Mobilization and differentiation of epicardial cells in murine development

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Grades

Doktor der Naturwissenschaften
(Dr. rer. nat.)

genehmigte Dissertation von
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geboren am 15.11.1977 in Winsen/Luhe

2013

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Tag der Promotion:	24.01.2013

Medizinische Hochschule
Hannover

Angefertigt am

Institut für Molekularbiologie

der Medizinischen Hochschule Hannover

unter der Betreuung von

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Für meine Mutter.

Summary

The murine epicardium is a monolayered epithelium that covers the outer surface of the heart. It protects the underlying myocardium and enables the frictionless movement of the beating heart within the pericardial cavity. During embryonic development the epicardium adding cells for the formation of the coronary vessels and the fibrous heart skeleton. Furthermore, the epicardium is a source of trophic signals for the development and maturation of the myocardium. The epicardium is derived from an extracardiac precursor cell population that is positioned at the venous pole of the linear heart tube of the early embryo. Cells of this proepicardium attach to the myocardial surface and form a contiguous epithelial lining within a short period of time. Single cells of this epicardium undergo an epithelial-mesenchymal transition (EMT), invade into the myocardium and differentiate terminally. In chick, cell lineage tracing experiments identified smooth muscle cells, perivascular and interstitial fibroblast as epicardium-derived cells (EPDCs) as well. In some reports an additional coronary endothelial cell fate of EPDCs has been indicated. Two independent genetic (cre/loxP-mediated) lineage tracing studies in mouse confirmed that the epicardium is a significant source of coronary smooth muscle cells and cardiac fibroblasts as well. A contribution of EPDCs to the cardiomyocyte lineage was reported in the mouse, but not in other vertebrates.

To unambiguously identify the fate of epicardial cells, an alternative genetic lineage tracing approach was to be performed using a *cre* knock-in allele of the *T-box gene 18* (*Tbx18*). Specific recombination in the right ventricular epicardium allowed to evaluate the final fates of EPDCs. Smooth muscle cells, perivascular and interstitial fibroblasts were confirmed as epicardium-derived. Re-evaluation of a *cre* knock-in allele of the *Wilms Tumor 1* gene (*Wt1*), which was previously used to confirm epicardial origin of cardiomyocytes, showed ectopic recombination within the heart. Thus, this mouse line cannot be used for epicardium-specific lineage tracing analysis and conditional knock-outs.

Furthermore, signaling pathways implicated in the differentiation and the mobilization of EPDCs was analyzed or re-evaluated. Conditional alleles of central mediators of these signalling pathways were analyzed using the $Tbx18^{cre}$ line.

With this approach the requirement of the Notch signalling pathway for the differentiation of perivascular smooth muscle cells and the necessity of Pdgfrα signalling for EMT and fibroblast differentiation was shown. However, the epicardium-specific loss of Ctnnb1, Fgfr1/2, Shh and the Hh receptor Smoothened did not affect the mobilization and differentiation of EPDCs. In fact, genetic gain-of-function experiments showed that the activation of the canonical Wnt and Hh pathways are deleterious for normal epicardial development.

In an independent project, the importance of Upk3b for the efficient sealing of luminal spaces was adressed in the mesothelium and urothelium. An *Upk3b* knock-out mouse was generated by homologous recombination in embryonic stem cells and subsequent injection into blastocysts. Neither cellular nor molecular changes where observed in the urothelia and mesothelia of these mutants. Thus, the loss of Upk3b did not reveal a specific function during embryogenesis and in homeostasis.

Keywords: murine heart development, lineage tracing, *Wt1*, *Tbx18*, epicardium, Ctnnb1, Pdgfrα, Shh, Fgfr

Zusammenfassung

Das Epikard ist ein einschichtiges Epithel, das dem Herzen von Wirbeltieren aufliegt. Es schützt das Myokard und ermöglicht die reibungsarme Bewegung des Herzens innerhalb des Herzbeutels (Perikard). Während der Embryonalentwicklung leistet das Epikard einen zellulären Beitrag zur Bildung des Koronargefäßsystems und des fibrösen Herzskeletts. Weiterhin sezerniert das Epikard trophische Faktoren, die für die Entwicklung und Reifung des unterliegenden Myokards essentiell sind. Das Epikard leitet sich aus einer extrakardialen Vorläuferzellpopulation ab, die mit Beginn der Schlaufenbildung des ursprünglich linearen Herzschlauches des frühen Embryos auf dem Septum transversum zu finden ist. Einzelne Zellen dieses so genannten Proepikards verlassen daraufhin den Zellverband, adhärieren am Myokard und bilden das einschichtige Epikard. Durch einen epithelial-mesenchymalen Übergang (EMT) werden einzelne Zellen des Epikards mobilisiert und wandern in den subepikardialen Raum und das Myokard ein. Zellschicksalsanalysen im Hühnchen identifizierten glatte Muskelzellen, perivaskuläre und interstitielle Bindegewebszellen des Herzens als Differenzierungsprodukte epikardial abgeleiteter Zellen (epicardium-derived cells - EPDCs). Zwei unabhängige, genetisch vermittelte (cre/*loxP*) Zellschicksalsanalysen, unter Verwendung der regulatorischen Sequenzen von *Tbx18* oder *Wt1,* bestätigten den epikardialen Ursprung kardiale Bindegewebszellen und glatter Muskelzellen in der Maus. Zusätzlich wurde die Differenzierung von EPDCs in Kardiomyozyten in der Maus beschrieben, wenngleich dies nicht in anderen Wirbeltieren bestätigt werden konnte.

Für die Beurteilung des endgültigen Schicksals epikardialer Zellen wurde eine alternative genetische Zellschicksalsanalyse mittels eines cre basierten Ansatzes durchgeführt. Hierbei wurde die regulatorische Sequenz des *T-box genes 18* (*Tbx18*) für die zellspezifische Expression der Cre-Rekombinase verwandt. Die spezifische Rekombination im Epikard des rechten Ventrikels erlaubte eine Zellschicksalsanalyse und es konnte bestätigt werden, dass glatte Muskelzellen, perivaskuläre und interstitielle Bindegewebszellen des Herzens epikardialen Ursprungs sind. In einer weiteren Studie konnte ich zeigen, dass die, in früheren Arbeiten zur Bestätigung des Kardiomyozytenschicksals epikardial abgeleiteter Zellen verwendete $Wt1^{cre}$ Linie aufgrund ektopischer Rekombination innerhalb des Herzens nicht geeignet ist das Schicksal epikardialer Zellen zu verfolgen oder zu manipulieren. Im Weiteren wurden Signalwege für die Differenzierung und Mobilisierung epikardialer Zellen in der Embryonalentwicklung untersucht beziehungsweise verifiziert. Hierzu wurden konditionelle Allele essentieller Komponenten einzelner Signalwege unter Verwendung der $Tbx18^{cre}$ Mauslinie untersucht. Diese Untersuchen zeigten, dass der Notch-Signalweg für die Differenzierung von perivaskulären glatten Muskelzellen und der Pdgfrα Signalweg für die EMT und Differenzierung von Bindegewebszellen notwendig sind. Im Gegensatz dazu

konnte bei der Analyse der Epikard-spezifischen Deletion von Ctnnb1, Fgfr1/2, Shh und des Hh Rezeports Smo keine Mobilisierungs- und Differenzierungsdefekte der EPDCs nachgewiesen werden. Vielmehr ergaben weitere Untersuchungen, dass die Aktivierung sowohl des kanonischen Wnt als auch des Hh Signalwegs schädlich für die epikardiale Entwicklung sind.

In einem unabhängigen Projekt sollte der Beitrag von Upk3b zur Barrierefunktion des Mesothels sowie des Urothels untersucht werden. Mittels homologer Rekombination in embryonalen Stammzellen der Maus und anschließender Bastozysteninjektion wurden Upk3b-Verlustmutanten generiert. Deren Analyse ergab jedoch keine zellulären und molekularen Veränderungen des Urothels und des Mesothels. *Upk3b* besitzt somit keine einzigartige Funktion in der Embryonalentwicklung und Homöostase von Urothelien und Mesothelien.

Schlagworte: murine Herzentwicklung, Zellschicksalsanalyse, *Wt1*, *Tbx18*, Epikard, Ctnnb1, Pdgfrα, Shh, Fgfr

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Introduction

The heart is a highly efficient muscle, pumping blood through the whole body all life long. Four chambers are separated by septa and valves to allow a unidirectional blood flow. The right ventricle receives the venous blood from the body and pumps it to the lungs, where it is oxygenated. The blood returns to the left atrium, which empties into the left ventricle and pumps the blood through the aorta into the arterial circuit of the body. The heart is the first organ to become functional in the developing embryo, as it is essential for the embryonic oxygen and nutrition supply. The developmental process to form a highly complex organ, whilst keeping up the constant blood circulation, is a tightly regulated process. Orchestration of important transcription factors and control of critical signaling pathways are vital for normal heart formation. Inherited heart malformations, i.e. alterations in developmental programs, affecting approximately one in hundred live births are and most often treated by surgical interventions to prevent postnatal death. Additionally, acquired heart diseases, i.e. myocardial infarction, are one of the main causes of death in industrialized nations.[1] The irreversible loss of cardiomyocytes and coronary vasculature in addition to the formation of scar tissue in the affected area, compromises heart function. Therapeutic approaches for acquired heart diseases are in crucial need of a sophisticated knowledge about the origin, generation and integration of cardiomyocytes. Curative therapies to regain the lost heart muscle are not available up to date, but are a topic of ongoing research.

Cardiac development

At embryonic day (E) 7.0-7.5, cardiac development in mice starts with the formation of two groups of cells located on either side of the embryonic midline directly below the head folds (Figure 1A). These groups extend across the midline, merge and form the cardiac crescent around E7.75. The cardiac cells coalesce in the ventral midline to form a linear heart tube until E8.25. It consists only of an endocardial lining and the myocardium, separated by a layer of extracellular matrix. From E8.25-10.5, the tubular heart elongates further and undergoes a complex looping process to position the atrium and caval veins in a - relative to the ventricle - dorso-cranial position. Moreover, the chambers emerge by ballooning out of the heart tube and the separation process starts by the formation of endocardial cushions and the interventricular septum. Additional remodeling processes result in the formation of the four chambered architecture of the mammalian heart (Figure 1).[2,3]

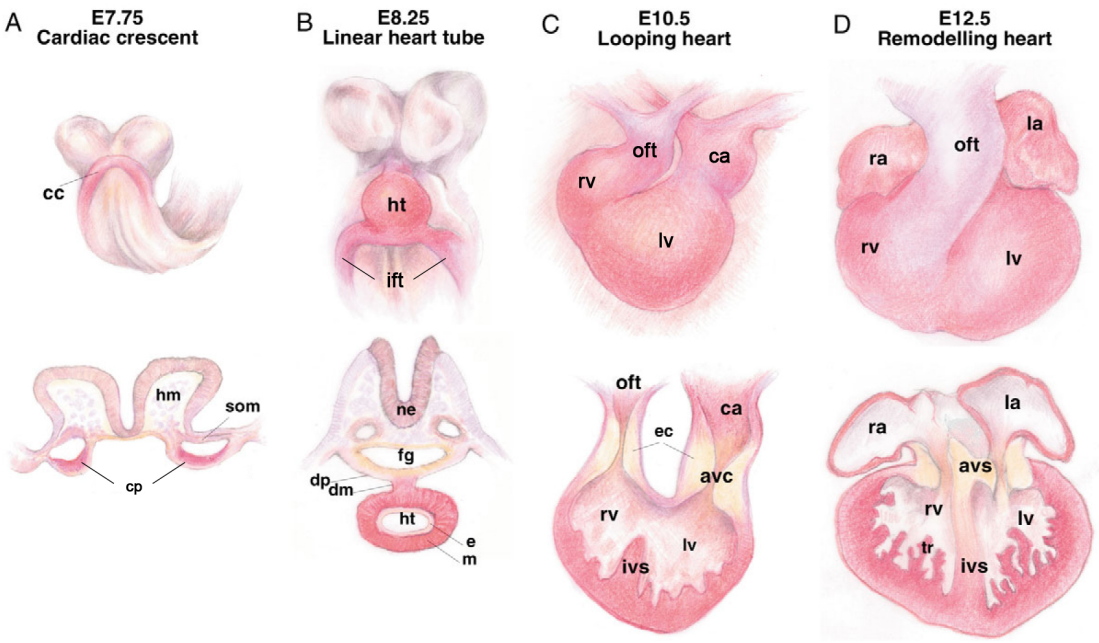

Figure 1 Schematic illustration of murine heart development

A-D, Anatomical drawings of consecutive stages of cardiac development at E7.75**(A)**, E8.25**(B)**, E10.5**(C)** and E12.5**(D)**. The upper panel shows whole embryos **(A, B)** and whole hearts **(C, D)** in a ventral view, cranial up. Corresponding sections are depicted below. See main text for additional information. avc, atrioventricular canal; avs, atrioventricular septum; ca, common atrium; cc, cardiac crescent; cp, cadiac progenitor; dm, dorsal mesocardium; dp, dorsal pericardium; e, endocardium; fg, foregut; hm, head mesoderm; ift, inflow tract; ivs, inter-ventricular septum; la, left atrium; lv, left ventricle; m, myocardium; ne, neural epithelium; oft, outflow tract; ra, right atrium; rv, right ventricle; som, somatic mesoderm; tr, trabeculae. Modified from (Stennard FA and Harvey RP,2005)[3].

The epicardium: A crucial source of cells and signals

The early linear heart is a simple tube consisting only of an endocardial lining and the myocardium. However at later stages in development the heart comprises a three-layered architecture. At that time the myocardium is covered by a mono-layered epicardium, being the outermost epithelium of the heart. The epicardium is a derivative of an extracadiac precursor cell population, known as the proepicardium. This transitory cell cluster can morphologically be identified as cuboidal cells on the sinus horns, facing towards the pericardial cavity, when cardiac looping is well under way.[4–7] These mesothelium-derived cells traverse the pericardial cavity and attach to the myocardium (Figure 2A). This second colonization of the heart is a feature found in all vertebrates. Nevertheless, specific differences between vertebrate classes can be seen. The process of cell transfer to the heart

is either a direct migration onto the heart via a matrix-rich tissue bridge (birds, amphibians and the rat)[8–10] or a release of free floating vesicles (mouse)[5,11,12] which pass the pericardial cavity establishing epicardial islands on the surface of the heart. Proliferation and migration of these cells results in the formation of a self-contained epicardium (Figure 2B). Underneath the epicardium forms a subepicardial mesenchyme, rich in extracellular matrix.[13] This collagen- and proteoglycan-containing milieu accumulates growth factors and provides a basis for the ingrowth of coronary vessels.[14,15] Further in development, individual cells of the epicardium undergo an epithelial-mesenchymal transition (EMT), leave the epithelial contiguity and either populate the subepicardial space or migrate into the compact myocardial wall (Figure 3).[13,16] During EMT, the cells loose their epithelial characteristics, the actin cytoskeleton reorganizes from an epithelial cortical alignment associated with cell-cell junctions into actin stress fibers that are anchored to focal adhesion complexes. These cells acquire a migratory behavior.[17] Mechanical or genetic ablation of the epicardium impairs myocardial growth, coronary vessel development and formation of cardiac interstitial cells.[9,18–22] Thus, the epicardium directly contributes to multiple cardiac lineages through EMT. In chick, cell lineage tracing experiments based on retroviral labeling identified smooth muscle cells, perivascular and interstitial fibroblasts as epicardium-derived cells (EPDCs).[10,16,23,24] An additional coronary endothelial fate of EPDCs has been indicated in some reports as well.[25,26] In mouse, cell lineage tracing analyses revealed contrasting results concerning the coronary endothelial fate of EPDCs. Endothelia of the coronary vessels where shown to arise from dedifferentiated angiogenic sprouts of the sinus venosus endothelium - the vein that returns blood to the embryonic heart. Furthermore, an endocardial origin of a subpopulation of coronary endothelia was hypothesized.[27]Two independent lineage tracing studies identified the murine epicardium as a relevant source of cardiac muscle for the developing mouse heart. A variable contribution of EPDCs to the cardiomyocyte lineage was observed, based on either the *Tbx18* or *Wt1* regulatory sequences, which permanently mark the developing epicardium with a genetic label.[28,29] Intensive epicardial proliferation and incorporation of EPDCs into the regenerating heart of zebrafish, which posses the outstanding ability for myocardial regeneration, have positioned the epicardium as an attractive potential target for effecting cardiac muscle regeneration.[30,31] Even though this natural regenerative capacity is not present in higher vertebrates, the enormous medical interest in a potential myocardial fate of epicardial cells in mammals forces to define their contributions in the settings of embryonic heart development and adult heart regeneration.

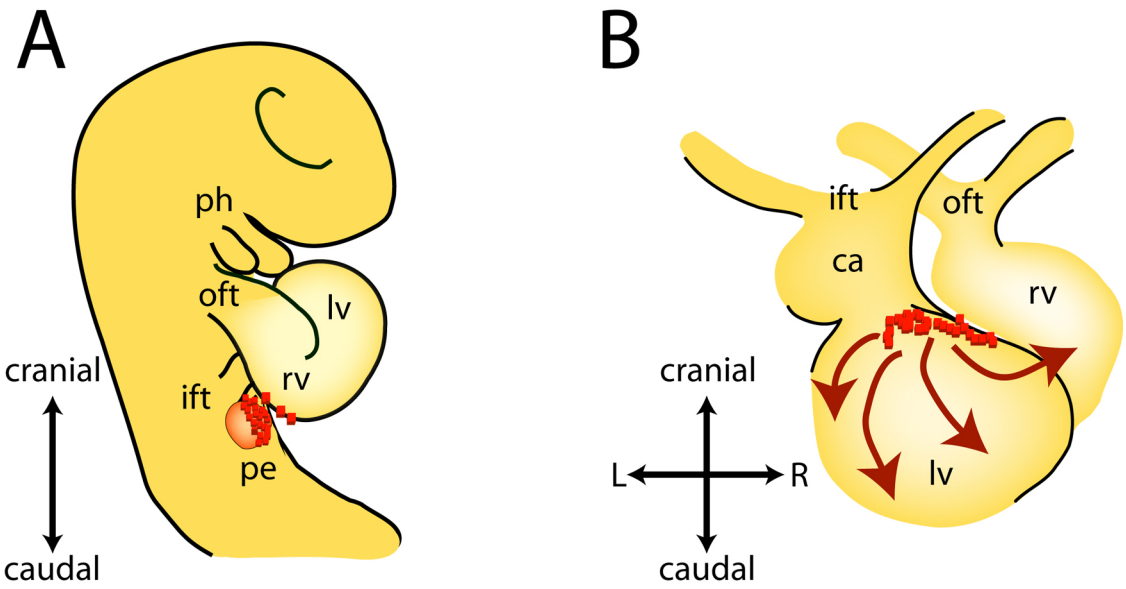

Figure 2 Proepicardial cells migrate onto the surface of the embryonic heart.
Schematic illustrations of (A) an E9.25 embryo, lateral view, and (B) an E9.75 isolated heart; dorsal view. The proepicardium (red) provides cells that attach to the surface of the embryonic heart at the region of the atrioventricular groove and later migrate until they completely cover the heart. ca, common atrium; ift, inflow tract; L, left; lv, left ventricle; oft, outflow tract; pe, proepicardium; ph, pharyngeal arches; R, right; rv, right ventricle.

Transcriptional regulation of epicardium development

The genetic control of epicardial EMT is carefully regulated and several transcription factors are expressed in the proepicardium and epicardium.

Wt1 is expressed in the (pro-)epicardium, the developing genitourinary system, and in the mesothelia covering the visceral organs.[32] Epicardial EMT is dramatically impaired in Wt1-null hearts[19,33,34] and canonical Wnt signaling was reduced, most likely due to a dysregulation of epicardial cell polarity.[35] *Wt1* inactivation also markedly decreases expression of *Raldh2* in the epicardium,[34,36] and thereby suppresses the differentiation of meschenchymal cells. Thus, *Wt1* interferes with epicardial EMT.

Epicardial EMT is also regulated by the *transcription factor 21 (Tcf21/capsulin/Pod1/epicardin). Tcf21* is expressed in mesenchymal cells of the respiratory, digestive, urogenital and cardiovascular system.[37–40] *Tcf21* null embryos fail to develop cardiac fibroblasts, as the *Tcf21*$^{+}$ subpopulation of epicardial cells cannot undergo EMT.[41] This is the first analysis which points to an epicardial cell fate decision (smooth muscle cells vs. fibroblasts) in the epicardium, before the process of EMT.

The *T-box-transcription factor 18 (Tbx18)* is expressed in the anterior part of the somites, the limb buds, the ureteric mesenchyme, the periotic mesenchyme and the (pro-)epicardium.[42–45]

Interestingly, the transcriptional repressor *Tbx18* is dispensable for epicardial development and the loss of *Tbx18* does not interfere with the mobilization and differentiation of EPDCs.[46]

Mobilization and differentiation of epicardial cells

Our knowledge about epicardial development increased in the recent years. Some signaling pathways were implicated to be involved in epithelial-mesenchymal transition.

Wnt (wingless-related MMTV integration site) signaling pathways regulate gene expression, proliferation, cell fate decisions, and cell polarity during embryonic development. Wnt proteins are secreted ligands that bind to Frizzled (Fzd) receptors, thereby stabilizing the protein beta-catenin (Ctnnb1). Ctnnb1 is the central mediator of canonical Wnt signaling, inducing transcription of target genes (such as *Axin2*), by forwarding Wnt signals to the TCF/LEF (Transcription factor/Lymphoid enhancer-binding factor) complex in the nucleus. During heart development, canonical Wnt signaling is tightly controlled to allow specification, self-renewal, and differentiation of various cardiac progenitor cell populations.[47]
A functional requirement of the canonical (Ctnnb1-dependent) Wnt-signaling pathway in epicardial EMT was shown by the paucity of EPDCs in embryos with epicardium-restricted Ctnnb1 inactivation.[34,48] Ctnnb1 is also an important component of intercellular adhesion complexes, and disruption of these complexes may contribute to the loss-of-function phenotype. Furthermore, EMT requires Ctnnb1 to establish epicardial cell polarity, direct mitotic spindle orientation, and thus interferes with the migratory behavior of EPDCs.[35] In addition to the reduced mobilization of EPDCs the differentiation of epicardium-derived coronary smooth muscle cells was impaired indicating for an involvement of Ctnnb1 in both events.[48]

Pdgfrα (platelet-derived growth factor receptor tyrosine kinase alpha) is important for embryonic development; driving the proliferation of undifferentiated mesenchyme, cell migration and angiogenesis.[49] The epicardium expresses Pdgfrα and a global or epicardium-restricted inactivation of Pdgfrα results in a cardiac fibroblast formation defect. In addition, Pdgfrα mutants show a reduced number of EPDCs within the myocardium, and thus this signaling pathway is involved in epithelial-mesenchymal transition.[50]

Fgfr1 and 2 (fibroblast growth factor receptor 1 and 2) are key players in the processes of proliferation and differentiation during embryonic development and often show functional redundancy.[51] Fgf signaling promotes angiogenesis by endothelial tube formation.[52] A epicardium-restricted combined inactivation of Fgfr1 and 2 results in a reduced number of EPDCs within the myocardium and a reduction in cardiac fibroblasts as well.[53] In addition,

epicardial and endocardial Fgf signals have been shown to induce the hedgehog signaling pathway.[54]

Hh (Hedgehog) signaling regulates coronary vascular formation and promotes neovascularization in the adult heart.[54,55] Hh signaling has been shown to promote the proliferation of adult stem cells from various tissues.[56,57] All three ligands (sonic, desert, indian hedgehog) are interchangeable, bind to the patched-receptors and induce the unique downstream effector smoothened (Smo). Epicardium-derived Fgf-signals have been shown to induce sonic hedgehog (Shh) signaling. Hh signaling to cardiomyoblasts is required for the development of coronary veins, while Hh signaling to perivascular cells is necessary for coronary arterial growth.[58]

The Notch signaling pathway is an important mediator of proliferation, stem cell maintenance, cell fate decisions and boundary formation in the development and homeostasis of various tissues.[59] Ligand binding to the Notch receptor releases 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 co-repressor complexes from this DNA-binding factor. Notch signaling has been associated with angiogenic growth of the blood vessels, arterial versus venous fate decisions, and vascular smooth muscle cell differentiation.[60] Furthermore, Notch1ICD is expressed in the (pro)epicardium, in forming coronary smooth muscle cells and endothelia of chicken embryos;[61] thereby suggesting a functional relevance in the developing murine epicardium.

The study of epicardial EMT revealed extensive signaling events in heart development and reiteration of these pathways might result in acquired heart diseases. In modern medicine the heart is easily accessible and drugs can be delivered directly to the epicardium. Therapeutic manipulations of epicardial cells are already tested *in vivo*. The G-actin monomer binding protein thymosin beta 4 (TB4) has been shown to reduce infarct size in experimental myocardial infarction[62]. Based on the possible potential of fetal EPDCs to differentiate into cardiomyocytes and modulation of epicardial activity by TB4, epicardial cells might represent a resident cardiac progenitor pool that could be recruited for therapeutic myocardial regeneration.

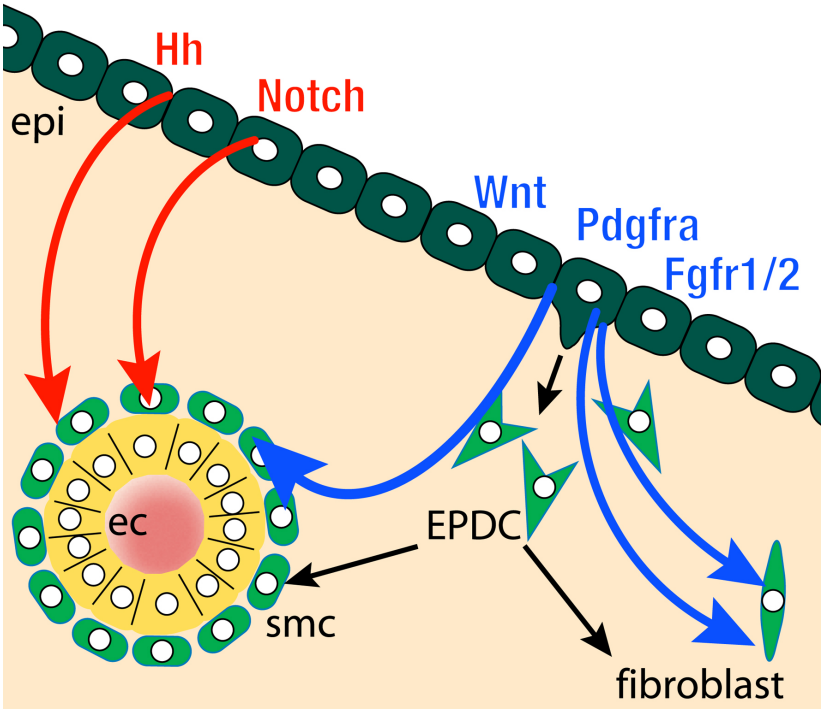

Figure 3 Molecular pathways in the mobilization and differentiation of epicardial cells. Individual cells of the epicardium (green) undergo an EMT, leave the epithelial contiguity and migrate into the myocardial wall. Signaling pathways implicated in either effecting mobilization and differentiation (blue arrows) or differentiation (red arrows) of epicardial cells. ec, endothelial cell; EPDC, epicardium-derived cell; epi, epicardium.

Mesothelia in development and homeostasis

The epicardium is an epithelium of mesodermal origin, thus correctly named a mesothelium. The mesothelium is the epithelial lining of mesodermic cavities. It develops from the mesoderm layer that lines the body cavity (coelom) of the early developing embryo.[63] Mesothelia form a monolayer of flattened, squamous-like cells, located on a thin basement membrane. They line the pleural and pericardial cavities of the chest, and the peritoneal cavity of the abdomen. The body wall is lined by the parietal mesothelium, whereas the visceral layer covers the organ in the respective cavity.[64]

In the adult, mesothelial cells secrete gycosaminogycans and surfactant to provide a frictionless free surface between the parietal and visceral layer.[65] During development single cells are able to leave the mesothelium, undergo a epithelial-mesenchymal transition and invade the underlying tissue. This feature is not only restricted to the epicardium, but mesothelium-derived fibroblasts and smooth muscle cells have been detected in the developing gut and lung as well.[16,23,66–69]

In various areas, including the adult omentum and the embryonic proepicardium, cuboidal mesothelial cells can be found.[70,4] Interestingly, the morphology of these precursor cells resembles cells that can be detected at the sides of injury in mesothelial tissues.[71] Additionally, it was suggested that free-floating mesothelial cells implant into the side of injury,[72] thus wound repair in these tissues might reflect a re-initiation of the embryonic gene program. In recent years the visceral pericardium, also known as epicardium, was in the focus of several studies addressing its potential function in the regenerative capacity of the heart. New insights into the function of mesothelial cells in homeostasis and repair might promote regenerative applications in the future.

Aim of the Thesis

The epicardium, the outermost lining of the heart, provides trophic signals and cells to the developing coronary vasculature and the fibrous heart skeleton. Genetic lineage tracing approaches in different model organisms provided conflicting results on the fate of epicardium-derived cells in embryonic development. Furthermore, the signaling pathways that mediate mobilization and differentiation of these cells have been only insufficiently defined. Tracing and manipulation of epicedium-derived cells in mouse can be performed using specific cre-lines.

In this thesis, the suitability of *Wt1*^{cre} and *Tbx18*^{cre} mouse lines for these purposes should be tested. Furthermore, an adequate line should be used for lineage tracing of epicardial cells by means of a sensitive reporter allele *in vivo*. In addition, signaling pathways effecting the mobilization and differentiation of epicardium-derived cells will be analyzed. Conditional alleles of unique mediators of the Notch-, canonical Wnt-, Hh-, Fgfr1/2- and Pdgfrα-signalling pathways will be crossed to the *Tbx18*^{cre} mouse line to test for their functional relevance in epicardial development *in vivo*. Furthermore, misexpression of constitutive active Wnt- and Hh- signaling in the epicardium should be analyzed.

Finally, mice, with genetically ablation of *Upk3b* were to be generated and analyzed for cellular and molecular changes in the urothelium and epicardium.

Together this study should provide us with unambiguous knowledge about the fate of epicardium-derived cells and should give more insights into signaling pathways effecting the mobilization and differentiation of epicardium-derived cells.

Tbx18 and the fate of epicardial progenitors

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Published in Nature (Nature. 2009;458;E8-E9).

NATURE|Vol 458|16 April 2009 BRIEF COMMUNICATIONS ARISING

Tbx18 and the fate of epicardial progenitors

Arising from: C.-L. Cai *et al. Nature* **454,** 104–108 (2008)

Uncovering the origins of myocardial cells is important for understanding and treating heart diseases[1,2]. Cai *et al.*[3] suggest that *Tbx18*-expressing epicardium provides a substantial contribution to myocytes in the ventricular septum and the atrial and ventricular walls. Here we show that the T-box transcription factor gene 18 (*Tbx18*) itself is expressed in the myocardium, showing that their genetic lineage tracing system does not allow conclusions of an epicardial origin of cardiomyocytes *in vivo* to be drawn.

During amniote embryogenesis, cells from the proepicardium, a mesothelial cell cluster located at the venous pole of the heart, emigrate onto the myocardium to form the epicardium—the epithelial outer lining of the heart. Lineage-tracing studies in chick demonstrated that epicardial cells invade the myocardium, giving rise to coronary vascular support cells and adventitial fibroblasts[4–6].

In mouse, genetic lineage studies can be used to assess the contribution of precursor cells to a mature tissue or organ[7]. For the proepicardium/epicardium, such an effort has now been undertaken by Cai *et al.*[3] based on the observation that the transcription factor gene *Tbx18* is expressed at high levels in these embryonic tissues[8]. Using a knock-in of the Cre recombinase gene in the *Tbx18* locus, epicardial cells were labelled irreversibly and their daughters were followed during development. The authors found that, in addition to known fates, *Tbx18*-positive epicardial cells contributed to the myocardium of the interventricular septum (IVS) and myocardial wall. Clearly, such a finding would define the epicardium as a previously unknown progenitor pool of cardiomyocytes complementing the earlier contributions of the first and second heart field[9].

Prerequisite to any genetic lineage study is the specificity of the gene that drives Cre recombinase. In this specific case, *Tbx18* (*Cre*) should not be expressed in the myocardium. In previous studies, we observed robust *Tbx18* expression in the IVS and left ventricular wall at embryonic day (E)12.5 (refs 10, 11). Further analysis revealed continuous *Tbx18* expression in the IVS and the myocardium of the left ventricle from E10.5 to at least E16.5 (Fig. 1a–d, f, g). *Tbx18* expression co-localized with myocardial (*Tnni3*) staining on adjacent sections (Fig. 1e). Immunofluorescent analysis using an anti-Tbx18 antibody confirmed that Tbx18 protein is continuously expressed in the IVS and the left ventricular cardiomyocytes (Nkx2-5-positive) from E10.5 onwards (Fig. 1i–m). Detection of pre-messenger RNA in epicardium-free IVS myocardium tissue obtained by laser capture microdissection from E11.5 wild-type hearts confirmed that *Tbx18* expression is initiated *de novo* in cardiomyocytes (Fig. 1h). A protein kinase C iota (*Prkci*) null mutant (a gift from M. Leitges), which completely fails to form epicardium around the ventricles and dies at E12.5, expressed Tbx18 in the IVS in a pattern similar to that in control embryos (Fig. 1n–q), further demonstrating that *Tbx18* expression in the myocardium of the IVS and left ventricle is independent from that in the epicardium.

We have generated a *Cre* knock-in allele of *Tbx18* (*Tbx18*^{Cre}), the Cre activity pattern of which faithfully reflects endogenous *Tbx18* expression[8] (Fig. 2a). Comparison of expression of *Tbx18* from the wild-type allele and *lacZ* from the R26R^{lacZ} reporter[12] on adjacent sections of hearts of *Tbx18*$^{Cre/+}$;R26R^{lacZ} embryos showed overlap of the Cre recombination pattern (*lacZ* expression) and endogenous *Tbx18* in the epicardium and in the IVS and left ventricular wall (Fig. 2b, c). Furthermore, we observed unchanged Cre activity from the *Tbx18*^{Cre} allele in the left ventricle and the septum region in mice mutant for the Wilms tumor 1 (*Wt1*) gene[13] (Fig. 2d, e). Formation of epicardium and subepicardial mesenchyme is severely compromised in these mice[14], again indicating that myocardial *Tbx18* expression is independent from the presence and functionality of the epicardium.

Together, our results unambiguously show that *Tbx18* expression is also present in cardiomyocytes of the IVS and the ventricular wall from E10.5 onwards.

The failure of Cai *et al.* to detect expression of *Tbx18* in the myocardium may result from lack of sensitivity in their expression assays and tools (nlacZ/nGFP knock-in allele)[3]. Their *Tbx18*^{Cre} allele is likely to be active in cardiomyocytes, similar to our *Tbx18*^{Cre} allele, but its pattern may be affected by the presence of the PGK-*neo* cassette[3] (compare dissimilarity in recombination in somites and head region in Fig. 2b in ref. 3 and Fig. 2a here), which could lead to ectopic Cre activity in the heart. A genetic lineage study similar to that in ref. 3, following the fate of *Wt1*^{Cre}-expressing epicardium, also indicated that epicardium differentiates to myocardium[15]. Our analysis,

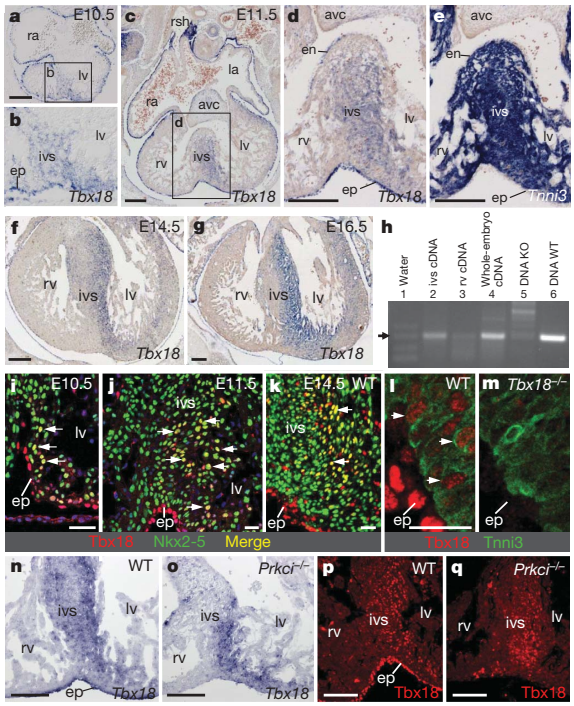

Figure 1 | Expression of *Tbx18* is not confined to the epicardium but is also found in cardiomyocytes of the interventricular septum and the left ventricle. a–g, Section *in situ* hybridization analysis for *Tbx18* (**a–d, f, g**) and cardiac troponin I (*Tnni3*, **e**) expression on paraffin sections of E10.5 (**a, b**), E11.5 (**c–e**), E14.5 (**f**) and E16.5 (**g**) hearts. Insets (**a, c**) are magnified (**b, d**). **h**, Pre-mRNA of *Tbx18* is detected in laser-captured epicard-free IVS from E11.5 wild-type (WT) hearts by RT–PCR analysis for intron 1 sequences (lane 2). Background signal is observed in tissue from the right ventricle of the same hearts (lane 3). KO, knockout. **i–m**, Immunofluorescent analysis of Tbx18 protein expression on paraffin sections of E10.5 (**i**), E11.5 (**j, l, m**) and E14.5 (**k**) hearts. Coexpression analyses of Nkx2-5 (yellow nuclei, white arrows in **i–k**). Absence of Tbx18 protein signals in sections of *Tbx18*$^{GFP/GFP}$ hearts (**m**). **n–q**, Section *in situ* hybridization analysis of *Tbx18* mRNA (**n, o**) and immunofluorescent analysis of Tbx18 protein (**p, q**) in E12.5 *Prkci* mutant hearts that lack an epicardium (**o, q**). avc, atrioventricular cushion tissue; en, endocardium; ep, epicardium; ivs, interventricular septum; la, left atrium; lv, left ventricle; ra, right atrium; rsh, right sinus horn; rv, right ventricle. Scale bars: 100 μm (**a–g, n–q**); 25 μm (**i–m**).

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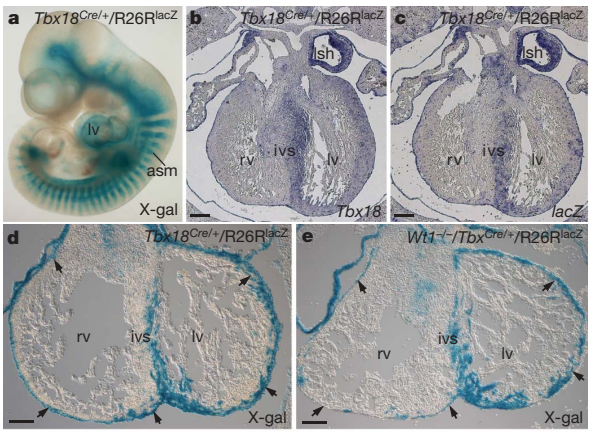

Figure 2 | Cre activity from a Cre-knock-in allele of *Tbx18* (*Tbx18*^{Cre}) recapitulates endogenous *Tbx18* expression. *Tbx18*^{Cre} mice were crossed to R26R^{lacZ} indicator mice[12] and expression and activity of the reporter was detected in compound *Tbx18*$^{Cre/+}$/R26R^{lacZ} embryos. **a**, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) staining on a whole-mount E10.5 embryo faithfully reflects endogenous *Tbx18* expression[8]. **b**, **c**, Section *in situ* hybridization analysis on adjacent paraffin sections of E14.5 hearts shows complete overlap of *Tbx18* (**b**) and *lacZ* reporter gene (**c**) expression. **d**, **e**, X-gal staining for *Tbx18*-driven Cre activity on transverse cryo-sections from E12.5 hearts of wild-type embryos (**d**) and a homozygous mutant for *Wt1* (**e**, ref. 14). The formation of the epicardium (arrows) is severely compromised in mutants. asm, anterior somite halves; lsh, left sinus horn. Other abbreviations are as in Fig. 1. Scale bars, 100 μm (**b**–**e**).

which shows that the genetic lineage study by Cai *et al.*[3] does not unequivocally demonstrate an epicardial origin of cardiomyocytes *in vivo*, supports the notion that independent approaches are required to assess the relationship of a specific progenitor cell to a specific differentiated cell type.

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Received 1 July 2008; accepted 3 February 2009.

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doi:10.1038/nature07916

Notch signaling regulates smooth muscle differentiation of epicardium-derived cells

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Published in Circulation Research (Circ. Res. 2011;108;813-823).

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License Number	2996350467236
License date	Sep 26, 2012
Licensed content publisher	Wolters Kluwer Health
Licensed content publication	Circulation Research
Licensed content title	Notch Signaling Regulates Smooth Muscle Differentiation of Epicardium-Derived CellsNovelty and Significance
Licensed content author	Thomas Grieskamp, Carsten Rudat, Timo H.-W. Lüdtke, Julia Norden, Andreas Kispert
Licensed content date	Apr 1, 2011
Volume Number	108
Issue Number	7
Type of Use	Dissertation/Thesis
Requestor type	Individual
Title of your thesis / dissertation	Mobilization and differentiation of epicardial cells in murine embryogenesis
Expected completion date	Nov 2012
Estimated size(pages)	100
Billing Type	Invoice
Billing address	Medizinische Hochschule Hannover Carl-Neuberg-Str. 1 Hannover, 30625 Germany
Customer reference info	
Total	0.00 USD
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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.

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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-

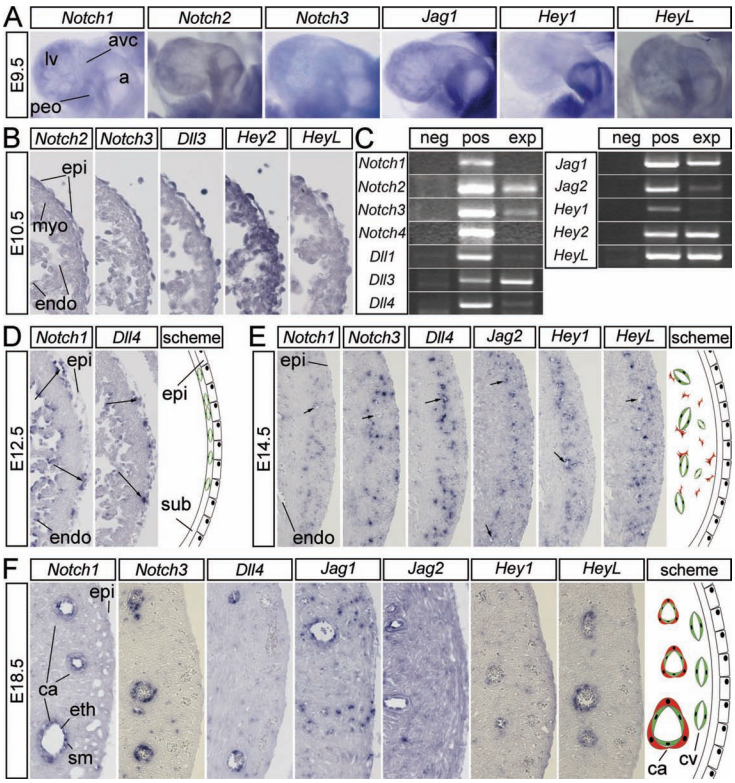

Figure 1. Expression of genes encoding Notch receptors, ligands, and targets in epicardial development. A through F, Expression of Notch receptor (*Notch1–4*), Notch ligand (*Dll1*, *Dll3*, *Dll4*, *Jag1*, *Jag2*), and Notch target gene expression (*Hey1*, *Hey2*, *HeyL*) by in situ hybridization analysis 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

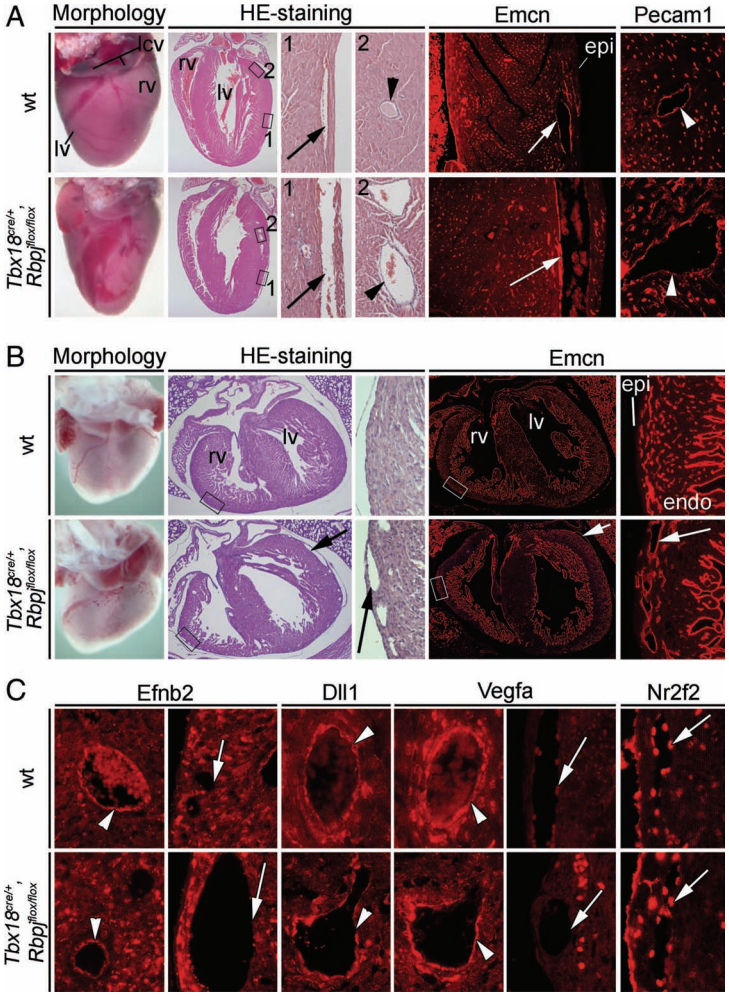

Figure 2. Defects of the coronary vasculature in mice with epicardial deletion of *Rbpj*. **A through C,** Analysis of coronary vessel formation in *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ mice by morphology of whole hearts, by hematoxylin/eosin staining and by immunofluorescence for endothelial marker 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.

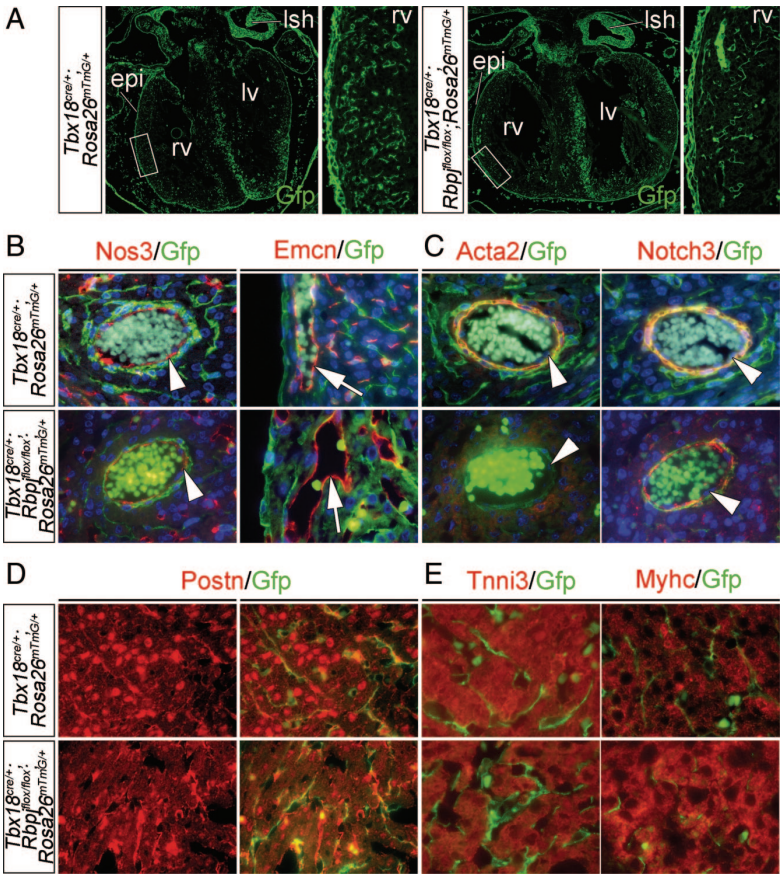

Figure 3. Disrupted cytodifferentiation of EPDCs in *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ mice. A, Analysis of formation and migration of EPDCs in *Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$; *Rbpj*$^{flox/flox}$ mice by immunofluorescence of the Gfp reporter on transverse sections of the whole E14.5 heart 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,

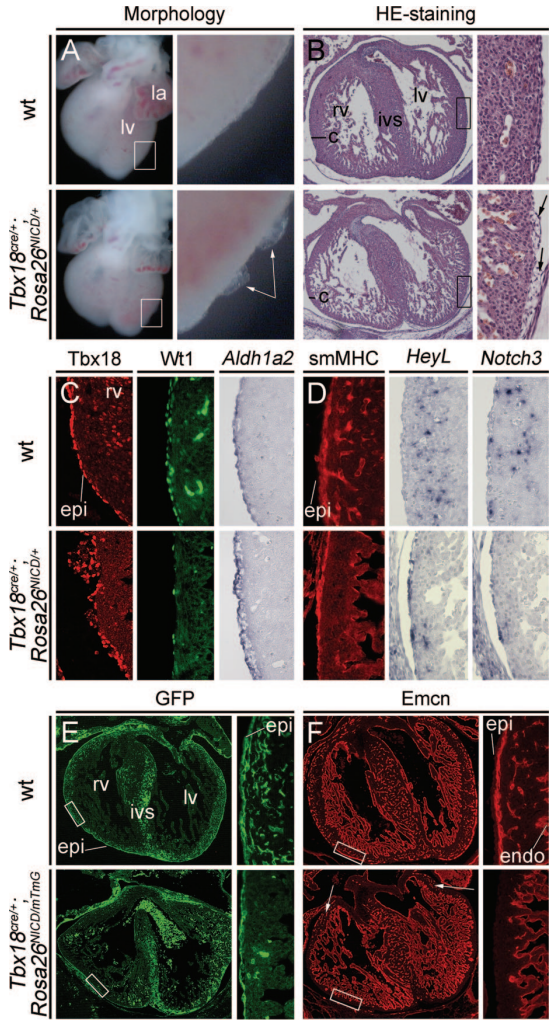

Figure 4. Disrupted epicardial development in mice overexpressing the Notch1 intracellular domain. A through E, Analyses were performed on E14.5 wild-type (wt) and *Tbx18*$^{cre/+}$; *Rosa26*$^{NICD/+}$ embryos for epicardial integrity and differentiation by morphological inspection of whole hearts **(A)**, 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

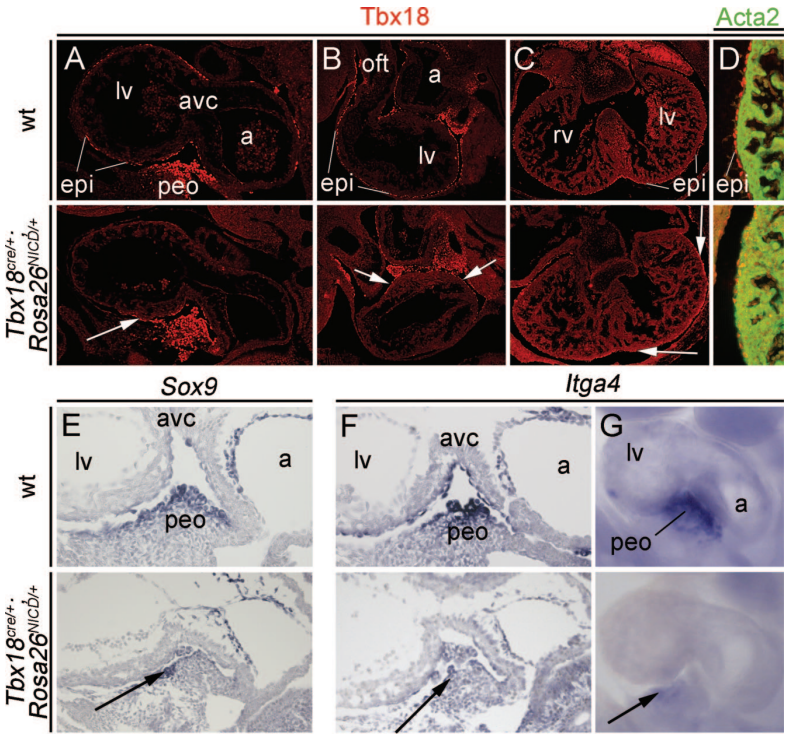

Figure 5. Early onset of epicardial defects in *Tbx18*$^{cre/+}$;*Rosa26*$^{NICD/+}$ embryos. A through D, Immunofluorescence analysis of Tbx18 protein expression (in **red**) in sections of whole hearts at E9.5 **(A)**, E10.5 **(B)**, E12.5 **(C)** and in a subregion of the right ventricle at E12.5 **(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,

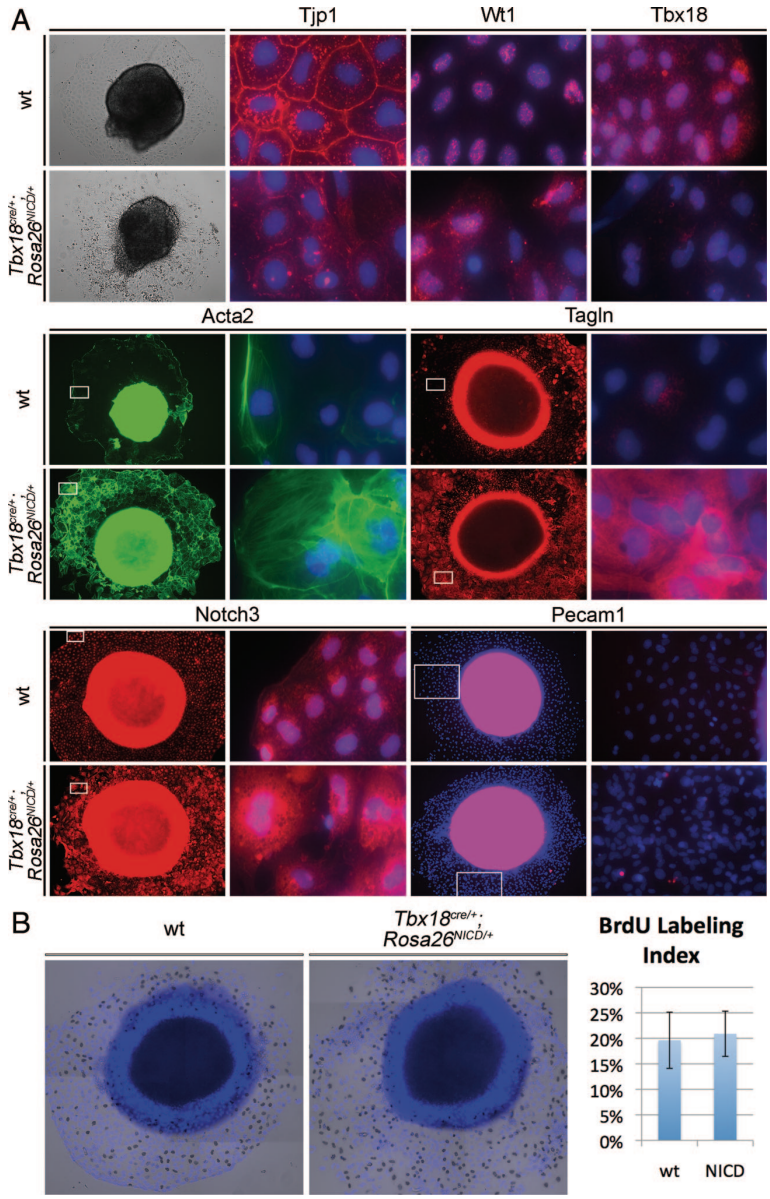

Figure 6. Induction of SM differentiation of primary epicardial cells by NICD overexpression. A, Cellular analysis of epicardial outgrowths of explants of right ventricles of wild-type (wt) and *Tbx18*$^{cre/+}$; *Rosa26*$^{NICD/+}$ embryos after 2 days of culture under serum-free conditions by bright 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

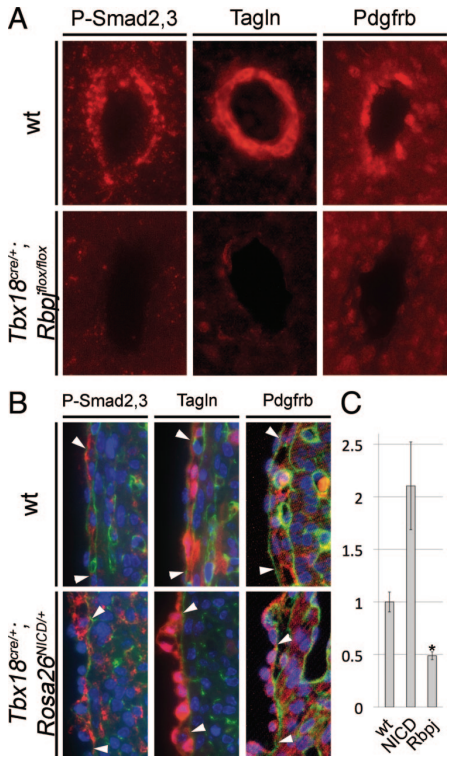

Figure 7. Tgfb signaling and Pdgfrb are downstream of the Notch pathway in SM differentiation of epicardial cells. A and B, Comparative immunofluorescence analysis of expression of P-Smad2, -3, Tagln, and Pdgfrb (red fluorescence) in coronary arteries of the right ventricle of wild-type (wt) and *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ 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.

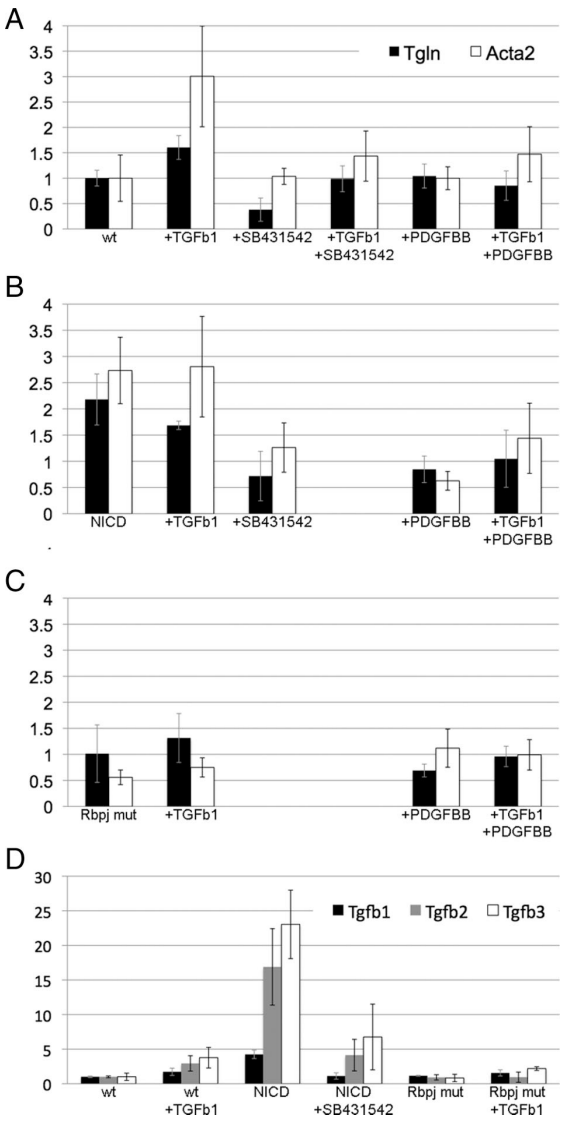

Figure 8. Notch and Tgfb signaling cooperate in SM differentiation of EPDCs. A through C, Semiquantitative RT-PCR analysis of *Acta2* and *Tagln* expression in differently treated pools of epicardial explants derived from wild-type **(A)**, *Tbx18*$^{cre/+}$;*Rosa26*$^{NICD/+}$ **(B)**, and *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/flox}$ **(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.

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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.

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*$^{flox/flox}$ mice were obtained from matings of *Tbx18*$^{cre/+}$;*Rbpj*$^{flox/+}$ males and *Rbpj*$^{flox/flox}$ 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*$^{NICD/NICD}$ females. The fate of epicardial cells was analyzed in *Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$ embryos (obtained from matings of *Tbx18*$^{cre/+}$ and *Rosa26*$^{mTmG/mTmG}$ mice), in *Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Rbpj*$^{flox/flox}$ embryos (obtained from matings of *Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Rbpj*$^{flox/+}$ males and *Rbpj*$^{flox/flox}$ females) and in *Tbx18*$^{cre/+}$;*Rosa26*$^{NICD/mTmG}$ 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^{o}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 DNaseI 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 (TagIn) (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*$^{cre/+}$*;Rosa*$^{26NICD/+}$ 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.

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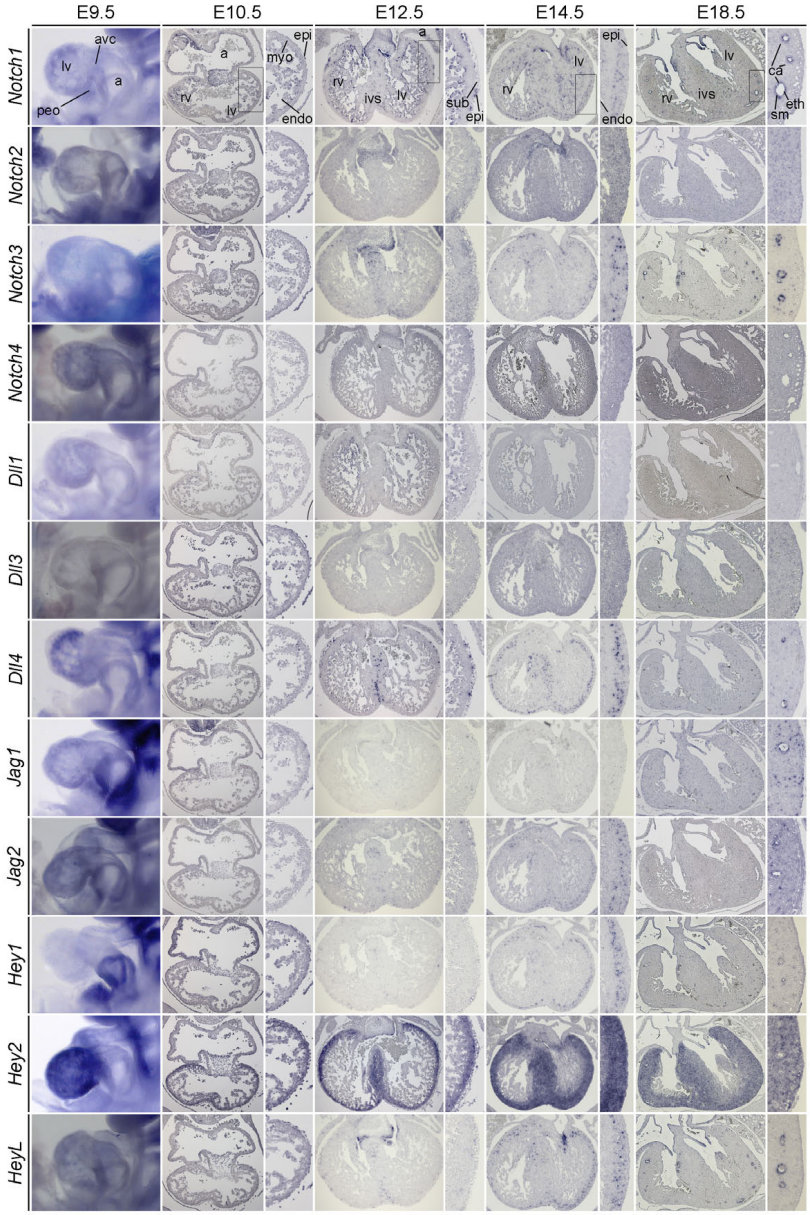
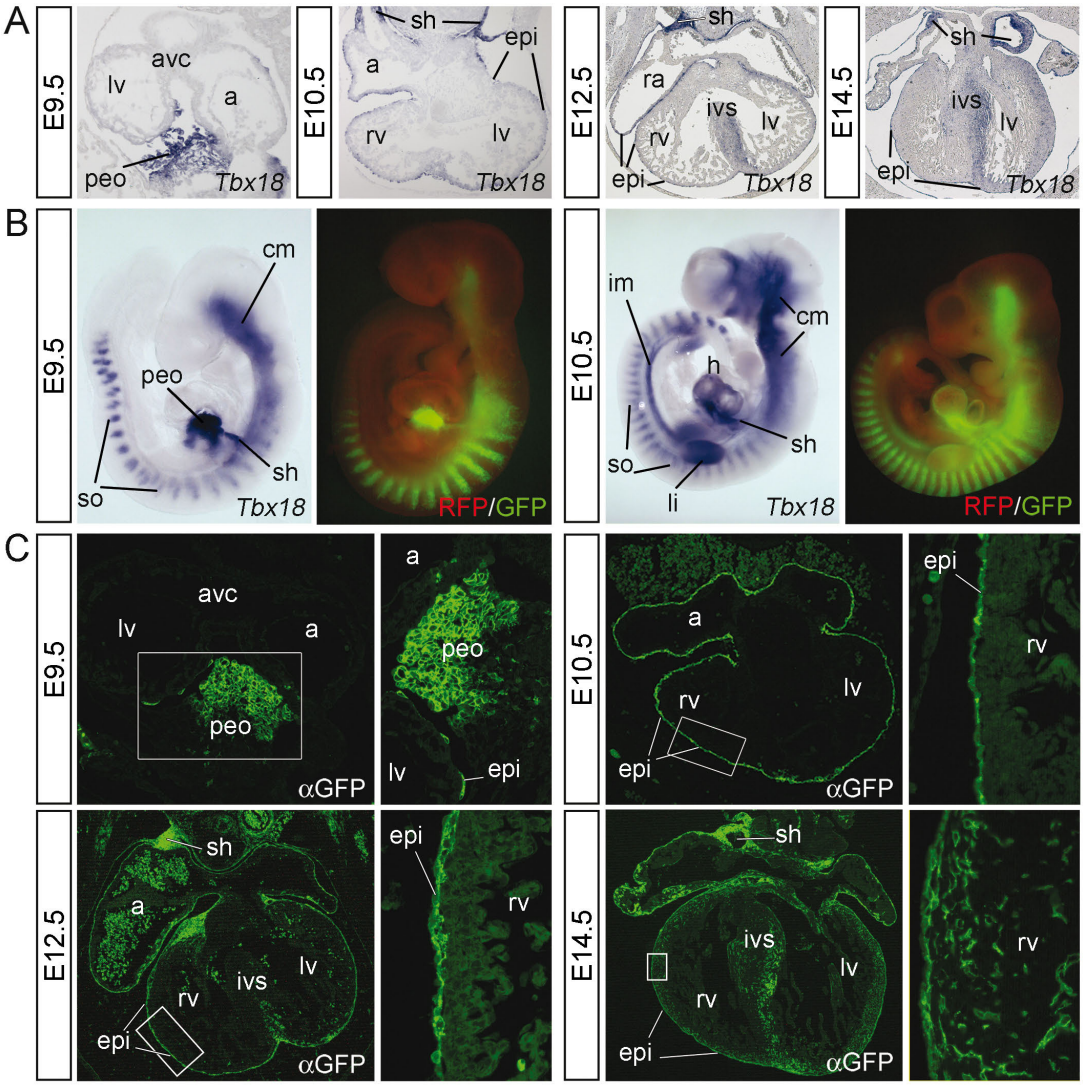
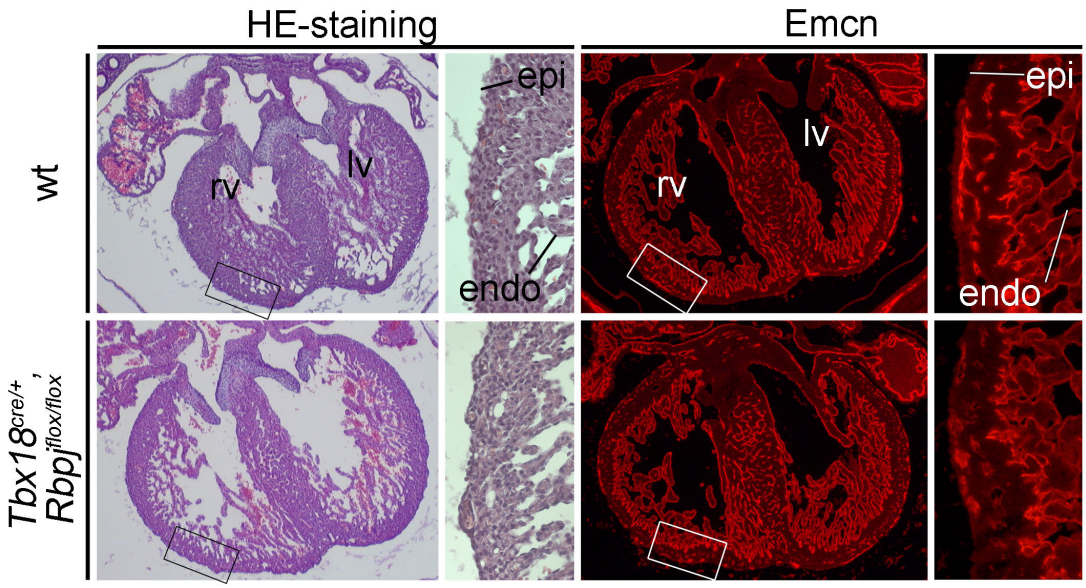
Supplemental Online Figures



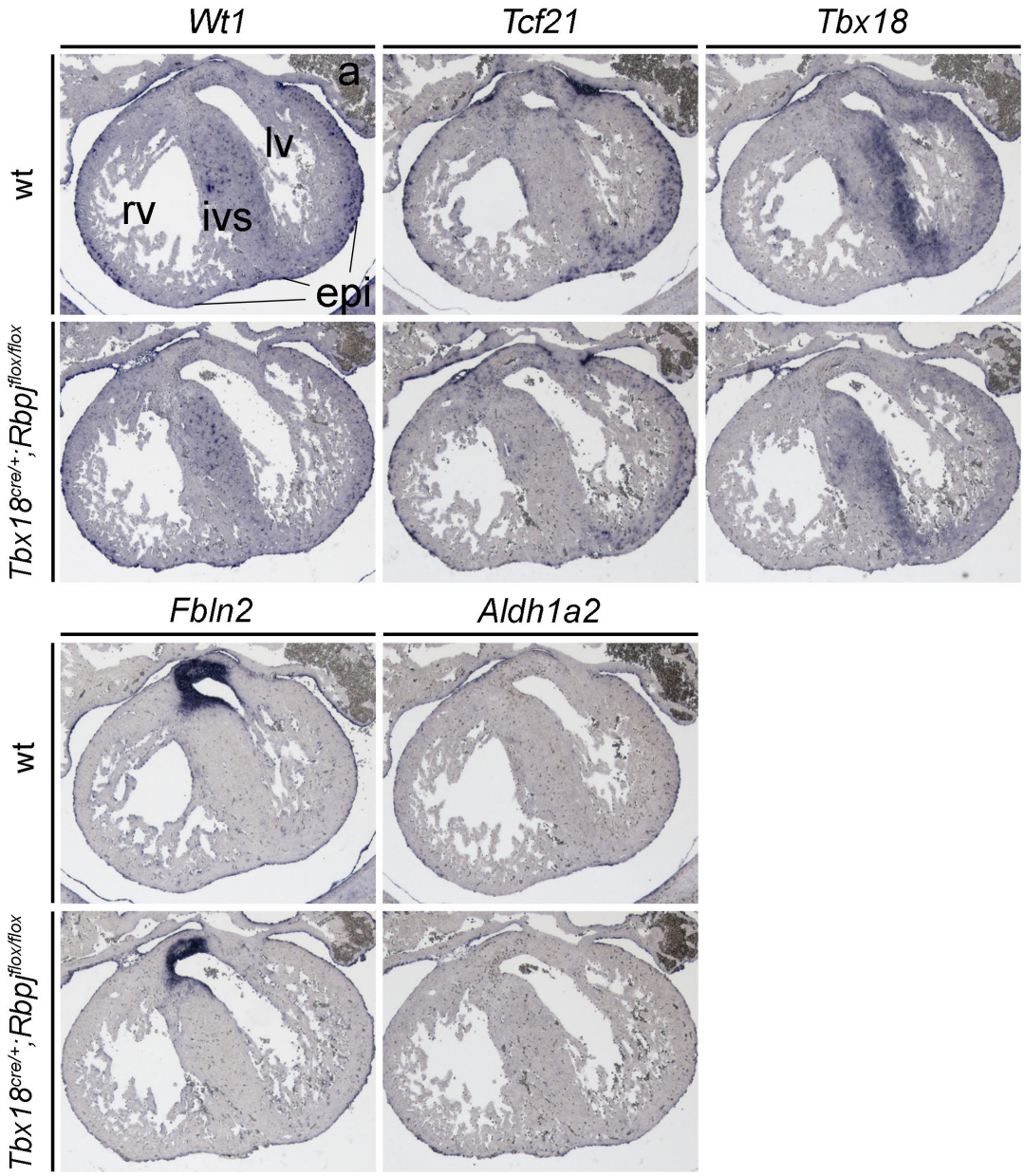
Online Figure I. Multiple genes encoding Notch receptors and ligands as well as Notch target genes are expressed in epicardial development. *In situ* hybridization analysis of Notch receptor (*Notch1-4*), Notch ligand (*Dll1*, *Dll3*, *Dll4*, *Jag1*, *Jag2*) and Notch target gene expression (*Hey1*, *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.



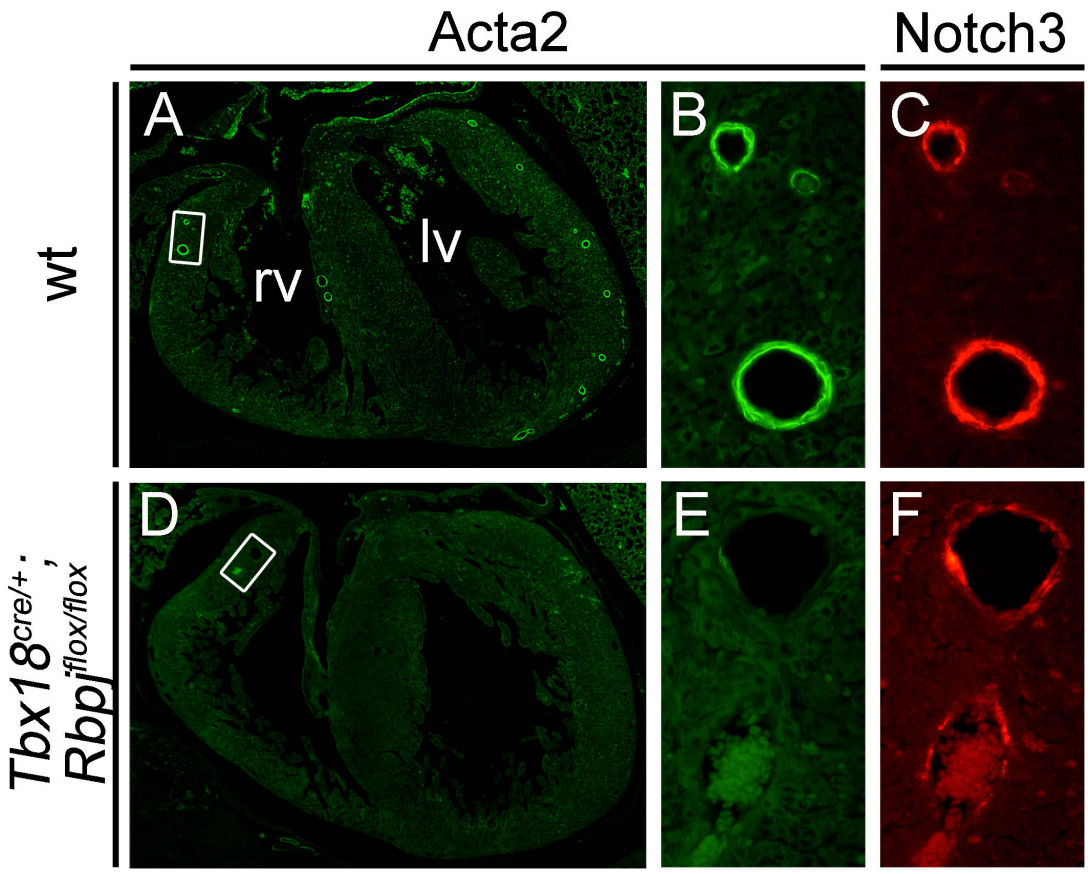
Online Figure II. *Tbx18*^{cre} mice - a tool for visualization, and manipulation and tracing of epicardial cells by genetic recombination. (A) *In situ* hybridization analysis of *Tbx18* expression during murine heart development. *Tbx18* expression is detected in the proepicardial organ at E9.5, and 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 *Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$ embryos of the same stages. The *Rosa26*^{mTmG} 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 *Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$ 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.



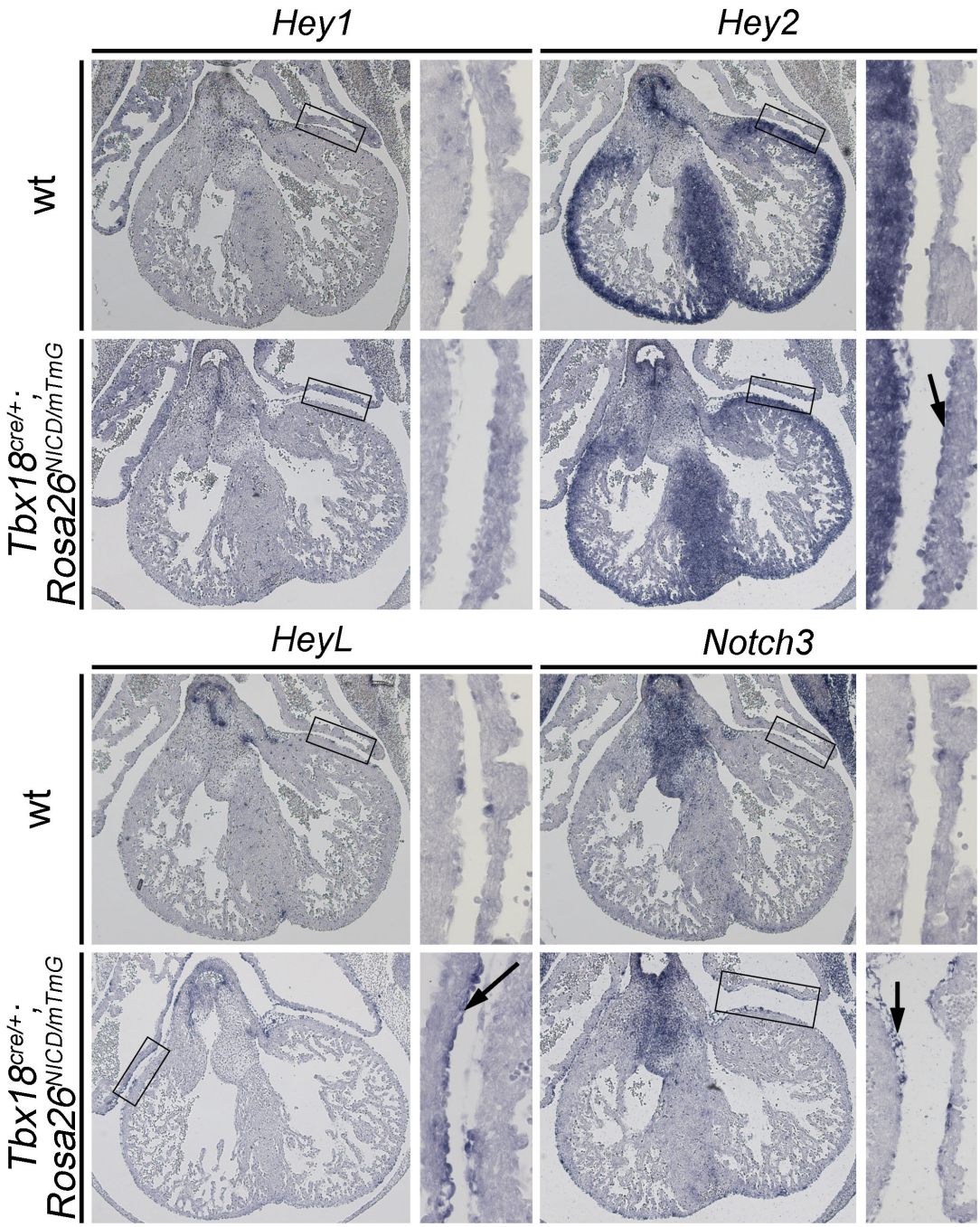
Online Figure III. Coronary endothelia are not affected by epicardial loss of *Rbpj* at E14.5. Histological analysis by haematoxylin/eosin (HE-) staining and immunofluoresence analysis of endomucin (Emcn) expression of transverse sections of wildtype (wt) and *Tbx18*$^{cre/+;}$*Rbpj*$^{flox/flox}$ hearts show that subepicardial blood vessels are distributed normally at this stage. endo, endocardium; epi, epicardium; lv, left ventricle; rv, right ventricle.



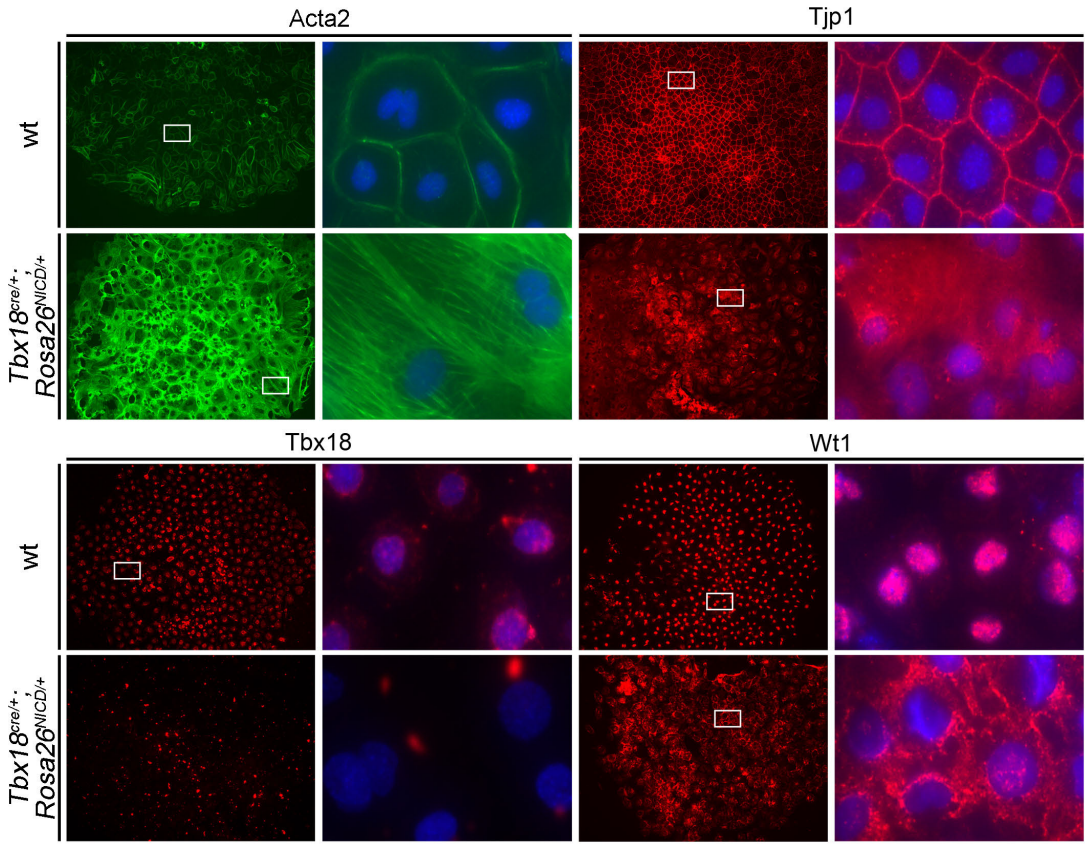
Online Figure IV. Loss of *Rbpj* does not affect epicardial identity and integrity. *In situ* hybridization analysis of epicardial marker genes in transverse section of the heart of E14.5 wildtype (wt) and *Tbx18*$^{cre/+;}$*Rbpj*$^{flox/flox}$ embryos. Epicardial expression of all marker genes is maintained 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 coronary arteries. 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 *Tbx18$^{cre/+}$;Rosa26$^{NICD/mTmG}$* embryos. Analysis of expression of Notch target genes *Hey1*, *Hey2*, *HeyL* and *Notch3* in hearts of E14.5 wildtype (wt) and *Tbx18$^{cre/+}$;Rosa26$^{NICD/mTmG}$* embryos by *in situ* hybridization analysis on transverse sections. *Hey2*, *HeyL* and *Notch3* are specifically induced in the epicardium of *Tbx18$^{cre/+}$;Rosa26$^{NICD/mTmG}$* 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 *Tbx18*$^{cre/+}$;*Rosa26*$^{NICD/+}$ 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

	mean ± SD		P-value compared with wt		P-value compared with wt + TGFb1	
	Tagln	*Acta2*	*Tagln*	*Acta2*	*Tagln*	*Acta2*
wt	1.00 ± 0.16	1.00 ± 0.46				
wt + TGFb1	1.60 ± 0.23	3.01 ± 0.99	**0.0004**	**0.0011**	not determined	
wt + SB431542	0.38 ± 0.23	1.03 ± 0.16	**0.0126**	0.9231	**0.0014**	**0.0377**
wt + TGFb1 + SB431542	0.99 ± 0.26	1.44 ± 0.49	0.9307	0.2932	**0.0190**	**0.0836**
wt + PDGF-BB	1.04 ± 0.24	1.00 ± 0.23	0.7130	0.9939	not determined	
wt + TGFb1 + PDGF-BB	0.85 ± 0.29	1.47 ± 0.54	0.2764	0.1036	not determined	
NICD	2.18 ± 0.49	2.73 ± 0.63	**0.0010**	**0.0049**	**P-value compared with NICD**	
NICD + TGFb1	1.69 ± 0.08	2.81 ± 0.96	**0.0005**	**0.0036**	0.1792	0.9293
NICD + SB431542	0.72 ± 0.47	1.26 ± 0.47	0.2028	0.4115	**0.0437**	**0.0385**
NICD + PDGFBB	0.85 ± 0.25	0.63 ± 0.18	0.2490	0.1216	**0.0038**	**0.0385**
NICD + TGFb1 + PDGF-BB	1.05 ± 0.54	1.44 ± 0.67	0.8328	0.3219	0.1595	0.1856
Rbpj mut	1.01 ± 0.55	0.56 ± 0.14	0.9701	0.1555	**P-value compared with Rbpj mut**	
Rbpj mut + TGFb1	1.32 ± 0.47	0.75 ± 0.18	0.3593	0.4018	0.6997	0.2263
Rbpj mut + PDGFBB	0.69 ± 0.12	1.12 ± 0.37	**0.0455**	0.7527	0.6806	0.0837
Rbpj mut + TGFb1 + PDGF-BB	0.96 ± 0.20	0.99 ± 0.29	0.7792	0.9785	0.9453	0.1030

	mean ± SD			P-value compared with wt		
	Tgfb1	*Tgfb2*	*Tgfb3*	*Tgfb1*	*Tgfb2*	*Tgfb3*
wt	1.00 ± 0.12	1.00 ± 0.13	1.00 ± 0.52	-	-	-
wt + TGFB1	1.72 ± 0.53	1.68 ± 1.11	3.76 ± 1.48	0.1817	**0.0190**	**0.0090**
NICD	4.25 ± 0.61	16.89 ± 5.53	23.03 ± 4.95	**0.0367**	**0.0199**	**0.0179**
NICD + SB431542	1.11 ± 0.46	4.13 ± 2.28	6.75 ± 4.75	0.7454	**0.0282**	0.0539
Rbpj mut	1.14 ± 0.08	0.91 ± 0.38	0.83 ± 0.53	0.4106	0.8439	0.6765
Rbpj mut + TGFb1	1.55 ± 0.45	0.94 ± 0.73	2.19 ± 0.27	0.0790	0.9511	0.3894
	P-value compared with NICD			**P-value compared with Rbpj mut**		
	Tgfb1	*Tgfb2*	*Tgfb3*	*Tgfb1*	*Tgfb2*	*Tgfb3*
wt		not determined			not determined	
wt + TGFb1		not determined			not determined	
NICD		not determined			not determined	
NICD + SB431542	**0.0045**	**0.0053**	**0.0115**		not determined	
Rbpj mut		not determined			not determined	
Rbpj mut + TGFb1		not determined		**0.0440**	0.9681	0.2181

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.

Wt1 and epicardial fate mapping

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Published in Circulation Research (Circ. Res. 2012;111;165-169).

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Wt1 and Epicardial Fate Mapping

Carsten Rudat, Andreas Kispert

Rationale: **The embryonic epicardium is a crucial cell source of the cardiac fibrous skeleton as well as of the coronary system. Genetic lineage tracing systems based on *Wt1* regulatory sequences provided evidence that epicardium-derived cells also adopt a myocardial fate in the mouse.**

Objective: **To define the adequacy of *Wt1*-based lineage tracing systems for epicardial fate mapping.**

Methods and Results: **Using in situ hybridization analysis and immunofluorescence on tissue sections, we detected endogenous expression of *Wt1* mRNA and Wt1 protein in the proepicardium and epicardium and also in endothelial cells throughout cardiogenesis. Expression analysis of a sensitive GFP reporter showed that recombination mediated by cre recombinase in the $Wt1^{creEGFP}$ line occurs randomly and sporadically in all cells of the embryo. Recombination in cardiomyocytes was found in the linear heart tube before establishment of a (pro)epicardium. In contrast, the tamoxifen-inducible $Wt1^{creERT2}$ mouse line mediated poor and variable recombination in the epicardium. Recombination in cardiomyocytes was not detected in this case.**

Conclusions: **Frequently used *Wt1* based cre-mediated lineage tracing systems are not suitable for epicardial fate mapping because of endogenous endothelial expression of Wt1, ectopic recombination ($Wt1^{creEGFP}$), and poor recombination efficiency ($Wt1^{creERT2}$) in the developing heart. We conclude that claims of a cardiomyocyte fate of epicardial cells in the mouse are not substantiated. (*Circ Res*. 2012;111:165-169.)**

Key Words: cardiomyocyte ■ epicardium ■ lineage tracing ■ Wt1

The outer lining of the mature vertebrate heart, the epicardium, develops after cardiac looping from a cluster of mesothelial cells of the septum transversum. Groups of cells detach from this proepicardium, adhere to the myocardium, and spread out to form a continuous epithelial layer.[1] Cell lineage tracings performed by retroviral reporter constructs and chimera analysis in the chick have shown that a subset of epicardial cells undergoes an epithelial–mesenchymal transition differentiates within the subepicardial space and myocardium into interstitial and perivascular fibroblasts and smooth muscle cells (SMCs) of the coronary arteries.[2–5] Some reports also suggested an additional coronary endothelial fate of epicardium-derived cells in birds.[6,7]

Two independent lineage tracings by cre/*loxP* technology confirmed fibroblasts and SMCs as epicardial derivatives but suggested an additional myocardial fate of epicardial cells in the mouse. Using a *cre* knock-in allele of the *T-box gene 18* (*Tbx18*), cardiomyocytes of the left ventricle and the interventricular septum were recognized as epicardial descendants,[8] and lineage tracing with two *cre* knock-in alleles of the *Wilms tumor 1* gene (*Wt1*; $Wt1^{creEGFP}$, $Wt1^{creERT2}$) detected a substantial contribution of epicardial cells to the myocardium of the chambers and the interventricular septum starting from embryonic day (E) 10.5.[9] Furthermore, using the same two $Wt1^{cre}$ lines, a myocardial differentiation of epicardial cells after myocardial infarction subsequent to pretreatment with thymosin $\beta 4$ was demonstrated.[10] However, other cre/*loxP*-based epicardial lineage tracing efforts did not find evidence for a myocardial fate of epicardial cells in the mouse.[11–13] In addition, it was recently reported that *Tbx18* exhibits endogenous expression not only in the proepicardium and epicardium but also in cardiomyocytes of the left ventricle and the interventricular septum starting at E10.5, ie, exactly in the regions claimed to be home of epicardium-derived cardiomyocytes in the study by Cai et al.[14]

Given the enormous medical interest in a potential myocardial fate of epicardial cells in mammals, we wished to reevaluate the adequacy of previously used *Wt1*-based cre/*loxP* lineage tracing systems. We provide evidence by expression and reporter analyses that an endothelial and myocardial fate of epicardial cells cannot be demonstrated in the mouse using this genetic tool.

Original received May 16, 2012; revision received May 25, 2012; accepted June 1, 2012.

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The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.112.273946/-/DC1.

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Non-standard Abbreviations and Acronyms	
E	embryonic day
GFP	green fluorescent protein
SMC	smooth muscle cell
Tbx18	T-box gene 18
Wt1	Wilms tumor 1

Methods

Mice and Genotyping

Mice with a knock-in of the *cre* recombinase gene in the *Wt1* locus ($Wt1^{tm1(EGFP/cre)Wtp}$ or $Wt1^{creEGFP}$),[9] mice with a knock-in of the tamoxifen inducible *cre*-recombinase in the *Wt1* locus ($Wt1^{tm2(cre/ERT2)Wtp}$ or $Wt1^{creERT2}$),[9] and the double fluorescent cre reporter line ($Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo/J}$ or $Rosa26^{mTmG}$)[15] were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained on an outbred (NMRI) background. Animal care was in accordance with national and institutional guidelines. Detailed Methods are provided in the Online Supplement.

Results

Wt1 Expression in the Heart Is Not Restricted to Epicardial Cells

Genetic lineage tracing by the cre/*loxP* technology relies on the fact that cre is expressed only in the precursor tissue but not in the differentiated cell type for which contribution should be tested. For *Wt1*-based epicardial fate mapping systems, it requires that *Wt1* is expressed in the (pro)epicardium but not in any other cell type of the developing and mature heart. Cardiac expression of *Wt1* has been reported for the (pro)epicardium, but also for cells of unknown nature in the subepicardium and the interventricular septum,[16–18] prompting us to reinvestigate *Wt1* expression during cardiac development (Figure 1).

In situ hybridization analysis on embryo sections did not detect *Wt1* mRNA in E8.5 hearts (Online Figure I). At E9.5, strong *Wt1* expression was found in the proepicardium and in the few epicardial cells that have settled onto the myocardium in the atrioventricular sulcus. At E14.5, the entire epicardial layer was positive for *Wt1,* as were individual cells in the interventricular septum and in the subepicardium and myocardium of the ventricles (Figure 1A, B).

Cardiac expression of Wt1 protein was not found in E8.5 embryos by immunofluorescence analysis (Online Figure I). At E9.5, high levels of Wt1 protein were confined to nuclei of the proepicardium and the first epicardial cells in the sulcus region. Weak Wt1 expression was occasionally observed in nuclei of the myocardium and endocardium (Figure 1C). At E14.5, all epicardial cells were positive for nuclear Wt1 protein, whereas in the interventricular septum the subepicardium and myocardium of the ventricles individual Wt1-positive cells were detected. Expression in the myocardium occurred mostly in tight association with luminal spaces (Figure 1D). Coexpression of Wt1 and the endothelial marker isolectin B4[19] at E9.5 and E14.5 confirmed endogenous expression of Wt1 in endothelial cells of the endocardium

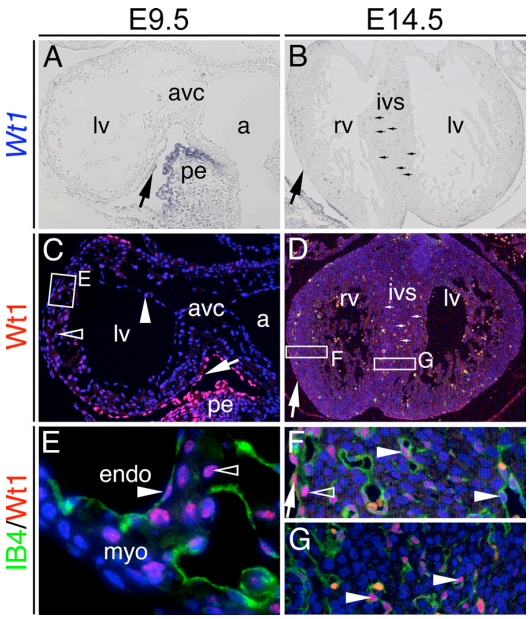

Figure 1. Expression of Wt1 in cardiac development. A and B, In situ hybridization analysis of *Wt1* mRNA. **C and D**, Immunofluorescence of Wt1 protein. **E–G**, Coimmunofluorescence of Wt1 and the endothelial marker isolectin B4 (IB4) on heart sections of wild-type mouse embryos at embryonic day (E) 9.5 and E14.5, as indicated. **Arrows** point to the epicardium. **Arrowheads** mark $Wt1^{+}IB4^{+}$ cells in the endocardium and vessels. **Open triangles** point to $Wt1^{+}IB4^{-}$ cells in the myocardium. **Small arrows** point to $Wt1^{+}$ cells in the interventricular septum. Nuclei are counterstained with 4′,6-diamidino-2-phenylindol. a, atrium; avc, atrioventricular canal; endo, endocardium; ivs, interventricular septum; myo, myocardium; lv, left ventricle; rv, right ventricle; pe, proepicardium.

and/or of the coronary vasculature (Figure 1E–G). Because of technical constraints (antibodies against Wt1 and the two well-established nuclear myocardial marker proteins Nkx2.5/Prox1 were all raised in rabbits), it was not possible to unambiguously demonstrate that $Wt1^{+}$ isolectin $B4^{-}$ cells in the E9.5 myocardium and the E14.5 subepicardium (open triangle in Figure 1E, F) are myocardial in nature. Thus, *Wt1* mRNA and Wt1 protein expression are not restricted to the (pro)epicardium in the developing heart; they are also found in differentiated endothelial cells. A weak myocardial expression at E9.5 cannot be excluded at this point.

$Wt1^{creEGFP}$ Mediates Sporadic and Ectopic Recombination in the Heart and the Rest of the Embryo

Although endogenous expression of *Wt1* in endothelial cells of the developing heart does not allow a *Wt1*-based lineage tracing system to claim an epicardial origin of this cell type, fibroblasts, SMCs, and (possibly) cardiomyocytes still may be analyzed for such a descent using this genetic tool. However, this requires that the cre activity pattern recapitulates endogenous expression of *Wt1* in a faithful manner. To stringently test if the $Wt1^{creEGFP}$ line that was previously used for this approach fulfills this criterion,[9] we performed a careful analysis of the recombination activity of this line

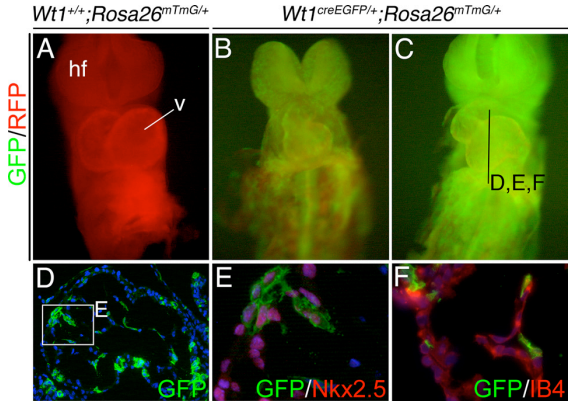

Figure 2. *Wt1*^{creEGFP}-mediated recombination in embryonic day (E) 8.5 embryos. Analysis of the recombination activity in *Wt1*$^{creEGFP/+}$*Rosa26*$^{mTmG/+}$ embryos and *Rosa26*$^{mTmG/+}$ control mice at E8.5. **A–C**, Epifluorescence analysis of green fluorescent protein (GFP) and red fluorescence protein (RFP) expression in whole embryos. **D–F**, Immunofluorescence analysis of GFP, the myocardial marker Nkx2.5, and the endothelial marker isolectin B4 (IB4) on heart sections of a *Wt1*$^{creEGFP/+}$*Rosa26*$^{mTmG/+}$ embryo as indicated. Nuclei are counterstained with 4′,6-diamidino-2-phenylindol. hf, head folds; v, ventricle.

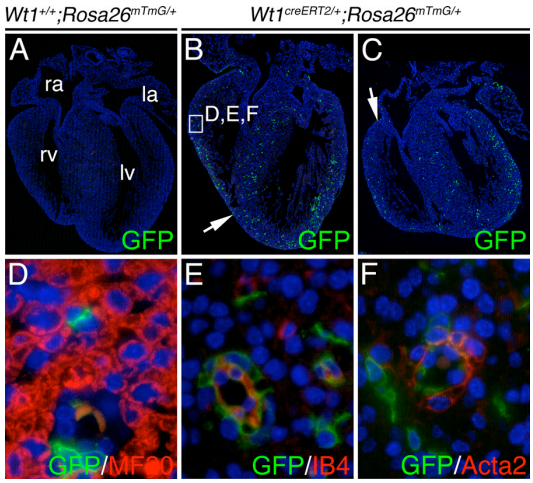

Figure 3. Inducible *Wt1*^{creERT2}-mediated recombination in late cardiac development. Pregnant mothers were injected at embryonic day (E) 11.5 with tamoxifen and hearts of *Wt1*$^{creERT2/+}$ *Rosa26*$^{mTmG/+}$ and *Rosa26*$^{mTmG/+}$ control mice analyzed for recombination activity at E16.5. **A–C**, Immunofluorescence of GFP expression in sections of whole hearts. **D–F**, Coimmunofluorescence of GFP, the myocardial marker MF20, the endothelial marker isolectin B4 (IB4) and the smooth muscle cell (SMC) marker Acta2 on sections of whole hearts. Shown are higher magnification images of a region in the right ventricle (**B**). **Arrows** point to the epicardium. Nuclei are counterstained with 4′,6-diamidino-2-phenylindol. la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle.

during cardiogenesis using the *Rosa26*^{mTmG} mouse line as a sensitive reporter. In this reporter line, recombination is easily visualized by bright membrane-bound green fluorescent protein (GFP) expression replacing a membrane-bound red fluorescent protein, and anti-GFP immunofluorescence analysis on sections additionally allows reliable cellular resolution of cre recombination events.[15]

To our surprise, *Wt1*$^{creEGFP/+}$*Rosa26*$^{mTmG/+}$ embryos exhibited highly variable patterns of green fluorescence in the entire embryo at E8.5 when neither *Wt1* mRNA nor Wt1 protein was detected (Figure 2A–C, Online Figure I). GFP expression analysis on embryo sections confirmed the stochastic presence and random distribution of GFP-positive cells in the heart at this stage. Recombination occurred in cardiomyocytes and endocardial cells, as shown by coexpression with the myocardial marker Nkx2.5 and the endothelial marker isolectin B4, respectively (Figure 2D–F, Online Figure II). At E9.5, GFP expression did not match endogenous Wt1 expression but was again highly variable in all embryonic tissues (Online Figures I and III). GFP-positive cardiomyocytes and endocardial cells were randomly distributed throughout the heart and also were found in regions where the epicardium had not yet settled (Online Figure III). Analysis of E12.5 and E18.5 embryos revealed a similar variability of GFP expression in all embryonic tissues; variable numbers of cardiomyocytes and endothelial cells were positive for GFP in the heart. At E18.5, we detected additional recombination in the heart in a fraction of periostin-expressing fibroblasts and Acta2-expressing SMCs (Online Figures IV and V). We conclude that *Wt1*^{creEGFP}-mediated reporter activity does not reflect endogenous expression of Wt1, but occurs randomly and sporadically in all embryonic tissues including the myocardium, endocardium, and vessels of the heart.

Wt1^{creERT2} Recombines Inefficiently in the Epicardium and Its Cellular Derivatives

Wt1^{creERT2} represents a second *Wt1* allele that was used for epicardial fate mapping as well as for "epicardium"-specific gene deletion experiments.[10,20,21] This allele mediates expression of a fusion protein of cre with a variant of the estrogen receptor that allows activation of cre activity by administration of tamoxifen in a temporally controlled fashion.[9] Our initial tests revealed that injection of high doses of tamoxifen before E11.5 results in embryonic lethality. Injection of 4 mg of tamoxifen intraperitoneally to pregnant dams at E11.5 led to earliest and highest recombination in our experience, although this procedure resulted in premature delivery at E16.5.

Analysis of GFP expression in *Wt1*$^{creERT2/+}$*Rosa26*$^{mTmG/+}$ hearts at E16.5 revealed low and highly variable levels of recombination in the heart. Epicardial recombination was incomplete, and limited GFP expression was detectable throughout the ventricular compact myocardium and the interventricular septum (Figure 3A–C). Double immunofluorescence analysis for GFP and MF20 failed to detect recombination within cardiomyocytes, whereas cells double-positive for GFP and isolectin B4 (endothelial cells) and GFP and Acta2 (SMCs) revealed rare recombination events in both cell types (Figure 3D–F). Thus, the *Wt1*^{creERT2} line is inadequate to follow the cellular descendents of the (pro)epicardial entity. The lack of recombination in cardiomyocytes indicates that these cells are not epicardium-derived or, alternatively, that *Wt1*-expressing cardiomyocytes are not present after

E10.5, even though the low recombination efficiency may conceal some rare events.

Discussion

Our results show that *Wt1* expression in the heart is not restricted to the epicardium and that available $Wt1^{cre}$ knock-in lines do not faithfully recapitulate endogenous expression of *Wt1* ($Wt1^{creEGFP}$) and recombine poorly ($Wt1^{creERT2}$), respectively.

Expression analyses of Wt1 on the level of mRNA and protein reported on subepicardial expression of the gene/protein in addition to strong (pro)epicardial expression during cardiac development. It was suggested that expression occurs in epicardium-derived cells and, hence, that epicardial cells maintain *Wt1* expression after they have left the epicardial continuity and become mesenchymal.[16–18] Our expression studies do not support this notion but strongly argue that subepicardial expression of *Wt1*/Wt1 is mainly found in endothelial cells of the endocardium and the coronary vasculature. Expression levels in endothelial cells are lower than in the epicardium but are likely to comprise the majority of coronary vessels that form as a plexus that grows from the sinus venosus toward the apex of the heart from E11.5 to E14.5. Notably, Wt1 expression is reactivated in endothelial cells of the coronary vasculature after myocardial infarction,[22] indicating a conserved regulatory module for endothelial *Wt1* expression in development and regeneration. We detected weak expression of Wt1 protein but not *Wt1* mRNA in the E9.5 myocardium. Alternative assays to in situ hybridization may address in the future whether low levels of *Wt1* transcription actually occur in cardiomyocytes at this stage. Endogenous expression of Wt1 in the endothelium/endocardium, and possibly the myocardium in the developing heart clearly excludes *Wt1*-based cre lines to trace an epicardial contribution to myocardial, endothelial, and endothelium derived cells in the mouse heart. Nonepicardial expression of Wt1 also was reported in the zebrafish heart, which prompted those investigators to exclude this gene from epicardial fate mapping efforts in this species.[23]

Based on the strong epicardial expression of *Wt1*, two *cre* lines have been developed to trace and manipulate epicardial cells and their descendants. Our present study clearly has shown that the $Wt1^{creEGFP}$ line mediates sporadic and highly variable recombination in the heart as well as in the rest of the embryo at stages when a (pro)epicardium is not present in the embryo, and in a pattern that is incompatible with endogenous *Wt1* expression. The underlying reason for this random activation of cre activity in this line remains unclear. We have excluded the presence of a *neo* cassette, which might interfere with proper transcriptional activation of *cre*. At present, we assume that integration of the targeting vector was imprecise or incorrect placing *cre* under different regulatory elements. In contrast, recombination efficiency obtained with the tamoxifen-inducible $Wt1^{creERT2}$ line in epicardial cells was extremely low, allowing for following the fate of a small set of epicardium-derived cells only. Furthermore, epicardial fate analysis was restricted to stages E11.5 onward because injection of tamoxifen at earlier stages resulted in embryonic death, as reported in other studies.[24,25]

We conclude that an epicardial origin of myocardial and endothelial cells in the heart cannot be deduced using *Wt1*-based cre/*loxP* technology. Our data additionally call for cautious interpretation and reinvestigation of data obtained by conditional gene deletion experiments using *Wt1*-based cre lines.

Sources of Funding

This work was supported by grants from the German Research Foundation (D.F.G.) for the Cluster of Excellence REBIRTH (From Regenerative Biology to Reconstructive Therapy) and for the Clinical Research Group KFO136 at Hannover Medical School (A.K.).

Disclosures

None.

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Novelty and Significance

What Is Known?

- The embryonic epicardium is a source of trophic signals that affects the myocardium. It also is a source of cells that form the coronary vasculature and the fibrous skeleton of the heart.
- During cardiac development, *Wt1* is expressed in the proepicardium and epicardium.
- Genetic lineage tracings based on *Wt1* regulatory sequences have provided suggestive evidence that epicardium-derived cells also adopt a myocardial fate in the mouse.

What New Information Does This Article Contribute?

- *Wt1* expression is not restricted to epicardial cells. During cardiac development, the protein also is expressed in endothelial and endocardial cells and, weakly, in cardiomyocytes.
- The *Wt1*^{creEGFP} line mediates sporadic and highly variable recombination in all cardiac cell types, including cardiomyocytes, as well as ectopic recombination at extracardiac sites.
- *Wt1*^{creERT2}-mediated recombination in the epicardium is incomplete and highly variable but does not occur in late cardiomyocytes.

The epicardium is an epithelial monolayer that covers and mechanically protects cardiac muscle. During embryogenesis, the epicardium also acts as a signaling center that promotes myocardial growth and coronary plexus formation, and is a source of cells for the fibrous skeleton of the heart, smooth muscle cells, and fibroblasts of coronary vessels. Although these functions have been confirmed for all vertebrates, a genetic (cre/*loxP*-mediated) lineage tracing study based on regulatory elements of the epicardially expressed *Wt1* gene have suggested that in the mouse, epicardial cells also substantially contribute to the myocardium. This finding is of high clinical relevance because it may open new avenues for deriving cardiomyocytes for myocardial cell therapy. Our study shows that *Wt1* expression not only is restricted to the epicardium but also is expressed in differentiated cells types of the heart, namely endothelial cells and cardiomyocytes. Also, we provide evidence that the *Wt1*^{creEGFP} line that was used for epicardial fate mapping before mediates ectopic and sporadic recombination in cardiomyocytes as well as in other cell types in which *Wt1* is not expressed. In addition, the inducible *Wt1*^{creERT2} line recombines poorly in the epicardium, making it difficult to follow all the descendants of this tissue. Thus, cardiomyocyte fate of epicardial cells could not be substantiated using this approach.

Supplemental Online Data

Wt1 and epicardial fate mapping

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Supplemental Online Methods

Mice and genotyping

Mice with a knock-in of the *cre* recombinase gene in the *Wt1* locus (*Wt1*$^{tm1(EGFP/cre)Wtp}$, synonym: *Wt1*^{creEGFP})[1], mice with a knock-in of the tamoxifen inducible *cre*-recombinase in the *Wt1* locus (*Wt1*$^{tm2(cre/ERT2)Wtp}$, synonym: *Wt1*^{creERT2})[1] and the double fluorescent cre reporter line *(Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo/J}$, synonym: *Rosa26*^{mTmG})[2] were obtained from the Jackson Lab and maintained on an outbred (NMRI) background. The fate of epicardial cells was analyzed in *Wt1*$^{creEGFP/+}$*;Rosa26*$^{mTmG/+}$ and *Wt1*$^{creERT2/+}$*;Rosa26*$^{mTmG/+}$ embryos. These were obtained from matings of *Wt1*$^{creEGFP/+}$ and *Wt1*$^{creERT2/+}$ males, respectively, with *Rosa26*$^{mTmG/mTmG}$ females. In the latter case, Tamoxifen (Sigma) was dissolved in ethanol at 100 mg/ml and then emulsified in corn oil (Sigma) to a final concentration of 12.5 mg/ml. 4 mg of Tamoxifen were intraperitoneally injected into mice at gestation day 11.5. Embryos for *Wt1* expression analysis were obtained from matings of NMRI wildtype mice.

For timed matings, vaginal plugs were checked in the morning after mating and noon was designated as embryonic day (E) 0.5. Female mice were sacrified by cervical dislocation. Embryos were harvested in PBS, decapitated, fixed in 4% paraformaldehyde overnight and stored in 100% methanol at -20^{o}C before further use. Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR (protocols are available upon request). H. Hedrich, state head of the animal facility, approved the care of animals and experiments at Medizinische Hochschule Hannover.

In situ hybridization analysis

In situ hybridization analysis of 10-µm paraffine sections with digoxigenin-labeled antisense riboprobes followed published protocols.[3] Details of used probes are available upon request.

Immunohistochemistry

For immunohistochemistry on 4-µm paraffine sections rabbit polyclonal antibody against Wt1 (1:100, CA1026, Calbiochem), rabbit polyclonal antibody against GFP (1:200, sc-8334, Santa Cruz), mouse monoclonal antibody against GFP (1:200, #814460001, Roche), mouse monoclonal antibody against Acta2 (1:200, C6198, Sigma), mouse monoclonal antibody against Acta2 (1:200, F3777, Sigma), Fluorescein-labeled GSL I – isolectin B4 (1:100, FL-1101, VectorLabs), mouse monoclonal antibody against MF 20 (1:200, Hybridoma Bank University of Iowa), rabbit polyclonal antibody against Nkx2.5 (1:200, ab35842, Abcam), rabbit polyclonal against Periostin (1:200, ab14041, Abcam) were used as primary antibodies.

Biotinylated goat-anti-rabbit (Dianova), Alexa488 goat-anti-rabbit (Invitrogen), Alexa488 donkey-anti-mouse (Invitrogen A21202), biotinylated goat-anti-mouse (Dianova 115-067-003), Alexa-Fluor555 goat-anti-mouse (Invitrogen A-21424) and Alexa-Fluor555 goat-anti-rabbit (Invitrogen A-21428) were used in a dilution of 1:400 as secondary antibodies. Nuclei were stained with 4',6-diamidino-2-phenylindol (DAPI) (Roth).

For antigen retrieval all sections were boiled for 3 min in antigen unmasking solution (H-3300, Vector Laboratories Inc) in a pressure cooker. Signal amplification was performed using the Tyramide Signal Amplification (TSA) system from Perkin-Elmer (NEL702001KT, Perkin Elmer LAS). For simultaneous detection of GFP and differentiation markers primary and secondary antibodies, respectively, were applied at the same time.

References

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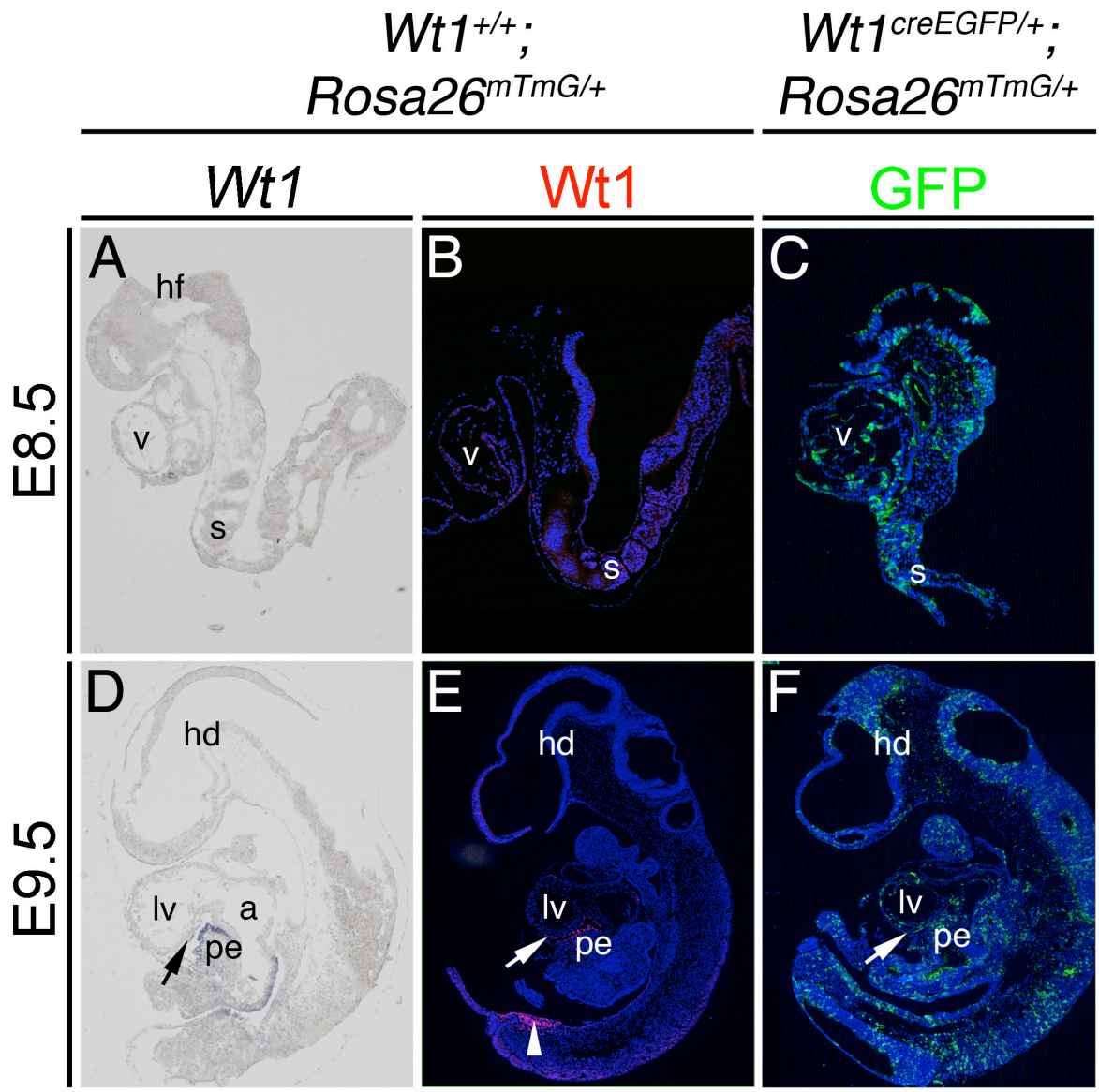
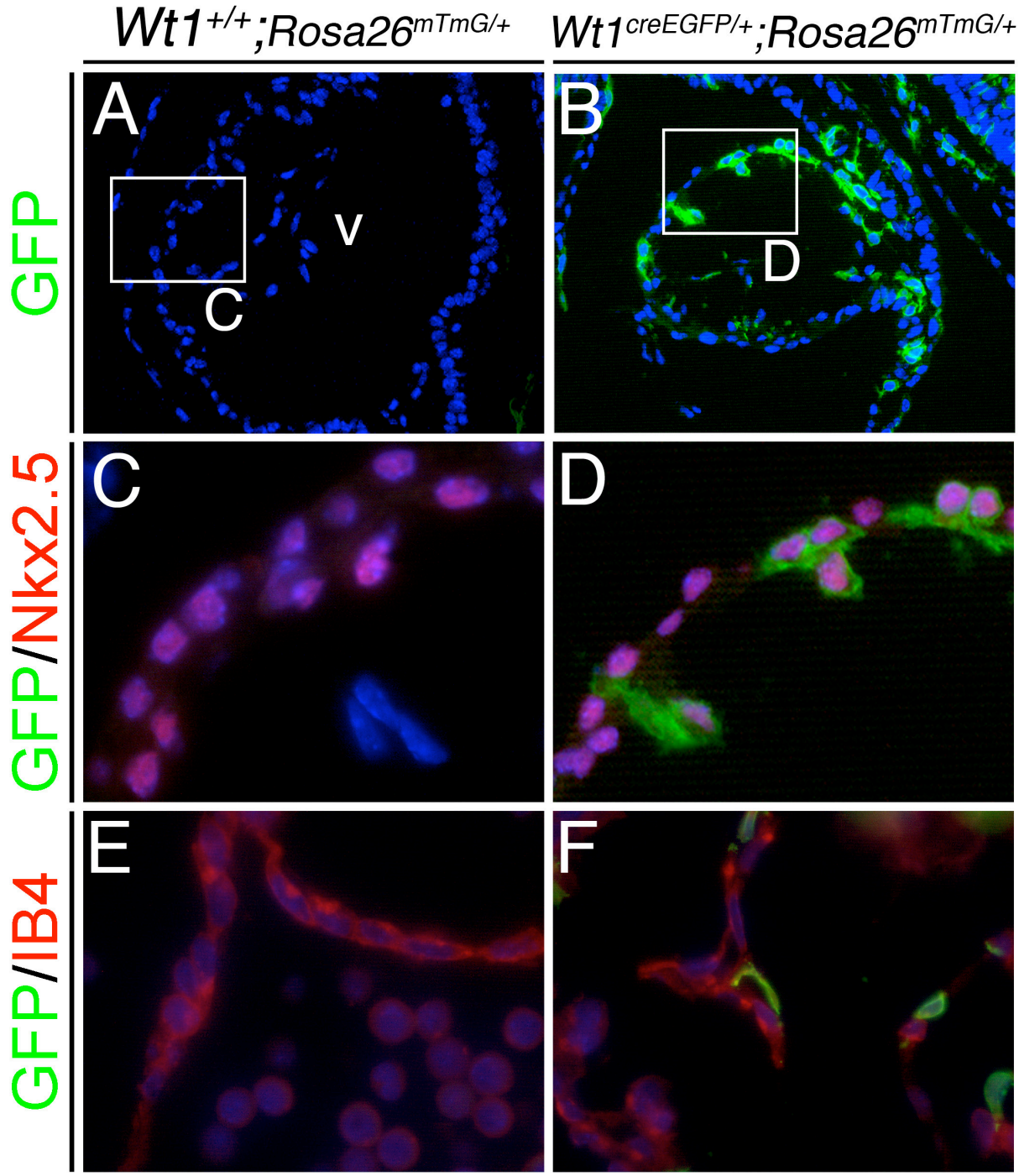
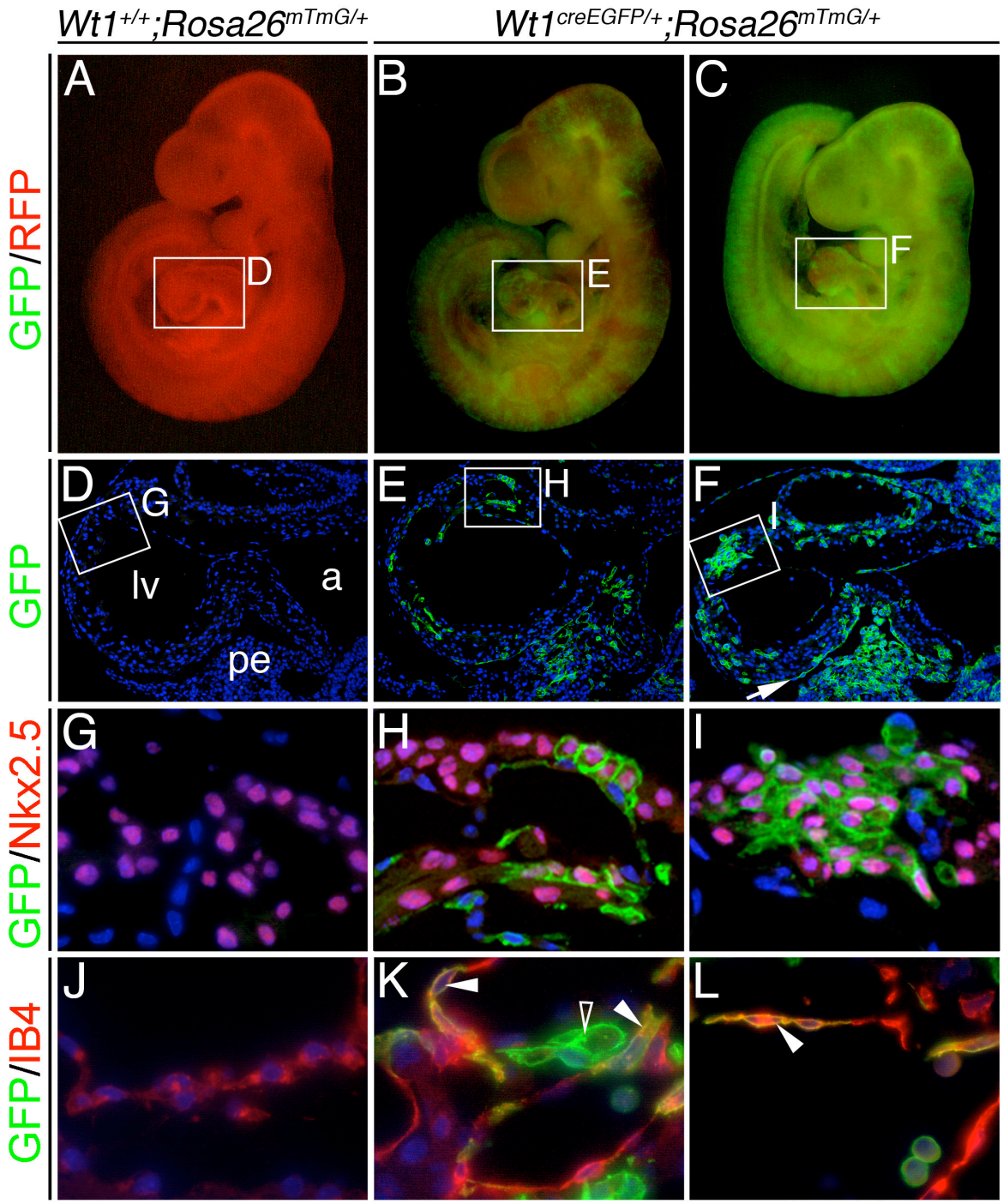
Supplemental Online Figures



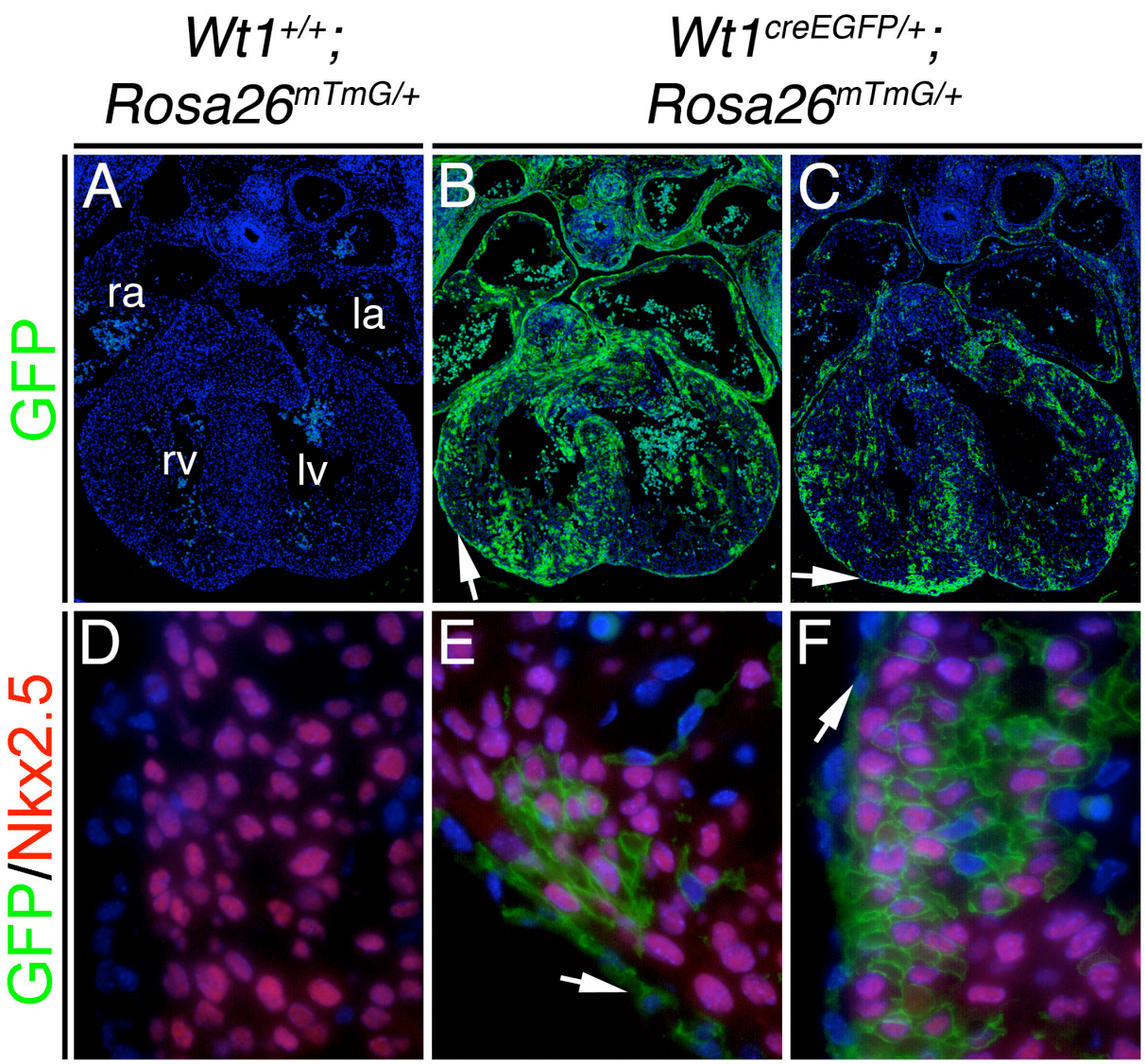
Online Figure I. Comparative analysis of endogenous expression of *Wt1* mRNA and Wt1 protein and *Wt1*^{creEGFP} mediated recombination in E8.5 and E9.5 embryos. A and **D**, *in situ* hybridization analysis of *Wt1* mRNA; **B** and **C**, **E** and **F**, immunofluorescence analysis of Wt1 (in red, **B** and **E**) and GFP protein expression (in green, **C** and **F**) on sagittal sections of *Wt1*$^{creEGFP/+}$*;Rosa26*$^{mTmG/+}$ and *Rosa26*$^{mTmG/+}$ control embryos at E8.5 and E9.5 as indicated. At E8.5, neither *Wt1* mRNA nor Wt1 protein is detected whereas sporadic GFP expression is found throughout the embryo. At E9.5, *Wt1* mRNA and Wt1 protein is found in the proepicardium, the forming epicardium (arrow in **D** and **E**) and the urogenital ridge (arrowhead in **E**). The GFP expression is highly variable throughout the heart and the whole embryo. a, atrium; hd, head; hf, head folds; lv, left ventricle; pe, proepicardium; s, somite; v, primitive ventricle.



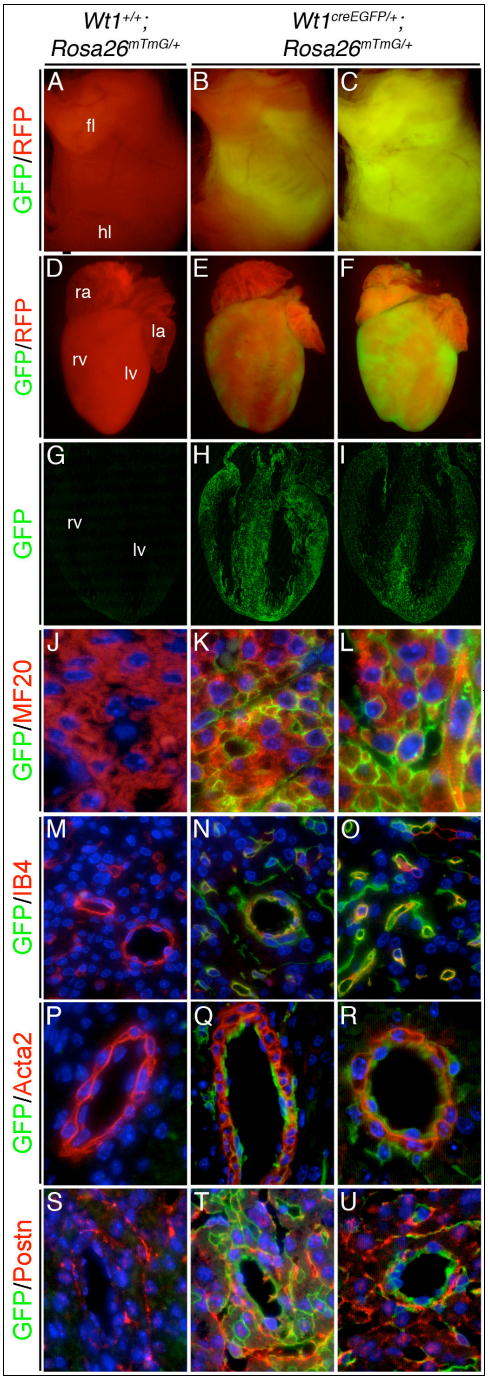
Online Figure II. *Wt1*^{creEGFP}-mediated recombination in E8.5 embryos. Analysis of the recombination activity in *Wt1*$^{creEGFP/+}$;*Rosa26*$^{mTmG/+}$ embryos and *Rosa26*$^{mTmG/+}$ control mice at E8.5. **A** and **B**, immunofluorescence analysis of GFP expression on heart sections. **C** through **F**, coimmunofluorescence analysis of expression of the lineage marker GFP, the myocardial marker Nkx2.5, and the endothelial marker IB4 on heart sections, magnified regions are indicated by white rectangles. Nuclei are counter-stained with 4',6-diamidino-2-phenylindol. v, ventricle.



Online Figure III. *Wt1*^{creEGFP}-mediated recombination in E9.5 embryos. Analysis of the recombination activity in *Wt1*$^{creEGFP/+}$;*Rosa26*$^{mTmG/+}$ embryos and *Rosa26*$^{mTmG/+}$ control mice at E9.5. **A** through **C**, epifluorescence analysis of GFP and RFP expression in whole embryos. **D** through **L**, (co-) immunofluorescence analysis of GFP, the myocardial marker Nkx2.5, and the endothelial marker IB4 on heart sections as indicated. Magnified regions are indicated by white rectangles. **Arrows** point to the epicardium. **Arrowheads** mark GFP^{+}IB4^{+} cells in the endocardium. **Open triangles** point to GFP^{+}IB4^{-} cells in the myocardium. Nuclei are counter-stained with 4',6-diamidino-2-phenylindol. a, atrium; lv, left ventricle; pe, proepicardium.



Online Figure IV. *Wt1*^{creEGFP} mediated recombination in E12.5 hearts. Analysis of the cre recombination activity by GFP expression in E12.5 hearts of *Wt1*^{creEGFP};*Rosa26*$^{mTmG/+}$ and *Rosa26*$^{mTmG/+}$ control embryos. **A** through **F**, (Co-)immunofluorescence analysis of GFP (in green, membranes) and the myocardial marker Nkx2.5 (in red, nuclei) on transverse heart sections as indicated. **D** through **F** show higher magnification images of the right ventricle. White arrows point to the epicardium. la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle.



Online Figure V. *Wt1*^{creEGFP}-mediated recombination in E18.5 embryos and hearts. Analysis of cre recombination activity in *Wt1*^{creEGFP};*Rosa26*$^{mTmG/+}$ embryos and *Rosa26*$^{mTmG/+}$ control mice. **A** through **F**, epifluorescence analysis of GFP and RFP expression in embryonic trunks (**A** through **C**) and hearts (**D** through **F**). **G** through **U**, (co-)immunofluorescence analysis of GFP (in green, membranes) and the myocardial marker MF20, the endothelial marker IB4, the SMC marker Acta2, and the fibroblast marker Postn (all in red) on sections of whole hearts as indicated. **J** through **U** show higher magnification images of the right ventricle. fl, fore limb; hl, hind limb; la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle.

Epicardial function of canonical Wnt-, Hh-, Fgfr1/2- and Pdgfrα-signaling

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Key words: epicardium, EMT, cardiac fibroblast, smooth muscle cell, coronary vasculature

Running title: Epicardial signaling pathways

Submitted manuscript.

Abstract

Rationale: The embryonic epicardium is a crucial source of cells for the cardiac fibrous skeleton and the coronary vasculature. Previous reports suggested that Wnt-, Hh-, Fgfr1/Fgfr2- and Pdgfrα-signaling pathways are required in the epicardium for mobilization and differentiation of epicardial cells, and/or for the formation of the coronary vasculature. However, the *cre* lines used for conditional ablation of these pathways might not have been specific for the epicardium.
Objective: To determine the relevance of canonical Wnt-, Hh-, Fgfr1/Fgfr2- and Pdgfrα-signaling in the developing epicardium.
Methods and Results: We used a *Tbx18*^{cre}-mediated conditional approach to delete and to stabilize, respectively, the unique downstream mediator of canonical Wnt-signaling, Ctnnb1, to delete and to stabilize, respectively, the unique downstream mediator of Hh-signaling, Smo, and to delete *Fgfr1/Fgfr2* and *Pdgfrα* in epicardial development. We show that epicardial loss of *Ctnnb1* does not affect cardiac development whereas epicardial expression of a stabilized version of Ctnnb1 results in formation of hyperproliferative epicardial cell clusters. Epicardial loss of *Shh* and *Smo* does not affect cardiac development whereas expression of a constitutively active version of Smo in the epicardium leads to epicardial thickening and loss of epicardial mobilization. Epicardial loss of *Fgfr1* and *Fgfr2* does not affect cardiac development either. In contrast, epicardial loss of *Pdgfrα* prevents differentiation of epicardium-derived cells into mature fibroblasts.
Conclusions: Our data question earlier reports on a role of canonical Wnt-, Hh- and Fgfr1/Fgfr2-signaling in epicardial development. They support the notion that Pdgfrα-signaling is crucial for differentiation of cardiac fibroblasts from epicardium-derived cells.

Non-standard abbreviations and acronyms

DAPI	4,6-diamidino-2-phenylindole
E	embryonic day
EMT	epithelial-mesenchymal transition
EPDC	epicardium-derived cell
SM	smooth muscle
SMC	smooth muscle cell

Introduction

The epicardium, the outer epithelial lining of the heart, exerts a crucial role both during development and disease as a source of signals and cells for the underling myocardium and the coronary vasculature. The epicardium arises from extracardiac precursor cells that constitute a grape-like aggregate at the venous pole of the developing heart. Groups of cells detach from this proepicardium, and adhere on the overlying myocardium from around embryonic day (E) 9.5 in the mouse. At E10.5, these cell clusters have spread out and formed a contiguous epithelial monolayer. Concomitant with the ingrowth of the coronary endothelium from the sinus venosus at E12.5, individual epicardial cells undergo an epithelial-mesenchymal transition (EMT) and migrate into the subepicardial space. These epicardium-derived cells (EPDCs) differentiate into interstitial and perivascular fibroblasts and into smooth muscle cells (SMCs) that surround the coronary vessels.[1,2] A contribution to coronary endothelia and cardiomyocytes has been reported in some lineage tracing studies in the mouse but was not confirmed in other studies or found in other vertebrates.[3,4,5,6,7,8,9] Intriguingly, in myocardial injury conditions the adult epicardium reactivates an embryonic gene program and supports myocardial healing (in zebrafish) and revascularization and scar formation (in the mouse) by secretion of angiogenetic factors and differentiation of fibroblasts.[10,11]

Mobilization and differentiation of epicardial cells is likely to be controlled by a number of autocrine and paracrine signals as suggested by the phenotypic consequences of mice with epicardium-specific (cre/loxP-mediated) deletion of crucial signaling components.[2,12] Using cre lines based on GATA binding protein 5 (*Gata5)*- and Wilms tumor 1 homolog (*Wt1)*-regulatory elements and a floxed allele of beta-catenin (*Ctnnb1*), a functional requirement of the canonical (Ctnnb1-dependent) wingless-related MMTV integration site (Wnt)-signaling pathway in epicardial EMT and coronary SMC differentiation was suggested.[13,14] Loss of platelet-derived growth factor receptor alpha (Pdgfrα) resulted in reduced EMT and a deficit in cardiac fibroblast formation,[15] which holds true as well for the combined conditional loss of fibroblast growth factor (Fgf) receptors 1/2 which was similarly achieved by a $Wt1^{cre}$-mediated recombination of floxed alleles in the epicardium.[16] Furthermore, epicardial derived Fgf-signals have been shown to induce sonic hedgehog (Shh) signaling, which in turn, regulates the formation of arterial and venous coronaries.[17] Finally, we showed by T-box18 ($Tbx18)^{cre}$-mediated epicardial deletion of the gene encoding the transcription factor recombination signal binding protein for immunoglobulin kappa J region (*Rbpj*) that Notch-signaling regulates SMC differentiation of EPDCs once they have reached a perivascular position.[6] Shared phenotypic consequences may argue for pathway cooperation in distinct epicardial subprograms including EMT (Wnt/Pdgfrα/Fgfr1,2) and SMC differentiation (Wnt/Notch). However, recent studies indicated that the *Gata5::cre* line and the $Wt1^{cre}$ lines,[18,4] that were used in these conditional gene targeting experiments, may not have been specific for the epicardium but mediated wide-spread recombination in other cardiac cell types as well.[19,20] Before embarking on genetic interaction studies of Notch-signaling with any of these pathways, we therefore wished to reevaluate the epicardial requirement of these pathways (canonical Wnt, Fgfr1/Fgfr2, Hh, Pdgfrα) using the $Tbx18^{cre}$ line which we have recently characterized to mediate specific recombination in the epicardium of the right ventricle.[6]

Here, we show that in mice with epicardial ($Tbx18^{cre}$-mediated) deletion of *Ctnnb1*, *Fgfr1/2*, *Shh* and of the gene encoding the Shh signal transducer smoothened (*Smo*) epicardial mobilization and differentiation is undisturbed. Using genetic gain-of-function approaches, we find evidence that epicardial activation of canonical Wnt- and Hh-signaling is actually deleterious for epicardial and myocardial development *in vivo*. Furthermore, we confirm that *Pdgfrα* is required for epicardial EMT and differentiation of cardiac fibroblasts.

Material and Methods

Mice

Mice with a knock-in of the cre-recombinase gene in the *Tbx18* locus (*Tbx18*$^{tm4(cre)Akis}$, synonym: *Tbx18*^{cre}) were previously generated in the laboratory at the Medizinische Hochschule Hannover.[21] Mice with *loxP* sites flanking the *Ctnnb1* locus from exon 2 to exon 6 (*Ctnnb1*^{tm2Kem} s*ynonym: Ctnnb1*^{fl}*)* were obtained from Rolf Kemler (Max-Planck-Institute for Immunobiology and Epigenetics, Freiburg/Germany),[22] mice with *loxP* sites flanking exon 3 of the *Ctnnb1* locus (*Ctnnb1*^{tm1Mmt}, synonym: *Ctnnb1*$^{(Ex3)fl}$) were obtained from Makoto Mark Taketo (Kyoto University, Kyoto/Japan).[23] Mice with *loxP* sites flanking exon 2 of the *Shh* locus (*Shh*^{tm2Amc}, synonym: *Shh*^{fl}),[24] mice with *loxP* sites flanking exon 4 of the *Fgfr1* locus (*Fgfr1*$^{tm5.1Sor}$, synonym: *Fgfr1*^{fl}),[25] mice with *loxP* sites flanking exons 7 to 10 of the *Fgfr2* locus (*Fgfr2*^{tm1Dor}, synonym: *Fgfr2*^{fl}),[26] mice with *loxP* sites flanking exon 1 of the *Smo* locus (*Smo*^{tm2Amc}, synonym: *Smo*^{fl}),[27] mice with a fusion protein of Enhanced Yellow Fluorescent Protein (EYFP) and the constitutively active W539L point mutation of the Smo protein and a *loxP*-flanked STOP fragment placed between its *Gt(ROSA)26Sor* promotor and the *Smo/EYFP* sequence (*Gt(ROSA)26Sor*$^{tm1(Smo/YFP)Amc}$, synonym: *Smo*^{GOF}),[28] mice with *loxP* sites flanking exons 1 to 4 of the *Pdgfrα* locus (*Pdgfrα*^{tm8Sor}, synonym: *Pdgfrα*^{fl}),[29] and the double fluorescent cre reporter line (*Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$, synonym: *R26*^{mTmG})[30] were all obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). All mice were maintained on an outbred (NMRI) background.
An expanded Materials and Methods section is available in the online data supplement.

Results

Tbx18^{cre} allows epicardium-specific recombination of *loxP*-flanked sequences

The T-box transcription factor gene *Tbx18* is strongly expressed in the proepicardium at E9.5 and in the epicardium until E16.5. Other cardiac expression domains include the sinus venosus, and the myocardium of the left ventricle and the interventricular septum.[31,6] Using *Rosa26*^{mTmG} reporter mice we recently demonstrated that *cre* expression from the *Tbx18* locus mediates recombination in all known *Tbx18* expression domains and their cellular descendants in the heart from E9.5 to E14.5 in a faithful manner. In the right ventricle, EPDCs were shown to differentiate into interstitial and perivascular fibroblasts and coronary SMCs but not into endothelial cells and cardiomyocytes.[6] Since a recent analysis suggested that Tbx18 expression is restricted to a subset of proepicardial cells, and might therefore not mark all epicardial cells and their descendants,[8] we reevaluated epicardial expression of Tbx18 at E10.5 when a contiguous epicardial layer is formed but EMT has not yet commenced. Immunofluorescence analysis of Tbx18 and DAPI nuclear counterstain showed that at this stage all epicardial cells (delineated by collagen (Col)4 staining) expressed Tbx18. Furthermore, *Tbx18*^{cre} mediated expression of membrane-bound GFP from the *Rosa26*^{mTmG} reporter in all epicardial cells at E10.5, confirming our previous results that *Tbx18*^{cre} represents a suitable tool for analysis of epicardial gene function (Online Figure I).

Canonical Wnt-signaling is dispensable for epicardial development

To analyze canonical Wnt-signaling in epicardial development, we investigated the expression of *Axin2*, a *bona fide* transcriptional target of this pathway,[32] by *in situ* hybridization on sections of E9.5 to E14.5 embryonic hearts (Online Figure II). Expression of *Axin2* was neither detected in the E9.5 proepicardium nor in the epicardium at subsequent stages although other sites of *Axin2* expression were readily identified. Since this assay cannot unambiguously exclude low levels of canonical Wnt-signaling in epicardial cells, we used our *Tbx18*^{cre} line and a floxed allele of *Ctnnb1* (*Ctnnb1*^{fl}),[22] the unique intracellular mediator of the canonical sub-branch of Wnt-signaling, to test for a functional requirement of this pathway in epicardial development. Immunofluorescence analysis on sections of hearts of E9.5 and E14.5 *Tbx18*$^{cre/+;}$*Ctnnb1*$^{fl/fl}$*;Rosa26*$^{mTmG/+}$ embryos revealed absence of Ctnnb1 from the proepicardium and epicardium but not from the myocardium confirming the suitability of the approach to specifically delete *Ctnnb1*, thus, canonical Wnt-signaling in all epicardial cells (Online Figure III).

Tbx18$^{cre/+;}$*Ctnnb1*$^{fl/fl}$*;Rosa26*$^{mTmG/+}$ mice survived embryogenesis but died due to skeletal malformations shortly after birth. On histological sections mutant ventricles seemed unaffected at E18.5; the ventricular wall thickness was normal and the integrity of the septa, valves and the epicardium was preserved (Figure 1). Since epicardial cells and signals direct the outgrowth of the coronary plexus, and the formation of coronary SMCs and the fibrous skeleton, we analyzed by immunofuorescence of marker proteins on transverse sections of E18.5 hearts for the presence and distribution of endothelial cells (isolectin-B4 staining (IB4)), of SMCs (transgelin (Tagln, also known as Sm22)) and of fibroblasts (periostin (Postn)). Stainings for all three markers and the capillary density in the right ventricle was indistinguishable between mutant and wildtype hearts, indicating that the coronary vasculature and the fibrous skeleton were not affected by epicardial deletion of *Ctnnb1*. Finally, immunofluorescent detection and subsequent quantification of the lineage reporter GFP from the *Rosa26*^{mTmG} allele visualized normal generation and distribution of epicardial cells and their descendants in mutant hearts both at E13.5 and E18.5 (Figure 1 and Online Figure IV).

To further investigate the character of *Ctnnb1*-deficient epicardial cells, we generated primary epicardial cell cultures from explants of right ventricles of E11.5 hearts. After four days in serum-free medium wildtype cells presented as a monolayer of tightly packed

hexagonal cells that showed membrane staining of the epithelial markers Ctnnb1 and tight junction protein 1 (Tjp1, also known as ZO1), nuclear staining of the epicardial marker Wt1 and weak cortical staining of the SM marker actin, alpha 2, smooth muscle, aorta (Acta2). In *Ctnnb1*-deficient cells Ctnnb1 was absent but the patterns of Tjp1, Wt1 and Acta2 expression were normal confirming their character as epicardial precursor cells (Online Figure VA). Irrespective of the genotype, addition of 10% FCS to the medium resulted in loss of Tjp1 and Wt1, and formation of Acta2-positive stress fibres, consistent with EMT and differentiation into SMCs (Online Figure VB). These results demonstrate that the epicardial loss of *Ctnnb1* does not affect the formation of the epicardium, EMT or differentiation of EPDCs *in vitro* and *in vivo*.

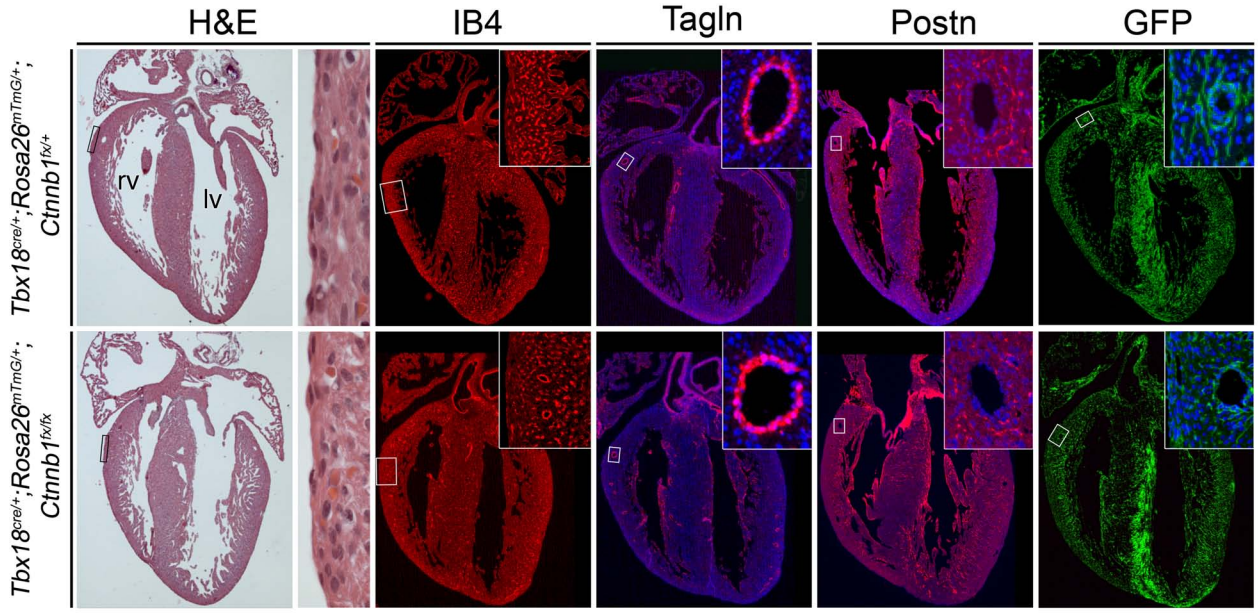

Figure 1. Epicardial loss of *Ctnnb1* does not lead to defects in the coronary vasculature, the ventricular myocardium or in epicardial derivatives. Histological analysis by hematoxylin and eosin staining (H&E) and immunofluorescence analysis of IB4, Tagln, Postn and the cell-lineage marker GFP on mid-transverse sections of E18.5 *Tbx18^{cre};Rosa26$^{mTmG/+}$;Ctnnb1$^{fl/+}$* (control) and *Tbx18^{cre};Rosa26$^{mTmG/+}$;Ctnnb1$^{fl/fl}$* (mutant) hearts. Insets show higher magnification images of the areas marked with rectangles. lv, left ventricle; rv, right ventricle.

Epicardial expression of a stabilized version of Ctnnb1 leads to formation of epicardial cell clusters

To further clarify the role of *Ctnnb1*-dependent Wnt-signaling in epicardial development, we used a gain-of-function approach with conditional (*Tbx18^{cre}*-mediated) overexpression of a stabilized form of *Ctnnb1* (*Ctnnb1$^{(Ex3)fl}$*).[23] *Tbx18$^{cre/+}$;Ctnnb1$^{(Ex3)fl/+}$* embryos died at E12.5 probably due to cardiovascular insufficiency as shown by formation of edema. Morphological examination of whole hearts at E11.5 using GFP epifluorescence revealed a reduced overall size, ballooned atria and clusters of fluorescent cells on the ventricular surface (Figure 2A). Histological inspection exposed a severely thinned ventricular myocardium, as well as defined subepicardial cell clusters (Figure 2B).

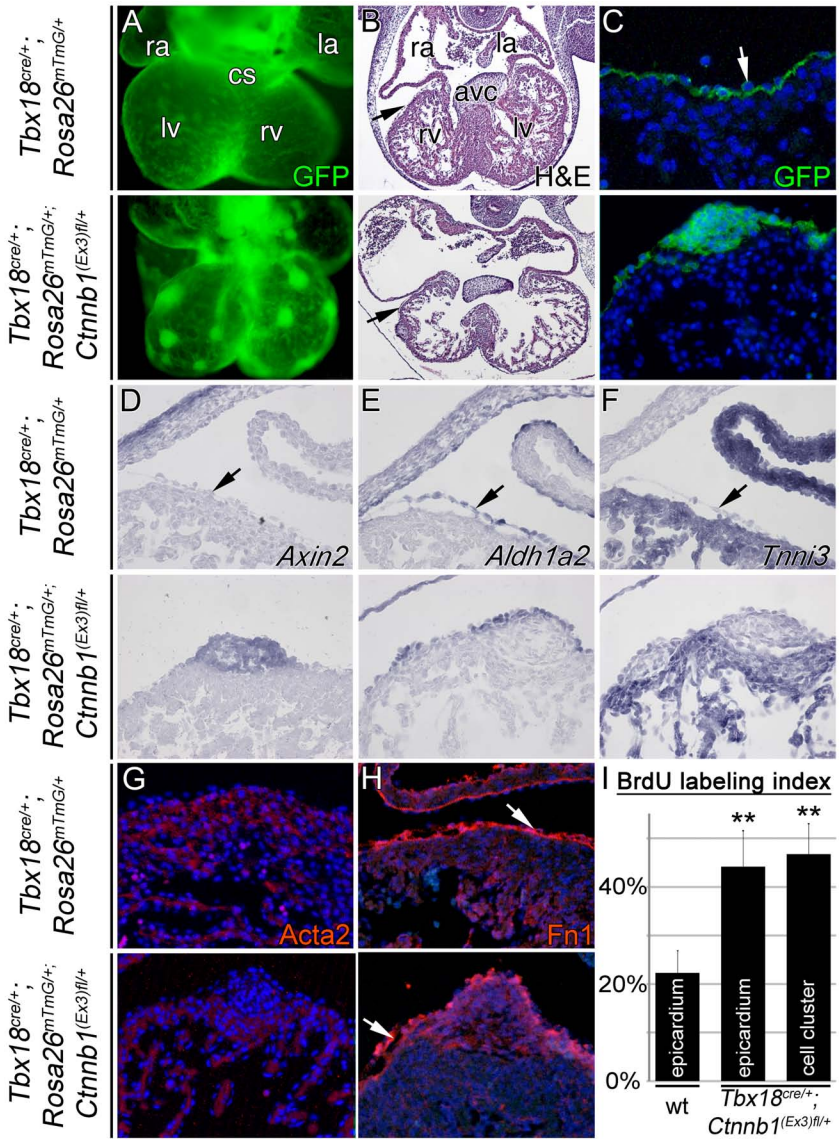

Figure 2. Epicardial expression of a stabilized version of Ctnnb1 induces formation of large cell aggregates on the surface of E11.5 hearts. (**A**) GFP epifluorescence in whole hearts in a dorsal view, (**B** through **H**) analysis of mid-transverse heart sections by hematoxylin and eosin (H&E) staining (**B**), by immunofluorescence of GFP (**C**), by *in situ* hybridization analysis for expression of *Axin2*, *Aldh1a2* and *Tnni3* (**D** through **F**), and by immunofluorescence of Acta2 and Fn1 (**G** and **H**) of control (*Tbx18^{cre};Rosa26$^{mTmG/+}$*) and mutant (*Tbx18^{cre};Rosa26$^{mTmG/+}$;Ctnnb1$^{(Ex3)fl/+}$* embryos. Arrows point to the epicardium. avc, atrioventricular canal; cs, coronary sinus; la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle. (**I**) Quantitative analysis of proliferation rates (% BrdU labeling index) of the epicardium and the cell clusters in genotypes as shown. Probability value is P=0.002.

Analysis of individual clusters by immunofluorescence for GFP proved the epicardial origin of these clusters (Figure 2C), which expressed as expected high levels of *Axin2* (Figure 2D), indicating active Ctnnb1-dependent Wnt-signaling. *In situ* hybridization analysis of the epicardial marker gene aldehyde dehydrogenase family 1, subfamily A2 (*Aldh1a2)* and the cardiomyocyte marker troponin I, cardiac 3 (*Tnni3)*, and immunofluorescence for Acta2 showed, that these cell clusters were neither epicardial nor myocardial in nature and did not

differentiate into SMCs. However, the clusters produced high levels of the extracellular matrix protein fibronectin (Fn)1 that is associated with fibroblast-like cells (Figure 2E-H). The BrdU proliferation assay uncovered a significantly (*P*=0.003) elevated proliferation rate at E11.5 both within the epicardial layer (44±7%) and the *Axin2*$^{+}$ cell clusters (47±6%) in the mutant compared to the control epicardium (22±5%) (Figure 2I and Online Figure VI). Thus, epicardium-specific expression of a stabilized form of Ctnnb1 results in hyperproliferation of epicardial cells and formation of cellular aggregates of fibroblast-like cells indicating that active canonical Wnt-signaling is deleterious for epicardial development.

Epicardial Hh-signaling is dispensable

To determine the presence of Hh-signaling in the developing epicardium, we investigated the expression of *patched homolog1 (Ptch1)*, a well established transcriptional target and a repressor of Hh-signaling[33] by *in situ* hybridization on sections of E9.5 to E14.5 embryonic hearts. Expression of *Ptch1* was neither detected in the E9.5 proepicardium nor in the epicardium at subsequent stages although other sites of *Ptch1* expression were clearly visible (Online Figure VII). To test for a functional requirement of this pathway in epicardial development, we used our *Tbx18*^{cre} line and a floxed allele of *smoothened (Smo*^{fl}*)*[27] which encodes a unique intracellular transducer of Hh-signaling. *Tbx18*$^{cre/+;}$*Smo*$^{fl/fl}$*;Rosa26*$^{mTmG/+}$ mice survived embryogenesis but died due to skeletal malformations shortly after birth. On histological sections mutant ventricles seemed unaffected at E18.5; the ventricular walls were of normal thickness, and valvuloseptal tissues and the epicardial lining were preserved in their integrity. Formation of IB4^{+} endothelial cells and of the endomucin (Emcn)$^{+}$ venous and capillary network of the coronary vasculature[34] was normal and Acta2^{+} SMCs surrounded the endothelial linings of the larger arteries as in the control situation. Postn was widely distributed throughout the ventricular walls of the mutant hearts indistinguishable from the control. Finally, the lineage reporter GFP from the *Rosa26*^{mTmG} allele confirmed normal generation and distribution of epicardial cells and their descendants in mutant hearts both at E14.5 and E18.5 (Figure 3 and Online Figure VIII).

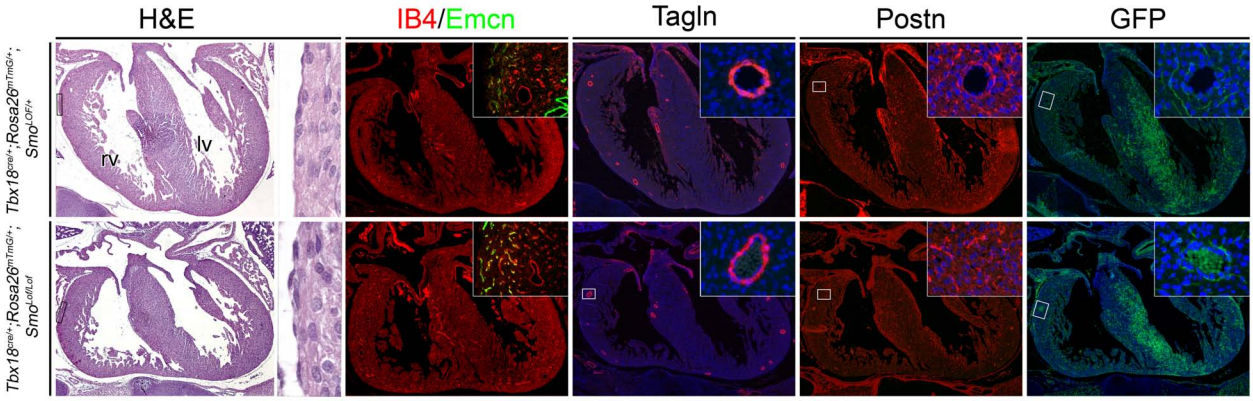

Figure 3. Epicardial loss of *Smo* does not lead to defects in the coronary vasculature, the ventricular myocardium or in epicardial derivatives. Histological analysis by hematoxylin and eosin staining (H&E) and immunofluorescence analysis for IB4/Emcn, Tagln, Postn and the cell-lineage marker GFP on mid-transverse sections of E18.5 *Tbx18*^{cre}*;Rosa26*$^{mTmG/+}$*;Smo*$^{fl/+}$ (control) and *Tbx18*^{cre}*;Rosa26*$^{mTmG/+}$*;Smo*$^{fl/fl}$ hearts. Insets show higher magnification images of the areas marked with rectangles. lv, left ventricle; rv, right ventricle.

Epicardial expression of a constitutive active version of Smo disrupts cardiac development

To investigate the consequences of epicardial activation of Hh-signaling, we (mis-)expressed a constitutively active form of Smo from the *Rosa26* locus (*Smo*^{GOF})[28] using a *Tbx18*^{cre}-mediated approach. *Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Smo*$^{GOF/+}$ embryos died after E13.5 showing severe edema formation. Histological sections of E13.5 mutant embryos revealed a severely hypoplastic ventricular myocardium, a thinned compact myocardial layer and a thickened epicardium (Figure 4A and B). The early coronary plexus formed (shown by IB4 staining), even though the vessels were not located in the deeper compact myocardium (Figure 4C). Immunofluorescent detection of the lineage marker GFP and the epicardial marker Wt1 showed an accumulation of epicardial cells and/or EPDCs and a severe reduction of EMT and immigration into the myocardium (Figure 4D through F). Surprisingly, epicardial proliferation, as detected by the BrdU incorporation assay, was significantly reduced, whereas myocardial proliferation was unaltered at E13.5 (Figure G and H). Thus, epicardium-specific expression of a constitutive active form of Smo results in an increase in epicardial cell density, lack of epicardial EMT and disturbed epicardial-myocardial signaling.

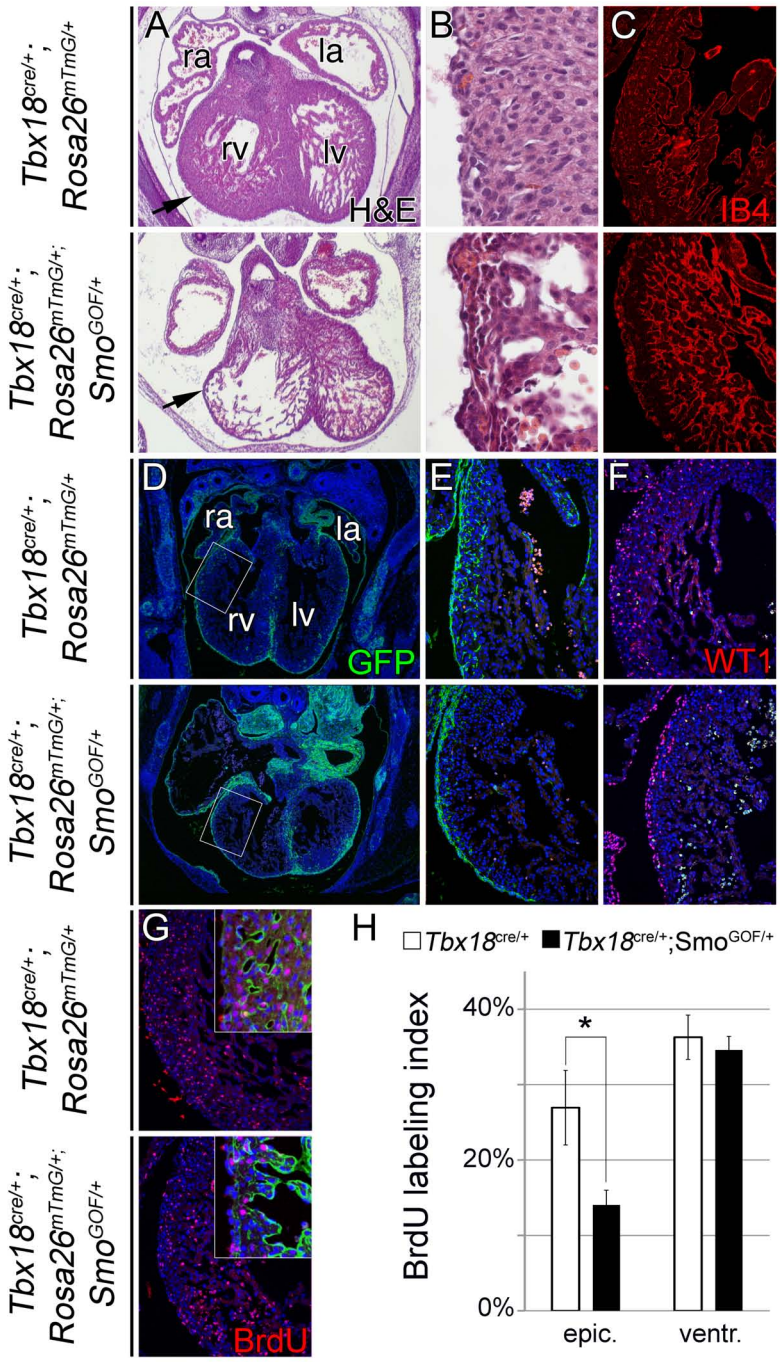

Figure 4. Expression of a constitutive active form of Smo affects epicardial and myocardial integrity in E13.5 hearts. (A through **G)** Mid-transverse sections of E13.5 hearts of control (*Tbx18*^{cre};*Rosa26*$^{mTmG/+}$) and mutant (*Tbx18*^{cre};*Rosa26*$^{mTmG/+}$;*Smo*$^{GOF/+}$) embryos were analyzed by hematoxylin and eosin (H&E) staining (**A** and **B**), by immunofluorescence analysis for IB4 (**C**), the lineage marker GFP (**D** and **E**), the epicardial marker Wt1 (**F**) and the proliferation marker BrdU (**G**). (**A** and **D**) show whole hearts, (**B** and **C**, **E** through **G**) magnified regions of the right ventricle. (**H**) Proliferation rates (% BrdU labeling index) of the epicardium in genotypes and stages as shown. Probability values epicardium, P=0.02; myocardium, P=0.6. la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle.

Epicardial *Shh* is dispensable for heart development

Although Hh-signaling within the epicardium is dispensable for normal heart development, epicardial Hh-signals may act onto the underlying myocardium and coronary system. In fact, it was previously suggested that epicardial Shh-signaling to cardiomyocytes and perivascular cells is required for the expression of vascular endothelial growth factors (VEGFs), thus, may promote growth of the vascular plexus.[17] Our *in situ* hybridization analysis on sections of E9.5 to E14.5 embryonic hearts (Online Figure IX) did not detect expression of the genes encoding the three Hh-ligands (*Shh*, *Dhh* and *Ihh*) although expression at other sites was easily detected. Furthermore, embryos with epicardial (*Tbx18*^{cre}-mediated) deletion of *Shh*[24] (*Tbx18*$^{cre/+}$;*Shh*$^{fl/fl}$;*Rosa26*$^{mTmG/+}$) survived embryogenesis, and exhibited hearts that were histologically indistinguishable from the wildtype control. The coronary network (IB4), the SM lining of the coronary arteries (Tagln), the fibroskeleton (Postn) and the formation of EPDCs (GFP) were all unchanged (Figure 5 and Online Figure X). Thus, epicardial loss of *Shh* does not affect embryonic development of the heart.

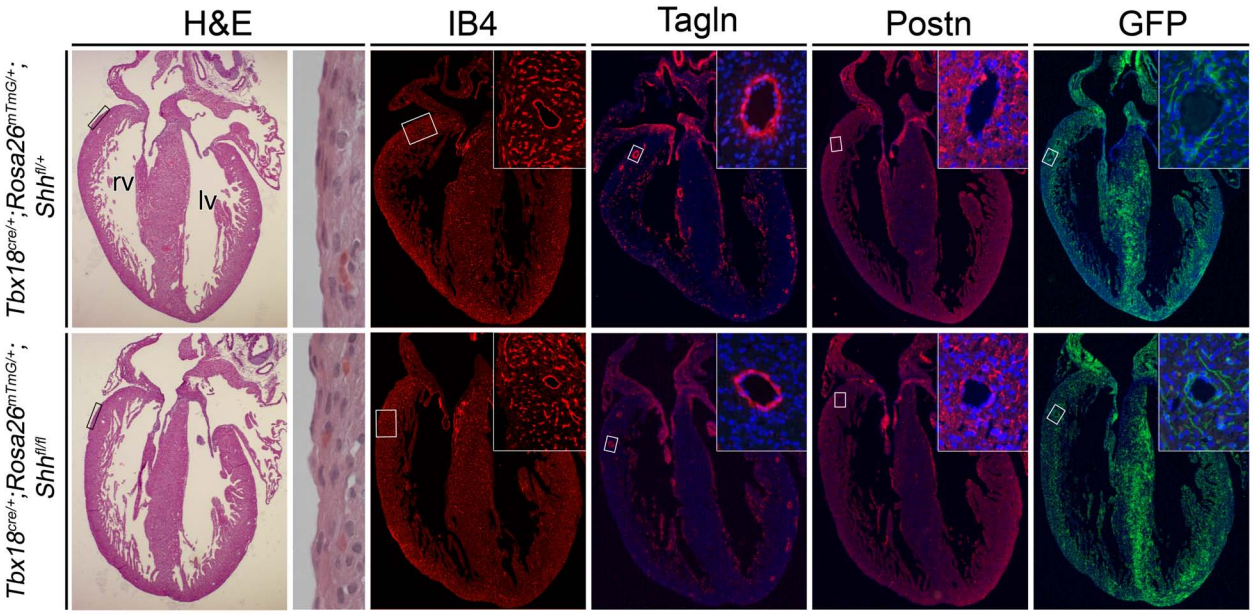

Figure 5. Epicardial loss of *Shh* does not lead to defects in the coronary vasculature, the ventricular myocardium or in epicardial derivatives. Histological analysis by hematoxylin and eosin staining (H&E) and immunofluorescence analysis of IB4, Tagln, Postn and the cell-lineage marker GFP on mid-transverse sections of E18.5 control (*Tbx18*^{cre};*Rosa26*$^{mTmG/+}$;*Shh*$^{fl/+}$) and mutant (*Tbx18*^{cre};*Rosa26*$^{mTmG/+}$;*Shh*$^{fl/fl}$) hearts. Insets show higher magnification images of the areas marked with rectangles. lv, left ventricle; ra, right atrium; rv, right ventricle.

Epicardial Fgfr1/Fgfr2-signaling is not required for heart development

Epicardial Fgfr1/Fgfr2-signaling was recently proposed to induce EPDC migration and fibroblast formation.[16] However, our *in situ* hybridization analysis did not detect (pro-)epicardial expression of *Etv4*, a *bona fide* transcriptional target of the Fgf signaling pathway,[35] on sections of E9.5 to E14.5 embryonic hearts (Online Figure XI). Moreover, embryos with *Tbx18*^{cre}-mediated epicardial deletion of *Fgfr1* and *Fgfr2*[25,26] (*Tbx18*$^{cre/+}$;*Fgfr1*$^{fl/fl}$;*Fgfr2*$^{fl/fl}$) survived embryogenesis and presented histologically normal hearts.

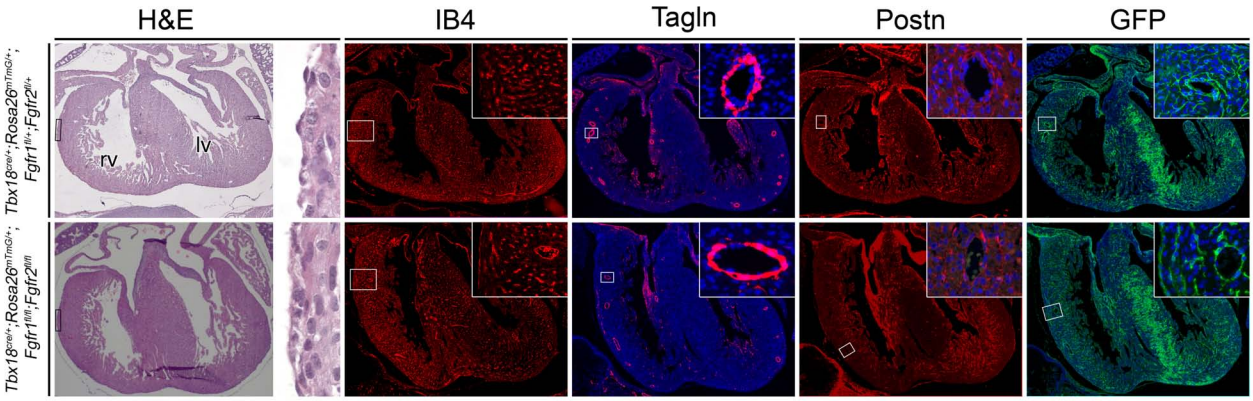

Figure 6. Combined epicardial loss of *Fgfr1* and *Fgfr2* does not affect the integrity of the coronary vasculature, the ventricular myocardium or epicardial derivatives. Histological analysis by hematoxylin and eosin staining (HE) and immunofluorescence analysis for IB4, Tagln, Postn and the cell-lineage marker GFP on mid-transverse sections of E18.5 control (*Tbx18*$^{cre/+}$;*Fgfr1*$^{fl/+}$;*Fgfr2*$^{fl/+}$;*Rosa26*$^{mTmG/+)}$ and mutant (*Tbx18*$^{cre/+}$;*Fgfr1*$^{fl/fl}$;*Fgfr2*$^{fl/fl}$;*Rosa26*$^{mTmG/+}$) hearts. Insets show higher magnification images of the areas marked with rectangles. lv, left ventricle; rv, right ventricle.

The networks of coronary endothelial cells (IB4), SMCs (Tagln), fibroblasts (Postn) and EPDCs (GFP) were not altered either (Figure 6 and Online Figure XII) suggesting that Fgfr1/Fgfr2-signaling in the epicardium is not essential for the development of the epicardium and the coronary system.

Epicardial *Pdgfra* is important for cardiac fibroblast differentiation

Finally, we analyzed the role of Pdgfrα-signaling[29] in epicardial development by a conditional approach. *Tbx18*$^{cre/+}$;*Pdgfra*$^{fl/fl}$;*Rosa26*$^{mTmG/+}$ mice survived embryogenesis. Histological and immunofluorescence analysis did not reveal changes of the ventricular walls (H&E), of the coronary endothelial network (IB4) and of the distribution of SMCs around the arteries (Tagln) between mutant and control hearts at E18.5.

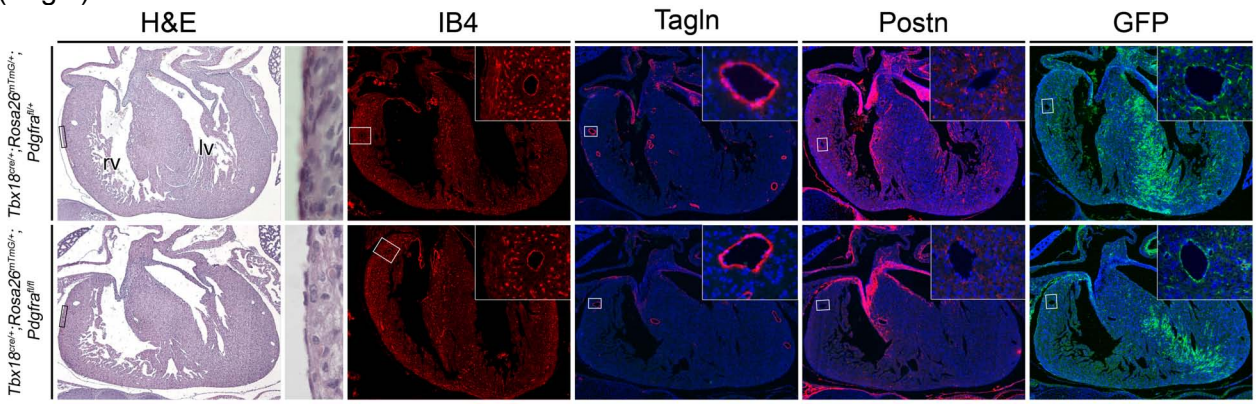

Figure 7. Epicardial *Pdgfra* is required for cardiac fibroblast differentiation. Histological analysis by hematoxylin and eosin staining (H&E) and immunofluorescence analysis for IB4, Tagln, Postn and the cell-lineage marker GFP on mid-transverse sections of E18.5 *Tbx18*$^{cre/+}$;*Pdgfra*$^{fl/+}$;*Rosa26*$^{mTmG/+}$ (control) *Tbx18*$^{cre/+}$;*Pdgfra*$^{fl/fl}$;*Rosa26*$^{mTmG/+}$ hearts. Insets show higher magnification images of the areas marked with rectangles. lv, left ventricle; rv, right ventricle.

Discussion

The embryonic epicardium is a crucial source of cells for the underlying myocardium and the coronary vasculature. Here, we have shown that mobilization and differentiation of epicardial cells does not depend on epicardial Wnt-, Fgfr1/2- and Shh/Smo-signaling but that differentiation of EPDCs into fibroblasts requires the Pdgfrα-signaling pathway. Discrepancies between our results and that of earlier reports may rely on the usage of different cre lines.

Canonical Wnt-signaling is deleterious for epicardial development
Canonical Wnt-signaling has been shown to regulate proliferation and differentiation of progenitor cells in various developmental contexts.[36] In the developing heart a crucial role for this pathway was uncovered in the proliferation of myocardial progenitors of the right ventricle, the atria, the outflow tract and the cardiac venous pole.[37] An additional requirement in epicardial mobilization and differentiation was suggested by a number of studies. First, active Wnt signaling was detected in the proepicardium and single cells of the epicardium using a transgenic Wnt reporter.[38,39] Second, reduced EMT and a failure of coronary SMC differentiation was reported upon conditional deletion of *Ctnnb1* using the transgenic *Gata5::cre* line.[13] Third, *Wt1^{cre}*-mediated deletion of *Ctnnb1* resulted in a complete absence of epicardial EMT[14]. However, a recent study mentioned that these mice survive to adulthood,[40] questioning the earlier conclusions. Our combined analysis on expression (using the faithful read-out by the Wnt-target gene *Axin2*), and *Tbx18^{cre}*-mediated loss- and gain-of-function of *Ctnnb1* does not only suggest that this pathway is irrelevant for epicardial EMT and SMC differentiation but that, in fact, an epicardial activity is deleterious for cardiac development. Since previous work showed that recombination by *Gata5::cre* is not restricted to epicardial cells but occurs in a widespread fashion in cardiomyocytes, cushion tissue and endothelial cells,[19] and that the *Wt1^{cre}* line mediates widespread recombination outside the epicardium in all cardiac and extracardiac tissues,[20] we assume that previous results can be explained by non-specificity of the used cre lines but also to a lack of suitable tracing methods and histochemical assays.

Hh-signaling is deleterious for epicardial development
The Hh-signaling pathway has been shown to be important for vasculogenesis in adult and embryonic development.[41] Claims for a specific role of the pathway in the epicardium and EPDCs were drawn from identification of *Shh* and *Ptch1* expression in the epicardium and from a conditional (*Dermo1^{cre}*-mediated) deletion of *Smo* that resulted in the reduction of coronary artery formation at the early coronary plexus stage.[42,17,43] Our investigations on the expression of Hh-ligands (*Shh*, *Ihh*, *Dhh*) and the Hh-pathway target gene (*Ptch1*) on cardiac sections revealed no presence of these components in the developing epicardium indicating the possibility that previous expression results on whole hearts were artefacts. Further, our conditional loss- and gain-of-function mutants of the Hh-signaling pathway do not confirm a functional relevance for this pathway in the epicardium but indicates deleterious consequences upon cardiac development once epicardially activated. Since the previously used *Dermo1^{cre}* line is additionally active in cushion tissue,[44] these experiments may point to an endothelial/endocardial requirement of Hh-signaling that was erroneously investigated. Furthermore, analysis of embryos at stages of early (highly variable) coronary plexus outgrowth rather that around birth when the coronary system is well established and functional might have contributed in erroneously assigning an epicardial function to Hh-signaling.

Fgfr1/Fgfr2-signaling is not required for epicardial development
Fgf-signals have been characterized as important paracrine regulators of proliferation, migration and differentiation of numerous cell types.[45] A crucial function for epicardial mobilization was suggested by several studies. First, Fgf ligands were shown to stimulate

EMT in cultured avian epicardial cells.[46] Second, pharmacological inhibition of Fgfr-signaling impaired epicardial EMT in avian proepicardial explants.[47] Third, reduced EPDC migration and formation of interstitial fibroblasts was reported upon conditional deletion of *Fgfr1* and *Fgfr2* using the *Wt1*^{cre}-line.[16] Our analysis of expression of *Etv4* does not support the notion that epicardial cells display active Fgf-signaling. Furthermore, combined (*Tbx18*^{cre}-mediated) loss of *Fgfr1* and *Fgfr2* was without phenotypic consequences for epicardial and myocardial development. We suggest that haploinsufficiency of *Wt1* might have interfered with formation and migration of EPDCs and has clearly reduced the sensitivity of Wt1 detection that was used as an EPDC marker in the previous study.[12] Further, endothelial expression of Wt1 in the coronary plexus might have further negatively impacted on the characterization of EPDCs in those experiments.[20]

Epicardial *Pdgfrα* is critical for cardiac fibroblast differentiation

Pdgfs have been characterized as crucial signals in the proliferation of mesenchymal precursor cells, as well as their differentiation and migration.[48] Reports on a functional involvement of Pdgfrα-signaling in epicardial development were based on the results from conditional deletion of *Pdgfrα* using the *Wt1*^{cre}-line.[15] Our conditional (*Tbx18*^{cre}-mediated) deletion experiment confirms that *Pdgfrα* is required for epicardial EMT, and more critically for differentiation of EPDCs in mature cardiac fibroblasts. Congruency of the results in this case is easily explained by the fact that extraepicardial recombination mediated by *Wt1*^{cre} in the heart is irrelevant for a gene the expression of which is restricted to the epicardium during cardiac development.

Acknowledgements

We thank Rolf Kemler for providing the *Ctnnb1*$^{fl/fl}$ mice and D. Vestweber for the Emcn antibody.

Sources of Funding

This work was supported by grants from the German Research Foundation (DFG) for the Cluster of Excellence REBIRTH (From Regenerative Biology to Reconstructive Therapy) and the Clinical Research Group KFO136 at Hannover Medical School to A.K.

Disclosures

None.

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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.
- Conditional gene targeting approaches have provided suggestive evidence that canonical Wnt-, Fgfr1/Fgfr2- and Pdgfrα-signaling regulate epicardial mobilization, and differentiation into smooth muscle cells and cardiac fibroblasts.
- Conditional gene targeting approaches have provided suggestive evidence that epicardial Hh-signaling regulates the formation of the coronary vasculature.

What new information does this article contribute?

- Canonical Wnt-signaling in the epicardium is not required epicardial development.
- Epicardial activation of canonical Wnt-signaling disrupts epicardial and myocardial integrity.
- Epicardial Hh-signaling is not required for the development for the epicardium and the coronary vasculature.
- Epicardial activation of Hh-signaling affects epicardial EMT and myocardial growth.
- Epicardial Fgfr1/Fgfr2-signaling is not required for epicardial development.
- Epicardial Pdgfrα-signaling regulates epicardial EMT and differentiation of cardiac fibroblasts from epicardium-derived cells.

Novelty and significance

The epicardium is an epithelial monolayer that covers and mechanically protects the cardiac muscle. During embryogenesis and in myocardial injury, the epicardium also acts as a signaling center that promotes myocardial growth and coronary plexus formation, and is a source of cells for the fibrous skeleton of the heart as well as for smooth muscle cells and fibroblasts of coronary vessels. Reactivation of embryonic epicardial programs by manipulation of relevant signaling pathways may provide a means to increase myocardial healing under injury conditions. Conditional gene targeting approaches have implicated a number of intracellular signaling pathways to regulate epicardial mobilization and differentiation during embryogenesis. Canonical (Ctnnb1-dependent) Wnt-signaling was suggested to regulate epicardial EMT and differentiation of coronary smooth muscle cells, Pdgfrα-signaling epicardial EMT and cardiac fibroblast formation, Fgfr1 and Fgfr2 epicardial EMT, and the Shh-pathway the formation of arterial and venous coronaries. However, cre lines based on *Gata5* and *Wt1* regulatory elements that were used in these experiments were recently shown to mediate wide-spread recombination in the early heart. For this reason we reinvestigated the requirement of these pathways in epicardial development, using conditional gene targeting approaches with a $Tbx18^{cre}$ line which efficiently and specifically mediates recombination in the epicardium. Our study shows that the canonical Wnt- and Hh-signaling pathways are not only dispensable for epicardial development but that they are in fact deleterious for epicardial and myocardial development. Epicardial loss of *Fgfr1*and *Fgfr2* does not affect epicardial and coronary vessel development either. However, we confirm a requirement for Pdgfrα signaling for mobilization of epicardial cells and subsequent differentiation in mature cardiac fibroblasts.

Supplemental Online Data

Epicardial function of canonical Wnt-, Hh-, Fgfr1/2- and Pdgfrα-signaling

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Running title: Epicardial signaling pathways

Mice

Mice with a knock-in of the cre-recombinase gene in the *Tbx18* locus (*Tbx18*$^{tm4(cre)Akis}$, synonym: *Tbx18*^{cre}) were previously generated in the laboratory at the Medizinische Hochschule Hannover.[1] Mice with *loxP* sites flanking the *Ctnnb1* locus from exon 2 to exon 6 (*Ctnnb1*^{tm2Kem} s*ynonym: Ctnnb1*^{fl}*)* were obtained from Rolf Kemler (Max-Planck-Institute for Immunobiology and Epigenetics, Freiburg/Germany),[2] mice with *loxP* sites flanking exon 3 of the *Ctnnb1* locus (*Ctnnb1*^{tm1Mmt}, synonym: *Ctnnb1*$^{(Ex3)fl}$) were obtained from Makoto Mark Taketo (Kyoto University, Kyoto/Japan).[3] Mice with *loxP* sites flanking exon 2 of the *Shh* locus (*Shh*^{tm2Amc}, synonym: *Shh*^{fl}),[4] mice with *loxP* sites flanking exon 4 of the *Fgfr1* locus (*Fgfr1*$^{tm5.1Sor}$, synonym: *Fgfr1*^{fl}),[5] mice with*loxP* sites flanking exons 7 to 10 of the *Fgfr2* locus (*Fgfr2*^{tm1Dor}, synonym: *Fgfr2*^{fl}),[6] mice with *loxP* sites flanking exon 1 of the *Smo* locus (*Smo*^{tm2Amc}, synonym: *Smo*^{fl}),[7] mice with a fusion protein of Enhanced Yellow Fluorescent Protein (EYFP) and the constitutively active W539L point mutation of the Smo protein and a *loxP*-flanked STOP fragment placed between its *Gt(ROSA)26Sor* promoter and the *Smo/EYFP* sequence (*Gt(ROSA)26Sor*$^{tm1(Smo/YFP)Amc}$, synonym: *Smo*^{GOF}),[8] mice with *loxP* sites flanking exons 1 to 4 of the *Pdgfrα* locus (*Pdgfrα*^{tm8Sor}, synonym: *Pdgfrα*^{fl}),[9] and the double fluorescent cre reporter line (*Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$, synonym: *R26*^{mTmG})[10] were all obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). All mice were maintained on an outbred (NMRI) background.

Embryos for expression analysis were derived form NMRI wildtype mice. Embryos for phenotypic analyses were obtained from matings of males triple heterozygous for *Tbx18*^{cre}, *R26*^{mTmG} and the floxed allele of the pathway mutant, and females homozygous for the same floxed allele of the pathway mutant to be analyzed. For timed pregnancies, vaginal plugs were checked in the morning after mating and noon was designated as embryonic day (E) 0.5. Female mice were sacrified by cervical dislocation. Embryos were harvested in PBS, decapitated, fixed in 4% paraformaldehyde overnight and stored in 100% methanol at -20^{o}C before further use. Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR. Hans Hedrich, state head of the animal facility, approved the care of animals and experiments at Medizinische Hochschule Hannover.

Histological analysis

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

Immunofluorescence

For immunofluorescence analysis, goat polyclonal antibody against Tbx18 (1:50, C-20, Santa Cruz), rabbit polyclonal antibody against GFP (1:200 sc-8334, Santa Cruz), mouse monoclonal antibody against GFP (1:200, 11 814 460 001, Roche), rabbit polyclonal antibody against SM22alpha (Tagln) (1:200, ab14106-100, Abcam), rabbit polyclonal antibody against Ctnnb1 (1:400, C2206, Sigma-Aldrich), Fluorescein-labeled GSL I – isolectin B4 (1:100, FL-1101, VectorLabs), rabbit polyclonal to periostin (1:200, ab14041, Abcam), monoclonal antibody against alpha-Smooth muscle actin (Acta2), FITC Conjugate (1:200, F3777, Sigma), rabbit polyclonal antibody against collagen type IV (1:200, AB756P, Millipore Corp.), rabbit polyclonal antibody against ZO1 (Tjp1) (1:200, 61-7300, Zymed Laboratories Inc.)

and rabbit polyclonal antibody against Wt1 (1:200, C-19, Santa Cruz) were used as primary antibodies.
Biotinylated goat-anti-rabbit (Dianova, 1:400), biotinylated donkey-anti-goat (Dianova, 1:400), Alexa488 goat-anti-rabbit (Invitrogen, 1:400), Alexa488 donkey-anti-mouse (Invitrogen A21202, 1:400), Alexa-Fluor555 goat-anti-mouse (Invitrogen A-21424, 1:400) and Alexa-Fluor555 goat-anti-rabbit (Invitrogen A-21428 1:400) were used as secondary antibodies. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Roth).
Immunofluorescence analysis against Ctnnb1 and Tbx18 was performed on cryosections. These were fixed overnight in 4% paraformaldehyde, embedded in tissue freezing medium (Jung, Germany), and sectioned to 4-µm. All other immunofluorescence analyses were done on 4-µm paraffin sections. All 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.

In situ hybridization analysis

In situ hybridization analysis on paraffin sections with digoxigenin-labeled antisense riboprobes was performed as described.[11] Details of used probes upon request.

Proliferation assay

Cell proliferation was analyzed by incorporation of 5-bromo-2-deoxyuridine (BrdU) on 5-µm sections of paraffin-embedded specimens similar to previously published protocols.[12] The quantification of proliferation in *Tbx18$^{cre/+}$;Ctnnb1$^{(Ex3)fl/+}$* and control wildtype littermates was performed with six sections each of four embryos of each genotype at E11.5. The BrdU-labeling index was defined as the number of BrdU-positive cells relative to the total number of nuclei (DAPI counterstain) within the epicardium or cell clusters, as indicated.

Quantification of right ventricular GFP$^+$ cells

Quantification of GFP$^+$ cells was performed on 4-µm paraffin sections of mutant and control hearts. Three sections each of three embryos were stained for GFP (as described above) and optical fields of a 20x magnification of the right ventricle were used to quantify the GFP$^+$ and DAPI$^+$ areas using the ImageJ (NIH) software. The calculated relative area (GFP$^+$ versus DAPI$^+$) in the corresponding control genotype was set to one and the normalized data expressed as mean±sd.

Epicardial cell culture

Primary epicardial cells were obtained from right ventricles dissected from E11.5 hearts. The ventricle was placed onto a gelatin-coated dish in serum-free DMEM supplemented with 2 mM Glutamax, 100 units/ml Penicillin, 100 µg/ml Streptomycin (Gibco). After 2 days the ventricle was carefully removed using forceps and the epithelial monolayer was further cultured in DMEM/Panserin401 (PAN Biotech) with or without 10% FCS.

Statistical analysis

Statistical analyses for BrdU incorporation, capillary density and GFP$^+$ cells were performed using the 2-tailed Student's t-test. Data were expressed as mean±SD.

Differences were considered not significant when the P-value was higher than 0.05, significant (*) when the P-value was below 0.05, highly significant (**) when the P-value was below 0.01, and extremely significant (***) if $P<0.001$.

Image analysis

Sections were photographed using a Leica DM5000 microscope with Leica DFC300FX digital camera. Immunofluorescence of cells, as well as mosaic merge pictures of sections where documented using a Leica DMI6000B microscope with a Leica FC350FX digital camera. The Leica LAS AF 2.3 software was used to generate a mosaic merge of 5x5 single pictures, allowing 10% overlap of neighboring pictures. Whole-mount specimens were photographed on Leica M420 with Fujix digital camera HC-300Z. All images were processed in ImageJ[13] and Adobe Photoshop CS4.

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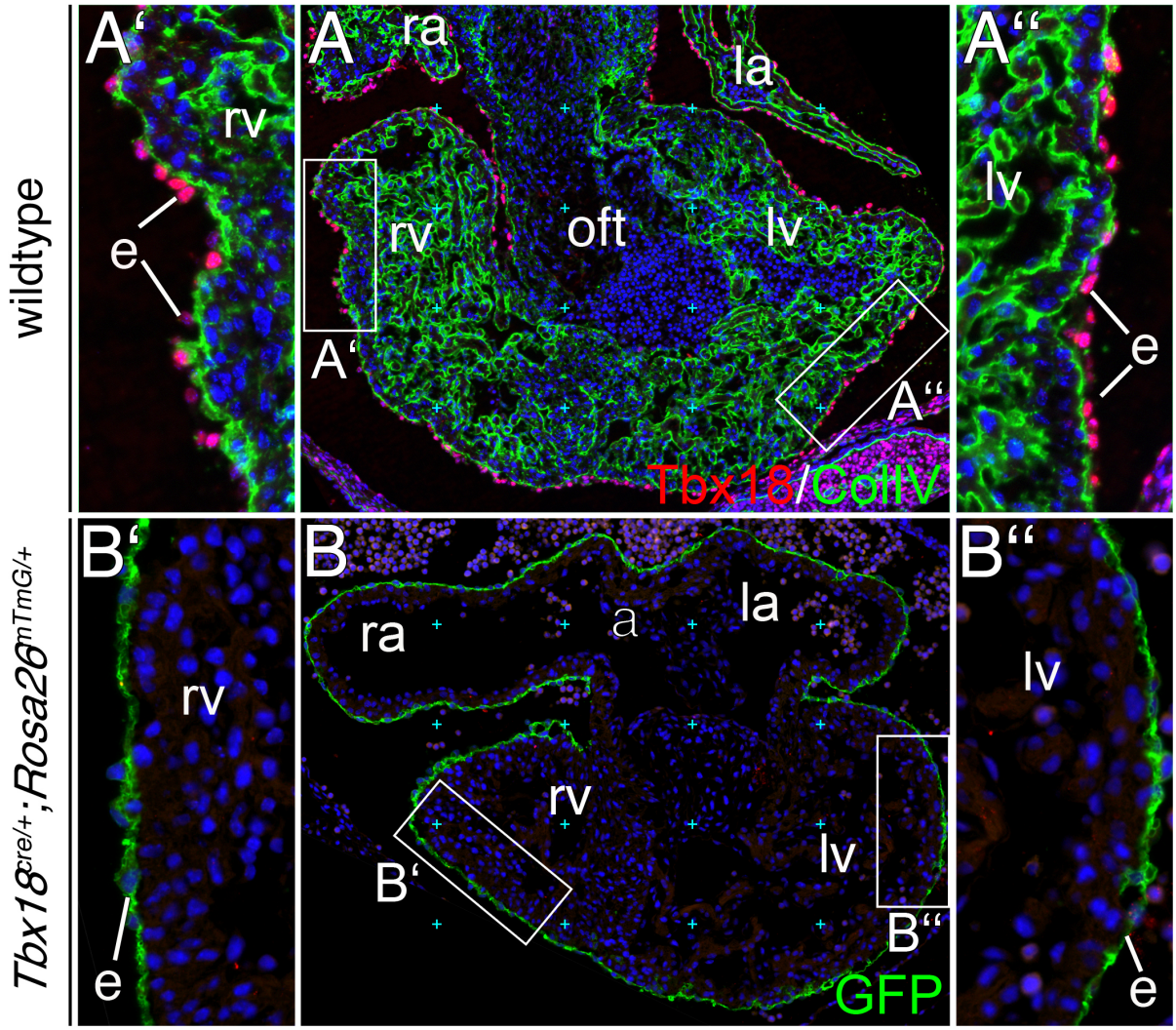
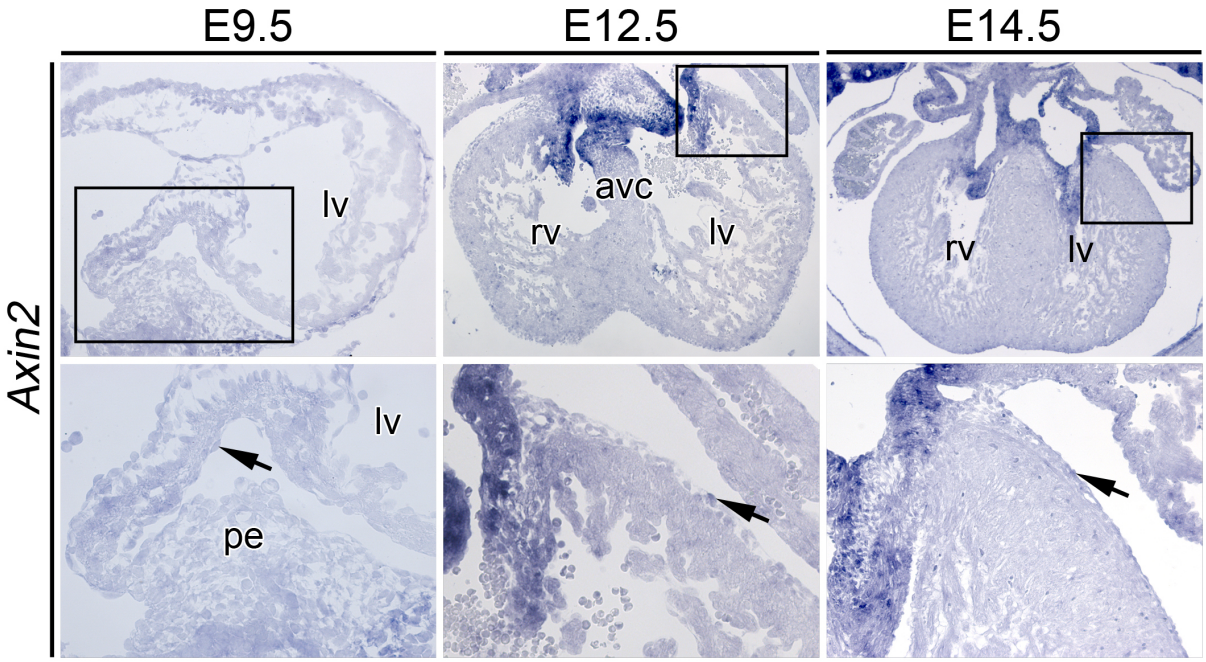
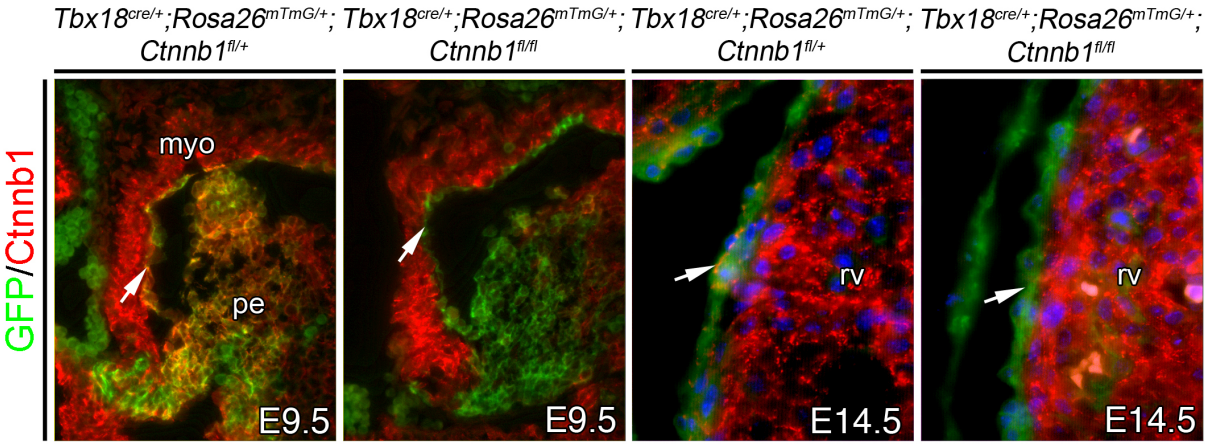
Supplemental Online Figures



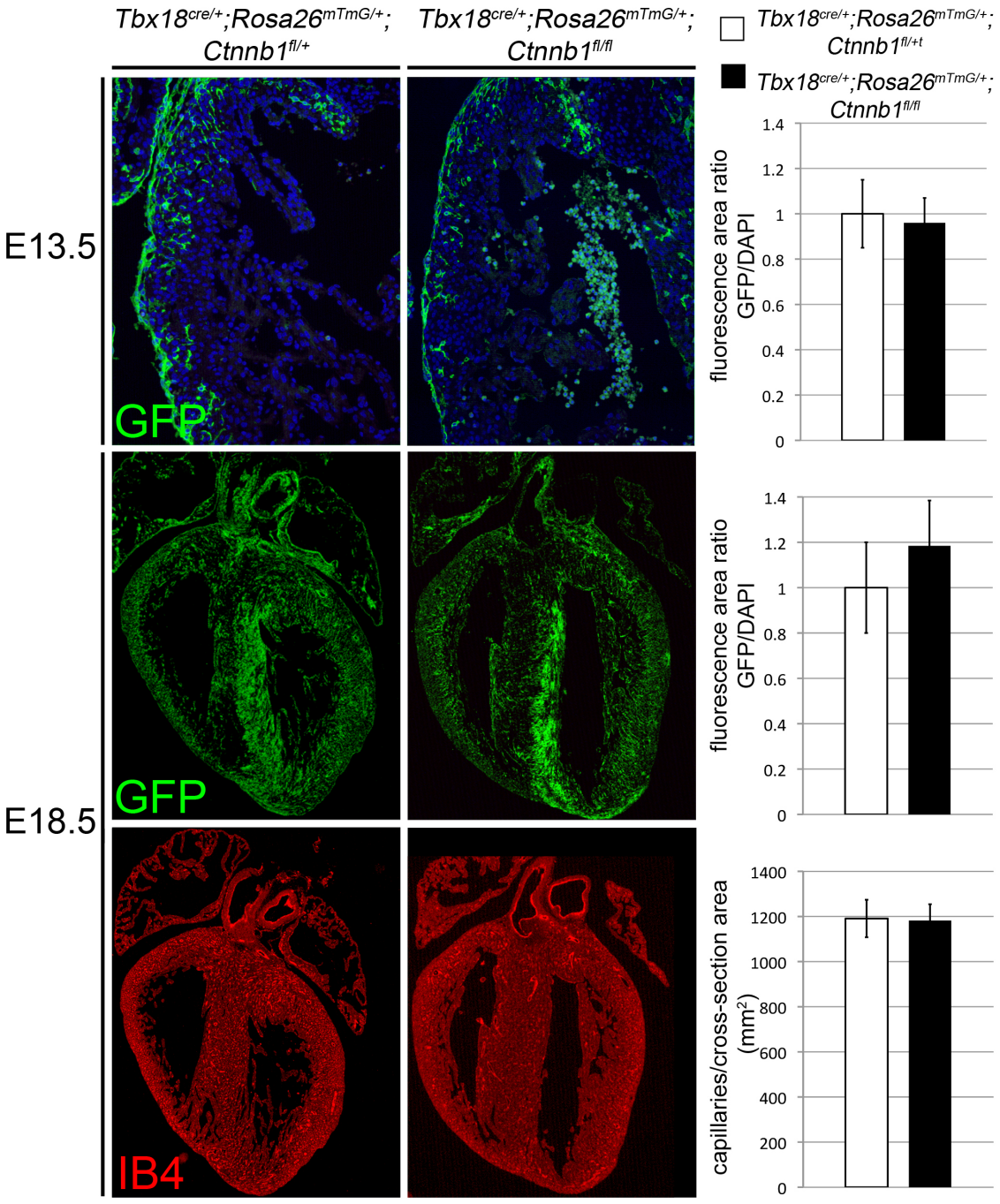
Online Figure I. Tbx18 expression and *Tbx18*^{cre}-mediated recombination in the epicardium of E10.5 hearts. (A, A’, A’’) Co-immunofluorescence analysis for expression of Tbx18 (red) and Col4 (green) with nuclear counterstaining (blue) on a transverse cryosection of an E10.5 wildtype heart. Tbx18 is found in the nucleus of all epicardial cells that are delineated by the ColIV-positive basal lamina. **(B, B’, B’’)** Immunofluorescence analysis for expression of membrane-bound GFP (green) of the recombination reporter *Rosa26*^{mTmG} on a transverse heart section of an E10.5 *Tbx18*$^{cre/+}$*;Rosa26*$^{mTmG/+}$ embryo. All epicardial cells express the GFP reporter. Magnified regions of the right ventricle (**A’**, **B’**) and of the left ventricle (**A’’**, **B’)** are indicated by rectangles (in **A**, **B**). Note that the overview figures were composed of 25 individual images. e, epicardium; la, left atrium; lv, left ventricle; oft, outflow tract; ra, right atrium; rv, right ventricle.



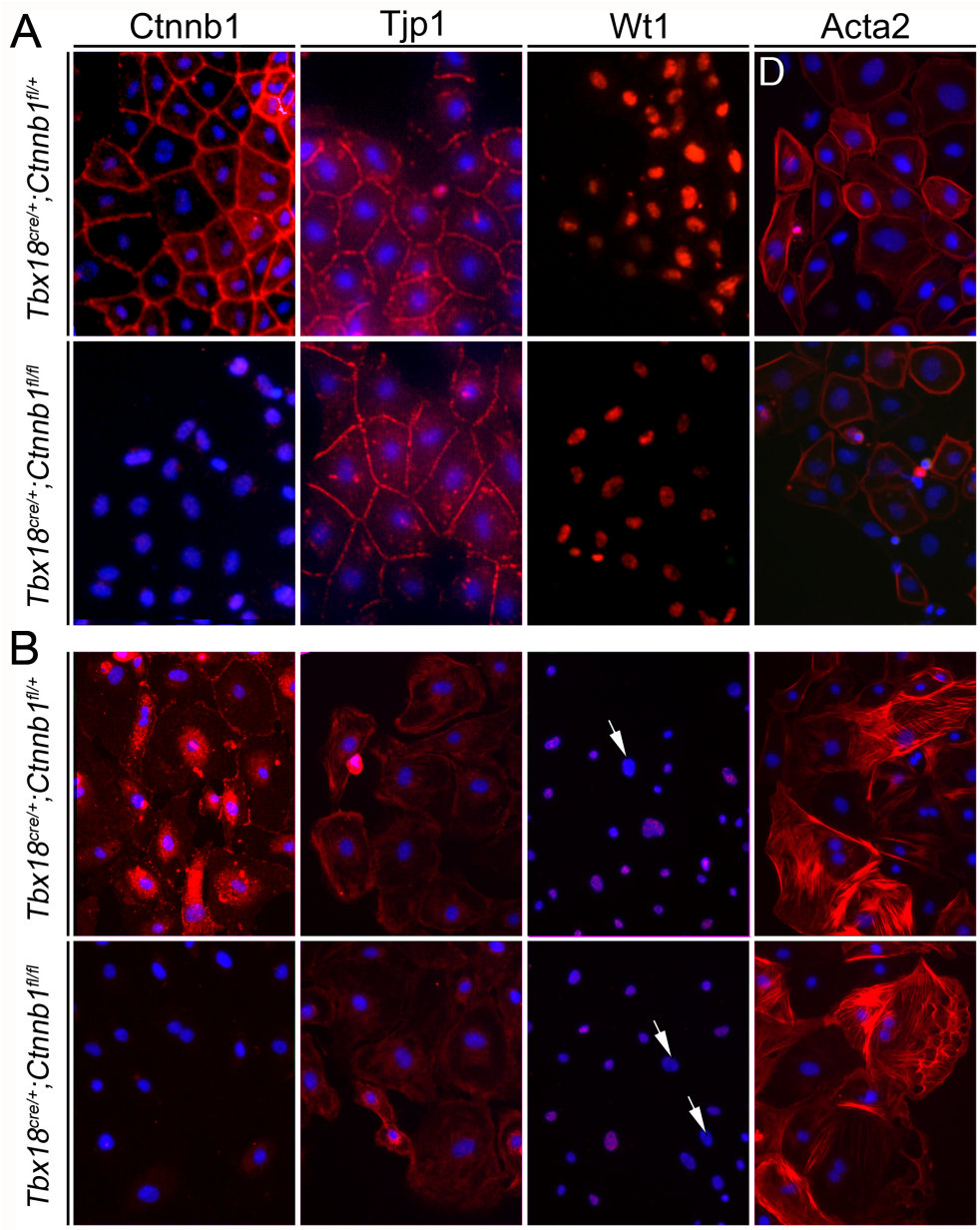
Online Figure II. Canonical Wnt signaling in epicardial development. *In situ* hybridization analysis for expression of *Axin2*, a *bona fide* transcriptional target of the canonical Wnt signaling pathway, on E9.5 sagittal as well as on E12.5 and E14.5 transverse sections of wildtype hearts. Rectangles display the regions shown in higher magnification in the images below at the indicated stages. Black arrows point to the epicardium. *Axin2* expression is not detected in the proepicardium and the epicardium. Note positive control regions within the hearts (e.g. the atrioventricular cushion tissue and valves). avc, atrioventricular canal; lv, left ventricle; pe, proepicardium; rv, right ventricle.



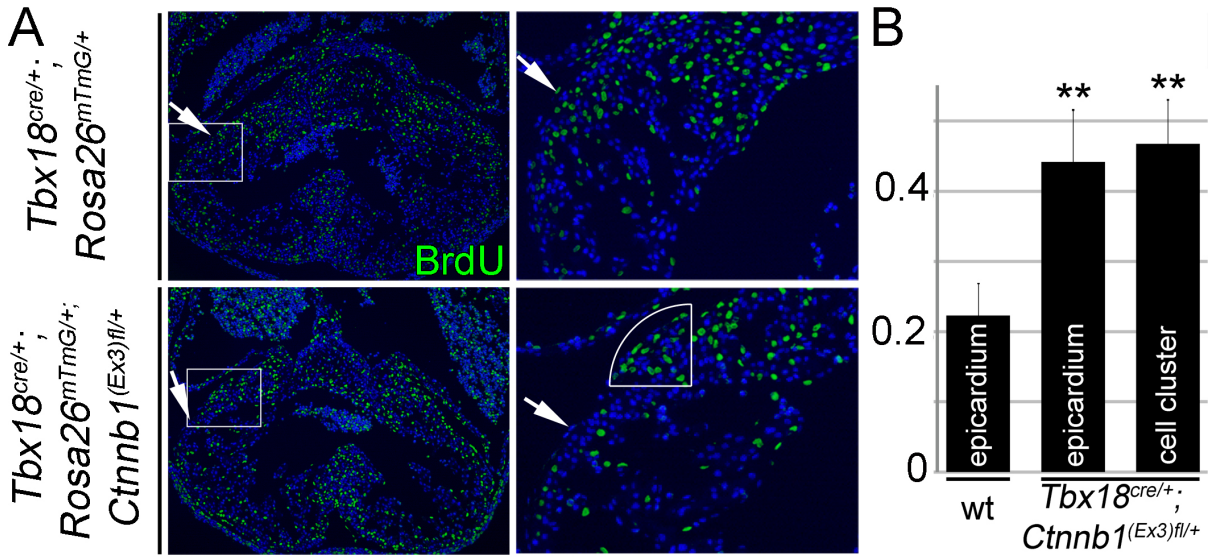
Online Figure III. Absence of Ctnnb1 in the (pro-)epicardium *of Tbx18$^{cre/+}$;Rosa26$^{mTmG/+}$;Ctnnb1$^{fl/fl}$* embryos. Co-immunofluorescence analysis of Ctnnb1 (red) and the recombination reporter GFP (green) in sagittal sections through the (pro-) epicardium at E9.5 and transverse sections of the right ventricle at E14.5 in control (*Tbx18$^{cre/+}$;Rosa26$^{mTmG/+}$;Ctnnb1$^{fl/+}$*) and mutant (*Tbx18$^{cre/+}$;Rosa26$^{mTmG/+}$;Ctnnb1$^{fl/fl}$*) embryos. Note the absence of Ctnnb1 expression in the mutant proepicardium and epicardium compared to the control. White arrows point to the epicardium. pe, proepicardium; myo, myocardium; rv, right ventricle.



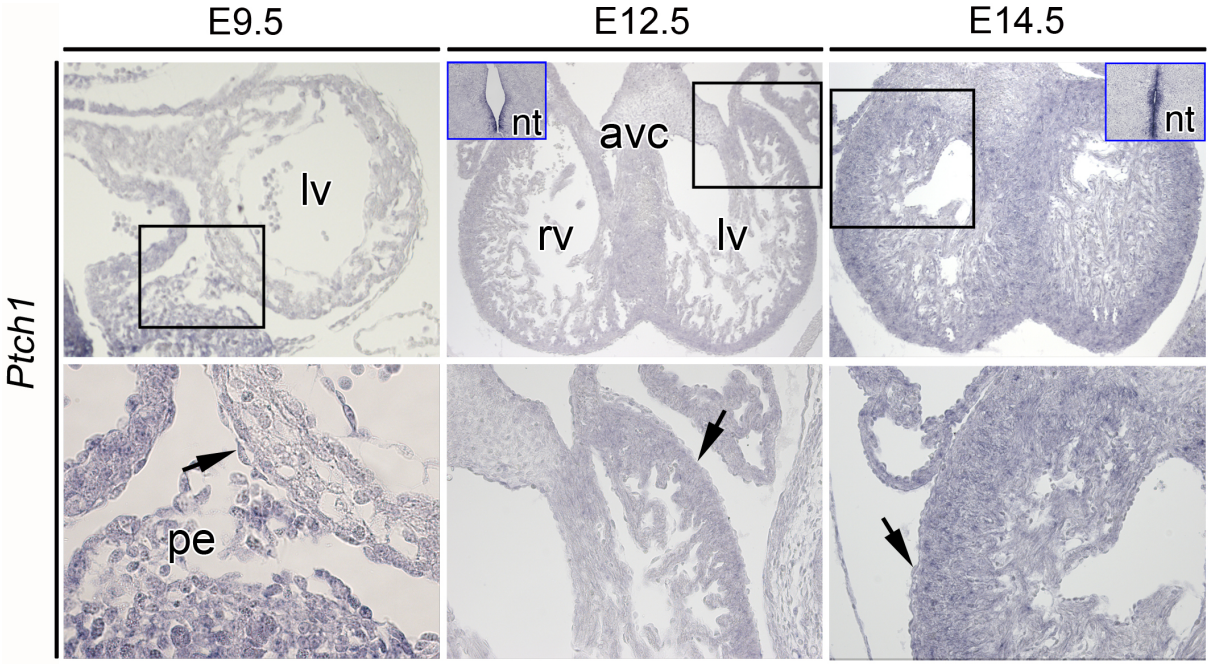
Online Figure IV. Epicardial loss of *Ctnnb1* does not affect EPDCs and coronary vessel formation. Immunofluorescence analysis of the recombination reporter GFP (green) and coronary vessel endothelium (IB4, red) in transverse sections of the heart at E13.5 and E18.5 in control (*Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Ctnnb1*$^{fl/+}$) and mutant (*Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Ctnnb1*$^{fl/fl}$) embryos. Quantification of EMT (GFP^{+} area vs DAPI^{+} area) at 13.5 (P=0.9) and at E18.5 (P=0.2) and vessel density (number of IB4^{+} vessels per area) at E18.5 (P=0.5) in the right ventricle does not reveal changes in the mutant.



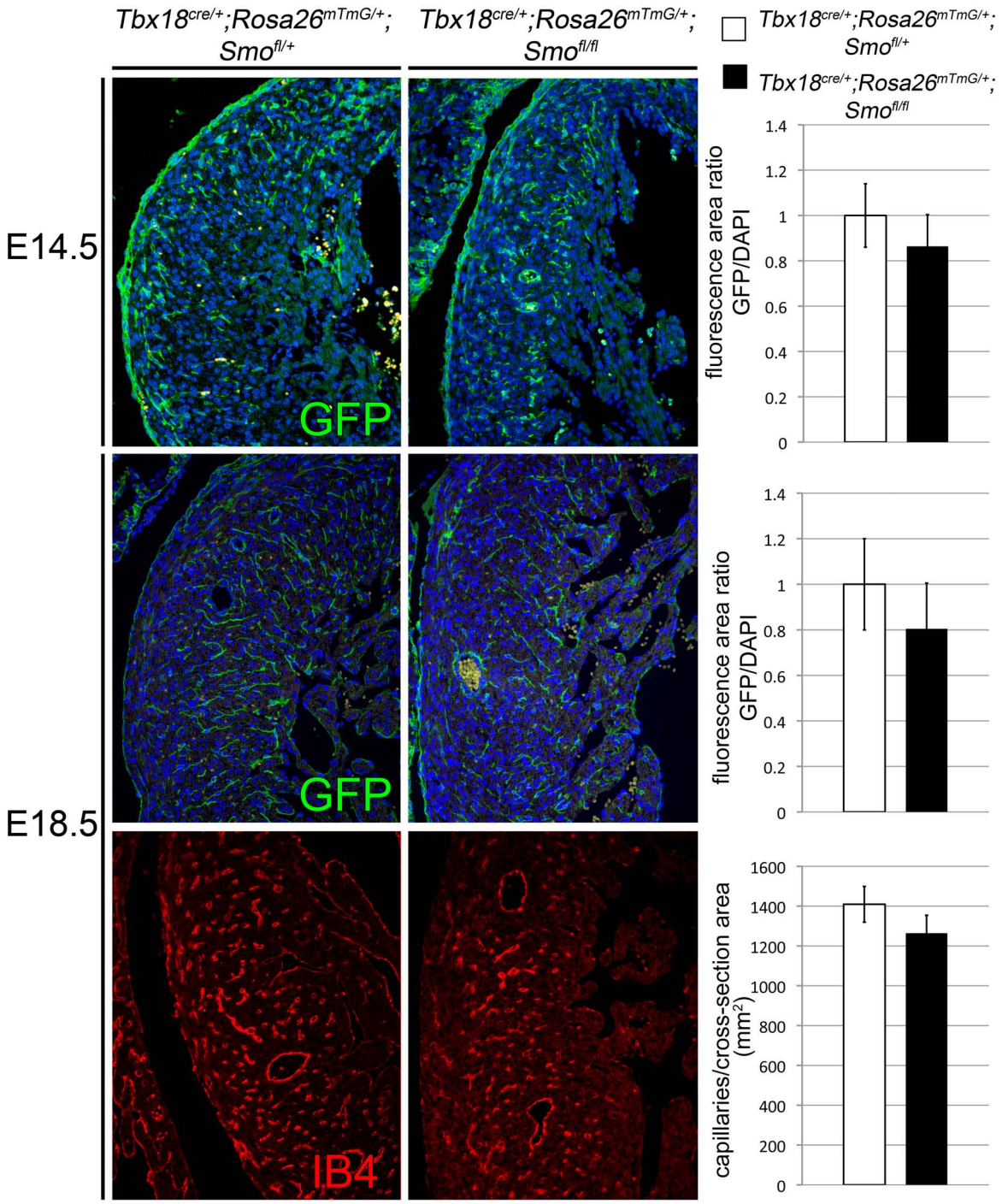
Online Figure V. *Ctnnb1*-deficient epicardial cells can be induced to differentiate into SMCs. Cellular analysis of epicardial outgrowths of explants of right ventricles of control (*Tbx18*$^{cre/+}$;*Ctnnb1*$^{fl/+}$) and *Ctnnb1*-deficient (*Tbx18*$^{cre/+}$;*Ctnnb1*$^{fl/fl}$) embryos. After 2 days of culture under serum-free conditions ventricles were removed and epicardial outgrowths cultures for another 2 days in a 1:1 mixture of DMEM and Panserin401 without serum **(A)** or they were cultured for 4 days in a 1:1 mixture of DMEM and Panserin401 containing 10% FCS **(B)**. Immunofluorescence analysis for Ctnnb1, Tjp1, Wt1 and Acta2 (red) with nuclear counter staining (blue). Shown are representative higher magnification of whole explants. Note the loss of Ctnnb1 immunoreactivity in the mutant cells. Tjp1, Wt1 and Acta2 stainings in the mutant are indistinguishable to the control. After addition of 10% FCS both normal and mutant epicardial cells underwent EMT and differentiated into SMCs.



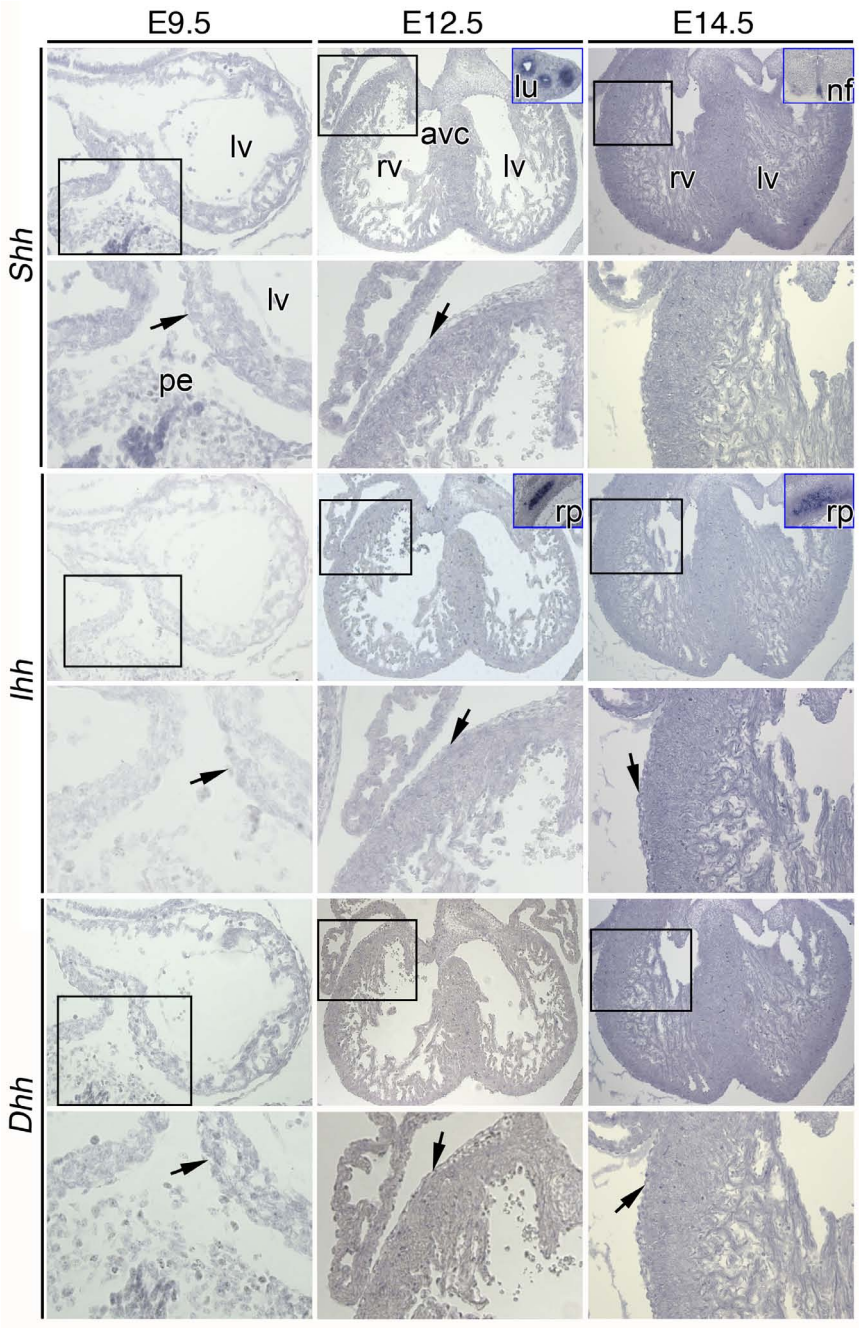
Online Figure VI. Constitutive Wnt-signaling in the epicardium leads to increased proliferation. (A) BrdU incorporation was analyzed by immunofluorescence on E11.5 transverse heart sections of control (*Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$)and *Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Ctnnb1*$^{(Ex3)fl/+}$ embryos. Rectangles display the regions shown in higher magnification. **Arrows** point to the epicardium, the **quadrant** marks an epicardial cell cluster. **(B)** Quantification of the BrdU labeling index shows significant enhanced proliferation in the mutant epicadium (P=0.003) and cell clusters (P=0.0004).



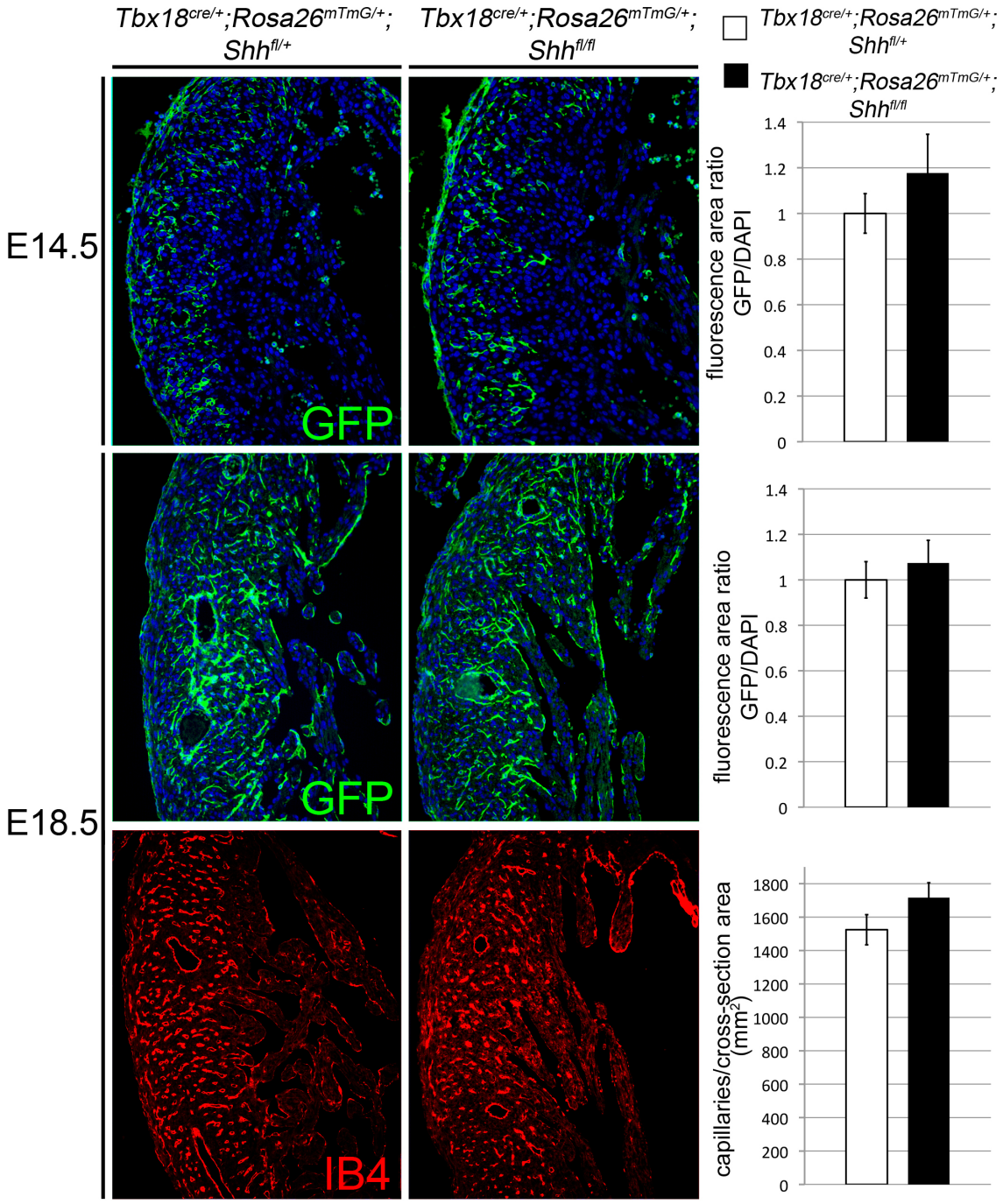
Online Figure VII. Hh-signaling in epicardial development. *In situ* hybridization analysis for expression of *Ptch1*, a *bona fide* transcriptional target of the Hh-signaling pathway, on E9.5 sagittal as well as on E12.5 and E14.5 transverse sections of wildtype hearts. Black rectangles display the regions shown in higher magnification in the images below at the indicated stages. Black arrows point to the epicardium. *Ptch1* expression is not detected in the proepicardium and the epicardium. Note positive control regions within the sections, shown as blue insets (e.g. notochord and floorplate). avc, atrioventricular canal; lv, left ventricle; nf, notochord/floorplate; pe, proepicardium; rv, right ventricle.



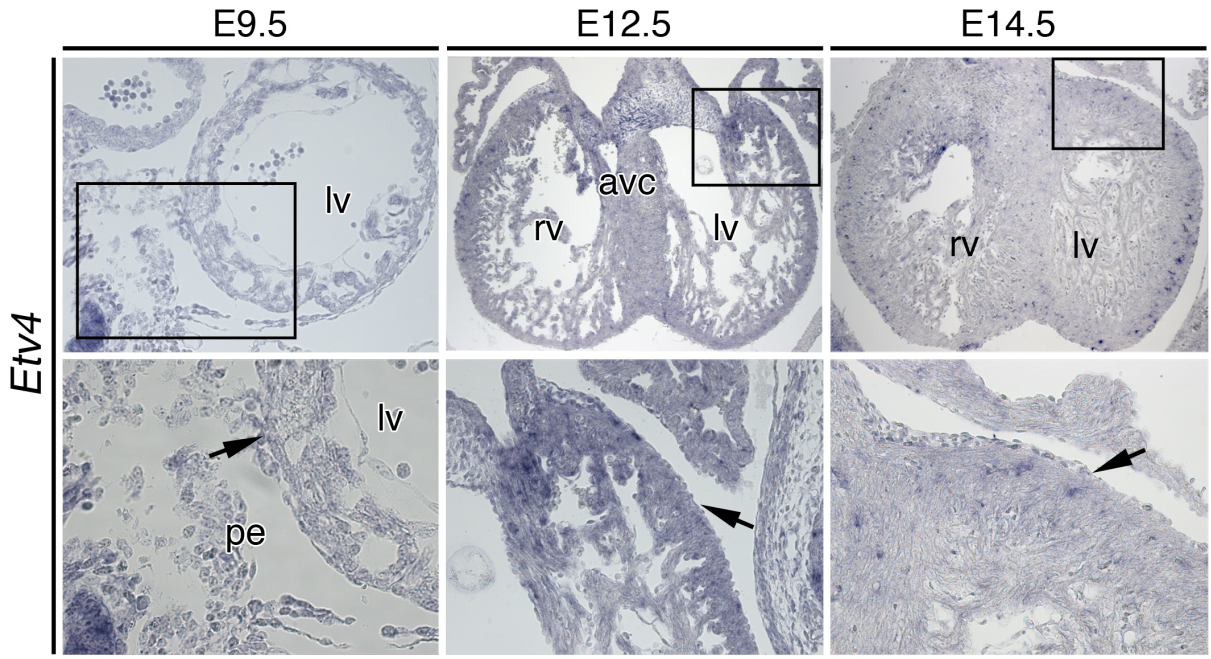
Online Figure VIII. Epicardial loss of *Smo* does not affect EPDCs and coronary vessel formation. Immunofluorescence analysis of the recombination reporter GFP (green) and of the coronary vessel endothelium (IB4, red) in transverse sections of the heart at E14.5 and E18.5 in control (*Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Smo*$^{fl/+}$) and mutant (*Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Smo*$^{fl/fl}$) embryos. Quantification of EMT (GFP^{+} area vs DAPI^{+} area) at 14.5 (P=0.7) and at E18.5 (P=0.4) and vessel density (number of IB4^{+} vessels per area) at E18.5 (P=0.1) in the right ventricle does not reveal changes in the mutant.



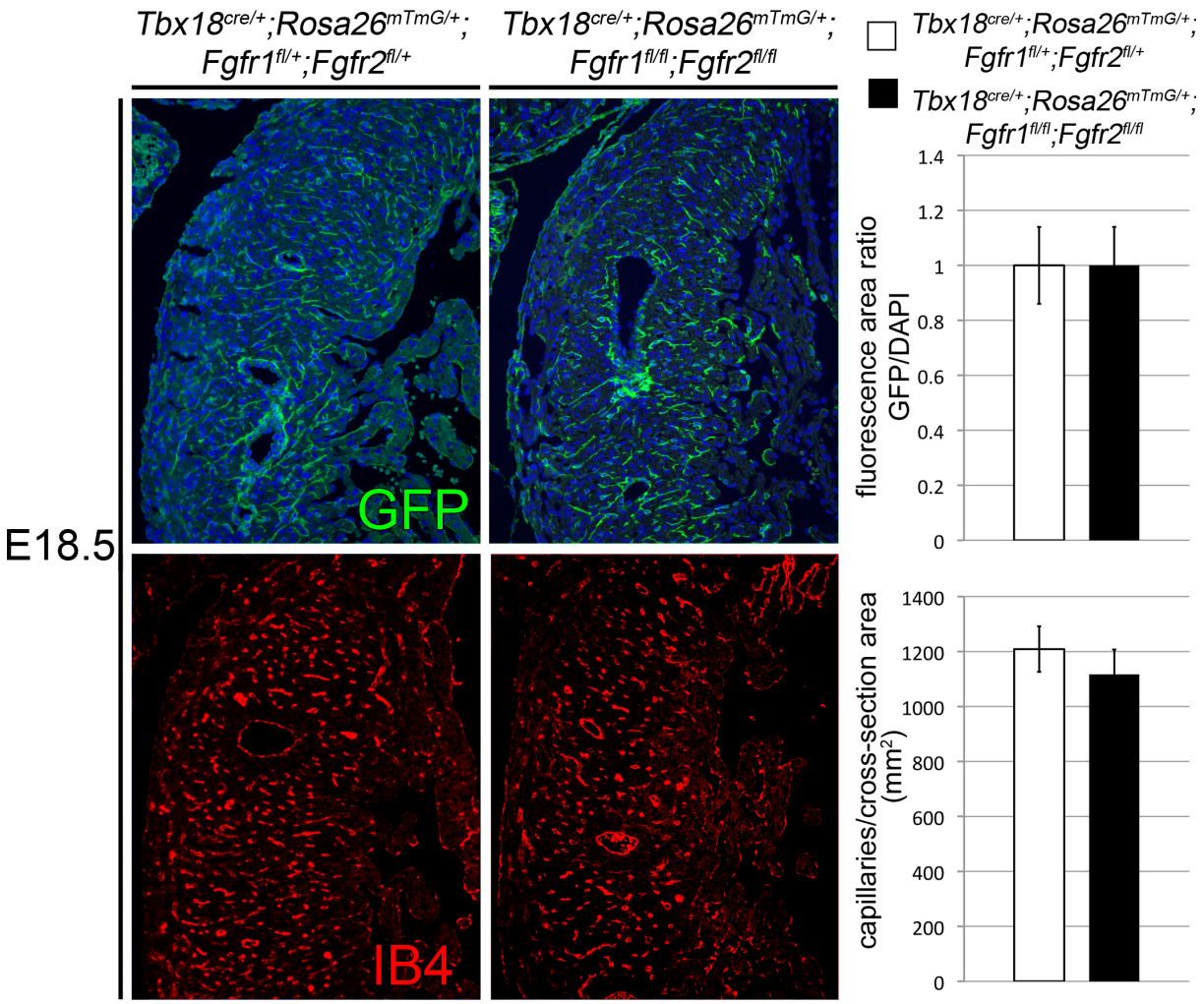
Online Figure IX. Expression of genes encoding Hh-ligands in epicardial development. *In situ* hybridization analysis for expression of*Shh, Dhh* and *Ihh* on E9.5 sagittal as well as on E12.5 and E14.5 transverse sections of wildtype hearts. Black rectangles display the regions shown in higher magnification in the images below at the indicated stages. Black arrows point to the epicardium. *Shh, Dhh and Ihh* expression is not detected in the proepicardium and the epicardium. Note positive control regions within the sections, shown as blue insets (e.g. *Shh* in the notochord and floorplate; *Ihh* in the rib primodium). avc, atrioventricular canal; lu, lung; lv, left ventricle; nf, notochord/floorplate; pe, proepicardium; rp, rib primodium; rv, right ventricle.



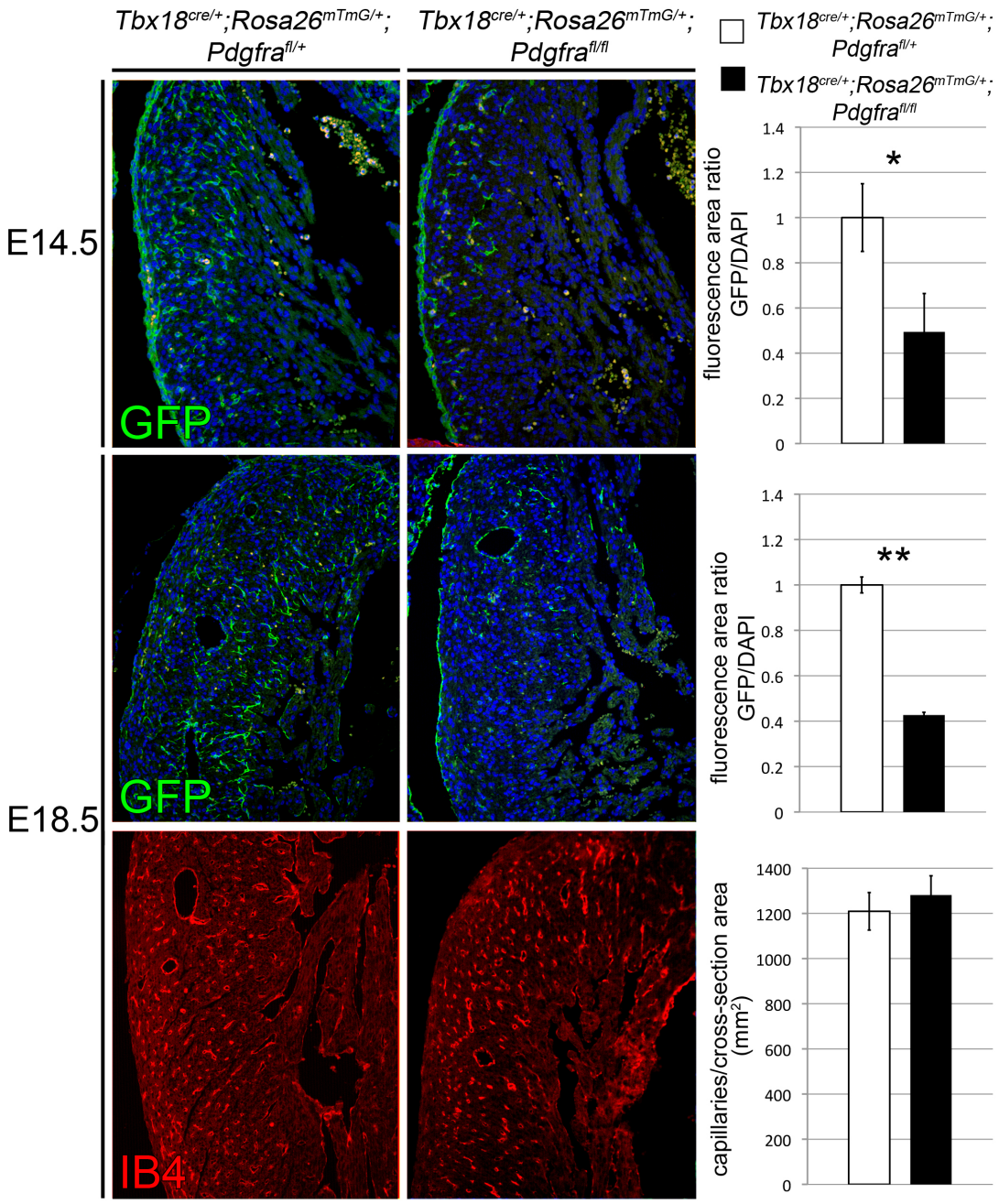
Online Figure X. Epicardial loss of *Shh* does not affect EPDCs and coronary vessel formation. Immunofluorescence analysis of the recombination reporter GFP (green) and of the coronary vessel endothelium (IB4, red) in transverse sections of the heart at E14.5 and E18.5 in control (*Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Shh*$^{fl/+}$) and mutant (*Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Shh*$^{fl/fl}$) embryos. Quantification of EMT (GFP^{+} area vs DAPI^{+} area) at E14.5 (P=0.4) and at E18.5 (P=0.5) and vessel density (number of IB4^{+} vessels per area) at E18.5 (P=0.1) in the right ventricle does not reveal changes in the mutant.



Online Figure XI. Fgf-signaling in epicardial development. *In situ* hybridization analysis for expression of *Etv4*, a *bona fide* transcriptional target of the Fgf-signaling pathway, on E9.5 sagittal as well as on E12.5 and E14.5 transverse sections of wildtype hearts. Boxed areas are shown in higher magnification in the lower row. Black arrows point to the epicardium. *Etv4* expression is not detected in the proepicardium and the epicardium. avc, atrioventricular canal; lv, left ventricle; pe, proepicardium; rv, right ventricle.



Online Figure XII. Combined epicardial loss of *Fgfr1* and *Fgfr2* does not affect EPDCs and coronary vessel formation. Immunofluorescence analysis of the recombination reporter GFP (green) and of the coronary vessel endothelium (IB4, red) in transverse sections of the heart at E18.5 in control (*Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Fgfr1*$^{fl/+}$;*Fgfr2*$^{fl/+}$) and mutant (*Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Fgfr1*$^{fl/fl}$;*Fgfr2*$^{fl/fl}$) embryos. Quantification of EMT (GFP^{+} area vs DAPI^{+} area) (P=0.6) and vessel density (number of IB4^{+} vessels per area) (P=0.2) in the right ventricle does not reveal changes in the mutant.



Online Figure XIII. Epicardial loss of *Pdgfra* leads to a reduced number of EPDCs. Immunofluorescence analysis of the recombination reporter GFP (green) and of the coronary vessel endothelium (IB4, red) in transverse sections of the heart at E14.5 and E18.5 in control (*Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Pdgfra*$^{fl/+}$) and mutant (*Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Pdgfra*$^{fl/fl}$) embryos. Quantification of EMT (GFP^{+} area vs DAPI^{+} area) at 14.5 (P=0.02) and at E18.5 (P=0.002) reveals a reduction of EPDCs whereas the vessel density (number of IB4^{+} vessels per area) (P=0.7) at E18.5 in the right ventricle is unchanged.

Upk3b is dispensable for urothelial and mesothelial development and integrity

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Key words: uroplakin, Upk3b, urothelium, mesothelium, EMT

Running title: *Upk3b* in urothelia and mesothelia

Manuscript in preparation.

Abstract

Rationale:
The mesothelial lining of the body cavities and the urothelial lining of the urinary tract are highly specialized epithelia that protect the underlying tissues from mechanical stress and toxic fluids. The development of these epithelia from simple precursors and the molecular characteristics of the mature tissues are poorly analyzed.

Objective:
To determine the spatiotemporal expression of *Upk3b* and analyze its function for differentiation and integrity of the mesothelial lining of the body cavities and the urothelial lining of the urinary tract.

Methods and Results:
Using *in situ* hybridization analysis on sections and in whole-mount specimens we show specific expression of *Upk3b* both in development as well as under homoeostatic conditions, in adult mice in the mesothelial lining of the body cavities including the epicardium and pericardium, as well as in the urothelium lining the urinary tract. We generated a *creERT2* knock-in allele of *Upk3b* by homologous recombination in embryonic stem cells and show that it represents a null allele despite the lack of *cre* expression from the mutated locus. Morphological, histological and molecular analyses of *Upk3b*-deficient mice do not detect changes in differentiation or integrity of the urothelium and the mesothelia that cover internal organs in *Upk3b*-deficient mice.

Conclusions:
We suggest that *Upk3b* does not have a unique function in development and homeostasis of urothelia and mesothelia in the mouse.

Introduction

The lining of the urinary tract, i.e. of the collecting duct system of the kidney, the ureter, the urinary bladder and the urethra, represents a highly specialized epithelium that is both flexible and tight to accommodate the varying intraluminal pressure and the toxicity of the urinary fluid. A compelling structural feature of this urothelium is the presence of an elaborated surface barrier, which is composed of extracellular matrix as well as of integral membrane proteins. Members of the uroplakin protein family have been identified as crucial building units of this surface barrier that exhibits an almost crystalline organization (urothelial plaques).[1] Uroplakins can be subdivided into three sub-groups that consist of Upk1a/Upk1b, Upk2 and Upk3a/Upk3b. The sub-groups are distinguished by the number of transmembrane domains (TMD), by their glycosylation pattern and by the size of their cytoplasmic domain. Upk3a and Upk3b proteins are characterized by a single TMD, a glycosylated N-terminal luminal domain and a relatively large cytoplasmic domain, that may anchor the urothelial plaques to the cytoskeleton.[2,3] Upk3a and Upk3b can form heterodimeric complexes with Upk1b, whereas Upk1a heterodimerizes with Upk2.[3,4] Functional analyses by gene targeting have uncovered a crucial role for *Upk2* and *Upk3a* in maintaining the impermeability of the urothelium.[5,6] In both mutants renal dysfunction and hydronephrosis develop, i.e. fluid-mediated dilatation of the renal pelvis, most likely due to a reduction of urothelial plaques and urinary leakage. Analysis of urothelial function of *Upk1a*, *Upk1b* and *Upk3b* has not yet been performed.

A restriction of uroplakin function to the epithelial lining of the urinary tract was recently questioned by the finding in microarray analyses that *Upk3b* is enriched in peritoneal, pleural and pericardial mesothelia of mice. Subsequent *in situ hybridization* analysis confirmed *Upk3b* expression in the mesothelial linings of the lung and heart, liver, kidney, spleen, intestine, testis and thymus in adult mice.[7] Mesothelia are monolayers of flattened squamous-like epithelial cells that line the pleural, pericardial and peritoneal cavities of the chest and the abdomen, respectively. They possess a parietal layer that covers the body wall and a visceral layer that covers the organ in the respective cavity. Adult mesothelia produce a lubricating fluid that allows the internal organs to slide over each other. During development individual cells of the mesothelia can undergo a mesenchymal transition and leave the epithelial integrity, invade the underlying space and differentiate into fibroblasts and smooth muscle cells. The visceral pericardium, also known as epicardium, has been particularly well studied in recent years since it turned out to provide precursors for the cardiac fibroskeleton as well as smooth muscle cells of the coronary vasculature.[8] Some studies reported endothelial and myocardial fates of epicardial cells[9,10] although these findings were criticized for technical ambiguities.[11,12]

Identification of *Upk3b* expression in mesothelial tissues raises the interesting possibility that mesothelia and urothelia share structural features that may relate to efficient sealing of luminal spaces. To gain deeper insight into the role of *Upk3b* in these tissues, we wished to determine its expression both in development and homeostasis and analyze its functional requirement using gene-knock-out technology in mice.

Here, we provide a detailed expression analysis of *Upk3b* and show that *Upk3b*-deficiency does not affect the development and integrity of the urothelia and mesothelia in mice.

Material and methods

Mice

For the generation of a *creERT2* knock-in allele of *Upk3b* a targeting vector was constructed to insert a *CreERT2* coding region (Addgene plasmid 14797)[13] followed by a PGK-neo cassette flanked by *loxP* sites[14] into the start codon of the *Upk3b* locus (Figure 3). The integrity of the targeting vector was confirmed by restriction mapping and sequencing before the plasmid was linearized and electroporated into 129/SvCast ES cells. 24 h after electroporation, selection of transgenic clones was started by addition of 125 µg/ml G418 to the medium. Surviving colonies were expanded and subsequently screened for correct integration of the 3'-homology arm by PCR and for correct 5'-integration by Southern blot analysis. Three ES clones with verified homologous recombination of both arms were microinjected into CD1 mouse morulae. Chimeric males were mated to a cre deleter line (*Tbx3*$^{tm1.1(cre)Vmc}$)[15] to remove the *neo* cassette. The double fluorescent cre reporter line (Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$, synonym: *R26*^{mTmG})[16] was obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). All mice were maintained on an outbred (NMRI) background.

Embryos for expression analysis were derived form NMRI wild-type mice. The cell fate was analyzed in *Upk3b*$^{creERT2/+}$*;Rosa26*$^{mTmG/+}$ embryos, obtained from matings of males double heterozygous for *Upk3b*^{creERT2} and *R26*^{mTmG} alleles and females heterozygous for *Upk3b*^{creERT2}. In the latter case, tamoxifen (Sigma) was dissolved in ethanol at 100 mg/ml and then emulsified in corn oil (Sigma) to a final concentration of 12.5 mg/ml. 4 mg of tamoxifen were intraperitoneally injected into mice at gestation day 9.5. For timed pregnancies, vaginal plugs were checked in the morning after mating and noon was designated as embryonic day (E) 0.5. Female mice were sacrified by cervical dislocation. Embryos and organs were harvested in PBS, decapitated, fixed in 4% paraformaldehyde overnight and stored in 100% methanol at -20^{o}C before further use. Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR. H. Hedrich, state head of the animal facility, approved the care of animals and experiments at Medizinische Hochschule Hannover.

Histological analysis

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

Immunofluorescence

For immunofluorescence analysis rabbit polyclonal antibody against GFP (1:200 sc-8334, Santa Cruz), mouse monoclonal antibody against GFP (1:200,11 814 460 001, Roche), rabbit polyclonal antibody against SM22alpha (Tagln) (1:200, ab14106-100, Abcam), Fluorescein labeled GSL I – isolectin B4 (1:100, FL-1101, VectorLabs), rat monoclonal antibody against endomucin (Emcn) (1:2, a kind gift of D. Vestweber, MPI Münster; Germany), rabbit polyclonal against periostin (1:200, Abcam, ab14041), mouse monoclonal antibody against alpha-Smooth muscle actin (Acta2), FITC Conjugate (1:200, F3777, Sigma) and mouse monoclonal antibody against MF20 (1:200, Hybridoma Bank University of Iowa) were used as primary antibodies.

Biotinylated goat-anti-rabbit (Dianova, 1:400), Alexa488 goat-anti-rabbit (Invitrogen, 1:400), Alexa488 donkey-anti-mouse (Invitrogen A21202, 1:400), Alexa-Fluor555 goat-anti-mouse (Invitrogen A-21424, 1:400) and Alexa-Fluor555 goat-anti-rabbit (Invitrogen A-21428 1:400) were used as secondary antibodies. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Roth).

Immunofluorescence analysis was done on 4-µm paraffin sections. All 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).

In situ hybridization analysis

In situ hybridization analysis on 10-µm paraffin sections and on whole embryos with digoxigenin-labeled antisense riboprobes was performed as described.[17]

Semi-quantitative reverse transcription PCR

For semiquantitative analysis of *Upk3b* expression three hearts each of wildtype, heterozygous and homozygous E12.5 embryos were pooled. RNA was extracted with RNAPure reagent (Peqlab) and DNaseI treated for 30min at 37°C. RNA was reverse transcribed with RevertAid H-minus M-MuLV Reverse Transcriptase (Fermentas). For semi-quantitative PCR, the pools were adjusted to yield the same *Gapdh* intensity at the mid-logarithmic phase and the *Upk3b* PCR was performed on these pools. Quantification was performed with ImageJ.[18]

Image analysis

Sections were photographed using a Leica DM5000 microscope with Leica DFC300FX digital camera. Whole-mount specimens were photographed on Leica M420 with Fujix digital camera HC-300Z. All images were processed in ImageJ[18] and Adobe Photoshop CS4.

Results

Upk3b is expressed in the urothelium and in mesothelial tissues during embryonic development

To determine the expression pattern of *Upk3b* during embryonic development, we performed *in situ* hybridization analysis on E9.5 to E16.5 mice (Figure 1). In E9.5 embryos expression of *Upk3b* was detected in the urogenital ridge, the proepicardium, the dorsal mesocardium and in single epicardial cells that were attached to the ventricular myocardium at this stage. At E10.5, *Upk3b* expression lined the contiguous epicardium in addition to all mesothelial linings of the body cavity. At E12.5 and subsequent embryonic stages, all mesothelial linings (i.e epicardium and pericardium, pleura, and peritoneum) expressed *Upk3b*. From E14.5 on, the epithelial lining of the developing urinay tract including the renal pelvis, the lumen of the bladder and the ureter were positive for *Upk3b* expression as well.

Upk3b is expressed in the pericardium and in the urothelium of adult mice

To test whether expression of *Upk3b* was maintained into adulthood, we performed *in situ* hybridization analysis on sections of organs obtained from 6-month old mice (Figure 2). In the heart *Upk3b* was confined to the epicardium that lined all chambers as well as in the pericardium covering the great coronary vessels. In the urinary system, the multi-layered urothelium lining the lumen of the renal pelvis, the ureter and the bladder expressed *Upk3b* in addition to the single layered outer peritoneal lining of these organs.

Generation of a *creERT2* knock-in allele of *Upk3b* by homologous recombination in ES cells

To elucidate the role of *Upk3b* both in the development and in the maintenance of urothelial and mesothelial tissues in adulthood, we wished to generate an *Upk3*b knock-in allele allowing tamoxifen inducible expression of the cre-recombinase gene under the control of endogenous *Upk3b* control elements (Figure 3). Mice with correct integration of a *creERT2* expression cassette in the *Upk3b* locus were obtained and subsequently tested for functionality of the creERT2 protein by injection of tamoxifen into pregnant *Upk3b*$^{creERT2/+}$*;Rosa26*$^{mTmG/+}$ dams at E9.5. To our surprise, *in situ* hybridization did not detect expression of the *cre* transcript in the epicardium, the pericardium or ureteric urothelium of E15.5 embryos although expression of *Upk3b* was still easily detected in these organs. Since this assay cannot unambiguously exclude the presence of low levels of *Cre* expression, we additionally performed a reporter gene analysis using GFP immunofluorescence to test for recombination on neighboring sections. Expression of GFP was not detected in any of the analyzed tissues (Figure 4 and data not shown). We, therefore, conclude that *creERT2* is not expressed in a correct manner from the *Upk3b*$^{creERT2/+}$ allele.

Upk3b$^{creERT2/creERT2}$ mice are *Upk3b* null mutants

To test if *Upk3b* is deleted in the *Upk3b*$^{creERT2/+}$ allele, we performed *in situ* hybridization analysis for the *Upk3b*-3'-untranslated region on transverse sections of the heart and ureter in E18.5 embryos homozygous for the *Upk3b*^{creERT2} allele. Expression of *Upk3b* was neither detected in mesothelia (e.g. lung, pleuropericardial membrane and epicardium) nor in the urothelium of the ureter. Furthermore, semiquantitative RT-PCR analysis of E12.5 isolated hearts derived from wildtype, heterozygous and homozygous mutant embryos confirmed the absence of *Upk3b* mRNA in *Upk3b*$^{creERT2/creERT2}$ embryos. We conclude that *Upk3b*$^{creERT2/creERT2}$ mice represent *Upk3b* null mutants, and thus, can be analyzed for phenotypic consequences of loss of *Upk3b*.

Upk3b is dispensable for development and integrity of the heart and the urinary bladder

Upk3b$^{creERT2/creERT2}$ mice were born in the expected Mendelian ratio, reached sexual maturity and became fertile and were unaltered in their behavior at 6-months of age. Morphologically,

the mutants exhibited no differences in the appearance of internal organs of the chest and the abdomen (data not shown). On histological sections the heart seemed unaffected; the ventricular wall thickness was normal and the integrity of the septa, valves and the epicardium was preserved (Figure 6).

Since epicardial cells give rise to the smooth muscle and fibroblast lineages, thus, contribute to the formation of the coronary vessels and fibrous skeleton of the heart, we analyzed by immunofluorescence of marker proteins the arrangement of vessels (isolectin-B4 endothelial staining), smooth muscle cells (transgelin (Tagln, also known as Sm22)) and of interstitial and perivascular fibroblasts (periostin (Postn)). Expression and distribution of these markers was indistinguishable between mutant and wildtype hearts, demonstrating that deletion of *Upk3b* is irrelevant for the integrity of the coronary vasculature and the cardiac fibrous skeleton at this level of resolution. Immunofluorescence analysis of myocardial marker MF20 revealed a normal myocardium excluding changes of the trophic function of the mutant epicardium as well.

The urinary bladder appeared normal on histological sections and the urothelium and the detrusor muscle were in sound condition. Given that the main function of the urinary bladder is to protect the underlying detrusor from the toxicity of the urine, we analyzed whether the urothelium (uroplakin1b (Upk1b)) was intact, and whether the muscle layer (transgelin, Tagln, also known as Sm22)) fibroblasts (periostin, Postn) and endothelial cells of the vessels (endomucin, Emcn) were affected. Expression of the urothelial, endothelial, fibroblast and muscle markers was indistinguishable between mutant and wildtype mice, indicating that *Upk3b* is dispensable for normal urinary bladder formation and homeostasis.

Discussion

Upk3b is expressed in the urothelium and mesothelial tissues during embryonic development and in adulthood

Our detailed expression analysis confirmed the urothelial presence of *Upk3b*. It additionally showed that onset of *Upk3b* expression in the epithelium of the urinary tract slightly precedes the onset of urine production in the renal pelvis around E16.0[19] compatible with the function of urothelial plaques to generate a permeability barrier against the toxic effects of the urine.[1]
Our expression analysis for the fist time identified *Upk3b* in all mesothelia from E9.5 to adulthood. Mesothelia are thought to be important for the protection of the underlying tissues, thus are likely to achieve a high degree of impermeability and flexibility at the same time. In analogy to the urothelium the occurrence of "mesothelial plaques" seems possible. To test this hypothesis, we performed additional expression analysis for the key components of the urothelial plaques *Upk1a*, *Upk2* and *Upk3a*. However, this analysis did not detect expression of any of these genes in mesothelial development (data not shown) making the formation of "mesothelial plaques" unlikely at this point. Nevertheless, luminal N-glycosylation of Upk3b protein[3] might account for the lubrication of mesothelial surfaces by binding of extracellular fluids. Furthermore, the potential interaction of Upk3b's cytoplasmatic domain with the cytoskeleton may participate in the maintenance of the apico-basal polarity of mesothelial tissues.

Upk3b^{creERT2} represents a *Upk3b* null allele but does not allow cre-mediated recombination

Our targeting construct was designed to allow for tamoxifen-controllable mesothelial and urothelial expression of a creERT2 fusion protein. However, to our surprise we failed to detect expression and activity of this protein in mutant animals. At this point, we do not know the reasons that might have caused this problem. We assume that promotor accessibility might be affected by the knocked-in sequence, and silencing of the locus resulted. Furthermore, reduced transcript stability, due to reduced polyadenylation, altered 5'- and 3'-untranslated regions and overall translational activity are possible factors for the lack of creERT2 protein. Finally, potential mutations acquired during the ES cell culture leading to non-sense mediated decay, cannot be excluded. Nonetheless, *Upk3b* was no longer expressed from the mutant allele allowing the characterization of the phenotypic consequences of *Upk3b* loss in mice.

Upk3b is dispensable for normal heart and urinary bladder formation.

The specific expression of *Upk3b* in all mesothelia of the developing murine embryo pointed towards a possible role in the separation of the body cavities, in which growth of mesothelia is of crucial importance.[20] Differences in the separation of the chest and abdomen and of pleural, pericardial and peritoneal cavities as well as the appearance of internal organs were not detected in the mutants (data not shown), excluding an important function for *Upk3b* in the formation of mesothelia.
Mesothelial cells of the heart, lung, intestine and liver can give rise to vascular smooth muscle cells and fibroblasts.[8,21–26] Our analysis of the epicardium, the best studied mesothelial tissue in vertebrates, did neither detect changes in the mesenchymal transition of epicardial cells nor in the subsequent differentiation into smooth muscle cells and cardiac fibroblasts. Furthermore, we noticed that the ventricular myocardium was of normal thickness and the coronary vasculature was well-elaborated excluding both a cellular and trophic role of Upk3b in epicardial development.
Interestingly, adult mesothelial cells of the omentum and epicardium have been reported to contribute to vascular smooth muscle cell and fibroblast lineages under chronic and/or acute injury conditions.[27,28] In peritoneal sclerosis, a submesothelial thickening of abdominal membranes[29] and in myocardial infarction new fibroblasts arise from the injured epicardium.[30] Often, these fibrotic conditions are additionally associated with inflammatory

processes. As the molecular mechanisms underlying this regenerative capacity derive from the reactivation of embryonic gene programs,[28,31] that were unaffected in *Upk3b*-deficient embryos, we deem it unlikely that *Upk3b* is implicated in the regenerative capacity of adult mesothelia. However, future work should test a requirement for Upk3b in pathological conditions in a more detailed fashion.

In urothelial plaques, Upk3b is present at low levels, amounting usually to less than 10% of Upk3a, the major plaque component. In *Upk3a*-deficient mice, urothelial plaques are present but smaller in size, *Upk3b* is up-regulated relative to other uroplakins.[53] Furthermore, co-immunoprecipitation experiments showed specific binding of Upk3b to Upk1b, the binding partner of Upk3a in plaques. Together with our finding that the urinary tract appears normal in *Upk3b*-deficient mice, this suggests that Upk3b acts redundantly with Upk3a in the urothelium. Analysis of mice double mutant for *Upk3a* and *Upk3b* may address the combined function of both factors in the future.

Acknowledgements

We thank D. Vestweber for the Emcn antibody. The MF20 hybridoma developed by Fischman, D.A. was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242.

Sources of Funding

This work was supported by grants from 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.

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Figures and legends

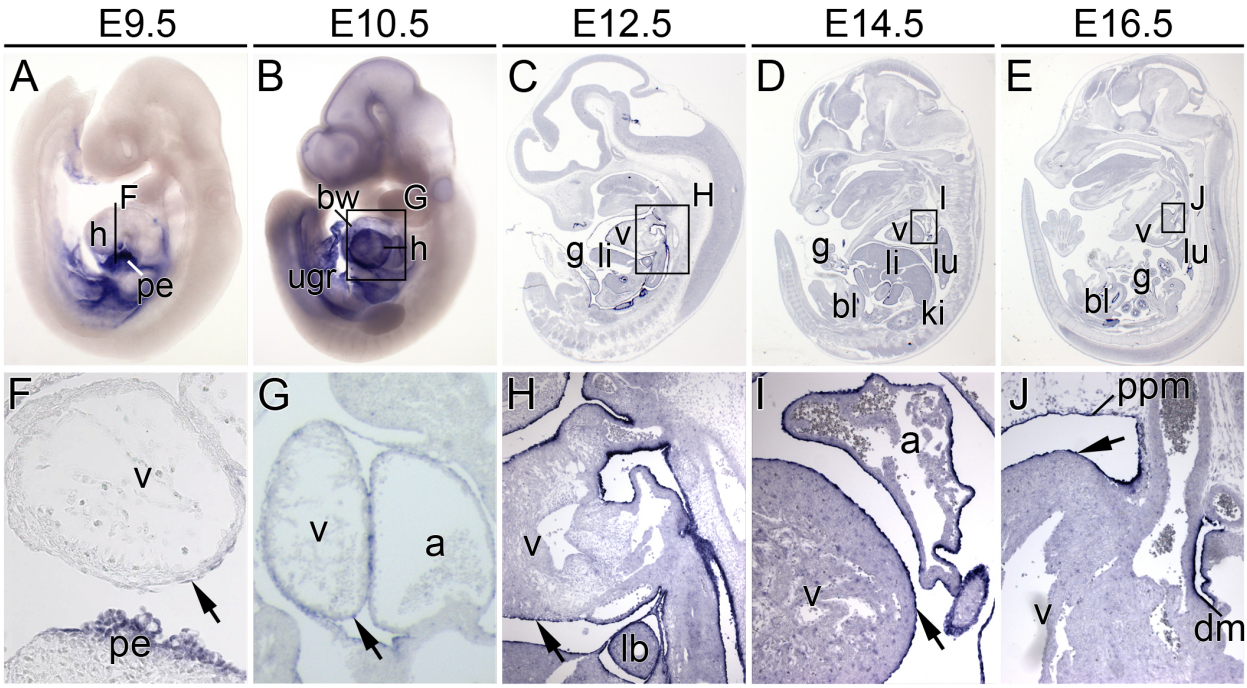

Figure 1. *Upk3b* expression in embryonic development. *In situ* hybridization analysis of *Upk3b* expression in whole wildtype embryos **(A and B)** and on sagittal embryo sections **(D through J)**. (**A through E**) Overview images of embryos, anterior is up, dorsal is to the right. (**F through J**) Higher magnification images of the regions marked by open rectangles (**in A through E**). Stages are as indicated. **Arrows** point to the epicardium. a, atrium; bl, urinary bladder; bw, body wall; dm, dorsal mesocardium; g, gut; h, heart; ki, kidney; lb, lung bud; li, liver; lu, lung; ugr, urogenital ridge; pe, proepicardium; ppm, pleuropericardial membrane; v, ventricle.

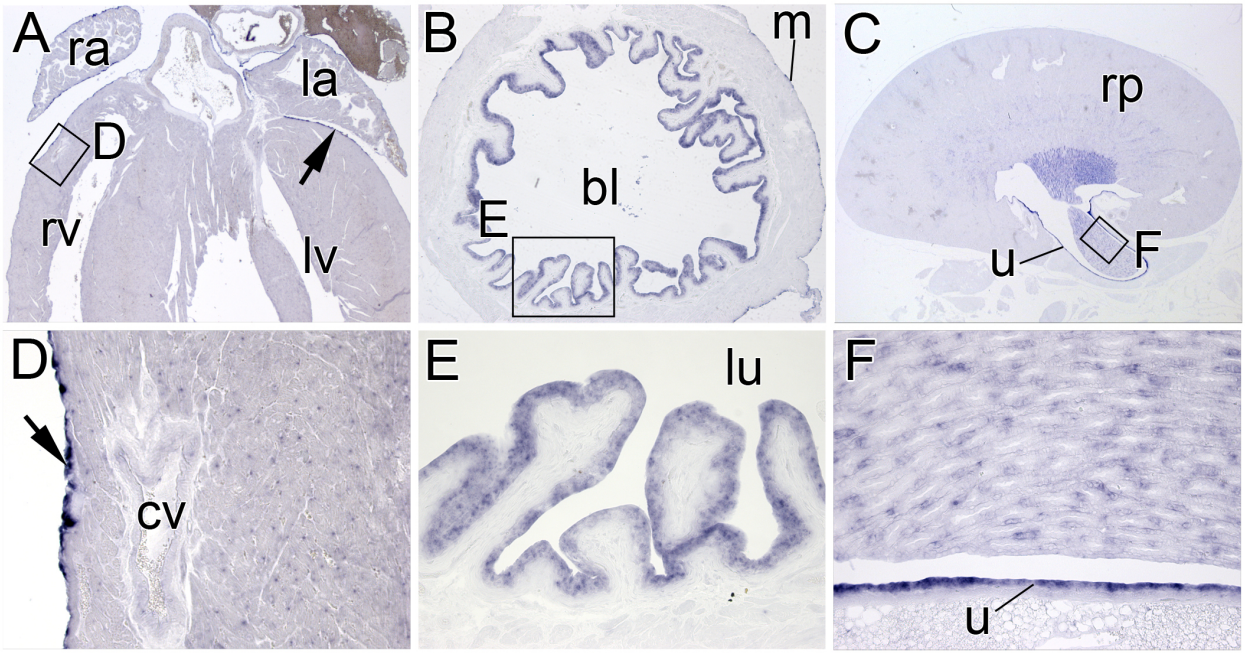

Figure 2. *Upk3b* expression in adult tissues. *In situ* hybridization analysis of *Upk3b* expression on sections of the adult heart **(A and D)**, the urinary bladder **(B and E)** and the kidney **(C and F)**. (**A through C**) Overview images of whole organ sections, (**D through F**) higher magnification images of the regions marked by open rectangles (**in A through C**). The **arrow** points to the epicardium. bl, urinary bladder; cv, coronary vessel; la, left atrium; lu, urinary bladder lumen; lv, left ventricle; m, urinary bladder mesothelium; ra, right atrium; rp, renal pelvis; rv, right ventricle; u, urothelium.

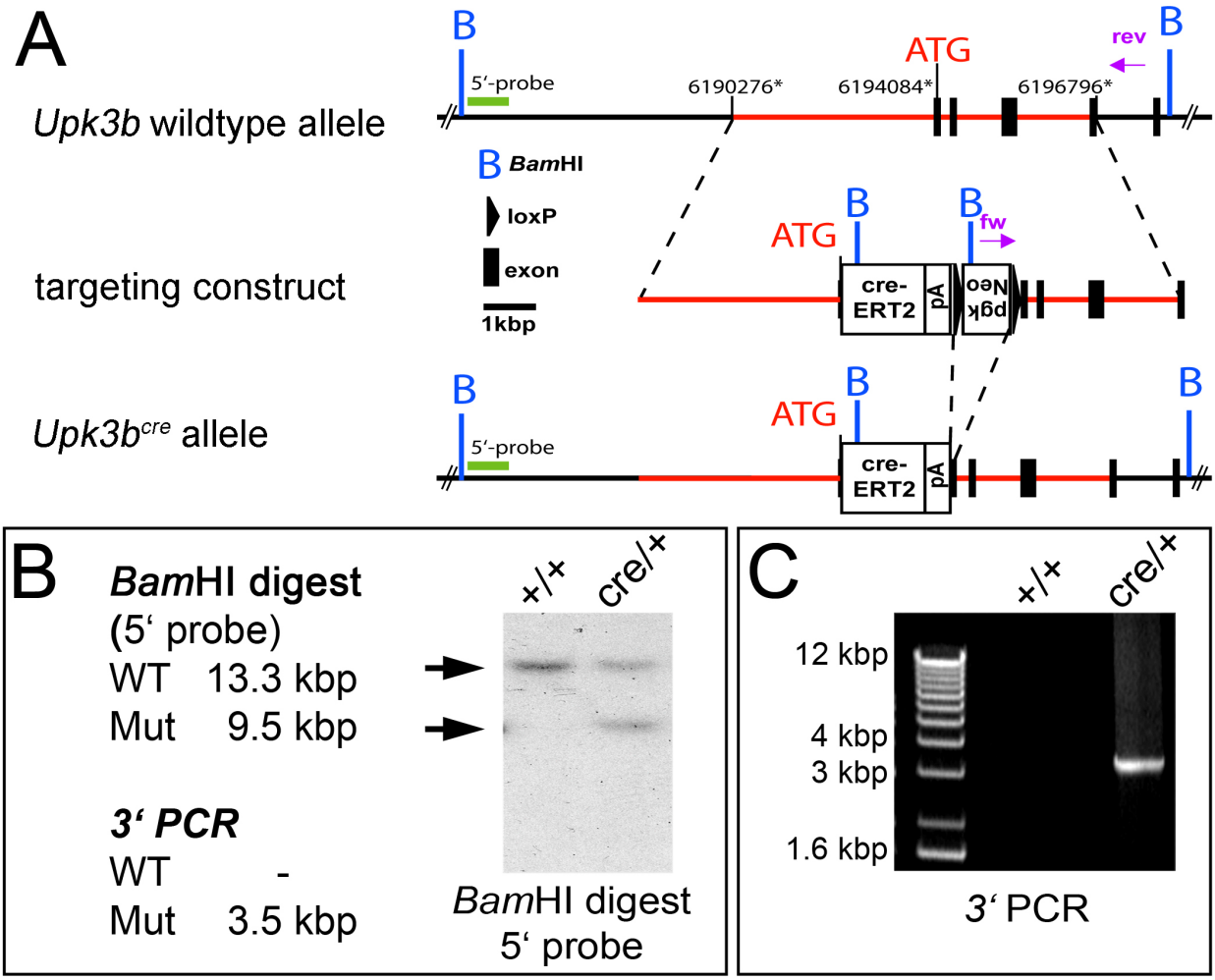

Figure 3. Generation and confirmation of a *creERT2* knock-in allele of *Upk3b*. (A) Scheme of the targeted insertion of a *creERT2* recombinase gene/*loxP*-flanked *neomycin* selection cassette in the *Upk3b* locus. Exons are shown in black, regions for homologous recombination in red. Screening for clones with correct integration of the *creERT2/neo* cassette was performed using a PCR for the 3'-region, primers are indicated in pink. A *Bam*HI restriction fragment length polymorphism (RFLP) with the indicated 5'-probe was used to check for correct 5'-integration **(B),** and a long-range PCR to verify the correct 3'-integration **(C)** of the targeting vector.ATG, transcriptional start codon; B, *Bam*HI; *creERT2*, *cre* recombinase fused to a triple mutant form of the human estrogen receptor expression cassette; kbp, kilo base pairs; *loxP*, locus of X-over P1; Neo, *neomycin* resistance gene; pA, polyadenylation signal; pgk, phosphoglycerate kinase I promoter.

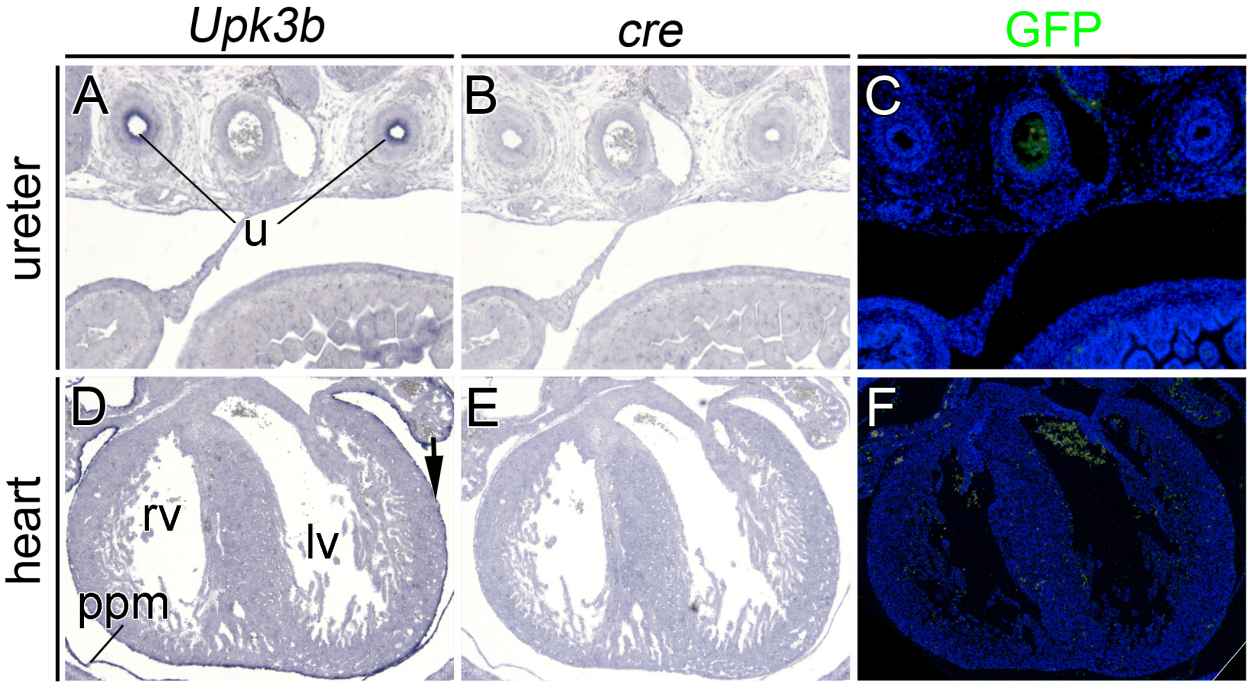

Figure 4. *Upk3b*$^{creERT2/+}$ mice neitherexpress cre nor mediate recombination of *loxP*-flanked sequences in the ureter and in the epicardium. Pregnant mothers were injected at E9.5 with tamoxifen and E15.5 *Upk3b*$^{creERT2/+}$;*Rosa26*$^{mTmG/+}$ embryos were analyzed by *in situ* hybridization for expression of *Upk3b* and *cre* on transverse sections of the ureter **(A and B)** and the heart **(D and E)**. Immunofluorescence analysis of the lineage marker GFP was performed on transverse sections of the ureter **(C)** and the heart **(F)**. **Arrows** point to the epicardium. lv, left ventricle; ppm, pleuropericardial membrane; rv, right ventricle; u, ureter. Nuclei are counter-stained with 4',6-diamidino-2-phenylindole.

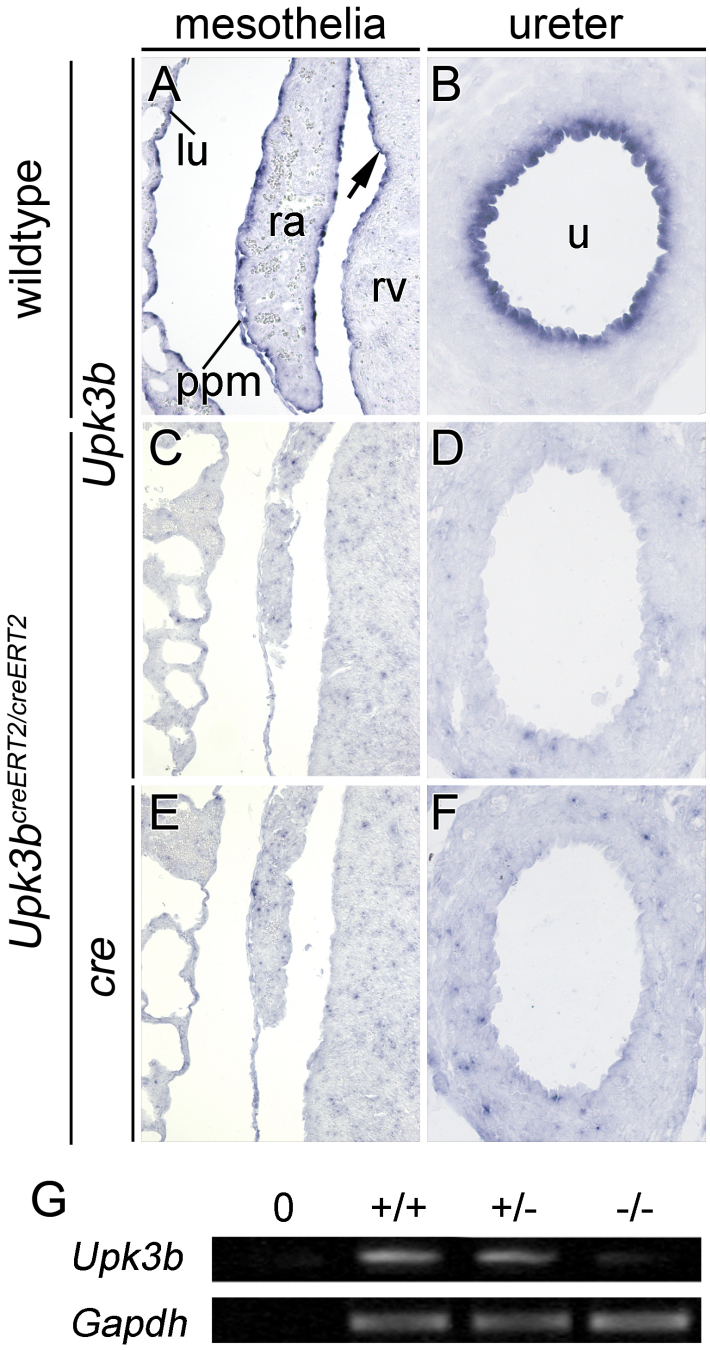

Figure 5. *Upk3b*$^{creERT2/creERT2}$ mice are *Upk3b* null mutants. *In situ* hybridization analysis for expression of *Upk3b* and *cre* on transverse sections of an E18.5 heart **(A through E)** and ureter **(B through F)** in wildtype and homozygous knock-out embryos. **(G)** Semiquantitative RT-PCR analysis of E12.5 isolated hearts derived from wildtype, heterozygous and knock-out (*Upk3b*$^{creERT2/creERT2}$) animals. lu, lung; ppm, pleuropericardial membrane; ra, right artrium; rv, right ventricle; u, ureter.

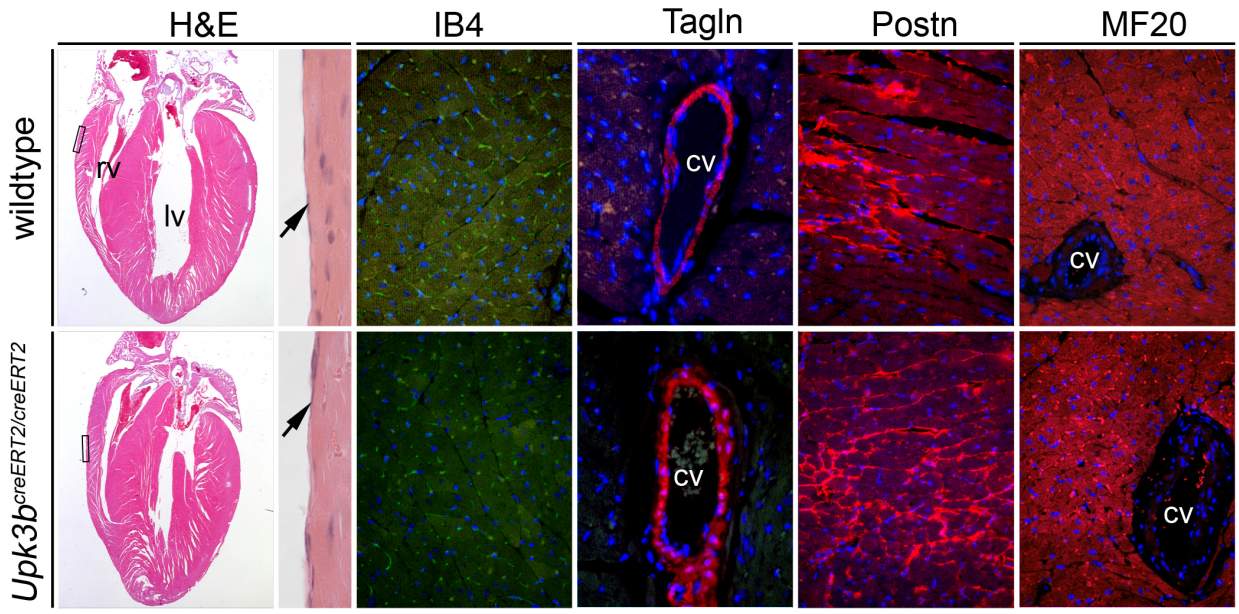

Figure 6. *Upk3b* is dispensable for normal heart formation. Hematoxylin and eosin staining (H&E) and immunofluorescence analysis of markers of capillary endothelia (IB4), cardiomyocytes (MF20), fibroblasts (Postn) and coronary smooth muscle cells (Tagln) on sections of 6-month old hearts of wildtype and homozygous knock-out mice. Magnified areas are indicated with rectangles. **Arrows** point to the epicardium. cv, coronary vessel; lv, left ventricle; rv, right ventricle.Nuclei are counter-stained with 4',6-diamidino-2-phenylindole.

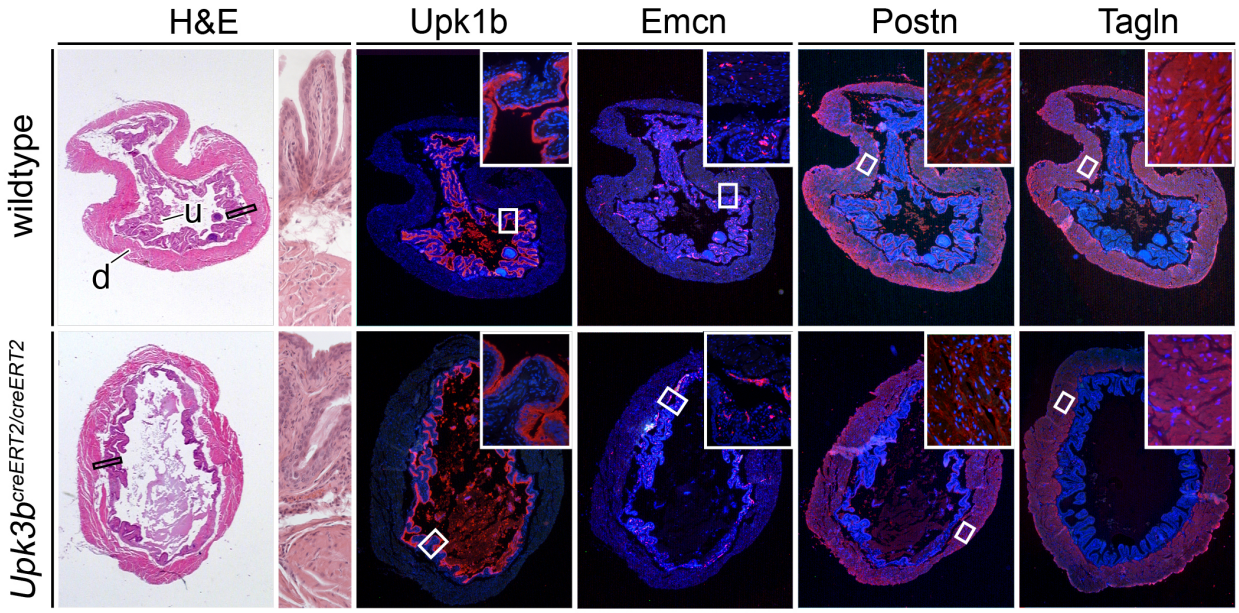

Figure 7. *Upk3b* is dispensable for normal urinary bladder formation. Hematoxylin and eosin staining (H&E) and immunofluorescence analysis for markers of the urothelium (Upk1b), vessel endothelium (Emcn), fibroblasts (Postn) and smooth muscle cells (Tagln) on sections of the bladder of 6-month old wildtype and homozygous knock-out animals. Magnified areas are indicated with rectangles. d, detrusor; u, urothelium.Nuclei are counter-stained with 4',6-diamidino-2-phenylindole.

Concluding remarks

In vertebrates the formation of the epicardium and addition of epicardium-derived cells to the developing heart is important for establishing a functional coronary vasculature and maturation of the myocardium. In this study, smooth muscle cells, perivascular and interstitial fibroblasts were confirmed as epicardium-derived cells in the right ventricle. This canonical epicardial lineage has been identified in zebrafish[73], chick[10,16,23,24] and mouse[28,29,74]. Further epicardial contribution to this myocardial and endothelial lineages was suggested by deployment of regulatory sequences of the epicardial marker genes *Wt1* and *Tbx18,*[28,29], but was disproven in this study and in independent publications.[41,73] Only recently two novel marker genes, *Scleraxis* (*Scx*) and *Semaphorin 3D* (*Sema3D*), have been identified in a subset of proepicardial cells, only overlapping in part with *Wt1* and *Tbx18* expressing cells. Interestingly, *Scx* and *Sema3D* labelled cells give rise to myocardial and endothelial cells additionally to the classical epicardial cell fates, in addition to the canonical epicardial lineage.[74] The proepicardium is obviously compartmentalized into genetically distinct subpopulations, shown by the largely non-overlapping expression of *Wt1/Tbx18* and *Scx/Sema3D*, and the observation, that some, but not all, proepicardial cells are able to differentiate into cardiac myocytes *in vitro.*[75,76] The latter might be substantiated in the common origin of at least some of the proepicardium-cells and the cardiomyocyte lineages, indicated by lineage tracings for early cardiogenic transcription factors.[29,77] Nevertheless, a contribution of epicardial cells to the cardiomyocyte lineage cannot be substantiated by the current state of scientific knowledge. The proposed endothelial fate of epicardial cells, based on the *Scx/Sema3D* lineage tracings, might be questioned, because a potential expression of these markers in the sinus venosus endothelium - which gives rise to the coronary endothelium[27] - has not been addressed so far. These data suggest that some cells within the proepicardium are not fully dedicated to an epicardial cell fate, however cells of the epicardium only give rise to the canonical epicardial lineage.

Complex signaling events govern the mobilization and differentiation of EPDCs. However, the reevaluation of the epicardium-specific loss of Ctnnb1, Fgfr1/2, Shh and the Hh receptor Smoothened did not reveal any contribution to these processes. Recombination in non-epicardium-derived cells may account for the detected phenotypes in previous studies.[34,48,53,58] Especially, the loss of Hh and canonical Wnt signaling in coronary endothelial cells might be causative for the observed coronary vasculature defects that in turn might influence smooth muscle cell differentiation. In addition, conditional approaches using $Wt1^{cre}$ imply already a heterozygous loss of *Wt1*, which possibly interferes with the mobilization of EPDCs. Thus the impaired EMT observed in $Wt1^{cre}$ mediated loss of Ctnnb1 and Fgfr1/2 can be questioned in this context. The requirement of the Notch signaling

pathway for the differentiation of perivascular smooth muscle cells was shown and the necessity of Pdgfrα signaling for EMT and fibroblast differentiation was confirmed in this study. This results in a simplified model of molecular signaling pathways in the mobilization and differentiation of epicardial cells in embryonic development.

Interestingly, *Tbx18* and *Wt1* – normally present only in the embryonic epicardium – are re-expressed in the murine heart after myocardial infarction. These studies on the one hand indicate that the canonical epicardial lineage is recapitulated in these conditions, but leave open that EPDCs might - at low frequencies - differentiate into mature cardiomyocytes in the presence of injury on the other.[78,79] In these experiments the fate of epicardial cells was followed with $Wt1^{cre}$ lines. However, in the infarcted rat heart *Wt1* is re-expressed additionally in coronary endothelial and smooth muscle cells in the infarct border zone.[80] Considering the fact that *Wt1* is upregulated by ischemic cues,[81] its expression in the border zone may reflect the ischemic conditions rather than being an indication of epicardial activation and EMT.

Wound healing after myocardial infarction might be dependent on cells of the canonical epicardial lineage, which secrete proangiogenic growth factors.[82] In independent studies Notch and Wnt signaling were found to be upregulated in the regions of injury and the loss of *Wnt1* impaired wound healing.[83,84] Given the potential of Wnt signaling in proliferation control and the requirement of Notch signaling in differentiation of coronary smooth muscle cells, the impact of these signaling pathways on EMT and secretion of growth factors in the adult epicardium ought to be analyzed in the future.

Although our findings indicate that epicardial cells are not a natural source of cardiomyocytes in development, the possibility remains that experimental manipulation of these cells may be used for regenerative therapies. For this purpose inducible epicardium-specific cre-lines like the $Upk3b^{creERT2}$ line are needed.

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Acknowledgements

First and foremost, I would like to thank my supervisor Prof. Andreas Kispert for this fascinating project and the great opportunity to work in his excellent research group. I am thankful for his continuous and enthusiastic support during the last years.

I would like to thank Prof. Dr. Hans-Jörg Jacobsen and Prof. Dr. Florian P. Limbourg for their kind acceptance as a referee during my dissertation.

Thanks also to all other members of the lab and the IfM, past and present, for discussions and fantastic team-work. In particular I would like to thank Dr. Thomas Grieskamp and Dr. Timo Lüdtke for accompanying my research project by sharing ideas and assistance in the lab.

Kindly acknowledged are Dr. Karin Schuster-Gossler and Rannar Airik, Ph.D. for generation of the *Tbx18*^{cre} mice and for their assistance in the generation of *Upk3b*^{creERT} mice, I used in my study.

Thanks to Dr. Anna Barbara Foik for being a pal in stern times.

Special thanks to Franziska Greulich, Dr. Marc-Jens Kleppa, and Dr. Julia Norden for support in my research project.

Thanks to Claudia. This work would not have been successful without you.

Finally, I want to say thank you to my parents who always backed me up whenever it was needed.

Curriculum Vitae

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Date of birth:	15/11/1977
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School education:

1984 – 1988	Grundschule Adendorf
1988 – 1990	Orientierungsstufe Adendorf
1990 – 1997	Gymnasium Scharnebeck

Military service:

1997 – 1998	Munster

Apprenticeship:

1998 – 2000	professional education as state-approved chemical- and biological technical assistant; LETTE-Verein Berlin

University studies:

2000 – 2004	Undergraduate studies of Biochemistry (“Vordiplom”) at the Freie Universität Berlin
2004 – 2007	Graduate studies of Biochemistry (“Diplom”) at the Freie Universität Berlin
2006 – 2007	Diploma thesis in the Institute for Pharmacology at the Freie Universität Berlin under supervision of Prof. Dr. Michael Schaefer Title of diploma thesis: “Translokationskinetik der ProteinkinaseCα in kleinen Zellkompartimenten”
2007 –	Research assistant in the laboratory of Prof. Dr. Andreas Kispert in the Institute for Molecular Biology at Hannover Medical School.

List of publications

Rudat C, Norden J, Taketo MM, Kispert A. Epicardial function of canonical Wnt-, Hh-, Fgfr1/2- and Pdgfrα-signaling. *Circulation Research*. Manuscript submitted.

Lüdtke T, Farin HF, **Rudat C**, Schuster-Gossler K, Petry M, Barnett P, Christoffels VM, KispertA. Tbx2 controls lung growth by direct repression of the cell cycle inhibitor genes Cdkn1a and Cdkn1b. *PLoS Genetics*. Manuscript submitted.

Rudat C, Kispert A. Wt1 and Epicardial Fate Mapping.
Circulation Research. 2012, 111:165–169.

Greulich F, **Rudat C**, Kispert A. Mechanisms of T-box gene function in the developing heart. *Cardiovascular Research*. 2011, 91:212 –222.

Norden J, Greulich F, **Rudat C,** Taketo MM, Kispert A. Wnt/β-Catenin Signaling Maintains the Mesenchymal Precursor Pool for Murine Sinus Horn Formation. *Circulation Research*. 2011, 109:e42 –e50.

Grieskamp T, **Rudat C**, Lüdtke TH-W, Norden J, Kispert A. Notch Signaling Regulates Smooth Muscle Differentiation of Epicardium-Derived Cells. *Circulation Research*. 2011, 108(7):813-23

Christoffels VM, Grieskamp T, Norden J, Mommersteeg MTM, **Rudat C**, Kispert A. Tbx18 and the fate of epicardial progenitors. *Nature*. 2009, 458:E8–E9.

Declaration

“I hereby declare and confirm that this thesis is entirely the result of my own work except where otherwise indicated. This thesis has not been used as part of any other examination.”

Erklärung zur Dissertation

Gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover

für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel
Mobilization and differentiation of epicardial cells in murine development
selbständig verfasst und die benutzte Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.