Notch Signaling Regulates Smooth Muscle Differentiation of Epicardium-Derived Cells

Thomas Grieskamp, Carsten Rudat, Timo H.-W. Lüdtke, Julia Norden, Andreas Kispert

Rationale: **The embryonic epicardium plays a crucial role in the formation of the coronary vasculature and in myocardial development, yet the exact contribution of epicardium-derived cells (EPDCs) to the vascular and connective tissue of the heart, and the factors that regulate epicardial differentiation, are insufficiently understood.**

Objective: **To define the role of Notch signaling in murine epicardial development.**

Methods and Results: **Using in situ hybridization and RT-PCR analyses, we detected expression of a number of Notch receptor and ligand genes in early epicardial development, as well as during formation of coronary arteries. Mice with epicardial deletion of *Rbpj*, the unique intracellular mediator of Notch signaling, survived to adulthood and exhibited enlarged coronary venous and arterial beds. Using a *Tbx18*-based genetic lineage tracing system, we show that EPDCs give rise to fibroblasts and coronary smooth muscle cells (SMCs) but not to endothelial cells in the wild type, whereas in *Rbpj*-deficient embryos EPDCs form and surround the developing arteries but fail to differentiate into SMCs. Conditional activation of Notch signaling results in premature SMC differentiation of epicardial cells and prevents coronary angiogenesis. We further show that Notch signaling regulates, and cooperates with transforming growth factor β signaling in SM differentiation of EPDCs.**

Conclusions: **Notch signaling is a crucial regulator of SM differentiation of EPDCs, and thus, of formation of a functional coronary system. (*Circ Res*. 2011;108:813-823.)**

Key Words: Tbx18 ■ Tgfb ■ Pdgfrb ■ coronary ■ smooth muscle cell

The epicardium, the outermost layer of the heart, develops after cardiac looping from a cluster of mesothelial cells of the septum transversum region at the cardiac venous pole. From embryonic day (E)9.0 on in the mouse, cells of this proepicardial organ (PEO) float through the pericardial space, adhere to the myocardium, spread out and form a continuous epithelial layer around E10.5. A subset of these epicardial cells undergoes an epithelial–mesenchymal transition (EMT), migrates into the subepicardial space or invades the underlying myocardium.[1] Cell lineage tracings mainly done in the chick have shown that subepicardial mesenchyme further differentiates in interstitial and perivascular fibroblasts, in smooth muscle cells (SMCs) and coronary endothelial cells.[2,3] Genetic fate-mapping studies in the mouse suggested that a substantial fraction of cardiomyocytes may also derive from epicardial cells.[4,5] Besides its role as cell source for the coronary vasculature and the myocardium, the embryonic epicardium acts as a center of paracrine signals that promote the maturation of other cardiac components including the embryonic myocardium. The genetic pathways that regulate the different steps of epicardial development are insufficiently understood.[1]

The Notch signaling pathway is an evolutionarily conserved regulator of local cell–cell interactions that mediate cell fate decisions, proliferation, apoptosis, boundary formation, and stem cell maintenance in a variety of tissues in development and homeostasis.[6] In mammals, 4 different transmembrane Notch receptors (Notch1 to -4) and 5 different transmembrane ligands (Delta-like [Dll]1, -3, -4; Jagged [Jag]1, -2) have been identified. Binding to a ligand on an adjacent cell induces 2 consecutive proteolytic cleavages of the Notch receptor to release the active Notch intracellular domain (NICD). NICD translocates to the nucleus, where its binding to the transcription factor recombination signal binding protein for immunoglobulin kappa J region (Rbpj) displaces corepressor complexes from this DNA-binding factor. Coactivators are recruited instead and transcription of target genes including members of the basic helix-loop-helix hairy/enhancer-of-split related with YRPW motif gene family (*Hey1*, *Hey2*, *HeyL*) is initiated.[6] Notch signaling has been implicated in numerous processes in cardiovascular development including endocardial cushion formation, maturation of the ventricular myocardium, establishment of atrioventricular canal boundaries, arterial-venous fate decisions, angiogenic

Original received July 21, 2010; revision received January 5, 2011; accepted January 11, 2011. In December 2010, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 14.5 days.

From the Institut für Molekularbiologie, Medizinische Hochschule Hannover, Germany.

Correspondence to Andreas Kispert, Institut für Molekularbiologie, OE5250, Medizinische Hochschule Hannover, Carl-Neuberg-Str.1, D-30625 Hannover, Germany. E-mail kispert.andreas@mh-hannover.de

Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/CIRCRESAHA.110.228809**

Non-standard Abbreviations and Acronyms

cSMC	coronary smooth muscle cell
DAPI	4,6-diamidino-2-phenylindol
Dll	Delta-like
E	embryonic day
EMT	epithelial–mesenchymal transition
EPDC	epicardium-derived cell
Hey	hairy/enhancer-of-split related with YRPW motif
IVS	interventricular septum
Jag	Jagged
NICD	Notch intracellular domain
Pdgfrb	platelet-derived growth factor receptor β polypeptide
PEO	proepicardial organ
Rbpj	recombination signal binding protein for immunoglobulin κJ region
SM	smooth muscle
SMC	smooth muscle cell
Tgfb	transforming growth factor β

growth of the blood vessel network, proliferation of endothelial cells and vascular SMC differentiation.[7,8] Expression of the Notch1ICD in mesothelial cells of the PEO and the epicardium, in nascent vessels, and in both endothelial and surrounding smooth muscle cells of the coronary arteries in the developing chick heart[9] suggested an additional role of this pathway in the developing epicardium.

Here, we analyze the role of Notch signaling in epicardial development by genetic loss- and gain-of-function experiments in the mouse. We show that Notch signaling regulates SM differentiation of EPDCs, and we define the epistatic relation with other signaling pathways implicated in coronary arteriogenesis.

Methods

Animal care was in accordance with national and institutional guidelines. Mice carrying a null allele of *Rbpj* (*Rbpj*$^{tm1.1Hon}$, synonym: *Rbpj*^{flox}),[10] mice with an integration of the intracellular domain of the *Notch1* receptor gene in the *Rosa26* locus (*Gt(ROSA)26Sor*$^{tm1(Notch1)Dam}$, synonym: *Rosa26*^{NICD}),[11] and mice with an insertion of the *cre* recombinase gene in the *Tbx18* locus (*Tbx18*$^{tm4(cre)Akis}$, synonym: *Tbx18*^{cre})[12] and the fluorescent reporter line (*Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J, synonym: *Rosa26*^{mTmG})[13] were all described previously. All mouse lines were maintained on an outbred (NMRI) background.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

Multiple Notch Receptors and Ligands Are Expressed During Development of the Epicardium and the Coronary System

To determine the temporal and spatial involvement of Notch signaling in mouse epicardial and coronary vessel development, we analyzed expression of genes encoding Notch receptors and ligands, and target genes of the Hey family by in situ hybridization from onset of (pro-)epicardial development at E9.5 to E18.5 when a functional coronary system has been established (Online Figure I). In whole E9.5 embryos, we detected strong expression of *Hey1*, weak expression of *Jag1* and very weak expression of *Notch1*, *Notch2* and *Notch3* in the PEO (Figure 1A). Expression of *Notch2* and *Notch3*, *Dll3*, *Hey1*, *Hey2* and *HeyL* was found in the epicardium at E10.5 (Figure 1B). Semiquantitative RT-PCR analysis on epicardial cell cultures confirmed transcription of these genes and provided evidence for additional expression of *Jag1* (Figure 1C). At E12.5 and later, expression of Notch pathway components was no longer found in the epicardium by in situ hybridization analysis. However, starting from E12.5 we detected expression of these genes in the subepicardial space and the underlying myocardium indicating an association with EPDCs and the forming coronary system. At E12.5, *Notch1* and *Dll4* were expressed in individual cells in the subepicardial mesenchyme, most likely representing endothelial cells of the sprouting coronary plexus (Figure 1D). *Hey2* expression was too prominent in the ventricular myocardium to distinguish an additional expression in the developing coronaries (Online Figure I). At E14.5, expression of Notch pathway constituents became more complex in the subepicardial region. We found expression of *Notch1*, *Notch3*, *Dll4*, *Jag2*, *Hey1* and *HeyL* in endothelial and associated perivascular cells (Figure 1E). At E18.5, expression of Notch components was exclusively associated with large coronary arteries that were distinguished by a larger lumen and the deep location from the smaller veins that were located subepicardially. *Notch1* was coexpressed with *Notch3* and *HeyL* in the outer ring of perivascular cells. In the inner endothelial layer *Notch1* was coexpressed with *Dll1* (very weakly), *Dll4*, *Jag1*, *Jag2* and *Hey1* (Figure 1F; Online Figure I). Thus, Notch expression (and signaling) occurs in a biphasic manner in epicardial development. First, in the PEO and early epicardium, later in EPDCs and/or endothelial cells during coronary artery formation.

Loss of Rbpj-Dependent Notch Signaling in the PEO and Epicardium Results in Severe Morphological Defects of the Coronary Vasculature

Given the complexity of Notch receptor and ligand expression during development of the epicardium and the coronary system, we assumed that redundancy between receptors and ligands, respectively, may necessitate the simultaneous removal of 2 or more genes to assign a Notch signaling requirement to these processes. We therefore decided to analyze the phenotypic consequences of removal of *Rbpj* that encodes a unique intracellular mediator of (canonical) Notch signaling.[14] Because *Rbpj*-deficiency results in early embryonic lethality in mice,[15] we used a tissue-specific inactivation approach using a *Tbx18*^{cre} line generated in our laboratory and a floxed *Rbpj* allele to analyze Notch signaling function in the PEO and epicardium.[10,12] The T-box transcription factor gene *Tbx18* is strongly expressed in the PEO at E9.5, and in the epicardium until E16.5. Other cardiac expression domains include the sinus horn mesenchyme/myocardium, and the myocardium of the left ventricle and the interventricular septum (IVS) (Online Figure II, A).[16,17] We used *Rosa26*^{mTmG} reporter mice[13] to demonstrate that *cre* expres-

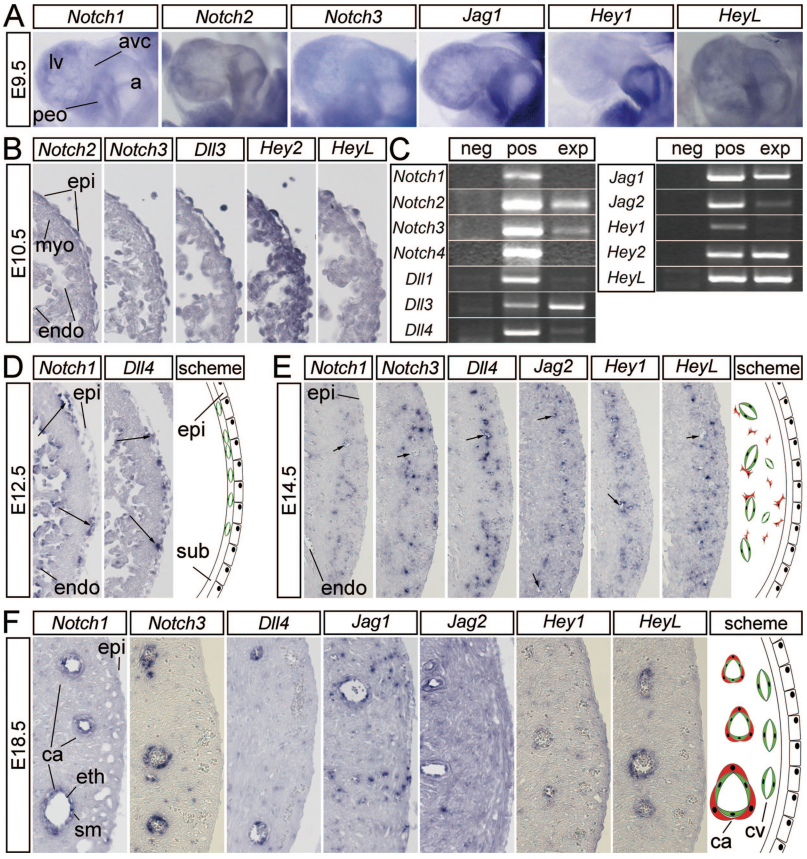

Figure 1. Expression of genes encoding Notch receptors, ligands, and targets in epicardial development. A through F, Expression of Notch receptor (*Notch1–4*), Notch ligand (*Dll1*, *Dll3*, *Dll4*, *Jag1*, *Jag2*), and Notch target gene expression (*Hey1*, *Hey2*, *HeyL*) by in situ hybridization analysis of whole hearts **(A)** and on sections of the left ventricle **(B and D through F)** at the indicated stages and by semiquantitative RT-PCR analysis **(C)** in epicardial explant cultures. Semiquantitative RT-PCR analysis was performed on water control (neg), on E16.5 embryonic lungs as positive control (pos), and on a pool of epicardial explants (exp). **Long arrows in D** indicate expression in subepicardial cells. **Short arrows in E** point to forming coronary vessels. **Schemes in D through F** depict subepicardial vessel formation with endothelial cells **(green)** and perivascular cells **(red)** that are associated with expression of Notch pathway genes. a indicates atrium; avc, atrioventricular canal; ca, coronary artery; cv, coronary vein; endo, endocardium; epi, epicardium; eth, endothelium; lv, left ventricle; myo, myocardium; peo, proepicardial organ; sm, smooth muscle cell layer; sub, subepicardial space.

sion from the *Tbx18* locus mediates recombination in all known *Tbx18* expression domains and their cellular descendants in whole E9.5 and E10.5 embryos, and in the heart from E9.5 to E14.5 in a faithful manner (Online Figure II, B and C). In the *Rosa26*^{mTmG} reporter line recombination is visualized by bright membrane bound GFP expression in a background of membrane bound red fluorescence. Anti-GFP immunofluorescence analysis on sections of *Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$ hearts provides additional cellular resolution of cre recombination events.[13]

To our surprise, *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ mice survived to 6 months of age without any obvious external defects. Hearts of 3-month-old mice had a normal shape but featured grossly abnormal coronary vessels with a dramatically enlarged vascular bed on their surface (Figure 2A). Histological sections revealed that the lumen of deeper vessels was increased as well albeit less dramatically compared to subepicardial vessels. Intramyocardial and subepicardial location suggested these vessels to be of arterial and venous identity, respectively.[18] We confirmed this notion by detecting expression of the venous and capillary marker endomucin (Emcn)[19] in the epithelial lining of these inflated vessels underneath the surface but not in the deeper intramyocardial vessels where the pan-endothelial marker Pecam1 indicated the presence of an endothelium (Figure 2A). To exclude that these coronary changes are merely a physiological adaptation to (unknown) stress but represent the consequences of a developmental defect, we analyzed mutant hearts during embryogenesis. Morphological and histological inspection revealed the presence of size increased intramyocardial and largely inflated superficial vessels in the *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ heart at E18.5. Emcn was again expressed in the endothelial linings of the superficial but not the deep vessels. Emcn staining also revealed reduction of intramyocardial capillaries suggesting that large coronary arteries and veins are expanded at the expense of these smaller vessels (Figure 2B). Arterial markers ephrin B2 (Efnb2), Dll1 and vascular endothelial growth factor A (Vegfa)[20] were expressed in the endothelium of the intramyocardial but not the superficial vessels whereas nuclear receptor subfamily 2, group F, member 2 (Nr2f2) was found in nuclei of venous but not of arterial endothelial cells (Figure 2C). In sections of E14.5 *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ hearts, the distribution and size of coronary vessels appeared relatively unaffected, and Emcn staining was not different from the wild type (Online Figure III). Thus, epicardial loss of *Rbpj* results in severe morphological changes of the coronary vasculature after onset of coronary circulation at E15.5. However, these changes are not caused or associated with an arterial-venous switch of coronary endothelial identity.

Rbpj-Dependent Notch Signaling Is Required for SM Differentiation of EPDCs

We next analyzed whether changes in identity or integrity of the epicardium, formation of subepicardial mesenchyme and/or subsequent differentiation of EPDCs may explain the observed defects in the coronary vascular system. In E14.5 *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ embryos, histological analysis confirmed the integrity of the epicardial epithelium (Online Figure III). Expression of epicardial marker genes Wilms

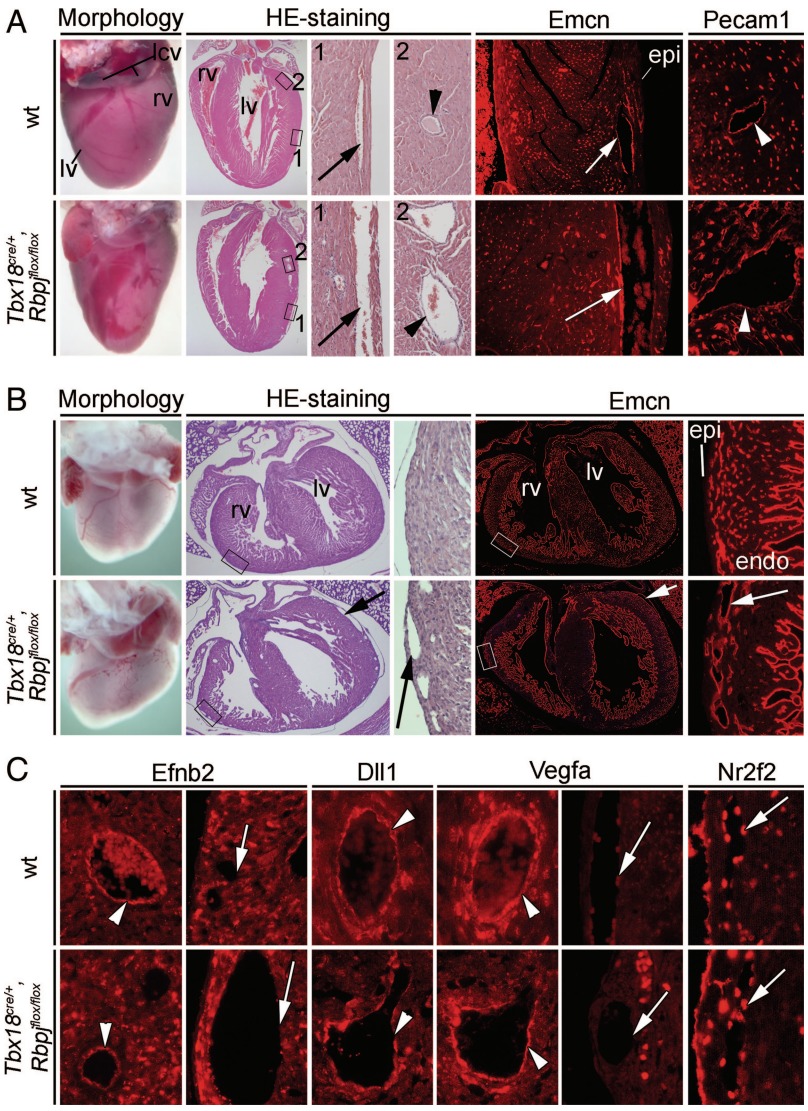

Figure 2. Defects of the coronary vasculature in mice with epicardial deletion of *Rbpj*. **A through C,** Analysis of coronary vessel formation in *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ mice by morphology of whole hearts, by hematoxylin/eosin staining and by immunofluorescence for endothelial marker proteins on transverse cardiac sections at 3 months of age **(A)** and at E18.5 **(B and C)**. **C,** Higher magnifications of individual coronary arteries and veins. Emcn stains endothelia of large veins and capillaries; Dll1, Efnb2, and Vegfa stain arterial endothelia; and Nr2f2 stains nuclei of venous endothelial cells. **Rectangles** in images of whole hearts are magnified in the images to the **right**. **Arrows** point to the endothelial lining of superficially located venous coronary vessels; **arrowheads** point to the arterial endothelial lining. endo indicates endocardium; epi, epicardium; lcv, left caval vein; lv, left ventricle; rv, right ventricle.

tumor 1 homolog (*Wt1*), transcription factor 21 (*Tcf21*), *Tbx18*, fibulin 2 (*Fbln2*), aldehyde dehydrogenase family 1, subfamily A2 (*Aldh1a2*) was unchanged confirming that epicardial cells retained their normal identity. Expression of *Wt1* and *Tcf21* was also found in subepicardial cells indicating that epicardial EMT occurred and EPDCs migrated into the subepicardial space and the myocardium (Online Figure IV). To further analyze the fate of epicardial cells, we performed *Tbx18*^{cre} based genetic lineage tracing in wild-type and *Rbpj* mutant background using the *Rosa26*^{mTmG} reporter line. Anti-GFP immunofluorescence on sections of E14.5 wild-type hearts labeled epicardial cells, thin and slender cells in the right ventricular myocardium, and cells surrounding superficial veins and deeply located arteries. A similar situation was found in *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ hearts at this stage confirming that loss of epicardial *Rbpj* function does not affect epicardial EMT and EPDC immigration (Figure 3A). Double immunofluorescence analysis for expression of Gfp and markers for arterial endothelial cells (nitric oxide synthase 3 endothelial cell [Nos3]), venous endothelial cells (Emcn), SMCs (actin α2 smooth muscle, aorta, Acta2, also known as SMaActin), prospective SMCs (Notch3), fibroblasts (periostin [Postn]), and cardiomyocytes (cardiomyocyte-specific troponin I cardiac 3,Tnni3, and myosin heavy chain cardiac muscle [Myhc]) confirmed that in wild-type conditions, EPDCs differentiated into fibroblasts and SMCs but not into endothelial cells (Figure 3B through 3D). Not a single cardiomyocyte in the right ventricle was GFP positive (Figure 3E) (in the left ventricle, endogenous myocardial expression of Tbx18 does not allow such an analysis).[17] However, differentiation of epicardium derived perivascular cells into SMCs selectively failed in *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ hearts (Figure 3C). Notably, Notch3 was still expressed in perivascular cells arguing that Notch3 expression is independent from Notch signaling but that it is required for SMC differentiation in coronary arteries (Figure 3C; Online Figure V). Thus, epicardial cells contribute to intramyocardial and perivascular fibroblasts, to coronary SMCs (cSMCs) but not to coronary endothelial cells. *Rbpj*-dependent Notch signaling is selectively required for differentiation of perivascular cells into SMCs. Changes of coronary morphology at later stages are a likely secondary physical consequence of the loss of the arterial SM coating.

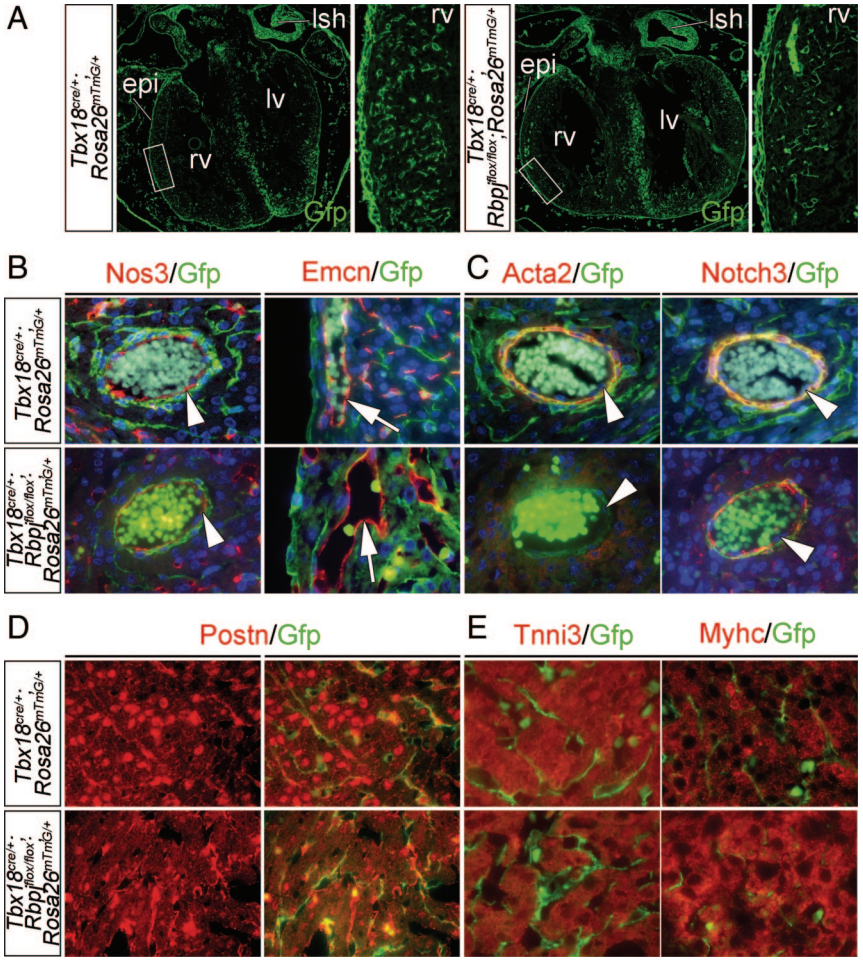

Figure 3. Disrupted cytodifferentiation of EPDCs in *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ mice. A, Analysis of formation and migration of EPDCs in *Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$; *Rbpj*$^{flox/flox}$ mice by immunofluorescence of the Gfp reporter on transverse sections of the whole E14.5 heart and on a higher magnification of the right ventricle **(white rectangles)**. **B through E,** Analysis of cytodifferentiation of EPDCs by double immunofluorescence of the Gfp reporter **(green)** and markers (in **red**) for arterial endothelial cells (Nos3), venous endothelial cells (Emcn) **(B)**, SMCs (Acta2), prospective SM cells (Notch3) **(C)**, fibroblasts (Postn) **(D)**, and cardiomyocytes (Tnni3, Myhc) **(E)** on serial transverse sections of the right ventricle of E18.5 *Tbx18*$^{cre/+}$; *Rosa26*$^{mTmG/+}$;*Rbpj*$^{flox/flox}$ hearts. Nuclei are counter-stained with DAPI. **Arrows** point to the endothelial lining of enlarged superficially located venous coronary vessels; **arrowheads** point to the arterial endothelial lining. epi indicates epicardium; lsh, left sinus horn; lv, left ventricle; rv, right ventricle.

Notch Signaling Is Sufficient to Induce Premature SM Differentiation of Epicardial Cells

Because our loss-of-function analysis showed that Notch signaling is required for SM differentiation of EPDCs, we wondered whether premature activation of the pathway in vivo might actually be deleterious for epicardial development. We achieved (pro-)epicardium specific activation of the pathway by *Tbx18*^{cre} mediated expression of the Notch1 intracellular domain (NICD) from a *Rosa26* knock-in allele (*Rosa26*^{NICD}).[11] *Tbx18*$^{cre/+}$;*Rosa26*$^{NICD/+}$ mice died shortly after E14.5 exhibiting edema formation and bleeding. Morphological inspection of the mutant hearts revealed local protrusions of the epicardium (Figure 4A). On histological sections, the epicardium appeared discontinuous, and a thick subepicardial matrix with intermingled mesenchymal cells detached the epicardium from the underlying myocardium. The myocardial compact layer was severely reduced in thickness (Figure 4B). Epicardial expression of Tbx18, Wt1 and *Aldh1a2* was discontinuous and/or reduced (Figure 4C). Expression of SM myosin heavy chain (smMHC), *HeyL*, *Hey2*, and *Notch3* was found in the epicardium but not in the subepicardium, intramyocardially, or in perivascular locations (Figure 4D; Online Figure VI). Gfp expression from the *Rosa26*^{mTmG} reporter in the *Tbx18*$^{cre/+}$;*Rosa26*$^{NICD/+}$ background was present in epicardial membranes but only in few subepicardial cells in the right ventricular myocardium (Figure 4E). This suggests that premature SM differentiation of epicardial cells prevents their immigration into the subepicardial space and the myocardium. Emcn expression was restricted to the endocardium, and venous endothelium in the atrial-ventricular groove region but did not extend subepicardially or intramyocardially toward the apex as in the wild type (Figure 4F).

We traced the developmental onset of these epicardial defects by analyzing Tbx18 and Acta2 expression at earlier stages. Tbx18 expression was present in the PEO at E9.5. In contrast to the wild-type situation, myocardial colonization by Tbx18-positive epicardial cells was severely delayed, and SM differentiation of epicardial cells prematurely induced in *Tbx18*$^{cre/+}$;*Rosa26*$^{NICD/+}$ embryos at E12.5 (Figure 5A through 5D). Expression of integrin α4 (*Itga4*) that is required for myocardial attachment of epicardial cells,[21] and of *Sox9* was downregulated in the E9.5 PEO (Figure 5E through 5G), explaining delayed myocardial colonization. Together, this analysis shows that (pro-)epicardial activation of Notch1 signaling leads to defects in the formation and differentiation of the epicardium that affect coronary plexus formation and myocardial growth.

SM Differentiation of Primary Epicardial Cells by NICD Overexpression

To obtain more detailed insight into the cellular and molecular consequences of epicardial activation of Notch signaling,

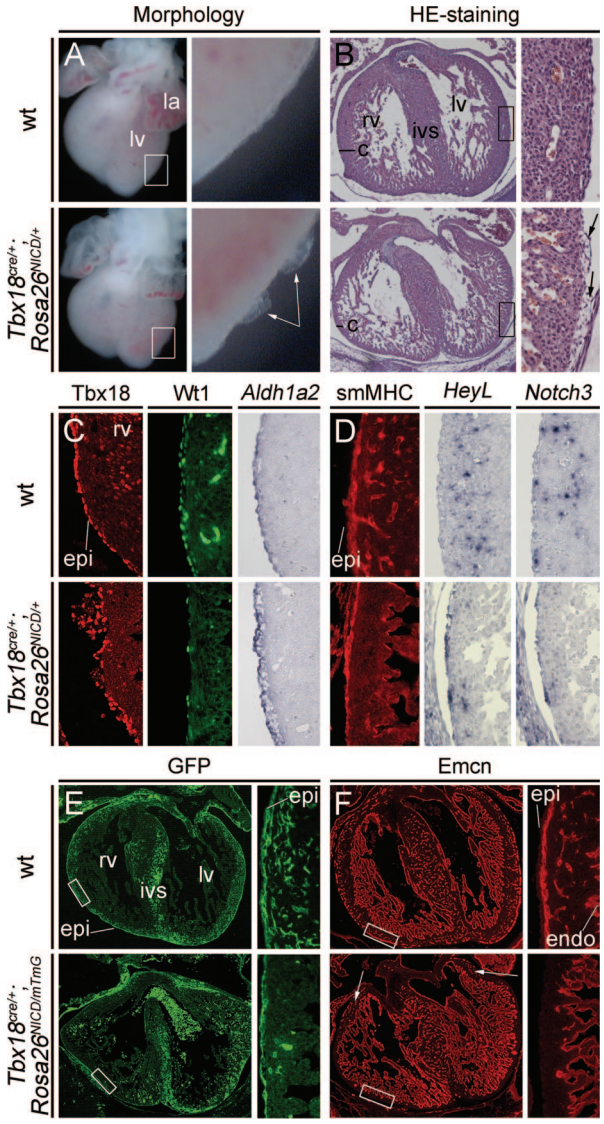

Figure 4. Disrupted epicardial development in mice overexpressing the Notch1 intracellular domain. A through E, Analyses were performed on E14.5 wild-type (wt) and *Tbx18*$^{cre/+}$; *Rosa26*$^{NICD/+}$ embryos for epicardial integrity and differentiation by morphological inspection of whole hearts **(A)**, by hematoxylin/eosin staining **(B)** (**arrows in A and B** point to epicardial blisters), by immunofluorescence of expression of Tbx18, Wt1, and smMHC, and by in situ hybridization of *Aldh1a2*, *HeyL*, and *Notch3* expression on transverse sections of the right ventricle **(C and D)**. **E,** Cell lineage tracing by anti-Gfp immunofluorescence on sections of the right ventricle in *Tbx18*$^{cre/+}$; *Rosa26*$^{NICD/mTmG}$ embryos. **F,** Emcn immunofluorescence to reveal the integrity of the coronary plexus. **White arrows** mark the restricted angiogenic sprouts of the coronary plexus close to the atrioventricular groove region. **Rectangles** mark the regions that are magnified in the images to the **right**. c indicates compact layer; endo, endocardium; epi, epicardium; ivs, interventricular septum; la, left atrium; lv, left ventricle; rv, right ventricle.

we analyzed cultures of highly enriched primary epicardial cells (Figure 6). These were obtained from right ventricular explants at E11.5. After 2 days in serum-free medium the outgrowth of wild-type ventricles presented as a monolayer of tightly packed hexagonal cells. Expression of the tight junction protein 1 (Tjp1, also known as ZO1) confirmed the epithelial character of these cells. Tbx18 was predominantly and Wt1 exclusively localized to the nucleus suggesting that these cells represent indeed epicardial "precursor" cells. Transgelin (Tagln), Acta2, and Notch3 were expressed at very low levels; Pecam1 was not expressed, confirming that these epicardial cells have not differentiated into the SM or endothelial cell lineage. In contrast, explants of *Tbx18*$^{cre/+}$; *Rosa26*$^{NICD/+}$ hearts provided a cellular outgrowth with a rugged front line, and cells with a more mesenchymal appearance and reduced cell contacts. Tjp1 was absent from the membrane but localized throughout the cytoplasm. Tbx18 expression was downregulated whereas Wt1 was redistributed from the nucleus to the cytoplasm. SM markers (Acta2, Tagln, and Notch3) were highly upregulated. Pecam1 expression was absent in these outgrowths (Figure 6A). Proliferation was not statistically affected by NICD overexpression (Figure 6B). The molecular changes were enhanced after 2 additional days of serum-free culture after removal of the ventricle (Online Figure VII). These results clearly show that NICD is sufficient to induce mesenchymal transition and SM differentiation of epicardial cells but does not affect cell proliferation.

Notch Signaling Acts Upstream of Tgfb Signaling and Pdgfrb in EPDCs

Previous work identified a requirement of transforming growth factor β (Tgfb) and platelet-derived growth factor β receptor (Pdgfrb) signaling in SM differentiation during coronary arteriogenesis in the mouse.[22,23] To analyze the epistasis between these pathways and Notch signaling, we determined whether expression of the intracellular mediator of Tgfb signaling, activated P-Smad2,3, and of Pdgfrb is affected by epicardial loss- or gain-of Notch signaling. In hearts of E18.5 *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ embryos, both P-Smad2 and -3 and Pdgfrb expression was specifically absent from perivascular cells of coronary arteries (Figure 7A). In contrast, expression of both proteins was induced in epicardial cells of *Tbx18*$^{cre/+}$;*Rosa26*$^{NICD/+}$ embryos at E14.5 (Figure 7B). Furthermore, semiquantitative RT-PCR analysis revealed twofold induction and reduction, respectively, of *Pdgfrb* mRNA expression in epicardial explant cultures of NICD-overexpressing and *Rbpj*-deficient hearts, respectively (Figure 7C). Together, these data show that Notch signaling is required and sufficient to induce Tgfb signaling and *Pdgfrb* expression in epicardium and EPDCs.

Notch Cooperates With Tgfb Signaling in inducing SM Genes in EPDCs

We further analyzed the interplay between these signaling pathways in epicardial explant cultures by scoring mRNA expression of the 2 smooth muscle marker genes *Acta2* and *Tagln* by semiquantitative RT-PCR after different pharmacological treatments in wild-type and mutant backgrounds (Figure 8; Online Table I). TGFb1 induced SM marker gene expression in wild-type epicardial cultures. The inhibitor of Tgfb receptor 1 (Alk5) SB431542 but also PDGF-BB abrogated this effect (Figure 8A). Expression of SM marker genes was similarly induced in NICD-expressing epicardial explants but could not be further enhanced by additional TGFb1

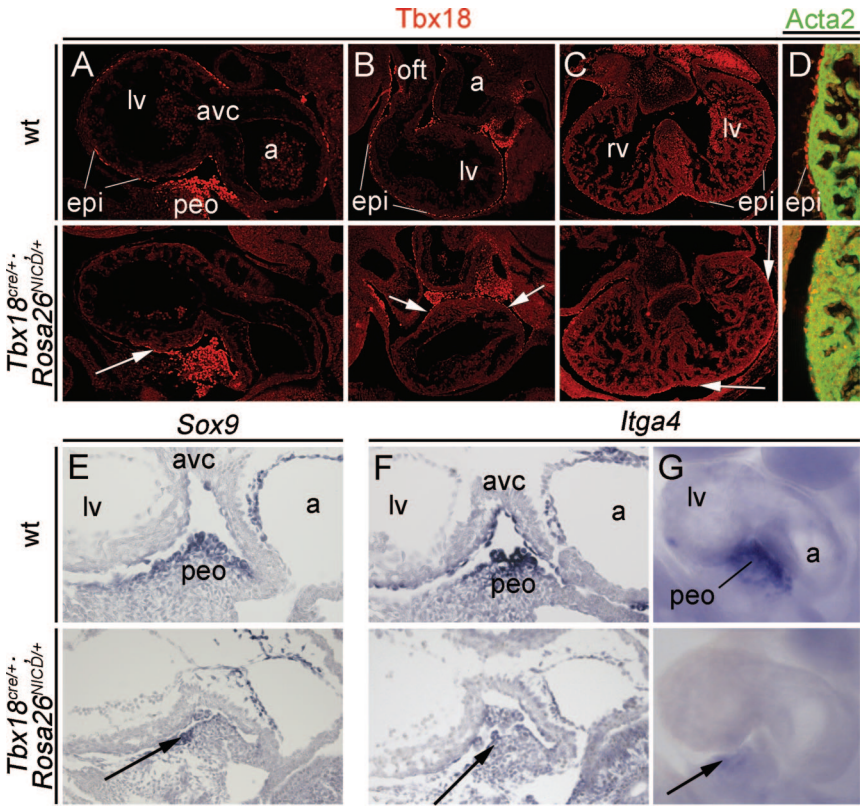

Figure 5. Early onset of epicardial defects in *Tbx18*$^{cre/+}$;*Rosa26*$^{NICD/+}$ embryos. A through D, Immunofluorescence analysis of Tbx18 protein expression (in **red**) in sections of whole hearts at E9.5 **(A)**, E10.5 **(B)**, E12.5 **(C)** and in a subregion of the right ventricle at E12.5 **(D)**. **White arrows** point to clusters of Tbx18-positive epicardial cells. Acta2 expression by immunofluorescence **(green in D)** is only present in the myocardial layer in the wild-type, whereas epicardial cells coexpress Tbx18 and Acta2 protein at this stage in the mutant. **E through G,** In situ hybridization analysis of *Sox9* and *Itga4* expression on saggital sections **(E and F)** and of *Itga4* in whole E9.5 hearts **(G)**. **Black arrows** indicate reduced expression of *Sox9* and *Itga4* in the mutant PEO. Genotypes are as indicated. a indicates atrium; avc, atrioventricular canal; epi, epicardium; lv, left ventricle; oft, outflow tract; peo, proepicardial organ; rv, right ventricle.

in the medium. Tgfb1-inhibitor and PDGF-BB both inhibited NICD-activated SM gene expression, suggesting that Tgfb signaling acts downstream of Notch signaling to induce SM differentiation (Figure 8B). Remarkably, TGFb1 was not sufficient to rescue loss of SM gene induction in absence of Notch signaling (in *Rbpj*-deficient cultures), arguing for cooperativity between these 2 pathways in SMC differentiation (Figure 8C). NICD expression strongly induced expression of *Tgfb1–3*, interestingly, again in a Tgfb-signaling–dependent manner (Figure 8D). Together, these in vitro experiments argue that Notch signaling induces Tgfb signaling by upregulating *Tgfb*-ligands. Notch and Tgfb signaling then cooperatively induce SM differentiation of EPDCs.

Discussion

The embryonic epicardium is a crucial cell source for the developing heart, yet the derived cell types, and the signals and factors that control their differentiation are incompletely understood. Here, we have shown by genetic experiments in vivo that cardiac fibroblasts and coronary SMCs derive from the epicardium, and that the canonical Notch pathway in conjunction with Tgfb signaling controls SM differentiation of EPDCs.

Fate of Epicardial Cells

Lineage studies in avian species originally demonstrated that the (pro-)epicardium is a source for cardiac fibroblasts and coronary vascular progenitors including mural and endothelial cells.[2,3,24] Genetic fate-mapping studies in the mouse questioned the epicardial origin of coronary endothelial cells but suggested that a subset of cardiomyocytes are epicardium derived. In fact, Cai et al reported that a substantial fraction of cardiomyocytes of the left ventricle and the IVS derive from Tbx18-positive epicardial cells,[4] whereas 7% to 18% ventricular, atrial and IVS cardiomyocytes were noted as descendants of Wt1-positive epicardial cells in another study.[5] Although a critical reevaluation of the Wt1-based epicardial lineage tracing has not yet been published, we have previously raised concerns on the validity of the Tbx18^{cre} approach based on the endogenous expression of Tbx18 in cardiomyocytes of the IVS and left ventricle from at least E10.5 in development.[17] The Tbx18^{cre} line used in our study allowed faithful activation of cre in all Tbx18 domains. Our experiments did not identify cardiomyocytes in the right ventricle to derive from the overlying epicardium questioning the results by Zhou et al.[5] We neither detected endothelial cells to be of epicardial origin corroborating a recent report that the coronary plexus develops by angiogenic sprouting of the sinus venosus region of the postlooped heart.[25] Decisive clarity on the definitive spectrum of epicardial fates requires additional experimental testing using independent genetic tracing systems that are based on genes selectively and exclusively expressed in the PEO/epicardium. Until this point, fibroblasts and SMCs may be considered as the 2 major if not exclusive cellular derivatives of the (pro-)epicardium in mammals.

Notch Signaling and cSMC Differentiation

Our expression analysis identified Notch expression and signaling in the PEO and epicardium, and in EPDCs and associated endothelial cells of the forming coronary vasculature. Characterization of mice with epicardial loss of Rbpj did not uncover phenotypic changes that would support a role for canonical Notch signaling in epicardium formation,

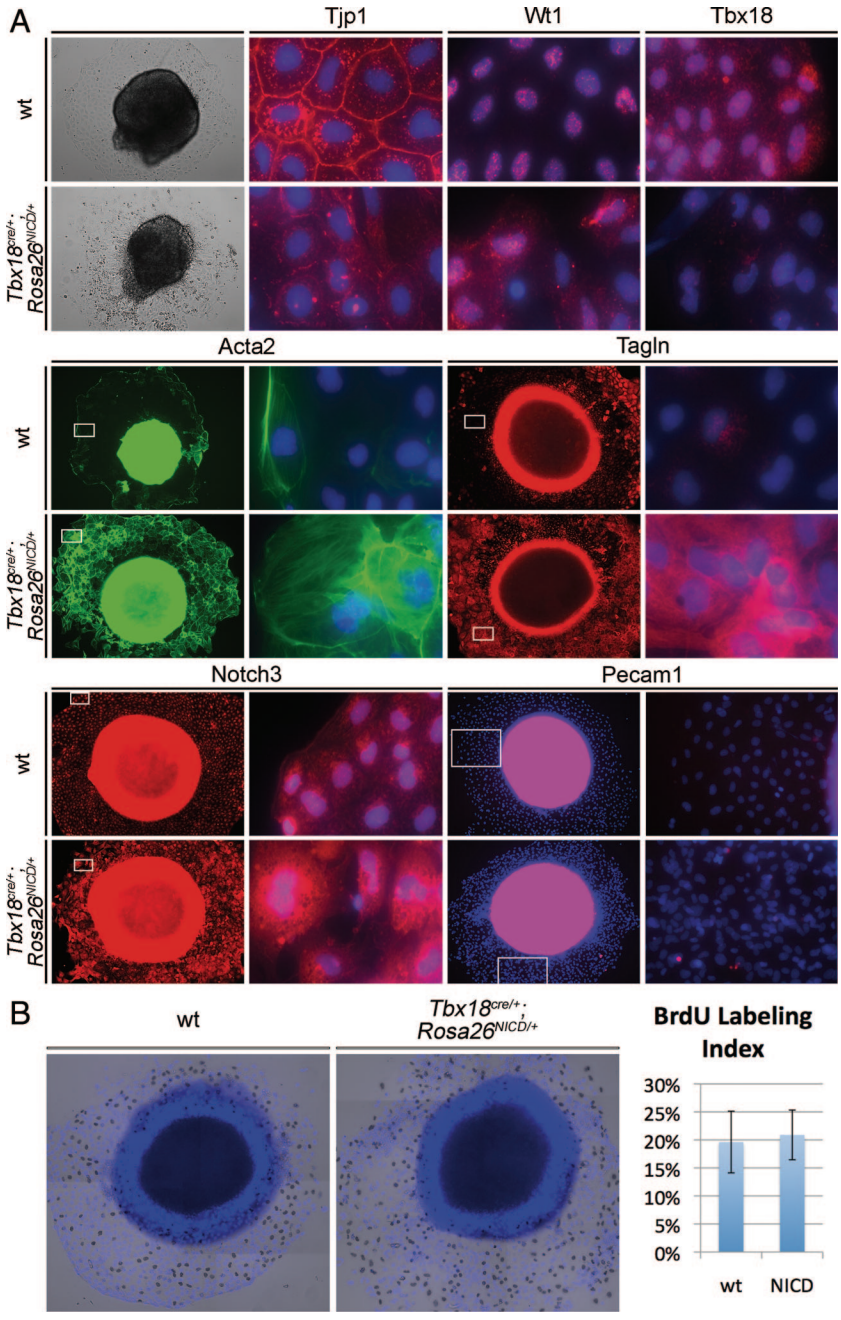

Figure 6. Induction of SM differentiation of primary epicardial cells by NICD overexpression. A, Cellular analysis of epicardial outgrowths of explants of right ventricles of wild-type (wt) and *Tbx18*$^{cre/+}$; *Rosa26*$^{NICD/+}$ embryos after 2 days of culture under serum-free conditions by bright field morphology, and immunofluorescent analysis of the tight junction protein Tjp1 (ZO1); epicardial transcription factors Wt1 and Tbx18; smooth muscle proteins Acta2, Tagln, and Notch3; and endothelial Pecam1. Shown are whole explants (bright field, Acta2, Tagln, Notch3, and Pecam1) and/or magnified regions **(white rectangles)** with nuclear counter-staining (DAPI) from the epicardial outgrowth only. **Red spots** occasionally found in the Pecam stainings are likely to present endocardial contaminants or unspecific staining of cell debris. **B,** Analysis of cell proliferation in epicardial explants by the bromodeoxyuridine (BrdU) incorporation assay, with quantification of bromodeoxyuridine-positive cells (wild type, 19.6±5.5%; NICD, 20.9±4.4%, P=0.733).

epicardial EMT, or fibroblast differentiation. In fact, forced epicardial expression of NICD led to defects of myocardial colonization and epicardial differentiation that strongly argue against Notch function at these stages of epicardial development. However, Notch signaling was specifically required for cSMC differentiation. Restricted expression of Notch1 and Notch3 in perivascular cells and of Jag1, Jag2, Dll1, and Dll4 in endothelial cells of coronary arteries suggests that multiple Notch ligands activate a redundant pair of Notch receptors in mural cells in trans. Notch ligand–receptor interaction may be additionally involved in the initial recruitment of EPDCs to arterial endothelial cells.[26] Expression of Notch3 in these cells was maintained, suggesting that ligand–receptor interaction mediates adhesion of the 2 cell types in an Rbpj-independent fashion. It also shows Notch3 expression in these cells is independent from Notch signaling. Loss of Rbpj in cSMC cells did not change the identity of coronary arterial endothelial cells in our mice. Hence, establishment and maintenance of arterial endothelial fate is independent of a functional SM coating as suggested by analysis of mice with endothelial specific loss of Jagged1.[27] Most likely, it is mediated by endothelial expression of Notch1, Dll1 and Dll4 as shown for large systemic arteries.[20,28]

Although the role of *Rbpj* in cSMCs had not been characterized before, the functional implication of canonical Notch signaling in the SM phenotype of perivascular cells of the systemic circulation was well known.[8,29] In fact, Notch1, Notch3, and Jag1 were identified as crucial

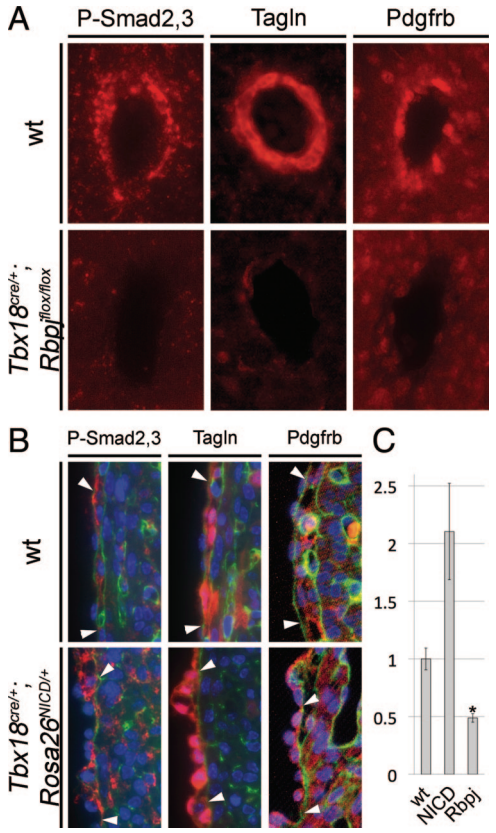

Figure 7. Tgfb signaling and Pdgfrb are downstream of the Notch pathway in SM differentiation of epicardial cells. A and B, Comparative immunofluorescence analysis of expression of P-Smad2, -3, Tagln, and Pdgfrb (red fluorescence) in coronary arteries of the right ventricle of wild-type (wt) and *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ embryos **(A)** and in epicardial and subepicardial cells in wild-type and *Tbx18*$^{cre/+}$;*Rosa26*$^{NICD/+}$ embryos **(B)** at E14.5. Green fluorescence for collagen IV allows visualization of the basement membrane underlying endothelial and epicardial cells **(white arrowheads)** and, thus, to delineate epicardium from underlying myocardium. Blue fluorescence marks DAPI-stained nuclei. Note absence of red fluorescence in epicardial cells in wild-type hearts. **C,** Semiquantitative RT-PCR analysis of *Pdgfrb* expression in pools of epicardial explants derived from wild-type (wt), *Tbx18*$^{cre/+}$;*Rosa26*$^{NICD/+}$ (NICD), and *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ (Rbpj) hearts. NICD: 2.1±0.42,P=0.06; Rbpj: 0.49±0.04; P=0.007.

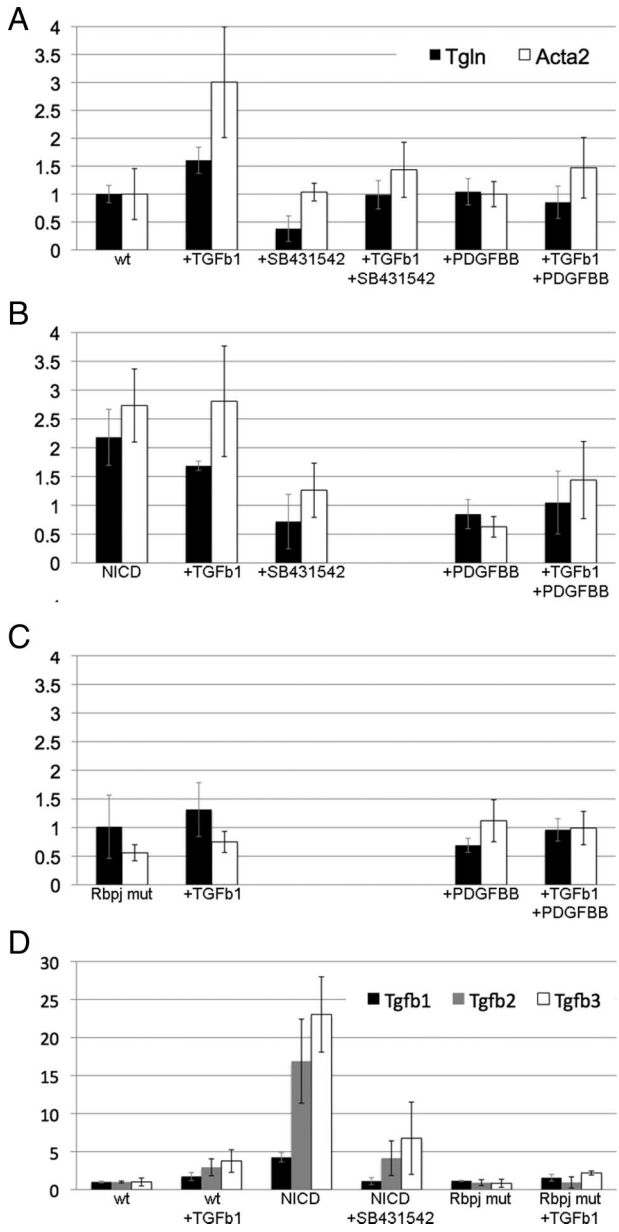

Figure 8. Notch and Tgfb signaling cooperate in SM differentiation of EPDCs. A through C, Semiquantitative RT-PCR analysis of *Acta2* and *Tagln* expression in differently treated pools of epicardial explants derived from wild-type **(A)**, *Tbx18*$^{cre/+}$;*Rosa26*$^{NICD/+}$ **(B)**, and *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ **(C)** embryos. **D,** Induction of *Tgfb1–3* in wild-type, *Rbpj*-deficient, and NICD-expressing epicardial explants under the indicated conditions. For values and statistical significance see Online Table I.

perivascular-endothelial receptors and ligand, respectively, in this tissue context as well. Hence, Notch signaling is likely to be a common regulator of SMC differentiation of perivascular cells independent from their developmental origin.

Notch Regulates and Cooperates With Tgfb Signaling in SM Differentiation of EPDCs

Previous work has established that canonical Wnt, Tgfb, and Pdgf signaling are required in vivo for formation of coronary SMCs from epicardial precursors. The Pdgfr pathway was implicated in proliferation and EMT of epicardial cells,[23] Wnt signaling in oriented epicardial cell division,[30,31] and Tgfb signaling both in epicardial EMT and later in SM differentiation of perivascular cells.[22,32] Although our analysis has not addressed interaction between Notch and Wnt signaling, we have shown that Notch pathway activation is required and sufficient for induction of Tgfb signaling and Pdgfrb expression in epicardial cells in vivo. Pdgfrb was reported as an immediate target of Notch function in vascular SMCs[33] but regulation of Tgfb signaling by Notch has only been noted in SM differentiation of mesenchymal stem cells[34] and in myofibroblast differentiation of alveolar epithelial cells.[35] Our findings suggest that NICD induction of Tgfb signaling in EPDCs is mediated by robust activation of Tgfb1–3 transcription, similar to the findings in cultures of alveolar cells.[35] Tang and coworkers recently demonstrated that Notch and Tgfb1 cooperatively activate SMC marker transcripts and

protein in primary human SMCs through parallel signaling axes.[36] Our failure to rescue SMC differentiation of primary epicardial cells by Tgfb1 clearly indicates that a similar cooperativity exists in EPDCs. Interestingly, Rbpj can directly activate transcription of the SMC marker Acta2.[37] Rbpj also binds and stabilizes P-Smad2/3 at Smad consensus binding sites within promoters of SM genes,[36] suggesting that molecular complex formation on adjacent DNA binding sites in promoters of SM genes as the basis for pathway cooperativity.

PDGF-BB efficiently inhibited induction of SM gene expression by Notch and Tgfb1 signaling in epicardial cells. The molecular mechanism of this inhibitory effect is unclear, but it suggests that Pdgfr signaling actively maintains the precursor character of EPDCs. Together, our findings support the model that Notch signaling activates and cooperates with Tgfb signaling to induce differentiation of vascular SMC from different progenitor pools. Pdgfrb is regulated by Notch signaling as well but may locally modulate or inhibit this differentiation program.

Acknowledgments

We thank Doug Melton and Tasuku Honjo for mice and Dietmar Vestweber and Robert Adelstein for antibodies.

Sources of Funding

This work was supported by grants from the European Community's Sixth Framework Programme contract ("HeartRepair" LSHM-CT-2005-018630) and the German Research Foundation (DFG) for the Cluster of Excellence REBIRTH (From Regenerative Biology to Reconstructive Therapy) and for the Clinical Research Group KFO136 at Hannover Medical School (to A.K.).

Disclosures

None.

References

1. Olivey HE, Svensson EC. Epicardial-myocardial signaling directing coronary vasculogenesis. *Circ Res*. 2010;106:818–832.
2. Dettman RW, Denetclaw W Jr, Ordahl CP, Bristow J. Common epicardial origin of coronary vascular smooth muscle, perivascular fibroblasts, and intermyocardial fibroblasts in the avian heart. *Dev Biol*. 1998;193:169–181.
3. Wilting J, Buttler K, Schulte I, Papoutsi M, Schweigerer L, Manner J. The proepicardium delivers hemangioblasts but not lymphangioblasts to the developing heart. *Dev Biol*. 2007;305:451–459.
4. Cai CL, Martin JC, Sun Y, Cui L, Wang L, Ouyang K, Yang L, Bu L, Liang X, Zhang X, Stallcup WB, Denton CP, McCulloch A, Chen J, Evans SM. A myocardial lineage derives from Tbx18 epicardial cells. *Nature*. 2008;454:104–108.
5. Zhou B, Ma Q, Rajagopal S, Wu SM, Domian I, Rivera-Feliciano J, Jiang D, von Gise A, Ikeda S, Chien KR, Pu WT. Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature*. 2008;454:109–113.
6. Schwanbeck R, Martini S, Bernoth K, Just U. The Notch signaling pathway: molecular basis of cell context dependency. *Eur J Cell Biol.* In press.
7. MacGrogan D, Nus M, de la Pompa JL. Notch signaling in cardiac development and disease. *Curr Top Dev Biol*. 2010;92:333–365.
8. Gridley T. Notch signaling in the vasculature. *Curr Top Dev Biol*. 2010;92:277–309.
9. Yang K, Doughman YQ, Karunamuni G, Gu S, Yang YC, Bader DM, Watanabe M. Expression of active Notch1 in avian coronary development. *Dev Dyn*. 2009;238:162–170.
10. Tanigaki K, Han H, Yamamoto N, Tashiro K, Ikegawa M, Kuroda K, Suzuki A, Nakano T, Honjo T. Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells. *Nat Immunol*. 2002;3:443–450.
11. Murtaugh LC, Stanger BZ, Kwan KM, Melton DA. Notch signaling controls multiple steps of pancreatic differentiation. *Proc Natl Acad Sci U S A*. 2003;100:14920–14925.
12. Trowe MO, Shah S, Petry M, Airik R, Schuster-Gossler K, Kist R, Kispert A. Loss of Sox9 in the periotic mesenchyme affects mesenchymal expansion and differentiation, and epithelial morphogenesis during cochlea development in the mouse. *Dev Biol*. 2010;342:51–62.
13. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. *Genesis*. 2007;45:593–605.
14. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell*. 2009;137:216–233.
15. Oka C, Nakano T, Wakeham A, de la Pompa JL, Mori C, Sakai T, Okazaki S, Kawaichi M, Shiota K, Mak TW, Honjo T. Disruption of the mouse RBP-J kappa gene results in early embryonic death. *Development*. 1995;121:3291–3301.
16. Kraus F, Haenig B, Kispert A. Cloning and expression analysis of the mouse T-box gene Tbx18. *Mech Dev*. 2001;100:83–86.
17. Christoffels VM, Grieskamp T, Norden J, Mommersteeg MT, Rudat C, Kispert A. Tbx18 and the fate of epicardial progenitors. *Nature.* 2009;458:E8–E9.
18. Lavine KJ, Long F, Choi K, Smith C, Ornitz DM. Hedgehog signaling to distinct cell types differentially regulates coronary artery and vein development. *Development*. 2008;135:3161–3171.
19. Morgan SM, Samulowitz U, Darley L, Simmons DL, Vestweber D. Biochemical characterization and molecular cloning of a novel endothelial-specific sialomucin. *Blood*. 1999;93:165–175.
20. Sorensen I, Adams RH, Gossler A. DLL1-mediated Notch activation regulates endothelial identity in mouse fetal arteries. *Blood*. 2009;113:5680–5688.
21. Yang JT, Rayburn H, Hynes RO. Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. *Development*. 1995;121:549–560.
22. Langlois D, Hneino M, Bouazza L, Parlakian A, Sasaki T, Bricca G, Li JY. Conditional inactivation of TGF-beta type II receptor in smooth muscle cells and epicardium causes lethal aortic and cardiac defects. *Transgenic Res*. 2010;19:1069–1082.
23. Mellgren AM, Smith CL, Olsen GS, Eskiocak B, Zhou B, Kazi MN, Ruiz FR, Pu WT, Tallquist MD. Platelet-derived growth factor receptor beta signaling is required for efficient epicardial cell migration and development of two distinct coronary vascular smooth muscle cell populations. *Circ Res*. 2008;103:1393–1401.
24. Mikawa T, Gourdie RG. Pericardial mesoderm generates a population of coronary smooth muscle cells migrating into the heart along with ingrowth of the epicardial organ. *Dev Biol*. 1996;174:221–232.
25. Red-Horse K, Ueno H, Weissman IL, Krasnow MA. Coronary arteries form by developmental reprogramming of venous cells. *Nature*. 2010;464:549–553.
26. Karsan A. Notch and integrin affinity: a sticky situation. *Sci Signal.* 2008;1:pe2.
27. High FA, Lu MM, Pear WS, Loomes KM, Kaestner KH, Epstein JA. Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. *Proc Natl Acad Sci U S A*. 2008;105:1955–1959.
28. Swift MR, Weinstein BM. Arterial-venous specification during development. *Circ Res*. 2009;104:576–588.
29. Morrow D, Guha S, Sweeney C, Birney Y, Walshe T, O'Brien C, Walls D, Redmond EM, Cahill PA. Notch and vascular smooth muscle cell phenotype. *Circ Res*. 2008;103:1370–1382.
30. Zamora M, Manner J, Ruiz-Lozano P. Epicardium-derived progenitor cells require beta-catenin for coronary artery formation. *Proc Natl Acad Sci U S A*. 2007;104:18109–18114.
31. Wu M, Smith CL, Hall JA, Lee I, Luby-Phelps K, Tallquist MD. Epicardial spindle orientation controls cell entry into the myocardium. *Dev Cell*. 2010;19:114–125.
32. Sridurongrit S, Larsson J, Schwartz R, Ruiz-Lozano P, Kaartinen V. Signaling via the Tgf-beta type I receptor Alk5 in heart development. *Dev Biol*. 2008;322:208–218.
33. Jin S, Hansson EM, Tikka S, Lanner F, Sahlgren C, Farnebo F, Baumann M, Kalimo H, Lendahl U. Notch signaling regulates platelet-derived growth factor receptor-beta expression in vascular smooth muscle cells. *Circ Res*. 2008;102:1483–1491.
34. Kurpinski K, Lam H, Chu J, Wang A, Kim A, Tsay E, Agrawal S, Schaffer DV, Li S. Transforming growth factor-beta and notch signaling

mediate stem cell differentiation into smooth muscle cells. *Stem Cells*. 2010;28:734–742.
35. Aoyagi-Ikeda K, Maeno T, Matsui H, Ueno M, Hara K, Aoki Y, Aoki F, Shimizu T, Doi H, Kawai-Kowase K, Iso T, Suga T, Arai M, Kurabayashi M. Notch induces myofibroblast differentiation of alveolar epithelial cells via TGF-ss/Smad3 pathway. *Am J Respir Cell Mol Biol*. In press.
36. Tang Y, Urs S, Boucher J, Bernaiche T, Venkatesh D, Spicer DB, Vary CP, Liaw L. Notch and transforming growth factor-beta (TGFbeta) signaling pathways cooperatively regulate vascular smooth muscle cell differentiation. *J Biol Chem*. 2010;285:17556–17563.
37. Noseda M, Fu Y, Niessen K, Wong F, Chang L, McLean G, Karsan A. Smooth muscle alpha-actin is a direct target of Notch/CSL. *Circ Res*. 2006;98:1468–1470.

Novelty and Significance

What Is Known?

- The embryonic epicardium is a source of trophic signals for the myocardium and a cellular source for the coronary vasculature and the fibrous skeleton of the heart.
- Notch signaling is a crucial regulator of common vasculogenesis.
- Transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF) signaling regulate smooth muscle (SM) differentiation of epicardium-derived cells (EPDCs).

What New Information Does This Article Contribute?

- In the mouse, the epicardium is a cellular source for cardiac fibroblasts and SMCs of the coronary arteries but not of cardiomyocytes and coronary endothelia.
- Notch pathway constituents are expressed during epicardial development.
- *Rbpj*-dependent Notch signaling is required for SMC differentiation of EPDCs in vivo.
- Notch signaling is sufficient to induce premature SMC differentiation of epicardial cells in vivo.
- Notch signaling acts upstream of TGF-β signaling and PDGFR-β expression in SMC differentiation of EPDCs.
- Notch and TGF-β signaling cooperate in SMC differentiation of EPDCs.
- PDGF signaling antagonizes Notch and TGF-β–induced SMC differentiation of EPDCs.

Coronary artery disease is a leading cause of death worldwide. The cellular and molecular programs that direct the formation of a functional coronary vasculature from simple precursors tissues are, however, only poorly understood. Here, we have identified fibroblasts and SMCs as the 2 cellular lineages derived from the epicardium and have uncovered Notch signaling as a major pathway required for SM differentiation of EPDCs in the mouse. Our study shows that epicardium is unlikely to be a cellular source of coronary endothelia, but that epicardial-derived signals play a crucial role in formation and elaboration of the coronary plexus. We identify TGF-β signaling as a downstream cooperator of Notch signaling in the development of the SM coating of the coronary arteries. Our results provide novel insight into the genetic and cellular pathways regulating the formation of the coronary vasculature that may aid in developing novel therapeutic avenues for cellular regeneration after coronary heart disease.