A Receptor-Specific Function for Notch2 in Mediating Vascular Smooth Muscle Cell Growth Arrest Through Cyclin-dependent Kinase Inhibitor 1B

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Rationale: Deregulated vascular smooth muscle cell (VSMC) proliferation contributes to multiple vascular pathologies, and Notch signaling regulates VSMC phenotype.

Objective: Previous work focused on Notch1 and Notch3 in VSMC during vascular disease; however, the role of Notch2 is unknown. Because injured murine carotid arteries display increased Notch2 in VSMC as compared with uninjured arteries, we sought to understand the impact of Notch2 signaling in VSMCs.

Methods and Results: In human primary VSMCs, Jagged-1 (Jag-1) significantly reduced proliferation through specific activation of Notch2. Increased levels of p27kip1 were observed downstream of Jag-1/Notch2 signaling and were required for cell cycle exit. Jag-1 activation of Notch resulted in increased phosphorylation on serine 10, decreased ubiquitination, and prolonged half-life of p27kip1. Jag-1/Notch2 signaling robustly decreased S-phase kinase-associated protein, an F-box protein that degrades p27kip1 during G1. Overexpression of S-phase kinase–associated protein before Notch activation by Jag-1 suppressed the induction of p27kip1. Additionally, increased Notch2 and p27kip1 expression was colocalized to the nonproliferative zone of injured arteries as indicated by staining with proliferating cell nuclear antigen, whereas Notch3 was expressed throughout normal and injured arteries, suggesting Notch2 may negatively regulate lesion formation.

Conclusions: We propose a receptor-specific function for Notch2 in regulating Jag-1–induced p27kip1 expression and growth arrest in VSMCs. During vascular remodeling, colocalization of Notch2 and p27kip1 to the nonproliferating region supports a model where Notch2 activation may negatively regulate VSMC proliferation to lessen the severity of the lesion. Thus, Notch2 is a potential target for control of VSMC hyperplasia. (Circ Res. 2013;113:975-985.)

Key Words: carotid intima-media thickness • cell proliferation • receptors, Notch • smooth muscle • vascular intima

Vascular smooth muscle cells (VSMCs) exhibit tremendous phenotype plasticity in response to injury, remodeling, shear stress, and other environmental cues. VSMC express contractile proteins necessary for maintaining vessel tone and function, including α-smooth muscle actin (SM actin), calponin1, SM22α, smooth muscle myosin heavy chain, and smoothelin B. In response to vascular injury, VSMCs downregulate contractile proteins and begin proliferating and migrating in response to secreted cytokines, potentially leading to the development and progression of neointimal hyperplasia, pulmonary hypertension, and other vascular diseases.

An intact Notch pathway is critical for normal vascular development and remodeling in response to vascular pathology. Notch3 was characterized as a major regulator of VSMC development and is mutated in human cerebral autosomal dominant arteriopathy with sub-cortical infarcts and leukoencephalopathy. We now understand that signaling through Notch2 and Notch1 also activate pathways required during embryonic vascular development and vascular repair. For example, mice with a homozygous hypomorphic mutation in Notch2 undergo abnormal development of the heart and eye vasculature, and Notch2 has been implicated in the development of aortic VSMC. Both Notch2 and Notch3 are required for normal VSMC development during embryogenesis where they play compensatory, yet nonoverlapping, roles. Several reports have investigated potential functions of Notch or Jagged-1 (Jag-1) in neointimal lesion formation during pathological remodeling in response to injury and demonstrated their regulation of VSMC phenotype. Interestingly, however, there have not been unique signaling functions identified downstream of Notch1, Notch2, or Notch3 in VSMCs.

Our work using human VSMCs identifies a novel, Notch2-specific signaling role in the regulation of VSMC proliferation. Although human VSMCs additionally express Notch1 and Notch3, and all can interact with Jag-1, only Notch2 signals to mediate cell cycle exit. We propose that this specific...
cell cycle regulatory pathway mediated by Jag-1 interaction with Notch2 is an important negative regulatory mechanism to prevent excessive VSMC proliferation in injured arteries.

Methods
An expanded Methods section is available in the Online Data Supplement.

Surgical Procedures
All mouse studies were performed with strict adherence to protocols approved by the Institutional Animal Care and Use Committee at Maine Medical Center. For the arterial ligation model, 8-week-old FVB male mice were subjected to common carotid artery ligation.10 Control mice received a sham operation. Carotid arteries were collected after 14 days.

Immunohistochemistry
Paraffin-embedded mouse carotid arteries were cut into 5 μm sections immediately adjacent to the site of injury. Sections were rehydrated, and antigen retrieval was performed using citric acid buffer and heat, permeabilized with 1% Triton X-100 for 30 minutes and blocked for 2 hours in a solution of 2% bovine serum albumin and 2% goat serum. Antibodies against Notch1, Notch2, Notch3, p27kip1 (Cell Signaling), PCNA (Santa Cruz), SM-actin (Sigma), or platelet-endothelial cell adhesion molecule 1 (PECAM1, CD31, from Abcam) were diluted 1:200–1:500 and incubated with sections overnight at 4°C. After washing in Tris-buffered saline containing 0.01% tween-20 (TBS-T, pH=7.4), sections were either incubated with HRP-conjugated goat antirabbit IgG for 2 hours, reacted with diaminobenzidine and counterstained with hematoxylin, or incubated with Alexa-fluor conjugated secondary antibodies (Invitrogen) for 2 hours, washed with TBS-T, counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and coverslipped.

Cell Culture
Human primary aortic VSMCs (Lonza) were used between passages 5 and 7. Human pulmonary arterial VSMCs and coronary artery VSMCs (Lonza) were used at passage 5. To activate VSMCs, we plated on dishes precoated with 3 μg recombinant rat Jag-1 fused to human Fc (R&D Systems) or with a human Fc control protein (Millipore) as described.11,12 Small-interfering RNAs or scrambled control (Qiagen) were transfected into VSMCs using the Amaxa nucleofector.2

Cell Cycle Analysis
Human aortic VSMCs were harvested by trypsinization, spun down, and washed in PBS before resuspension in ice-cold 70% ethanol and incubation at −20°C overnight. The next day, the cells were centrifuged, washed in ice-cold PBS, and resuspended in MUSE cell cycle reagent (Millipore), a propidium iodide-based staining kit compatible with the MUSE cell analyzer. DNA content was analyzed using the MUSE cell analyzer.

Statistical Analysis
F-scores were generated for experiments containing multiple comparisons using ANOVA. Student’s 2-tailed t-test was used for pairwise analysis. Statistical significance was considered at P<0.05.

Results
Notch2 Expression Is Increased in VSMC of Remodeling Arteries
To determine the levels of Notch receptors in VSMCs of normal and injured vessels, we used the carotid artery ligation model as a reproducible means to generate neointimal lesion formation.10 Carotid arteries from 8-week-old FVB male mice were studied 14 days after left carotid artery ligation or sham surgery. Expression of Notch3 was localized to the media of sham arteries, whereas Notch1 and Notch2 were undetectable (Figure 1A, left columns). Consistent with previous studies,13 vascular injury resulted in robust upregulation of Notch2, predominantly localized to the medial VSMCs (arrowheads). Notch3 expression was high in both the medial and neointimal VSMCs, whereas Notch1 was marginally elevated 14 days after vascular injury (Figure 1A, right columns). Cells with increased Notch2 protein in the ligated artery were also positive for smooth muscle actin and SM22α, markers of VSMCs (data not shown). This expression pattern in injured arteries suggests an enhanced function for Notch2 in response to vascular remodeling.

Previous studies found that Jag-1 activation of Notch3 in VSMCs leads to maturation and quiescence.14 To determine whether Jag-1 also signals through other Notch receptors, we activated VSMCs with recombinant Jag-1 fused to a human Fc domain15 and analyzed whole-cell lysates by immunoblot for Notch. Notch1, Notch2, and Notch3 were detected in cultured human aortic VSMCs; however, only Notch2 and Notch3 intracellular domains (ICD) were increased by stimulation with Jag-1 as compared with Fc (Figure 1B). Notch2 activation after Jag-1 stimulation was further verified by immunostaining (Figure 1C). Before ligand treatment, Notch2 was localized to the cell membrane (arrowheads) but was predominantly nuclear after Jag-1 stimulation. These experiments confirm accumulation of Notch2 in VSMCs after vascular injury and its expression and activation in cultured human aortic VSMCs.

Jag-1-Selective Activation of Notch2 Is Required to Inhibit VSMC Proliferation
Proliferation of VSMCs contributes to neointimal hyperplasia during pathological remodeling, and antiproliferative agents have proven efficacious in reducing restenosis.14 We previously reported that Jag-1 activation of Notch receptors in VSMCs significantly reduced cell proliferation in addition to inducing differentiation.12 To identify the receptor mediating the cell proliferation effect, we silenced Notch1, Notch2, or Notch3 in VSMCs using small-interfering (si) RNA. Confirmation of Notch1, Notch2, or Notch3 knockdown was performed by immunoblot analysis for ICD compared with nontargeting RNA (ntRNA) control (Figure 2A). We found each siRNA to reduce its Notch target specifically and effectively. We then analyzed Notch target gene Hes1 by quantitative reverse transcription polymerase chain reaction after Jag-1 stimulation to validate suppression of Notch signaling (Online Figure IA–C). Knockdown of each Notch receptor significantly reduced the level of Hes1 transcript induced by Jag-1 stimulation. Finally, we assessed the effect of Notch knockdown on the VSMC differentiated phenotype. Jag-1–induced SM-actin transcripts were significantly reduced with knockdown of Notch1, Notch2, or Notch3 (Online Figure ID). Additionally,
reduction in the basal levels of Notch2 and Notch3 was sufficient to reduce the ability of cells to contract a collagen gel (Online Figure IE).

VSMCs transfected with ntRNA or siRNA probes against Notch1, Notch2, or Notch3 receptors in sham control and injured murine carotid arteries 14 days after ligation. Slides were counterstained with hematoxylin. NC indicates, nonimmune control; IEL, internal elastic lamina; and EEL, external elastic lamina. Scale bar=100 μm for columns 1 and 3, and 50 μm for columns 2 and 4, n=5 per group. B. Immunoblot of whole-cell lysates for the intracellular domain (ICD) of Notch1 (N1ICD), Notch2 (N2ICD), or Notch3 (N3ICD) in human aortic VSMCs plated on Fc or Jag-1 Fc for 6 hours. C. VSMCs were plated on Fc or Jag-1 Fc for 15 hours, and Notch2 was analyzed using immunofluorescence (green). DAPI and phalloidin were used to visualize cell nuclei and actin filaments, respectively. Scale bar=50 μm.

Figure 1. Notch2 is produced in remodeling vascular smooth muscle cells (VSMCs) and responsive to Jag-1 ligand. A. Immunohistochemical analysis of Notch1, Notch2, or Notch3 receptors in sham control and injured murine carotid arteries 14 days after ligation. Slides were counterstained with hematoxylin. NC indicates, nonimmune control; IEL, internal elastic lamina; and EEL, external elastic lamina. Scale bar=100 μm for columns 1 and 3, and 50 μm for columns 2 and 4, n=5 per group. B. Immunoblot of whole-cell lysates for the intracellular domain (ICD) of Notch1 (N1ICD), Notch2 (N2ICD), or Notch3 (N3ICD) in human aortic VSMCs plated on Fc or Jag-1 Fc for 6 hours. C. VSMCs were plated on Fc or Jag-1 Fc for 15 hours, and Notch2 was analyzed using immunofluorescence (green). DAPI and phalloidin were used to visualize cell nuclei and actin filaments, respectively. Scale bar=50 μm.

However, no change was observed in Notch2 knockdown cells (Figure 2E and 2F). The suppression in cell proliferation correlated with cell number. Cells transfected with ntRNA, siNotch1, or siNotch3 had significantly reduced cell number, whereas transfected siNotch2 populations had high cell density (Figure 2G). These data show that Jag-1 signals exclusively through Notch2 to inhibit VSMC proliferation in vitro.

Nuclear Notch2 ICD Is Downregulated During Entry Into S-Phase

Because Jag-1–specific activation of Notch2 is required to inhibit VSMC proliferation, we analyzed whether endogenous Notch2ICD expression varies during cell cycle progression. We used propidium iodide (PI) staining of total DNA content and analyzed the cells to quantify proportions in different phases of the cell cycle. To validate our system, VSMCs were plated on Fc or Jag-1 Fc for 48 hours, and the cell cycle was analyzed using

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PI staining (Online Figure IIA). Quantification of cells activated by Jag-1 Fc as compared with Fc revealed ≈14% increase in the G0/G1 population, whereas the S-phase and G2/M populations were reduced by ≈5% and ≈7%, respectively (Online Figure IIB). To study Notch2ICD expression throughout cell cycle progression, VSMCs were serum starved for 30 hours to synchronize the cells in G0 and then released using freshly prepared SmGM medium. Cells were harvested at 0 (30 hours starvation time point), 12, 15, 18, 24, and 30 hours after release and were simultaneously processed for cell cycle analysis (Figure 3A–G) or nuclear and cytoplasmic fractionation for Notch2ICD levels (Figure 3H and 3I). Nuclear and cytoplasmic levels of Notch2ICD were at their lowest from 12 to 15 hours after release, concomitant with entry of the G0/G1 population into S-phase (Figure 3G). At 18 hours after release, the S-phase population began moving into G2/M, and simultaneous upregulation of nuclear Notch2ICD was observed (Figure 3I, blue line). After increased nuclear Notch2ICD expression at 18 hours, the population of cells in S-phase rapidly and steadily declined until 24 hours. Nuclear Notch2 steadily decreased through 30 hours as the cells normalized their proliferation rates. Steadily decreasing Notch2ICD coincided with a steady increase in the cytoplasm, suggesting nuclear export of the protein after transition of the population from S-phase to G2/M at 18 hours. Thus, nuclear Notch2ICD in VSMC changes during progression through the cell cycle, is lowest during entry into S-phase, and peaks during exit from S-phase.

**Selective Regulation of p27kip1 by Jag-1/Notch2 Signaling Inhibits VSMC Proliferation**

To identify cell cycle regulatory proteins targeted by Jag-1 via Notch2, we analyzed p27kip1, p21cip1/waf1, cyclin E1, and its associated cyclin-dependent kinase 2 (CDK2), all important regulators of VSMC cell cycle.18,19 Although p21 cip1/waf1 was slightly downregulated by activation with Jag-1 Fc for 48 hours, p27kip1 levels doubled (Figure 4A). Additionally, Jag-1 Fc activation inhibited expression of cyclin-dependent kinase 2 and cyclin E1. One function of p27 kip1 is to bind cyclin E1/cyclin-dependent kinase 2 complexes and prevent cell cycle progression.20 To determine whether Jag-1 Fc promotes increased nuclear levels of p27 kip1, we stimulated VSMC with Jag-1 Fc or Fc for 48 hours before fractionating the cells into nuclear and cytoplasmic components. Immunoblot analysis to detect p27kip1 protein showed increases in both nuclear and cytoplasmic levels in response to Jag-1 Fc (Figure 4B and 4C), suggesting that enhanced nuclear p27kip1 expression may mediate the cell cycle inhibitory effects.

To determine whether p27kip1 is required for Jag-1 to suppress VSMC proliferation, we used an siRNA targeting p27kip1 (si-p27kip1) to suppress the induction by Jag-1 signaling. Quantification of knockdown efficiency showed that 125 pmol of si-p27kip1 reduced levels of p27kip1 and p-p27kip1 S10 by ≈38% and 45%, respectively (Figure 4D and 4E). Phosphorylation of p27kip1 on S10 is known to promote its stability and considerably increase its half-life.21 Using this
system, we seeded ntRNA and si-p27kip1–transfected VSMCs on Fc or Jag-1 Fc for 42 hours before pulsing with BrdU for 6 hours. Quantification of BrdU–positive nuclei showed a significant reduction in proliferation in ntRNA receiving cells plated on Jag-1 Fc at 48 hours as compared with Fc (Figure 4F), whereas even a moderate reduction in p27kip1 protein rescued the Jag-1–induced suppression of proliferation. These results were confirmed using propidium iodide staining.

Figure 3. Nuclear Notch2 intracellular domain (ICD) is downregulated during entry into S-phase. Vascular smooth muscle cells (VSMCs) at passage 5 were synchronized in serum-free smooth muscle basal medium (SmBM) for 30 hours to induce G0. Freshly prepared smooth muscle growth media were added, and the cells were harvested for cell cycle analysis at 0 (A), 12 (B), 15 (C), 18 (D), 24 h (E), and 30 hours (F). G, Graphical representation of the percentage of cells in G0/G1, S, and G2/M at each time point was analyzed. Graphed are means±SD. H, VSMCs were harvested at the same time points after release from starvation and fractionated into their nuclear (left) and cytoplasmic (right) constituents for immunoblot analysis of Notch2ICD. Total histone H3 and pyruvate kinase PKM 1/2 were used as loading controls for nuclear and cytoplasmic fractions, respectively. Notch2ICD was normalized to loading control and quantified for each time point (I). Graphed are means±SD.

Figure 4. Selective regulation of p27kip1 by Jag-1/Notch2 signaling inhibits vascular smooth muscle cell (VSMC) proliferation. A, VSMCs were plated on Fc or Jag-1 Fc for 48 hours, and cell lysates were collected for immunoblot to detect total p27kip1, p21cip1/waf1, cyclin E1, and cyclin-dependent kinase 2 (CDK2). B, Cell lysates were also fractionated into a nuclear and cytoplasmic pool, and immunoblotted to detect total p27kip1. Pyruvate kinase (PKM1/2) and TATA box binding protein (TBP) were used as cytoplasmic and nuclear markers, respectively. C, Quantification of nuclear and cytoplasmic expression of p27kip1 in response to activation by Fc or Jag-1 Fc for 48 hours. D, VSMCs were transfected with 125 pmol of small-interfering targeting p27kip1 (si-p27kip1), and efficiency was analyzed by immunoblot (D and E). F, VSMCs transfected with nontargeting RNA or si-p27kip1 were plated on Fc or Jag-1 Fc for 42 hours before pulsing with 10 μM 5-bromo-2′-deoxyuridine (BrdU) for 6 hours and quantifying the percentage of BrdU-positive nuclei. G–J, Immunoblot analysis and quantification of p27kip1 levels after 48 hours activation by Fc or Jag-1 Fc in control, Notch1, Notch2, or Notch3 knockdown cells, respectively. Graphed are means±SD.
in conjunction with cell cycle analysis (data not shown). These
data show that the increase in p27\textsuperscript{kip1} is required for Jag-1 to
suppress VSMC proliferation.

Because Notch2 selectively mediates Jag-1 signaling to re-
duce cell proliferation, we tested whether regulation of p27\textsuperscript{kip1} is
also specific to Notch2. VSMCs were transfected with
ntRNA, siNotch1, siNotch2, or siNotch3, and plated on Jag-1 Fc for 48 hours before harvesting to analyze p27\textsuperscript{kip1} protein.
We found that Jag-1 Fc upregulated expression of p27\textsuperscript{kip1} in
control, Notch1 and Notch3 knockdown cells but not in cells
lacking Notch2 (Figure 4G–J). These data indicate that Jag-1
induces p27\textsuperscript{kip1} expression exclusively through Notch2 to pro-
mote cell cycle arrest.

Jag-1 Signaling via Notch2 Stabilizes p27\textsuperscript{kip1}
Protein in VSMCs

Canonical Notch signaling involves cleavage and transloca-
tion of NotchICD to the nucleus where it alleviates repression
of the transcriptional regulator C promoter binding factor-1
(CBF-1) to promote transcriptional activation. We evaluated
the levels of p27\textsuperscript{kip1} transcript after 24- and 48-hour activation
by Jag-1 in VSMCs. Using quantitative reverse transcription
polymerase chain reaction, we did not observe any signifi-
cant changes in p27\textsuperscript{kip1} mRNA levels after Jag-1 stimulation
(Figure 5A). Another common level of p27\textsuperscript{kip1}regulation is post-translational, including phosphorylation events at
specific residues that affect protein stability. S10 phosphorylated
p27\textsuperscript{kip1} is the primary form in G0/G1 cells, representing \approx70%
of the phosphorylated protein.\textsuperscript{22} Importantly, S10 phosphory-
lration increases the stability of p27\textsuperscript{kip1} in vivo.\textsuperscript{23} In addition,
p27\textsuperscript{kip1} can be phosphorylated on threonine (T187), which
promotes its turnover.\textsuperscript{23} To determine whether Jag-1 signaling
affects levels of these phosphorylated forms, we plated
VSMCs on Fc or Jag-1 Fc for 48 hours and analyzed whole-
cell lysates for total and phosphorylated forms of p27\textsuperscript{kip1}. Jag-
1 activation resulted in increased p-p27\textsuperscript{kip1} S10, a decrease in
p-p27\textsuperscript{kip1} T187 and, as anticipated, increased total p27\textsuperscript{kip1} at
48 hours (Figure 5B). A profile of decreased phosphorylation
on T187 and increased phosphorylation on S10 is expected
to increase the stability of the protein. To determine whether
the overall increase in p27\textsuperscript{kip1} was indeed a result of protein
stabilization, we measured its half-life. VSMCs were plated
on Fc or Jag-1 Fc for 24 hours before adding 200 \mu\text{M of fi-
nal concentration cycloheximide (CHX) to inhibit de novo

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**Figure 5.** Jag-1 signaling via Notch2 stabilizes p27\textsuperscript{kip1} in vascular smooth muscle cell (VSMC). A, VSMCs were stimulated with Jag-1 Fc or Fc for 24 or 48 hours, and RNA were collected for quantitative reverse transcription polymerase chain reaction analysis of p27\textsuperscript{kip1} transcript. Graphed are the fold changes relative to Fc. B, Lysates were collected from VSMCs stimulated with Fc or Jag-1 Fc for 48 hours and analyzed by immunoblot for phosphorylated and total p27\textsuperscript{kip1}. C, VSMCs were plated on Fc or Jag-1 Fc for 24 hours before addition of dimethyl sulfoxide (DMSO) or cycloheximide (CHX). Lysates were collected at indicated times. D–G, Control, Notch1, Notch2, or Notch3 knockdown cells were plated on Fc or Jag-1 Fc for 48 hours, and lysates were collected for immunoblot analysis of p-p27\textsuperscript{kip1}
S10 (top) and respective quantification relative to \( \beta \)-tubulin (bottom). H, VSMCs were stimulated with Fc or Jag-1 Fc for 48 hours, and cell lysates were immunoprecipitated (IP) to pull down total p27\textsuperscript{kip1}. Analysis of whole-cell lysates and remaining supernatant after p27\textsuperscript{kip1} IP confirms robust upregulation of p27\textsuperscript{kip1} by Jag-1 Fc at 48 hours and subsequent depletion of p27\textsuperscript{kip1} after IP [H, input and supernatant, respectively]. I, Immunoprecipitated p27\textsuperscript{kip1} was analyzed by immunoblot for ubiquitin, and levels were normalized to immunoprecipitated p27\textsuperscript{kip1} for quantification (J). Graphed are means±SD.
protein synthesis or vehicle (DMSO). Lysates were collected at 0, 8, or 15 hours to quantify p27kip1. Representative immunoblots (Figure 5C) indicated that the normal half-life of p27kip1 was within 8 hours. However, after Jag-1 Fc stimulation, the protein level was unchanged at 8 hours, and had an extended half-life, decreasing only by 15 hours. These data demonstrate that Jag-1 activation of Notch2 likely results in stabilization of the existing pool of p27kip1.

We then tested whether increased levels of p-p27kip1 S10 stimulated by Jag-1 Fc was mediated via Notch2 signaling. Similar to total p27kip1 expression (Figure 4G–J) Jag-1 requires Notch2 to increase p-p27kip1 S10 protein. Suppression of Notch1 or Notch3 did not affect the ability of Jag-1 Fc to increase p-p27kip1 S10 (Figure 5D–G). Ubiquitination of p27kip1 marks the protein for turnover.24 Because of enhanced phosphorylation on S10 and prolonged half-life in response to Jag-1 Fc, we analyzed ubiquitination of p27kip1. VSMCs were activated with Jag-1 Fc or Fc for 48 hours before immunoprecipitation (IP) of p27kip1. Before and after IP, a small amount of whole-cell lysate from Fc and Jag-1 Fc conditions was analyzed by immunoblot for p27kip1 (Figure 5D–G). Ubiquitination of p27kip1 increases the half-life of p27kip1 observed in Figure 5C. These experiments suggest that Jag-1/Notch2 signaling does not regulate p27kip1 by inducing de novo transcription, but instead, stabilizes the existing species by promoting extensive post-transcriptional modifications. Increased S10 phosphorylation and decreased ubiquitination likely account for enhanced p27kip1 stability and VSMC cell cycle arrest.

**Jag-1/Notch2 Regulation of p27kip1 Is via Downregulation of Skp2**

Skp2 is a potent regulator of p27kip1 levels via ubiquitination and proteosomal degradation.25 Notch signaling regulates Skp2 expression in T-cell acute lymphoblastic leukemia cell lines23 and cell cycle progression via Skp2-dependent regulation of p27kip1 in adult stem cells.26 Additionally, Skp2-mediated ubiquitination of p27kip1 regulates VSMC proliferation in culture and in response to vascular injury.27,28 In light of reduced p27kip1 ubiquitination (Figure 5I–J), and the regulation of p27kip1 by Skp2 in VSMC, we investigated whether Jag-1/Notch2 signaling regulates Skp2. VSMCs were stimulated with Jag-1 Fc for 24 and 48 hours, and Skp2 mRNA and protein levels were analyzed. Although no change in Skp2 transcript was apparent at either time (Figure 6A), Skp2 protein was robustly suppressed (Figure 6B). In Fc-stimulated cells, Skp2 expression was mainly nuclear, and although Jag-1 did not affect the localization of Skp2, it significantly reduced its levels after 24 and 48 hours (Figure 6C; arrowheads). Reduced Skp2 expression in the nucleus is consistent with increased nuclear p27kip1 (Figure 4B and 4C).

To determine whether Jag-1 regulates Skp2 expression via Notch2 exclusively, we plated control, Notch1, Notch2, or Notch3 knockdown cells on Fc or Jag-1 Fc for 48 hours and analyzed expression of Skp2 and p27kip1 by immunoblot (Figure 6D and 6E). Knockdown of Notch2 rescued suppression of Skp2 by Jag-1 observed in control, Notch1, and...
Notch3 knockdown cells. In addition, decreased Skp2 by Jag-1 was associated with increased p27\(^{kip1}\) under all conditions except when Notch2 receptors were silenced.

VSMC response to stimuli varies depending on the source from which they are derived and can even vary within the same artery because of differential origins during development.\(^{29}\) To determine whether Jag-1 regulation of Skp2 and p27\(^{kip1}\) is a common pathway in VSMC derived from other vascular beds, primary human pulmonary artery or coronary artery VSMC was plated on Fc or Jag-1 Fc for 48 hours and assessed for levels of p27\(^{kip1}\), p-p27\(^{kip1}\) S10, and Skp2 (Online Figure III). Consistent with human aorta-derived VSMCs, VSMCs from these sources responded to Jag-1 with increased total p27\(^{kip1}\), p-p27\(^{kip1}\) S10, and decreased Skp2 protein compared with Fc. Thus, Jag-1 regulation of Skp2 and p27\(^{kip1}\) may be a common pathway in human VSMCs from multiple origins. We also tested the effect of overexpression of a constitutively active Notch1ICD, Notch2ICD, or Notch3ICD on VSMCs. A Skp2 adenoviral (Ad) expression construct\(^{30}\) was used (Online Figure VE and VF). In injured arteries, Notch1 expression was high in the medial VSMCs (Figure 7C–F, green channel), with co-localization of Notch receptors and p27\(^{kip1}\) in injured and normal arteries. Wild-type, 8-week-old male FVB mice were subjected to complete ligation of the left common carotid artery directly adjacent to the bifurcation or a sham surgery. After 14 days, carotid arteries were harvested for immunohistochemistry analysis. Histological staining revealed extensive vascular remodeling characterized by neointima formation and reduced luminal size in the ligated artery compared with sham surgery (Figure 7A). At higher magnification, the subendothelial neointima appears ~8 to 12 layers thick, and the medial layer is hypertrophic (Figure 7B).

To study Notch and p27\(^{kip1}\) expression in the proliferative and nonproliferative regions of the remodeling artery, we used costaining with proliferating cell nuclear antigen (PCNA) to label proliferating cells.\(^{32}\) In injured arteries after 14 days, PCNA staining was predominantly localized to the neointimal VSMCs (Figure 7C–F, green channel, dotted white line marks the internal elastic lamina), whereas no PCNA positive cells were present in the sham arteries (Online Figure VA–D, green channel). Additionally, Notch1, Notch2, and p27\(^{kip1}\) expression was undetectable in sham arteries; however, prominent Notch3 levels were observed in the medial VSMCs (Online Figure VA–D). Staining for smooth muscle marker SM-actin and endothelial marker CD31 was performed to identify vessel structure and composition, and a negative control for antibody specificity was used (Online Figure VE and VF). In injured arteries, Notch1 was detectable in the endothelium and trace amounts in neointimal VSMCs (Figure 7C). In stark contrast to uninjured arteries, Notch2 levels were high in the medial VSMCs (Figure 7D, white arrows). Interestingly, Notch2 expression was high in the nonproliferating VSMC as indicated by staining in regions that were negative for PCNA staining (Figure 7D, overlay). Only trace amounts of Notch2 were detectable in the endothelium and neointimal VSMCs, whereas Notch3 was expressed throughout the injured vascular wall (Figure 7E). Similar to Notch2 protein, high levels of p27\(^{kip1}\) localization was high in the nonproliferative VSMCs (Figure 7F white arrows) and outside of the proliferative zone. SM-actin and CD31 staining are shown to indicate cell type(s) and vessel structure (Figure 7G). This localization of Notch receptors is consistent with our model that Notch2 and p27\(^{kip1}\) are up-regulated and colocalized to the nonproliferative VSMCs of the vascular wall after injury. Notch2 may be one regulator of p27\(^{kip1}\) expression in the injured vasculature that leads to re-establishment of vascular quiescence during remodeling.
Discussion

Proper Notch signaling is required for the maturation of the cardiovascular system during development, and in humans, mutations of components of the Notch pathway lead to vascular disease (reviewed in Boucher et al3). Quiescent VSMCs in vivo express high levels of Notch3 and Jag-1, whereas injury or pathology promotes expression of Notch1 and Notch2 within the VSMCs13 (Figure 1). The specific roles and signaling functions of each of the 4 Notch receptors is not well understood. Our study is the first to identify a Notch2-specific signaling function in human vascular cells, which when activated, is predicted to suppress smooth muscle hyperproliferation. Because of the association of impaired Notch signaling and vascular disorders, there is an appreciation for targeting the Notch pathway in the treatment of cardiovascular diseases.33 The most widely used Notch antagonist is γ-secretase inhibitor, which is being tested in clinical trials of patients with cancer. However, the lack of specificity of this enzyme for the Notch pathway34 presents a complex challenge when targeting diseases where multiple Notch receptors are active. Previous studies suggest that inhibition of some Notch pathways, including Notch1, may be effective in decreasing neointimal lesion formation.13,31 However, our findings suggest that selectively enhancing Notch2 function under conditions of VSMC hyperproliferation may promote cell cycle exit. Current attempts are indeed geared toward more selective targeting of individual Notch receptors.55 We suggest that it is critical to understand the roles of each Notch receptor in specific disease processes to successfully apply targeted therapeutic interventions.

We identified a specific requirement for Notch2 in negatively regulating VSMC proliferation downstream of Jag-1. Although cooperative roles can be shared between receptors, our data suggest Notch2-specific signaling roles that are unique (Figure 8). To our knowledge, this is the first study to identify a receptor-specific function for Notch2 in VSMCs. Notch2 is required for Jag-1–induced VSMC differentiation via targeting of Skp2 and p27kip1 to decrease cell proliferation (Figure 8). This cell cycle regulation is not mediated via either Notch1 or Notch3 receptors, although both of those receptors can respond to a Jag-1 signal. Thus, we hypothesize that one function of Notch2 is to provide crucial negative feedback on VSMC proliferation in response to vascular injury. Loss of differentiation of medial VSMCs and subsequent migration and proliferation to the subendothelial compartment in response to injury have been reported.36 Based on the in vitro mechanisms presented in this report, and the enhanced expression and co-localization of Notch2 and p27kip1 to medial VSMCs after injury, one could speculate that Notch2 activation antagonizes excessive proliferation of medial VSMCs to the neointimal layer, thereby acting to regulate lesion formation and vascular occlusion negatively.

Little is known about the contributions of Notch2 signaling during VSMC development and in response to vascular injury. Proliferation of VSMCs derived from cardiac neural crest cells requires Notch2 signaling. This result is in contrast to our model that Notch2 suppresses proliferation in VSMCs from the adult injured vessel. It is possible that Notch2 proliferative signals are sensed differently in an embryonic vascular progenitor cell versus an adult differentiated VSMCs. Also embryonically, a delay in VSMC differentiation is observed in developing blood vessels of Notch2-deficient mice, and these effects are severely exacerbated by dual knockout of
Notch2 and Notch3\(^8\). Constitutive expression of Notch2 and Notch3 are in the neointimal and medial VSMCs after injury, and Notch1 expression is the highest in neointimal VSMCs.\(^{13}\) Although associated with many pathologies, pulmonary stenosis is often observed in patients with Allagile syndrome, caused by mutations in Jag-1 or Notch2 in humans.\(^{37}\) and is consistent with Jag-1/Notch2 negatively regulating VSMC proliferation. Although there are many overlapping functions for Notch receptors, their differences in expression in time and space in response to vascular injury suggest the possibility of distinct receptor-specific functions. The diverse origin of VSMC progenitors during development may also strongly influence the nonoverlapping functions of Notch receptors in VSMCs, and sensitivity to Notch2 signaling could vary during homeostasis or remodeling, and at different anatomic sites. Further identification of receptor-specific roles for Notch in large elastic arteries and smaller arterioles will be required to gain a more comprehensive picture of vascular function. Our findings are important in advancing our understanding of the molecular events contributing to arterial stenosis and other proliferative vascular disorders.

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**Disclosures**

None.

**References**

vascular tone and homeostasis. During vascular obstructive injury and during vascular disease.

**What New Information Does This Article Contribute?**

- **Jagged-1** activates Notch1, Notch2, and Notch3 in human VSMCs, however, only Notch2 induces stabilization and nuclear accumulation of p27kip1, causing cell cycle exit.
- S-phase associated kinase (Skp2) is suppressed by Jagged-1/Notch signaling in VSMCs and is required for the induction of p27kip1.
- Notch2 is induced in VSMCs after vascular injury, and colocalizes with p27kip1 in nonproliferative regions of the remodeling artery.

Arterial vascular smooth muscle cells (VSMCs) contribute to vascular tone and homeostasis. During vascular obstructive diseases, VSMC hyperproliferation leads to lumen occlusion. Notch signaling regulates lesion formation after injury; however, specific signaling mechanisms for each of the 4 Notch proteins are not clear. We identified a unique function of Notch2 in VSMCs, downstream of Jagged-1 stimulation. We found that Notch2 specifically suppresses proliferation via induction of the cell cycle inhibitor p27kip1, a function that cannot be mediated via Notch1 or Notch3. The mechanism includes the suppression of Skp2, a negative regulator of p27kip1, leading to reduced proliferation of VSMCs during homeostasis. Notch2 is not expressed downstream of Jagged-1 stimulation. We found that Notch2 specifically suppresses proliferation via induction of the cell cycle inhibitor p27kip1, a function that cannot be mediated via Notch1 or Notch3. The mechanism includes the suppression of Skp2, a negative regulator of p27kip1, leading to reduced proliferation of VSMCs during homeostasis.
A Receptor-Specific Function for Notch2 in Mediating Vascular Smooth Muscle Cell Growth Arrest Through Cyclin-dependent Kinase Inhibitor 1B
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Supplemental Material

A Receptor-Specific Function for Notch2 in Mediating Vascular Smooth Muscle Cell Growth Arrest Through p27kip1, Boucher et al.

Supplemental Methods

Surgical procedures- All mouse protocols were evaluated and approved by the Institutional Animal Care and Use Committee at Maine Medical Center, and personnel were trained in the care and ethical use of vertebrate animals, as well as surgical techniques. For the arterial ligation model, 8 week old FVB male mice were subjected to common carotid artery ligation as described. Briefly, mice were anesthetized by intra-peritoneal injection of 2.5% avertin (2,2,2-tribromoethanol and tertiary amyl alcohol) in PBS (dosage 125-150mg/kg body weight). The left common carotid was surgically isolated and sutured just below the bifurcation. Control mice were subjected to sham operation in which the carotid artery was isolated but not sutured. After 14 days, mice were anesthetized by intra-peritoneal injection of 2.5% avertin before perfusion of the vasculature with 4% paraformaldehyde (PFA) through the left ventricle and carotid artery dissection. Once isolated, the carotid arteries were post fixed in 4% PFA for 6 hours before embedding in paraffin for sectioning.

Immunohistochemistry- Mouse artery sections were rehydrated using a rundown of Americlear followed by decreasing percentages of alcohol. Once rehydrated, sections were subjected to antigen retrieval using citric acid and steam for 30 minutes. After cooling, the sections were permeabilized using 1% Triton X-100 in PBS for 1h and blocked for 2h in a solution of 2% BSA and 2% goat serum. Antibodies against Notch1, Notch2, Notch3, p27kip1 (Cell Signaling), PCNA (Santa Cruz), SM-actin (Sigma) and PECAM (CD31, Abcam) were diluted 1:200-1:500 in blocking buffer and incubated with the sections overnight at 4°C. After washing 3x in PBS-T (pH=7.4), sections were incubated with HRP-conjugated goat anti-rabbit IgG for 2h in blocking buffer, washed 3x in PBS-T and reacted with diaminobenzidine (DAB, Thermo Scientific). For immunofluorescence, alexa-fluor 488 or 564 conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies (Invitrogen) were used in place of HRP conjugated antibodies. Alexa-fluor conjugated antibodies were diluted 1:1000 in TBS-T and incubated with the slides for 3h at room temperature. DAPI staining was used to visualize nuclei.

Cell culture- Primary VSMC derived from the human aorta, pulmonary arteries or coronary artery were obtained from Lonza, cultured in SmGM and used between passages 5 to 7. Ligand stimulation of Notch receptors was achieved by pre-coating 6-well culture dishes (Grainer) with 3µg of Fc-specific, goat anti human IgG in PBS for 3h followed by 3µg recombinant rat Jagged1 fused to human Fc (R&D Systems) or human Fc control protein (Millipore) overnight in PBS as previously described. Approximately 90,000 cells were plated per well for all experiments.

Immunofluorescence in vitro-Cells were fixed using 4% PFA for 30 minutes and permeabilized with 1% Triton X-100 in PBS for 15 minutes before addition of blocking buffer (2% goat serum/2% BSA) for 1h. Notch2 primary antibodies were diluted 1:250 while SM22α and αSM-actin primary antibodies were diluted 1:500 in blocking buffer and incubated at 4°C overnight. Cells were then washed 3x in PBS and incubated with alexa-fluor conjugated secondary antibodies (as described above) for 1h at RT. Cell were then washed 3x in PBS and stained with DAPI (Calbiochem) diluted 1:5,000 in PBS for 30 minutes to visualize nuclei and alexa-fluor 568 phalloidin diluted 1:100 in PBS for 30 minutes to visualize the cytoskeleton.

Cell cycle analysis- Cells stimulated with 5-bromo-3’deoxyuridine (BrdU) were stimulated with Jag-1 Fc or Fc control for 42h before addition of BrdU to a final concentration of 10µM for 6h. After 6h, the
media was removed, cells washed 2x in PBS and subjected to immunofluorescence analysis as described above with the exception of the use of mouse anti-BrdU Alexa Fluor 488 conjugated (clone BU-1) primary antibodies (Millipore) diluted 1:250 in blocking buffer overnight.

For MUSE analysis of cell cycle, Fc or Jag-1 Fc stimulated cells were trypsinized, spun down at 300g and washed in PBS. The cells were spun down again at 300g and the pellet was resuspended in 70% ethanol and placed at -20°C overnight for fixation. After fixation, cells were spun down, washed in PBS and re-suspended in MUSE cell cycle reagent (propidium iodide) for 30 minutes at room temperature. Stained cells were analyzed using the cell cycle program within the MUSE software in 3 separate readings of 2500 cells. Experiments were repeated in triplicate.

**Transfection**- Small interfering RNAs (Qiagen Flexitube Gene Solution and Origene) or scrambled control (Qiagen non-targeting RNA) were transfected into VSMC using the Amaza nucleofector as described. Briefly, 10⁶ cells were suspended in 100µl human aortic nucleofector solution (Lonza) and mixed with appropriate siRNAs or non-targeting controls before transferring to a cuvette for electroporation using Amaza nucleofector program V-025 (Lonza). Knockdown of Notch1, Notch2, Notch3, was obtained using a final concentration of 200pmol specific siRNA, while 150pmol siRNA was used to knockdown p27kip1.

**Western Blot**-Detection of proteins using immunoblot was performed as described. Briefly, whole cell lysates were run on polyacrylamide gels (7.5% to 12% depending on the proteins being investigated). Proteins were transferred to PVDF membranes, blocked using 5% skim milk and probed overnight with primary antibodies diluted in a solution of 5% BSA/TBS w/0.2% tween-20. Antibodies were against the following proteins were used: Notch1, Notch2, Notch3, CBF-1, cylinE1, cyclinD1, CDK2, p27kip1, p21cip, PKM1/2 and Skp2 (Cell Signaling); TATA box binding protein (TBP), phosphorylated p27kip1 on S10 and SM22α (Abcam); β-tubulin, smooth muscle α-actin (Sigma), phosphorylated histone H3 on S10 (Millipore); phosphorylated p27kip1 on T187 (Santa Cruz). Goat anti-mouse and goat anti-rabbit HRP conjugated secondary antibodies (Cell Signaling) were diluted 1:2000 in 5% milk and incubated for 1h at RT. Nuclear fractionation prior to immunoblot analysis in certain experiments was performed using the Nuclear Extract Kit (Active Motif) protocol.

**Immunoprecipitation**-To detect ubiquitinated p27kip1, whole cell lysates were collected in non-denaturing lysis buffer (20mM Tris-HCl pH=8, 137mM NaCl, 1% triton X-100, 2mM EDTA) containing protease and phosphatase inhibitors (Cell Signaling). A small amount of lyasate was set aside for analysis (input). The remaining lysates were incubated with mouse anti-p27kip1 (Santa Cruz) overnight at 4°C on a tumbler. The next day, 50μl of protein A/G agarose beads (Santa Cruz) were added to each of the lysates and incubated for 4h at 4°C on a tumbler. Beads were isolated by centrifugation at 2500rpm and the supernatant set aside for analysis of pull-down efficiency. The beads were washed 3x in fresh lysis buffer, before boiling for 10 minutes in Laemmli sample buffer. The input, supernatant and bead extracts were run on a 12% SDS denaturing gel as described above. Rabbit anti-ubiquitin antibodies (Cell Signaling) were used to detect ubiquitinated p27kip1. The blots were stripped and re-probed for total p27kip1 using rabbit anti-p27kip1 (Cell Signaling).

**Adenoviral Transduction**-Viruses encoding GFP, the ICD of Notch1, Notch2 and Notch3 or Skp2 were diluted 1000 from their original stocks in serum free SmBM in the presence of 3% Gene Juice (Invitrogen) for 30 minutes at room temperature prior to addition to cells. Adenoviral Skp2 over expression constructs were generously donated by Dr. Dennis Bruemmmer and colleagues at the University Of Kentucky School of Medicine. Appropriate volumes of viral solution were calculated based on the number of cells in culture to obtain desired viral titer. VSMC cultures were washed and SmBM was
added just prior to addition of the virus. Viral cultures were incubated at 37°C and 5% CO₂ for 6h. After 6h, the viral media was removed; the cells were washed and replenished with growth media (SmGM).

**Quantitative PCR**—For mRNA analysis, RNA was extracted using the cell and plant extraction kit (Exiqon), quantified and reverse transcribed to cDNA using qSRIPT (Quantabio). The following primer sequences were used for quantitative PCR: Hes1 F-5’-TGGATGCGAGTCTACGATG-3’, R-5’-TAAGGCCACTTGGCCACCTTC-3’; p27kip F-5’-AAGAGCGAGCCAGCGCAAG-3’, R-5’-GGCCGCCGTTCTGTAGTA-3’; Skp2 F-5’-GGCTGAGAGCAAGGGAGT-3’, R-5’-GGCAATCACCCCTTGAGACA-3’; Hey1 F-5’-GCCAGCATGGAAGCGGTACAG-3’, R-5’-GGCAATCACCCCTTGAGACA-3’; SM F-5’-TTCAATGTCCAGCCATGTA-3’, R-5’-GAAGGAATAGCCACGCTCAG-3’, β-actin F-5’-GGCTGAGAGCAAGGGAGT-3’, R-5’-GGCAATCACCCCTTGAGACA-3’. Cycling conditions were 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds. The comparative CT method was used to calculate fold change.

**Collagen Contraction Assay**—For collagen contraction assays, approximately 35,000 VSMC were embedded in a collagen gel in a 24 well plate according to the manufacturer’s protocol (Cell Biolabs). After 1h, SmGM was added and the cells were given 18h to recover before releasing the gels from the sides of the well. Collagen gels were measured 24h later to quantify contraction.

**Statistical Analysis**—F-scores were generated for experiments containing multiple comparisons using ANOVA. Student’s two tailed t-test was used for pair wise analysis. Statistical significance was considered at p<0.05.

Online Figure I

Online Figure I. Jag-1 signals through Notch1, Notch2 and Notch3 receptors in VSMC, and each contribute to the differentiation phenotype. VSMC were transfected with 200pmol of non-targeting RNA (ntRNA), or siRNA targeted to Notch1, Notch2, and Notch3 as shown in Fig. 2A. RNA was collected from control, Notch1, Notch2 or Notch3 knockdown cells plated on Fc or Jag-1 Fc for 24h and Hes1 expression analyzed by qRT-PCR (A-C). Because Jag-1 activation of Notch induces differentiation of VSMC, control and Notch knockdown VSMC were stimulated with Fc or Jag-1 Fc for 48h and SM-actin levels analyzed by qRT-PCR (D). Graphed are the fold changes relative to Fc ± SD. E) VSMC with knockdown of Notch2 and Notch3 or ntRNA control were plated in a collagen gel, and allowed to contract for 24h. Graphed are the relative mean areas of the collagen gels ± SD, and p values represent comparison to ntRNA group. Control indicates cells that were treated with 2, 3-butanedione monoxime (contraction inhibitor).
Online Figure II. **Jag-1 inhibits VSMC proliferation in vitro.** A) VSMC were stimulated with Fc (left) or Jag-1 Fc (right) for 48h before harvesting for cell cycle analysis using propidium iodide staining in conjunction with a MUSE cell analyzer. Blue=G0/G1 gate, red=S gate and green=G2/M gate. B) The percentage of cells in G0/G1, S and G2/M were quantified after stimulation with Fc or Jag-1 Fc for 48h. Graphed are means ± SD.
Online Figure III. Jag-1 inhibits proliferation of VSMC from diverse anatomical sites. A) Primary human pulmonary artery (A) or primary human coronary artery (B) smooth muscle cells were plated on Fc or Jag-1 Fc for 48h before harvesting for immunoblot analysis of p27\textsuperscript{kip1}, p-p27\textsuperscript{kip1} S10 and Skp2 (left) and quantification of their expression relative to Fc (right). Graphed are the means ± SD.
Online Figure IV

Online Figure IV. The effects on p27^{kip1}, Skp2 and proliferation are unique to endogenous Jag-1/Notch2 signaling. A) VSMC were transduced with 5000vp/cell human adenovirus encoding GFP or a V5-tagged constitutively active ICD of Notch1, Notch2 or Notch3 and harvested 48h later for immunoblot analysis of p27^{kip1}, Skp2 and Ki67 (marker of proliferation). Changes in protein expression relative to GFP-transduced cells were quantified (B). C) VSMC were plated on recombinant rat Delta-like-1 Fc for 48h and analyzed for p27^{kip1}. Graphed are the means ± SD.
Online Figure V. **Notch2 and p27\textsuperscript{kip1} are not expressed in uninjured arteries.** Carotid arteries from sham surgeries were sectioned and processed for immunofluorescence analysis of A) PCNA (green) and Notch1 (red), B) PCNA (green) and Notch2 (red), C) PCNA (green) and Notch3 (red), D) PCNA (green) and p27\textsuperscript{kip1} (red), E) SM actin (green) and CD31 (red), or F) non-immune antibody control (NC) and counterstained with DAPI to visualize nuclei. Notch3 (C) is the predominant Notch receptor expressed in the normal adult carotid artery wall (n=3).