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ORIGINAL PAPER

CD34 marks angiogenic tip cells in human vascular endothelial cell cultures

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Abstract The functional shift of quiescent endothelial cells into tip cells that migrate and stalk cells that proliferate is a key event during sprouting angiogenesis. We previously showed that the sialomucin CD34 is expressed in a small subset of cultured endothelial cells and that these cells extend filopodia: a hallmark of tip cells in vivo. In the present study, we characterized endothelial cells expressing CD34 in endothelial monolayers in vitro. We found that CD34-positive human umbilical vein endothelial cells show low proliferation activity and increased mRNA expression of all known tip cell markers, as compared to CD34-negative cells. Genome-wide mRNA profiling analysis of CD34-positive endothelial cells demonstrated enrichment for biological functions related to angiogenesis and migration, whereas CD34-negative cells were enriched for functions related to proliferation. In addition, we found an increase or decrease of CD34-positive cells in vitro upon exposure to stimuli that enhance or limit the number of tip cells in vivo, respectively. Our findings suggest cells with virtually all known properties of tip cells are present in vascular endothelial cell cultures and that they can be isolated based on expression of CD34. This novel strategy may open alternative avenues for future studies of molecular processes and functions in tip cells in angiogenesis.

Keywords Angiogenesis · Endothelial tip cell · Vascular endothelial cell cultures · CD34

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Introduction

Angiogenesis is crucial in the pathogenesis of many chronic and potentially fatal diseases [1, 2]. In order to promote vascular network expansion, sprouting angiogenesis requires a subset of specialized endothelial cells while the majority of cells remains quiescent in the pre-existing blood vessel. Among these highly specialized cells are tip cells, which coordinate multiple critical processes in a hierarchical way. Located at the leading edge of the vascular sprout, tip cells form cellular protrusions or filopodia to guide migration towards a source of angiogenic growth factors [3]. Simultaneously, they signal to adjacent endothelial cells via Delta-like ligand (DLL)-Notch interactions

not to adapt the tip cell phenotype, but to maintain the proliferative stalk cell phenotype and to form a vascular lumen [3, 4]. Because tip cells comprise a distinct subpopulation of endothelial cells with a unique molecular signature, they constitute an attractive target for pro- and anti-angiogenic therapy. Yet to develop such therapeutic strategies, a thorough understanding of the regulation of tip cell functions and the genes involved is needed.

Research on tip cells predominantly relies on in vivo models of angiogenesis [3–11], especially on developmental retinal angiogenesis during the first postnatal week in mice. These studies have shown that the fate of endothelial tip cells is controlled by a number of different signaling pathways, including that of vascular endothelial growth factor (VEGF) [2, 3, 9, 11] and the Notch ligand DLL4 [4]. In vitro tests can provide critical information on many factors regulating angiogenesis in vivo and are essential first steps for preclinical validation of potential therapeutic targets. Whether the tip cell phenotype is present in 2-dimensional vascular endothelial cell cultures, and whether this subpopulation of cells can be isolated is experimentally unexplored.

We hypothesized that, within the context of vascular endothelial cell cultures, CD34 is a novel marker to select for endothelial cells with a tip cell phenotype in vitro. CD34 is a highly glycosylated transmembrane cell surface glycoprotein, expressed by hematopoietic stem and progenitor cells [12–14] and, on the luminal cell membrane of quiescent endothelial cells of small blood vessels and lymphatics [7, 15–20]. Consequently, freshly-isolated human vascular endothelial cells are CD34-positive ($CD34^+$), but expression is rapidly lost when resting cells are cultured and forced into cell cycle [21, 22]. Interestingly, a small subset of $CD34^+$ cells remains present in later passages of primary endothelial cell cultures and also in immortalized endothelial cell lines [23]. In human umbilical vein endothelial cell (HUVEC) cultures, we previously observed that these $CD34^+$ cells extend filopodia [7]. CD34 expression on endothelial tip cell filopodia was also detected during angiogenesis in human tumors and embryogenesis, and in mouse embryogenesis and wound healing [7, 19, 20].

In order to further unravel the molecular mechanisms that induce and maintain tip cell identity and regulate their specific functions, we investigated these $CD34^+$ cells in cultures of vascular endothelial cells. We show that this $CD34^+$-subset of endothelial cells have filopodia, low proliferation activity and that the $CD34^+$ phenotype in endothelial cell cultures is regulated similarly to mechanisms that regulate the tip cell phenotype in vivo. We exploited positive selection of $CD34^+$ vascular endothelial cells in vitro and compared their transcriptome to CD34-negative ($CD34^-$) cells. Real-time PCR and microarray data analysis identified increased expression of all known genes previously associated with tip cells in vivo. Our results open alternative avenues of research that may help to understand the molecular processes and functions in angiogenesis in general, and of the specialized endothelial tip cell in particular.

Materials and methods

Cells and cell cultures

Primary HUVECs were isolated from umbilical veins by collagenase digestion according to a published method [24] and routinely grown in M199 basal medium (Gibco, Grand Island, NY, USA) supplemented with 10% human serum, 10% fetal bovine serum (Biowhittaker, Walkersville, MD, USA) and 1% penicillin-streptomycin-glutamine (Gibco). Umbilical cords were obtained from the Department of Obstetrics and Gynaecology (Academic Medical Center, Amsterdam, The Netherlands). HUVEC cultures between passages 1 and 3 were used for all experiments. EC-RF24 [23] (derived from HUVECs) and HMEC-1 [23] (derived from human dermal microvascular endothelial cells) were routinely grown in EGM-2 MV BulletKit (Lonza, Walkersville, MD, USA). EC-RF24 and HMEC-1 between passages 30 and 40 were used for all experiments. All cells were cultured in 0.2% gelatin-coated culture flasks at 37°C and 5% CO_2. HMEC-1 was a kind gift of Prof. Dr. P. Hordijk (Sanquin, Amsterdam, The Netherlands) and EC-RF24 was a kind gift of A.M. Klous (Academic Medical Center, Amsterdam, The Netherlands).

When indicated, cells were treated with VEGF (R&D Systems, Minneapolis, MN, USA), basic fibroblast growth factor (bFGF) (Invitrogen, Carlsbad, CA, USA), or tumor necrosis factor-alpha (TNF-α) (10 ng/ml; R&D Systems) or tissue culture plates were coated with 0.2% gelatin (w/v) in PBS containing DLL4 (1 μg/ml; R&D Systems) or bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) as a control according to Harrington et al. [25].

Immunostaining of cell cultures

For immunofluorescence microscopy, third passage HUVECs were grown to confluence on gelatin-coated coverslips (Thermo Scientific, South Logan, UT, USA). Cells were fixed for 20 min with freshly-prepared 2% paraformaldehyde in PBS and blocked for 30 min in 10% normal goat serum in PBS at room temperature. For double immunostaining, cells were incubated overnight at 4°C with antibodies directed against CD34 [7] (clone QBEND-10; Monosan, Uden, The Netherlands) and cadherin 5 (CDH5; also known as vascular endothelial cadherin (VE-Cadherin)) (Abcam, Cambridge, UK). Secondary antibodies were goat anti-mouse fluorescein isothiocyanate (FITC) and goat

anti-rabbit Cyanine-3 (Cy3) (Jackson Laboratories, Bar Harbor, ME, USA). The coverslips were then mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) containing DAPI and examined using a wide-field fluorescence microscope (Leica, Heidelberg, Germany). As negative controls, primary antibody was omitted.

Immunohistochemistry of tissue samples

Vibratome sections from paraformaldehyde-fixed normal and pathologic human tissues were stained with anti-CD34 or anti-CD31 (clone EN4; Abcam) antibody as described previously [7].

The spheroid-based angiogenesis model

HUVECs (750 cells/spheroid) were seeded in methyl cellulose (Sigma-Aldrich) containing medium to form cell spheroids (http://spherogenex.de) [26]. After 24 h, cells were embedded in collagen gel with or without bFGF (20 ng/ml, R&D Systems) and allowed to sprout for 24 h. Gels containing the spheroids were fixed with 2% paraformaldehyde in PBS overnight and immunostained as described above.

Flow cytometric analysis

Cell suspensions were obtained by TrypLE (Gibco) treatment of adherent endothelial cell monolayers. All immunofluorescent labeling and washing was performed in PBS containing 0.1% bovine serum albumin. Cells were fixed in 2% paraformaldehyde in PBS for 15 min at room temperature and incubated with anti-CD34-phycoerythrin (anti-CD34-PE; clone QBend-10; Monosan), anti-CD31-allophycocyanin (anti-CD31-APC; eBioscience, San Diego, CA, USA), anti-CD146-FITC (Abcam), anti-CD14-FITC (Becton Dickinson, Mountain View, CA, USA), anti-CD133-APC (Miltenyi Biotec, Bergisch Gladbach, Germany), and anti-CD117-APC (Becton Dickinson). Cells were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson) in combination with FlowJo software (Tree Star, San Carlos, CA, USA).

Cell sorting and RNA isolation

HUVECs of passage 3 were sorted for CD34 expression at 3×10^7 cells/h under sterile conditions on a FACSAria (Becton Dickinson) and total RNA from both fractions ($CD34^+$ and $CD34^-$) was extracted using TRIzol (Invitrogen). The amount of total RNA was approximately 1 µg/10^5 cells, as measured on a nanodrop (ND-1000; NanoDrop Technologies, Wilmington, DE, USA), and RNA quality was determined by Bioanalyzer 2100 traces (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocols.

Quantitative PCR

A 1-µg aliquot of total RNA was DNase-I treated (amplification grade; Invitrogen) and reverse transcribed into first-strand cDNA using Superscript III and oligo$(dT)_{12-18}$ (Invitrogen). Primer details are provided in Supplementary Table 1. NCBI BLAST confirmed specificity of the primers. The presence of a single PCR product was verified by both the presence of a single melting temperature peak and detection of a single band of the expected size on agarose gels. Non-template controls were included to verify the method and the specificity of the primers. Mean primer efficiency was 96% ± 3%. Real-time quantitative PCR (qPCR) was performed as described previously [27, 28], using a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Gene expression data was normalized with the geomean of β-actin and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, and zeta polypeptide (YWHAZ) gene expression data, as determined by NormFinder [29].

Microarray analysis

The GeneChip HT HG-U133+ PM Array Plate (Affymetrix, Santa Clara, CA, USA) system was used for microarray analysis. Technical handling of the microarrays was performed at the MicroArray Department (MAD) at the Swammerdam Institute for Life Sciences (University of Amsterdam, Amsterdam, The Netherlands). In short, biotin-labeled cRNA samples were prepared as described in the GeneChip 3'IVT express kit user manual (Affymetrix) using 0.1 µg purified total RNA as template for the reaction and a 16 h incubation time. Hybridization, staining and scanning were performed in a GeneTitan MC Instrument (Affymetrix). The raw data are available at the Gene Expression Omnibus (GSE 34850) (http://www.ncbi.nlm.nih.gov/geo/). After passing array quality control, expression values for each array were calculated using the robust multi-array average (RMA) algorithm, (Affy package version 1.22.0) [30] available from the Bioconductor project (http://www.bioconductor.org) for the R statistical language (http://cran.r-project.org). The normalized data was statistically analyzed for differential gene expression using ANOVA (Maanova package, version 1.14.0) [31] with a model that considered the presence of CD34 as a fixed effect and individual as a random effect. In order to quantify the effect of the presence of CD34 on gene expression, a permutation based statistical test (2,000 permutations) was performed, and all P values were adjusted for false discovery-rate correction [32]. Corrected P values of less than 0.15

were considered to indicate significant differences. Model-based log2 ratios of $CD34^+$ signal to $CD34^-$ signal were generated for each probe set. A list was generated that contained those probe sets for which an average absolute fold change of at least 2.0 was observed between $CD34^+$ and $CD34^-$ samples.

Gene ontology analysis and statistics

Gene ontology (GO) term enrichment was performed with the two sets that were ≥2-fold upregulated or downregulated with statistical significance, using the DAVID algorithm [33] with the total set of genes of the Affymetrix HG-U133A chip as background. The following GO terms were selected: GOTERM_BP_ALL (biological process), GOTERM_CC_ALL (cellular component) and GOTERM_MF_ALL (molecular function), with classification strategy 'high'. GO terms were assumed to be biologically relevant with an enrichment score of at least 2.5 and a Benjamini–Hochberg–corrected modified Fisher's exact *P* value of less than 0.02. Benjamini–Hochberg statistics were used to correct *P* value to be more conservative in order to lower family-wise false discovery rate [34].

Gene set enrichment analysis

Changes in the expression of functionally related genes at the genome-wide expression profile level were detected using gene set enrichment analysis (GSEA) (version 2.07; Broad Institute, Cambridge, MA, USA). For the analysis, genes represented by more than one probe were collapsed to the probe with the maximum value using the gene symbols [35]. Gene sets were generated from gene lists published by Strasser et al. [8], Del Toro et al. [5], and Harrington et al. [25]. Mouse gene symbols were converted to their human homologues (http://ncbi.nlm.nih.gov/homologene). Genes of which no human homologue could be found were excluded from the list. In this way, five gene lists were generated (1) Strasser et al. ≥2-fold overrepresented in a microdissected tip-cell fraction; (2) Del Toro et al. ≥2-fold upregulated genes in *dll4+/−* mouse retina; (3) Del Toro et al. ≥2-fold downregulated genes in *dll4+/−* mouse retina; (4) Harrington et al. ≥1.5-fold upregulated genes in DLL4-retrovirally-infected HUVECs; (5) Harrington et al. ≥1.5-fold downregulated genes in control empty vector-infected HUVECs. Our data was then tested for enrichment in these five gene sets using GSEA, with the number of permutations set to 1,000. False discovery rate (FDR) *Q* value <25% was used as criteria for significantly enriched gene sets.

Live cell imaging

Live cell imaging was performed according to a published method [36]. In brief, equal yields of $CD34^+$ and $CD34^-$sorted HUVECs were seeded separately into a 6-well plate. After 12 h of incubation, the culture medium above the cells was layered with mineral oil (Sigma-Aldrich) to prevent evaporation of the medium. The 6-well plate was placed at 37°C in an atmosphere containing 5% CO_2 under an inverted microscope (Leica) and phase contrast images were acquired at time intervals of 10 min for 48 h at 10 fixed spots in each cell culture. Time lapse movies were analyzed using custom-made software.

Statistical analysis

All cell culture experiments were performed in triplicate. Analysis of variance with significance $\alpha = 0.05$ was used for processing the data. Statistical significance was determined using a two-tailed Mann Whitney *U* test for qPCR data or a (paired) Student *t* test for FACS and live cell imaging data. Prism 5 software (GraphPad Software Inc, San Diego, CA, USA) was used for statistical analysis.

Results

CD34 is expressed on filopodia of endothelial cells in angiogenesis in vivo

Luminal endothelial CD34 staining was observed to be ubiquitous in quiescent small blood vessels and capillaries in non-pathologic human tissues. However, in human colon carcinoma, in addition to the luminal endothelial membrane staining, a striking CD34 staining of abluminal filopodia was observed, extruding from endothelial cells at sites of active angiogenesis (Fig. 1). In serial sections

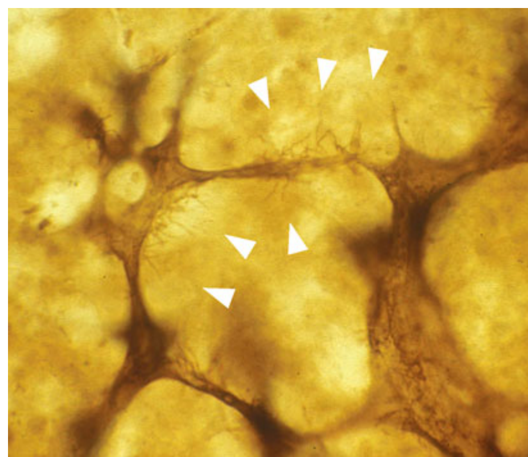

Fig. 1 CD34 is expressed on endothelial filopodial extensions at sites of active angiogenesis in vivo. Immunoperoxidase staining with anti-CD34 monoclonal antibody QBEND-10 of a section of surgically removed human colon carcinoma lesion. Anti-CD34 antibody stains capillaries and numerous endothelial filopodia (*arrowheads*). ×450

stained with anti-CD31 antibody, staining of filopodia was not observed (data not shown).

Morphological characterization of $CD34^+$ cells in vascular endothelial cell cultures

Employing flow cytometry, we found that the mean proportion of $CD34^+$ cells in HUVEC cultures from 5 different donors was 11.9% (SD 3.0; range 7.4–15.9%) at passage 3 (Fig. 2a). To study the morphological characteristics of the $CD34^+$ HUVECs in vitro, we examined HUVECs in a confluent monolayer. Immunofluorescence microscopy showed a small fraction of $CD34^+$ cells, whereas the vascular endothelial cell marker VE-cadherin was readily detectable in every cell (Fig. 2b). In the fraction of HUVEC cells that expressed CD34, CD34 staining was detected on all cell membrane domains, but these $CD34^+$ HUVEC cells had a distinct morphological appearance. They were often elongated, and demonstrated a polarized extension of CD34-marked filopodia located in one or two areas of their plasma membrane (Fig. 2b). These observations are in agreement with earlier reports describing that $CD34^+$ HUVECs have morphological characteristics similar to tip cells in vivo, which have filopodia at the leading front of the cells [7].

To rule out the possibility that the $CD34^+$ cells in HUVEC cultures were contaminating hematopoietic stem/progenitor cells and to confirm the endothelial nature and purity of cells, we showed co-expression of the endothelial markers CD31, VE-cadherin and CD146, and excluded expression of stem cell markers CD133 and CD117 and the myelomonocytic marker CD14 (Fig. 3).

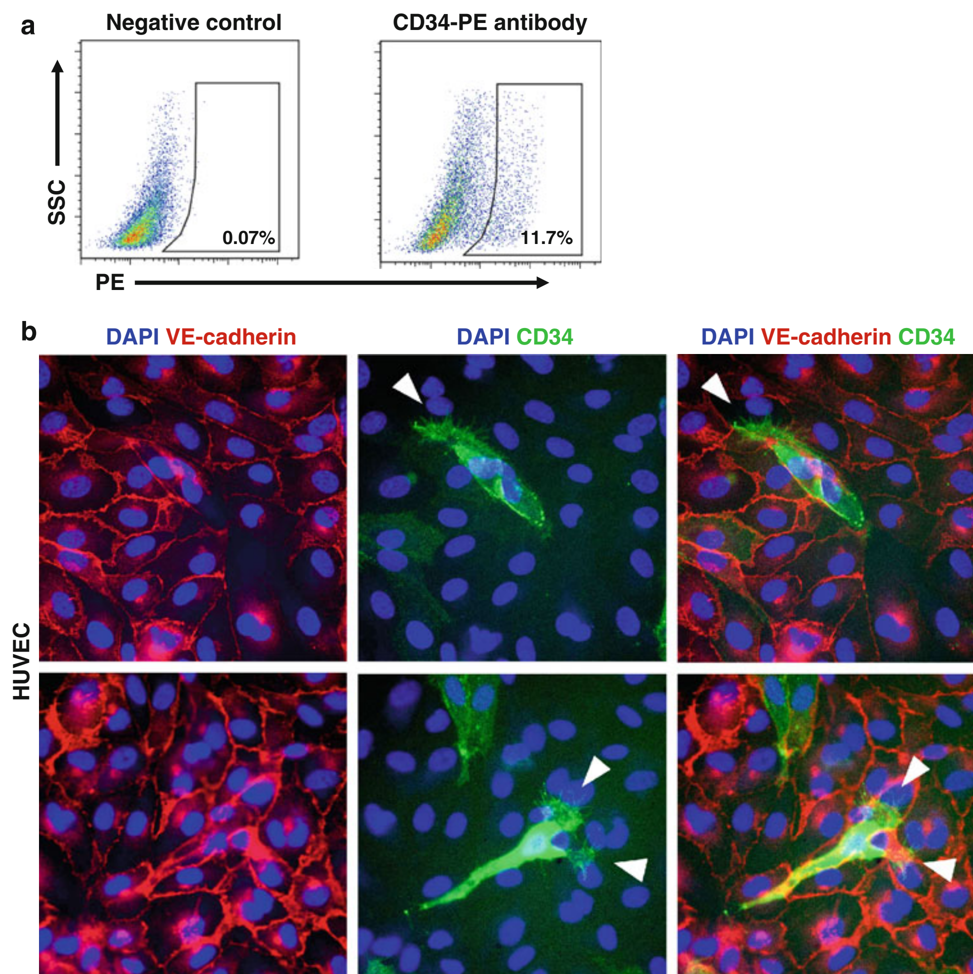

Fig. 2 Morphology of $CD34^+$ cells in HUVEC cultures shows a strong resemblance with that described for tip cells in vivo. **a** Flow cytometric dot plots of HUVECs of one representative donor demonstrating the proportion of $CD34^+$ cells in a HUVEC culture at passage 3. **b** Third passage HUVECs expressed VE-cadherin (*red*), but only a subset was stained for CD34 (*green*). Note filopodia extending from elongated $CD34^+$ HUVECs (*arrowheads*), whereas adjacent $CD34^-$ HUVECs show cobblestone morphology and lack of filopodia. DNA was stained with DAPI (*blue*). (Color figure online)

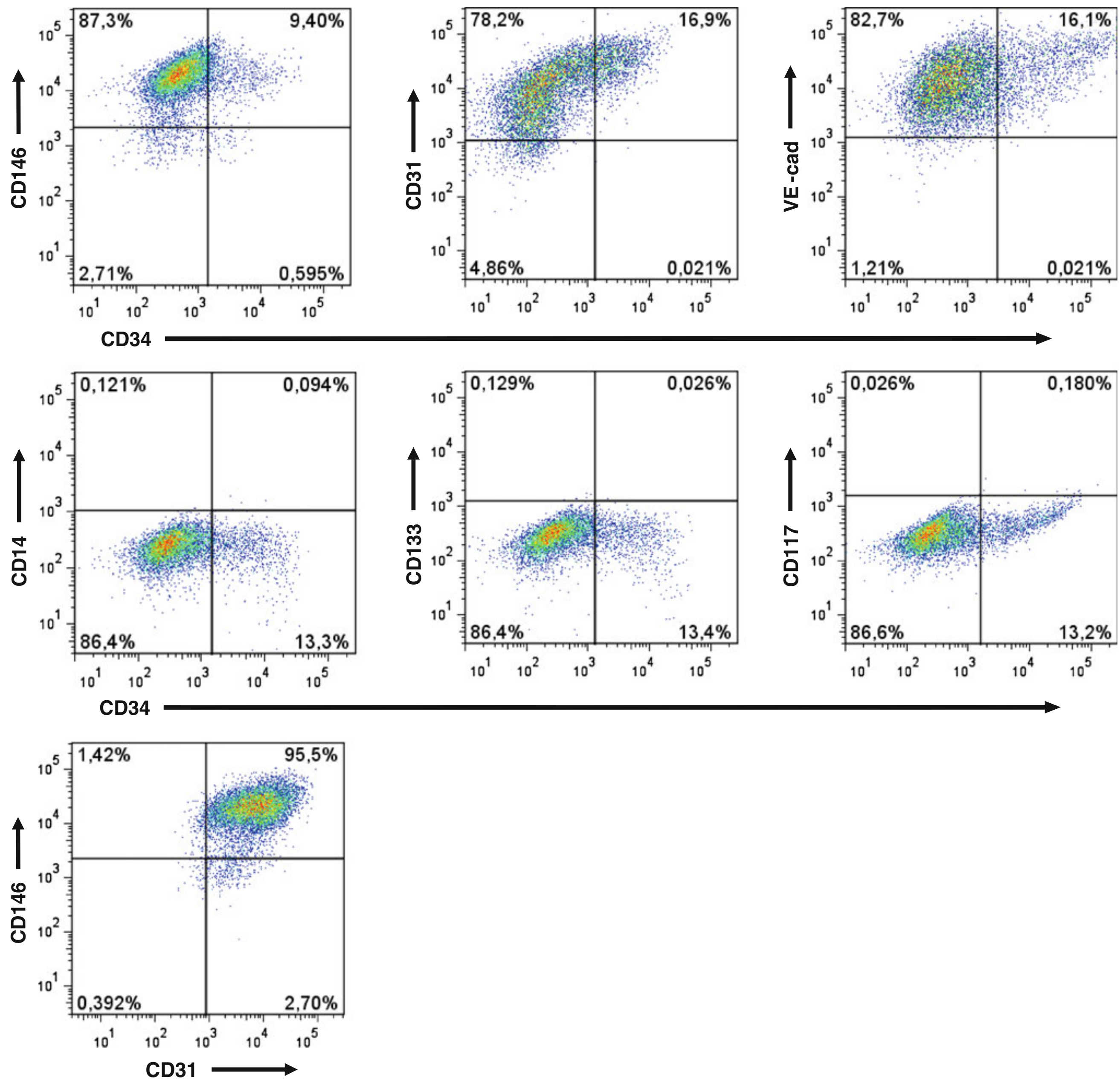

Fig. 3 $CD34^+$ cells in HUVEC cultures co-express endothelial markers. $CD34^+$ HUVECs were analyzed by 2-color flow cytometry for the expression of CD31, CD146 and VE-cadherin (endothelial cell specific markers), CD133 and CD117 (stem cell and endothelial progenitor cell specific markers), and CD14 (myelomonocytic specific marker). The results demonstrate that all $CD34^+$ cells are positive for CD31, CD146 and VE-cadherin, but negative for CD133, CD117 and CD14

$CD34^+$ cells are actively generated in endothelial cell cultures and show low proliferation activity

We investigated whether the $CD34^+$ phenotype in culture is reversible, as recent observations in vivo have demonstrated that the positions and phenotypes of tip cells and stalk cells in sprouting vessels are reversible [37]. We found that $CD34^-$ cells isolated from primary human endothelial cells were able to re-express CD34 when these cells were cultured again. After 10 days of culture, the relative subpopulation of $CD34^+$ cells was of a similar magnitude compared to unsorted HUVECs (Fig. 4a, b). Re-expression of CD34 in $CD34^-$ FACS-sorted cell cultures was also observed in 2 immortalized human endothelial cell lines, HMEC-1 and ECRF24 (data not shown).

Tip cells in vivo have low proliferation activity [3]. To further characterize the $CD34^+$ cell population with respect to proliferation activity, we sorted HUVECs by FACS,

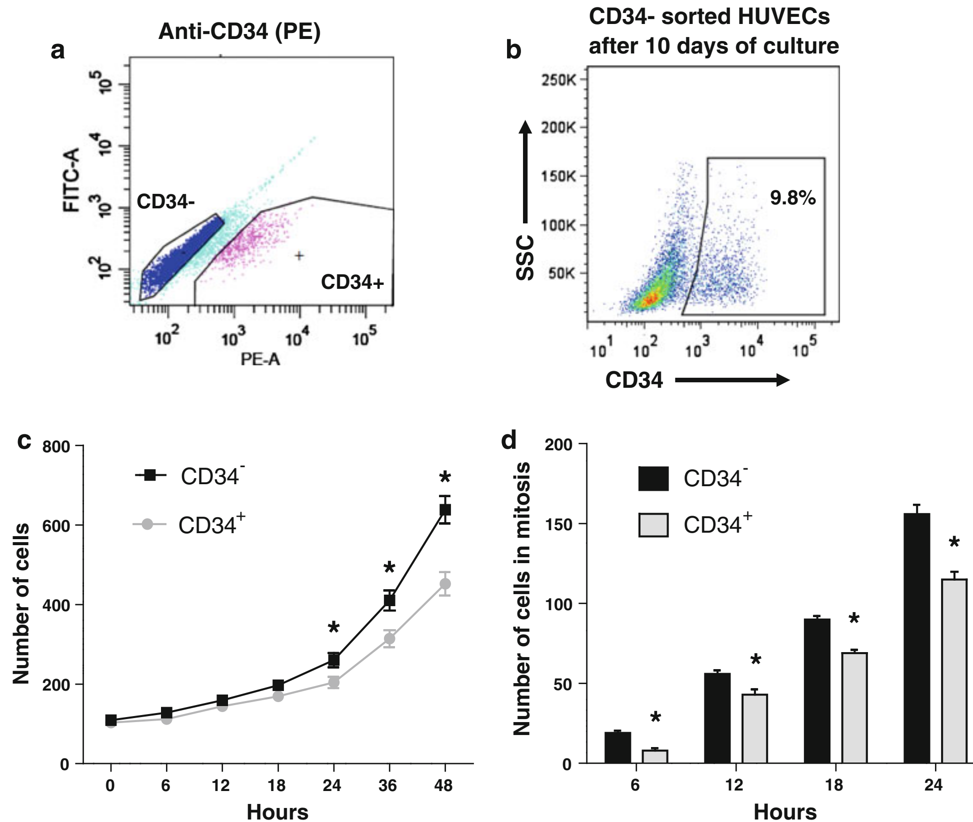

Fig. 4 CD34$^+$ cells in HUVEC cultures are actively generated and show a lower proliferation rate. **a** CD34$^-$ cells were sorted from HUVEC cultures using FACS and then cultured again. **b** After 10 days of culture, cells were analyzed for CD34 expression by FACS showing a subpopulation of 9.8% of CD34$^+$ cells. SSC, side scatter. CD34$^+$ and CD34$^-$ FACS-sorted HUVECs were cultured separately for 48 h and images were taken every 15 min at 10 fixed spots in each culture. Time-lapse movies were analyzed by counting the total amount of cells and the number of cells in mitosis, as recognized by the division of one cell into 2 daughter cells. **c** Numbers of cells at the given time points. **d** Total number of cells in mitosis. Shown are means and standard error of the means (SEM); * indicates $P < 0.05$, relative to CD34$^-$, determined by Student t test

cultured CD34$^+$ and CD34$^-$ cells separately and analyzed cell proliferation through live-cell imaging. CD34$^+$ HUVECs showed fewer cells in mitosis as compared to CD34$^-$ HUVECs (Fig. 4c, d). We detected no differences in cell cycle time between CD34$^+$ and CD34$^-$ cells.

CD34$^+$ phenotype in endothelial cell cultures is regulated by similar mechanisms that regulate the tip cell phenotype in vivo

Differentiation into the tip cell phenotype in vivo is controlled by a number of signaling pathways, including that of VEGF [2, 3, 38], TNF-α [39], and Notch ligand DLL4 [4]. In addition, binding of VEGF-A to VEGF receptor 2 (VEGFR2) in tip cells induces expression of DLL4, and subsequently DLL4 binds to Notch that is expressed on adjacent cells providing a signal inducing the stalk cell phenotype [4]. We determined whether the relative amount of CD34$^+$ cells in vitro is also controlled by these 3 mechanisms. Third-passage HUVECs stimulated with VEGF showed an increased percentage of CD34$^+$ cells whereas bFGF stimulation did not change the percentage of CD34$^+$ cells (Fig. 5a). For VEGF, the response was dose dependent with a maximum effect at 25 ng/ml. HUVECs continuously stimulated with TNF-α showed a reduced number of CD34$^+$ cells (Fig. 5b). In addition, we promoted the stalk cell phenotype by activating Notch signaling by culturing HUVECs on DLL4-coated dishes [25]. As predicted, this resulted in a decrease in the percentage of CD34$^+$ cells (Fig. 5c). However, the γ-secretase inhibitor DAPT, which inhibit Notch receptor cleavage and signaling and leads to elevated numbers of tip cells in vivo [9], did not increase the percentage of CD34$^+$ cells when added to HUVEC cultures.

Because monolayer HUVEC cultures may reflect only certain aspects of the angiogenic cascade, we subsequently studied CD34 expression on HUVEC in a 3-dimensional spheroid-based tube formation model [26, 40, 41]. Before embedding in collagen gel, HUVEC spheroids did not stain with anti-CD34 antibody (data not shown). After

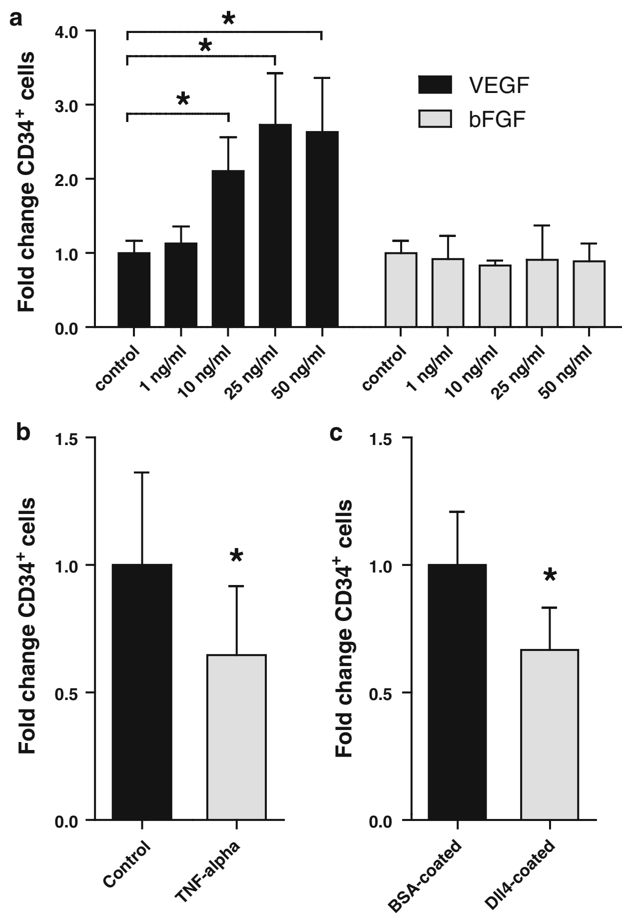

Fig. 5 The number of CD34$^+$ cells on HUVECs in vitro is regulated by mechanisms known to induce or inhibit the tip cell phenotype in vivo. The proportion of CD34$^+$ HUVECs was determined by flow cytometry after culturing of cells in the presence or absence of VEGF, bFGF, TNF-α or DLL4 for 48 h. **a** Dose-dependent increase of CD34 expressing cells when stimulated with VEGF but not bFGF. **b** Continuous stimulation with TNF-α decreased the number of CD34$^+$ HUVECs. **c** HUVECs cultured on DLL4-coated plates showed a lower number of CD34$^+$ cells. Shown are mean and SD; * indicates $P < 0.05$ relative to control, determined by Student *t* test for paired samples

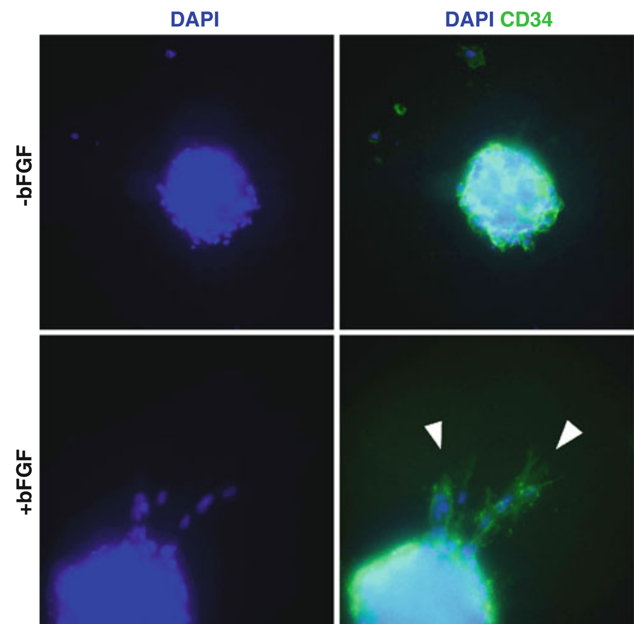

Fig. 6 CD34 on HUVECs in vitro is upregulated during angiogenesis and tip cell formation. 3-Dimensional in vitro angiogenesis of collagen-gel embedded HUVEC spheroids cultured in the presence or absence of bFGF after fixation and staining for the expression of CD34 (*green*). *Arrowheads* point at filopodial extensions of tip cells. (Color figure online)

embedding in collagen gel containing FCS, only cells at the surface of the spheroids re-expressed CD34 (Fig. 6). After exposure to bFGF to induce sprouting angiogenesis, CD34 was expressed in the newly formed sprouts and on the filopodial extensions of the leading tip cells (Fig. 6), mimicking the expression pattern observed in angiogenesis in vivo [7, 17, 19, 20].

Gene expression of tip cell markers is increased in CD34$^+$ endothelial cells in vitro

Besides expression of VEGFR2 and DLL4, other genes have been associated with the tip cell phenotype in vivo, including VEGFR3 [9, 11, 42], platelet-derived growth factor B (PDGFB) [3], unc-5 homolog B (UNC5B) [43], roundabout homologue 4 (ROBO4) [44], ephrin-B2 (EFNB2) [6, 10], and neuropilin-1 (NRP1) and NRP2 [45, 46]. Two more recent studies identified expression of chemokine receptor type 4 (CXCR4), apelin (APLN), and angiopoietin-2 (ANGPT2) to be enriched in tip cells [5, 8]. We analyzed the expression of these genes in FACS-sorted CD34$^+$ and CD34$^-$ subpopulations of HUVECs by real-time qPCR. mRNA expression levels of all 13 presently known tip cell markers appeared to be increased in CD34$^+$ cells, and 9 out of 13 were significantly ($P < 0.05$) over-expressed (more than 2-fold) as compared to CD34$^-$ cells (Table 1). We found a 121.8-fold overexpression of CD34 in CD34$^+$ HUVECs, but no significant changes in expression levels of the other two CD34-family members podocalyxin-like 1 (PODXL1) and PODXL2 (Supplementary Table 2) [14].

CD34$^+$ cells in HUVEC cultures are a model to study tip cell phenotype and function in vitro

To further test our hypothesis that CD34 uniquely marks endothelial cells with a tip-cell phenotype, and to further characterize gene regulation in CD34$^+$ HUVEC cells, we performed Affymetrix cDNA microarray analysis in CD34$^+$ versus CD34$^-$ cells. We found 441 genes to be differentially expressed (more than 2-fold) with statistical

Table 1 qPCR analysis comparing CD34$^+$ to CD34$^-$ sorted HUVECs revealed statistically significant enriched expression of 9 genes previously identified as tip cell markers in vivo. In bold, significant change

Gene symbol	Full name	Mean fold difference CD34$^+$ compared to CD34$^-$ HUVEC	*P* value
VEGFR2	Kinase insert domain receptor	5.9	**0.002**
VEGFR3	Fms-related tyrosine kinase 4	1.8	0.180
NRP1	Neuropilin 1	2.5	**0.013**
NRP2	Neuropilin 2	4.8	**0.003**
PDGFB	Platelet-derived growth factor beta polypeptide	2.1	0.180
DLL4	Delta-like 4	7.0	0.085
ANGPT2	Angiopoietin 2	8.1	**0.004**
APLN	Apelin	5.0	**0.025**
UNC5B	Unc-5 homolog B	4.1	0.475
ROBO4	Roundabout homolog 4, magic roundabout	2.0	**0.013**
PLXND1	Plexin D1	3.9	**0.006**
EFNB2	Ephrin-B2	4.4	**0.009**
CXCR4	Chemokine (C-X-C motif) receptor 4	41.9	**0.017**
CD34	CD34 molecule	121.4	**0.001**

significance (*Q* value <0.15), of which 172 were upregulated and 269 downregulated in the CD34$^+$ population (Fig. 7a, Supplementary Table 3). The series of 172 upregulated genes was enriched for GO terms consistent with the tip cell phenotype in vivo, such as angiogenesis, regulation of cell migration and blood vessel morphogenesis (Fig. 7b). GO term analysis of the series of 269 downregulated genes showed enrichment for cell cycle processes, an observation that is consistent with the absent proliferation activity observed in tip cells (Fig. 7b) [3]. Transcriptional levels of a selection of over 25 differentially expressed genes selected from the microarray data were validated and confirmed by qPCR in 7 HUVEC donors sorted for CD34 (Supplementary Table 2). The upregulated expression levels of known tip cell markers were confirmed in the microarray analysis, as 9 out of 13 were significantly (*Q* value <0.15) overexpressed in CD34$^+$ HUVECs (Supplementary Table 2).

We examined whether our microarray data show overlap with data of 2 microarray studies which investigated the tip and stalk cell transcriptome in vivo but employed different strategies [5, 8], and with a microarray study which investigated the effects of DLL4-Notch signaling in HUVECs [25]. GSEA showed that the gene sets positively correlating with a tip cell phenotype were significantly enriched ($P < 0.05$) in our CD34$^+$ gene set, confirming that at least some of the genes that are upregulated in CD34$^+$ HUVECs in our study are potentially bone fide tip cell markers. The highest correlation ($P < 0.001$) was found with the gene set of Del Toro et al. [5], who compared retinal endothelial cells isolated from the eyes of *Dll4+/−* and wild type mice.

Among the genes that showed upregulated expression in CD34$^+$ HUVECs were the secreted molecules insulin growth factor-2 (IGF2), insulin-like growth factor binding protein 4 (IGFBP4) and adrenomedullin (Supplementary Table 2). Furthermore, we found a marked upregulation of CXCR4 and its ligand CXCL12 (also known as SDF-1) (Supplementary Table 2).

Discussion

In this study, we demonstrate that cells with virtually all the known properties of tip cells are present in 2-dimensional vascular endothelial cell cultures and that they can be isolated on the basis of CD34 expression. We found CD34 expression in a subset of HUVEC, HMEC-1 and ECRF24 cultures, in comparable numbers as observed in previous studies [7, 22, 23]. We demonstrated that the morphology of CD34$^+$ cells in HUVEC cultures shows a strong resemblance to the appearance of tip cells in human tumors and developing mouse retina [3, 4, 6, 7, 9, 10]. Key features of this similarity include their highly polarized nature, numerous filopodia probing the environment, and migration toward an angiogenic stimulus. Many in vivo angiogenesis models rely on these typical morphological aspects when studying tip cells, but to our knowledge, tip cells have not previously been identified in endothelial cultures in vitro.

We show that the percentage of cells expressing CD34 in endothelial cell monolayers in vitro appears to be regulated, at least partly, by similar mechanisms reported to regulate the endothelial tip cell phenotype in vivo. These findings strongly support our hypothesis that CD34$^+$ cells in vitro represent true tip cells. In fact, stimulation of HUVECs with all three main known regulatory molecules of the tip cell phenotype, i.e. VEGF [3], TNF-α [39], and Notch ligand DLL4 [4], resulted in an increase or decrease of CD34$^+$ cells in HUVEC cultures as predicted by our hypothesis. The maximum number of CD34$^+$ cells that can be generated in a HUVEC monolayer by adding VEGF appeared to be limited in our model.

Both VEGF and bFGF are known to have potent angiogenic activities in vivo and in vitro. We observed in HUVEC monolayers that VEGF but not bFGF signaling promotes the tip cell phenotype. Untreated HUVECs in

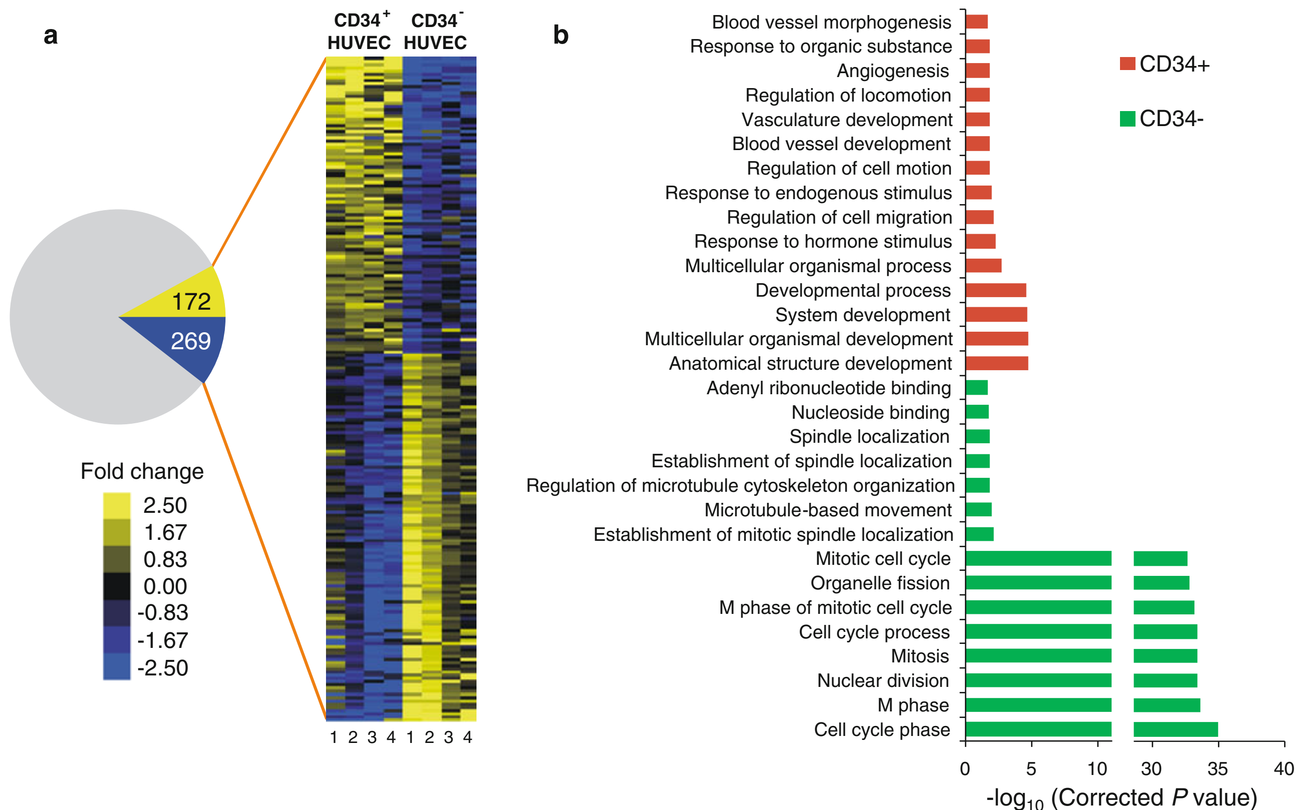

Fig. 7 Gene expression profiles of CD34$^+$ HUVECs show features of a tip cell phenotype. **a** Pie chart (*left*) illustrating the number of genes with significantly (Q value <0.15) altered expression (*total circle*) between CD34$^+$ and CD34$^-$ fractions, and the 441 genes with significantly (Q value >0.15) increased (*yellow part*) or decreased (*blue part*) expression by more than 2-fold. Heat map (*right*) of the 441 differentially expressed genes in 4 HUVEC donors sorted for CD34 (*yellow*, induced; blue, repressed; $\log_2$ based scale). **b** GO analysis reveals a significant association of CD34$^+$ cells with terms that are characteristic for tip cell functions and association of CD34$^-$ cells with terms of cell proliferation. *P* values represent a Benjamini–Hochberg–corrected EASE score. (Color figure online)

spheroids do not express CD34 [26, 40]. When we embedded HUVEC spheroids in collagen gel-containing FCS, CD34 expression was induced in endothelial cells at the surface of the spheroid by both VEGF and bFGF. Exposure to VEGF or bFGF induced three-dimensional in vitro sprouting angiogenesis in this assay, with CD34 expression on tip cell filopodia. This demonstrates that the tip cell phenotype and CD34 expression co-occur in angiogenesis in vitro. Our findings suggest that bFGF signaling alone stimulates angiogenesis but not necessarily the formation of tip cells. These differences in the effects of VEGF and bFGF stimulation in angiogenesis models in two and three dimensions require further study.

By culturing the FACS sorted CD34$^-$ and CD34$^+$ fractions of HUVECs, we found that the expression of CD34 and maintenance of the CD34$^+$ phenotype in vitro is an active and reversible process. Our results further indicate that formation of a subset of CD34$^+$ cells in vitro is a common feature of endothelial cell cultures, even in immortalized cell lines after many passages. These findings are in accordance with data from in vivo studies on tip cells, which showed that tip cells are actively generated and that a dynamic competition between endothelial cells to become a tip cell occurs continuously [37]. It also shows that in general, culture conditions employed in endothelial cell cultures can be regarded as a pro-angiogenic environment.

Comparison of isolated CD34$^+$ and CD34$^-$ HUVEC populations showed increased gene expression of all presently known tip cell markers in CD34$^+$ cells by qPCR. These results are again consistent with our hypothesis that this CD34$^+$ population represents tip cells in vitro. Microarray analysis of the CD34$^+$ and CD34$^-$ HUVECs further demonstrated that CD34$^+$ cells display a gene expression profile that is consistent with a tip cell phenotype. CD34$^+$ cells were enriched for genes associated with biological functions such as “angiogenesis”, “blood vessel development” and “regulation of cell migration”, and CD34$^-$ cells were enriched for genes associated with biological functions related to proliferation, including “cell cycle process” and “mitosis”. Furthermore, we found a significant correlation between our microarray analysis and

three gene sets from recent studies that investigated genomic profiles of vascular endothelial tip cells of various sources [5, 8, 25].

Our microarray analysis of the tip cell transcriptome as identified in CD34$^+$ HUVECs has yielded a wealth of new data. For example, inhibition of CXCR4 signaling was recently shown by others to lead to defects in retinal tip cell morphology and reduction of intercellular connections between tip cells [8]. Our gene expression profile of CD34$^+$ HUVECs confirmed this association. We observed co-expression of CXCL12 and CXCR4 which is consistent with the recent evidence indicating that the CXCL12-CXCR4 signaling axis has an important role in tip cell formation and promotes an autocrine signaling mechanism in endothelial tip cells [8]. The secreted molecules IGF2, IGFBP4 and adrenomedullin were among the genes upregulated in CD34$^+$ HUVECs. Consistent with this finding, secreted molecules also formed the largest cluster of tip cell-enriched genes identified by del Toro et al. [5], who suggested that these proteins are retained in the basal lamina surrounding stalk cells following tip cell to stalk cell transdifferentiation.

Our present study has characterized CD34$^+$ cells in HUVEC cultures and established an in vitro model for the study of tip cell biology. Our results confirmed previous findings that CD34 is expressed in endothelial cell filopodia at sites of active angiogenesis in vivo. We showed in normal and pathological human tissues, in addition to the overall luminal vascular staining, striking CD34 staining of endothelial filopodial extensions at sites of active angiogenesis in vivo. Our study did not aim at unraveling the function of CD34 expression in tip cells. Recent evidence has indicated that CD34 acts as an anti-adhesive molecule during lumen formation in developing blood vessels, by maintaining or promoting the separation between contralateral apical endothelial cell surfaces in a luminal tube [14, 47]. Although the function of CD34 in resting endothelial cells and in tip cells during angiogenesis was not addressed in the present study, we speculate that the proposed anti-adhesive property of CD34 may also play a role in maintaining vascular lumina in resting tissues, as well as in allowing the migration of tip cells and the movement and probing of their filopodia through the tissue matrix.

In summary, our findings suggest that the tip cell phenotype and CD34 expression co-occur in endothelial monolayers in vitro. Our results show that these CD34$^+$ cells in vitro are a valid tip cell model and that differential gene expression, functional characteristics and regulatory mechanisms of these cells are likely to predict properties of tip cells during angiogenesis. Along with the relative ease to obtain vascular endothelial cell cultures, our model allows for a convenient way to study effects of various types of blockers or promoters on tip cell phenotype and tip cell number. Furthermore, studies of the characteristics of CD34$^+$ cells in vascular endothelial cell cultures will open new avenues for the study of molecular processes and functions in angiogenesis in general, and of the specialized endothelial tip cells in particular. By characterizing the transcriptome of tip cells in vitro we have identified a large number of tip cell-associated genes not previously published, in addition to the known genes previously associated with tip cells in vivo. These findings expand the tip cell gene expression profile and may contribute significantly to our future understanding of tip cell biology and angiogenesis.

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Conflict of interest The authors declare no competing financial interests.

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