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Endothelial cells expressing low levels of CD143 (ACE) exhibit enhanced sprouting and potency in relieving tissue ischemia

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Abstract The sprouting of endothelial cells from pre-existing blood vessels represents a critical event in the angiogenesis cascade. However, only a fraction of cultured or transplanted endothelial cells form new vessels. Moreover, it is unclear whether this results from a stochastic process or instead relates to certain endothelial cells having a greater angiogenic potential. This study investigated whether there exists a sub-population of cultured endothelial cells with enhanced angiogenic potency in vitro and in vivo. First, endothelial cells that participated in sprouting, and non-sprouting cells, were separately isolated from a 3D fibrin gel sprouting assay. Interestingly, the sprouting cells, when placed back into the same assay, displayed a sevenfold increase in the number of sprouts, as compared to control cells. Angiotensin-converting enzyme (CD143) was significantly down regulated on sprouting cells, as compared to regular endothelial cells. A subset of endothelial cells with low CD143 expression was then prospectively isolated from an endothelial cell culture. Finally, these cells were found to have greater potency in alleviating local ischemia, and restoring regional blood perfusion when transplanted into ischemic hindlimbs, as compared to unsorted endothelial cells. In summary, this study indicates that low expression of CD143 can be used as a biomarker to identify an endothelial cell sub-population that is more capable to drive neovascularization.

Keywords Therapeutic angiogenesis · 3D sprouting assay · Cell transplantation · Alginate

Introduction

Angiogenesis describes the sprouting and stabilization of new blood vessels from pre-existing vessels [1]. This process involves a cascade of events, including endothelial cell activation, migration and proliferation, followed by interactions with mural cells to stabilize the initially immature new vasculature. Endothelial cell sprouting occurs in a direct response to spatially and temporally graded microenvironmental cues, including oxygen deprivation [2], soluble growth factor gradients [3] and insoluble matrix signals [4]. Sprouting cells include both "tip cells" and "stalk cells" (or "trunk cells") [5]. Endothelial tip cells are the leading cells of a sprout, are highly polarized, migratory, and minimally proliferative, and display numerous extended filopodia [6]. Endothelial stalk cells follow the tip cells, and are characterized by fewer filopodia, higher proliferative capacity and lumen formation and coordination [7]. Although there exists plasticity and reversibility between these phenotypes during sprouting [8], very little is known about whether cells that participate in formation of new sprouts, as compared to those that do

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not, were previously committed to a more angiogenic phenotype, or if this is a stochastic process.

Endothelial cell sprouting has been studied both in vitro and in vivo [9]. Distinct in vitro methods have been used to study sprouting and tube formation, including the 2D Matrigel tube formation [10], 3D collagen gels [11, 12], 3D fibrin gels [13] and 3D droplet assay [14]. These assays have been mainly used to probe the endothelial cell functional response to angiogenic stimulators, inhibitors or regulators [15, 16]. The quantification typically includes number of sprouts or capillary-like tubes formed and length of sprouts. From these in vitro studies, it is possible to estimate that only $\sim$9 % of the cells participate in sprout formation [13]. However, no studies have yet specifically investigated the key characteristics and mechanisms that distinguish sprouting cells from non-sprouting cells. Endothelial cell transplantation studies have also been an important tool to study the in vivo participation of exogenous endothelial cells in new sprout formation. These in vivo studies typically involve either simple cell infusions [17, 18] or the use of a material carrier [15, 19]. Although the transplantation of endothelial cells demonstrates significant therapeutic benefit in animals models, only a very small fraction of these cells participate in the creation of functional vessels [20], and it is again unclear what distinguishes those cells that do and do not participate in the formation of new vessels networks.

In this study, we investigate whether the cells that participate in sprouting have distinct angiogenic capacity, as compared to non-sprouting endothelial cells. Primary human microvascular endothelial cells (HMVEC) were utilized in this study as in vivo angiogenesis typically occurs at the microvasculature level [9, 21]. To first separate cells that participated in sprouting and non-sprouting cells, a method was developed to isolate sprouting endothelial cells in the 3D in vitro sprouting assay. The angiogenic capacity of the sprouting cells was then analyzed by placing these cells back into the in vitro sprouting assay, and their expression of angiogenic genes was also analyzed. Finally, endothelial cells expressing low levels of CD143, a cell surface marker found to be expressed at low levels in sprouting cells, were prospectively isolated and transplanted into ischemic hindlimbs of rodents, to test their in vivo potency in orchestrating neovascularization.

Materials and methods

Endothelial cell culture

Human dermal microvascular endothelial cells (HMVEC-d, Lonza) between passages 3 and 8 were used in all experiments. These cells are a purified homogenous population obtained from blood vessels from skin tissue. For all the experiments in this study, the cells used were from a single donor (not pooled) and were not obtained from a mixed population of lymphatic and blood vessels. Cells were cultured in EGM-2MV media (Lonza) and typically cultured in tissue culture flasks (75 cm^2, BD Bioscience) in a humidified 5 % CO_2 atmosphere, and the cell media was changed every day. Cells were passaged upon reaching 70–80 % confluence using 0.05 % trypsin–EDTA (Invitrogen–Gibco).

Proliferation assay

Endothelial cell proliferation was quantified by determining the cell number under each test condition. 5×10^4 cells in 2 mL of EGM-2MV media were seeded into 6-well plates (BD Biosciences) overnight. The wells were washed twice with Dulbecco's phosphate-buffered saline (PBS) (Ca^{2+}, Mg^{2+}-free, Gibco) and cultured for 3 days using 2.5 mL of "no growth factor media" or "no growth factor media" supplemented only with 50 ng/mL of human recombinant $VEGF_{165}$ (R and D Systems). The "no growth factor media" was made of basal EGM-2MV media supplemented with fetal bovine serum (FBS) (5 %), ascorbic acid, hydrocortisone and GA-1000 (gentamicin, amphotericin B) (as supplied by Lonza BulletKit). After the three-day period, cells in each well were detached using trypsin and counted with a Z2 particle Coulter Particle Counter (Beckman Corp.).

Sprouting assay

Cytodex 3 microcarriers (Amersham Biosciences) were hydrated in PBS for three hours (0.2 mL/mg of dry microcarriers). The PBS supernatant was decanted and replaced by fresh PBS (0.1 mL/mg), and microcarriers were sterilized by autoclave. HMVEC-d passage 4 through passage 6 were suspended in EGM-2MV media, mixed with 50 mg of sterilized microcarriers at a 9:1 (cell/microcarrier) ratio and placed in a 50-mL spinner flask (Bellco Glass Inc.) with a 3.57:1 (mg of microcarriers/mL of EGM-2MV) ratio. After a 3-h stirring cycle (28 min stirring, 2 min stagnant) at 60 rpm, additional EGM-2MV media was added to make a 2:1 (mg of microcarriers/mL of EGM-2MV) ratio. The microcarriers with cells remained continuously stirring for 21 more hours. After this time elapsed, the microcarriers were transferred to a tissue culture flask (25 cm^2) and cultured for 2–3 days, until the beads were completely covered with cells.

For the sprouting assay, the following percentages by volume were used to formulate the fibrin gel: (Solution 1) Fibrinogen solution (4 mg/mL, Sigma) 68.2 %, aprotinin (500 μg/mL, Sigma) 9.1 % and microcarriers with cells solution 22.7 %. (Solution 2) Thrombin (22.72 units/mL,

Sigma) 8.3 % and PBS (Gibco) 91.7 %. Solution 1 (0.25 mL) was mixed in a 24-well plate with 0.20 mL of solution 2. The gel solution was placed in an incubator for 30 min to allow for gel coagulation. Cultures were fed daily with 0.8 mL of fresh EGM-2MV media for 5 days. Following the five-day period, gels were washed twice with PBS and fixed in 4 % formaldehyde overnight in 4 °C. The formaldehyde solution was then aspirated, and gels washed twice with PBS. The fibrin gels were visualized under an Olympus IX2-UCB microscope at 10× magnification. Sprouting was defined as branching structures originating from the microcarriers that contained multiple, connected endothelial cells [22, 23]. For each well, the total number of sprouts was divided by the total number of beads and the averages of each condition were calculated. For the re-challenge sprouting assays, both sprouting and non-sprouting cells were incubated with microcarriers (as described above). Sprouting cells adhered to the beads before sprouting and were then combined with fibrin gels. Non-sprouting cells were incapable of adhering to microcarriers and so could not be used in these studies (experiment tried five times, with all exhibiting poor adhesion of non-sprouting cells to microcarriers).

Isolation of sprouting and non-sprouting cells

The fibrin gel containing the microcarriers and sprouts was washed twice with PBS, and mechanically cut up into pieces that were 2–3 mm in diameter. The fibrin gel sections were then exposed to 187.5 μg/mL solution of plasmin (Sigma) and placed on an orbital shaker for 30 min at 37 °C. EGM-2MV media was subsequently added to quench the plasmin solution. After the microcarriers had settled to the bottom, the supernatant was centrifuged at 700 rpm for 6 min. The supernatant was decanted, and the pellet of "sprouting cells" was suspended in EGM-2MV media, transferred to a cell culture flask and grown until confluence. The remaining microcarriers were incubated in PBS for 5 min. The microcarriers were allowed to settle down and the supernatant was decanted. The remaining endothelial cells were removed from the microcarriers via trypsinization on an orbital shaker at 37 °C for 15 min. EGM-2MV was used to quench the microcarriers, and the solution was briefly agitated to remove cells that were not adherent to the microcarriers. The microcarriers were then allowed to settle to the bottom while the supernatant containing the "non-sprouting cells" was collected and added to a cell culture flask in order to grow until confluence.

Control for sprouts

Endothelial cells (HMVEC-d—P5) were cultured in a tissue culture flask (75 cm^2) until confluence and then seeded into a fibrin gel. EGM-2MV media (0.8 mL) was added on top of the fibrin gel. After the second day, the fibrin gel was degraded using the technique mentioned earlier and the control cells were placed in a cell culture flask in order to grow until confluence.

RNA isolation and analysis

RNA was isolated from 2,285,000 endothelial cells for each condition, including sprouting cells and regular HMVEC-d at the same passage number. Cell cultures were detached using trypsin and washed using PBS. RNeasy (Qiagen, Valencia, CA), RNAse-free DNase (Qiagen) and QIAshredder homogenizer (Qiagen) kits were used to isolate RNA according to the manufacturer's protocol.

Reverse transcription reactions were carried out using 3 μg of RNA from endothelial cells (HMVEC-d—P5) in 20 μl volumes with the RT^2 PCR Array First Strand Kit (SuperArray) and the corresponding manufacturer's protocol.

The cDNA was analyzed using the RT^2 PCR Array (SuperArray), which examines 96 genes related to human endothelial cell biology. Preparation for real-time PCR was done according to the manufacturer's protocol, and readings were taken on the MJ Opticon 2 (Bio-Rad). Data analysis was done using SuperArray's Excel-based PCR Array template to compare gene expression levels among genes.

Quantification of ACE/CD143 activity via ELISA

Control endothelial cells (HMVEC-d), sprouting cells and non-sprouting cells were seeded on 6-well plates (5,000 cells/cm^2) and cultured with EGM-2MV for 3 days. The EGM-2MV was removed on day 3 and concentrated tenfold before stored at −20 °C. Cell counting and viability for each well was assessed using Countess automated cell counter (Life Technologies). Quantitative determination of human ACE/CD143 concentrations present in the conditioned medium was performed following the protocol of the Human ACE Quantikine ELISA Kit (R&D Systems).

Immunocytochemistry of CD143 on sprouting assay

A sprouting assay was performed for 5 days as described previously. After overnight fixation, fibrin gels were blocked for non-specific staining with 10 % normal donkey serum (Sigma), incubated overnight (4 °C) with unconjugated primary antibodies against CD143 (R&D Systems) and then incubated for 1 h with the secondary antibody (Northern-Lights 493 Fluorochrome-labeled) (R&D Systems). Hoechst

33342 counterstain was also used to localize cell nuclei. An Axio Vert.A1 microscope (Zeiss) was used to visualize different fields at 100× and 400× and connected to an AxioCam ICm1 digital image capture system (Zeiss).

Quantification of telomerase activity

Endothelial cells (HMVEC-d) between passage 7 and passage 8 were cultured in EGM-2MV until confluence and then detached using trypsin. One million cells were washed once with PBS, lysed and incorporated into a real-time PCR premix. Telomerase activity tests were done according to the Quantitative Telomerase Detection Kit (US Biomax Inc.), and the MJ Opticon 2 was used to read samples.

Antibody array

Endothelial cells (HMVEC-d) between passage 7 and passage 9 were cultured in a 6-well plate using EGM-2MV media without growth factors and changing the media every day for 3 days. On the third day, 2 mL of the culture media was collected and protein secretions of the cells were analyzed using the TranSignal^{TM} Angiogenesis Antibody Array (Panomics). The array membrane was exposed to Hyperfilm^{TM} ECL for 1 min. Signal intensities were compared from the developed array image.

Flow cytometry

Endothelial cells (HMVEC-d—P5) were detached from plates using Hank's balanced salt solution (HBSS, Gibco) with 5 mM EDTA (Sigma) and 25 mM of HEPES (Sigma). The cell suspension was quenched with EGM-2MV media and centrifuged at 700 rpm for 6 min. The cell pellet was washed twice with Pharmingen stain buffer (BD Biosciences) and centrifuged for 5 min at 300×g, 4 °C. The cells were resuspended with stain buffer (FBS) (BD Biosciences) to a final concentration of 4×10^6 cells/mL and treated with 1 μg of Human IgG/10^5 cells (Sigma) for 5 min at room temperature. Cells were incubated with anti-human CD143-FITC (AbD Serotec) for 45 min on ice. The concentration of isotype control and the CD143 antibodies were exactly the same (0.25 μg/10^5 cells). Cells were sorted (3×10^7 cells/h) using a Beckman Coulter MoFlo XDP Cell Sorter, and appropriate gating was used to obtain the 10 % of cells expressing the highest and lowest CD143.

Random distribution model

A sprouting assay was performed for 5 days, and a random sample of over 360 microcarriers was analyzed for each condition. Microcarriers were grouped together based on the number of sprouts that each one possessed. A histogram was created where microcarriers were characterized as having either 0 through 5 sprouts. A Poisson distribution was used as a theoretical model that created a discrete probability distribution based on the population mean of sprouts/bead for a given condition. The two models were compared using a chi-square test with $p \leq 0.05$ being considered as statistically significant.

Animals and surgical procedures

All animal work was performed in compliance with NIH and institutional guidelines. Female SCID mice aged 7 weeks were purchased (Taconic) and used for these studies. Mice were anesthetized with an intraperitoneal injection of a mixture of ketamine 80 mg/kg and xylazine 5 mg/kg prior to all surgical procedures. Hindlimb ischemia was induced by unilateral external iliac and femoral artery and vein ligation as previously described [24, 25].

After the vessel ligation, mice were treated with alginate macroporous scaffolds containing oligopeptides (GGGGRGDSP—two oligopeptides per alginate molecule) and 3 μg of recombinant human $VEGF_{121}$ (R&D Systems) as previously described [26]. Cells were cultured in vitro for 8 days under daily feeding with EGM-2MV media. The alginate scaffolds loaded with control (unsorted) ECs or the 10 % of sorted cells that expressed the lowest CD143 ($n = 6$ per condition; 1.5×10^6 cells/scaffold) were implanted on the medial side of thigh muscle, and incisions were subsequently surgically closed and animals monitored over time.

Perfusion, ischemic grade, immunohistochemistry and blood vessel density quantification

Before surgery and 0, 1, 3 and 7 days, and 2, 4, and 6 weeks post-surgery measurements of the ischemic/normal limb blood flow ratio were performed on isoflurane (2 % v/O_2)-anesthetized animals ($n = 6$/time point/experimental condition) using a Periscan system blood perfusion monitor laser Doppler equipment (Perimed, Sweden). Perfusion measurements were obtained from the right (ischemic) and left (non-ischemic) limb. To minimize variability due to ambient light and temperature, the index was expressed as a ratio of ischemic to non-ischemic limb blood flow.

The distribution of hindlimb ischemia severity was monitored at 1, 2, 4 and 6 weeks post-surgery. The animal hindlimbs, while under isoflurane, were closely examined, including toe discolorations. Each animal was given an ischemic score according to the level of ischemia present, ranging from 0 to 5, representing no necrosis (5), toe discoloration (4), one necrotic toe (3), two or more necrotic toes (2), necrotic foot (1) and autoamputation (0).

Hindlimb muscle tissues were retrieved, fixed, paraffin-embedded, and stained for mouse CD31 (BD Biosciences Pharmingen) by using a previously validated protocol [25–29]. For measurement of capillary densities, 30 randomly chosen high-power fields of the tissue were analyzed. The number of positively stained blood vessels was manually counted and normalized to the tissue area. Sections from each sample were visualized at 200× and 400× with a Nikon Eclipse E800 light microscope (Japan) connected to an Olympus DP70 digital image capture system (Japan) and analyzed using IPLab 3.7 software (Scanalytics).

Statistical analysis

All statistical analysis was performed using Student's *t* test (two-tail comparisons) and one-way analysis of variance (ANOVA) unless stated and analyzed using InStat 3.0b (Graphpad) software. Differences between conditions were considered significant if $p < 0.05$.

Results

Characterization of sprouting cells and control cells

The cells that directly participate in the formation of endothelial sprouts (Sprouting cells) in a 3D fibrin gel were collected and separated from the cells that remained at the microcarriers (non-sprouting cells) through a combination of fibrin gel degradation and centrifugation (Fig. 1). The cells still adherent to the microcarriers detached only after 15 min treatment with trypsin, ensuring that the sprouting cell population did not contain non-sprouting cells with this separation procedure. The proliferation ability of both populations of cells (sprouting cells and non-sprouting cells), and control cells that were subjected to the same enzyme exposure but never used in a sprouting assay, was next tested (Fig. 2a). The sprouting cells showed a lower mitogenic potential as compared to all other cells, including regular endothelial cells cultured on tissue culture plastic at all times (ECs), the control endothelial cells that were submitted to the same fibrin gel culture and isolation conditions as cells in the sprouting assay and the non-sprouting cells. In contrast with all other cell populations, the presence or absence of VEGF did not alter the proliferation rate of the sprouting cells when placed in 2D culture conditions. However, when these same sprouting cells were placed again in a new sprouting assay, their ability to create new sprouts from the microcarriers was remarkably higher, as compared to regular endothelial cells (Fig. 2b). The sprouting cells exhibited a sevenfold increase in the number of sprouts, even in the absence of growth factors, as compared to regular endothelial cells (Fig. 2c). No side-by-side comparison between sprouting cells and non-sprouting cells was performed in this assay, because non-sprouting cells consistently failed (repeated five times) to adhere to microcarriers after isolation. The distribution profiles of the frequency of sprouts per bead for both the microcarriers loaded with sprouting cells and regular endothelial cells were also quantified (Fig. 2d). The beads loaded with the sprouting cells exhibited a twofold, fivefold and fourfold increase in the percentage of beads that displayed one, two and three independent sprouts, respectively, when compared with beads loaded with regular endothelial cells. The Poisson distribution for the number of sprouts/bead was calculated for each condition, using the average sprouts/bead at that condition and compared to the actual experimental results. Interestingly, the experimental results demonstrated a higher percentage of beads with two or more sprouts, as compared to the theoretical Poisson distribution that would be expected for both conditions. In particular, beads loaded with sprouting cells exhibited a threefold increase in the percentage of beads displaying three independent sprouts, as compared to a Poisson distribution, and a fourfold increase as compared to the distribution observed for beads loaded with regular endothelial cells.

Genotypic characterization of sprouting cells and cell sorting

The gene expression of sprouting cells was next analyzed and compared to regular endothelial cells. A higher level of telomerase activity was observed for the sprouting cells, as compared to regular endothelial cells, but the difference was not statistically significant (Fig. 3a). No gross qualitative differences were noted between these two different endothelial cell populations in terms of their secretion of 38 angiogenic factors (Supporting material). However, the genotypic character of these cells was further evaluated by comparing the expression profile of 84 genes involved in modulating angiogenesis (Fig. 3b). From the 84 genes tested, 54 genes maintained relatively constant levels of regulation (i.e., differences in expression <1.5 fold), 18 genes were upregulated and 12 genes were downregulated on sprouting cells as compared to regular ECs. CD62L, CD143, Apo2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and natriuretic peptide receptor A (NPRA) were more highly expressed (>2.5-fold) on regular ECs. Granulocyte–macrophage colony-stimulating factor (GM-CSF), tissue plasminogen activator (PLAT) and chemokine (C–C motif) ligand 5 (CCL5) were more heavily expressed (>2.5-fold) on sprouting cells. Strikingly, two genes that displayed low levels of expression on sprouting cells, selectin L (CD62L) and ACE/CD143, are cell surface molecules that could possibly be utilized to prospectively sort regular endothelial cell cultures in order

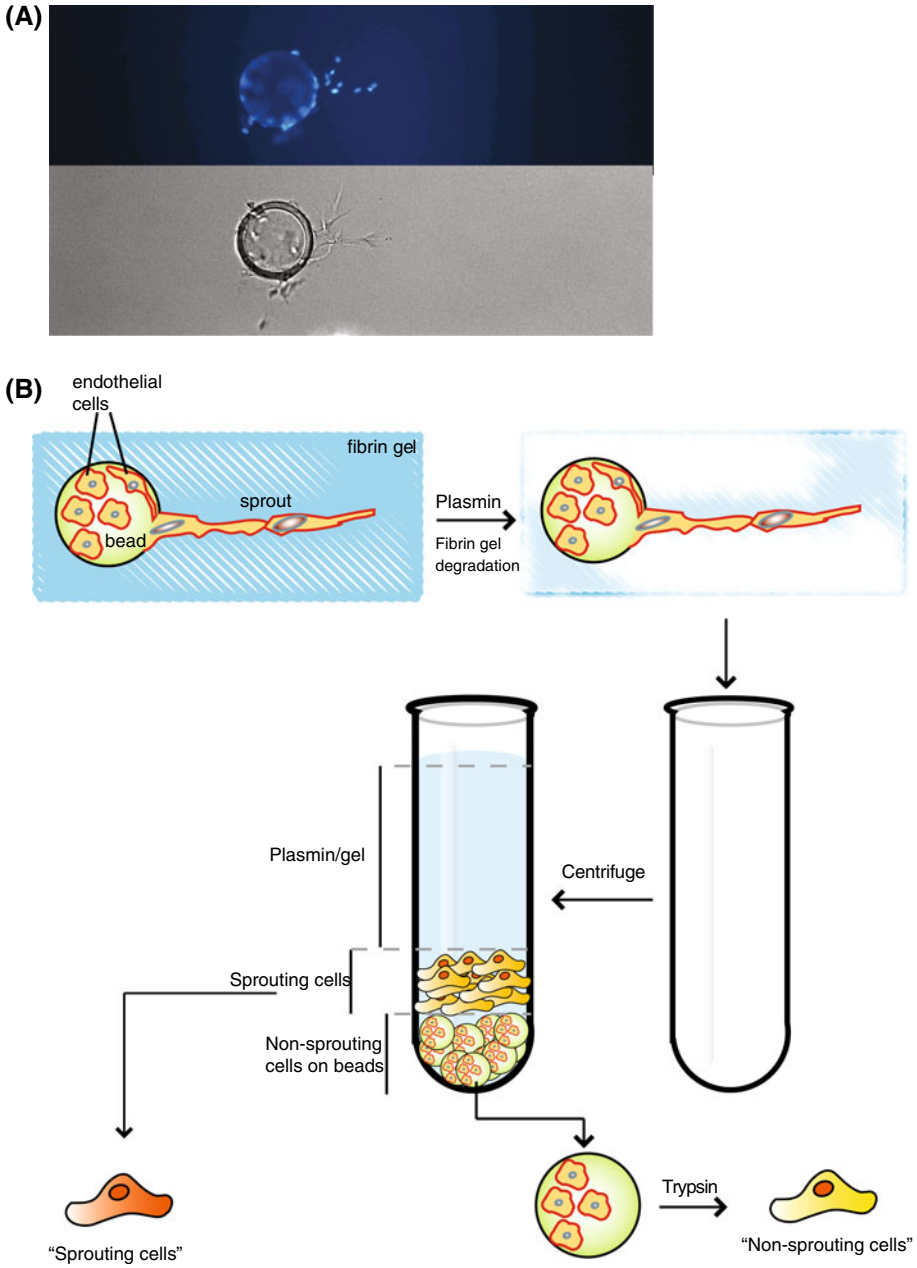


Fig. 1 Depiction of endothelial cell sprouts formed in a fibrin-based gel sprouting assay. **a** Fluorescent (*top*) and phase-contrast (*bottom*) photomicrographs of HMVEC-d cells sprouting from microcarriers at day 3. Cells were labeled with DAPI for nucleus localization and further cell quantification. **b** Diagram describing the approach utilized to isolate HMVEC-d cells that formed sprouts ("sprouting cell") from cells that remained on the microcarriers ("non-sprouting cell"). A combination of fibrin gel degradation mediated by plasmin, and centrifugation was utilized to separate the sprouting cells from the cells remaining adherent to the microcarrier beads. Non-sprouting cells were subsequently removed from microcarrier beads using trypsinization. Photomicrographs in 1A are at ×200

to obtain a subset of cells with potentially greater angiogenic ability. To test whether changes of CD143 expression are differentially associated with sprouting and non-sprouting cells during a 3D sprouting assay, sprouting cultures were stained with antibodies against CD143 (Fig. 3c). Cells that were residing on the microcarriers displayed positive CD143 staining, but in contrast no expression of CD143 was observed on the cells that were sprouting from the microcarriers. Next, the levels of ACE/CD143 expression between ECs, sprouting and non-sprouting cells were evaluated on conditioned medium via ELISA. Sprouting cells secreted significantly lower levels of ACE/CD143 as compared to ECs and non-sprouting cells (Fig. 3d).

To test whether a sub-population of endothelial cells with greater angiogenic ability could be prospectively isolated, cells were treated with antibodies against CD143, and the 10 % of cells with the lowest and highest level were sorted (Fig. 4) and further expanded in culture. Endothelial cells sorted for CD143-low expression demonstrated similar proliferative capacity as regular endothelial cells (Fig. 4c). These cells also displayed a significant increase in the ability to form new sprouts when compared with regular endothelial cells of the same passage number (Fig. 4d). The CD143-high

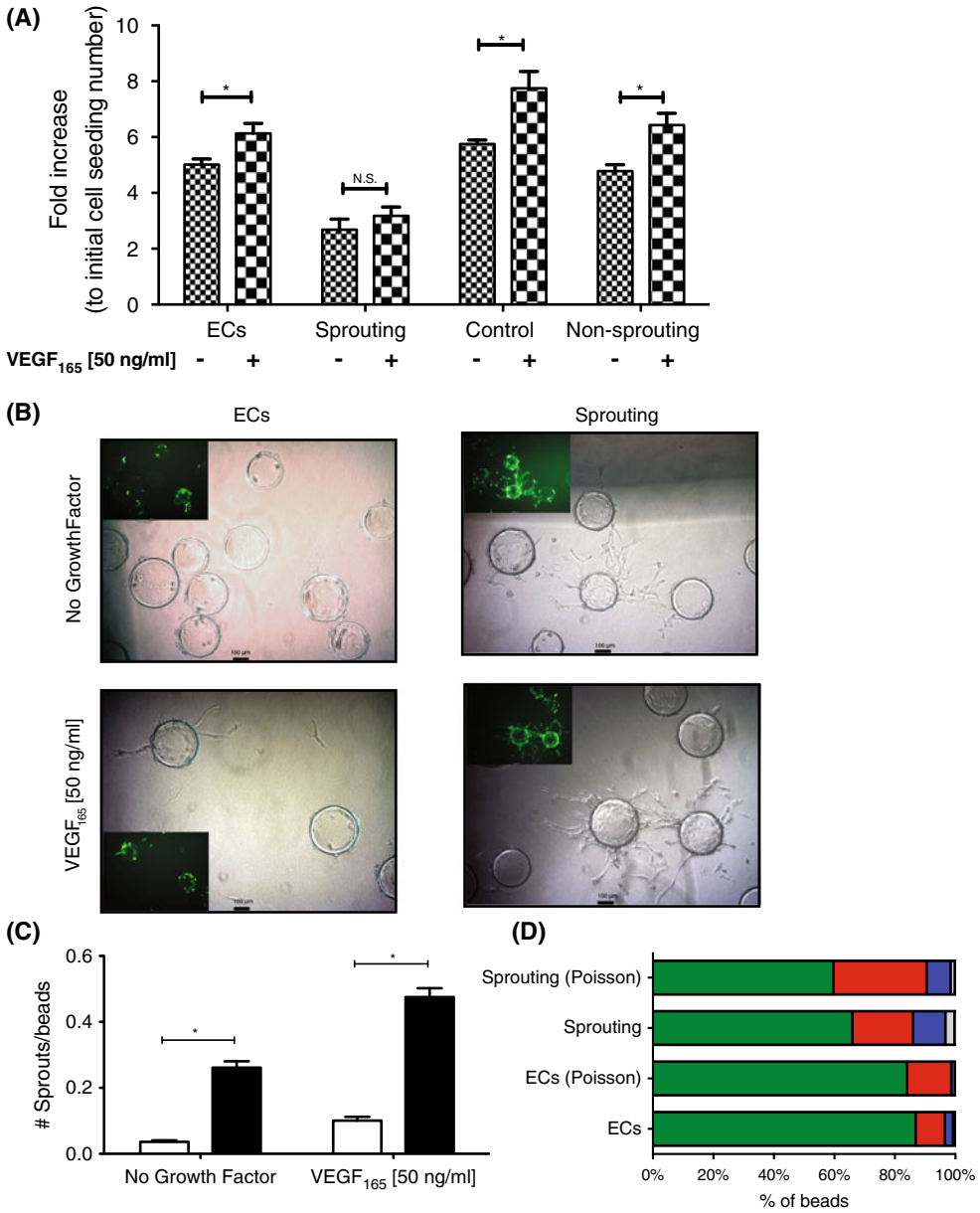
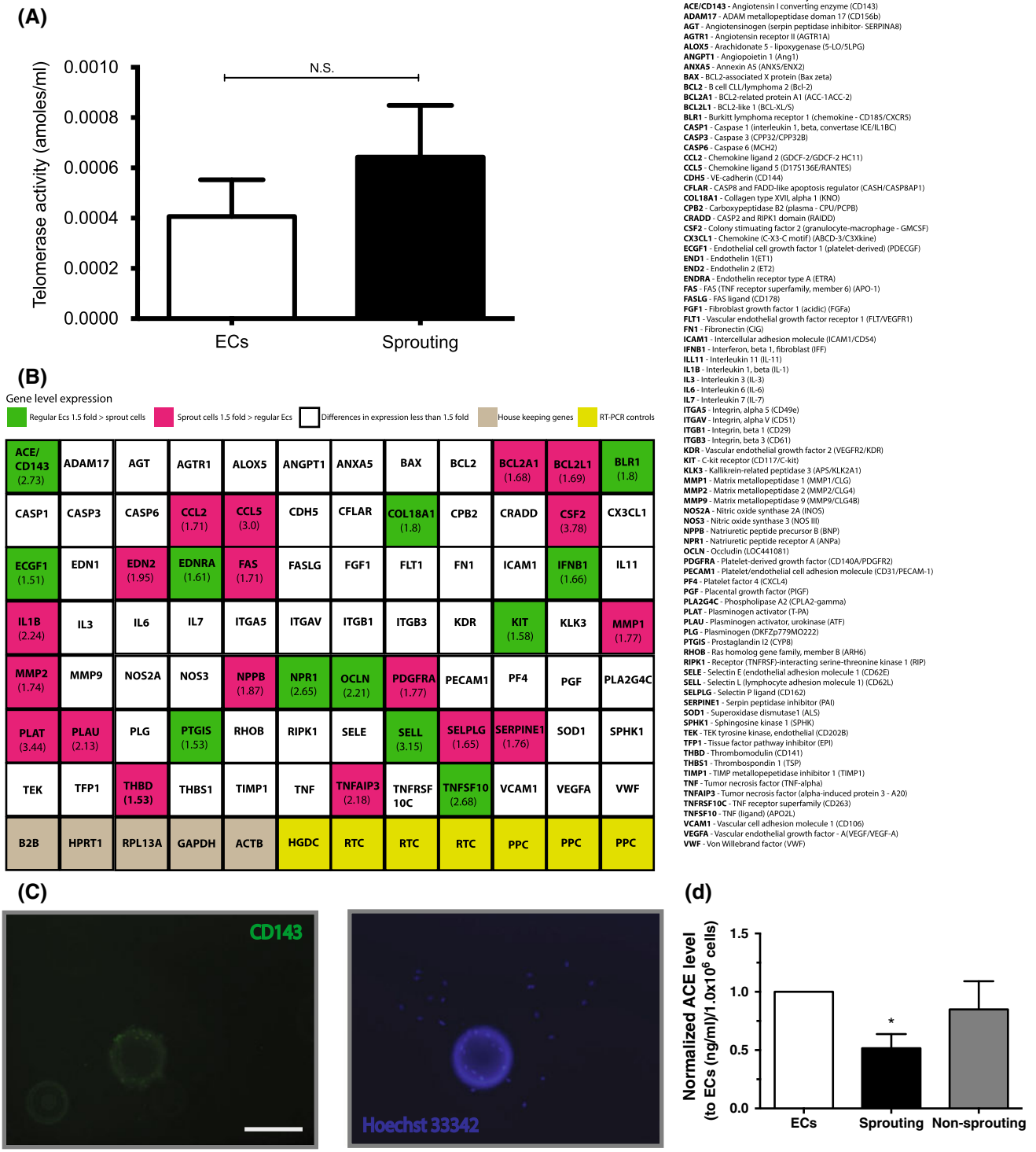


Fig. 2 Characterization of sprouting and non-sprouting cells. **a** Proliferation of cells isolated from sprouts (sprouting) and cells that remained on the beads (Non-sprouting), as compared to regular HMVEC-d (ECs) (not placed in fibrin gels) and HMVEC-d cells that were cultured in the fibrin gels and were subject to the exact same process of isolation as the sprouting and non-sprouting cells (Control). All cell types were tested in media containing either no growth factors, or this media supplemented with $VEGF_{165}$ [50 ng/mL]. An equal number of each type of cell was seeded in each well, and the resultant cell number at 3 days quantified. **b** Representative fluorescent (*inserts*) and phase-contrast photomicrographs displaying the density and quantity of sprouts formed both from the regular HMVEC-d (ECs) and the sprouting cells under both cell media containing VEGF and no growth factors. **c** Cells that formed sprouts (sprouting cells; *dark bars*) displayed a considerable greater ability to make new sprouts when placed back into the assay, as compared to regular HMVEC-d (*white filled bars*), both under $VEGF_{165}$-supplemented media and no growth factor media. **d** Histogram of the distribution of number of sprouts formed by regular ECs (both observed and theoretical—Poisson values) and sprouting cells (both observed and theoretical—Poisson values) for microcarriers displaying zero sprouts (*green stack bar*), one sprout (*red stack bar*), two sprouts (*blue stack bar*), three sprouts (*gray stack bar*), four sprouts (*orange stack bar*). Mean values are presented with standard deviations, asterisk indicates statistically significant differences ($p < 0.05$) between conditions and N.S. displays no statistically significant difference between conditions. All photomicrographs (insert included) in 2B are at ×200, and calibration bar represents 100 μm. (Color figure online)



cells exhibited comparable proliferation ability but they demonstrated very low adhesion to the microcarrier beads (tested for five independent trials) and so were not further tested. The endothelial cell sub-population that demonstrated no expression of CD143 ($\sim$3 % of endothelial cells) was also isolated, but this subset of endothelial cells did not proliferate in vitro after sorting and so was not further examined.

◀**Fig. 3** Characterization of sprouting cells. **a** Telomerase activity of control HMVEC-d endothelial cells (ECs) as compared to HMVEC-d endothelial cells that had previously formed sprouts (sprouting). **b** Expression levels of 84 signature angiogenic genes in control HMVEC-d and HMVEC-d that formed sprouts. The number in parenthesis represents the fold change between the two cell populations, and each gene is color-coded to represent the magnitude of change and whether an increase or decrease was noted with sprouting cells. **c** Microcarriers seeded with HMVEC-d were embedded in a 3D fibrin gel and fed daily for 5 days with EGM-2MV. After fixation, gels were incubated with a nuclear stain (Hoechst 33342—*right panel*) and CD143 antibody (*left panel*). Differential CD143 staining was observed between sprouting cells and cells residing on the microcarriers (non-sprouting cells). **d** The levels of ACE/CD143 in conditioned medium from HMVEC-d endothelial cells (ECs), sprouting cells and non-sprouting cells. Mean values are presented with standard deviations. Asterisk indicates statistically significant differences ($p < 0.05$) between ECs and non-sprouting cells (**e**) and N.S. displays no statistically significant difference between conditions (**a**). *Calibration bar* represents 200 μm

Therapeutic effect of CD143-low cells in rescuing ischemic hindlimbs

The angiogenic potential of the CD143-low sub-population of cells was next examined in a mouse model of peripheral artery disease (PAD). In particular, the ability of regular endothelial cells and the CD143-low sub-population, to promote vascularization, tissue perfusion and reversion of limb ischemia was quantified (Fig. 5). Both cell populations were transplanted using a macroporous alginate system, as this has been previously demonstrated to enhance their engraftment and angiogenic potential, as compared to simple injection into tissue [26]. Muscle tissue from the ischemic hindlimbs regions was retrieved after 6 weeks, sectioned, immunostained against the endothelial marker CD31, and the blood vessel density quantified (Fig. 5a, b). Animals treated with alginate scaffolds loaded with CD143-low cells exhibited a twofold increase in blood vessel density as compared to the transplantation of regular endothelial cells (Fig. 5b). A positive therapeutic effect of the CD143-low cells was also evidenced by a significant increase both in the recovery of regional blood flow over time and in the reversion of ischemia severity. Animals treated with CD143-low cells recovered ~75 % of limb perfusion, as compared to ~50 % recovery for the animals treated with regular endothelial cells (Fig. 5c). Animals treated with regular endothelial cells had an autoamputation rate of 30 %, while animals treated with CD143-low cells had no autoamputation, and 50 % of these animals only experienced toe necrosis (Fig. 5d).

Discussion

The results of this study demonstrate that the sub-population of cultured endothelial cells that participate in sprouting display an intrinsic increased ability to form new sprouts, as compared to the overall endothelial cell population. These sprouting cells, after isolation, exhibited relatively low proliferation and low CD143 expression. Furthermore, the results of this study indicate that a sub-population of endothelial cells prospectively enriched for low levels of CD143 demonstrate increased sprouting in vitro and are capable of enhancing neovascularization in a commonly used pre-clinical model of hindlimb ischemia. This latter finding suggests that CD143 could be used as a biomarker to identify more potent subsets of endothelial cells for use in cell-based therapeutic strategies.

A technique was first developed to isolate and separate sprouting endothelial cells from non-sprouting cells in an in vitro 3D sprouting assay, and these cells were found to have distinct characteristics that correlate with an enhanced, intrinsic ability to participate in angiogenesis. There has been significant research to understand the mechanisms leading to the formation of new vascular sprouts [30], but very little is known about the plasticity and commitment of cells that participate in de novo sprout formation, specifically when rechallenged to initiate and commit to sprouting. The sprouting assay used in this study is often used with supporting cells [23, 31], but supporting cells were not used in the current experiments. Also, the endothelial cell population used in the current studies was also distinct from many past studies. Supporting cells were not used in order to avoid confounding influences from these cells. The endothelial cells used in this study (HMVEC-d) were obtained from a homogeneous population of cells of a single donor and may be more relevant to capillary endothelial cell biology than HUVEC [32]. Methods that allow one to isolate and separate sprouting and non-sprouting cells have previously been lacking, limiting investigation of differences between these sub-populations. Laser capture microdissection can be used to isolate sprouting cells from non-sprouting cells [13]; however, this method requires the fixation of captured cells. One cannot subsequently further probe the ability of the sprouting cells, isolated in this manner, to proliferate and participate again in sprouting. In contrast, the method developed in this report allows the isolation of viable cells and has revealed that these cells demonstrate an enhanced ability to again form new sprouts. Surprisingly, these cells actively sprouted, even in the absence of VEGF stimulation. This highly robust sprouting could be explained by several factors, including a higher density of receptors for angiogenic factors such as neuropilin-1 (NRP1) [33, 34], leading to a response to very low levels in the medium even in the absence of supplementation, or the upregulation of genes related to migration and motility, including RhoA and RhoB [35–37]. However, it should be noted that the VEGFR1 and VEGFR2 gene expression levels on the

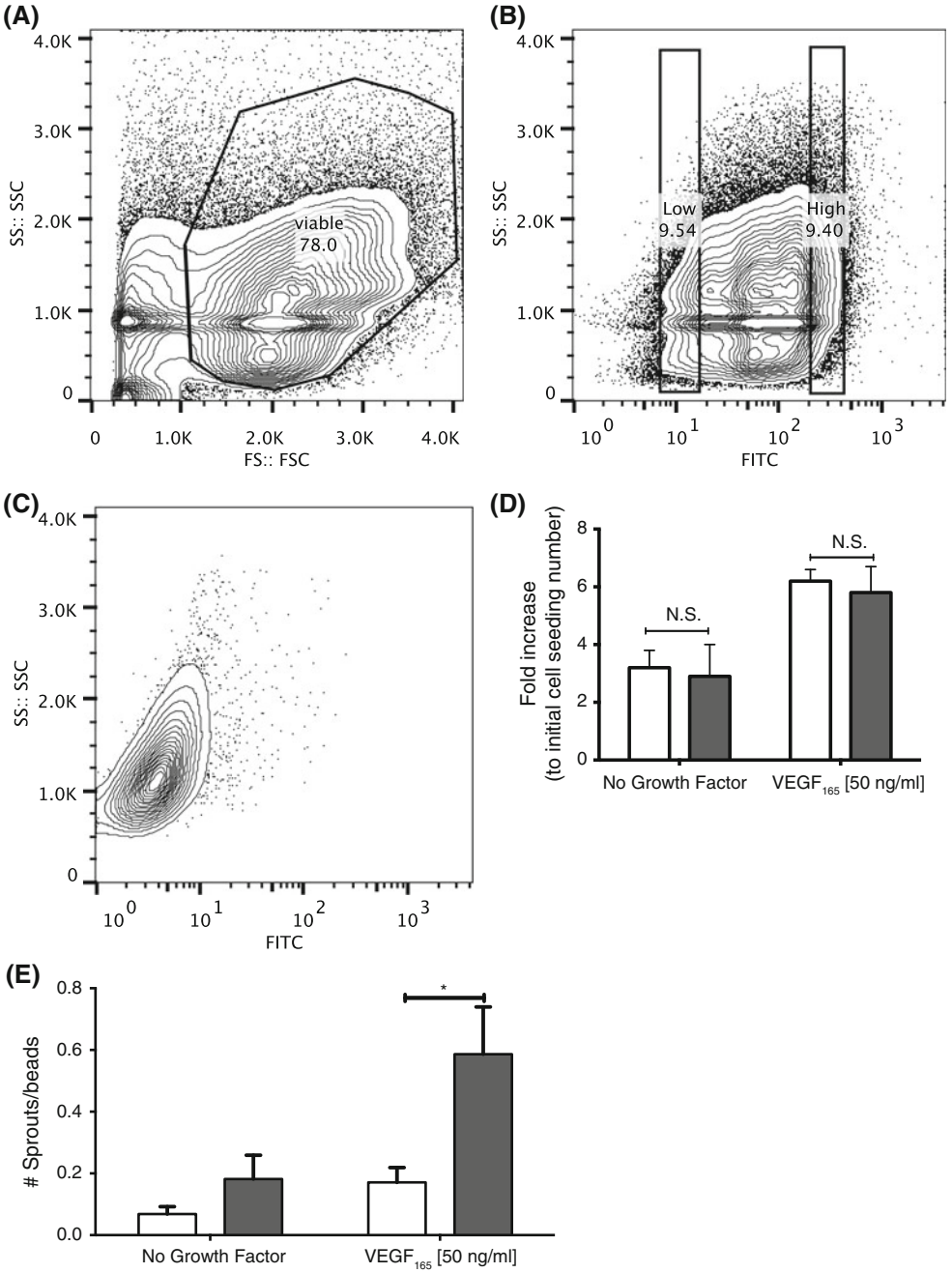


Fig. 4 FACS analysis and sorting of CD143-high- and low-expressing HMVEC-d, and the proliferation and sprouting of CD143-low cells. **a** Cells were stained with CD143 antibodies, and first subjected to initial gating for the viable cell population using forward scatter (FS) and side scatter (SS); *Black outline* on plot represents gating for viable cell population. **b** Viable cells were then sorted for the 10 % of cells with the highest and lowest expression of CD143. *Black rectangles* represent these two cell populations. **c** HMVEC-d were stained with isotype control antibodies matching the host species of the primary antibody allowing an assessment of the level of background staining. **d** Proliferation of endothelial cells sorted for CD143 low (*dark bars*) as compared to regular (non-sorted) endothelial cells (*white bars*) under media containing either no growth factors or media supplemented with $VEGF_{165}$ [50 ng/mL]. **e** Sprouting of endothelial cells expressing low levels of CD143 (*black bars*), as compared to regular HMVEC-d (*white filled bars*), in media with no growth factors or media that contains no growth factors except $VEGF_{165}$. Mean values are presented with standard deviations. N.S. indicates no statistically significant difference between conditions, and asterisk indicates statistically significant differences ($p < 0.05$) between conditions

sprouting cells were similar to the control endothelial cell population. Furthermore, the frequency of distribution of sprouts per bead observed for sprouting cells did closely fit the frequency predicted by a Poisson distribution, suggesting that this enhanced ability to form new sprouts is not the result of a random event.

The limited proliferation displayed by sprouting cells after isolation could be attributed to the fact that this cell population entails both tip cells and stalk cells, and tip cells have been reported to exhibit low proliferation [6, 38, 39]. Moreover, the profile of cytokines secreted by the sprouting cells was very similar to that observed for non-sorted endothelial cells, which is again consistent with previous studies [26]. Several genes and pathways have been previously described to exhibit increased expression in sprouting cells, including Notch signaling [40], VEGF receptors [41], various adhesion molecules [42] and cell surface receptors [7, 43]. However, in our study, VEGFR1 and VEGFR2 were in the group of 54 genes that displayed similar levels of expression in the sprouting cells and non-sorted endothelial cells. It is also important to note that in our isolation approach, both the tip and stalk cells [44] are

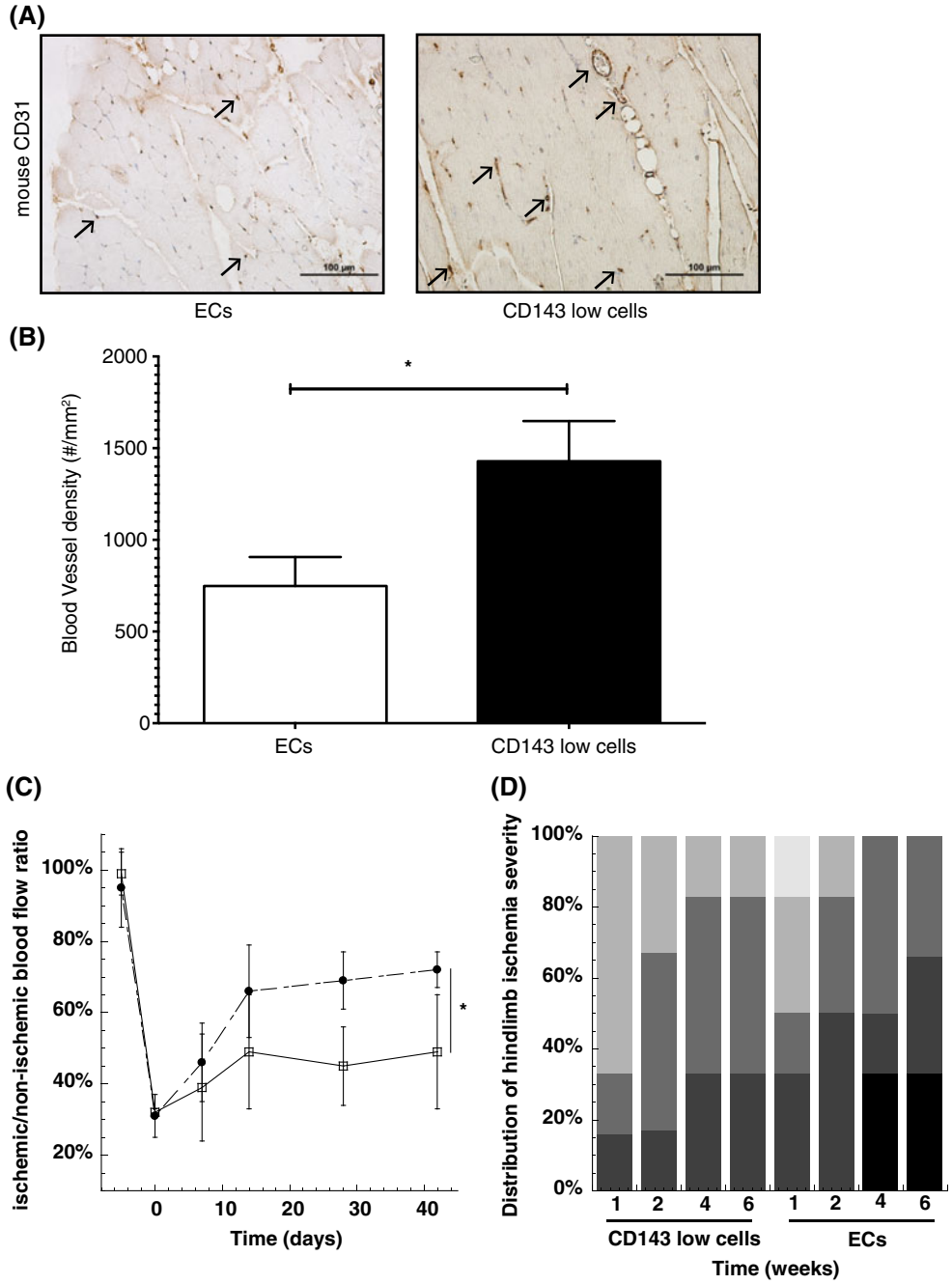


Fig. 5 The ability of CD143-low expression endothelial cells to drive neovascularization in murine ischemic hindlimbs was compared with regular (unsorted) endothelial cells. **a** Representative images of muscle tissue stained for the endothelial cell marker CD31 following transplantation of regular endothelial cells, or CD143-low cells at 6 weeks post-treatment. **b** The blood vessel density in muscle tissue was quantified for these two experimental conditions. **c** Perfusion of ischemic hindlimbs over time following transplantation of CD143-low cells (*filled circle*) or regular endothelial cells (*open square*). **d** The distribution of severity of ischemia was monitored over time for both the animals treated with CD143-low cells and the animals treated with regular endothelial cells. Ischemia was graded as no necrosis ((□)), toe discoloration ((▫)), one necrotic toe ((▫)), two or more necrotic toes ((▫)), necrotic foot ((▫)) and autoamputation ((■)). Mean values are presented with standard deviations, asterisk indicates statistically significant differences ($p < 0.05$) between conditions. *Arrows* in (**a**) highlight positive CD31 cells

present in the sprouting cell population, which likely accounts for the difference from past reports. Importantly, of the set of genes that exhibited a significant alteration in sprouting cells, as compared to non-sorted endothelial cells, a number of these genes are known to play a role during inflammation [45–48], including GM-CSF, CCL5,

PLAT and selectin L. This observation is consistent with previous studies that underscore a close correlation between signals and cells related to inflammation, and endothelial sprouting [49, 50].

The findings of this study suggest that CD143 could be used as a specific biomarker to identify a subset of endothelial cells highly capable of driving neovascularization. First, gene expression analysis revealed that sprouting cells display low expression of CD143, as compared to a non-sorted cell population. Moreover, endothelial cells sorted for a low surface level of CD143 were more capable of forming sprouts in vitro, as compared to non-sorted endothelial cells. Consistent with these findings, much high levels of CD143 expression were found on non-sprouting cells as compared to cells in the act of sprouting. The role of CD143 inhibitors in vascular diseases is well established [51], but this correlation between the ability of an endothelial cell to sprout and the level of expression of CD143 is novel. Several studies have correlated high levels of CD143 activity with several cardiovascular complications [52–55], and inhibitors of CD143 and the angiotensin II type 1 receptor are now being widely used clinically, including in the treatment of hypertension and heart failure [56]. Further, it has been also reported that CD143 inhibitors favor endothelial cell survival and increase sprouting [57], which is consistent with our findings. The levels of proliferation of CD143-low endothelial cells were similar to the pre-sorted endothelial cells. These results could be interpreted as being in conflict with other results from our studies that demonstrated that sprouting cells have reduced proliferation. The reasons for these differences were not mechanistically evaluated in this study, but there are at least two possible reasons. One is related with the limited replicative lifespan of primary cells in vitro [58]. The sprouting cells will likely have proliferated during sprouting, perhaps limiting further proliferation. In contrast, the cells that are prospectively isolated (CD143-low endothelial cells) have likely proliferated to a lower extent. The second, and more likely, possibility is that sprouting alters the mitogenic capacity of endothelial cells permanently once the cells have sprouted. Although it has been established that tip cells display low proliferative capacity, very little is known about the reversibility of this behavior [6, 38]. Finally, the cell culture medium from sprouting cells displayed a significant decrease in the levels of CD143, as compared to both non-sprouting and endothelial cells. The levels of CD143 expression obtained for the endothelial cells under normal cell culture conditions are consistent with previous work [59].

Importantly, our results demonstrate that a subset of CD143-low endothelial cells is more efficient in restoring regional blood perfusion and reversing local ischemia in a rodent model, than the unsorted endothelial cell population. A previously validated macroporous alginate system [26] was used to deploy both sets of human endothelial cells in a murine model of severe peripheral ischemia. In that study, the negative control of no added ECs led to severe ischemia and accelerated autoamputation. Due to the severity of tissue necrosis, that negative control was not replicated in this study for humane reasons. Overall, the present findings suggest that clinical strategies based on endothelial cell or endothelial cell progenitor transplantation to drive therapeutic angiogenesis [26, 43, 60] may in the future be improved by the use of CD143 as a biomarker to identify and isolate a subset of vascular cells with enhanced therapeutic potency.

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Conflict of interest None.

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