Human Adult Vena Saphena Contains Perivascular Progenitor Cells Endowed With Clonogenic and Proangiogenic Potential

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Background—Clinical trials in ischemic patients showed the safety and benefit of autologous bone marrow progenitor cell transplantation. Non–bone marrow progenitor cells with proangiogenic capacities have been described, yet they remain clinically unexploited owing to their scarcity, difficulty of access, and low ex vivo expansibility. We investigated the presence, antigenic profile, expansion capacity, and proangiogenic potential of progenitor cells from the saphenous vein of patients undergoing coronary artery bypass surgery.

Methods and Results—CD34-positive cells, negative for the endothelial marker von Willebrand factor, were localized around adventitial vasa vasorum. After dissection of the vein from surrounding tissues and enzymatic digestion, CD34-positive/CD31-negative cells were isolated by selective cell sorting. In the presence of serum, CD34-positive/CD31-negative cells gave rise to a highly proliferative population that expressed pericyte/mesenchymal antigens together with the stem cell marker Sox2 and showed clonogenic and multilineage differentiation capacities. We called this population “saphenous vein–derived progenitor cells” (SVPs). In culture, SVPs integrated into networks formed by endothelial cells and supported angiogenesis through paracrine mechanisms. Reciprocally, endothelial cell–released factors facilitated SVP migration. These interactive responses were inhibited by Tie-2 or platelet-derived growth factor-BB blockade. Intramuscular injection of SVPs in ischemic limbs of immunodeficient mice improved neovascularization and blood flow recovery. At 14 days after transplantation, proliferating SVPs were still detectable in the recipient muscles, where they established N-cadherin–mediated physical contact with the capillary endothelium.

Conclusions—SVPs generated from human vein CD34-positive/CD31-negative progenitor cells might represent a new therapeutic tool for angiogenic therapy in ischemic patients. (Circulation. 2010;121:1735-1745.)

Key Words: CD34 antigen ■ pericytes ■ angiogenesis factors ■ ischemia ■ cell therapy

Recent evidence indicating the presence of progenitor cells in arteries and veins has inspired hope for their application in regenerative vascular medicine. In fetal and postnatal vessels, putative proangiogenic progenitors reside in the vasculogenic niche, which comprises adventitial stromal cells and mature vascular cells of the vasa vasorum. We previously showed that CD34-positive (CD34pos) cells from the human fetal aorta coexpress stem cell markers such as CD133 and c-Kit, are clonogenic, and give rise to vascular cells and skeletal myocytes. Local implantation of fetal aorta–derived CD34pos/CD133-positive (CD133pos) cells promoted reparative neovascularization in models of ischemia and diabetic ulcers through incorporation into nascent vessels and paracrine stimulation of resident vascular cells.

Ethical concerns and immunogenic/tumorigenic problems limit the clinical use of embryonic/fetal stem cells. On the other hand, CD34pos progenitor cells from adult vessels show low coexpression of CD133 and show limited expansibility and differentiation potential. To date, no preclinical data are available on the in vivo regenerative potential of adult vessel–derived progenitor cells. The establishment of standardized protocols for isolation of proangiogenic cells from adult tissues and the demonstration of therapeutic activity are therefore needed.

Clinical Perspective on p 1745

The saphenous vein is the conduit of choice in coronary and peripheral artery bypass operation. During the surgical procedure, a large portion of the vein is excised, but usually,
only part of it is transplanted. Here, we demonstrate the presence of progenitor cells with proangiogenic capacity in saphenous vein leftovers from elderly cardiovascular patients. Furthermore, we show the ability of these progenitor cells to give rise to a clonogenic population able to stimulate in vitro angiogenic activity of endothelial cells (ECs) and to improve in vivo reparative neovascularization.

**Methods**

An expanded Methods section is provided in the online-only Data Supplement.

**Vein Collection**

Veins were collected during bypass operations and dissected carefully from the surrounding tissue, washed in PBS with antibiotics, and then processed for immunohistochemistry or cell culture.

**Immunohistochemical Analysis of Human Saphenous Veins**

Sections were stained with antihuman CD34 in combination with von Willebrand factor (vWF) or with CD31 and either NG2 or platelet-derived growth factor receptor-β (PDGFRβ). Alexa Fluor-conjugated secondary antibodies were used. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

**Cell Isolation and Culture**

Venous samples were minced and digested with Liberase 2 (Roche, Basel, Switzerland). For culture selection, 2×10^5 cells/mL were plated in the presence of Human Medium (HM; complete NeuroCult medium, StemCell Technologies Inc, Vancouver, British Columbia, Canada) that contained basic fibroblast growth factor (10 ng/mL) and epidermal growth factor (20 ng/mL; both from R&D Systems, Minneapolis, Minn) on uncoated wells (n=5).

CD34pos/CD31-negative (CD31neg) cells were isolated from saphenous vein digests either by magnetic bead–assisted cell sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany; n=6) or by fluorescence-activated cell sorting (n=2). Fluorescence-activated cell sorting was performed with a high-speed cell sorter (MoFlo, Beckman Coulter, Fullerton, Colo). Purity of the preparations was assessed by flow cytometry analysis. Differentiation/expansion was performed by seeding CD34pos/CD31neg cells obtained with each method on fibronectin-coated plates in endothelial growth medium (EGM2, Lonza, Basel, Switzerland) that contained 2% fetal bovine serum (Lonza).

**Flow Cytometry Analysis**

Single-cell suspensions of freshly prepared vein digests were stained with a combination of fluorescence-labeled antibodies against CD34, CD31, CD133, CD117/Kit, chemokine (C-X-C motif) receptor 4 (CXCR4), kinase insert domain–containing receptor (KDR), CD45, CD11b, PDGFRβ, and NG2. Cultured cells were stained for CD44, CD90, CD105, CD29, CD49a, CD49b, CD13, CD59, CD73, and CD146 in addition to the antibodies reported above. Data were acquired in a flow cytometric sorting Canto II (BD Biosciences, Franklin Lakes, NJ) flow cytometer and analyzed with flow cytometric sorting Diva software (BD Biosciences).

**Immunocytochemical Characterization**

Cultured cells were analyzed by immunocytochemistry for expression of NG2, PDGFRβ, desmin, vimentin, nestin, sex-determining region Y-box2 (Sox2), CD90, CD44, α-sarcormeric actin, α-smooth muscle actin, CD31, osteocalcin, β3-tubulin, acetylated microtubule, the dopamine transporter, tyrosine hydroxylase, and N-cadherin. Unconjugated primary antibodies were followed by the appropriate fluorescent secondary antibody. Nuclei were stained with DAPI.

### Single-Cell Cloning

Single-cell cloning was accomplished by use of a motorized device connected to the flow cytometric sorter (Cyclone, Beckman Coulter).

### Multilineage Differentiation

The ability of saphenous vein–derived progenitor cells (SVPs) to differentiate into osteoblasts, adipocytes, chondrocytes, ECs, hepatocytes, myocytes, and neuronal cells was tested as described previously and as detailed in the online-only Data Supplement.

**Coculture and Conditioned Media Studies**

SVPs were labeled with CM-Dil (Molecular Probes [Invitrogen], Eugene, Ore) and saphenous vein–derived ECs (SVECs) were either stained with FITC-conjugated Ulex europaeus agglutinin I (Sigma-Aldrich Corp, St Louis, Mo) or left unlabeled. Cells were then cocultured (1:4 ratio of SVPs to SVECs) on Matrigel (3D; BD Biosciences) or on gelatin-coated coverslips (2D). Conditioned culture medium (CCM) was obtained from 70% confluent SVPs or SVECs cultured for 48 hours in fresh medium. The unconditioned culture medium served as control. Matrigel angiogenesis assay and cell migration, proliferation, and apoptosis assays were performed on SVECs and SVPs as described previously.

### Hindlimb Ischemia Model

Male 8-week-old CD1 Foxn1nu/nu mice (Charles River Laboratories, Wilmington, Mass) underwent unilateral limb ischemia as described previously. One day after induction of ischemia, 8×10^6 Dil-labeled SVPs (passage 6), so-called early-culture endothelial progenitor cells, or vehicle (DMEM, 30 μL) was injected into 3 different points of the ischemic adductor muscle (n=7 mice per group). Blood flow recovery was followed up by laser Doppler flowmetry as reported previously.

### Assessment of Neovascularization in Ischimic Muscles

Sections (3 μm) from paraffin-embedded adductor muscles, harvested at day 14 after ischemia (n=4 mice per group), were stained with isocitbin B4 and α-smooth muscle actin to recognize ECs and smooth muscle cells, respectively, and to calculate capillary and arteriole density. SVP phenotype in vivo was confirmed by staining with anti-human CD44. Proliferating cell nuclear antigen was used as a marker for proliferation.

Position of SVPs in the muscle was assessed in 100-μm-thick sections after staining with directly fluorophore-labeled isocitbin B4 and anti-N-cadherin antibody. Z-stack images were taken by confocal microscope and 3D reconstructed by use of Velocity imaging software (Improvision, Coventry, United Kingdom).

### Statistical Analysis

Values are presented as mean±SEM. Analyses were performed with GraphPad Prism 5 software (GraphPad Software, San Diego, Calif). Statistical significance was assessed by use of paired or unpaired t test for comparison between 2 groups. For analysis of blood flow recovery and cell biology assays in which measurements were repeated on the same subject at different time points, repeated-measures ANOVA was used, followed by Bonferroni post hoc test. Finally, 2-way ANOVA for block design was applied when variables repeated on the same subject at different time points, repeated-measures ANOVA was used, followed by Bonferroni post hoc test. Finally, 2-way ANOVA for block design was applied when variables repeated on the same subject at different time points. P<0.05 was interpreted to denote statistical significance.

### Results

**Human Saphenous Veins Contain CD34pos Progenitor Cells**

A total of 4×10^5 to 10×10^5 cells were obtained from digestion of single saphenous veins (4 to 5 cm length). Flow cytometry analysis of 12 veins demonstrated the presence of...
CD34\(^{pos}\)CD31\(^{neg}\) cells selected either by culture, MACS, or flow cytometric sorting (n=2 to 3 veins for each method) were plated on fibronectin in the presence of serum. Five to 10 days after plating, all preparations gave rise to small colonies composed of elongated and fast-growing cells. Of note, the morphology of CD34\(^{pos}\)CD31\(^{neg}\)-derived cells was independent from the isolation procedure (online-only Data Supplement Figure IIA through IIC).

Cells at passage 4 (n=4 preparations) were assayed for their antigen expression by flow cytometry, which showed that serum-induced differentiation was associated with the loss of CD34. In addition, cells expressed the mesenchymal stem cell markers CD44, CD90, CD105, CD29, CD49a, CD49b, CD13, CD59, and CD73, although they were negative for CD31, CD133, c-Kit, CD146, and CD45 (Figure 3A).

Immunocytochemical analysis was performed (n=3 preparations) to further define the cell characteristics (Figure 3B). Cells obtained by all 3 procedures consistently expressed desmin (≈99%), vimentin (95±5%), NG2 (62±6%), and PDGFR\(\beta\) (48±2%). Of note, the expression of NG2 and PDGFR\(\beta\) was increased remarkably upon differentiation of CD34\(^{pos}\)CD31\(^{neg}\) cells in SVPs (online-only Data Supplement Figure IID).

As illustrated in Figure 3B, immunocytochemical analysis further showed that most of the CD34\(^{pos}\)CD31\(^{neg}\)-derived cells were positive for Sox2 (75±17%) but did not express other embryonic-like transcription factors, such as Oct3/4 and NANOG (not shown). The muscle-specific \(\alpha\)-sarcomeric actin was expressed in 12±10% of the cells. Interestingly, the early neuronal markers B3-tubulin and nestin were expressed by 58±15% and 20±9% of the population, respectively. In contrast, although isolated from the vessel wall, cells did not express CD31 or \(\alpha\)-smooth muscle actin. They were also negative for epithelial cell--associated cytokeratins 8, 18, and 19 and the astrocyte marker glial fibrillary acidic protein (not shown). Thus, the antigenic phenotype of the CD34\(^{pos}\)CD31\(^{neg}\)-derived progeny, henceforth termed SVPs, indicates similarities with undifferentiated cells of the mesenchymal/pericyte lineage.9,10

Importantly, SVPs could be expanded with a doubling time of ≈45 hours (calculated at passage 5), progressively growing in size and decelerating their proliferation rate after passage 10. Expansion of SVPs would potentially allow the generation of 30 to 50\(\times\)10\(^6\) cells starting from an average culture (at passage 3) of 2 to 3\(\times\)10\(^3\) cells. Furthermore, SVPs were routinely stored in liquid nitrogen and subsequently defrosted during the present study, which illustrates the feasibility of cryopreservation.

### Clonogenic and Differentiation Potential

Next, we investigated whether SVPs possess 2 fundamental properties of progenitor cells, clonogenicity and multipotency. For the cloning assay, 1363 CD34\(^{pos}\)CD31\(^{neg}\)-derived SVPs from 4 different populations were sorted into 96-well Terasaki plates (1 cell per well; Figure 4A). One week after sorting, 19.8±2.1% of the cells had formed colonies that contained 5 to 20 cells; however, only one third of these early developed clones (=7% of the initially seeded cells) could be expanded to 1 to 2\(\times\)10\(^5\) cells, whereas ≈2% of the initially plated cells did grow further to approximately 3 to 5\(\times\)10\(^5\) cells and were subcloned with an efficiency of

### Table. Flow Cytometry Analysis of Cells Obtained From Digested Saphenous Veins (n=12)

<table>
<thead>
<tr>
<th>Population</th>
<th>% of Total*</th>
<th>% of CD34 Population†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+</td>
<td>27.6±4.1</td>
<td>100</td>
</tr>
<tr>
<td>CD133+</td>
<td>4.8±1.6</td>
<td>5.6±1.7</td>
</tr>
<tr>
<td>c-Kit+</td>
<td>2.4±1.3</td>
<td>4.5±1.6</td>
</tr>
<tr>
<td>KDR+</td>
<td>1.1±0.5</td>
<td>2.0±0.3</td>
</tr>
<tr>
<td>CXCR4+</td>
<td>16.9±4.9</td>
<td>22.1±9.1</td>
</tr>
<tr>
<td>NG2+</td>
<td>4.5±0.9</td>
<td>2.0±1.4</td>
</tr>
<tr>
<td>CD31+</td>
<td>12.0±4.6</td>
<td>13.8±6.8</td>
</tr>
<tr>
<td>CD45+</td>
<td>9.7±3.1</td>
<td>7.1±1.4</td>
</tr>
<tr>
<td>CD11b+</td>
<td>8.8±6.5</td>
<td>6.7±4.7</td>
</tr>
</tbody>
</table>

*Values are expressed as mean±SEM.
†Values in this column are percentages of each marker in the CD34\(^{pos}\) fraction.

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Figure 1. Identification of CD34<sup>pos</sup> cells in saphenous veins. Representative scattergrams (A and B) showing the relative abundance of CD34<sup>pos</sup>/CD31<sup>neg</sup> cells (C, red box) within saphenous vein digests. Forward-sideward scatterplot (A) and isotype control are shown (B). FSC indicates forward scatter; SSC, side scatter; PE, phycoerythrin; and FITC, fluorescein isothiocyanate. Di, Low-magnification picture shows morphology of saphenous vein and distribution of vasa vasorum in adventitia (delimited by dashed lines). Sections were stained for progenitor/EC marker CD34 (red; Dii), EC marker vWF (green; Diii), and nuclei (DAPI, blue, Div). Representative confocal image merge reveals the presence of CD34<sup>pos</sup>/vWF<sup>neg</sup> progenitor cells located in the perivascular zone of the vasa vasorum (white arrowheads in Dv). Furthermore, in the same region, a few CD34<sup>pos</sup> cells (green; E and F) coexpressed NG2 (red, E) or PDGFRβ (red, F). Double-positive cells are indicated by white arrows. CD31 (white; E and F) stained ECs of vasa vasorum (yellow arrowheads; E and F). Higher-magnification panels in E and F show adventitial CD34<sup>pos</sup> cells, negative for CD31 and coexpressing NG2 or PDGFRβ. Scale bar=50 μm (D) and 10 μm (E and F).
≈18%, which generated highly plastic subclones (Figure 4A). Primary clones conserved the mesenchymal/pericyte immunophenotype of the original population (Figure 4B). Finally, bulk populations of SVPs, clones, and subclones were tested for their ability to differentiate into derivatives of all 3 germ layers. When exposed to differentiation stimuli, SVPs were able to generate mesodermic lineages, such as osteoblasts, adipocytes (Figure 4C), and α-sarcomeric actin-positive myocytes (Figure 4D), whereas no chondrocyte or EC differentiation was obtained. Additionally, SVPs gave rise to neuron-like cells, which expressed early marker β3-tubulin, arrayed in filaments and bundles, and more differentiated markers such as acetylcholine transferase, the dopamine transporter, and tyrosine hydroxylase (Figure 4E). No differentiation toward hepatocytic cell lineage (endodermic differentiation) was detected (not shown). Interestingly, all subclones displayed a wide differentiation capacity, which gave rise to mesodermic lineages (adipocytes, osteoblasts, and myocytes) and neuron-like cells. Taken together, the results demonstrated that SVPs are capable of self-renewal and that their clones possess a broad differentiation capacity.

SVPs Directly Interact With ECs Supporting Their Network Formation Capacity

Perivascular mesenchymal cells, namely, pericytes, play a fundamental role in angiogenesis by sustaining the organization of provisional EC structures and strengthening intercellular connections. To demonstrate the supportive capacity of SVPs, we cocultured them with SVECs on Matrigel. As shown in Figure 5A, SVPs alone were able to form complex networks, composed of nodes connected by a number of thin branches. Culturing SVPs together with SVECs remarkably improved the network-forming capacity of SVECs (Figure 5B through 5D). We further investigated the connections established between the 2 cell types in coculture. SVECs formed continuous branches that were surrounded by SVPs, we cocultured them with SVECs on Matrigel. As shown in Figure 5E; online-only Data Supplement Movie I). Similarly, bidimensional coculture indicated that each SVP connected to multiple SVECs (Figure 5F; online-only Data Supplement Figure III). N-cadherin is expressed by both ECs and pericytes and is implicated in EC/pericyte interaction during vessel formation. In the present coculture model, N-cadherin was polarized on the SVP pseudopodia in contact with SVECs. In SVECs, N-cadherin was uniformly distributed on the cellular membrane (Figure 5G). These data indicate the establishment of physical interactions between the 2 populations that favored network enhancement and stabilization.

SVPs Support EC Proliferation and Network Formation via Paracrine Mechanisms

By reverse-transcription polymerase chain reaction, we verified that SVECs and SVPs express complementary components of the angiopoietin (Ang)-1/Tie-2 and PDGF-BB/
PDGFβ systems, which are strongly implicated in EC/pericyte cross talk (Figure 5H).9 Furthermore, by ELISA, we confirmed that SVPs release Ang-1 (704 ± 232 pg/mL) and SVECs release PDGF-BB (20.3 ± 1.2 pg/mL) in their respective conditioned medium.

We therefore investigated whether the stimulatory action of SVPs on SVEC angiogenic activity is mediated by paracrine mechanisms. Accordingly, SVP-CCM remarkably supported the persistence of the EC network in the Matrigel assay compared with unconditioned medium (online-only Data Supplement Figure IV). Preincubation of SVECs with Tie-2–blocking antibody abrogated the stimulatory effect of SVP-CCM on network formation (Figure 5I).

Next, we investigated the effect of CCM from SVPs and SVECs on distinct cellular processes, namely, survival, proliferation, and migration. Incubation of SVECs with SVP-CCM significantly increased the rate of bromodeoxyuridine incorporation (Figure 5J) but did not modify the migratory activity of SVECs in the scratch assay (Figure 5K). Furthermore, application of SVP-CCM in a Caspase-Glo assay did not improve the survival of starved SVECs (data not shown).

Conversely, SVEC-CCM improved SVP migration (Figure 5L) but did not show any effect on proliferation compared with unconditioned culture medium (Figure 5M). The addition of PDGF-BB–blocking antibody to the SVEC-CCM abrogated its promotinal effect on SVP migration (Figure 5L).

**Intramuscular Injection of SVPs Improves Blood Flow Recovery in a Model of Hindlimb Ischemia**

We then determined whether the proangiogenic activity of SVPs translates into improvement of reparative neovascularization in vivo. For this purpose, we injected SVPs or unconditioned medium (vehicle) intramuscularly in immunodeficient mice subjected to unilateral limb ischemia. We found that SVP administration significantly accelerated foot perfusion recovery compared with vehicle. In the SVP-treated group, full recovery was obtained at day 7 after ischemia; the same level was reached 7 days later in the vehicle-injected group (Figure 6A).

Immunohistochemistry demonstrated that the density of capillaries and small arterioles was significantly higher in SVP-injected than in vehicle-injected muscles (Figure 6B and...
Figure 5. Interactions between SVPs and SVECs. SVPs formed peculiar structures in Matrigel (A). Network formation was strongly enhanced in SVP-SVEC coculture (C) compared with culture of SVECs alone (B). D, Bar graph showing average tube length; values are mean±SEM of 4 experiments performed in quadruplicate. VF indicates view field. ****P<0.0001 vs SVECs. Confocal microscopy image shows tubes formed by SVECs (isolectin, green) and covered by SVPs (DiI, red; E). F, Phase-contrast microscopy image showing the interaction of SVPs (DiI, red) with multiple SVECs (unstained) in 2D coculture. G, Representative confocal microscopy image showing different patterns of N-cadherin staining (green fluorescence) in SVPs (localized at filopodia, white arrowheads) and ECs (diffuse to all of the cell membrane). Scale bars: 500 μm (A, B, and C); 50 μm (F and G); 20 μm (E). H, Expression of Ang-1/Tie-2 and PDGF-BB/PDGFRβ in SVECs and SVPs. NC indicates negative control. I through M, Bar graphs show the reciprocal paracrine effect of SVECs and SVP-CCM. SVP-CCM influenced SVEC network-formation capacity (I) and proliferation (J) but not migration (K). Tie-2 blockade inhibited the stimulatory action on the network-formation capacity of SVECs (I). SVP-CCM enhanced SVEC migration (L), this effect being inhibited by PDGF-BB blockade, but did not alter their proliferation (M). Positive control was medium that contained 10% serum, whereas negative control was serum-deprived medium. Values are mean±SEM of 3 experiments performed in quadruplicate. *P<0.05 and **P<0.01 vs unconditioned culture medium; ###P<0.001 vs CCM. UCM indicates unconditioned medium; BrdU, bromodeoxyuridine; and RFU, relative fluorescence unit.
To verify whether the effect of SVPs on arteriole formation was due to a paracrine action on vascular smooth muscle cells, murine vascular smooth muscle cells were stimulated with SVP-CCM in in vitro assays. We found that SVP-CCM improved vascular smooth muscle cell viability and survival but did not affect bromodeoxyuridine incorporation or migration (online-only Data Supplement Figure V).

Transplantation of early-culture endothelial progenitor cells derived from peripheral blood monocytes of healthy subjects8 improved postischemic neovascularization without accelerating limb reperfusion (online-only Data Supplement Figure VI).

**Engraftment of Transplanted SVPs Into Ischemic Tissues**

At 14 days after injection, we still detected a large number of SVPs deeply engrafted in the injected adductor muscles (31.3±8.8 cells/section; Figure 6D). Furthermore, we confirmed that SVPs retained their phenotype in vivo, as shown by positivity for human CD44 (Figure 6E). Importantly, we detected diffuse proliferating cell nuclear antigen staining in engrafted SVPs (32.7±2.5%), which indicates that the cells were not just surviving in the tissue but were also actively proliferating (Figure 6F). At this late stage, it was still possible to detect proliferating murine capillary ECs and myofibers, but although a trend was evident, no difference in proliferation was detected between the SVP-injected and vehicle-injected groups (online-only Data Supplement Figure VII).

To study the localization of injected cells and the establishment of 3D contacts between host and recipient cells, we analyzed whole-mount preparations of adductor muscles. 3D reconstruction of confocal microscopy images confirmed the presence and abundance of SVPs in the injected tissue (Figure 7A) and indicated that cells were localized mainly around host capillaries (Figure 7B through 7D). Staining for N-cadherin showed polarization of the protein in the junctions formed between SVPs and capillary ECs, thus confirming the physical contact between them (Figure 7E and 7F). Taken together, these data suggest that SVPs have the capacity to incorporate in the host tissue and to localize around the capillaries as natural mural components.

**Discussion**

Seminal reports described the presence of mesenchymal precursors in vessels from young organ donors12 or donors of
unspecified age\textsuperscript{1,13}; however, to the best of our knowledge, the present study is the first to demonstrate the isolation of progenitor cells from saphenous veins of aged patients with coronary artery disease. Furthermore, whereas previous methods relied on plastic adhesion for isolation/selection,\textsuperscript{12,13} we adopted specific surface markers, namely, CD34 and CD31, to achieve enhanced purity and reproducibility. The differentiation of CD34\textsuperscript{pos}CD31\textsuperscript{neg} cells gave rise to a clonogenic and multipotent cell population that could be expanded for at least 10 passages and could be cryopreserved easily. As a result, a relatively small number of CD34\textsuperscript{pos}CD31\textsuperscript{pos} cells from a patient’s vein could potentially generate 30 to 50 million viable cells, which could be stored to create a bank of ready-to-use cells for autologous therapy. Application to preclinical models of peripheral ischemia demonstrated that the isolated cells possessed a potent reparative action, behav-

Figure 7. Interaction of SVPs with the host vasculature. Z-stack of confocal images showing numerous SVPs (CM-Dil; red) surrounding the host vasculature (isolectin B4; green; A and B). 3D reconstruction of the field shows physical contact between SVPs and ECs (C and D; white arrowheads). Direct interaction was also confirmed by N-cadherin staining (green), which accumulated in the junction between SVPs (red) and capillary ECs (blue; E and F). Nuclei were stained with DAPI (blue in A through D; white in E and F). Scale bar=25 μm.
ing like perivascular supportive cells and promoting neovascularization by physical and paracrine interaction with ECs.

We previously reported the presence of multipotent CD34<sup>pos</sup>CD133<sup>pos</sup> cells in the outer layer of the aortic stroma of human fetuses<sup>2,4</sup>. After stimulation with growth factors, fetal progenitors generated a population of mature ECs and mural cells. Furthermore, transplantation of fetal aorta CD34<sup>pos</sup>CD133<sup>pos</sup> cells improved healing of ischemic muscles and diabetic ulcers<sup>2,4</sup>. Our new findings suggest that CD34<sup>pos</sup> progenitors persist in the vasculogenic zone of adult veins, although they appear to be more differentiated, expressing low levels of CD133 and showing limited plasticity compared with their fetal counterparts. Sorted or culture-selected CD34<sup>pos</sup>CD31<sup>neg</sup> cells also showed restricted expandability, but, on stimulation with serum, they produced highly proliferating cells, which abundantly expressed typical markers of mesenchymal/pericyte lineage. Because those markers were natively expressed at low abundance in vein-resident and freshly isolated CD34<sup>pos</sup>CD31<sup>neg</sup> cells, the newly emerging phenotype might originate from serum-dependent transcriptional induction or expansion of mesenchymal/pericyte clones within the bulk population of CD34<sup>pos</sup>CD31<sup>neg</sup> cells. Perpetuation of the mesenchymal/pericyte phenotype in clones and subclones of SVPs supports the second hypothesis. The dual nature of the SVP phenotype recalls recent reports showing strong similarities between pericytes and tissue-specific mesenchymal stem cells<sup>14,15</sup>. For instance, mesenchymal stem cells and pericytes are commonly defined as CD34<sup>neg</sup><sup>15,16</sup> and, in fact, SVPs quickly stop expressing CD34 on differentiation. Furthermore, SVPs retain a certain level of “stemness,” as indicated by their expression of Sox2; they possess clonogenic capacity and show osteogenic, myogenic, adipogenic, and neuroectodermal potential as previously described for resident mesenchymal progenitor cells<sup>17,18</sup> and pericytes<sup>19,20</sup>.

We showed that SVPs establish interactions with multiple ECs, both in coculture systems and in vivo, recapitulating the natural function of perivascular support cells<sup>21</sup>. We also documented a reciprocal paracrine cross talk between the 2 cell types that resulted in stimulation of EC network formation capacity and proliferation and SVP migration. During angiogenesis, ECs sprout and proliferate to form the capillary lumen, stimulated by factors released by the surrounding cells. This process is followed by the migration of mural cells, which improves the stability and functionality of the vessel<sup>22</sup>. Importantly, the present results highlight the involvement of the Ang-1/Tie-2 and PDGF-BB/PDGFR<sub>B</sub> systems in the interactive responses of SVPs and ECs.

We found that transplantation of SVPs promotes neovascularization and accelerates blood flow recovery of ischemic limbs, which is relevant to clinical applications. Because no SVP-derived vascular structures were detected, but numerous proliferating SVPs were present in the muscles, we hypothesize that the reparative effect is mainly due to paracrine mechanisms, in line with previous reports on mesenchymal stem cell therapy<sup>23–26</sup>. Vascular smooth muscle cells may represent an additional target for the paracrine factors released by SVPs, as documented by in vitro studies that showed increased viability of murine vascular smooth muscle cells exposed to SVP-CCM. However, with respect to the effect of SVP transplantation on arterioles, we also hypothesize an indirect action, consisting of stimulation of capillary sprouting, which then undergoes muscularization.

We were unable to compare SVPs with other tissue-resident or circulating progenitor cells from the same patients because access to other sources for exclusive research finalities was ethically unjustified in those critically ill subjects. Interestingly enough, however, patients’ SVPs were therapeutically equivalent in terms of neovascularization promotion and were superior with regard to blood flow recovery compared with early-culture endothelial progenitor cells from young healthy donors, a cell population originally shown to improve the healing of ischemic limbs<sup>27</sup>.

Further studies are warranted to establish the relative potency and potential synergism of SVPs with other cell types in models of peripheral and myocardial ischemia. From a clinical perspective, we speculate that SVPs might have potential therapeutic application in pathologies such as diabetic retinopathy that are characterized by dysfunction of perivascular support cells<sup>28</sup>. Furthermore, resident CD34<sup>pos</sup>CD31<sup>neg</sup> cells and their progeny might take part in vein graft remodeling and adaptation by supporting the establishment of vascular connections between vasa vasorum of the graft and the recipient<sup>29</sup>.

In conclusion, we have provided a method to isolate and expand proangiogenic cells from the saphenous vein of aged cardiovascular patients. This is of high clinical relevance, because the starting material is usually available during bypass surgery without additional risk or burden to the patient. The method allows easy preparation and expansion of the cells to provide progenitor cells that accomplish important requirements for autologous cell therapy, being present in an easily accessible adult tissue, expandable in vitro, and able to engraft into host neovascularization in vivo with high efficiency.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

This study is the first to demonstrate that saphenous leftovers, designed to be discarded after bypass surgery, are indeed an invaluable source of perivascular progenitor cells. These cells can be expanded and stored for subsequent autologous transplantation with the aim of promoting therapeutic angiogenesis. Other potential therapeutic exploitations include use in diseases characterized by impairment of perivascular support cells, such as diabetic microangiopathy, and the application of saphenous vein–derived progenitor cells for the promotion of vein graft adaptation, by seeding them on perivascular stents. These potential applications will require proof of concept and further preclinical validation.
Human Adult Vena Saphena Contains Perivascular Progenitor Cells Endowed With Clonogenic and Proangiogenic Potential
Paola Campagnolo, Daniela Cesselli, Ayman Al Haj Zen, Antonio Paolo Beltrami, Nicolle Kränkel, Rajesh Katare, Gianni Angelini, Costanza Emanueli and Paolo Madeddu

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Supplemental Material

Supplemental methods

Ethical issues

Studies complied with the ethical principles stated in the “Declaration of Helsinki” and were covered by ethical approval number 06/Q2001/197 from Bath Research Ethics Committee. Five to ten centimeter long leftover segments of saphenous veins were collected from coronary artery by-pass patients (average age 69±2 years), who gave written informed consent to the procedure. Peripheral blood samples (50-70 mL) were collected from healthy donors (30±2 years) by vein puncture for isolation of MNCs. The experiments involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and with the prior approval of the UK Home Office and the University of Bristol Ethics Committee.

Vein collection and preparation

Human saphenous veins were collected in PBS containing penicillin and streptomycin at the end of by-pass operations. Veins were then carefully dissected from surrounding tissues using a sterile scalpel and the thoroughly washed in abundant PBS containing antibiotics. Venous samples were then either processed for embedding and immunohistochemistry or stored in DMEM with 10% FBS at 4°C and processed for cell culture. Before digestion with enzymes, saphenous veins were again washed in PBS and antibiotics.
**Immunohistochemical analysis**

Sections, 3µm thick, were obtained from formalin-fixed paraffin-embedded saphenous vein specimens, dewaxed and blocked with 10% goat serum prior to staining with mouse anti-human CD34 (1:50, Dako) and rabbit anti-human vWF (1:100, Dako) followed by secondary antibody anti mouse-Alexa 568 and anti rabbit-Alexa 488 (Molecular Probe). Sections, 3µm thick, were obtained from fresh frozen, OCT-embedded (Sigma-Aldrich) saphenous vein specimens and stained with the combination of mouse monoclonal CD34 (1:100, Dako), goat polyclonal CD31 (1:50, Santa Cruz) and either rabbit polyclonal PDGFRβ (1:25, Cell Signaling) or NG2 (1:100, Chemicon). Nuclei were counterstained with DAPI (Vector Laboratories). Negative controls were performed by replacing the primary antibody with Ig-matched isotype or PBS.

**Isolation of the CD34$^{pos}$ CD31$^{neg}$ population**

Specimens were extensively washed in PBS and then manually minced with a scalpel before being incubated for 4 hours with 3.7mg/mL Liberase 2 (Roche). Remaining aggregates were eliminated through filtration with 40µm cell strainer.

Culture selection was obtained by plating 2x10$^5$ cells/mL in HM medium (complete NeuroCult medium-StemCell Technologies- containing 10ng/mL of b-FGF and 20ng/mL of EGF, both R&D) on uncoated wells.$^1$ Culture was followed for up to ten days and half of the medium was changed regularly.

Magnetic beads isolation was performed on freshly obtained cell suspensions, after filtration through a 30µm cell strainer. Cells were incubated with anti-CD31 conjugated beads (Miltenyi) and passed through a magnetic column, following manufacturer’s instruction. This allowed efficient removal of mature endothelial cells. After depletion of CD31 positive cells, remaining cells were further incubated with anti-CD34 beads (Miltenyi) for 30 min at 4°C.
and then processed to obtain purified CD34 positive cells.

Cell sorting was performed on freshly digested veins, after staining with directly conjugated antibodies for CD34 and CD31. Cells were firstly gated for their size to exclude aggregates and debris and then CD34<sup>pos</sup> CD31<sup>neg</sup> and CD34<sup>neg</sup> CD31<sup>neg</sup> populations were sorted in two different tubes. The sorting was performed using a high speed cell sorter (MoFlo, Beckman Coulter, USA) and Summit software (Dako, Denmark) was employed to analyze data and to set sort decisions. Purity of the populations obtained was assessed by staining with CD34-FITC and CD31-PE antibodies (both Miltenyi), followed by FACS analysis.

**Culture of SVPs**

CD34<sup>pos</sup> cells isolated by culture selection, magnetic beads and FACS sorting were plated on fibronectin (10µg/mL) coated plates in presence of differentiation medium (EGM2-2% FBS, Lonza). Adherent cells appeared 5-10 days later; they were fed every three days with fresh medium and passaged to new culture dishes once they reached 60-70% confluence. TrypLE (Invitrogen) was utilized to detach cells from the growth substrate.

Population doubling time (PDT) was calculated by plating SVPs at passage 5 on fibronectin coated plates at the concentration of 2,000 cell/mm<sup>2</sup> and counting cell number every 24h. Experiment was repeated 3 times in duplicates. PDT was calculated in the logarithmic phase using the formula PDT = t/log<sub>2</sub>(N<sub>t</sub>/N<sub>0</sub>), where N<sub>t</sub> is the number of cells at time t and N<sub>0</sub> the initial number of cells, t is the time in between the counts.

**Flow cytometry analysis**

Staining for flow cytometry purposes was performed either on freshly isolated cells or on in vitro expanded cells. Cells isolated from the digestion of saphenous vein samples were labeled with directly conjugated antibodies against CD34 (BD Bioscience), CXCR4, CD11b,
CD31 (all from Caltag Laboratories), CD45, CD133 (all from Miltenyi), KDR, c-kit (all from R&D System) or with primary rabbit anti-human NG2 (1:100, Chemicon) and PDGFRβ (1:100, Abcam) for 15 min, the latter followed by goat anti-rabbit secondary antibody.

Cultured cells were instead detached from the growth substrate through a short incubation in TrypLE (Invitrogen, USA). Single cell suspensions were incubated with directly labeled antibody against for CD44, CD90, CD105, CD29, CD49a, CD49b, CD13, CD59, CD73, CD146 (all from Becton Dickinson) in addition to the antibodies mentioned above and analyzed shortly thereafter.

**RT-PCR**

Total RNA was obtained from freshly isolated CD34\textsuperscript{neg}CD31\textsuperscript{neg} CD34\textsuperscript{pos}CD31\textsuperscript{neg}, cultured SVPs and SVECs (RNeasy, Qiagen, Crawley, UK) and reverse transcribed (Quantitech Reverse transcription kit, Qiagen). For qualitative analysis of gene expression, PCR was performed using 2X PCR mix (Promega) and the primer pairs specified below in a PTC-200 thermal cycler (MJ Research). After 25-30 cycles, products were separated on 1% agarose gels.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>18S rRNA</strong></td>
<td>forward: 5’- TAGAGGGACAAGTGGCGTTC -3’</td>
</tr>
<tr>
<td></td>
<td>reverse: 5’- TGTACAAAGGGCAGGG ACTT -3’</td>
</tr>
<tr>
<td><strong>NG2</strong></td>
<td>forward: 5’- GCTGAGATCAGAAGGGACCA -3’</td>
</tr>
<tr>
<td></td>
<td>reverse: 5’- GTACAGGACCTGCCTGTGT -3’</td>
</tr>
<tr>
<td><strong>PDGFRβ</strong></td>
<td>forward: 5’- GCTCACACTGACCAACCTCA -3’</td>
</tr>
<tr>
<td></td>
<td>reverse: 5’- TCTTCTCGTGCAGTGCACC -3’</td>
</tr>
<tr>
<td><strong>PDGF-BB</strong></td>
<td>forward: 5’- CCAGGTGAGAAAGATCGAGATTG -3’</td>
</tr>
<tr>
<td></td>
<td>reverse: 5’- ATGCAGTGCTCTGAATTCCG -3’</td>
</tr>
<tr>
<td>Angiopoietin-1 forward: 5’- ACGATGGCAACTGTGCAG -3’</td>
<td>reverse: 5’- TCCGACTTCATGTTTCCACAA -3’</td>
</tr>
<tr>
<td>Tie-2 forward: 5’- CCAACGTGATTGACACTGG -3’</td>
<td>reverse: 5’- TGTGAAGCGTCTCACAGGTC -3’</td>
</tr>
</tbody>
</table>

**Immunocytochemistry**

For immunofluorescence, adherent cells were fixed in 4% buffered paraformaldehyde and permeabilized (in case of intra-cytoplasmatic antigens) with 0.1% TritonX prior to incubation with primary antibody for PDGFRβ (1:50, Cell signaling), Vimentin (1:20, ABCAM), NG2 (1:150), Desmin (1:20), Nestin (1:100), Sox2 (1:100, all from Chemicon), CD90 (1:50), CD44 (1:50, Becton Dickinson), α-smooth muscle actin (1:50, Dako), CD31 (1:100, Sigma), α-sarcomeric actin (1:200, Sigma), GATA-4 (1:20, Santa Cruz), Cytokeratins 8-18-19 (1:100, Dako), osteocalcin (1:50, Santa Cruz), β3-tubulin (1:1000, Abcam), acetylcholine transferase (1:2000, Abcam), glial fibrillary acidic protein (1:100, Dako), tyrosine hydroxilase (1:100, Sigma-Aldrich), and the dopamine transporter (1:100, Abcam). AlexaFluor labeled secondary antibodies (Molecular Probes/Invitrogen, USA) were employed to detect primary antibodies. DAPI (Vector Laboratories, Inc, Burlingame, CA, USA) was used to identify nuclei.

Immunocytochemistry was performed utilizing the peroxidase-conjugated EnVision system (Dako).

**Single cell cloning**

Cells derived from 4 different cell lines (n=1 culture selected, n=1 MACSorted, n=2 FACSsorted cells) were detached and used for single cell cloning at passage 5. We plated 1,363 cells, one per well in a fibronectin/gelatin coated 96-well Terasaki plates in EGM2 2% (Lonza), using a motorized system connected to the FACS sorter (Cyclone, Beckman).
Coulter). Sorted cells were expanded and maintained as described for SVPs.

**Multilineage differentiation**

Osteoblast differentiation capacity was tested by plating cells at high density (2-3x10⁴/cm²) in DMEM supplemented with 5% FBS, 10mM β-glycerophosphate, 10⁻⁷M dexamethasone, and 0.2mM ascorbic acid (all from Sigma). Medium was changed every 3 to 4 days for 14 days. Differentiation was assessed by osteocalcin expression and von Kossa staining. Adipocytic differentiation was induced by plating cells at high density (2x10⁴/cm²) in DMEM-high glucose supplemented with 5% horse serum, 10⁻⁶M dexamethasone, 0.2mM indomethacin, 0.01mg/mL insulin, and 0.5mM 3-isobutyl-1-methyl-xanthine (all from Sigma). Differentiation was assessed by Oil Red O staining. Chondrocytic potential was tested stimulating cells in presence of serum-free medium without supplement with the addition of 100 ng/mL TGF-β1. Differentiation was assessed by pH=2 Alcian Blue Staining. To verify endothelial cell differentiation capacity, cells were seeded at high density on fibronectin coated dishes in Endocult (Stem Cell Technologies, USA). Differentiation was assessed by the expression of CD31. Hepatocytic differentiation was induced growing cells for two weeks at high density onto fibronectin coated dishes in a medium containing 0.5% FBS, 10ng/mL FGF-4 and 20ng/mL HGF (both from Peprotech). After this period, FGF-4 and HGF were substituted with 20ng/mL OncostatinM for other 14 days (Peprotech). Hepatocytic differentiation was assessed by the expression of GATA-4 and cytokeratins 8-18-19. Myocyte differentiation capacity was tested plating 0.5 to 1x10⁴ cell/cm² in expansion medium containing 5% FCS (Sigma), 10 ng/mL bFGF, 10 ng/mL VEGF, and 10 ng/mL IGF-1 (all from Peprotech). Cells were allowed to become confluent and cultured for up to 2 weeks with medium exchanges every 4 days. Myogenic differentiation was assessed by the expression of α-sarcomeric actin. For neurogenic differentiation, cells were plated in DMEM-
high glucose (Invitrogen), 10% FBS (Sigma-Aldrich). After 24h medium was replaced with DMEM-high glucose, 10% FBS containing B27 (Invitrogen), 10ng/mL EGF and 20ng/mL bFGF (both from Peprotech). After 5 days, cells were washed and incubated with DMEM containing 5μg/mL insulin, 200μM indomethacin and 0.5mM IBMX (all from Sigma), in the absence of FBS, for 5 hours. Neurogenic differentiation was assessed by the expression of β3 tubulin, acetylcholine transferase, dopamine transporter and tyrosine-hydroxilase.

_Culture of “early-culture EPCs”_

Early-culture EPCs were isolated following a protocol previously published.²⁻³ Briefly, mononuclear white blood cells (MNCs) were isolated from peripheral blood (PB) by gradient centrifugation on Histopaque-1077 density medium (Sigma) and plated 1x10⁶ cell/cm² on fibronectin (Sigma)-coated plates with EGM-2 MV SingleQuots and 10% FBS (EGM-2MV; Lonza). Medium was changed the second day and the culture was extended to 5-7 days. In line with the current literature, cultured “early EPCs” showed hematopoietic characteristics (CD45, CD11b and CD14 expression) and low-positivity for stem/progenitor cells markers (CD133 and CD34). They as well express EC markers (KDR, CXCR4, eNOS) and did uptake acetylated low density lipoprotein (acLDL) and bind Ulex europaeus agglutining I.

_Isolation of SVECs and co-culture with SVPs_

To obtain SVECs, saphenous veins were clamped and filled with digestion solution (0.5% collagenase I, Gibco) and then incubated for 30min at 37°C. Detached cells were then collected and plated on fibronectin coated plates with EGM2 2% (Lonza).

For co-culture experiments cells were firstly stained: SVPs were labeled by incubating them for 5min at 37°C, followed by 15min at 4°C, with CM-DiI diluted 1:1000 in culture medium (Molecular Probe); SVECs were labeled for 30min at 37°C with FITC-conjugated Ulex
europaeus agglutining I (Sigma), diluted 10µg/mL in medium, or left unlabeled. Cells were plated together in proportion 1:4 (SVPs:SVECs) on a fibronectin/gelatin coated 13mm coverslip or on complete Matrigel (BD). Matrigel pictures were taken using a brightfield inverted microscope after 24h to quantify network formation. Experiment was performed in quadruplicates and repeated three times. Three-dimensional pictures of the branches were taken using confocal microscopy (Leica). Two-dimensional co-cultures were fixed and stained for N-cadherin (1:80, Sigma) and images were taken using fluorescence microscope (Olympia).

**Conditioned media preparation**

To obtain conditioned culture medium (CCM), cells were grown to 70% confluence and incubated with fresh medium for 48h, then CCM was collected and cells were counted in a standard manner. For studies on VSMC functionality, CCM was obtained incubating SVPs with EBM2 (Lonza), 2% FBS and without the addition of growth factors, for 48h.

**Multiplex cytokine analysis**

Secretion of Angiopoietin-1 and PDGF-BB by SVECs (n=4) and SVPs (n=9) in their supernatants was quantified using the Luminex 100 system (Luminex Corp., Austin, Texas, USA). Samples were run in duplicate.

**Functional studies on the conditioned media**

To study the ability of CCM to stimulate tube network formation, 25,000 SVECs were plated on each well of an 8-well chamber slide coated with Matrigel, in presence of unconditioned culture medium (UCM) or CCM. Structure formation was assessed after 24 and 48h as described before by measuring total tube length and number of branches per view field.² For
proliferation assay and MTT assay 2,000 SVECs or 1,500 SVPs or 1,000 VSMCs were plated on 96-well plates and stimulated with control medium (UCM) or CCM for 24 or 48h (in case of VSMCs). BrdU incorporation was assessed using Cell proliferation ELISA, Roche. MTT assay (Promega) was performed following manufacture’s instruction. Migration was measured in a scratch assay as described before. Briefly cells were plated at confluence in fibronectin-coated 48-well plate and then a scratch was produced in the center of each well using a p1000 tip. Distance between the two edges was measured straight after the creation of the scratch (D₀) and after overnight incubation (D_ON) with conditioned or unconditioned medium. Percentage of gap closure (%GAP) was calculated as following: %GAP=100-(100*D_ON/D₀).

Apoptosis was measured using CaspaseGlo 3/7 (Promega) following manufacturer’s instructions. Blocking antibody against Tie-2 and PDGF-BB were mixed to the CCM at 10 and 50ng/mL, respectively. To ensure the blocking effect, SVECs were incubated for 30 minutes with anti-Tie2 antibody prior to the exposure to the CCM. Similarly, anti-PDGF-BB was added to the SVE C-CCM 30 minutes before its addition to SVPs.

Experiments were performed at least in quadruplicate and repeated three times.

Hindlimb ischemia model and cell transplantation

Anesthesia was induced in 8 week-old male CD1 Foxn1nu/nu (Charles River) with intraperitoneal injection of 0.3g/kg of 2,2,2-tribromoethanol. Hindlimb ischemia was performed on the left limb by ligation and electrical cauterization of the femoral artery. One day after ischemia SVPs or “early-culture EPCs” were detached and stained using CM-DiI (Molecular Probes) following manufacturer’s protocol and resuspended in 30µL of DMEM. With mice under anesthesia, 80,000 cells or vehicle medium (DMEM, 30µL total volume) were injected into 3 different points of the operated adductor muscle (n=7 animals per group). Blood flow
recovery was followed up by Laser Doppler flowmetry measurements at 3, 7, 14 days after ischemia.5

Histology

Two weeks after induction of hindlimb ischemia, mice were euthanized and perfused with 4% formalin. Adductors were excised and either paraffin embedded (n=4 mice per group) or further dissected to obtain whole mount preparation of the adductors (n=3 mice per group). Capillary density was calculated on sections stained with Isolectin B4 (1:100, Invitrogen). Twenty to thirty fields at 1000x were counted per slide. Arteriole density was calculated by counting and measuring the diameter of α-SMA stained vessels in the whole section and then normalized to the total area of the muscle. Proliferating SVPs, endothelial cells and muscle fiber fractions were calculated after staining of the sections with proliferating nuclear antigen (1:100 PCNA, Dako) together with Isolectin B4. PCNA positive cells were counted in the whole section; number of positive SVPs was normalized on total number of SVPs, number of positive fibers was normalized on total area of the muscles, whereas number of positive capillaries was normalized on total nucleated capillaries. Staining for CD44 was performed to confirm SVP phenotype in vivo using mouse rabbit anti human CD44 antibody (Thermo Scientific). Whole mount preparation of the muscles was performed by fixing the excised muscles in PFA 2%; adductors were then stored at -80°C in OCT until processing. 100µm thick sections were stained with directly conjugated Isolectin B4 and N-cadherin. Three-dimensional images were taken using confocal microscopy (Leica).
Supplemental Figure 1
Supplemental Figure 2

A
B
C

D

NC  CD34-  CD34+  CD31-  CD31-  SVP  P2  P3  P7

NG2

PDGFRβ

18s
Supplemental Figure 3
Supplemental Figure 4

24h

A

B

48h

C

D

E

F

Cumulative tube length per VF (mm)

UCM   SVP-CCM   UCM   SVP-CCM

24h   48h

Number of branches per VF

UCM   SVP-CCM   UCM   SVP-CCM

24h   48h
Supplemental Figure 7

A

PCNA\textsuperscript{pos} capillaries

\begin{align*}
\text{Vehicle} & \quad 10 \\
\text{SVPs} & \quad 15
\end{align*}

B

PCNA\textsuperscript{pos} fibers/mm\textsuperscript{2}

\begin{align*}
\text{Vehicle} & \quad 1 \\
\text{SVPs} & \quad 2
\end{align*}
**Figure legends**

**Supplemental Figure 1: FACS sorting of \textit{CD34}^{pos} \textit{CD31}^{neg} cells.** Cells obtained from vein digestion were gated for their size (A) and then \textit{CD34}^{pos}\textit{CD31}^{neg} population was sorted by FACSsorting (B). Flow cytometry characterization of the selected population showed that sample purity was routinely >99% (C, n=2).

**Supplemental Figure 2: Morphology and characterization of cultured SVPs.** Phase contrast photographs showing morphological phenotype of SVPs derived from HM selection (A), magnetic beads isolation (B) and FACS sorting (C). Scale bar: 200μm. Gene expression analysis of \textit{CD34}^{pos}\textit{CD31}^{neg} sorted cells showed native expression of NG2 and PDGFRβ. (D). Differentiation into SVPs gradually increased the expression of the two markers. NC: negative control; PCR reaction performed substituting cDNA with water.

**Supplemental Figure 3: Interactions between SVPs and SVECs in bi-dimensional co-culture.** Phase contrast microscopy image showing the morphology of a 2D co-culture between SVPs, large cells with fibroblast-like morphology (white arrows), and SVECs, smaller cobblestone-shaped cells. Of note, SVPs form membrane extensions that allow the interaction with multiple SVECs. Scale bar: 200μm.

**Supplemental Figure 4: Effect of SVP-CCM on network formation by SVECs.**

Representative pictures taken from structures formed by SVECs on Matrigel upon treatment with unconditioned (A, B) or conditioned (C, D) culture medium at 24 and 48h. Bar graph showing the quantification of total tube length (E) and number of branches (F) at 24 and 48 hours from seeding. Scale bar: 500μm. \#P<0.05 and \#\#P<0.01 vs. 24h and *P<0.05 and **P<0.01 vs. UCM.

**Supplemental Figure 5: Effect of SVP-CCM on VSMCs in vitro.** Bar graphs illustrating the effect of SVP-CCM on cell number (A), apoptosis (B), proliferation (C) and migration (D) of murine VSMC. Controls consist of normal VSMC medium with (A, B, D) without serum (C).
*P<0.05 and **P<0.01 vs. UCM.

**Supplemental Figure 6: Effect of early-culture endothelial progenitor cells (EPCs) transplantation.** Line graph showing blood flow recovery after induction of unilateral limb ischemia in vehicle- or EPC-injected mice (A, arrow indicates time of cell/vehicle injection). Bar graphs showing capillary (B) and arteriole density (C) in muscles injected with EPCs as compared to vehicle at 14 days after ischemia. Values are mean±SEM n=7 animals per group. *P<0.05 and **P<0.01 vs. Vehicle. ^P<0.05, ^^P<0.01 and ^^^P<0.001 vs. day 1.

**Supplemental Figure 7: Cell proliferation in ischemic muscles.** Bar graphs showing the number of proliferating endothelial cells and myofibers as assessed by PCNA. Values are mean±SEM; n=4 animals per group.

**Supplemental Video 1: Three-dimensional reconstruction of a structure formed by co-cultured cells in Matrigel.** Z-stack of confocal images of a portion of the network formed by SVECs and SVPs in Matrigel were taken and reconstructed. The software elaboration created a rotating solid structure that shows the connections between the branch, formed by SVECs (stained with Isolectin B4, green), and the overlaying SVPs (stained red with CM-DiI).

**Supplemental references**


