Epigenetic Profile of Human Adventitial Progenitor Cells Correlates With Therapeutic Outcomes in a Mouse Model of Limb Ischemia

Miriam Gubernator,* Sadie C. Slater,* Helen L. Spencer,* Inmaculada Spiteri, Andrea Sottoriva, Federica Riu, Jonathan Rowlinson, Elisa Avolio, Rajesh Katare, Giuseppe Mangialardi, Atsuhiko Oikawa, Carlotta Reni, Paola Campagnolo, Gaia Spinetti, Anestis Touloumis, Simon Tavaré, Francesca Prandi, Maurizio Pesce, Manuela Hofner, Vierlinger Klemens, Costanza Emanueli, Gianni Angelini, Paolo Madeddu

Objective—We investigated the association between the functional, epigenetic, and expressional profile of human adventitial progenitor cells (APCs) and therapeutic activity in a model of limb ischemia.

Approach and Results—Antigenic and functional features were analyzed throughout passaging in 15 saphenous vein (SV)–derived APC lines, of which 10 from SV leftovers of coronary artery bypass graft surgery and 5 from varicose SV removal. Moreover, 5 SV-APC lines were transplanted (8×10^5 cells, IM) in mice with limb ischemia. Blood flow and capillary and arteriole density were correlated with functional characteristics and DNA methylation/expressional markers of transplanted cells. We report successful expansion of tested lines, which reached the therapeutic target of 30 to 50 million cells in ≈10 weeks. Typical antigenic profile, viability, and migratory and proangiogenic activities were conserved through passaging, with low levels of replicative senescence. In vivo, SV-APC transplantation improved blood flow recovery and revascularization of ischemic limbs. Whole genome screening showed an association between DNA methylation at the promoter or gene body level and microvascular density and to a lesser extent with blood flow recovery. Expressional studies highlighted the implication of an angiogenic network centered on the vascular endothelial growth factor receptor as a predictor of microvascular outcomes. *FLT-1* gene silencing in SV-APCs remarkably reduced their ability to form tubes in vitro and support tube formation by human umbilical vein endothelial cells, thus confirming the importance of this signaling in SV-APC angiogenic function.

Conclusions—DNA methylation landscape illustrates different therapeutic activities of human APCs. Epigenetic screening may help identify determinants of therapeutic vasculogenesis in ischemic disease. (***Arterioscler Thromb Vasc Biol*****. 2015;35:675-688. DOI: 10.1161/ATVBAHA.114.304989.)**

Key Words: angiogenesis inhibitors ■ epigenomics ■ pericytes

The recognition of multipotent stem cells residing nearby or within the blood vessel wall has inspired the novel concept of the vascular niche being a determinant site for endogenous repair processes and a source of therapeutic cells for regenerative medicine applications.[1] Two distinct stem cell populations associated with the vascular system, namely pericytes and adventitial progenitor cells (APCs), are the focus of intense research. Pericytes wrap around endothelial cells in microvessels and express a spectrum of antigens, such as CD146, PDGFRβ, and NG2.[2,3] The second cell subtype, the APC, has been recently described by different groups to be located in the blood vessel wall in close vicinity to the adventitial vasa vasorum.[4–6] APCs express typical pericyte markers (NG2 and PDGFRβ) and mesenchymal markers (CD44, CD90, CD73, and CD29), as well as stem cell antigens (Oct-4, Sox-2, and KLF4), but are negative for myogenic (α-smooth muscle actin, hematopoietic (CD45), and endothelial markers (CD31 and CD146). In addition, the expression of transmembrane glycoproteins, such as CD133, CD34, and CD105, has been used for in situ identification and immunomagnetic isolation of APCs from fetal and adult vessels.[6–8] The expression of CD133/CD34 is reportedly lost during early culture

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From the Bristol Heart Institute, School of Clinical Sciences, University of Bristol, Bristol, UK (M.G., S.C.S., H.L.S., F.R., J.R., E.A., R.K., G.M., A.O., C.R., C.E., G.A., P.M.); The Institute of Cancer Research, Evolutionary Genomics and Modelling Team, Centre for Evolution and Cancer, Sutton, UK (I.S., A.S.); Imperial College, London, UK (P.C., C.E., G.A.); MultiMedica Research Institute, Milan, Italy (G.S.); Cancer Research UK Cambridge Institute, Cambridge, UK (A.T., S.T.); Centro Cardiologico Monzino, Milan, Italy (F.P., M.P.); and Austrian Institute of Technology, Vienna, Austria (M.H., V.K.).

*These authors contributed equally to this work.

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Correspondence to Paolo Madeddu, MD, FAHA, Bristol Heart Institute, School Of Clinical Sciences, University of Bristol, Upper Maudlin St, Bristol BS2 8HW, UK. E-mail mdprm@bristol.ac.uk

Nonstandard Abbreviations and Acronyms

APC	adventitial progenitor cell
CABG	coronary artery bypass graft
CV	coefficient of variation
CREB	cAMP response element-binding protein
EGR	early growth response gene
FLT-1	VEGF Receptor 1
HLA	human leukocyte antigen
HUVEC	human umbilical vein endothelial cells
LI	limb ischemia
MAZ	MYC-associated zinc finger protein
NC	no evidence of cardiovascular disease
RUNX1	Runt-related transcription factor 1
STRING	Search Tool for the Retrieval of Interacting Genes
SV	saphenous vein
VEGF	vascular endothelial growth factor

of founder cells, whereas pericyte/mesenchymal markers and CD105 are retained throughout long-term expansion of polyclonal or single clone APCs.[6]

Recently, we reported that transplantation of APCs, which were derived from saphenous vein (SV) leftovers of coronary artery bypass graft (CABG) surgery, improves anatomic and functional indexes of recovery in models of peripheral and myocardial ischemia.[6,9] In the latter model, the therapeutic activity was similar in immunocompetent and immunodeficient recipient mice, suggesting that the cell product is tolerogenic.[9] SV-APCs exert therapeutic effects through direct incorporation around and stabilization of the host's neovasculature, as well as through release of angiocrine proteins. We also demonstrated that the microRNA-132 is constitutively expressed and secreted by SV-APCs and remarkably upregulated, together with its transcriptional activator cAMP response element-binding protein (CREB) on exposure to hypoxia/starvation. Secreted microRNA-132 acts as a paracrine activator of healing by stimulating angiogenesis and reducing interstitial fibrosis.[9]

On the basis of these promising results, we are now proposing SV-APCs for the treatment of patients with myocardial or peripheral ischemia. We envisage using autologous SV-APCs as the safest and directly amenable therapeutic option. However, interindividual variability and age-related decrease in regenerative capacity are matters of concern with autologous cell therapy.[10] In addition, epigenetic and expressional changes occurring during stem/progenitor cell isolation and expansion might result in substantial phenotypic modifications, ultimately impacting on therapeutic activity of the cell product. Epigenetic mechanisms can induce plastic, short-term modification on chromatin structure by histone tail modifications, as well as rigid, long-term effects by DNA methylation. Currently, much attention is being paid to the effects of CpG methylation on stem cell self-renewal, differentiation, and cancerous transformation.[11,12] However, in comparison with the large information obtained from embryonic stem cells, the role of CpG methylation in regulating the biology of adult stem cells has been less extensively examined.[13] Furthermore, to the best of our knowledge, no previous study has investigated whether the epigenetic and expressional trait of adult stem/progenitor cells correlates with their therapeutic activity.

In the present study, we examined different SV-APC populations to assess antigenic and functional characteristics and therapeutic performance in a murine model of limb ischemia (LI). To determine whether a cardiovascular impacted background will influence the cell regenerative capacity, we sourced SV-APCs from 2 groups of subjects. SV-APC populations were derived, as previously reported, from vein leftovers of patients with coronary artery disease undergoing CABG surgery (CABG SV-APCs)[6,9] and control without coronary artery disease subjects undergoing saphenectomy for varicosity (NC SV-APCs). After addressing the feasibility of production and quality of the cell therapy product, we investigated the underlying mechanisms that enable such therapy, specifically asking the question whether epigenetic/expressional markers of transplanted cell populations are associated with the varied outcome of reparative processes in vivo.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Maintenance of Typical Antigenic Profile During Expansion

Clinical characteristics of all patients used in this study are reported in Table I in the online-only Data Supplement. Under the current standard operating protocol, we report successful cell expansion in 22 of 35 SV specimens (68%), resulting in 30 to 50 million viable SV-APCs in each preparation at P8 in ≈ 10 weeks. We found that the success of expansion is strictly dependent on the amount of vein tissue used for cell extraction. With tissue weight >1.6 g, the efficiency was consistently 100%.

Having documented the feasibility of SV-APC expansion for therapeutic usage, we next verified the antigenic phenotype of 15 SV-APC lines using a panel of typical markers reported previously.[6,9] Immunocytochemical analyses of SV-APCs at P4 revealed the characteristic expression of pericyte/mesenchymal markers, PDGFRβ, NG2, desmin and vimentin, while α-smooth muscle actin was not detected or was detected in low quantity (Figure 1A). The expression of NG2 and PDGFRβ was verified through subsequent passages by immunocytochemistry using InCell Analyzer. Of note, both markers were equally conserved during expansion of CABG and NC preparations (Figure 1B). In addition, flow cytometry confirmed that, at P4, SV-APCs express high levels of CD44 and CD105 and variable quantities of CD90. In contrast, CD34 was virtually lost during initial passages (Figure 1C). Furthermore, 5 CABG and 3 NC SV-APCs were assessed in the same sitting at P4 and P7 in triplicate experiments (Figure 1D). Considering all the 8 samples together, we found CD44 and CD105 to be expressed at 98.7±1.0% and 91.7±2.1%, which remained unchanged at P7 (97.9±0.6% and 90.7±1.2%, respectively). The coefficient of variation (CV) was 3% (P4) and 2% (P7) for CD44 and 6% (P4) and 4%

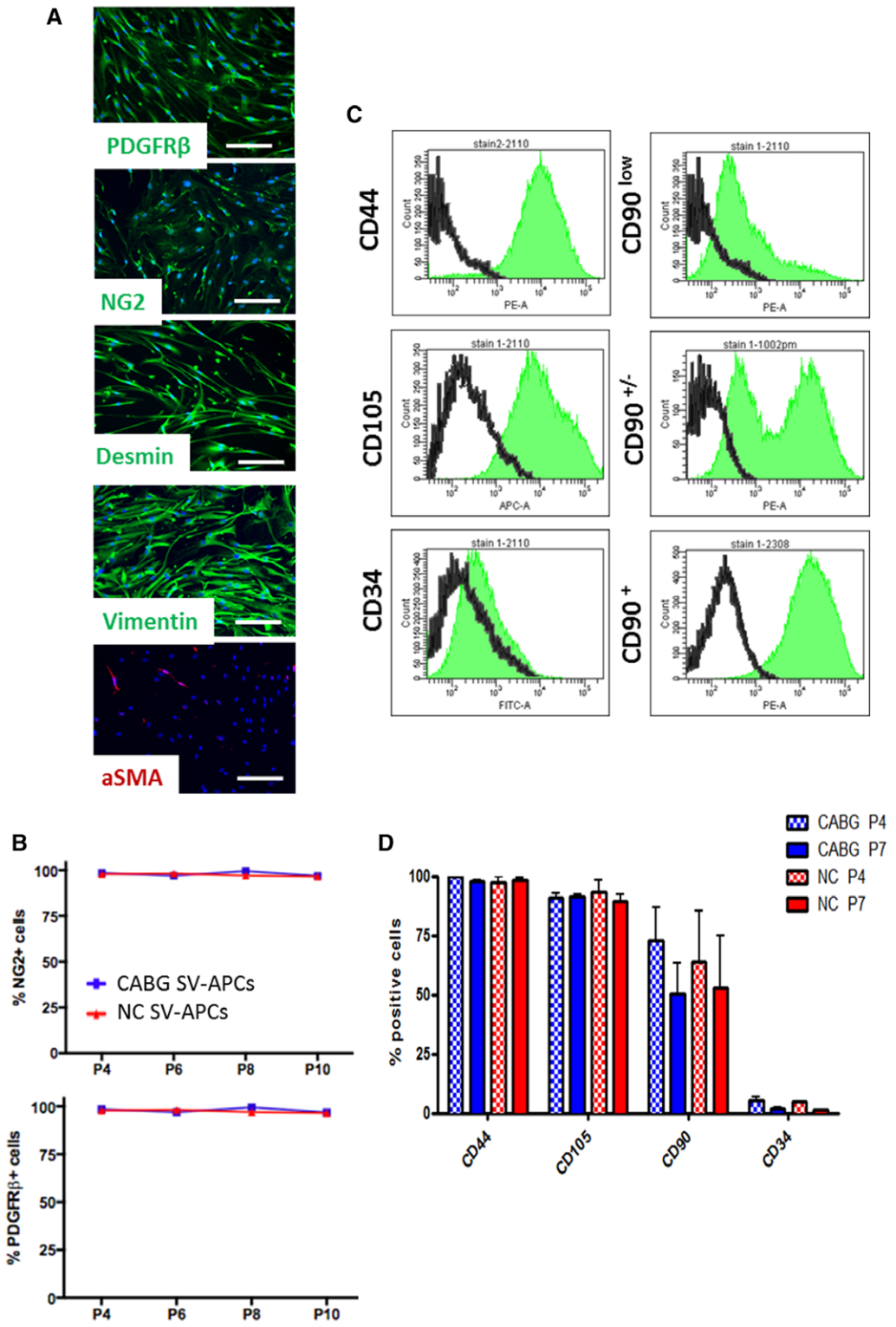

Figure 1. Phenotypic analysis of the patient's saphenous vein (SV)–adventitial progenitor cells (APCs). **A**, Immunocytochemistry of cultured SV-APCs (P4, n=15) reveals conserved presence of PDGFRβ, NG2, desmin, and vimentin and low incidence/absence of α-smooth muscle actin (α-SMA; scale bar 200 μm). **B**, Conservation of pericyte features was seen by quantification of NG2 and PDGFRβ expression by InCell Analyzer (n=5 coronary artery bypass graft [CABG] SV-APCs; n=3 no evidence of cardiovascular disease [NC] SV-APCs). **C** and **D**, Each cell population was also assessed by FACS, as illustrated here as an example (**C**). This analysis showed high CD44/CD105 and low to no CD34 expression after P4 in vitro. CD90 was variably expressed, allowing us to classify SV-APCs into CD90dim and CD90bright. Cell lines were also compared in triplicate analysis side-by-side (**D**), to show conserved phenotype over culture from P4 to P7 for CABG SV-APCs and NC SV-APCs (n=5 and 3, respectively). Data are mean and standard error. APC-A indicates adventitial progenitor cell; FITC-A, fluorescein isothiocyanate; and PE, phycoerythrin.

(P7) for CD105. CD90 decreased from 69.4±11.2% at P4 to 51.2±10.6% at P7 and showed a marked variability (CV, 46% and 69%, respectively). In addition, we observed a further downregulation of the CD34 antigen from P4 (5.2±0.7%) to P7 (1.4±0.4%). Two-way ANOVA did not detect any difference for each single antigen, when comparing CABG- and NC-APCs at P4 and P7 (Figure 1D), indicating that cardiovascular background does not impact on SV-APC antigenic features during expansion to obtain clinically relevant amounts of the cell product.

SV-APC Functional Behavior During Expansion

Functional features were next assessed to determine the variability in quality among different SV-APC populations. First, we performed a battery of in vitro assays to address differences in growth potential by analyzing proliferation and viability and quantifying the level of apoptosis and senescence throughout the expansion (Figure 2A–2C; n=10 for CABG SV-APCs and n=5 for NC SV-APCs). Proliferation activity seen by bromodeoxyuridine incorporation remained stable from P4 to P6 and then showed a trend to decrease with further passaging, especially for cells from CABG patients; however, this change did not reach a statistical significance in either group (Figure 2A). Viability (MTS assay; Figure 2B), apoptosis (activated caspase; Figure 2C), senescence (β-galactosidase; Figure 2D), and migratory activity (gap closure; Figure 2E) remained unchanged during SV-APC passaging. Overall, these data are reassuring with regard to the quality of SV-APCs, which show conserved viability and motility and low levels of replicative senescence during expansion in culture.

Angiogenic Activity of Expanded SV-APCs

We next investigated the effect of SV-APCs in sustaining the organization of endothelial cell networks in Matrigel (Figure 3A–3C). SV-APCs from both CABG and NC similarly improved the network size by increasing average branch length, as well as average branch thickness, compared with human umbilical vein endothelial cells (HUVECs) alone. The variability was slightly lower for the effect of SV-APCs on branch thickness (CV, 15% and 21% for NC and CABG, respectively) when compared with average branch length (CV, 21% and 24%).

Promotion of Blood Flow Recovery and Reparative Neovascularization by SV-APC Injection

Next, we compared the therapeutic activity of 5 SV-APCs populations (P7) with respect to vehicle, in an immunocompetent mouse model of unilateral LI (n=7 mice per each cell therapy

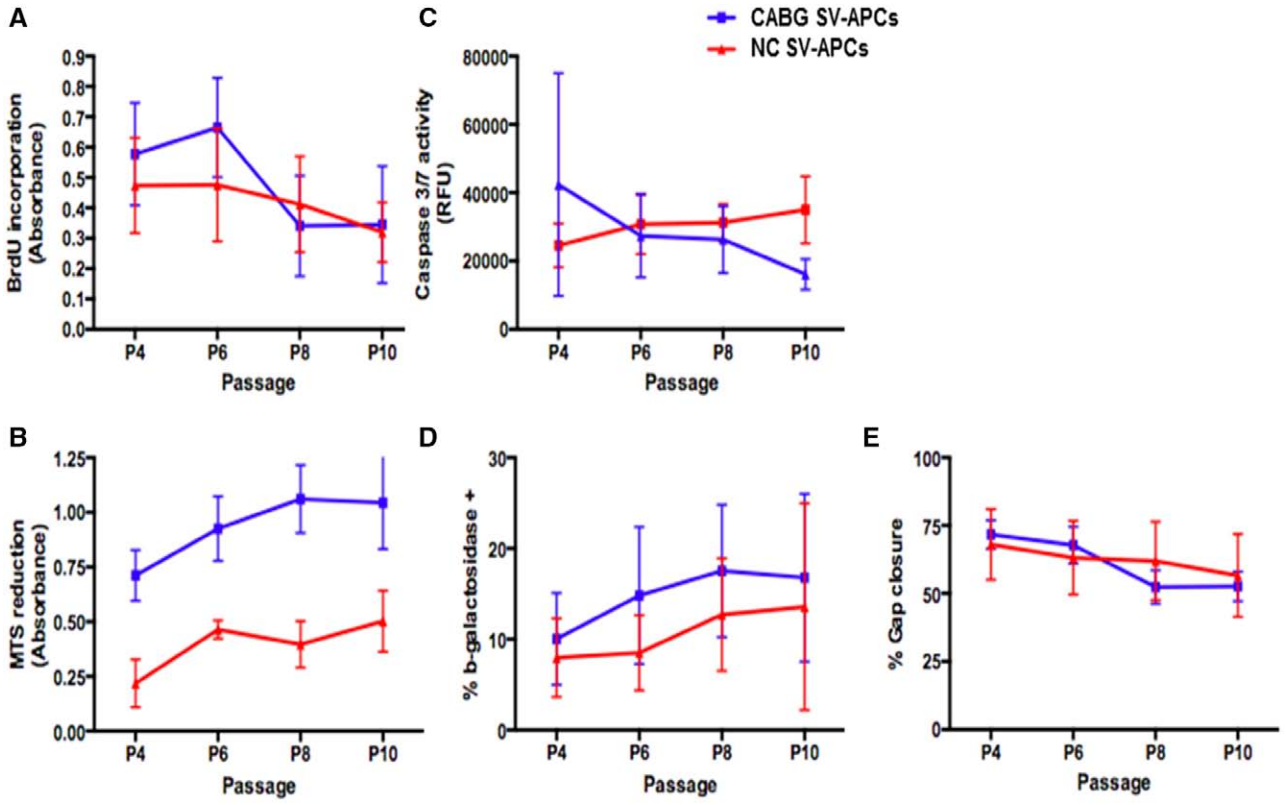

Figure 2. In vitro assessments during saphenous vein (SV)–adventitial progenitor cell (APC) expansion. To allow for a rolling process of different cell population analysis, each SV-APC population was individually assessed at 4 time points in the same experimental sitting and repeated in triplicate. Multiple in vitro assays were chosen to provide an insight into the differences in the expansion capacity and quality of cell product. Data shown here include analysis of (**A**) proliferation activity seen by BrdU incorporation, (**B**) viability/metabolic activity seen through MTS assay, and incidence of cell death and senescence by caspase 3/7 (**C**) and β-galactosidase activity (**D**). Additional, functional capacity over the expansion was addressed by migratory capacity in a scratch assay (**E**). n=10 for coronary artery bypass graft (CABG); n=5 for no evidence of cardiovascular disease (NC) in all assays except for scratch assay that was performed in n=7 CABG and 4 NC. Data shown as mean and standard error.

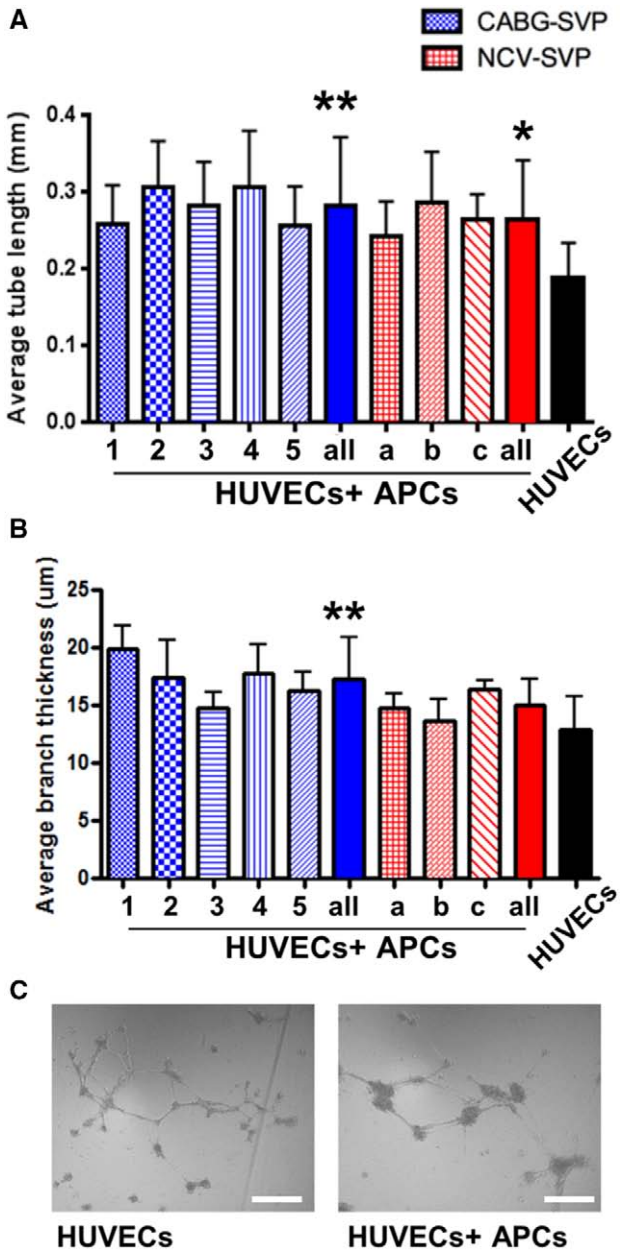

Figure 3. Network supporting capacity. Proangiogenic activity on Matrigel revealed consistently increased average length (**A**) and thickness of network (**B**). Variability existed among the cell populations, but overall no difference was observed between cells from coronary artery bypass graft (CABG; indicated by progressive numbers) and no evidence of cardiovascular disease (NC) subjects (indicated by letters). Hence, saphenous vein (SV)–adventitial progenitor cells (APCs) induced a network remodeling with stronger branches, as can be seen by representative images of human umbilical vein endothelial cells (HUVECs) vs HUVECs+SV-APCs (**C**). Data are means and standard error. n=3 for all figures. **P*<0.05 and ***P*<0.01 vs HUVECs alone. Statistical analysis comparing all SV-APCs (CABG and NC) and HUVECs alone showed a *P*=0.006 for tube length and *P*=0.002 for tube thickness.

or vehicle group). The choice of immunocompetent mice was based on the established knowledge that the immune system plays a key role in the native reparative response to ischemia and on previous demonstration that SV-APCs have tolerogenic properties in a mouse model of myocardial infarction.[9] In-line with this, we found SV-APCs express intermediate levels of major histocompatibility complex class I human leukocyte antigens (HLAs) A, B, and C and are negative for class II HLA-DR, CD80, and Fas ligand as assessed by reverse transcription polymerase chain reaction (RT-PCR; P. Madeddu, MD, FAHA, unpublished data, 2014). Furthermore, using a flow cytometry–based cytotoxicity method, we found that coincubation of SV-APCs with activated murine lymphocytes (1:6 ratio) induces low levels of SV-APC death compared with cell death observed after coincubation of human endothelial cells and murine lymphocytes (2.12% versus 7.12%, respectively; *P*<0.05).

Blood flow analysis during 28 days postischemia indicates an overall enhancement of reperfusion in SV-APC–treated limbs compared with vehicle-injected ones (*P*=0.007; Figure 4A–4C; Figure I in the online-only Data Supplement). In addition, a similar improvement was observed when examining the blood flow recovery at the level of the foot plantar region (*P*=0.003 versus vehicle). This effect was equivalent to that observed in a previous study in immunodeficient mice with LI.[6] The therapeutic response varied widely across groups as indicated by the assessment of the mean time to reach peak blood flow (from 15 to 24 days, average 20 days, in SV-APC–treated mice versus 25 days in vehicle-injected mice), the percentage increase in blood flow from the induction of ischemia to final measurement at 28 days postischemia (from 535±119% to 828±167%, average 616±73%, in SV-APC–treated mice versus 434±171% in vehicle), and the time-weighted blood flow recovery (from 0.38±0.04 to 0.61±0.08 Doppler units, average 0.48±0.02, in SV-APC–treated groups versus 0.32±0.05 Doppler units in vehicle; Figure 4C). Interestingly, intragroup variability was less in groups receiving SV-APC therapy (time-weighted blood flow recovery: mean 26 CV, 33%; range, 14–32%) compared with those receiving vehicle (CV, 39%). Furthermore, improved perfusion by SV-APC therapy resulted in reduced muscle fibrosis, as illustrated by Azan-Mallory staining (Figure 4D).

Measurement of capillary and arteriole density at 28 days postinjection indicates the benefit of SV-APCs on reparative neovascularization (Figure 4E–4G). No change in microvascular density was instead observed in contralateral muscles (data not shown). Contrary to Doppler blood flow recovery, cell therapy increased the variability of microvascular response to ischemia in individual groups (median capillary density CV, 28% [range, 19–31%] versus 9% in vehicle; median arteriole density CV, 27% [range, 22–38%] versus 17% in vehicle). In addition, no association was found between the Doppler blood flow data and the capillary or arteriole density in ischemic muscles, possibly because of the fact that perfusion was measured sequentially during recovery from ischemia, whereas vascular profiles were assessed at euthanasia. Hence, these parameters were considered as separate outcome indexes in subsequent studies on molecular and epigenetic predictors of therapeutic efficacy.

Assessment of SV-APC Persistence in Ischemic Muscles

The persistence of injected SV-APCs, a secondary end point of the in vivo study, was determined by flow cytometry

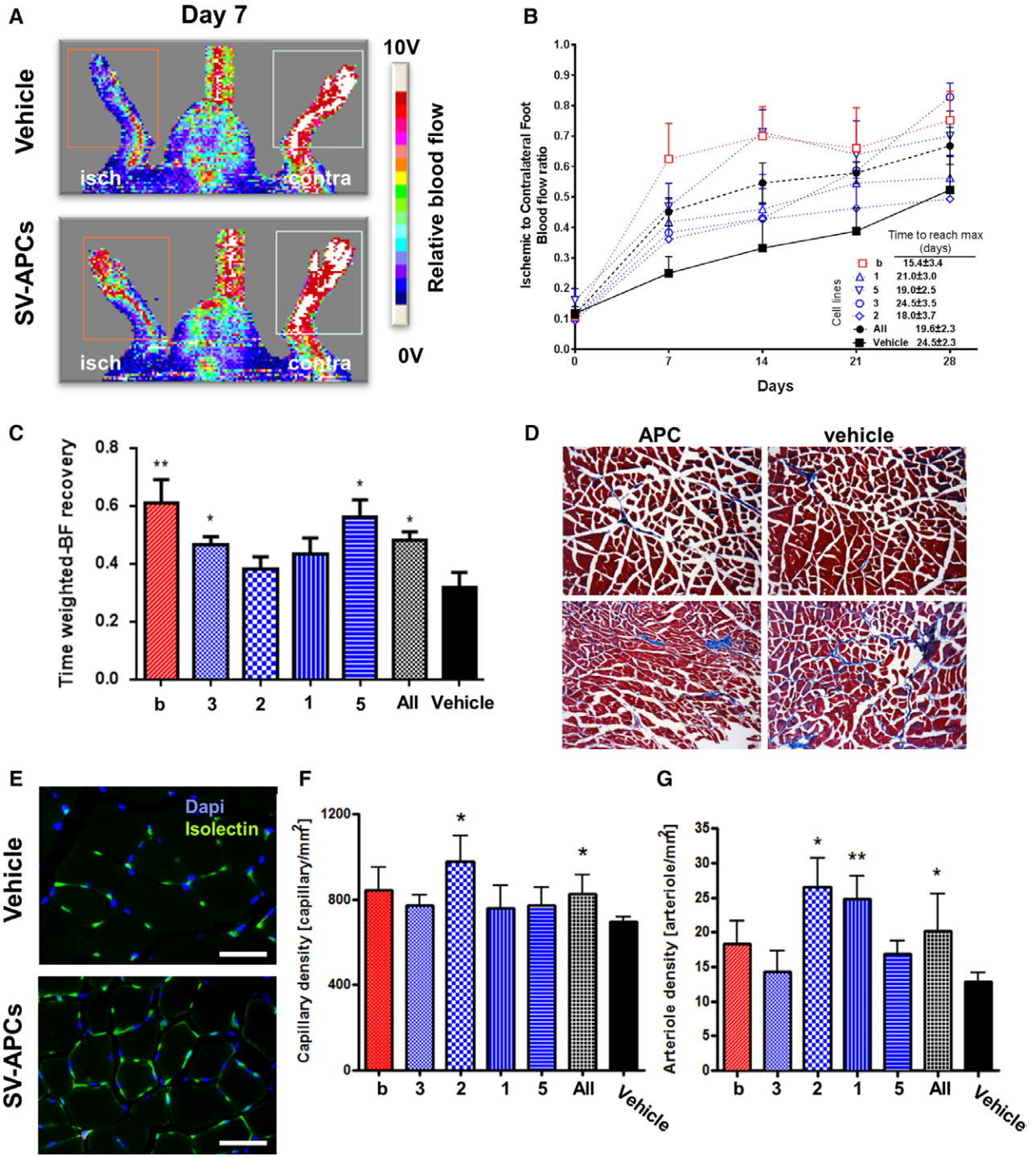

Figure 4. Therapeutic effect in a mouse model of limb ischemia. **A–C**, Intramuscular injection of different saphenous vein (SV)–adventitial progenitor cell (APC) populations (4 representative of coronary artery bypass graft [CABG] donors, blue, and 1 control from saphenectomy, red; n=7 per group assessed) in immunocompetent mice subjected to unilateral limb ischemia promotes acceleration and time-weighted enhancement of blood flow (BF) recovery. **A**, Images of laser Doppler flowmetry at 7 days from induction of ischemia depicting areas of interest where measures were performed (squares delimited by dotted lines). **B**, Time course of the ischemic/contralateral foot BF ratio of mice grouped according to received cell therapy or vehicle (n=7 in each group assessed). **C**, Bar graph representation of time-weighted BF recovery. **D**, Azan-Mallory staining showing reduced muscle fibrosis in SV-APC–injected muscles compared with vehicle. **E–G**, Immunohistological analysis showing the capillary and arteriole density by representative images (**E**, 50 μm scale bar) and mean values in SV-APC (each cell line and all cell lines averaged) and vehicle-injected groups (**F** and **G**; n=5 mice per group). Data are means and standard error.*P<0.05 and **P<0.01 vs vehicle.

analysis of human-specific antigen HLA-ABC and the 2 highly expressed markers CD44 and CD105. Briefly, mouse adductor muscles were collected 0, 2, or 7 days after SV-APC injection and digested to single cells (n=3 per group). Next, to clean up gating of target population (shown in detail in Figure II in the online-only Data Supplement), single cell

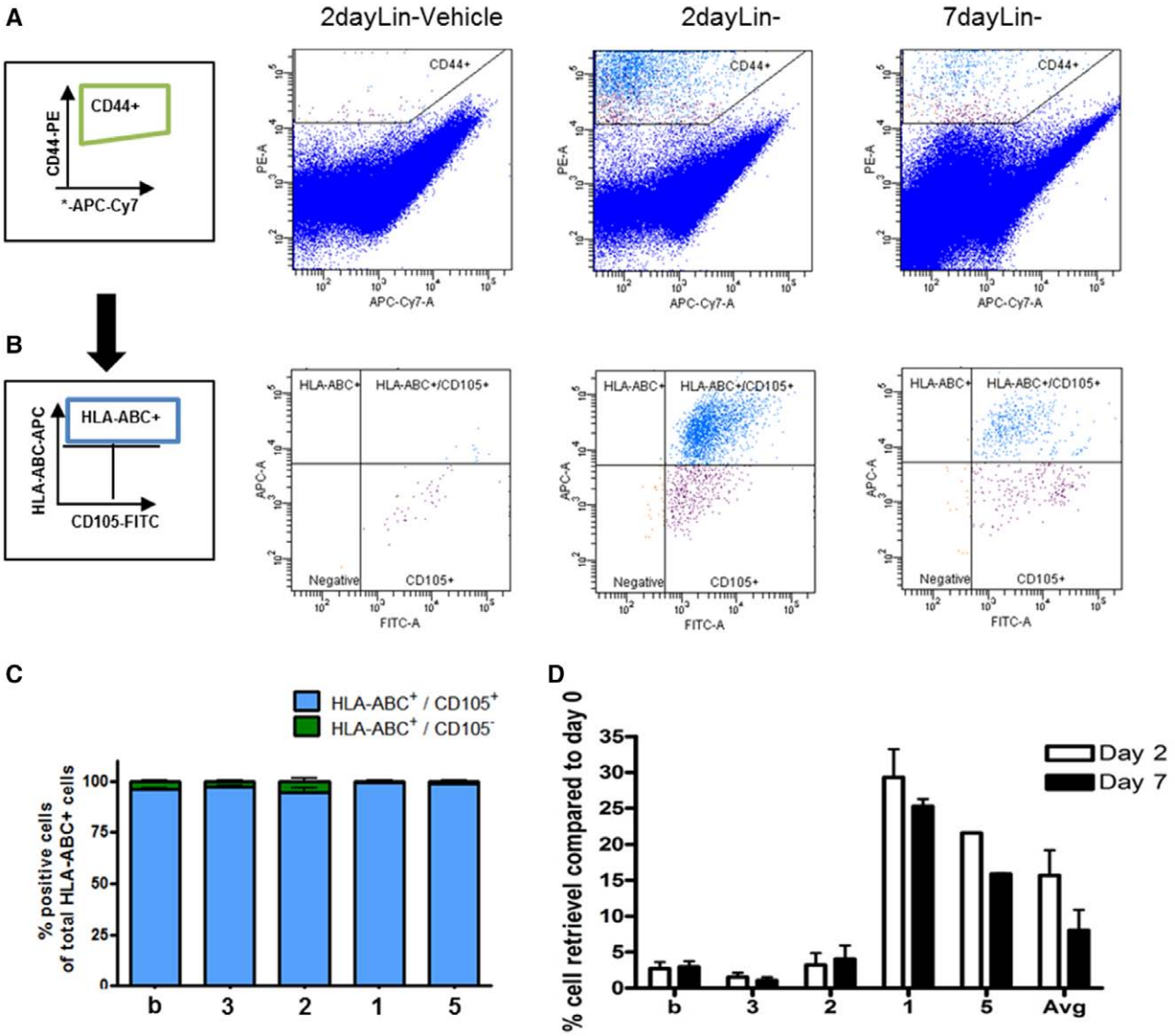

Figure 5. Retrieval of injected cells from ischemic muscles. **A–D**, After mouse lineage depletion, living cells (adventitial progenitor cell [APC]-Cy7 negative) were selected from cells of the ischemic adductor muscle according to CD44⁺ positivity. Gated cells were further selected for human leukocyte antigen (HLA)-ABC and assessed for conservation of CD105 phenotype. n=3 mice for each of 6 groups (5 saphenous vein [SV]–APC-injected and 1 vehicle-injected). Shown here are representative FACS dot plots for the selection process for vehicle and day 2 and day 7 postinjection (**A** and **B**). Live cells were accounted for by the absence of live/dead staining (APC-Cy7). **C**, Abundance of CD44⁺/HLA-ABC⁺ cells within the retrieved population. **D**, Average data of cell retrieval compared with day 0 expressed as means and standard error. FITC-A indicates fluorescein isothiocyanate.

suspensions were mouse lineage depleted. Live/dead staining confirmed the presence of a viable population (APC-Cy7). Then, human cells were selected as CD44⁺ (Figure 5A, green box) and HLA-ABC⁺ (Figure 5B, blue box). Specificity of the staining procedure was verified in vehicle-injected samples, which show low background signal (Figure 5B; Figure II in the online-only Data Supplement). Furthermore, retrieved cells using CD44/HLA-ABC were >95% positive for CD105, thus indicating the high retention of original phenotype after transplantation (Figure 5C). Cell persistence was finally quantified by normalizing the absolute cell numbers (using counting beads) by the number of cells extracted at day 0. Variable quantities of human SV-APCs were found in adductor muscles at day 2 postinjection, ranging from 1.9% to 29.3% (mean 15.7%) of the quantity retrieved at time 0. The average cell retrieval was further reduced to 8%, at day 7 postinjection (Figure 5D). There was no association between the number of SV-APCs in ischemic muscles at 2 days postinjection and the primary end points (blood flow recovery and microvascular density).

Correlation Between Clinical and Functional Data and Primary Outcome End Points

Having shown that the benefit elicited by SV-APCs in the LI model varies among different cell lines, we next asked whether clinical data of the donor subjects and functional in vitro properties of the injected cells can anticipate the primary outcome end points. We found that cells from smoker donors are less effective in improving blood flow recovery (time-weighed blood flow: 0.37±0.03 Doppler units) compared with cells from nonsmokers (0.52±0.05 Doppler units;

$P<0.01$). Furthermore, there was a significant inverse correlation between age of the donor and capillary density outcome ($R^2=0.88$; $P<0.02$). Importantly, we found a direct correlation between the ability of SV-APCs to promote in vitro angiogenesis on Matrigel and the capillary density outcome ($R^2=0.80$; $P<0.05$; Figure III in the online-only Data Supplement), which indicates that the in vitro assay is an efficient predictor of the vasculogenic effect in vivo.

Correlation Between DNA Methylation Profile of SV-APCs and Primary Outcome End Points

To identify new epigenetic predictors of therapeutic activity, we next performed a whole genome DNA methylation array of the SV-APC populations, which were injected in the mouse model of LI. We identified 936 unique genes (106 of these involving promoter regions), whose methylation status is correlated with the time-weighted blood flow outcome data ($P<0.001$). In addition, 5461 genes (930 in the promoter region) had a methylation status that correlates with capillary density, and 784 unique genes (of these 89 in the promoter region) were associated with arteriole count ($P<0.001$ for both the outcome indexes).

We next used the TRANScription FACtor (TRANSFAC) database to identify the transcription factors that regulate the expression of genes emerging from the association between DNA methylation and outcome data. Results indicate that KROX (a component of the early growth response genes [EGR] family) regulates a significant amount of the genes whose methylation status correlates with blood flow, capillary density, and arteriole counts (418, 1979, and 369, respectively).[14,15] Moreover, 536 genes associated with blood flow, 2805 genes associated with capillary density, and 460 genes associated with arteriole counts are regulated by MYC-associated zinc finger protein (MAZ), which is involved in cell proliferation and mediates vascular endothelial growth factor (VEGF)–induced angiogenesis.[16] Hence, interference of DNA methylation with KROX/EGR1 and MAZ might result in large effects on gene transcription and possibly on promotion of tissue repair by SV-APCs.

We next integrated all the differentially methylated genes emerging from blood flow (936), capillary density (5461), and arteriole count (784) analyses and found that 304 of them are common to the 3 outcome end points. In keeping with our previous finding, 158 (52%) of these genes bear KROX-/EGR1-binding sites ($P<0.001$). In addition, an analysis of the genomic locations of these 304 shared genes identified a significant enrichment of the 6p21 loci (17 of the 304 genes; $P<0.0001$). Furthermore, using the Search Tool for the Retrieval of Interacting Genes (STRING) database, we found that these shared genes are interconnected in a network centered on CREB-binding protein (Figure IV in the online-only Data Supplement), a nuclear protein that binds to CREB. Furthermore, in the described network, CREB-binding protein seemed to be associated with Runt-related transcription factor 1 (RUNX1), which also locates on chromosome 21 and correlates with outcomes end points at both the methylation (Figure V in the online-only Data Supplement) and the gene expression level (vide infra).

We next restricted the analysis on the methylation status to known angiogenic genes. Gene ontology analysis revealed that 12 angiogenesis-related genes correlate with capillary density (Figure 6). Of these 12 genes, neurogenic locus notch homolog 4 and prokineticin-2 also correlate with blood flow recovery and arteriole count (Figure VI in the online-only Data Supplement). Analysis of gene interaction revealed a network of 5 genes, comprising neurogenic locus notch homolog 4, endothelial PAS domain-containing protein 1 (also known as hypoxia-inducible factor-2α), neuropillin-2, placental growth factor, and VEGF receptor 1 (FLT-1), the latter being the core molecule within the network (Figure 6). Altogether, these data lately indicate that complex epigenetic mechanisms may influence the therapeutic activity of the cell product from different donors.

Concerns have been raised that, after isolation and culture expansion, stem cells accumulate stochastic mutations, which may favor malignant transformation. Therefore, we used PubMeth (http://www.pubmeth.org), a cancer methylation database combining text mining and expert annotation, to investigate whether the DNA methylation profile of SV-APCs denotes epigenetic features associated with cancer. We found that genes encoding for cell cycle regulators have methylation patterns that do not resemble that of a cancer state (data not shown). Moreover, a pathway analysis did not reveal any enrichment within our significant genes of currently annotated cancer pathways in the Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/kegg). Therefore, the DNA methylation profile of SV-APCs is reassuring with regard to safety.

Correlation Between the Expressional Profile of SV-APCs and Outcome End Points

We next investigated the expressional profile of SV-APCs using gene arrays (GEO accession number: GSE57964) and RT-PCR analyses. From gene array raw data (45 018 probes), we extracted genes with a significant correlation with outcome end points ($P<0.01$). We found that 494 genes significantly correlated with blood flow (259 with positive correlation and 235 with negative correlation), 420 genes correlated with capillary density (297 positively and 123 negatively), and 225 genes correlated with arteriole density (115 positively and 110 negatively). The lists of genes are given in Table II in the online-only Data Supplement.

Similar to the study on the DNA methylation data, we performed a gene set enrichment analysis to identify transcription factors whose targets are significantly enriched among genes correlating with outcome end points. Using GATHER, we identified 38 transcription factors that are significantly enriched among those genes correlating with capillary density (Table III in the online-only Data Supplement). The nuclear respiratory factor 2, which regulates genes containing antioxidant response elements in their promoters, showed the highest *p* value within the transcription factor list. In addition, MAZ, a transcription factor that emerged from the DNA methylation analysis described earlier, was identified to be associated with a high number (139) of differentially expressed genes.

Gene ontology analysis of the genes related to capillary density revealed a cluster of biological functions related to blood circulation, namely to circulatory system process,

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Gene_ID	Gene Name	Function
PGF	placental growth factor	Growth factor active in angiogenesis, and endothelial cell growth, stimulating their proliferation and migration. It binds to receptor VEGFR-1/FLT1. PLGF-2 binds neuropilin-1 and 2 in a heparin-dependent manner
FLT1	fms-related tyrosine kinase 1 (vascular endothelial growth factor receptor 1)	Receptor for VEGF, VEGFB and PGF. Has a tyrosine-protein kinase activity. The VEGF-kinase ligand/receptor signaling system plays a key role in vascular development and regulation of vascular permeability.
KRT1	keratin 1	May regulate the activity of kinases such as PKC and SRC via binding to integrin beta-1 (ITB1) and the receptor of activated protein kinase C (RACK1/GNB2L1)
PROK2	prokineticin 2	May function as an output molecule from the suprachiasmatic nucleus (SCN) that transmits behavioral circadian rhythm. May also function locally within the SCN to synchronize output.
NOTCH4	Notch homolog 4 (Drosophila)	Functions as a receptor for membrane-bound ligands Jagged1, Jagged2 and Delta1 to regulate cell-fate determination. Upon ligand activation through the released notch intracellular domain (NICD) it forms a transcriptional activator complex with RBP-J kappa and activates genes of the enhancer of split locus. May regulate branching morphogenesis in the developing vascular system (By similarity)
IL17F	interleukin 17F	Stimulates the production of other cytokines such as IL- 6, IL-8 and granulocyte colony-stimulating factor, and can regulate cartilage matrix turnover. Stimulates PBMC and T-cell proliferation. Inhibits angiogenesis
EPAS1	endothelial PAS domain protein 1	Transcription factor involved in the induction of oxygen regulated genes. Binds to the hypoxia response element (HRE) of target gene promoters. Regulates the vascular endothelial growth factor (VEGF) expression and seems to be implicated in the development of blood vessels and the tubular system of lung. May also play a role in the formation of the endothelium that gives rise to the blood brain barrier
HAND2	heart and neural crest derivatives expressed 2	Essential for cardiac morphogenesis, particularly for the formation of the right ventricle and of the aortic arch arteries. Required for vascular development and regulation of angiogenesis, possibly through a VEGF signaling pathway. Plays also an important role in limb development, particularly in the establishment of anterior-posterior polarization, acting as an upstream regulator of sonic hedgehog (SHH) induction in the limb bud.
NRP2	neuropilin 2	High affinity receptor for semaphorins 3C, 3F, VEGF-165 and VEGF-145 isoforms of VEGF, and the PLGF-2 isoform of PGF

B

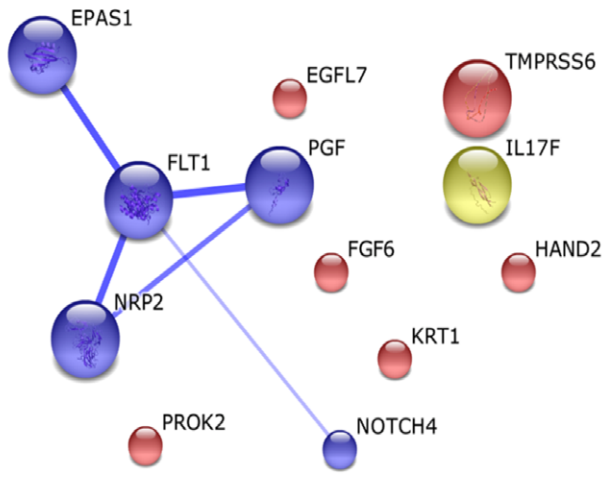

Figure 6. Association of the methylation status of genes involved in regulation of angiogenesis with microvascular end point. **A**, The methylation of 12 genes correlates with capillary counts, 2 of which (neurogenic locus notch homolog 4 [NOTCH4] and prokineticin-2 [PROK2]) also correlate with blood flow and arteriole density outcomes. **B**, Gene interaction network analysis of the 12 angiogenesis-related genes revealed vascular endothelial growth factor receptor 1 (FLT-1) as the core molecule within the network.

vascular process in circulatory system, regulation of tube size, vasoconstriction, and regulation of blood vessel size. Other, less significant functions are related to negative regulation of steroid biosynthetic process, vesicle-mediated transport, and response to muramyl dipeptide. According to this analysis, 18 genes associated with vascular biology were found significantly correlated with capillary density, 9 positively (ACE, ABAT, CBS, GUCI1B3, IRX5, KCNG2, MC3R, NANOS2, and LSC6A4) and 9 negatively (ADRB2, EDN1, GSTM2, HTR7, KCNMB4, MYLK, RCAN1, TBXA2R, and TTN; Table IV in the online-only Data Supplement).

A similar analysis of the genes correlated with arteriole density shows clusters of functions related to positive regulation of transforming growth factor-β production, cytokine secretion, positive regulation of angiogenesis, and positive regulation of prostaglandin biosynthetic process. From this analysis, 7 genes were positively correlated (AVPR1A, GDNF, CCL19, CHI3L1, IL26, PTGS2, and UTS2) and 4 negatively correlated with arteriole density (CD34, CASP1, RUNX1, and TLR1; Table V in the online-only Data Supplement). In contrast, gene ontology analysis using genes correlated with blood flow outcome did not yield any significant enrichment or interconnection.

Finally, we performed RT-PCR studies to validate the results of gene array analysis and also to investigate the predictive value of specific genes that were found to be associated with pluripotency and resistance to oxidative stress in previous studies on SV-APCs (Table VI in the online-only Data Supplement).[9,17] We found that the expression of the stemness marker KLF4 by SV-APCs correlates with the capillary density outcome, whereas SOD2 correlates with both capillary and arteriole density. In-line with data from methylation arrays, RT-PCR confirmed the positive correlation between FLT-1 and capillary density outcome. There was also a positive correlation between the expression of plasminogen, which is reportedly implicated in postischemic arteriogenesis,[18] and the arteriole density outcome. Furthermore, we investigated the expression levels of 2 microRNAs, namely microRNA-132, which we have previously reported to be implicated in the in vivo healing effects of SV-APCs in a mouse model of myocardial infarction,[9] and microRNA-125b, which plays crucial roles in many different cellular processes, such as cell differentiation, proliferation, and apoptosis, associated with angiogenesis through inhibition of its target gene MAZ.[16,19–21] Consistent with an antiangiogenic action of microRNA-125, we found this microRNA to be inversely correlated with capillary density and arteriole density outcomes, whereas no association was found for microRNA-132 (Table VI in the online-only Data Supplement). Finally, we verified the expression of 3 genes emerging from ontology analysis of gene array data, namely prostaglandin-endoperoxide synthase 2/cyclooxygenase 2, the enzyme that converts arachidonic acid to prostaglandin H2, chitinase-3–like protein 1, also known as cartilage glycoprotein 39 (YKL-40), which is implicated in mural cell–mediated angiogenesis,[22] and RUNX1, which has been associated with proangiogenic activity of endothelial progenitor cells.[23] In a previous study, we reported that chitinase-3–like protein 1 and RUNX1 are abundantly expressed in SV-APCs compared with human endothelial cells.[17] We found a trend for positive correlation between the expression levels of chitinase-3–like protein 1 and capillary density, although this did not reach statistical significance. Similarly, the association of prostaglandin-endoperoxide synthase 2/cyclooxygenase 2 with microvascular density suggested by gene array data was not confirmed by RT-PCR. In-line with DNA methylation and gene array data, we found that RUNX1 correlates with arteriole density in an inverse manner.

Functional Impact of FLT-1 Silencing on Network Formation

Because FLT-1 showed a positive correlation with the capillary density, we next investigated the effect of FLT-1 silencing on SV-APC capacity to form networks and support similar properties in HUVECs. SV-APCs were treated with FLT-1 siRNA, scramble-siRNA, or left untransfected and seeded on Matrigel alone or with HUVECs (Figure 7; Figure VII in the online-only Data Supplement). Silencing was confirmed by quantitative PCR and Western blotting analysis (Figure 7A and 7B). *FLT-1* silencing also reduced the amount of sFLT-1 in SV-APC–conditioned media (Figure 7C). Interestingly, *FLT-1*–silenced SV-APCs showed a remarkably reduced capacity to form tubes in Matrigel (Figure 7D). In addition, SV-APCs increased the average tube length compared with HUVECs alone (by 27% in untransfected and 28% in control siRNA). In contrast, this supporting capacity of SV-APCs was totally abrogated following *FLT-1* silencing (Figure 7E). These data demonstrate that FLT-1 expression is fundamental for the angiogenesis-promoting activity of SV-APCs.

Discussion

Pericytes exhibit distinct properties, including progenitor activity and angiogenic factor secretion, which render these cells attractive for regenerative medicine. We have recently identified a population of clonogenic pericytes, alias SV-APCs, in the adventitia of human SV and documented their ability to induce reparative processes in preclinical ischemic models.[6,9] In view of clinical application, this study investigated the feasibility of SV-APC expansion for the production of a consistent therapeutic cell product. We also tested whether clinical characteristics of the donor, data from functional assays, and DNA methylation and gene expression arrays can predict the in vivo effects of injected SV-APCs on microvascular and blood flow recovery in a mouse model of peripheral ischemia. Results indicate that small leftovers of SV give rise to large amounts of therapeutic cells. Furthermore, we lately show that multiple interactions at the epigenome and transcriptome level may contribute to the variability of therapeutic outcomes in vivo and demonstrated that the reduction of these highlighted genes can impact on network formation ability in vitro. Silencing studies verified FLT-1 as a core determinant of the proangiogenic action of SV-APCs.

Clinical stem cell trials of patients with myocardial or critical LI indicate that the threshold therapeutic dosage is ≈30 million cells.[24,25] The expansion capacity of our standard operating protocol is compatible with those cell dosages and allows retention of the typical antigenic and functional profile, independent of clinical characteristics of the donor. However, because the success of expansion is dependent on the amount of vein tissue, vein leftovers from CABG

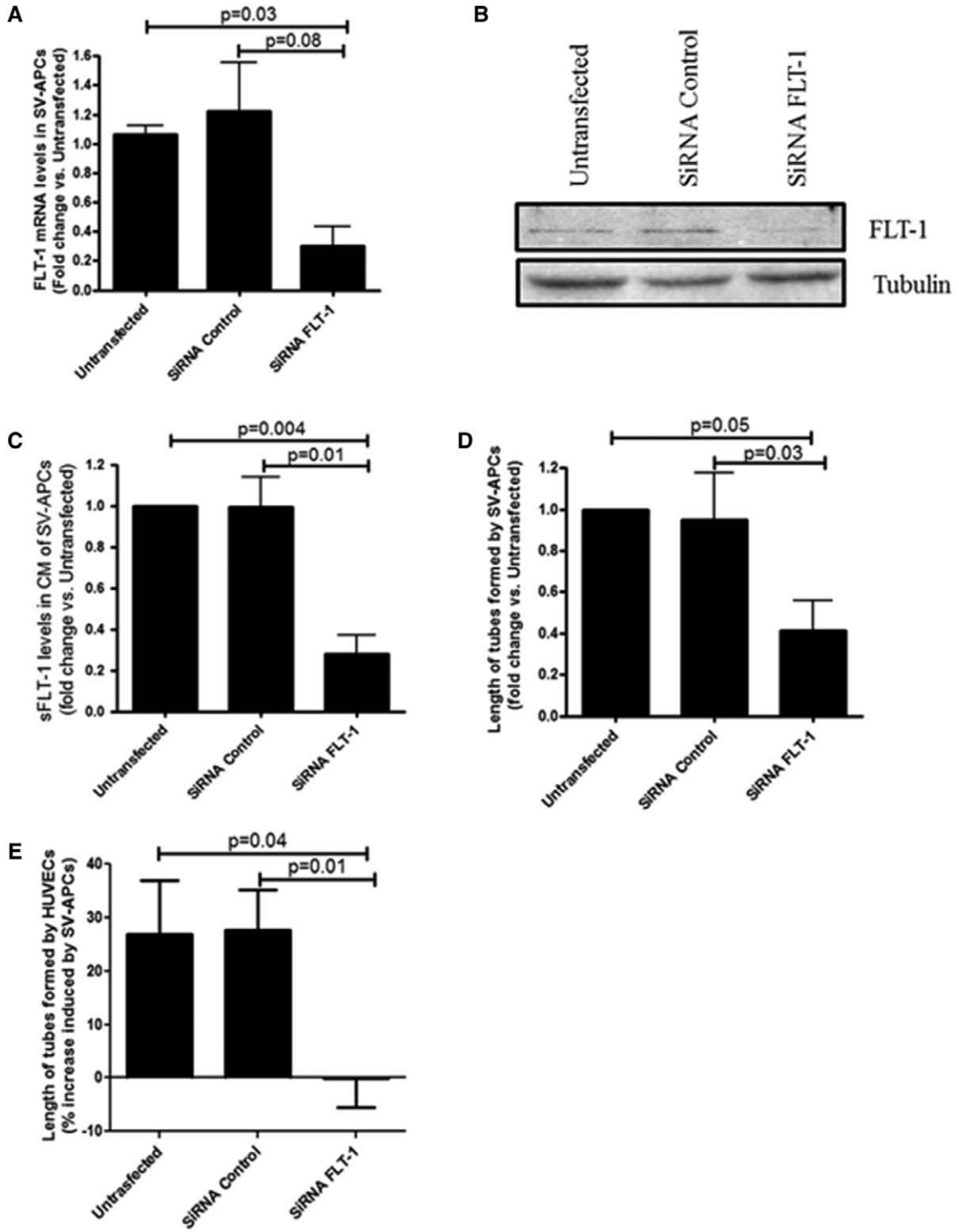

Figure 7. Inhibition of tube formation by silencing of the vascular endothelial growth factor receptor 1 (FLT-1) gene in saphenous vein (SV)–adventitial progenitor cells (APCs). **A**, Quantitative polymerase chain reaction (PCR) confirmation of reduction in FLT-1 transcripts after silencing of FLT-1 by siRNA in SV-APCs. Relative quantification of FLT-1 compared with house-keeping gene UBC. Controls are SV-APCs untrasfected or scramble-siRNA transfected (n=4). **B**, Western blotting analysis of FLT-1 protein in SV-APCs. Densitometry using Bio-Rad Image laboratory 5.1 software confirmed reduction in FLT-1 protein levels (untransfected 602628, control siRNA 657956, and FLT-1 siRNA 200460 A.U.). **C**, Levels of soluble FLT-1 (sFLT-1) in conditioned media (CM) from SV-APCs (n=4). **D**, Length of tubes formed by SV-APCs in a Matrigel assay (n=4). **E**, Increase in the length of tubes formed by human umbilical vein endothelial cells (HUVECs) in a Matrigel assay following coculture with SV-APCs (n=4).

surgery may be occasionally too small to produce clinically relevant numbers of therapeutic cells. For this reason, and because of the time required for cell expansion, we plan to perform a clinical trial in patients with refractory angina, in which a vein segment will be electively harvested for cell procurement.

Individual variability in drug efficacy and safety is a major challenge in current clinical practice. In the case of autologous cell therapy, the medicinal product is not homogeneous, and its therapeutic activity may vary among cell lines. Interestingly, we found that patient's age and smoking habit are negatively associated with therapeutic outcomes and that in vitro angiogenesis is a predictor of SV-APC–induced improvement in reparative angiogenesis. Furthermore, we confirm that injected cells persist for a few days in the injected ischemic muscle. This might be attributed to a combined effect of the ischemic environment and rejection of xenogenic cells. Using a flow cytometry method for precise quantification of human cells, we were able to show that the number of retrieved human cells from injected muscle is not associated with differences in outcome end points. This is compatible with the notion that the paracrine component is a major determinant of SV-APC therapeutic activity in vivo.

Risk factors may affect cell functionality and therapeutic activity by interfering with gene expression. Furthermore, epigenetic modifications accrue through repeated passaging during cell expansion. To the best of our knowledge, no previous study has verified whether the epigenetic landscape predicts the benefit of stem/progenitor cell therapy. This study focuses on DNA methylation, which represents a major modifier of the epigenome. According to current understanding, CpG islands at promoters of genes are normally unmethylated, allowing transcription. Increased DNA methylation of CpG islands results in transcriptional inactivation.[26,27] Methylation also occurs in intergenic sequences and gene bodies, where it reportedly regulates gene expression by several mechanisms, including inhibition of alternative promoters, antagonism of polycomb-mediated repression, and induction of chromosome compaction.[28,29] We have investigated the DNA methylation status of different SV-APC lines using both promoter and nonpromoter methylation probes. Considering microvascular and blood flow recovery outcomes separately, we found that capillary density was associated with the largest number of differentially methylated genes, exceeding the number of genes associated with blood flow recovery and arteriole density by 5.8- and 7.0-fold, respectively. Furthermore, a larger proportion (17%) of genes associated with capillary density is methylated at the promoter region compared with genes associated with the other 2 outcomes (11% in both). These results suggest a strong impact of epigenetic variability on capillary responses.

Differences in DNA methylation may be functionally irrelevant in resting cells, but they could lead to important alterations of functional phenotype after induction of gene expression on cells exposure to an ischemic or inflammatory environment. One mechanism by which DNA methylation can modulate gene expression is by impeding transcription factors to bind to regulatory regions of target genes or facilitating the assembly of repressor complexes at the methylated regions.[30] Searching in TRANSFAC database, we found that the inducible transcription factors MAZ and KROX/ERG1 regulate several hundred genes whose methylation state correlates with capillary (2805 and 1979, respectively) and arteriole density (460 and 369, respectively). Furthermore, MAZ emerged as a transcription factor regulating a large number of genes differentially expressed according to microvascular outcomes. MAZ is a zinc finger transcription factor that binds to GpC-rich *cis*-elements in the promoter regions of numerous mammalian genes and is also able to recruit different proteins, such as methylases and acetylases, to the transcriptional complex, thereby acting as an initiator or terminator of transcription.[31] The transcription factor has been implicated in VEGF-induced angiogenesis[16,32,33]; this effect being negatively controlled by microRNA-125b, of which MAZ is an inhibitory target.[16] Of note, we found that the expression of microRNA-125b in SV-APCs is inversely correlated with their ability to induce reparative vascularization in the mouse LI model.

The transcription factor KROX/EGR1 couples short-term changes in the extracellular milieu to long-term changes in gene expression. It is induced by different growth factors and chemokines, including VEGF and SDF-1,[34,35] and stimulates microvascular endothelial cell growth and neovascularization through FGF-mediated mechanisms.[36] We found that 52% of genes whose methylation status correlates with all outcome end points bear KROX-/EGR1-binding sites, suggesting a potent effect of this transcription factor on the genes associated with the therapeutic action of SV-APCs. These shared genes are interconnected in a network centered on CREB-binding protein, which plays a role in VEGF- and FGF-dependent angiogenesis signaling and in epigenetic control of cell proliferation,[37,38] and are significantly enriched at the 6p21 loci. The mechanisms that direct methylation to specific sequences and loci in the genome remain mainly unknown, although an interaction between DNA methyltransferases and other epigenetic factors has been proposed.[26] Importantly, we found that the VEGF receptor FLT-1, which is under the regulatory control of KROX/EGR1,[39] constitutes the core molecule within a network of angiogenic genes whose methylation is associated with microvascular outcomes. Altogether, these results highlight that the complex epigenetic modulation of SV-APC therapeutic activity is under the control of a limited number of transcriptional regulators. In addition, another important finding from epigenetic studies is that no cancer-related transformation was detected in the methylome of the analyzed cell lines, suggesting that those may be safe for patient use.

Results from gene array data also highlight a multiplicity of expressional changes associated with microvascular improvements induced by SV-APCs transplantation. We previously reported that SV-APCs express high levels of stemness markers and antioxidant enzymes. Here, we document that KLF4, a core component of the pluripotency transcriptional network, correlates with capillary density according to both the DNA methylation and the PCR validation studies. Furthermore, the expression of SOD2, which transforms toxic superoxide from the mitochondrial electron transport chain into hydrogen peroxide and oxygen, correlates with both capillary and arteriole density.

Although we could not find an association between the expression levels of microRNA-132 and outcome end points, this does not exclude its participation in the therapeutic action of SV-APCs, as documented previously using a gene silencing approach.[9] Similarly, there was a positive association between

the prostaglandin synthase enzyme prostaglandin-endoperoxide synthase 2/cyclooxygenase 2 and CH3L1 and capillary density outcome in the gene array study, and this association was only confirmed as a trend but did not reach statistical significance in the RT-PCR validation study. The association of CH3L1 with reparative angiogenesis is intriguing as this adhesion factor is implicated in inducing adhesive contacts between mural cells and endothelial cells through polarization of N-cadherin and activation of the β-catenin/vascular smooth muscle actin complex.[22] We previously showed that SV-APCs establish N-cadherin–positive adhesive contacts with proliferating endothelial cells.[6]

In-line with the methylation data, FLT-1 expression was found to be positively correlated with capillary density in our validation studies using RT-PCR. Unlike the FLK1/KDR VEGF receptor, the role of FLT-1 is not as well understood. Two major splice variants of the *FLT-1* gene encode the full-length transmembrane receptor and a soluble, secreted, truncated receptor. A recent study showed that sFLT-1 regulates pericyte function in vessels and that the deletion of *FLT-1* from specialized glomerular pericytes, known as podocytes, causes reorganization of their cytoskeleton with massive proteinuria and kidney failure, characteristic features of nephrotic syndrome in humans.[40] Importantly, our study lately shows that a reduction in *FLT-1* transcripts remarkably impacts on SV-APC capacity to form tubes and stabilize HUVEC networks in vitro, thus indicating for the first time an important role of FLT-1 in SV-APC proangiogenic action.

In conclusion, our study is the first to show that diversities in the epigenetic and expressional profile of human adventitial stem cells can significantly impact on microvascular and hemodynamic outcomes in a model of peripheral ischemia. These data open up new perspectives for future studies to potentially predict therapeutic response of cell therapy using a high-throughput screening of stem cell epigenome.

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Disclosures

None.

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Significance

This work represents an essential step in our long-term strategy to bring human adventitial progenitor cells to clinical application in patients with ischemic disease using an autologous approach. Results indicate the feasibility of expanding adventitial progenitor cells to amounts sufficient to clinical exploitation and persistence of a consistent antigenic and functional phenotype through expansion. For the first time, we show that whole genomic screening at the DNA methylation and mRNA levels provides clues into the therapeutic activity in a model of peripheral ischemia. This opens up new perspectives for refinement of cell therapy using epigenetic screening of the cell product.