Activation Dynamics and Signaling Properties of Notch3 Receptor in the Developing Pulmonary Artery

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Notch3 signaling is fundamental for arterial specification of systemic vascular smooth muscle cells (VSMCs). However, the developmental role and signaling properties of the Notch3 receptor in the mouse pulmonary artery remain unknown. Here, we demonstrate that Notch3 is expressed selectively in pulmonary artery VSMCs, is activated from late fetal to early postnatal life, and is required to maintain the morphological characteristics and smooth muscle gene expression profile of the pulmonary artery after birth. Using a conditional knock-out mouse model, we show that Notch3 receptor activation in VSMCs is Jagged1-dependent. In vitro VSMC lentivirus-mediated Jagged1 knockdown, confocal localization analysis, and co-culture experiments revealed that Notch3 activation is cell-autonomous and occurs through the physical engagement of Notch3 and VSMC-derived Jagged1 in the interior of the same cell. Although the current models of mammalian Notch signaling involve a two-cell system composed of a signal-receiving cell that expresses a Notch receptor on its surface and a neighboring signal-sending cell that provides membrane-bound activating ligand, our data suggest that pulmonary artery VSMC Notch3 activation is cell-autonomous. This unique mechanism of Notch activation may play an important role in the maturation of the pulmonary artery during the transition to air breathing.

Lung development involves the integration of multiple signaling pathways to ensure coordinated growth and differentiation of airway and vascular structures (1). The initial event in vessel development is the formation of basic tubular structures from endothelial precursors (2). Under the influence of PDGF-β and Wnt signaling (3, 4), formation of mature vessels occurs by the stepwise recruitment, growth, and differentiation of mesenchymal precursors into a circumferential mural cell layer composed of smooth muscle cells and pericytes (5).

It is noteworthy that the lung vascular system is subjected to a dramatic switch at birth, from a low-flow, low-pressure fetal state to a high-flow, higher pressure postnatal state (1). Although it is recognized as an essential feature of the transition from intrauterine life, the timing and identification of signals that control how lung vascular cells adapt to this changeover remain unclear. The involvement of Notch signals in multiple aspects of vessel development led us to investigate the role of this pathway during this transition period.

The Notch signaling pathway is highly conserved across species, controlling cell fate decisions and tissue patterning during development (6). In classical Notch signaling, a cell surface Notch receptor is activated by binding with a membrane-bound ligand delivered by a neighboring signal-sending cell (7). This interaction initiates a series of proteolytic cleavages that release the Notch intracellular domain into the cytoplasm of the signal-receiving cell before translocation to the nucleus and induction of target gene transcription (8, 9).

Of the four mammalian Notch receptors, Notch3 is expressed predominantly in vascular smooth muscle cells (VSMCs) (10, 11). Notch3 regulates arterial specification of VSMCs in fetal systemic arteries (12). In the developing retinal vasculature, Notch3 deletion impairs mural cell recruitment, resulting in progressive loss of vessel coverage (13). Its role, however, in pulmonary artery development has not yet been examined. Of note, Notch3 serves as a sensor of hemodynamic stress (14) and is involved in vascular tone regulation (15), two properties unique to this receptor. Furthermore, Notch3 has several structural features that differ from other Notch family members, including a lack of transactivation domain, a smaller number of EGF-like repeats, and a distinctive ligand-binding domain (16), thereby raising the possibility that Notch3 activation may occur through a mechanism that is distinct from classical Notch signaling. In this context, recent studies in Drosophila demonstrated an alternative mode of Notch activation that is cell-autonomous and occurs inside the cell (17, 18).

In this study, we show that Notch3 is expressed selectively in pulmonary artery VSMCs and is active from late fetal to early postnatal life. Our data point to a key role for Notch3 in the final stages of VSMC maturation during the adaptation to postnatal life. Most notably, we provide evidence indicating that Notch3 signaling in pulmonary artery VSMCs occurs through a unique cell-autonomous mechanism that is dependent on an interaction with Jagged1 in the interior of VSMCs.

3 The abbreviations used are: VSMC, vascular smooth muscle cell; hrGFP, humanized R. reniformis GFP; α-SMA, α-smooth muscle actin; N3ECD, Notch3 extracellular domain; RPASMC, rat pulmonary artery smooth muscle cell; qPCR, quantitative real-time PCR; eGFP, enhanced GFP; E, embryonic day; N3ICD, Notch3 intracellular domain.
EXPERIMENTAL PROCEDURES

**Animals**—Pregnant CD1 mice were obtained from Charles River Laboratories (Wilmington, MA). Notch3−/− mice were provided by Dr. Thomas Gridley (The Jackson Laboratory, Bar Harbor, ME). A transgenic mouse (α-SMA-hrGFP) that expresses humanized Renilla reniformis GFP (hrGFP; Stratagene, Cedar Creek, TX) under the control of the rat α-smooth muscle actin (SMA) gene promoter was generated. In brief, the rat SMA promoter was ligated into the multiple cloning site of the pHRGF vector (Stratagene) immediately upstream of sequences that encode hrGFP. The promoter fragment consists of 2.6 kb of sequence upstream of the transcription start site and all of intron 1 from the rat SMA gene. A linearized construct was microinjected into pronuclei of fertilized C57/B6 eggs before implantation in foster mothers. A founder line containing two copies of the transgene (as determined by quantitative Southern hybridization) was chosen. These mice selectively express hrGFP in smooth muscle cell populations in vivo. Notch3−/− α-SMA-hrGFP mice were generated by crossing Notch3−/− and α-SMA-hrGFP mice. Jagged1flox/flox mice were provided by Dr. Nadean Brown (Cincinnati Children’s Hospital, Cincinnati, OH). To selectively delete VSMC Jagged1, Jagged1flox/flox mice were crossed with sma22α−/−Cre (Sma-22cre) mice (The Jackson Laboratory). Animal studies were approved by the Boston University Institutional Animal Care and Use Committee.

**Antibodies**—A goat polyclonal antibody directed to the mouse Notch3 extracellular domain (N3ECD; Sf21 recombinant mouse Notch3 amino acids 40–486) was purchased from R&D Systems (Minneapolis, MN). Goat anti-Notch3 polyclonal antibodies directed to the extracellular (Q-14) and intracellular (M-134) domains and goat anti-Jagged1 polyclonal antibody (C-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A directly conjugated hamster anti-mouse N3ECD monoclonal antibody (HMN3-133) was purchased from BioLegend (San Diego, CA). For Western blot analysis of rat cells, rat anti-N3ICD monoclonal antibody (8G5) from Cell Signaling Technology (Danvers, MA) was used. Antibodies directed to CD45 (30-F11) and CD31 (MEC 13.3) were obtained from Pharmingen. Biotinylated anti-α-SMA antibody (1A4) was purchased from Thermo Scientific (Waltham, MA). Cy2-conjugated donkey anti-rabbit IgG and Cy3-conjugated anti-goat IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa Fluor 488-, 568-, and 647-conjugated anti-rabbit and anti-goat antibodies were purchased from Invitrogen. For Western blot analysis, anti-Jagged1 antibody obtained from Cell Signaling Technology was used. For all flow cytometry studies, conjugated isotype antibodies were used as controls.

**Immunohistochemistry and Immunofluorescence**—Lungs were fixed with 4% formalin, paraffin-embedded, sectioned, deparaffinized, and subjected to antigen retrieval before application of primary antibodies and incubation with HRP-conjugated or fluorescence-conjugated antibodies. Details are provided in supplemental Table 1.

**In Situ Hybridization**—In situ hybridization was performed on 10-μm lung sections using digoxigenin-UTP-labeled Hey1 and Hey2 riboprobe as described previously (19). See supplemental Table 2 for more details.

**Cell Isolation**—Cell suspensions were obtained by digesting lungs with 0.1% collagenase A (Roche Diagnostics), 2.4 units/ml Dispase (Roche Diagnostics), and 6 units/ml DNase I (Qiagen, Valencia, CA) at 37 °C for 1 h. Neonatal rat pulmonary artery smooth muscle cells (RPASMCs) were isolated as described (20).

**Confocal Microscopy**—Cells were cultured for 48 h, fixed with 4% paraformaldehyde, and permeabilized with 0.25% Triton X-100 in PBS before addition of primary and fluorochrome-conjugated secondary antibodies. Images were captured using a Zeiss LSM 510 confocal laser microscope and analyzed using NIH ImageJ browser software. Further details are provided in supplemental Table 3.

**Western Blotting and Immunoprecipitation**—Lung cells were obtained as described above. CD45− and CD31− cells were depleted from lung preparations by incubation with biotinylated anti-mouse CD45 and CD31 antibodies for 30 min on ice. After washing, biotin-binding magnetic beads (Dynabeads, Invitrogen) were added before submission to a magnetic field. The non-binding cell fraction was lysed in radioimmunoprecipitation assay buffer in the presence of protease inhibitors (1 mm phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A) for 30 min at 4 °C. Protein concentration was measured in the supernatant using the Bradford assay. For analysis, 80 μg of protein was separated through a 4–12% SDS-polyacrylamide gel before electrophoretic transfer to a PVDF membrane. Membranes were blocked with 2% BSA in TBS for 1 h. Blots were probed with anti-N3ICD (1:200) or anti-Jagged1 (1:200) antibody. Antigen-antibody complexes were identified with HRP-conjugated secondary antibodies. Enhanced chemiluminescence was used for detection. For immunoprecipitation, equal amounts of protein lysates were incubated with anti-N3ECD antibody for 3 h at 4 °C. Immune complexes were collected by incubation with protein A-Sepharose for 2 h before washing with PBS, separation by SDS-PAGE, and electrophoretic transfer to a PVDF membrane. Western blotting was performed with anti-Jagged1 or anti-N3ECD antibody as described above.

**Flow Cytometry and Cell Sorting**—Non-permeabilized and permeabilized cells were stained with fluorochrome-conjugated mouse-specific monoclonal antibodies prior to analysis. Permeabilization was achieved using BD Cytofix/Cytoperm solution (BD Biosciences). Flow cytometry was performed on an LSR II cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR). Antibody conditions are detailed in supplemental Table 4. hrGFP+ cells were collected from lung cell suspensions of α-SMA-hrGFP mice using a MoFlo cell sorter (Beckman Coulter, Fullerton, CA).

**Real-time PCR Analysis**—RNA was isolated using an RNeasy mini kit (Qiagen). cDNA was transcribed using the Promega reverse transcription system. Quantitative real-time PCR (qPCR) was performed in a StepOne Plus instrument (Applied Biosystems, Foster City, CA). All assays were performed in triplicate at two different cDNA concentrations to ensure that the amplification efficiencies for reference genes and genes of...
interest were the same. Reactions were normalized to 18 S rRNA. All TaqMan probes were obtained from Applied Biosystems. For contractile smooth muscle-related genes, qPCR was performed with fast SYBR reagent (Invitrogen) as described (21). GAPDH was used as an internal control. After PCR, a melting curve was constructed in the range of 60–95 °C to evaluate the specificity of the amplification. See supplemental Table 5 for TaqMan primer details.

**Cell Culture**—Primary RPASMCs were cultured in DMEM supplemented with 10% FBS. The rat alveolar macrophage NR8383 cell line was purchased from American Type Culture Collection and maintained as recommended. For inhibition of /H9253-secretase activity, RPASMCs were cultured in the presence of either 10 M DAPT dissolved in Me2SO or only Me2SO for 72 h.

**Lentivirus-mediated Jagged1 Knockdown**—Jagged1 shRNA or scrambled shRNA (22) was cloned into the pLVTHM lentiviral vector (23), a kind gift of Dr. D. Trono (Ecole Polytechnique Federale, Lausanne, Switzerland). Replication-incompetent lentivirus was created using a five-plasmid transfection procedure (24). All viral supernatants were concentrated ~100-fold by ultracentrifugation. Titering of all vectors was performed by infection of FG293 cells. RPASMCs were transduced with a single exposure at a multiplicity of infection of 100. Infected cells were identified by enhanced GFP (eGFP) expression. Jagged1 knockdown was confirmed by qPCR and Western blot analysis. Co-culturing of the wild type with Jagged1 or scrambled shRNA-transduced cells was performed at a 1:1 ratio for 72 h prior to collection by cell sorting.

**Cell Transfection**—Plasmids containing cDNAs encoding the mouse N3ICD or full-length Notch3 fused to GFP (25) were gifts of Dr. J. Arboleda-Velasquez (Harvard Medical School, Boston, MA). Neonatal RPASMCs and 293T cells were transiently transfected using Lipofectamine 2000 (Invitrogen). After transfection, cells were stained with anti-GFP antibody (Millipore) for microscopic analysis.

**Cell Surface Biotinylation**—To enrich for surface proteins, cell surface biotinylation of RPASMCs was performed using a cell surface protein biotinylation kit (Pierce). During the biotinylation procedure, all reagents and cultures were kept on ice. In brief, RPASMC cultures were washed with ice-cold PBS and then incubated in EZ-Link NHS-SS-biotin reagent for 30 min. Excess biotin was blocked with glycine. Cells were washed again with glycine buffer and lysed. Lysates were rocked for 1 h on a NeutrAvidin-agarose column. Non-biotinylated internal fractions were collected by washing the column with wash buffer, and biotinylated external proteins were collected by elution with 50 M DTT. Western blotting was performed using anti-N3ECD, anti-Jagged1, and anti-p53 antibodies in the biotinylated extracellular fraction, non-biotinylated intracellular fraction, and total cell lysates.
Statistical Analysis—Data are presented as means ± S.D. Statistical analysis was performed using Student’s t test. Significant differences were determined as p < 0.05, p < 0.01, and p < 0.001.

RESULTS

Abnormal Pulmonary Artery Morphology and Smooth Muscle Marker Gene Expression in Notch3−/− Mice—To evaluate the contribution of Notch3 receptor signaling in pulmonary artery development, we compared the morphology of vessels stained with anti-α-SMA antibody in WT and Notch3−/− mice. The pulmonary arteries in WT and Notch3−/− embryos at embryonic day (E) 18.5 appeared grossly similar (Fig. 1A). At postnatal day 3, however, VSMCs in Notch3−/− mice were dysmorphic, non-cohesive, and vacuolated and showed a disorganized distribution of SMA (Fig. 1A). Alterations in the α-SMA staining pattern and the morphology of smooth muscle cells persisted into adulthood, although the changes were less pronounced than at postnatal day 3 (Fig. 1A). To determine whether these morphological abnormalities are associated with changes in expression of smooth muscle-related genes in the Notch3−/− mouse, we quantified mRNAs for transgelin (Sma22), α-SMA, γ-SMA, smoothelin, and calponin as described (21). At all ages examined, down-regulation of these genes was observed in Notch3−/− mice (Fig. 1B). In contrast, the morphology of bronchial smooth muscle was not affected in Notch3−/− mice (data not shown), consistent with the restricted expression of Notch3 to VSMCs (supplemental Fig. S1A).

Temporal Expression and Activation of Notch3 in the Developing Lung—Using an antibody directed against the N3ECD, we found that this receptor is expressed in lung arterial VSMCs from E14.5 into adulthood (Fig. 2A). To determine the kinetics of Notch3 activation, the cellular localization of the Notch3 intracellular domain (N3ICD) was determined. As discussed, the N3ICD arises from a γ-secretase-dependent cleavage that follows receptor activation, before translocation to the nucleus (26). For this analysis, we employed an antibody previously shown to identify the mouse N3ICD (27); the specificity of this antibody was further confirmed by the lack of positive VSMC staining in the Notch3−/− mouse lung (supplemental Fig. S1B). We found that the N3ICD was distributed in the cytoplasm of lung VSMCs at E14.5 (Fig. 2B) but in the nucleus at E18.5, indicating active Notch3 signaling at late gestation (Fig. 2B, inset). N3ICD nuclear localization persisted until postnatal day 5 (data not shown) before reverting to a perinuclear distribution in adult VSMCs (Fig. 2B). Consistent with the timing of nuclear localization of the N3ICD (Fig. 2B), cleaved N3ICD protein was found in E18.5 lung lysates but was absent in adult lysates as determined by Western blot analysis (Fig. 2C).
Notch3 activation has been shown to promote RBP-Jk-mediated induction of Hey1 and Hey2 mRNAs in VSMCs in vitro and in vivo (28). Therefore, to confirm the temporal dynamics of Notch3 signaling in the pulmonary artery, we evaluated the relative expression of Hey1 and Hey2 in isolated smooth muscle cells. For isolation of pulmonary artery VSMCs, a transgenic mouse that expresses hrGFP under the control of the rat \( \alpha \)-SMA promoter (\( \alpha \)-SMA-hrGFP) was generated. In the \( \alpha \)-SMA-hrGFP mouse, hrGFP co-localized exclusively with \( \alpha \)-SMA-expressing smooth muscle cells of the pulmonary artery and bronchial tubes (supplemental Fig. S2). hrGFP cells were collected from E18.5 and adult lungs, and the levels of Hey1 and Hey2 were determined by qPCR. Hey1 and Hey2 mRNA levels were significantly higher at E18.5 compared with E14.5 or adult lungs (Fig. 2D), consistent with the timing of Notch3 activation (Fig. 2B and C).

To further validate that the time-restricted up-regulation of Hey1 and Hey2 in VSMCs reflects Notch3 activation, we assessed expression of other Notch family members in E18.5 VSMCs. We found that Notch1 and Notch4 mRNAs were nearly undetectable, as determined by qPCR (data not shown). Although there was a low level of Notch2 mRNA in VSMCs, Notch2 protein expression was not observed in E18.5 pulmonary artery VSMCs but was observed in adventitial cells surrounding adult pulmonary arteries (supplemental Fig. S3). Moreover, in situ hybridization confirmed that Hey1 and Hey2 were expressed only in VSMCs, but not in E18.5 bronchial smooth muscle cells (Fig. 2E).

To definitively prove that Hey1 and Hey2 mRNA expression in E18.5 VSMCs is reflective of Notch3 signaling, a Notch3\(^{-}\) mouse was generated. In isolated E18.5 Notch3\(^{-}\) VSMCs, Hey1 and Hey2 mRNA levels were decreased by \( \sim \)75% relative to WT VSMCs (Fig. 2F). Taken together, these observations show that Notch3 signaling is active in VSMCs from late fetal to early postnatal life.

Notch3 Activation Is Dependent on VSMC-derived Jagged1—Based on in vitro co-culture studies, endothelium-derived Notch ligands have been proposed to engage Notch receptors expressed in VSMCs (29). However, the presence of an intervening basement membrane between the endothelium and VSMC layer raises questions regarding the feasibility of this mode of activation in vivo. To identify the activating Notch3 ligand in VSMCs, we assessed the relative mRNA expression of Delta1, Delta4, Jagged1, and Jagged2 in sorted hrGFP\(^{+}\) cells from E18.5 and adult lungs. We found that Jagged1 was the most abundantly expressed ligand at E18.5 and was down-regulated in the adult (Fig. 3D, inset). Taken together, these findings demonstrate that lung VSMCs...
smooth muscle gene expression was nearly identical to what muscle marker genes in the absence of VSMC-derived Jagged1 intriguingly, we also observed down-regulation of contractile smooth muscle-specific transgelin promoter (Sma22-Cre) under the control of the smooth muscle-specific transgelin promoter (Sma22-Cre) (30). Lungs derived from E18.5 Jagged1 flox/flox Sma22-Cre embryos exhibited widespread loss of Jagged1 selectively in VSMCs. In contrast, Jagged1 expression in the endothelium was intact in this mouse (Fig. 4, A and B), confirming the tissue-specific expression of the Cre recombinase. Jagged1 deletion in VSMCs resulted in suppression of Notch3 activation, as assessed by diminished N3ICD nuclear localization in VSMCs (Fig. 4, C and D) and diminished Hey1 and Hey2 expression (Fig. 4E).

In recent work, expression of contractile smooth muscle marker genes was found to be down-regulated in the ductus arteriosus of Jagged1 flox/flox Sma22-Cre embryos (21). Interestingly, we also observed down-regulation of contractile smooth muscle marker genes in the absence of VSMC-derived Jagged1 (supplemental Fig. S4) in the embryonic lung. This pattern of smooth muscle gene expression was nearly identical to what was observed in the Notch3−/− mouse lung (Fig. 1B), providing further evidence that endothelium-derived Jagged1 is not involved in VSMC Notch3 activation but rather requires the action of VSMC-derived Jagged1.

In view of these observations, we sought to demonstrate that Notch3 and Jagged1 are physically associated in the E18.5 lung. For this, immunoprecipitation of Notch3 was performed on lysates isolated from CD45/CD31-depleted E18.5 whole lung preparations, followed by Western blot analysis using anti-Jagged1 antibody. Jagged1 was detected in immunoprecipitates obtained with anti-Notch3 antibody, but not in a preimmune rabbit serum control (Fig. 4F). This finding shows that Jagged1 is engaged with Notch3 at E18.5.

Evidence for Cell-autonomous Notch3 Cell Activation in Pulmonary Artery VSMCs—Our observations are thus far consistent with two possible modes of Notch3 activation by Jagged1 in lung VSMCs. Notch3 may be activated through (a) a cell-autonomous pathway or (b) a homotypic interaction between two neighboring VSMCs. To help distinguish between these possibilities, we established an in vitro culture system with RPASMCs isolated from neonatal rats. Similar to murine VSMCs, cultured RPASMCs expressed Jagged1, Notch3, and the cleaved N3ICD (supplemental Fig. S5, A and B). Interestingly, Western blot analysis indicated that the majority of Notch3 in these cells was found in the cleaved form (supplemental Fig. S5B). Importantly, the diminished intensity of the band corresponding to the cleaved N3ICD in lysates from cells incubated with a γ-secretase inhibitor (DAPT) confirmed that expression of this protein fragment reflects Notch3 activation (supplemental Fig. S5C).

To evaluate whether Notch3 activation in RPASMCs is cell-autonomous, N3ICD cellular localization was determined in sparsely plated cells by confocal microscopy. As shown in Fig. 5 (A–C), individual RPASMCs exhibited a nuclear N3ICD despite a lack of contact with neighboring cells, compatible with cell-autonomous activation. It is noteworthy that the N3ICD was predominantly localized in nuclei, consistent with our assessment of Notch3 activation by Western blot analysis (supplemental Fig. S5B).

To further address whether cell-cell contact contributes to Notch3 activation, we designed a functional in vitro assay. We hypothesized that 1) if Jagged1 activates Notch3 independently of cell-to-cell contact, then we will observe down-regulation of target genes after knocking down Jagged1 in RPASMCs and 2) this down-regulation will not be rescued by co-culturing Jagged1 knockdown cells with WT RPASMCs. To evaluate this, RPASMCs were transduced with a lentivirus expressing Jagged1 shRNA or a control scrambled shRNA coupled to the reporter gene eGFP. RPASMC transduction with the Jagged1 shRNA lentivirus resulted in Jagged1 knockdown (supplemental Fig. S6, A and B), as well as a significant reduction in Hey1 and Hey2 gene expression (supplemental Fig. S6, C and D).

To test whether WT RPASMCs could rescue Hey1 and Hey2 expression in knockdown cells, control scrambled shRNA or Jagged1 knockdown RPASMCs were co-cultured with WT RPASMCs at a 1:1 ratio for 72 h. Cells were then separated by cell sorting using eGFP as a selection marker, and Hey1 and Hey2 expression was assessed by qPCR. Consistent with a cell-
autonomous signaling model, determination of Hey1 and Hey2 in WT, scrambled shRNA, and Jagged1 knockdown cells showed that WT cells were unable to rescue the down-regulation of Hey1 or Hey2 mRNA in Jagged1 knockdown cells (Fig. 5, D and E).

To confirm that RPASMCs are capable of activating Notch signaling in neighboring cells in vitro, we co-cultured RPASMCs with a rat alveolar macrophage cell line that expresses detectable cell surface Notch3 (supplemental Fig. S7), but neither Jagged1 (data not shown) nor Hey1 or Hey2 mRNA (Fig. 5, F and G). We found that WT cells and RPASMCs expressing scrambled shRNA stimulated Hey1 and Hey2 mRNA expression in co-cultured macrophages to a greater level than Jagged1 knockdown RPASMCs (Fig. 5, F and G). These findings confirm that RPASMC-derived Jagged1 is competent to activate neighboring cells. These observations also raise the possibility that Notch3 may not be expressed on or activated from the surface of VSMCs.

Evidence for Intracellular Localization of Notch3 in VSMCs—To investigate the cellular localization of Notch3 in E18.5 mouse lung cells, flow cytometry was performed. We were unable to detect the N3ECD on the surface of E18.5 lung cells (Fig. 6A). In contrast, the N3ECD was detected in permeabilized lung cells (Fig. 6A). To more specifically evaluate N3ECD expression in VSMCs, non-permeabilized and permeabilized E18.5 hrGFP+ cells were analyzed by flow cytometry. As observed with whole lung cells, the N3ECD was detected only in permeabilized cells (Fig. 6B), consistent with an intracellular localization of Notch3 in VSMCs.

To examine this further, confocal microscopy of isolated E18.5 hrGFP+ cells was performed. Analysis of individual cells stained with anti-N3ECD antibody revealed a cytoplasmic distribution of Notch3, with no evidence of cell surface expression (Fig. 6C). In accordance, individual cells simultaneously stained with anti-N3ECD and anti-N3ICD antibodies showed co-localization of these two domains only in the cytoplasm of E18.5 VSMCs (Fig. 6D).

To rule out the possibility of Notch3 epitope masking or disruption by the enzymatic cell isolation procedure, Notch3 expression in adult lung CD45+ cells isolated by the same method was determined. We observed that Notch3 was detectable on the surface in this cell population (supplemental Fig. S8), consistent with published work (31) and confirming that this procedure does not affect Notch3 integrity or antibody binding. To demonstrate that the intracellular localization of Notch3 is not due to receptor internalization during cell processing, E18.5 hrGFP+ cells were fixed and stained at 4 °C.
these conditions, the N3ECD was observed only inside VSMCs, thus ruling out receptor recycling from the cell surface.

To evaluate whether Notch3 cellular localization in RPASMCs is similar to that in mouse VSMCs, confocal microscopy was employed. As in the mouse, Notch3 was not detected on the cell surface of RPASMCs but rather localized to the perinuclear region (Fig. 7, A and B). To further corroborate these findings, biotinylated proteins from RPASMCs were analyzed. In agreement, we were unable to detect Notch3 in a biotinylated surface protein fraction by Western analysis (supplemental Fig. 7C). In contrast, Notch3 was detected in the non-biotinylated fractions and total cell lysates. As expected, Jagged1 was found in biotinylated and non-biotinylated fractions and whole cell lysates. On the other hand, the intracellular protein p53 was markedly enriched in the non-biotinylated internal fraction (Fig. 7C). Additionally, Ponceau S staining revealed approximate equal protein loading (data not shown). Taken together, these results demonstrate Notch3 expression restricted to the interior of pulmonary artery VSMCs.

To determine whether the restricted intracellular Notch3 receptor localization is dependent on cellular context, recombinant GFP, N3ICD-GFP, and Notch3-GFP fusion proteins were transfected into RPASMCs and human kidney epithelial 293T cells. Confocal microscopy showed cytoplasmic GFP expression in both cell types with the control plasmid (Fig. 8, A and B).
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FIGURE 8. Localization for recombinant Notch3-GFP fusion proteins in transfected RPASMCs and 293T cells as determined by confocal microscopy. A and B, cytoplasmic GFP distribution in cells transfected with a control plasmid. C and D, nuclear GFP localization in cells transfected with the N3ICD-GFP construct. E, cytoplasmic and cellular membrane GFP distribution in 293T cells transfected with the full-length Notch3-GFP construct. F, perinuclear GFP distribution in RPASMCs cells transfected with the full-length Notch3-GFP construct. Green, GFP; red, β-tubulin; blue, nuclei. Scale bars = 20 μm.

and B). GFP was localized in the nuclei of both RPASMCs and 293T cells transfected with N3ICD-GFP (Fig. 8, C and D). Consistent with previous reports (32), GFP was detected on the cell surface and cytoplasm of 293T cells transfected with Notch3-GFP (Fig. 8E). In RPASMCs transfected with Notch3-GFP, however, GFP localized exclusively to the perinuclear region without evidence of surface expression (Fig. 8F). These findings show that, even under conditions of overexpression, Notch3 localizes to the interior of VSMCs.

DISCUSSION

In this study, we found that Notch3 expression is restricted to pulmonary artery VSMCs in the developing lung. We observed that activation of this receptor occurs during a discrete time period spanning late fetal to early postnatal life and is dependent upon VSMC-derived Jagged1. This time-restricted signal regulates pulmonary artery cell phenotype, as evidenced by alterations in expression of smooth muscle-related genes and VSMC morphology in the Notch3−/− mouse.

Importantly, we were able to detect Notch3 cell surface expression in lung VSMCs by a variety of assays. Rather, we demonstrated that this receptor displays an intracellular localization that appears unique to VSMCs. The differential targeting of overexpressed Notch3 to the interior of RPASMCs and to the cell surface of human kidney epithelial 293T cells and macrophages underscores the importance of cellular context in determining the cellular localization of Notch3. The relative basis for how particular structural elements of the Notch3 protein facilitate targeting to an intracellular compartment in VSMCs, as opposed to the surface of macrophages, is not yet evident but is a focus of ongoing investigation. On the basis of our overexpression data, we speculate that differences in associated Notch3 targeting molecules underlie these observations. Overall, these data argue for a cell-autonomous signaling mode of Notch3 signaling that involves an engagement with the Jagged1 ligand in the interior of VSMCs.

It is important to emphasize the functional differences between the ligand-dependent intracellular Notch pathway described in Drosophila (17) and the pathway we identified in VSMCs. In Drosophila, this pathway is active during mitosis and plays a defining role in determining the differential cell fates of the two daughter cells that arise from the asymmetric cell division of sensory organ progenitor cells. This process involves the asymmetric sorting of a subtype of endosomes containing Notch and Delta, which leads to release of the Notch intracellular domain to only one daughter cell (17). In contrast, during active intracellular Notch3 signaling, we found no evidence that VSMCs are proliferating (data not shown). Furthermore, cell-autonomous Notch3 signaling occurs in cells with an already established fate during late gestation.

There are differences in the structure of Notch3 compared with other family members. One speculation is that these structural features mediate selectivity for Jagged1 (33). There are, however, unique functional properties for Notch3. In systemic vessels, Notch3 may serve as a direct regulator of hemodynamic tone (15). One possibility is that Notch3 is a direct regulator of tone in the pulmonary artery during the transition to postnatal life.

Previous work has indicated Notch-ligand interactions occurring in cis, resulting in inhibition of Notch signaling (34). Rather, we found in VSMCs that Notch3-Jagged1 interaction in cis leads to activation. In ongoing work, we found that Notch3 and Jagged1 reside in the same endosomal compartments, suggesting the possibility of receptor-ligand interaction and proteolytic release of the N3ICD at this site.

In the context of our findings, this leads us to propose two models of cell-autonomous intracellular Notch3 activation in VSMCs. In one model, membrane-bound Jagged1 interacts with membrane-bound Notch3 in the interior of the same endosome, thereby generating the force required to expose the γ-secretase target site in the cytoplasm for cleavage. In the second model, membrane-bound Jagged1 and membrane-bound Notch3 interact on the cytoplasmic side of two contiguous endosomes, thereby exposing the Notch intracellular domain cleavage site in the endosomal interior. Of note, although γ-secretase substrates are processed at or near the cell surface, this enzyme complex also resides in the endosomal reticulum, Golgi network, and intermediate compartments (17). Its optimal activity is at low pH, indicating that γ-secretase could function within the acidic milieu of late endosomes (35).

A broader biological question relates to why this alternative mode of Notch activation is operative in pulmonary artery VSMCs. Although unclear at this time, cell-autonomous Notch3 signaling may have involved, in part, to ensure the efficient and homogeneous adaptation of pulmonary artery VSMCs to the sudden and dramatic increase in blood flow and pressure associated with birth and air breathing. The restricted timing of Notch3 activation is consistent with this possibility, thereby pointing to a key role for this pathway in regulating the final stages of VSMC maturation. The emer-
gence of dysmorphic VSMCs early after birth in Notch3−/− mice, which is highly suggestive of an injury response (36), further underscores a role for Notch3-dependent signals in the adaptation from a fetal to a postnatal pulmonary vasculature. The improved morphological appearance of the adult as opposed to the postnatal day 3 vessels in Notch3−/− mice suggests the presence of an active repair process.

Finally, we posit that our findings may have relevance to understanding two pathological conditions. Recently, dysregulation of Notch3 activation has been implicated as a key factor in the pathogenesis of adult pulmonary hypertension (27). Whether Jagged1-dependent cell-autonomous Notch activation is involved in this pathophysiological process will need to be clarified, particularly if targeted Notch3-dependent therapies are considered. Furthermore, in view of the late timing of Notch3 activation during development and its effects on VSMC phenotype, we wonder whether relative Notch3 signaling deficiency plays a role in the pathogenesis of the pulmonary vasculopathy associated with prematurity.

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