Angiogenic responses in a 3D micro-engineered environment of primary endothelial cells and pericytes

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Original Paper

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Abstract

Angiogenesis plays a key role in the pathology of diseases such as cancer, diabetic retinopathy, and age-related macular degeneration. Understanding the driving forces of endothelial cell migration and organization, as well as the time frame of these processes, can elucidate mechanisms of action of important pathological pathways. Herein, we have developed an organ-specific microfluidic platform recapitulating the in vivo angiogenic microenvironment by co-culturing mouse primary brain endothelial cells with brain pericytes in a three-dimensional (3D) collagen scaffold. As a proof of concept, we show that this model can be used for studying the angiogenic process and further comparing the angiogenic properties between two different common inbred mouse strains, C57BL/6J and 129S1/SvlmJ. We further show that the newly discovered angiogenesis-regulating gene Padi2 promotes angiogenesis through Dll4/Notch1 signaling by an on-chip mechanistic study. Analysis of the interplay between primary endothelial cells and pericytes in a 3D microfluidic environment assists in the elucidation of the angiogenic response.

Keywords Microfluidic · Angiogenesis · Primary endothelial cell · Pericycle · 3D cell culture

Introduction

Angiogenesis is the process of forming new capillary vessel sprouts from an existing blood vessel. The process plays a critical function during development and in a number of diseases such as cancer, cardiovascular disease, and age-related macular degeneration. Complex signaling networks are involved in regulating angiogenesis and therapeutic targets have been discovered either to stimulate or to suppress these networks. The most well-known stimulator is vascular endothelial growth factor (VEGF), an angiogenic factor that acts in a concentration-gradient-driven manner. The VEGF family has many members but VEGF-A plays the most important role in endothelial responses [1–4]. This growth factor stimulates robust chemotaxis in which endothelial cells migrate from low-to-high VEGF-A concentration. VEGF-A is essential for the induction and growth of angiogenic tip cells through the Notch signaling pathways [2, 5]. Notch signaling pathways are evolutionarily conserved and determine cell fate and differentiation. They are activated via cleavage of Notch intracellular domain (NICD) by γ-secretase, causing the translocation of NICD to the nucleus in which it regulates transcription of its target genes [6]. Notch signaling through Notch1 and Notch4 plays a key role in angiogenesis and is regulated by several Notch ligands including Dll1, 2, 4, and Jagged1, 2 [7]. During angiogenesis, Dll4 and Jagged1 have different roles [8]. In response to VEGF, Dll4 is mainly expressed on angiogenic tip cells that make up the leading front of the vascular sprout. Dll4 expression defines tip cell identity by regulating expression of cell surface receptors such as VEGFR2/3, NRP1, and PDGF-BB. In addition, Dll4 expression in tip cells suppresses the tip phenotype in neighboring...
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Diagram a shows the process involving EC and PC, with time points: EC at -5 d, -4 d (puromycin), -1 d (media), 0 d (harvest), and PC at >-20 d, >-15 d (passage), 0 d (harvest).

Diagram b includes FACS analysis of EC and PC for VEGFR2, PDGFRβ, CD31, and NG2.

Diagram c shows images of 129S1 EC and C57BL/6 EC, along with 129S1 PC and C57BL/6 PC.

Diagram d displays images of 129S1 EC, C57BL/6 EC, 129S1 PC, and C57BL/6 PC.

Diagram e illustrates the use of PDMS and glass coverslip to facilitate cell culture and visualization.

Diagram f includes images of CD31 and NG2, with annotations for mature vessel covered with PC, PC detached from migrating tip cells, new vessel growth, pericyte formed filopodial-like structure, and pericytes migrated along newly formed vessel sprouts.
endothelial cells wherein stalk cells become new tip cells. Jagged1 promotes proliferation and a sprouting phenotype by antagonizing Dll4/Notch signaling. The balance and specific selection of ligands binding to Notch receptors among endothelial cells are still not clear [8].

Although cell-based models play a key role in biomedical research and drug discovery, most in vitro 2D cell models do not adequately address the topology of the tissue components, reducing their ability to recapitulate the in vivo processes. 3D cultures better mimic in vivo growth and interaction with the surroundings, as well as polarization and differentiation [9]. Collagen which is derived from intrinsic extracellular matrix (ECM) has a high-level of biocompatibility and can be employed [10] to facilitate cellular adhesion and support the interplay between cells and their stroma. Despite the advantages of in vivo systems, most models lack high-throughput capability and are time consuming.

Microfluidic cell-based 3D models complement in vivo systems by providing a more streamlined approach, while implementing complexities not achievable in 2D in vitro systems [11–13]. These 3D models have been utilized for a number of different cell culture applications, with advantages of real-time imaging in a controlled geometrical, physical, and biochemical microenvironment [14–16]. Previous reports of microfluidic cell-based 3D models used in studying angiogenesis involve spatial control, lumen formation, gradient formation, and perfusion for vasculature applications [17–20] as well as interaction with different types of tissues [21–23]. However, these microfluidic cell culture models mainly rely on transformed cell lines that have been previously propagated outside of their physiological environment for an extended time, and thus may not be useful for particular applications due to accumulating genetic alterations during passaging and contamination by other aggressive cell types [24]. While primary cells have a limited life span, they benefit from functional and genetic fidelity [25]. Thus, primary cells will be of better use to study heterogeneous cells isolated from different tissues, or different genetic backgrounds. In addition, endothelial cell lines isolated from various tissues behave differently in terms of their junction complexes, endothelium fenestra, morphology, and permeability [1]. Hence, it is important to isolate tissue-specific endothelial cells to recapitulate the in vivo microphysiological systems of interest.

To allow maximum preservation of in vivo signaling networks, herein we describe a 3D microfluidic construct with primary endothelial cells and co-cultured pericytes isolated simultaneously from murine brain tissue. We used this model system to study angiogenesis in two strains of mice with different genetic backgrounds. We have previously shown that the ability to respond to angiogenic stimuli is controlled by genetic variation [26] and performed genome-wide association studies (GWAS) in common inbred mouse strains to identify novel genes responsible for differences in angiogenic response [27]. Peptidyl arginine deiminase type 2 (Padi2), whose involvement has been linked to many human diseases such as cancer [28] and neurodegenerative diseases [29], has been recently identified as a novel angiogenesis-regulating gene. Using our 3D microfluidic construct, here we derived primary endothelial cells and pericytes from either a “high (129S1/SvJ)” or a “low (C57BL/6J)” angiogenic strain [26, 27]. As a proof of concept, we examined the differences in angiogenic response to VEGF. The potential mechanism of Padi2 was also investigated using our novel microfluidic system. Understanding these processes can aid and expand our knowledge of the pathways that directly mediate angiogenic response.

Results

Isolation of primary murine brain endothelial cells and pericytes to mimic 3D angiogenic microenvironment

We successfully isolated and cultured primary brain endothelial cells from C57BL/6J and 129S1/SvJ and used these cells to create a three-dimensional angiogenic microenvironment. Figure 1a is a descriptive flow chart for cell culture preparations. We improved the existing method [30] for optimizing the cell yield and survival as described in the Method section. Puromycin selection was used to eliminate all other cell types while selecting only for brain endothelial cells expressing P-glycoprotein [31]. The same starting culture system was used for growing pericytes. However, as pericytes were passaged, they outgrew endothelial cells and...
become dominant in the culture. To verify endothelial cells and pericytes, we examined the expression of two specific markers for both endothelial cell (CD31 and VEGFR2) and pericytes (PDGFRβ and NG2) via flow cytometry analyses. While there is no specific marker available to identify pericytes, usually a combination of markers of NG2 and PDGFRβ is used to phenotype these cells. Our results showed that over 95% and 92% of isolated primary brain endothelial cells express high levels of CD31 and VEGFR2, respectively, and over 89% and 81% of pericytes express...
Fig. 2 Comparison of Three-dimensional neovessels and microenvironment formed by C57BL/6J and 129S1/SvlmJ cells in microfluidic device. a. Immunofluorescent staining of self-assembled vessel structure by co-culture of endothelial cells and pericytes. b. Immunofluorescent staining of angiogenic sprouts induced from C57BL/6J and 129S1/SvlmJ endothelial cells and pericytes co-culture (red: CD31-stained endothelial cells; green: NG2-stained pericytes; blue: nuclei). Angiogenesis was induced by 25 ng/mL VEGF for 7 days. c. Quantitative analysis of angiogenic sprouting length for four groups: 129S1/SvlmJ endothelial cell culture only; 129S1/SvlmJ endothelial cells in co-culture with 129S1/SvlmJ pericytes; C57BL/6J endothelial cell culture only; C57BL/6J endothelial cells in co-culture with C57BL/6J pericytes. d. Quantitative analysis of tip cell numbers for four groups (as in e). e Quantitative analysis of vessel diameters for four groups (as in e). f. Blood vessel lumen formation, pericyte adherence to and migration along the endothelial cell sprouts from a representative image (129S1/SvlmJ co-culture). g. Concentration profiles and representative images at 0 min and 5 min of fluorescent dextran from which permeability can be quantified, for endothelial cell only and endothelial cell-pericyte co-culture. 129S1/SvlmJ. h. Quantitative analysis of the diffusion permeability across the vessel sprouts, for four groups (as in e). Average values across n=10 regions within a single device. i. Schematic demonstration on endothelial cell sprouting and pericyte migration in two strains. j. Immunofluorescent staining on pericyte migration. k. Quantification on pericyte migration. Scale bars: 100 µm. Estimated diameter was obtained by dividing area of capillary sprouting by skeletal length. Tip regions were defined as the regions from the very tip to the first or second vascular loop.

For this study, a previously reported microfluidic system (Fig. 1e) [22] was used with minor changes. Primary endothelial cells and pericytes were seeded together in the fluidic channel and allowed to assemble in order to mimic the vascular capillary structure (pericyte migration shown in Supplementary Figure S1). We confirmed this by immunostaining the cells using their specific markers CD31 and NG2, respectively (Fig. 1c). Cell morphology is shown in Fig. 1d.

For this study, a previously reported microfluidic system (Fig. 1e) [22] was used with minor changes. Primary endothelial cells and pericytes were seeded together in the fluidic channel and allowed to assemble in order to mimic the vascular capillary structure (pericyte migration shown in Supplementary Figure S2). In this study, we also optimized the pericyte-to-endothelial cell ratio (Supplementary Figure S3) to 1:20. Approximately 30,000–50,000 endothelial cells and 1500–2000 pericytes per device were seeded. After 7 days of VEGF stimulation, vessel sprouts started to grow into the central collagen gel channel. Along with vessel growth, pericytes were recruited and migrated along the surface of new sprouts (Fig. 1e). This system mimics physiological angiogenesis (Fig. 1f). Initially, blood vessels are covered with pericytes and a single pericyte can interact with multiple endothelial cells. In the presence of VEGF-A, pericytes start to detach from the migrating tip cells (arrow), then the endothelial cells sprout as filopodial-like structures followed by tube formation. Eventually, pericytes migrate along the endothelial tubes and stabilize them.

**C57BL/6J and 129S1/SvlmJ isolated endothelial cells and pericytes respond differently to angiogenic stimuli**

After cells were seeded in the devices, pericytes and endothelial cells self-organized into lumen-like structures at 12 h post seeding. Visualization of pericytes revealed that they were localized surrounding these tubular structures, rather than forming a mosaic monolayer. Initially, the endothelial cells formed a single layer (red layer) surrounded by pericytes (green layer). The results demonstrate that the setup has the correct configuration of an inner lining of ECs and outer lining of PCs (Fig. 2a, a 3-section view image is shown in Supplementary Figure S4). The presence of pericytes also influences the functionality of the system via formation of vascular plexus and continuous endothelium, which is required for developing mature vasculature (Supplementary Figure S5). We have previously shown that there are significant differences in angiogenic response among different common inbred strains and demonstrated that this trait is genetically controlled by different loci [27]. Here, as a proof of concept, we compared the angiogenic response of primary endothelial cells isolated from two different strains, C57BL/6J and 129S1/SvlmJ with different angiogenic responsiveness. Endothelial cells isolated from the high angiogenic strain, 129S1/SvlmJ, exhibited a higher level of angiogenesis in terms of sprouting length, tip cell numbers, and average vessel diameters when compared to C57BL/6J (low angiogenic) (Fig. 2b; Supplementary Figure S6 for phase-contrast image comparison). In both the isolated endothelial cell culture as well as the co-culture with pericytes system, the sprout length of medium-sized vessels from the 129S1/SvlmJ was approximately 1.6-fold higher compared to the C57BL/6J in response to VEGF (Fig. 2c). The average tip cell numbers in 129S1/SvlmJ was likewise higher than C57BL/6J (11 ± 2.2 vs. 7 ± 1.7) in the absence of pericytes and also when co-cultured with pericytes (14 ± 2.6 vs. 9 ± 2.8, Fig. 2d). Vessels composed of endothelial cells from C57BL/6J or 129S1/SvlmJ showed similar diameters (16.6 µm and 17.0 µm, respectively). However, when these cells were co-cultured with their respective pericytes, a slightly larger vessel diameter (1.25 fold) was observed in 129S1/SvlmJ compared to C57BL/6J (Fig. 2e). In flow conditions, we have also observed this trend of angiogenesis in C57BL/6J versus 129S1/SvlmJ endothelial cells (Supplementary Figure S7, Supplementary Methods), as VEGF works together with mechanical fluid forces to mediate angiogenesis [32]. To mimic the physiological shear stress subjected by blood vessels (5–10 dyn/cm², post-capillary venules to arteries [33]), the flow rate was set to 1000 µl/h.
giving the existing flow channel height and width (See Supplementary Figure S7).

Pericyte recruitment and endothelial vessel lumen formation are depicted in a 3-section view in Fig. 2f. The white arrow identifies a new vessel with formed intact lumen (red) that is surrounded by a few pericytes (green). Vessel permeability was quantified by testing the diffusion pattern of 70 kDa dextran rhodamine (Fig. 2g). A decrease in fluorescence intensity indicated the presence of a barrier. As expected, vessels formed by endothelial cells alone showed significantly more leakage than vessels formed by co-cultured endothelial cells and pericytes. There was no difference in permeability between C57BL/6J and 129S1/SvImJ under either condition (Fig. 2h, calculation of diffusive permeability shown in Supplementary Method). However, 129S1/SvImJ exhibited a higher level of angiogenesis with more migration of endothelial cells and pericytes (Fig. 2i, j, k).

**High PADI2 expression in endothelial cells and angiogenic tip cells from 129S1/SvImJ**

We found that PADI2 is expressed at higher levels mainly in the primary endothelial cells from high angiogenic mouse strain 129S1/SvImJ when compared to C57BL6/J (Fig. 3a). This is consistent with the in vivo finding in murine corneal models [20]. Higher PADI2 expression was found in the tip cells for both strains (Fig. 3a), but more prominent in the 129S1/SvImJ. Similar observation with endothelial cells alone is shown in Supplementary Figure S8. Figure 3b demonstrates the presence of the VEGF-enhanced PADI2 expression in endothelial cells from both C57BL/6J and 129S1/SvImJ. Notably, in the absence of VEGF with minimal vessel sprouting, PADI2 expression was also minimal, suggesting PADI2 expression may be induced by VEGF and may also play a role in the angiogenic tip cell phenotype. Notably, no difference was observed in PADI2 expression in the presence of flow compared to static condition (Supplementary Figure S9).

**PADI2 inhibition reduces angiogenesis in a 3D system**

In order to determine whether PADI2 expression levels affect angiogenesis in a 3D system, we assessed the effect of Padi2 knock-down by siRNA using electroporation. The knock-down process is demonstrated in Supplementary Figure S10. Figure 4a shows significant Padi2 knock-down in 129S1/SvImJ primary endothelial cells. We observed significant reduction in vessel sprouting length (94.3 µm vs. 63.2 µm), tip cell numbers (9 ± 2.0 vs. 4 ± 1.4), and vessel diameters (22.8 µm vs. 11.1 µm) in siRNA-treated groups compared to vehicle control (Fig. 4b–e). We then treated the primary endothelial cells in the 3D assay with BB-Cl-Amidine, a potent inhibitor of PADI2, and found a significant decrease in sprouting length (Fig. 4g), number of tip cells (Fig. 5h), and vessel diameters (Fig. 4i) in a dose-dependent manner (Citrulline expression was also analysed, as citrullination is a byproduct of Padi enzymatic activity and is inhibited by BB-Cl-Amidine). This drug was tested at multiple concentrations in both 3D assays (Fig. 4f) and in standard 2D cell culture (Fig. 4j) to determine the concentration needed to produce adequate inhibition. We observed the largest effect of BB-Cl-Amidine with no toxicity at a concentration of 500 nM in the 3D assay (Fig. 4f). In standard cell culture, a significantly impaired endothelial cell monolayer is present at 1 µM. The results suggest that Padi2 is involved in angiogenesis through regulating tip cell formation and migration. Interestingly, pericyte migration pattern in BB-Cl-Amidine-treated group was similar with control group, with pericytes still migrating along vessel sprouting trunks (Fig. 4k, l). However, pericyte migration distance was reduced in the BB-Cl-Amidine-treated group compared to control, which is likely due to the inhibition of endothelial cell migration. Among all the PADIs, Padi2 and Padi4 are the most common isoforms in brain tissue and were shown to have nuclear activity [34]. To exclude any effect of Padi4, we performed immunostaining of Padi4 in BB-Cl-Amidine and DAPT treatment groups (Supplementary Figure S11A) and found no change in PADI4 expression compared to corresponding control groups. Padi4 siRNA knock-down also did not affect the phenotype of angiogenesis (Supplementary Figure S11B, C). These results suggest that Padi4 is not involved in angiogenesis regulation in this system.

**PADI2 expression is upregulated when Notch signaling pathway is inhibited**

Due to high expression of PADI2 in tip cells, we investigated if there was a possible interplay between PADI2 expression and Notch signaling. Notch signaling is known to play an important role in angiogenesis by regulating differentiation of tip cells and stalk cells [35]. In the presence of VEGF stimulation, Notch signaling is involved in a complex crosstalk between tip cells and stalk cells in creating a new branching point [2]. C57BL/6J primary endothelial cells treated with γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyler ester (DAPT) resulted in an elevated level of PADI2, specifically more significant in the enlarged tip cell regions compared to an untreated group (Fig. 5a). We found similar results in endothelial cells treated with DAPT from 129S1/SvImJ mice (Fig. 5b). PADI2 appeared to accumulate in the tip cell regions in DAPT-treated group (Fig. 5c). We next assessed the level of citrullination...
in these cells and found a correlation (Fig. 5a, c). Interestingly, PADI2 expression in the tip cell nuclei was absent when treated by DAPT together with BB-Cl-Amidine treatment. In a previous study, PADI2 was detected in the nucleus and cytoplasm and has been found to citrullinate nuclear histone and several RNA-binding proteins, thus regulating transcription through epigenetic mechanisms [36, 37]. This may suggest that Notch signaling is associated with PADI2 expression (Fig. 5c).

**Padi2 promotes angiogenesis through crosstalk with Dil4/Notch1 signaling pathway**

To elucidate the role of Notch signaling and Padi2-related pathways in angiogenesis, Notch1 intracellular domain (N1ICD) level was evaluated in the sprouting vessel branches (Padi2 knock-down endothelial cell from 129S1/SvmlJ mice). N1ICD was upregulated in both Padi2 knock-down cells and BB-Cl-Amidine-treated cells compared to the corresponding control groups (Fig. 6a), and increased
N1ICD levels in the nuclei were also observed by immunofluorescence (Fig. 6b). In addition, the expression of Notch downstream genes Hey1 and Hey2 was upregulated in the spraying vessel branches in the Padi2 knock-down group (Fig. 6c and enlarged view, d), indicating Notch signaling was elevated when Padi2 was inhibited. Figure 6e shows that DAPT abolished the BB-Cl-Amidine effects by restoring spraying formation (Fig. 6d), the number of tip cells (Fig. 6e), and vessel diameters (Fig. 6f, g), in comparison to control. Figure 6h was representative image for each group.

To investigate the effect of Notch ligands, we examined Dll4, Jagged1 and Dll1. We observed a slightly higher DLL4 expression in the BB-Cl-Amidine-treated group (Fig. 7a, b), while JAGGED1 and DLL1 expression showed no significant differences by either Western blot or immunostaining. We then examined the effect of applied recombinant mouse DLL4 protein. An overview of neovascular stabilization and morphogenesis is shown in Fig. 7c. The DLL4-stimulated group had reduced spraying length (Fig. 7d), vessel diameter (Fig. 7e), and fewer tip cell numbers (Fig. 7f) compared to untreated endothelial cells. Additional BB-Cl-Amidine treatment further reduced spraying angiogenesis. However, DAPT treatment counters the effects of inhibiting PADI2.

Discussion

In this study, we created a novel 3D microfluidic angiogenic model with primary mouse brain microvascular endothelial cells and pericytes. With this model, we compared in vitro spraying angiogenesis using cells derived from two inbred mouse strains to investigate the mechanisms behind the differences reported in their angiogenesis in vivo. A key inspiration for our investigation was to develop a 3D in vitro system to recapitulate different angiogenic phenotypes observed in vivo. Lack of complex cellular and structural microenvironments in conventional 2D in vitro assays poorly replicates the desired phenotypes. Furthermore, this microfluidic-based platform is capable of validating candidate genes identified by GWAS in vitro by performing mechanistic studies.

In a previous study, we addressed the genetic contribution to different responses of angiogenesis in mouse models. In this study, we have isolated microvascular endothelial cells and pericytes from the same organ to capture the unique angiogenic microenvironment that is lost within non-primary cells and cells from different tissues. We selected brain tissue for our 3D angiogenesis model, in part, because pericytes and microvascular endothelial cells are abundantly present in the brain microvasculature and are well-differentiated. Most importantly, cerebrovascular disease such as stroke [38] is one of the most prevalent life-threatening diseases world-wide. Successful development of a primary endothelial cell-based, central nervous system (CNS) vascular model would benefit future studies of CNS vascular diseases. We have improved the current methods in isolating mouse primary endothelial cells from brain by using a different enzyme for digestion and modifying the protocols as mentioned in the Methods section. Our improved protocol has increased the recovery of primary endothelial cells growing in microfluidic devices.

We have previously shown that 129S1/SvlmJ mice exhibit more robust angiogenesis compared to C57BL6/J in response to growth factors bFGF and VEGF in a cerebral microcircuit assay [27]. Here, we find that 129S1/SvlmJ endothelial cells are highly angiogenic characterized by elongated, enlarged vessels, and increased numbers of tip cells under both static and flow conditions. From our results, pericytes had minimal effect on alternating spraying trends in both strains, but they played a key role in stabilizing lumen-like vessel structures in our system and employed an essential role on endothelial cell proliferation, migration, and stabilization. They assist in developing the vessel plexus by initiating vascular anastomoses. As well, pericytes reinforce vascular structures via forming continuous endothelium with non-interrupted stress fibers. In addition, they prevent vessel leakage while promoting vessel maturation, similar to previous reports that pericytes play a key role in controlling cerebral perfusion and restricting the brain microvasculature permeability [39, 40]. Notably, our system with endothelial cells and pericytes recapitulates the basic unit of vasculature and mimics physiological angiogenesis through the process of pericyte detachment, tip cells migration, pericyte re-adhesion, and pericyte migration following neovessel sprouts. We concluded that pericytes are essential
in the 3D systems to retain normal vessel function in order to mimic angiogenesis process.

*Padi2* is part of the peptidyl arginine deiminase family of enzymes that catalyze the post-translational conversion of peptidyl arginine residues to citrulline in the presence of Ca^{2+} [41]. *Padi2* has shown to be involved in tumor progression [42] and neurodegenerative human disorders including Alzheimer’s disease and multiple sclerosis [43]. We recently showed the potential role of *Padi2* as an angiogenesis-regulating gene [27]. Herein, we recapitulated the unique in vivo vascular microenvironment in vitro in order to study the function of *Padi2* in a simpler system. In addition, we were able to perform cell specific knock-down experiments on endothelial cells which is more difficult to achieve in animal models. In this 3D model, PADI2 inhibitor inhibited angiogenesis in a dose-dependent manner.

The connection between *Padi2* and Notch signaling has not been previously explored. Here, we found PADI2 expression higher in endothelial tip cells compared to stalk cells (non-tip cells) in the presence of VEGF. We then found a strong association between Notch signaling and PADI2 expression in our model. In both *Padi2* knock-down and BB-Cl-Amidine-treated endothelial cells, N1ICD was elevated compared to the corresponding controls. This resulted in reduced sprouting angiogenesis in both strains tested in this study. This reduced angiogenic phenotype was abrogated

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*Fig. 5* *Padi2* was negatively associated with Notch signaling in co-culture. 

**a** Immunofluorescent staining (with enlarged tip cell regions) of neovessel morphology and PADI2/citrulline expression on C57BL/6 cells versus those cells treated by 20 µM DAPT. **b** PADI2 expression in 129S1/SvImJ cells versus those cells treated by 20 µM DAPT or 20 µM DAPT + 500 nM BB-Cl-Amidine. **c** PADI2/citrulline expression in control versus 20 µM DAPT or 20 µM DAPT + 500 nM BB-Cl-Amidine treatment groups within tip cell area (129S1/SvImJ). White arrow indicates PADI2 accumulation in tip cell region. Red: CD31; green: PADI2; magenta: citrulline; blue: nuclei. Scale bars: 100 µm
by blocking Notch signaling. Our results reveal a previously unknown Padi2 endothelial function through crosstalk with Dll4/Notch1 signaling. Previous studies have shown that Dll4/Notch1 signaling inhibition leads to increased filopodia protrusion and vessel diameter in animal studies [44]. In addition, angiogenesis requires a tightly coordinated balance between EC sprouting (tip cells) and the maintenance of existing vasculature (stalk cells) [8] and this balance can be controlled by Dll4 expression in endothelial tip cells [2, 45]. Here, our observation indicated that when Padi2 is highly expressed, it leads to lower levels of Dll4 in the stalk cells resulting in an angiogenic “shift” toward a more proangiogenic state. Our results suggest that high Padi2 expression in 129S1/SvImJ may be partially responsible for the high angiogenic response due to its involvement of Dll4/Notch1 signaling in the regulation of sprouting. We found that downregulation of Padi2 correlated with higher Dll4/Notch1 signaling and resulted in N1ICD being released, which led to reduced tip cell formation and lower angiogenesis. In contrast, upregulation of Padi2 resulted in lower Dll4/Notch1 signaling with increased tip cell formation and accelerated angiogenesis. Based on our data, N1ICD is upregulated when Padi2 was knocked down. This may be due to Padi2 being involved in Notch cleavage and the release of N1ICD, followed by the activation of Notch signaling and increased N1ICD nuclei translocation. Future studies are required to show the detailed mechanism of Padi2 involvement in Notch signaling pathway.

In this study, a continuum of pericyte cellular phenotype and lack of a specific pericyte marker were limitations of primary pericyte isolation from passage 1. However, we still believe this primary-cell-based microfluidic prototype is useful to study cellular mechanisms and to address the complex interplay of networks via multi-cellular microenvironment settings. Future work will investigate further the reciprocal role of Padi2 and Notch signaling. This microfluidic vasculature surrogate facilitates the study of potential pathways involved in angiogenic response. In addition, many studies develop personalized therapies via customizing genetic interventions within genetic models. Murine models are feasible to study specific gene mutations; however, one can use the primary murine cells from specific tissues with various phenotypic features to investigate disease mechanisms which has potential for personalized medicine.

Conclusions

This study describes a microfluidic-based platform, integrating primary endothelial cells and pericytes isolated from the same organ. We have compared the genetic differences between mouse strains and investigated the interaction between angiogenic stimuli and cellular responses through a high-resolution confocal imaging. Herein, we have shown that Padi2, a novel angiogenesis gene recently identified in a mouse GWAS study, is highly expressed in angiogenic tip cells and interacts with VEGF-A and Notch signaling pathways. Further, a possible mechanism for this effect appears to be through Padi2 association with the Dll4/Notch1 signaling pathway. Our model enables the investigation of genetic phenotypes in a 3D microphysiological system and further elucidates the role of Padi2 in angiogenesis.

Methods

Mouse primary endothelial cells/pericyte isolation and culture

Primary cultures of mouse brain capillary endothelial cells were prepared from 7-week-old mice. Briefly, 4–5 mice were euthanized via CO2 and brain tissue was minced under aseptic conditions into approximately 1 mm³ pieces. The pulp was digested for 1 h in 37 °C using 9 mL of 1 mg/mL Collagenase A (Roche) with 40 μg/mL DNase-I, prepared in an ice-cold Dulbecco’s modified Eagle medium (DMEM). The mixture was then dissociated by approximately 25 gentle inversions. The cell pellet was separated by centrifugation in 14–15 mL of 20% bovine serum albumin (BSA)-PBS (2000 rpm, 15 min with soft deceleration setting). After that, the cell pellet was further digested with 5 mL additional enzyme (same as above) for another 15 min in 37 °C. Digestion was stopped by neutralizing with 5 mL Fetal Bovine Serum (FBS) and centrifuged for 5 min (2000 rpm). The harvested endothelial cells were grown in two 75 cm² tissue culture flasks (pre-coated with 1% BD collagen-I and dissolved in 0.02 N acetic acid), and EGM-2MV media (Lonza) with 20% vol/vol FBS and 1 × pen-strep for overnight. Cells were maintained in fresh cell culture media on the second day with additional 3 μg/mL puromycin (final concentration) for 3 days. The medium was changed every other day. At day 5, cells were grown in fresh media without puromycin for 24 h and were harvested with 0.05% Trypsin-EDTA on day 6 for culturing on microfluidic devices. For primary pericytes, the culture steps were identical except for puromycin being involved in Notch cleavage and the release of N1ICD, followed by the activation of Notch signaling and increased N1ICD nuclei translocation. Future studies are required to show the detailed mechanism of Padi2 involvement in Notch signaling pathway.

Microfluidic device fabrication and angiogenesis microenvironment generation

The microfluidic device for this study was fabricated using polydimethyl siloxane (PDMS). The PDMS replica was made by soft lithography from SU-8 patterned wafers. The
device has one gel channel in the middle and two outer fluidic channels. A coverslip was washed with glacial acetic acids for 1 h, then washed with DI water and ethanol before bonded to the micropattern to form a closed chamber. The endothelial cell/pericyte-growth channel was formed via injection of collagen gel solution (Invitrogen, 2.5 mg/mL.
Fig. 6 Padi2 promotes angiogenesis through coordinated Notch signaling inhibition in co-culture. a Notch1 intracellular domain (N1ICD) level with PADI2 functional (left) and expression (right) inhibition. b N1ICD nucleus level with PADI2 functional (up) and expression inhibition (down). White arrow indicates nuclear N1ICD distribution (enlarged of a). c Level of HEY1 and HEY2 with PADI2 expression inhibition. d. HEY1 and HEY2 expressions on angiogenic vessel branches with PADI2 expression inhibition (enlarged of c). e Quantitative analysis of angiogenic sprouting length on control, 500 nM BB-Cl-Amidine, versus 20 µM DAPT + 500 nM BB-Cl-Amidine. Bars: 100 µm.

Characterization of endothelial-cell-pericyte co-culture permeability

Fluorescent dextran 70 kDa Texas Red (Life Tech, D-1830) was added to culture media at 12.5 µg/mL [46]. The mixture was applied to the endothelial cell channel. Using fluorescence microscopy, the concentration fields were captured at 0 min and 5 min, respectively (No flow was applied during a permeability measurement). Their raw intensity profiles were analyzed using ImageJ (LOCI, University of Wisconsin). Permeability calculation was described in Supplementary Method.

Electroporation

Primary endothelial cells were washed with PBS, harvested with Trypsin-EDTA, and suspended 100 µL in Nucleofector™ Solution in a cell density of 7 x 10⁵ cells with or without siRNA (On-target smart pool siRNA for mouse Padi2, 300 nM, Dharmacon). The RNA-cell mixtures were transferred into cuvettes provided in Basic EC Nucleofector Kit (Lonza), and electroporated using pre-set conditions for endothelial cells in Nucleofector™ 2 Devices (Lonza). After transfection, EGM-2MV media were added and cells were immediately replated into the microfluidic device (See Supplementary Figure 10).

Immunocytochemistry of cells

Primary endothelial cells and pericytes were stained with CD31 (BD Biosciences, 553373), NG2 (Millipore, AB5320), PADI2 (ProteinTech, 12110-1), citrulline (Fisher Scientific, MABN328), NOTCH1 intracellular domain—N1ICD (Novus Biologicals, NB100-78486), HEY1 (Abcam, ab154077), HEY2 (Abcam, ab167280), JAGGED1 (Abcam, ab109536), DLL1 (Abcam, ab85346), and DLL4 (ProteinTech, 21584-1). Briefly, cell culture media were removed from the fluidic channels, and the channel containing the cells was rinsed in PBS and fixed in 4% Paraformaldehyde (PFA) (Sigma-Aldrich) for 15 min at room temperature. 0.1% Triton X-100 (Sigma-Aldrich) was then added for 15 min before blocking by 5% BSA dissolved in 1xPBS for 45 min-1 h at room temperature. After that, the cells were stained with primary antibodies (1:100) overnight at 4 °C. Secondary antibodies (1:200), Alexa Fluor 488 (goat anti-rabbit IgG (H+L), Invitrogen), or Alexa Fluor 647 (goat anti-mouse IgG (H+L), Invitrogen) were added for 1 h at room temperature as needed. Fluorescent images were obtained by confocal microscopy (Zeiss LSM880). For stimulating Notch signaling, recombinant mouse DLL4 protein was used at 10 μg/mL (R&D Systems, 1398-D4-050).

Image processing and analysis

Three-dimensional image stacks (80 μm range) of individual endothelial cell sprouts were acquired by confocal microscopy using a 20 x objective lens. Images of sprouting length, tip cell numbers, and vessel diameters were quantified by Imaris 7.0 software (Bitplane) and ImageJ (LOCI, University of Wisconsin). Sprouting length was defined by the distance of the tip of the furthest migrated cells to the trunk vessel for every individual sprout in a particular region. A region was defined by one 20 x image view containing two collagen spaces in between every three pillars, and tip cell number was quantified within a region. Quantification of vessel diameter was performed by analyzing individual skeletonized 3D projection images using ImageJ plugin AnalyzeSkeleton of the average diameter in a region. Estimated vessel diameter was obtained by dividing area of capillary sprouting by skeletal length.

Flow cytometry

Primary endothelial cells and pericytes were harvested by 0.05%Trypsin-EDTA and were washed once with PBS.
and resuspended in FACS buffer (PBS containing 5% (vol/vol) BSA). They were then stained with fluorochrome-conjugated Abs for 1 h at room temperature. Labeled cells were washed once with PBS and analyzed using BD FACS Calibur (BD Biosciences). Cells were also stained with isotype-matched antibodies to exclude background staining. All data were analyzed by the FlowJo software (Tree Star Inc.). The antibodies used in this study were PE-conjugated CD31 (BD Biosciences, 553373), FITC-conjugated PDGFR-β (Santa Cruz, sc-432), PE-conjugated VEGFR2 (R&D Systems, FAB357P), and NG2 (Abcam, AB5320) + Alexa Fluor 488 (goat anti-rabbit, IgG (H+L), Invitrogen), all with the ratio of 1:100.

Fig. 7 *Padi2* promotes angiogenesis through crosstalk with Dll4/Notch1 signaling in vitro. a Western blot analysis of DLL4, DLL1, and JAGGED1 expression on control versus 500 nM BB-Cl-Amidine treatment. b Immunofluorescent images represent DLL4, DLL1, and JAGGED1 expression level on control versus BB-Cl-Amidine treatment. c Neovessel morphology on control, BB-Cl-Amidine, DLL4 + BB-Cl-Amidine groups versus DLL4 + BB-Cl-Amidine + DAPT groups (500 nM BB-Cl-Amidine, 20 µM DAPT, 10 µg/mL recombinant mouse DLL4). d Quantitative analysis of angiogenic sprouting length for four groups (as in c). e Quantitative analysis of tip cell numbers for four groups (as in c). f Quantitative analysis of vessel diameters for four groups (as in c). Red: CD31; green: DLL4; magenta: DLL1; gray: JAGGED1; blue: nuclei. Scale bars: 100 μm.
Western blot

Total protein lysates extracted from primary endothelial cell cultures were used in western blot analysis. Protein separation was performed using 12% SDS-PAGE and transferred to nitrocellulose membrane. Non-specific binding was blocked by 3% non-fat milk in Tris-buffered saline (TBS; 25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) containing 0.1% Tween-20. Primary antibodies were used at 1:1000 to incubate blots overnight at 4 °C, including PADI2 (ProteinTech, 12110-1), JAGGED1 (Abcam, ab109536), DLL1 (Abcam, ab85346), and DLL4 (ProteinTech, 21584-1). Peroxidase-conjugated anti-mouse, anti-rabbit, and anti-goat immunoglobulins (GE Healthcare) were used as secondary antibodies. The developed X-ray film was then scanned using Densitometer (Bio-Rad) and analyzed using QualityOne software (Bio-Rad). Western blotting was performed on two individual sets of designed experiments—One was to determine Padi2 knock-down level, the other was to examine DLL4 expression on BBCl-Amidine treatment.

Statistical analysis

All data are expressed as mean ± S.E.M. Comparison between multiple groups was performed by one-way ANOVA and indicated by following Tukey’s mean comparison tests. Unpaired Student’s t test was used when comparing two groups (defined as: *p < 0.05; **p < 0.01; ****p < 0.0001). Analysis was performed by Prism 7 (GraphPad). All measurements were calculated by averaging the mean values of at least three microfluidic devices, obtained at least two independent experiments with at least 3 measurements made for each device.

Author contributions  JB, RJD, MK, HIF, STK, and AEB contributed to the design and implementation of the research, JB, LFS, and LB performed the experiments, and JB, RJD, RDK, and MK contributed to the interpretation of the results and the preparation of the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Data availability  All data generated or analyzed during this study are included in this article (and its supplementary information files), and are available from the corresponding author upon request.

Compliance with ethical standards

Conflict of interest  The author(s) declare(s) that there is no conflict of interest regarding the publication of this article.

Ethics approval  All animal studies were conducted in compliance with the protocols approved by the Institutional Animal Care and Use Committee of Boston Children’s Hospital (approval number 15-08-2998R for mouse experiments).

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