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Cardiogenic Genes Expressed in Cardiac Fibroblasts Contribute to Heart Development and Repair

Milena B. Furtado[1], **Mauro W. Costa**[1], **Edward Adi Pranoto**[1], **Ekaterina Salimova**[1], **Alex Pinto**[1], **Nicholas T. Lam**[2], **Anthony Park**[3], **Paige Snider**[4], **Anjana Chandran**[1], **Richard P. Harvey**[5], **Richard Boyd**[3], **Simon J. Conway**[4], **James Pearson**[6], **David M. Kaye**[2], and **Nadia A. Rosenthal**[1]

[1]Australian Regenerative Medicine Institute, Monash University, VIC, Australia

[2]Baker IDI Heart and Diabetes Institute, VIC, Australia

[3]Department of Anatomy and Developmental Biology, Monash University, VIC, Australia

[4]Indiana University School of Medicine, IN, USA

[5]Victor Chang Cardiac Research Institute, NSW, Australia

[6]Monash Biomedical Imaging, Monash University, VIC, Australia

Abstract

Rationale—Cardiac fibroblasts are critical to proper heart function through multiple interactions with the myocardial compartment but appreciation of their contribution has suffered from incomplete characterization and lack of cell-specific markers.

Objective—To generate an unbiased comparative gene expression profile of the cardiac fibroblast pool, identify and characterize the role of key genes in cardiac fibroblast function, and determine their contribution to myocardial development and regeneration.

Methods and Results—High-throughput cell surface and intracellular profiling of cardiac and tail fibroblasts identified canonical MSC and a surprising number of cardiogenic genes, some expressed at higher levels than in whole heart. Whilst genetically marked fibroblasts contributed heterogeneously to interstitial but not cardiomyocyte compartments in infarcted hearts, fibroblast-restricted depletion of one highly expressed cardiogenic marker, Tbx20, caused marked myocardial dysmorphology and perturbations in scar formation upon myocardial infarction.

Conclusions—The surprising transcriptional identity of cardiac fibroblasts, the adoption of cardiogenic gene programs and direct contribution to cardiac development and repair provokes alternative interpretations for studies on more specialized cardiac progenitors, offering a novel perspective for reinterpreting cardiac regenerative therapies.

Address correspondence to: Dr. Milena B. Furtado, Australian Regenerative Medicine Institute, Monash University, Clayton campus, Wellington Rd, VIC, 3800, Australia, Tel: 61-3-99029736, FAX: 61-3-99029601, milena.furtado@monash.edu.

DISCLOSURES
None.

Keywords

Heart; fibroblast; transcription factors; transcriptional network; cardiac fibroblasts

NTRODUCTION

Until recent years, cardiovascular studies have focused almost exclusively on the muscular component of the heart, with less attention paid to the non-myocyte stromal cell populations that comprise more than 50% of cardiac cell types. Studies demonstrating that the mammalian heart maintains a population of tissue-resident mesenchymal progenitors (variously termed CSCs CPCs, CFU-F) have increased appreciation for their potential contribution to multiple aspects of myocardial function and pathophysiology that must be redressed to achieve successful therapy[1]. Yet the field is plagued by vague definitions that fail to distinguish subsets of stromal cells with specialized functions in diverse tissues[2].

Loosely designated by their mesenchymal origin, capacity to adhere to plastic and to secrete extracellular matrix, fibroblasts are a stromal element of almost all tissues where they play a role in tissue homeostasis and scaffolding support, as well as regulating self-tolerance, organ development, wound healing, inflammation and fibrosis[3]. They represent a principal component of the cardiac tissue bed and share many generic properties with more well studied mesenchymal stem cells (MSCs), yet remain relatively poorly characterized. A common embryonic lineage ancestry for cardiac fibroblasts and other cardiac stromal cells can be traced to the endocardium and the pro-epicardial organ, both of which give rise to migratory cell populations that undergo epithelial-to-mesenchymal transition (EMT)[4]. Fibroblasts and MSCs likely play similar functions, acting as sources of growth factors, generating coordinate systems for site-specific cell interactions critical for the development, differentiation, morphogenesis and renewal, and providing the appropriate niche conditions for stem cell maintenance and differentiation[3]. Progress in distinguishing the role of the cardiac fibroblast in normal and pathological settings has been hampered by the heterogeneity of the stromal cell populations in the heart, each presumably with specialised functions, and the lack of tools to define their specific roles in myocardial sub-compartments.

In this study, we reveal that a high proportion of adult mouse cardiac fibroblasts express defined mesenchymal stem cell (MSC) markers including Sca1 and PDGFRα. They also display an unexpected, organ-specific spectrum of cardiogenic genes that have been broadly implicated in multiple human cardiovascular defects, in particular *TBX20* and *GATA4*. Although cardiac fibroblasts do not contribute significantly to cardiomyocyte renewal after infarction, conditional genetic ablation of *Tbx20* in fibroblasts has adverse consequences for both normal cardiac development and post-infarct repair. These findings underscore the potential artificiality of semantic distinctions between fibroblasts and other stromal cell types and progenitor pools in the heart, and shed new light on the roles played by cardiac fibroblasts and the cardiogenic genes they express in cardiac homeostasis and disease.

METHODS

All animal experimentation conformed with local (Monash University) and national guidelines in Australia. Handling of patient samples was performed with the approval of the Alfred hospital Human Research and Ethics Committee and patients gave written informed consent. An expanded Methods section is available in the Online Data Supplement.

RESULTS

Cardiac fibroblast mesenchymal signature

Facing the lack of a pan-fibroblast marker, we used a non-biased pre-plating approach to isolate a global organ-specific fibroblast population. The strategy required short-term non-confluent primary cultures to avoid artefacts from long culture conditions. Tail fibroblasts were concomitantly isolated under similar conditions for comparative analysis. Short-term cultured cardiac fibroblasts displayed distinct morphology (Fig1A), with abundant cytoplasmic protusions resembling lamellipodial and filopodial processes onto the plastic surface, unlike tail fibroblasts. Molecular analysis showed that both heart and tail cells expressed a suite of known fibroblast markers (Fig1B), including extracellular matrix (ECM) components collagen1α1/1α2, filamin A, tenascin C, periostin and cell surface receptors CD90 (Thy1), DDR2 and CD140a/b, as well as the intermediate filament vimentin[5].

To further define cardiac fibroblast identity, we performed a high-throughput cell surface profiling. CD90 was used as a positive control for fibroblasts from various organs as previously described[6, 7]. CD90 stained ~65% of heart and ~86% of tail cells, but not the entire population (Fig1C). While neither fibroblast population showed significant staining for hematopoietic and endothelial markers CD45, CD31 and CD34, among others (Online FigI), we observed consistently high expression of bona-fide MSC markers[8] (Fig1C). SCA1 was found in 79% of cardiac and 87% of tail cells, CD49e was present in 93% of heart and 99% of tail fibroblasts, CD51 stained over 95% of both heart and tail cells, CD29 was virtually expressed in all cells. CD44 showed a more modest distribution (around 20% in heart and tail). The less canonical MSC marker CD140a[9, 10] was also found in a smaller fraction of tail and heart fibroblasts. To characterize this fraction in more depth, triple staining using CD90, CD140a and SCA1 was performed (Fig1D). Markers showed distinct distribution between $CD90^+$ and $CD90^-$ populations (Fig1D) specially in tail fibroblasts, which displayed much lower levels of CD140a and virtually no cell singly stained by CD140a in both $CD90^+$ and $CD90^-$ fractions, or concurrently negative for Sca1 and CD140a. Our combined flow cytometry analysis further supported the heterogeneity of the fibroblast population, and confirmed the lack of a unique pan-fibroblast marker to isolate uniform cell populations of varied embryological origins.

Core cardiogenic transcriptional network in cardiac fibroblasts

To identify unique genes expressed in cardiac vs. tail fibroblasts, a comparative microarray analysis was performed. Cardiac fibroblasts showed an unexpected enrichment in cardiogenic transcription factors commonly associated with cardiomyocyte (CM)

development and function (Fig 2A), in particular Tbx20 variant 1 (~10fold), Tbx20 variant 2 (~ 7.4 fold), Gata4 (~9fold), Gata6 (~5fold) and Hand2 (~6fold). In addition, epicardial Tcf21 (10~fold) and Wt1 (5~fold) were amongst the highest up-regulated genes. The endocardial Tie1 gene was significantly up-regulated (~3.4 fold), while other genes involved in endocardial EMT (Tie2, Nfatc, Sox9, Vegfs and others) were not significantly regulated. In fact, genetic tracing experiments indicated that the endocardial compartment may be a minor contributor (12%) to the overall cardiac fibroblast population found in the homeostatic adult mouse heart (Online FigII). Other CM-specific transcripts were found in both samples or were not differentially regulated, such as Tbx2, Tbx5, Mef2c and Nkx2-5 (Online FigIII). Importantly, no structural CM genes were differentially regulated in these samples (Online. FigIV). Analysis of sorted $CD90^{+}$; $CD31^{-}$, $Cd45^{-}$ cell fraction corroborated the presence of these newly identified transcription factors in freshly isolated cells (Online. FigV).

Microarray findings were further validated by qPCR (Fig 2B), where whole heart was used as a positive control. Gata4/6 transcripts were significantly higher in cardiac fibroblasts when compared to whole heart (2.7/2.1 fold), while Tbx5, Gata5, Mef2c, and Nkx2-5 transcripts were enriched in the heart. Tbx20 and Hand2 transcripts were highly up-regulated in cardiac fibroblasts (13.3 and 3.5 fold, respectively). Their putative embryological provenance from the epicardium is supported by the dramatic up-regulation of Tcf21 (159 fold over whole heart and tail fibroblasts) and Wt-1 (54 Fold over tail fibroblasts). CM-specific Myh6 and Tnnt2 genes were not found in cardiac or tail fibroblasts, while Thy-1 levels were 18.4 to 47 fold higher in both fibroblast lineages.

Heterogeneity of the cardiogenic program in fibroblasts

Given the known heterogeneity of fibroblasts, we sought to deepen our transcription factor analysis to explore individual expression fluctuations within the cardiac population. Using a combination of immunofluorescence (IF), β-gal staining and flow cytometry (Fig3A), we observed high staining for GATA4 (99%), MEF2C (91%) and TBX20 (100%) in mouse cardiac fibroblasts. NKX2-5 was found in only 41% of cells, most of which showed very low staining levels. To corroborate these findings, we performed single cell qPCRs (Fig3B). All cardiac fibroblasts analysed displayed high levels of Gata4 and Tbx20. Only a proportion of cells expressed Tbx5 (27%) and Mef2c (89%), both of which were similarly detected in tail in our previous analyses (Fig1–2). Tbx2 and Nkx2-5 were also chimeric in their expression. No Myh6 transcripts were detected. The heat map showed that 86% of cells were triple positive for Tbx20, Gata4 and Mef2c; 61% were positive for the 4 factor combination Tbx20, Gata4, Mef2c and Tbx2, while only 25% of cells were positive for the direct reprogramming cocktail Gata4, Mef2c and Tbx5[11, 12].

To relate the variability of transcription factor expression with positional information, expression of some factors was further investigated within different chamber compartments (Fig3C). Most factors were found within all chambers [right atrial (RAF); left atrial (LAF); interventricular septal (IVS), right ventricular (RVF) and left ventricular (LVF) fibroblasts], although 3 patterns were observed: 1. Gata4 and Gata6 were similarly distributed among all chambers; 2. Tcf21, Hand2 and Tbx20 were higher in all ventricular compartments when

compared with atrial ones; 3. Gata5 and Wt1 were highly enriched in atrial compartments when compared with ventricular ones. This dataset indicates that cardiac fibroblast identity is at least partially subject to regional specification.

Further exploration of spatial differences in primary human fibroblasts (Fig3D) revealed that as for the mouse cells, THY1 was enriched in fibroblasts isolated from right atrial appendages (hAF) and interventricular septum (hVF), although POSTN and PDGFRA did not seem particularly enriched when compared with whole atrial appendage (hA) or ventricular septum (hV). CM specific structural markers MYH6, MYH7 and TNNT2 were not found in isolated fibroblasts. Transcriptional network comparison between heart, cardiac fibroblasts and BJ foreskin fibroblasts demonstrated commonalities and differences between mouse and human cells. In particular, GATA4, GATA6, TBX20, MEF2C, TCF21, HAND2, WT1 were found in all heart samples. GATA5 was enriched in hAFs but also in 2 out of 3 hVFs, reflecting some variability in ventricular pattern. NKX2-5 was also found in low levels in 2 hVF samples, while TBX5 could be detected in hA samples but not in any other compartment. BJ cells expressed TBX5 and HAND2, both involved in limb patterning, and MEF2C, also related to muscle formation and neurogenesis. This analysis confirmed a similar profile of factors in human cardiac fibroblasts, demonstrating the evolutionary conservation of the core cardiogenic transcriptional network in these cells.

Fibroblast Tbx20 expression in heart development and maturation

Throughout the analysis above, *Tbx20* was among the highest and most consistently expressed gene in the fibroblasts of all heart compartments. We have previously shown that constitutive loss of Tbx20 results in death of embryos at early gestation with grossly abnormal heart morphogenesis and a severely compromised cardiac transcriptional program[13]. Using a fibroblast-specific Cre transgene driven by periostin regulatory elements (Postn-Cre)[14] and a floxed allele of the Tbx20 locus in which Cre-mediated Tbx20 gene deletion brings a *lacZ* reporter under the control of Tbx20 cis-regulatory elements[13], we generated $Postn^{cre/+};Tbx20^{f/+}$ x $Tbx20^{f/+}$ crosses to obtain mutant $Postn^{cre/+};Tbx20^{f/f}$ (CKO) and heterozygous control $Postn^{cre/+};Tbx20^{f/+}$genotype littermates (Fig4). A null $Tbx20^{lacZ}$ allele was further introduced to control animals ($Postn^{cre/+};Tbx20^{f/lacZ}$) to provide a readout of constitutive Tbx20 expression through β Galactosidase (β-gal) staining. $Postn^{cre}$activated β-gal expression in $Postn^{cre/+};Tbx20^{f/+}$ and $Postn^{cre/+};Tbx20^{f/f}$ hearts at 11.5 days post-coitum (dpc) in outflow tract (OFT) and atrioventricular canal (AVC) endocardial cushions, followed by a salt-and-pepper pattern in the myocardial chambers, absent in wild-type control littermates (Fig4A–C). As expected $Postn^{cre/+};Tbx20^{f/lacZ}$ embryos exhibited ubiquitous staining (Fig4D), consistent with full Tbx20 expression pattern in the heart[13].

Heterozygous ($Postn^{cre/+};Tbx20^{f/+}$) and mutant ($Postn^{cre/+};Tbx20^{f/f}$) hearts isolated at 16.5 dpc displayed β-gal staining in OFT and AVC valvular areas (Fig4E–G), and a mottled pattern in atrial and ventricular chambers. No major gross morphological abnormalities were observed in whole mounts of $Postn^{cre/+};Tbx20^{f/f}$ CKO embryonic hearts, except for a slight decrease in ventricular chamber size (Fig4G; N=5). Histological analyses revealed a partial ventricular septal defect (VSD) (Fig4K; N=5) and immature hyperplastic valves (Fig4L)

when compared to *Postn*$^{cre/+}$;*Tbx20*$^{f/+}$controls (Fig4I and J, respectively). CKO embryos also displayed hypoplastic IVS (compare bars in Fig4L′ and J′) and a reduction in compact myocardium thickness (compare bars in Fig4L″ and J″).

At the molecular level, qPCR analysis of whole hearts showed a 30% reduction in overall Tbx20 levels in CKO hearts when compared with controls (Fig4M), reflecting fibroblast-specific deletion, while myocardial expression remained intact. A compromise in CM maturation was indicated by a significant increase in expression of BMP10 (Fig4M), a trabeculation marker, suggesting that compact layer thinning in CKO hearts was linked to altered CM differentiation, as no alterations in CM proliferation were seen at 16.5 dpc (nmyc – data not shown). Notably, only a proportion of fibroblasts isolated from *Postn*$^{cre/+}$;*Tbx20*$^{f/f}$ CKO embryonic hearts activated the lacZ reporter when compared with *Postn*$^{cre/+}$;*Tbx20*$^{f/lacZ}$ ubiquitous controls (Fig4N left panel). Correspondingly, qPCR analysis of isolated cardiac fibroblasts revealed 40% less Tbx20 transcripts in *Postn*$^{cre/+}$;*Tbx20*$^{f/f}$ CKO fibroblasts vs. 80% less Tbx20 transcripts in fibroblasts from *Postn*$^{cre/+}$;*Tbx20*$^{f/lacZ}$ control hearts. This variable Cre activity amongst the presumably heterogeneous fibroblast pool presumably contributed to protection of *Postn*$^{cre/+}$;*Tbx20*$^{f/f}$ CKO embryos from lethal prenatal and perinatal morphogenetic abnormalities, as CKO mutants were found in Mendelian ratios to adulthood (Online Table I). Nevertheless, CKO mutant fibroblasts displayed reduced Tcf21 and Ddr2 expression, as well as lower retinoic acid activity (Aldh1a2), accompanied by an increase in Flna and Acta2, suggestive of increased ECM production and myofibroblast activity. Transcripts encoding the gap junction gene Gja1, known to be involved in fibroblast:fibroblast and fibroblast:CM coupling[15, 16], were also reduced. Taken together, these data provide evidence that Tbx20-activated gene programs in fibroblasts are important for proper maturation of myocardial as well as non-myocardial compartments in heart development.

Profile of cardiac fibroblasts in the regenerative response

To assess the participation of cardiac fibroblasts in an injury/regeneration scenario, we performed myocardial infarction (MI) in Postn-Cre;Rosa26R reporter mice. Although only ~10% of fibroblasts expressed Postn-Cre in the adult mouse heart in homeostasis (data not shown), fibroblast Gal expression was strongly enhanced upon MI (N=2), showing robust staining 3 days post-infarct (dpi) (Fig5A–B; N=2), which was then localised to the injury/scar area at 7 dpi and 14 dpi (Fig5C–D; N=2). Stained cells were mostly seen in intra-myocardial interstitial spaces at all time-points analysed (Fig5E–H). A second reporter mouse (RosaZsgreen) which provided more robust fluorescent staining was used to further refine Postn-Cre driver activity after MI. RosaZsgreen (Ai6) has been shown to enhance visualisation of Cre driver activity[17], presumably through increased reporter transcript expression, brighter protein fluorescence and/or improved locus recombination. At 14 dpi, abundant Zsgreen^{+} fibroblasts were detected in scar tissue (Fig5J; N=3). No Zsgreen staining was detected in Troponin T positive myocytes in the infarct border zone, indicating that *Postn*$^{cre/+}$;*Rosazsgreen*$^{f/+}$ fibroblasts do not contribute new CMs to infarcted tissue. In addition, no Zsgreen^{+} fibroblasts were present in the endocardial compartment (Fig5J, J′), and only rare Zsgreen^{+} fibroblasts were seen in the epicardium after injury (Fig5K, K′). Comparison of proliferation rates between Zsgreen^{-} and Zsgreen^{+} cells at the injury site at

14 dpi revealed low proliferation rates (8.6% and 2.12%, respectively), suggesting that the remodelling process is already well advanced (Fig5L).

Fibroblasts isolated from MI hearts by FACS and plated for 5 days (Fig5K; N=2) displayed similar morphology to that of the overall cardiac fibroblast population (Fig1) and expressed Thy1, Postn, Ddr2, Pdgfra (Cd140a) and Pdgfrb (CD140b) as well as Tbx5, Tbx20, Gata4 and Mef2c. While core transcription factors were unchanged in MI hearts, the cardiac stem cell marker SCA1 was expressed in the majority (65%) of Zsgreen^{+} cells freshly isolated from mouse hearts in homeostasis and in 72% of Zsgreen^{+} cells isolated at 14 dpi (Fig5L,M; N=5). Zsgreen^{+}; SCA1^{+} cells also showed increased expression of Thy1 and Ddr2 in both homeostasis (Fig5L) and injury (Fig5M) compared to Zsgreen^{+};SCA1^{-} cells. The myofibroblast marker Acta2 was reduced in the Zsgreen^{+};SCA1^{+} population, suggesting that SCA1^{+} fibroblasts are more undifferentiated or have a lower potential to form myofibroblasts.

Fibroblast Tbx20 activation controls scar formation in infarcted hearts

Given the presence of cardiogenic gene in fibroblasts of the infarcted adult heart, *Postn$^{cre/+}$; Tbx20$^{f/f}$* CKO mice were tested for altered response post-MI. Whilst these mice had normal heart function under homeostatic conditions as assessed by MRI (Fig6A – pre), they presented decreased end diastolic (EDV) and systolic (ESV) volumes (87.87 μl ±13.96 and 57.66 μl ±12.5, respectively) at 30 dpi when compared with controls (114.1μl±11.79 and 78.2 μl ±11.24, respectively). This resulted in a discrete increase in left ventricular ejection fraction (LV-EF − 36.08%±3.3 for CKO mutants and 32.95%±3.0 for controls). Although statistical significance in functional changes was not reached due to experimental variability, MRI measurement of wall thickness (WT) from apex to base revealed increased thickness of scar area (left ventricular free wall – LV-FW) in *Postn$^{cre/+}$; Tbx20$^{f/f}$* CKO hearts when compared with controls (1.07±0.12 and 0.58mm±0.08, respectively; p=0.006) with no changes in septal wall thickness (LV-SW). These results were confirmed by histological analysis using trichrome staining (Fig6B) of the infarcted area marked by the presence of stitch knots (circles). The thicker scars and reduced dilation *in Postn$^{cre/+}$; Tbx20$^{f/f}$* CKO mutant hearts was consistent with the observed increases in ejection fraction and dysregulation of fibroblast function.

DISCUSSION

In addition to their interstitial tissue scaffolding functions, fibroblasts have been shown to regulate organ development, wound healing and fibrosis, maintain stem cell niche, and play important immunomodulatory role in inflammation and self-tolerance. Here we outline an organ-specific gene expression profile for an unbiased cardiac fibroblast population subjected to short-term culture conditions, and highlight the integral role played by cardiac fibroblasts by defining a cell-specific function for the cardiogenic transcription factor Tbx20 in myocardial repair and myofibroblast differentiation. These results uncover an important function for cardiac fibroblasts in regulation of genes broadly implicated in multiple congenital heart diseases (CHDs). Mutations in the GATA family of cardiac transcription factors, vital regulator of heart development and heart failure[18], have also been associated

with a number of congenital defects in humans[19]. Gata4 was expressed at high levels in rat[20] as well as in mouse cardiac fibroblasts. Our observation that Tbx20 is expressed at significantly higher levels in cardiac fibroblasts than in whole adult heart sheds new light on the mechanistic basis of *Tbx20* mutations in mice[21, 22] and in patients with CHD that include defects in septation, valvulogenesis and dilated cardiomyopathy[22–26]. The compromised scar formation in infarcted mouse hearts with Tbx20 deletion in the fibroblast compartment suggests a further role for this gene in the control of interstitial myofibroblast generation, influencing the outcome of recovery from myocardial damage.

Despite the coexistence of MSC and fibroblasts in almost all tissues, with similar morphology and shared molecular characteristics, their functional relationship has remained obscure. Characterisation of the unfractionated fibroblast component of the heart revealed high levels of MSC markers, most of which interact with ECM components: CD29 and CD49e are fibronectin receptors, CD44 is a receptor for hyaluronic acid and other ECM ligands, while CD51 is also an integrin, promoting adhesion and signal transduction capabilities. Communication with ECM is a conventional aspect of fibroblast activity, and may therefore not reflect pluripotency, but rather the capacity to adhere and interact to the microenvironment. The SCA1 surface marker has been used extensively to define cardiac progenitors [9, 10, 27] but is also expressed in differentiated cells[28]. As only a small percentage of cardiac $SCA1^+$ cells can be efficiently converted to differentiated CMs[9, 10, 27], it is plausible that the principal role of $SCA1^+$ mesenchymal cells in the adult heart is to provide support to the myocardium in an injury scenario[5]. Our results support this hypothesis, as Periostin-Cre^+ cells were mostly $SCA1^+$ and found in scar areas after MI (Fig 6). Although a recently reported adult cardiac MSC population characterized on the basis of CD140a, SCA1 and CD90 expression (CFU-F)[10] did not show the cardiogenic factors identified here, those cells most likely represent a sub-population of our fibroblast pool. The $CD140a^+$, $SCA1^+$, $CD90^+$ freshly isolated fibroblast fraction was found to express most transcription factors uncovered by our analysis (Online FigV), suggesting that the cells described by Chong et al[10] could have been affected by long-term culture conditions, therefore losing their original transcriptional profile. The present analysis of the entire cardiac mesenchymal population, as opposed to sub-selected cell clones based on specific MSC markers, implicates a broader spectrum of functions for cardiac fibroblasts as mesenchymal support and myofibroblast formation, representing a putative source of progenitors that can differentiate into multiple cell types in certain disease states[29]. Our study underscores the close relationship between stromal cells in the myocardial context, and represents a necessary first step towards assigning functions to specific interstitial cell subsets in the heart.

The embryological origin of cardiac fibroblasts can be traced back to both endocardial and epicardial layers of the heart[30]. Interestingly, Tcf21 and Wt1, both epicardial markers, were up-regulated in adult cardiac fibroblasts in homeostasis, with no obvious enrichment found for well-known genes involved in endocardial EMT, except for Tie1. A relatively small contribution of endocardial cells to the resting adult heart interstitial compartment was further supported by Tie2-cre genetic tracing (Online FigII), suggesting that the majority of cells isolated in this study were epicardially derived. Tcf21 is also essential for epicardial

EMT[31, 32], while Wt1 has been further implicated in control of proliferation and EMT[33]. Interestingly, Tcf21 was up-regulated in fibroblasts isolated from ventricular compartments (Fig3C), while Wt1 was enriched in atrial compartments only, suggesting complex spatial distinctions amongst cardiac fibroblasts that warrant further investigation.

The identification of a core cardiac transcriptional network in heart fibroblasts calls for a more precise definition of a functional CM progenitor in vitro. This is particularly important for protocols of differentiation where the definition of CM has been attributed to non-beating cells that express cardiogenic transcription factors, and underscore the need for standardized criteria based on well-structured sarcomere markers combined with electrophysiological activity as true indicators of cardiomyocyte fate.

The direct reprogramming of cardiac fibroblasts into CM-like cells using factors Gata4, Mef2c and Tbx5 in mice and further validation in humans represents a promising CM replacement strategy[11, 34, 35]. The potential plasticity of the cardiac fibroblast population is not surprising in light of the current results showing that Tcf21, Tbx5, Tbx20, Gata4, Gata5, Gata6, Tie1, Hand2, Mef2c, Nkx2-5 and Wt1 genes were all significantly up-regulated in cardiac fibroblasts when compared with tail fibroblasts. This indicates that at least a subpopulation of cardiac fibroblasts are primed for trans-differentiation by expression of cardiogenic factors, which is likely to lie behind the beneficial effects seen in recent trans-differentiation studies, prompting novel strategies to target specific cardiac cell populations with enhanced trans-differentiative capacities.

Although in the present and analysis cardiac fibroblasts do not contribute substantially to heart muscle in injury settings, our findings raise the intriguing possibility that these cells play a central role in the development of cardiovascular disease associated with deregulation of key cardiogenic factors. Conditional deletion of Tbx20 in the fibroblast compartment affected myocardial development in a substantial manner (Fig4). The hypoplasticity of ventricular chambers was accompanied by a significant increase in BMP10, indicating a cell non-autonomous role for fibroblasts in the control of myocardial growth. This was supported by the impaired activity of mutant embryonic fibroblasts, measured as increased myofibroblast and ECM producing activity, reduced coupling properties and altered retinoic acid signaling (Aldh1a2). Interestingly, retinoic acid (RA) has been implicated in epicardial activation in mice expressing transgenic IGF1 in the heart[36, 37], which display improved healing properties. This is further supported by experiments in zebrafish, where RA is required in the endocardium and epicardial compartments for proper injury response and cardiomyocyte regeneration in adult hearts[38]. RA has also been recently shown to regulate cardiac progenitor differentiation. We have also observed improved performance of Tbx20 mutant hearts upon challenge using MI (Fig6), most likely due to increased ECM production and myofibroblast activity observed in mutant fibroblasts (Fig4). This resulted in reduced dilation and thicker walls in scar areas of mutant hearts. Our results are consistent with previous observations by Takeda et al[39], in which deletion of the transcription factor Klf5 in cardiac fibroblasts ameliorated the hypertrophic response in a pressure overload model. The field now requires a directed dissection of the roles played by cardiac fibroblasts and the cardiogenic genes they express in heart homeostasis and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard abbreviations and Acronyms

Acta2	smooth muscle actin
Aldh1a2	aldehyde dehydrogenase 1, subfamily a2
BMP	bone morphogenetic protein
CD29	cluster of differentiation 29 (integrin beta1; fibronectin receptor)
CD31	cluster of differentiation 31 (platelet endothelial cell adhesion molecule – PECAM)
CD34	cluster of differentiation 34 (hematopoietic progenitor cell antigen)
CD44	cluster of differentiation 44 (hyaluronate receptor)
CD45	cluster of differentiation 45 (protein tyrosine phosphatase, receptor type C)
CD49e	cluster of differentiation 49e (integrin alpha5; fibronectin receptor)
CD51	cluster of differentiation 51 (integrin alpha v; vitronectin receptor)
CD90	cluster of differentiation 90 (thymus cell antigen 1)
CD140a	cluster of differentiation 140a (platelet-derived growth factor receptor alpha)
CD140b	cluster of differentiation 140b (platelet-derived growth factor receptor beta)
CHD	congenital heart disease
CM	cardiomyocyte
DDR2	discoidin domain receptor 2
dpi	days post-infarct
ECM	extracellular matrix
EDV	end diastolic volume
EMT	epithelial to mesenchymal transition
ESV	end systolic volume

GATA4/5/6	GATA binding protein 4/5/6
Hand2	heart and neural crest derivatives expressed transcript 2
Hprt	hypoxanthine-guanine phosphoribosyltransferase
HBSS	Hanks' balanced salt solution
IB4	isolectin B4
LV-EF	left ventricle ejection fraction
LV – FW	left ventricle free wall
LV – SW	left ventricle septal wall
Mef2c	myocyte enhancer factor 2c
MI	myocardial infarction
MSC	mesenchymal stem cell
Myh6	myosin heavy chain 6, alpha isoform
Myh7	myosin heavy chain 7, beta isoform
PBS	phosphate buffered saline
PFA	paraformaldehyde
Postn	periostin
RA	retinoic acid
SCA1	stem cell antigen 1 (Lys6a/e gene)
Tbx2/5/20	T-box transcription factor 2/5/20
Tcf21	transcription factor 21 (epicardin)
Tie1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1
VEGF	vascular endothelial growth factor
WT	wall thickness
Wt1	Wilms tumour 1

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Novelty and Significance

What Is Known?

- Cardiac fibroblasts comprise highly heterogeneous cell populations in body tissues.
- Fibroblasts are close relatives to mesenchymal stem cells.
- Cardiac fibroblasts can be reprogrammed into cardiomyocytes using a cocktail of 3 cardiogenic transcription factors: Gata4, Mef2c and Tbx5.

What New Information Does This Article Contribute?

- Unbiased cell surface analysis showed that the majority of cardiac fibroblasts express bona-fide mesenchymal stem cell markers.
- High-throughput molecular analysis revealed a robust cardiogenic program in fibroblasts isolated from mouse heart, which was conserved in human heart cells.
- A conditional knockout strategy revealed the importance of the cardiogenic program for fibroblast and cardiomyocyte homeostasis in heart development and disease.

Cardiac fibroblasts play an important role in cardiovascular biology due to their manifold activities, including the modulation of myocardial homeostasis. To date, the organ-specific properties of these cells has been poorly characterized. We have uncovered a mesenchymal stem cell signature, but also a remarkable cardiogenic transcriptional profile exclusively in cardiac fibroblasts, perturbation of which severely compromises heart function. These findings call for a revised measure of cardiomyocyte differentiation from varied stem cells, involving functional assays. Our work paves the way for the design of more appropriate applications for pharmacological screening platforms and patient-tailored cell therapy for heart disease.

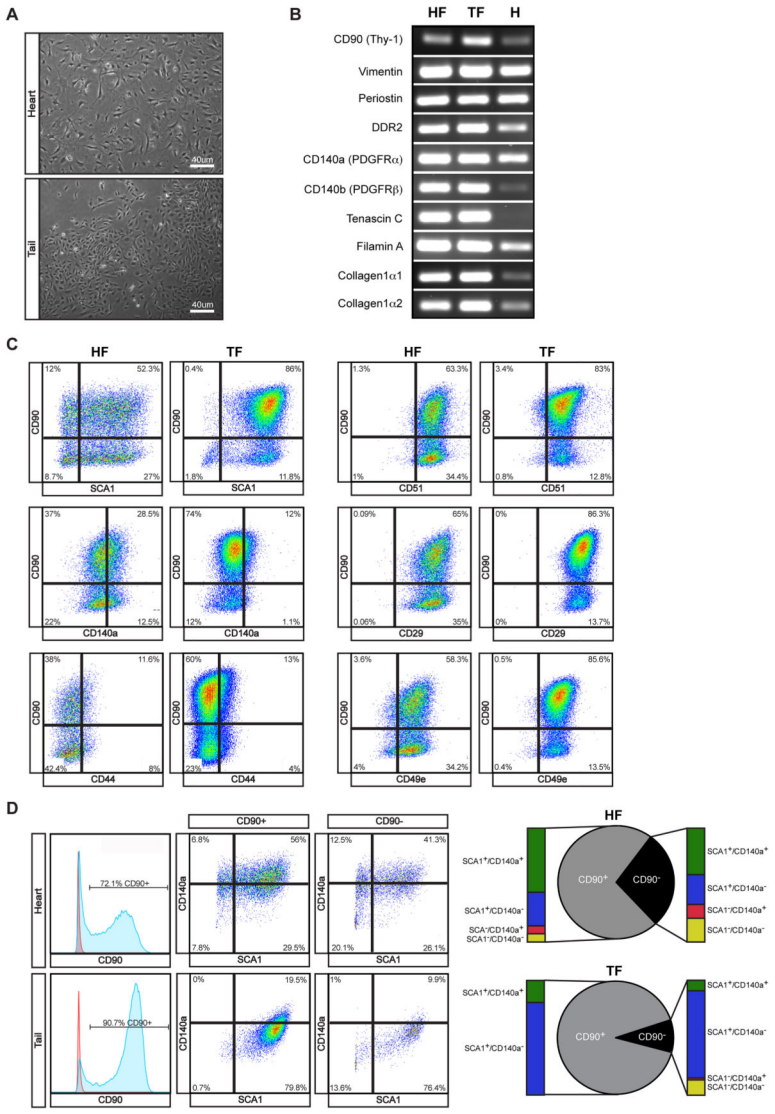

Figure 1. Comparative characterisation of cardiac fibroblasts

A. Plated heart and tail fibroblasts in culture for 5 days. **B.** RT-PCR analysis of heart (HF) or tail (TF) fibroblasts showing expression of known fibroblast markers. Whole heart (H) was used as positive control. **C.** Cell surface immuno-profiling reveals expression of SCA1, CD29, CD44, CD49e, CD51 and CD140a plotted against CD90. **D.** Triple staining using CD90, CD140 and SCA1 and pie chart depicting relationships among previously described cardiac MSC antigens CD90, Sca1 and CD140a.

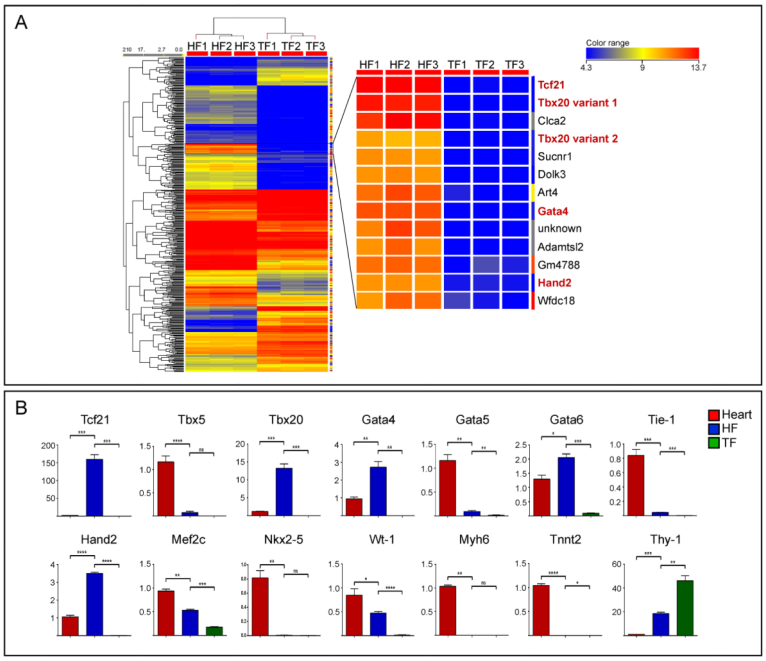

Figure 2. High throughput molecular analysis reveals cardiogenic genes in fibroblasts

A. Heat map of microarray analysis showing global differences between heart and tail cells and zoom in cluster of highly up-regulated genes in heart fibroblasts, including cardiogenic transcription factors Tcf21, Tbx20, Hand2 and Gata4. Heatmap shows enrichment in heart fibroblasts in red and reduction in blue. **B.** Validation of cardiogenic genes through qPCR. Heart tissue was used as comparison. Tcf21, Tbx20, Gata4, Gata6 and Hand2 were up-regulated in heart fibroblasts when compared with heart or tail fibroblasts. Tbx5, Gata5 and Mef2c showed lower expression in heart fibroblasts when compared with heart. Message for Myh6 and Tnnt2 was virtually negative for both heart and tail fibroblasts. Three biological replicates were used; experiments were performed in triplicates. Two-tailed T test was used for statistical analysis ($p<0.05$*; $p<0.01$**; $p<0.001$***; $p<0.0001$****).

HF – heart fibroblast; TF – tail fibroblast

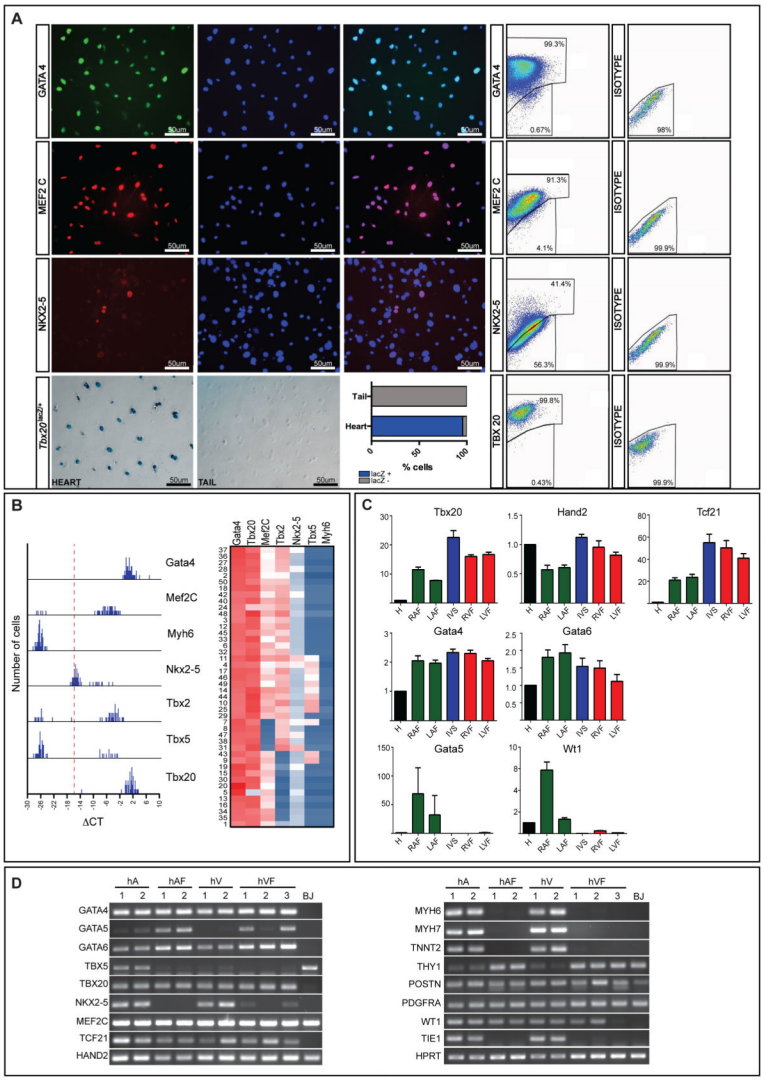

Figure 3. Heterogeneity of core transcriptional network

A. Immunofluorescence for Gata4, Mef2c and Nkx2-5 or β-gal staining for Tbx20 on fibroblast cultures. DAPI (blue) stains nuclei. **B.** Population analysis by flow cytometry depicting proportion of cells within the cardiac fibroblast population expressing respective factors. **C.** Single cell qPCR showing fluctuation of expression of individual transcription factors within the overall heart fibroblast population. Results expressed as ΔCT using Hprt as normalisation control. Height of bars represents number of cells expressing similar transcript levels. Dashed red line represents the negative cut off CT (35 cycles). Heat map (high expression-red; low expression-blue) shows combined transcription factor expression levels per cell (numbers denote cell identity). **C.** Spatial distribution of transcription factor expression within different cardiac compartments. Three patterns are observed: consistently higher levels in ventricular compartments (top panels), similarly distributed over all compartments (mid panels) and enrichment in atrial compartments (lower panels). **D.** Expression of core network in primary isolated human fibroblasts. BJ primary foreskin fibroblasts were used as non-cardiac control.

H – heart; RAF – right atrial fibroblasts; LAF – left atrial fibroblasts; IVS – interventricular septal fibroblasts; RVF – right ventricular fibroblasts; LVF – left ventricular fibroblasts; hA – human atrium; hAF – human atrial fibroblast; hV- human ventricle; hVF – human ventricular fibroblast.

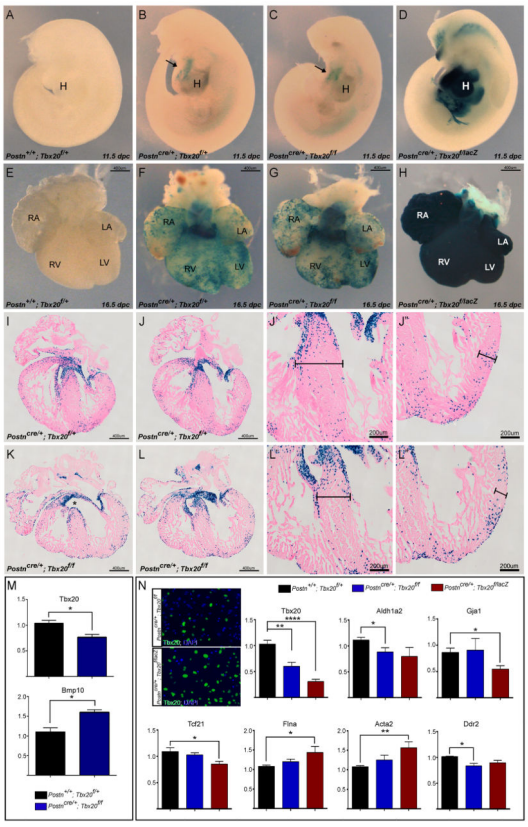

Figure 4. Morphogenetic defects in hearts with Tbx20 deleted cardiac fibroblasts

A–D. Postn-Cre deletes Tbx20 in OFT (arrow) and AVC regions from 11.5 dpc, as observed by β-gal staining (blue) in *Postn*$^{cre/+}$;*Tbx20*$^{f/+}$ (B) and *Postn*$^{cre/+}$;*Tbx20*$^{f/f}$ (C) embryos. *Postn*$^{cre/+}$;*Tbx20*$^{f/lacZ}$ embryos (D) showed full expression pattern for Tbx20-lacZ throughout heart and body. Control hearts (A) had no staining. **E–H.** Dissected hearts showed strong β-gal staining in valvular components in *Postn*$^{cre/+}$; *Tbx20*$^{f/+}$ (F) and *Postn*$^{cre/+}$;*Tbx20*$^{f/f}$ (G) embryos, as well as salt-and-pepper staining throughout myocardial chambers, while *Postn*$^{cre/+}$;*Tbx20*$^{f/lacZ}$ embryos (H) had uniform heart staining. Control hearts (E) had no staining. **I–L.** Histological analysis revealed Tbx20-lacZ cells in all cushions and valve leaflets, the atrioventricular groove and all myocardial chambers, specially in areas of compact myocardium and in close contact with the epicardial surface. *Postn*$^{cre/+}$;*Tbx20*$^{f/f}$ embryos had VSD (asterisk in K), immature AVC valves (L) and hypoplastic septal and free ventricular walls (L′, L″ - bars) when compared with controls (I, J, J′, J″, respectively). **M.** pPCR analysis showed reduced Tbx20 and increased BMP10 transcripts in *Postn*$^{cre/+}$;*Tbx20*$^{f/f}$ as compared to *Postn*$^{cre/+}$; *Tbx20*$^{f/+}$ hearts. **N.** (Left) Tbx20 expression imaged with β-gal staining in fibroblasts isolated from 16.5dpc hearts of *Postn*$^{cre/+}$;*Tbx20*$^{f/f}$ and *Postn*$^{cre/+}$;*Tbx20*$^{f/lacZ}$ mice. (Right) qPCR quantitation of transcripts in fibroblasts isolated from 16.5dpc hearts of *Postn*$^{cre/+}$;*Tbx20*$^{f/f}$, *Postn*$^{cre/+}$;*Tbx20*$^{f/+}$ and *Postn*$^{cre/+}$;*Tbx20*$^{f/lacZ}$ mice.

H – heart; RA – right atrium; LA – left atrium; RV- right ventricle; LV – left ventricle; OFT – outflow tract; AVC – atrioventricular canal; VSD – ventricular septal defect

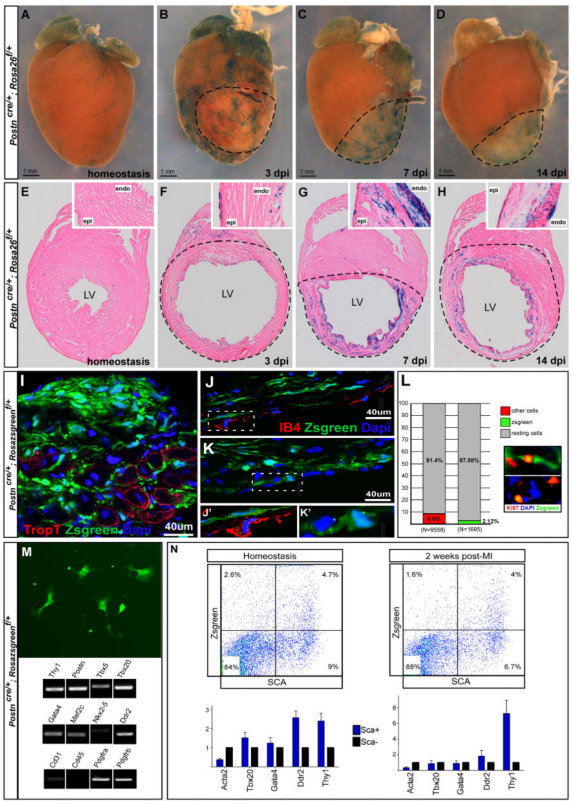

Figure 5. Contribution of cardiac fibroblasts to infarcted hearts

A–J. *Postn*$^{cre/+}$*;Rosa26*$^{f/+}$ mouse hearts before (A), 3 days post-infarct (dpi) (B), 7 dpi (C) and 14 dpi showing β-gal staining in whole-mount (A–D) and sections (E–H). Dashed black areas mark infarct border zones. **I.** *Postn*$^{cre/+}$*;Rosazsgreen*$^{f/+}$ (green) in injured myocardium showing robust contribution of fibroblasts to scar formation 14 dpi. CM marker TroponinT (TropT) shown in green, nuclei in blue (DAPI). **J, K.** Fibroblasts were found in sub-endocardial region **(J–J′)**, highlighted by IB4 (red) and in rare cells at the epicardial surface **(K–K′).** L. Proliferation index measured as Ki67+ cells over total number of nuclei (DAPI) for Zsgreen^{+} and Zsgreen^{-} cells, showing low proliferation rates in injured area 14 dpi. **M.** Sorted *Postn*$^{cre/+}$*;RosaZsgreen*$^{f/+}$cell morphology and molecular markers (RT-PCR) confirm fibroblast identity. **N.** Freshly sorted Zgreen^{+}/SCA^{+} or Zsgreen^{+}/SCA^{-} cell expression profiles before (L) or 14dpi (M) showing both fractions display similar levels of transcription factors before and after injury, although SCA^{+} cells display higher message for Ddr2 and Thy1. dpi – days post-infarct; LV – left ventricle; endo – endocardium; epi – epicardium.

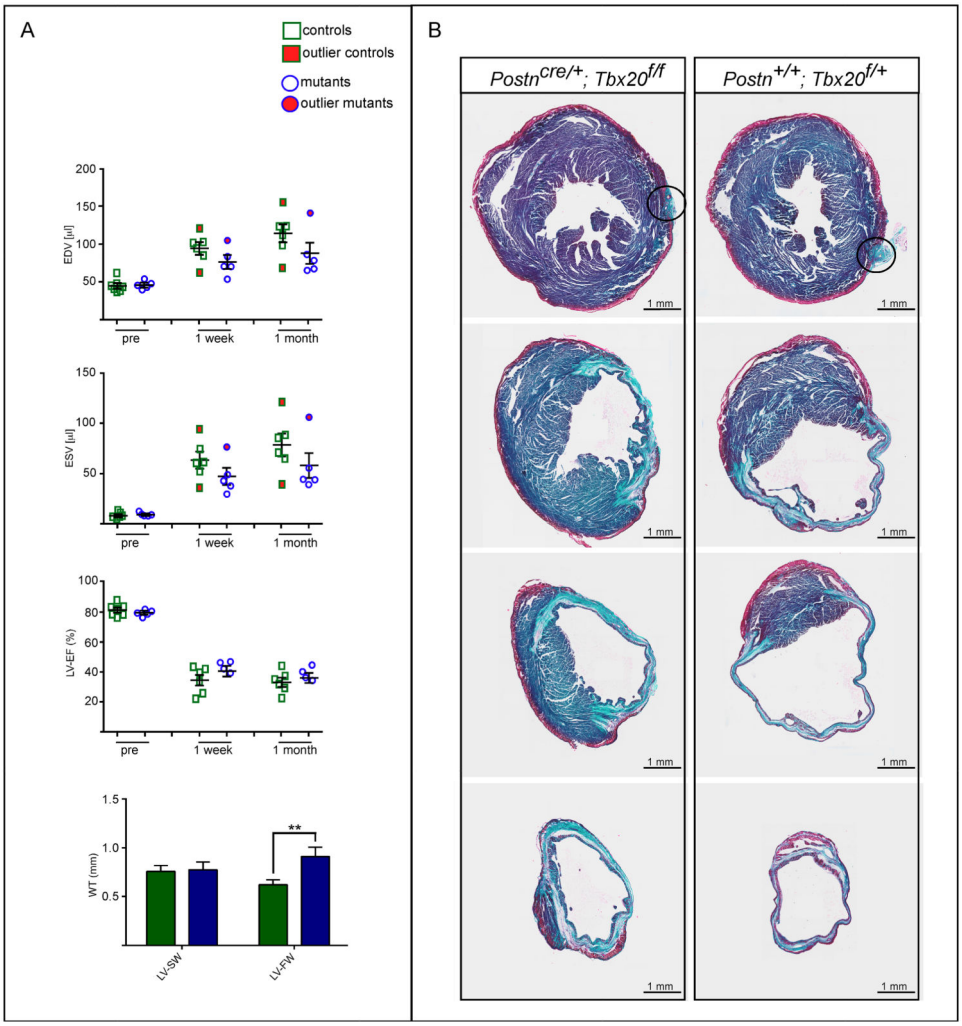

Figure 6. Increased scar thickness and reduced dilation in myocardial infarcts of *Postn*$^{cre/+}$;*Tbx20*$^{f/f}$ adult hearts

A. MRI analysis revealed reduced end systolic volume (ESV) and end diastolic volume (EDV) in *Postn*$^{cre/+}$;*Tbx20*$^{f/f}$ mutants 1 month after infarction (N=6 controls; N=5 mutants). These changes discretely improved left ventricular function, measured as left ventricular ejection fraction (LV-EF). Data did not reach statistical significance (Two-way ANOVA), due to experimental variability and the presence of outlier animals, represented in graphs in red filled squares (controls) or circles (mutants). Control animals had *Postn*$^{+/+}$;*Tbx20*$^{f/+}$ or *Postn*$^{cre/+}$;*Tbx20*$^{f/+}$ genotypes. Wall thickness was significantly increased in scar areas of *Postn*$^{cre/+}$;*Tbx20*$^{f/f}$ mutant hearts (Two-tailed T-test; $p<0.01$). B. Trichrome staining confirmed increased scar thickness (green) and reduced dilation of left ventricular free wall in *Postn*$^{cre/+}$;*Tbx20*$^{f/f}$ mutant animals. Black circle in upper panels marks stitches at upper infarct border.