A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human Organs

Mihaela Crisan,[1,2] Solomon Yap,[1,2] Louis Casteilla,[1,2,4] Chien-Wen Chen,[1,2,8] Mirko Corselli,[1,2,8] Tea Soon Park,[1,2,8] Gabriella Andriolo,[3,8] Bin Sun,[1,2] Bo Zheng,[1,2] Li Zhang,[5] Cyrille Norotte,[1,2] Pang-Ning Teng,[1,2] Jeremy Traas,[6] Rebecca Schugar,[1,2] Bridget M. Deasy,[1,2] Stephen Badylak,[5] Hans-Jörg Bűhring,[7] Jean-Paul Giacobino,[1,2] Lorenza Lazzari,[3] Johnny Huard,[1,2] and Bruno Péault[1,2,5,*]
[1]Department of Pediatrics
[2]Department of Orthopaedic Surgery
Stem Cell Research Center, Children's Hospital of Pittsburgh, Pittsburgh, PA 15213, USA
[3]Cell Factory, Department of Regenerative Medicine, Fondazione Ospedale Maggiore Policlinico, Milan 20122, Italy
[4]UMR 5018 CNRS-UPS, Toulouse 31059, France
[5]McGowan Institute for Regenerative Medicine, Pittsburgh, PA 15213, USA
[6]Division of Surgery, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA
[7]Department of Internal Medicine, University Clinic of Tübingen, Tübingen 72076, Germany
[8]These authors contributed equally to this work
*Correspondence: bruno.peault@chp.edu
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SUMMARY

Mesenchymal stem cells (MSCs), the archetypal multipotent progenitor cells derived in cultures of developed organs, are of unknown identity and native distribution. We have prospectively identified perivascular cells, principally pericytes, in multiple human organs including skeletal muscle, pancreas, adipose tissue, and placenta, on CD146, NG2, and PDGF-Rβ expression and absence of hematopoietic, endothelial, and myogenic cell markers. Perivascular cells purified from skeletal muscle or nonmuscle tissues were myogenic in culture and in vivo. Irrespective of their tissue origin, long-term cultured perivascular cells retained myogenicity; exhibited at the clonal level osteogenic, chondrogenic, and adipogenic potentials; expressed MSC markers; and migrated in a culture model of chemotaxis. Expression of MSC markers was also detected at the surface of native, noncultured perivascular cells. Thus, blood vessel walls harbor a reserve of progenitor cells that may be integral to the origin of the elusive MSCs and other related adult stem cells.

INTRODUCTION

Subsets of cells that participate in the architecture of blood vessels exhibit developmental potentials beyond angiogenesis. Vascular endothelium generates hematopoietic cells in the embryo (Jaffredo et al., 1998; Oberlin et al., 2002; Zambidis et al., 2006), and multipotent mesoangioblasts have been derived from the embryonic aorta and other blood vessels (DeAngelis et al., 1999; Cossu and Bianco, 2003; LeGrand et al., 2004). Pericytes, aka Rouget cells or mural cells, also called mesangial cells in the kidney and Ito cells in the liver, closely encircle endothelial cells in capillaries and microvessels (Andreeva et al., 1998). Some contain α-smooth muscle actin (α-SMA) and regulate microvessel contractility (Nehls and Drenckhan, 1991; Boado and Pardridge, 1994). Pericytes can also inhibit, via TGF-β activation, the division of endothelial cells (reviewed in Betsholtz et al., 2005). In addition, pericytes are believed to include progenitors of different cell types, although this assumption relies mostly on indirect evidence. In one study, bovine retinal pericytes implanted in diffusion chambers in nude mice differentiated into chondrocytes and adipocytes (Farrington-Rock et al., 2004). Pericytes are also believed to be osteogenic and, therefore, be responsible for the pathologic calcification of blood vessels, heart valves, and skeletal muscle (reviewed in Collett and Canfield, 2005). The appearance, in pericyte-rich cultures of human dental pulp, of dentin-expressing cells also suggested that odontoblasts can be derived from perivascular cells (Alliot-Licht et al., 2005). However, each of the aforementioned differentiation experiments was performed on pericyte-containing cultures and not on purified pericytes. As a recent exception, Dellavalle et al. (2007) have demonstrated that pericytes sorted from human skeletal muscle by alkaline phosphatase expression can regenerate skeletal myofibers in dystrophic immunodeficient mice. Mesoangioblasts, the blood vessel-associated myogenic stem cells previously identified in vitro (DeAngelis et al., 1999), may therefore be related to perivascular cells. Some of us have previously discovered in "preplate" cultures of skeletal muscle a similar population of muscle-derived stem cells (MDSCs) of unknown identity that regenerate myofibers more efficiently than committed satellite cells (Qu-Petersen et al., 2002; Péault et al., 2007). Mesoangioblasts and MDSCs may be related to other elusive, multilineage progenitor cells encountered in bone marrow and other tissues, such as MSCs (mesenchymal stem cells) (Caplan, 1991; Pittenger et al., 1999) and multipotent adult progenitor cells (MAPCs) (Jiang et al., 2002). White adipose tissue (WAT) also contains progenitors of multiple mesodermal cell lineages and is therefore an attractive source of autologous cells for transplantation. Zuk

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et al. (2002) found a putative MSC population in human WAT and drove its differentiation into adipocytes, osteoblasts, chondrocytes, and myoblasts. Gronthos et al. (2001) further showed similarities between human WAT stroma vascular cells and bone marrow stromal cells. Whether these multipotent cells, all of which were isolated retrospectively in culture, share a common ancestor in multiple developed organs is unknown. In order to further explore the presence of multilineage progenitors among human perivascular cells, we first validated a combination of markers to typify these cells, which were then purified from skeletal muscle, pancreas, adipose tissue, and other organs. We demonstrate that human perivascular cells, pericytes in particular, are myogenic in vivo and ex vivo, irrespective of their tissue of origin. Moreover, human perivascular cells sorted from diverse human tissues and cultured over the long term give rise to adherent, multilineage progenitor cells that exhibit the features of MSC.

RESULTS

Immunohistochemical Detection of Perivascular Cells in Human Tissues

As a prerequisite to human perivascular cell sorting by flow cytometry, we used immunohistochemistry to establish a relevant combination of markers for this elusive cell population. To this end, we examined each of the following: (1) ten adult (16–78 years of age) and 18 fetal skeletal muscle samples (16–24 weeks of gestation); (2) 5 adult (33–61 years) and 6 fetal pancreases (17–24 weeks); (3) 28 placentas (20 at 17–24 weeks and 8 at term); and (4) 15 adult WAT as well as (5) 3 fetal hearts, 3 fetal skins, 5 lungs, 1 brain, 2 eyes, 1 gut, 1 bone marrow, and 3 term umbilical cords. In all organs, we observed the ubiquitous expression, by perivascular cells surrounding capillaries (diameter <10 μm) and arterioles (diameter from 10 to 100 μm), of NG2, a proteoglycan associated with pericytes during vascular morphogenesis (Ozerdem et al., 2002) and CD146 (aka S-endo1, Mel-CAM, Muc18, or gicerin), an endothelial cell antigen also expressed at the surface of pericytes (Li et al., 2003; Middleton et al., 2005; Sacchetti et al., 2007) (Figures 1A–1C and 1G). CD146 was indeed present on endothelium in capillaries, but not on microvessel endothelial cells (data not shown). CD146 was also detected on perivascular cells around venules. In order to ascertain that CD146+ cells detected around small vessels are indeed pericyte-like cells, tissue sections were double stained with antibodies to CD146 and NG2. As illustrated by Figures 1G–1I, CD146+ perivascular cells are also positive for NG2 expression. Furthermore, all perivascular cells express PDGF-Rβ (Figure 1D), and α-SMA was reproducibly detected in cells surrounding venules and arterioles, but not around most capillaries (Figure 1F and data not shown). In contrast, perivascular cells do not express endothelial cell markers such as CD144 (VE-cadherin) (Figure 1E), von Willebrand factor (vWF) (Figure 1D), CD34 (Figures 1A–1C), the *Ulex europaeus* lectin ligand (Figure 1F), or CD31 (data not shown). The same pericyte/perivascular cell phenotype was found in all other human organs and tissues tested. Most importantly, cells exhibiting this surface phenotype were, in all tissues analyzed, exclusively perivascular.

Flow Cytometry Sorting of Perivascular Cells

We next proceeded to analyze and sort perivascular cells by using multicolor fluorescence-activated cell sorting (FACS). Twenty-eight fetal (17–23 weeks), 6 adult skeletal muscles (50–78 years), and 8 fetal myocardiums (17–22 weeks) were first processed independently. Twenty fetal (19–22 weeks) and 8 term placentas were also used since microdissected placental blood vessels transplanted intact into SCID/mdx mouse muscles regenerated myofibers (data not shown). Eight fetal (17–23 weeks) and 2 adult human pancreases, 2 fetal skins, 4 fetal brains, 24 fetal bone marrows, and 32 adult WAT samples were used as organs devoid of regular myogenic cells. CD56+ cells were initially gated out of muscle cell suspensions in order to avoid contamination by regular myogenic cells (Péault et al., 2007) and later on eliminated from all tissues used for FACS analysis and sorting (Figure 2B), as were hematopoietic cells (CD45+) (Figure 2C). Perivascular cells were identified and sorted by high CD146 expression and lack of CD34, the latter in order to ascertain the absence of endothelial cells within sorted cells. The same population of CD146+ CD34− CD45− CD56− cells was detected in all tissues analyzed (Figures 2D–2J), amounting to 0.88% ± 0.18% in fetal skeletal muscle, 0.29% ± 0.09% in adult skeletal muscle, 1.4% ± 0.33% in fetal pancreas, 0.65% ± 0.10% in adult pancreas, and 1.79% ± 1.10% in placenta. These cells were more frequent in adipose tissue (14.6% ± 1.02%; Figure 2F), since analysis was performed on the stromal vascular fraction, which is markedly enriched in blood vessels. Perivascular cells from multiple tissues were also observed to express alkaline phosphatase (Figures 2K–2N), a marker used recently to typify pericytes in human skeletal muscle (Dellavalle et al., 2007). Sorted perivascular cells were confirmed by RT-PCR analysis in each experiment, not to include detectable hematopoietic cells (CD34, CD45), endothelial cells (CD34, vWF) or regular myogenic cells (CD56, Pax7) (data not shown).

Generalized Myogenic Potential of Perivascular Cells

Perivascular cells sorted from four distinct fetal skeletal muscles were first tested for myogenic potential in culture. Sorted cells were cultured for 8–10 days in muscle proliferation medium, then for 5–10 more days in muscle fusion medium. Typical myotubes containing three to five nuclei appeared after 8–10 days and further developed, enclosing up to 15 nuclei 10 days later. All myotubes developed in these conditions expressed human myosin heavy chain (Figure 3A), desmin (Figure 3B), and dystrophin (Figure 3C). Perivascular cells sorted from human muscle were then injected into the skeletal muscles of immunodeficient SCID-NOD mice that had been injured by intramuscular injection of cardiotoxin. Three weeks later, immunohistochemistry on injected muscle sections revealed the presence of myofibers expressing human spectrin (Figure 3D). A fluorescent probe for a human pancentromeric sequence was hybridized on some chimeric muscle sections, confirming the presence of central human nuclei within human spectrin expressing, regenerating myofibers (Figure 3D, insert). These results confirmed that perivascular cells sorted stringently from human skeletal muscle are endowed with myogenic potential. In three independent experiments, the myogenic potentials in vivo of freshly sorted perivascular cells ($CD146^{high}$ CD45− CD34− CD144− CD56−), myoblasts (CD146− CD45− CD34− CD144− CD56+) and total skeletal muscle cells were compared. These three populations generated, respectively, 20.1 ± 11.9, 13.3 ± 5.7, and 3.0 ± 2.5 myofibers expressing human spectrin per 10^3 cells injected

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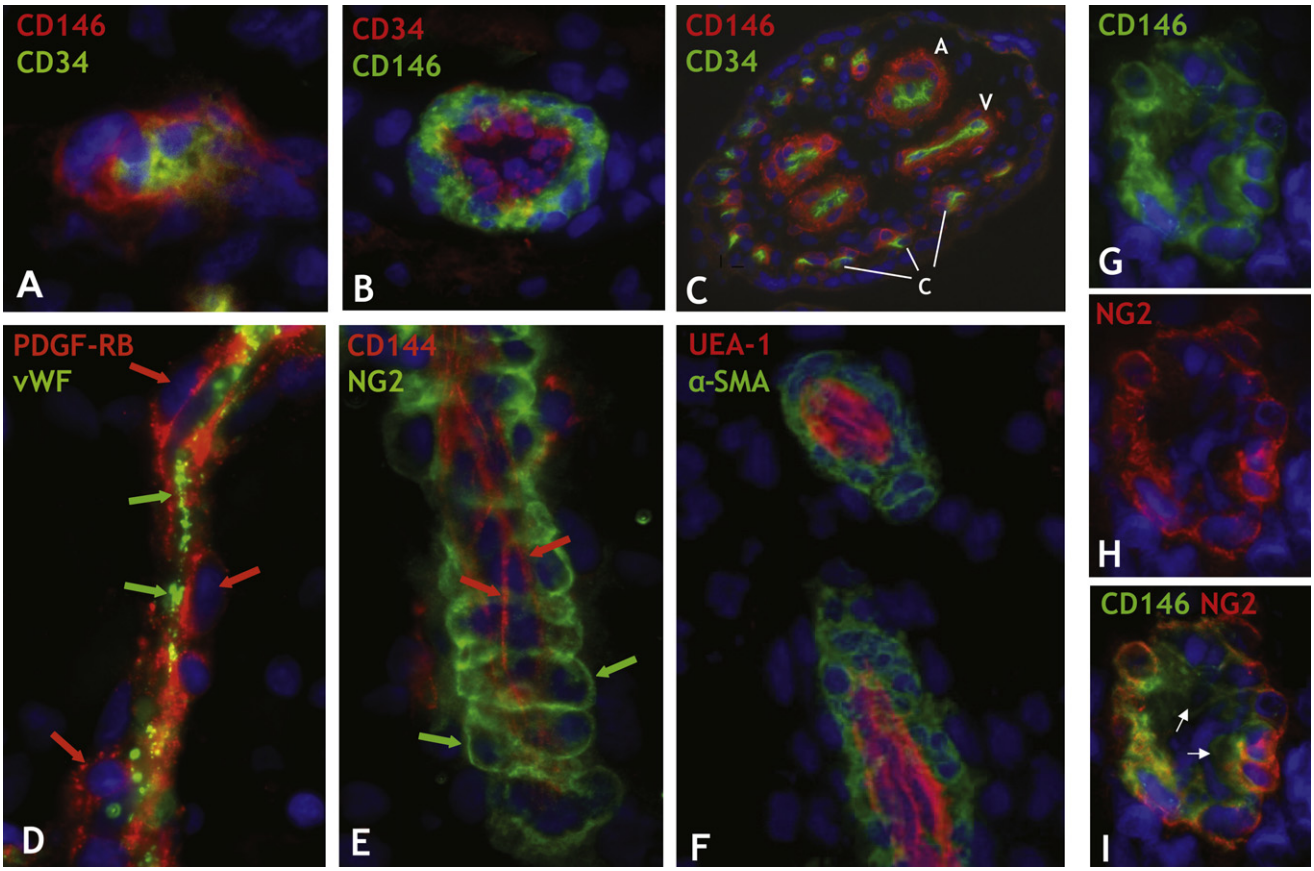

Figure 1. Immunodetection of Perivascular Cells in Human Organs

Sections were indirectly immunostained with antibodies to mural and endothelial cell markers. (A) Myocardium: a capillary endothelial cell in transverse section marked by CD34 expression (green) is closely surrounded by a CD146+ pericyte (red) (×900). (B) Skeletal muscle: small vessel transverse section; CD146+ perivascular cells (green) surround CD34+ endothelial cells (red) (×400). (C) Placental villus: arterioles (A), venules (V), and capillaries (C) are all lined with CD34+ endothelial cells (green) surrounded by CD146+ perivascular cells (×100). (D) Skeletal muscle: small vessel longitudinal section; PDGF-Rβ-positive perivascular cells (red; red arrows) surround vWF+ endothelial cells (green; green arrows) (×400). (E) Fetal pancreas: small vessel longitudinal section; NG2-positive perivascular cells (green; green arrows) surround CD144+ endothelial cells (red; red arrows) (×400). (F) Transverse and longitudinal sections of placental small blood vessels. Endothelial cells marked by the *Ulex europaeus* lectin (red) are surrounded by perivascular cells expressing α-SMA (green) (×400). (G–I) Transverse section of a skeletal muscle blood vessel double stained with antibodies to CD146 (G) and NG2 (H). (G) and (H) have been merged to create (I). Perivascular cells marked by CD146 expression also express NG2. Cells in a more central location express exclusively CD146 (arrows) and likely represent endothelial cells (×300). (A) Fetal myocardium; (B, D, G–I) skeletal muscle; (E) fetal pancreas; (C and F) placenta. Nuclei have been labeled blue with DAPI.

into the cardiotoxin conditioned mouse muscle (Figure 3H, red columns). Sorted perivascular cells produced at least as many myofibers as did purified CD56+ myoblasts, which rules out the possibility that the myogenic potential observed in the former is a consequence of their contamination by rare myoblasts.

These results indicate that at least some mural cells associated with muscle blood vessels are endowed with myogenic potential and may play a role in muscle ontogeny and regeneration, and confirm recently published results (Dellavalle et al., 2007). The important question as to whether this myogenic potential can be generalized to perivascular cells residing in nonmuscle tissues was then addressed by examining, in a similar experimental setting, mural cells extracted from pancreas, WAT, and placenta. CD146^{high} CD34− CD45− CD56− cells were sorted from four placentas, 25 WAT samples, and 5 fetal pancreases along an antigen profile very similar to that encountered in the skeletal muscle (Figures 2E, 2F, and 2I). Purity of the sorted cells was confirmed by RT-PCR analysis (data not shown). Strikingly, placenta-, WAT- and pancreas-derived perivascular cells also exhibited potential, as did skeletal muscle perivascular cells, to generate myotubes in culture (data not shown) and to yield human dystrophin- and spectrin-expressing myofibers upon transplantation into SCID-mdx (Figure 3G) or cardiotoxin-treated SCID-NOD mouse muscles (Figures 3E and 3F). The muscle regeneration index of fat-derived perivascular cells was even higher than that of muscle perivascular cells assayed in the same conditions (Figure 3H, yellow bars).

Cultured Perivascular Cells Retain Myogenic Potential and Exhibit Migratory Ability

Since both mesoangioblasts and MDSC are culture-adapted cell lines (Qu-Petersen et al., 2002; Cossu and Bianco, 2003), we next asked whether purified perivascular cells can be cultured

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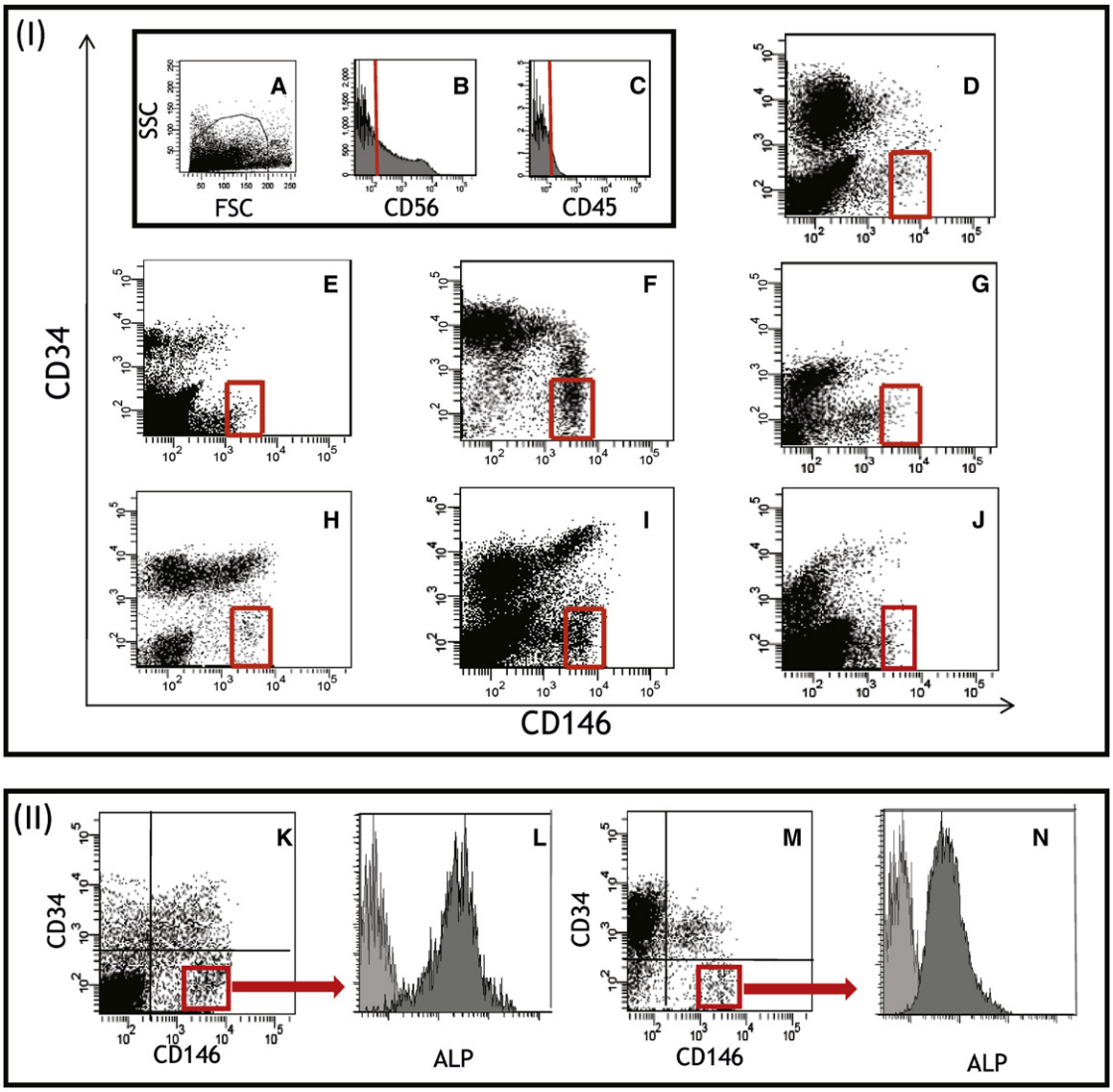

Figure 2. Flow Cytometry Analysis and Sorting of Perivascular Cells

Fresh tissues were dissociated enzymatically, and cell suspensions were stained with directly or indirectly labeled antibodies and run on a FACSAria cell sorter. Following double-scatter cell selection (A) and exclusion of CD56+ cells (B) and CD45+ cells (C), CD146^{high} CD34− perivascular cells were sorted inside the gates represented in red. A similar cell subset was detected and sorted in skeletal muscle (D), placenta (E), adipose tissue (F), skin (G), myocardium (H), pancreas (I), and bone marrow (J). (A), (B), and (C) are from skeletal muscle. In box II, perivascular cells were identified (red gates) as described above in adipose tissue (K) and muscle (M). Additional staining of the same cell suspensions with an anti-ALP antibody revealed that virtually all perivascular cells coexpress ALP (L and N).

over the long term and still sustain myogenic potential. Perivascular cells sorted from fetal and adult muscle, midterm placenta, fetal skin, fetal pancreas, bone marrow, and adipose tissue and subsequently seeded into culture adhered and proliferated in a similar manner. Subconfluent perivascular cells generally exhibited an elongated, grossly rectangular shape and displayed short arms at their smaller ends (Figure 4A). At confluence, cells were more polygonal (Figure 4B). Contact inhibition was not obvious, since cells at confluence occasionally grew into spherical structures above the plastic adherent monolayer (data not shown). Figure 4C illustrates the cumulative number of population doublings (PDs) during 20 weeks of continuous perivascular cell culture from a 23-week-old fetal muscle: sorted cells could undergo more than 40 PDs before the onset of senescence. We calculated the perivascular cell population doubling time (PDT) as previously described (Deasy et al., 2005). Growth was slow in the first 6 weeks: approximately one PD per week or PDT = 162 hr. We observed the fastest growth rate during weeks 6–16 (approximately 5–13 passages), during which the PDT was approximately 60 hr. After 17 weeks of culture, perivascular cell growth again slowed down to a PDT of about 106 hr (Figure 4C). The same growth kinetics was observed for perivascular cells sorted from pancreas, skin, adipose tissue, and placenta (data not shown). Gene expression in cultured perivascular cells was analyzed by RT-PCR. After either 4, 8, or 14 passages, muscle-derived perivascular cells stably expressed NG2, CD146, and

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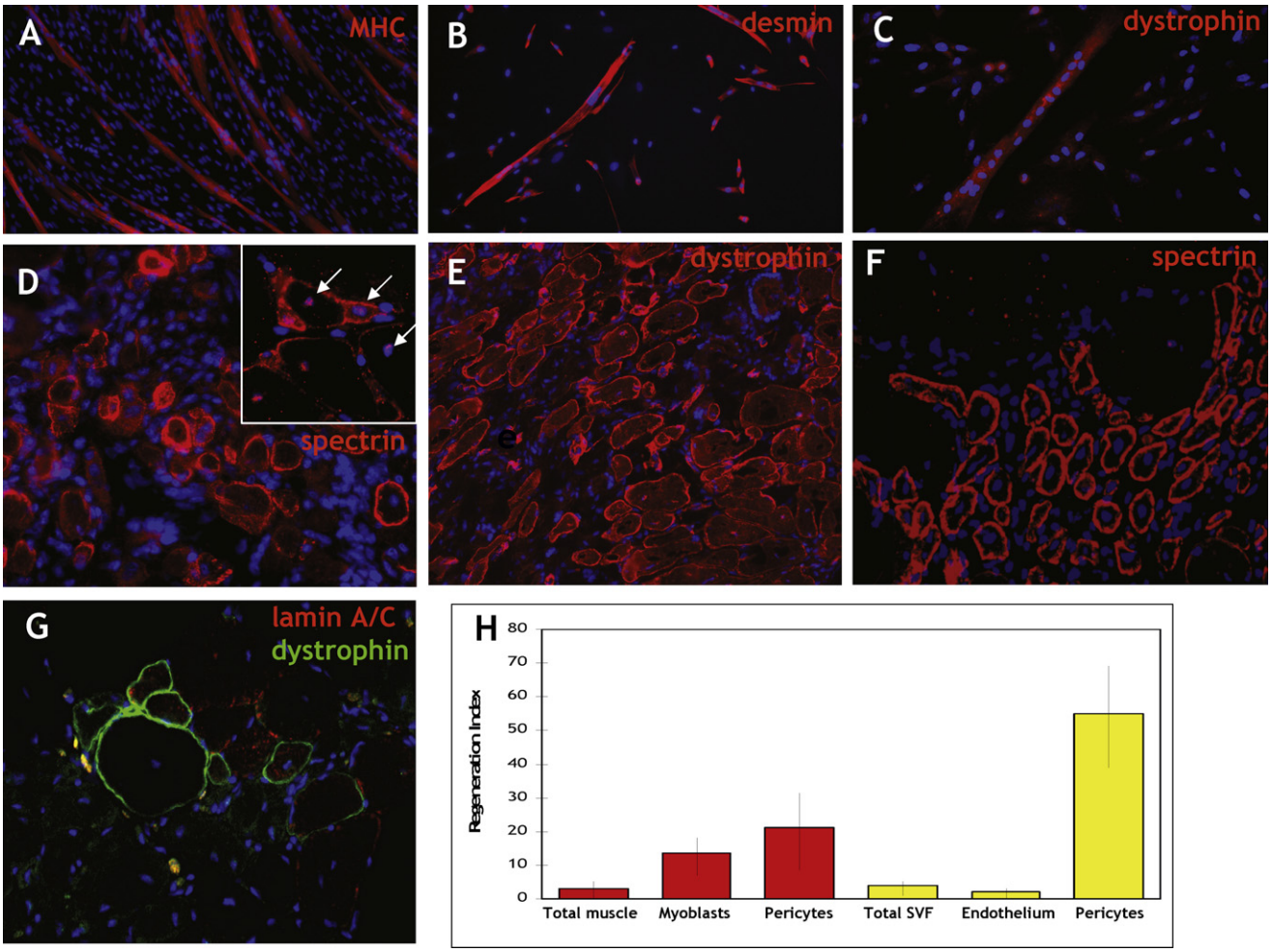

Figure 3. Myogenic Potential of Perivascular Cells

Two thousand CD146+ CD56− CD34− CD45− cells sorted from skeletal muscle were seeded per well into a 48-well plate in myogenic culture conditions. Two weeks later, numerous multinucleated muscle fibers expressing slow myosin heavy chain (MHC) (A, ×100), desmin (B, ×100), and dystrophin (C, ×200) had developed. Nuclei have been labeled blue with DAPI. 5×10^3 perivascular cells sorted from skeletal muscle (D), pancreas (E), or 10^4 from WAT (F) were injected into cardiotoxin-conditioned SCID-NOD mouse muscles. Three weeks later, recipient muscle frozen sections were stained with antibodies specific to human spectrin (D and F) and human dystrophin (E). Numerous human spectrin- and dystrophin-expressing muscle fibers (red) are present (×200). In one experiment ([D], insert), sections from a recipient mouse muscle were immunostained with an antibody to human spectrin, then exposed to a probe specific for a human pancentromeric sequence. Hybridization products appear conspicuously (pink) in the central nuclei of human spectrin expressing myofibers (arrows). (G) 2.5×10^4 perivascular cells sorted from the midgestation placenta were injected into dystrophin-deficient SCID-mdx mouse muscles that were harvested 4 weeks later. Frozen sections were stained with antibodies to mouse/human dystrophin (green) and human lamin A/C (red). Clusters of human dystrophin-expressing myofibers are present, and human cells reside near the regenerated myofibers (yellow). Nuclei have been labeled blue with DAPI (×200). (H) Red columns: unfractionated adult skeletal muscle cells, myoblasts (CD146- CD45- CD34- CD56+), and perivascular cells ($CD146^{high}$ CD45− CD34− CD56−) sorted therefrom were injected into the cardiotoxin-treated skeletal muscles of SCID-NOD mice. Yellow columns: the same experiment was performed with the WAT unfractionated stromal vascular fraction (SVF), endothelial cells (CD45− CD34+ CD56−), or perivascular cells ($CD146^{high}$ CD45− CD34− CD56−) purified from the same WAT. Three weeks later, human spectrin-expressing myofibers were counted on sections of recipient mouse muscles, and the regeneration index was calculated as the number of human myofibers produced per 10^3 donor cells injected (three independent experiments). Error bars represent the standard error of the mean.

α-SMA, but not CD31, CD34, CD45, or CD144, excluding the growth of contaminating endothelial or hematopoietic cells (Figure 4D).

The migration of progenitor cells at the site of injured or missing tissue is logically important for successful organ reconstruction. Degradation products of mammalian extracellular matrix (ECM) are chemotactic for progenitor cells, both in vivo and in culture (Reing et al., 2008). Cultured perivascular cells exhibited strong chemotaxis toward both a papain and a pepsin digest of ECM harvested from porcine urinary bladder (UBM) (Figure 4E).

Importantly, perivascular cells extracted from either muscle, placenta, or pancreas and cultured remained capable, at all passage times tested (5, 9, 11), of differentiating into myotubes in culture (data not shown) and regenerating myofibers in vivo (Figure 5A). Cultured perivascular cells from a 23 week fetal muscle were efficiently infected with recombinant lentiviruses encoding green fluorescent protein (GFP), close to 100%

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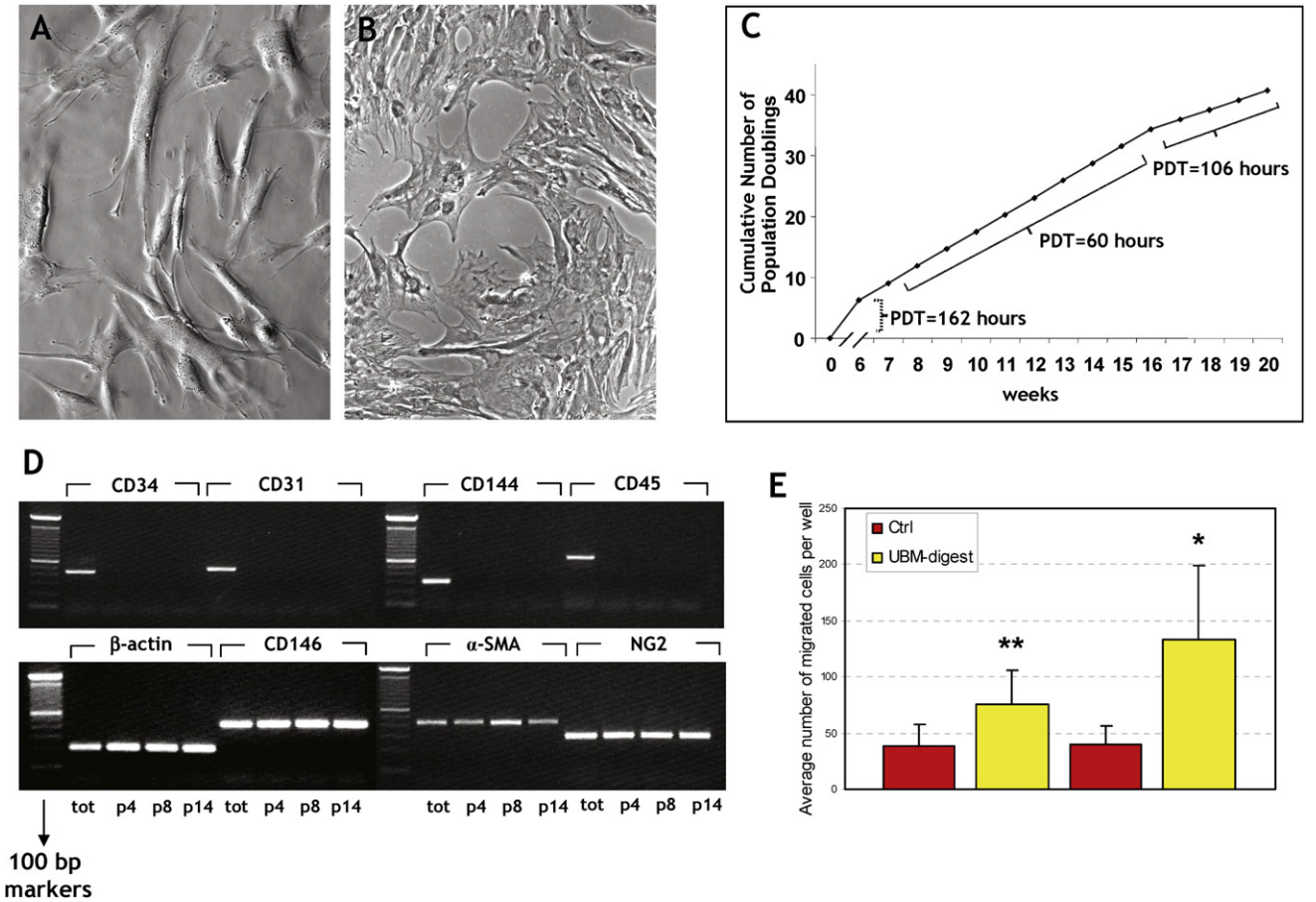

Figure 4. Long-Term Culture of Purified Perivascular Cells

Perivascular cells sorted from skeletal muscle and seeded in culture adhere and proliferate ([A], ×400) to confluence ([B], ×100). (C) PDs during 20 weeks of continuous muscle-derived perivascular cell culture. PDTs are indicated on the plot. The fastest growth rate is observed during weeks 6–16, (PDT of about 60 hr). (D) RT-PCR analysis was performed on perivascular cells sorted from the same muscle and cultured for 4, 8, and 14 passages (p4, p8, p14) and total fresh dissociated skeletal muscle cells (tot) to detect expression of markers of endothelial cells (CD31, CD34, CD144), hematopoietic cells (CD45), and perivascular cells (CD146, α-SMA, NG2). Only the latter are detected at all stages of the culture. (E) Perivascular cell migration toward pepsin-digested (left columns) and papain-digested (right columns) ECM was measured in a chemotaxis chamber. Migrations in response to matrix degradation products (yellow columns) and control buffer (red columns) were compared. $^{**}p \leq 0.02$ and $^{*}p \leq 0.05$, respectively. The y axis represents the number of cells per well that have migrated through the filter toward the chemoattractant.

cultured cells expressing GFP upon transduction. When injected into cardiotoxin-injured SCID-NOD mouse skeletal muscles, GFP-transgenic cells gave rise to discrete green fluorescent myofibers, confirming the myogenic potential of cultured perivascular cells (Figure 5B).

Cultured Perivascular Cells Give Rise to MSCs

We assessed, by flow cytometry and RT-PCR, antigens expressed by long-term cultured human perivascular cells (Figure 6). This confirmed that all perivascular cells still display, over extended culture, the markers their ancestors natively expressed in the tissue of origin (CD146, α-SMA, NG2, PDGF-Rβ, alkaline phosphatase) and that no cells in these cultures express markers of endothelial cells (CD34, CD144, CD31, and vWF), hematopoietic cells (CD45), or myogenic cells (myogenin, m-cadherin, myf-5, and Pax7) (Figures 6A–6C). We also investigated the expression in these cultures of antigens that typify MSCs derived from human bone marrow. Intriguingly, cultured human perivascular cells from either skeletal muscle, pancreas, adipose tissue, placenta, bone marrow, or other organs expressed all recognized markers of MSC, including CD10, CD13, CD44, CD73, CD90, and CD105 (Figure 6A) as well as CD108 (Sema L), CD109 (platelet activation factor), CD140b (PDGF-Rβ), CD164 (MGC 24), CD166 (ALCAM), CD318 (CDCP1), CD340 (HER-2), CD349 (frizzled-9), SSEA-4, and HLA-CL I (data not shown). In contrast, cultured perivascular cells, similar to MSC, do not express CD56, CD106, CD133, CD324 (E-cadherin), CD326 (Ep-CAM), CD344 (frizzled-4), and HLA-DR (Figure 6 and data not shown). These results suggested that the elusive MSCs are derived from perivascular cells. To further document this possible affiliation, we explored whether the developmental potential of cultured perivascular cells reflects that of MSCs. Indeed, when cultured in appropriate inductive conditions, perivascular cells from all tissue sources tested, including bone

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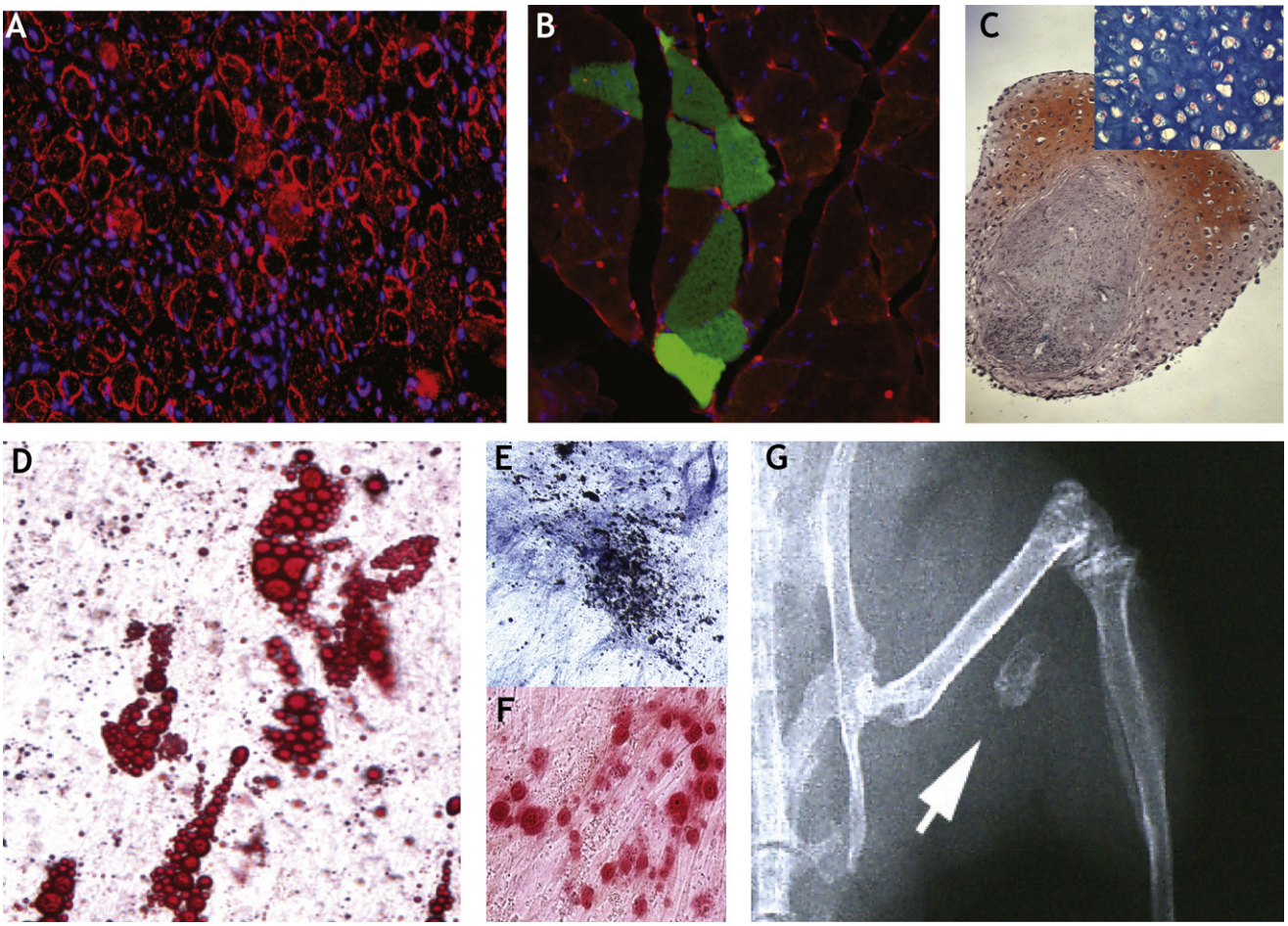

Figure 5. Multilineage Potential of Long-Term Cultured Perivascular Cells

(A) Twenty thousand perivascular cells sorted from skeletal muscle and cultured for 9 weeks were injected into the cardiotoxin-treated skeletal muscles of SCID-NOD mice. Three weeks later, human spectrin-expressing myofibers generated by injected perivascular cells are present in the host muscle (red). Nuclei have been labeled blue with DAPI (×200).

(B) 10^6 GFP-expressing cultured perivascular cells were injected into a cardiotoxin-damaged tibialis anterior skeletal muscle. Fourteen days later, clusters of GFP-expressing myofibers (green) are present throughout the recipient muscle (×30).

(C) Cultured perivascular cells were centrifuged into high-density cell pellets and cultivated in chondrogenic medium in the presence of TGF-β1. Twenty-one days later, fixed pellet sections were stained with safranin O (×20). (Insert) Pellet section stained with Alcian blue and nuclear fast red for the detection of s-GAG and nuclei, respectively (×150).

(D) Cultured perivascular cells were further cultivated in adipogenic medium for 14 days, then fixed and incubated with Oil red O for detection of lipids (×250).

(E) Cells cultivated in osteogenic medium for 21 days were fixed and treated for the detection of alkaline phosphatase, then incubated in a 2.5% silver nitrate solution (von Kossa staining). Alkaline phosphatase-positive cells are blue, and mineral deposits appear in black (×150).

(F) Cells cultivated in osteogenic medium as in (E) were incubated for 10 min in alizarin red at pH 4.2. Calcium deposits are stained in red (×150).

(G) Cultured perivascular cells immobilized in a Gelfoam sponge were implanted in a skeletal muscle pocket cut in the hind limb of a SCID-NOD mouse. X-ray analysis, at day 30 after implantation, reveals a high-density area in the recipient muscle, demonstrating ectopic bone formation by the implanted cells.

marrow, differentiated into chondrocytes (Figure 5C) and multilocular adipocytes (Figure 5D). Cultured perivascular cells also gave rise to osteocytes in vitro (Figures 5E and 5F) and developed into bony nodules when transplanted into a skeletal muscle pocket (Figure 5G) in an immunodeficient mouse. In order to ascertain that individual cultured perivascular cells are multipotent, cells from two independent cultures of muscle pericytes were cloned by limiting dilution. For each starting pericyte population, 480 wells were seeded with single cells, out of which 120 and 48 clones developed, respectively (i.e., 25% and 10% of single seeded perivascular cells grew clonally). Ten randomly chosen clones from each donor tissue were further cultured and split to analyze separately adipogenic, chondrogenic, and osteogenic differentiation potentials. Each one of the 20 perivascular cell clones analyzed differentiated into the three cell lineages, demonstrating that cloned perivascular cells exhibit the typical developmental potential of MSCs (data not shown).

Perivascular Cells Natively Express MSC Markers

Having shown that perivascular cells sorted to homogeneity from diverse human organs yield in culture a progeny of MSCs, we wished to determine whether this affiliation can be further

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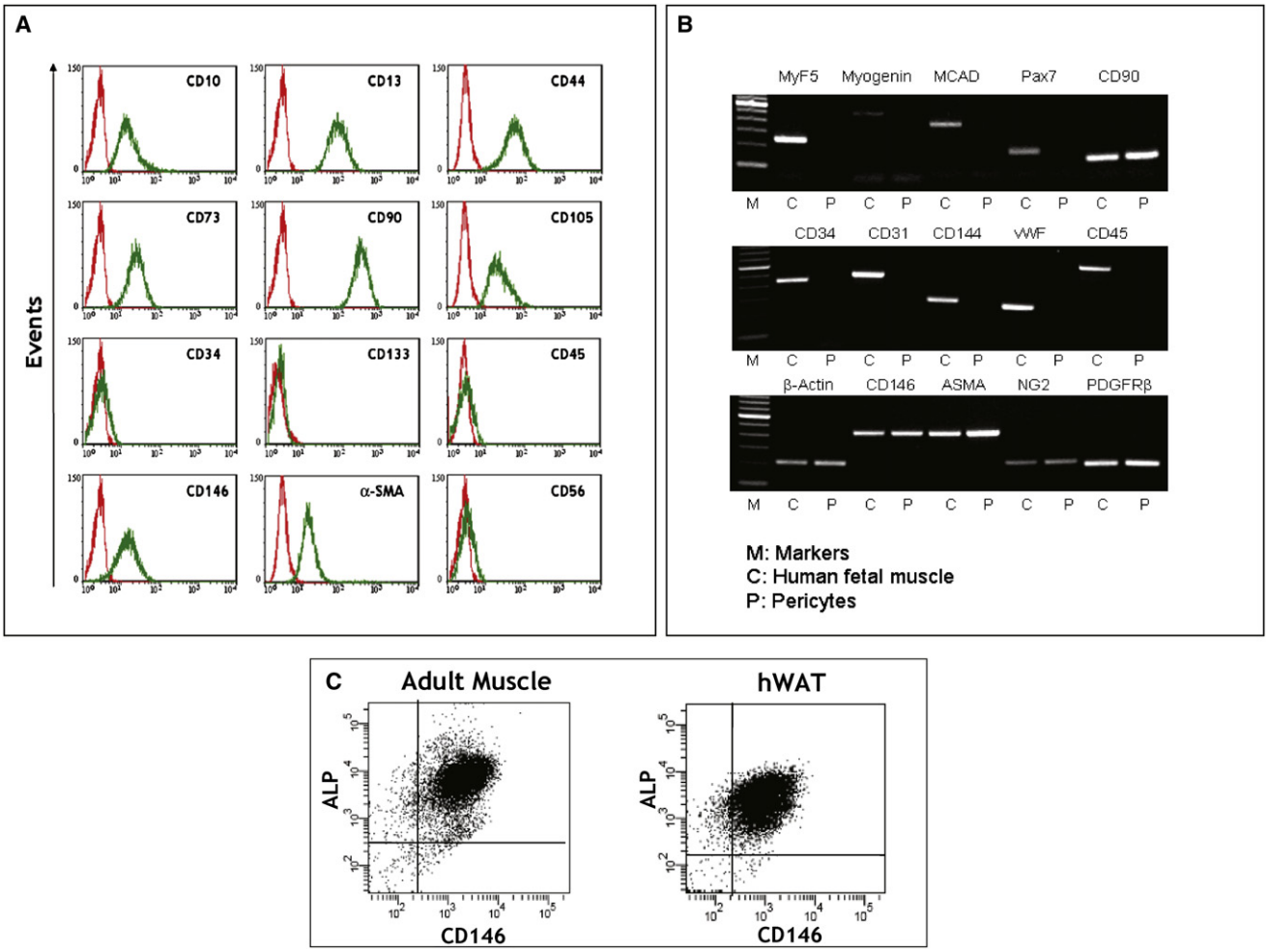

Figure 6. Long-Term Cultured Perivascular Cells Express Markers of MSCs

(A) Cells were detached from the culture flask with trypsin, permeabilized with Triton X-100 when required, and stained with directly labeled monoclonal antibodies (green histograms) or exposed to isotype-matched nonimmune directly labeled immunoglobulins (red histograms).

(B) Perivascular cells purified from skeletal muscle and cultured for 7 passages (9 weeks) were analyzed by RT-PCR. Presence of messengers expressed in endothelial cells (CD34, CD144, CD31, and vWF), perivascular cells (CD146, α-SMA, NG2, PDGF-Rβ), hematopoietic cells (CD45), MSC (CD90), and myogenic progenitors (M-cadherin, myogenin, Myf5, and Pax7) was compared between cultured pericytes (P) and total muscle (control, C).

(C) Perivascular cells sorted from adult skeletal muscle and hWAT and cultured for 4 weeks were detached and double stained with antibodies to CD146 and ALP.

supported by showing that mural cells, in their native perivascular arrangement within intact tissues, already express MSC markers. To this end, antibodies to CD44, CD73, CD90, and CD105 were added, in a five-color FACS analysis, to those used to typify perivascular cells as described in Figure 2. One fetal (23 weeks) and four adult skeletal muscles (56–86 years), three midgestation and two term placentas, and two adult WAT samples (27 and 42 years) were analyzed along this strategy. CD146^{high} CD34− CD45− CD56− perivascular cells were found to express CD44, CD73, CD90, and CD105 (Figures 7A–7E), which was confirmed by immunohistochemical staining of tissue sections (Figures 7F–7J). The perivascular location of the cells expressing MSC markers was confirmed in confocal microscopy by triple immunohistostaining with anti-CD146 (Figures 7I and 7J) and anti-α-SMA (data not shown).

DISCUSSION

We have refined the analysis and purification of cells that constitute the human blood vessel wall. Confirming and extending previous observations (Ozerdem et al., 2002; Middleton et al., 2005; Sacchetti et al., 2007), we have validated the CD146+ NG2+ PDGFRβ+ ALP+ CD34− CD45− vWF− CD144− phenotype as an indicator of pericyte/perivascular cell identity throughout human fetal and adult organs. We demonstrate that perivascular cells isolated prospectively from skeletal muscle and, less expectedly, nonmuscle tissues are myogenic in culture and in vivo. We can rule out that the myogenic potential present in perivascular cells sorted from skeletal muscle is due to contamination by regular myogenic cells since (1) no Pax7 or CD56 expression was ever detected by RT-PCR analysis within

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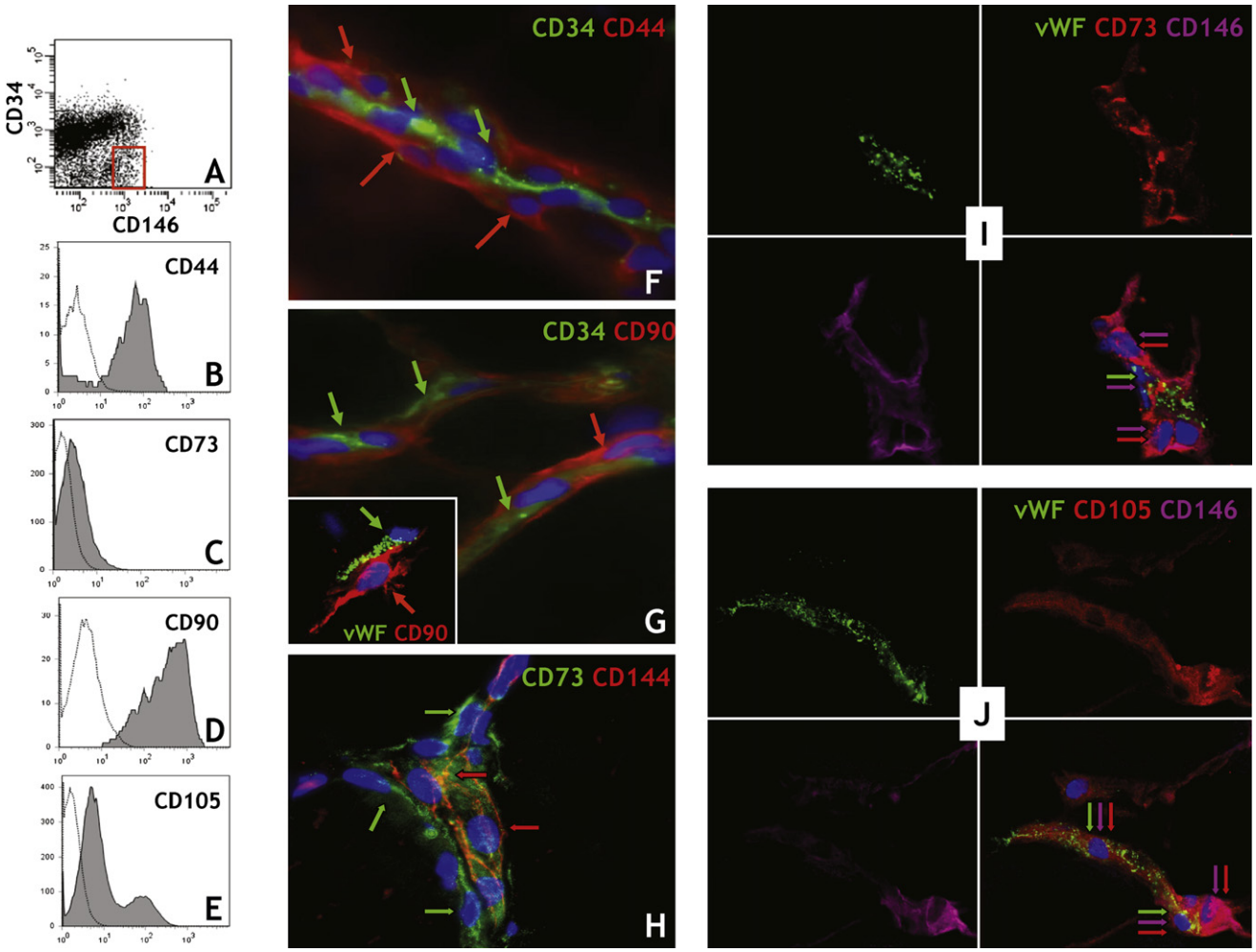

Figure 7. Perivascular Cells Natively Express MSC Markers

(Left column) The stromal vascular fraction isolated from human WAT was stained simultaneously with antibodies to perivascular cells and MSCs and analyzed by flow cytometry. CD45− CD56− CD146^{high} CD34− cells were gated (A) and analyzed for coexpression of CD44 (B), CD73 (C), CD90 (D), and CD105 (E). Clear histograms in (B)–(E) represent control cells incubated with unrelated isotype-matched antibodies. (Middle column) Frozen sections of human WAT were costained with antibodies to CD34 (green) or von Willebrand factor (vWF, green) to reveal endothelial cells (green arrows) and either CD44 (red, [F]) or CD90 (red, [G]. Pericytes lining small blood vessels express both CD44 (F) and CD90 ([G], main) (red arrows). Pericytes surrounding capillaries also strongly express CD90 ([G], inlet ×1000) (red arrow). (H) Frozen sections of adult human muscle were costained with antibodies to CD144 (red) to reveal endothelial cells (red arrows) and CD73 (green). Pericytes lining the small blood vessel express CD73 ([H], ×600, green arrows). (Right column) Frozen sections of human WAT were colabeled with antibodies to vWF (green), CD146 (purple), and CD73 ([I], red) or CD105 ([J], red). Confocal microscopy revealed that pericytes lining capillaries express both CD73 ([I], ×1000) and CD105 ([J], ×1000) in addition to CD146 (purple/red arrows). In contrast, endothelial cells express vWF and lower levels of CD146 (I) (green/purple arrows) as well as CD105 (J) (green/purple/red arrows).

extracts of freshly sorted cells or by immunocytochemistry on the long-term cultured progeny thereof and (2) the average muscle regeneration index of perivascular cells was at least as high as that of CD56+ myogenic cells purified from the same muscles. We also confirmed that cultured muscle pericytes, despite their vigorous myogenic potential, do not express any known myogenic cell marker such as MyoD, myogenin, m-cadherin, myf-5, and Pax7. This is an important point inasmuch as some activated myoblasts in fetal muscle express CD146 (Cerletti et al., 2006). These results confirm that pericytes in human skeletal muscle represent a myogenic cell compartment that is distinct from that of satellite cells (Dellavalle et al., 2007) but, importantly, also extend this potential to perivascular cells present in nonmuscle tissues such as pancreas, fat, bone marrow, and placenta, indicating the existence of a ubiquitous myogenic potential throughout the organism. We suppose that this developmental potential is circumscribed to pericytes, i.e., perivascular cells associated with capillaries and microvessels, which build up most of, if not all, the vasculature in some of the tissues analyzed, such as fetal pancreas, muscle, and WAT. However, the presence of myogenic progenitors at the periphery of larger veins and arteries cannot be ruled out, since two of the pericyte markers used in this work, NG2 and CD146, are also expressed around larger blood vessels.

Multilineage progenitor cells have been previously identified in multiple adult human and rodent tissues to include (1) MSCs,

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which are derived from bone marrow, placenta, umbilical cord, and cord blood and adipose tissue and can differentiate into mesoderm lineage cells, including myoblasts (Caplan, 1991; Pittenger et al., 1999); (2) muscle-derived MDSCs, which some of us have previously characterized (Qu-Petersen et al., 2002; Péault et al., 2007); and (3) bone marrow-derived MAPCs, which can contribute to mesodermal, endodermal, and ectodermal cell lineages and have equivalents in mouse brain (Jiang et al., 2002), pancreas (Seaberg et al., 2004), and dermis (Toma et al., 2001) as well as in human skin (Shih et al., 2005) and WAT (Zuk et al., 2002). The existence of all these adult multipotent progenitors has been, however, revealed only retrospectively in long-term cultures of the source tissues; therefore, their identity and anatomic distribution in native organs could not be determined. Using a large panel of known and novel cell markers (Battula et al., 2007; Gang et al., 2007), we observed that cultured perivascular cells from a variety of tissues exhibit a phenotype that is strikingly similar to that of MSCs derived from bone marrow. Moreover, we show that long-term cultured perivascular cells sorted from diverse fetal and adult tissues can differentiate into osteocytes, chondrocytes, and adipocytes, which is the cardinal property of MSCs. Most importantly, single cultured perivascular cells seeded in vitro developed at high frequency into clones, which all yielded osteocytes, chondrocytes, and adipocytes when seeded in the appropriate differentiation conditions. This demonstrates that each individual perivascular cell has multilineage mesodermal potential and therefore adheres to the strict definition of a MSC. Conversely, our unpublished observations indicate that the same cultured human perivascular cells can neither differentiate into neural cells nor develop into teratomas when transplanted into mouse brain and testis, respectively (data not shown). These cells therefore appear to be developmentally closer to MSCs than MAPCs or ESCs. In support of a perivascular origin of MSCs, we have also observed that perivascular cells express natively, before culture, CD44, CD73, CD90, and CD105, four surface molecules commonly used as MSC markers. This indicates that perivascular cells—or a subset thereof—have not merely acquired MSC antigens in culture but natively expressed these markers, and therefore further suggests a developmental affiliation between perivascular cells and MSCs. Interestingly, Schwab and Gargett (2007) have also suggested CD146 and PDGFRβ as markers of MSC-like cells in the endometrium, and Traktuev et al. (2008) described multipotent cells in WAT that coexpress some pericyte and MSC markers; that cell subset is, however, marked by CD34 expression and therefore clearly distinct from the cells we describe herein.

Overall, our data support the hypothesis that an ancestor of the MSC is natively associated with the blood vessel wall and, more precisely, belongs to a subset of perivascular cells (da Silva Meirelles et al., 2008), even though it remains possible that some MSCs originate in other cell subsets. The omnipresence of mural cells would explain why such multilineage progenitors have been found in a multitude of organs. In agreement, pericytes surrounding brain blood vessels constitutively express nestin, a marker of neuronal progenitor cells (Alliot et al., 1999), and therefore may play a role in neuronal regeneration. Chemoablated Leydig cells in the rat testis are regenerated by adjacent vascular pericytes which, during this process, transiently express nestin (Davidoff et al., 2004). A study performed in mice also showed that perivascular cells expressing Annexin 5 represent multilineage mesenchymal progenitors (Brachvogel et al., 2005). Interestingly, a population of CD146+ subendothelial cells in human bone marrow contains osteogenic progenitors that are also at the origin of the stromal cells that support hematopoiesis (Sacchetti et al., 2007).

A physiological role of perivascular cells in human skeletal muscle regeneration remains, however, to be formally demonstrated, especially with respect to the observed presence of a similar myogenic potential within pancreas, placenta, or fat. Satellite cells are undifferentiated myoblasts that are integral to myofiber regeneration during postnatal life (Morgan and Partridge, 2003; Péault et al., 2007), and experiments in quail-chicken chimeras have revealed the origin of adult satellite cells in the dermomyotome (Gros et al., 2005). This makes it unlikely that, at least in birds, cells from the vessel wall contribute significantly to the pool of satellite cells.

On the other hand, we have recently identified a population of myoendothelial cells in human skeletal muscle that coexpress markers of both satellite and endothelial cells and exhibit robust myogenic potential (Zheng et al., 2007). While the physiologic role of vascular mesodermal progenitors in tissue development, repair, and homeostasis remains to be elucidated, their practical use to treat medical conditions can be already envisioned, since these cells can be easily purified from convenient tissue sources such as skeletal muscle and adipose tissue and multiplied in culture to therapeutically relevant numbers with no significant loss of developmental potential.

EXPERIMENTAL PROCEDURES

Human Tissues

Human fetal tissues were obtained following spontaneous, voluntary, or therapeutic pregnancy interruptions performed at Magee-Womens Hospital (University of Pittsburgh), in compliance with Institutional Review Board protocol number 0506176. Developmental age (16–24 weeks of gestation) was estimated by measuring foot length. Informed consent for the use of fetal tissues was obtained from patients in all instances. Adult human pancreas and muscle were procured by the Center for Organ Recovery and Education (CORE, Pittsburgh) from multiorgan donors. Abdominal subcutaneous fat was obtained anonymously from female patients (mean age of 51 years) undergoing abdominoplasty at the Department of Surgery of the University of Pittsburgh Medical Center.

Immunohistochemistry and Cytochemistry

Fresh tissues were gradually frozen by immersion in isopentane (Merck) cooled in liquid nitrogen and embedded in tissue freezing medium (Triangle Biomedical Sciences). WAT was impregnated in gelatin/sucrose and frozen in the same conditions. Sections (5–9 μm) were cut on a cryostat (Microm) and fixed for 5 min with 50% acetone (VWR International) and 50% methanol (Fischer Chemical) or for 10 min in 4% paraformaldehyde (PFA, Sigma). Sections were dried for 5 min at room temperature (RT), washed three times for 5 min in PBS, and blocked with 5% goat serum (GIBCO) in PBS for 1 hr at RT. Sections were incubated with uncoupled primary antibodies overnight at 4°C or for 2 hr at RT in the case of directly coupled antibodies. After rinsing, sections were incubated for 1 hr at RT with a biotinylated secondary antibody, then with fluorochrome-coupled streptavidin, both diluted in 5% goat serum in PBS. For intracellular stainings, cells were first permeabilized with PBS 0.1% Triton X-100 (Sigma). Cultured cells were fixed inside wells as described above, then washed three times in PBS 0.1% Triton X-100 and incubated for 1 hr in PBS, 5% goat serum. Cultured cells were then stained as described above, in the presence of 0.1% Triton X-100. The following uncoupled

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anti-human primary antibodies were used: anti-CD140b (PDGF-Rβ; clone 28D4; Monoclonal Antibody Facility of the University Clinic of Tübingen, undiluted), anti-CD146 (BD Pharmingen, 1:100), anti-CD31 (DAKO, 1:100), anti-CD34 (Serotec, 1:50), anti-CD44, anti-CD90 (both from Becton Dickinson (BD), 1:20), anti-CD73 and anti-CD105 (both from Invitrogen, 1:50), anti-NG2 (BD Pharmingen, 1:300), and anti-lamin A/C (Novo Castra, 1:100). Coupled antibodies used included the following: anti-CD146-Alexa 488 (Chemicon, 1:200), anti-α-SMA-FITC (Chemicon, 1:100), anti-CD34-FITC (DAKO, 1:50 or Miltenyi, 1:20), anti-vWF-FITC (US Biological, 1:100), biotinylated anti-CD144 (BD, 1:100), and biotinylated anti-CD146 (Miltenyi Biotec, 1:11). Streptavidin-Cy3 (Sigma, 1:500) and Streptavidin-Cy5 (CyDye, 1:500) were used in conjunction with biotinylated antibodies. Skeletal muscle proteins were detected with anti-skeletal myosin heavy chain (fast) (Sigma 1:100), anti-skeletal myosin heavy chain (slow) (Sigma 1:100), anti-spectrin and anti-dystrophin (Novocastra, 1:20) and anti-desmin (1:50, Sigma). Rabbit anti-mouse dystrophin (Abcam, 1:100) was used to detect dystrophin positive myofibers in SCID/mdx mice. Directly biotinylated *Ulex europaeus* lectin (UEA-1) was also used as an endothelial cell marker (Vector, 1:200). Secondary goat anti-mouse antibodies were biotinylated (DAKO and Immunotech, 1:1000) or coupled to Alexa 488 (Molecular Probes, 1:500). Streptavidin-Cy3 (Sigma, 1:1000) was used. Nuclei were stained with DAPI (4′, 6-diamino-2-phenylindole dihydrochloride, Molecular Probes, 1:2000) for 5 min at RT. An isotype-matched negative control was performed with each immunostaining. Slides were mounted in glycerol-PBS (1:1, Sigma) and observed on an epifluorescence microscope (Nikon Eclipse TE 2000-U). Alternatively, sections were analyzed on an Olympus Fluoview 1000 confocal microscope equipped with 100× oil immersion optics.

Fluorescent in situ hybridization, used in one experiment, is described in the Supplemental Data available online.

Flow Cytometry

Fresh tissues were cut into small pieces in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 20% fetal calf serum (FCS, GIBCO), 1% penicillin-streptomycin (PS, GIBCO), and collagenases I, II, and IV (1 mg/mL, Sigma), then incubated at 37°C for 1 hr on a shaker. Final cell dissociation was achieved between ground glass slides. Adipose tissue was minced, then digested in DMEM containing 3.5% bovine serum albumin (Sigma) and collagenase II (1 mg/ml, Sigma) for 70 min under agitation at 37°C. Mature adipocytes were separated from pellets by centrifugation (2000 rpm, 10 min). Pellets were resuspended in erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) and incubated for 10 min at RT. Placenta was minced and agitated in DMEM, 1% PS, and 1 mg/ml collagenases I, II, and IV (1 g tissue/1 ml solution) for 30 min at 37°C, 120 rpm; 0.05% trypsin (GIBCO) was added, and the suspension was agitated for 10 min more. Cells were centrifuged and resuspended in DMEM, 1% PS. Cells were passed through a 70 μm cell strainer, centrifuged, and resuspended in erythrocyte lysis buffer and incubated for 15 min at RT.

Cells from all tissues were then processed for immunofluorescence staining as previously described (Zheng et al., 2007). Cells (10^5 for analysis and around 30×10^6 for sorting) were incubated with a combination of the following directly coupled mouse anti-human antibodies: anti-CD34-PE (DAKO, 1:100), anti-CD45-APC-Cy7 (Santa Cruz Biotechnologies, 1:200), or anti-CD45-APC (BD, 1:100), anti-CD56-PE-Cy7 and anti-CD146-FITC (Serotec, 1:100) in 1 ml DMEM, 20% FCS, and 1% PS at 4°C for 15 min in the dark. Cells were then incubated for 15 min with 7-amino-actinomycin D (7-AAD, 1:100, BD) for dead cell exclusion and run on a FACSAria flow cytometer (BD). Five-color analysis of ALP and MSC marker expression by perivascular cells is described in the Supplemental Data.

Cultured cells were labeled at different passages with the following commercial antibodies: anti-CD13-PE, -CD34-PE, -CD44-FITC (BD), anti-CD45-PE-Cy7 (Beckman Coulter), anti-CD56-PE (Chemicon), anti-CD73-PE (BD), anti-CD90-PE (Chemicon), anti-CD105 FITC (ImmunoTools), anti-CD133-2-APC (Miltenyi Biotec), anti-CD146-PE (BioCytex), anti-α-SMA-FITC (Sigma), anti-HLA-ABC-FITC (Immunotech), and anti-HLA-DR-PE, -CXCR4, -NGF-R, and -BB9 (BD), the latter being revealed with anti-mouse IgG1-FITC (Exalpha). The following reagents from the monoclonal antibody facility of the University Clinic of Tübingen were also used for staining of cultured cells: anti-CD10 (CALLA; clone 97C5), anti-CD34 (clone 43A1), anti-CD56 (N-CAM; clone 39D5), anti-CD105 (endoglin; clone 1G2C2), anti-CD109 (clone W7C5), anti-CD133 (clone W6B1C3), anti-CD140b (PDGF-RB; clone 28D4), anti-CD164 (clone 67D2), anti-CD318 (CDCP1; clone CUB1), anti-CD324 (E-cadherin; clone 67A4), anti-CD326 (Ep-CAM; clone 9C4), anti-CD340 (HER-2; clone 24D2), anti-CD344 (frizzled-4; clone CH3A4), and anti-CD349 (frizzled-9; clone W3C4E11). Anti-CD166 was purchased from BD Pharmingen, and the PE conjugates against CD31, CD106, and CD108 were a kind gift from Dr. Gene Lay (BioLegend, San Diego, CA). Full information on the human cell markers used in this study can also be found at: http://www.hcdm.org. Isotype control immunoglobulins used were IgG1-PE, IgG1-FITC (both from Chemicon), IgG1-PE-Cy7 (Beckman Coulter), and IgG1-APC (BD). Resuspended cultured cells were stained as follows. Cells were permeabilized with 0.1% Triton X-100 (Sigma) when necessary. After washing the cells twice with PBS containing 1% FCS and 0.01% NaN_3 (FACS buffer), cells were incubated with polyglobin to block nonspecific binding. Cells were then incubated with the indicated primary antibodies for 15 min on ice. After washing in FACS buffer, cells were incubated with a $F(ab)_2$ fragment of goat anti-mouse secondary antibody conjugated with R-PE (Dako Cytomation) for 15 min. Finally, cells were washed twice, and at least 50,000 events were acquired on a FACSCanto II cytometer (BD), using FCS express software for analysis.

RT-PCR

Total RNA was extracted from 10^4 cells using the Absolutely RNA Nanoprep Kit (Stratagene). cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen). PCR was performed for 30 cycles at 58°C annealing temperature with Taq polymerase (Invitrogen), and PCR products were electrophoresed on 1% agarose gels. The primers used for PCR are listed in Table S1. Each set of oligonucleotides was designed to span two different exons so that genomic DNA contamination is of no concern.

Cell Culture and Gene Transduction

Sorted perivascular cells were seeded at 2×10^4 cells per cm^2 in Endothelial Cell Growth Medium 2 (EGM-2, Cambrex BioScience) and cultured at 37°C for 2 weeks in plates coated with 0.2% gelatin (Calbiochem). Confluent cells were then detached by treatment with trypsin-EDTA (GIBCO) for 10 min at 37°C, then split 1:3 in uncoated plates in DMEM high glucose (GIBCO), 20% FCS, 1% PS (GIBCO). After the fifth passage, cells were then passaged 1:6 in the same conditions, and culture medium was changed every 4 days. We calculated the PDT as previously described (Deasy et al., 2005). Perivascular cells were cloned in 96-well cloning plates (BD) by serial limiting dilution down to a concentration of one cell/well in DMEM, 20% FCS. Medium was changed twice a week. Plates were examined daily under the microscope in order to evaluate cell growth. Each clone was expanded until enough cells were present to test differentiation potentials as described below. For gene transfer, cultured perivascular cells at passage 12 were detached with 0.25% trypsin/EDTA and seeded at a density of 100,000 cells/cm^2. After 16 hr, medium was replaced with transduction medium (DMEM high glucose, 20% FCS, 1% PS, 8 μg/mL polybrene), and an EIAV-based CMV-driven eGFP expression vector (Endo et al., 2007) was added at an moi of 100. After 3 days, nearly 100% of cells expressed GFP and were returned to culture medium.

Myogenesis in Culture

Cells (2×10^3 cells per cm^2) were cultured for 7 days in proliferation medium (DMEM high glucose, 10% FCS, 10% horse serum [HS, GIBCO], 1% CEE [chicken embryo extract, Accurate], 1% PS) and then for 7–10 days in fusion medium (DMEM high glucose, 1% FCS, 1% HS, 0.5% CEE, 1% PS [GIBCO]). Half of the medium was renewed every 4 days. Myogenesis was induced by lowering serum concentration to 2%, and medium was changed every 4 days until elongated, multinucleated myofibers appeared.

Muscle Regeneration In Vivo

Eight- to twelve-week old SCID-NOD mice were anaesthetized by inhalation of isofluorane/O_2. Cardiotoxin (15 μg; CTX, Molecular Probes) was injected into the gastrocnemius muscle 3 hr prior to cell transplantation. Mice were reanaesthetized, and cells suspended in 35 μl PBS, or the same volume of PBS as a control, were then slowly injected into the injured muscle. Mice were sacrificed 3 weeks later and muscle was harvested. Alternatively, 8- to 12-week old dystrophic SCID-mdx mice were used as recipients. Muscles that had

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received GFP+ cultured cells were fixed in 1.5% PFA, mounted in embedding medium (Tissue-Tek, Sakura), and sectioned as 10 μm thick transverse sections. After three 15 min washes in PBS, slides were mounted with 50% glycerol/DAPI solution. GFP was directly visualized by fluorescence microscopy using both standard bandpass as well as ratiometric techniques to eliminate background autofluorescence (Leica DMRBE).

Adipogenesis, Osteogenesis, and Chondrogenesis in Culture

For adipogenic differentiation, cultured cells at 70% confluence were switched to DMEM, 10% FCS, 1 μM dexamethasone, 0.5 μM isobutylmethylxanthine, 60 μM indomethacine, and 170 μM insulin (all from Sigma-Aldrich). After 14 days, cells were fixed in 2% PFA at RT, washed in 60% isopropanol, and incubated with oil red O for 10 min at RT for the detection of lipids.

For chondrogenesis, pellets were prepared by spinning down 3×10^5 cultured cells and grown in serum-free DMEM containing an insulin-transferrin-selenious (ITS) acid mix (BD Biosciences), 50 μg/ml L-ascorbic acid 2-phosphate (WAKO), 100 μg/ml sodium pyruvate, 40 μg/ml L-proline (both from Invitrogen), 0.1 μM dexamethasone (Sigma-Aldrich), and 10 ng/ml transforming growth factor β1 (TGF-β1; Peprotech). After 21 days, pellets were fixed in 10% formalin, dehydrated in ethanol, and embedded in paraffin. Sections 5 μm thick were rehydrated and stained with Alcian blue and Nuclear Fast Red for the detection of sulfated glycosaminoglycans and nuclei, respectively.

For in vitro bone formation, cells at 70% confluence were cultivated in DMEM, 10% FCS, 0.1 μM dexamethasone, 50 μg/mL L-ascorbic acid, and 10 mM β-glycerophosphate. After 21 days, cells were fixed in 4% formaldehyde for 2 min and incubated for 10 min with alizarin red (pH 4.2) for the detection of calcium deposits. For detection of alkaline phosphatase activity, fixed cells were incubated for 45 min in a mixture of naphtol AS-BI alkaline solution with Fast Blue BB. Cells were then rinsed with deionized water and incubated in a 2.5% silver nitrate solution for 30 min in order to detect mineral deposition (all reagents from Sigma-Aldrich).

For osteogenesis in vivo, 5×10^5 cells in 100 μl were seeded on the surface of a 6 × 6 mm piece of sterile gelatin sponge (Gelfoam), which was then placed in a 24-well plate. After the cell suspension was absorbed, 3 ml of DMEM, 10% FCS was added to the well, which was incubated overnight. The Gelfoam scaffold was then implanted into a pocket in the gluteofemoral muscle of a SCID-NOD mouse. X-ray analysis was performed 30 days later to evaluate bone formation.

Cell Migration Assay

Cultured cells were starved for 15–18 hr in medium containing 0.5% FCS, resuspended in serum-free medium at 6×10^5 cells/mL, and preincubated for 1 hr at 37°C. Polycarbonate chemotaxis filters (8 μm pore size, Neuro Probe, Gaithersburg, MD) were coated with 0.05 mg/mL collagen I (BD Biosciences, San Jose, CA). Pepsin or papain ECM degradation products (Reing et al., 2008) were added to the bottom wells (100 ug/ml porcine urinary bladder matrix [dry weight] pepsin digest and 100 ug/ml UBM papain digest) of a Neuro Probe 48-well micro chemotaxis chamber (Neuro Probe Inc., Gaithersburg, MD). Thirty thousand cells were then added to each upper well and placed in the incubator for 3 hr. Nonmigrating cells on the top side of the membrane were removed, and migrating cells on the bottom side were stained with Diff Quik (Dade AG Liederbach, Germany). Three fields were counted from each well. Each condition was tested in quadruplicate wells, and the average number of migrating cells was determined. Each experiment was repeated three times. The Student's t test was used to assess differences in cell migration toward ECM degradation products and appropriate control buffer. P values ≤ 0.05 were considered significant.

SUPPLEMENTAL DATA

The Supplemental Data include supplemental text and one table and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/3/3/301/DC1/.

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