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The epigenetic profile of human adventitial progenitor cells correlates with therapeutic outcomes in a mouse model of limb ischemia

Gubernator: DNA methylation and cell therapy benefit

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ABSTRACT

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

Methods: Antigenic and functional features were analysed throughout passaging in 15 saphenous vein-derived APC lines, of which 10 from saphenous vein (SV) leftovers of coronary artery bypass graft surgery and 5 from varicose SV removal. Moreover, 5 SV-APC lines were transplanted ($8x10^5$ cells, IM) in mice with limb ischemia. Blood flow (BF) and capillary and arteriole density were correlated with functional characteristics and DNA methylation/expressional markers of transplanted cells.

Results: We report successful expansion of tested lines, which reached the therapeutic target of 30-50 million cells in ~10 weeks. Typical antigenic profile, viability and migratory and pro-angiogenic activities were conserved through passaging, with low levels of replicative senescence. *In vivo*, SV-APC transplantation improved BF 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 centred on the VEGF receptor FLT-1 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 HUVECs, thus confirming the importance of this signalling 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.

Non-standard Abbreviations and Acronyms

ANOVA	analysis of variance
APC	adventitial progenitor cells
Ang1	angiopoetin1
αSMA	α smooth muscle actin
CABG	coronary artery bypass graft surgery
CHI3L1	Chitinase-3-like protein
CREB	cyclic AMP response element-binding protein
CREBBP	CREB binding protein
CV	coefficient of variation
EGM	endothelial growth media
EGR	early growth response gene
EPAS1	Endothelial PAS domain-containing protein 1
FBS	fetal bovine serum
FLT-1	VEGF Receptor 1
HLA	human leukocyte antigen
HUVEC	human umbilical vein endothelial cells
LI	limb ischemia
LIN	lineage
MAZ	MYC-associated zinc finger protein
miR	microRNA
NC	no evidence of cardiovascular disease
NRP2	Neuropillin-2
NOTCH4	Neurogenic locus notch homolog 4
NRF2	Nuclear respiratory factor 2
PBS	phosphate buffered saline
PTGS2/COX2	Prostaglandin-endoperoxide synthase 2/cyclooxygenase 2
PGF	Placental growth factor
PROK2	Prokineticin-2
RUNX1	Runt-related transcription factor 1 (RUNX1)
STRING	Search Tool for the Retrieval of Interacting Genes
SV	saphenous veins
TRANSFAC	TRANScription FACtor
VEGF	vascular endothelial growth factor

INTRODUCTION

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, like 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, αSMA), hematopoietic (CD45) and endothelial markers (CD31 and CD146). In addition, the expression of trans-membrane glycoproteins, like CD133, CD34 and CD105, has been employed for *in situ* identification and immunomagnetic isolation of APCs from foetal and adult vessels.[6-8] The expression of CD133/CD34 is reportedly lost during early culture of founder cells, whereas pericyte/MSC 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 anatomical and functional indexes of recovery in models of peripheral and myocardial ischemia.[6, 9] In the latter model, the therapeutic activity was similar in immune-competent and immune-deficient recipient mice, suggesting 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 (miR-132) is constitutively expressed and secreted by SV-APCs and remarkably upregulated, together with its transcriptional activator cyclic AMP response element-binding protein (CREB) upon exposure to hypoxia/starvation. Secreted miR-132 acts as a paracrine activator of healing by stimulating angiogenesis and reducing interstitial fibrosis.[9]

Based on these promising results, we are now proposing SV-APCs for treatment of patients with myocardial or peripheral ischemia. We envisage using autologous SV-APCs as the safest and directly amenable therapeutic option. However, inter-individual 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 if 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. To determine if a cardiovascular impacted background will influence the cell regenerative capacity, we sourced SV-APCs from two 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 to the varied outcome of reparative processes *in vivo*.

METHODS

Expanded methods are provided as online supplement.

Ethics

Studies using human cells complied with the principles stated in the "Declaration of Helsinki" and were covered by Research Ethics Committee approval (06/Q2001/197). Patients gave written informed consent to be recruited in the study. Clinical characteristics of all patients used in this study are reported in **Supplementary Table 1**. Experiments involving live animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (the Institute of Laboratory Animal Resources, 1996) and with approval of the British Home Office and the University of Bristol.

Cell culture and in *vitro* analyses

Cell culture: SV-APCs were isolated and continuously cultured on fibronectin/gelatin in EGM-2 with 2%FBS (Lonza) as previously described. [6, 9]

Antigenic profile: SV-APCs were stained for flow cytometry analyses using anti-CD44 (ebioscience), anti-CD-105 (Life Technologies), anti-CD90 and anti-CD34 (both BD Biosciences) and analyzed using a FACS Canto II flow cytometer and FACS Diva software (both BD Biosciences). Immunocytochemistry included NG2 (1:100, Millipore), PDGFRβ (1:200, Santa Cruz), desmin (1:40, Sigma-Aldrich), vimentin (1:100, ebioscience) and α-SMA (1:400, Sigma-Aldrich). Images and quantification was performed by InCell Analyser (GE Healthcare).

In vitro assays: Cell proliferation (BrdU incorporation, Roche Technologies), apoptosis (caspase 3/7, Promega), viability (MTS assay, Promega), senescence (β-galactosidase, Calbiochem) were performed according to manufacturer's instructions. Migration (scratch assay) was measured as described[6] on gelatin/fibronectin coated multi-well plates and manual scratch distance was measured after 24 hours. Percentage of gap closure (%GAP) was calculated as following: $\%GAP=100-(100*D^1/D^0)$. Formation of tubes by SV-APCs alone or in association with human umbilical vein endothelial cells (HUVECs, Lonza) was assessed using a matrigel assay (BD Biosciences).

siRNA silencing: siRNA targeting *FLT-1* was transfected in SV-APCs. FLT-1 knock-down was confirmed using western blot and qPCR analysis. Soluble FLT-1 (sFLT-1) levels in SV-APC conditioned media were analysed by ELISA (R and D Systems).

Genomic screening

Gene array: Extracted total RNA was reverse transcribed and labelled by *in vitro* transcription. Labelled cRNA was hybridised onto 4x44k Agilent whole human genome microarrays overnight, washed and scanned, and the obtained 16bit tiff images were analysed using Agilent Feature Extraction software. All steps were performed according to manufacturer's instructions (Agilent Technologies).

Validation of gene expression by RT-PCR: RNA was reverse-transcribed using a High Capacity RNA-to-cDNA Kit (Life Technologies) or using specific primers provided with the Taqman miRNA assay and microRNA Reverse Transcription Kit (Life Technologies), followed by amplification of cDNA using QuantiTect primers (Qiagen) or Taqman probes (Life Technologies) for *UBC (Hs00824723_m1), U6 (001973), NRP1 (Hs00826128_m1), NRP2 (Hs00187290_m1) VEGFc (Hs00153458_m1), ANGPT1 (Hs00375822_m1), PLG (Hs00264877_m1), ENG (Hs00923996_m1)* and *FLT-1 (HS01052961_m1), C-KIT (QT00080409), SOX2 (QT00237601), KLF-4 (QT00061033), VEGFa (QT01010184), hsa-miR132 (MS00003458), hsa-miR-125b (MS00006629), Chitinase 3 like-1 protein (Hs00609691_m1), RUNX1 (Hs01021971_m1) and PTGS1 (Hs00153133_m1), FGF1 (Hs00265254_m1), FGF2 (Hs00266645_m1) and PGF (Hs00182176_m1)*. Primers for SODs gene and catalase have been previously described.[14] Quantitative PCR was performed on a LightCycler480 Real-Time PCR system (Roche Technologies). The mRNA expression level was determined using the $2^{-\Delta C}{}_t$ method. Each reaction was performed in triplicate.

Methylation arrays: Illumina 450k methylation arrays were used to analyze the methylome at the single CpG level. The "methylumi" R package was utilized to extract the beta-values (i.e. percentage of methylationper CpG) as reported at http://watson.nci.nih.gov/bioc_mirror/packages/2.13/bioc/manuals/methylumi/man/methylumi.pdf.

In vivo experiments

Unilateral limb ischemia was performed by ligation and electrical cauterization of the left femoral artery. This was followed by injection of SV-APCs ($8x10^4$ cells in 30μL, P7) or phosphate buffered saline (PBS, vehicle) 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 immediately, and followed at 7, 14, 21, 28 days after ischemia. Adductors were excised (n=5 mice per group) and whole mount preparation of the muscles was performed by fixing them in PFA 2% and stored in OCT until processing. Sections were stained with directly conjugated antibodies against Isolectin B4 (1:100, Life Technologies) and α-SMA (Roche Technologies). Fibrosis was determined using Azan Mallory staining.[15]

In order to investigate the time course of cell retention after transplantation, SV-APCs ($2x10^5$) were injected into ischemic muscles as indicated above and retrieved at 2 or 7 days from induction of ischemia. Dispase II/collagenase A (both from Roche Technologies) digested muscles were mouse lineage depleted (Miltenyi Biotec), and FACS sorted using CD105 (Life Technologies), CD44 (eBioscience) and HLA-ABC (W6/32, Biolegend). The number of cells retrieved from ischemic muscles was corrected by the efficiency of the extraction procedure, which is calculated by taking into account the number of injected cells and the number of cells retrieved from muscles immediately after injection (time 0).

Statistical Analysis

Comparison of multiple groups was performed by analysis of variance (ANOVA) and Dunnet's or Bonferroni post-test multiple comparison tests as appropriate. Two-group analysis was performed by Student's t-test. Values were expressed as means± standard error of the mean. Probability values (p) less than 0.05 were considered significant. Correlation between variables and microvascular endpoints (capillary and arteriole density) was calculated by regression analysis. The time-weighted average of blood flow recovery was calculated for each animal and used as normalization measure of BF over multiple time points. This value takes into account not only the numerical levels of the variable but also the amount of time where the numerical variable was maintained, i.e. the overall pattern, while controlling variability from repeated measures. The use of a single normalized measure of BF allows determining correlations with predictor variables without the need of multiple measures adjustments and with sample sizes compatible with the 3R requirement.

Processing and statistical analysis of gene arrays was done in R/Bioconductor[16] and gene set enrichment was performed using ClueGO [17] and GATHER.[18] To verify the correlation between methylation probes and the respective blood flow or angiogenesis outcome values, we used the Generalized Estimation Equation Solver package in R (GEE [Carey, V.J., Lumley, T., and Ripley, B.D. (2012), gee: Generalized Estimation Equation Solver, URL http://CRAN.R-project.org/package=gee, R package version 4.13-18.]). Methylation probes with p <0.01 were first selected. Only those gene promoter regions and gene bodies with at least 3 CpGs correlating with outcome, independently on the direction of the correlation, were considered. Promoter regions were identified as within the 1500bp preceding the gene body.

RESULTS

Maintenance of typical antigenic profile during expansion

Under the current standard operating protocol, we report successful cell expansion in 22 out of 35 SV specimens (68%), resulting in 30-50 million viable SV-APCs in each preparation at P8 in ~10 weeks. We found that the success of expansion is strictly dependent upon the amount of vein tissue used for cell extraction. With tissue weight above 1.6 grams, 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-SMA was not detected or detected in low quantity (**Figure 1A**). NG2 and PDGFRβ expression 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% (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 behaviour 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 2 A-C**, 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-C**). SV-APCs from both CABG and NC similarly improved the network size by increasing average branch length as well as average branch thickness compared to 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) as 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 limb ischemia (n=7 mice per each cell therapy 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 A, B, and C and are negative for class II human leukocyte antigen HLA-DR, CD80 and Fas

ligand as assessed by RT-PCR (Madeddu, unpublished data). Furthermore, using a flow cytometry-based cytotoxicity method, we found that co-incubation of SV-APCs with activated murine lymphocytes (1:6 ratio) induces low levels of SV-APC death as compared with cell death observed after co-incubation of human endothelial cells and murine lymphocytes (2.12 vs. 7.12% respectively, $P<0.05$).

Blood flow analysis over 28 days post-ischemia indicates an overall enhancement of reperfusion in SV-APC-treated limbs compared to vehicle-injected ones (p=0.007) (**Figure 4A-C and Supplementary Figure I**). In addition, a similar improvement was observed when examining the blood flow recovery at the level of the foot plantar region (p=0.003 vs. vehicle). This effect was equivalent to that observed in a previous study in immunodeficient mice with limb ischemia.[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 vs. 25 days in vehicle-injected mice), the percent increase of blood flow from the induction of ischemia to final measurement at 28 days post-ischemia (from 535±119 to 828±167% - average, 616±73% - in SV-APC-treated mice vs. 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 vs. 0.32±0.05 Doppler Units in vehicle) (**Figure 4C**). Interestingly, intra-group variability was less in groups receiving SV-APC therapy (time-weighted blood flow recovery: mean 26 CV, 33%; range, 14-32%) as compared to 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 day 28 days post-injection indicates the benefit of SV-APCs on reparative neovascularization (**Figure 4E-G**). 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%) vs. 9% in vehicle; median arteriole density CV, 27% (range 22-38%) vs. 17% in vehicle). In addition, no association was found between the Doppler blood flow data and capillary or arteriole density in ischemic muscles, possibly due to the fact that perfusion was measured sequentially during recovery from ischemia, while vascular profiles were assessed at sacrifice. 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 endpoint of the *in vivo* study, was determined by flow cytometry analysis of human-specific antigen HLA-ABC and the two 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 **Supplementary Figure II**), single cell 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 very low background signal (**Figure 5B** and **Supplementary Figure II**). 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 post-injection, 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 post-injection (**Figure 5D**). There was no association between the number of SV-APCs in ischemic muscles at 2 days post-injection and primary endpoints (blood flow recovery and microvascular density).

Correlation between clinical and functional data and primary outcome endpoints

Having shown that the benefit elicited by SV-APCs in the limb ischemia model varies among different cell lines, we next asked if clinical data of the donor subjects and functional *in vitro* properties of the injected cells can anticipate the primary outcome endpoints. 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) as

compared with cells from non-smokers (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 capillary density outcome ($R^2=0.80$, $p<0.05$, **Supplementary Figure III**), 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 endpoints

In order 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 limb ischemia. We identified 936 unique genes (106 of these involving promoter regions), whose methylation status is correlated to 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).[19, 20] Moreover, 536 genes associated with blood flow, 2805 genes associated with capillary density and 460 genes associated with arteriole counts are regulated by MAZ (MYC-associated zinc finger protein), which is involved in cell proliferation and mediates VEGF-induced angiogenesis. [21] 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 three outcome endpoints. 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 very significant enrichment of the 6p21 loci (17 out 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 centred on CREBBP (**Supplementary Figure IV**), a nuclear protein that binds to CREB. Furthermore, in the described network, CREBBP appeared to be associated to Runt-related transcription factor 1 (RUNX1), which also locates on chromosome 21 and correlates with outcomes endpoints at both the methylation (**Supplementary Figure V**) and 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 (NOTCH4) and PROK2 also correlate with blood flow recovery and arteriole count (**Supplementary Figure VI**). Analysis of gene interaction revealed a network of 5 genes, comprising NOTCH4, Endothelial PAS domain-containing protein 1 (EPAS1, also known as Hypoxia-inducible factor-2alpha or HIF-2alpha), Neuropillin-2 (NRP2), placental growth factor (PGF) and VEGF Receptor 1 (FLT-1), the latter being the core molecule within the network (**Figure 6**). Altogether, these data newly indicate that complex epigenetic mechanisms may influence the therapeutic activity of the cell product from different donors.

Concerns have been raised that, following isolation and culture expansion, stem cells accumulate stochastic mutations, which may favour malignant transformation. Therefore, we used PubMeth (http://www.pubmeth.org), a cancer methylation database combining text-mining and expert annotation, to investigate if 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 (KEGG) 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 endpoints

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 to outcome endpoints ($p<0.01$). We found 494 genes significantly correlated to blood flow (259 with positive correlation and 235 with negative correlation), 420 genes correlated to capillary density (297 positively and 123 negatively), and 225 genes correlated to arteriole density (115 positively and 110 negatively). The lists of genes are given in **Supplementary Table 2**.

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 amongst genes correlating with outcome endpoints. Using GATHER we identified 38 transcription factors which are significantly enriched amongst those genes correlating with capillary density (**Supplementary Table 3**). The nuclear respiratory factor 2 (NRF2), which regulates genes which contain antioxidant response elements in their promoters, showed the highest *p* value within the transcription factor list. In addition, MAZ, a transcription factor which also emerged from the DNA methylation analysis described above, 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, 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 to 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) (**Supplementary Table 4**).

A similar analysis of the genes correlated to arteriole density show clusters of functions related to positive regulation of transforming growth factor beta 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) (**Supplementary Table 5**). In contrast, gene ontology analysis using genes correlated with blood flow outcome did not yield any significantly enriched and interconnected GO terms.

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 (**Supplementary Table 6**).[9, 14] We found that the expression of the stemness marker KLF4 by SV-APCs correlates with the capillary density outcome, while 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 post-ischemic arteriogenesis, [22] and the arteriole density outcome. Furthermore, we investigated the expression levels of 2 microRNAs, namely miR-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 miR-125b, which plays crucial roles in many different cellular processes, like cell differentiation, proliferation and apoptosis, associated with angiogenesis through inhibition of its target gene MAZ. [21, 23-25] Consistent with an anti-angiogenic action of miR-125, we found this microRNA to be inversely correlated with capillary density and arteriole density outcomes, while no association was found for miR-132 (**Supplementary Table 6**). Finally, we verified the expression of 3 genes emerging from ontology analysis of gene arrays data, namely Prostaglandin-endoperoxide synthase 2/cyclooxygenase 2 (PTGS2/COX2), the enzyme that converts arachidonic acid to prostaglandin H2, Chitinase-3-like protein (CHI3L1), also known as cartilage glycoprotein 39 (YKL-40) which is implicated in mural cell mediated angiogenesis,[26] and RUNX1, which has been associated with pro-angiogenic activity of endothelial

progenitor cells.[27] In a previous study, we reported that CHI3L1 and RUNX1 are abundantly expressed in SV-APCs as compared with human endothelial cells.[14] We found a trend for positive correlation between the expression levels of CHI3L1 and capillary density, though this did not reach statistical significance. Similarly, the association of PTGS2/COX2 with microvascular density suggested by gene array data was not confirmed by RT-PCR. In line with DNA methylation and gene arrays data, we found that RUNX1 correlates with arteriole density in an inverse manner.

Functional impact of FLT-1 silencing on network formation

As 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 and Supplementary Figure VII**). Silencing was confirmed by qPCR and western blotting analysis (**Figure 7A&B)**. *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**). The addition, SV-APCs increased the average tube length compared to 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 very attractive for regenerative medicine. We have recently identified a population of clonogenic pericytes, alias SV-APCs, in the adventitia of human saphenous vein and documented their ability to induce reparative processes in pre-clinical ischemic models.[6, 9] In view of clinical application, this study investigated the feasibility of SV-APC expansion for production of a consistent therapeutic cell product. We also tested if 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 saphenous vein give rise to large amounts of therapeutic cells. Furthermore, we newly show that multiple interactions at the epigenome and transcriptome level may contribute to the variability of therapeutic outcomes *in vivo* and demonstrated that reduction of these highlighted genes can impact on network formation ability *in vitro.* Silencing studies verified FLT-1 as a core determinant of the pro-angiogenic action of SV-APCs.

Clinical stem cell trials of patients with myocardial or critical limb ischemia indicate that the threshold therapeutic dosage is around 30 million cells.[28, 29] 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, since the success of expansion is dependent on the amount of vein tissue, vein leftovers from CABG 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 of reparative angiogenesis. Furthermore, we confirm that injected cells persist for a few days in the injected ischemic muscle. 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 endpoints. 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 if 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.[30, 31] Methylation also occurs in inter-genic 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. [32, 33] We have investigated the DNA methylation status of different SV-APC lines using both promoter and non-promoter 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 as compared with genes associated with the other two 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 following induction of gene expression upon 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 and/or facilitating the assembly of repressor complexes at the methylated regions. [34] Searching in TRANSFACdatabase 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.[35] The transcription factor has been implicated in VEGF-induced angiogenesis,[21, 36, 37] this effect being negatively controlled by miR-125b, of which MAZ is an inhibitory target.[21] Of note, we found that the expression of miR-125b in SV-APCs is inversely correlated with their ability to induce reparative vascularization in the mouse limb ischemia 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 [38 39] and stimulates microvascular endothelial cell growth and neovascularization through FGF-mediated mechanisms.[40] We found that 52% of genes whose methylation status correlates with all outcome endpoints bear KROX/EGR1 binding sites, suggesting a potent effect of this transcription factor on the genes associated to the therapeutic action of SV-APCs. These shared genes are interconnected in a network centred on CREBBP, which plays a role in VEGF- and FGF-dependent angiogenesis signalling and in epigenetic control of cell proliferation,[41, 42] 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 have been proposed.[30] Importantly, we found that the VEGF receptor FLT1, which is under the regulatory control of KROX/EGR1,[43] 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 analysed cell lines, suggesting that those may be safe for patient use.

Results from gene arrays 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 anti-oxidant 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 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 miR-132, and outcome endpoints, 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 PTGS2/COX2 and CH3L1 and capillary density outcome in the gene arrays 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.[26] We previously showed that SV-APCs establish N-Cadherin positive adhesive contacts with proliferating endothelial cells.[6]

In line with the methylation data, FLT1 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 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.[44] Importantly, our study newly shows that a reduction in *FLT-1* transcripts remarkably impacts on SV-APC capacity to form tubes and stabilise HUVEC networks *in vitro*, thus indicating for the first time an important role of FLT-1 in SV-APC pro-angiogenic 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
Noting to be disclosed

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, 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 provide 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.

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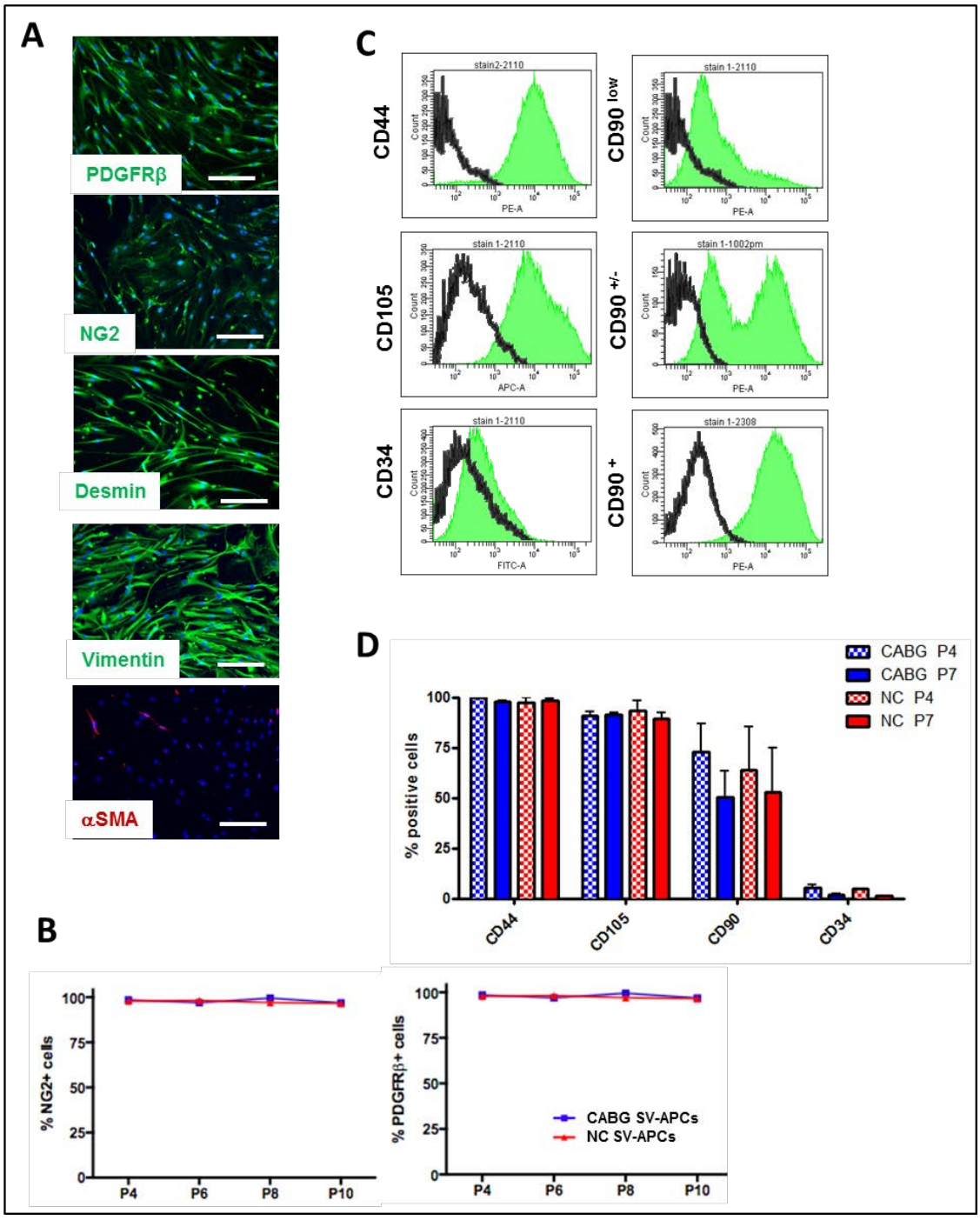


Figure 1: Phenotypic analysis of the patient's SV-APCs. A) Immunocytochemistry of cultured SV-APCs (P4, n=15) reveals conserved presence of PDGFRβ, NG2, desmin and vimentin and low incidence/absence of α-SMA, scale bar 200μm. **B**) Conservation of pericyte features was seen by quantification of NG2 and PDGRRβ expression by InCell Analyzer (n=5 CABG SV-APCs, n=3 NC SV-APCs). **C&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 $CD90^{dim}$ and $CD90^{bright}$. 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.

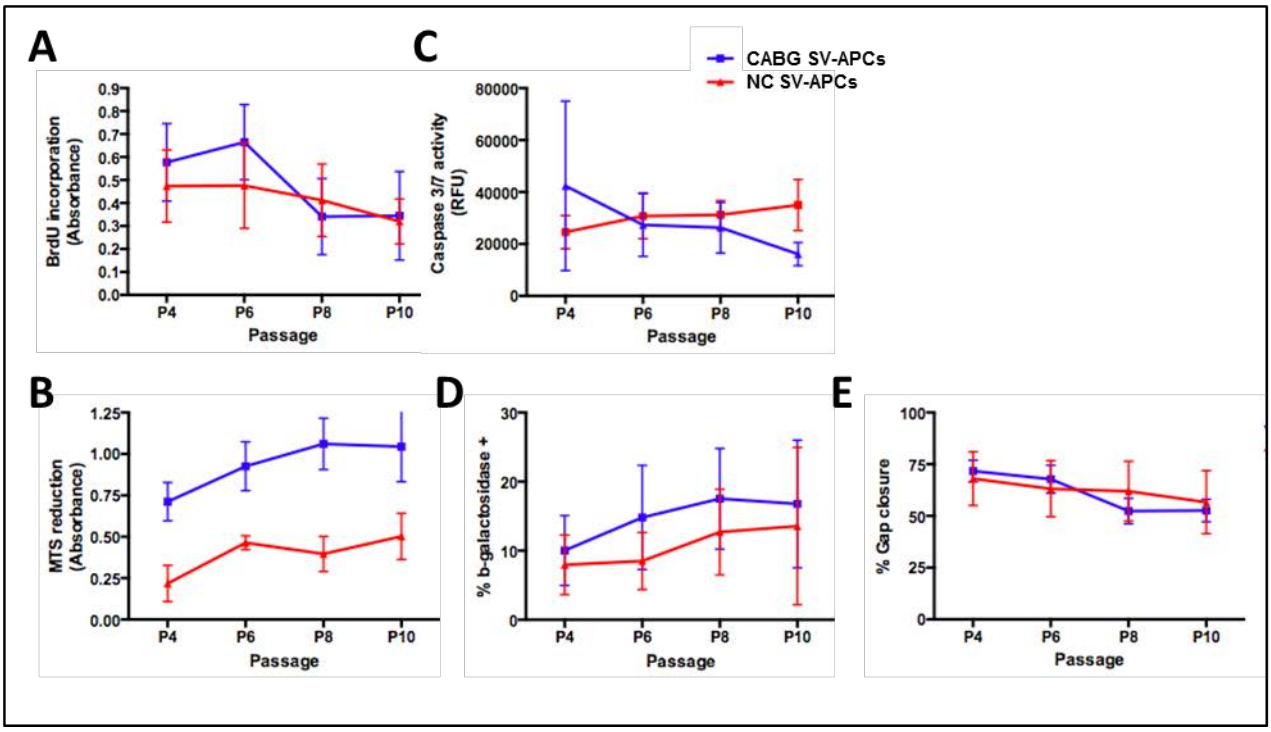


Figure 2: *In vitro* assessments during SV-APC expansion. In order to allow for a rolling process of different cell populations analysis, each SV-APC population was individually assessed at 4 time-points in the same experimental sitting and repeated in triplicate. Multiple *in vitro* assays were chosen to provide an insight into the differences of expansion capacity and quality of cell product. Data shown here includes analysis of: **A**) proliferation activity seen by BrdU incorporation, **B**) viability/metabolic activity seen through MTS assay, **C&D**) 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 CABG, n=5 for NC in all assays except for scratch assay which was performed in n=7 CABG and 4 NC. Data shown as mean and standard error.

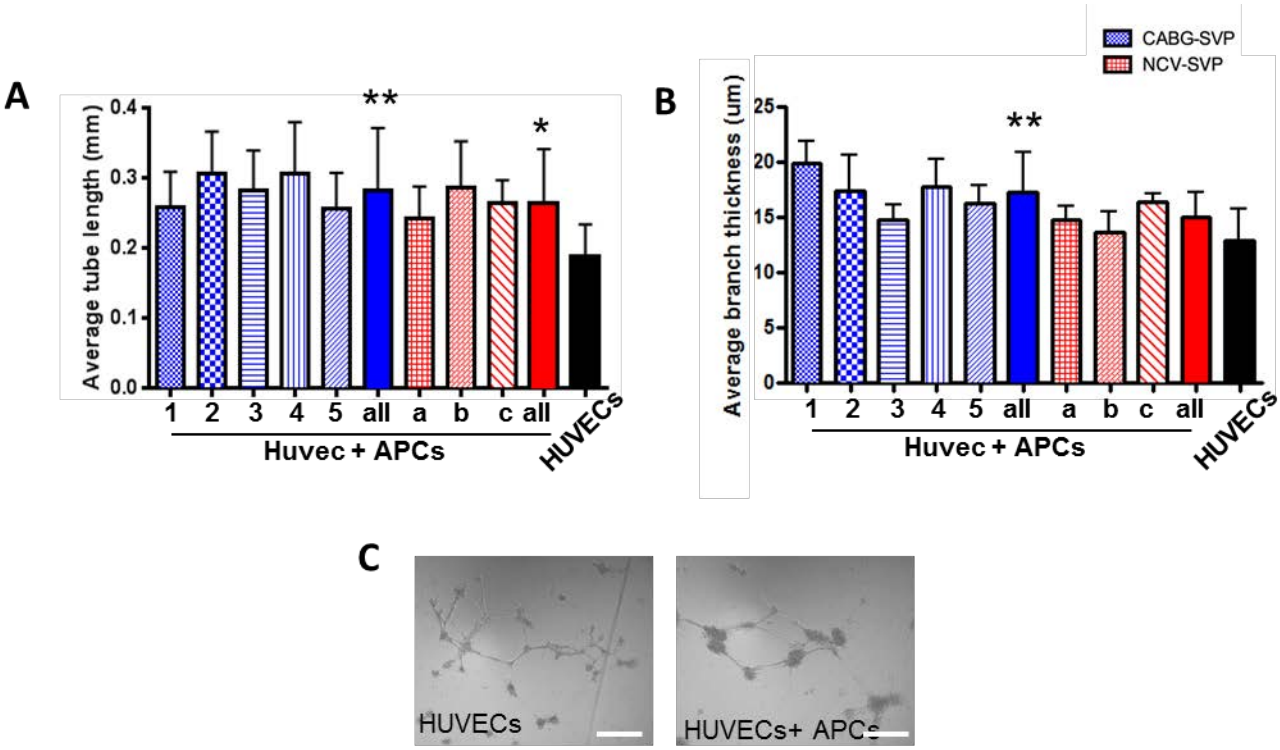


Figure 3: Network supporting capacity. Pro-angiogenic activity on Matrigel revealed consistently increased average length (**A**) and thickness of network (**B**). Variability existed among the cell populations, but overall no difference was observed between cells from CABG (indicated by progressive numbers) or NC subjects (indicated by letters). Hence, SV-APCs induced a network remodelling with stronger branches as can be seen by representative images of 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.

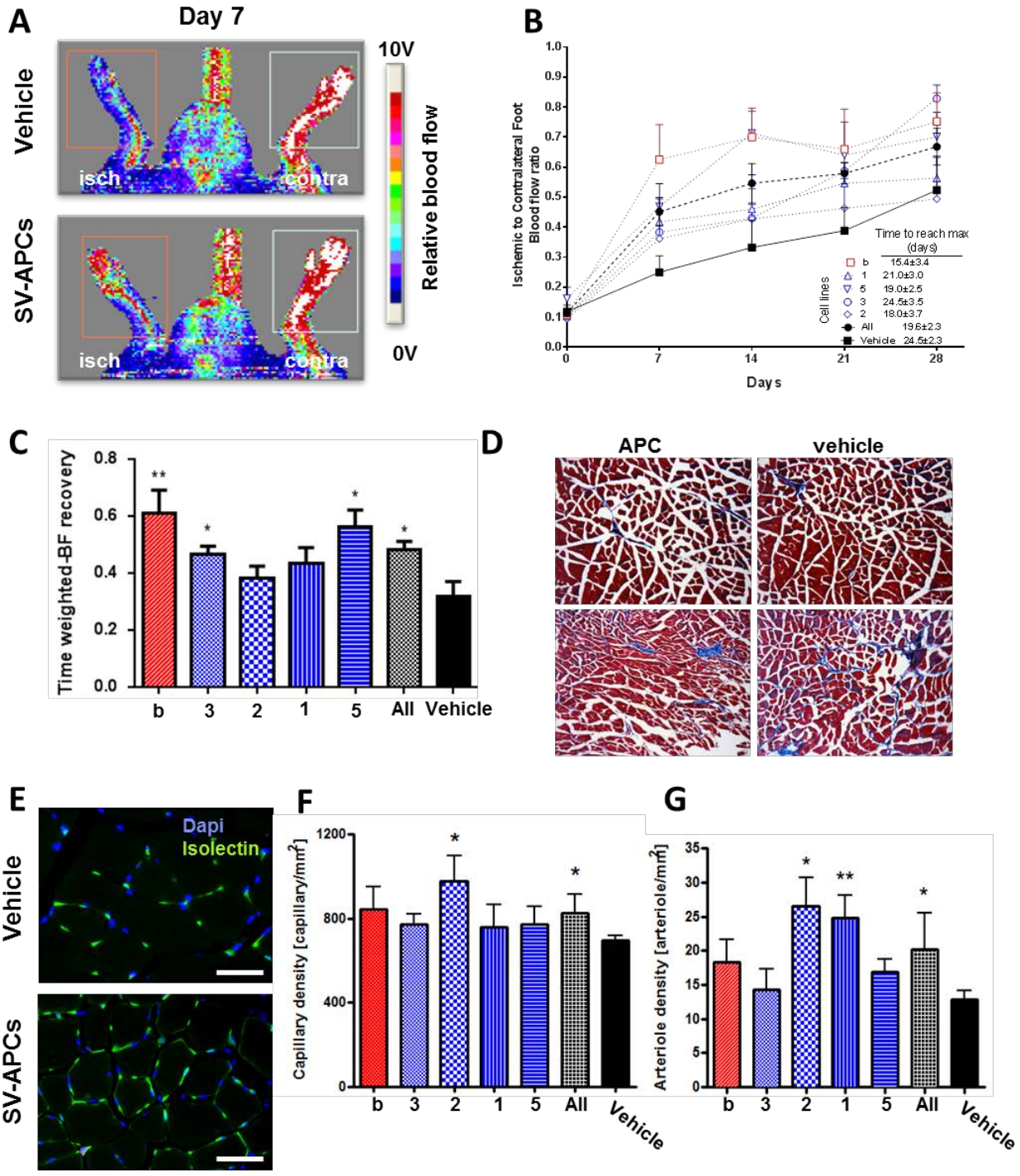


Figure 4: Therapeutic effect in a mouse model of limb ischemia. **A-C**) Intramuscular injection of different SV-APC populations (4 representative of CABG donors -blue- and 1 control from saphenectomy -red- n=7 per group assessed) in immunocompetent mice subjected to unilateral limb ischemia promotes acceleration and time-weighted enhancement of blood flow 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-to-contralateral foot blood flow ratio of mice grouped according they received cell therapy or vehicle (n=7 in each group assessed). (**C**) Bar graph representation of time-weighted blood flow recovery. **D**) Azan Mallory staining showing reduced muscle fibrosis in SV-APC-injected muscles as compared with vehicle. **E-G**) Immunohistological analysis showing the capillary and arteriole density by representative images (**E**, 50um scale bar) and mean values in SV-APC (each cell line and all cell lines averaged) and vehicle injected groups (**F&G**) (n=5 mice per group). Data are means and standard error..*p<0.05 and **p<0.01 vs. vehicle.

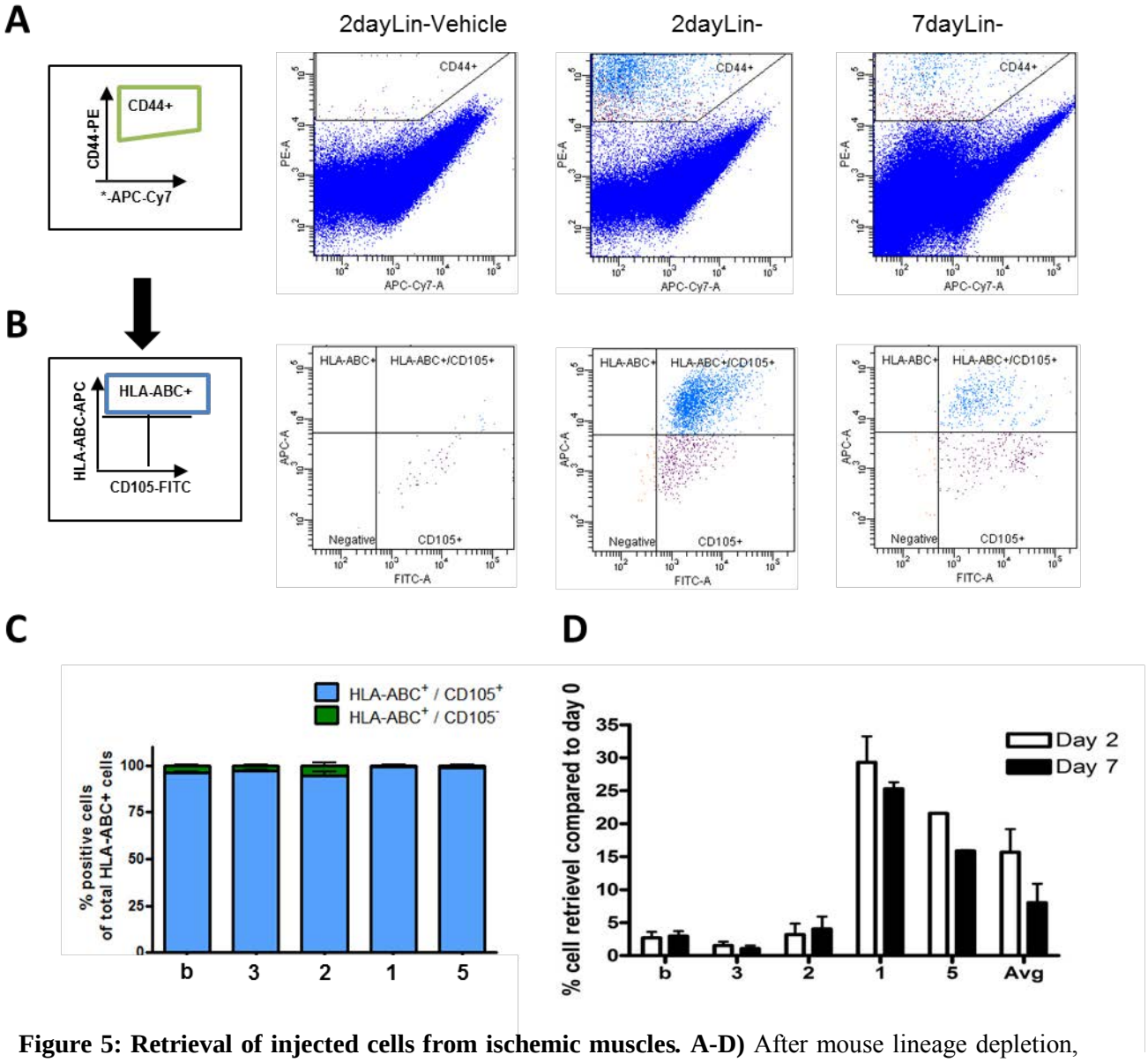


Figure 5: Retrieval of injected cells from ischemic muscles. A-D) After mouse lineage depletion, living cells (APC-Cy7 negative) were selected from cells of the ischemic adductor muscle according to CD44+ positivity. Gated cells were further selected for HLA-ABC and assessed for conservation of CD105 phenotype. N=3 mice for each of 6 groups (5 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 post-injection (**A&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 to day 0 expressed as means and standard error.

A

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

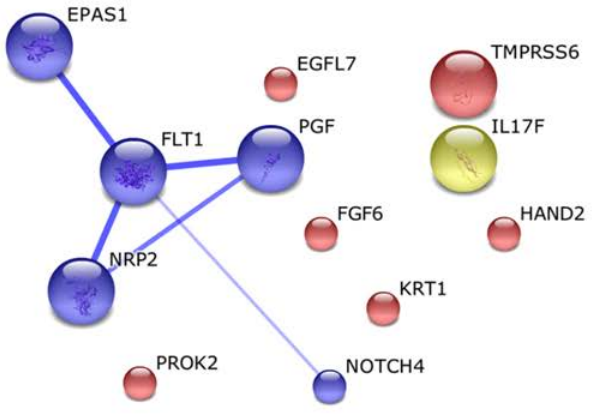
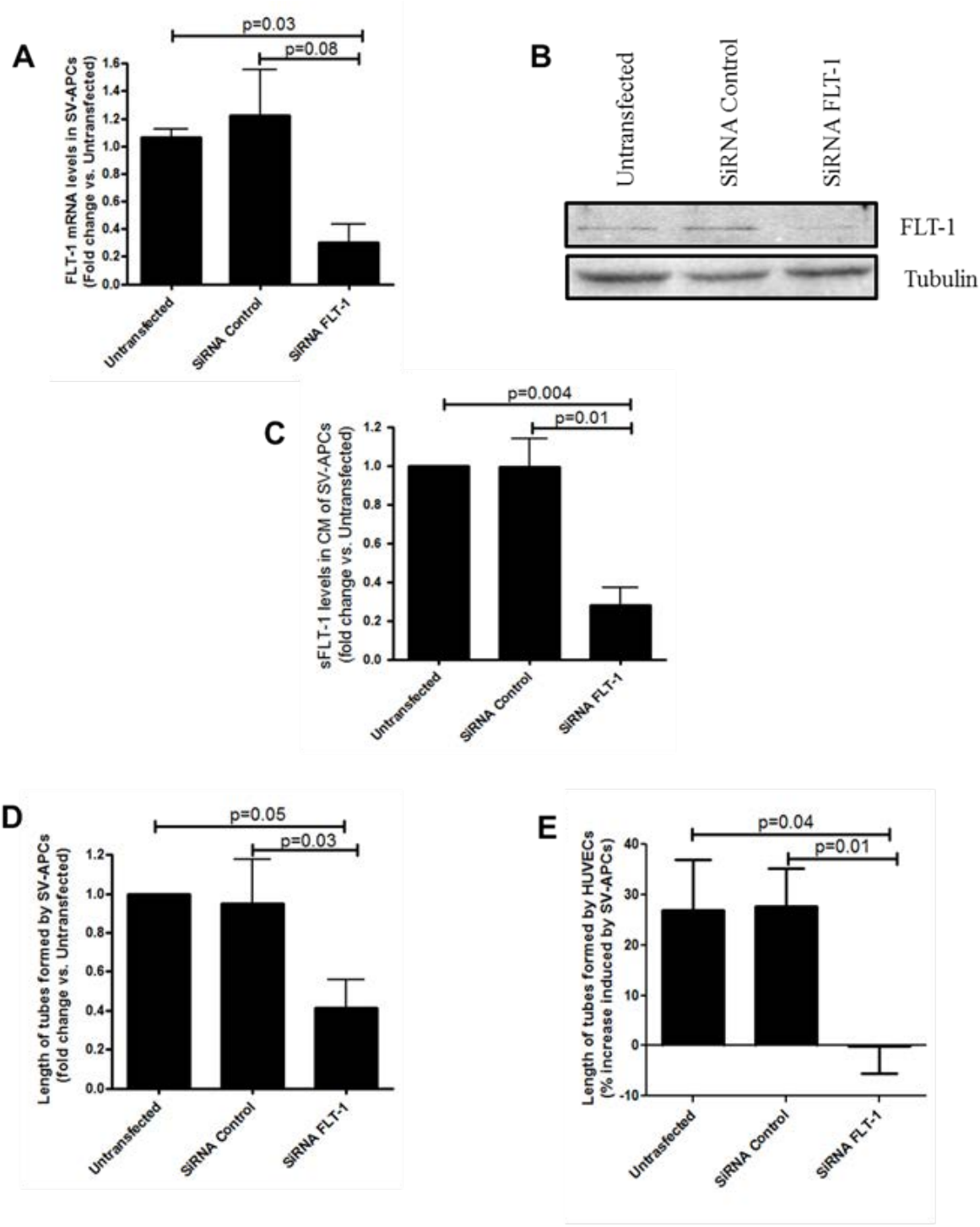


Figure 6: Association of the methylation status of genes involved in regulation of angiogenesis with microvascular endpoint. (A) The methylation of 12 genes correlates with capillary counts, two of which (NOTCH4 and PROK2) also correlate with blood flow and arteriole density outcomes**. (B)** Gene interaction network analysis of the 12 angiogenesis-related genes revealed FLT1 as the core molecule within the network.



Supplementary Figure VI: Inhibition of tube formation by silencing of the *FLT-1* gene in SV-APCs. (**A**) qPCR confirmation of reduction in *FLT-1* transcripts following 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 blot analysis of FLT-1 protein in SV-APCs. Densitometry using BioRad Image lab 5.1 software confirmed reduction in FLT-1 protein levels (untransfected 602628, control siRNA 657956, *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 HUVECs in a Matrigel assay following coculture with SV-APCs (n=4).