Notch Signaling Regulates Smooth Muscle Differentiation of Epicardium-Derived Cells

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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...
growth of the blood vessel network, proliferation of endothelial cells and vascular SMC differentiation. 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 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^flfr::Cre,J) mice, and mice with an insertion of the cre recombinase gene in the Tbx18 locus (Tbx18^cre::ACTB-DTomato::GFP^tm1,H9260, synonym: Tbx18^Cre),11 and mice with an insertion of the cre recombinase gene in the Tbx18 locus (Tbx18^Cre::ACTB-DTomato::GFP^tm1,H9260, synonym: Tbx18^Cre) were described previously. All mouse lines were maintained on an outbred (NMRI) background.

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 1). In whole E9.5 embryos, we detected strong expression of Hey1, weak expression of Jag1 and very weak expression of Notch1, Notch2 and Jag2 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, mostly 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 biphase 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::Actb-DTomato::Gfp^tm1,H9252 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 mice13 to demonstrate that cre expres-
tion 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<sup>cre</sup> 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 ground of membrane bound red fluorescence. Anti-GFP immunized by bright membrane bound GFP expression in a backsion of the venous and capillary marker endomucin (Emcn)<sup>19</sup> respectively.<sup>18</sup> We confirmed this notion by detecting expres-
sions in nuclei of venous but not of arterial endothelial cells.

To our surprise, Tbx18<sup>cre<sup>/</sup>+</sup>;Rbpj<sup>flox/flox</sup> 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.<sup>18</sup> We confirmed this notion by detecting expression of the venous and capillary marker endomucin (Emcn)<sup>19</sup> 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<sup>cre<sup>/</sup>+</sup>;Rbpj<sup>flox/flox</sup> 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)<sup>20</sup> 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<sup>cre<sup>/</sup>+</sup>;Rbpj<sup>flox/flox</sup> 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<sup>cre<sup>/</sup>+</sup>;Rbpj<sup>flox/flox</sup> embryos, histological analysis confirmed the integrity of the epicardial epithelium (Online Figure III). Expression of epicardial marker genes Wilms

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**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, endothelium; epi, epicardium; eth, endothelium; lv, left ventricle; myo, myocardium; peo, proepicardial organ; sm, smooth muscle cell layer; sub, subepicardial space.
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 Tbx18cre based genetic lineage tracing in wild-type and Rbpj mutant background using the Rosa26mTmG 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 Tbx18cre;Rbpjfoxflox hearts at this stage confirming that loss of epicardial Rbpj function does not affect epicardial EMT and EPDC immigration (Figure 3A). 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 Tbx18cre;Rbpjfoxflox 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 2. Defects of the coronary vasculature in mice with epicardial deletion of Rbpj. A through C, Analysis of coronary vessel formation in Tbx18cre;Rbpjfoxflox 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. Emmn 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.
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<sup>cre</sup> mediated expression of the Notch1 intracellular domain (NICD) from a Rosa26 knock-in allele (Rosa26<sup>NICD</sup>).<sup>11</sup> Tbx18<sup>cre</sup>/H11001; Rosa26<sup>NICD</sup>/H11001 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<sup>mTmG</sup> reporter in the Tbx18<sup>cre</sup>/Rosa26<sup>NICD</sup> 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<sup>cre</sup>/Rosa26<sup>NICD</sup> embryos at E12.5 (Figure 5A through 5D). Expression of integrin α4 (Itga4) that is required for myocardial attachment of epicardial cells<sup>21</sup> 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,
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+; Rosa26Nicked 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 Tgfβ Signaling and Pdgfrb in EPDCs**

Previous work identified a requirement of transforming growth factor β (Tgfβ) and platelet-derived growth factor β receptor (Pdgfrb) signaling in SM differentiation during coronary arteriogenesis in the mouse. To analyze the epistasis between these pathways and Notch signaling, we determined whether expression of the intracellular mediator of Tgfβ signaling, activated P-Smad2,3, and of Pdgfrb is affected by epicardial loss- or gain-of Notch signaling. In hearts of E18.5 Tbx18cre+; Rbpjfllox/lox 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+; Rosa26Nicked hearts 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 Tgfβ signaling and Pdgfrb expression in epicardium and EPDCs.

**Notch Cooperates With Tgfβ 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). TGFβ1 induced SM marker gene expression in wild-type epicardial cultures. The inhibitor of Tgfβ 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 TGFβ1

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 Tbx18cre+; Rosa26Nicked 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+; Rosa26Nicked 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.

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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 SM 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. 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, whereas 7% to 18% ventricular, atrial and IVS cardiomyocytes were noted as descendants of Wt1-positive epicardial cells in another study. 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 Tbx18cre approach based on the endogenous expression of Tbx18 in cardiomyocytes of the IVS and left ventricle from at least E10.5 in development. The Tbx18cre 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. 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 venous region of the postlooped heart. 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,
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. 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. Most likely, it is mediated by endothelial expression of Notch1, Dll1 and Dll4 as shown for large systemic arteries.

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. In fact, Notch1, Notch3, and Jag1 were identified as crucial
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 Pdgf pathway was implicated in proliferation and EMT of epicardial cells, Wnt signaling in oriented epicardial cell division, and Tgfb signaling both in epicardial EMT and later in SM differentiation of perivascular cells. 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 Pdgf expression in epicardial cells in vivo. Pdgf was reported as an immediate target of Notch function in vascular SMCs but regulation of Tgfb signaling by Notch has only been noted in SM differentiation of mesenchymal stem cells and myofibroblast differentiation of alveolar epithelial cells. Our findings suggest that NIDC induction of Tgfb signaling in EPDCs is mediated by robust activation of Tgfb1–3 transcription, similar to the findings in cultures of alveolar cells. Tang and coworkers recently demonstrated that Notch and Tgfb 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 Tgfβ1 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 Tgfβ1 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 Tgfβ 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.

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Disclosures
None.

References


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 partner 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.
Supplemental Online Data

Notch signaling regulates smooth muscle differentiation of epicardium-derived cells

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Supplemental Online Materials and Methods

Mice and genotyping
Embryos for Notch expression analysis were derived from matings of NMRI wild-type mice. Tbx18^{cre/+};Rbpj^{floxflox} mice were obtained from matings of Tbx18^{cre/+};Rbpj^{floxflox} males and Rbpj^{floxflox} females. Single heterozygous littermates were used as controls for mutant embryos. Tbx18^{cre/+};Rosa26^{NICD/+} mice were obtained from matings of Tbx18^{cre/+} males and Rosa26^{NICD/+} females. The fate of epicardial cells was analyzed in Tbx18^{cre/+};Rosa26^{mTmG/+} embryos (obtained from matings of Tbx18^{cre/+} and Rosa26^{mTmG/+} mice), in Tbx18^{cre/+};Rosa26^{mTmG/+};Rbpj^{floxflox} embryos (obtained from matings of Tbx18^{cre/+};Rosa26^{mTmG/+};Rbpj^{floxflox} males and Rbpj^{floxflox} females) and in Tbx18^{cre/+};Rosa26^{NICD/NICD} mice (obtained from matings of Tbx18^{cre/+}Rosa26^{mTmG/+} males and Rosa26^{NICD/NICD} females). For timed pregnancies, vaginal plugs were checked in the morning after mating, noon was taken as embryonic day (E) 0.5. Embryos were harvested in PBS, fixed in 4% paraformaldehyde overnight and stored in 100% methanol at –20°C before further use. Wildtype littermates were used as controls. Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR (protocols upon request). H. Hedrich, state head of the animal facility, approved the care of animals and experiments at Medizinische Hochschule Hannover.

Epicardial cell culture
To obtain cultures of primary epicardial cells, E11.5 hearts were dissected and half of one right ventricle plated onto a gelatin-coated dish in serum-free DMEM supplemented with 2mM Glutamax, 100 units/ml Penicillin, 100 µg/ml Streptomycin (Gibco). Epicardial cells migrated from the ventricle onto the dish and formed an epithelial monolayer. After 2 days, the ventricle was carefully removed using forceps. The highly enriched epicardial cells were further cultured in DMEM/Panserin401 with or without 10% FCS, 250 pM recombinant human TGFb1 (#100-21, PeproTech or R&D Systems), recombinant human PDGF-BB (250pM, Cell Signaling) and the Tgf receptor 1 (Alk5) inhibitor SB431542 (2.5 µM, Sigma).

Histological analysis
For histological stainings embryos were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned to 4 or 10 µm. Sections were stained with haematoxylin and eosin, following standard procedures.

Semi-quantitative reverse Transcription PCR
For expression analysis of Notch pathway components 18 wildtype (NMRI) epicardial cell cultures were pooled. We used E16.5 embryonic lungs as a positive control in this experiment since this tissue is known to express all Notch pathway components.1-3 For analysis of smooth muscle differentiation 2-4 cultures were combined. 2-5 individual pools of each experiment were used for RT-PCR analysis. Total RNA was extracted with RNAPure reagent (Peqlab) and DNasel treated for 30min at 37°C. RNA was reverse transcribed with RevertAid H-minus M-MuLV Reverse Transcriptase (Fermentas). For semi-quantitative PCR, the number of cycles was adjusted to the mid-logarithmic phase. Quantification was performed with Quantity One software (Bio-Rad). Normalization was against Gapdh. Assays were performed at least twice in duplicates. P-values were calculated using the unpaired two tailed Student's t-Test. Primers and PCR conditions are available on request.
**In situ** hybridization analysis

*In situ* hybridization analysis of whole embryos and of sections with digoxigenin-labeled antisense riboprobes followed published protocols.\(^4,^5\) Details of used probes upon request.

**Immunodetection of proteins**

For immunohistochemistry rabbit polyclonal antibody against Wt1 (1:200, C-19, Santa Cruz), goat polyclonal antibody against Tbx18 (1:200, C-20, Santa Cruz), rabbit polyclonal antibody against ZO1 (Tjp1) (1:200, 61-7300, Zymed Laboratories Inc.), monoclonal antibody against CD31 (Pecam1) (1:50, 550274, BD Pharmingen), rabbit polyclonal antibody against GFP (1:200, sc-8334, Santa Cruz), monoclonal antibody against GFP (1:200, 11814460001, Roche), goat polyclonal antibody against P-Smad2/3 (Ser433/435) (1:50, sc-11769, Santa Cruz), monoclonal antibody against alpha-Smooth muscle actin (Acta2), Cy3 Conjugate (1:200, C 6198, Sigma), monoclonal antibody against alpha-Smooth muscle actin (Acta2), FITC Conjugate (1:200, F3777, Sigma), goat polyclonal antibody against cardiac troponin I (Tnni3) (1:200, 4T21/2, HyTest), monoclonal antibody against heavy chain cardiac myosin (Myhc) (1:200, ab15, Abcam), rabbit polyclonal against SM22alpha (Tagln) (1:200, ab14106, Abcam), goat polyclonal antibody against ephrinB2 (Efnb2) (1:200, AF496, R&D Systems), monoclonal antibody against CoupTFII (Nr2f2) (1:100, PP-H7147-10, R&D Systems), rat monoclonal antibody against endomucin (Emcn) (1:2, a kind gift of D. Vestweber, MPI Münster; Germany), rabbit polyclonal antibody against Notch3 (1:200, ab23426, Abcam), rabbit polyclonal antibody against periostin (Postn) (1:50, ab14041, Abcam), rabbit polyclonal antibody against Delta-like1 (Dll1) (1:100, H-265, sc9102, Santa Cruz), rabbit polyclonal antibody against Vegf (Vegfa) (1:100, sc-507, Santa Cruz), rabbit polyclonal antibody against eNOS (Nos3) (1:200, Affinity BioReagents), rabbit polyclonal antibody against bovine aortic smooth muscle myosin (BASM, smooth muscle myosin heavy chain 204/200, smMHC) (1:200, a kind gift of R. Adelstein, Bethesda, USA), rabbit polyclonal antibody against collagen type IV (1:200, #AB756P, Millipore Corp.) and rabbit monoclonal antibody against Pdgf receptor beta (1:100, #3169, Cell Signaling) were used as primary antibodies. Alexa488 goat-anti-rabbit (Invitrogen, 1:250), Alexa488 donkey-anti-mouse (Invitrogen, 1:200), Rhodamin goat-anti-rabbit (Dianova, 1:200), Red-X donkey-anti-mouse (Dianova, 1:200), R-PE em575 swine-anti-goat (Invitrogen, 1:200), biotinylated donkey-anti-goat (Dianova, 1:200), biotinylated goat-anti-mouse (Dianova, 1:200), biotinylated goat-anti-rat (Dianova, 1:200), DyLight 488-conj. Fab fragment donkey-anti-rabbit (Dianova, 1:200) and biotinylated goat-anti-rabbit (Dianova, 1:200) were used as secondary antibodies. Nuclei were stained with 4,6-Diamidino-2-phenylindol (DAPI) (Roth). For antibodies against Tbx18, P-Smad2/3, Efnb2, Emcn, Notch3, Postn, Dll1, Vegfa, Nr2f2, Nos3 and Pdgf receptor beta, paraffin sections were pressure cooked for 3 min in antigen unmasking solution (H-3300, Vector Laboratories Inc). The signal was amplified using the Tyramide Signal Amplification (TSA) system from Perkin-Elmer (NEL702001KT, Perkin Elmer LAS). For double staining with GFP, secondary antibody was added during the biotinylated secondary antibody step of the TSA protocol.

**Proliferation assay**

Epicardial cell cultures obtained from explants of E11.5 right ventricles were incubated for 4h with BrdU (5-Bromo-2´-deoxyuridine, 0.05 mg/ml) and fixed in methanol. Cultures were treated with mouse-on-mouse blocking reagent according to the manufacturer (VectorLabs) before the antigen was detected by successive incubations with anti-BrdU antibody (1:200, Roche), biotinylated anti-mouse IgG (Vector-Labs), and VectastainABC (VectorLab) reagent. The detection reaction was performed using diaminobenzidine and hydrogen peroxide as substrates. Nuclei were stained with 4,6-Diamidino-2-phenylindol (DAPI) (Roth). The BrdU-labeling rate was defined as the number of BrdU-positive nuclei relative to the total number of nuclei as detected by DAPI counterstain on four epicardial explant cultures each of wildtype and Tbx18<sup>Cre/</sup>:Rosa<sup>26Nicd14</sup> hearts. The mean values and standard deviation were determined and the Student’s t-test was performed to test for statistical relevance.
Documentation
Sections and Immunofluorescence were photographed using a Leica DM5000 microscope with Leica DFC300FX digital camera. Immunofluorescence of cells was documented using a Leica DMI6000B microscope with Leica DFC350FX. All images were processed in Adobe Photoshop CS.

References
Multiple genes encoding Notch receptors and ligands as well as Notch target genes are expressed in epicardial development. *In situ* hybridization analysis of Notch receptor (*Notch1-4*), Notch ligand (*Dll1*, *Dll3*, *Dll4*, *Jag1*, *Jag2*) and Notch target gene expression (*Hey1*, *Hey2*, *HeyL*) in the whole hearts of E9.5 embryos (first column), and in the E10.5 epicardium, in subepicardial cells at E12.5, in forming coronary vessels at E14.5, and in coronary arteries at E18.5 on transverse sections of the heart. Black rectangles indicate the region shown in higher magnification images to the right. This analysis reveals two waves of Notch expression and Notch signaling during epicardial development, from E9.5 to E10.5 in the proepicardium and early epicardium; from E12.5 onwards in endothelial and perivascular cells of the forming coronary arteries. a, atrium; avc, atrioventricular canal; ca, coronary artery; endo, endocardium; epi, epicardium; eth, endothelium; ivs, interventricular septum; myo, myocardium; lv, left ventricle; peo, proepicardial organ; rv, right ventricle; sm, perivascular smooth muscle cell layer; sub, subepicardial space.
**Online Figure II.** *Tbx18cre* mice - a tool for visualization, and manipulation and tracing of epicardial cells by genetic recombination. (A) *In situ* hybridization analysis of *Tbx18* expression during murine heart development. *Tbx18* expression is detected in the proepicardial organ at E9.5, and from E10.5 to E14.5 in the epicardium, the sinus horns, the interventricular septum and the left ventricular myocardium. (B) Comparison of *Tbx18* expression in whole E9.5 and E10.5 embryos as detected by *in situ* hybridization analysis and GFP reporter fluorescence in whole *Tbx18cre/+;Rosa26mTmG/+* embryos of the same stages. The *Rosa26mTmG* reporter provides red fluorescence in unrecombined cells, and green fluorescence after cre mediated recombination. Note the perfect overlap of *Tbx18* expression and GFP activity from the reporter. (C) Anti GFP immunofluorescence on sections of the developing heart at the indicated stages from *Tbx18cre/+;Rosa26mTmG/+* embryos. Note the perfect match of GFP and *Tbx18* expression as shown in (A) and the presence of epicardium-derived cells in the right ventricular myocardium at E14.5. White rectangles indicate areas of the heart that are magnified in the images to the right. a, atrium; avc, atrioventricular canal; cm, cranial paraxial mesoderm; epi, epicardium; h, heart; im, intermediate mesoderm; ivs, interventricular septum; li, limb bud; lv, left ventricle; peo, proepicardial organ; ra, right atrium; rv, right ventricle; sh, sinus horn; so, somite.
Online Figure III. Coronary endothelia are not affected by epicardial loss of Rbpj at E14.5. Histological analysis by haematoxylin/eosin (HE-) staining and immunofluorescence analysis of endomucin (Emcn) expression of transverse sections of wildtype (wt) and Tbx18^{cre/+};Rbpj^{flox/lox} hearts show that subepicardial blood vessels are distributed normally at this stage. endo, endocardium; epi, epicardium; lv, left ventricle; rv, right ventricle.
Online Figure IV. Loss of *Rbpj* does not affect epicardial identity and integrity. *In situ* hybridization analysis of epicardial marker genes in transverse section of the heart of E14.5 wildtype (wt) and *Tbx18cre/+;Rbpjflox/flox* embryos. Epicardial expression of all marker genes is maintained in the mutant. a, atrium; epi, epicardium; ivs, interventricular septum; lv, left ventricle; rv, right ventricle.
Online Figure V. cSMCs are absent in hearts with epicardial loss of Rbpj. (A-F) Immunofluorescence analysis of Acta2 and Notch3 expression in transverse heart sections of wildtype (wt) and $Tbx18^{cre/+};Rbpj^{flox/flox}$ embryos at E18.5. (A,D) Overview of the heart with white rectangles to indicate the magnified region. (B,C,E,F) Magnified images of the heart with white rectangles to indicate the magnified region. Acta2 expression is completely lost in the mutant, whereas Notch3 is still found in cells surrounding coronary arterial endothelial cells. lv, left ventricle; rv, right ventricle.
Online Figure VI. Epicardium specific activation of Notch signaling in \textit{Tbx18}^{cre+/+};\textit{Rosa26}^{NICD\text{tmG}} embryos. Analysis of expression of Notch target genes \textit{Hey1}, \textit{Hey2}, \textit{HeyL} and \textit{Notch3} in hearts of E14.5 wildtype (wt) and \textit{Tbx18}^{cre+/+};\textit{Rosa26}^{NICD\text{tmG}} embryos by \textit{in situ} hybridization analysis on transverse sections. \textit{Hey2}, \textit{HeyL} and \textit{Notch3} are specifically induced in the epicardium of \textit{Tbx18}^{cre+/+};\textit{Rosa26}^{NICD\text{tmG}} hearts (arrows). Rectangles indicate regions that are magnified in the images on the right side.
Online Figure VII. NICD expression induces SM differentiation of epicardial cells. Cellular analysis of epicardial outgrowths of explants of right ventricles of wildtype (wt) and Tbx18cre/+;Rosa26NICD/+ embryos. After two days of culture under serum-free conditions in DMEM the ventricle was removed and the culture continued for two more days in 50% DMEM/50%Panserin under serum-free conditions. Immunofluorescence analysis of expression of the smooth muscle proteins Acta2, the tight junction protein Tjp1 (ZO1), and the epicardial transcription factors Wt1 and Tbx18. Shown are whole explants and magnified regions (white rectangles) with nuclear counterstaining (DAPI). Acta2 expression is upregulated and relocalized from the cortical region into stress fibers, Tjp1 is localized from the cell membranes into the cytoplasm, Tbx18 is lost and Wt1 relocalized to the cytoplasm in epicardial cells overexpressing NICD. Together, this shows that epicardial cells underwent EMT and differentiated into SMCs.
Supplemental Online Table I

The table contains mean values and standard deviations (SD) as well as calculated P-values for Fig. 8. Values refer to indicated columns of the graph. Statistical significance is assumed if the P-value is below 0.05. Significant changes are indicated in bold.

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<th>Tagln</th>
<th>Acta2</th>
<th>P-value compared with wt</th>
<th>Tagln</th>
<th>Acta2</th>
<th>P-value compared with wt + TGFb1</th>
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<tr>
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<td>1.44 ± 0.49</td>
<td>0.7130</td>
<td>0.9939</td>
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<tr>
<td>wt + PDGF-BB</td>
<td>1.04 ± 0.24</td>
<td>1.00 ± 0.23</td>
<td>0.7130</td>
<td>0.9939</td>
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<td></td>
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<tr>
<td>wt + TGFb1 + PDGF-BB</td>
<td>0.85 ± 0.29</td>
<td>1.47 ± 0.54</td>
<td>0.2764</td>
<td>0.1036</td>
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<tr>
<td>NICD</td>
<td>2.18 ± 0.49</td>
<td>2.73 ± 0.63</td>
<td>0.0010</td>
<td>0.0049</td>
<td>P-value compared with NICD</td>
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Online Table I.