Notch3 Is Critical for Proper Angiogenesis and Mural Cell Investment

Hua Liu, Wenbo Zhang, Simone Kennard, Ruth B. Caldwell, Brenda Lilly

Rationale: The heterotypic interactions of endothelial cells and mural cells (smooth muscle cells or pericytes) are crucial for assembly, maturation, and subsequent function of blood vessels. Yet, the molecular mechanisms underlying their association have not been fully defined.

Objective: Our previous in vitro studies indicated that Notch3, which is expressed in mural cells, mediates these cell–cell interactions. To assess the significance of Notch3 on blood vessel formation in vivo, we investigated its role in retinal angiogenesis.

Methods and Results: We show that Notch3-deficient mice exhibit reduced retinal vascularization, with diminished sprouting and vascular branching. Moreover, Notch3 deletion impairs mural cell investment, resulting in progressive loss of vessel coverage. In an oxygen-induced retinopathy model, we demonstrate that Notch3 is induced in hypoxia and interestingly, pathological neovascularization is decreased in retinas of Notch3-null mice. Analysis of oxygen-induced retinopathy mediators revealed that angiopoietin-2 expression is significantly reduced in the absence of Notch3. Furthermore, in vitro experiments showed that Notch3 is sufficient for angiopoietin-2 induction, and this expression is additionally enhanced in the presence of hypoxia-inducible factor 1α.

Conclusions: These results provide compelling evidence that Notch3 is important for the investment of mural cells and is a critical regulator of developmental and pathological blood vessel formation. (Circ Res. 2010;107:860-870.)

Key Words: Notch3 ■ retina ■ angiogenesis ■ smooth muscle cell ■ pericytes ■ blood vessel
patterning and maturation. In fact, recent findings of several research groups revealed a vital role for endothelium-dependent Notch signaling in tip cell formation and sprouting. Yet, it is unclear to what extent Notch-regulated vascular patterning events are consequences of homotypic or heterotypic signaling.

In contrast to Notch1, which is widely expressed, Notch3 is exclusively expressed in mural cells in the vasculature. In humans, Notch3 gene mutations give rise to CADASIL, an inherited early stroke syndrome leading to dementia caused by systemic vascular degeneration and eventual loss of VSMCs within the arterial wall. In the mouse, genetic studies have implicated Notch3 in the regulation of smooth muscle maturation, modulation of vascular physiology, and response to ischemia. Although these studies shed light on the importance of Notch3 in blood vessel structure and function, they did not investigate the direct role of Notch3 in governing blood vessel formation. In this study, we explored the contribution of Notch3 in developmental and pathological angiogenesis using the mouse retina as a model. Our findings imply that Notch3 facilitates heterotypic interaction between endothelial and mural cells that in turn dictates blood vessel patterning. Furthermore, Notch3 appears to play a role in response to hypoxia by regulating the expression of Ang-2.

**Methods**

**Animals**

Notch3+/− mice were a generous gift from Dr Tom Gridley. Notch3+/−, Notch3−/−, and wild-type littermates were obtained by crossing Notch3+/− and Notch3−/− mice. All experimental procedures on mice were performed according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee for the Use of Animals in Research.

**Cell Culture**

Human aortic smooth muscle cells from Lonza were grown in DMEM supplemented with 10% FBS, 100 IU/mL penicillin-streptomycin. Cells between passages 6 and 8 were used for experiments. Mouse aortic smooth muscle cells were isolated from aortas of Notch3+/− and Notch3−/− mice. Cells between passages 3 and 8 were used for experiments. Lentiviral and adenoviral transduction was performed as described previously.

**Immunostaining on Whole-Mount Retinas**

The immunostaining in retinas was performed as described previously. Briefly, eyes were isolated from Notch3−/− and Notch3+/− mice at indicated time points and fixed in 4% paraformaldehyde for 30 minutes. The cornea, sclera, lens, vitreous, and hyaloid vessels were removed to isolate retinas and radial incisions were made at equal intervals along the retinal edge. Subsequently, retinas were placed in cold methanol for 20 minutes, blocked, and permeabilized in PBS containing 5% donkey serum and 0.3% Triton X-100 for 1 hour. Primary antibody, Notch3 (1:100) (Santa Cruz Biotechnology), smooth muscle (SM) α-actin (1:1000) (Sigma), NG2 (1:200) (Millipore), or Ang-2 (1:200) (Zymed), collagen IV (1:200) (Millipore), or phospho-histone H3 (1:200) (Millipore) was co-stained with 10 μg/mL TRITC-labeled isocitrate B4 (Griffonia simplicifolia) (Invitrogen). Retinas were flat-mounted in Vectashield (Vector Laboratories). Confocal images were captured and vessels were quantified with NIH ImageJ software by a blind observer.

**Cell Death Detection ELISA**

For the detection of apoptosis, a cell death detection ELISA PLUS kit (Roche) was used. Isolated retinas were homogenized in 100 μL of lysis buffer, and 20 μL of the supernatant of each retina was assayed. For cells, aortic smooth muscle cells were serum-starved in DMEM with 0.25% FBS. An equal number of cells were used for cell death detection.

**Aortic Ring Assay**

Aortic-sprouting assays were performed as described previously with modification. Briefly, aortas from Notch3+/− and Notch3−/− littermates at postnatal day (P)11 were dissected, cut into 1-mm-thick rings, and incubated overnight in complete EBM-2 media (Lanza) containing 10% FBS before being placed into a rat tail collagen gel (1.5 mg/mL). Forty-eight hours after embedding, rings were stained with 10 μg/mL TRITC-labeled isocitrate B4 and NG2 and imaged by confocal microscopy. Vessels were quantified with ImageJ software.

**Oxygen-Induced Retinopathy**

Oxygen-induced retinopathy (OIR) was induced as described previously, with minor modifications. In brief, at P7, pups, along with nursing mothers, were placed in 70% oxygen. At P12, they were returned to 21% oxygen for 5 days. From P12 to P17, the animals were anesthetized, their retinas were collected, and isocitrate B4 immunostaining on whole-mount retinas was performed. Avascular area was quantified as a percentage of whole retinal area. Neovascularization was quantified using ImageJ software by calculating the area of isocitrate B4 staining of neovascular tufts as reported.

**RNA Isolation and Quantitative RT-PCR**

Total RNA was extracted from mouse retinas using RNAqueous-4PCR kit (Invitrogen) and reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) to generate cDNA. Quantitative PCR was performed using a StepOne PCR system (Applied Biosystems) with Power SYBR Green.

**Statistical Analysis**

Data analyses were performed using PrismGraph, and comparisons between data sets were made using Student’s t test. Differences were considered significant if P<0.05, and data are presented as means±SEM. Data shown are representative of at least 3 independent experiments.

**Results**

**Notch3 Protein Is Expressed in Retinal Mural Cells**

Using an in vitro angiogenesis assay, we demonstrated recently that Notch3 mediates mural cell–enhanced blood vessel formation. From these studies, we predicted that Notch3 would have a similar effect in vivo and would act to modulate blood vessel formation in a mural cell–dependent fashion. To address this premise, we examined angiogenesis in retinas of mice deficient for Notch3. Earlier reports had
shown that Notch3 mRNA is expressed in the retinal vasculature and appeared localized to mural cells.\textsuperscript{35} We confirmed expression of Notch3 protein in the retinal blood vessels by immunostaining. Whole-mount retinas stained with a Notch3 antibody in conjunction with endothelial-specific isolectin B4 demonstrated that Notch3 protein is specifically expressed in mural cells and is absent from endothelial cells within the retinal vascular network (Figure 1A). Furthermore, Notch3 protein is observed in mural cells at the angiogenic front, where endothelial sprouting activity is abundant (Figure 1B).

**Notch3 Deletion Influences Development of the Retinal Vasculature**

Notch3-null mice were previously shown to be viable and fertile, but adult mice exhibit structural defects in the distal arteries, including enlargement of the vessels and defective smooth muscle maturation.\textsuperscript{28} To determine whether the expression of Notch3 in retinal mural cells regulates the process of blood vessel formation, we performed whole-mount isolectin B4 staining of mouse retinas and evaluated the pattern of the vasculature from Notch3\textsuperscript{-/-}, Notch3\textsuperscript{+/-}, and wild-type littermates at different developmental stages. Retinal vessels begin to sprout from the optic disc right after birth and extend out to the periphery at P8. At P3, we found that retinas from Notch3\textsuperscript{-/-} mice displayed diminished vascularization. Coverage of the superficial retinal plexus was decreased by 30% in Notch3\textsuperscript{-/-} mice compared with Notch3\textsuperscript{+/-} and Notch3\textsuperscript{+/-} retinas (Figure 2A and 2C). Examination of vessel patterning revealed a 30% reduction in vessel branch points (Figure 2B and 2D), leading to an overall decline in vascular density. No difference was apparent in comparison of Notch3 heterozygote and wild-type animals. At P5 and P7, similar defects were observed in Notch3-null retinas, albeit to a lesser extent (Online Figure I). In adult mice (P43), the vascular patterning defects associated with the absence of Notch3 were only slight (data not shown). These findings indicate that Notch3 acts as a regulator of vessel formation in vivo and may have a more pronounced role in early angiogenesis.

The blood vessel–forming phenotype in Notch3-null retinas indicated a defect in vessel sprouting, which resulted in an overall decrease in vascular density. To determine whether the angiogenic defects observed in the Notch3-null mice might be caused by an absence of mural cells at the angiogenic front, we performed colocalization studies with isolectin B4 and the mural cell marker NG2.\textsuperscript{36} These results showed that the mural cells in the Notch3-null retinas were normally distributed and appeared to associate with the developing vessels in a similar fashion to wild-type and heterozygous littermates (Online Figure II). Furthermore, to exclude the possibility that reduced vessel density was a result of increased vessel regression, we stained for collagen IV to detect empty collagen sleeves.\textsuperscript{37} We observed no differences between the Notch3-null mice and controls (Online Figure III). To directly evaluate the influence of Notch3 on vessel sprouting, we performed ex vivo sprouting assays with isolated aortic rings. Thoracic aortas from Notch3-null mice exhibited reduced vessel sprouts when cultured for 2 days compared with heterozygous controls, whereas mural cell recruitment was not affected (Figure 3). Taken together, these results demonstrate that mural cell–expressed Notch3 can regulate vessel sprouting, not only in developing retinal capillaries but also in large elastic arteries prompted to undergo angiogenesis.

**Notch3 Deletion Impairs the Investment of Mural Cells**

Given that prior analysis of Notch3-deficient mice uncovered defects in smooth muscle maturation,\textsuperscript{28} we sought to investigate how pericytes and smooth muscle cells of the retina...
were invested in the vascular network in the absence of Notch3. To do so, we undertook SM α-actin and NG2 staining to highlight retinal pericytes and smooth muscle cells, in conjunction with isoelectric B4. In early developmental stages, mural cells appeared to be normally distributed in the vascular plexus of Notch3+/−/− mice (Online Figure II). However, the null animals showed significant reduction in SM α-actin staining at P8 and P15 (Figure 4). Additionally, at P15, it was evident that the density of surrounding mural cells in arteries was dramatically decreased in Notch3+/−/− mice, with the formation of visible gaps between adjacent cells (Figure 4). The decrease in SM α-actin staining was even more pronounced in P43 adults, where mural cells were found sparsely surrounding the arteries (Figure 4 and Online Figure IV). These results indicate that the absence of Notch3 progressively impacts the investment of pericytes and smooth muscle cells over time. Because Notch3 is known to affect smooth muscle differentiation and particularly the expression of SM α-actin,28 we used an additional mural cell marker, NG2 to highlight these cells. Consistent with the expression of SM α-actin, we observed a similar pattern of NG2 staining in null mice (Figure 5A). Thus, these findings show that the lack of SM α-actin staining was not attributable to an absence of marker expression but, rather, was caused by the progres-
sive loss of mural cells. To further verify the absence of pericyte and smooth muscle cell coverage in adult retinas lacking Notch3, we costained with DAPI to mark nuclei within individual blood vessels. As predicted, the Notch3-null vessels had an average of 58% fewer mural cell nuclei compared with wild-type and heterozygous littermates (Figure 5B and Online Figure IV).

We reasoned that the loss of mural cells from the retina had to occur by 1 of 2 processes: by mural cells becoming disassociated from the vessels and migrating away3,38 or by apoptosis. From careful analysis, we did not observe migrating mural cells in retinas of Notch3-null mice. Cells that remained were closely attached to the endothelial abluminal surface as in control retinas (H.L., B.L., unpublished observation, 2009). Prior evidence linking Notch3 to cell survival led us to pursue apoptosis as a mechanism for cell loss in the retinas.39,40 Apoptosis was assessed by a cell death ELISA kit using whole retinas from mice at P10. We observed that Notch3−/− mice exhibited a statistically significant increase in apoptosis (Figure 6A). Assessment of proliferation in the Notch3-null and control retinas did not reveal any obvious differences (Online Figure V). Although the difference in apoptosis was small, the progressive loss of mural cells suggests that Notch3 acts to suppress cell death, and, in its absence over time, cells gradually succumb, resulting in considerable deficiency of mural cells. To further confirm this, we performed in vitro apoptosis assays using mouse aortic smooth muscle cells isolated from heterozygous and homozygous animals. Consistent with our in vivo data, Notch3-deficient smooth muscle cells exhibited an increase in apoptosis compared with control cells (Figure 6B).

**Notch3 Modulates Pathological Angiogenesis**

To investigate the role of Notch3 in pathological angiogenesis, we used a mouse model of OIR.33 In this model, P7 pups were exposed to 70% oxygen (hyperoxia) for 5 days (P7 to P12) to induce vascular regression and obliteration in the central retina. At P12, mice were returned to room air for 5
days, during which time period, relative hypoxia initiates rapid vessel growth and pathological neovascularization. We examined the vascular phenotypes of mice subjected to OIR. At the height of vascular regression in P12 retinas, Notch3−/−H11002 mice and Notch3−/−H11001/−/−H11002 littermates had a comparable vascular obliteration, which quantitatively occupied 35% of the total retinal surface (Online Figure VI). These data show that Notch3 deficiency does not affect the susceptibility of retinal vasculature to oxygen-induced vessel regression. In contrast, during the neovascularization phase, Notch3−/−H11002 retinas exhibited a diminished number of new blood vessels, compared with Notch3+/−H11001/−/−H11002 littermates (Figure 7A through 7C). In the null mice, there was a 35% decrease in neovascularization. Moreover, the number and the size of vascular tufts were reduced in Notch3−/−H11002 animals. To characterize the vascular tufts in more detail, retinas were costained for isolectin B4 and NG2.41 These images revealed that NG2-positive staining was seen in both the heterozygous and null tufts at similar intensities (Figure 7D). Thus, under pathological conditions, Notch3 deletion has a negligible effect on vascular regression but substantially impairs the process of neovascularization, as evidenced by a decrease in the size and number of vascular tufts.

Notch3 Expression Is Increased During Oxygen-Induced Neovascularization and Regulates Angiopoietin-2

To further explore the role of Notch3 in retinal neovascularization, we examined its expression in retinas of wild-type mice subjected to OIR. Compared with mice in normoxia, Notch3 mRNA was increased in relative hypoxia, as assessed by quantitative PCR (Figure 8A). At P12, just after hyperoxia
Notch3 exhibits reduced hypoxia-driven angiogenesis, similar to growth factor (VEGF) and the angiopoietins. Given that an increase in angiogenic factors such as vascular endothelial more than 2-fold. These observations signify that Notch3 control levels and at P17 exceeded control expression by more than 2-fold. These observations suggest that Notch3 might have a unique role in modulating vascular development under pathological conditions.

It is known that pathological angiogenesis is stimulated by an increase in angiogenic factors such as vascular endothelial growth factor (VEGF) and the angiopoietins. Given that Notch proteins function as transcription coactivators, and Notch3 is increased in hypoxia, we theorized that it might act to regulate the expression of certain other factors during hypoxia. Examination of known Notch target genes in hypoxia-treated retinas at P17 revealed a significant decrease in Htr3 mRNA expression in Notch3-null mice (Online Figure VII). We next evaluated mRNA levels of angiogenic growth factors and their receptors in these same hypoxia-treated retinas. Expression of VEGF-A, VEGF-C, Ang-1, and Tie-2 all exhibited similar expression levels in Notch3+/− and Notch3−/− littermates (Online Figure VII). Conversely, Ang-2 mRNA expression was decreased by approximately 40% in the null retinas compared with control (Figure 8B). The expression of Ang-2 was additionally assessed by immunostaining, which showed the Ang-2 protein was significantly reduced in Notch3 deficient mice (Figure 8C). In the retina, Ang-2 expression is upregulated during both physiological and pathological neovascularization (Online Figure VIII and elsewhere). Mice that are deficient for Ang-2 exhibit reduced hypoxia-driven angiogenesis, similar to that observed in our Notch3-null animals. These combined results suggest that one mechanism in which Notch3 governs pathological angiogenesis is by regulation of Ang-2. To evaluate whether the Notch3-dependent reduction in Ang-2 is unique to the pathological conditions produced by hypoxia, we examined the expression of Ang-2 in Notch3-null and heterozygous mice under normoxia. In P17 retinas isolated from mice exposed to room air, the Notch3 deletion had no effect on Ang-2, Ang-1, or Tie-2 expression, or that of Hes1, Hr2, and Hrt3 (Online Figure IX). These experiments imply that Ang-2 is a target of Notch3 only during oxygen-induced neovascularization and suggest that crosstalk between Notch3 and hypoxic signaling triggers this distinct expression.

To determine whether Notch3 could directly induce Ang-2 expression, we overexpressed a constitutively active form of Notch3 (NICD3) and measured Ang-2 mRNA. Lentiviral transduction of the NICD3 in primary cultures of human aortic smooth muscle cells caused Ang-2 mRNA expression to be significantly increased compared with control cells transduced with a GFP-expressing lentivirus (Figure 8D). Hypoxia-inducible factor (HIF)α is a key regulator of hypoxia-induced signaling, and was previously shown to directly interact with Notch1 to activate transcription. To determine whether HIF1α and Notch3 cooperate to regulate the expression of Ang-2, we cotransduced the NICD3 with a HIF1α cDNA and measured Ang-2 transcript levels. Indeed, whereas Ang-2 expression was augmented by NICD3, addition of HIF1α with NICD3 produced a greater increase in Ang-2 (Figure 8D). Interestingly, HIF1α transduction in aortic smooth muscle cells did not invoke an increase in Ang-2 expression on its own and required the NICD3. Thus, as suggested by previous reports, the regulation of Ang-2 expression in mural cells differs from that in endothelial cells and likely depends on Notch3 in hypoxia. Overall, these results indicate...
that Ang-2 is a direct target of Notch3 in mural cells, and Notch3 specifically regulates its expression under pathological conditions, possibly in conjunction with HIF1α. In summary, our data support the notion that Notch3 modulates oxygen-induced neovascularization by regulating the expression of Ang-2 from mural cells, which then acts on adjacent Tie-2–expressing endothelial cells to modulate blood vessel assembly.

**Discussion**

Notch signaling can regulate cell-to-cell communication and control cell fate decisions in a variety of cell types. In the vascular endothelium, signaling between Dll4 and Notch1 in tip and stalk cells, respectively, prevents stalk cell sprouting. This Notch-dependent homotypic interaction helps to pattern blood vessels. In this study, we asked how Notch signaling might facilitate heterotypic interactions between mural cells and endothelial cells that help shape the vasculature. Previous work has suggested an important role for pericytes in modulating tube formation, and an in vitro study by our laboratory demonstrated that mural cell–expressed Notch3 affects angiogenesis. Here, we show that under physiological conditions, Notch3 deletion decreases vascularization in the mouse retina at early developmental stages. These angiogenic defects are not a result of enhanced regression, which points to growth retardation as the likely cause. The decline in angiogenesis is associated with a reduction in branching, and accordingly, these data reveal that Notch3 receptor signaling from mural cells instructs endothelial cells to modulate blood vessel assembly.

![Figure 8. Notch3 regulates Ang-2. A, Quantitative PCR analysis of Notch3 mRNA expression in the retinas of wild-type mice at indicated time points (P12 to P17) in OIR or normal condition (Con). B, mRNA expression of Ang-2 in P17 retinas of Notch3+/− and Notch3−/− mice subjected to OIR. C, Retinas at P17 from OIR-treated Notch3+/− and Notch3−/− mice were stained with an Ang-2 antibody (green), isoelectin B4 (blue), and mural cell marker SM α-actin (red). Images were taken at ×630 by confocal microscopy. Scale bar, 20 μm. D, Induction of Ang-2 expression by Notch3 and HIF1α in vitro. Human aortic smooth muscle cells were transduced with lentivirus expressing the human Notch3 intracellular domain (NICD3) or GFP. Seventy-two hours after transduction, cells were infected with adenovirus expressing GFP (AdGFP) or HIF1α (AdHIF1α) as indicated for 48 hours. Cells were then collected and analyzed by quantitative RT-PCR to evaluate Ang-2 expression. *P<0.05 compared with relevant control.](http://circres.ahajournals.org/doi/pdf/10.1161/CIRCRESAHA.117.311733)
However, previous studies have offered insight into a potential mechanism. One study showed that an endothelial-specific knockout of Jagged1 has reduced retinal angiogenesis, including branching defects that resemble those we observed in Notch3-deficient mice. Another demonstrated that mice lacking endothelial-expressed Jagged1 have severe mural cell defects. In addition, previous data from our laboratory showed that Jagged1 promotes Notch activity and mural cell differentiation. In light of these previously published findings, one possible model to explain the Notch3-dependent angiogenic phenotype is that endothelial-expressed Jagged1 signals to Notch3 on mural cells to promote mural cell investment. The invested mural cells then influence endothelial growth and branching by producing secreted factors. Indeed, additional studies will be required to elucidate the exact mechanism through which Notch3 influences vessel patterning.

Adult Notch3-null mice exhibit impaired arterial differentiation, enlargement of vessels, and defective maturation of VSMCs. In addition, Notch3 deficiency impacts vascular tone in resistance arteries, indicative of functional defects in smooth muscle cells. To investigate more thoroughly the vascular smooth muscle defects associated with loss of Notch3, we examined them in the retinal vasculature. This vascular bed is formed postnatally and is conductive to stepwise analysis of mural cell recruitment and differentiation. From our experiments, mural cells initially appeared to be recruited normally in Notch3-null mice, albeit the expression of SM α-actin was slightly decreased. However, over time, mural cells surrounding the vessels were progressively lost. Apoptosis assays revealed a small but significant increase in cell death, consistent with previous reports that linked Notch3 to cell viability. These results are in contrast to those published by Domenga et al, who reported no detectable apoptosis in the tail arteries of Notch3 deficient mice. This discrepancy could be attributable to differences in the vascular beds that were analyzed. Yet, in vitro apoptosis assays that we performed on aortic smooth muscle cells from Notch3 heterozygous and null mice were consistent with our in vivo results from retinal tissue. We believe that in the absence of Notch3, the small increase in apoptosis leads to an incremental loss of mural cells over time. Thus, Notch3 not only functions to regulate arterial differentiation and maturation but also controls the investment and stability of mural cells contributing to functional blood vessels.

Our data indicate that the loss of pericytes has little effect on retinal angiogenesis, because mural cells begin to be lost after P8 in Notch3-null mice; yet, the angiogenic phenotypes we observed were most pronounced before this stage. The loss of Notch3 appears to have no effect on recruitment. Our data show that mural cells are present and distributed normally at the angiogenic front. These would imply that it is not the absence of Notch3-deficient mural cells that affects vessel growth but a defect in their function. Currently, we do not know the exact mechanism through which Notch3 conveys angiogenic signals. Possibilities include heterotypic signaling to endothelial cells or Notch-regulated gene expression of angiogenic factors in mural cells. At later stages after the vessels are formed, mural cell loss does not seriously impact their stability or regression. We did observe an overall enlargement of arteries in Notch3-deficient retinas at later stages (data not shown), similar to the findings in the study by Domenga et al. Taken together, these findings imply that pericyte-expressed Notch3 impinges on endothelial cell vessel-forming capabilities early on to regulate assembly, and once the vessel network is established, mural cells have little effect on modulating blood vessel structure. Importantly, however, we cannot exclude the possibility that compensatory mechanisms are activated in the null mice, which mask additional roles of Notch3 in mural cells. Perhaps other Notch receptors, such as Notch1, take over in the absence of Notch3.

Notch3 function has been linked to ischemic diseases. Arboleda-Velasquez et al showed that Notch3-deficient mice were more susceptible to ischemic stroke, whereas Li et al revealed a protective effect of Notch3 deficiency for the development of pulmonary hypertension. In the present study, using an ischemia-induced retinopathy model, we demonstrate that Notch3 plays a direct role in proliferative neovascularization. In the absence of Notch3, vascular tufts are decreased, suggesting that in ischemia, Notch3 acts to promote blood vessel formation, possibly as a maladaptive response to hypoxia. Similar to physiological conditions, Notch3 does not appear to be required for recruitment of mural cells in hypoxic conditions. Our experiments did not address whether Notch3 is required for mural cell stability and investment in this disease state, as it is during development. The Notch pathway is known to intersect with hypoxic signaling, and Notch1 and HIF1α can directly interact to regulate transcription. Consequently, it is not surprising that Notch3 affects blood vessel formation in disease states, although the transcriptional targets of Notch signaling in ischemia have not been described previously. Here, our results show for the first time that Ang-2 is a target of Notch3 under ischemic conditions. Moreover, we show that Notch3 cooperates with HIF1α to regulate Ang-2. Indicative of a mutual pathway, Ang-2–deficient mice share phenotypic similarities with Notch3-null mice subjected to OIR. Therefore, the convergence of Notch3 and HIF1α onto Ang-2 likely serves to precisely control this vital signaling factor exclusively in ischemia.

In summary, our data conclusively show that mural cell–expressed Notch3 is important for modulating distinct aspects of developmental and pathological blood vessel formation. Notch3 signaling to endothelial cells regulates vessel branching, and its expression on mural cells is critical for their differentiation and viability. Furthermore, in pathological conditions, Notch3 regulates Ang-2 expression under hypoxia, demonstrating a role for this Notch family member in governing neovascularization. Our results suggest that Notch3 uses multiple mechanisms to control different aspects of blood vessel formation and function, and they differ under normal and aberrant states. Thus, therapeutic targeting of Notch3 might be a novel approach for disrupting angiogenesis in certain vascular-related diseases.

Acknowledgments

The support from the Metabolic Vascular Disease Group of the Vascular Biology Center is greatly appreciated.
Sources of Funding
This work was supported by NIH grant R01 HL076428 (to B.L.) and American Heart Association predoctoral fellowship 09PRE2220351.

Disclosures
None.

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**Novelty and Significance**

**What Is Known?**
- Blood vessel formation or angiogenesis is a complex process that relies on cell–cell interaction.
- Mural cells are known to regulate angiogenesis by modulating the activity of endothelial cells.
- Notch signaling has been implicated in the regulation of angiogenesis.

**What New Information Does This Article Contribute?**
- The expression of Notch3 in mural cells influences blood vessel patterning in the retina.
- Notch3 acts to maintain vascular integrity by controlling mural cell investment.
- Notch3 regulates pathological angiogenesis and regulates angiopoietin-2 expression.
Notch3 Is Critical for Proper Angiogenesis and Mural Cell Investment
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_Circ Res._ 2010;107:860-870; originally published online August 5, 2010;
doi: 10.1161/CIRCRESAHA.110.218271

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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SUPPLEMENTAL MATERIAL

Detailed Methods

Animals. Notch3^{-/-} mice in a C57BL/6 background were a generous gift from Dr. Tom Gridley.1 Notch3^{-/-}, Notch3^{+/-} and wild type littermates were obtained by crossing Notch3^{+/-} and Notch3^{-/-} mice. All experimental procedures on mice were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Committee for the Use of Animals in Research.

Cell culture. Human aortic smooth muscle cells from Lonza were grown in DMEM supplemented with 10% FBS, 100I.U./ml penicillin-streptomycin. Cells between passages 6 and 8 were used for experiments. Mouse aortic smooth muscle cells were isolated from aortas of Notch3^{+/-} and Notch3^{-/-} mice. Cells between passages 3 and 8 were used for experiments. All cultures were maintained in humidified 5% CO₂ at 37 °C. Lentiviral and adenoviral transduction was performed as described previously.2, 3 Briefly, recombinant lentiviruses were produced by transient transfection of TN-293 cells (Clontech). Subconfluent TN-293 cells were transiently co-transfected with pCDF1-MCS2-EF1-copGFP-NICD3 or the control plasmid without insert, and the lentiviral packaging plasmids pFIV34N and pVSV-G overnight. 48 hours after transfection, viral supernatant was harvested and used for infection. The human HIF1α cDNA was cloned into the pAdTrack shuttle vector in front of the CMV promoter using KpnI and EcoRI restriction sites. The adenovirus plasmids containing GFP alone (AdGFP) and GFP together with HIF1α (AdHIF1α) were transfected into HEK293 cells, and the viral particles were amplified and purified. For infection, human aortic smooth muscle cells were seeded in a 12-well plate at a density of 2x10⁴ cells per well 24 hours prior to viral infection. To each well, 0.5ml of lentivirus suspension diluted in 0.5ml DMEM with 10% FBS was added. Polybrene was supplemented at a final concentration of 6μg/ml. 24 hours later, cells were transferred to fresh DMEM containing 10% FBS. 72 hours after infection, cells were infected with adenoviral particles, which were diluted in 400μl Opti-MEM (Invitrogen). 2 hours after incubation, 600μl normal growth media was supplemented. The following day, the virus-containing media was replaced with fresh media. Efficiency of transduction was monitored by GFP expression.

Immunostaining on whole-mount retinas. The immunostaining in retinas was performed as previously described.3 Briefly, eyes were isolated from Notch3^{-/-} and Notch3^{+/-} mice at indicated time points and fixed in 4% paraformaldehyde for 30 minutes. The cornea, sclera, lens, vitreous, and hyaloid vessels were removed to isolate retinas and radial incisions were made at equal intervals along the retinal edge. Subsequently retinas were placed in cold methanol for 20 minutes, blocked and permeabilized in PBS containing 5% donkey serum and 0.3% Triton-X-100 for 1 hour. Primary antibody, Notch3 (1:100) (Santa Cruz), sm α-actin (1:1000) (Sigma), NG2 (1:200) (Millipore),
Ang-2 (1:200) (Zymed), Collagen IV (1:200) (Millipore), or phospho-histone H3 (1:200), was costained with 10μg/ml TRITC labeled-isolectin B4 (*Griffonia simplicifolia*) (Invitrogen) for 2 hours at 37°C. Incubations with fluorescently tagged secondary antibodies were performed at 4°C overnight, including Alexa-Fluor 488 donkey anti-mouse (1:250), Alexa-Fluor 594 goat anti-rabbit and Alexa-Fluor 488 goat anti-rabbit (Invitrogen). Retinas were flat mounted in Vectashield (Vector Laboratories). Confocal images were captured and vessels were quantified with NIH ImageJ software by a blind observer.

**Cell death detection ELISA.** For the detection of apoptosis, a cell death detection ELISA<sup>PLUS</sup> kit (Roche) was used to quantify DNA fragmentation according to the manufacturer’s instructions. Briefly, isolated retinas were homogenized in 100μl lysis buffer, incubated for 30 minutes at room temperature, and centrifuged for 10 minutes at 2000rpm. 20μl of the supernatant of each retina were assayed. For cells, aortic smooth muscle cells from Notch3<sup>+/−</sup> and Notch3<sup>−/−</sup> mice were plated at a density of 5x10<sup>4</sup> cell in a 12-well plate, starved in DMEM with 0.25% FBS for 72 hours. An equal number of cells were lysed in 100μl lysis buffer and were utilized for cell death detection.

**Aortic ring assay.** Aortic sprouting assays were performed as previously described with modification.<sup>4</sup> Briefly, aortas from Notch3<sup>+/−</sup> and Notch3<sup>−/−</sup> littermates at P11 were dissected, cut into 1-mm-thick rings, and incubated overnight in complete EBM-2 media (Lonza) containing 10% FBS before being placed into a rat tail collagen gel (1.5mg/ml). 48 hours after embedding, rings were stained with 10μg/ml TRITC labeled-isolectin B4 and NG2, and imaged by confocal microscopy. Vessels were quantified with ImageJ software.

**Oxygen-induced retinopathy (OIR).** OIR was induced as previously described, with minor modifications.<sup>5</sup> In brief, at postnatal day (P)7 pups, along with nursing mothers, were placed in 70% oxygen. At P12, they were returned to 21% oxygen for 5 days. From P12 to P17, the animals were anesthetized, their retinas were collected and iso-lectinB4 immunostaining on whole-mount retinas were performed. Avascular area was quantified as percentage of whole retinal area. Neovascularization was quantified using ImageJ software by calculating the area of iso-lectinB4 staining of neovascular tufts as reported.<sup>6</sup>

RNA isolation and quantitative reverse transcriptase-polymerase chain reaction (qPCR). Total RNA was extracted from mouse retinas using RNAqueous-4PCR kit (Invitrogen) according to the manufacturer’s instruction, and reverse transcribed with M-MLV reverse transcriptase (Invitrogen) to generate cDNA. Quantitative PCR was performed using a StepOne PCR system (Applied Biosystems) with Power SYBR Green. The fold difference in various transcripts was calculated by the ΔΔCT method using 18S as the internal control. After PCR, a melting curve was constructed in the range of 60°C to 95°C to evaluate the specificity of the amplification products. Primer sequences for mouse transcripts were as follows: 18S For-5’-GTT GGT TTT CGG AAC TGA GGC-3’; 18S Rev-5’-GTC GGC ATC GTT TAT GGT CG -3’; Notch3 For-5’-TTG TCT GGA TGG AAG CCC ATG T-3’; Notch3 Rev-5’-ACT GAA CTC TGG CAA ACG CCT-3’; Angiopoietin-2 For-5’-ACA CCG AGA AGA TGG CAG TGT-3’; Angiopoietin-2 Rev-
5'-CTC CCG AAG CCC TCT TTG TA-3'; Angiopoietin-1 For-5’-GGG CTG GAA GGA GTA TAA AAT GG-3’; Angiopoietin-1 Rev-5’-GAA CTC GTT CCC AAG CCA ATA T-3’; Tie-2 For-5’-CAA TCA GGC CTG GAA ATA CAT TG-3’; Tie-2 Rev-5’-TCC GCG GCT CCA AGT AGT T-3’. For human Angiopoietin-2 For-5’-AAC AGG AGG CTG GTG GTT TG-3’; Rev-5’-TGT GGA TAG TAC ATT CCG TTC AAG TT-3’. Hrt3 For-5’-CGC AGA GGG ATC ATA GAG AAA CG-3’; Hrt3 Rev-5’-GCC AGG GCT CGG GCA TCA AAG AA-3’; Hes1 For-5’-CCC CAG CCA GTG TCA ACA C-3’; Hes1 Rev-5’-TGT GCT CAG AGG CCG TCT T-3’; Hrt2 For-5’-CAC ATC AGA GTC AAC CCC ATG T-3’; Hrt2 Rev-5’-GCC ATG AGC AGA AGG CAC TT-3’.

**Statistical analysis.** Data analyses were performed using PrismGraph and comparisons between data sets were made using Student’s t test. Differences were considered significant if P < 0.05, and data are presented as mean ± standard error of the mean (SEM). Data shown are representative of at least three independent experiments.
Supplemental References


Online Figure I

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Online Figure I. Notch3 deletion compromises retinal angiogenesis in vivo. (A, B) Retinal vasculature from Notch3+/+, Notch3+/− and Notch3−/− littermates were stained with iso-lectin B4 at P5 and P7. Graphs show quantification of vascularized area and branch points in retinas. * P < 0.05 compared to heterozygous and wild type. Images were taken at 50X by confocal microscopy. Bar, 25 μm. (C, D) Superficial and deep vascular plexus from Notch3+/+ and Notch3−/− littermates were stained with iso-lectin B4 at P10 and P14. Images were taken at 100X by confocal microscopy. Bar, 100 μm.
Online Figure II
Online Figure II. Mural cells in the angiogenic front of the retina. Retinas isolated from Notch3+/+, Notch3+/− and Notch3−/− at P3 (A) and P5 (B) were stained with iso-lectin B4 (red) and mural cell marker NG2 (green). Confocal images were taken at 200X magnification. Bar, 50μm.
Online Figure III. Notch3 deletion does not increase vessel regression. Retinas were isolated from Notch3+/− and Notch3−/− littermates at P3, and stained with iso-lectin B4 (red) and anti-collagen IV (green). Confocal images were taken at 250X magnification. Bar, 25μm. Arrows indicate the empty sleeves (collagen IV+ iso-lectin B4−) resulting from vessel regression.
Online Figure IV
Online Figure IV. Notch3 deletion decreases the coverage of mural cells in retinal vasculature. Retinas were isolated from Notch3+/+, Notch3+/− and Notch3−/− littermates at P43, and stained with mural cell marker sm α-actin (green) and iso-lectin B4 (red) (A). Confocal images were taken at 100X magnification. Bar, 100μm. Retinas were stained with sm α-actin (green), DAPI (blue, for nuclei) as well as iso-lectin B4 (red) (B). Arrows indicate the nuclei of mural cells. Confocal images were taken at 630X magnification. Bar, 20μm. (C) Quantification of mural cell nuclei in retinal arteries of Notch3+/+, Notch3+/− and Notch3−/− at P43. * P < 0.05 compared to relevant controls.
Online Figure V. Notch3 deletion does not affect proliferation of mural cells in retinal arteries. (A) Retinas were isolated from Notch3+/+, Notch3+/− and Notch3−/− littermates at P10, and stained with iso-lectin B4 (red), anti-phospho-histone H3 (pH-H3, green, for proliferation) and DAPI (blue, for nuclei). Confocal images were taken at 400X magnification. Bar, 20μm. (B) Quantification of proliferating mural cells in retinal arteries.
Online Figure VI. Notch3 deletion does not affect vessel obliteration at P12 in the OIR model. Retinas from Notch3+/− and Notch3−/− littermates were examined at P12 by microscopy after oxygen-induced retinopathy. (A) Representative retinal vasculature from Notch3+/− (left) and Notch3−/− (right) littermates at P12. White lines outline the area of vaso-obliteration. Bar, 500μm. (B) Graph represents vaso-oblitration in Notch3+/− and Notch3−/− mice at P12 (n=6 per group); ns, not significant.
Online Figure VII. Gene expression comparison in P17 retinas of Notch3+/- and Notch3-/- mice subjected to OIR. Quantitative PCR analysis of Hes1, Hrt2, Hrt3, Angiopoietin-1, Tie-2, VEGF-A and VEGF-C mRNA expression in the retinas of Notch3+/- and Notch3-/- mice at P17 after oxygen-induced retinopathy (n=3); ns, not significant; * P < 0.05 compared to relevant control.
Online Figure VIII. Gene expression in the course of OIR and normal conditions. Quantitative PCR analysis of Angiopoietin-1, Angiopoietin-2 and Tie-2 mRNA expression in the retinas of wild type mice at indicated time points (P12-P17) in OIR model and normal condition (Con) (n=3).
Online Figure IX. Gene expression comparison in P17 retinas of Notch3+/- and Notch3-/- mice under normal conditions. Quantitative PCR analysis of Hes1, Hrt2, Hrt3, Angiopoietin-1, Angiopoietin-2 and Tie-2 in P17 retinas of Notch3+/- and Notch3-/- mice under normal conditions (n=3); ns, not significant.