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Differential expression of embryonic epicardial progenitor markers and localization of cardiac fibrosis in adult ischemic injury and hypertensive heart disease

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Abstract

During embryonic heart development, the transcription factors Tcf21, Wt1, and Tbx18 regulate activation and differentiation of epicardium-derived cells, including fibroblast lineages. Expression of these epicardial progenitor factors and localization of cardiac fibrosis was examined in mouse models of cardiovascular disease and in human diseased hearts. Following ischemic injury in mice, epicardial fibrosis is apparent in the thickened layer of subepicardial cells that express Wt1, Tbx18, and Tcf21. Perivascular fibrosis with predominant expression of Tcf21, but not Wt1 or Tbx18, occurs in mouse models of pressure overload or hypertensive heart disease, but not following ischemic injury. Areas of interstitial fibrosis in ischemic and hypertensive hearts actively express Tcf21, Wt1, and Tbx18. In all areas of fibrosis, cells that express epicardial progenitor factors are distinct from CD45-positive immune cells. In human diseased hearts, differential expression of TCF21, WT1, and TBX18 also is detected with epicardial, perivascular, and interstitial fibrosis, indicating conservation of reactivated developmental mechanisms in cardiac fibrosis in mice and humans. Together, these data provide evidence for distinct fibrogenic mechanisms that include Tcf21, separate from Wt1 and Tbx18, in different fibroblast populations in response to specific types of cardiac injury.

Keywords

Heart disease; Fibrosis; Epicardium-derived cells; Tcf21; Wt1; Tbx18; Mouse; Human

1. Introduction

Cardiac fibrosis, the accumulation of fibrillar collagen in the heart, contributes to the progression of cardiovascular disease (CVD) towards heart failure, which is a leading cause of death in the United States [1, 2]. Fibrotic remodeling is induced following various cardiac insults and can lead to compromised heart function after myocardial infarction (MI) or with

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Disclosure statement

There are no conflicts to disclose.

Appendix A. Supplementary Information

hypertensive heart disease (HHD) [2, 3]. Recent evidence suggests that not all fibrosis is the same, in that different types of cardiac injury or disease lead to variable levels of fibrillar collagen deposition in different regions of the heart [4]. Following MI, the fibrotic response initially compensates for the injury with the formation of a scar, concurrent with a transient inflammatory response [5, 6]. Subsequently, myocardial remodeling following MI becomes maladaptive with diffuse extracellular matrix (ECM) deposition throughout the heart [3]. With HHD, the hemodynamic pressure overload causes pervasive activation of myofibroblasts and inflammation throughout the heart, which leads to myocardial stiffening and ischemia secondary to excessive ECM deposition [7]. The molecular and cellular mechanisms regulating differential accumulation of fibrillar collagen in different types of CVD are unknown.

During embryonic heart development, the transcription factors Tcf21, Wilms' tumor 1 (Wt1), and Tbx18 are expressed in the epicardium and epicardium-derived cells (EPDCs) [8-11]. EPDCs include progenitors of cardiac fibroblasts and vascular smooth muscle (SM) cells, and the epicardial transcription factors Tcf21, Wt1, and Tbx18 have distinct and critical functions in these EPDC lineages [8, 10, 12-15]. The bHLH transcription factor Tcf21 (Pod1/Epicardin/Capsulin) promotes cardiac fibroblast development and inhibits SM differentiation of EPDCs [8, 12]. The zinc finger transcription factor Wt1 is required for epicardial epithelial-mesenchymal transition (EMT), is expressed in undifferentiated EPDCs, and is downregulated during differentiation of fibroblast and SM epicardial derivatives [11, 16]. The T-box transcription factor Tbx18 also is expressed in embryonic EPDCs but is not required for epicardial activation or lineage maturation during embryonic heart development [10, 17, 18]. Epicardial reactivation of Wt1 and Tbx18 following MI has been reported, but the functions of these embryonic epicardial progenitor transcription factors in adult heart disease have not yet been determined [19, 20]. Less is known regarding adult expression and function of Tcf21, which is required for prenatal fibroblast development [8, 12]. In addition, it is not known if EPDC regulatory programs are activated with the development of cardiac fibrosis in different regions of the heart in response to specific pathologic conditions.

In order to investigate activation of EPDC developmental programs in regionalized cardiac fibrosis in adult CVD, expression of epicardial progenitor transcription factors was examined in mouse models of ischemic or hypertensive heart disease and in human diseased hearts. Differential accumulation of cardiac fibrosis was observed with predominant epicardial fibrosis apparent after ischemia/reperfusion (I/R), in contrast to predominant perivascular fibrosis resulting from hypertensive thoracic aortic constriction (TAC) or Angiotensin II (AngII) infusion. Tcf21, Wt1, and Tbx18 all are expressed in the thickened fibrotic layer of subepicardial cells following I/R injury in mice. In contrast, Tcf21, but not Wt1 or Tbx18, is expressed in perivascular fibrosis resulting from HHD. Tcf21, Wt1, and Tbx18 also are expressed in interstitial fibrotic regions of the heart in mouse models of ischemic or hypertensive heart disease. In diseased human hearts, differential progenitor transcription factor expression also is detected in regionalized cardiac fibrosis, indicating conservation of epicardial progenitor transcription factor expression during fibrogenesis in mouse and human CVD.

2. Materials and methods

2.1 Mice

16-week-old FVBN mice heterozygous for *Tcf21LacZ* [8, 21] were subjected to transverse aortic constriction (TAC) to produce pressure overload [22, 23]. Sham surgery performed on *Tcf21LacZ* heterozygous mice entailed sternotomy alone. 10-week-old FVBN mice heterozygous for *Tcf21LacZ* and wild type littermates were subjected to cardiac ischemia/

reperfusion (I/R) injury [24, 25]. Alternatively, hypertensive cardiac remodeling was induced by chronic AngII infusion as previously described [22, 23]. Mice were sacrificed by CO₂ asphyxiation, hearts were collected for histology or immunostaining, and heart weight/body weight ratios were recorded at the time of collection.

All animal procedures were approved by the Cincinnati Children's Hospital Medical Center Institutional Animal Care and Use Committee and performed following institutional guidelines.

2.2 Histology and morphometry

Hearts were isolated, fixed, dehydrated, paraffin-embedded, and sectioned as previously described [26]. Histological sections (5 μ m) were analyzed for fibrosis by Masson's trichrome staining using the Masson's Trichrome 2000 kit per manufacturer's instructions (American Mastertech). Images were obtained using a Nikon SMZ1500 microscope, DXM1200F digital camera, and NIS-Elements D 3.2 software. Quantification of fibrosis was calculated using NIS-Elements Basic Research software (Nikon).

Epicardial thickness was quantified as described previously [27]. Briefly, epicardial thickness, defined as the epicardial epithelial cell layer together with intervening subepicardial cells overlying the myocardium, was measured perpendicular to the ventricular surface in comparable trichrome-stained sections of diseased and control hearts. The epicardium overlying the infarct was excluded from analysis, but the border zone and the remote left ventricle (LV) and right ventricular (RV) epicardium were included in the quantifications. Five measurements of epicardial thickness were obtained per image. The average epicardial thickness was calculated per image and used for statistical analysis. 3-5 regions (images) of the ventricular surface were analyzed per section. For each heart, the average epicardial thickness was calculated for three sections, each separated by at least 20 μ m. Approximately the same mid-ventricular position of the LV and RV was analyzed in each heart.

Perivascular fibrosis was quantified as described previously [28]. Briefly, following trichrome staining, images of intramyocardial coronary blood vessels ranging from 50 to 200 μ m in diameter were obtained. Adventitial (perivascular) fibrosis was identified by blue fibrillar collagen staining. Blood vessel smooth muscle (tunica media) was identified by red staining. The area of adventitial fibrosis was calculated by subtracting the area of the blood vessel (lumen + smooth muscle) from the area of the adventitia + blood vessel, using NIS-Elements Basic Research software (Nikon). In order to normalize for vessel size, a ratio of adventitial area to vessel area was calculated. This ratio represents normalized perivascular fibrotic area. Oblique or non-round blood vessels were excluded from analysis. In each heart, 8-28 images of intramyocardial coronary arteries were measured.

Epicardial thickness and area of perivascular fibrosis were measured and analyzed in at least three hearts per treatment group ($n=3-6$). Statistical significance was determined by Student's *t*-test with $P < 0.01$. Data are reported as mean with standard error of the mean (s.e.m.).

2.3 X-Gal staining

X-Gal staining was performed as previously described with modifications [29]. Briefly, hearts were harvested, snap frozen, cryosectioned (10 μ m), fixed in 4% paraformaldehyde (PFA) for 10 min on ice, and washed in PBS 4 $\times$ 5 min at room temperature. Sections were incubated overnight at 37°C in X-Gal staining solution [1 mg/ml X-Gal (Invitrogen, 15520-018), 5 mM K-Ferricyanide/K-Ferrocyanide, 0.02% Igelal, 0.01% deoxycholate, and

2 mM MgCl₂ in 0.1 M PBS]. Sections were washed in PBS 4 × 15 min, post-fixed in 4% PFA for 30 min on ice, washed in PBS, and coverslipped.

2.4 Immunofluorescence

Antibody labeling for immunofluorescence (IF) after antigen retrieval was performed as previously described [8]. Briefly, antigen retrieval was performed in boiling citric acid based antigen unmasking solution (1:100, Vector Laboratories) for 5 min under pressure. The following primary antibodies were used as previously described: βGalactosidase (βGal) (Abcam, ab9361), WT1 (CalbioScience, CA1026), TCF21/POD1 (Santa Cruz Biotechnology, sc-32914), TBX18 (Santa Cruz, sc-17869), α-Smooth Muscle Actin (αSMA) (Sigma Aldrich, IMM12-1KT), and Smooth Muscle Myosin (Myh11) (Biomedical Technologies, BT-562) [8]. Immunostaining for Vimentin (Abcam, ab45939) was performed as described in [30]. Primary antibodies against Collagen Type III (Rockland Immunochemicals, 600-401-105 [1:100]) and CD45 (R&D Systems, AF114 [1:200]) were used. Corresponding Alexa conjugated secondary antibodies (Invitrogen) were applied as previously described [31]. Alternatively, Renaissance Tyramide Signal Amplification Plus Tetramethylrhodamine kits (Perkin Elmer) were used as described previously [32]. For CD45 antibody labeling, biotinylated donkey anti-goat (Santa Cruz, sc-2042 [1:200]) and Alexa conjugated streptavidin secondary [1:500] antibodies were used. Nuclei were counter stained with 4', 6-diamino-2-phenyl-indole, dihydrochloride (DAPI) (Invitrogen [1:10,000]).

IF was detected using a Nikon A1-R LSM confocal microscope. For each experiment, images were captured with NIS-Elements D 3.2 software in parallel using identical confocal laser settings, with constant PMT filters and integration levels.

2.5 Quantification of immunoreactivity

Confocal images obtained after IF were used to quantify transcription factor expression in mouse heart sections. The number of cells expressing each marker was quantified using NIS-Elements D 3.2 software. Single-channel images were used, a specific threshold value was set, and expression above this threshold value was used to quantify the number of cells expressing each antigen, including βGal, Tcf21, Wt1, and Tbx18. Positive nuclei were counted in the epicardium and subepicardial mesenchyme, in the perivascular adventitia, and within the myocardial interstitium. The number of expressing cells per field was determined for 2-3 fields from 3-6 sections per heart (n=3-6).

2.6 Human cardiac specimens

Human diseased cardiac specimens were procured postmortem from the National Disease Research Interchange (NDRI, Philadelphia, PA). Donors diagnosed with congestive heart failure (CHF) were requested, and donor age ranged from 71-91 years old. Diagnostic data provided by the NDRI included CHF, hypertension, and coronary artery disease, as described in Table S1. Ventricular myocardium was fixed in 10% formalin prior to shipment by the NDRI. Explants from epicardial, interstitial, and endocardial regions (1 cm × 1 cm × 3 mm) were isolated from the LV and RV myocardium, dehydrated through ethanol and xylene, and paraffin embedded. Sections (5 μm) were processed and analyzed using Masson's trichrome staining and IF as described above. Anti-WT1 (Santa Cruz, sc-192 [1:100]) and anti-TBX18 (R&D Systems, MAB63371 [10 μg/ml]) antibodies were used on human sections for IF analysis. Immunoreactivity for each transcription factor was confirmed in tissue sections from at least 3 donors (n=3). Institutional Review Board exemption and permission to perform these studies were approved by Cincinnati Children's Hospital Medical Center.

2.7 Statistical analysis

Statistical significance was determined by Student's *t*-test with $P < 0.01$ or $P < 0.05$ as indicated. Data are reported as mean with standard error of the mean (s.e.m.).

3. Results

3.1 Differential localization of cardiac fibrosis in mouse models of ischemic and hypertensive heart disease

Mice were subjected to I/R, TAC, or chronic AngII infusion, all of which lead to cardiac fibrosis and heart failure [3, 33-35]. The location and extent of cardiac fibrosis for ischemic (I/R) and hypertensive (TAC and AngII) models of heart failure were evaluated by histological analysis of sectioned hearts after Masson's trichrome staining (Figs. 1, S1). Epicardial, perivascular, and interstitial regions of the LV myocardium were examined in detail at histological and cellular levels in order to evaluate differences in the fibrogenic response (fibrogenesis) induced in distinct types of heart disease. Epicardial and perivascular fibrosis, defined as collagen accumulation in the adventitia of coronary arteries, were quantified from trichrome-stained sections of hearts for each experimental model.

Epicardial fibrosis was examined in hearts from mice subjected to I/R, TAC, or AngII-induced injury. In hearts subjected to 24-hour ischemia followed by 7 days reperfusion, fibrillar collagen is apparent on the surface of the heart in the thickened epicardium located away from the scar (Figs. S1B boxed region; 1B, arrowhead). Increased epicardial fibrosis and epicardial thickening also are apparent overlying remote right ventricle myocardium (data not shown). In contrast to I/R injury, fibrillar collagen and epicardial thickness are comparable to quiescent sham epicardium (Figs. S1A; 1A) following 14 days of TAC-induced pressure overload (Figs. S1C; 1C) or AngII-induced hypertensive cardiac remodeling (Figs. S1D, 1D). Morphometric analysis demonstrates increased fibrillar collagen on the surface of I/R hearts, indicated by increased epicardial thickness, while the epicardial thickness of TAC and AngII hearts is unchanged relative to sham (Fig. 1M). Thus ischemic injury, but not pressure overload or hypertension, specifically promotes the epicardial fibrotic response in the adult mouse heart.

Fibrillar collagen accumulates around the coronary vasculature in TAC (Figs. S1C, 1G, arrowhead) and AngII-treated hearts (Figs. S1D, 1H, arrowhead), indicative of perivascular fibrosis in models of pressure overload and hypertensive cardiac remodeling. By contrast, excluding the infarct scar, fibrillar collagen surrounding the coronary vessels does not accumulate 7 days post-I/R (Figs. S1B, 1F). Morphometric analysis indicates increased collagen deposition surrounding coronary vessels in TAC and AngII-treated hearts, but no change in vessels in I/R hearts relative to sham (Fig. 1N). In addition fibrillar collagen accumulates within the myocardial interstitium in all three disease models (Fig. 1J-L, arrowheads).

3.2 Tcf21 expression is activated with epicardial, perivascular, and interstitial fibrogenesis in ischemic or hypertensive heart disease

Expression of embryonic epicardial progenitor transcription factors was examined in adult cardiac fibrosis differentially localized in the diseased myocardium and resulting from distinct types of cardiac injury. In particular, we sought to determine if Tcf21, required for cardiac fibroblast differentiation during development [8, 12], also is active in adult fibrotic heart disease. In order to determine if Tcf21 expression is induced following different types of cardiac injury, adult mice heterozygous for the *Tcf21*LacZ knock-in reporter allele were subjected to I/R or TAC [21]. Histological analysis was performed on sectioned mouse hearts using X-Gal staining to visualize Tcf21βGal expression in epicardial, perivascular and

myocardial interstitial regions of fibrogenesis. In normal adult hearts, Tcf21 β Gal is expressed in a small subpopulation of epicardial cells (Fig. 2A, arrowhead), and baseline Tcf21 β Gal expression is detected in a subset of perivascular (Fig. 2D) and interstitial cells (Fig. 2G). Seven days after I/R injury, Tcf21 β Gal expression is increased on the surface of the heart (Fig. 2B, arrowheads) and in the myocardial interstitium (Fig. 2H) where fibrosis is induced. Epicardial Tcf21 β Gal expression also is increased one day after I/R, prior to the detection of fibrosis (Fig. S2). Thus Tcf21 β Gal expression is activated in the epicardium prior to fibrosis in response to I/R injury.

In contrast to epicardial activation, Tcf21 β Gal expression is similar to baseline in regions surrounding coronary vessels of I/R hearts (Fig. 2E) where fibrosis is not apparent. Following TAC-induced pressure overload, increased Tcf21 β Gal expression is apparent surrounding coronary vessels (Fig. 2F) and in the interstitium (Fig. 2I) where fibrosis occurs in this model. However, epicardial Tcf21 β Gal expression is similar to baseline expression (Fig. 2A) in mice subjected to TAC (Fig. 2C), consistent with the lack of a fibrogenic response on the surface of the heart in these animals. Therefore, increased Tcf21 β Gal expression coincides with the differential localization of fibrosis (Fig. 1) in I/R versus TAC mouse models of heart disease. Together, these data demonstrate that Tcf21 expression is activated during fibrogenesis following injury.

3.3 Tcf21, Wt1, and Tbx18 are expressed in subepicardial cells induced with cardiac ischemic injury

The transcription factors Tcf21, Wt1, and Tbx18 are expressed in overlapping and distinct subsets of epicardial cells and EPDCs during cardiac development [8, 19, 36]. To determine if epicardial progenitor markers are induced in the context of cardiac fibrosis, double antibody immunofluorescent labeling and confocal microscopy were performed using β Gal, Tcf21, Wt1, and Tbx18 specific antibodies on sectioned hearts from mice subjected to I/R (ischemic injury) or TAC (pressure overload). Tcf21 β Gal is expressed in the thickened layer of subepicardial cells on the heart surface 7 days after I/R injury (Fig. 3B, D). Wt1 is co-expressed with Tcf21 β Gal (Fig. 3B, arrowheads, E) in some, but not all, cells in the subepicardium following I/R consistent with colocalization of these factors in subpopulations of EPDCs in the developing embryo [8]. Likewise, endogenous Tcf21 (Fig. 3G, I) and Tbx18 (Fig. 3K, M) also are expressed in activated subepicardial mesenchymal cells on the surface of the heart after I/R. Thus, Tcf21, Wt1, and Tbx18 are expressed in overlapping and distinct populations of subepicardial mesenchymal cells following I/R, similar to their expression during embryonic development [8]. In contrast, in hearts subjected to TAC, Tcf21 β Gal and Wt1 are expressed in sparse cells of the quiescent epicardium (Fig. 3C) similar to sham (Fig. 3A), and widespread expression on the heart surface and EPDC activation are not observed (Fig. 3D, E). Likewise, Tcf21 (Fig. 3H, I) and Tbx18 (Fig. 3L, M) are expressed at low baseline levels in the quiescent epicardium of TAC hearts, similar to sham (Fig. 3F, J). Together, these data indicate that ischemic injury leads to increased epicardial progenitor marker expression in subepicardial mesenchymal cells associated with epicardial fibrosis. However, epicardial fibrosis and progenitor marker transcription factor expression are relatively inactive with cardiac pressure overload after TAC.

3.4 Tcf21, but not Wt1 or Tbx18, is expressed in regions of perivascular fibrosis resulting from pressure overload

To determine if developmental mechanisms are reactivated in regions of perivascular fibrosis, expression of the epicardial progenitor markers Tcf21, Wt1, and Tbx18 was examined in mice subjected to cardiac TAC or I/R. In perivascular fibrotic regions in mouse hearts subjected to TAC, Tcf21 β Gal expression (Fig. 4C, D) is increased in regions of

adventitial fibrosis as indicated by the fibroblast markers Vimentin (Fig 4C) and Collagen type III (Col3) (Fig. S3C). In contrast, perivascular expression of Tcf21 β Gal in I/R injured hearts (Fig. 4B, D) is comparable to the sparse expression detected in sham-operated animals (Fig. 4A, arrowheads). As expected, endogenous Tcf21 protein expression also is increased in regions of perivascular fibrosis after TAC (Fig. 4G, H) or Ang-II infusion (data not shown), but not following I/R (Fig. 4F). In the developing heart, Tcf21 differentially promotes fibroblasts, while inhibiting SM cells [8]. However, Tcf21 expression is not highly induced in the remodeling coronary smooth muscle layer in hearts subjected to TAC (Fig. S3), suggesting that Tcf21 does not have a similar role in adult perivascular fibrogenesis. Strikingly, in contrast to Tcf21, expression of the epicardial progenitor markers Wt1 (open arrowheads, Fig. 4K, L) and Tbx18 (Fig. 4O, P) is not detected in regions of perivascular fibrosis following TAC or AngII infusion (data not shown). Thus, Tcf21 is induced in perivascular fibrosis after pressure overload, as well with epicardial fibrosis after I/R, in contrast to Wt1 and Tbx18, which are restricted to epicardial fibrosis and are not expressed in perivascular cardiac fibrotic disease.

3.5 Tcf21, Wt1, and Tbx18 are expressed in regions of interstitial fibrosis in mouse models of heart disease

To determine if embryonic epicardial progenitor markers are induced during interstitial fibrogenesis, expression of Tcf21, Wt1, and Tbx18 was examined in the fibrotic myocardial interstitium of adult mouse hearts following I/R or TAC. As determined by IF analysis, Tcf21 β Gal-positive cells (Fig. 5B,C) and Tbx18-positive cells (Fig. 5N,O) in the fibrotic interstitium are surrounded by Col3-positive extracellular matrix (ECM), indicating that Tcf21 β Gal and Tbx18 are expressed with induction of interstitial cardiac fibrosis. Tbx18-positive fibroblasts surrounded by Col3 also are detected in the control mouse interstitium (arrowhead, Fig. 5M), indicating that Tbx18-expressing fibroblasts are present in the fibrous matrix of a normal adult heart. Similar to Tcf21 β Gal and Tbx18, Tcf21 (Fig. 5F-H) and Wt1 (Fig. 5J-L) expression increases with interstitial fibrosis in I/R hearts relative to control hearts. Increased endogenous Tcf21 was not detected in hearts after TAC (Fig. 5H), but the distinction between Tcf21 β Gal and Tcf21 immunopositivity is likely due to the weakness of the Tcf21 antibody. Interestingly, interstitial Wt1 expression is increased with I/R (Fig. 5L), but not TAC, suggesting further selectivity in developmental transcription factors activation with specific cardiac lesions. Tcf21 and Wt1 are expressed in interstitial cells, but they are not colocalized with the myofibroblast marker α SMA, which suggests that Tcf21 and Wt1 are active in differentiated fibroblasts, not myofibroblasts, in the fibrotic interstitium. The prevalence of differentiated fibroblasts is consistent with more widespread expression of Col3 than α SMA in the fibrotic interstitium at 7 days post I/R or 14 days post-TAC (Fig. S4). Thus, expression of the epicardial progenitor markers Tcf21, Wt1, and Tbx18 is activated with induction of interstitial fibrogenesis in hearts subjected to TAC or I/R.

3.6 Infiltrating immune cells are distinct from cells expressing embryonic epicardial progenitor markers in differentially localized regions of cardiac fibrosis

The inflammatory response is crucial for scar formation and cardiac repair following injury [37]. As detected by IF analysis, CD45-positive immune cells are present in epicardial, perivascular, and interstitial regions of fibrosis indicated by Col3-positive fibrotic ECM mouse hearts 7 days post-I/R and 14 days post-TAC (Fig. S5). CD11b and CD68, which mark granulocytes and macrophages, respectively, are similarly expressed throughout the fibrotic epicardium of the I/R heart (data not shown). To determine if Tcf21, Wt1, and Tbx18 are expressed in the infiltrating immune cell population, double IF analysis was performed. Interestingly, CD45-positive immune cells, present throughout the fibrotic epicardium of the I/R heart, are distinct from the Tcf21-expressing EPDCs (Fig. 6B). A few CD45-expressing immune cells are detected on the epicardial surface of TAC or sham

hearts, but they are distinct from Tcf21-positive epicardial cells (Fig., 6A, C). CD45-positive immune cells also are detected in perivascular fibrotic regions in TAC hearts (Fig. 6F), but not surrounding coronary vessels in I/R or sham (Fig. 6E, D) where fibrosis is not apparent. Similar to the epicardium, the CD45-positive immune cells are distinct from the Tcf21-positive fibroblasts surrounding coronary vessels following pressure overload, as well as in the fibrotic interstitium (Fig. 6H, I) of hearts subjected to I/R or TAC. Therefore, in localized regions of cardiac fibrosis, the CD45-positive immune cell population is separate from the Tcf21-positive fibroblast population. In addition, Wt1-positive cells are distinct from CD45-positive immune cells in the epicardium and interstitium of I/R and TAC hearts (Fig. S6). Together these data indicate that embryonic epicardial markers are expressed in cell populations that are distinct from infiltrating inflammatory cells.

3.7 Fibrosis and transcription factor expression in human diseased myocardium from patients with CHF and hypertension

Myocardium samples from deceased donors aged 71 to 91 years, with a mean age of 81.8 years were obtained. Sample donors had histories of myocardial infarction (MI) ($n=2$), hypertension ($n=6$), and/or CHF ($n=2$) (Table S1). CHF tissue donors frequently presented with co-morbidities in addition to MI and hypertension, including cardiomyopathy, atrial fibrillation, coronary artery disease, and/or stroke. Aged-matched “control” donors, lacking a diagnosis of CHF, also presented with epicardial, perivascular, and interstitial fibrosis (Table S1), consistent with accumulation of cardiac fibrosis with age in epicardial, perivascular, and interstitial regions.

Fibrotic regions within each specimen were examined by histological and IF analyses. Fibrillar collagen accumulates in epicardial (Fig. 7A, J), perivascular (Fig. 7B, K), and interstitial regions (Fig. 7C, L) of human hearts, as detected by trichrome staining and COL3 IF analysis. A major difference noted between mouse and human hearts is that the subepicardial layer contains extensive adipose tissue (Fig. 7A) in humans but not mice [38]. Similar to mice, TCF21 (Fig. 7D), WT1 (Fig. 7G), and TBX18 (Fig. 7J) are expressed in the thickened epicardial layer on the heart surface in human CHF. Likewise, a few TCF21-expressing fibroblasts (arrowheads, Fig. 7E) are detected surrounding the hypertensive coronary artery. Notably, WT1 (Fig. 7H) and TBX18 (Fig. 7K) expression was not detected with perivascular fibrosis in hypertensive donors, consistent with the lack of perivascular expression in mice subjected to TAC. However, WT1 expression is apparent in the coronary vessel endothelium as noted previously [39]. Within the myocardial interstitium, TCF21, WT1, and TBX18 (Fig. 7F, I, L) are expressed in regions of interstitial fibrosis in diseased human hearts as was also observed in mice subjected to I/R or TAC. Compilation of all samples analyzed demonstrates that TCF21, WT1, and TBX18 are predominantly expressed in epicardial and interstitial regions of fibrosis, but that TCF21 is more prevalent in regions of perivascular fibrosis than WT1 or TBX18 (Table S2). These relative differences in epicardial progenitor marker expression in epicardial versus perivascular fibrosis is similar to that observed in mice subjected to TAC or I/R, supporting a conservation of fibrotic mechanisms in mammalian CVD.

4. Discussion

Here we show that fibrosis is differentially localized and that epicardial progenitor markers are differentially expressed with distinct types of cardiac injury. As shown in the model (Fig. 8), ischemic injury leads to accumulation of epicardial fibrosis on the surface of the adult mammalian heart, as indicated by fibrillar collagen (blue). In the I/R mouse model and in human CHF tissue, epicardial fibrosis is characterized by expression of Tcf21, Wt1, and Tbx18 in subepicardial mesenchymal cells. In contrast, pressure overload and HHD lead to

accumulation of perivascular fibrosis with increased expression of Tcf21, but not Wt1 or Tbx18, in mice subjected to TAC or AngII infusion. Similarly, Tcf21, but not Wt1 or Tbx18, is expressed in regions of perivascular fibrosis in human CVD, consistent with conservation of embryonic epicardial progenitor marker expression in fibrogenesis. Cardiac ischemic injury results in interstitial fibrosis with expression of Tcf21, Wt1 and Tbx18 in Col3-positive areas in mouse and human CVD. In contrast, interstitial Wt1 expression is not increased during pressure overload, demonstrating further fibroblast diversity with distinct types of cardiac injury. In addition, infiltrating immune cells positive for CD45 are separate from cells expressing Tcf21, Wt1, and/or Tbx18 in fibrotic regions in mouse models of CVDs. Together, these data indicate that multiple developmental mechanisms are differentially activated upon cardiac fibrotic remodeling. In addition, we provide evidence for distinct fibrogenic mechanisms that include Tcf21, distinct from Wt1 and Tbx18, in different fibroblast populations in response to specific types of cardiac injury.

Tcf21, Wt1, and Tbx18 are expressed in subepicardial mesenchyme following I/R, as well as in interstitial fibrosis resulting from ischemic injury or HHD. By contrast, Tcf21, but not Wt1 or Tbx18, is expressed in perivascular fibrosis in pressure-overloaded mouse hearts. Similar differential patterns of transcription factor expression also are observed in localized fibrosis in human CHF. During heart development Tcf21, Wt1, and Tbx18 all have been implicated in epicardial EMT necessary for generation of subepicardial progenitors of fibroblasts and smooth muscle cells [12, 40, 41]. After EMT, expression of Tcf21, Tbx18 and Wt1 is maintained in EPDCs as they invade the heart prior to differentiation into fibroblast and smooth muscle lineages [9]. Epicardial EMT also occurs after cardiac ischemic injury, and the presence of Tcf21, Wt1 and Tbx18 in the epicardium and subepicardial mesenchyme after I/R or MI supports a role for these factors in this process [20, 27, 41, 42]. Likewise all three factors also are expressed during interstitial fibrosis, but the activation mechanisms and origins of these cells are yet to be determined. Tcf21, unlike Wt1 and Tbx18, has been implicated specifically in differentiation of fibroblasts from EPDCs in the developing heart [8, 12, 43]. After cardiac injury, expression of Tcf21 is detected in epicardial, perivascular, and interstitial fibrosis, in contrast to more restricted expression of Wt1 and Tbx18. Thus, Tcf21 likely has a critical role in fibrosis in response to a variety of cardiac lesions and is a potential target for manipulation in fibrogenic progenitors that contribute to heart failure.

Reactivation of embryonic epicardial regulatory mechanisms has been demonstrated in animal models of MI and has been implicated in cardiac repair associated with increased angiogenesis or myogenesis after cardiac injury [19, 20, 27, 44-46]. However, a potential involvement of developmental regulatory mechanisms in cardiac fibrosis remains relatively unexamined. Since fibroblasts are the most abundant derivatives of the epicardium during development [47], the reactivation of EPDC progenitor markers on the heart surface and in activated populations within the heart may reflect regulatory roles in cell proliferation and differentiation of fibroblast precursor cells in adult fibrotic disease [20, 27]. In adult disease, cardiac fibrosis can contribute to heart failure but also is necessary to preserve cardiac function after certain types of injury. For example, fibrous matrix deposition is crucial during scar formation, as evident by increased mortality of *Periostin*^{-/-} mice with reduced fibrogenesis after MI [48]. Conversely, *Periostin*^{-/-} mice have improved cardiac function with decreased fibrosis following TAC-induced pressure overload [48], supporting the idea that there are distinct fibrogenic mechanisms in different types of CVD. Thus, the differential activation of epicardial progenitor gene expression in different regions of the heart with specific types of cardiac injury may contribute to adaptive and maladaptive mechanisms of cardiac remodeling.

The activation mechanisms and sources of cardiac fibroblasts in various types of cardiac disease are not well-characterized. During heart development, epicardium-derived mesenchymal cells on the surface of the heart invade the myocardium and contribute to fibroblast and smooth muscle lineages [15, 49]. However in adult ischemic heart disease, activated EPDCs arising from EMT do not invade the myocardium, but remain on the heart surface, as demonstrated by adenovirus-mediated lineage tracing [20, 27, 50]. Therefore, it is likely that interstitial and perivascular fibroblast progenitor cells detected in adult diseased hearts are not newly derived from the epicardium. Coronary vessel endothelial-mesenchymal transition (EndMT) has been reported as a potential source of perivascular fibroblasts, but these cells also could arise from activation of a resident adventitial population as occurs with atherosclerosis [51, 52]. In addition, Wt1 and Tbx18, required for EMT in the epicardium, are not expressed in adventitial fibroblasts surrounding coronary vessels, suggesting that perivascular fibrosis occurs via a mechanism distinct from epicardial fibrosis. Myeloid (bone marrow) derived cells and mesenchymal stem cells also have been implicated in cardiac fibrosis [52-55]. However, these cells appear to be distinct from cells expressing epicardial progenitor markers in epicardial, perivascular, and interstitial fibrosis and therefore are likely to contribute to distinct repair or pathologic mechanisms. Together, our data support the existence of different fibrogenic cell populations, distinct from immune cells, in the epicardium, adventitia, and interstitium, consistent with the idea that not all fibrosis is the same. Further studies are necessary to determine the specific mechanisms of embryonic epicardial marker gene induction and cellular origins of various populations of cardiac fibroblasts.

The differential localization of Wt1, Tbx18, and Tcf21 in epicardial, perivascular, and interstitial fibrosis is similar in mouse and human fibrotic heart disease, supporting the use of mouse models for the investigation of therapeutic approaches. The finding that epicardial progenitor markers gene expression is induced with cardiac injury in human and mouse systems could be exploited in the development of new therapeutics directed toward preventing and reversing cardiac fibrosis. The current treatments for CVD slow, but do not reverse, cardiac fibrosis and are not targeted specifically towards fibroblast activation mechanisms [56, 57]. Activated fibroblast progenitor cells in the epicardium, adventitia, and interstitium are likely to be of different origins and regulated by different fibrogenic mechanisms in response to ischemic or hypertensive heart disease. Thus development of targeted anti-fibrotic therapies for specific types of cardiac lesions seems necessary. Since epicardial developmental mechanisms are reactivated in adult fibrotic heart disease, it is possible that these cells contribute to the progression of heart failure. Thus the potential of activated epicardial progenitors to contribute to cardiac fibrosis must be considered in efforts directed towards harnessing this cell population for cardiac repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

αSMA	alpha Smooth Muscle Actin
AngII	angiotensin II
βGal	Beta-Galactosidase
BMI	body mass index
CVD	cardiovascular disease
COD	cause of death
CHF	congestive heart failure
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
EndMT	endothelial-mesenchymal transition
EPDC	epicardium-derived cell
HHD	hypertensive heart disease
IF	immunofluorescence
I/R	ischemia/reperfusion
LV	left ventricle
MI	myocardial infarction
MSC	mesenchymal stem cell
PFA	paraformaldehyde
RV	right ventricle
SM	smooth muscle
TAC	transverse aortic constriction
Wt1	Wilms' tumor 1

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Highlights

Cardiac fibrosis is differentially localized with ischemic injury or hypertensive disease.

Tcf21, Wt1, and Tbx18 are expressed in epicardial and interstitial fibrosis following ischemic injury in adult mice.

Tcf21, but not Wt1 or Tbx18, is actively expressed in perivascular fibrosis resulting from pressure overload in adult mouse hearts.

Cells expressing embryonic epicardial progenitor markers are distinct from CD45-positive immune cells.

Figure 1. Ischemia/reperfusion (I/R) leads to epicardial fibrosis, while pressure overload and hypertension result in perivascular remodeling and fibrosis
 (A-L) Cardiac fibrosis was evaluated in mouse models of heart disease at the following timepoints: seven days (7d) post-I/R, 14d post-transverse aortic constriction (TAC), and 14d post-chronic Angiotensin II infusion (AngII). Masson's trichrome staining was used to visualize fibrosis (blue) in sections of mouse hearts. Epicardial fibrosis in the left ventricle (LV) away from the scar (B, arrowhead) is increased in I/R hearts, relative to sham (sternotomy only) controls (A). Perivascular fibrosis is visibly increased surrounding large coronary vessels in the LV free wall in hearts subjected to TAC (arrowhead, G) and AngII infusion (arrowhead, H), relative to sham control (E). Interstitial fibrosis in the LV myocardium is apparent in all disease models (arrowheads, J-L), compared to sham (I). (M) Fibrotic epicardial thickness was measured in comparable LV regions for 3 sections of 3-4 hearts per group and is increased in hearts subjected to I/R but not TAC or AngII infusion. (N) Perivascular fibrosis, quantified as the ratio of the area of adventitial fibrosis surrounding the blood vessel to the area of the tunica media, is increased in hearts subjected to TAC or AngII infusion but not I/R. Data are presented as mean+s.e.m. Statistical

significance of observed differences relative to sham was determined by Student's *t*-test ($n=3-4$). **P* 0.01.

Figure 2. Tcf21βGal is expressed in differentially localized fibrotic regions of the heart in mouse models of heart disease

(A-I) XGal staining was performed to visualize Tcf21βGal expression in sections of hearts from adult mice heterozygous for the *Tcf21LacZ* knock-in allele. Mice were subjected to I/R or TAC and analyzed one to two weeks later. Increased Tcf21βGal expression is apparent in the epicardium (arrowheads, B) of hearts after I/R, and surrounding a coronary vessel (F, arrowheads) from a heart subjected to TAC. Interstitial Tcf21βGal expression is increased in hearts subjected to either I/R (H, arrowheads) or TAC (I, arrowheads) relative to unoperated controls (G, arrowhead).

Figure 3. Tcf21βGal, Wt1, Tcf21, and Tbx18 are expressed in the thickened subepicardial mesenchyme on the heart surface following I/R
 (A-C,F-H,J-L) Tcf21βGal and transcription factor expression in the epicardium and EPDCs in hearts from adult mice subjected to I/R, TAC, or sham operations was assessed by immunofluorescence (IF) analysis using the following antibodies: (A-C) anti-βGal (red, indicative of Tcf21βGal expression) + anti-Wt1 (green); (F-H) anti-Tcf21 (red); (J-L) anti-Tbx18 (red). (B,G,K) Arrowheads indicate transcription factor expression in subepicardial mesenchymal cells in mouse hearts away from the scarred region 7d post-I/R. The dotted line delineates the subepicardial mesenchyme-myocardial boundary. Tcf21βGal and Wt1 (B) are co-expressed (yellow) in some but not all cells in the subepicardium of mouse hearts following I/R. Tcf21 (G) and Tbx18 (K) are expressed in subepicardial mesenchymal cells in mouse hearts following I/R. (A,C,F,H,J,L) Tcf21βGal, Tcf21, WT1 and Tbx18 are expressed in the relatively quiescent epicardium (insets) of hearts from mice subjected to sham (sternotomy only) and TAC operations. (D,E,I,M) Quantification of the average number of Tcf21βGal+ (D), Wt1+ (E), Tcf21+ (I), or Tbx18+ (M) epicardial/subepicardial mesenchymal cells per microscopic field is shown. Error bars indicate standard error of the mean (s.e.m.). Statistical significance of observed differences relative to sham control was determined by Student's *t*-test (*n*=3-6). * *P*<0.01, # *P*<0.05. Epi, epicardium.

Figure 4. Tcf21, but not Wt1 or Tbx18, is expressed in regions of perivascular fibrosis in adult mouse hearts subjected to pressure overload

(A-C) Double IF was performed using anti-βGal (red, indicative of Tcf21βGal expression) and anti-Vimentin (green, indicative of fibroblasts). A few βGal-positive cells (A,B) (insets, arrowheads) are detected near blood vessels in hearts from sham (sternotomy only) and I/R operations. Tcf21βGal and Vimentin are co-expressed (C) (inset, arrowheads) in cells surrounding a coronary artery in a mouse heart 14 days post-TAC. (E-G) Endogenous Tcf21 expression (arrowheads, red) recapitulates that of Tcf21βGal. (I-K,M-O) In contrast, Wt1 (K) and Tbx18 (O) are not expressed (open arrowheads, lack of red staining) in regions of perivascular fibrosis following pressure overload. Note that epicardial expression of Wt1 and Tbx18 was detected in these same sections. All images are of large coronary vessels in the LV free wall. Dotted lines delineate vessel lumen. (D,H,L,P) Quantification of the average number of Tcf21βGal+ (D), Tcf21+ (H), Wt1+ (L), and Tbx18+ (P) adventitial cells per microscopic field is shown. Error bars indicate s.e.m. Statistical significance of the observed differences relative to sham control was determined by Student's *t*-test ($n=3-6$). * $P<0.01$. L, lumen.

Figure 5. Tcf21, Wt1, and Tbx18 are expressed in regions of interstitial fibrosis in I/R and TAC mouse models of cardiac disease

(A-C,E-G,I-K,M-O) Double IF analysis was performed using the following antibodies: (A-C) anti-βGal (red, indicative of Tcf21βGal expression) + anti-Collagen (Col) 3 (green); (E-G) anti-Tcf21 (red) + anti-αSmooth Muscle Actin (αSMA) (green); (I-K) anti-Wt1 (red) + anti-αSMA (green); (M-O) anti-Tbx18 (red) + anti-Col3 (green). Arrowheads indicate positive interstitial cells. Tcf21βGal (B,C) and Tbx18-positive (N,O) cells are surrounded by Col3 (insets, arrowheads) in the interstitium of both disease models as well as in sham controls. (F,G,J,K) Tcf21 and Wt1-expressing interstitial cells (arrows) are distinct from cells positive for αSMA expression. (D,H,L,P) Quantification of the average number of Tcf21βGal+ (D), Tcf21+ (H), Wt1+ (L), and Tbx18+ (P) interstitial cells per microscopic field is shown. Expression of Tcf21βGal (B,C) and Tbx18 (N,O) is increased in the interstitium of hearts from both I/R and TAC models. Wt1 expression is increased in the fibrotic interstitium following I/R (J), but not TAC (K). Error bars indicate s.e.m. Statistical significance of the observed differences relative to sham control was determined by Student's *t*-test ($n=3-6$). * $P<0.01$, # $P<0.05$.

Figure 6. Tcf21-expressing cells are distinct from CD45-positive immune cells in fibrotic regions of hearts from mice subjected to I/R or TAC

(A-I) Double IF analysis was performed using anti-Tcf21 (red) and anti-CD45 (green). White arrowheads indicate Tcf21 positivity, and open arrowheads indicate CD45 positivity. (B,C,F,G,H,I) CD45-positive immune infiltrate, distinct from Tcf21 expressing cells, is detected in regions of epicardial (B, inset), perivascular (F, inset), and interstitial (H,I) (inset) fibrosis. Epi, epicardium; L, lumen.

Figure 7. TCF21, WT1, and TBX18 are expressed in human cardiac fibrosis

(A-C) Localized cardiac fibrosis was evaluated in tissue from human donors diagnosed with hypertension and other co-morbidities. Masson's trichrome staining was performed to visualize fibrosis (blue) in sections of diseased human cardiac tissue. (A) Epicardial fibrosis (black arrowhead) with a thick layer of subepicardial mesenchyme is apparent. (B) Perivascular fibrosis (black arrowhead) is detected surrounding a coronary vessel. (C) Interstitial fibrosis (black arrowhead) is apparent. (D-L) IF analysis was performed to visualize (D-F) TCF21 (red), (G-I) WT1 (red), and (J-L) TBX18 (red) + COL3 (green) expression in human cardiac tissue. White arrowheads indicate immunopositivity. (D-F) TCF21 is expressed in the epicardium (D, inset), perivascular adventitia (E, inset), and myocardial interstitium (F, inset). The dotted line in (E) delineates the vessel lumen. (G-I) WT1 expression is detected in the (G) epicardium (inset) and subepicardial mesenchymal cells, and in interstitial cells (I, inset). (H) WT1 is not expressed in perivascular adventitial

cells, although WT1 expression is detected in the coronary endothelium (open arrowhead, inset). (J-L) TBX18 immunopositivity is detected in COL3-expressing regions of the epicardium (J, inset) and the interstitium (L, inset), but not in perivascular fibrosis, positive for COL3 (K). Specimens depicted in each panel are as follows: (A,D,G) specimen 69128; (J) specimen 69126; (B,E,H,K) specimen 69164; (C,F,I) specimen 69109; (L) specimen 69194. See Table S1 for clinical diagnostic information. Epi, epicardium; L, lumen.

Figure 8. Model of embryonic epicardial marker gene induction in localized regions of cardiac fibrosis

In a normal heart, the epicardial layer is quiescent and fibroblasts are present in perivascular adventitia and myocardial interstitium. Ischemic injury causes accumulation of epicardial fibrosis, as indicated by fibrillar collagen (blue lines) with increased expression of the embryonic epicardial progenitor genes *Tcf21*, *Wt1*, and *Tbx18*. Pressure overload or hypertensive heart disease leads to perivascular fibrosis characterized by *Tcf21*, but not *Tbx18* or *Wt1* expression. Fibrotic tissue in the myocardial interstitium predominantly expresses EPDC markers *Tcf21* and *Tbx18*, and to a lesser extent, *Wt1*. CD45-positive immune infiltrating cells are present in all three regions of fibrosis, but are distinct from cells that express embryonic epicardial progenitor markers.