Notch promotes vascular maturation by inducing integrin-mediated smooth muscle cell adhesion to the endothelial basement membrane

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Running Title: Notch induces vascular maturation via αvβ3
Abstract

Vascular development and angiogenesis initially depend on endothelial tip cell invasion, which is followed by a series of maturation steps, including lumen formation and recruitment of perivascular cells. Notch ligands expressed on the endothelium and their cognate receptors expressed on perivascular cells are involved in blood vessel maturation, though little is known regarding the Notch dependent effectors that facilitate perivascular coverage of nascent vessels. Here, we report that vascular smooth muscle cell (VSMC) recognition of the Notch ligand Jagged1 on endothelial cells leads to expression of integrin αvβ3 on VSMCs. Once expressed, integrin αvβ3 facilitates VSMC adhesion to von Willebrand Factor (vWF) in the endothelial basement membrane of developing retinal arteries, leading to vessel maturation. Genetic or pharmacological disruption of Jagged1, Notch, αvβ3, or vWF suppresses VSMC coverage of nascent vessels and arterial maturation during vascular development. Therefore, we define a Notch-mediated interaction between the developing endothelium and VSMCs leading to adhesion of VSMCs to the endothelial basement membrane and arterial maturation.
Introduction

The process of angiogenesis must be highly regulated in order to produce a patent vascular system with adequate perfusion to support the surrounding tissue. While multiple cell types, secreted proteins, and growth factors coordinately regulate angiogenesis and vessel stabilization, the Notch signaling pathway is unique in that it is involved at multiple stages of angiogenesis, from initial vascular plexus formation and artery/vein patterning, to vascular smooth muscle cell (VSMC) recruitment and vascular remodeling.

Notch signaling, which is characterized by heterotypic cell-cell interactions between Notch-ligand and -receptor expressing cells, represents an evolutionarily conserved mechanism that is known to be important for cell fate decisions during embryogenesis and, more recently, angiogenesis. Mammals express four Notch receptors, Notch1-4, and five Notch ligands, Delta-like ligand (Dll) 1, Dll3, Dll4, Jagged1 (Jag1), and Jagged2 (Jag2). Notch1, Dll4, and Jag1 are required for vascular development, and genetic deletion of each of these genes results in embryonic lethality\(^1\)\(^-\)\(^5\). The loss of Notch3, whose expression is restricted primarily to VSMCs in the vasculature, is not lethal, but rather demonstrates that Notch3 is required for arterial differentiation and VSMC maturation\(^6\). While Notch3 seems to be the critical receptor for mural cell differentiation, Jag1 is the corresponding ligand that has been shown to be most important for this process\(^7\). Endothelial-specific deletion of Jag1 results in severe mural cell defects, whereas the expression of Jag1 on endothelial cells promotes
mural cell differentiation\textsuperscript{8-10}. While there has been research into the role that Notch signaling plays in vessel patterning and VSMC differentiation, the role of Notch signaling in vascular maturation has not been explored because the mouse models used to date are embryonic lethal.

The recruitment of pericytes and VSMCs to nascent vessels is critical to vessel maturation\textsuperscript{11}. In the absence of perivascular cell coverage, newly formed vessels are subject to regression and are dependent upon growth factor stimulation from the environment for their survival\textsuperscript{12}. However, once invested with pericytes, vessels are stabilized and resistant to regression. One mechanism by which Notch signaling regulates VSMC recruitment to vessels is by the upregulation of PDGFR\textbeta\textsuperscript{13}, however other Notch-downstream effector genes involved in the recruitment or retention of VSMCs in vessel maturation have not yet been described.

In addition to mural cell recruitment, the deposition of an endothelial basement membrane also regulates vessel maturation. Basement membranes are thin layers (50-100 nm) of specialized extracellular matrix shared by endothelial and epithelial cells which provide structure and support for those cells\textsuperscript{14}. The endothelial basement membrane is unique in its accumulation of the protein von Willebrand Factor (vWF), which is derived from endothelial Weibel-Palade body secretions\textsuperscript{15}. Following the formation of a quiescent vasculature, vWF plays an essential role in hemostasis. The lack of vWF, or dysfunctional vWF, leads to the
congenital bleeding disorder von Willebrand Disease\textsuperscript{16}. Additionally, vWF in the endothelium acts in the recruitment, adhesion, and migration of leukocytes\textsuperscript{17,18}. It has also recently been shown to play a role in vessel patterning\textsuperscript{19}. Here we provide evidence that vWF regulates the recruitment of VSMCs to mature arteries.

In this report we identified a novel Notch-downstream effector, integrin $\alpha v\beta 3$, on VSMCs. Furthermore, we demonstrate that Notch-induced upregulation of $\alpha v\beta 3$ enables VSMC interaction with vWF in the endothelial basement membrane. We propose that Notch ligand expression on endothelial cells, in combination with vWF accumulation in the endothelial basement membrane, promote arterial maturation via $\alpha v\beta 3$ expression on VSMCs.

**Material and Methods**

**In vivo assays**

Mouse experiments were performed under approval by the University of California San Diego Institutional Animal Care and Use Committee. Balb/c mice (Jackson Labs) were used for DAPT treatment, anti-CD61 injection, and the vWF/$\alpha$SMA time course. DiYF mice were a gift from T. Byzova (The Cleveland Clinic) and vWF KO mice were a gift from D. Wagner (Harvard). Both DiYF and vWF KO mice were on a C57BL/6 background, and age-matched C57BL/6 mice (Jackson Labs) were used as controls.
For injection of anti-CD61, postnatal day (P)5 Balb/c pups received a single intravitreal injection of purified NA/LE hamster anti-mouse CD61 (BD Biosciences) (0.5 ug in 0.5 ul volume) using a 2.5 ul Hamilton syringe (Hamilton cat# 87942) fitted with a 33 GA 0.5-inch removable needle (point style 4; Hamilton) into one eye. The contralateral eye received a single injection of purified NA/LE Hamster IgGκ isotype control. Retinas were harvested on day P8 and processed for immunohistochemistry.

For injection of DAPT, DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butylester) (Tocris) was dissolved in 20% DMSO and 80% corn oil (Sigma). Balb/c pups were injected subcutaneously over three consecutive days, at 100 mg/kg in a volume of 10 μl/g daily, either from P2-P5 or from P3-P6.

**Whole mount immunohistochemical staining of retinas**

Retinas were harvested from postnatal pups aged P1-P28, fixed in 4% PFA for 10 min and dissected in PBS. The retinas were permeabilized in ice-cold methanol for 10 min then blocked in 20% FBS/20% NGS (or 20% NDS)+0.3% Triton X-100 in PBS for 2 hr. Primary antibodies were added to antibody diluents (10% FBS/10% NGS or 10%NDS) + 0.3% Triton X-100 in PBS) O/N at 4°C. The next day, retinas were washed for 2 hr and secondary antibodies were added. Primary antibodies: Blood vessels were labeled with either isolectin B4 directly conjugated to Alexa 647 (Invitrogen) or with rat anti-mouse CD31/PECAM-1 (BD Biosciences). VSMCs were labeled with mouse-αSMA directly conjugated to
either FITC or Cy3 (Sigma-Aldrich). Other primary antibodies used: rabbit anti-human vWF (Millipore), rabbit anti-NG2 chondroitin sulfate proteoglycan (Millipore), rabbit anti-Jagged1 (Santa Cruz), goat anti-Jagged1 (Santa Cruz), goat anti-Notch3 (Santa Cruz). Secondary antibodies: goat anti-rabbit A568 or A647, goat anti-rat A488, and donkey anti-goat A568 or A647 (Invitrogen). All antibodies were used at a 1:200 dilution.

**Immunohistochemistry of retinal frozen sections**

Retinas were harvested as described, fixed in 4% PFA in PBS O/N at 4°C, incubated in 20% sucrose in PBS for 2 hr, and embedded in OCT (Tissue-Tek). 10 um thick frozen sections were cut, post-fixed in acetone, rehydrated in PBS, then blocked with 5% BSA, 2% NGS in PBS for 2 hr. Sections were incubated with primary antibodies: rat anti-mouse CD31, 1:200 (BD Biosciences), mouse anti-SMA FITC-conjugated, 1:200 (Sigma-Aldrich), and hamster anti-mouse CD61, 1:10 (BD Biosciences) overnight in 5% BSA, 2% NGS in PBS. After washing, sections were incubated with goat anti-rat A546 and goat anti-hamster A647 (Invitrogen) for 2 hr and washed again.

**Confocal fluorescence microscopy**

Imaging was performed on a Nikon Spectral C1 confocal microscope (Nikon C1si with EZC1 acquisition software, Nikon Instruments) with Plan Apo 10×/0.45 air, Plan Apo 20×/0.75 air and Plan Apo 60×/1.40 oil objective lenses (Nikon). All images were recorded with a sequential acquisition of the fluorescent channels to
prevent fluorescence bleed-through. Images were analyzed with MetaMorph software (Molecular Devices) for determination of VSMC coverage and with ImageJ for VSMC-EC coalignment (RG2B colocalization plugin).

**Transmission electron microscopy**

Samples were immersed in modified Karnovsky’s fixative (1.5% glutaraldehyde, 3% paraformaldehyde and 5% sucrose in 0.1 M sodium cacodylate buffer, pH 7.4) for at least 8 hours, post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour and stained en bloc in 1% uranyl acetate for 1 hour. Samples were dehydrated in ethanol, embedded in epoxy resin, sectioned at 60 to 70 nm, and picked up on Formvar and carbon-coated copper grids. Grids were stained with uranyl acetate and lead nitrate, viewed using a JEOL 1200EX II (JEOL, Peabody, MA) transmission electron microscope and photographed using a Gatan digital camera (Gatan, Pleasanton, CA).

**Cell culture**

HUVECs (Lonza) were cultured in EBM-2 media (Lonza) supplemented with a bullet kit containing endothelial-specific growth factors (Lonza) and 10% FBS (Hyclone). Human aortic vascular smooth muscle cells (VSMCs) from a young donor (Invitrogen, lot #200707) were cultured in Media 231 with smooth muscle cell growth supplement (Invitrogen).
Jag-1 stimulation of VSMC in vitro

VSMCs between passages 3-7 were cultured on 6-well TCT-dishes coated with 5 μg/ml IgG or Jagged1 for 18 hr as previously described13.

Microarray analysis

VSMCs were cultured on Jag1 or IgG-coated plates as described above. RNA was extracted using RNA isolation kit (Qiagen). Reverse-transcription PCR was done with RNA-to-cDNA kit (Applied Biosystems). cDNA was run on a TaqMan Low-Density Array, human angiogenesis panel (Applied Biosystems, Cat# 4378725), amplified on a 7900 HT Fast Real Time PCR system (Applied Biosystems) according to manufacturer’s instructions. All microarray data is available at the Gene Expression Omnibus (GEO) database under accession number GSE34024.

RNA extraction, reverse transcription PCR

VSMC were cultured on Jag1 or IgG-coated plates as described. RNA was harvested with TRIzol (Invitrogen) according to manufacturer’s instructions. cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Individual quantitative RT-PCRs were performed with Absolute qPCR mix (Thermo Scientific) and SYBR Green (Invitrogen) on 20 μg cDNA on a SmartCycler (Cepheid) according to the manufacturers’ instructions. Primers: Hey2 (CAACCCCTTGTGCTCCTCTC / CCGTGGATGGCATTCGGAG, PrimerBank ID# 6912414a3); Hes1 (CAGGAATGTTTTGACCGAGTCT /
AACGTGCTCAGGTTTTTAGCC), PrimerBank ID# 5031691a2); integrin β3 (GTGACCTGAAGGAGAATCTGC / TCACTCAGTGGAACTCGATG, PrimerBank ID#2443452a3)12, β-actin (GGAGGAGCTGGAAGCAGCC / GCTGTGCTACGTCGCCCTG)14.

**Tube formation assay**

HUVECs at passage 2 were trypsinized and coated onto Cytodex3 microcarrier beads (GE Healthcare) as previously described12. Following overnight culture in EGM-2 (Lonza), HUVEC-coated beads were suspended in fibrinogen (Sigma, 2 mg/ml) with aprotinin (Sigma, 0.15 U/ml) in PBS at a concentration of 500 beads/ml. VSMCs were labeled with DilC12(3) (1:1000 in culture media for 2 hr; BD Biosciences) and added to bead solution at 1x10⁵ cells/ml. Fibrin gels were made in 24-well TCT-plates by mixing 0.5 ml fibrinogen solution with thrombin (final concentration 0.625 U/ml). After gels solidified, 1 ml EGM2 was added to each well with *Ulex europaeus* Agglutinin I conjugated to FITC (1:200; Vector Labs) to visualize endothelial tubes. For IHC, 1 ml gels were made on glass chamber slides. Fibrin gels were fixed with 4% PFA O/N, permeabilized with methanol (4°C) for 15 min. Staining proceeded as described for the retinas, with all antibody incubations done overnight.

**Endothelial cell-VSMC co-alignment quantification**

The overlap between endothelial cells and VSMCs was taken as a percentage of the endothelial tubes in each image analyzed. To do this, stacks of confocal
fluorescent images were analyzed in ImageJ with the RG2B colocalization plugin. The resultant colocalization images, which show only pixel overlap, were converted to 8-bit images and thresholded. These are the “colocalization” images. Separately, the corresponding RGB confocal images were split into individual color channels and processed to binary. Of these binary images, the channel of interest is that of the endothelial tubes. These are the “EC tubes” images. For analyzing colocalization in the bead assay, the pixels corresponding to the beads themselves were deleted from all images analyzed because we were interested in colocalization along the tubes. Finally, the ImageJ “analyze particles” function was run on all images, with the readout being Area Fraction. To determine colocalization, the following calculation was used:

\[
\frac{\text{Area Fraction}_{(\text{colocalization})}}{\text{Area Fraction}_{(\text{EC tubes})}} = \text{colocalization}
\]

**Adhesion assay**

16-well E plates (Roche) were coated with vWF (gift from Z. Ruggeri) at 10 μg/ml overnight and blocked with 1% heat denatured BSA (Hyclone). VSMCs in Opti-MEM with 0.25 mM MnCl₂, 1 mM MgCl₂, 1 mM CaCl₂, 20 mM Hepes were added to wells, each condition was run in triplicate. Data was collected on the xCelligence system (Roche) according to manufacturer’s instructions. Impedance was measured, resulting in a cell index number that correlated to VSMC adhesion.
Inhibition of Notch, vWF, αvβ3, and β3 in vitro

A 100 mM stock solution of DAPT (Tocris) in DMSO was added to VSMC culture media at a final concentration of 25 μM, vehicle was DMSO. A vWF blocking antibody specific to the β3 RGD binding site (α-152B, gift from Z. Ruggeri) was added to the culture media at a final concentration of 30 μg/ml; the αvβ3 blocking antibody LM609 and the integrin β1 blocking antibody P4C10 were each added to the culture media at a final concentration of 20 μg/ml. Vehicle was mouse IgG (BD Biosciences).

siRNA knockdown in VSMC

VSMCs were transfected with siRNAs against Notch3 (Qiagen; Notch3-3) or All Stars control siRNA (Qiagen) using human aortic SMC nucleofector kit (Lonza) in the Amaxa nucleofector system (Lonza) according to manufacturer’s instructions. Knockdown was confirmed by western blot with rabbit anti-Notch3 (Sigma), rabbit anti-Hey2 (ProteinTech Group), mouse anti-HSP60 (Santa Cruz) was used as a loading control.

Statistical analyses

All statistical analyses were performed with Excel (Microsoft). Two-tailed Student's t test was used to calculate statistical significance. A P value < 0.05 was considered to be significant.
Results

Notch is required for VSMC coverage during postnatal angiogenesis

To understand the role of Notch signaling in the perivascular compartment during postnatal developmental angiogenesis, we examined vascular mural cell coverage in the developing mouse retina following pharmacological inhibition of Notch signaling activity. The gamma-secretase inhibitor DAPT blocks the cleavage and release of the intracellular domain of Notch, which is the critical effector that controls gene expression and as such acts as a global Notch inhibitor both in vivo and in vitro. As previously reported, DAPT treatment from P2-P5 leads to the formation of an immature vascular plexus in the periphery of the mouse retina (Figure 1A, B). Because we are interested in the effect of Notch inhibition on vessel maturation, we examined VSMC coverage in the arterial region closest to the optic nerve, which is more mature. Not only did we observe a reduction in the arterial length covered by VSMCs (Figure 1A, B, E), we also found many areas of incomplete VSMC coverage (Figure 1C, D) following DAPT treatment, indicative of an immature artery. The effect of Notch inhibition on mural cell coverage was limited to αSMA+ perivascular cells and did not impact αSMA+/NG2+ pericytes (Supplemental Figure 1).

Endothelial Jag1 is required for arterial VSMC coverage and maintenance

Endothelial cell Jag1, a Notch ligand recognized by VSMCs, is predominantly expressed in the arterial endothelium during murine retinal development (Supplemental Figure 2) and is required for VSMC differentiation during...
embryogenesis\textsuperscript{8}. Genetic ablation of Jag1 from the endothelium of mice using a Tie2-Cre mouse line resulted in embryonic lethality with severe VSMC defects\textsuperscript{8}. To investigate the role of Jag1 in postnatal arterial maturation we used a genetic murine model that bypasses the critical requirement for Jag1 in early endothelial development (VE-Cadherin-Cre; Jag1 ECKO mice)\textsuperscript{27}. In the retinas of these mice, we observed weak arterial \( \alpha \)SMA\textsuperscript{+} perivascular staining in P7 pups compared to controls, indicating that VSMC recruitment was deficient, leading to less mature arteries during development (Figure 2A, B). Staining with an alternative marker for VSMCs, SM\( 22\alpha \), confirmed that it is the VSMC compartment which is affected by loss of endothelial Jag1 (Supplemental Figure 3). Pericyte coverage was not affected (Supplemental Figure 4). In adults, we observed a loss of VSMCs in the arteries immediately adjacent to the Jag1\textsuperscript{−} endothelium (Figure 2C-H). These findings suggest that Jag1 is an endothelial cell Notch ligand capable of regulating arterial maturation and maintaining arteries in a mature state, without loss of VSMCs.

**Interaction with Jag1 induces integrin \( \alpha \upsilon \beta 3 \) expression on VSMCs**

To identify Jag1-dependent Notch-mediated genes that might contribute to arterial maturation, VSMCs were seeded onto plates coated with or without immobilized recombinant Jag1. Eighteen hours later we analyzed changes in gene expression using a low-density array featuring a panel of genes associated with human angiogenesis (Applied Biosystems). Cell interaction with Jag1
altered the expression of several angiogenesis-related genes in VSMCs, including PDGFRβ, integrin β3, and the Notch-downstream gene Hey1 (Table 1).

Integrin β3, which associates with αv to form the integrin heterodimer αvβ3, is known to play an important role in vascular cell adhesion, migration, and development28,29. We confirmed that integrin β3 mRNA expression was increased 5.3-fold by RT-PCR (±0.7 s.e.m.; n=6) (Figure 3A) and observed a 3-fold increase in αvβ3 surface expression by FACS on VSMCs following interaction with immobilized Jag1 (Figure 3B). We also verified that integrin αvβ3 was expressed on arterial-associated VSMCs during retinal development, and that this expression was dependent on Notch signaling (Figure 3C). Blockade of Notch signaling by DAPT administration selectively prevented the expression of αvβ3 on VSMCs of maturing arteries, but did not impact the expected pattern of β3 expression on angiogenic sprouting tip cells (Figure 3C). These findings reveal that VSMC integrin αvβ3 expression depends, in part, on a Jag1/Notch interaction.

**Integrin αvβ3 regulates arterial VSMC coverage *in vivo***

We next considered whether αvβ3 expression on VSMCs might contribute to arterial VSMC coverage during retinal development. We used pharmacological and genetic approaches to block β3 expression and function *in vivo*. First, we characterized retinas from the β3 knockin “DiYF mice,” which express an inactive β3 subunit with two critical tyrosines converted to phenylalanines, producing
deficient αvβ3 signaling30,31. Retinas were harvested at P3, P5, and P7 and blood vessels were labeled using an endothelial-specific lectin and αSMA to visualize endothelial cells and VSMCs, respectively (Figure 4A). VSMC coverage was quantified by measuring the arterial αSMA labeling as a percentage of endothelial outgrowth from the optic nerve to the vascular plexus periphery, along each arterial branch. At each time point, we observed significantly less αSMA+ perivascular cell coverage in DiYF compared to wild type mice (Figure 4B). The reduced mural cell coverage seems specific to VSMCs, as pericyte coverage did not differ between DiYF and wild type animals (Supplemental Figure 5). Arteries in retinas from mice completely deficient in αvβ3 showed a delay in VSMC coverage compared to age-matched controls at P4, yet by P7 we observed normal vascular patterning and maturation (data not shown).

To confirm a role for αvβ3 in arterial maturation, a β3 integrin function-blocking antibody was injected intravitreally into wild type mice at P5 and retinas were examined at P8 for the extent of vessel maturation and VSMC arterial coverage. We chose to initiate treatment at P5 since arterial patterning has been established at this developmental stage, but with minimal arterial VSMC coverage. The β3 function-blocking antibody suppressed angiogenesis in the deep vascular plexus of the retina, consistent with previous reports of the anti-angiogenic activity of β3 integrin antagonists34,35 (Supplemental Figure 6). Following treatment with anti-β3, we observed reduced αSMA+ coverage of the
developing arteries of P8 mice relative to control (IgG)-injected mice (Figure 4C-E). Thus, both genetic and pharmacological approaches indicate that \( \alpha \nu \beta_3 \) expression on VSMCs is required for the coverage and maturation of newly developing arteries.

**von Willebrand Factor regulates arterial VSMC coverage in vivo**

During vascular development both endothelial cells\(^{28}\) and VSMCs\(^{36}\) transiently express integrin \( \alpha \nu \beta_3 \), which is a receptor for von Willebrand Factor (vWF). Importantly, vWF is an RGD-containing adhesion protein that is unique to the vascular basement membrane\(^{37,38}\). Previous studies have revealed that endothelial cells and VSMCs share a common basement membrane in mature arteries\(^{39,40}\). Since endothelial cells and VSMCs both upregulate integrin \( \alpha \nu \beta_3 \), a receptor for their shared basement membrane during angiogenesis, we considered whether vWF might regulate VSMC-dependent arterial maturation. To test this *in vivo*, we first characterized the vWF expression pattern during retinal development in mice from P1 to P21. Whole-mount staining revealed that vWF accumulated in the endothelial basement membrane during vessel maturation (Supplemental Figure 7). We observed a strong accumulation in major arteries and veins, but only minimal accumulation in the capillary beds. During the first month of development, vWF accumulated along arteries in a pattern that completely co-aligned with the perivascular outgrowth of VSMCs (Supplemental Figure 7).
To determine the functional role of basement membrane vWF in arterial maturation, we examined retinas from vWF KO mice. Arterial VSMC coverage was quantified by measuring the arterial αSMA labeling as a percentage of endothelial outgrowth, from the optic nerve to the vascular plexus periphery, along each arterial branch. During the early stages of developmental angiogenesis, vWF KO mice showed significantly less VSMC coverage compared with wild type controls (Figure 5A, B). However, pericyte coverage did not differ between vWF KO and wild type animals (Supplemental Figure 5). Similar to our observations in DiYF mice (Figure 4), early defects in developmental angiogenesis were resolved by three weeks of age, suggesting the existence of compensatory/redundancy mechanisms. These finding suggest that vWF regulates an early step in VSMC coverage of developing arteries.

**Notch upregulates VSMC adhesion to endothelial vWF via αvβ3**

To explore the link between Notch signaling in VSMCs and their adhesion to vWF, VSMCs treated with or without Jag1 were allowed to attach to a vWF-coated substrate *in vitro*. Jag1-stimulated VSMCs, which upregulate αvβ3 expression (Figure 3A, B), showed enhanced adhesion to vWF compared with control cells not exposed to Jag1 (Figure 6A, B). This increased adhesion could be disrupted by addition of function-blocking antibodies specific to either αvβ3 or to the RGD-binding site on vWF, but not the addition of β1 integrin function-blocking antibodies (Figure 6A, B). Also, inhibition of Notch signaling with DAPT suppressed VSMC adhesion to vWF (Supplemental Figure 8A). Together, these
findings suggest that Jag1 ligation leads to upregulation of integrin αvβ3, allowing VSMCs to adhere to the vWF-containing vascular basement membrane.

To examine the functional relevance of vWF in the basement membrane in the context of a VSMC-endothelial cell interaction, we adapted an in vitro model system in which VSMCs associate with outgrowing endothelial vWF-producing tubes, a process which could be readily visualized in real time\textsuperscript{23}. Microcarrier beads coated with primary human umbilical vein endothelial cells (HUVECs) were suspended in a fibrin gel containing human aortic VSMCs. The endothelial cells form large lumen-containing vascular networks within the fibrin gel. The vascular networks that form deposit vWF into a basement membrane-like structure (Figure 6C). VSMCs migrate towards the outgrowing tubes and associate with them, approximating the relationship between endothelial cells and mural cells observed in vivo. The addition of a vWF function-blocking antibody, specific to the RGD site on vWF (anti-152b)\textsuperscript{38}, to the culture media reduced VSMC interaction with outgrowing endothelial tubes by approximately 70% compared to control IgG (Figure 6D-G, L). These findings indicate that vWF can serve as a common adhesion protein for both endothelial cells and VSMCs, providing a mechanism for these cell types to engage a common basement membrane and thereby enable specific perivascular coverage during arterial development.
To investigate the impact of Notch signaling on VSMC interaction with vWF in the endothelial basement membrane, we examined the effects of blocking Notch signaling on co-patterning during tube formation in vitro. When Notch signaling was inhibited with DAPT, we observed decreased co-patterning between VSMCs and endothelial tubes by approximately 50% relative to control cells (Supplemental Figure 8B-F). When Notch signaling was specifically blocked in VSMCs by knock-down of Notch3 gene expression (Figure 6M), we found that VSMCs lacking Notch3 showed significantly less co-patterning with endothelial tubes compared with control VSMCs (Figure 6H-L). Knockdown of Notch3 decreased both the mRNA and protein expression of integrin β3 by approximately 40%, as measured by qPCR and western blot (Supplemental Figure 9). Similar to our observations that vWF was required for VSMC-endothelial interaction (Figure 6D-G), we found that Notch signaling in VSMCs, which upregulates αvβ3, was also required for this interaction. Taken together, these findings reveal a central role for Notch signaling leading to αvβ3-mediated adhesion between VSMC and vWF in the endothelial basement membrane.
Discussion

Vascular development and angiogenesis depend first on endothelial tip cell invasion, followed by a series of maturation steps, which include recruitment of perivascular cells and the deposition of a mature basement membrane. Notch signaling has been linked to artery/vein patterning, endothelial tip and stalk cell formation, as well as VSMC coverage of newly formed arteries, leading to the formation of functional vascular plexus. However, it is unclear how Notch signaling facilitates vessel maturation, specifically arterial VSMC coverage. Previous studies have implicated Notch in PDGFRβ expression, a receptor critically involved in pericyte function. In the current study we have identified a new role for Notch that appears to regulate the specific recruitment of VSMCs to maturing arteries.

We present several lines of evidence that Notch signaling is critical for VSMC recruitment to arteries due to its regulation of αvβ3, leading to VSMC interaction with vWF in the endothelial basement membrane. First, integrin αvβ3 was among the most upregulated genes following VSMC interaction with the Notch ligand Jag1 in vitro. Integrins are known to play a critical role in cell adhesion and cell migration. Importantly, αvβ3 expression gives a nucleated cell the unique ability to bind to vWF, which is the only tissue-restricted αvβ3 ligand available in the endothelial basement membrane. When Notch signaling was inhibited in neonatal pups, we observed discontinuous arterial VSMC coverage of arteries and a VSMC morphology that was consistent with an adhesion or
migratory defect. Additionally, mice specifically lacking the Notch ligand Jag1 in the endothelium showed a paucity of VSMCs immediately adjacent to the endothelium. Second, mice expressing a defective integrin αvβ3 or those deficient in vWF showed a significant delay in VSMC arterial coverage, suggesting that this receptor-ligand pair plays a critical role in VSMC recruitment and subsequent arterial maturation. However, it is important to note that this phenotype was transient and that by day 21 of development the arteries appeared normal, suggesting a redundant or compensatory mechanism might be involved. This is not entirely surprising since adult mice deficient in vWF16 or αvβ332 do not show a discernable vascular defect; this has been linked in part to compensation45. Injection of an αvβ3 antagonist into wild type mice at postnatal day 5 resulted in a disruption of VSMC arterial coverage measured on day 8, corroborating the developmental phenotype observed in the genetically altered mice.

From their restricted location on the arterial endothelium, Notch ligands and receptors ensure the maturation of arterial vessels. This process is specific to arterial development since arterial vWF staining and VSMC coverage were coincident, and this was not observed in developing veins within the retina. This cascade ensures that VSMCs are selectively localized to mature arterial regions, rather than regions that continue to undergo extensive vascular remodeling.
Previous *in vivo* studies point to a critical role for Notch in the VSMC compartment\(^6,8,9\). Notch3-deficient mice have thinner VSMC coverage and a discontinuous layer of non-cohesive VSMCs\(^6\), reminiscent of what we observed in mice with deficiencies in β3 signaling or vWF expression. Furthermore, VSMCs from Notch3\(^{-/-}\) mice displayed migratory deficiencies, whereas Notch3 over-expression affected actin cytoskeleton dynamics\(^6\). These observations are consistent with our description that Notch signaling in VSMCs regulates integrin αvβ3, thereby affecting cell adhesion and migration.

It was recently reported that endothelial vWF regulates angiogenesis\(^19\). The work described herein reveals that vWF is acting not only on endothelial cells\(^19\), but is also critical for VSMCs and their ability to interact with the endothelial basement membrane. Our findings raise the possibility that vWF secreted into the basement membrane by endothelial cells could participate in VSMC outside-in signaling. As such vWF in the endothelial basement membrane may serve a dual purpose, regulating both endothelial network formation as well as VSMC recruitment leading to arterial maturation.

Vessel maturation, or the lack thereof, has relevance to cancer, diabetic retinopathy, restenosis, wound healing, and stroke, among other pathologies. During pathological angiogenesis associated with tumor growth, blood vessels lack an intact basement membrane and are highly disorganized, leading to
increased vascular permeability\textsuperscript{46}. Interestingly, these vessels show minimal expression of vWF and are relatively devoid of perivascular cell coverage\textsuperscript{47}.

While tumor vessels have reduced perivascular coverage and generally lack VSMCs, other pathological vessels, such as those formed during wound repair, suffer from the recruitment of too many VSMCs\textsuperscript{48}. Accumulation of VSMCs in the tunica intima leads to coronary atherosclerosis and restenosis following angioplasty, and Notch signaling likely plays a role in this recruitment\textsuperscript{49}. Additionally, integrin $\alpha_v\beta_3$ is upregulated on VSMCs during restenosis, and is considered a therapeutic target to prevent arterial narrowing\textsuperscript{50}. In addition to osteopontin\textsuperscript{50}, Notch signaling could be a driving factor in the well-described upregulation of $\alpha_v\beta_3$ on VSMCs in restenosis. In this case, inhibitors of Notch signaling as well as $\alpha_v\beta_3$ antagonists are potential therapeutics worth considering.

In the current study we have identified new roles for both Notch and vWF in regulating arterial maturation. Our findings suggest that endothelial cells display the ligand Jag1 to increase the expression of integrin $\alpha_v\beta_3$, a receptor for vWF, on VSMCs. We found that arterial vWF is coincident with VSMC patterning during development and is required for endothelial VSMC coverage \textit{in vitro}, suggesting that vWF in the endothelial basement membrane is utilized by $\alpha_v\beta_3$-expressing VSMCs. We propose a model in which developing arteries utilize Notch signaling between endothelial cells and local perivascular cells to generate
integrin β3 expressing VSMCs that gain the ability to engage vWF within the endothelial basement membrane and potentiate the maturation of newly formed arteries (Supplemental Figure 10).

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Authorship
Contribution: L.S. and D.A.C. designed the project; L.S. carried out the experiments, except the microarray which was carried out by E.A.M. and D.J.S. and the immunohistochemistry of Jag1 ECKO adult tissue which was carried out and analyzed by J.J.H. and M.L.I. A.Z., A.M., T.V.B., and Z.M.R. contributed new reagents. S.M.W., E.A.M., and D.J.S. contributed to experimental analysis. L.S. and D.A.C. analyzed the data and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
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References:


Table 1

VSMC mRNA fold-change following culture on Jag1

<table>
<thead>
<tr>
<th>Down &gt; 3-fold</th>
<th>Up &gt; 3-fold</th>
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<tr>
<td>Follistatin</td>
<td>Angiopoietin 2</td>
</tr>
<tr>
<td>ADAMTS1</td>
<td>ECGF1</td>
</tr>
<tr>
<td>TGFa</td>
<td>Hey1</td>
</tr>
<tr>
<td>VEGFC</td>
<td>Integrin beta3</td>
</tr>
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<td>PDGFR beta</td>
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<td>Platelet factor 4</td>
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Figure Legends

Figure 1

**Notch signaling is required for arterial maturation *in vivo***. Representative image of P6 retinas, following (A) vehicle (DMSO) or (B) γ-secretase inhibitor (DAPT) treatment to block Notch signaling from P2-P5, immunostained with antibodies specific to CD31 (red) and αSMA (green). DAPT treatment results in an immature vascular plexus, represented by enhanced vascular branching (arrows) and reduced αSMA⁺ perivascular cell coverage of arteries (arrowheads). (C) Vehicle treated pups show continuous VSMC coverage of arteries, marked by αSMA, unlike (D) DAPT-treated pups where individual VSMCs are clearly visible (arrowhead) and arterial coverage is interrupted (arrows). (E) DAPT treatment prevents arterial maturation, measured as a 40% decrease in arterial VSMC (αSMA⁺) coverage compared to controls. Values represent means ± s.e.m. DMSO (n=17), DAPT (n=25), where n is the number of arteries analyzed. *P*=0.004. Scale bars: A, B, 600 μm; C, D, 25μm

Figure 2

**Endothelial Jag1 maintains arterial VSMC coverage in development and adulthood.** (A, B) Representative images of vessels in P7 retinas from mice with (Jag1 lox/lox VECAD-Cre negative, “wild type”) and without (Jag1 lox/lox VECAD-Cre positive, “JAG1 ECKO”) Jag1 in the endothelium. Retinas were immunostained with antibodies to CD31 (red) and αSMA (green). (C-H) Large vessels from young adult mice were immunostained with antibodies to CD31
(red), αSMA (green), and TOPRO (blue). Aorta from wild type (C, D) and Jag1 ECKO (E, F) adult mouse. Arrows (F) indicate loss of smooth muscle cells in the layer immediately below the Jag1-negative endothelium. (G, H) Mesenteric vessel from Jag1 ECKO mouse, arrow (H) notes lack of smooth muscle cells. (I) Scale bars, A, B 100 μm; C-F, 50 μm; G, 20 μm.

Figure 3

A Jagged1/Notch interaction drives integrin αvβ3 expression in VSMC

(A) VSMC culture on a Jag1-coated substrate increased mRNA expression of integrin β3 5.3-fold by qRT-PCR (±0.7; n=6). Culture on Jag1 also upregulated canonical Notch-downstream genes Hes1 (3.4 ± 1.4, n=3) and Hey2 (2.4 ±0.5, n=4). (B) FACS analysis of VSMCs found that surface expression of integrin αvβ3 increased 3-fold in response to culture on a Jag1-coated substrate. (C) Representative images of retinas from mice treated with vehicle (DMSO) or DAPT to block Notch signaling from P3-P6, and analyzed P7. Retinas were immunostained with antibodies against integrin β3 (green) and αSMA (red) to label VSMCs, and CD31 (blue) to label endothelial cells. DAPT treatment inhibited the expression of αvβ3 in arterial-associated VSMCs, but did not inhibit the expression of αvβ3 in the sprouting angiogenic tip cells. Values represent means ± s.e.m., 2^ΔΔCT analysis. Scale bar, 50 μm.
Figure 4

Integrin β3 is required for VSMC coverage in developmental angiogenesis.

(A) Representative images of retinas undergoing early postnatal retinal developmental angiogenesis at three different time points: P3, P5, and P7. Mice with deficient downstream αvβ3 signaling (DiYF) have less arterial VSMC coverage at each time point than age-matched wild type (C57BL/6) controls. Retinas were immunostained with isolectin GS-IB4 from Griffonia simplificonia (lectin, red) to label the endothelium and αSMA to label VSMC (green). (B) VSMC coverage was measured along each artery as a percentage of the outgrowth of the vascular plexus (dotted lines). Arterial coverage is reduced by 30% at p3, 22% at p5, and 14% at P7. (C, D) Intravitreal injection of blocking antibodies against integrin β3 (D) reduces arterial αSMA staining compared to control (C). (E) The β3 function-blocking antibody reduced arterial VSMC coverage by 74% (P = 0.01). Values represent means ± s.e.m. C57BL/6 (n=12 (P3), n=23 (P5), n=19 (P7)), DiYF (n=15 (P3), n=23 (P5), n=21 (P7)), Isotype control-injected, n= 5; anti-β3 injected, n= 6, where n is the number of retinas analyzed. For DiYF time course, P = 1.4x10^{-4} (P3), P = 7.5x10^{-9} (P5), P = 1.0x10^{-3} (P7). Scale bars, A, 150 μm (P3); 300 μm (P5); 600 μm (P7); C, D, 50 μm.
Figure 5

vWF is required for VSMC coverage in developmental angiogenesis. (A) Representative images of retinas undergoing early postnatal retinal developmental angiogenesis at three different time points: P3, P5, and P7. Retinas were immunostained with CD31 (red) to label the endothelium and αSMA to label VSMCs (green). (B) vWF KO mice have significantly less VSMC coverage compared to age-matched controls, measured along each artery as a percentage of the outgrowth of the vascular plexus (dotted lines). Arterial coverage is reduced 37% at P3, 21% at P5, and 17% at P7. Values represent means ± s.e.m. C57BL/6 (n=12 (P3), n=23 (P5), n=19 (P7)), vWF KO (n=14 (P3), n=25 (P5), n=14 (P7)), where n is the number of retinas analyzed. P = 9.3x10^{-4} (P3), P = 1.7x10^{-9} (P5), P = 7.3x10^{-3} (P7). Scale bars, 150 μm (P3); 300 μm (P5); 600 μm (P7).

Figure 6

VSMC adhesion to vWF is due to a Notch-dependent upregulation of integrin αvβ3. (A, B) The ability of VSMCs to adhere to a vWF-coated substrate was quantified following 18-hour culture on control (IgG) or Jag1 coated plates (to stimulate Notch signaling). Blocking antibodies specific to αvβ3 (A, LM609) and the RGD-binding site of vWF (B, anti-152B) prevented Notch-dependent adhesion to vWF, whereas a blocking antibody specific to β1 integrins (A, anti-P4C10) did not. (C-K) Representative images of in vitro fibrin bead angiogenesis
assays in which HUVECs (green) were cultured on microcarrier beads and suspended in a fibrin gel with VSMCs (red). (C) A fibrin gel was fixed and immunostained with a fluorescein-labeled Ulex lectin specific to endothelial cells and with antibodies to vWF showed that the in vitro endothelial tubes produced a vWF-rich basement membrane. The addition of a blocking antibody to vWF (anti-152B) significantly reduced co-patterning, as quantified by pixel overlap in ImageJ, between VSMCs and endothelial tubes (F, G) compared to controls (D, E). Blocking Notch signaling specifically in VSMCs by siRNA knockdown (J, K) significantly reduced co-patterning compared to transfection with All Stars negative control siRNA (H, I). Arrows point to regions magnified, shown in E, G, I, K. (L) VSMC-endothelial co-patterning was reduced 66% when vWF blocking antibody was added to culture media and 69% when Notch3 was transfected into VSMCs. (M) Notch3 receptor knockdown in VSMCs was confirmed by western blot in comparison to VSMCs transfected with All Stars negative control siRNA, as was the resultant decrease in Notch signaling as measured by decreased Hey2 expression, a canonical Notch downstream transcription factor. Molecular weight (MW) of N3 precursor: 280 kDa; MW of truncated N3: 120 kDa. Values represent means ± s.e.m. IgG control (n=14); α-152b (n=17); control siRNA (n=23), Notch3 siRNA (n=23); where n is the number of beads analyzed; \( P=2.5\times10^{-13} \) (α-152b); \( P=8.4\times10^{-13} \) (N3 siRNA). Scale bars, D, F, H, J, 325 μm; C, E, G, I, K, 150 μm.
Figure 3

A  mRNA fold-change

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<thead>
<tr>
<th>Gene</th>
<th>Fold-change (2^ddCT)</th>
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<tr>
<td>Hes1</td>
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<tr>
<td>Hey2</td>
<td>2.5 ± 0.3</td>
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<tr>
<td>β3</td>
<td>6.0 ± 0.7</td>
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B  VSMC surface expression of αvβ3

- No treatment
- 18 hr culture on ctl IgG
- 18 hr culture on Jag1

C  β3 integrin  αSMA  CD31  Merge

Vehicle Control artery

DAPT artery

DAPT sprouting EC
Figure 4

A

<table>
<thead>
<tr>
<th></th>
<th>P3</th>
<th>P5</th>
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<tr>
<td>DiYF</td>
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B

- % Arterial VSMC coverage

C

- Vehicle

D

- anti-β3

E

- VSMC-EC colocalization

area fraction (pixel)

- Control
- anti-β3
Figure 5

A  P3  P5  P7

Wild type

CD31

αSMA

vWF KO

Wild type

B

Arterial αSMA+ perivascular coverage

Wild type - - vWF KO

% of artery αSMA+ postnatal day

0  20  40  60  80

3  5  7
Figure 6

A. Adhesion to vWF over time for different conditions: blue line (Jag1, anti-β1), green line (Jag1, anti-αvβ3), purple line (Ctl, anti-β1), and pink line (Ctl, anti-αvβ3).

B. Adhesion to vWF over time for different conditions: blue line (Jag1, vehicle), red line (Ctl, vehicle), green line (Jag1, anti-vWF), and purple line (Ctl, anti-vWF).

C. Lectin vWF stained images showing different vWF expression levels.

D. Control images showing neuron-like structures.

E. vWF Blocking Ab images showing neuron-like structures with red and green fluorescence.

F. vWF Blocking Ab images showing neuron-like structures with red and green fluorescence.

G. Negative control siRNA images showing neuron-like structures with red and green fluorescence.

H. Notch3 siRNA images showing neuron-like structures with red and green fluorescence.

I. Negative control siRNA images showing neuron-like structures with red and green fluorescence.

J. Notch3 siRNA images showing neuron-like structures with red and green fluorescence.

K. Negative control siRNA images showing neuron-like structures with red and green fluorescence.

L. Bar graph showing vSMC-EC tube co-patterning with percentage of co-alignment for different conditions:

- ctl
- anti-vWF
- ctl siRNA
- N3 siRNA

M. Western blot images for MW and IB: Notch3, Hsp60, Hey2.
Notch promotes vascular maturation by inducing integrin-mediated smooth muscle cell adhesion to the endothelial basement membrane