Vascular Smooth Muscle Notch SignalsRegulate EndothelialCell Sensitivity to Angiogenic Stimulation*

Ke Yang and Aaron Proweller

From the Department of Medicine, Case Cardiovascular Research Institute, and University Hospitals Harrington-McLaughlin Heart and Vascular Institute, Case Western Reserve University, Cleveland, Ohio 44106

The evolutionarily conserved Notch signaling pathway is required for normal vascular development and function, and genetic associations link select Notch receptors and ligands to human clinical syndromes featuring blood vessel abnormalities and stroke susceptibility. A previously described mouse model engineered to suppress canonical Notch signaling in vascular smooth muscle cells (vSMCs) revealed surprising anatomical defects in arterial patterning and vessel maturation, suggesting that vSMCs have the functional capacity to influence blood vessel formation in a Notch signaling-dependent manner. In further analyses using this model system, we now show that explanted aortic ring tissue and Matrigel implants from the smooth muscle Notch signaling-deficient mice yield markedly diminished responses to angiogenic stimuli. Furthermore, cultured Notch signaling-deficient primary vSMCs have reduced proliferation and migration capacities and reveal diminished expression of PDGF receptor β and JAGGED1 ligand. These observations prompted a series of endothelial cell (EC)-vSMC co-culture experiments that revealed a requirement for intact vSMC Notch signals via JAGGED1 for efficient EC Notch1 receptor activation and EC proliferation. Taken together, these studies suggest a heterotypic model wherein Notch signaling in vSMCs provides early instructive cues to neighboring ECs important for optimal postnatal angiogenesis.

Formation of the mammalian vascular system requires distinct and phasic processes that are highly regulated yet incompletely understood. Current developmental models feature the coalescence and differentiation of endothelial cell (EC)2 precursors that generate a tubular network through a process termed vasculogenesis. Subsequent “angiogenic” remodeling through EC proliferation, sprouting, branching, and regression overlaps with “vascular myogenesis,” characterized by the participation of vascular smooth muscle cells (vSMCs)/pericytes, providing structural and functional support for assembly of competent blood vessels (1). The myogenic influence is viewed as a relatively late contribution to vessel building, relying principally on the release of paracrine factors affecting EC behavior (2, 3). However, in some contexts, vSMCs may have a more pioneering role during early angiogenesis, serving either as guide posts for sprouting ECs or as modulators of the angiogenic response (4–7). As such, the temporal contribution of neighboring vSMCs in angiogenesis is highly complex and requires further scrutiny.

Canonical Notch signaling describes a pathway through which binding of four transmembrane Notch receptors (N1–N4) by JAGGED or DSL (Delta/Serrate/Lag-2) ligands results in γ-secretase-mediated cleavage and release of the Notch receptor intracellular domain and its translocation to the nucleus, wherein it partners with the potent transcriptional activator CSL/RBP-Jκ (8). This heterotrimeric complex induces the expression of Notch target genes, including members of the basic helix-loop-helix family, leading to cell context-dependent effects (9).

Among an elite group of angiogenic signaling axes, including those governed by VEGF, PDGF, Ephrin/Eph, TGFβ, and Ang1/2, the Notch signaling pathway is also required for pre- and postnatal vasculogenesis and/or angiogenesis (10–12). The angiogenic functions for individual Notch ligands and their receptors have been predominantly explored within ECs, highlighting a critical requirement for Notch1 and Dll4 proteins to interact in trans between stalk and tip ECs, respectively. This arrangement appears to confer a regulatory control of VEGF signal sensitivity that promotes competent vessel assembly during sprouting angiogenesis (13–17). Furthermore, JAGGED1 ligand expression in ECs has been linked to promoting efficient smooth muscle differentiation in vivo and more recently shown to be a negative regulator of Notch signaling by antagonizing Dll4-mediated EC Notch activation (18, 19). As such, a critical role for Notch signaling exists in ECs through interactions reminiscent of the lateral inhibition mechanism typical of this signaling pathway, in which juxtaposed cells establish altered fates from instructional Notch signals (20, 21).

Despite the relative expression of multiple Notch receptors and ligands in vSMCs, much less is known of the influences that individual smooth muscle Notch pathway components or total signaling output confers upon angiogenic processes (22–25). As a parallel to EC Notch signaling, the capacity for vSMCs to promote and engage in heterotypic contact and functionally influence neighboring cells in a Notch signaling-dependent...
manner is conceptually appealing, and recent reports utilizing Notch3-deficient tissues have begun to illuminate this possibility (26–28). As reported previously, an adult murine model (SM22-Cre+/DNMAML1−) engineered to suppress canonical Notch signaling in smooth muscle exhibited defects in arterial maturation and cerebroarterial patterning, suggesting that vSMC-derived Notch signals can have a profound influence on proper arterial vessel formation (29). An important strength of this model is that it relies on the elaboration of a dominant-negative form of MAM (DNMAML1) to inhibit Notch signals transduced by all Notch receptors on vSMCs. Therefore, the sum output of canonical Notch activity is significantly blunted (29). By overcoming potential functional redundancies among Notch receptors, SM22-Cre+/DNMAML1+ mice may provide a more comprehensive characterization of the net effect of Notch signaling loss in vSMCs.

Herein, we have begun to elucidate potential mechanisms underlying the vascular phenotypes observed in SM22-Cre+/DNMAML1+ animals. Surprisingly, we found that vascular tissues derived from Notch signaling-deficient mice exhibit markedly diminished angiogenic capacity as observed in explanted aortic ring and Matrigel implant assays. Examination of Notch signaling-deficient primary vSMCs revealed defective proliferation and migration with reduced levels of PDGF receptor β (PDGFRβ) and JAGGED1 ligand. Furthermore, co-culture of primary vSMCs with ECs revealed a surprising Notch signaling-dependent influence on EC growth potential. Taken together, our findings suggest a mechanistic model wherein vSMCs modulate early EC responses to angiogenic stimuli through instructional Notch signals.

EXPERIMENTAL PROCEDURES

Animals—The generation and initial characterization of SM22-Cre+/DNMAML1+ (DNMAML1-expressing) and control SM22-Cre−/DNMAML1+ (non-DNMAML1-expressing) littermate mice were reported previously (29). All mice were maintained in a C57BL/6 genetic background. Animal experimentation was performed under the approved protocols of the Case Western Reserve University Animal Care and Use Committee and National Institutes of Health guidelines.

Antibodies and Growth Factors—As indicated for immunostaining and Western blotting, antibodies included rabbit monoclonal anti-mouse CD31 (Pharmingen); mouse monoclonal anti-smooth muscle α-actin (SMA) 1A4 and Cy3-conjugated anti-smooth muscle SMA (Sigma); rabbit polyclonal anti-NG2 and anti-γ-actin (SMA) 1A4 and Cy3-conjugated anti-γ-actin (SMA) 1A4 (Cell Signaling); and rabbit polyclonal anti-cleaved Notch1, and anti-β-actin (Cell Signaling); and anti-phosphohistone H3, anti-phospho-PDGFRβ, anti-phospho-ERK1/2, anti-Akt, anti-phospho-Akt, anti-JAGGED1, anti-cleaved Notch1, and anti-β-actin (Cell Signaling); and anti-proliferating cell nuclear antigen (PCNA; Abcam). Secondary antibodies included Alexa Fluor® 594- or rhodamine-labeled anti-rabbit/rabbit IgG (Jackson ImmunoResearch Laboratories) and Alexa Fluor 488-conjugated goat anti-rat IgG (H+L) secondary antibody. Mural cells were identified by reaction with primary antibodies against SMA or the proteoglycan NG2 (1:200). Tissues were mounted with DAPI (Dako) and imaged using a high resolution inverted fluorescence microscope platform (Leica DMi6000B) with bright-field optics. Vessel quantification was performed by counting neovessel numbers within the corresponding regions of interest from both control and mutant rings. Where indicated, EC and mural cell association in neovessel structures was identified by whole mount immunostaining. Briefly, aortic ring explants were formalin-fixed, blocked with 1% BSA and 5% normal goat serum, and permeabilized with 0.5% Triton X-100. ECs were detected using rat monoclonal anti-mouse CD31 primary antibody (1:50) with Alexa Fluor 488-conjugated goat anti-rat IgG (H+L) secondary antibody. Mural cells were identified by reactivity with primary antibodies against SMA or the proteoglycan NG2 (1:200). Tissues were mounted with DAPI (Dako) and imaged using a high resolution inverted fluorescence microscope platform (Leica DMi6000B).

Matrigel Plug Angiogenesis Assay—Growth factor-reduced Matrigel (BD Biosciences) was combined in solution with recombinant VEGF (200 ng/ml), FGF2 (1 μg/ml), and 60 units/ml heparin (Sigma). The gel solutions (500 μl each) were uniformly injected subcutaneously into the abdominal region of anesthetized mice. Matrigel plugs were harvested on days 3, 5, and 7 and either fixed overnight in 4% paraformaldehyde for paraffin embedding and hematoxylin and eosin staining or directly frozen in O.C.T. compound (Tissue-Tek) for immunohistochemistry. To aid visualization of neovessel organization and competency, 100 μl of FITC-conjugated dextran (25 mg/ml, Mr = 2 × 10⁸, Sigma) was administered intravenously via the right jugular vein 10 min before plug harvest. Immunohistochemistry of Matrigel Plugs—Frozen Matrigel plugs were sectioned at 10-μm thickness, followed by brief fix-
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Proliferation Studies—Primary aortic vSMCs from SM22-Cre+/DNMAML1- or SM22-Cre+/DNMAML1+ mice were seeded at a density of 1 × 10^4/cm² onto 96-well plates (Falcon) in medium containing 50% Ham's F-12 + 50% DMEM, 0.1 or 0.5% FBS, 1% penicillin/streptomycin (Invitrogen), 1% l-glutamine (Invitrogen), and 2% Hepes (20 mM; Invitrogen). Cell proliferation was assessed at day 3 using routine anti-phospho-Akt and ERK1/2 colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma) following the manufacturer’s instructions. MTT samples were read on a Molecular Devices kinetic plate reader using a test wavelength of 570 nm and a reference wavelength of 450 nm.

Migration Studies—Aortic ring explants in Matrigel were prepared similarly as for angiogenesis studies except that the culture medium contained 2.5% FBS rather than autologous serum. PDGF-BB (25 ng/ml) was added to the explant cultures, and the medium was replaced every 2 days. At the indicated time points, vSMC outgrowth promoted by PDGF-BB was identified by whole mount SMA staining. A modified Transwell assay was used to measure the migration capacities of isolated primary vSMCs. Briefly, aortic SMCs derived from SM22-Cre+/DNMAML1- or SM22-Cre+/DNMAML1+ mice were seeded at a density of 5 × 10^4 cells/well in the upper compartment of the Transwell insert coated with type I collagen (0.1 μg/cm²). Following 18 h of serum starvation, cells were permitted to migrate for 4 h in the presence or absence of 25 ng/ml PDGF-BB in migration medium (DMEM + Ham’s F-12 and 0.1% BSA) in the lower chamber compartment. The cells on the upper side were removed by a cotton tip before fixing the insert membrane with 4% paraformaldehyde. DAPI mounting medium was used to identify migrated cells present on the bottom side of the membrane. Cells were counted in five randomly chosen fields at high magnification (×100) using fluorescence microscopy. Each treatment was performed in triplicate.

Where indicated, cell migration studies were performed using SM22-Cre+/DNMAML1+ -derived vSMCs infected with adenovirus encoding human PDGFRβ (Vector Biolabs). 5 × 10^5 cells were infected with 5 μl of virus particles (per the manufacturer’s titer) in 2 ml of vSMC growth medium to achieve a multiplicity of infection of 10. After 48 h, infected cells were collected and submitted to Transwell migration studies as described above.

Protein Analyses—Cultured primary vSMCs were harvested at the indicated time points, and total cell protein lysates were collected in radioimmune precipitation assay buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM PMSE, and 1 × Roche Complete miniprotease inhibitor). Lysates were subjected to denaturing gel electrophoresis, semidry Western transfer, and immunoblotting. Proteins were detected by chemiluminescence (HRP), and films were digitally scanned for protein quantification using NIH Image software. Relative protein levels were normalized to the β-actin signal.

PDGFRβ Effector Stimulation Studies—For measurement of PDGFRβ signaling activity, serum-starved SM22-Cre+/DNMAML1- and control SM22-Cre+/DNMAML1+ vSMCs were incubated with or without PDGF-BB (40 ng/ml) for 5 min, 30 min, or 4 h, followed by lysis in ice-cold radioimmune precipitation assay buffer. Total cell protein was submitted to Western blot analyses for detection of phosphorylated (activated) PDGFRβ and downstream effectors Akt and ERK1/2.

Heterotypic Co-culture Assay—Primary aortic SMCs derived from SM22-Cre+/DNMAML1- or control SM22-Cre+/DNMAML1+ mice were seeded at a density of 5 × 10^5 cells/well, corresponding to >90% confluency upon adherence, and maintained in medium containing 45% Ham’s F-12, 45% DMEM, and 10% FBS. Mouse brain-derived ECs (1 × 10^5; bEnd.3; American Type Culture Collection) were layered onto the vSMC monolayer, and the co-cultures were propagated for 48 h. Cultures were trypsinized, followed by the addition of ICAM2-conjugated Dynabeads (Pharmingen) to the cell suspension and magnetic separation of ECs from vSMCs. Selectivity of recovered EC and SMC populations was highly specific based on the failure to detect SMA and PECAM protein by Western blotting in the EC and vSMC lysates, respectively (data not shown). Cell lysates were prepared and subjected to protein analyses as described above.

JAGGED1 Knockdown in Primary Aortic Smooth Muscle Cells—Lentiviral shRNA particles targeting mouse JAGGED1 were purchased from Santa Cruz Biotechnology (sc-37203-V). Briefly, control SM22-Cre-/DNMAML1-derived vSMCs (5 × 10^4) were incubated with 5 μl of particles in 2 ml of vSMC growth medium. After 48 h, the medium was replaced with fresh medium and supplemented with 5 μg/ml puromycin to select positive shRNA-expressing cells. Selected cells were replated and co-cultured with bEnd.3 ECs as described under “Heterotypic Co-culture Assay.”

Statistical Analysis—Data are presented as means ± S.E. Statistical analysis was performed using Student’s paired one-tailed t test. p values <0.05 were considered significant.

RESULTS

Aortic Rings Derived from Mice with Smooth Muscle Cell Notch Signaling Deficiency Display Impaired Vascular Sprouting—Mouse aortic ring explant assays were employed to determine the angiogenic capacity of postnatal SM22-Cre+/DNMAML1+ animals. Aortic rings dissected from SM22-Cre+/DNMAML1+ (mutant) or SM22-Cre-/DNMAML1+ (control) littermate mice were embedded in Matrigel with the requisite inclusion of 2.5% mouse serum to stimulate vessel sprouting. Compared with control rings, which exhibited robust angiogenesis, explants derived from SM22-Cre+/DNMAML1+ mice displayed a >70% reduction in neovessel formation.
formation (Fig. 1, compare A–D with F–H and J). Whole mount staining for CD31 and SMA in control neovessels revealed expected alignment of ECs and vSMCs, respectively, reflecting mature vessel structures (Fig. 1 J).

To circumvent the need for serum stimulation and therefore to judge responses to select growth factors more specifically, aortic ring explant cultures were performed in rat collagen gel in the presence of 20 ng/ml VEGF. Under these select conditions, VEGF elicited the maximal number of neovessels by day 7 in control aortic explants (Fig. 2, A–D). These vessels displayed a mature luminal arrangement of CD31+/H11001 ECs enveloped by NG2+/H11001 (and SMA+/H11001; data not shown) mural cells consistent with vSMCs (Fig. 2, J and K). In contrast, aortic rings derived from SM22-Cre+/DNMAML1mice displayed a dramatically poor response to VEGF (Fig. 2, E–H). As summarized in Fig. 2 I, the average number of microvessels observed in control versus mutant aortic ring explants was 12.2 ± 1.9 and 1.8 ± 0.8, respectively. In addition, the few sprouts detected in mutant ring cultures were predominantly CD31+ structures lacking mural coverage (Fig. 3, compare A–D with E–H). Quantification of the vSMC number per unit vessel length revealed a significant difference of 3.2 ± 0.7 versus 0.8 ± 0.5 between control and mutant rings, respectively (Fig. 3 I). These data demonstrate poor angiogenic capacity of aortic rings with intrinsic vSMC Notch signaling deficiency. Furthermore, the suboptimal angiogenic response to serum stimulation in Matrigel was recapitulated under the select influence of VEGF in collagen-based explants, suggesting impaired EC sensitivity to this growth factor.

Matrigel Plug Implants Reveal Poor Vascular Engraftment in SM22-Cre+/DNMAML1 Mice—To extend our observations from aortic ring explantation, assessment of in vivo postnatal angiogenesis capacity in SM22-Cre+/DNMAML1 mice was performed using a Matrigel-based implant system. Matrigel supplemented with FGF2 and VEGF was subcutaneously injected into SM22-Cre+/DNMAML1 or control SM22-
Cre-^+/DNMAML1^+ mice following procedures optimized for inducing functional blood vessel engraftment characterized by luminal red blood cell content and FITC-dextran filling. At 7 days post-implantation, a well organized vascular network was observed in control plugs, with vessel competency confirmed by intact FITC-dextran filling (Fig. 4, A–D). In contrast, blood- or dextran-filled conduits were poorly delineated in Matrigel plugs derived from SM22-Cre-^+/DNMAML1^+ animals and typically displayed randomly diffuse red blood cell and dextran deposition (Fig. 4, M–P). In control plugs, close inspection by histological analyses revealed an organized assembly of dextran-filled conduits lined by both ECs and vSMCs determined by CD31 and SMA immunohistochemical detection, respectively (Fig. 4, E–H and I–L, respectively). In contrast, mutant-derived plugs exhibited a qualitative absence of competent vessels, with loosely associated ECs and vSMCs bearing little spatial relationship to FITC-dextran perfusion (Fig. 4, Q–T and U–X, respectively). These contrasting findings were also apparent in SM22-Cre-^+/DNMAML1^+- and control mouse-derived Matrigel plugs harvested earlier on day 5 (supplemental Fig. 1), suggesting that impaired vessel assembly is present at the earliest detectable stages in mutant plugs.

To examine whether cellular apoptosis was contributing to the observed paucity of competent vessel structures in mutant plugs, TUNEL staining and caspase-3 immunoreactivity were applied to day 7 plug sections but did not reveal evidence consistent with enhanced apoptosis (supplemental Fig. 2). In summary, the Matrigel plug studies provide evidence for altered postnatal angiogenesis in SM22-Cre-^+/DNMAML1^+ mice, in which early vessel assembly is compromised with the apparent absence of vessel regression or apoptosis. Together with the findings from aortic ring studies, the data support a role for Notch signaling in vSMCs as a modulator of early EC responses to angiogenic stimulation both ex vivo and in vivo.

Notch Signaling Deficiency in vSMCs Inhibits Serum-stimulated Proliferation and Growth Factor-induced Migration—To investigate behavioral features of Notch signaling-deficient vSMCs that may contribute to defective postnatal angiogenesis, cell proliferation and migration capacities were initially measured in primary vSMCs derived from control and SM22-Cre-^+/DNMAML1^+ mice. Methods for isolating primary aortic vSMCs yielded expectant GFP^+ (DNMAML1-expressing) cells from mutant mouse aortas (supplemental Fig. 3). vSMC growth capacity was assessed by measuring mitotic DNA

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**FIGURE 2.** Refractory VEGF-induced angiogenesis in collagen-based aortic ring explants derived from SM22-Cre-^+/DNMAML1^+ mice. A–D, representative control aortic ring explants cultured for 7 days in either serum-free MCDB131 medium (A) or medium enriched with human recombinant VEGF (20 ng/ml) (B–D). E–H, representative SM22-Cre-^+/DNMAML1^+ mouse-derived aortic ring explants cultured for 7 days in either serum-free MCDB131 medium (E) or medium enriched with human recombinant VEGF (20 ng/ml), resulting in relatively few sprouting vessels (F–H). I, microvessels were quantified as described in the Fig. 1 legend (12.2 ± 1.9 versus 1.8 ± 0.8 for WT versus mutant, respectively; *, p < 0.01; n = 6). J and K, in control explants, the maturity of microvessels was determined by CD31 (green) and NG2 (red) staining of EC- and vSMC-lined conduits, respectively. Scale bars = 100 μm (A–H and J) and 10 μm (K).
content in cultured cells using antibodies directed against phosphohistone 3. Immunostaining revealed a significant reduction in phosphohistone H3-positive cell numbers as early as day 3 in Notch signaling-deficient vSMCs compared with control cell cultures (10.1 ± 3.1 versus 32.2 ± 12.4, respectively) (Fig. 5A). These findings were further confirmed using an established spectrophotometric assay in which tetrazolium salt (MTT) is reduced to an insoluble purple formazan crystal in metabolically active proliferating cells. As shown in Fig. 5B (purple line), a significant reduction in the absorbance at 570 nm was observed in serum-stimulated Notch signaling-deficient vSMCs compared with control cells (0.07 ± 0.01 versus 0.10 ± 0.01 at day 3 and 0.09 ± 0.02 versus 0.28 ± 0.06 at day 6, respectively).

The migration capacity of vSMCs from control and Notch signaling-deficient mice was also determined first by chemotactic responses to PDGF-BB in Matrigel-based aortic ring explants. PDGF-BB supplementation elicited extensive SMA-positive cellular outgrowth (occupying nearly 45% of the defined region of interest) in control aortic rings but only minimal outgrowth (5%) in SM22-Cre+/DNMAML1+ derived aortic ring cultures (Fig. 6, compare A–C with D–F and G). In addition, exposure of cultured primary vSMCs to PDGF-BB (20 ng/ml) for 4 h stimulated the migration of control aortic vSMCs by 9.5-fold in a standard Transwell assay system (Fig. 6H). In contrast, only a 4.7-fold increase in migration was observed in Notch signaling-deficient vSMCs (Fig. 6H). Taken together, these data indicate that intrinsic Notch signaling is important for proliferation and migration of mouse primary vSMCs in vitro.

Notch Signaling-deficient Primary vSMCs Exhibit Reduced PDGFRβ and JAGGED1 Protein Levels—The observation that vSMCs from SM22-Cre+/DNMAML1+ mice displayed defective PDGF-induced migration prompted an assessment of the levels of the receptor, PDGFRβ, which was recently identified as a novel transcriptional target of Notch activation (31). Protein lysates were prepared from control and SM22-Cre+/DNMAML1+ derived aortic vSMCs propagated under serum and serum-free (base-line) conditions. Western blot analysis revealed decreased levels of PDGFRβ...
FIGURE 4. Poor vascular engraftment in Matrigel plug implants from SM22-Cre+/DNMAML1+ mice. VEGF/FGF2-supplemented Matrigel plugs from control and mutant mice were harvested 7 days post-implantation. Low magnification bright-field (A and M) and fluorescence (FITC-dextran-infused; green) (B and N) images reveal abnormal vascular structures and patterning in mutant plugs (M and N). C/D and O/P, hematoxylin/eosin-stained sections from control and mutant plugs, respectively. E–H and I–L, organized assembly of dextran-filled conduits lined by CD31+/ECs and SMA+/vSMCs, respectively, in Matrigel plugs from control mice. Q–T and U–X, mutant plugs reveal abnormal dextran deposition without closely associated ECs and vSMCs, respectively. Note that F–H are high magnification views of random regions in E; likewise, R–T are high magnifications from Q, J–L from I, and V–X from U. Scale bars = 100 μm (C, E, I, O, Q, and U) or 10 μm (A, B, D, F–H, J–N, P, R–T, and V–X).
in Notch signaling-deficient cells compared with controls under all growth conditions tested (Fig. 7, A and B). Consistent with reduced PDGFRβ content in SM22-Cre⁺/DNMAML1⁺ vSMCs, interrogation of the downstream PDGFRβ signaling axis in these cells following PDGF-BB stimulation revealed significant decreases in phosphorylated (activated) ERK1/2 and Akt in addition to phospho-PDGFRβ compared with control cells (supplemental Fig. 4).

To further establish a functional linkage between abnormal migration and decreased PDGFRβ levels in Notch signaling-deficient vSMCs, rescue experiments were performed in which adenoviral delivery of human PDGFRβ (Ad-hPDGFRβ) into SM22-Cre⁺/DNMAML1⁺ vSMCs restored ~80% PDGFRβ content relative to wild-type control cells infected with adenovirus bearing GFP (Ad-GFP) (Fig. 8A). In contrast to Ad-GFP-infected SM22-Cre⁺/DNMAML1⁺ cells, Ad-hPDGFRβ-infected vSMCs displayed a significantly improved migratory response to PDGF-BB at levels comparable with wild-type Ad-GFP-infected cells (Fig. 8B). These findings support the conclusion that impaired migration of Notch signaling-deficient vSMCs results, at least in part, from reduced endogenous PDGFRβ levels.

PDGFR signaling and Notch receptor stimulation have been shown to modulate levels of the Notch ligand JAGGED1 in vSMCs (27, 32). Therefore, expression of this ligand was also measured in cultured SM22-Cre⁺/DNMAML1⁺ cells. Western blotting revealed a dramatic (~90%) decline in JAGGED1 levels in the Notch signaling-deficient cells compared with controls in the presence or absence of serum (Fig. 7, A and B). These collective data confirm in mouse primary vSMCs that the expression of PDGFRβ and JAGGED1 is influenced by intrinsic cell Notch signaling.

**FIGURE 5.** **Reduced proliferation capacity of Notch signaling-deficient primary aortic smooth muscle cells.** A, a significantly fewer number of phosphohistone H3-positive (PH3⁺) cells were recorded in serum-stimulated cultures of vSMCs derived from SM22-Cre⁺/DNMAML1⁺ mice compared with controls (10.1 ± 3.1 versus 32.2 ± 12.4, respectively; *, p < 0.01; n = 3). **B**, MTT assay. Serum stimulation of quiescent vSMCs for 3 or 6 days resulted in significantly less absorbance at 570 nm (purple line) in Notch signaling-deficient smooth muscle cells compared with controls (0.07 ± 0.011 versus 0.10 ± 0.01 on day 3 and 0.09 ± 0.02 versus 0.28 ± 0.06 on day 6, respectively; p < 0.01; n = 3).

**ECs Exhibit Reduced Growth Rates Concomitant with Lower Levels of Activated Notch1 Receptor (Notch1 Intracellular Domain (NICD)) after Co-culture with Notch Signaling-deficient vSMCs**—Given the relative loss of JAGGED1 expression, experiments were performed to address 1) whether Notch signaling-deficient vSMCs would fail to trigger Notch receptor stimulation on ECs in a heterotypic co-culture system and 2) if EC growth is altered under such conditions. In these experiments, mouse brain-derived ECs (bEnd.3) were utilized to preserve species uniformity in the co-cultures and were determined to express multiple Notch receptors, including Notch1 (Fig. 9A, middle panel) and JAGGED ligand (data not shown). Co-culture conditions were designed to overcome growth and migration deficiencies inherent to Notch signaling-deficient vSMCs, thereby eliminating disparities in vSMC number during the incubation period. This was achieved by plating a sufficient and equivalent number of either control or Notch signaling-deficient vSMCs to form a confluent monolayer within a few hours of plate adherence. Thereafter, bEnd.3 ECs were applied to the vSMC monolayer of either control or Notch signaling-deficient vSMC cultures, followed by further incubation (Fig. 9A, left panel). Cultures were subsequently disrupted, and ECs were isolated by Dynabeads selection. Total protein from recovered ECs was subjected to Western blotting for the determination of cell-replicative capacity (PCNA detection) and levels of activated Notch1 receptor (NICD). Compared with ECs co-cultured with control vSMCs, ECs undergoing contact incubation with Notch signaling-deficient vSMCs revealed an ~40% reduction in both cleaved Notch1 and the proliferation marker PCNA (Fig. 9A, middle and right panels). These data suggest that in a heterotypic two-dimensional co-culture system, intrinsic EC growth responses and levels of Notch1 activity depend on the fidelity of neighboring vSMC Notch signals and
implicate JAGGED1 in vSMCs as an important instructional ligand in this context.

To corroborate these findings, shRNA knockdown of JAGGED1 was performed in control SM22-Cre+/DNMAML1+ vSMCs, which were re-deployed in co-culture with bEnd.3 ECs. Fig. 9B shows that JAGGED1 levels in vSMCs were reduced by nearly 50% following JAGGED1-specific shRNA treatment compared with scrambled control shRNA. In contrast to incubation with control shRNA-treated vSMCs, bEnd.3 ECs co-cultured with JAGGED1 shRNA-containing vSMCs revealed significant reductions in both endogenous NICD and PCNA expression (~25 and 40%, respectively) (Fig. 9C). These results phenotypically copy the protein expression pattern observed in ECs co-cultured with SM22-Cre+/DNMAML1+ vSMCs, supporting the concept that modulation of EC Notch activity and proliferative gene expression is a feature of instructional Notch signaling cross-talk from vSMCs to ECs in part through vSMC JAGGED1.

**DISCUSSION**

Vascular formation involves a complex interplay of signals from ECs, vSMCs, and non-vascular cells required for proper vessel assembly and function. These interactions are both temporally and spatially regulated. In this study, the output of
Notch signaling in vSMCs was found to have an early regulatory role in influencing EC responses to angiogenic stimuli. First, in a tissue explant system, we demonstrated that sprouting angiogenesis and neovessel formation are strikingly compromised in SM22-Cre^+/DNMAML1^−/DNMAML1^− vSMCs, which are capable of optimal association and functional contact with ECs. Second, the postnatal vessel-building capacity of SM22-Cre^+/DNMAML1^− vSMCs was also tested in vivo using Matrigel implants preconditioned with angiogenic growth factors VEGF and FGF2. These studies further revealed a remarkable incapacity for competent angiogenesis in the Notch signaling-deficient animals. Whereas a mature, highly organized, and well perfused neovascular system representing successful engraftment with the host circulation was identified in control mice, perfused neovascular system representing successful engraftment with the host circulation was identified in control mice. Hence, together with the aortic ring studies, it appears that Notch signaling in vSMCs confers an important regulatory role during early angiogenesis, possibly through modulation of EC responses to growth factor stimuli.

Moreover, SM22-Cre^+/DNMAML1^−/DNMAML1^− vSMCs also exhibited a marked reduction in JAGGED1 protein, which may have implications for the capacity for mutant vSMCs to engage Notch receptors displayed on neighboring vascular cells in either a homotypic (vSMC-vSMC) or heterotypic (vSMC-EC) manner. This observation in mouse primary vSMCs extends a previous report of Notch signaling regulation of JAGGED1 expression in which transduction of the Notch3 receptor by EC JAGGED1 ligand resulted in the modulation of JAGGED1 levels in vSMCs (27). The altered JAGGED1 protein levels in SM22-Cre^+/DNMAML1^−/DNMAML1^− vSMCs could be explained by insufficient JAGGED1 ligand engagement in mutant vSMCs. Notably, Ecs co-cultured with JAGGED1-depleted wild-type vSMCs revealed comparable reductions in endogenous Notch1 receptor cleavage and PCNA levels, thus recapitulating the effects of Notch signaling-deficient SM22-Cre^+/DNMAML1^− cells. Collectively, these findings raise the possibility that through the output of Notch signaling, vSMCs provide instructional cues that alter EC growth responses. We propose that during early angiogenesis, vSMCs with normal PDGFRβ levels and JAGGED1 expression are capable of optimal association and functional contact with ECs, where ligand engagement of EC Notch receptors promotes EC Notch signaling and proliferation required for efficient angiogenesis. In contrast, Notch signaling-deficient vSMCs bearing reduced PDGFRβ and JAGGED1 protein may display inefficient heterotypic association and limited transduction of EC Notch receptors, resulting in poor EC sensitivity to angiogenic stimuli.
Our model extends recently reported findings from Liu et al. (28) that the absence of the Notch3 receptor in mice rendered inefficient retinal angiogenesis despite the leading-edge presence of vSMCs during early phases of vascular growth. Although the precise mechanism of angiogenic regulation remains unclear, our finding that EC growth capacity is perturbed in association with lower levels of EC Notch1 activity may provide a mechanistic link between dysregulated vSMC Notch signaling and EC function required for efficient angiogenic growth responses.

However, our studies do not exclude the possibility that Notch signaling in wild-type vSMCs controls expression of a secretory factor promoting EC responses to angiogenic stimuli independent of JAGGED1 or EC Notch signaling activity. For instance, in the study by Liu et al. (28), Notch3 activity appeared necessary for Ang2 expression in vSMCs specifically during pathological retinal angiogenesis. Moreover, it is also possible that other Notch ligands present in vSMCs contribute to intercellular cross-talk. Two recent studies reported the initial characterization of mice with smooth muscle-specific JAGGED1 deletion. Hofmann et al. (34) observed that JAGGED1 deficiency resulted in partial perinatal lethality from hepatobiliary development failure. In contrast, Feng et al. (33) observed perinatal lethality resulting from patent ductus arteriosus. A comprehensive examination of the pre- and postnatal systemic vasculature in these animals has yet to be described, although there was an indication of reduced hepatic artery content in the study by Hofmann et al. A more detailed re-exploration of these animal models may help clarify the role of vSMC JAGGED1 in heterotypic cell interactions in the context of angiogenic processes.

To date, a complete understanding of the role of select Notch receptors and ligands in vSMCs is lacking. Although Notch3 receptor knock-out mice have been subjected to numerous vascular analyses and have exposed key roles in SMC function and vascular reactivity, other Notch receptors are present in vSMCs, supplying residual Notch signaling (22, 24, 35–37). The use of mouse models (such as SM22-Cre+/DNAML1+/−) designed to repress canonical Notch signaling activity at the level of the Notch-CSL-MAM transcription complex provides a useful tool to understand how the total output of canonical Notch signaling in vSMCs might regulate key processes, including angiogenesis. SM22-Cre+/DNAML1+/− mice survive into adulthood despite altered vascular patterning and arterial mat-
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uration, suggesting that Notch signaling in vSMCs is necessary for proper vascular formation but not for survival into adulthood (29). The observed defects in the angiogenesis studies reported here suggest that the disrupted arterial patterning previously identified in the Notch signaling-deficient animals may be partially a consequence of dysfunctional EC responses during normal angiogenic processes both in utero and in the adult animal. Further investigation will be necessary to clarify the

FIGURE 9. Reduced levels of proliferation marker PCNA and activated Notch1 receptor (NICD) in ECs co-cultured with either Notch signaling- or JAGGED1-deficient primary vSMCs. A, left panel, the schematic illustrates the heterotypic co-culture assay system as described under “Experimental Procedures.” Briefly, mouse brain-derived ECs (bEnd.3) were layered onto either control or mutant vSMC monolayers and incubated for 48 h, followed by EC selection and protein isolation. Middle panel, a representative immunoblot analysis displayed reduced PCNA detection and levels of activated Notch1 receptor (NICD) in ECs co-cultured with SM22-Cre+/DNMAML1+/H11001 mouse-derived vSMCs compared with control SM22-Cre+/DNMAML1+/H11001 vSMCs. Right panel, immunoblot quantification (normalized to the β-actin signal) revealed a 40% relative reduction in both cleaved Notch1 and PCNA levels (*, p < 0.01; n = 3). B, a representative immunoblot (left panel) and quantification analyses (right panel) of wild-type SM22-Cre+/DNMAML1+/H11001 cell lysates revealed a nearly 50% reduction in JAGGED1 protein after delivery of JAGGED1-targeted lentiviral shRNA versus a control scrambled shRNA (Con). Protein levels were normalized to the β-actin signal and to JAGGED1 in control shRNA cells (*, p < 0.05; n = 3). C, representative immunoblot (left panel) and quantification analyses (right panel) displaying levels of PCNA and activated Notch1 receptor (NICD) in ECs co-cultured with vSMCs containing JAGGED1 shRNA versus control shRNA. Normalization to the β-actin signal revealed 25 and 40% relative reductions in cleaved Notch1 and PCNA levels, respectively (*, p < 0.01; n = 3).
interacting functions provided by vSMCs and ECs in early angiogenesis.

Notch signaling within ECs has a critical role during developmental angiogenesis, providing instructive cues to neighboring ECs through Notch ligand-receptor interactions typical of the canonical Notch signaling pathway. Current models dictate that Dll4 ligands displayed on tip ECs engage relatively abundant Notch1 receptors on stalk ECs, suppressing their migratory behavior while promoting a proliferation capacity necessary for the emerging sprout. Interestingly, EC JAGGED1 has recently been shown to inhibit the Dll4-Notch signaling axis in ECs during mouse retinal angiogenesis, adding to the complexity of molecular interactions between EC populations (18). Given the juxtaposition of vSMCs and ECs in differing angiogenic contexts, it may not be surprising that vSMC Notch ligands (and receptors) could be important inputs modulating early EC angiogenic responses.

Herein, we provide novel experimental evidence suggesting that vSMCs modulate EC growth responses in a Notch signaling-dependent manner. These findings support a growing body of literature uncovering an important vSMC influence during the initial phases of vessel building.

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