Tbx18 function in epicardial development

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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 molecular circuits that direct the temporal and spatial generation of these cell types from epicardium-derived cells are only partly known. We aimed to elucidate the functional significance of the conserved epicardial expression of the T-box transcription factor gene Tbx18 using transgenic technology in the mouse.

Methods and results
We show by cellular and molecular analyses that in Tbx18-deficient mice the epicardium is formed normally and that epicardial cells undergo an epithelial–mesenchymal transition, differentiate into smooth muscle cells and fibroblasts, and form a normal coronary vasculature and fibrous skeleton. Prolonged expression of Tbx18 in epicardium-derived cells by a transgenic approach in vivo does not affect the differentiation and migratory behaviour of these cells. In contrast, epicardial misexpression of a transcriptional activator version of Tbx18, Tbx18VP16, results in premature smooth muscle differentiation of epicardial cells. Inhibition of Notch and transforming growth factor beta receptor signalling in Tbx18VP16 expressing epicardial cells in explant cultures reverts this phenotype.

Conclusion
Tbx18 is dispensable for epicardial development, yet a repressive T-box function may be required to prevent premature smooth muscle cell differentiation by repressing transforming growth factor beta receptor and Notch signalling in the embryonic epicardium.

Keywords
Epicardium • Tbx18 • Smooth muscle cell differentiation • Notch • Tgfrbr1

1. Introduction
The epicardium, the epithelial monolayer that covers the cardiac muscle, plays a pivotal role both during development and disease as a source of signals and cells for the underlying myocardium and the coronary vasculature. The epicardium arises from an extracardiac cell population at the venous pole of the developing heart, the proepicardium. Proepicardial cells leave the cluster and settle on the overlying myocardium starting at around embryonic day (E) 9.5 in the mouse. Shortly thereafter, at E10.5, these cell clusters spread out and form 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 A contribution to coronary endothelia and cardiomyocytes can be reported in some lineage tracing studies in the mouse but was not confirmed in other studies nor found in other vertebrates.2–7 Intriguingly, under myocardial injury conditions, the adult epicardium reactivates an embryonic gene program to support myocardial healing (in zebrafish) and revascularization and scar formation (in the mouse).8,9

A number of signalling pathways have been described to regulate epicardial EMT and differentiation. Transforming growth factor beta receptor (Tgfrbr) and platelet-derived growth factor receptor alpha (Pdgfra) signalling have been implicated in epicardial EMT, canonical Wnt-, Notch- and platelet-derived growth factor receptor beta (Pdgfrb) signalling in differentiation of EPDCs into SMCs, and Pdgfra signalling in fibroblast differentiation.10–13 To date, we have no knowledge about how these signalling pathways interact, and how they are regulated in time and space to precisely balance epicardial precursor proliferation and differentiation.

T-box 18 (Tbx18), a member of the conserved family of T-box transcription factors, is expressed in the proepicardium, the epicardium, the interventricular septum, the left-ventricular myocardium, and the sinus horns (the intrapericardial myocardialized aspects of the cardinal veins) in the developing murine heart.4,14,15 In the latter region, Tbx18 is required to maintain the mesenchymal precursor pool for sinus horn formation.15 Although epicardial expression of Tbx18 is conserved from zebrafish to man,16–18 a requirement of the gene for the development of this tissue has not yet been analysed.
Here, we address the epidermal function of Tbx18 by combining several genetic approaches. We show that Tbx18 is dispensable for epidermal development, but that a transcriptional activator version of the Tbx18 protein induces epidermal EMT and SMC differentiation by interfering with Tgfrbr and Notch signalling.

2. Methods

2.1 Mice and genotyping

Hannover Medical School is a holder of a PHS approved animal welfare assurance (AS919-01) 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 German legislation.

Transgenic mice harbouring a knock-in of the lacZ gene in the Tbx18 locus (Tbx18lacZ, synonym: Tbx18tm1Tkac), were mated with a mouse with an insertion of the cre recombinase gene in the Tbx18 locus (Tbx18tm1Rb26R2),24 mice carrying a null allele of Rbpj (Rbpjtm1.1Her, synonym: RbpjK),26 and the fluorescent reporter line (Gt(Rosa)26SorCre/ACTB-tdTomato,Egfp,LSL-Cre),27 were all described before. For the production of a conditional misexpression allele of Tbx18 and Tbx18VP16, a ‘knock-in’ strategy into the X-chromosomal Hypoxanthine guanine phosphoribosyl transferase (Hprt) gene locus was employed.23 Construction of the targeting vector, ES cell work, and generation of chimeras followed exactly the procedure of the Rbpj mutant.24

Embryos were harvested in PBS, decapitated, fixed in 4% paraformaldehyde overnight, and stored in 100% methanol at −20°C before further use. Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR (see Supplementary material online for protocols).

2.2 Histological analysis

Embryos were fixed as mentioned earlier, embedded in paraffin, and sectioned to 5 μm for histological stainings. Sections were stained with haematoxylin and eosin following standard procedures. At least three embryos of each genotype were used for each analysis.

2.3 In situ hybridization analysis

Non-radioactive in situ hybridization analysis with digoxigenin-labelled antisense riboprobes was performed as described.24 At least three embryos of each genotype were used for each analysis. Details of used probes upon request.

2.4 Immunohistochemistry, proliferation, and TUNEL assays

Cell proliferation was investigated by the BrdU incorporation assay. Briefly, pregnant mice were injected with 10 μL of 10 mg/mL BrdU (#10280879001, Roche) per gram body weight and sacrificed after 1 h. Embryos were harvested as described earlier. Five-micrometre paraffin sections were stained with a mouse monoclonal anti-BrdU antibody (1:200, 1170376, Roche) using the M.O.M. Basic kit as outlined by the manufacturer (BMK-2202, Vector Laboratories). Wild-type littersmates were used as controls.

The evaluation of apoptotic cells was performed on 5 μm paraffin sections using the ApopTag Plus fluorescein in situ apoptosis detection kit (S7111, Millipore).

For immunohistochemistry, 5 μm sections of paraffin-embedded embryos were stained with rabbit polyclonal anti-Tagln (1:300, ab14106, Abcam), with rabbit polyclonal anti-Notch3 (1:300, ab23426, Abcam), with goat polyclonal anti-Myh11 (1:200, a kind gift of R. Adelstein, Bethesda, USA), with mouse monoclonal anti-GFP (1:200, 1181446001, Roche), with rabbit polyclonal anti-GFP (1:300, sc-8334, Santa Cruz), with goat polyclonal anti-Tbx18 (1:50, C-20, Santa Cruz), with FITC-conjugated mouse monoclonal anti-Acta2 (1:200, F3777, Sigma), with rabbit polyclonal anti-Postn (1:300, ab14041, Abcam), with goat polyclonal anti-Tnn3 (1:300, 47212, HyTest), with rabbit polyclonal anti-Wt1 (1:400, C-19, Santa Cruz), or with FITC-conjugated isocitrate dehydrogenase (B4; 1:500, FL-1101, VectorLabs) as well as secondary antibodies. Nuclei were stained with 4,6-diamidino-2-phenylindol (DAPI) (Roche). For antibodies against Tbx18, Notch3, Tagln, Myh11, GFP (rabbit IgG), Postn, Tnn3, and Wt1, the signal was amplified by the use of the tyramide signal amplification kit (NEL702001KT, Perkin Elmer) as described in the manufacturer’s manual. Before staining, paraffin sections were deparaffinized and pressure-cooked for 3 min in antigen unmasking solution (H-3300, Vector Laboratories Inc.). For double staining with GFP, the second antibody was added after the staining for the first one. At least three embryos of each genotype were used for each analysis. For the statistical evaluation, three section planes in at least three embryos were counted. The mean value and standard variation was determined. The unpaired two-tailed Student’s t-test was performed to determine the statistical significance.

2.5 Epidermal explant cultures

Primary epidermal explant cultures were obtained as described before.5 For the inhibitor studies, 2.5 μM SB431542 (Sigma), a compound antagonising type I receptors of the Tgfb superfamily (Alk4, Alk5, Alk7),28 was added to the medium and replaced after 2 days as well. After 4 days, the epidermal explants were washed in PBS, fixed in 5 min in 100% methanol at −20°C, and stained for Tip1 (1:300, rabbit polyclonal anti-Tip1, 61-7300, Zymed Laboratories Inc.), Acta2 (1:200, FITC-labelled mouse monoclonal anti-Acta2, F3777, Sigma), and Tagln (1:200, rabbit polyclonal anti-Tagln, ab14106, Abcam). The Tip1 staining was amplified using the tyramide signal amplification kit (NEL702001KT, Perkin Elmer). Biotinylated goat anti-rabbit IgG (1:250, Dianova), biotinylated donkey anti-goat IgG (1:250, Dianova), biotinylated goat anti-mouse IgG (1:250, Dianova), and Alexa488-labelled donkey anti-mouse IgG (1:250, Invitrogen) were used as secondary antibodies. Nuclei were stained with 4,6-diamidino-2-phenylindol (DAPI) (Roeh). For antibodies against Tbx18, Notch3, Tagln, Myh11, GFP (rabbit IgG), Postn, Tnn3, and Wt1, the signal was amplified by the use of the tyramide signal amplification kit (NEL702001KT, Perkin Elmer) as described in the manufacturer’s manual. Before staining, paraffin sections were deparaffinized and pressure-cooked for 3 min in antigen unmasking solution (H-3300, Vector Laboratories Inc.). For double staining with GFP, the second antibody was added after the staining for the first one. At least three embryos of each genotype were used for each analysis. For the statistical evaluation, three section planes in at least three embryos were counted. The mean value and standard variation was determined. The unpaired two-tailed Student’s t-test was performed to determine the statistical significance.

2.6 Image analysis

Sections were photographed using a Leica DMS5000 microscope with Leica DFC300FX digital camera and afterwards processed in Adobe Photoshop CS3. Quantification of migration and proliferation assays was performed using ImageJ and the Cellcounter plugin (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/, 1997–2011).
3. Results

3.1 Tbx18 is dispensable for epicardial development

To address the requirement of Tbx18 in epicardial development, we analysed mice homozygous for a null allele of Tbx18 (Tbx18<sup>−/−</sup> or Tbx18<sup>−/−<m>cre</m></sup>, see Supplementary material online, Figure S1). Tbx18<sup>−/−<m>cre</m></sup> (Tbx18KO) mice maintained on an outbred genetic background died shortly after birth due to skeletal defects and respiratory distress. Morphological inspection did not reveal gross-morphological differences between ventricles of Tbx18KO and wild-type hearts and the associated coronary vasculature at E18.5 (Figure 1A and A'). On histological sections, the thickness of the compact and trabeculated myocardium, and the localization and number of coronary vessels in superficial and deeper locations of the myocardium were not significantly affected in the mutant (Figure 1B and B', see Supplementary material online, Figure S2). The mutant epicardium was tightly attached to the surface of the heart and expressed the epicardial marker gene Wilms tumor 1 (Wt1) as in the wild-type (Figure 1C and C, D and D'). We traced EPDC formation and localization by using the R26<sup>emG<sub>Tm</sub></sup> reporter mouse line in combination with the Tbx18<sup>−/−</sup> driver line. After cre-mediated recombination, a membrane-bound form of EGFP is expressed that can be visualized at the subcellular level by anti-GFP immunofluorescence. Due to endogenous expression of Tbx18 in the myocardium of the interventricular septum and the left ventricle, we restricted our lineage analysis to the right ventricle. Analysis of reporter gene expression in Tbx18KO hearts revealed normal formation and migration of EPDCs into the myocardium where they intermingled with cardiomyocytes and surrounded coronary vessels (Figure 1E and F). Expression of the SMC marker transgelin (Tagln) and actin, alpha 2, smooth muscle, aorta (Acta2) was found in deeper vessels (Figure 1F and F', G and G'), whereas the fibroblast marker periostin (Postn) was expressed in interstitial cells as well as in a perivascular position both in wild-type and in Tbx18KO hearts (Figure 1H and H'). Co-staining for the lineage marker GFP confirmed the epicardial origin of both SMCs and fibroblasts (see Supplementary material online, Figure S3). Endothelial cells and cardiomyocytes, as visualized by isolectin B4 (IB4) staining against carbohydrates specifically found on non-primate endothelial cells and expression of cardiac troponin I (Tnnt3), respectively, were not positive for GFP indicating that they do not derive from EPDCs in either genotype (see Supplementary material online, Figure S3). Thus, loss of Tbx18 does neither affect epicardial integrity and EMT nor the subsequent differentiation of EPDCs.

3.2 EPDC fate is independent from Tbx18

Tbx18 expression is found in the pro-epicardium and the epicardium, but lost upon formation of EPDCs. We addressed the significance of this down-regulation by a conditional Cre/loxP-based transgenic approach to maintain Tbx18 expression in EPDCs in vivo. For this purpose, we generated an Hprt<sup>Tbx18<sub>−/−</sub></sup> allele by integration of a bicistronic transgene-cassette containing the mouse Tbx18 open-reading frame followed by IRES-GFP in the ubiquitously expressed X-chromosomal Hypoxanthine guanine phosphoribosyl transferase (Hprt) locus. Transgene expression was driven in the epicardium and its descendants by the Tbx18<sup>−/−</sup> line (see Supplementary material online, Figure S1). Due to random X-chromosome inactivation, female embryos possessed a mosaic expression. Male embryos expressed the transgene in a uniform manner and were subsequently used for phenotypic analysis. Expression of Tbx18 in EPDCs of these Tbx18<sup>cre<sup>−/−</sup></sup>−<sup>Hprt<sub>Tbx18</sub>+/y</sup> mice (referred to as epiTbx18) was confirmed by anti-Tbx18 immunofluorescence (see Supplementary material online, Figure S4). Tbx18<sup>cre<sup>−/−</sup></sup>−<sup>Hprt<sub>Tbx18</sub>+/y</sup> mice were present in the expected Mendelian ratio at E18.5 but were not recovered after birth. The reason for this post-natal lethality is unclear but could relate to over-expression of Tbx18 at extracardiac sites, most notably the vertebral column. Morphologically, no differences between epiTbx18 and wild-type hearts were detected at E18.5 (Figure 2A and A'). On histological sections, the myocardium possessed a normal thickness and coronary vessels were formed as in wild-type (Figure 2B and B', see Supplementary material online, Figure S2); the epicardium presented as a thin epithelial layer and expressed the marker gene Wt1 as in the wild-type (Figure 2C and C', D and D'). In lineage tracing experiments using the R26<sup>emG<sub>Tm</sub></sup> reporter mouse line, we confirmed the formation of EPDCs, their migration into the myocardium and differentiation into coronary Tagln- and Acta2-positive SMCs as well as Postn-positive cardiac fibroblasts but not into coronary endothelial cells and cardiomyocytes in right ventricles of epiTbx18 hearts indistinguishable from the situation in the wild-type (Figure 2E–H and E'–H', see Supplementary material online, Figure S3). As the loss of Tbx18 in the epicardium...
Figure 1. Epicardial expression of Tbx18VP16 affects myocardial and epicardial integrity at E14.5. (A, B, A’, and B’) Analysis of heart morphology (A and A’) and of histology of transverse heart sections by haematoxylin and eosin (HE) staining (B, C, B’, and C) do not reveal phenotypic changes in the myocardium (B and B’) and epicardial tissue layers (C and C) in E18.5 epiTbx18 (Tbx18VP16; HprtTbx18/y; Rosa26tmTmG+/N2cre) embryos. (D–H, D’–H’) Immunofluorescence analysis of the epicardial marker Wt1 (D and D’), of the reporter GFP (E and E’), of the SMC proteins Tagln (F and F’) and Acta2 (G and G’), and of the fibroblast marker Postn (H and H’) shows that epicardial identity is established, and formation and differentiation of EPDCs into SMCs and fibroblasts occurs normally in E18.5 epiTbx18 hearts. (I–K) Immunofluorescence analysis of GFP expression does not detect changes in epicardial EMT and EPDC formation in Tbx18KO and epiTbx18 hearts at E13.5. (I, J) Quantitative analysis of EPDC formation by counting GFP-positive cells invading the right ventricle (subepicardial and intermyocardial) at E12.5, E13.5, E14.5, and E18.5 does not reveal significant changes between wild-type (E12.5: 43 ± 23; E13.5: 113 ± 25; E14.5: 213 ± 40; E18.5: 350 ± 21), Tbx18KO (E12.5: 37 ± 24; E13.5: 116 ± 29; E14.5: 189 ± 51; E18.5: 297 ± 53), and epiTbx18 (E12.5: 57 ± 11; E13.5: 121 ± 20; E14.5: 181 ± 41; E18.5: 381 ± 61) hearts. Three embryos per genotype and age were counted. The standard deviation is displayed as error bars. Abbreviations are as in Figure 1. Scale bars are 500 μm in B and B’, 20 μm in C–H, C’–H’, and 50 μm in I–K.

3.3 An activating form of Tbx18 induces premature SMC differentiation in epicardial cells

T-box proteins encode transcription factors that are characterized by a highly conserved DNA-binding motif, the T-box. Although differences in binding site specificity may exist, most if not all T-box proteins recognize a common DNA-motif, the T-box element. Co-expression of closely related family members may therefore result in functional redundancy. In fact, we have recently shown that Tbx2 and Tbx3 redundantly regulate development of the atrioventricular canal. To circumvent the generation of a large number of double

or the maintenance of Tbx18 in EPDCs may also affect the timing of epicardial EMT, we examined the invasion of EPDCs that were marked with the lineage label GFP in Tbx18KO, epiTbx18, and wild-type mice at different developmental stages. Cells started to migrate into the right ventricle from E12.5, where they were mostly localized to the subepicardial space, and invaded the myocardium from E13.5 starting at the atrioventricular junction and progressing towards the apex (Figure 2I–K). Statistical analysis of GFP-positive cells invading into the right ventricle of Tbx18KO, epiTbx18, and wild-type mice revealed that the temporal profile of EPDC formation was independent from the presence or absence of Tbx18 in the epicardium and in EPDCs (Figure 2L).
mutants of Tbx18 and other family members that are expressed in the epicardium including Tbx2, Tbx5, and Tbx20.\textsuperscript{28–30} we used a biochemical variant of Tbx18 which no longer represses but activates gene transcription to test for a possible redundancy of Tbx18 and other repressive T-box proteins. This was achieved by fusing the strong activation domain of the Herpes simplex virus encoded protein VP16 to the C-terminus of Tbx18.\textsuperscript{31} For epicardial misexpression of this fusion protein in vivo, we generated an Hprt\textsuperscript{Tbx18VP16} allele similar to the Hprt\textsuperscript{Tbx18} allele and crossed the line to Tbx18\textsuperscript{-/-} mice; mutant male embryos (Tbx18\textsuperscript{mut+/-};Hprt\textsuperscript{Tbx18VP16}) generated by this breeding are referred to as epiTbx18VP16 mice (see Supplementary material online, Figure S1). EpiTbx18VP16 mice died at E14.5 probably due to severe cardiovascular deficiencies. The hearts of these mice were hypoplastic and exhibited pericardial and sinus horn defects similar to Tbx18KO hearts (Figure 3A and A').\textsuperscript{15} Unlike Tbx18KO and wild-type mice, the right ventricular myocardial compact layer of epiTbx18VP16 hearts was visibly thinner (Figure 3B and B'). Statistical analyses confirmed that myocardial thinning reached significant levels at E12.5 and highly significant levels at E14.5 (Figure 3C). A significantly reduced proliferation of the right-ventricular myocardium at E12.5 and E13.5 as detected by the BrdU incorporation assay may contribute to this phenotype (Figure 3D). Apoptosis and cardiomyocyte size were unaltered when compared with wild-type hearts, and thus, do not contribute to the reduced ventricular wall thickness (see Supplementary material online, Figure S5). Immunohistochemistry against the endothelial marker protein endomucin (Emcn)\textsuperscript{32} detected an incomplete ingrowth of the coronary plexus into the right ventricle of epiTbx18VP16 hearts (Figure 3E and E'), which like the reduced myocardial thickness may relate to disturbed epicardial signalling. The epicardium was variably detached (occasionally forming small blisters) from the myocardium but expressed the epicardial marker genes Wt1 and Aldh1a2 (Figure 3F–H) suggesting that an epicardium is formed but that it is functionally impaired in epiTbx18VP16 mice.

We traced epicardial EMT and EPDC formation in epiTbx18VP16 mice by GFP immunofluorescence after increasing the R26\textsuperscript{mTmG} reporter line in the mutant background. At E14.5, a large number of GFP-positive cells were found in the subepicardial space and myocardium of the right ventricle in wild-type hearts. In epiTbx18VP16 mice, GFP expression was confined to the epicardium (Figure 4A and B). Surprisingly, SMC markers Notch3, Tagln, Myh11, and Acta2 were ectopically expressed in the epicardium of epiTbx18VP16 hearts suggesting that epicardial cells differentiated prematurely into SMCs and subsequently failed to invade into the right-ventricular myocardium (Figure 4A). Analysis at E12.5 showed upregulation of Notch3 and Tagln but not of Myh11 or Acta2 in the epicardium of epiTbx18VP16 mice (Figure 4B). Note that Tagln and Acta2 were still expressed in early cardiomyocytes of wild-type and epiTbx18VP16 hearts at E12.5 (Figure 4B).

Thus, an activating T-box function may be sufficient to trigger premature SM differentiation of epicardial cells indicating that a repressive T-box function controls the timing of epicardial EMT.

3.4 Notch and Tgfbr signalling mediate EMT and SMC differentiation of epicardial cells expressing Tbx18VP16

To obtain more detailed insight into the cellular and molecular consequences of epicardial expression of Tbx18VP16, we analysed cultures of highly enriched primary epicardial cells (Figure 5). These were obtained from right-ventricular explants at E11.5. After 4 days in serum-free medium, the outgrowth of wild-type ventricles presented as a monolayer of tightly packed hexagonal cells that expressed the tight junction protein 1 (Tjp1, also known as ZO1) in their membranes and only occasionally showed cortical staining of Acta2 (Figure 5A and E) and only weak expression of Tagln (see Supplementary material online, Figure S6). In contrast, epicardial cells of epiTbx18VP16 hearts were larger and had less cell contacts, expressed much lower levels of Tjp1 and formed Acta2- and Tagln-positive stress fibres implying that these cells have lost their epithelial character and differentiated into SMCs (Figure 5B and F, see Supplementary material online, Figure 6).

Previous work has shown that Tgfbr and Notch signalling is necessary and sufficient for epicardial EMT (Tgfbr) and SMC differentiation (Tgfbr, Notch), and that the two pathways tightly interact with each

![Figure 4](image-url) Premature SMC differentiation upon epicardial misexpression of Tbx18VP16 in vivo. (A and B) Immunofluorescence analyses of the lineage reporter GFP and of SMC markers Notch3, Tagln, Myh11, and Acta2 in wild-type and in epiTbx18VP16 (Tbx18\textsuperscript{mut+/-};Hprt\textsuperscript{Tbx18VP16}\textsuperscript{+/};Rosa26\textsuperscript{mTmG/+/}) embryos show that epicardial misexpression of Tbx18VP16 prevents formation of EPDCs and results in premature expression of SMC markers. Dashed lines indicate the epicardial–myocardial border. Stages are as indicated. Scale bars are 20 μm. Arrows indicate Acta2-positive cells within the epicardium of epiTbx18VP16 hearts at E14.5. Turquoise-stained dots are auto-fluorescing blood cells.
To address the functional involvement of both of these pathways in the premature SMC differentiation of \( \text{epiTbx18VP16} \) epicardial cells, we inhibited these pathways and analysed the expression of membrane-bound Tjp1 and the formation of Acta2- or Tagln-positive stress fibres in epicardial explants. Addition of SB431542, a commercially available compound that inhibits the type I receptors of the Tgfbr superfamily (Alk4, Alk5, Alk7), led to decreased expression and membrane localization of Tjp1 and to reduced levels of Acta2 and Tagln in wild-type cultures compatible with the inhibition of EMT and SMC gene expression in these cells (Figure 5I and M, see Supplementary material online, Figure 6). In \( \text{epiTbx18VP16} \) epicardial cells, Acta2- and Tagln-positive stress fibres were lost, and a cortical Acta2 staining similar to the staining observed in untreated wild-type cells was visible upon this treatment (Figure 5J and N, see Supplementary material online, Figure 6). Together with the observation that the increased size of \( \text{epiTbx18VP16} \) cells remained unaffected (Figure 5B and J), we conclude that activation of the Tgfbr signalling pathway contributes to premature EMT and SMC differentiation in \( \text{epiTbx18VP16} \) epicardial cells. To disrupt canonical Notch signalling, we introduced a mutant allele of Rbpj, which encodes the unique intracellular mediator of this pathway, into the mutant background to obtain \( \text{Tbx18cre/+;} \text{HprtTbx18VP16/y;} \text{Rosa26mTmG/+} \) (\( \text{epiTbx18VP16RbpjKO} \)) hearts for expression of the epithelial marker protein Tjp1 (red fluorescence), and the SMC marker Acta2 (green fluorescence) in untreated cultures (DMSO control) and in cultures treated for 4 days with a Tgfbr inhibitor (+SB431542). Inhibition of both Tgfbr and Notch signalling rescues the character of epithelial precursor cells. Note that epicardial outgrowth in \( \text{epiTbx18VP16} \) hearts is severely reduced. Scale bar is 50 \( \mu \)m.

From these experiments, we conclude that both Tgfbr and Notch signalling are functionally implicated in EMT and SMC differentiation of Tbx18VP16-expressing epicardial cells. Components of both pathways may be targeted by repressive T-box factors in the epicardium to ensure a proper timing of EMT and differentiation of epicardial cells.

4. Discussion
The embryonic epicardium is a crucial cell source for the developing heart, yet the molecular factors that control formation and differentiation of epicardium-derived mesenchymal cells in time and space are incompletely understood. Here, we have shown by genetic experiments in the mouse that Tbx18 is dispensable for epicardial development but that a transcriptional activator form of Tbx18 is sufficient to induce premature SM differentiation of epicardial cells.

4.1 Tbx18 may act redundantly with other T-box proteins in epicardial development
Tbx18 is a vertebrate-specific member of the Tbx1-subfamily of T-box transcription factors that play pivotal roles in patterning and differentiation of a large number of embryonic tissues. Functional analyses in the mouse have unraveled important functions of Tbx18...
at most of its sites of expression including the somites, the ureter, and the inner ear where the gene is required for patterning of fields of mesenchymal precursors. Expression of Tbx18 in the epicardium has been reported from all vertebrate models analysed to date including zebrafish, Xenopus, chick, and mouse embryos. Intriguingly, epicardial expression is not only found in the embryo, but is also reactivated as part of an embryonic gene program in the mature epicardium after myocardial injury both in zebrafish and in mouse. Given the strong conservation of epicardial expression in the vertebrate lineage, it was tempting to assume a functional implication in the development of this tissue. Analysis of Tbx18KO mice did not reveal changes of formation of the epicardium, nor in the differentiation of cell types that arise from EPDCs after epicardial EMT, fibroblasts, and SMCs. Although we cannot exclude minor changes in the fibrous skeleton and the coronary vasculature in the mutant, we can state that Tbx18 does not play a major unique role in the development of these cardiac components. Furthermore, maintenance of Tbx18 expression after epicardial EMT in EPDCs as well as in fibroblasts and SMCs did not affect their differentiation suggesting that down-regulation of Tbx18 in these cells is not of any functional significance. Restricted expression of Tbx18 in the epicardium may merely reflect an evolutionarily conserved regulatory module of activation by an epicardially restricted upstream factor and reactivation of Tbx18 after myocardial injury may, thus, have no influence on scarring or revascularization.

The lack of phenotypic consequences for epicardial development in Tbx18KO mice could also reflect compensation by a related factor. In fact, a number of T-box proteins, including Tbx2, Tbx5, and Tbx20 are co-expressed with Tbx18 in the proepicardium and epicardium. To overcome repression of possible target genes by Tbx18 and another transcriptional repressor, we misexpressed a Tbx18VP16 fusion protein that is able to bind to Tbx18 target sequences and activate gene transcription. These Tbx18VP16 mice completely recapitulated the phenotypic consequences of loss of Tbx18 in the heart (pericardium, sinus horns) as well as in extracardiac tissues (somites, ureter) confirming the assumption that Tbx18 indeed acts as a transcriptional repressor in vivo.

Within the heart, epiTbx18VP16 mice exhibited a thinned myocardium and a lack of coronary plexus formation compatible with disturbed epicardial signalling as seen in RXRa- and Raldh2-deficient mice. Furthermore, epiTbx18VP16 mice featured premature SMC differentiation that is likely to result in failure of EPDCs to invade the myocardium as seen in another mouse model. Hence, an activating version of Tbx18 is sufficient to trigger SM differentiation of epicardial cells. This, in turn, suggests that Tbx18 in combination with another T-box protein maintains the precursor character of the epicardium to allow proper mobilization and differentiation of epicardial cells, and subsequent formation of the fibroskeleton and the coronary vasculature. It remains to be analysed by double mutant analysis which other T-box protein may account for this role.

In the light of the described function of Tbx18VP16 in inducing SM differentiation of epicardial cells, lack of repression of SMC differentiation by maintained expression of Tbx18 in epiTbx18 mice may seem contradictory. However, these results can easily be reconciled if one assumes that Tbx18 has to interact with a co-factor which is normally restricted to the epicardium to exert repression of SMC differentiation. In fact, Tbx18 like other T-box transcription factors interacts with a number of other transcription factors in the heart as well as in extracardiac regions of the embryo.

### 4.2 Activation of Notch and Tgfbr signalling by an activating form of Tbx18

Irrespective of whether Tbx18VP16 mimics the loss of an endogenous repressive T-box activity in the epicardium in vivo, its function allows insight into the molecular mechanisms that mediate SM differentiation of epicardial cells. Our inhibition experiments in epicardial explants clearly showed that combined inhibition of Tgfbr as well as Notch signalling reverts the phenotype to an epicardial precursor state. Individual inhibition of Tgfbr signalling resulted in reduced formation of Acta2/Tagln-positive cells but did not affect the mesenchymal character, whereas inhibition of canonical Notch signalling led to an epithelial character of cells that expressed some stress fibres. This argues against a simple epistatic relationship between the two pathways but suggests that Tbx18VP16 activates both pathways independently and that they then synergize in the elaboration of the SM phenotype.

Genetic experiments in the mouse have shown that loss of the unique mediator of Notch signalling, RBPj, in the epicardium, resulted in normal EMT and fibroblast formation but failure of EPDCs to differentiate into SMCs. In contrast, loss of the Tgfbr1 (Alk5) in epicardial cells impaired EMT and SM differentiation of epicardial cells but also interfered with epicardial—myocardial signalling. Intriguingly, expression of the Notch intracellular domain (NICD) similar to Tbx18VP16 induced premature SM differentiation of epicardial cells in vivo as did administration of Tgfbr1 to epicardial explant cultures in vitro. Furthermore, we showed that activated Notch signalling in a Tgfbr1-dependent fashion induced expression of Tgfbr1-T3 arguing for a feed-forward activation loop between the two pathways in SM differentiation. We suggest that SMC differentiation can be independently induced in epicardial cells by both pathways on a low level but is dramatically potentiated by feed-forward activation between the two pathways.

Thus, simultaneous inhibition of both pathways by a repressive T-box function may dramatically aid in maintaining the epithelial precursor character of epicardial cells.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

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