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# Epicardial function of canonical Wnt-, Hedgehog-, Fgfr1/2-, and Pdgfra-signalling

**Carsten Rudat[1], Julia Norden[1], Makoto Mark Taketo[2], and Andreas Kispert[1]***

[1]Institut für Molekularbiologie, OE5250, Medizinische Hochschule Hannover, Carl-Neuberg-Str.1, Hannover D-30625, Germany; and [2]Department of Pharmacology, Graduate School of Medicine, Kyoto University, Sakyo, Kyoto 606-8501, Japan

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**Aims** The embryonic epicardium is a source of smooth muscle cells and fibroblasts of the coronary vasculature and of the myocardium, but the signalling pathways that control mobilization and differentiation of epicardial cells are only partly known. We aimed to (re-)evaluate the relevance of canonical Wnt-, Hedgehog (Hh)-, Fibroblast growth factor receptor (Fgfr)1/2-, and platelet-derived growth factor receptor alpha (Pdgfra)-signalling in murine epicardial development.

**Methods and results** We used a T-box 18 (*Tbx18)*$^{cre}$-mediated conditional approach to delete and to stabilize, respectively, the downstream mediator of canonical Wnt-signalling, beta-catenin (Ctnnb1), to delete and activate the mediator of Hh-signalling, smoothened (Smo), and to delete *Fgfr1*/*Fgfr2* and *Pdgfra* in murine epicardial development. We show that epicardial loss of *Ctnnb1*, *Smo*, or *Fgfr1*/*Fgfr2* does not affect cardiac development, whereas the loss of *Pdgfra* prevents the differentiation of epicardium-derived cells into mature fibroblasts. Epicardial expression of a stabilized version of Ctnnb1 results in the formation of hyperproliferative epicardial cell clusters; epicardial expression of a constitutively active version of Smo leads to epicardial thickening and loss of epicardial mobilization.

**Conclusion** Canonical Wnt-, Hh-, and Fgfr1/Fgfr2-signalling are dispensable for epicardial development, but Pdgfra-signalling is crucial for the differentiation of cardiac fibroblasts from epicardium-derived cells.

**Keywords** Epicardium • EMT • Cardiac fibroblast • Smooth muscle cell • Coronary vasculature

## 1. 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 at E12.5, individual epicardial cells undergo an epithelial–mesenchymal transition (EMT) and migrate into the subepicardial space. These epicardium-derived cells (EPDCs) largely differentiate into the interstitial and perivascular fibroblasts and into the smooth muscle cells (SMCs) that surround the coronary vessels.[1–3]

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 signalling components.[2,4] 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) Wnt-signalling pathway in epicardial EMT and coronary SMC differentiation, was suggested.[5,6] Loss of platelet-derived growth factor receptor alpha (Pdgfra) resulted in reduced EMT and a deficit in cardiac fibroblast formation,[7] which holds true as well for the combined conditional loss of the genes encoding fibroblast growth factor (Fgf) receptors 1/2 which was similarly achieved by a *Wt1*$^{cre}$-mediated recombination of floxed alleles in the epicardium.[8] Furthermore, epicardial derived Fgf-signals have been shown to induce sonic hedgehog (Shh) signalling, which in turn, regulates the formation of arterial and venous coronaries.[9] Finally, we showed by *T-box18* (*Tbx18)*$^{cre}$-mediated epicardial deletion of the gene encoding the transcription factor Rbpj that Notch-signalling regulates SMC differentiation of EPDCs once they have reached a perivascular position.[10] Shared phenotypic consequences may argue for pathway cooperation in distinct epicardial subprograms including EMT (Wnt/Pdgfra/Fgfr1,2) and SMC differentiation (Wnt/Notch).

* Corresponding author. Tel: +49 511 5324017; Fax: +49 511 5324283, Email: kispert.andreas@mh-hannover.de

However, recent studies indicated that the *Gata5::cre* line and *Wt1*-based *cre* lines, that were used in these conditional gene targeting experiments,[11,12] may not have mediated specific and/or efficient recombination in the epicardium.[13,14] Before embarking on genetic interaction studies of Notch-signalling with any of these pathways, we therefore wished to re-evaluate the epicardial requirement of these pathways (canonical Wnt, Fgfr1/Fgfr2, Hh, Pdgfra) using the *Tbx18*$^{cre}$ line which we have recently characterized to mediate epicardial recombination.[10]

Here, we show that in mice with epicardial (*Tbx18*$^{cre}$-mediated) deletion of *Ctnnb1*, *Fgfr1/2*, 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 activation of canonical Wnt- and Hh-signalling in the *Tbx18*$^{cre}$ lineage is actually deleterious for epicardial and myocardial development *in vivo*. Furthermore, we confirm that *Pdgfra* is required for the differentiation of cardiac fibroblasts.

# 2. Methods

## 2.1 Mice and genotyping

Medizinische Hochschule Hannover is a holder of a PHS approved animal welfare assurance (A5919-01) in compliance with the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). Permission for the generation of transgenic animals was provided by the authorities of the state of Niedersachsen (AZ 33.9-42502-04-08/1518). All animal work conducted for this study was approved by H. Hedrich, state head of the animal facility at Medizinische Hochschule Hannover and performed according to German legislation.

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.[15] Mice with *loxP* sites flanking the *Ctnnb1* locus from exon 2 to exon 6 (*Ctnnb1*$^{tm2Kem}$ *synonym: Ctnnb1*$^{fl}$) were obtained from Rolf Kemler (Max-Planck-Institute for Immunobiology and Epigenetics, Freiburg/Germany),[16] mice with *loxP* sites flanking exon 3 of the *Ctnnb1* locus (*Ctnnb1*$^{tm1Mmt}$, synonym: *Ctnnb1*$^{(Ex3)fl}$) were provided by Makoto Mark Taketo (Kyoto University, Kyoto/Japan).[17] Mice with *loxP* sites flanking exon 2 of the *Shh* locus (*Shh*$^{tm2Amc}$, synonym: *Shh*$^{fl}$),[18] mice with *loxP* sites flanking exon 4 of the *Fgfr1* locus (*Fgfr1*$^{tm5.1Sor}$, synonym: *Fgfr1*$^{fl}$),[19] mice with *loxP* sites flanking exons 7 to 10 of the *Fgfr2* locus (*Fgfr2*$^{tm1Dor}$, synonym: *Fgfr2*$^{fl}$),[20] mice with *loxP* sites flanking exon 1 of the *Smo* locus (*Smo*$^{tm2Amc}$, synonym: *Smo*$^{fl}$),[21] 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*$^{GOF}$),[22] mice with *loxP* sites flanking exons 1–4 of the *Pdgfra* locus (*Pdgfra*$^{tm8Sor}$, synonym: *Pdgfra*$^{fl}$),[23] and the double fluorescent cre reporter line (*Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$, synonym: *R26*$^{mTmG}$)[24] were all obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained on an outbred (NMRI) background.

NMRI wild-type embryos were used for expression analysis. 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 analysed. Timed pregnancies were obtained, by checking vaginal plugs in the morning after mating and noon was designated as embryonic day (E) 0.5. Female mice were sacrificed by cervical dislocation, uteri were harvested in PBS, and embryos decapitated. Tissues were fixed in 4% paraformaldehyde overnight and stored in 100% methanol at −20°C. Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR.

## 2.2 Histological analysis

For histological stainings, embryos were fixed as stated earlier, paraffin-embedded, and sectioned to 4 μm. Sections were stained with haematoxylin and eosin, following standard procedures.

## 2.3 Immunofluorescence

For immunofluorescence analysis, goat polyclonal antibody anti-Tbx18 (1:50, C-20, Santa Cruz), rabbit polyclonal antibody anti-GFP (1:200 sc-8334, Santa Cruz), mouse monoclonal antibody anti-GFP (1:200, #11814460001, Roche), rabbit polyclonal antibody anti-Tagln (1:200, ab14106-100, Abcam), rabbit polyclonal antibody anti-Ctnnb1 (1:400, C2206, Sigma-Aldrich), Fluorescein-labelled isolectin-B4 (1:100, FL-1101, VectorLabs), rabbit polyclonal anti-Postn (1:200, ab92460, Abcam), monoclonal antibody anti-Acta2, FITC-conjugate (1:200, F3777, Sigma), rabbit polyclonal antibody anti-Col type IV (1:200, AB756P, Millipore Corp.), rabbit polyclonal antibody anti-ZO1 (1:200, 61-7300, Zymed Laboratories Inc.), and rabbit polyclonal antibody anti-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 as stated earlier, 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.) and signals were 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.

## 2.4 *In situ* hybridization analysis

*In situ* hybridization analysis on paraffin sections with digoxigenin-labelled antisense riboprobes was performed as described.[25]

## 2.5 Proliferation assay

Cell proliferation was analysed by incorporation of 5-bromo-2-deoxyuridine (BrdU) on 5 μm sections of paraffin-embedded specimens similar to previously published protocols.[26] The quantification of proliferation in *Tbx18*$^{cre/+}$;*Ctnnb1*$^{(Ex3)fl/+}$, *Tbx18*$^{cre/+}$;*Smo*$^{GOF/+}$, and control wild-type littermates was performed with six sections each of four embryos of each genotype at E11.5. The BrdU-labelling 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.

## 2.6 Quantification of GFP$^+$ and Postn$^+$ cells and of the capillary density in the right ventricle

Three 4 μm paraffin sections of mutant and control hearts of three embryos each were stained for GFP, Postn, and IB4 and optical fields of a 20× magnification of the right ventricle were used to quantify the GFP$^+$, Postn$^+$, and DAPI$^+$ areas using the ImageJ (NIH) software.[27] The calculated relative area (GFP$^+$, Postn$^+$ vs. DAPI$^+$) in the corresponding control genotype was set to one and the normalized data expressed as mean ± SD. Capillaries were counted and depicted per cross-section heart area (mm$^{-2}$).

## 2.7 Epicardial cell culture

Explant cultures of primary epicardial cells were obtained as described before.[10] For the wound closure assay, the culture medium was supplemented with 10% FBS, and a scratch was made with a P10 tip after 4 days. Imaging

**Table 1 Comparison of mouse models used to elucidate the epicardial function of canonical Wnt-, Hh-, Fgfr1/2-, and Pdgfra-signalling**

| Floxed allele | Cre line | Phenotype | Background | Recombination score | Potential explanation | Ref. |
|---|---|---|---|---|---|---|
| *Ctnnb1*$^{tm2Kem}$ | *Tg(GATA5-cre) 1Krc* | Impaired EPDC migration Impaired coronary artery formation Impaired cSMC differentiation | nd | Absence of Ctnnb1 in the epicardium (IHC) | Recombination of *Gata5::cre* in CM, cushion tissue, and EC points to a requirement of Ctnnb1 signalling pathway in those tissues | 5 |
| | *Wt1*$^{tm2(cre/ERT2)Wtp}$ | Deficient epicardial EMT at E13.5 | Mixed | Absence of Ctnnb1 in the epicardium (IF) and *Wt1*$^{cre/ERT2}$;*R26*$^{mTmG}$; *Ctnnb1*$^{fl}$ fate at E13.5 | *Wt1*$^{cre/ERT2}$ recombines inefficiently in the epicardium leading to a variability in the fate mapping analysis, haploinsufficiency of Wt1 might interfere with the formation of EPDCs | 6 |
| | *Tbx18*$^{tm4(cre)Akis}$ | No developmental phenotype with respect to the right ventricle | Outbred (NMRI) | Absence of Ctnnb1 in the epicardium (IF) and *Tbx18*$^{cre}$;*R26*$^{mTmG}$; *Ctnnb1*$^{fl}$ fate at E9.5–18.5 | Ctnnb1 pathway is dispensable for developmental epicardial EMT and EPDC differentiation | This study |
| *Smo*$^{tm2Amc}$ | *Twist2*$^{tm1(cre)Dor}$ | Reduction of coronary artery formation at the early coronary plexus stage | C57/Bl6J | *Twist2*$^{cre}$; ROSA26R fate at E12.5 and P10 | *Twist2*$^{cre}$ line is additionally active in the cushion tissue; experiments may point to an endothelial/-cardial requirement of Hh-signalling | 9 |
| | *Tbx18*$^{tm4(cre)Akis}$ | No developmental phenotype with respect to the right ventricle | Outbred (NMRI) | *Tbx18*$^{cre}$;*R26*$^{mTmG}$;*Smo*$^{fl}$ fate at E14.5+ E18.5 | Hh pathway is dispensable for developmental epicardial EMT and EPDC differentiation | This study |
| *Fgfr1*$^{tm5.1Sor}$ *Fgfr2*$^{tm1Do}$ | *Wt1*$^{tm1(EGFP/cre)Wtp}$ | Reduced EPDC migration Reduced interstitial fibroblasts Reduced myocardial proliferation | nd | Reduced expression of FGFR2 in the epicardium (IHC) | Haploinsufficiency of Wt1 might have interfered with formation and migration of EPDCs and their detection via Wt1. Additional myocardial KO might interfere with myocardial proliferation | 8 |
| | *Tbx18*$^{tm4(cre)Akis}$ | No developmental phenotype with respect to the right ventricle | Outbred (NMRI) | *Tbx18*$^{cre}$;*R26*$^{mTmG}$;*Fgfr1*$^{fl}$;*Fgfr2*$^{fl}$ fate at E14.5 and E18.5 | Fgf pathway is dispensable for developmental epicardial EMT and EPDC differentiation | This study |
| *Pdgfra*$^{tm8Sor}$ | *Tg(GATA5-cre) 1Krc*, *Wt1*$^{tm2(cre/ERT2)Wtp}$ | Reduced epicardial EMT Reduced fibroblast differentiation | Mixed C57/ Bl6129SV | *Wt1*$^{cre/ERT2}$;R26R$^{tdT}$ fate at E14.5 | *Pdgfra* is required for EMT, and the differentiation of EPDCs in mature cardiac fibroblasts *Pdgfra* expression restricted to the epicardium | 7 |
| | *Tbx18*$^{tm4(cre)Akis}$ | Reduced fibroblast differentiation | Outbred (NMRI) | *Tbx18*$^{cre}$; *R26*$^{mTmG}$;*Pdgfra*$^{fl}$ fate at E14.5 and E18.5 | *Pdgfra* is required for the differentiation of EPDCs in mature cardiac fibroblasts | This study |

CM, cardiomyocyte; cSMC, coronary smooth muscle cell; E, embryonic day; EC, endothelial cell; EMT, epithelial–mesenchymal transition; EPDC, epicardium-derived cell; IF, immunofluorescence; IHC, immunohistochemistry; P, postnatal day.

was performed at 0 and 24 h, and wound closure was determined on bright field images in ImageJ[27] by dividing the scratch area at 0 h at 24 h.

### 2.8 Statistical analysis

Statistical analyses for BrdU incorporation, capillary density, and $GFP^+$ cells were performed using the two-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 $P < 0.001$. For statistics for individual experiments see Supplementary material online, *Table 1*.

### 2.9 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 5 × 5 single pictures, allowing 10% overlap of neighbouring pictures. Whole-mount specimens were photographed on Leica M420 with Fujix digital camera HC-300Z. All images were processed in ImageJ[27] and Adobe Photoshop CS4.

## 3. Results

### 3.1 *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. Expression in the latter, however, is weak and scattered.[10,28,29] 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, that lacks endogenous myocardial expression of *Tbx18*, EPDCs were shown to differentiate into interstitial and perivascular fibroblasts and coronary SMCs but not into endothelial cells and cardiomyocytes.[10] 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,[30] we re-evaluated 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 (see Supplementary material online, *Figure S1*). However, phenotypic analyses of (*Tbx18*$^{cre}$-mediated) conditionally mutant mice were restricted to the right ventricle throughout this study to account for the endogenous myocardial expression of *Tbx18* in the left ventricle but the lack thereof in the right ventricle.

### 3.2 Canonical Wnt-signalling is dispensable for epicardium and EPDC development

To analyse canonical Wnt-signalling in epicardial development, we investigated the expression of *Axin2*, a bona fide transcriptional target of this pathway,[31] by *in situ* hybridization on sections of E9.5–E14.5 embryonic hearts (see Supplementary material online, *Figure 2*). 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-signalling in epicardial cells, we used our *Tbx18*$^{cre}$ line and a floxed allele of *Ctnnb1* (*Ctnnb1*$^{fl}$),[16] the unique intracellular mediator of the canonical sub-branch of Wnt-signalling, to test for a functional requirement of this pathway in epicardial development. Immunofluorescence analysis on the sections of hearts of E9.5 and E14.5 *Tbx18*$^{cre/+}$;*Ctnnb1*$^{fl/fl}$;*Rosa26*$^{mTmG/+}$ embryos revealed the 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-signalling in all epicardial cells (see Supplementary material online, *Figure S3*).

*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 analysed by immunofluorescence 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)], and of fibroblasts [periostin (Postn)]. Stainings for all three markers and quantification of $Postn^+$ and $IB4^+$ cells in the right ventricle did not reveal differences between mutant and wild-type hearts, indicating that the coronary vasculature and the fibrous skeleton were not affected by conditional deletion of *Ctnnb1*. Finally, immunofluorescent detection and subsequent quantification of the *Tbx18*$^{cre}$ 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*, see Supplementary material online, *Figure S4*).

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 4 days in serum-free medium, wild-type 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. Irrespective of the genotype, addition of 10% FCS to the medium resulted in the loss of Tjp1 and Wt1, and formation of Acta2-positive stress fibres, consistent with EMT and differentiation into SMCs (see Supplementary material online, *Figure S5*). These results demonstrate that the *Tbx18*$^{cre}$-mediated loss of *Ctnnb1* does not affect the formation of the epicardium, EMT or differentiation of EPDCs *in vitro* and *in vivo*.

### 3.3 Epicardial expression of a stabilized version of Ctnnb1 leads to the formation of cell clusters

To further clarify the role of *Ctnnb1*-dependent Wnt-signalling in epicardial development, we used a gain-of-function approach with conditional (*Tbx18*$^{cre}$-mediated) overexpression of a stabilized form of *Ctnnb1* (*Ctnnb1*$^{(Ex3)fl}$).[17] *Tbx18*$^{cre/+}$;*Ctnnb1*$^{(Ex3)fl/+}$ embryos died at E12.5 probably due to cardiovascular insufficiency as shown by the formation of

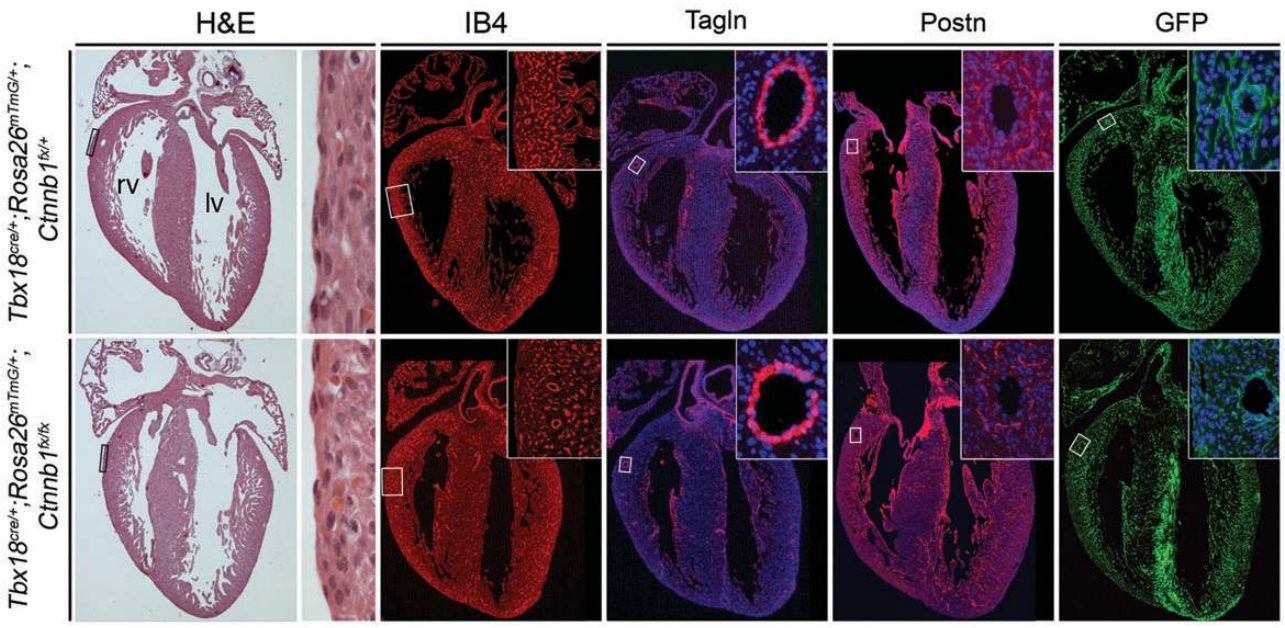


**Figure 1** Epicardial loss of *Ctnnb1* does not lead to defects in the coronary vasculature, the ventricular myocardium, or in epicardial derivatives. Histological analysis by haematoxylin and eosin staining (H&E) and immunofluorescence analysis of IB4, Tagln, Postn, and the cell-lineage marker GFP 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. For statistics for this and all other experiments see Supplementary material online, *Table 1*.

oedema. 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*). Analysis of individual clusters by immunofluorescence for GFP proved the epicardial origin of these clusters, which expressed as expected high levels of *Axin2* (*Figure 2C* and *D*), indicating active Ctnnb1-dependent Wnt-signalling. *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 ($44 \pm 7\%$) and the $Axin2^{+}$ cell clusters ($47 \pm 6\%$) in the mutant compared with the control heart ($22 \pm 5\%$) (*Figure 2I*, see Supplementary material online, *Figure S6*). Hence, epicardium-specific expression of a stabilized form of Ctnnb1 results in the hyperproliferation of epicardial cells and formation of cellular aggregates of fibroblast-like cells indicating that active canonical Wnt-signalling is deleterious for epicardial development.

## 3.4 Epicardial Hh-signalling is dispensable for epicardial development

To determine the presence of Hh-signalling in the developing epicardium, we investigated the expression of the three ligand genes *Shh*, *Dhh*, and *Ihh* and of *patched homolog1 (Ptch1)*, a well-established transcriptional target and a repressor of Hh-signalling,[32] by *in situ* hybridization on sections of E9.5–E14.5 embryonic hearts. Expression of *Shh*, *Dhh, Ihh*, and *Ptch1* was neither detected in the E9.5 proepicardium nor in the epicardium at subsequent stages although other sites of expression were clearly visible (see Supplementary material online, *Figure S7* and *S8*). To test for a functional requirement of this pathway in epicardial development, we used our $Tbx18^{cre}$ line and a floxed allele of *smoothened* ($Smo^{fl}$)[21] which encodes a unique intracellular transducer of Hh-signalling. $Tbx18^{cre/+};Smo^{fl/fl};Rosa26^{mTmG/+}$ 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[33] was normal and $Tagln^{+}$ 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 $Tbx18^{cre}$ 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*, see Supplementary material online, *Figure S9*).

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

To investigate the consequences of epicardial activation of Hh-signalling, we (mis-)expressed a constitutively active form of Smo from

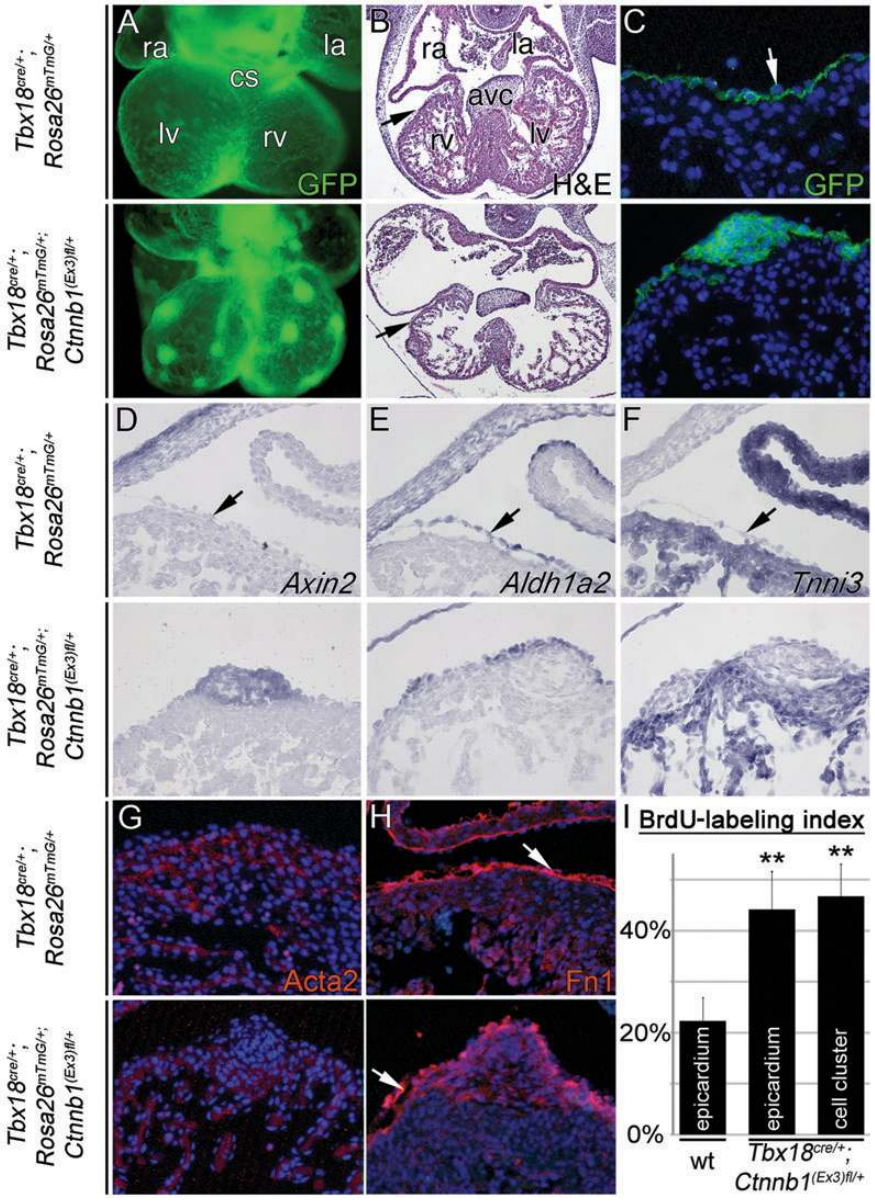


**Figure 2** Epicardial expression of a stabilized version of Ctnnb1 induces formation of large cell aggregates on the surface of E11.5 hearts. (*A*) GFP epifluorescence in whole hearts in a dorsal view, (*B*–*H*) analysis of mid-transverse heart sections by haematoxylin and eosin (H&E) staining (*B*), by immunofluorescence of GFP (*C*), by *in situ* hybridization analysis for expression of *Axin2*, *Aldh1a2*, and *Tnni3* (*D*–*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-labelling index) of the epicardium and the cell clusters in genotypes as shown.

the *Rosa26* locus (*Smo*$^{GOF}$)[22] using a *Tbx18*$^{cre}$-mediated approach. *Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Smo*$^{GOF/+}$ embryos died after E13.5 showing severe oedema 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. IB4 expression in the endocardium appeared increased possibly due to endocardial thickening (*Figure 4C*). Immunofluorescent detection of the lineage marker GFP and the epicardial marker Wt1 revealed an accumulation of epicardial cells and/or EPDCs and a severe reduction of EMT and of immigration into the myocardium (*Figure 4D –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, *Tbx18*$^{cre}$-mediated expression of a constitutive active form of Smo results in an increase in epicardial cell density, lack of epicardial EMT, and disturbed epicardial-myocardial/endocardial signalling.

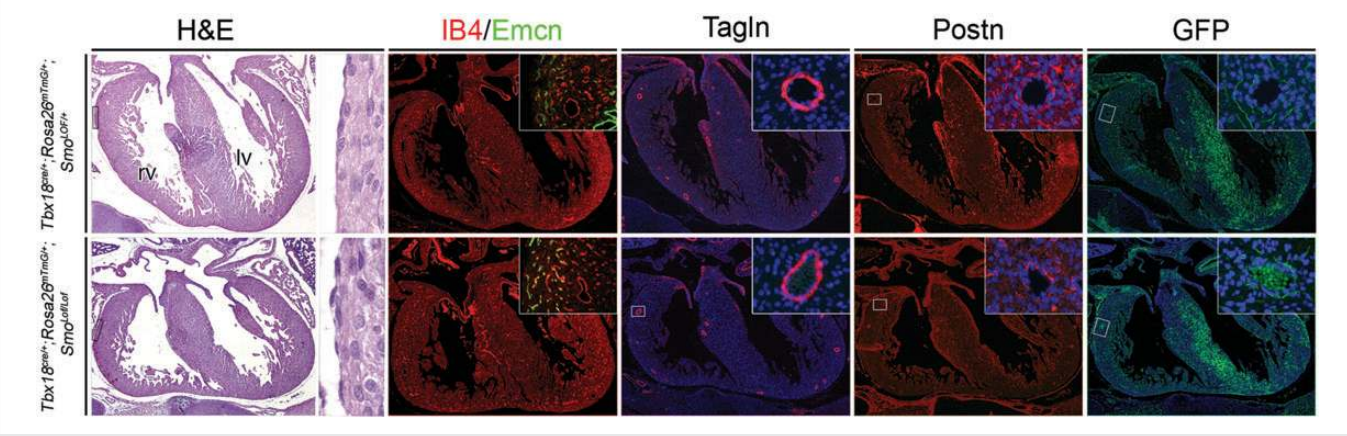


**Figure 3** Epicardial loss of *Smo* does not lead to defects in the coronary vasculature, the ventricular myocardium, or in epicardial derivatives. Histological analysis by haematoxylin and eosin staining (H&E) and immunofluorescence analysis for IB4/Emcn, Tagln, Postn, and the cell-lineage marker 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.

## 3.6 Epicardial Fgfr1/Fgfr2-signalling is not required for heart development

Epicardial Fgfr1/Fgfr2-signalling was recently proposed to induce EPDC migration and fibroblast formation.[8] However, our *in situ* hybridization analysis did not detect (pro-)epicardial expression of *Etv4*, a bona fide transcriptional target of the Fgf-signalling pathway,[34] on sections of E9.5–E14.5 embryonic hearts (see Supplementary material online, *Figure 10*). Moreover, embryos with $Tbx18^{cre}$-mediated epicardial deletion of *Fgfr1* and *Fgfr2* [19,20] ($Tbx18^{cre/+}$;$Fgfr1^{fl/fl}$;$Fgfr2^{fl/fl}$;$R26^{mTmG/+}$) survived embryogenesis and presented histologically normal hearts. In E18.5 hearts, the networks of coronary endothelial cells (IB4) and of fibroblasts (Postn) were neither qualitatively nor quantitatively affected; SMCs (Tagln) of coronary arteries were unaltered. The distribution of the epicardial lineage tracer GFP in the right ventricular myocardium was unchanged both at E14.5 and at E18.5 (*Figure 5*, see Supplementary material online, *Figure S11*). Together, these findings suggest that Fgfr1/Fgfr2-signalling in the $Tbx18^{cre}$ lineage is not required for the mobilization and differentiation of epicardial cells.

## 3.7 Epicardial *Pdgfra* is important for cardiac fibroblast differentiation

Finally, we analysed the role of Pdgfra-signalling[23] 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. However, expression of Postn was dramatically reduced and $GFP^+$ cells (from the lineage reporter $Rosa26^{mTmG}$) showed a morphological change from slender, longitudinal to a shortened, circular phenotype throughout the ventricles (*Figure 6*). The $GFP^+$ area in the right ventricular myocardium was significantly and highly significantly reduced at E14.5 and E18.5, respectively. Quantification confirmed highly significant reduction of Postn expression, whereas $IB4^+$ cells were unaffected in number at E18.5 (see Supplementary material online, *Figure S12*). To distinguish if the reduction of subepicardial GFP expression reflects a defect in EPDC formation/migration or derives from a subsequent defect in EPDC differentiation, we analysed primary epicardial cell cultures from explants of right ventricles of E11.5 hearts. In FCS-treated explant cultures, a 'wound' in the monolayer was reduced by ~50% after 24 h and was completely closed after 72 h, irrespective of the genotype (see Supplementary material online, *Figure S13*) indicating that differentiation but not formation and migration of EPDCs may be primarily affected in *Pdgfra*-deficient epicardial cells.

# 4. Discussion

The embryonic epicardium is a crucial source of cells for the underlying myocardium and the coronary vasculature. Here, we have provided genetic data that suggest that mobilization and differentiation of epicardial cells does not depend on Wnt-, Fgfr1/2-, and Hh/Smo-signalling in the $Tbx18^{cre}$ lineage but that differentiation of EPDCs into fibroblasts requires the Pdgfra-signalling pathway. Discrepancies between our results and that of earlier reports may rely on the usage of different cre lines, different genetic backgrounds, and/or assay systems (*Table 1*).

## 4.1 Canonical Wnt-signalling is deleterious for epicardial development

Canonical Wnt-signalling has been shown to regulate proliferation and differentiation of progenitor cells in various developmental contexts including myocardial progenitors in the heart.[35] A requirement in epicardial mobilization and differentiation was suggested by two independent studies. First, reduced EMT and a failure of coronary SMC differentiation was reported upon conditional deletion of *Ctnnb1* using the transgenic *Gata5::cre* line.[5] Secondly, $Wt1^{creERT2}$-mediated deletion of *Ctnnb1* resulted in a complete absence of epicardial EMT.[6] 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* suggests that this pathway is not involved in epicardial EMT and SMC differentiation but that an epicardial activity may actually be deleterious for cardiac development. These divergent findings may stem from the

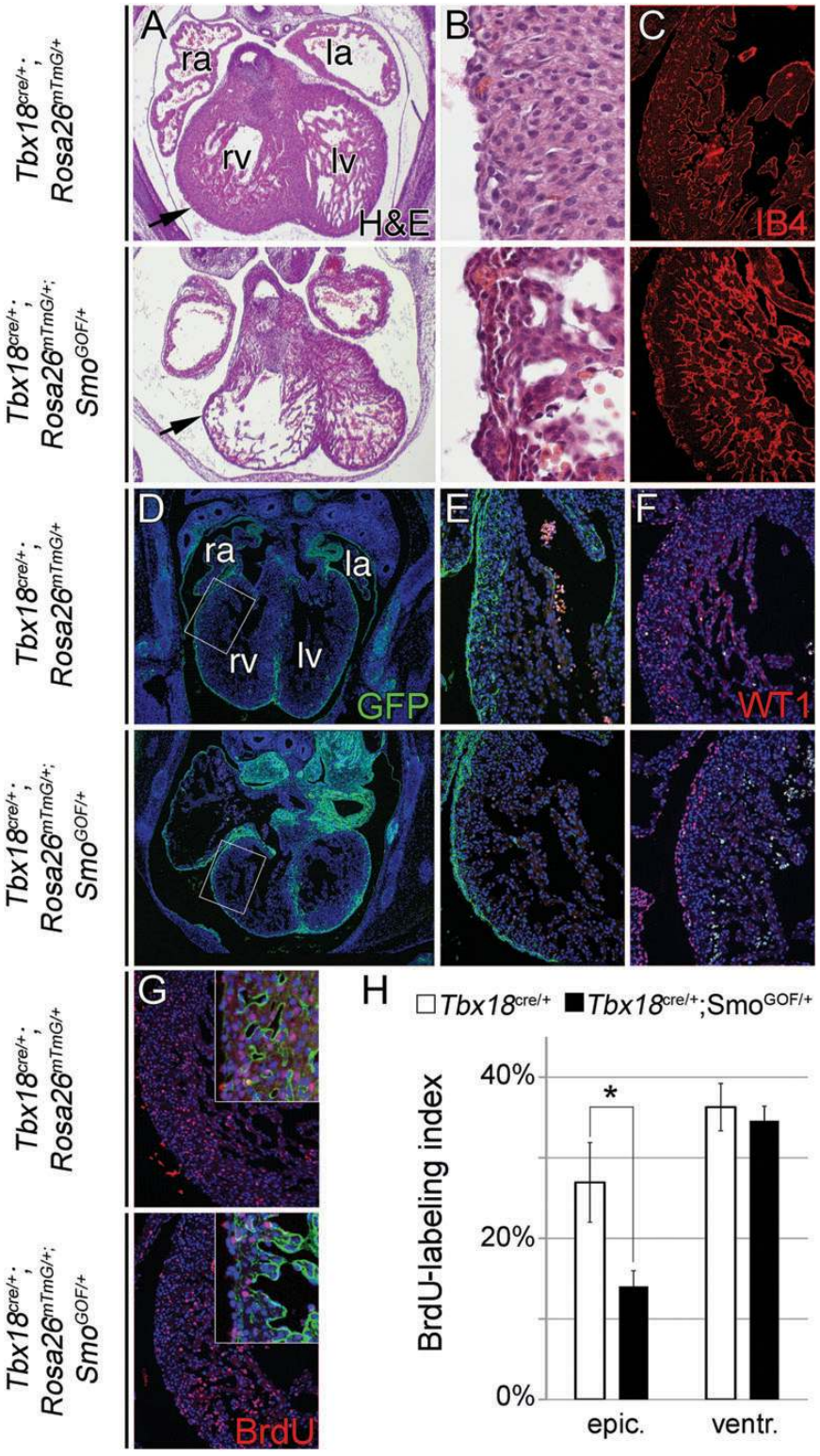


**Figure 4** Expression of a constitutive active form of Smo affects epicardial and myocardial integrity in E13.5 hearts. (*A*–*G*) Mid-transverse sections of E13.5 hearts of control (*Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$) and mutant (*Tbx18*$^{cre/+}$;*Rosa26*$^{mTmG/+}$;*Smo*$^{GOF/+}$) embryos were analysed by haematoxylin 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*), magnified insets show Col4 in green. (*A* and *D*) show whole hearts, (*B* and *C*, *E*–*G*) magnified regions of the right ventricle. (*H*) Proliferation rates (% BrdU labelling index) of the epicardium in genotypes and stages as shown. la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle.

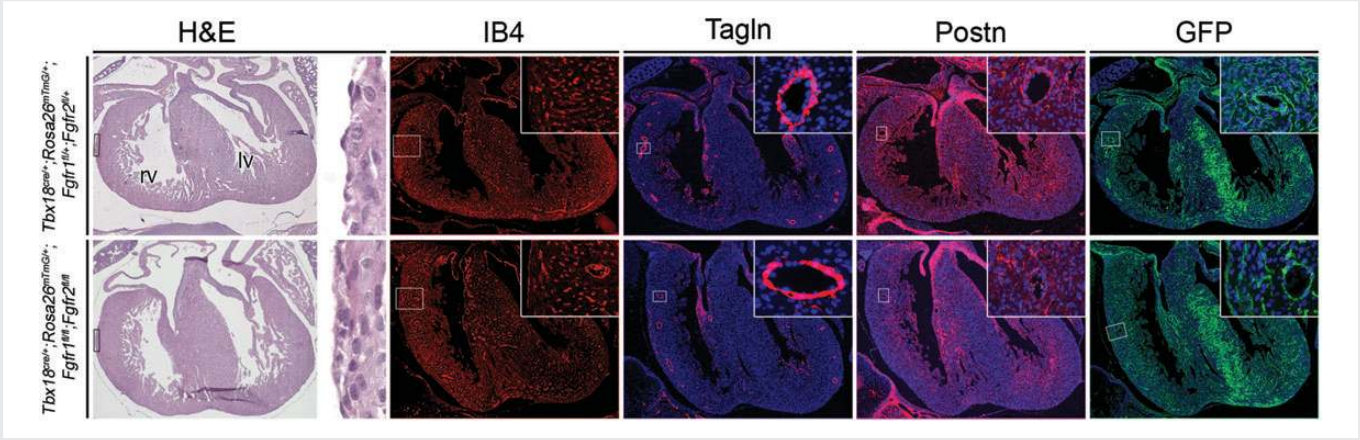


**Figure 5** Combined epicardial loss of *Fgfr1* and *Fgfr2* does not affect the integrity of the coronary vasculature, the ventricular myocardium, or epicardial derivatives. Histological analysis by haematoxylin and eosin staining (HE) and immunofluorescence analysis for IB4, Tagln, Postn, and the cell-lineage 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.

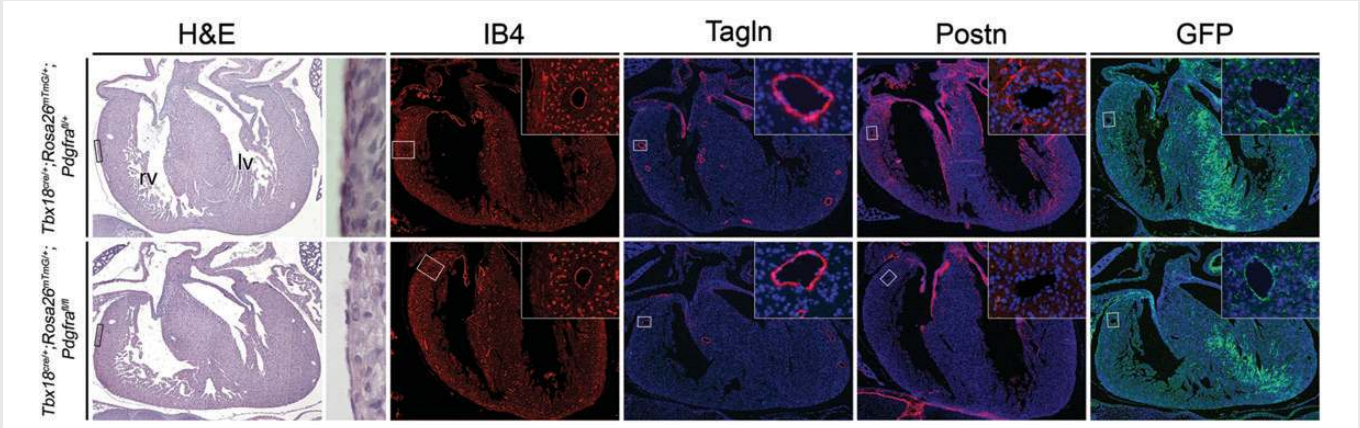


**Figure 6** Epicardial *Pdgfra* is required for cardiac fibroblast differentiation. Histological analysis by haematoxylin and eosin staining (H&E) and immunofluorescence analysis for IB4, Tagln, Postn, and the cell-lineage marker GFP on mid-transverse sections of E18.5 *Tbx18*$^{cre/+}$;*Pdgfra*$^{fl/+}$;*Rosa26*$^{mTmG/+}$ (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.

different cre lines used for conditional targeting of *Ctnnb1*. In fact, 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,[13] suggesting that epicardial defects may be secondary to changes in these cell types. On the other hand, defects in epicardial EMT as observed with the *Wt1*$^{creERT2}$ line may relate to the variability and incompleteness of epicardial recombination.[14,6] In contrast, the *Tbx18*$^{cre}$ line used in our experiments mediates robust and complete recombination in epicardial cells and seems to be a reliable tool for phenotypic analysis in the right ventricle that lacks any myocardial/endocardial expression of Tbx18. Virally induced epicardial overexpression of a stabilized form of Ctnnb1 was previously reported to lead to increased epicardial EMT.[36] We did not detect epicardial EMT in *Tbx18*$^{cre/+}$;*Ctnnb1*$^{ex3/+}$ mice but observed epicardial clustering instead. Lethality around E12.5 prevented to analyse epicardial EMT at later time-points. Both experimental gain-of-function set-ups are very different making a direct comparison difficult. As in the loss-of-function case, independent efforts with additional epicardium-specific cre lines may clarify the findings.

## 4.2 Hh-signalling is deleterious for epicardial development

The Hh-signalling pathway has been shown to be important for vasculogenesis in adult and embryonic development.[37] 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 of whole hearts 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.[9,38,39] Our investigations on the expression of the Hh-pathway target gene (*Ptch1*) on cardiac sections revealed no presence in the developing epicardium. Further, our conditional loss- and gain-of-function mutants of the Hh-signalling pathway did not reveal a functional relevance for this pathway in the epicardium but indicated deleterious

consequences upon cardiac development once epicardially activated. Since the previously used *Dermo1*$^{cre}$ line is additionally active in cushion tissue,[40] these experiments may point to an endothelial/endocardial requirement of Hh-signalling. Furthermore, analysis of embryos at stages of early coronary plexus outgrowth rather that around birth when the coronary system is well established may have detected a defect that may have been transient in nature.

### 4.3 Fgfr1/Fgfr2-signalling in epicardial development

Fgf-signals have been characterized as important paracrine regulators of proliferation, migration, and differentiation of numerous cell types.[41] A crucial function Fgf-signalling in epicardial development *in vivo* was suggested by a conditional deletion of *Fgfr1* and *Fgfr2* using the *Wt1*$^{cre}$ line.[8] In that study, the authors observed reduced EPDC migration and formation of interstitial fibroblasts. Our analysis of expression of the general Fgf target gene *Etv4* does not support the notion that epicardial cells display active Fgf-signalling. Furthermore, combined (*Tbx18*$^{cre}$-mediated) loss of *Fgfr1* and *Fgfr2* was without phenotypic consequences for epicardial and myocardial development. It is conceivable that haploinsufficiency of *Wt1* has interfered with formation and migration of EPDCs and has reduced the sensitivity of Wt1 detection that was used as an EPDC marker in the previous study.[4,8]

### 4.4 Epicardial *Pdgfra* 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.[42] Reports on a functional involvement of Pdgfra-signalling in epicardial development were based on the results from systemic and conditional deletion of *Pdgfra* using the *Wt1*$^{creERT2}$ line.[7] Our conditional (*Tbx18*$^{cre}$-mediated) deletion experiment confirms that *Pdgfra* is required for the differentiation of EPDCs in mature cardiac fibroblasts.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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