Differential Regulation of NOTCH2 and NOTCH3 Contribute to Their Unique Functions in Vascular Smooth Muscle Cells

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Background: Notch receptors govern vascular smooth muscle phenotypes, yet their distinct mechanisms are undefined.

Results: NOTCH2 and NOTCH3 are differentially regulated and uniquely control smooth muscle cell proliferation and survival.

Conclusion: NOTCH2 and NOTCH3 have discrete functions in vascular smooth muscle cells that are linked to their expression.

Significance: These findings highlight functional differences between the predominant Notch receptors in vascular smooth muscle cells.

Notch signaling is a key regulator of vascular smooth muscle cell (VSMC) phenotypes, including differentiation, proliferation, and cell survival. However, the exact contribution of the individual Notch receptors has not been thoroughly delineated. In this study, we identify unique roles for NOTCH2 and NOTCH3 in regulating proliferation and cell survival in cultured VSMCs. Our results indicate that NOTCH2 inhibits PDGF-B-dependent proliferation and its expression is decreased by PDGF-B. In contrast, NOTCH3 promotes proliferation and receptor expression is increased by PDGF-B. Additionally, data show that NOTCH3, but not NOTCH2 protects VSMCs from apoptosis and apoptosis mediators degrade NOTCH3 protein.

We identified three pro-survival genes specifically regulated by NOTCH3 in cultured VSMCs and in mouse aortas. This regulation is mediated through MAP kinase signaling, which we demonstrate can be activated by NOTCH3, but not NOTCH2. Overall, this study highlights discrete roles for NOTCH2 and NOTCH3 in VSMCs and connects these roles to specific upstream regulators that control their expression.

Notch receptors belong to an evolutionarily conserved family of cell surface receptors (Notch1–4) that transduce signals between neighboring cells via cell-to-cell contact (1, 2). Notch receptor activation is triggered when a ligand (Jagged-1, -2 or Delta-like-1, -3, -4 in mammals) expressed on an adjacent cell surface binds to one of the receptors. The Notch receptor then undergoes cleavage, which allows the intracellular domain (NICD)2 to translocate to the nucleus. In the canonical pathway, the NICD binds with the transcription factor CSL (named after a trio of related proteins CBF, Suppressor of Hairless, Lag-1) to regulate downstream gene expression (3, 4). There is also evidence of non-canonical signaling by Notch receptors through direct interaction of the receptors or NICDs with members of other signaling pathways such as TGFβ (5, 6), Wnt (7–9), and MAPK (10, 11). In vascular smooth muscle cells (VSMCs), the Notch2 and Notch3 receptors predominate (12–15). Notch signaling is critical for growth regulation and cell fate determination in many cell types, including vascular cells (16–18). The regulation of contractile differentiation and extracellular matrix synthesis by Notch activity has been well defined (12, 15, 19–24), but what is less clear is the exact role of Notch receptors in VSMC proliferation and cell survival. Notch signaling has been shown to both increase and decrease VSMC proliferation, suggesting context dependence and/or receptor-selective functions (14, 24–26). A recent report provided some clarity on this issue, by identifying a specific and unique role for Notch2 in inhibiting VSMC proliferation (27). Thus, individual Notch receptors may possess unique roles in regulating VSMC proliferation, which could be linked to distinct signaling cues that control cell fate.

Notch signaling has also been closely associated with apoptosis and cell survival, especially in cancer cells (9, 28–36) and smooth muscle cells (11, 14, 25, 37, 38). Notch3 specifically has been shown to promote cell survival in several different cell types (9, 11, 25, 33–35, 37) and is a poor prognostic biomarker in several cancers (29, 30, 39). The role of NOTCH3 in cell survival is less clear, but has been shown to enhance apoptosis in certain cellular contexts (31, 40), in contrast to the effect of NOTCH3. There is also some evidence that Notch3 can promote cell survival by enhancing or inducing MAP kinase signaling, though the exact mechanism and role for Notch3 in regulating VSMC survival is unclear (11, 33).

In this study we sought to better define the specific roles of NOTCH2 and NOTCH3 in the regulation of proliferation and cell survival in smooth muscle cells. Additionally, we investigated the expression profiles of these two receptors in response to proliferation and apoptosis cues.
NOTCH2 and NOTCH3 Have Unique Functions in Smooth Muscle

Experimental Procedures

Cell Culture—Primary cultures of human aortic smooth muscle cells (HAoSMCs) were purchased from Vascularife and maintained in SMC medium: DMEM (Thermo Fisher Scientific) supplemented with 5% FBS (HyClone), insulin (4 ng/ml), EGF (5 ng/ml), ascorbic acid (50 ng/ml), 2 mM glutamine, 1 mM sodium pyruvate, and 100 units/ml penicillin/streptomycin. Primary cells between passages 6 and 8 were used for all experiments. All cultures were maintained in humidified 5% CO2 at 37 °C. “Serum-starved” cells were cultured in DMEM supplemented with 0.2% FBS, 2 mM glutamine, 1 mM sodium pyruvate, and 100 units/ml penicillin/streptomycin for 48 h before treatment. 20 ng/ml of PDGF-B (Peprotech) were added to this medium where specified. Inhibitors used were all dissolved in DMSO and used at the following concentrations: 5 μM PDGFR receptor tyrosine kinase inhibitor I (Fisher), 10 μM U0126 (Calbiochem), 10 μM JNK Inhibitor II (Calbiochem), and 10 μM MG-132 proteasome inhibitor (ApexBio). Hydrogen peroxide (Kroger) was added at concentrations indicated. For phosphorylation experiments, cells were serum-starved for 24 h, and then cultured in 10% FBS DMEM for 1 h before collection.

Quantitative RT-PCR (qPCR)—Total RNA was isolated from cells using RiboZol reagent (Ameresco) according to the manufacturer’s instructions and reverse transcribed with M-MLV reverse transcriptase (Promega) to generate cDNA. Real-time PCR was performed using a StepOne PCR system (Applied Biosystems) with SYBR Green and 50 ng of cDNA as template. The fold difference in target gene mRNA levels was calculated using the △△CT method and normalized to GAPDH mRNA from the same sample. Primer sequences were as follows: NOTCH3, 5′-AGA TCA GGT CGG AGA TG (reverse) and 5′-GGC AGA TCT GCT GAG CAT (forward) (reverse); NOTCH2, 5′-ACA AAG TGT ATG CGT GGA A (forward) and 5′-GGC AGA TCT GCT GAG CAT (reverse); NOTCH2, 5′-ATG GAA ATC CCA TCA CCA TCT T (forward) and 5′-CGC CCC CTC TGA TTT TGG (reverse); Bcl2, 5′-TGG TTC CGC AGT TTC CTC (forward) and 5′-GAG TTT CTC CGC AGT TTC CTC (reverse); GAPDH, 5′-GAA ACC ATT CAC ACC GTT GAT (forward) and 5′-GAG TTT CTC CGC AGT TTC CTC (reverse); Notch3, 5′-AAC UGC GAA GUG AAC AUU G. Control siRNA was purchased from Invitrogen. All siRNAs were transfected at 20 nM. 24 h post-transfection, cells were used in assays as described. Efficacy of knockdown was confirmed by Western blot, as shown in Fig. 5.

Lentiviral Transduction—The lentivirus plasmids containing GFP, NICD2-FLAG, and NICD3-FLAG constructs were made as described previously (22). The lentivirus plasmids were transfected into TN-293 cells using PolyJet (SignaGen), and the viral particles were amplified and purified as described previously (41), with the following changes. Virus-containing supernatant collected from a 10-cm plate was concentrated by adding 10% polyethylene glycol (PEG) and 150 mM NaCl, rocking overnight at 4 °C, pelleted by centrifugation at 3000 rcf, and resuspended in 150 μl of DMEM. For transduction of GFP or NICDs, HAoSMCs were seeded in a six-well plate at a density of 1 × 10^5 cells per well, 24 h before viral infection. 20 μl of lentivirus suspension diluted in 2 ml of media with 5% FBS was added to each well. Polybrene was supplemented at a final concentration of 6 μg/ml. Twenty-four hours later, cells were transferred to fresh media containing 5% FBS for additional 24 h incubation. Cells were then collected for mRNA, protein, or used in assays as described. The efficiency of transduction was evaluated using GFP expression and qPCR. Viral particles were titrated to achieve 90 to 100% transduction. Expression of cDNAs was confirmed using qPCR (not shown) and Western blot analysis using a FLAG antibody (Figs. 3 and 4).

Western Blotting—Cells were homogenized in RIPA buffer containing 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% SDS, and protease inhibitors (Sigma). For detection of phosphorylated proteins, 1 mM Na3VO4 and 1 mM NaN3 phosphotase inhibitors were added. Protein concentrations were determined by the Bradford assay (Bio-Rad). Equivalent amounts of protein were run on 10% SDS-polyacrylamide gels and proteins were transferred to nitrocellulose membranes (GE Healthcare). The membranes were incubated for 1 h at room temperature with 5% nonfat dry milk, then with primary antibodies against NOTCH3 (1:1,000, sc-5593, Santa Cruz Biotechnology), NOTCH2 (1:2,000, C651.6DbHN, Developmental Studies Hybridoma Bank), tubulin (1:20,000, T7816; Sigma), MKI67 (1:1,000, ab66155, ABCAM), FLAG (1:1,000, M2, Fisher), caspase3 (1:1,000, 8G10, Cell Signaling Technology, Inc.), ERK1/2 (1:1,200, C651.6DbHN, Developmental Studies Hybridoma Bank), tubulin (1:20,000, T7816; Sigma), MKI67 (1:1,000, ab66155, ABCAM), FLAG (1:1,000, M2, Fisher), caspase3 (1:1,000, 8G10, Cell Signaling Technology, Inc.) in 5% nonfat dry milk overnight at 4 °C. Secondary antibodies conjugated to the horseradish peroxidase (HRP), (1:5,000; Amersham Biosciences) were incubated for 1 h at room temperature. Proteins were detected by enhanced chemiluminescence (Thermo Fisher Scientific) and films were either digitally scanned or chemiluminescence was directly measured using the Bio-Rad ChemiDoc XRS+. Protein was quantified using ImageLab software (Bio-Rad). Relative protein expression was calculated by normalization to tubulin.

Proliferation Assay—Relative proliferation was determined by methylene blue assay as described previously (42), using 3000 HAoSMCs per well of a 96-well collagen-coated plate.

UV-B Irradiation—HAoSMCs were plated in a 6-well plate at 1 × 10^5 cells/well the day before irradiation. Medium was
removed and the cells were washed in PBS, before irradiation with 25–100 J/cm² with the Spectrolinker XL-1500 (Spectronics Corporation), with bulbs producing UV-B radiation at the wavelength 254 nm. Cells were collected 9-h postirradiation.

Caspase3 Apoptosis Assay—Lysates were prepared by suspension of cells in KPM buffer (50 mM KCl, 50 mM PIPES, 10 mM EGTA, 1.92 mM MgCl₂ pH 7.0, 1 mM DTT, 1 mM PMSF, 10 μg/ml cytochalasin B (Acros Organics), 2 μg/ml protease inhibitors (Sigma)) and 5–10 freeze/thaw cycles. Lysates were then used in a caspase3 enzymatic activity assay using AFC-DEVD substrate (MP Biomedicals) as described previously (43). Fluorescence was measured by Spectramax M5 (Molecular Devices, filters: excitation, 400 nm; emission, 508 nm). \( V_{\text{max}} \) values were calculated by Softmax Pro software (Molecular Devices).

Mouse Lines, Genotyping, and Crosses—All strains were maintained in C57Bl/6 background. Notch3 \( ^{-/-} \) (B6;129S1-Notch3tm1Grid/J) (44) mutant mice were generated and generously provided by Dr. Thomas Gridley. Notch2fl/fl (B6.129S-Notch2tm3Grid/J)(JAX 010525) (45) and Myocardin-Cre (Myocdtm1(cre)Jomm/J)(JAX 014180) (46) were purchased from the Jackson Laboratory. Notch2fl/fl and Myocardin-Cre mice were crossed to produce smooth muscle-deficient Notch2 mice. Genotyping of mice and embryos was carried out by PCR with the following primers: Notch3wt1: 5'-CCA TGA GGA TGC TAT CTG TGA C, Notch3wt2: 5'-CAC ATT GGC ACA AGA ATG AGC C, Notch3dl1: 5’-GCT ACT GAG CAA ACT CAG C, Notch3dl2: 5’-CCT TCT ATC GCC TTC TTG, Notch2fox1: 5’-TAG GAA GCA GCT CAG CTC ACA G, Notch2fox2: 5’-ATA ACG CTA AAC GTG CAC TGG AG, Myocardin-Cre forward: 5’-TCC TGC CCT GCT GGT TAA TTA GCC TCG, Wild-type reverse: 5’-TCA GCA AAG AGT GCA GAC CCC AGG AG, Myocardin-Cre reverse: 5’-AAC CTC ATC ACT CTT GCA TCG ACC GG. All mouse studies were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the Research Institute at Nationwide Children’s Hospital. The Nationwide Children’s Hospital Research Institute IACUC specifically approved this study.

Collection of Mouse Aortas and Isolation of Aortic Smooth Muscle Cells—Aortas were removed from euthanized mice and stripped of their adventitia and endothelial cells. For RNA isolation, aortic tissue was placed in RiboZol and lysed using the Tissuelyser II (Qiagen). For isolation of primary aortic smooth muscle cells, aortic tissue was digested in HBSS (CellGro) with 175 units/ml Collagenase II (Sigma), 175 units/ml Elastase (Sigma), and 1% Antibiotic-Antimycotic Solution (CellGro). After 45 min at 37 °C, aortas were homogenized by pipetting, pelleted, and grown in SMC medium supplemented with 1% Antibiotic-Antimycotic Solution.

Statistical Analysis—Data analyses were performed using GraphPad Prism, and comparisons between data sets were made using Student’s t test, one-way ANOVA, or two-way ANOVA as noted. Differences were considered significant if \( p < 0.05 \). Data are presented as mean ± S.D. unless otherwise noted. Data shown are representative of at least three independent experiments.

Results

NOTCH2 and NOTCH3 Expression Are Uniquely Regulated by PDGF-B—To identify potential functional differences between NOTCH2 and NOTCH3 in VSMCs, we first sought to detect expression changes in response to a well-known mediator of VSMC phenotypes, PDGF-B. Treatment of serum...

![FIGURE 1. NOTCH2, but not NOTCH3 receptor expression is decreased by PDGF-B. A and B, HAoSMCs were serum-starved for 48 h, then treated with 20 ng/ml PDGF-B and collected to isolate mRNA for qPCR (A), or protein for Western blot analysis (B), at designated timepoints. qPCR shown as expression relative to GAPDH, \( n = 5 \). NOTCH2 and NOTCH3 band intensity quantified relative to tubulin, \( n = 6 \). C, serum-starved HAoSMCs were treated with/without 20 ng/ml PDGF-B, and vehicle control, 5 μM PDGF-receptor inhibitor (PDGFRI), 10 μM MEK inhibitor (U0126), or 10 μM JNK inhibitor (JNKII) for 48 h. qPCR shown as expression relative to GAPDH, \( n = 4 \). Significance determined by one-way ANOVA. *, \( p < 0.05 \), n.s., not significant.]
starved human aortic smooth muscle cells (HAoSMCs) with PDGF-B produced a progressive loss of NOTCH2 mRNA and protein, but did not significantly decrease NOTCH3 expression (Fig. 1, A and B). NOTCH2 transcript expression measured by quantitative PCR (qPCR) showed significant decrease at 12 h and beyond, while the protein decrease by Western analysis lagged, exhibiting a significant reduction at the 24 and 48-hour time points. In contrast, although NOTCH3 mRNA expression was not affected, protein levels were increased by 2-fold at 48 h. To determine which pathway(s) downstream of PDGF-B might be responsible for NOTCH2 downregulation, we co-treated HAoSMCs with PDGF-B and select pathway inhibitors. PDGF-B-associated NOTCH2 decrease was ablated by both a PDGF receptor inhibitor (PDGFR1) and the MEK/ERK inhibitor U0126, but not by a JNK inhibitor (JNKII) (Fig. 1C). Overall, these data demonstrate that NOTCH2 is specifically down regulated by PDGF-B at the RNA and protein level, while NOTCH3 transcript expression remains unaffected, and protein levels were increased in marked contrast to NOTCH2.

**NOTCH2 and NOTCH3 Have Unique Functions in Smooth Muscle**
NOTCH2 and NOTCH3 Have Unique Functions in Smooth Muscle

ation assay. For receptor ablation, cells were transfected with siRNA directed against each receptor to knockdown expression and compared with control siRNA in the presence or absence of PDGF-B. For overexpression studies, the NOTCH2 (NICD2) and NOTCH3 (NICD3) intracellular domains were transduced via a lentivirus and compared with control GFP-expressing virus with and without PDGF-B treatment.

In the absence of NOTCH2 (siNOTCH2), smooth muscle cells without PDGF-B exhibited increased proliferation compared with control siRNA-transfected cells. In the presence of 20 ng/ml of PDGF-B, while there was an overall increase in the rate of proliferation, no difference was observed between control and siNOTCH2 cells (Fig. 2A). This lack of a difference is likely due to the ability of PDGF-B to decrease NOTCH2 expression similar to the effects of siRNA knockdown. Overexpression of NICD2 produced complementary results, as NICD2 overexpression inhibited PDGF-B associated proliferation (Fig. 2B). In complete contrast to the inhibitory effects of NOTCH2 on cell proliferation, manipulation of NOTCH3 revealed a pro-proliferation activity. In response to 20 ng/ml PDGF-B, cells treated with siNOTCH3 had a reduced proliferation rate (Fig. 2C), and NICD3-expressing cells showed increased proliferation (Fig. 2D). These data were further supported by Western blots measuring the expression of the proliferation marker, MKI67 (Ki67), which had stronger induction in siNOTCH2 cells, and weaker in siNOTCH3 samples, compared with control siRNA-treated cells (Fig. 2E). Consistently, NICD2 overexpression caused reduced MKI67 expression, while NICD3 promoted its expression in PDGF-B-treated smooth muscle cells (Fig. 2F). Collectively, these data demonstrate opposite functions of the NOTCH2 and NOTCH3 receptors on smooth muscle proliferation.

To further validate our proliferation results, we isolated aortic smooth muscle cells from mice with a global knock-out of Notch3 (N3⁻/⁻), and a smooth muscle-specific knock-out of Notch2 (N2fl/fl, MCC⁻/⁻). These mouse primary cells were utilized in our proliferation assays as described for the HAoSMCs and compared with wild-type (WT) cells derived from control mice. Similar to the siRNA knockdown findings with human smooth muscle cells, Notch3-null smooth muscle cells exhibited reduced proliferation, and Notch2-deleted cells exhibited enhanced proliferation (Fig. 3). However, in these Notch-deficient cells both the untreated and PDGF-B-treated cells had significantly different proliferation rates compared with wild-type cells. Thus, in both human and mouse aortic smooth muscle cells, Notch2 is anti-proliferative and Notch3 acts in a pro-proliferative manner. Overall, these results demonstrate that Notch2 and Notch3 have different roles in regulating VSMC proliferation in response to PDGF-B.

**NOTCH3 Is Uniquely Regulated by Inducers of Apoptosis—**

NOTCH3 has been strongly linked to cell survival, thus we asked if NOTCH3 might be regulated by cell death signals, similar to how PDGF-B down-regulates NOTCH2. To test this, cells were treated with hydrogen peroxide or ultraviolet (UV) irradiation to promote apoptosis, followed by RNA and protein isolation to measure Notch receptor expression. At the transcript level there was no significant changes in either receptor’s expression (Fig. 4, A and B). However, Western analysis of NOTCH3 protein revealed a precipitous decline when HAoSMCs are treated with either apoptosis inducer, while NOTCH2 protein expression remained unchanged (Fig. 4, C and D). Given that NOTCH3 protein, but not mRNA expression was decreased by two different apoptosis inducers, we hypothesized it was due to a common mechanism involving protein degradation. Indeed, when we treated cells with the proteasomal inhibitor MG-132 the UV-induced decrease of NOTCH3 protein was ablated (Fig. 4E). The loss of NOTCH3 by UV irradiation could also be seen using the overexpressed Flag-tagged-NICD3, whose degradation was similarly blocked by MG-132 (Fig. 4F). In contrast, Flag-tagged-NICD2 was not affected by UV irradiation or MG-132 treatment. Taken together, these data indicate that the intracellular domain of NOTCH3 is specifically targeted for proteasomal degradation following apoptosis induction.

**NOTCH3 Protects VSMCs from Induction of Apoptosis—**

Since NOTCH3 protein is targeted for degradation after apoptosis stimulation we hypothesized that NOTCH3 down-regulation is induced to allow VSMCs to enter apoptosis. If this is true, NOTCH3 signaling should be protective against apoptosis induction. To test this, we utilized the same siRNA knockdown and overexpression strategies as described for proliferation assays to measure the effects of NOTCH2 and NOTCH3 on...
UV-induced apoptosis. Apoptosis was monitored by Western analysis to measure activated cleaved caspase3, and an enzymatic caspase assay utilizing the fluorescent caspase3 substrate, AFC-DEVD. Overexpression of NICD3 in the presence of UV irradiation showed a protective effect by decreasing caspase3 activity (Fig. 5, A and C). Accordingly, siRNA knockdown of NOTCH3 resulted in an increase in cleaved caspase3 protein and caspase3 activity (Fig. 5, B and D). Conversely, NOTCH2 overexpression or knockdown had no effect on smooth muscle cell apoptosis as measured by caspase3 activity. These data reveal that, as with proliferation, NOTCH2 and NOTCH3 have differing effects on cell survival, with NOTCH3 acting as a protective factor against UV-induced apoptosis.

NOTCH3 Promotes Transcription of Cell Survival Genes via Activation of MAPK—To determine the mechanism by which NOTCH3 inhibits apoptosis, we measured the expression of known pro-survival genes in HAoSMCs with altered Notch receptor expression. From this we identified three pro-survival genes that were positively associated with NOTCH3 expression. Pro-survival genes, BCL2 (47), BIRC5 (Survivin) (48), and CFLAR (cFLIP) (49) showed a significant increase in transcript expression in the presence of lentivirally expressed NICD2 or NICD3 were UV-irradiated and cultured for 9 h with 10 \( \mu \)M MG-132. Western blots to detect FLAG-tagged proteins, relative to tubulin, \( n = 3 \). Significance determined by one-way ANOVA, \( * p < 0.05 \), n.s., not significant.
MAPK pathway (50–52), therefore we tested to see if NOTCH3 induction of these genes was through MAPK. HAoSMCs overexpressing control GFP, NICD2, or NICD3 were treated with the MEK inhibitor, U0126 and RNA was collected for expression analysis. As predicted, the MEK inhibitor caused a general decrease in the pro-survival genes mRNA expression in all conditions, but importantly completely blocked the induction of these genes by NICD3 (Fig. 6B).

Notch receptors have been previously reported to interact with other signaling pathways both directly and via their transcriptional targets (5–11). To test if NOTCH3 could activate the MAPK pathway in smooth muscle cells, we measured the phosphorylation of the MAPK signaling mediator ERK in HAoSMCs with overexpressed or knocked down NOTCH2 or NOTCH3 (Fig. 6, C and D). Knockdown of NOTCH3 decreased ERK phosphorylation (phospho-ERK), while overexpression of NICD3 caused an increase. Interestingly, although knockdown of NOTCH2 did not affect phospho-ERK levels, overexpression of NICD2 caused a strong decrease. From these results we demonstrate a causal link between NOTCH3’s ability to induce pro-survival gene expression and VSMC survival. The data indicate that NOTCH3 acts through the MEK/ERK signaling pathway to up-regulate these pro-survival genes.

Genetic Deletion of Notch3, but Not Notch2 Has Unique Effects on Cell Survival of Primary Aortic Smooth Muscle Cells—To validate the regulation of pro-survival genes by NOTCH3, we measured the mRNA expression of the three identified pro-survival genes in the aortas of Notch-deficient mice. We found that transcript expression of all three genes was significantly decreased in the aortas of Notch3-null (Notch3−/−) mice, but not the Notch2-mutant (Notch2−/−; MCC−/−) mice (Fig. 7A). Accordingly, we found that primary aortic smooth muscle cells with genetic deletion of Notch3 mirrored the Notch3 siRNA results in HAoSMC (Fig. 5D), showing increased caspase3 activity in response to apoptosis induction (Fig. 7B). Additionally, these mouse aortic smooth muscle cells exhibited a similar
phospho-ERK profile to their human counterpart (Fig. 6C), where Notch3-deficient cells had dramatically reduced ERK phosphorylation (Fig. 7C). These results reinforce our hypothesis that NOTCH3 promotes VSMC survival by inducing ERK signaling and promoting expression of pro-survival genes.

Overall, the data presented herein illustrate unique aspects of NOTCH2 and NOTCH3 function and expression in VSMC. Our results demonstrate a direct interaction between their expression and function in governing proliferation and cell survival. Moreover, these findings reveal receptor-specific relationships between the Notch pathway and ERK signaling, which suggests complexities that contribute their unique functions.

**Discussion**

Data presented here provide new insight into how the Notch signaling pathway is regulated within VSMCs, and exerts its effects to influence phenotypic properties. Our results demonstrate that the NOTCH2 and NOTCH3 receptors have different roles in VSMC proliferation and survival (Fig. 8). NOTCH3 acts via the MEK/ERK pathway to promote cell survival, and its protein is targeted for proteasomal degradation in response to apoptosis cues. The growth factor PDGF-B increases NOTCH3 protein expression, which in turn promotes proliferation, possibly through MEK/ERK signaling. In contrast, NOTCH2 acts to inhibit proliferation, and PDGF-B signals through the MEK/ERK pathway to remove this inhibition by down-regulating the NOTCH2 receptor. Overall, these findings identify NOTCH2 as anti-proliferative and NOTCH3 as pro-proliferative and pro-survival in smooth muscle cells, with MEK/ERK signaling serving a pivotal role in these functional outcomes.

One interesting conclusion from our results is that the expression of the two receptors is linked to the phenotypes they promote. NOTCH2 is anti-proliferative and a proliferation
inducer, PDGF-B, reduces its expression. Likewise, NOTCH3 is pro-survival and apoptosis inducers decrease its protein expression. This reciprocal regulation of NOTCH2 and NOTCH3 by mediators of the very processes they influence suggests that these inducers exert their effects, at least in part, through controlling Notch receptor expression. In fact, one could envision NOTCH2 as a brake that must be removed before proliferation can proceed and a similar role for NOTCH3 as an impediment to the progression of apoptosis. Indeed, in a report demonstrating NOTCH2’s inhibitory role in smooth muscle proliferation, the Liaw laboratory showed NOTCH2 mRNA expression is sharply decreased after S-phase allowing the cell to undergo mitosis (27). Our data showing rapid proteasomal degradation of NOTCH3 protein by apoptosis mediators suggests a similar strategy, in which NOTCH3 must be decreased to allow apoptosis to proceed. Thus, in mature smooth muscle cells, where both Notch receptors are expressed, they not only contribute to a contractile differentiated phenotype, but also maintain a quiescent cell that is protected from apoptosis.

Our results identify MAPK signaling as being intricately connected to the NOTCH2 and NOTCH3 receptors. We show that MEK/ERK signaling acts upstream of NOTCH2 and contributes to its down-regulation by PDGF-B. Conversely, we demonstrate that the MEK/ERK pathway is downstream of NOTCH3, and its activation is critical for NOTCH3-dependent up-regu-

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**FIGURE 7.** Genetic deletion of Notch3 has unique effects on the survival of mouse aortic smooth muscle cells. A, mRNA expression of pro-survival genes in aortas of wild-type (WT), Notch2-deficient (Notch2fl/fl; MCC+/−), and Notch3-mutant (Notch3−/−) adult mice. qPCR with relative expression compared with GAPDH, n = 4. B, SMCs were isolated and cultured from aortas of wild-type, Notch2, and Notch3-deficient mice and used in enzymatic caspase3 activity assays, n = 4. C, total and phosphorylated (phospho)-ERK was measured in cultured smooth muscle cells following serum challenge, with tubulin used as loading control, n = 4. Significance determined by one-way ANOVA, *, p < 0.05.

**FIGURE 8.** NOTCH2 and NOTCH3 have unique roles in proliferation and cell survival related to their expression.
loration of pro-survival genes. MAPK signaling has long been linked to cell survival (53, 54), but NOTCH3's ability to activate it to enhance survival is a novel finding. Additionally, the large disparity in phospo-ERK levels between NICD2 and NICD3 overexpression reveal significant differences in how NOTCH2 and NOTCH3 interact with the MAPK pathway. Since the MAPK pathway is also a strong promoter of cell growth and proliferation, it is likely that the regulation of MEK/ERK signaling by NOTCH2 and NOTCH3 is at least partially responsible for their observed effects on proliferation. One possible mechanism for NOTCH3's regulation of proliferation and/or MEK/ERK signaling is by influencing growth factor receptors. Notch signaling has been shown to increase expression of PDGFRβ (55), the receptor for PDGF-B. However, we did not see any difference in PDGFRβ expression between NOTCH2 and NOTCH3 in our overexpression or knockdown studies (data not shown), suggesting additional mechanisms contribute to their unique effects on proliferation.

VSMC phenotypes are tightly regulated by a number of factors that contribute to how these cells function and respond to stimuli. With this in mind, the varied expression of these two receptors in different phases of vascular development, injury, and disease (14, 56) implicates the balance of NOTCH2 and NOTCH3 signaling as a key determinant of VSMC phenotypes. One could imagine that an overabundance of NOTCH3 activity leads to rapid expansion and maintenance of a smooth muscle population, while a surplus of NOTCH2 activity leads to quiescence. The classic understanding of VSMC phenotype holds that a differentiated contractile cell is quiescent, and a proliferating cell is dedifferentiated. Given that Notch signaling is a known promoter of differentiation (12, 19, 20, 57), and we have shown that both NOTCH2 and NOTCH3 stimulate expression of contractile genes this indicates a more complex picture of these phenotypes. Our data suggests that NOTCH3 may contribute to an intermediate VSMC phenotype capable of both proliferating and expressing contractile genes. This distinct function is likely important for development, but also might contribute to disease. Pulmonary arterial hypertension exemplifies this concept, where VSMCs have excessive proliferation, resist apoptosis, and have increased expression of contractile genes, all correlated with increased NOTCH3 expression. Interestingly, these disease phenotypes are ameliorated with a reduction of NOTCH3 signaling (58).

The advent of CRISPR-Cas9 technology is quickly making traditional knockdown methods obsolete. The recent discovery that knockdowns by traditional methods do not always match the phenotypes seen by deletion with CRISPR-Cas9 systems (59), suggests it may be a better alternative to avoid off-target effects (60, 61). While we are confident in the efficiency of our siRNA knockdowns, given our results are corroborated by data using both over-expression and genetic deletion systems, in moving forward one must consider deletion of Notch2/3 using CRISPR-Cas9. Additionally, an exciting avenue of investigation made easier by CRISPR-Cas9 is the potential identification of the structural domains of the Notch receptors that harbor their unique effects. It is now relatively simple to create chimeric proteins composed of different regions of individual proteins. Introduction of Notch2/Notch3 chimeric receptors into cells and animal models would significantly advance our understanding of modularity and distinct functions of the Notch family of receptors.

Taken together, our results show for the first time that NOTCH2 and NOTCH3 can uniquely regulate VSMC phenotypes and demonstrate that their expression is closely related to their functions. These results clearly show that Notch signaling is an important mediator of smooth muscle biology and can exert unique forces through expression of different receptor combinations.

Acknowledgments—We thank Dr. Andrea Doseff and Daniel Arango for their help in performing the caspase3 apoptosis assays. The authors declare that they have no conflicts of interest with the contents of this article.

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NOTCH2 and NOTCH3 Have Unique Functions in Smooth Muscle

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