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VEGF/PKD-1 signaling mediates arteriogenic gene expression and angiogenic responses in reversible human microvascular endothelial cells with extended lifespan

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Abstract

Microvascular ECs (MVECs) are an ideal model in angiogenesis research. The aim of this study was to determine vascular endothelial growth factor (VEGF)/protein kinase D1 (PKD-1) signaling in expression of arteriogenic genes in human MVECs. To achieve this aim, we transduced specific SV40 large T antigen and telomerase into primary human dermal MVECs (HMVEC-D) to establish reversible HMVECs with extended lifespan (HMVECi-D). HMVECi-D was then exposed to VEGF/VEGF-inducer GS4012 or transduced with constitutively active protein kinase PKD-1 (PKD-CA). Quantitative RT-PCR was performed to detect arteriogenic gene expression. Furthermore, the angiogenic capacity in response to VEGF pathway was evaluated by Matrigel tube-formation and proliferation assays. We observed that VEGF/PKD-1 signaling axis significantly stimulated the expression of arteriogenic genes and promoted EC proliferation, along with downregulation of CD36 expression. Intriguingly, overexpression of PKD-CA also resulted in formation of tip cell morphology, accompanied by increased mRNA of delta-like ligand 4 (DLL4). In conclusion, we have successfully established and characterized HMVECi-D, and showed that VEGF/PKD-1 signaling axis increases angiogenic and arteriogenic gene expression. These studies suggest that the axis may regulate arteriolar differentiation through changing MVEC gene expression.

Keywords Angiogenesis · Arteriolar differentiation · CD36 · Microvascular endothelial cells · Protein kinase D · VEGF

Introduction

The endothelial cells (ECs) constituting the endothelium are morphologically and functionally different in order to meet the unique needs of the underlying tissues [1–3] through arteriogenesis (formation of small arteries), venogenesis (formation of new veins), or angiogenesis (formation of capillaries) [4–6]. Different organs have different types of ECs: heart ECs are different from lung and brain ECs; capillary ECs are different from arterial and venous ECs in terms of molecular markers and phenotypes [1, 4, 7–9]. The EC heterogeneity determines whether vascular network is developed into arteries, veins, or capillaries and is associated with VEGF signaling [4, 7, 8, 10–12]. EC differentiation plays an important role in these processes. Our recent studies suggest that protein kinase D1 (PKD-1)-mediated transcriptional reprogramming is essential for microvascular EC (MVEC) differentiation into arteriolar ECs and improvement of blood perfusion in ischemic tissues via the formation of arterioles [9, 13]. However, it remains largely unknown as to whether and how terminally differentiated ECs are reprogrammed

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and differentiated into other types of ECs for arteriolar differentiation and functional angiogenesis. Furthermore, MVECs may be used as an important model in angiogenesis research because angiogenic processes mainly occur in the microvasculature.

To further understand molecular mechanisms of EC differentiation and discover an affordable and ideal in vitro EC model system for studying angiogenesis, we transduced both large T antigen of SV40 [14] and telomerase [15] into primary human dermal MVECs (HMVEC-D), and successfully established a reversible HMVEC line with extended life spans (HMVECi-D). This conditional immortalization of HMVECi-D not only showed long lifespan but also was able to be reversed to primary HMVECs by removing the transduced genes via expression of a Cre recombinase. Moreover, VEGF/PKD-1 signaling significantly increased the expression of a few key arteriogenic genes including delta-like ligand 4 (DLL4) and neuropilin 1 in HMVECi-D, along with downregulation of CD36 expression. These results suggest that the VEGF/PKD-1 signaling axis may be critical to EC transdifferentiation into arteriolar ECs. Furthermore, HMVECi-D showed typical angiogenic functions in response to angiogenic factors, indicating that HMVECi-D may be an optimal in vitro model system in studying EC differentiation and angiogenesis.

Materials and methods

Reagents and antibodies

VEGF and Matrigel were from BD Biosciences. FITC anti-human CD36 (Clone 5-271) antibody was purchased from the BioLegend. The anti-VEGFR2 antibody (SAB4501645) was purchased from the Sigma-Aldrich. Anti-PECAM/CD31 antibody was kindly provided by Peter Newman laboratory (Blood Research Institute, BloodCenter of Wisconsin). The PKD inhibitor CID 755673 was purchased from Selleck Chemicals, and GS4012 from Calbiochem. Lentivirus was prepared by Vector Core at Blood Research Institute, BloodCenter of Wisconsin and Medical College of Wisconsin. The RNeasy Mini Kit, the cDNA preparation kit, RT^2 qPCR Primer Assay for human NRP1, DLL4, CD36, PPIA, PrimeTime® qPCR probe-based assays, GAPDH, and HPRT1 were from QIAGEN or Integrated DNA Technologies. qPCR Master Mix was purchased from ABI, MIDSCI or Integrated DNA Technologies. Alexa Fluor secondary antibodies were from Invitrogen. Mounting Medium with 4, 6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories.

Reversible extension of EC lifespan by lentiviral transduction of SV40 antigen and telomerase

The approach was established in reference to previous studies [16, 17]. Briefly, HUVECi and HMVECi cells were prepared by two rounds of lentiviral transduction using first pLOX-Ttag-IRES-TK (Addgene plasmid 12246), followed by pLOX-TERT-IRES-TK (Addgene plasmid 12245), which were provided by Didier Trono laboratory. Approximately 2×10^5 cells were plated in one well of a 6-well plate and transduced with pLOX-Ttag-IRES-TK Lentivirus at a multiplicity of infection (MOI) of 10. Two weeks post transduction, approximately 2×10^5 cells were plated in one well of a 6-well plate and transduced with pLOX-TERT-IRES-TK at an MOI of 10. Doubly transduced cells were kept in culture, with aliquots being frozen at passage 15 and passage 18 for long-term storage and experiments. Reversal of immortalization was achieved by transduction with pLM-CMV-R-Cre, which was provided by Michel Sadelain laboratory (Addgene plasmid 27546). Cells were transduced at MOI 10. An EVOS®FL cell imaging system was used to determine transduction efficiency by observing cherry expression.

Endothelial and tumor cell culture

Primary HMVEC-Ds were grown in a condition as described previously [8]. Primary HUVECs were obtained from Hybridoma Core, Blood Research Institute, the BloodCenter of Wisconsin and grown in media for HUVEC culture (Lonza). The transformed cells HMVECi-D and HUVECi were grown in the same media for primary HMVECs and HUVECs. Highly aggressive skin cancer cells with both CD44 and ALDH1A1 positive were screened from malignant melanoma cell line B16/F10 (ATCC), and aggressive estrogen-positive breast adenocarcinoma cells E0771 were provided by Dr. Kamalakannan Rajasekaran (Blood Research Institute, BloodCenter of Wisconsin) and selected with both CD44 and ALDH1A1 positive staining after sustained starvation for two weeks. These cells were cultured as previously described [10, 18].

Plasmid transduction

A non-inducible lentiviral system was used to establish stable transduction of GFP control and constitutively active PKD-1 (PKD-CA) conjugated to GFP in ECs. ECs were transduced with lentiviral particles containing PKD-CA: GFP or GFP of 5–10 MOI. An EVOS®FL cell imaging system was used to determine transduction efficiency.

Three-dimensional cell culture

Warm 1% agarose in 1× PBS for EC culture (Certified™ Low Range Ultra Agarose, Bio-Rad Laboratories, Inc.) solution was put into the wells in a six-well plate with a hot micropipette tip and underwent gelation at room temperature. Cells of 0.5–1 × 10^6/mL were then suspended in warm agarose, dispensed onto the pre-coated wells, and incubated at 37 °C, during which cells got embedded within the matrix upon gelation [9, 19, 20]. The cells at indicated time points were directly used for imaging with an EVOS®FL cell imaging system.

DiI-Ac-LDL uptake assays

Ac-LDL uptake in HMVECi-D and HUVECi was examined based on the protocol from an assay kit (Cell Applications, Inc.). Cells were grown in an extracellular matrix-coated 96-well plate and grown to approximately 75–95% confluence, and were incubated with DiI-Ac-LDL at 37 °C in EC media for 4 h. The media were then removed; the cells were washed, and fixed with 4% PFA (Electron Microscopy Sciences, PA). Images were taken using an EVOS®FL cell imaging system.

Matrigel tubule formation assays

Approximately 2500 cells were plated on 48-well culture plates coated with growth factor reduced Matrigel (100 µl/well; BD). The cells were cultured in complete EC growth media. After 48 h, the formed cellular networks were imaged either directly or after staining with Alexa Fluor 568 Phalloidin (1:200 dilutions; Invitrogen) and visualized by an EVOS®FL cell imaging system.

Real-time RT-qPCR

Gene expression was assessed by real-time RT-qPCR as previously described [21]. Relative quantitation (RQ) experiments were designed and performed in a QuantStudio 6 PCR system. The RT^2 qPCR primer assays or PrimeTime® qPCR probe-based assays for the target genes and housekeeping genes were used for PCR reactions and data acquisitions. Total RNA was isolated from ECs using the RNeasy Mini Kit and then subjected to real-time RT-qPCR. GAPDH, HPRT1, or PPIA transcripts were amplified in separate wells for normalization of variances in input RNA. The relative Ct value was used to compare the fold or quantitative change of mRNA expression.

Immunofluorescence assays

Cultured cells were fixed in 4% paraformaldehyde in 1× PBS for 2 h at room temperature, and permeabilized in 0.1% Triton X-100 and blocked in 1× PBS containing 3–5% BSA for 60 min at room temperature. Cells were then incubated with primary antibodies (1:250 to 1:500 dilutions) overnight at cold room followed by incubation with alexa-conjugated secondary antibodies (1:500 dilutions) for 1 h at room temperature. The cells without incubation with the primary antibodies served as a negative control. The presence of PECAM/CD31, VEGFR2, and CD36 in ECs was detected using relevant antibodies after fixing with 4% PFA and mounted with aqueous mounting medium with DAPI. The images were acquired by using an EVOS®FL cell imaging system.

Proliferation assay

The HMVECi-Ds were seeded onto 48- or 96-well plates in the presence of VEGF or GS4012. The proliferation assays were performed by using almarBlue® Cell Viability assay kit (Invitrogen). The fluorescence intensity was measured with EnSpire® Multimode Plate Reader (PerkinElmer) by fluorescence detection at excitation and emission wavelength of 570 nm and 585 nm, respectively. Two independent experiments were carried out, and the data were expressed as the mean ± S.D. of assays performed in triplicate wells.

Statistics

Quantitative data are presented as mean ± SD. Comparisons were done by ANOVA with Bonferroni's multiple comparison tests or Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

Angiogenic signaling-mediated angiogenic and arteriogenic gene expression

To establish immortalized human EC lines, lentiviral transduction of pLOX-Ttag-IRES-TK (Addgene plasmid 12246) followed by pLOX-TERT-IRES-TK (Addgene plasmid 12245) was performed in primary HMVEC-D and primary HUVECs as previously described [16, 17] (Supplementary Fig. 1A, B). HMVECi-D was developed, showing far more passaging than primary ECs. Under microscope, we observed that cell morphology of both continuously passaged HMVECi-D and HUVECi were similar to their parental primary cells (Supplementary Fig. 2A).

To assess whether expression of hTERT and SV40 large T induces neoplastic transformation of ECs, we grew the HMVECi-D and HUMVEi-D clones in three-dimensional agarose gel, with skin and breast cancer cells that formed syngeneic cancer in vivo [10, 22] as controls. The skin and breast cancer cells formed tumor spheres (Supplementary Fig. 2B, C), and vascular tubule-like structures were formed in the breast cancer spheres (Supplementary Fig. 2C).

Unlike cancer cells, HMVECi-D and HUVECi did not form any tumor spheres with prolonged two-dimensional or three-dimensional cell culture (Supplementary Fig. 2D, E). The transformed ECs did not survive culture of agarose matrix, either. Yet, long-term culture of transformed ECs showed contact inhibition (Supplementary Fig. 2D). In addition, the transformed ECs were maintained for over 100 population doublings. Forced expression of Cre recombinase in HMVECi-D or HUVECi to remove the transduced genes resulted in a slow growth or arrest of cell growth, and an increase in the numbers of aging and dead or apoptotic cells (Supplementary Fig. 2F, G, H).

Since MVECs can be used as an ideal in vitro model system in angiogenesis [23] and arteriolar differentiation [4, 9], we focused on HMVECi-D for pilot mechanistic studies in expression of arteriogenic genes and proliferation. To determine whether HMVECi-D expresses typical markers that distinguish ECs from other cell types, we detected the expression of PECAM-1 (CD31) and VEGFR-2. Consistent to primary HMVEC-D, HMVECi-D expressed the two key EC-specific markers as shown by immunoreactivity of PECAM-1 (Fig. 1a) and VEGFR 2 (Fig. 1b) by using immunofluorescence microscopy.

Delta-like ligand 4 (DLL4) is a tip cell marker of ECs and arterial EC marker [24–27], which is important in MVEC differentiation [5, 7, 9]. We examined mRNA expression of DLL4 in response to angiogenic signaling in HMVECi-Ds. Consistent to previous studies [9, 28], the transcript levels of DLL4 were slightly but significantly increased in HMVECi-Ds when PKD-CA was overexpressed (Fig. 2a). Intriguingly, overexpressing PKD-CA induced tip cell-like morphology with significant branching morphogenesis in some of these cells (Fig. 2a).

Since PKD-1 signaling is critical for vascular endothelial cell growth factor (VEGF)-mediated angiogenic gene expression and MVEC functions [29], we examined whether VEGF also has an impact on angiogenic and arteriogenic gene expression in HMVECi-D. When we exposed these cells to VEGF, we observed that VEGF significantly promoted mRNA expression of DLL4 (Fig. 2b). Moreover, the expression of arteriogenic gene neuropilin 1 (NRP1) was induced by exposure to VEGF or overexpression of PKD-CA (Fig. 2c, d).

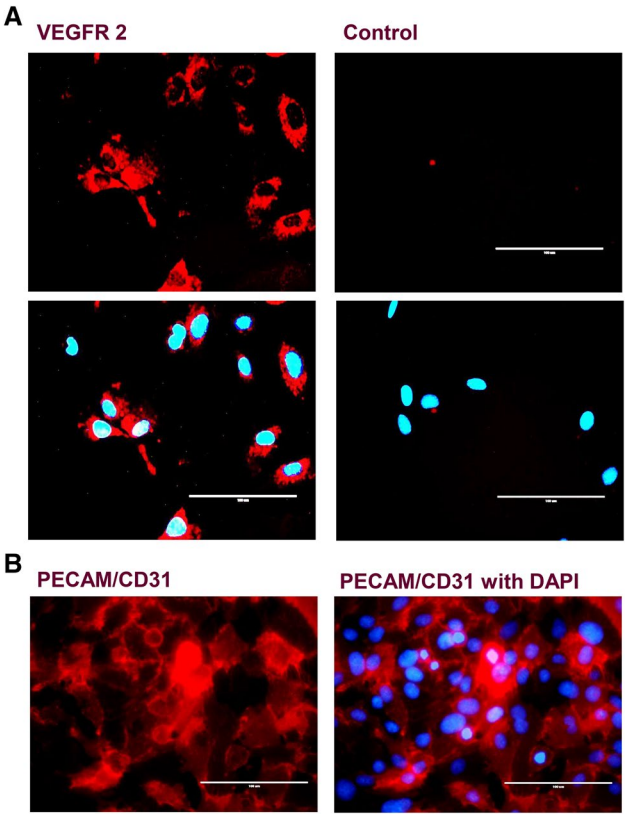

Fig. 1 Expression of typical EC markers in transformed HMVECi-Ds. **a** VEGFR 2 expression was detected in HMVECi-D (P20) by immunofluorescence microscopy. VEGFR2 expression is shown (left panel) and negative experimental control without adding primary antibodies (right panel). **b** Expression of PECAM/CD31 in HMVECi-D (P20) was detected by immunofluorescence microscopy. Nuclei were stained with DAPI (blue). Bar = 100 μm. All pictures were taken by using an EVOS™ imaging system. (Color figure online)

CD36 expression is downregulated in response to VEGF signaling in HMVECi-D

CD36 is highly expressed in HMVEC-D and considered as an important angiogenic regulator and molecular marker in capillary or microvascular ECs [8, 9, 30–32]. To determine whether the HMVECi-D expresses CD36, we examined CD36 protein levels by using immunofluorescence microscopy. We observed that most HMVECi-D expressed CD36, and deletion of the transgenes by Cre recombinase did not appear to change the levels of CD36 expression significantly (Fig. 3a). To define transcriptional expression of CD36, we performed real-time qRT-PCR, and observed that the expression levels of CD36 mRNA were moderately high in these cells (Fig. 3b, *Ct* value data not shown), though the relative mRNA levels in primary HMVECs were higher than the transformed HMVECs. Importantly, when the transformed HMVECs were treated with either VEGF or GS4012

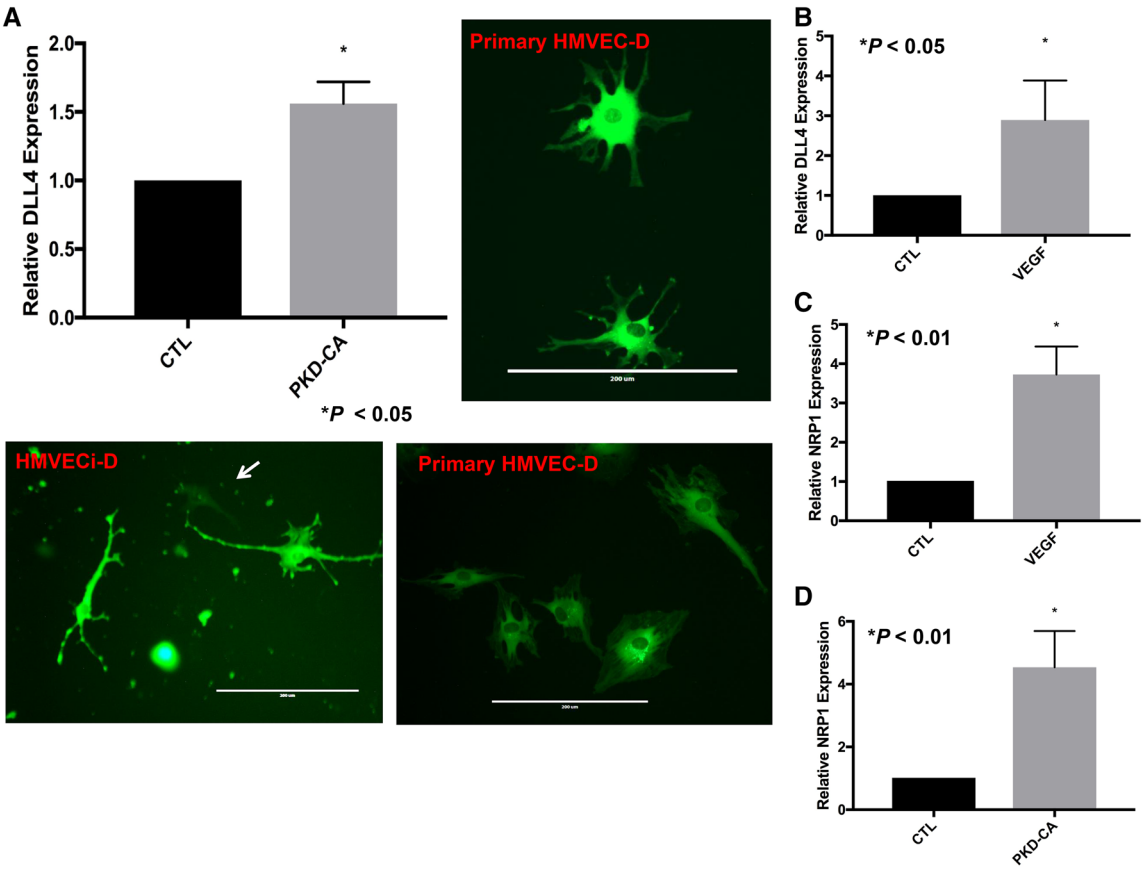

Fig. 2 Increase in expression of arteriogenic genes in response to VEGF/PKD-1 signaling in HMVECi-Ds. **a** PKD-CA overexpression promoted mRNA expression of DLL4 (upper left panel). Tip cell morphology was observed in some primary and transformed microvascular ECs transduced with PKD-CA. Approximately 100 cells were observed and representative images are shown: PKD-CA transduction in primary human MVECs (upper right panel); PKD-CA transduction in HMVECi-D (lower left panel; arrow indicates a cell without any transduction); GFP transduction in primary HMVEC-D (lower right panel). Bar = 200 µm. **b** and **c** mRNA levels of DLL4 and Neuropilin 1 (NRP1) in HMVECi-D exposed to VEGF (100 ng/ml) for 24 h as assayed by qPCR. **d** mRNA expression of NRP1 in HMVECi-D transduced with PKD-CA

(a VEGF inducer), the expression levels of the CD36 transcripts were significantly reduced (Fig. 3b).

Different angiogenic responses of HMVECi-D to VEGF and GS4012

DiI-Ac-LDL can be used to metabolically label and identify ECs [33]. To functionally identify and characterize the HMVECi-Ds, we visualized the interactions of Ac-LDL with the cells by incubating cell cultures with DiI-Ac-LDL, and examined uptake of acetylated LDL by fluorescence microscopy. Similar to primary ECs, the uptake occurred in HMVECi-D as shown by bright red staining (Fig. 4a).

Since in vitro Matrigel assays are regarded as representative of the later stages of angiogenesis (differentiation), and used as a model to mimic in vivo capillary development, we performed two-dimensional Matrigel assays to define angiogenesis function of these cells. We observed the formation of tubule-like structures in HMVECi-D in complete EC culture media containing VEGF (Fig. 4b,c) in response to matricellular signals. HMVECi-Ds underwent morphogenic differentiation to form capillary-like structures, suggesting that these cells maintained their differentiated phenotype of primary ECs in vitro [7, 8].

It is known that VEGF as a vascular permeability factor significantly acts on microvasculature to promote angiogenesis [34]. We thus assessed proliferation of HMVECi-D in response to VEGF. As expected, the growth rates were significantly increased when the cells were exposed to VEGF. Intriguingly, GS4012, which is important in arterial differentiation as a VEGF-inducer [35, 36], inhibited HMVECi-D proliferation (Fig. 4d).

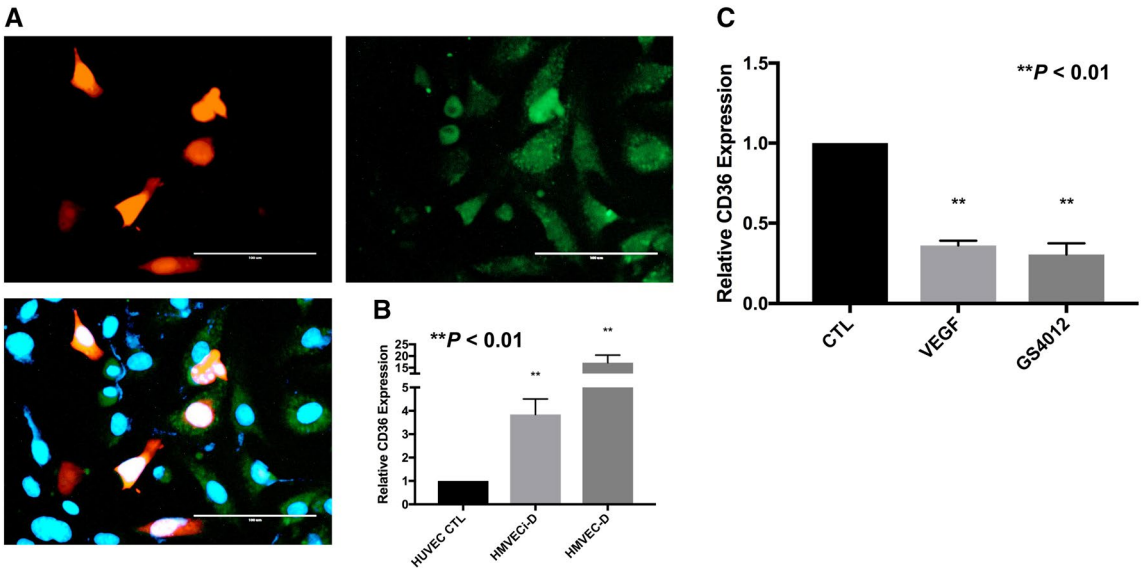

Fig. 3 Downregulation of CD36 expression in HMVECi-Ds exposed to angiogenic factors. **a** HMVECi-Ds were transduced with Cre recombinase tagged with cherry, and CD36 protein expression was stained with anti-human CD36: FITC antibody (green). CD36 expression was detected in cells with or without Cre recombinase transduction by fluorescence microscopy. Pictures were taken in an EVOS™ imaging system. Cre transduced cells are shown in red (cherry). Bar = 100 μm. **b** CD36 mRNA expression in HMVECi-D. Comparative qPCR assays were performed for CD36 expression relative to a HUVEC control with primary HMVEC-Ds used as a positive control ($P < 0.01$). **c** mRNA expression of CD36 in HMVECi-D following treatment with VEGF and GS4012. Comparative qPCR assays were performed for CD36 expression in HMVECi-D treated with VEGF (100 ng/ml) or GS4012 (10 μg/ml) relative to an untreated HMVECi-D controls ($P < 0.01$). (Color figure online)

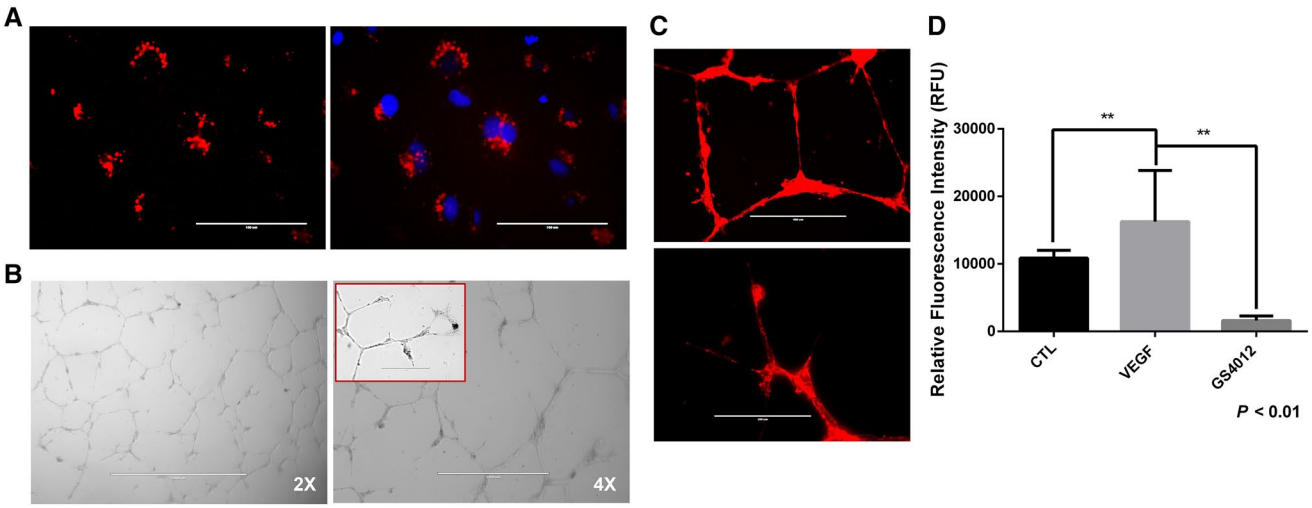

Fig. 4 Proangiogenic functions of VEGF and GS4012 in HMVECi-Ds. **a** Uptake of acetyl-LDL by HMVECi-D. DiI-Ac-LDL uptake (in red) occurred in HMVECi in EC culture media containing VEGF, with nuclei stained by DAPI (in blue). Representative pictures were taken in an EVOS™ imaging system. Bar = 100 μm. **b** Vascular network-like structure was formed by HMVECi-D in two-dimensional Matrigel. Black and white pictures for tube formation assays in cells grown on growth factor reduced Matrigel and EC culture media containing VEGF. Insert is the magnification. **c** Fluorescence images for tube formation assays for HMVECi-D. The cells were stained by F-actin with Alexa Fluor conjugated phalloidin. For black and white images, bar = 2000 μm (×2) or 1000 μm (×4); for fluorescence images, bar = 400 μm (upper panel) or 200 μm (lower panel). **d** HMVECi-D increased proliferation in response to VEGF treatment while GS4012 inhibited HMVECi-D proliferation. HMVECi-Ds in EBM-2 containing 2% FBS for 2 days were exposed to VEGF 100 ng/ml or GS4012 10 μg/ml for 24 h, the cell growth and proliferation was analyzed. (Color figure online)

Discussion

In this study, we highlight that the VEGF/PKD-1 signaling axis promotes angiogenic and arteriogenic gene expression in HMVECi-Ds. This finding has important implications in arteriolar differentiation of microvascular ECs, further supporting and extending the function of PKD-1 signaling in proangiogenic and proarteriogenic reprogramming [9].

Protein kinase PKD-1 is essential for angiogenic gene expression, VEGF signaling, and angiogenesis [29, 37, 38]. PKD-1 signaling is upstream of PI3/Kinase/AKT and MAPK/Erk1/2, and signals through Erk1/2-PLCϒ1 axis, thus critical to arterial differentiation [9, 29, 39]. We have shown that this signaling axis stimulates the expression of DLL4 and neuropilin 1, two important molecules in arterial differentiation, along with CD36 downregulation and endothelial tip cell morphology. These studies strongly suggest that the VEGF/PKD-1 signaling axis plays a critical role in arteriolar differentiation, blood vessel specification, and remodeling [9, 29, 39, 40].

More importantly, we have shown that the transformed HMVECi-Ds maintain CD36 expression at transcriptional and translational levels, an essential feature in microvascular ECs for microvascular functions and differentiation [4, 7–9, 31, 32, 35, 36]. Intriguingly, we have demonstrated that GS4012, a small chemical molecule and VEGF inducer that is important in arterial differentiation, influences MVEC proliferation differently from VEGF. This suggests that GS4012 tends to determine arterial fate [36] rather than inducing a proangiogenic response. The angiogenic functions of this small molecule are beyond VEGF, though it acts as a VEGF-inducer [12].

Similar to our recent studies [9, 13, 41], VEGF/PKD-1 signaling activation stimulates the expression of arteriogenic genes but inhibits CD36 expression. Therefore, it is tempting to speculate that the VEGF might determine an arteriolar fate of MVECs via a VEGFR2-PLCϒ-PKD-1-dependent pathway once CD36 signaling is downregulated in MVECs. If the suitable extracellular signaling is incorporated into the VEGF pathway, the PKD-1 signaling will be activated and biased to MAPK/Erk1/2-PLCϒ upstream of VEGF for arterial specification as previously reported [7, 9, 12, 35, 42].

Furthermore, we have achieved conditional extension of lifespan of primary MVECs through lentivirus-mediated gene transfer by using specific vector combinations of SV40 large T antigen and telomerase. The developed HMVECi-Ds retain not only fundamental properties of ECs, including key phenotypic and morphological characteristics, but also moderate expression of CD36. They also show the potential to be immortalized (the current passage of cells is 35). More importantly, they can be reverted to the primary cell status of original generations as the transgenes are removed by Cre-mediated excision [16]. Therefore, HMVECi-Ds may be used as an ideal in vitro model system in the study of angiogenesis and mechanisms of MVEC differentiation. However, further characterization and study on metabolism of MVECs may be of significance, because CD36 is not only an angiogenesis regulator but also regulator of cellular metabolism as a fatty acid receptor and because EC metabolism is considered as a driver rather than a bystander effect of angiogenesis [43].

Additionally, cell-based therapeutic angiogenesis is considered as an approach to restoring blood perfusion in ischemic tissue [44], which is of significance in the treatment of peripheral artery disease such as in diabetes, because of lacking effective pharmacological and interventional re-vascularization therapies [45]. The approaches of establishing reversible HMVECi-D with differentiation potential could be exploited therapeutically to engineer ischemic tissue-specific ECs or generate functional arteriolar ECs from HMVECs. This may produce therapeutic ECs at clinical grade and scale for developing functional vascular networks under diseased conditions.

In summary, due to heterogeneity and changes in phenotypes of ECs under certain microenvironment [9, 46, 47], the unique HMVECs we established can aid in understanding the formation of complex vascular system and underlying mechanisms in adult angiogenesis. Furthermore, our studies suggest that diverse microenvironmental cues may influence EC phenotypes through transcriptional and epigenetic reprogramming, especially in MVECs [9, 10, 41]. Therefore, HMVECi-Ds can be used in establishing angiogenesis models, which is likely to be more representative of the in vivo situation. Specifically, we have shown that the VEGF/PKD-1 signaling axis is probably critical for arteriolar differentiation in HMVECs under certain cellular conditions. Inducing arteriolar differentiation of HMVEC via the signaling axis may open exciting perspectives in promotion of functional arterioles and in cell transplantation for ischemic cardiovascular diseases, tissue engineering, and regenerative medicine [48].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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