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The bHLH transcription factor Tcf21 is required for lineage-specific EMT of cardiac fibroblast progenitors

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SUMMARY

The basic helix-loop-helix (bHLH) family of transcription factors orchestrates cell-fate specification, commitment and differentiation in multiple cell lineages during development. Here, we describe the role of a bHLH transcription factor, Tcf21 (epicardin/Pod1/capsulin), in specification of the cardiac fibroblast lineage. In the developing heart, the epicardium constitutes the primary source of progenitor cells that form two cell lineages: coronary vascular smooth muscle cells (cVSMCs) and cardiac fibroblasts. Currently, there is a debate regarding whether the specification of these lineages occurs early in the formation of the epicardium or later after the cells have entered the myocardium. Lineage tracing using a tamoxifen-inducible Cre expressed from the *Tcf21* locus demonstrated that the majority of Tcf21-expressing epicardial cells are committed to the cardiac fibroblast lineage prior to initiation of epicardial epithelial-to-mesenchymal transition (EMT). Furthermore, *Tcf21* null hearts fail to form cardiac fibroblasts, and lineage tracing of the null cells showed their inability to undergo EMT. This is the first report of a transcription factor essential for the development of cardiac fibroblasts. We demonstrate a unique role for Tcf21 in multipotent epicardial progenitors, prior to the process of EMT that is essential for cardiac fibroblast development.

KEY WORDS: *Tcf21*, Epicardium, Cardiac fibroblast, Epithelial-to-mesenchymal transition, Epicardial-derived cells, Mouse

INTRODUCTION

Cardiac fibroblasts are a major non-myocyte population of the heart that regulates structural, mechanical, biochemical and electrical properties during normal heart function (Souders et al., 2009). Although they play a role in normal heart physiology, the deposition of excess extracellular matrix (ECM) by fibroblasts is associated with most cardiac diseases (Weber, 2000). Despite their importance, few studies have focused on genes and pathways that are important for the embryonic development and fate specification of this cell population. Recent studies have identified several genes and signaling pathways that contribute to the development of coronary vascular smooth muscle cells (cVSMCs) (Merki et al., 2005; Mellgren et al., 2008; Sridurongrit et al., 2008; del Monte et al., 2011; Grieskamp et al., 2011). However, relatively little is known about factors that are essential for differentiation along the cardiac fibroblast lineage (Smith et al., 2011). Some studies suggest that cardiac fibroblasts are a heterogeneous population that originates from multiple sources in the body, including endothelial cells, bone marrow-derived cells and the epicardium (Zeisberg and Kalluri, 2010), but it is unclear what portion of fibroblasts are derived from exogenous sources and how each population participates in matrix deposition and heart pathology.

Studies in the avian system first suggested that the epicardium is the primary source of interstitial fibroblasts in the heart (Mikawa and Gourdie, 1996; Dettman et al., 1998; Gittenberger-de Groot et al., 1998; Manner, 1999; Winter and Gittenberger-de Groot, 2007). The epicardium, a multipotent mesothelial layer of cells, spreads over the myocardium soon after the heart has looped at approximately embryonic day (E)9.5. Through a process of epithelial-to-mesenchymal transition (EMT), a subpopulation of epicardial cells migrates into the myocardium and gives rise to epicardial-derived cells (EPDCs). EPDCs differentiate predominantly into either cVSMCs or interstitial and adventitial cardiac fibroblasts (Mikawa and Gourdie, 1996; Dettman et al., 1998; Gittenberger-de Groot et al., 1998). However, retroviral-labeling experiments in chick embryos also suggested that clonal identities of EPDCs are probably established in the proepicardium (Mikawa and Gourdie, 1996). More extensive fate-tracing approaches with current genetic tools could further refine when EPDC fate is actually specified.

Cell type-specific basic helix-loop-helix (bHLH) transcription factors are key regulators of organ morphogenesis (Weintraub et al., 1991; Lee et al., 1995; Porcher et al., 1996; Srivastava et al., 1997). Tcf21 (capsulin/Pod1/epicardin) is a Class II bHLH transcription factor that binds DNA through the consensus E box sequence (CANNTG) as a heterodimer (Lu et al., 1998). Throughout embryogenesis, robust expression of Tcf21 is noted in mesenchymal cells of the respiratory, digestive, urogenital and cardiovascular systems (Hidai et al., 1998; Lu et al., 1998; Quaggin et al., 1998; Robb et al., 1998). Loss of *Tcf21* results in perinatal lethality, and published reports document roles for this transcription factor in lung, kidney, spleen, gonad and facial skeletal muscle development (Quaggin et al., 1999; Lu et al., 2000; Lu et al., 2002; Cui et al., 2004). Although Tcf21 has been extensively utilized to identify proepicardial and epicardial cells, its function in cardiac development remains uninvestigated.

Here, we describe that *Tcf21* null embryos fail to develop cardiac fibroblasts and we identify a role for this bHLH family member in EPDC fate specification and EMT. Using mice that express Cre

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from the *Tcf21* locus, we show that, initially, Tcf21-expressing epicardial cells are multipotent with the ability to form either cVSMCs or cardiac fibroblasts. Over time, however, Tcf21 becomes restricted to the cardiac fibroblast lineage. These data demonstrate that epicardial cell fate decisions occur in the epicardium before the process of EMT and, because *Tcf21* null hearts form no cardiac fibroblasts, that Tcf21 is an essential transcription factor for cardiac fibroblast cell fate determination.

MATERIALS AND METHODS

Mice

Tcf21$^{lacZ/+}$ (Lu et al., 2000), *Tcf21*$^{iCre/+}$ (Acharya et al., 2011), *Pdgfra*$^{GFP/+}$ (Hamilton et al., 2003) and *Gata5Cre*$^{Tg/0}$ (Merki et al., 2005) mice have been previously described. Reporter strains used in the study include: *R26R*^{YFP} (Srinivas et al., 2001), *R26R*^{tdT} (Madisen et al., 2010), *XlacZ4* (Tidhar et al., 2001) and *Col1-GFP*$^{Tg/0}$ mice (Lin et al., 2008). All procedures described in this study were approved by the Institutional Animal Care and Use Committees of UT Southwestern Medical Center and conformed to NIH guidelines for care and use of laboratory animals. Mice were maintained on a mixed C57BL6/129SV background and data for each experiment were deduced from a minimum of three nulls and three littermate controls.

In situ hybridization

Digoxigenin-labeled probes against *Tbx18* (from Sylvia Evans, University of California, San Diego, CA, USA), *Col1a1*, *Col3a1* (from Benoit Crombrugghe, MD Anderson Cancer Center, TX, USA), *Tcf21* (from Anthony Firulli, Indiana University-Purdue University Indianapolis, IN, USA), *Pdgfra* (from Christer Betsholtz, Karolinska Institute, Stockholm, Sweden) and *Raldh2* (*Aldh1a2* – Mouse Genome Informatics), which was synthesized using the sequence information from accession number BC075704, were used according to standard in situ hybridization protocols (Smith et al., 2011).

Tamoxifen induction and immunohistochemistry

Tamoxifen (MP Biomedicals 156708) was administered by gavage (0.1 mg/g body weight) to pregnant dams. For adult inductions, tamoxifen (0.2 mg/g body weight) was administered by gavage for five consecutive days. No reporter activity was detected at any time in the absence of tamoxifen. Briefly, hearts were isolated in PBS, fixed in 4% paraformaldehyde for 1 hour, frozen embedded, and sectioned. Immunohistochemistry was performed (Acharya et al., 2011) using the following antibodies: anti-GFP (Molecular Probes, Invitrogen, A11122; 1:250); anti-Pdgfrβ (EM Bioscience, 14-1402-81; 1:200); anti-Acta1 (Sigma, A7811; 1:400); anti-PECAM (BD Pharmingen, 553370; 1:200); biotinylated Isolectin B4 (Vector Laboratories, B1205; 1:500); and anti-SM22α (Abcam, ab14106-100; 1:500). Immunohistochemistry on paraffin sections was performed for the following antibodies: anti-periostin (Santa Cruz Biotechnology, Sc-49480; 1:50) and anti-β-galactosidase (Cappel, 559761; 1:500). β-Galactosidase staining was performed as described previously (Acharya et al., 2011).

RNA isolation and qRT-PCR

For quantitative RT-PCR, RNA was either isolated from E18.5 hearts (atria and the conotruncal region removed) using Trizol (Invitrogen) or from cells using RNAqueous Micro Kit (Ambion). Following DNase1 treatment, cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) and random hexamers (Roche). Gene expression profiles were generated using standard qPCR methods (according to manufacturer's recommendations, Bio-Rad) with iTAQ SYBR Green master mix (Bio-Rad) on a CFX96 instrument (Bio-Rad). Samples were run in triplicate and normalized to cyclophilin expression. Primer sequences for qRT-PCR are listed in supplementary material Table S1.

Western blotting

Whole-cell extracts from E18.5 hearts (atria and conotruncal regions removed) were immunoblotted with the following antibodies: anti-periostin (Santa Cruz SC-49480; 1:500); anti-goat HRP (Sigma, 1:1000); anti-β-tubulin (BD 556321; 1:1000); anti-mouse IgM (Zymed, 1:3000); and anti-SM22α (Abcam, 1:200).

Adenoviral production

AdGFP and Adβgal were kindly provided by Robert Gerard (University of Texas Southwestern, Dallas, TX, USA). For AdTcf21, full length mouse *Tcf21* cDNA was synthesized from mouse epicardial cells (MECs) total RNA by reverse transcription and cloned into pcDNA3.1 as an *Eco*RI and *Xho*I fragment to generate N-terminal Myc-tagged Tcf21 overexpression plasmid. The myc-tagged cDNA was subsequently mobilized into pAd/CMV/DEST (Invitrogen) for adenoviral production.

Imaging and statistical analysis

A Zeiss Axiovert 200 with a Hamamatsu ORCA-ER camera was used for fluorescence imaging, a Zeiss Axiovert 200 with an Olympus DP71 camera for color imaging and a Zeiss Stemi SV 11 Apo with an Olympus DP71 camera for whole-mount imaging. Higher magnification images were captured using an LSM510 META laser scanning confocal microscope. All images and figures were edited and created in Photoshop CS4. All statistical calculations were performed using Prism 5 (Graph Pad). *P* values for statistical significance were obtained using Student's *t*-test for single variables between control and test samples.

Flow cytometry

Hearts from 3-month-old *Pdgfra*$^{GFP/+}$, *Col1GFP*$^{Tg/0}$ and *Tcf21*$^{iCre/+}$:*R26R*^{tdT} (induced with tamoxifen at E14.5 and delivered by Cesarean section) were processed to generate single cell suspensions for fluorescence-activated cell (FAC) sorting as described previously (Russell et al., 2011). Cells were sorted with a MoFlo flow cytometer (Cytomation) using Summit software. For transcript analysis, sorted cells were collected into lysis buffer for RNA extraction (RNAqueous Micro Kit from Ambion). A fraction of each sample was also collected into PBS for post-sort assessment of purity.

Cell culture and isolation

Primary epicardial cells were cultured from E12.5 hearts as published previously (Mellgren et al., 2008). To assay for EMT following Tcf21 overexpression in primary epicardial cultures, wild-type E12.5 hearts (atria and cushions removed) were cultured in 10% FBS, 1:1 DMEM:M199 supplemented with basic fibroblast growth factor (2 ng/ml). After 2 days, the hearts were removed and cells were allowed to grow for an additional 3 days. Cultures were then transduced with either the control virus (Adβgal) or AdTcf21 for 2 days followed by immunostaining for cell-cell junctional marker (β-catenin; BD Transduction Laboratories, 610153; 1:200) and cytoskeletal actin (phalloidin; Invitrogen, A12379; 1:500). Primary cardiac fibroblasts were isolated from E18.5 and adult hearts (after

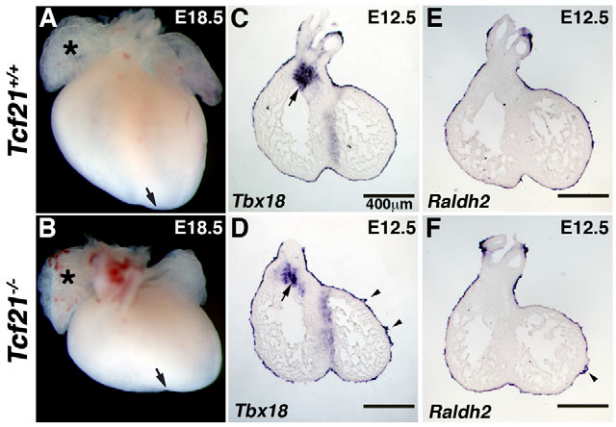

Fig. 1. Epicardial development in *Tcf21* null hearts. (**A**,**B**) Whole-mount images of E18.5 wild-type (A) and *Tcf21* null (B) hearts. Arrows and asterisks indicate the absence of a distinct apex and an enlarged right atrium, respectively, in the null hearts. (**C-F**) Epicardial-specific gene expression at E12.5. Arrowheads indicate regions of epicardial detachment in null hearts and arrows point to atrioventricular cushion region.

removing the atria, outflow tract and the cardiac valves) using Dulbecco's Modified Eagle Medium (DMEM) containing 0.05% bovine serum albumin (BSA), 500 U/ml collagenase 2 (Worthington Biomedical Corporation) and 0.003% trypsin at 37°C with continuous shaking for an hour. Dissociated cells were filtered through a cell strainer (70 μm) and pelleted by centrifuging at 1000 ***g*** for 3 minutes before plating.

RESULTS

Tcf21 null embryonic heart phenotype

Even though *Tcf21* is expressed in the developing cardiovascular system as early as E8.5 (Lu et al., 1998), the exact role of this transcription factor in cardiac development is poorly understood. Using the *Tcf21*$^{lacZ/+}$ allele (Lu et al., 2000), we investigated its function in the epicardium. *lacZ* expression from the *Tcf21* locus in the heart was first detectable in the proepicardium and was evident subsequently in the growing epicardium, atrioventricular canal and an interstitial cell population within the heart (supplementary material Fig. S1A,B) (Lu et al., 1998). We generated *Tcf21* null embryos, and they had dysmorphic hearts that lacked a distinct apex. Notably, the right atrium was enlarged (Fig. 1A,B). Nulls also displayed hemorrhaging in the pericardial cavity (observed at E17.5 and later) and reduced myocardial thickness (supplementary material Fig. S1C,D; data not shown). Because the epicardium is the most prominent site of *Tcf21* expression within the heart, we investigated epicardial gene expression and morphology. Although nulls exhibited regions of epicardial detachment around midgestation (supplementary material Fig. S1E,F), in situ hybridization demonstrated normal expression of epicardial markers, including *Tbx18* and *Raldh2* (Fig. 1C-F). Thus, despite the phenotypic abnormalities in *Tcf21*$^{-/-}$ hearts, epicardial formation was normal.

Defective epicardial EMT in *Tcf21* nulls

EMT is a key biological process integral to the development of many tissues, including the epicardium. It is characterized by a combination of loss of epithelial properties (for example, polarized organization, cell-cell junctions) and acquisition of mesenchymal traits that lead to migratory and invasive properties. As epicardial spreading was unaffected in *Tcf21* nulls, we assayed for epicardial cell migration into the myocardium using β-galactosidase expression driven by the *Tcf21*^{lacZ} allele. At E12.5, when expression of Tcf21 is primarily confined to the epicardium, control and null hearts exhibited indistinguishable β-galactosidase expression (Fig. 2A,B; supplementary material Fig. S2A). However, at E13.5 and all subsequent time points, fewer β-galactosidase-expressing (β-gal^{+}) cells were observed in the null hearts (supplementary material Fig. S2C-H; data not shown). Examination of coronal sections from E13.5 to E18.5 revealed that

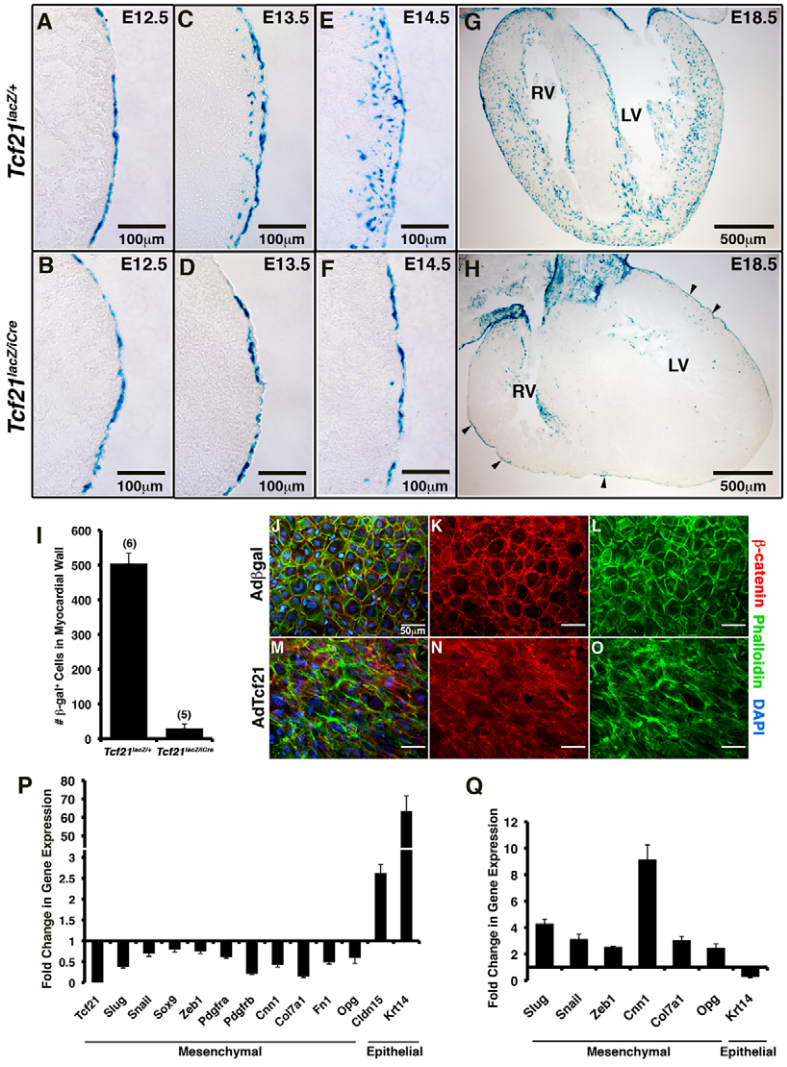

Fig. 2. Defective epicardial EMT in *Tcf21* null hearts. (**A-H**) β-Galactosidase staining in heart sections of the indicated stages and genotypes. A-F show high magnification images of the left ventricle (LV). Arrowheads in H point to null cells in the epicardium. RV, right ventricle. (**I**) Quantification of *lacZ*$^{+}$ cells in the myocardial wall. A 20× field of view, spanning the entire LV (three sections per heart) was quantified for total number of *lacZ*$^{+}$ cells. *n* values are indicated in parentheses. (**J-O**) Wild-type primary epicardial cultures were transduced with either Adβgal (J-L) or AdTcf21 (M-O) and stained for adherens junctions using β-catenin (red), and actin cytoskeleton using phalloidin (green). Nuclei were stained with DAPI (blue). (**P**) EMT-related gene expression changes in E12.5 primary epicardial cultures. Fold change in gene expression with respect to wild type (normalized to 1) is shown. (**Q**) Gene expression changes upon overexpression of Tcf21 (AdTcf21) in primary epicardial cultures. AdGFP served as control (normalized to 1). Data are mean ± s.d.

whereas control hearts had β-gal$^+$ cells within the myocardium, null hearts lacked interstitial cells with *Tcf21* promoter activity (Fig. 2A-I; data not shown). Because we observed no alteration in apoptosis or epicardial cell proliferation in *Tcf21* null hearts (supplementary material Fig. S3A-C), loss of β-gal$^+$ cells inside the heart was probably caused by a failure of epicardial cell migration. To test whether *Tcf21* lineage cells migrate into the heart, we indelibly tagged Tcf21-expressing cells using the genetic combination of an inducible Cre driven from the *Tcf21* promoter (Acharya et al., 2011) and the *R26R^{tdT}* reporter allele. Cre recombinase was induced at E10.5, when Tcf21 expression is restricted to the epicardium, and hearts were isolated at specified time points. As shown (supplementary material Fig. S2I-L), *Tcf21^{iCre}*-labeled epicardial cells (tomato$^+$) are present within the myocardium soon after epicardial EMT occurs (E13.5). Therefore, the lack of β-gal$^+$ cells in the myocardium of *Tcf21$^{-/-}$* hearts probably resulted from a defect in epicardial cell migration.

We then tested whether Tcf21 could induce EMT in primary epicardial cultures (Fig. 2J-O). As shown in Fig. 2J-L, control epicardial cultures transduced with only Adβgal showed junctional β-catenin and cortical phalloidin staining. However, upon overexpression of Tcf21 (Fig. 2M-O), these epithelial characteristics were lost, i.e. cells no longer displayed junctional β-catenin and acquired actin stress fibers. To evaluate EMT in *Tcf21* nulls further, we examined gene expression changes. As shown in Fig. 2P, expression of mesenchymal genes representative of epicardial EMT (Smith et al., 2011), including *Slug* (*Snai2* – Mouse Genome Informatics), *Snail* (*Snai1* – Mouse Genome Informatics), *Sox9*, *Zeb1*, *Pdgfra*, *Pdgfrb*, *Cnn1* (calponin), *Col7a1*, *Fn1* (fibronectin 1) and *Opg* (osteoprotegerin; *Tnfrsf11b* – Mouse Genome Informatics) was downregulated in *Tcf21$^{-/-}$* cultures. Concomitantly, expression of epithelial markers, such as *Cldn15* (claudin 15) and *Krt14* (keratin 14), was retained in null hearts. These data are indicative of an EMT defect in null epicardial cultures. Conversely, we also analyzed the gene expression changes upon *Tcf21* overexpression (AdTcf21). Interestingly, overexpression of *Tcf21* in primary epicardial cultures induced the expression of a subset of mesenchymal markers, including *Slug*, *Snail*, *Zeb1*, *Cnn*1, *Col7a1* and *Opg*, and downregulated the epithelial marker *Krt14* (Fig. 2Q). Taken together, these data suggest that Tcf21 is required for epicardial EMT, at least in a subset of epicardial cells.

Tcf21$^{-/-}$ hearts form cVSMCs but lack epicardial-derived cardiac fibroblasts

Published reports suggest an interstitial, non-myocyte pattern of Tcf21 expression in late gestation embryonic and adult hearts, implicating expression in cardiac fibroblasts (Lu et al., 1998; Acharya et al., 2011). To determine whether Tcf21 expression was limited to a subpopulation of EPDCs after EMT, we compared *Tcf21^{lacZ}* reporter expression with that of endothelial and cVSMC markers. As shown in Fig. 3A-D, Tcf21 expression is clearly excluded from endothelial (PECAM$^+$) and cVSMC (Pdgfrβ$^+$) cell populations.

Guided by multiple growth factor signaling cues, the epicardium gives rise primarily to two cell fates: cVSMCs and cardiac fibroblasts (Winter and Gittenberger-de Groot, 2007). To determine how the defect in migration affects the development of these two EPDC fates, we investigated the formation of cVSMCs in *Tcf21* nulls, using mice possessing an *XlacZ4* transgene that drives nuclear β-galactosidase expression in populations of smooth muscle cells (Tidhar et al., 2001). Figure 3E,F shows that cVSMCs

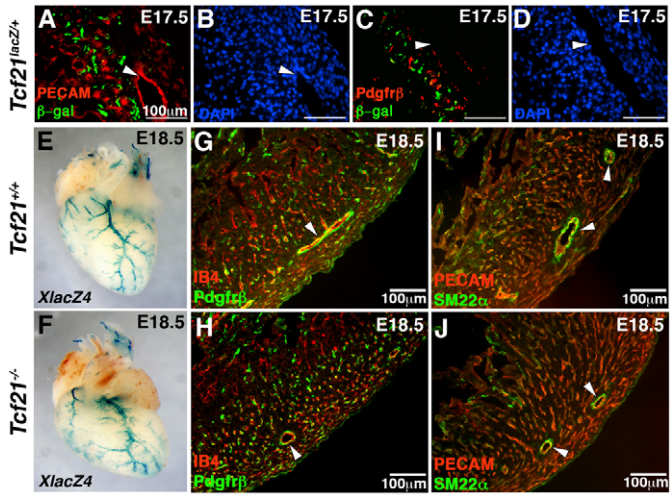

Fig. 3. *Tcf21* null hearts form cVSMCs. (**A-D**) Immunohistochemistry for β-galactosidase (green) in E17.5 *Tcf21$^{lacZ/+}$* hearts co-stained (in red) for PECAM (A) or Pdgfrβ (C). Nuclear counterstain with DAPI (blue) for images A and C is shown in B and D, respectively. Arrowheads point to vessels. (**E**,**F**) Development of coronary vasculature detected using whole-mount β-galactosidase staining of wild-type (E) and null (F) E18.5 hearts carrying the *XlacZ4* transgene. (**G-J**) Immunohistochemistry for SMC marker expression in wild-type (G,I) and *Tcf21$^{-/-}$* (H,J) hearts. E18.5 hearts were co-stained for SMC (Pdgfrβ or SM22α in green) and endothelial (isolectin B4 or PECAM in red) markers to detect vessels. Arrowheads point to vessels.

were detectable in both null and control hearts. Similar results were obtained when expression of smooth muscle (SM) markers Pdgfrβ and SM22α (Tagln – Mouse Genome Informatics) was examined (Fig. 3G-J). Thus, despite defective epicardial EMT of Tcf21 reporter-tagged cells, *Tcf21* null hearts develop cVSMCs. These data suggest that Tcf21 could be required in the development of the other EPDC population, namely cardiac fibroblasts.

Perhaps the best known function of cardiac fibroblasts is their ability to synthesize and secrete ECM proteins. Hence, to investigate a defect in the development of the cardiac fibroblast population, we first analyzed *Tcf21* null hearts for extracellular matrix deficiencies. We examined expression of periostin (*Postn*), a TGF-β inducible protein secreted by cardiac fibroblasts that is also significantly upregulated in response to pathological stress (Norris et al., 2008). *Tcf21* null hearts maintained expression of periostin in the cardiac valves (Fig. 4A′,B′), but expression in the ventricular myocardium was reduced compared with wild-type controls (Fig. 4A,B). We then analyzed collagen expression to indicate the presence of cardiac fibroblasts. Col1a1 and Col3a1 are reportedly the most highly secreted collagens by cardiac fibroblasts. Col1 expression was initially analyzed using a *Col1GFP* transgenic reporter (Lin et al., 2008). In control hearts, GFP-expressing cells were abundantly detected in the epicardium, myocardial interstitium and the AV canal (Fig. 4C,E). By contrast, interstitial Col1GFP expression was completely lost in the *Tcf21* null hearts (Fig. 4D,F). Nulls, however, retained GFP expression in the epicardium and the AV canal (Fig. 4D). In situ hybridization with a *Col1a*-specific probe yielded comparable results (Fig. 4G,H). *Col1a* transcript was greatly diminished in *Tcf21$^{-/-}$*, although some residual expression was maintained around the vessels, in agreement with its reported expression in the VSMC population (Ponticos et al., 2004). Additionally, we also compared

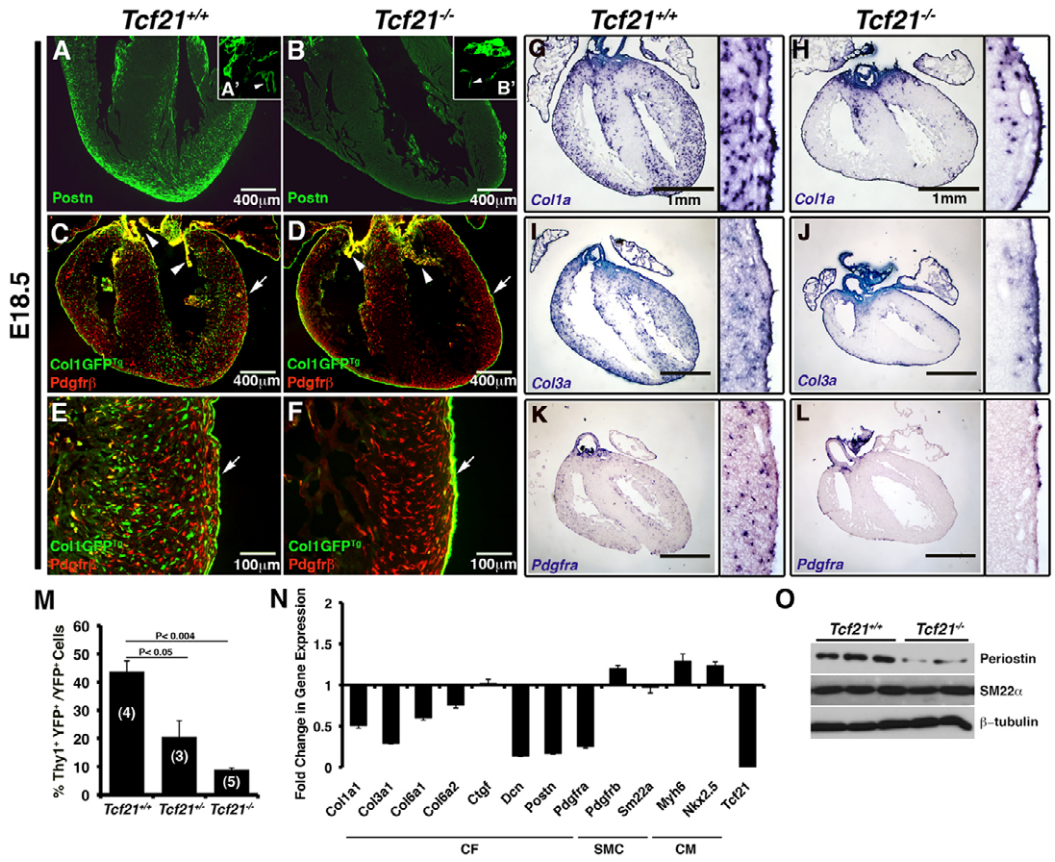

Fig. 4. *Tcf21* null hearts lack epicardial-derived cardiac fibroblasts. (**A-B′**) Periostin immunohistochemistry on wild-type (A) and *Tcf21*$^{-/-}$ (B) hearts. Inset images (A′,B′) show staining of the atrioventricular valve region. Arrowheads indicate valves. (**C-F**) Expression of *Col1GFP* transgene (green) in wild-type (C,E) and null (D,F) hearts. Hearts were co-stained for Pdgfrβ (red). E and F represent higher magnification LV images of C and D, respectively. Arrows and arrowheads indicate epicardium and valves, respectively. (**G-L**) In situ hybridization of wild-type (G,I,K) and *Tcf21* null (H,J,L) hearts. Expression of cardiac fibroblast specific genes *Col1a1* (G,H), *Col3a1* (I,J) and *Pdgfra* (K,L) is shown. Corresponding higher magnification images of the LV are shown to the right. (**M**) Percentage of epicardial-derived cardiac fibroblasts (Thy1^{+}:YFP^{+}/YFP^{+}) from wild-type, *Tcf21*$^{+/-}$ and *Tcf21*$^{-/-}$ hearts. CD31^{+} endothelial cells were excluded from the analysis. *n* values are indicated in parentheses. (**N**) Quantitative RT-PCR showing expression of cardiac fibroblast (CF), vascular smooth muscle cell (SMC), and cardiomyocyte (CM) differentiation markers in *Tcf21*$^{-/-}$ hearts relative to wild-type littermate controls. Fold change with respect to control (normalized to 1) is shown. Data represented are mean ± s.d. (**O**) Immunoblots comparing cardiac fibroblast (periostin) and VSMC (SM22α) protein expression in control versus *Tcf21*$^{-/-}$ hearts. Protein amounts were normalized using β-tubulin. All experiments were performed using E18.5 hearts.

Col3a1 expression by in situ hybridization and the results were very similar (Fig. 4I,J). Taken together, these data demonstrate that *Tcf21* null hearts are highly deficient in several fibroblast-enriched ECM proteins.

Thus far, the data indicated that matrix production by cardiac fibroblasts was impaired in *Tcf21* null hearts, but the EMT data suggested a complete failure of these EPDCs to enter the myocardium. To investigate the presence of a defined cardiac fibroblast population, we utilized two cell surface receptors as markers of this cell population. Recent data demonstrate a requirement for PDGF receptor tyrosine kinases in epicardial EMT and epicardial-derived cell fates (Smith et al., 2011). Although Pdgfrβ has been shown to regulate cVSMC development (Mellgren et al., 2008), loss of Pdgfrα results in a specific loss of only the cardiac fibroblast population. As shown in Fig. 3G,H and Fig. 4C-F, Pdgfrβ expression was unaltered in *Tcf21* null hearts. However, when we compared control and *Tcf21* null hearts for *Pdgfra* expression by in situ hybridization, we observed reduced *Pdgfra* expression in *Tcf21*$^{-/-}$ hearts (Fig. 4K,L). Furthermore, flow cytometry data from E18.5 hearts demonstrated a significant reduction in Thy1^{+} epicardial-derived cardiac fibroblasts in *Tcf21*$^{-/-}$ hearts. Utilizing *Gata5 Cre* transgenic mice (Merki et al., 2005) and a *R26R*^{YFP} reporter (Srinivas et al., 2001), we tagged epicardial-derived cells and examined their numbers in the presence or absence of Tcf21. We generated single cell suspensions from E18.5 hearts and used a cell strainer to remove cardiomyocytes. Using Thy1.1, a membrane glycoprotein expressed on the surface of cardiac fibroblasts (and also endothelial cells) and YFP detection, we compared the proportion of Thy1^{+}; YFP^{+}; CD31^{-} cells with the total number of YFP^{+} cells in the heart. This double-positive population was the epicardial-derived cardiac fibroblasts. As shown in Fig. 4M, *Tcf21*$^{-/-}$ nulls had far fewer epicardial-derived cardiac fibroblasts compared with both wild type and heterozygotes.

To understand the gene expression changes resulting from the loss of Tcf21, we analyzed E18.5 null hearts for a variety of cell differentiation markers by quantitative RT-PCR. As shown in Fig. 4N, comparison of gene expression in E18.5 wild-type and null hearts revealed significant downregulation of multiple cardiac fibroblast-enriched genes including *Col1a1*, *Col3a1*, *Col6a1*,

Col6a2, *Postn* (periostin), *Dcn* (decorin) and *Pdgfra*. By comparison, VSMC (*Pdgfrb* and *SM22a*) and cardiomyocyte (*Nkx2.5* and *Myh6*) genes appeared unchanged. Similar results were obtained upon immunoblotting whole-cell extracts for select cell type markers (Fig. 4O). We also generated primary cardiac fibroblast cultures from control ($Tcf21^{lacZ/+}$) and null ($Tcf21^{lacZ/iCre}$) hearts (supplementary material Fig. S2M,N). Far fewer cardiac fibroblasts were obtained from null hearts. In summary, these data demonstrate a specific loss of an epicardial-derived cell population in the *Tcf21* null hearts, namely the cardiac fibroblasts.

$Tcf21^{iCre}$ labels cardiac fibroblasts

It was intriguing to note that even though *Tcf21* reporter activity appeared in the majority of epicardial cells, its absence affected only cardiac fibroblast development. Interestingly, when embryonic hearts (atria and conotruncal region removed) were analyzed for *Tcf21* expression by quantitative RT-PCR, a decrease in expression was noted at E12.5 and E13.5 relative to E11.5 (supplementary material Fig. S4A). In situ hybridization studies showed a similar trend with some epicardial cells downregulating *Tcf21* expression (supplementary material Fig. S4B-F). Together, these observations hinted at a potential role for this bHLH family member in the subset of epicardial cells that will become cardiac fibroblasts.

To determine whether this was the case, we performed lineage tracing of Tcf21-expressing cells. To indelibly label this population of cells and its descendents, we used the $Tcf21^{iCre/+}$ line described above. Tamoxifen induction results in Cre recombination and in the presence of a *R26R* reporter, either YFP ($R26R^{YFP}$) or tomato ($R26R^{tdT}$), identification of Tcf21-expressing cells and all of their progeny. Co-staining with cell type-specific markers [platelet/endothelial cell adhesion molecule (PECAM), endothelial; Pdgfrβ and SM22α, VSMC; Acta1, cardiomyocyte; $Pdgfr\alpha^{GFP}$ and Col1GFP, cardiac fibroblast] enabled lineage identification of tagged cell populations. In contrast to several reports that suggest that epicardial cells can become cardiomyocytes (Cai et al., 2008; Zhou et al., 2008), we found that cells expressing Tcf21 at E14.5, a time point immediately after epicardial EMT, did not co-stain with Acta1. In fact, Tcf21-traced cells were essentially excluded from the endothelial, cVSMC and cardiomyocyte populations (Fig. 5A-C, Fig. 6A-D; supplementary material S5A-D,I-L). However, when we examined the traced cells using the $R26R^{tdT}$ reporter for Pdgfrα expression ($Pdgfra^{GFP/+}$ allele) (Hamilton et al., 2003), a fraction of the tomato$^+$ cells overlapped with GFP$^+$ Pdgfrα-expressing cells (Fig. 5D), suggesting that the tagged cells belonged to the cardiac fibroblast lineage. We also analyzed whether Tcf21 lineage-traced cells had acquired a cardiac fibroblast fate based on *Col1GFP* reporter activity. As shown in Fig. 5E (and in supplementary material Fig. S6A-D), $R26R^{tdT}$ reporter activity overlapped with Col1GFP-expressing cells. Using the same markers, cardiac fibroblast-specific labeling was also observed in $Tcf21^{iCre}$ mice following adult induction (supplementary material Fig. S6I-N). Acta1 co-staining on these adult induced hearts

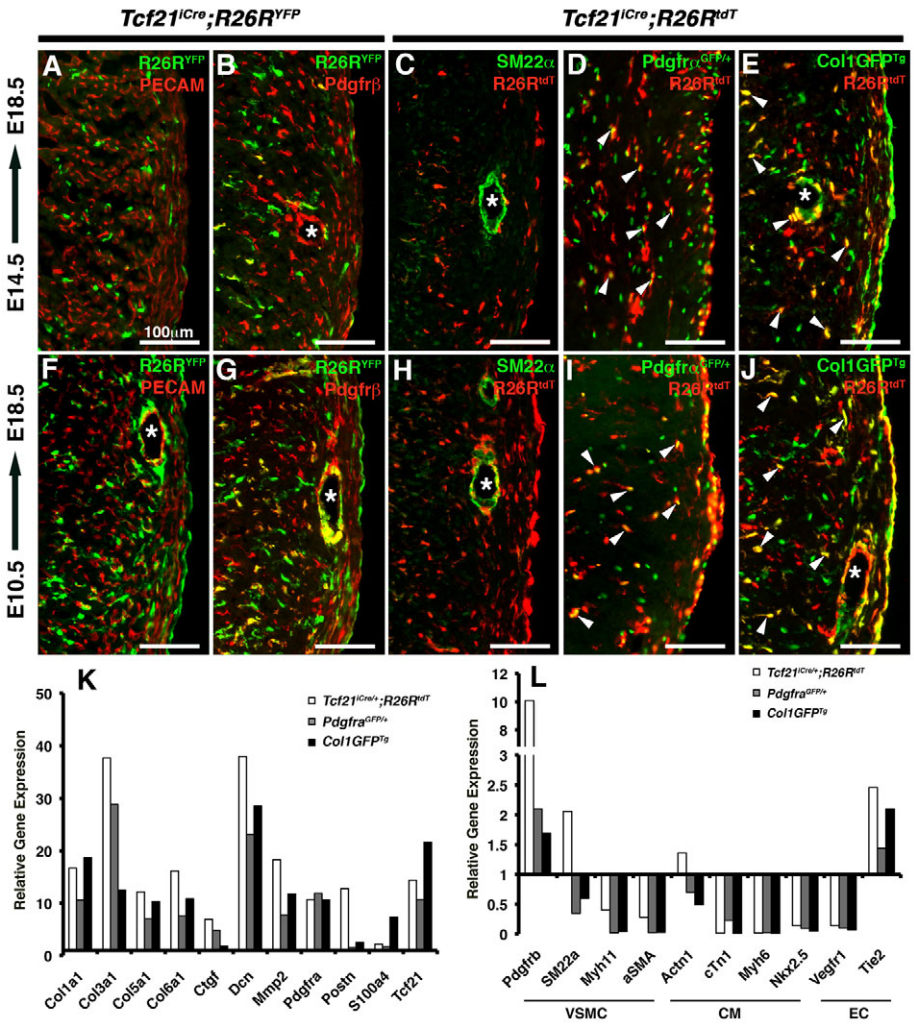

Fig. 5. Lineage tracing with $Tcf21^{iCre}$ labels cardiac fibroblasts. (**A-J**) Lineage tracing using $R26R^{YFP}$ (green; A,B,F,G) and $R26R^{tdT}$ (red; C-E,H-J) in E18.5 $Tcf21^{iCre}$ embryonic hearts induced at E14.5 (A-E) or E10.5 (F-J). PECAM labels endothelial cells, Pdgfrβ and SM22α label smooth muscle cells, and $Pdgfr\alpha^{GFP}$ and Col1GFP label cardiac fibroblasts. Arrowheads in D, E, I and J show examples of cells that are positive for both GFP and $R26R^{tdT}$ reporter activity. Asterisks indicate the lumen of vessels. (**K**) Quantitative gene expression profiling for cardiac fibroblast markers following FAC sorting of $Tcf21^{iCre/+}$:$R26R^{tdT}$ lineage traced, $Pdgfra^{GFP/+}$ and *Col1GFP* transgenic hearts. Flow sorted tomato$^+$ cells from $Tcf21^{iCre/+}$:$R26R^{tdT}$ lineage-traced hearts derived from embryos induced at E14.5 are compared by qRT-PCR with sorted GFP$^+$ cardiac fibroblast population from $Pdgfr\alpha^{GFP/+}$ and *Col1GFP* transgenic hearts. Fold change in gene expression relative to unsorted population (normalized to 1) is shown. (**L**) Quantitative gene expression profiling of sorted cell populations shown in K for vascular smooth muscle cell (VSMC), cardiomyocyte (CM) and endothelial cell (EC) marker genes. Fold change in gene expression compared with the unsorted population (normalized to 1) is shown.

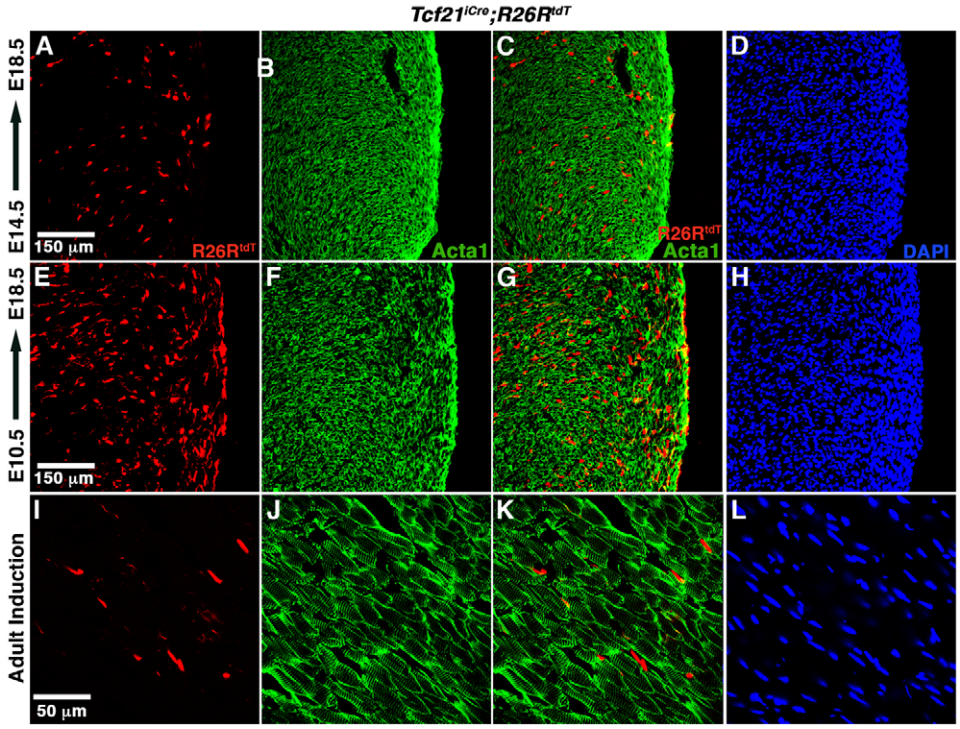

Fig. 6. *Tcf21*^{iCre} lineage-traced epicardial cells do not give rise to cardiomyocytes. (**A-L**) Representative images of lineage-traced E18.5 *Tcf21*^{iCre}*;R26T*^{tdT} embryonic (A-H) or adult (I-L) hearts induced at E14.5 (A-D), at E10.5 (E-H) or in the adult (I-L). Co-stain is cardiomyocyte marker, Acta1 (in green).

demonstrated further that the Tcf21-lineage cells were excluded from the cardiomyocyte population (Fig. 6I-L). These tracing experiments thus indicate that Tcf21-expressing cells at E14.5 and later are restricted to the cardiac fibroblast lineage.

To analyze the lineage-traced cells in greater detail, we performed gene expression profiling on RNA isolated from labeled cells. Cre recombination was induced in *Tcf21*^{iCre}*:R26R*^{tdT} embryos at E14.5, followed by Cesarean birth at E18.5. At 3 months of age, hearts were harvested to generate a single cell suspension (after removing atria and conotruncal region), and FACS analysis was performed. Tcf21 lineage-traced cells (tomato^{+}) constituted ~6% of the single cell suspension. Sorted cells were then assayed for cardiac fibroblast-, SMC-, cardiomyocyte- and endothelial-specific gene expression. As shown in Fig. 5K, quantitative RT-PCR on RNA isolated from the lineage-traced tomato^{+} cells revealed significant enrichment of several genes expressed by cardiac fibroblasts including *Col1a1*, *Col3a1*, *Col5a1*, *Col6a1*, *Pdgfra*, *Postn*, *Dcn*, *Mmp2* and *Tcf21*, relative to the unsorted population. By contrast, the *Tcf21*$^{iCre/+}$*:R26R*^{tdT}-labeled cell lineage was not enriched for smooth muscle-, endothelial- or cardiomyocyte-specific genes (Fig. 5L). Although *Pdgfrb* expression was enriched in the lineage-traced population, we believe this is the result of a tagged population present in the ventricular septal cells, which also express Tcf21 (supplementary material Fig. S7). We then compared this gene expression profile with that of GFP^{+} cells from hearts expressing the *Col1GFP* transgene and *Pdgfra*$^{GFP/+}$. Analysis of the same set of candidate genes using RNA isolated from FACS GFP^{+} cells revealed a very similar pattern of enrichment for cardiac fibroblast genes (Fig. 5K,L), allowing us to conclude that lineage tracing with the *Tcf21*^{iCre} lineage-tagged cells at E14.5 marks cardiac fibroblasts.

To determine whether Tcf21-expressing cells are predisposed to acquiring a cardiac fibroblast fate, we traced cells before epicardial EMT at E10.5. Tamoxifen induction at this time point resulted in tagging of the entire epicardium (supplementary material Fig. S2I), and the fate of these cells at E18.5 was determined. Unlike Tcf21-expressing cells tagged at E14.5, which were primarily excluded from SMCs, at E10.5, a fraction of *Tcf21*^{iCre}*;R26R* labeled cells overlapped with the Pdgfrβ^{+} and SM22α^{+} populations (Fig. 5G,H; supplementary material Fig. S5E-H,M-P), indicating that cells expressing Tcf21 at this earlier time point (a time point prior to epicardial EMT) retained the potential to differentiate into cVSMCs and were not committed to the cardiac fibroblast lineage. E10.5 lineage-traced cells also differentiated into cardiac fibroblasts based on overlap with GFP^{+} cells expressing *Pdgfra*$^{GFP/+}$ allele or the *Col1GFP* transgene (Fig. 5I-J; supplementary material Fig. S6E-H). These lineage-tracing experiments suggest that early in development, Tcf21-expressing epicardial cells retain the ability to give rise to either of the two epicardial-derived cell fates. However, as development proceeds, they become restricted to the fibroblast lineage. One report suggesting that epicardial cells can contribute to the cardiomyocyte population, found that they did so only before E11.5 (Cai et al., 2008). When we examined E10.5 lineage-traced cells, we found no overlap of traced cells with the cardiomyocyte marker Acta1 (Fig. 6E-H). In addition, when we co-stained for the endothelial marker PECAM, no overlap was observed (Fig. 5F). This result is in agreement with recent reports demonstrating that coronary vascular endothelial cells are derived from the septum transversum (Red-Horse et al., 2010) and other cell populations within the proepicardium (Katz et al., 2012). Thus, it appears that Tcf21 epicardial progenitors, at the time points we examined, do not contribute to the cardiomyocyte or endothelial lineage.

Temporal loss of Tcf21 expression in cells destined to become cVSMC

As shown earlier (Fig. 2A-I), epicardial cells with *Tcf21* promoter activity that lack Tcf21, are severely impaired in epicardial EMT. To examine the fate of *Tcf21* null cells, we utilized lineage tracing in the presence and absence of Tcf21 at different gestational ages

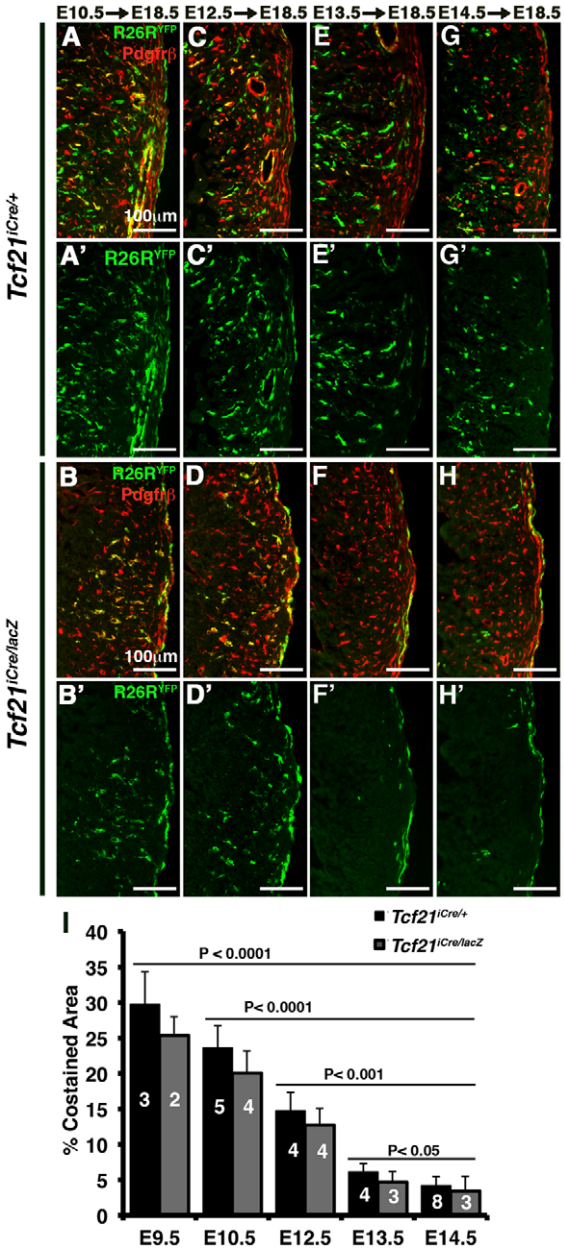

Fig. 7. Lineage tracing with *Tcf21*^{iCre} in null hearts. (**A-H′**) Lineage tracing in E18.5 control (*Tcf21*$^{iCre/+}$) and null (*Tcf21*$^{iCre/lacZ}$) embryonic hearts following Cre induction at indicated ages. Panels A′-H′ represent lineage-traced cells showing *R26R*^{YFP} activity (in green), and co-staining with the VSMC marker Pdgfrβ (red) is shown in panels A-H. (**I**) Quantification of percentage of migrated lineage-traced cells that are Pdgfrβ$^+$ in A-H. Percentage of overlap between YFP$^+$ (green) and Pdgfrβ$^+$ (red) area was determined relative to the total Pdgfrβ$^+$ area using Image J software. The epicardium was excluded from this analysis. An average of four sections per heart were utilized for the quantification and *n* values are indicated on the bars. Data shown is mean ± s.d. and *P* values represent statistical significance of differences between E14.5 and each of the earlier time points indicated for the same genotype. Differences noted between control and null hearts of the same time point were not statistically significant.

(Fig. 7A-H′). Cells expressing Tcf21 were followed using one-day inductions (supplementary material Fig. S8). Cre recombinase was induced around the time of epicardial EMT, and Tcf21 lineage-traced cell fates were examined at E18.5. In the controls, tamoxifen induction at E10.5 (a time point prior to EMT) resulted in significant labeling of the epicardium, in addition to cells that had migrated into the ventricle (Fig. 7A,A′). By contrast, although *Tcf21*$^{iCre/lacZ}$ showed similar tagging of the epicardium, a reduced number of cells had migrated into the myocardium (Fig. 7B,B′,I). Similar results were obtained following Cre induction at E12.5, although by this time, the number of cells within the ventricle was further reduced compared with E10.5 Cre induction (Fig. 7C-D′,I). Strikingly, almost all of the *Tcf21*$^{iCre/lacZ}$ labeled cells were Pdgfrβ$^+$, indicating that null cells with *Tcf21* promoter activity before E12.5 can become VSMCs.

Next, we traced the fate of null cells at the time of (E13.5) and after (E14.5) epicardial EMT (Fig. 7E-H′). Whereas control hearts possessed labeled EPDCs in the ventricle similar to Cre induction at earlier time points (Fig. 7E,E′,G,G′), *Tcf21*$^{iCre/lacZ}$ littermate hearts had almost no migrated cells within the ventricle when induced at E13.5 and later (Fig. 7F,F′,H,H′). We then quantified the percentage of migrated cells that were Pdgfrβ$^+$ and lineage tagged (Fig. 7I). Starting at E13.5, the lack of migrated cells in the null hearts was concomitant with a significant decline in the number of Pdgfrβ$^+$ cells that were lineage tagged in the control hearts. In addition, the Tcf21 lineage-tagged cells remained in the epicardium, as seen by the increased number of labeled cells in the null epicardium (supplementary material Fig. S3D), suggesting impaired EMT. The overall epicardial cell numbers between control and null hearts appeared unchanged (supplementary material Fig. S3E). Taken together with the observation that lineage-traced null cells before E12.5 also give rise to cVSMCs, these data suggest that by E13.5, Tcf21 lineage-traced epicardial cells are committed to the fibroblast lineage. More importantly, the defect in migration of the cardiac fibroblast population in the null hearts did not result in a fate switch. As shown in Fig. 7I, the proportion of migrated Pdgfrβ$^+$ cells relative to the entire labeled population in control and null hearts was comparable throughout the entire time course, indicating no increase in the cVSMC population. In conclusion, these data strongly support an essential role for Tcf21 in cardiac fibroblast lineage-specific EMT and demonstrate that EPDC lineage commitment precedes the initiation of EMT and migration of cells into the myocardium.

DISCUSSION

Although it has been established that the epicardium is the main source of resident cardiac fibroblasts (Dettman et al., 1998; Gittenberger-de Groot et al., 1998; Vrancken Peeters et al., 1999; Cai et al., 2008; Zhou et al., 2010; Smith et al., 2011), the molecular mechanisms that govern the lineage diversification of the multipotent epicardial progenitors are not understood. In the present study, we have uncovered a lineage-specific role for Tcf21 in epicardial development (Fig. 8). Our results demonstrate that in the absence of Tcf21, cardiac fibroblast progenitors remain in the epicardium and fail to undergo EMT. Furthermore, we show that, initially, the Tcf21-expressing cell population is multipotent with the ability to differentiate into either cardiac fibroblasts or cVSMC. As the epicardium develops, a subpopulation of these cells lose Tcf21 expression and either become cVSMC or remain as undifferentiated epicardium.

Recent studies utilizing Cre drivers under the control of *Tbx18* and *Wt1* cis-regulatory elements suggest that epicardial progenitors contribute to the cardiomyocyte lineage (Cai et al., 2008; Zhou et al., 2008). This observation is a matter of debate as even low levels of Cre in the cardiomyocyte lineage can result in Cre reporter-expressing cells (Christoffels et al., 2009). Our results suggest that

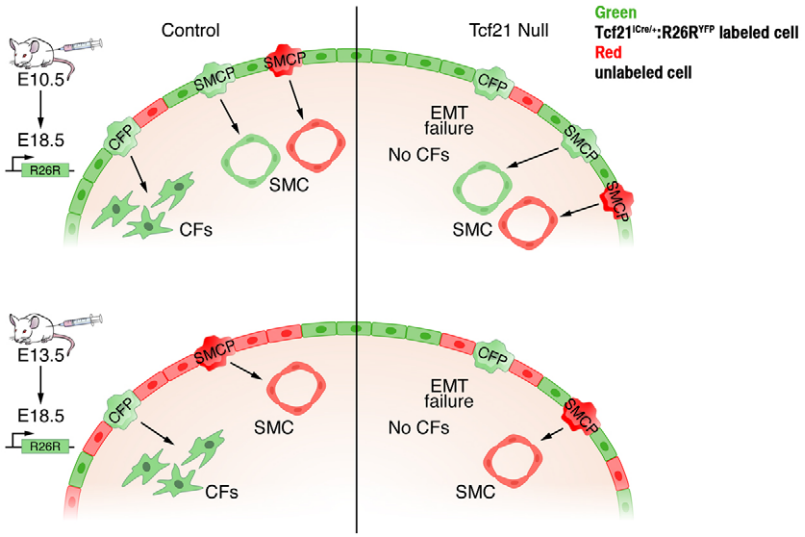

Fig. 8. Proposed function of Tcf21 in epicardial progenitor cell fate determination. Prior to epicardial EMT (E10.5), Tcf21-expressing progenitors (in green) can develop into either cardiac fibroblasts (CFs) or SMCs. As Tcf21 is essential for EMT of the cardiac fibroblast progenitors, null hearts lack cardiac fibroblasts, but *Tcf21*^{iCre} reporter-labeled cVSMCs are detected. Around E13.5, Tcf21 expression is downregulated in smooth muscle cell progenitors (SMCPs) but is maintained in cardiac fibroblast progenitors (CFPs). Labeled cells in wild-type hearts form predominantly cardiac fibroblasts, but in *Tcf21* null hearts the cardiac fibroblast progenitors remain in the epicardium. SMC, smooth muscle cell.

Tcf21-expressing epicardial cells do not contribute significantly to the cardiomyocyte lineage at the embryonic time points examined. This does not completely rule out an epicardial origin for cardiomyocytes as we are only examining the Tcf21-expressing component of the epicardium. Here, we have demonstrated heterogeneity of epicardial cells with regard to their cell fate, therefore it is possible that a small component of the epicardium never expresses Tcf21 and is therefore excluded from our tagged population.

There are two competing models regarding the timing of epicardial cell fate determination. The first suggests that EPDC fates are determined at the stage of the proepicardium (Mikawa et al., 1992; Mikawa and Gourdie, 1996). Using a replication-defective retrovirus, individual cells of the proepicardium were marked and the resulting labeled cell types examined. Analysis of hearts bearing clones presumably derived from a single cell demonstrated that only one cell type was present in those clones, either cVSMC or endothelial cells. No clones had a mixed population of cell types, suggesting that the fate of cells within the proepicardium was already determined. The second model argues that EPDC cell fates are not determined until the EPDCs reach the vasculature and that the epicardium comprises multipotent progenitors. These conclusions were drawn after transplanted avian epicardial cells gave rise to both cVSMC and interstitial cells (presumably cardiac fibroblasts) (Dettman et al., 1998).

One reason for the difficulty in reaching a consensus regarding EPDC fate determination is that the tools for unambiguously labeling and identifying the fibroblast cell type have not existed. In addition, there is still a debate regarding the origin of the coronary vascular endothelial cells. If these cells are from an origin other than the proepicardium or epicardium, as has been recently suggested (Red-Horse et al., 2010; Zhou et al., 2010), any interpretation regarding multipotency that includes this cell type could potentially be misleading. Here, we show that the determination of EPDC fate occurs within the epicardium around the time of EMT, wherein cells become progressively restricted to a single cell fate. Interestingly, this specification does not occur at a discrete time point or location but, instead, appears to be a gradual loss of multipotency that begins as early as E10.5.

These data do not rule out the possibility that a population of cells might be determined as early as the proepicardium stage, nor do they show that all cells have lost their multipotency. They simply show that the majority of cells becomes restricted to a specific lineage by E13.5. Further evidence that this critical time window occurs before the process of EMT is that fibroblast-fated cells never exit the epicardium in the *Tcf21* null. If fate determination occurred after EMT, one would expect to see undifferentiated cells within the myocardium or possibly an increase in cVSMC. Neither of these scenarios occurs. Instead, *Tcf21* null cells destined to be fibroblasts are retained within the epicardium.

The failure of only the cardiac fibroblast lineage to undergo EMT presents an intriguing circumstance, in which epicardial EMT might be driven by the acquisition of a mesenchymal gene program that is directly linked to differentiation. If differentiation drives EMT, this would be a unique mode of EMT progression. In most systems of EMT, the specific type of the mesenchymal cell (smooth muscle or fibroblast, for example) is not evaluated. This is because the mesenchymal cell phenotype during the evaluation of EMT is usually defined by a generic examination of gene expression, which includes EMT transcription factors such as Twist and Snail, and cytoskeletal elements including vimentin, αSMA and filamentous actin expression (Thiery and Sleeman, 2006). Arguably, many of these will be expressed by a cVSMC or a cardiac fibroblast. Thus, the possibility exists that differentiation or loss of multipotency precedes epicardial EMT. Currently, it is difficult to separate differentiation from EMT because we have not identified any unambiguous marker that is expressed in the epicardium by a lineage-committed cardiac fibroblast. Indirect evidence that *Tcf21* null epicardium has disrupted cardiac fibroblast lineage commitment is that expression of a cardiac fibroblast marker, such as Pdgfrα, is lost. When we generated *Tcf21* null hearts with one copy of the *Pdgfra*^{GFP} reporter, which is expressed in the epicardium and cardiac fibroblast progenitors (Hamilton et al., 2003; Smith et al., 2011), GFP expression was lost within the epicardium after E12.5. In fact, no GFP-expressing cells were present within the ventricular myocardium (A.A. and M.D.T., unpublished). This observation could indicate that the gene expression program of the cardiac fibroblast is disrupted in the *Tcf21* null. This is consistent with the idea that cardiac fibroblast progenitor differentiation program occurs within the epicardium before EMT.

Identification of transcription factors that drive fibroblast differentiation has been difficult. One possible reason for this is there are different developmental sources for these cells, and each organ

could have its own unique gene program to drive differentiation of its specific fibroblast population (Souders et al., 2009). As stated above, another difficulty is the lack of distinct markers for these cells. Very few markers are unique to fibroblasts, and often none of these markers are constantly expressed by cardiac fibroblasts. Often an activated fibroblast is referred to as a myofibroblast with the predominant molecular markers being αSMA and vimentin (Santiago et al., 2010; Rohr, 2011). These, of course, are also expressed by other mesenchymal cells, including VSMC. In fact, only one transcription factor has been described with a potential role in cardiac fibroblast function (Takeda et al., 2010). Cardiac fibroblast specific deletion of this transcription factor, *Kruppel-like factor 5* (*Klf5*), suppresses cardiac fibrosis and hypertrophy elicited in response to pressure overload injury. Targeted deletion of *Klf5* is embryonic lethal, thus limiting the investigation into a potential role in fibroblast development and differentiation. However, the study by Takeda et al., did suggest a likely role of this transcription factor in modulating fibroblast proliferation.

Tcf21 is expressed in the mesenchyme of many different tissues (Lu et al., 1998; Robb et al., 1998; Quaggin et al., 1999), and chimeric analysis has suggested a cell autonomous role for Tcf21 in the differentiation of stromal cells into peritubular interstitial cells and pericytes. However, lack of specific markers made it difficult to determine whether there was a defect in differentiation of one lineage over another (Cui et al., 2004). In other tissues, Tcf21 might direct gene programs distinct from that of the fibroblast. For example, Tcf21, along with a closely related gene (*MyoR*; *Msc* – Mouse Genome Informatics), is expressed in facial skeletal muscle and regulates specification of a subset of head muscles (Lu et al., 2002). Therefore, identification of Tcf21 targets within the multipotent epicardial progenitors, as well as the committed fibroblasts, would provide important details regarding this elusive cell population. These investigations are now possible given the information and reagents described here.

Based on its expression pattern, Tcf21 is likely to be a competency factor for cardiac fibroblast formation. Because it is initially expressed in all epicardial cells, it cannot be the sole factor directing cardiac fibroblast specification. Other transcription factors must interact either directly or indirectly with Tcf21 to control differentiation of the fibroblast lineage. Another example of this pattern of transcriptional regulation in lineage specification of multipotent progenitors is observed in the pancreas. In mice, the transcription factor Pdx1 (pancreatic and duodenal homeobox 1) is expressed in all of the progenitors of the pancreatic lineages: exocrine, endocrine and ductal. However, after E12.5 following a series of binary cell fate-mediated lineage specifications, Pdx1 expression is restricted to and required in islet β cells (Jensen, 2004).

It is proposed that EPDCs recapitulate their embryonic programs during disease and, thus, could potentially serve as an adult stem cell population for cardiac regeneration (Winter and Gittenberger-de Groot, 2007). If reactivation of the embryonic program is indeed one of the major mechanisms of myocardial regeneration and neovascularization following injury, then a better understanding of factors that lead to undesirable fibrosis would certainly contribute to better therapeutic outcomes.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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