDGFRB*): Fw CAGTAAGGAGGACTTCCTGGAG; Rev CCTGAGAGATCTGTGGTTCCAG; alkaline phosphatase (*ALP*): Fw CCTCCTCGGAAGACACTCTG; Rev CACCACCTGTAGCCAGGCC; *MYOD*: Fw GAAGCTAGGGGTGAGGAAGC; Rev CCCGGCTGTAGATAGCAAAG; *MYF5*: Fw CTATAGCC TGCCGGGACA; Rev TGGACCAGACAGGACTGTTACAT; β -actin: Fw CAACTCCATCATGAAGTGTGAC; Rev GCCATGCCAATCTCATCTTG; PDZ-binding motif (*TAZ*): Fw AGATGACCTTCACGGCCACTG; Rev TGAGGCACTGGTGTGGAAGT; Runt-related transcription factor 2 (*RUNX2*): Fw GCAGCACGCTATTAAATCCAA; Rev ACAGATTCATCCATTCTGCCA; Osterix (*OSX*): Fw GCCA GAAGCTGTGAAACCTC; Rev GCAACAGGGGATTAACTGA; PW1/paternally expressed gene 3 (*PEG3*): Fw GATCCAAGAGAAGTGCCTACC; Rev GGAAGATTCATCTTACAAATCCC; hsa-miR-1: UGGAUGUAAAGAA GUAUGUAU; hsa-miR-133b: UUUGGUCCCCUUAACCA GCUA; has-miR-206: UGGAUGUAAGGAAGUGUG UGG.

Protein extraction and western blot

Cell pellets were lysed for 30 min at 4°C using RIPA buffer (TRIS 50 mM, NaCl 150 mM, SDS 0.1%, sodium deoxycholate 0.5%, Triton X-100 or NP40 1%) supplemented with protease inhibitor cocktail. Centrifugation at 11,000 rcf for 10 min at 4°C allowed the removal of insoluble materials. Protein concentration was assessed using Bradford protein assay (Bio-Rad, Hercules, CA). Fifty micrograms of total proteins were loaded on 12.5% SDS-polyacrylamide electrophoresis gel. Separated proteins were transferred onto PVDF membrane that was blocked with 5% nonfat milk in TRIS-buffered saline and Tween 20 for 1 h at RT.

Primary antibody incubation was performed overnight at 4°C with the following antibodies: polyclonal anti-neural/glia antigen 2 (NG2, 1:500; Chemicon, EMD Millipore, Billerica, MA), monoclonal anti-desmin (1:3,000; Millipore), monoclonal anti- α -Tubulin (1:5,000), polyclonal anti-SM22- α (1:5,000; Abcam), monoclonal anti- α -SMA (1:2,000). Secondary anti-rabbit-, anti-mouse-, and anti-goat-IgG

antibodies conjugated to horseradish peroxidase (1:3,000; GE-Healthcare, Little Chalfont, United Kingdom) were kept 1 h at RT. Immunoreactive bands were detected using the Amersham ECL Western Blotting Detection Reagent (GE-Healthcare). Quantification of the intensities of immunoreactive bands was performed by ImageJ software (National Institutes of Health).

Flow cytometry

Flow cytometry (FC) analysis was performed on undifferentiated cells at early passages (p2–p5). Cells were collected by trypsinization and then washed in ice-cold PBS containing 0.5% bovine serum albumin (FC buffer). 1×10^5

cells were resuspended in FC buffer and incubated for 30 min at 4°C with the following monoclonal antibodies: phycoerythrin (PE)-conjugated CD13 (1:50; DAKO, Agilent Technologies, Santa Clara, CA); PE-conjugated CD31 (1:50; Santa Cruz, Dallas, TX); allophycocyanin (APC)-conjugated CD34 (1:50; BD, Franklin Lakes, NJ); APC-conjugated CD44 (1:50; e-Bioscience, San Diego, CA); fluorescein isothiocyanate (FITC)-conjugated CD45 (1:50; BD); CD73 (1:50; BD); APC-conjugated CD90 (1:50; BD); PE-conjugated CD105 (1:50; Ancell, Bayport, MN); PE-conjugated CD146 (1:50; BD); FITC-conjugated NG2 (1:50; e-Bioscience). PE-conjugated anti-mouse IgG secondary antibody (1:50; BD) was used for the detection of CD73-positive cells. To set fluorescence background,

FIG. 1. Characterization of cultured PCs from skeletal and smooth muscle tissues. **(A)** Flow cytometry analysis on cultured skeletal muscle-derived human pericytes (sk-hPCs) and cultured smooth muscle-derived human pericytes (sm-hPCs). Representative plots of a panel of markers (green: CD13, CD31, CD34, CD44, CD45, CD146, CD73, CD90, CD105, and NG2) are shown for isogenic cell populations isolated from donors #3 and #5. Ig isotype-matched antibodies are shown in red. **(B)** Phase contrast images of isogenic sk-hPCs (a, b) and sm-hPCs (c, d) from donors #3 and #5. Scale bars: 100 μm. **(C)** RT-PCR analysis of the expression of *PDGFR-β*, *ALP*, *MYOD*, and *MYF5* genes in isogenic cultured PCs from skeletal (sk) and smooth (sm) muscle of donors #1 and #3. Total RNA from human bone marrow-derived mesenchymal stem cells (MSCs) and no-template samples were used as positive (+) and negative (–) controls, respectively, for *PDGFR-β* and *ALP*. Total RNA from human myoblasts and terminal differentiated osteocytes were used as positive (+) and negative (–) controls, respectively, for *MYF5* and *MYOD*. The expression of β-actin was used as control. β-actin expression of human bone marrow MSCs and no-template samples are not shown. **(D)** Western blot analysis of NG2 and desmin expression in isogenic cultured PCs from skeletal (sk) and smooth (sm) muscle of donors #2 and #4. Expression of β-actin was used as loading control. **(E)** Immunofluorescence microscopy analysis with anti-desmin (a–d) and anti-α-SMA (e–h) antibodies (both revealed in green) in sk-hPCs and sm-hPCs from donors #2 and #6. Nuclei were counterstained with TO-PRO[®]-3 Iodide. Scale bars: 20 μm. α-SMA, smooth muscle actin-α; ALP, alkaline phosphatase; NG2, neural/glial antigen 2; PCs, pericytes; PDGFR-β, platelet-derived growth factor receptor-β; RT-PCR, reverse transcription–polymerase chain reaction. Color images available online at www.liebertpub.com/scd

FIG. 2. Smooth muscle, osteogenic, and adipogenic differentiation of sk-hPCs and sm-hPCs. **(A)** Immunofluorescence analysis with anti-SM22- α (red) of isogenic sk-hPCs and sm-hPCs from all donors (#1–#6), following 8 days of smooth muscle differentiation. Nuclei were counterstained with DAPI. **(B)** Western blot (a) and relative densitometric analysis (b) of the expression of α -SMA and SM22- α of isogenic sk-hPCs and sm-hPCs from donors #4 and #6 before (undifferentiated, u) and following 8 days of smooth muscle differentiation (differentiated, d). α -Tubulin was used as loading control. Densitometric values relative to undifferentiated cells that are arbitrarily set as one, are reported as mean $\pm$ SEM ($n=2$). **(C)** Alizarin Red staining of calcified bone mineral matrix deposited by sk-hPCs and sm-hPCs from all donors (#1–#6). Sk-hPCs (a–f) and sm-hPCs (g–l) were stained 2 and 4 weeks after the induction of osteogenic differentiation, respectively. **(D)** Oil Red O staining of lipid droplets in sk-hPCs (a–f) and sm-hPCs (g–l) from all donors (#1–#6). All cell populations were stained 2 weeks after the induction of adipogenic differentiation. For all cell populations, three experimental replicates were performed in each differentiation assay. Scale bars: 20 μ m (A), 4.35 mm (C), and 100 μ m (D). DAPI, 4',6-diamidino-2-phenylindole; SM22- α , smooth muscle protein-22 α . Color images available online at www.liebertpub.com/scd

identical Ig isotypes conjugated to PE, FITC, or APC (all from BD) were used.

Statistical analyses

Statistical analyses were performed using the Student's *t*-test or the two-way analysis of variance using GraphPad

Prism6 software. Wilcoxon matched pairs, nonparametric *T*-test was used to compare microRNAs expression during myogenic differentiation of given cell populations, setting target expression of undifferentiated cells as internal reference. Mann–Whitney unpaired, nonparametric *T*-test was used to compare microRNAs expression between isogenic sk-hPCs and sm-hPCs during both proliferation and

differentiation, setting target expression of sk-hPCs as internal reference. Results were calculated as mean $\pm$ SEM of at least three different pairs of isogenic sk-hPCs and sm-hPCs. Differences in target expression were considered statistically significant when *P* value was lower than 0.05.

Results

Sk-hPCs and sm-hPCs have comparable immunophenotype and molecular pattern

Biopsies from skeletal and smooth muscle were obtained from the same patients and were processed in parallel to isolate isogenic sk-hPCs and sm-hPCs from 42 donors, exploiting a previously reported isolation procedure [31]. All data presented throughout the article are from six representative isogenic sk-hPCs and sm-hPCs (donors #1, #2, #5, #6: Cesarean section; donors #3, #4: hysterectomy). The remaining 36 isogenic pairs were also analyzed and gave similar results. Cells were expanded for two/three passages, and morphologically indistinguishable (Fig. 1B) sk-hPCs and sm-hPCs were analyzed in parallel to assess the presence of pericytic markers and the absence of both endothelial and myogenic markers. FC analysis indicated that all cell populations stained positive for the surface antigens CD13, CD44, CD146, and NG2 (Fig. 1A), in agreement with previously reported analysis on *in vitro* [12,25] and *in vivo* [13] microvascular PCs from skeletal muscle and adipose tissue.

The percentage of cells expressing CD146 and NG2 varied across cell populations, ranging between 12%–71% and 48%–85%, respectively. Variable expression of NG2 across cell populations was also noted in western blot experiments (Fig. 1D). In addition, mesenchymal immunophenotype of sk-hPCs and sm-hPCs was corroborated by the expression of CD73, CD90, and CD105 (Fig. 1A). Finally, the endothelial and/or hematopoietic markers CD31, CD34, and CD45 were never detected (Fig. 1A). To further confirm that isolated cells expressed microvascular PCs' markers, expression of *PDGFRB* and *ALP*, two markers expressed by vessel-associated PCs [35], was verified by reverse transcription–polymerase chain reaction (RT-PCR) in all sk-hPCs and sm-

hPCs (Fig. 1C). Conversely, the expression of myogenic factors such as *MYOD* and *MYF5* was never detected (Fig. 1C). Western blot analysis indicated that all sk-hPCs and sm-hPCs expressed desmin (Fig. 1D), whose expression was also confirmed by immunofluorescence analysis (Fig. 1E, a–d). Finally, all cell populations expressed α -SMA, although different levels of expression and pattern of organization were noted in sm-hPCs (Figs. 1E, e–h, and 2B, a).

Sk-hPCs and sm-hPCs have distinct differentiation potentials

Smooth muscle, osteogenic, and adipogenic potentials of isogenic sk-hPCs and sm-hPCs were evaluated to assess the multipotential properties of the isolated cell populations. Smooth muscle differentiation was induced by continuous TGF- β treatment for 8 days. Both sk-hPCs and sm-hPCs were able to differentiate in smooth muscle cells as confirmed by the expression of smooth muscle protein-22 α (SM22- α) and α -SMA (Fig. 2A, a–l and 2B, a). Both smooth muscle markers were upregulated following 8 days of differentiation (Fig. 2B, b).

All sk-hPCs exposed to osteogenic medium differentiated in calcified mineral matrix-secreting cells as early as 15 days following the induction of osteogenic differentiation, as assessed by Alizarin Red staining (Fig. 2C, a–f). In marked contrast, sm-hPCs were not able to secrete extracellular ossified mineral matrix even 28 days after the induction of differentiation (Fig. 2C, g–l).

Finally, sk-hPCs readily differentiated into adipocytes, as revealed by Oil Red O staining of lipid droplets into the cytoplasm (Fig. 2D, a–f). In contrast, sm-hPCs were not able to accumulate lipid droplets, indicating that they failed adipogenic differentiation (Fig. 2D, g–l).

Sk-hPCs, but not sm-hPCs differentiate in skeletal muscle cells

The myogenic potential of sk-hPCs has attracted the interest of several researchers given the potential clinical

FIG. 3. Skeletal muscle (SKM) differentiation of sk-hPCs and sm-hPCs. (A) Immunofluorescence analysis of α -Actinin expression in isogenic sk-hPCs (a–f) and sm-hPCs (g–l) from all donors (#1–#6), 10 days following induction of skeletal myogenic differentiation. Scale bars: 20 μ m. (B) Detailed immunofluorescence analysis of SKM differentiation of sk-hPCs (a–c, g–i, m–o) and sm-hPCs (d–f, j–l, p–r) following 2 days (a–f), 7 days (g–l), and 15 days (m–r) of differentiation. MYOD (a, c, d, f; red) and Myogenin (b, c, e, f; green) double-positive nuclei were detected after 2 days of differentiation only in sk-hPCs. α -Actinin (h, i, k, l; violet)-positive myotubes that contain several myogenin-positive nuclei (g, i, j, l) were identified at 7 days of differentiation only in sk-hPCs. Fifteen days after the induction of differentiation, α -Actinin-positive myotubes were detected in differentiating sk-hPCs (n, o), but not in sm-hPCs (q, r). To visualize cell morphology, F-actin cytoskeleton of 15 days differentiated cells was stained by rhodamine-conjugated phalloidin (m, o, p, r; red). All nuclei were counterstained with DAPI. For all cell populations three experimental replicates were performed in each differentiation assay. Scale bars: 20 μ m. (C) Relative expression analyses of mir-1, mir-133b, and mir-206 in sk-hPCs and sm-hPCs following 3 days of SKM differentiation. The expression of each target microRNA in undifferentiated cells was used as internal reference and arbitrarily set as one. (D) Relative expression analysis of mir-1, mir-133b, and mir-206 in undifferentiated sk-hPCs and sm-hPCs, and in differentiating sk-hPCs and sm-hPCs 3 days after the induction of SKM differentiation. MyomiRs expression in sk-hPCs was used as internal reference and arbitrarily set as one in both undifferentiated and differentiating cells. (E) Quantitative analysis of PW1/Peg3 in undifferentiated sk-hPCs and sm-hPCs. For the analysis of relative expression, PW1/Peg3 expression in sk-hPCs was used as internal reference and arbitrarily set as one. (C–E) $2^{-\Delta\Delta Ct}$ method was used to calculate the relative fold change of target expression. Data reported in (C, D) are mean $\pm$ SEM of six pairs of isogenic sk-hPCs and sm-hPCs. Data reported in E are the mean $\pm$ SD of three different experiments performed on each cell population. Statistical analyses were performed by Wilcoxon matched pairs, nonparametric *T*-test (C) and Mann–Whitney unpaired, nonparametric *T*-test (D, E). Significant *P* value <0.05; **P* < 0.05; ***P* < 0.01; ****P* < 0.001. PW1/Paternally expressed gene 3. Color images available online at www.liebertpub.com/scd

application of these cells. In fact, sk-hPCs are currently used in a phase I/II clinical trial aimed to ameliorate the conditions of patients affected by Duchenne muscular dystrophy (EudraCT no. 2011-000176-33). To better understand if skeletal myogenic potential is confined to skeletal muscle PCs or it is also a property of PCs residing in other tissues, we performed a more detailed analysis of skeletal muscle differentiation, comparing sk-hPCs and sm-hPCs.

Undifferentiated cells were thus exposed to skeletal muscle-differentiation medium for 10 days. Sk-hPCs fused and formed multinucleated α -actinin-positive myotubes (Fig. 3A, a–f) that were already formed as early as 7 days after the induction of differentiation (Fig. 3B, g–i). In contrast, sm-hPCs did not fuse and α -actinin-positive cells were never detected during the same time course of differentiation (Fig. 3A, j–l). To exclude a mere delay of myogenesis in differentiating sm-hPCs, skeletal muscle differentiation was carried on for five additional days. Nonetheless, even following 15 days of differentiation, sm-hPCs were still unable to fuse and form myotubes (Fig. 3B, p–r). Similarly, neither α -Actinin-positive cells nor multinucleated myotubes were observed when sm-hPCs were even cocultured with the mouse C2C12 cells (data not shown), which enhance the ability of human adipose tissue-derived PCs to form myotubes [25].

Skeletal myogenic differentiation relies on a fine-tuned mechanism based on the interplay between known myogenic factors, including MYOD and Myogenin, and a recently identified set of muscle-specific microRNAs, called muscle-specific miRNAs (myomiRs) [36].

Interestingly, following the exposure to myogenic medium, the expected sequential activation of MYOD and Myogenin was observed in differentiating sk-hPCs (Fig. 3B, a–c, g–i), whereas no expression of MYOD and Myogenin was observed in sm-hPCs (Fig. 3B, d–f, j–l). We also analyzed the expression of three major promyogenic myomiRs, namely mir-1, mir-133b, and mir-206, during the early phases of myogenic differentiation of both sk-hPCs and sm-hPCs. As expected, the expression of mir-1, mir-133b, and mir-206 was upregulated in differentiating sk-hPCs, whereas the expression of the three myomiRs was unaffected or even decreased in sm-hPCs induced to differentiate (Fig. 3C). Accordingly, direct comparison of myomiRs expression between sk-hPCs and sm-hPCs showed that sk-hPCs displayed higher expression level of mir-1, mir-133b, and mir-206 with respect to sm-hPCs in both undifferentiated and differentiating conditions (Fig. 3D).

Finally, we also found that the expression of PW1/Peg3, recently identified as a key regulator of human mesoangioblasts (hMABs) stem cell competence [37], was reduced by about 50% in sm-hPCs with respect to the levels observed in sk-hPCs, in agreement with the results obtained from the differentiation analysis of sk-hPCs and sm-hPCs (Fig. 3E).

Notch signaling inhibition affects sk-hPCs, but not sm-hPCs differentiation abilities

The results obtained from the differentiation analysis of sk-hPCs and sm-hPCs indicated that cultured PCs isolated from skeletal and smooth muscle tissues are endowed with distinct differentiation abilities, although they can be isolated from the microvascularized portion of the tissues and share similar immunophenotypic and molecular pattern.

Given the ability of Notch to regulate and direct myogenic and osteogenic potential of undifferentiated cells [27–30], we next investigated whether the inhibition of Notch signaling would affect the differentiation potential of sk-hPCs and sm-hPCs. Accordingly, isogenic sk-hPCs and sm-hPCs were cultured in the presence of DAPT, a selective inhibitor of γ -secretase [33], for two consecutive passages, before the induction of differentiation. Following DAPT addition for two passages, we observed a three-fold reduction of the amount of Notch intracellular domain in treated cells, as assessed by western blot analysis (data not shown). Cells were then induced to differentiate toward myogenic and osteogenic lineages in the presence of DAPT.

The inhibition of Notch signaling during both proliferation and differentiation of sk-hPCs enhanced the recruitment of nuclei into myotubes (Fig. 4A, a, b), resulting in a three-fold increase of overall fusion index compared to untreated cell populations (Fig. 4A, c). Accordingly, the expression of the early myogenic factors *MYOD* and *MYF5* (Fig. 4C) and the expression of promyogenic microRNAs, mir-1, mir-133b, and mir-206 (Fig. 4D), were significantly upregulated in DAPT-treated sk-hPCs. Interestingly, the enhancement of myogenic efficiency of DAPT-treated sk-hPCs was effective only when DAPT was continuously administered in both growth and differentiation media. In fact, either DAPT-treated undifferentiated sk-hPCs induced to differentiate in the absence of DAPT or untreated undifferentiated sk-hPCs induced to differentiate in the presence of DAPT, did not display altered fusion index with respect to control cells (Fig. 4E). At variance of sk-hPCs, all DAPT-treated sm-hPCs remained unable to fuse and form myotubes (Fig. 4B, a–c). Accordingly, the expression of *MYOD*, *MYF5*, mir-1, mir-133b, and mir-206 in sm-hPCs was unaffected by drug treatment (data not shown).

The effect of DAPT treatment on the osteogenic potential of sk-hPCs and sm-hPCs was also investigated. The ability of sk-hPCs to differentiate toward osteogenic lineage was strongly reduced following continuous DAPT treatment during both proliferation and differentiation (Fig. 5A, a, b). Quantitative analysis of cell differentiation performed by means of Alizarin Red extraction indicated that the amount of extracellular mineral matrix produced by DAPT-treated sk-hPCs was about 20-fold lower than that measured in untreated sk-hPCs (Fig. 5A, c). Accordingly, quantitative RT-PCR analysis of transcription factors involved in the onset of osteogenic differentiation such as *TAZ*, *RUNX2*, and *OSX* revealed that the expression of all these genes was significantly downregulated following DAPT treatment.

Interestingly, DAPT was able to inhibit osteogenesis of sk-hPCs when present only during differentiation. Addition of DAPT to only undifferentiated cells was not sufficient to inhibit osteogenesis (Fig. 5D). Finally, the analysis of Notch signaling inhibition on osteogenic potential of sm-hPCs revealed that DAPT-treated sm-hPCs were not able to differentiate toward osteogenic lineage, in strict analogy with the results obtained with untreated sm-hPCs (Figs. 5B, a–c and 2C, g–l). The effect of DAPT treatment on sk-hPCs and sm-hPCs was also assessed on adipogenic differentiation. Notch signaling inhibition did not alter the adipogenic potential of both cell populations, yielding results comparable to those obtained from control cell populations (data not shown and Fig. 2D, a–l).

FIG. 4. Effects of Notch pathway inhibition on myogenic ability of sk-hPCs and sm-hPCs. Representative immunofluorescence analysis of α -Actinin expression in sk-hPCs (**A**) and sm-hPCs (**B**) cultured in standard myogenic differentiation medium (CTRL, **a**) or in myogenic differentiation medium supplemented with N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, **b**), the γ -secretase inhibitor, for 12 days. Nuclei were stained with DAPI. Relative fusion index of CTRL and DAPT-treated cells are reported in the histograms in (**A**, **c** and **B**, **c**). Fusion index of CTRL cells was arbitrarily set as one. Plotted data are the mean ratio $\pm$ SEM of fusion index of six DAPT-treated cell populations over six CTRL cell populations. Fusion index was calculated as the number of nuclei inside α -actinin-positive multinucleated cells, divided by the total number of nuclei. ND, not detected. (**C**) Quantitative analysis by real-time PCR of *MYOD* and *MYF5* expression in sk-hPCs from all donors ($n=6$), 2 and 5 days after the induction of SKM differentiation. Relative expression in CTRL and DAPT-treated cells was calculated by $2^{-\Delta\Delta C_t}$ method. *MYOD* and *MYF5* expression in CTRL cells was arbitrarily set as one. Reported data are mean $\pm$ SEM. (**D**) Relative expression analysis of mir-1, mir-133b, and mir-206 in CTRL and DAPT-treated undifferentiated sk-hPCs from all donors ($n=6$). Relative expression between CTRL and DAPT-treated cells was calculated by $2^{-\Delta\Delta C_t}$ method. MyomiR expression in CTRL cells was arbitrarily set as one. Statistical analyses were performed by Mann-Whitney unpaired, nonparametric *T*-test. Reported data are mean $\pm$ SEM. (**E**) Relative myogenic differentiation efficiency of sk-hPCs cultured with (+) or without (–) DAPT administration during proliferation and/or SKM differentiation. Plotted data are the mean ratio $\pm$ SEM of fusion index of three treated sk-hPCs populations over three CTRL cell populations. Fusion index of CTRL cells (–/–) was arbitrarily set as one. Significant *P* value <0.05 ; **P* <0.05 ; ***P* <0.01 . Scale bars: 20 μ m.

FIG. 5. Effects of Notch pathway inhibition on osteogenic ability of sk-hPCs and sm-hPCs. Osteogenic differentiation ability of sk-hPCs (**A**) and sm-hPCs (**B**) was visualized by Alizarin Red staining of bone mineral matrix 4 weeks after the induction of differentiation. Representative micrographs of control (CTRL) and DAPT-treated sk-hPCs and sm-hPCs cultured in standard osteogenic medium (CTRL, **a**) or in osteogenic medium supplemented with DAPT (DAPT, **b**). Relative quantification analysis of osteogenesis by Alizarin Red extraction in CTRL and DAPT-treated cells are reported in the histograms in (**A**, **c** and **B**, **c**). The absorbance of extracted Alizarin Red was converted in μg and normalized to DNA content. Normalized Alizarin Red content of CTRL cells was arbitrarily set as one. Plotted data are the mean ratio $\pm$ SEM of three different cell populations per treatment. (**C**) Quantitative analysis by real-time PCR of *TAZ*, *RUNX2*, and *OSX* expression of three sk-hPCs populations, before induction of differentiation and 2 and 5 days after osteogenesis induction. Relative expression between CTRL and DAPT-treated cells was calculated by $2^{-\Delta\Delta C_t}$ method. *TAZ*, *RUNX2*, and *OSX* expression in control cells was arbitrarily set as one. Reported data are mean $\pm$ SEM. (**D**) Relative quantification analysis of sk-hPCs cultured with (+) or without (-) DAPT administration during proliferation and/or osteogenic differentiation. Plotted data are the mean ratio $\pm$ SEM of normalized Alizarin Red content of three treated sk-hPCs over three control cell populations. Significant *P* value <0.05; **P* <0.05; ***P* <0.01. Scale bars: 100 μm . *RUNX2*, Runt-related transcription factor 2; *TAZ*, PDZ-binding motif. Color images available online at www.liebertpub.com/scd

Discussion

Multilineage mesodermal differentiation potential has been proven for human PCs isolated from skeletal muscle (sk-hPCs), adipose (at-hPCs), and cardiac (ct-hPCs) adult tissues [12,13,26]. However, whether hPCs from distinct tissues are equivalent in terms of differentiation abilities is not yet clear. To further address this point, cultured PCs derived from smooth muscle (sm-hPCs) were compared to isogenic sk-hPCs.

Based on the initial characterization reported in Fig. 1, we concluded that sk-hPCs and sm-hPCs share the same immunophenotypic and molecular profile. According to previous data on PCs from adipose tissue [25], these findings indicate that cultured PCs isolated from different sources without exploiting any additional cell selection are virtually indistinguishable. However, when we compared multipotent differentiation abilities of sk-hPCs and sm-hPCs, we observed that sk-hPCs were able to differentiate into adipocytes, osteocytes, skeletal, and smooth muscle cells, whereas sm-hPCs were only able to give rise to smooth muscle cells.

The restricted differentiation abilities of sm-hPCs is surprising compared to those of sk-hPCs, at-hPCs, or ct-hPCs, where at least adipogenic and osteogenic potentials have been always observed [12,13,26]. Interestingly, stem cells from myometrium endowed with adipogenic, osteogenic, and chondrogenic differentiation abilities, and thus resembling putative MSCs, have been reported [38]. However, sm-hPCs and myometrial MSCs differ in their morphology, molecular marker profiles and differentiation abilities. Notably, such differences are not observed in adipose tissue, where both MSCs and PCs are capable to differentiate into smooth muscle cells, but also into adipocytes and osteocytes.

Lack of multipotent abilities of sm-hPCs with respect to sk-hPCs is in agreement with a recent report showing that cardiac PCs, although able to differentiate toward osteocytes and adipocytes, fail to differentiate in skeletal muscle cells [26]. In addition, we recently reported that hPCs isolated from skeletal and adipose tissues are yet endowed with multipotent mesodermal abilities, but sk-hPCs differentiate at much more efficient rate than at-hPCs [25]. Taken together, all these evidences indicate that hPCs isolated from different tissues cannot be considered equivalent in terms of their differentiation potentials.

It has been recently shown that stem cell competence of adult sk-hPCs, and in particular their skeletal myogenic potential, is positively regulated by the expression of PW1/Peg3 [37]. The authors also showed that knockdown of PW1/Peg3 abrogated skeletal muscle differentiation of hMABs. PW1/Peg3 expression has been also detected in PICs (PW1⁺/Pax7⁻ interstitial cells), a myogenic cell population apparently distinct from PCs, which has been identified in the interstitium of postnatal skeletal muscle [39], and that is also able to support skeletal myogenesis of satellite cells [40]. In this study, we report that PW1/Peg3 expression in sm-hPCs is only about 50% of that observed in sk-hPCs. Why sm-hPCs, although able to express considerable levels of PW1/Peg3, are unable to differentiate into skeletal muscle cells remains to be determined.

It is worth noting that spontaneous differentiation into skeletal muscle cells has been observed only in sk-hPCs. In fact, skeletal muscle differentiation of at-hPCs, even if

not as efficient as that of sk-hPCs, was dependent on coculture with rodent myoblasts [25]. On the other hand, sm-hPCs were not able to differentiate into skeletal muscle cells even following induction of differentiation in coculture with C2C12 cells (data not shown).

Furthermore, inhibition of Notch signaling, known to stimulate skeletal muscle differentiation of myogenic cells [41], was unable to stimulate skeletal myogenic differentiation in sm-hPCs. This agrees with the molecular analysis revealing that sm-hPCs, at variance of sk-hPCs, were not able, upon induction of skeletal muscle differentiation, to activate the expression of myogenic transcription factors or muscle-specific microRNAs such as mir-1, mir-133b, and mir-206, further confirming that sm-hPCs do not have any potential to differentiate into skeletal muscle cells.

Interestingly, failure of both cardiac and adipose tissue PCs to undergo spontaneous myogenic differentiation has also been linked to lack of coordinated expression of *MYOD*, *MYF5*, and muscle-specific microRNAs [25,42]. Notably, we observed that skeletal myogenic differentiation of sk-hPCs was enhanced by inhibition of Notch signaling, in agreement with previous studies on murine C2C12 and satellite cells [41,43–45].

Here, we provide evidence that Notch pathway inhibition enhances skeletal myogenic differentiation of sk-hPCs acting on the regulation of the expression of myogenic factors and myomiRs. This, however, contrasts with a recent study reporting that AP-positive hMABs require Dll1-induced canonical Notch signaling to achieve a more efficient skeletal muscle differentiation [46]. These apparently conflicting results might reflect the isolation protocol used by Quattrocchi et al. [46], who selects a subpopulation of ALP-positive cells from the bulk cell population of hPCs used in their work. Indeed, the effective role of Notch signaling in skeletal muscle differentiation still needs to be fully elucidated [47].

Interestingly, we also found that Notch signaling inhibition completely abolished osteogenic potential of sk-hPCs. To our knowledge, this is the first study that describes the effect of inhibition of Notch pathway on the osteogenic fate of adult sk-hPCs. Different reports show that Notch signaling is a regulator of osteogenic differentiation [29,30], although opposite effects, likely depending on cell types and/or temporal context and stage of commitment, have been reported [48–50].

In conclusion, the results reported in this study support the notion that hPCs from different adult tissues, even when isolated using the same protocol, show distinct differentiation potentials. Skeletal muscle PCs can spontaneously differentiate into skeletal muscle cells, but also in osteocytes, adipocytes, and smooth muscle cells. Human PCs from adipose and cardiac tissues are able to differentiate into osteocytes, adipocytes, and smooth muscle cells. PCs from adipose tissue can differentiate into muscle cells, but only when cocultured with myogenic cells. As reported here, hPCs from myometrium display only a restricted unipotent potential to differentiate toward smooth muscle cells. Further work will help to define whether this restricted potential may reflect the acquisition of physiological features of myometrial cells, like those observed in pregnancy. This latter aspect agrees with the emerging hypothesis that the hPCs may be committed and recruited to fulfill the specific growth and/or repair requirements of the tissue where they reside [51,52].

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Author Disclosure Statement

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