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Notch Activation Results in Phenotypic and Functional Changes Consistent With Endothelial-to-Mesenchymal Transformation

Michela Nosedà,* Graeme McLean,* Kyle Niessen, Linda Chang, Ingrid Pollet, Rachel Montpetit, Réza Shahidi, Katerina Dorovini-Zis, Linheng Li, Benjamin Beckstead, Ralph E. Durand, Pamela A. Hoodless, Aly Karsan

Abstract—Various studies have identified a critical role for Notch signaling in cardiovascular development. In this and other systems, Notch receptors and ligands are expressed in regions that undergo epithelial-to-mesenchymal transformation. However, there is no direct evidence that Notch activation can induce mesenchymal transdifferentiation. In this study we show that Notch activation in endothelial cells results in morphological, phenotypic, and functional changes consistent with mesenchymal transformation. These changes include downregulation of endothelial markers (vascular endothelial [VE]-cadherin, Tie1, Tie2, platelet-endothelial cell adhesion molecule-1, and endothelial NO synthase), upregulation of mesenchymal markers (α -smooth muscle actin, fibronectin, and platelet-derived growth factor receptors), and migration toward platelet-derived growth factor-BB. Notch-induced endothelial-to-mesenchymal transformation does not seem to require external regulation and is restricted to cells expressing activated Notch. Jagged1 stimulation of endothelial cells induces a similar mesenchymal transformation, and Jagged1, Notch1, and Notch4 are expressed in the ventricular outflow tract during stages of endocardial cushion formation. This is the first evidence that Jagged1-Notch interactions induce endothelial-to-mesenchymal transformation, and our findings suggest that Notch signaling may be required for proper endocardial cushion differentiation and/or vascular smooth muscle cell development. (*Circ Res.* 2004;94:910-917.)

Key Words: endothelial-to-mesenchymal transformation ■ Notch ■ Jagged1 ■ endocardial cushion

The Notch signaling pathway plays a critical role during development. Four mammalian Notch receptors (Notch1 through 4) and 5 Notch ligands (Delta-like [Dll]-1, Dll3, Dll4, Jagged1, and Jagged2) have been identified. Notch receptor-ligand interaction results in a series of proteolytic cleavages of the Notch receptor, producing a C-terminal intracellular fragment (NotchIC) that translocates to the nucleus. In the nucleus, NotchIC binds to the transcriptional repressor CBF1/RBP-J κ , thereby derepressing or coactivating the expression of various lineage-specific genes.¹

Perturbation of the Notch pathway has been implicated in the pathogenesis of various cardiovascular diseases in humans.² Of interest, patients with Jagged1 mutations (Alagille syndrome) display congenital cardiovascular anomalies that seem to be secondary to faulty endocardial cushion formation.³⁻⁶ In the mouse, Notch1-deficient embryos demonstrate

severe vascular developmental defects, which are exacerbated in Notch1/Notch4 double-mutant embryos.⁷ Constitutive activation of Notch4 also causes defects in vascular remodeling.^{8,9} Mice that are rendered null for Jagged1 die from hemorrhage early during embryogenesis, whereas mice that are doubly heterozygous for a Jagged1-null allele and a Notch2 hypomorphic allele exhibit cardiac anomalies similar to those seen in Alagille syndrome.^{10,11} Genes that lie downstream of Notch activation, such as the basic helix-loop-helix factor, HRT2/HEY2, have also been implicated in cardiovascular development.^{12,13}

Notch receptors and their ligands have been localized to the vasculature.¹⁴ Notch receptors have also been observed in the endocardium, and the Notch ligand Jagged1 is present on endocardial and periendocardial cells of the cardiac cushions.^{15,16} The endocardial cushion is a specialized embryonic

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tissue that gives rise to the cardiac valves and membranous septa. A critical event in cardiac cushion formation is a differentiation process referred to as endothelial-to-mesenchymal transformation (EMT), which is a specific form of epithelial-to-mesenchymal transformation.^{17,18} In the cardiovascular system of the adult, mesenchymal cells derived from the transformation of a subset of cardiac valve endothelial cells may also be necessary for maintenance of the leaflet architecture.¹⁹ Furthermore, EMT may play a role in the development of neointimal lesions in transplant atherosclerosis and restenosis.²⁰

Intercellular signaling between Notch receptors and ligands is critical for cell fate determination by influencing cell proliferation, differentiation, and apoptosis.²¹ Notch members and their ligands are expressed in various regions that undergo EMT in order for development to proceed appropriately.^{22,23} Our studies demonstrate that Notch activation in endothelial cells promotes mesenchymal transformation and suggest that Jagged1-Notch interactions may participate in endocardial cushion formation by inducing EMT.

Materials and Methods

Cell Culture and Reagents

The HMEC-1 microvascular endothelial cell line, hereafter referred to as HMEC, was provided by the Centers for Disease Control and Prevention (Atlanta, Ga) and cultured as previously described.⁹ Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described.²⁴ Human aortic endothelial cells (HAECs) were purchased and cultured in supplemented endothelial growth media (Clonetics). Ovine endocardial cells (OECs) were isolated from sheep cardiac ventricles by treatment with collagenase (45 minutes at 37°C). DiI-acetylated LDL uptake and expression of endothelial markers (vascular endothelial [VE]-cadherin and platelet-endothelial cell adhesion molecule [PECAM-1]) were confirmed. OECs were maintained in Waymouth's media (Gibco) with 10% FBS and antibiotics.

Gene Transfer

Endothelial cells were transduced using the retroviral vector MSCV-IRES-YFP (MIY) (gift from R.K. Humphries, British Columbia Cancer Agency, Vancouver, BC). cDNA constructs encoding the C-terminal HA-tagged Notch4 intracellular domain, Notch1 intracellular domain (gift of S. Artavanis-Tsakonas, Harvard Medical School, Charlestown, Mass), and full-length Jagged1 were cloned into MIY. Endothelial cells were transduced as previously described.²⁵

Transmission Electron Microscopy

Endothelial cultures were processed as previously described.²⁶ Briefly, cultures were washed with M199, fixed in 2.5% glutaraldehyde/2% paraformaldehyde in 0.2 mol/L sodium cacodylate buffer for 1 hour, post-fixed in 1% OsO₄ for 1 hour, stained en bloc with uranyl magnesium acetate, dehydrated, and embedded in Epon-Araldite. Blocks cut from the embedded cultures were reembedded for cross-sectioning. Thin sections were stained with uranyl acetate and lead citrate and viewed on a Zeiss EM 10 microscope.

Immunoblotting

Cells were lysed, and 50 µg of total protein was analyzed by SDS-PAGE. The monoclonal antibody against the HA epitope was purchased from BabCo, and anti-VE-cadherin, anti-PECAM-1, anti-Tie1, and anti-Tie2 antibodies were all from Santa Cruz Biotechnology. Anti-endothelial NO synthase (eNOS/NOS type III) and anti-fibronectin antibodies were purchased from Transduction Laboratories, and anti- α -smooth muscle actin (SMA) antibody was

obtained from Cymbus Biotechnology (Hampshire, UK). Anti-phospho-Smad2 and anti-total-Smad2 antibodies (gift from N. Khalil, University of British Columbia, Vancouver, BC) were manufactured by UBI.

Migration Assay

The ability of endothelial cells to migrate toward platelet-derived growth factor (PDGF)-BB (20 ng/mL) was measured by a modified Boyden chamber assay as previously described.⁹

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and blocked/permeabilized in 4% FCS/0.2% Triton X-100/PBS. Secondary antibodies were Alexa 594-conjugated. To quantitate the proportion of SMA-positive cells, at least five high-power fields (comprising at least 200 cells) were evaluated, and the proportion of SMA-positive cells expressed were as a percentage of the total number of DAPI-stained nuclei in each field.

Flow Cytometry

Cells were trypsinized, washed in PBS, and fixed in 4% paraformaldehyde. Cells were blocked/permeabilized, stained with anti-SMA antibody, and analyzed on an EPICS ELITE-ESP flow cytometer (Beckman Coulter).

Luciferase Assays

Endothelial cells were transfected by electroporation (1.5×10^6 cells) with 1 to 5 µg of plasmid DNA as previously described.²⁷ Forty-eight hours after transfection, dual-luciferase reporter assays were performed according to manufacturer's recommendations (Promega Corporation). The CBF1-dependent reporter, 4xCBF1wt-LUC, was a gift from S.D. Hayward (Johns Hopkins School of Medicine, Baltimore, Md).²⁸ The SMA promoter-reporter construct encompassing a 5.4-kb region comprising -2555 to +2813 of the rat SMA gene was a gift from F. Dandre and G.K. Owens (University of Virginia, Charlottesville, Va). Transfections were normalized by transfecting cells with 50 ng of the *Renilla* luciferase plasmid pRL-CMV (Promega).

In Situ Hybridization

The murine Jagged1 probe was a gift from S.E. Egan (Hospital for Sick Children, Toronto, Ontario), and the Notch1 and Notch4 probes were a gift from J. Rossant (Samuel Lunenfeld Research Institute, Toronto, Ontario). For whole-mount in situ hybridization, embryonic hearts were fixed overnight at 4°C in 4% paraformaldehyde in PBS, dehydrated in methanol, and stored at -20°C. For hybridizations, embryonic hearts were processed as described.²⁹

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated using TRIzol Reagent (Invitrogen), DNase-treated, and reverse-transcribed to cDNA, followed by polymerase chain reaction (PCR) (see the online data supplement for primers and annealing conditions, available at <http://circres.ahajournals.org>). A control reaction, omitting reverse transcriptase (RT), was performed for each RNA sample to verify the absence of genomic DNA.

Results

Activated Notch Induces Endothelial-to-Mesenchymal Transformation

During the course of a previous study, we noted that HMECs expressing Notch4IC (HMEC-Notch4IC) lost the characteristic cobblestone morphology of confluent endothelial cells.⁹ As seen in Figure 1A, HMEC-Notch4IC formed multilayered cultures suggesting loss of endothelial phenotype and potential transformation to mesenchymal cells. Transmission electron microscopy confirmed that HMEC-Notch4IC failed to form monolayers and proper cell-to-cell junctions and

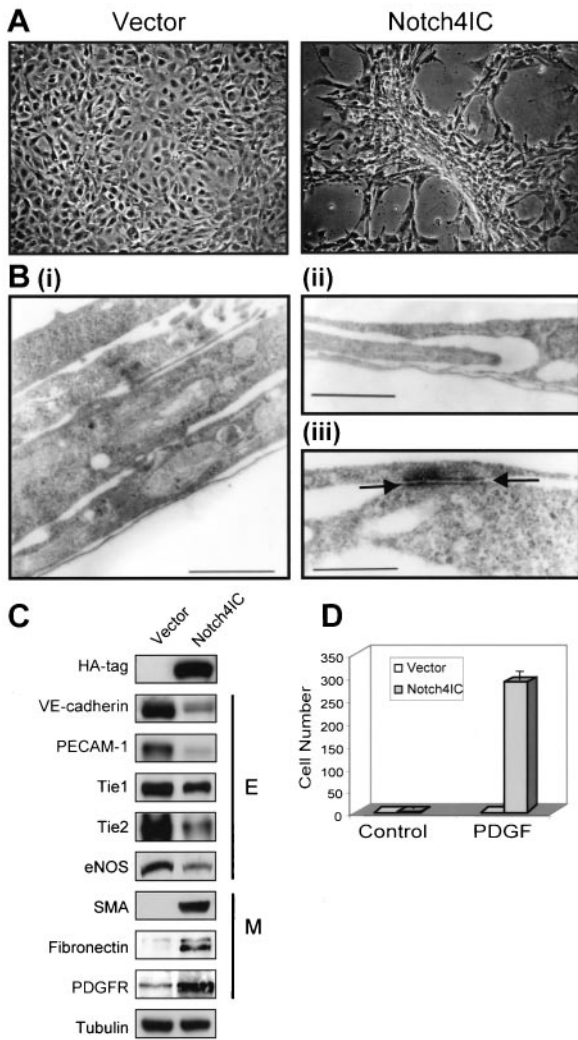


Figure 1. Activated Notch4 induces EMT. A, Phase-contrast micrographs of HMECs transduced with either the empty vector or Notch4IC. B, Transmission electron micrographs of cultures in A. HMEC-Notch4IC are arrayed in overlapping cell layers (i, magnification $\times 51\,000$, bar = $1\ \mu\text{m}$) with no evidence of intercellular junctions between adjacent cells (ii, magnification $\times 18\,000$, bar = $2\ \mu\text{m}$). HMEC-vector (iii, magnification $\times 61\,000$, bar = $0.75\ \mu\text{m}$) cells are focally apposed and form junctional complexes (arrows). C, Immunoblots probed for endothelial (E) and mesenchymal (M) markers on cell lysates harvested from HMEC-vector or HMEC-Notch4IC. D, Migration of HMECs transduced with either the empty vector or Notch4IC toward medium (control) or 20 ng/mL PDGF-BB was measured using a modified Boyden chamber assay. Results are mean \pm SD of 3 independent experiments.

showed marked overlapping with infrequent, rudimentary cell contacts (Figures 1B, panel i, and 1B, panel ii). In contrast, HMEC-vector cells retained their capacity to form junctions (Figure 1B, panel iii).

VE-cadherin is a cell adhesion molecule that is localized to the interendothelial region and is required for the formation of adherens junctions.³⁰ Immunoblotting for VE-cadherin demonstrated significant downregulation of this critical junctional molecule, suggesting mesenchymal transformation (Figure 1C). Furthermore, we noted a reduction in expression, to varying degrees, of several other endothelial-specific

proteins (PECAM-1, Tie1, Tie2, and endothelial NOS) (Figure 1C). In addition to the loss of endothelial phenotype, EMT implies the acquisition of mesenchymal markers.^{19,31–35} To determine whether Notch4-activated cells upregulate mesenchymal proteins, we examined expression of SMA, fibronectin, and PDGF receptors in HMEC-Notch4IC. Immunoblotting shows induction of all three proteins in HMEC-Notch4IC (Figure 1C).

PDGF is a known chemotactic factor for mesenchymal cells, and in particular, PDGF-BB plays a role in recruitment of mesenchymal cells during vascular development.^{35,36} Hence, we examined the chemotactic response of HMEC-Notch4IC or HMEC-vector to PDGF-BB. HMEC-Notch4IC was able to migrate toward PDGF-BB in a modified Boyden chamber assay, whereas vector-transduced cells did not (Figure 1D). Thus, Notch4 activation in endothelial cells induces morphological, phenotypic, and functional changes observed during EMT.

Several studies have shown that aortic endothelium can differentiate into mesenchymal-like cells *in vitro*.^{31,37} More recent work has demonstrated that HUVECs also retain the potential to differentiate into mesenchymal cells.^{19,38} In addition to Notch4, Notch1 is also expressed in endothelial cells.¹⁴ Hence, we transduced HMECs, HAECs, and HUVECs with Notch4IC, Notch1IC, or empty vector. Our data demonstrated that activated Notch1, as well as Notch4, had the potential to induce EMT in endothelial cells from different vascular beds, as determined by morphological, immunophenotypic, and functional criteria (online Figure 1 and data not shown). Because of the critical requirement for EMT during cardiac cushion formation, we determined whether activated Notch was also able to induce EMT in cardiac endothelial cells. OECs were transduced with the empty vector, Notch4IC, or Notch1IC. OECs also underwent a morphological transformation, as witnessed by loss of uniform cell shape, loss of intercellular contacts, cellular polarization, formation of filopodia (Figure 2A), and the induction of SMA (Figure 2B). Downregulation of endocardial proteins such as PECAM-1 was also confirmed (data not shown).

To determine the efficiency of Notch-induced EMT in different endothelial types, we quantitated SMA-positive cells by immunofluorescent staining 10 to 14 days after transduction. HAECs and OECs demonstrated the greatest capacity to undergo Notch-induced EMT (Figure 2C). Interestingly, in all endothelial types, Notch1 showed greater efficacy in inducing EMT. Taken together, these results indicate that activated Notch is able to induce mesenchymal transformation in endothelial cells from various vascular beds and suggest that different Notch members may play similar functional roles in EMT.

Notch-Induced EMT Is Restricted to Cells Expressing Activated Notch

Activated Notch may induce EMT in conjunction with activation of other signaling molecules. In this regard, transforming growth factor- β (TGF- β) has been suggested to play an important role in EMT in the endocardial cushion, as well as in ovine and human valvular and bovine aortic endothelial

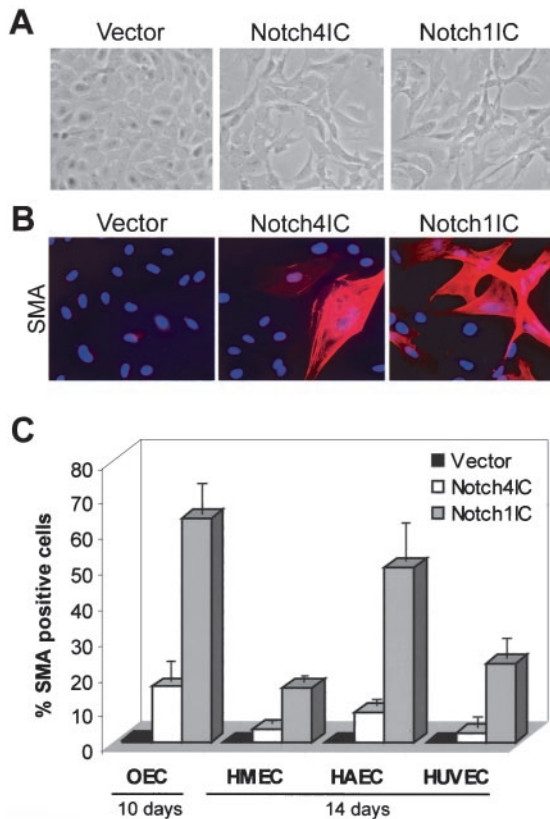


Figure 2. Activated Notch4 and Notch1 induce transdifferentiation of endothelial cells from various vascular beds. OECs were transduced with the empty vector, Notch4IC, or Notch1IC and analyzed by phase-contrast microscopy (A) or immunofluorescence for SMA expression (B) 10 days after transduction. C, Endothelial cells from various sources were transduced with empty vector, Notch4IC, or Notch1IC. At 10 days (OECs) or 14 days (HMECs, HAECs, or HUVECs) after transduction, the proportion of SMA-positive cells was assayed and expressed as a percentage of the total cell number (mean \pm SD). Results are representative of 3 independent experiments.

cells.^{19,31,39} Primary human endothelial cells transduced with Notch4IC or Notch1IC showed variable levels of TGF- β expression with no consistent increase induced by Notch activation, although HMEC-Notch4IC did show increased TGF- β_2 secretion (data not shown). To determine whether TGF- β stimulation was sufficient to induce EMT, we treated HMECs and HAECs with recombinant TGF- β_1 or TGF- β_2 (5 ng/mL) for up to 28 days. Consistent with studies performed on human vascular endothelial cells, treatment of HMECs and HAECs with exogenous TGF- β_1 or TGF- β_2 did not induce morphological changes of EMT or expression of SMA (data not shown).¹⁹ Furthermore, inhibition of TGF- β activity with a pan-anti-TGF- β -neutralizing antibody did not inhibit or reduce the Notch4IC- or Notch1IC-induced morphological changes or SMA expression in either HMECs or HAECs despite the ability of this antibody to inhibit phosphorylation of Smad2 (Figure 3).

To determine whether NotchIC-transduced endothelial cells secrete other soluble factors that are capable of inducing phenotypic changes, we added 3-day conditioned medium from HMEC-vector and HMEC-Notch4IC to parental

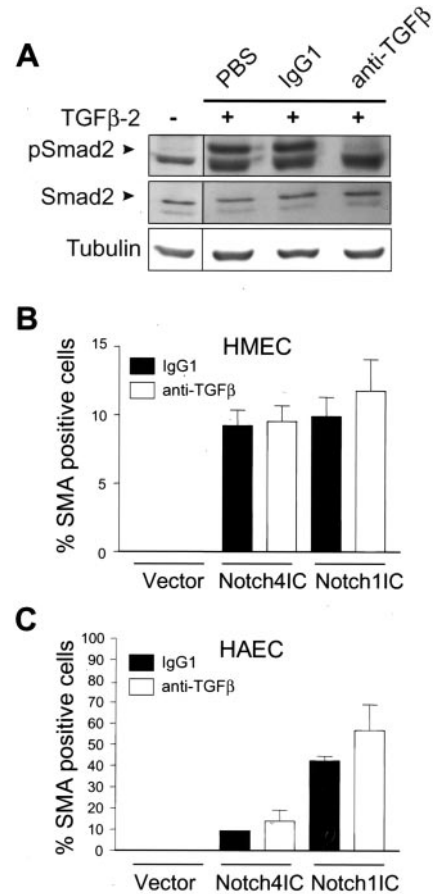


Figure 3. TGF- β is not required for Notch-induced EMT. HMECs and HAECs were treated with either 10 μ g/mL control IgG1 or a pan-anti-TGF- β neutralizing antibody during transduction with the empty vector, Notch4IC, or Notch1IC. Subsequently, medium was changed daily with the addition of fresh IgG1 (10 μ g/mL) or pan-anti-TGF- β antibody (10 μ g/mL). Total cell lysates were probed with an anti-phospho-Smad2 antibody and tubulin (A). HMECs (B) and HAECs (C) were analyzed by immunofluorescence for the expression of SMA-positive cells (mean \pm SD), enumerated 14 days after transduction, as described in Materials and Methods. Results are representative of 2 independent experiments.

HMECs or primary HAECs. We did not observe morphological changes or SMA expression in HMECs or HAECs treated daily with conditioned medium from Notch4IC-transduced HMECs over a 28-day period (data not shown). The above findings suggest that Notch-induced mesenchymal transformation does not depend on paracrine factors and is likely restricted to cells expressing activated Notch.

To confirm that Notch-induced mesenchymal transformation occurs only in cells expressing activated Notch, HMECs were infected with a retroviral vector (MIY) that contains yellow fluorescence protein (YFP) linked to the transgene through an internal ribosomal entry site. Only the YFP-positive (Notch4IC-expressing) cells expressed SMA, as determined by flow cytometry (Figure 4A). This finding was confirmed by high-purity cell sorting of YFP-positive and YFP-negative subpopulations of both Notch4IC- and vector-transduced cells. Immunoblotting of the sorted populations showed downregulation of endothelial-specific proteins and

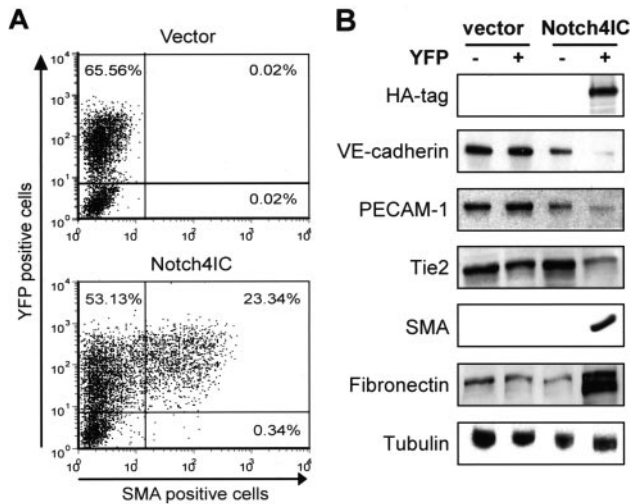


Figure 4. Notch-induced mesenchymal transformation is cell autonomous. A, HMECs were transduced with the empty vector or Notch4IC and analyzed by flow cytometry for YFP and SMA expression after 21 days. Results are representative of 3 independent experiments. B, HMECs were transduced with the empty vector or Notch4IC and sorted into YFP-positive and YFP-negative populations. The sorted cells were then analyzed by immunoblotting for the expression of endothelial and mesenchymal markers. Results are representative of 2 experiments.

upregulation of mesenchymal markers only in YFP-positive HMEC-Notch4IC (Figure 4B). Studies using Notch1IC-expressing HUVECs similarly demonstrated that only YFP-positive (Notch1IC-expressing) HUVECs expressed SMA (online Figure 2). Thus, only endothelial cells that express activated Notch undergo EMT, and external factors do not seem to be required for this process in vitro. However, additional studies assessing the role of TGF- β family members will be required to understand the interaction of this pathway with Notch signaling during EMT.

Jagged1 Induces EMT

The Notch ligand, Jagged1, is expressed on endothelial and smooth muscle cells during development and in the adult.¹⁴ Jagged1 mutations are associated with congenital cardiac anomalies that may be secondary to defective endocardial cushion development.³⁻⁶ To determine whether Jagged1-Notch interactions can induce EMT, we expressed wild-type Jagged1 in HMECs (HMEC-Jagged1). HMEC-Jagged1 or HMEC-vector were cocultured with parental HMECs, and expression of endothelial markers was analyzed by immunoblotting. In cocultures with HMEC-Jagged1, VE-cadherin, PECAM-1 and Tie2 were downregulated and fibronectin was upregulated (Figure 5A). We verified that Jagged1-Notch interactions induced SMA expression by immunofluorescent staining (Figure 5B). To confirm activation of the Notch pathway by Jagged1, HMEC-Jagged1 cocultures and HMEC-vector cocultures were transiently transfected with a CBF1-dependent promoter-luciferase construct. HMEC-Jagged1 cocultures demonstrated 4.5-fold higher CBF1-luciferase activity compared with HMEC-vector cocultures, demonstrating that enforced expression of Jagged1 activates CBF1-dependent Notch signaling (Figure 5C). The presence of a

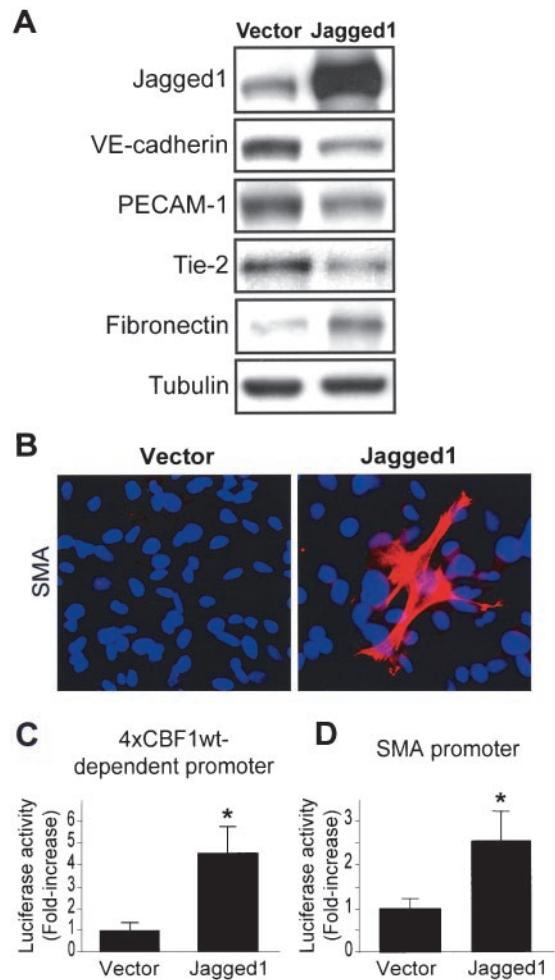


Figure 5. Jagged1 induces EMT. A, HMEC-vector or HMEC-Jagged1 cocultures were examined for expression of endothelial and mesenchymal markers by immunoblotting. B, Expression of SMA was tested by immunofluorescence. HMEC-vector or HMEC-Jagged1 cocultures were transiently cotransfected with a constitutively active *Renilla* luciferase plasmid and a CBF1-dependent promoter-luciferase construct (C) or an SMA promoter-luciferase construct (D), and relative luciferase activity was determined, as described in Materials and Methods. The graphs show a single experiment done in triplicate and are representative of 3 independent experiments (mean \pm SD). * P <0.05.

conserved consensus CBF-1 binding site that is located 64 to 59 nucleotides upstream of the cap site in the SMA promoter suggests that activation of Notch may directly induce SMA transcription. Promoter-luciferase assays using Notch4IC- or Notch1IC-transduced HMECs demonstrated a Notch-dependent increase in SMA promoter activity that was confirmed by induction of SMA mRNA by RT-PCR (data not shown). Examination of HMEC-Jagged1 cocultures also demonstrated a 2.5-fold increase in SMA promoter-luciferase activity over HMEC vector, indicating that Jagged1-Notch interactions are capable of upregulating SMA expression (Figure 5D). These results do not prove a direct transcriptional regulation of SMA by putative Notch-CBF1 complexes but suggest that increased expression of SMA may be attributable to induction of transcription. Taken together, our results support the hypothesis that Jagged1-Notch interac-

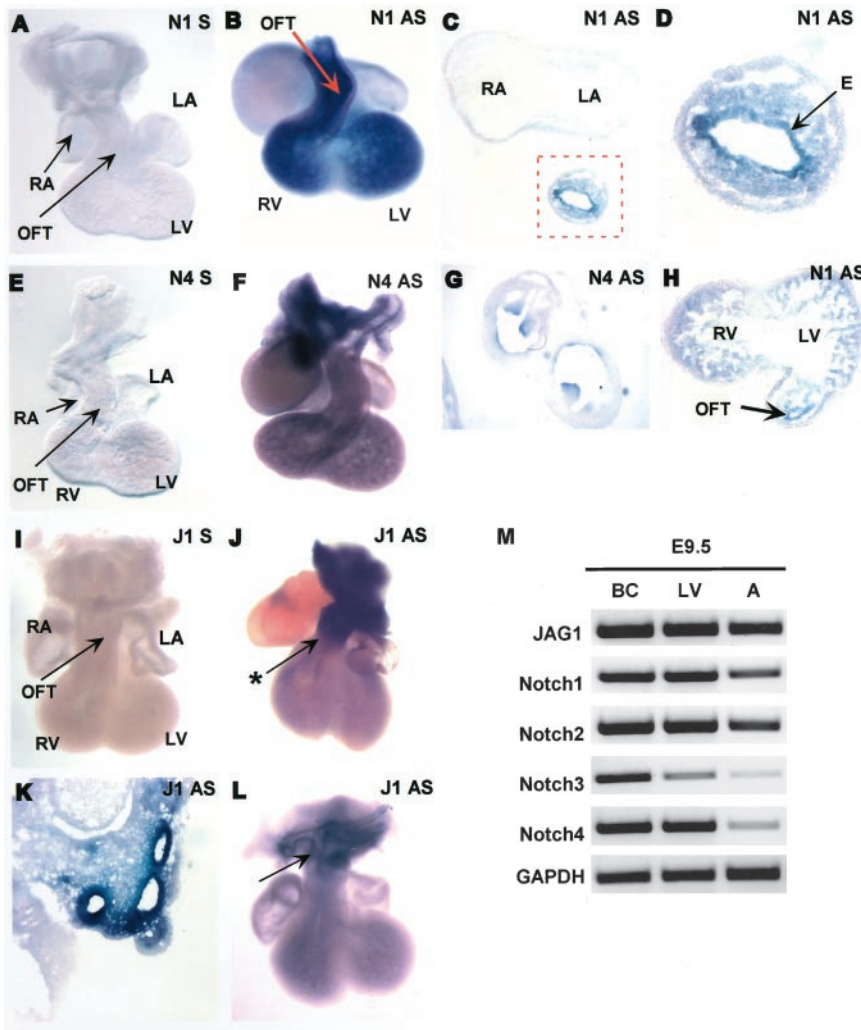


Figure 6. Notch1, Notch4, and Jagged1 are expressed during heart development. Expression of Notch1 mRNA was examined by whole-mount in situ hybridization of E10.5 mouse hearts using sense (A) and anti-sense (B through D and H) RNA probes. Cross sections of an E10.5 heart hybridized for Notch1 expression at the level of the atria (C and D) and ventricles (H) are shown. The region outlined in red in C is shown enlarged in D. The arrow in H points to expression of Notch1 in the endothelial lining of the outflow tract. Notch4 expression was examined in E10.5 hearts using sense (E) and anti-sense (F and G) RNA probes. Notch4 expression is also shown in cross sections from the outflow tract of E11.5 mouse hearts (G). Jagged1 expression (I through L) is shown in both E10.5 (I through K) and E11.5 (L) mouse hearts. The Jagged1 sense control is shown at E11.5 (I). Cross section of an E10.5 heart showing Jagged1 expression is shown in K. The asterisk in panel J indicates the point in the outflow tract where the aorta and pulmonary arteries have begun to separate. M, Semiquantitative RT-PCR was used to examine the expression of Jagged1 and Notch 1 to 4 in E9.5 hearts. E9.5 hearts were dissected and divided into 3 regions, the atria, the left ventricle, and the bulbus cordis, which included the outflow tract and the right ventricle. RA indicates right atria; LA, left atria; RV, right ventricle; LV, left ventricle; OFT, outflow tract; BC, bulbus cordis; N1, Notch1 probe; N4, Notch4 probe; J1, Jagged1 probe; S, sense probe; AS, anti-sense probe; and E, endothelium.

tions contribute to the transdifferentiation of endothelial cells to a mesenchymal phenotype.

Notch1, Notch4, and Jagged1 Are Expressed in the Early Developing Heart

Because our data demonstrate that in vitro Jagged1-Notch interactions induce EMT, we sought to determine whether Notch1, Notch4, and Jagged1 were expressed in areas where EMT is occurring in the developing heart. EMT begins at approximately embryonic day (E) 9.5 in the atrioventricular endocardial cushions and at E10.5 in the ventricular outflow tract.⁴⁰ At E10.5, Notch1 is expressed throughout the ventricles in the forming trabeculae (Figure 6H). Low-level expression is also observed in the inner endocardial layer of the atria (Figure 6C and data not shown). The highest level of Notch1 expression is present in the endothelium of the outflow tract at the time when EMT is commencing in this region (Figures 6C, 6D, and 6H). Notch4 exhibits low levels of expression throughout the heart at E10.5 (Figure 6F) and is selectively observed in regions of presumptive valve leaflet development in the outflow tract at E11.5 (Figure 6G).

Jagged1 expression can be detected as early as E9.5 in the bulbus cordis of the developing heart (data not shown), the region of the heart that will form the right ventricle and the

outflow tract. By E10.5, Jagged1 expression can be detected throughout the heart, including the atria and ventricles, with significantly higher levels observed in the outflow tract (Figure 6J). In the outflow tract, expression is observed in both the endothelial cells and in the surrounding mesenchyme, with notably higher expression in the anterior portion of the outflow tract (Figure 6K). A clear boundary between high expression and low expression is seen at the region of the outflow tract where separation of the pulmonary artery and the aorta begins. Expression of Jagged1 in the outflow tract is maintained at E11.5 (Figure 6L). To examine expression of Notch receptors and ligands earlier in cardiac development, RT-PCR was performed on mouse hearts harvested at E9.5 and separated into atria, ventricles, and bulbus cordis (outflow tract). As seen in Figure 6M, Jagged1 and all four Notch receptors are strongly expressed in the bulbus cordis.

Discussion

Our findings demonstrate that expression of activated Notch1 or Notch4 in endothelial cells causes transdifferentiation to a mesenchymal phenotype and implicate Jagged1-Notch interactions in promoting EMT. One role for Notch-mediated EMT may occur during endocardial cushion formation. Endocardial cushion development is a key process in the

formation of the valves and membranous septum, which partitions the heart tube into an atrium and ventricle and the common ventricular outflow tract into dorsal aorta and pulmonary artery.^{17,18} Given that Notch receptors and the ligand Jagged1 are expressed in regions of the heart where EMT is required for proper development, the results described herein support a role for Jagged1-Notch interactions in promoting mesenchymal transformation during cardiac cushion formation and subsequent valvuloseptal development.^{15,16} Interestingly, $\approx 70\%$ of patients affected by Alagille syndrome, a disorder with pleiotropic developmental abnormalities, have mutations in the Jagged1 gene.³⁻⁶ The most frequent cardiac anomaly in these patients is peripheral pulmonic stenosis. However, abnormalities that implicate defective endocardial cushion development are also described.³⁻⁶ In particular, a recent study reported that 13% of patients with Alagille syndrome are affected by tetralogy of Fallot.⁵ Furthermore, mutations of Jagged1 are associated with defects of cardiac cushion development independent of the other developmental anomalies reported by Alagille.^{6,41} We speculate that defective Notch activation and consequent attenuation of EMT may play a role in the pathogenesis of atrioventricular defects associated with Jagged1 mutations.

There is accumulating evidence that endothelial cells can transdifferentiate into smooth muscle cells during developmental and pathological processes.⁴² Labeling studies in quail embryos showed that injected endothelial cells integrate into the dorsal aorta and become subendothelial mesenchymal cells expressing SMA.⁴³ Several groups have shown that Flk1+ (vascular endothelial growth factor receptor-2) cells derived from embryonic stem cells can differentiate into endothelial cells and subsequently into smooth muscle cells with loss of Flk1 expression.⁴⁴⁻⁴⁶ Smooth muscle cells derived from endothelial cells have been shown to incorporate into sites of neovascularization in the adult.^{46,47} Endothelial to smooth muscle cell transdifferentiation is not restricted to embryonic cells, because several groups have shown that adult endothelial cells can differentiate into smooth muscle cells.^{31,37,38}

Other studies have observed that neointimal smooth muscle cells in atherosclerosis and restenosis seem to arise from endothelial cells.^{20,31,48} Notch receptors and Jagged1 are expressed in both endothelial and periendothelial cells, and a marked increase in expression of Jagged1 and Jagged2 and Notch2 through 4 has been described in the regenerating endothelium after balloon injury of the rat carotid artery.⁴⁹ Mature vascular endothelium can give rise to smooth muscle cells via EMT using a mechanism that requires cell-cell contact.³¹ This is in line with our findings showing that Jagged1-Notch interactions on adjacent cells induce EMT. It is tempting to speculate that Notch activation may play a role in smooth muscle formation in neointimal growth or during vascular development.

In mammals, members of the TGF- β superfamily as well as various extracellular matrix proteins seem to play an important role in EMT during endocardial cushion formation.^{18,50} However, both TGF- β -dependent and TGF- β -independent events are involved, and TGF- β does not induce SMA in microvascular endothelial cells and umbilical vein

endothelium.^{19,51} In our in vitro system, Notch-mediated EMT is restricted to cells expressing activated Notch, and TGF- β_1 and - β_2 do not seem to be essential for Notch-mediated transformation. However, additional investigation is required to verify whether other members of the TGF- β superfamily are involved and whether EMT requires crosstalk between the Notch and TGF- β pathways in vivo. Nevertheless, our data indicate a potential role for the Notch pathway in endocardial cushion development and possibly in vascular smooth muscle development and neointimal formation.

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