Developmental Heterogeneity of Cardiac Fibroblasts Does Not Predict Pathological Proliferation and Activation


Rationale: Fibrosis is mediated partly by extracellular matrix–depositing fibroblasts in the heart. Although these mesenchymal cells are reported to have multiple embryonic origins, the functional consequence of this heterogeneity is unknown.

Objective: We sought to validate a panel of surface markers to prospectively identify cardiac fibroblasts. We elucidated the developmental origins of cardiac fibroblasts and characterized their corresponding phenotypes. We also determined proliferation rates of each developmental subset of fibroblasts after pressure overload injury.

Methods and Results: We showed that Thy1+CD45−CD31−CD11b−Ter119− cells constitute the majority of cardiac fibroblasts. We characterized these cells using flow cytometry, epifluorescence and confocal microscopy, and transcriptional profiling (using reverse transcription polymerase chain reaction and RNA-seq). We used lineage tracing, transplantation studies, and parabiosis to show that most adult cardiac fibroblasts derive from the epicardium, a minority arises from endothelial cells, and a small fraction from Pax3-expressing cells. We did not detect generation of cardiac fibroblasts by bone marrow or circulating cells. Interestingly, proliferation rates of fibroblast subsets on injury were identical, and the relative abundance of each lineage remained the same after injury. The anatomic distribution of fibroblast lineages also remained unchanged after pressure overload. Furthermore, RNA-seq analysis demonstrated that Tie2-derived and Tbx18-derived fibroblasts within each operation group exhibit similar gene expression profiles.

Conclusions: The cellular expansion of cardiac fibroblasts after transaortic constriction surgery was not restricted to any single developmental subset. The parallel proliferation and activation of a heterogeneous population of fibroblasts on pressure overload could suggest that common signaling mechanisms stimulate their pathological response. (Circ Res. 2014;115:625-635.)

Key Words: aging ■ cardiac fibroblasts ■ fibrosis ■ growth and development

Cardiac fibrosis underlies the pathological response of the mammalian heart to certain insults, such as a pressure overload injury, and this process is largely mediated through cardiac fibroblasts. The functions and characteristics of cardiac fibroblasts in scar formation and tissue remodeling are being actively explored.1 However, the study and analysis of cardiac fibroblasts are limited by imprecise definitions for this cell type and, concomitantly, by lack of specific markers to aid in their identification. Several other interstitial cell types, such as mesenchymal and inflammatory cells, express certain markers commonly used for identifying cardiac fibroblasts. One such example is fibroblast-specific protein 1 that is expressed in both fibroblasts and macrophages in the liver.2 Therefore, it is imperative to delineate and validate a robust set of markers to isolate cardiac fibroblasts.

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It is thought that injury induces the expression of α-smooth muscle actin (α-SMA) in a fraction of cells expressing fibroblast markers leading to their activation. These activated cells, termed myofibroblasts, secrete extracellular matrix to increase collagen content and hence cause histological fibrosis. Accumulating evidence supports the existence of heterogeneity among cardiac fibroblasts.1 For example, several developmental origins have been suggested for cardiac fibroblasts, such as the bone marrow (BM),3 embryonic endothelium,4 circulation,5 neural crest,6,7 and epicardium.8 Nevertheless, it is not known whether certain fibroblast subtypes, on the basis of their developmental origin, have a propensity toward proliferation and activation after...
profibrotic cardiac injuries. Indeed, it remains unclear whether fibroblasts from different lineages exhibit phenotypic or transcriptional disparities. Understanding the developmental origin of cardiac fibroblasts may enable identification of fibroblast subsets that are directly involved in fibrosis and may thus help to develop novel treatment strategies to target fibrosis.

Herein, we define a set of surface markers that distinguish cardiac fibroblasts from other cell types in the heart. We use fate-mapping models, BM transplantation, and parabiosis studies in the setting of physiological aging as well as pressure overload injury to determine the developmental sources of cardiac fibroblasts and to elucidate whether pathological activation of fibroblasts is developmentally determined. We demonstrate that cardiac fibroblasts derive primarily from the epicardium, whereas a smaller fraction originates from the endothelial lineage, and a minority from the neural crest. Our work further shows that circulating, hematopoietic, and stromal cells do not contribute to the cardiac fibroblast pool. Interestingly, we did not observe any differences in proliferation rate among cardiac fibroblasts of different developmental origins after pressure overload injury. Rather, our findings show that fibroblasts from distinct lineages have a similar phenotype and gene expression profile in a given context, such as aging and injury.

**Methods**

An expanded Materials and Methods is available in the Online Data Supplement.

**Mice**

Pax3Cre, Tie2Cre, Wt1CreERT2, Myh11Cre-GFP, Vav1Cre, CAGdsRed, and C57Bl/6 mice strains were obtained from the Jackson Laboratory and have been described previously. B6C3F1 transgenic mice, Myh6-GFP, and R26R<LoxP/>reporter mice were gifts from Sylvia Evans (San Diego, CA), Deepak Srivastava (San Francisco, CA), and Liqun Luo (Stanford, CA), respectively. All procedures were performed with the approval of the University of California, Los Angeles Animal Research Committee or the Institutional Animal Care and Use Committee at Stanford University. Two operators blinded to the genotype and experimental design performed all animal surgeries.

**Isolation of Cardiac Fibroblasts**

Mice were injected with heparin and euthanized, then the heart was dissected out and perfused with Hank’s balanced salt solution. The hearts were cut into small pieces and digested with Liberase Blendzyme TH and TM (Roche) in Medium 199 plus DNase I and poloxamer at 37°C for 1 hour. Cells were passed through a 70-μm cell strainer (BD Falcon) and centrifuged. The cell pellet was resuspended in staining buffer (3% fetal bovine serum in Hanks’ balanced salt solution) containing the relevant Thy1+HE− (HE refers to hematopoietic and endothelial lineages) surface marker antibodies (Online Table I) and incubated in the dark for 30 minutes at room temperature.

**Parabiosis**

Mice of the same weight, size, and sex were paired ≥2 to 3 weeks before the surgery, after being observed for at least a week to make sure that they were compatible. Mice were anesthetized by isoflurane in O2. Toe pinch was used as an indicator of the pain response. After shaving the corresponding lateral aspects of each mouse, the site was disinfected using an alcohol swab and povidone/iodine. Matching skin incisions are made from the olecranon to the knee joint of each mouse, and the subcutaneous fascia was bluntly dissected to create ≈0.3 cm of free skin. One partner had the procedure done on the right side and the other on the left. The right olecranon of 1 animal is attached to the left olecranon of the other by a single 4-0 silk suture and tie. The partners’ knee joints were similarly connected. The dorsal and ventral skin were then approximated by staples or by continuous suture, and the animals were warmed with heating pads and monitored until recovery. Parabiotic pairs were housed 1 pair per cage and given acidified water (pH 2.5). After 4 weeks of anastomosis, blood samples from each animal in a parabiotic pair were analyzed using flow cytometry. Animal pairs with <90% blood chimerism were excluded from our studies. Biological replicates were used, and the experiment was repeated 3 times in the laboratory (n=4–6 per sample; minimum n=3 to observe statistical significance, with more to account for possible animal death).

**BM Transplant**

C57Bl/6 mice (H2b, Thy1.1+CD45.2−dsRed+) served as donors for hematopoietic stem cells (HSCs), green fluorescent protein (GFP)-expressing or dsRed-expressing C57Bl/6 mice (H2b; Thy1.2+CD45.2+GFP) were used as donors for nonhematopoietic BM cells for transplantation into C57Bl/6 (H2b, Thy1.1+CD45.2−) recipients. Donors were 6 to 12 weeks old, recipients were 8 weeks old at transplant. BM was flushed from tibiae and femora into Hanks’ balanced salt solution/2% fetal bovine serum, enriched for c-Kit (3C11) cells by magnetic column separation (CD117 MicroBeads, MACS Separation Columns LS; Miltenyi Biotec, Auburn, CA), and KTLS (c-Kit+, Thy1.1lo, lineage marker+, Sca-1+) HSC were purified by FACS (fluorescence-activated cell sorting)-sorting, selecting for c-Kit+, Thy1.2+−, Sca-1+, Lin− (CD3, CD4, CD5, CD8α, B220, Gr1, Mac1, and Ter119). A total of 1000 FACS-purified HSCs were infused per mouse. For cotransfer of nonhematopoietic cells, BM was flushed from tibiae, femora, and pelvis. Nonhematopoietic cells were extracted by magnetic column depletion of CD45+ cells (CD45 MicroBeads; Milltenyi Biotec). C57Bl/6.CD45.2 recipients received a lethal 1050 cGy dose total body γ-irradiation, +5 hours before tail-vein injection of a radioprotective dose of 1000 KTLS-HSC. In cotransfer experiments, 5×105 CD45− stromal/nonhematopoietic cells were injected simultaneously with the HSCs. Biological replicates were used, and the experiment was performed 3 times.

**Statistical Analysis**

Statistical testing was performed with Microsoft Excel version 12.2.8 and GraphPad Prism. Results are presented as mean±SEM and were compared using a 2-tailed Student’s t test or 2-way ANOVA (significance was assigned for P<0.05).

**Results**

**Thy1+HE− Cells Exhibit a Fibroblast Phenotype In Vitro and In Vivo and Encompass the Majority of Cardiac Fibroblasts**

We applied a panel of surface markers to identify cardiac fibroblasts: Thy1 as an inclusive surface protein (Thy1+) given its association with cardiac fibroblasts,1011 and exclusion of hematopoietic cells (CD45+Ter119−), macrophages (CD11b+),2 and endothelial cells (CD31−). In accordance with the reported proportion of cardiac fibroblasts in mice,12 this combination (hereafter called Thy1+HE−) marked >30% of the cells in adult mouse hearts (Figure 1A). Using cardiomyocyte-specific and smooth muscle reporter transgenic mice, we show that cardiomyocytes and vascular smooth muscle cells are excluded from the Thy1+HE− population (Online Figure IA and IB). In comparison with age-matched whole heart controls, Thy1+HE− cells exhibit markedly elevated expression of fibroblast- and...
extracellular matrix (ECM)-associated genes (Figure 1B), whereas cardiomyocyte and vascular cell–related genes are expressed at low levels (Figure 1B, inset). Because individual fibroblast markers may be found on multiple cell types, we hypothesized that individual fibroblasts may not express every fibroblast-associated marker. We quantified the fraction of Thy1+HE− cells from whole hearts coexpressing known fibroblast markers using flow cytometry, and we demonstrate an enriched expression of collagen I (Col1), discoidin domain-containing receptor 2 (DDR2), and platelet-derived growth factor receptor α (PDGFRα) in the Thy1+HE− population compared with the whole heart by flow cytometry. Sorted cells retain a mesenchymal morphology and maintain expression of these proteins after 3 passages. Exposure to transforming growth factor (TGF)-β1 induces expression of α-smooth muscle actin (α-SMA) in cultured Thy1+HE− cells in a dose-dependent manner (scale bar, 50 μm). All error bars represent SEM. DAPI indicates 4′,6-diamidino-2-phenylindole.

**Figure 1.** Characterization of Thy1+HE− cells. A, After gating for cells on forward and side scatter axes, and excluding doublets, cells that were negative for CD45, Ter119, CD11b, and CD31 could be divided into Thy1+ and Thy1− populations. Of these 2 fractions, the Thy1+ cells were designated as fibroblasts and selected for analysis in all experiments. B, Relative gene expression in Thy1+HE− cells from 8-week-old mice with reference to age-matched whole heart controls for fibroblast- and nonfibroblast-associated genes (inset; using GAPDH as reference gene; n=3 mice). C, Thy1+HE− cells highly express the fibroblast markers collagen I (Col1), platelet-derived growth factor receptor α (PDGFRα), and discoidin domain-containing receptor 2 (DDR2) in comparison with the whole heart by flow cytometry. Sorted cells retain a mesenchymal morphology and maintain expression of these proteins after 3 passages. D, Exposure to transforming growth factor (TGF)-β1 induces expression of α-smooth muscle actin (α-SMA) in cultured Thy1+HE− cells in a dose-dependent manner (scale bar, 50 μm). All error bars represent SEM. DAPI indicates 4′,6-diamidino-2-phenylindole.

**Figure 2.** Proliferation of cardiac fibroblasts after pressure overload injury. A, The percent of Thy1+HE− cells undergoes a small, nonsignificant increase upon transaortic constriction (TAC) injury, but plateaus after 7 days post injury. Similarly, the percentage of bromodeoxyuridine (BrdU+) fibroblasts increases from a baseline of 2% to 10% at 7 days after TAC, then remains constant through 30 days post TAC (n=4 mice per group). B, Immunohistochemistry of heart sections in A shows colocalization of BrdU with cells expressing fibroblast and myofibroblast markers in TAC (bottom) compared with sham sections (top; scale bar, 50 μm). All error bars represent SEM.
pericytes, respectively) was rarely detected in the Thy1+HE− cells (Online Figure IC). Sorted Thy1+HE− cells were cultured in vitro for several passages (Online Figure ID), and they retained expression of fibroblast-associated ECM and structural proteins. Cultured cells also expressed the fibroblast markers Thy1, DDR2, and PDGFRα (Figure 1C), further demonstrating the fibroblast phenotype of Thy1+HE− cells (fibroblast-specific protein 1 marked a heterogeneous population of cells, including some hematopoietic cells. We therefore restricted our analysis to the aforementioned fibroblast-associated markers).

In culture, treatment of Thy1+HE− cells with transforming growth factor-β1 resulted in upregulation of α-SMA in a dose-dependent manner (Figure 1D). Furthermore, RNA-seq of Thy1+HE− cells isolated 7 days after transaortic constriction (TAC) or sham operation revealed an increased expression of ECM and ECM regulatory genes, activated fibroblast genes, and cell cycle genes in the TAC relative to sham specimens (Online Figure IE, IF, and IG), as would be predicted following fibrosis. This provides additional validation for the use of the Thy1+HE− panel of markers to isolate cardiac fibroblasts.

To determine whether the Thy1+HE− population encompasses all cardiac fibroblasts, we isolated Thy1+HE− cells from adult wild-type (WT) mouse hearts and examined the fibroblast phenotype of Thy1+HE− cells (fibroblast-specific protein 1 marked a heterogeneous population of cells, including some hematopoietic cells. We therefore restricted our analysis to the aforementioned fibroblast-associated markers). Cells were intravenously delivered in a host mouse after myeloablation. B, GFP− and RFP− cells are not present in the Thy1+HE− fraction 30 days after transaortic constriction (TAC) or sham in BM-recipient mice (n=4). C, Immunohistochemistry (IHC) shows that RFP− cells stain primarily with CD45. Representative TAC sections were stained with Periostin and α-smooth muscle actin (α-SMA) from the fibrotic areas in the subepicardium of the free ventricular wall. D, Schematic demonstrates surgical anastomosis of unlabeled and GFP-labeled adult female mice to achieve parabiosis. E, Thy1+HE− sorted cells from wild-type (WT) mouse hearts 7 and 30 days after parabiosis do not express GFP (n=4). F, IHC staining for CD45, collagen I (Col1), α-SMA and Vimentin (Vim; arrows indicate cells that costain for the marker of interest, and arrowheads indicate cells that do not costain with the marker). Scale bar 50 μm for merged panel and 12.5 μm for the inset. DAPI indicates 4′,6-diamidino-2-phenylindole.

Next, we sought to determine whether increased fibrosis on pressure overload TAC injury occurs through fibroblast proliferation as opposed to an increase in ECM production from a fixed number of fibroblasts (Online Figure IIA and IIB). Adult mice (8 weeks old) underwent TAC or sham operation and were analyzed. Masson’s trichrome staining of the respective tissue revealed sparse global fibrosis at 4 days after injury that increased by day 7 and persisted at 2 weeks (Online Figure IIC) in the TAC hearts. Using flow cytometry, we observed an increase in the number of Thy1+HE− cells as well as an increase in the fraction of Thy1+HE− cells on TAC injury relative to sham (Figure 2A; Online Figure IID). In accordance, ≈10% of the Thy1+HE− cells derived from the operated mice were bromodeoxyuridine (BrdU+) at 7 days after TAC and remained at this level at 4 weeks, compared with 2% in the sham mice (P=0.008; n=3 mice/group/time point; Figure 2A). Immunohistochemistry of TAC specimens showed BrdU+ cells that costained with the fibroblast markers DDR2 and PDGFRα. BrdU+ cells also costained with α-SMA and Col1 (Figure 2B). Together, these data suggest that fibroblast proliferation in response to TAC occurs primarily during the first week after injury.

To determine whether apoptosis of fibroblasts occurs after TAC injury, we analyzed for Annexin V and propidium iodide (PI) expression on Thy1+HE− cells from WT mice at 1, 4, 7, and 14 days after injury. We observed a significant increase in fibroblasts undergoing late apoptosis (defined by Annexin V′PI′...
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staining) 4 days post TAC (Online Figure IIE) compared with sham. At all other time points, the extent of apoptosis was equivo-
cal between the 2 surgical groups (Online Figure IIE). It is pos-

sible, however, that nonapoptotic pathways (necrosis, elimination

by the reticulo-endothelial system, etc) also lead to clearance of

fibroblasts. We therefore examined for the presence of necrotic

cells among the Thy1+HE- fibroblasts by analyzing the uptake

of PI. We observed rare necrosis of fibroblasts at the time points

studied (7 and 14 days after sham and TAC), with no significant

increase in necrosis in the TAC relative to the sham fibroblasts

(Online Figure IIIF). These data further support that the first week

after pressure overload injury is the most dynamic period with

respect to cell turnover. Fibroblast apoptosis peaks during this
time but is counteracted by a more robust generation of new fi-
broblasts. The time course of these cellular changes suggests a
discrete temporal period for potential therapeutic interventions.

BM and Circulating Cells Do Not Generate Cardiac Fibroblasts During Development, Aging, or After Pressure Overload Injury

Hematopoietic cells have been reported to contribute to the formation of scar tissue after cardiac injury.5 To rigorously test

this hypothesis, we transplanted GFP-labeled whole BM cells

or red fluorescent protein (RFP)-labeled HSCs along with

CD45-depleted GFP-labeled BM stromal cells into adult mice

after myeloablation.15 Eight weeks after engraftment, TAC

served labeled cells grossly in both surgical groups16 (Online

Figure IIID). Flow cytometric analysis (Figure

3B). Moreover, HSC-derived RFP+ cells expressed the hematopoietic marker CD45 rather than fibroblast markers (Figure 3C).

To further confirm whether circulating cells can be a cell of

origin for cardiac fibroblasts, we surgically paired WT and GFP-
labeled female mice to achieve parabiosis (Figure 3D). Blood

chimerism was obtained by 4 weeks after anastomosis (Online

Figure IIB and IIIC) and GFP+ cells were observed in the hearts

of both mice. To determine whether pressure overload injury fa-
cilitates fibroblast or fibroblast-like cell generation by circulating
cells, we performed TAC injury on the WT mouse of WT-GFP

parabiotic pairs 4 weeks after surgical anastomosis. Heart/body

weight ratio demonstrated cardiac hypertrophy after TAC injury

(Online Figure IID). Flow cytometric analysis (Figure 3E) and

immunohistochemistry (Figure 3F; Online Figure IIIE) at 7 and

30 days after injury did not reveal any GFP+ circulating cells

expressing the Thy1+HE- set of markers; instead, the hematopoi-
etsic marker CD45 could be detected in GFP+ cells (Figure 3F).

Analysis of the BM transplantation and parabiosis models

excludes a hematopoietic or BM stromal contribution to cardiac fibrosis during pressure overload injury. However, a

potential developmental contribution of hematopoietic progeni-

tors or lineages to cardiac fibroblasts cannot be excluded; such a

phenomenon could reconcile the transplantation data with previous studies.17 To examine whether cardiac fibro-

blasts have an embryonic hematopoietic origin, we generated

VavCreER2;R26RmTmG mice, in which GFP labels hematopoietic

progenitors and their progeny (Online Figure IIIF).18 We

did not observe any GFP expression in the Thy1+HE- cell

compartment (Online Figure IIIG). Collectively, these distinct

models exclude a hematopoietic, BM stromal, or circulating
cell origin for cardiac fibroblasts during development, physi-
osological aging, or on pressure overload.19

Endothelial-Derived Cells Generate Cardiac Fibroblasts

It has been reported that endothelial-to-mesenchymal transition

(EndMT) contributes to cardiac fibrosis on TAC injury.4 Whether

EndMT, which is known to occur during development to gener-

ate heart valves,20 also contributes to cardiac fibroblasts during

aging and injury remains unclear. We generated Tie2CreER2;R26RmTmG mice, in which Tie2-derived progeny is indelibly labeled with

GFP (Figure 4A). At 7 and 30 days after sham and TAC op-

erations, ≈20% of Thy1+HE- cells were GFP+ in both groups

(P=0.35; n=5 mice/TAC/time point and n=3 mice/sham/time point; Figure 4B). Therefore, the relative proportion of endothel-

ial-derived fibroblasts remains the same despite proliferation

after pressure overload, suggesting that fibroblast division on

injury is not restricted to the EndMT-derived fibroblast subset.

Immunohistochemistry demonstrated that GFP+ cells ex-

pressed DDR2 and PDGFRα, as well as Coll1 and Vim, in both

sham and TAC hearts (Figure 4C; Online Figure IVA and IVB).

Tie2-derived fibroblasts were found in association with most

blood vessels, whereas no single vascular territory exhibited a

disproportionate number of GFP+ fibroblasts. Some GFP+ cells,

predominantly in the fibrotic areas of TAC hearts, co-

localized with α-SMA expression, demonstrating the capacity

of this lineage to express myofibroblast markers (Figure 4C).

Pax3-Derived Cells Contribute to Cardiac Fibroblasts

The neural crest is a migratory population of cells that origi-
nates from the dorsal aspect of the neural tube. Neural crest–
derived lineages have been fate-mapped using the Pax3Cre

transgenic model21 and the cardiac neural crest has been pos-
tulated to contribute to the cardiac mesenchyme.22 Thy1+HE-

fibroblasts from WT hearts after sham and TAC operations
did not express Pax3, as evidenced by RNA-seq analysis

(data not shown). We therefore generated Pax3CreER2;R26RmTmG

mice to ascertain the cardiac contribution of neural crest cells

(Figure 5A). We observed many GFP+ cells in the outflow

tract, consistent with the previously described contribution of

neural crest cells (Figure 5B).23

The fraction of Thy1+HE- cells that were GFP+ remained

≈5% in both TAC and sham groups at 7 and 30 days post in-

jury (Figure 5C; P=0.4; n=5/TAC/time point and n=3/sham/
time point). Most GFP+ cells expressed the neural markers,

Sm32 and NF160, on immunohistochemistry (Online Figure

VA). However, we also observed rare, scattered GFP+ cells

in the atria and ventricles that stained with Coll1 and Vim

(Figure 5D). We did not detect expression of Pax3 in the GFP-

cells at both the mRNA (data not shown) and protein levels in

adult hearts (Online Figure VB), which argues against ectopic

expression of Pax3 in cardiac fibroblasts. However, we can-
not exclude transient Pax3 expression in cardiac fibroblasts

during development. These data support a limited contribution

of Pax3-expressing neural crest cells to cardiac fibroblasts,

which warrants further inquiry.
Epicardial Cells Form Cardiac Fibroblasts During Development But Not After Pressure Overload Injury

It has been reported widely that cardiac fibroblasts may be derived from the epicardium, which forms from the migrating cells of the transient proepicardium after E9.5 in mice. We generated Tbx18Cre/+;R26RmT/mG mice (Figure 6A) to determine whether epicardium-derived fibroblasts preferentially proliferate in response to pressure overload injury. The GFP+ cells in the Thy1+HE− population remained at ≈75% in both sham and TAC groups at 7 and 30 days post injury (P=0.35; n=5 mice/TAC/time point and n=3 mice/sham/time point; Figure 6B). We observed dispersed...
GFP+ cells that costained with Vim, DDR2, PDGFRα, and Col1 in sham and TAC heart sections (Figure 6C; Online Figures VIA, VIIA, and VIIB). We also observed GFP-labeled cells whose phenotype (CD146+CD31−) and anatomic location (intimate proximity to CD31+ endothelial cells of small capillaries) is characteristic of pericytes (Figure 6C). In the TAC hearts we also observed a significant amount of GFP+ cells remote from the vasculature (ie, not associated with blood vessels) that costained with α-SMA. Moreover, the majority of epithelial-to-mesenchymal transition (EMT)–derived fibroblasts were observed throughout the interstitium of left and right ventricular free walls.

To determine whether there is reactivation of the epicardium and subsequent EMT on pressure overload injury, we administered tamoxifen to adult Wt1CreERT2/+;R26RmT/mG mice before a sham or TAC operation, followed by tamoxifen delivery during the week after surgery (Online Figure VIB and VIC). On analysis 1 week after the sham operation, we observed rare epicardial and myocardial GFP expression, which did not colocalize with expression of fibroblast markers (Figure 6D; Online Figure VID). Notably, 1 week after the operation, we observed a 5-fold increase in the abundance of GFP+ cells in both the epicardium and the myocardium of TAC when compared with that in sham hearts (Online Figure VIDA). As in the sham model, these cells did not express fibroblast markers or α-SMA: many were CD146+CD31+ (endothelial markers) and a smaller fraction were CD146+CD31− (pericyte markers; Figure 6D), suggesting that in contrast to the effects of ischemic injury, Wt1-expressing cells may not generate fibroblasts in adulthood or on pressure overload injury. These data therefore argue against EMT as the signaling mechanism that underlies proliferation in the epicardial-derived fraction of fibroblasts after injury. On the contrary, the observed pattern of costaining suggests the following explanations: (1) endogenous endothelial expression of WT1 is induced after injury, (2) expression of WT1 in pericytes, and (3) potential generation of pericytes from the epicardium in adulthood and after injury (the expression of Wt1 in endothelial cells has been reported previously).27

Characterization of Pax3-, Tie2-, and Tbx18-Derived Fibroblasts In Vitro and In Vivo
To compare the phenotypic characteristics and gene expression profiles of fibroblasts from different developmental sources, we isolated GFP+ Thy1+HE- cells from each of the 3 Cre models and expanded them in culture. All fibroblast subsets exhibited a spindle-like morphology characteristic of mesenchymal cells and expressed Col1 (Figure 7A). On addition of transforming
growth factor-β1 to the culture medium, fibroblasts from all subsets expressed the activated fibroblast marker, α-SMA (Figure 7B). In vitro BrdU uptake assays showed that the relative proliferation rate (based on BrdU optical density) is similar among all 3 developmental subsets at individual time points after BrdU exposure, and this rate increases in parallel in all subsets over time (Online Figure VIIIA).

We next studied the anatomic distribution of each fibroblast subset in sham and TAC hearts 7 days after injury (Figure 7C; Online Figure VIIIB): Tbx18-derived fibroblasts seemed evenly distributed throughout all cardiac chambers, whereas Tie2-derived fibroblasts exhibited a trend toward preferential localization in the ventricles and septum. The majority of Pax3-derived fibroblasts were localized in the right atrium. Interestingly, the relative abundance of each subset with respect to individual chambers did not change on TAC injury.

To directly measure the in vivo proliferative capacity of the fibroblast subsets on TAC injury, we administered BrdU to Tbx18Cre/+;R26RmT/mG and Tie2 Cre/+;R26RmT/mG mice starting at the time of surgery until analysis 7 days later (Figure 7D).
fraction of BrdU+ fibroblasts in the sham group was similar between the 2 models (≈5% for Tbx18 versus ≈6% for Tie2; P=0.13; n=3 mice/group) and significantly increased after TAC injury (≈13% for Tbx18, ≈15% for Tie2; P=0.07; n=3 mice/group). Similar percentages of BrdU+ fibroblasts were observed in WT hearts (Figure 2A). These data directly confirm that on pressure overload injury, there is a balanced proliferation in each developmental subset of fibroblasts. Collectively, these findings suggest that the developmental origin affects the anatomic distribution of cardiac fibroblasts but does not result in phenotypic differences with respect to the expression of fibroblast-associated genes, the propensity towards activation in response to appropriate stimuli, or their proliferation rate.

**Tbx18- and Tie2-Derived Fibroblasts Have Similar Transcriptional Profiles Upon Pressure Overload Injury**

In an effort to determine phenotypic similarities among the different fibroblast developmental subsets, we performed RNA-seq of Tie2-derived and Tbx18-derived fibroblasts isolated 7 days post TAC or sham operation. To quantify and compare the gene expression level within and between the sham and TAC groups (ie, Tie2-derived and Tbx18-derived fibroblasts), we calculated and normalized the expression level of the genes through reads per kilobase of exon model per million mapped reads as described previously. The total number of expressed genes (reads per kilobase of exon model per million mapped reads, ≥1) in Tie2 sham and Tbx18 sham was 9198 and 8845, respectively (Figure 7E). Among them, 1950 genes in Tie2 sham and 2277 genes in Tbx18 sham showed a high level of expression (reads per kilobase of exon model per million mapped reads, ≥11), with 1851 common genes between these 2 groups (Figure 7F). Of 10079 genes expressed in Tie2 TAC, 1828 genes showed a high level of expression, whereas 2711 genes of 8968 genes expressed in Tbx18 TAC were highly expressed (Figure 7F). Among the common genes expressed in sham and TAC fibroblasts were fibroblast- and fibrosis-related markers, whose expression increased after TAC operation (Figure 7G; Online Table III). For example, fibroblast-related genes, such as Col1a1, Col1a2, Postn, Thy1, and DDR2, were expressed at approximately the same level between Tie2 sham and Tbx18 sham fibroblasts; expression of these genes increased in the respective TAC fibroblasts. ECM homeostasis-related genes showed a dynamic variation between sham and TAC fibroblasts: Sparc, Timp1, Mmp14, and Mmp2 showed upregulation in TAC compared with sham fibroblasts, whereas Timp2 and Mmp18 expression levels did not show a significant change (Online Table III). Upregulation of transforming growth factor, tumor necrosis factor, LIF (leukemia inhibitory factor) signaling components on TAC injury suggests a role for these factors during and after pressure overload injury and cardiac hypertrophy. These data further attest that developmentally distinct fibroblasts have parallel gene expression patterns that change similarly in response to pressure overload injury, providing evidence for comparable phenotypes.

### Discussion

The lack of cardiac fibroblast–specific markers has made it challenging to investigate the lineage origin of these cells and their response to injury. The previously reported markers are either not specific for fibroblasts because they are expressed by other interstitial cells or they only mark a subpopulation of fibroblasts. Here, we identified a panel of surface markers (Thy1+HE+) and validated its use for prospective identification of cardiac fibroblasts using several approaches: (1) immunohistochemical and flow cytometric analysis using a combination of fibroblast markers in the context of their anatomic location, (2) global- and fibroblast-specific gene expression analysis, (3) in vivo proliferation in response to injury, and (4) in vitro characterization. This set of core surface markers may form the basis for further refinement and fractionation of cardiac fibroblasts to delineate phenotypically or pathologically distinct fibroblast subsets in future studies.

We used several experimental tools to demonstrate that the progeny of BM and circulating cells can migrate to the heart, especially in the context of injury, but they do not adopt a fibroblast phenotype. Use of the Thy1+HE+ markers may exclude fibrocytes, which are putative circulating hematopoietic-derived cells that participate in the process of fibrosis and scar formation in the heart after injury. However, given previous reports of inflammatory cells that express fibroblast markers but do not deposit ECM (and thus phenotypically are not fibroblasts), better characterization of this cell population is needed, especially given the results from our BM transplantation, parabiosis, and the lineage-tracing hematopoietic models described above. Moreover, a recent study demonstrated the near-complete absence of hematopoietic-associated genes (eg, CD45, CD34) in cardiac fibroblasts. However, it is possible that pressure overload injury may not be the ideal injury model to study the possible recruitment of circulating progenitors to the heart (to generate fibroblasts), perhaps, because of a less severe inflammatory response as compared with other injury models. Although it has been shown previously that epicardial cells generate cardiac fibroblasts, our work demonstrates that although EMT is an important developmental process, its induction after injury does not generate fibroblasts (unlike during development). We found rare cells staining for epicardial markers after TAC injury dispersed throughout the myocardium rather than localized to the fibrotic regions, but this likely represents ectopic expression by interstitial and endothelial cells as these cells did not coexpress fibroblast markers. It is possible, however, that epicardial cells undergo EMT on injury and generate mesenchymal cells that are confined to the subepicardial location without migration to the area of fibrosis. It is technically possible that inefficient recombination in the used inducible system or the short time course before analysis may have led to underrepresentation of fibroblasts being generated from EMT in the W1-CreERT2 model (although we show that proliferation peaks by 1 week post injury).

Our data corroborate some of the findings of Zeisberg et al in identifying endothelial-derived fibroblasts as a subset of cardiac fibroblasts but differs in the absence of evidence for active EndMT on TAC injury. We show that the relative proportion of endothelial-derived fibroblasts remains the same after injury in the setting of a global increase in fibroblasts. By itself, this finding could certainly be explained by EndMT occurring after pressure overload, as this could lead to increased labeling using the Tie2Cre model. However, based on the data obtained with the Tbx18Cre and Pax3Cre models, as well as the fact that the percentage of endothelial-derived fibroblasts remains the same post injury, it can be inferred that proliferation of existing fibroblasts, rather than
EndMT, is the primary response to pressure overload. The differences between the 2 studies could be explained by the use of different transgenic fate-mapping mouse models. In our study, we used the Tie2Cre rather than the Tie1Cre model. Developmental studies have demonstrated that the Tie1 promoter drives gene expression in endothelial cells from embryonic day E10 until birth as well as in a small fraction of hematopoietic cells (≥12%–20% of the adult erythroid, myeloid, and lymphoid cells). Although the expression pattern is similar to Tie1Cre mice, Tie2 expression starts as early as embryonic day E8.5, hence making it potentially a more suitable mouse model to lineage trace a greater proportion of endothelial cells. Moreover, experimental differences such as the length of time from injury to analysis as well as the use of fibroblast-specific protein 1 and α-SMA as markers for the identification of cardiac fibroblasts could explain the discrepancies observed between the 2 studies. Fibroblast-specific protein 1 marks the myeloid lineage in addition to fibroblasts, which may confound interpretation of fibroblast lineage studies given the abundance of BM-derived cells after TAC injury.

Recently, a comprehensive study by Moore-Morris et al was published that corroborates our findings herein using a collagen1a1-GFP reporter mouse, which could identify cardiac fibroblasts based on the expression of GFP. Although they used an alternate method for labeling cardiac fibroblasts, they used similar genetic models (Tbx1Cre, Wt1Cre, Wt1CreER, Tie2Cre, and others) and sought to clarify the embryonic lineages that generate fibroblasts in the heart. In line with our data, they showed that the epicardial and endothelial lineages are the primary contributors (ie, no apparent contribution from hematopoietic cells). On aortic banding injury of adult mice, moreover, they did not observe reactivation of EMT or EndMT; furthermore, fibroblasts of epicardial and endothelial origins had similar gene expression patterns in the setting of aortic banding. Therefore, the independent report by Moore-Morris et al that takes advantage of a unique transgenic reporter model for cardiac fibroblasts validates the findings in this study.

In spite of their developmental heterogeneity, fibroblasts from disparate subsets proliferate at a parallel rate on TAC. The developmental programs that generate fibroblasts in utero do not seem to mediate proliferation after injury, as confirmed by Moore et al. These data suggest that ontogeny does not determine the pathological proliferation of cardiac fibroblasts elicited by pressure overload. It is more likely that a shared mechanism stimulates division of embryonically distinct fibroblast subsets. Moreover, the data derived from the RNA-seq studies could lead to a precise understanding of the signaling pathways that regulate each developmental subset and the entire fibroblast population in response to injury.

Prospective isolation of cardiac fibroblasts using our panel of surface markers may facilitate future studies to characterize signaling pathways that regulate the response of this pathologically important cell type to injury and especially to determine the molecular mechanisms that drive proliferation on injury. Identification of such mechanisms would be therapeutically relevant, as inhibiting fibroblast proliferation may prevent deposition of scar tissue and, thereby, minimize fibrosis.

Acknowledgments

We acknowledge Konstantina-Ioanna Sereti for critical reading of the article and Dr Matt Schibler for assistance with confocal images. We would also like to acknowledge Dr Xinmin Li and Jian Zhou of the University of California, Los Angeles (UCLA) Clinical Microarray Core with their assistance in RNA sequencing. Flow Cytometry experiments were performed in UCLA Broad Stem Cell Research Center Flow Cytometry Core Resource. S.R. Ali, S. Ranjbarvaziri, and R. Ardehali conceived the project and designed the experiments. S.R. Ali, S. Ranjbarvaziri performed the majority of the experiments and analyzed data from all experiments with R. Ardehali. Figures were prepared by S.R. Ali, S. Ranjbarvaziri, and M. Talkhabi. M. Talkhabi, P. Kamran, and Z. Tang assisted with immunohistochemistry. A. Subat assisted with experimental breeding, and A. Hojjat assisted with RNA-seq experiments. K.S. Volz performed the BrdU experiments. P. Zhao performed the surgeries. A.M.S. Müller performed bone marrow transplantation. K. Red-Horse provided Tbx1Cre/+;R26RmTmG mice. S.R. Ali, S. Ranjbarvaziri, and R. Ardehali wrote the article.

Sources of Funding

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Disclosures

None.

References

On injury, cardiac fibroblasts from different lineages exhibit similar function and proliferation rates and gene expression patterns, suggesting that cardiac fibroblasts are functionally identical in spite of distinct developmental origins. Our findings indicate that epicardial- and endothelial-derived fibroblasts proliferate at a similar rate and have a similar pattern of gene expression that epicardial or circulating cells. Importantly, we discovered that epicardial fibroblasts and studied their developmental origins using lineage-tracing experiments. We found that most cardiac fibroblasts are derived from the epicardium and the endothelium during development. Using multiple models, we show that hematopoietic cells do not generate cardiac fibroblasts. Importantly, we discovered that epicardial- and endothelial-derived fibroblasts proliferate at a similar rate and have a similar pattern of gene expression in response to pressure overload—induced stress/injury. These findings suggest that developmental origin of cardiac fibroblasts does not affect their pathological propensity; rather, cardiac fibroblasts may be functionally similar. Our findings suggest that therapeutic targets for pathological cardiac fibrosis should aim to identify the common signaling pathways that are activated on injury in all fibroblasts.
Developmental Heterogeneity of Cardiac Fibroblasts Does Not Predict Pathological Proliferation and Activation


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SUPPLEMENTAL MATERIAL.

**Mice**
Pax3<sup>Cre/+</sup>, Tie2<sup>Cre/+</sup>, Wt1<sup>CreERT2/+</sup>, Myh11<sup>cre/+;GFP</sup>, Vav1<sup>Cre/+</sup>, CAG-dsRed and C57Bl/6 mice strains were obtained from the Jackson Laboratory and have been described previously. Tbx18<sup>Cre</sup> transgenic mice<sup>6</sup>, Myh6<sup>-GFP</sup> and R26R<sup>mT/mG</sup> reporter mice were gifts from Sylvia Evans (San Diego, CA) and Liqun Luo (Stanford, CA) respectively. All procedures were carried out with the approval of the University of California, Los Angeles (UCLA) Animal Research Committee or the Institutional Animal Care and Use Committee at Stanford University. Two operators blinded to the genotype and experimental design performed all animal surgeries.

**Tamoxifen.**
Tamoxifen (Sigma) was dissolved in corn oil (Sigma) by sonication at a concentration of 20mg/ml. Tamoxifen was administered by gavage over two weeks starting one week prior to surgery to the Wt1<sup>CreERT2/+;R26R</sup><sup>mT/mG</sup> mice. Mice received a total of six doses of 4mg during this period.

**Genotyping.**
The following primers were used for genotyping:
Cre: 5' GCGGTCTGGCAGTAAAAACTATC -3' and 5' GTGAAACAGCATTGCTGTCACTT -3';
mT/mG: 5' CTCTGCTGCCTCCTGGCTTCT 3', 5' CGAGGCGGATCAACAAGCAATA -3' and 5' TCAATGGGCGGGGGTGC -3'

**Transaortic Constriction**
Adult mice weighing 25± 5 g were randomly divided into sham and TAC groups (n=4-6 per group; minimum n=3 to observe statistical significance, with more to account for possible animal death). Biological replicates were used, and the experiments were repeated 2-3 times in the lab. The number of animals used for each group was estimated based on mortality rate of 4-8% upon transaortic constriction, as well as in accordance with past studies using this injury model. Although there was a small variation in the severity and extent of cardiac fibrosis, there was a near-uniform degree of LV dysfunction. Animals were anesthetized by i.p. injection of ketamine/xylazine (100 mg/10 mg/kg). Endotracheal intubation was performed using a blunt 20-gauge needle that was then connected to a volume-cycled rodent ventilator (SAR-830/P; CWE, Inc.) with a tidal volume of 0.2 ml and a respiratory rate of 120/min. The chest was entered in the second intercostal space at the top left aortic arch, the transverse aorta was isolated, and aortic constriction was performed by tying a 7-0 nylon suture ligature against a 27-gauge blunt needle. The needle was then removed to yield a constriction 0.4 mm in diameter. In sham-operated control mice, the entire procedure was identical except that aortic constriction was not performed. The chest tube was used to evacuate the pneumothorax, and it was removed once negative pressure was re-established. The chest was closed in layers using 5-0 Vicryl sutures. Ventilation was maintained until sufficient spontaneous breathing occurred, followed by extubation and removal of the chest tube. The whole surgical procedure was
performed under aseptic conditions. For analysis, animals were randomly divided to 1, 2, and 4 weeks post-operation groups.

**BrdU detection by flow cytometry for in vivo proliferation studies**
BrdU powder (Sigma) was dissolved in saline (10mg/ml). BrdU was injected intraperitoneally after aortic constriction surgery. Mice were supplied BrdU-added water (1 mg/ml) until analysis (as described earlier). BrdU water was changed every two days. BrdU intracellular staining was performed according to instructions of the BD Pharmingen™ BrdU Flow Kits. In short, cells were fixed and permeabilized in Cytofix/Cytoperm Buffer (BD), followed by incubation in Cytoperm Permeabilization Buffer plus (BD) and DNase treatment (BD) and exposure to fluorescent anti-BrdU antibody. Cells were washed and resuspended in staining buffer and analyzed using flow cytometry.

**Echocardiography**
In vivo cardiac morphology was assessed by transthoracic echocardiography in live mice as described (23), including the following indices: left ventricular size (end-diastolic and end-systolic dimension), wall thickness (posterior wall thickness), and ventricular function (ejection fraction). ECHO was performed on mice sedated with isoflurane vaporized (2.5% for induction, 1.0% for maintenance) in oxygen. The animal's chest was shaved and positioned in the left lateral decubitus position for ultrasonic imaging with Vevo 770 high-resolution ECHO system equipped with a 35 MHz transducer. LV chamber dimensions and PWT were obtained from M-mode images; LV systolic function is also assessed from these measurements by calculating EF. All mice underwent echocardiography one week after TAC or sham. Studies and analysis were performed blinded to heart condition.

**Isolation and culture of Cardiac Fibroblasts (CF)**
Before sacrifice, mice were injected with heparin, then the heart was dissected out and perfused with HBSS. The hearts were cut into small pieces and digested with Liberase Blendzyme TH and TM (Roche) in Medium 199 plus DNAse I and polaxamer in 37°C for 1h. Cells were passed through a 70µm cell strainer (BD Falcon) and centrifuged. The cell pellet was resuspended in staining buffer (3% FBS in HBSS) containing the relevant Thy1+HE- surface marker antibodies (Supplemental Table 1) and incubated in the dark for 30 min at room temperature. Thy1+HE- cells were cultured on a 0.1% gelatin-coated 6-well plates in DMEM supplemented with 15% FBS and antibiotics. The medium was changed 24 hours after the primary culture, followed by changes every 48h. After first passage cells were washed and cultured in serum-free culture medium supplemented with 0.5 mg/ml insulin and 0.5 mg/mL transferrin. At confluence, cells were randomly treated either with 0, 10, 25 or 50 ng/mL TGF-β1. After 4 days, untreated and TGF-β treated cells were washed, fixed in 4% paraformaldehyde and stained for expression of Col1 and α-SMA. Cell counts were performed on ImageJ using the ‘Cell Counter’ plug-in and performed by two people blinded to the cell type and condition.

**Flow cytometry analysis**
Digested cells from adult mice hearts were suspended in staining buffer (3% FBS in HBSS) containing the relevant surface marker antibodies (Supplementary Table 1). These were incubated in the dark for 30 minutes at room temperature. After
three washing steps, a secondary antibody conjugated to Alexa flour 488 was added. For intracellular staining of Col1, cells were fixed with 4% PFA for 15 min at room temperature before staining. In all steps, a permeabilization buffer containing 0.2% saponin (Sigma) was used. To analyze the anatomic localization of fibroblasts, after perfusion with HBSS, we separate left and right atria, and also left and right ventricles and septum from each heart under the dissecting microscope (Leica). Biological replicates (n=3) were used.

**Quantitative RT-PCR**

Total RNA from hearts and sorted cardiac fibroblasts (Thy1+HE- cells) were extracted using TRI reagent (Sigma) and RNeasy MinElute Cleanup Kit (Qiagen) according to manufacturer’s instructions. The concentration and quality of extracted RNA were measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Complementary DNAs (cDNAs) were synthesized using an Omniscript RT Kit (Qiagen). For quantitative RT-PCR, we used a QuantiTect SYBR Green PCR Kit (Qiagen), amplified cDNA and gene specific primers (Supplemental Table 2) on a PRISM 7900HT Sequence Detection System (Applied Biosystems). PCR conditions included initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72 °C for 45 s, followed by a final extension at 72°C for 10 min. The mean cycle threshold (Ct) values from triplicate measurements were used to calculate gene expression, with normalization to GAPDH as an internal control. We used the ddCt method to analyze relative gene expression in sorted samples compared to whole-heart control samples. Technical replicates (n=3) and biological replicates (n=3) were performed. The experiment was repeated three times in the lab.

**RNA Sequencing**

Thy1+HE- cells were isolated from sham and TAC operated hearts 7 days after operation (n=3 per group), and then sorted again directly into RNA Later solution (Ambion). (Number of mice was based on risk of surgical survival as well as expense). Total RNA was extracted using RNeasy miniElute Cleanup Kit (Qiagen) according to manufacturer’s instructions. The cDNA library was generated using the reagents provided in the Illumina® TruSeq™ RNA Sample Preparation v2 Kit (Illumina). The clustering was performed on a cBot cluster generation system using an Illumina HiSeq single read cluster generation kit (Illumina) according to the manufacturer’s instructions. The amplified cDNA library was sequenced on an Illumina HiSeq 2500 for estimating transcript abundance (1X50 reads and >12 million reads/sample). The raw reads were generated by HiSeq 2500 sequencing control software, and Fastq files were analyzed using NextGene software (Softgenetics, PA) for calculating Reads Per Kilobase of transcript per Million mapped reads (RPKM). The analyses were performed by the UCLA Clinical Microarray Core Facility, which was blinded to the identity of cell type and surgical group. The statistical analyses were performed using Partek genomics Suite 6.6. Thresholds for selecting significant genes were set at >= 1.5-fold and p<0.05. Genes that met both criteria simultaneously were considered as significant changes. Global functional analyses, network analyses and canonical pathway analyses were performed using Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). The RNA sequencing data were submitted to GEO and were assigned the following accession numbers: GSE51620.
**In vitro proliferation assay**

The relative proliferation of Thy1+HE- cells derived from each lineage (Tie2Cre, Pax3Cre and Tbx18Cre) was analyzed with a BrdU cell proliferation kit following the product manual (Millipore). In brief, $10^3$ Thy1+HE- cells were seeded into a well of a 96 well culture plate. BrdU was added into culture after cells were attached at day 0 and at day 3. BrdU incorporation was detected 1, 3 and 5 days later using a microplate reader (BIO-RAD Model 3550). The proliferation absorbance were calculated using the following formula:

\[ \text{Proliferation absorbance} = \text{actual absorbance} - \text{background absorbance}. \]

**Apoptosis assay**

PI and Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen) were used according to the manufacturer's instructions to assess cell death in Thy1+HE- cells. Briefly, adult mouse hearts were digested and sorted for Thy1+HE- cells at 1, 4, 7, and 14 days after TAC and sham surgery. Immediately after sorting cells, FACS Calibur (BD Biosciences) was used to analyze late apoptotic cells as Annexin V+/PI+ double-positive cells or PI+ cells for necrotic cells.

**Cytospun preparation**

Aliquots of $10^5 - 10^6$ cells in 200 µl PBS were cytocentrifuged at 800 rpm for 4 minutes on glass slides. Cytospin slides were dried for 10 minutes and were fixed with Paraformaldehyde 4% for 15 minutes.

**Histology and immunohistochemistry**

Heart tissues were fixed for 6 hours at 4°C in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS (Fisher Scientific), washed in PBS, immersed in a solution of 30% sucrose (Sigma) in PBS at 4°C overnight, embedded in Tissue-Tek OCT Compound (Sakura), and transferred to a bath of 2-Methylbutane (Fisher Scientific) on dry ice. Frozen tissue slides were prepared at 7-µm thickness using a cryostat (Leica) and stored at -80°C. Masson’s trichrome staining (Sigma) was performed according to manufacturer’s instructions. For immunohistochemistry, slides were dried at room temperature for 20 minutes, washed 3 times for 10 minutes each in PBS, permeabilized in PBS containing 0.25% Triton X-100 (Fisher Scientific) for 10 minutes at room temperature followed by washing in PBS-T (PBS containing 0.05% Tween-20 (Fisher)) twice for 5 minutes. The tissue slides were then incubated with blocking buffer (10% normal goat serum (Sigma) in PBS) for 30 minutes at room temperature. The tissue slides were then incubated with primary antibodies (Supplemental Table 1) diluted in blocking buffer overnight at 4°C followed by 1 hour at room temperature. After washing three times for 10 minutes with PBS-T, the tissue slides were incubated with the secondary antibody (Supplemental Table 1) for 1 hr at room temperature, washed three times for 10 minutes with PBST, and then mounted with DAPI-containing mounting media (Vector). We used mounting media without DAPI for slides stained with the secondary antibody Alexa Fluor 350. Tyramide Signal Amplification Kit (Invitrogen) was used for double staining using Alexa Fluor 350 according to the manufacturer’s protocol. The immunostained slides were observed and analyzed using a Leica fluorescent microscope (LEICACTR6500).
**Parabiosis**
Mice of the same weight, size and sex were paired at least two to three weeks before the surgery, to make sure that they are compatible. Mice are anesthetized by isoflurane in O$_2$. Toe pinch was used as an indicator of the pain response. After shaving the corresponding lateral aspects of each mouse, the site was disinfected using an alcohol swab and povidone/iodine. Matching skin incisions are made from the olecranon to the knee joint of each mouse, and the subcutaneous fascia was bluntly dissected to create approximately 0.5 cm of free skin. One partner had the procedure done on the right side, and the other on the left. The right olecranon of one animal is attached to the left olecranon of the other by a single 4-0 silk suture and tie. The partners’ knee joints were similarly connected. The dorsal and ventral skins were then approximated by staples or by continuous suture, and the animals were warmed with heating pads, and monitored until recovery. Parabiotic pairs were housed one pair per cage, and given acidified water (pH 2.5). After 4 weeks blood samples from each animal in a parabiont pair were analyzed using flow cytometry. Animal pairs with lower 90% blood chimerism were excluded from our studies.

**Bone Marrow Transplant**
C57BL/6 mice (H2b, Thy1.1+CD45.2+ dsRed+) served as donors for hematopoietic stem cells (HSC), GFP- or dsRed-expressing C57BL/6 mice (H2b; Thy1.2+CD45.2+GFP+) were used as donors for non-hematopoietic bone marrow (BM) cells for transplantation into C57BL/6 (H2b, Thy1.1+CD45.2+) recipients. Donors were 6-12 weeks old, recipients were 8 weeks old at transplant. BM was flushed from tibiae and femora into HBSS/2% FBS, enriched for c-Kit (3C11) cells by magnetic column separation (CD117 MicroBeads, MACS Separation Columns LS; Miltenyi Biotec, Auburn, CA), and ‘KTLS-HSC’ were purified by FACS-sorting, selecting for c-Kit+, Thy1.2$^{lo-neq}$, Sca-1+, Lin- (CD3, CD4, CD5, CD8α, B220, Gr1, Mac1 and Ter119). 1000 FACS-purified HSC were infused per recipient mouse. For co-transfer of non-hematopoietic cells, BM was flushed from tibiae, femora, and pelvis. Non-hematopoietic cells were extracted by magnetic column depletion of CD45+ cells (CD45 MicroBeads; Miltenyi Biotec). C57BL/6.6.CD45.2 recipients received a lethal 1050 cGy dose total body gamma irradiation, ~5h prior to tail-vene injection of a radioprotective dose of 1,000 KTLS HSC. In co-transfer experiments 5x10$^6$ CD45- non-hematopoietic/stromal cells were injected simultaneously with the HSC. Biological replicates were used, and the experiment was performed three times.
# Online Table I: Antibodies used for Flow Cytometry and Immunofluorescence

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### Online Table II: qPCR Primers

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**Online table III.**
Differentially expressed genes in fibroblasts derived from Tie2Cre and Tbx18Cre mice after sham and TAC operations based on RPKM values (RPKM > 11).

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<th>Gene</th>
<th>Sham</th>
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Online Figure I. Characterization of Thy1+HE- cells. (A-B) Flow cytometry of Myh6-GFP (n=3) (A) and Myh11Cre-eGFP (n=3) (B) hearts for Thy1+HE- cells. (C) Immunofluorescence staining of 50,000 cytospun Thy1+HE- cells isolated from freshly digested hearts. These cells express PDGFRα and DDR2, but rarely express CD146 or α-SMA. (D) Sorted Thy1+HE- cells were plated and observed at various time points. Scale bar: 200 µm. (E) Principal component analysis (PCA) of RNA-sequencing data (performed on Thy1+HE- cells isolated from sham and TAC mice (n=3 each) 7 days after the operations) demonstrates reproducibility between biological replicates of the samples. (F) Heat map of differentially expressed genes (using hierarchical clustering) between the two groups. Red indicates up-regulated genes and blue indicates down-regulated genes. There is increased expression of genes associated with ECM regulation, signaling pathways, fibroblasts function and activation, and cell cycle progression and decreased expression of cell-death and cell cycle arrest proteins in TAC (using fold change >1.5, p<0.05 as cut-off). (G) A listing of the top 16 biofunction terms enriched in TAC relative to sham. Analysis of differentially-expressed genes between Thy1+HE- cells from TAC (n=3) and sham (n=3) at day 7 after TAC operation was performed using Ingenuity Pathway Analysis software. The y-axis is the log-transformed p value, p=0.05 is equivalent to y value of 1.3 (yellow line). (H) After isolation from sham and TAC hearts 7 days after the operation, some cytospun Thy1-HE- cells co-express PDGFRα and DDR2. (I) A fraction of Thy1-HE-CD146+ and Thy1-HE-CD105+ cells isolated from sham and TAC hearts 7 days after surgery express either DDR2 or PDGFRα; few cells express both. Scale bar: 50µm.
Online Figure II. Characterization of TAC injury and resultant Thy1+HE cell death. (A) TAC surgery consistently resulted in cardiac hypertrophy as evident by increased ratio of heart weight/body weight. The surgery also resulted in a reduction of the ejection fraction to approximately 35-45% by echocardiography (n=3 hearts/surgical group). (B) Immunostaining of gross heart sections with Col1 antibody showed diffused fibrosis 7 days after TAC injury. Scale bar: 1 mm. (C) Masson's trichrome staining of sham and TAC hearts at 4, 7, and 14 days post-injury. Scale bar, top: 1 mm, bottom: 100 μm. (D) The total number of Thy1+HE-cells slightly increases after injury in sham and TAC models, and a plateaus after the first week in the TAC model. (E) The number of late apoptotic (Annexin V+/PI+) cells in the Thy1+HE-population was higher 4 days after TAC operation than the sham surgery (p<0.05). (F) Necrotic cells (PI+) are rare among Thy1+HE-fibroblasts in both sham and TAC hearts at 7 and 14 days following the operation (p>0.05). All error bars represent s.e.m.
Online Figure III. Bone marrow and parabiosis models do not reveal hematopoietic contribution to cardiac fibroblasts. (A) Gross images of sham and TAC hearts in (GFP-labeled and RFP-labeled BM) BMT-recipient mice show transplanted cells in the heart. Scale bar: 2 mm. (B) Gross images of parabiosed wild-type singleton heart show GFP-labeled circulating cells in the vasculature. Scale bar: 2 mm. (C) Blood chimerism is achieved in parabiont pairs, analyzed 4 weeks after surgery. (D) Shared circulation in parabiotic mice does not alter loading of the TAC’ed heart. The TAC’ed heart showed an expected increase in heart weight-to-body weight ratio at the end of the study when the parabiont pair was separated (n=3 hearts/surgical group). (E) IHC of the wild-type heart in the parabiont pair with CD31, CD146, and Periostin. (F) Schematic representation of the VavCre/+;R26RmTmG/+ . (G) Flow cytometry of Thy1+HE- cells for GFP and RFP (n=3 hearts/surgical group). Scale bar: 50 µm for merged panel and 12.5 µm for the inset. All error bars represent s.e.m.
Online Figure IV. Confocal characterization of Tie2-derived cardiac fibroblasts. (A) Representative sections of Tie2Cre+;R26R\textsuperscript{mT/mG} sham hearts stained with Col1 and PDGFR\textalpha. (B) TAC sections stained for DDR2, Col1, and PDGFR\textalpha. (Arrows indicate GFP+ Tbx18-derived cells that co-stain for the marker in red). Scale bar: 50 µm for merged panel and 12.5 µm for the inset.
Online Figure V. Characterization of Pax3-derived cells. (A) Tissue sections stained for Smi32 and NF160, markers of neural tissue. (B) IHC of wild-type E9.5, wild-type adult heart, and adult Pax3Cre/+;R26RmTmG/+ heart sections to detect Pax3 expression (a: atrium and v: ventricle). Arrows indicate GFP+ cells that co-stain for the marker in red, and arrowheads indicate GFP+ (i.e. Pax3-derived) cells that do not stain with the marker in red. Scale bar: 50µm for merged panel and 12.5 µm for the inset.
Online Figure VI. Epicardial contribution to cardiac fibroblasts. (A) Staining of Tbx18<sup>Cre/+;</sup>R26R<sup>mT/mG</sup> with Col1 and Vim. (Arrows indicate GFP+ cells that co-stain for the marker in red). (B) Schematic of Wt1<sup>CreERT2/+;</sup>R26R<sup>mT/mG</sup> model. (C) Schematic of TM administration strategy. (D) Labeled cells in sham and TAC hearts (n=4 per group). (E) IHC of TAC sections with Col1. Scale bar: 50 µm for merged panel and 12.5 µm for the inset.
Online Figure VII. Confocal characterization of Tbx18-derived cardiac fibroblasts. (A) Immunohistochemistry of sham Tbx18Cre^{mT/mG} sections with Col1. (B) Representative TAC sections were stained for Col1, DDR2, and PDGFRα. (Arrows indicate GFP+ cells that co-stain for the marker in red). Scale bar: 50 µm for merged panel and 12.5 µm for the inset.
Online Figure VIII. Comparison of proliferation, localization and gene expression in Tbx18-, Tie2- and Pax3-derived fibroblasts. (A) No significant difference among relative BrdU incorporation in each lineage-derived fibroblasts at 1, 3 and 5 days post BrdU exposure. (B) Localization of Thy1+DDR2+ cells in each lineage-traced fibroblast subset. (C) PCA of RNA-seq data performed on GFP+Thy1+HE- cells isolated from Tie2 and Tbx18 mice 7 days after sham and TAC showed sham- and TAC-derived cells were clustered separately, confirming the similarity between Tie2 sham and Tbx18 sham, as well as between Tie2 TAC and Tbx18 TAC. Discrepancy between shams and TACs indicated different gene expression profile after TAC operation in Tie2 and Tbx18. (D) Heat map of the RNA-seq data shows differentially expressed genes (using hierarchical clustering) between the two groups (sham and TAC). Red indicates up-regulated genes and blue indicates down-regulated genes. (E) A listing of the top 18 bio-function terms enriched in TAC relative to sham mice. Analysis of differentially-expressed genes between Thy1+HE- cells from TAC and sham at day 7 after TAC operation was performed using Ingenuity Pathway Analysis software. The y-axis is the log-transformed p value. p=0.05 is equivalent to y value of 1.3 (yellow line). Scale bar: 50 µm.


