Biochemical and Biophysical Research Communications 443 (2014) 700–705

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Generation of functional endothelial-like cells from adult mouse germline-derived pluripotent stem cells

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ARTICLE INFO

Article history:
Received 3 December 2013
Available online 12 December 2013

Keywords:
Germline-derived pluripotent stem cells
Endothelial cells
Ischemia
Cell-based therapy
Arteriogenesis
Angiogenesis

ABSTRACT

Functional endothelial cells and their progenitors are required for vascular development, adequate vascular function, vascular repair and for cell-based therapies of ischemic diseases. Currently, cell therapy is limited by the low abundance of patient-derived cells and by the functional impairment of autologous endothelial progenitor cells (EPCs). In the present study, murine germline-derived pluripotent stem (gPS) cells were evaluated as a potential source for functional endothelial-like cells.

Cells displaying an endothelial cell-like morphology were obtained from gPS cell-derived embryoid bodies using a combination of fluorescence-activated cell sorting (FACS)-based selection of CD31-positive cells and their subsequent cultivation on OP9 stromal cells in the presence of VEGF-A. Real-time reverse transcriptase polymerase chain reaction, FACS analysis and immunofluorescence staining showed that the gPS cell-derived endothelial-like cells (gPS-ECs) expressed endothelial cell-specific markers including von Willebrand Factor, Tie2, VEGFR2/Flk1, intercellular adhesion molecule 2 and vascular endothelial-cadherin. The high expression of ephrin B2, as compared to Eph B4 and VEGFR3, suggests an arterial rather than a venous or lymphatic differentiation. Their capability to take up Dil-conjugated acetylated low-density lipoprotein and to form capillary-like networks on matrigel confirmed their functionality.

We conclude that gPS cells could be a novel source of endothelial cells potentially suitable for regenerative cell-based therapies for ischemic diseases.

1. Introduction

Vascular development, adequate vascular function and vascular repair are depending on endothelial cells. Accordingly, for vascular regenerative cell-based therapies, irrespective whether these are relying on single cell preparations [1] or tissue-engineered vascular grafts [2] functional endothelial cells are required. Currently, such applications are predominantly based on endothelial progenitor cells (EPCs) [1]. However, the purification of EPCs from peripheral blood or bone marrow is laborious. Furthermore, patient-derived EPCs [3,4] required for immunocompatible cell-based therapies for ischemic diseases such as coronary heart disease and peripheral artery disease are often functionally impaired, low in number and have a limited proliferation potential *in vitro* [5,6]. Therefore, the evaluation of alternative sources of therapeutically suitable endothelial cells is required.

The prototypical pluripotent embryonic stem (ES) cells have been demonstrated already to be a suitable source for the derivation of endothelial cells. The transplantation of such cells generated from murine [7] as well as human embryonic stem cells [8] improved blood perfusion in mouse models of cardiac [7] and hindlimb [8] ischemia. However, concerns remain with respect to their use in clinical applications due to ethical implications and immunological problems. Germline-derived pluripotent stem (gPS) cells [9] could be an alternative source for endothelial cells. The transplantation of gPS cell-derived cells is supposed to be basically devoid of the risk of immune rejection, as their establishment would be based on immunocompatible donor testicular tissue or even autologous testicular biopsies. Therefore, we investigated whether functional endothelial-like cells potentially suitable for therapeutic applications can be derived from murine gPS cells as a model for the human counterparts which still remain to be established.

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http://dx.doi.org/10.1016/j.bbrc.2013.12.035

2. Materials and methods

2.1. Cell culture

Murine gPS cells and OP9 cells were cultivated as previously described [9,10]. To induce differentiation, embryoid bodies (EBs) were generated. On day 0 of differentiation, gPS cells were seeded at a density of 1×10^6 cells/ml in 10-cm bacteriological dishes (Sarstedt, Nümbrecht, Germany) in differentiation medium consisting of Iscove's medium (PAA, Cölbe, Germany) supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Biowest, Nuaillé, France), L-glutamine with penicillin/streptomycin (stock solution: 100×; PAA, Cölbe, Germany), 10 μM β-mercaptoethanol (Gibco, Berlin, Germany), and 1% (v/v) NEA non-essential amino acids stock solution (100×; Gibco, Berlin, Germany).

2.2. Enrichment of gPS-ECs

On day 5 of differentiation, the EBs were incubated with acutase (PAA, Cölbe, Germany) to create a single-cell suspension. CD31-positive cells were isolated from the cell suspension by fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) using a phycoerythrin (PE)-conjugated anti-CD31 antibody (Biozol, Eching, Germany). Subsequently, the isolated cells were plated onto OP9 cells and maintained in alpha-Minimum Essential Medium supplemented with 10% FBS, L-glutamine with penicillin/streptomycin (100× stock solution), 10 μM β-mercaptoethanol, and 50 ng/ml of recombinant mouse vascular endothelial growth factor-A (VEGF-A_{164}) (Prospec, Rehovot, Israel). Endothelial colonies were then plated onto gelatin- or collagen IV-coated cell culture dishes and maintained in the above-mentioned medium.

2.3. Real-time RT-PCR

RNA was extracted from gPS cells, 2-day-old EBs, 5-day-old EBs, and gPS-ECs using the RNeasy Mini-kit (Qiagen, Hilden, Germany) and reverse transcribed using a cDNA synthesis kit (Applied Biosystems, CA, USA). The amplification was performed on the ABI prism 7500 Fast Sequence Detection System (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Real-time RT-PCR analysis was carried out for each sample in triplicates. GAPDH was used as an internal control. Relative gene expression values were calculated by normalizing Ct (threshold cycle) values of the target genes with that of the housekeeping gene (GAPDH) using the $\Delta\Delta C_t$ method. Specific primers used are listed in Table 1.

2.4. Flow cytometric analysis

Flow cytometric analysis of the cells was performed on a FACS Calibur (BD Bioscience, Heidelberg, Germany). The antibodies used in these FACS experiments were: PE-conjugated anti-stage-specific embryonic antigen1 (SSEA1; BD Bioscience, Heidelberg, Germany), PE-conjugated anti-CD31 (Biozol, Eching, Germany), PE-conjugated anti-Flk1 (eBioscience, Hatfield, United Kingdom), anti-vascular endothelial-cadherin (VE-Cadherin; gift from Prof. Vestweber, Max-Planck-Institute for Molecular Biomedicine; Münster, Germany), anti-Tie2 (gift from Prof. Vestweber), anti-von Willebrand Factor (vWF; DAKO, Glostrup, Denmark), PE-conjugated IgG control, rat IgG control (BD Bioscience, Heidelberg, Germany), and Alexa 488-conjugated anti-rat IgG antibody (Invitrogen, Karlsruhe, Germany).

2.5. Immunofluorescence staining

The cells were fixed with 4% paraformaldehyde (PFA) for 10 min and permeabilized with 0.5% triton-X for 5 min at room temperature (RT). The fixed cells were incubated with 3% BSA in PBS for 30 min to block non-specific binding and stained with the primary antibodies anti-VE-Cadherin (gift from D. Vestweber, Münster) and anti-vWF (DAKO, Glostrup, Denmark) for 1 h at RT. Bound antibodies were visualized using Alexa 488-conjugated goat anti-rat IgG secondary antibody (Invitrogen, Karlsruhe, Germany). Incubations with the secondary antibody were carried out for 1 h at RT. The stained cells were then examined under a Leica microscope (Leica Microsystems, Heidelberg, Germany).

2.6. In vitro angiogenesis assay in matrigel and staining with BS-1 lectin

To induce tube formation, the cells were plated in matrigel-coated 24-well culture dishes [11] and cultivated in medium containing 50 ng/ml of VEGF. Cell morphology was observed under a Leica microscope and cell staining was performed using tetramethylrhodamine isothiocyanate (TRITC)-conjugated *Bandeiraea simplicifolia*-1 (BS-1) lectin (Sigma–Aldrich, Schnelldorf, Germany) [12].

2.7. Cellular uptake of Dil-acetylated low-density lipoprotein (Dil-Ac-LDL) [13]

The cells were incubated with 10 μg/ml of acetylated low-density lipoprotein labeled with 1, 1′ dioctadecyl-3, 3, 3′,

Table 1
Sequences of oligonucleotide primers.

Gene	Forward primer	Reverse primer
GAPDH	TGGTTCCAGTATGACTCCACTCAC	GATGACAAGCTTCCCATTCTCG
Tie2	TTGAAGTGACGAATGAGAT	ATTTAGAGCTGTCTGGCTT
VE-Cadherin	GAATGACAACCCTCCGGAAT	TCCTCGTTCTTCAGGGCAAA
CD31	TGTCATTGGAGTGGTCATCG	GGCTTCCACACTAGGCTCAG
Flt1	CTCTGATGGTGATCGTGG	CATGCGTCTGGCCACTTG
vWF	AGGGCTGGAGTGTGCTAAGA	TACCAATGGCAGAtGCAAGTG
ICAM2	ACTCCACAGACCCCACAGAC	ATGGCAAAAGAAGACCGTGT
Flk1	CCCGCATGAAATTGAGCTAT	AAACATCTTCGCCACAGTCC
VEGFR3	CGATGCCCTGTACCTGCAGTG	CCGCTGGCAGAACTCCTCATTG
EphB4	GTCCACCGAGACCTGGCTG	GGGCCGGGCATTCCGGTC
Ephrin B2	AACCAGGAGGGAGGGGTGTG	GACAGCGTGGTCGTGTGCTG
Nkx2.5	CAAGTGCTCTCCTGCTTTCC	CATCCGTCTCGGCTTTGT
SM22α	CACCTGGCACTCTCCACCTTC	GATTTCATCCCACTACCGAAAG
Oct4	AGTATGAGGCTACAGGGACACCTTTC	GGACTGAGTAGAGTGTGGTGAAGTGG
Nanog	CTGCTCCGCTCCATAACTTCG	AATGCGCATGGCTTTCCCTA
CD34	ATGTCCGGCCTTCTCCTATT	CCCAAAGGTCAGAGATTGGA

3′-tetramethyl-indo-carbocyanine-perchlorate (Dil-Ac-LDL; Biomedical Technologies Inc., Stoughton, MA, USA) for 4 h at 37 °C. After washing three times with PBS, the cells were fixed with 4% paraformaldehyde for 10 min at RT and mounted with 4′-6-Diamidino-2-phenylindole (DAPI)-containing mounting medium [14]. The images of Dil-labeled cells were obtained using a Leica microscope.

3. Results

3.1. Differentiation of gPS cells into EC-like cells

CD31-positive cells obtained by FACS on day 5 of differentiation (see Section 2) were subcultured on OP9 cells in the presence of VEGF. After 43 days, several colonies adopted a uniform cobblestone-like morphology similar to that of endothelial cells. These endothelial cell-like colonies were transferred into gelatin-coated cell culture dishes, cultured with VEGF supplementation for another 13 days and expanded further on gelatin- or collagen IV-coated cell culture dishes, again with VEGF supplementation. The cells readily formed monolayers and displayed a cobblestone morphology, typical of endothelial cells [15] (Fig. 1A and B). They were maintained in culture for up to 11 passages without loss of their specific characteristics.

3.2. Expression of endothelial cell-specific markers in gPS-ECs

Real-time RT-PCR analysis revealed the expression of the mRNAs encoding various proteins typical for endothelial cells including VE-Cadherin (CD144), Flk1 (Fig. 1C), ICAM2, Tie2, Flt1, CD31 and vWF (data not shown) in gPS-ECs. Furthermore, an increased level of the mRNA encoding the arterial marker ephrin B2 as compared to those of the mRNAs encoding the lymphatic marker VEGFR3 or the venous marker Eph B4, respectively, were detected in gPS-ECs (Fig. 1D). In contrast to the general and arterial endothelial markers, the mRNAs encoding the smooth muscle cell marker SM22α, the cardiomyocyte progenitor cell marker Nkx2.5, the EPC marker CD34 and the pluripotency markers Oct4 and Nanog were either not or only minimally expressed in gPS-ECs (not shown).

3.3. Flow cytometric and immunofluorescence analysis of gPS-ECs

FACS analysis showed that gPS cell-derived cells expressed endothelial cell-specific markers including Tie2, Flk1, CD31 (Fig. 2), VE-Cadherin and vWF (data not shown), whereas the monocytic/EPC markers CD14 (Fig. 2) and CD34 (not shown) were not detected. Furthermore, the murine ES cell marker SSEA1 was not detected (Fig. 2) probably excluding a significant contamination with undifferentiated cells. Consistent with the FACS data, immunofluorescence staining demonstrated that VE-Cadherin was expressed at the cell adherens junctions and vWF (Fig. 3), a glycoprotein synthesized by endothelial cells, was also expressed by the gPS-ECs [16].

3.4. In vitro vasculogenic potential of gPS-ECs

To test the functionality of gPS-ECs *in vitro*, LDL uptake [17] and tube formation assays [18] were performed. The cells incorporated Dil-Ac-LDL (Fig. 4A–C), and formed tube-like networks in matrigel in the presence of VEGF (Fig. 4D). These tubular networks displayed BS-1 lectin binding (Fig. 4E), another characteristic of endothelial cells [19].

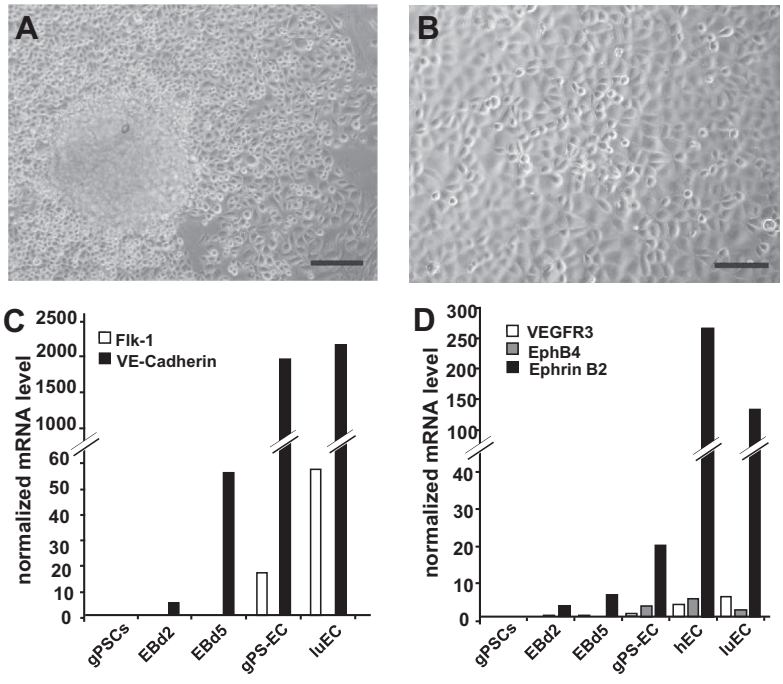

Fig. 1. Morphology and real-time RT-PCR analyses of the expression of marker genes in gPS-ECs. The gPS-ECs show a cobblestone-like morphology, typical of endothelial cells (A, B; scale bars indicate 250 μm). RT-PCR analysis revealed the expression of the indicated general endothelial cell marker genes (C) as well as endothelial subtype-specific marker genes (D). Analyses were performed in gPS-ECs, undifferentiated gPS cells, day 2 and day 5 gPS cell-derived EBs (EB d2/d5), and (where indicated) in heart and/or lung endothelial cells (h/lu ECs). All indicated mRNA levels represent relative values that were calculated by normalization to the levels of the respective mRNAs in undifferentiated gPS cells.

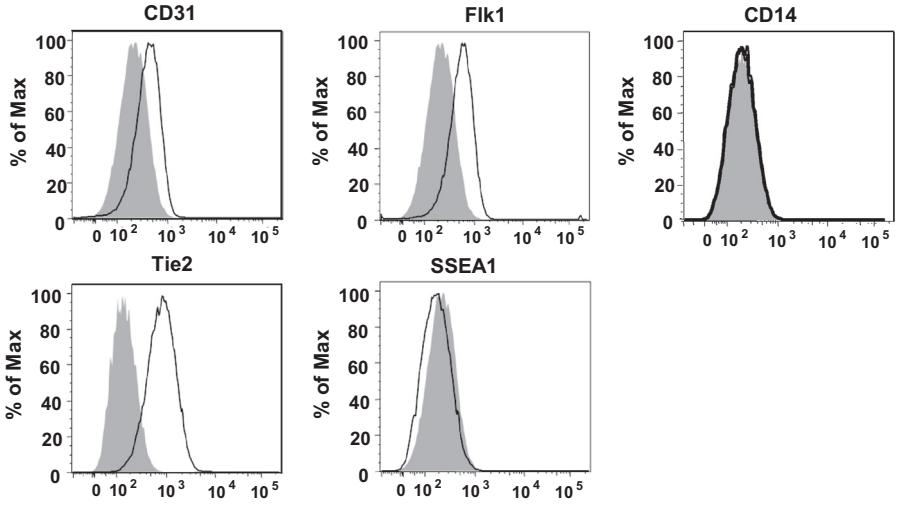

Fig. 2. FACS analyses. Detection of the indicated proteins on the surface of gPS-ECs by FACS analysis; grey: IgG isotype controls.

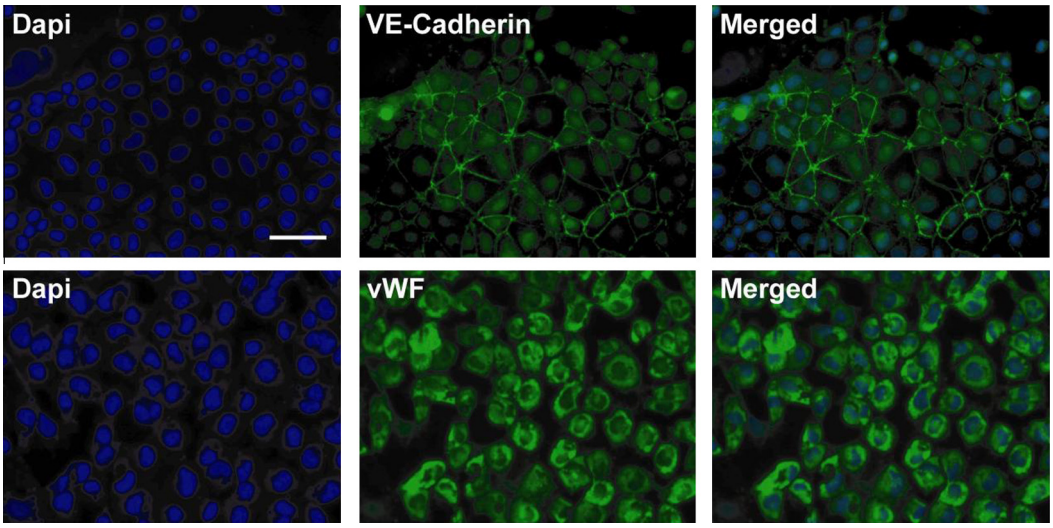

Fig. 3. Immunofluorescence analyses. Immunofluorescence detection of vascular endothelial-cadherin (VE-Cadherin) and von Willebrand Factor (vWF). Scale bar indicates 50 μm.

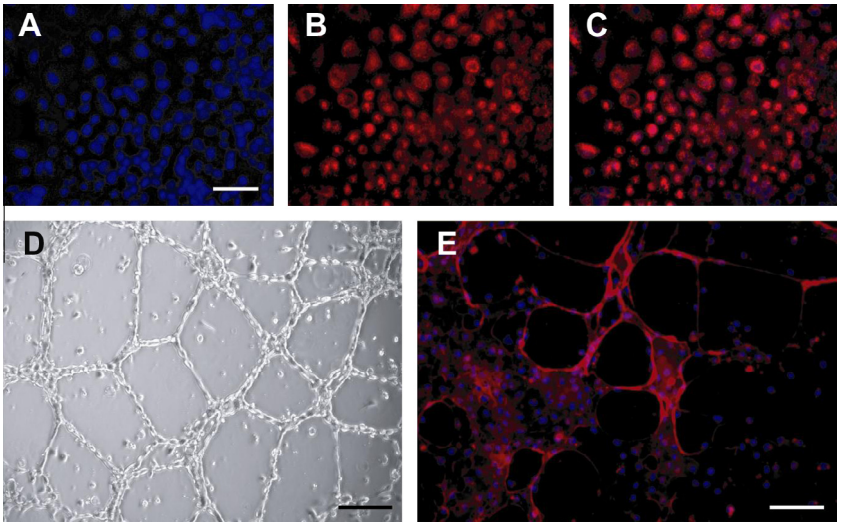

Fig. 4. *In vitro* functional analyses. Internalized DiI-conjugated acetylated low-density lipoprotein (DiI-Ac-LDL) in gPS-ECs. Panel A, DAPI-stained nuclei, B, DiI-Ac-LDL fluorescence, C, merged images. Tube-like structures in matrigel (D), stained by fluorescently labelled *Bandeiraea simplicifolia*-1 (BS-1) lectin (E). Scale bars indicate 50 μm (A–C) and 250 μm (D, E).

4. Discussion

In contrast to the differentiation of mouse- and human ES cells into endothelial cells, which has been reported repeatedly [7,8], the derivation and characterization of EC-like cells from gPS cells is reported here for the first time. This was achieved by the application of an embryoid body-based differentiation protocol in combination with FACS, relying on the detection of the endothelial marker CD31, and subsequent cultivation of the selected cells on OP9 stromal cells with VEGF-A supplemented medium to further support differentiation. CD 31 is a widely used marker for endothelial cells [20] and the potential of mouse ES cell-derived CD31-positive cells to differentiate into endothelial cell-like cells *in vitro* has been shown already [21,16].

The gPS cell-derived cells display a cobblestone-like morphology which resembles the morphology of primary endothelial cells and clearly differs from that of smooth muscle cells, which can also be derived from pluripotent stem cells [22,23] but have a more spindle-like shape [24]. This is consistent with the significant expression of various endothelial cell markers including Tie2, CD31, VE-Cadherin, vWF, Flk1, Flt1, and ICAM2 [25] and the very low degree or even lack of expression of smooth muscle cell markers including αSMA(α-smooth muscle actin) and SM22α [22] on the RNA and/or protein level, respectively.

In previous studies, CD14 and/or CD34 have been used as markers for the identification of human [26,27] as well as murine [28,29] EPCs. As gPS-ECs do neither express relevant levels of CD14 nor CD34, they are closer to mature endothelial cells than to immature EPCs. Furthermore, the lack/low level of CD14 expression argues against a monocytic phenotype of the gPS-ECs [30].

With respect to the endothelial subtype of the gPS cell-derived cells the realtime RT-PCR data for ephrin B2 (arterial marker), Eph B4 (venous) and VEGFR3 (lymphatic) mRNAs suggest a more arterial than venous or lymphatic character of the cells. It has been shown for ES cell-derived EC-like cells that the supplementation of the medium with VEGF-A alone preferentially supports (default) venous differentiation whereas arterial differentiation appears to require activation of Notch signaling which, in turn, can be experimentally achieved by stimulating the cAMP pathway using the cAMP homolog 8bromo-cAMP [31] (for review see [32]). As the protocol applied here did not include an experimental activation of Notch signaling it can be assumed that it was endogenously activated for unknown reasons. Lymphatic endothelial differentiation of ES cells on OP9 stromal cells has been shown to require VEGF-C supplementation [32]. This might explain why this direction of differentiation was not supported by the protocol employed in this work.

In addition to the endothelial cell-typical pattern of marker gene expression, gPS derived cells displayed the uptake of Dil-ac-LDL and formed endothelial tubes in matrigel. Both, LDL-uptake and tube formation *in vitro* are related to important functional properties of endothelial cells which are not necessarily displayed by endothelial-like cells derived from pluripotent stem cells [33].

In summary, apparently functional endothelial cell-like cells potentially suitable for basic research on vascular function and development as well as for investigations providing proof of concept for the use of human gPS-ECs in therapeutic applications were derived from gPS cells.

Acknowledgments

This work was supported by the Kompetenznetzwerk Stammzellforschung NRW (S.N., R.K.), Innovative Medical Research IMF (S.E., S.N., R.K.), the Bundesministerium für Bildung und Forschung BMBF (K.K., S.N., R.K.). We thank Prof. Hans R. Schöler, Max Planck Institute for Molecular Biomedicine, Münster, Germany, for financial support (DFG grant SPP 1356 "Pluripotency and Cellular Reprogramming"; SCHO 340/5-1) as well as for scientific discussions and proof reading. The anti-VE-Cadherin antibody was a kind gift from D. Vestweber, Max Planck Institute for Molecular Biomedicine, Münster, Germany. The funding sources were not involved in study design, the collection, analysis and interpretation of data, the writing of the report and the decision to submit the article for publication.

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