BACKGROUND: A hallmark of heart failure is cardiac fibrosis, which results from the injury-induced differentiation response of resident fibroblasts to myofibroblasts that deposit extracellular matrix. During myofibroblast differentiation, fibroblasts progress through polarization stages of early proinflammation, intermediate proliferation, and late maturation, but the regulators of this progression are poorly understood. Planar cell polarity receptors, receptor tyrosine kinase–like orphan receptor 1 and 2 (Ror1/2), can function to promote cell differentiation and transformation. In this study, we investigated the role of the Ror1/2 in a model of heart failure with emphasis on myofibroblast differentiation.

METHODS AND RESULTS: The role of Ror1/2 during cardiac myofibroblast differentiation was studied in cell culture models of primary murine cardiac fibroblast activation and in knockout mouse models that underwent transverse aortic constriction surgery to induce cardiac injury by pressure overload. Expression of Ror1 and Ror2 were robustly and exclusively induced in fibroblasts in hearts after transverse aortic constriction surgery, and both were rapidly upregulated after early activation of primary murine cardiac fibroblasts in culture. Cultured fibroblasts isolated from Ror1/2 knockout mice displayed a proinflammatory phenotype indicative of impaired myofibroblast differentiation. Although the combined ablation of Ror1/2 in mice did not result in a detectable baseline phenotype, transverse aortic constriction surgery led to the death of all mice by day 6 that was associated with myocardial hyperinflammation and vascular leakage.

CONCLUSIONS: Together, these results show that Ror1/2 are essential for the progression of myofibroblast differentiation and for the adaptive remodeling of the heart in response to pressure overload.

Key Words: fibroblasts  ■ fibrosis  ■ heart failure  ■ inflammation  ■ myocardial inflammation

Excessive fibrosis during pathological cardiac remodeling is a hallmark of heart failure and the response to cardiac injury. Cardiac fibrosis is the result of resident cardiac fibroblasts that undergo myofibroblast differentiation to promote inflammation and secrete extracellular matrix in response to various forms of cardiac injury.1–4 Although injury-induced inflammation and matrix deposition is an adaptation to acute cardiac injury that can prevent the heart from rupture,5,6 excessive fibrotic deposition leads to ventricle stiffness that impairs cardiac function.7–9 Recently, myofibroblast differentiation has been characterized as a transition through what is referred to as 3 phenotypic polarization stages: an initial proinflammatory phenotype, an intermediate proliferative phenotype, and a final mature phenotype.10 These polarization stages have specific functional differences. The proinflammatory fibroblast recruits inflammatory cells to the cardiac...
tissue in response to injury, the proliferative fibroblast undergoes cell division and deposits extracellular matrix in response to transforming growth factor beta (TGF-β), and the mature myofibroblast maintains and strengthens the fibrotic deposits by expressing matrix remodeling proteins.11 A recent study using single-cell RNA sequencing to analyze murine interstitial cells after myocardial infarction revealed fibroblast populations consistent with these polarization states.12 Despite its importance, we have limited knowledge of the factors that control the passage of myofibroblast through the different stages of differentiation.

The receptor tyrosine kinase–Like orphan receptors 1 and 2 (Ror1 and Ror2) have been evaluated for their roles in development and cell transformation.13–15 These membrane proteins are most highly expressed in developing tissues,16 and mice lacking Ror1 display skeletal defects,17 whereas mice lacking Ror2 have impaired heart and limb development.18 Ror1 and Ror2 expression can be reactivated in various cancers,19–24 and knockdown or Ror1, Ror2, or both Ror1/2 in various transformed cell lines reduces their proliferation and migration.25–28 Additionally, Ror1 expression in satellite cells promotes proliferation and skeletal muscle regeneration after injury.29 These functions of Ror1/2 may be linked to the ability of this receptor system to control the planar cell polarity signaling pathway. Planar cell polarity is the asymmetrical alignment of cells to coordinate directionality with neighboring cells and extracellular matrix.30 Proteins in the planar cell polarity pathway, including Ror1/2 and others (tyrosine-protein kinase–like 7; prickle homologue 1; Vang-like [Vangl] 1 and 2, disheveled1, 2, and 3), regulate cell polarity by organizing the actin cytoskeleton and segregating proteins to opposite sides of the cell.31,32 In specific cell types, planar cell polarity can regulate proliferation, migration, and cell differentiation.33–38 Thus, it is tempting to speculate that planar cell polarity-mediated actin organization may be critical to myofibroblast function, and that it also functions as an integral step in the actin alignment-mediated signal transduction that promotes locomotion, contraction, and matrix reorganization during fibroblast differentiation.39

Components of the planar cell polarity pathway have been implicated in clinical and experiment heart failure.40–46 Most recently, Ror1 has been reported to be robustly upregulated in ischemic cardiomyopathy,47 and Ror2 has been shown to be upregulated in right ventricular remodeling and heart failure.48 However, the role of Ror1/2 cell surface receptors in cardiac remodeling has not been addressed. While many of the functions attributed to Ror1 and Ror2 are shared by the process of cardiac fibroblast activation and differentiation, the roles of Ror1 and Ror2 in myofibroblasts have not been investigated previously. In the course of our studies, we found that Ror1 and Ror2 were generally expressed at low levels in the nonchallenged adult mouse tissues. However, the expression of these proteins become markedly increased in activated fibroblast in response to injurious stimuli. Thus, in this study, we investigated the role of Ror1 and Ror2 in cardiac remodeling through in vivo mouse models of heart failure and in vitro cell culture models of cardiac myofibroblast differentiation.

**METHODS**

**Data and Method Availability**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Mouse Strains**

All animal experiments were approved by the Animal Care and Use Committees at Boston University and the
University of Virginia. Ror1/2/ubiquitin C-driven Cre recombinase with tamoxifen-inducible mutant human estrogen receptor (Ubc-CreER\textsuperscript{ERT2}) mice were generated by combining Ror1 flox (Jackson Labs #018353\textsuperscript{,49}), Ror2 flox (Jackson Labs #018354\textsuperscript{,49}), and Ubc-CreER\textsuperscript{ERT2} (Jackson Labs #008085\textsuperscript{,50}) alleles, which are all in the B6 mouse background. Additionally, Ror2 expression was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1. Immunofluorescent staining of Acta2 (Cell Signaling Technologies Catalog #240-B) was assessed as described above using primers listed in Table S1.
with PBS-T (PBS+0.1% Tween20), incubation with fluorescent Acta2 antibody, then counterstain with DAPI. Immunostained cells were imaged by confocal microscopy (Leica SP8). Acta2 fiber alignment and Acta2 fluorescence intensity were quantified by ImageJ (version 2.0.0).

### Bulk RNA Sequencing

Primary cardiac fibroblasts were isolated as described above from either control mice (Ror1/2^fl/fl^) or Ror1/2 knockout mice (Ror1/2^fl/fl^+Ubc-CreERT2) after injection with tamoxifen (Sigma-Aldrich Catalog #T5648) when mice were 6 to 8 weeks old. Each isolation pooled 4 hearts from mice of the same litter, with the same male:female ratio variation between control and Ror1/2 knockout samples. Mice were bred between a Ror1/2^fl/fl^ genotype and a Ror1/2^fl/fl^+Ubc-CreERT2 genotype to generate ~50% control mice and ~50% Ror1/2 knockout mice per litter, allowing for littermate paired samples. Specifically, isolations of control and Ror1/2 knockout mice were performed on 4 different litters with paired samples from the same litters (Ex: control sample 1 and Ror1/2 knockout sample 1 used hearts from mice of the same litter). Primary cardiac fibroblasts were grown for 9 days in culture, then RNA lysate was isolated and purified. Purified RNA was submitted to the University of Virginia Genome Analysis and Technology Core for whole transcriptome sequencing by first library preparation with NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB Catalog #E7760) and then sequencing by Illumina NextSeq 500 Sequencing System for paired-end 75-base pair reads. Programs were used with standard inputs for bioinformatic analysis: raw read data was quality checked with FastQC (Babraham Bioinformatics), preprocessed to filter out Illumina primer sequences, and aligned with Kallisto59 to generate estimated counts, normalized by log2 transformation and filtered low-abundance genes with Sleuth,60 analyzed for gene expression by transcripts per million reads and differential expression with Sleuth by the likelihood ratio test, and analyzed for gene ontology term enrichment with Generally Accepted Gene-set Enrichment.61 Aligned and normalized sequencing results and gene ontology term enrichment results are provided in Data S1 and S2.

### Immunophenotyping of Cardiac Tissue

Cardiac tissue was isolated after sham or TAC surgery. Tissue was digested with collagenase I (450 U/mL), collagenase XI (125 U/mL), DNase I (60 U/mL), and hyaluronidase (60 U/mL), and the isolated cells from digested tissue were immunostained with fluorescently conjugated antibodies against CD45-Pacific Blue, 30-F11 (BioLegend Catalog #103126), Ly6G-PE, 1A8 (BioLegend Catalog #127618), CD11b-APC-Cy7, M1/70 (BioLegend Catalog #101226), F4/80-PE-Cy7, BM8 (BioLegend Catalog #123114), and Ly6C-FITC, HK1.4 (BioLegend Catalog #128006). Dead cells were excluded by staining with DAPI. Immunostained fluorescent cells were analyzed by flow cytometry using a BD LSR II flow cytometer (BD Bioscience). Additionally, isolated cardiac tissue was lysed and analyzed for inflammatory cytokines interleukin-1β, interleukin-6, and C-C motif chemokine ligand 2 by quantitative reverse transcription PCR. Real-time PCR primers are listed in Table S1.

### Evans Blue Staining

Vascular permeability of the heart after pressure overload was evaluated by the extent of the leakage of Evans blue dye (Sigma-Aldrich Catalog #E2129). Mice were euthanized 30 minutes after tail-vein injection of 1% w/v in 0.9% saline. Dyes were allowed to circulate throughout the body during this period.

### Statistical Analysis

Analysis of means between groups with a sample size of 4 to 6 in each group was performed using either a Mann-Whitney test for comparison between 2 groups or a Kruskal-Wallis test followed by Dunn’s multiple comparison corrected post hoc test for comparison among 3 or more groups by Prism (GraphPad Software, Inc., San Diego, CA). Otherwise, statistical analysis of means between groups was performed using either a standard 2-tail Student t test or a 2-way ANOVA test followed by a Tukey’s multiple comparison corrected post hoc test by Prism. Statistical analysis of gene set enrichment in gene ontology analysis used the statistical tests in the Generally Accepted Gene-set Enrichment package. Other statistical tests are described in figure legends. All statistical analysis of RNA sequencing data sets was performed through computational analysis packages, which contain statistical corrections for large data sets.

### RESULTS

#### Transverse Aortic Constriction Induces Early Ror1/2 Expression in Cardiac Tissue

Cardiac pressure overload in the mouse model of TAC induces fibroblast activation and myocardial remodeling, including initial inflammation (1–3 days after TAC), extracellular matrix remodeling (3–14 days after TAC), and eventual heart failure (14–28 days after TAC).62 Thus, cardiac tissue was analyzed before and after TAC surgery to investigate the expression patterns of Ror1 and Ror2 during the remodeling time course. The TAC surgery model was initially performed on Ror2-LacZ mice that has the LacZ reporter gene knocked in to the
There was no detectable Ror2-mediated LacZ expression in uninjured, or sham-operated mice (Figure 1A and not shown), but β-galactosidase expression was markedly induced at 7 days after TAC (Figure 1B). Immunohistochemical analysis revealed that Ror2 protein was concentrated in cells of the interstitial space of the myocardial tissue at 7 days after TAC (Figure 1C). Next, we determined the time course of Ror1 and Ror2 protein induction after TAC surgery by western blot analysis of protein lysates from cardiac tissue. The expression of both Ror1 and Ror2 protein was increased by 3 days after TAC, peaking at 7 days, and then decreasing at 14 and 28 days after TAC (Figure 1D).

To determine the cell type(s) that express Ror1 and Ror2, RNA was isolated from endothelial cells, leukocytes, and fibroblasts from sham-operated and TAC-treated hearts 7 days after surgery, and quantitative PCR was performed to detect the levels of relevant transcripts. Ror1 and Ror2 transcript expression was detected in the activated cardiac fibroblasts at 7 days after TAC, but not in the endothelial or leukocyte cell populations (Figure 1E, Figure S1). Ror2 protein expression was also detected in fibroblasts after TAC surgery by flow cytometry (Figure S2). Overall, the timing, location, and cell-specific gene expression patterns suggest that both Ror1 and Ror2 are induced during the activation and expansion of cardiac fibroblasts that occurs in response to pressure overload–induced myocardial remodeling.

### Ror1 and Ror2 Are Induced During Early Cardiac Fibroblast Activation

The induction of Ror1/2 during myofibroblast differentiation was also investigated in cultured cells using isolated murine cardiac fibroblasts. Fibroblasts from wild-type murine hearts were attached to cell culture plates, flattened, and expanded over 9 days after isolation (Figure S3). RNA expression of key genes increased at different rates during this activation time course (Figure 2A). As expected, Fsp1 (fibroblast-specific protein 1) was induced by day 3 and the fibroblast activation genes Slug and Snail increased at day 9. Both Ror1 and Ror2 increased over this time course, but the induction of Ror2 preceded that of Ror1. The planar cell polarity protein transcript tyrosine-protein kinase–like 7 increased by day 3 and continued to increase at days 6 and 9, but Prickle and Vangl2 displayed no statistically significant change in expression. Protein analysis of Ror1 and Ror2 by western immunoblot in cardiac fibroblasts at day 0 and day 9 after isolation confirm the upregulation of both proteins in cell culture (Figure S4).
Next, myofibroblast differentiation was stimulated in these cultures by treatment with TGF-β1. Myofibroblast differentiation could be detected by cell enlargement and SMα-actin filament elongation (Figure 2B and 2C). However, in contrast to the earlier stage of fibroblast activation, TGF-β1 treatment did not lead to a further increase in Ror1 or Ror2 expression. On the other hand, hallmarks of myofibroblast differentiation could be observed following TGF-β1 treatment including the increased transcript expression of myofibroblast differentiation proteins Acta2 and Postn, and decreased expression of the cytokines interleukin-6 and tumor necrosis factor-α (Figure 2D). Together, these data suggest that the induction of Ror1/2 is an early event during fibroblast activation, and that their expression is not altered at the later stages of myofibroblast differentiation.

Figure 3. Ror1/2-mediated early fibroblast activation in primary murine cardiac fibroblasts.
Primary cardiac fibroblasts were isolated from healthy control and transgenic Ror1/2 double-knockout mice transcriptional phenotype was assessed by bulk RNA sequencing. A, RNA transcript reads of specific genes related to planar cell polarity, inflammatory cytokines, proliferation and cell division, extracellular signal-related kinase 1/2 signaling, matrix remodeling, natriuretic signaling, and myofibroblast differentiation were quantified as normalized Z score (4 samples for each genotype). B, Gene ontology analysis of differential gene expression between control and Ror1/2 knockout cardiac fibroblasts showed the top 10 upregulated and downregulated terms by q value, with terms grouped by color: cell activation in orange, inflammation in red, proliferation in green, and microtubule regulation in black (bars represent mean±SEM, q values obtained through Generally Applicable Gene-set Enrichment for Pathway Analysis, or GAGE, statistical algorithms). C, Significance vs fold change of each gene between control and Ror1/2 knockout samples was visualized by volcano plot, with genes in each gene ontology term highlighted in corresponding colors (all other genes in gray), and specific genes related to planar cell polarity, inflammation, and proliferation were highlighted and labeled. D, 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay after 2 hours of EdU incubation determined rate of proliferation in control and Ror1/2 knockout cardiac fibroblasts (bars represent mean±SD, sample size=3 per group, Student t test P=0.0054). ERK, extracellular signal-regulated kinase; GO, gene ontology; KO, knock out; pos reg, positive regulation; and Ror indicates receptor tyrosine kinase-like orphan receptor.
*p<0.05.
Ror1/2 Double-Knockout Fibroblasts Exhibited an Immature Cardiac Myofibroblast Phenotype

We next investigated the role of Ror1/2 in fibroblast activation in the fibroblast cell culture system using primary cardiac fibroblast cells isolated from control or Ror1/2 knockout mice. Ror1/2 was eliminated in adult mice through an inducible transgenic knockout strategy by crossing Ror1/2<sup>lox/lox</sup> and Ubc-CreERT<sup>2</sup> mice and treating the progeny with tamoxifen for 2 weeks. These mice displayed no observable phenotype, and they had a normal cardiac phenotype 3 months after the induction of gene ablation (Figure S5). Primary cardiac fibroblasts were isolated from control or Ror1/2 knockout mice and grown to confluence. RNA was then isolated,
and next-generation RNA sequencing was performed. Many genes associated with a manually curated network of fibrosis and fibroblasts were differentially regulated between control and Ror1/2 knockout fibroblasts (Figure 3A). As expected, cells were largely void of Ror1 and Ror2 transcripts, and the planar cell polarity gene Ptck, Vangl2, Prickle1, Dvl1, Dvl2, Dvl3) were generally reduced in the Ror1/2 knockout fibroblasts. Notably, Ror1/2 knockout fibroblasts showed an increase in inflammatory cytokine gene expression (Il1a, Il6, Tnfa, Ccl2) and a decrease in transcripts associated with cell proliferation (Pcna, Mki67, Cdk1, Cdk2, Cdk4, Cdk6). Additionally, transcripts associated with the promotion of fibrosis in the extracellular signal-related kinase 1/2 signaling and matrix remodeling pathways were downregulated (Map2k3, Map3k1, Map3k5, Mapk14, Mapk8, Timp1, Timp2), and transcripts associated with the inhibition of fibrosis in the matrix remodeling and natriuretic signaling pathways were upregulated (Mmp2, Mmp9, Mmp14, Nppa, Nppb, Npr1, Npr2, Npr3). Analysis of the top 10 upregulated and downregulated gene ontology terms between control and Ror1/2 knockout fibroblasts revealed the strong induction of inflammation-related pathways and an inhibition of proliferation-related and microtubule organization-related pathways (Figure 3B). The specific genes included in these groupings were highly differentially expressed between control and Ror1/2 knockout fibroblasts, along with planar cell polarity genes, inflammatory genes, and proliferation gene Mki67 also shown (Figure 3C). The reduction in cell proliferation in the Ror1/2 knockout fibroblasts was documented by EdU incorporation assay, showing a 10% reduction in EdU+ cells during the 2-hour Edu incubation period (Figure 3D). To the extent that the cell culture system models the early activation of fibroblasts, Ror1/2 dual deficiency led to the upregulation of inflammation pathways and downregulation of pathways associated with matrix production, proliferation, and microtubule organization. Collectively, these data are consistent with a cellular fibroblast phenotype that appears to be stalled in the proinflammatory polarization state, suggesting that Ror1 and Ror2 are required for the progression of fibroblasts from this immature state to the intermediate proliferative state.10

Ror1/2 Double-Knockout Fibroblasts Are Less Responsive to TGF-β1–Induced Myofibroblast Maturation

Previous findings showed that activated fibroblasts in the early proinflammatory state are less responsive to TGF-β stimulation than fibroblasts in the intermediate proliferative state. Thus, to investigate whether the Ror1/2 knockout fibroblasts are functionally stalled in an initial proinflammatory phenotype, we compared the responsiveness of control and Ror1/2-knockout fibroblasts to TGF-β1–induced myofibroblast differentiation. In control fibroblasts, TGF-β1 treatment led to gene expression changes that are consistent with myofibroblast differentiation (increased Acta2 and Postn, decreased interleukin-6). However, Ror1/2 knockout fibroblasts displayed diminished Acta2 and Postn induction and no decrease in interleukin-6 after TGF-β1 treatment when compared with control fibroblasts (Figure 4A, Figure S6A and S6B). Western blot analysis of interleukin-6 protein levels confirmed that interleukin-6 protein is upregulated in Ror1/2 knockout fibroblasts and that TGF-β1 treatment reduced interleukin-6 expression in control but not in Ror1/2 knockout fibroblasts (Figure 4B and 4C).

Next, TGF-β1–treated control and Ror1/2 knockout fibroblasts were visualized after Acta2 immunostaining. Although treatment with TGF-β1–induced similar fibroblast cell enlargement between control and Ror1/2 knockout fibroblasts (control=0.073±0.016 mm², Ror1/2 knockout=0.071±0.010 mm², P=0.92), the Acta2 fibers appeared misaligned and disorganized in the Ror1/2 knockout fibroblasts compared with the Acta2 fiber alignment in the control fibroblasts (Figure 4D). Quantification of the directional angle of individual Acta2 fibers within each cell showed that the Acta2 fibers in the Ror1/2 knockout fibroblasts were significantly less aligned with each other than the Acta2 fibers in the control fibroblasts (Figure 4E). Quantification of overall Acta2 fluorescent intensity revealed that Ror1/2 deficiency led to diminished TGF-β1–induced Acta2 expression at the protein level (Figure S6C). Taken together, these results show that Ror1/2 knockout fibroblasts are less responsive to TGF-β1, a feature that is consistent with the notion that these cells are in the less mature, proinflammatory state. These results further suggest that the planar cell polarity signaling pathway may be critical for actin alignment during fibroblast maturation.

TAC Induces Hyperinflammation, Rapid Heart Failure, and Death in Ror1/2 Double-Knockout Mice

To investigate the role of Ror1/2 in cardiac tissue remodeling in vivo, the inducible Ror1/2 double-knockout mice (Ror1/2(fl/fl)+Ubc-CreER(T2)) were treated with tamoxifen for 2 weeks and subjected to TAC. Ror1/2 double-knockout mice displayed a rapid decline in heart function after TAC, as observed by echocardiography measurements at 3 days after TAC (Figure 5A and 5B). This rapid development of systolic dysfunction was associated with death of the Ror1/2 double-knockout mice between 4 and 6 days after TAC (Figure 5C). Consistent with the heart failure phenotype, the TAC-treated, Ror1/2 double-knockout mice...
displayed marked increases in heart and lung weights (Figure S7).

Hematoxylin and eosin staining of heart sections revealed a massive inflammatory infiltrate in the Ror1/2 double-knockout mice after TAC (Figure 6A). Inflammatory cell infiltration was further characterized by flow cytometry analysis of cells isolated from digested cardiac tissue at 3 days after TAC. This

**Figure 4.** Ror1/2-mediated response to myofibroblast differentiation induced by TGF-β1.

Myofibroblast differentiation was induced by treatment with 10 ng/mL TGF-β1 for 4 days. A, RNA expression of myofibroblast-related (Acta2 and Postn) and inflammation-related (interleukin-6) genes was quantified in control and Ror1/2 knockout fibroblasts (bars represent mean±SD, sample size=3 per group, Kruskal-Wallis P values: Acta2=0.0006, Postn<0.0001, Il6=0.0006). B, Protein levels of interleukin-6 were visualized by Western blot in control and Ror1/2 knockout fibroblasts after TGF-β1 induction (representative blot shown), quantification of 3 replicates in (C) (bars represent means±SD, sample size=3 per group, Kruskal-Wallis P=0.0006). D, α-Smooth muscle actin filaments were visualized by immunofluorescent staining of subconfluent cells in control and Ror1/2 knockout fibroblasts, and (E) alignment of α-smooth muscle actin filaments was quantified and normalized per cell (461 filament angles in control, 717 filament angles in Ror1/2 knockout, lines on graph represent contingency table of 10-degree bins for 19 total bins, chi-square P value for testing numbers in bins <0.0001). A.U., arbitrary units; KO, knock out; ns, not significant; Ror, receptor tyrosine kinase-like orphan receptor; and TGF-β1, transforming growth factor beta. *p<0.05, ****p<0.0001.
analysis revealed a large increase in the number of CD11b+Ly6G+ neutrophils, CD11b+Ly6G-F4/80+ macrophages, and CD11b+Ly6G-Ly6C+ monocytes in the Ror1/2 double-knockout mice compared with wild-type mice treated by TAC (Figure 6B and 6C). In Ror1/2 double-knockout mice, TAC also caused a large induction of proinflammatory cytokine gene transcripts including Il1b, Il6, and Ccl2 (Figure 6D). Notably, vascular leakage could also be detected after TAC in the Ror1/2 double-knockout mice as indicated by the permeability of the myocardium to Evans Blue dye (Figure 6E). These data reveal that the hearts of TAC-treated, Ror1/2 double-knockout mice are hyperinflammatory. This phenotype is consistent with the notion that Ror1/2 is essential for the progression of fibroblast maturation from the initial proinflammatory stage during their activation sequence.

**DISCUSSION**

Through these experiments, we examined the roles of the cell surface receptors Ror1 and Ror2 in cardiac myofibroblast differentiation. We found that Ror1 and Ror2 are upregulated in fibroblasts early after activation, both in vivo by pressure overload cardiac injury induced by TAC surgery and in cardiac fibroblast cell culture. Multiple lines of data indicate that dual Ror1/2 deficiency impairs myofibroblast differentiation.
Figure 6. Inflammation response in transgenic Ror1/2 double-knockout mice after TAC surgery. Inflammatory profile of control and transgenic Ror1/2 double-knockout mice was assessed after TAC or sham surgery (n=5 mice were used per group in each experimental condition). A. Hematoxylin and eosin staining of cardiac tissues 3 days after TAC were imaged. B, Cells were isolated from cardiac tissue and relative quantity of leukocyte populations were determined by flow cytometry, (C) quantified by absolute number (bars represent mean±SEM, sample size=5 per group, Mann-Whitney P values: neutrophil=0.0079, monocyte=0.0079, macrophage=0.0079). D, Gene expression of proinflammatory cytokines in cardiac lysate was measured (bars represent mean±SEM, sample size=5 per group, Kruskal-Wallis P values: Il1b=0.0004, Il6=0.0030, Ccl2=0.0011). E, Vascular permeability at 1 day after TAC was assessed by Evans Blue dye injection, with vascular leakage visualized by blue dye in the cardiac tissue. Ror indicates receptor tyrosine kinase–like orphan receptor; TAC, transverse aortic constriction; and Ubc-CreERT2, ubiquitous cre recombinase estrogen receptor (tamoxifen-inducible). *p<0.05.
Specifically, cultured Ror1/2 knockout fibroblasts exhibited an immature, proinflammatory state, and they were impaired in their response to TGF-β1 stimulation. Additionally, while mice lacking Ror1/2 did not exhibit a baseline phenotype, they were unable to acutely adapt to pressure overload cardiac injury. In response to TAC, these mice underwent profound decompensated remodeling by 3 days, and they typically died within 6 days of surgery. Collectively, these results suggest that the early induction of Ror1/2 in fibroblasts is essential for the appropriate myofibroblast differentiation and required for the myocardium to adapt to the initial stages of pressure overload injury.

The phenotype of the Ror1/2 double-knockout mice highlighted a critical role for Ror1/2 in controlling the inflammatory phenotype of fibroblasts during the early stages of cardiac remodeling. The rapid upregulation of cytokines in response to TAC injury leads to the infiltration of neutrophils and monocyte-derived macrophages. Several recent studies have highlighted the role of fibroblasts in promoting inflammation during the initial phases of the cardiac remodeling response to myocardial infarction and pressure overload. Our results expand on these studies by showing that fibroblasts lacking Ror1/2 appear to be phenotypically similar to fibroblasts in the transient, proinflammatory state that occurs early in the fibroblast differentiation continuum following experimental myocardial infarction. Transcriptional analyses of cultured cardiac fibroblasts revealed that Ror1/2 deficiency was associated with a decrease in pathways that promote fibrosis (proliferation genes, extracellular signal-related kinase 1/2 signaling genes, tissue inhibitors of matrix metalloproteinases, and mothers against decapentaplegic homolog 2/3) and an increase in pathways that inhibit fibrosis (inflammatory cytokines, matrix metalloproteinases, and natriuretic signaling genes). These differences in gene expression suggest that the Ror1/2 knockout fibroblasts are in an immature state that is proinflammatory, leukocyte-recruiting and less responsive to fibrotic stimuli. We confirmed this by showing that Ror1/2 knockout fibroblasts were less responsive to TGF-β1 compared with control fibroblasts, displaying reduced induction of Acta2 and Postn gene expression and diminished repression of Il6 gene expression. Thus, we propose that Ror1/2 deficiency causes activated fibroblasts to stall in an early proinflammatory state of differentiation such that they are unable to efficiently transition to the intermediate proliferative state and mature homeostatic state. Consistent with this hypothesis, we found that Ror1/2 double-knockout mice exhibit a hyperinflammatory phenotype associated with elevated cytokine transcript expression, exuberant inflammatory cell infiltration, and vascular leakage within 3 days of TAC surgery. We speculate that the premature cardiac mortality observed in Ror1/2 knockout mice after TAC is attributable to fibroblast-mediated vascular dysfunction that leads to excessive leukocyte recruitment and the subsequent impairment of cardiac contraction. This hypothesis can be rigorously tested by models that ablate these genes in specific fibroblast subtypes. While recent studies using single-cell RNA sequencing have highlighted the large diversity of fibroblast cell types in the heart, our study does not identify the fibroblast subtype(s) that upregulate Ror1/2. Future studies should address this important issue. Collectively, the results in this study highlight the critical role of early Ror1/2 induction in the control of the early inflammatory response in the injured myocardium, and they suggest that hyperinflammatory activated fibroblasts can have a detrimental role in myocardial remodeling.

In myofibroblasts, appropriate α-smooth muscle actin stress fiber organization is required for the transduction of signaling responses to external stimuli, and the misalignment of α-smooth muscle actin stress fibers is associated with dysregulated myofibroblast differentiation. Consistent with the notion that Ror1/2 induction is essential for myofibroblast differentiation, we find that Ror1/2 deficiency leads to α-smooth muscle actin filament misalignment in TGF-β1-treated fibroblasts. Ror1/2 signaling can control stress fiber alignment, myofibroblast differentiation, and other cellular phenotypes through the regulation of upstream and downstream planar cell polarity components. This system involves the participation of inner plasma membrane proteins associated with Ror1/2 that control cell polarity and asymmetric cell division through the regulation of actin filament organization by the action of the small guanosine triphosphatases RhoA and Rac. Our work is consistent with other studies that have implicated planar cell polarity proteins in myofibroblast differentiation, including the reported induction of Frizzled2 in myofibroblasts after experimental myocardial infarction and hypoxia-mediated suppression of myofibroblast differentiation through RhoA inhibition. Furthermore, ablation of mothers against decapentaplegic homolog 3, a key signaling protein downstream of TGF-β cardiac fibroblasts, will lead to the suppression of RhoA and a disruption of actin alignment. The coregulation of Ror1/2 and components of planar cell polarity signaling in activated cardiac fibroblasts suggests that asymmetrical cell division plays a role in myofibroblast differentiation. Consistent with this notion, the planar cell polarity pathway has been shown to influence actin filament assembly, cell structure, and asymmetrical cell division in various cell types and organisms. Together, these results suggest a potential role of planar cell polarity signaling in mediating stress fiber organization and TGF-β responses in activated cardiac fibroblasts.
We acknowledge that Ror1/2 double-deficient mice were constructed using a global knockout strategy that employed ubiquitin-CreER<sup>13</sup>. As discussed, these mice lack a baseline phenotype, yet undergo rapid decompensated heart failure and death in response to pressure overload. Although we cannot rule out the contribution of other cell types lacking Ror1/2 to this phenotype, we note that Ror1/2 expression appears specific to activated cardiac fibroblasts under these conditions. Our experimental evidence in support of this include that expression of Ror1/2 was greatly increased after TAC-induced cardiac injury in the interstitial cells of the myocardium, and the TAC-induced upregulation of Ror1/2 gene expression was specific to fibroblasts with no Ror1/2 induction in endothelial cells or leukocytes. Consistent with these findings, a number of other studies have reported Ror1 and Ror2 expression in activated fibroblasts or fibroblast-like cells, and a recent proteomic analysis of the human heart reported that ROR1 expression was 200-fold higher in than cardiac fibroblasts than other cardiac cell types. In addition, studies with isolated fibroblasts further corroborate the in vivo observations. These in vitro studies documented the robust expression of Ror1/2 and associated planar cell polarity protein transcripts (tyrosine-protein kinase–like 7; Vangl1 and 2; prickle homologue 1; and disheveled 1, 2 and 3) in activated cardiac fibroblasts, and showed that the ablation of Ror1/2 leads to a proinflammatory phenotype that is consistent with the cardiac phenotype of the Ror1/2-deficient mouse following TAC surgery.

**CONCLUSIONS**

Cardiac fibroblasts are sentinel cells in the heart that respond to early injury by adopting a proinflammatory and leukocyte-recruiting phenotype, followed by their transition to a reparative/proliferative phase that is proangiogenic and profibrotic, and followed by a homeostatic phase. Our study reveals the critical role that the cell surface receptors Ror1 and Ror2 play in allowing myofibroblasts to appropriately transition through these phases. While the deficiency of these proteins has no detectable baseline phenotype, Ror1/2-deficient fibroblasts activated by pressure overload appears stalled in the early phase of the fibroblast differentiation continuum—a stage of differentiation that is highly proinflammatory. Further analysis of the injury-induced, hyperinflammatory phenotype of the Ror1/2 double-knockout mice may provide a greater window of understanding of how excessive inflammation contributes to pathological cardiac remodeling. Elevated inflammation is predictive of worse outcomes in patients with heart failure, and recent clinical trials of anti-inflammatory therapies targeting interleukin-1β and interleukin-1R1 have shown promising results in the treatment of this condition in some patient groups. Our results suggest that impairments in planar cell polarity-mediated regulation of myofibroblast differentiation could have a causative role in the development of excessive inflammation that is associated with heart failure. Therefore, a better understanding of the molecular mechanisms that regulate the progression of myofibroblast differentiation could provide opportunities for the development of therapies that more effectively reduce cardiac inflammation.

**ARTICLE INFORMATION**

Received October 22, 2020; accepted March 22, 2021.

**Affiliations**

Cardiovascular Research Center, School of Medicine (N.W.C., S.S., Y.W., H.O., K.H., M.S., A.N., K.S., T.V., J.J.S., K.K.H., K.W.); Department of Cell Biology, School of Medicine (N.W.C., K.K.H.); Hematovascular Biology Center, School of Medicine (S.S., Y.W., K.K.H., K.W.); Department of Cardiology, School of Medicine (S.S., K.W.); Department of Pharmacology (A.N.) and Department of Biomedical Engineering (J.J.S.), University of Virginia, Charlottesville, VA; Molecular Cardiology (S.S., Y.W., K.O., M.S., S.M., K.W.) and Vascular Biology (Y.W.), Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, MA (N.O.); Department of Cardiology, Graduate School of Medicine, Osaka City University, Osaka, Japan (S.S., H.O., K.H., M.S.); Department of Cardiology, Xinqiao Hospital, Army Medical University, Chongqing, China (Y.W.); and Cardiovascular Research Center, School of Medicine, Yale University, New Haven, CT (K.K.H.).

**Acknowledgments**

The authors thank the University of Virginia Genome Analysis and Technology Core for advice and technical support on bulk RNA sequencing, and the University of Virginia Health Sciences Library and Research Computing Center for assistance with computational analysis.

**Sources of Funding**

This study was supported by grants to Dr Chavkin (NIH T32 HL007224, NIH T32 HL007284), Dr S. Sano (NIH R01 HL152174), Dr Wang (China Scholarship Council), Dr Ogawa (Japan Heart Foundation), A. Nelson (NIH T32 HL007284), Dr Saucerman (NIH R01 HL137355), Dr Hirschi (RO1 HL146056, U2ES01703), Dr Walsh (NIH R01 HL138014, 139819 and HL141256), and Drs Gokce and Walsh (NIH R01 HL142650).

**Disclosures**

None.

**Supplementary Material**

Data S1–S2
Table S1
Figures S1–S7

**REFERENCES**

1. Fan D, Takawale A, Lee J, Kassiri Z, Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease. Fibrogenesis Tissue Repair. 2012;5:15. DOI: 10.1186/1755-1536-5-15.
2. van den Borne SW, Diez J, Blankesteijn WM, Verjans J, Hofstra L, Narula J. Myocardial remodeling after infarction: the role of myofibroblasts. Nat Rev Cardiol. 2010;7:30–37. DOI: 10.1038/nrccardio.2009.199.
3. Tallquist MD, Molkentin JD. Redefining the identity of cardiac fibroblasts. Nat Rev Cardiol. 2017;14:484–491. DOI: 10.1038/nrccardio.2017.57.
4. Kanisicak O, Khalil H, Ivey MJ, Karch J, Malken BD, Correll RN, Brody MJ, J. Lin S-C, Aronow BJ, Tallquist MD, et al. Genetic lineage tracing defines myofibroblast origin and function in the injured heart. Nat Commun. 2016;7:12260. DOI: 10.1038/ncomms12260.
transversal aortic constriction.

Zeigler AC, Nelson AR, Chandrabhattach A, Bazzinika O, Holmes JW, Saucerman JJ. Computational model predicts paracrine and intracellular drivers of fibroblast phenotype after myocardial infarction. Matrix Biol. 2020;91-92:136–151. DOI: 10.1016/j.matbio.2020.06.007.

Patel B, Bansal SS, Ismaha M, Hamid T, Rokosh G, Mack M, Prabhoo SC, CR2—monocyte-derived infiltrating macrophages are required for adverse cardiac remodeling during pressure overload. JACC Basic Transl Sci. 2018;3:230–244.

Undurthi SD, Nasal DM, Patel NJ, Thomas E, Yu J, Pierson CG, Bansal SS, Mohler PJ, Hund T. Fibroblast growth factor-inducible 14 mediates macrophage infiltration in heart to promote pressure overload-induced cardiac fibrosis. Life Sci. 2020;247:117440. DOI: 10.1016/j.lfs.2020.117440.

Suzuki K, Satoh K, Ikeda S, Sunamura S, Otsuki T, Satoh T, Kikuchi N, Nomura J, Kurosawa R, Nogi M, et al. Basigin promotes cardiac fibrosis and failure in response to chronic pressure overload in mice. Antioxiders and Redox Signal. 2016;36:636–646. DOI: 10.1161/ATVBHA.115.306686.

Skelly DA, Squiers GT, McLellan MA, Boisette MT, Robson P, Rosenthal NA, Pinto AR. Single-cell transcriptional profiling reveals cellular diversity and intercommunication in the mouse heart. Cell. 2018;22:600–610. DOI: 10.1016/j.celrep.2017.12.072.

Gladka MM, Molenar B, de Ruter H, van der Eer T, Tsui H, Versteeg D, Langen G, Hubers M, Emmanouilides A, van Rooij E. Single-cell sequencing of the healthy and diseased heart reveals cytokine-associated protein 4 as a new modulator of fibroblasts activation. Circulation. 2018;138:166–180. DOI: 10.1161/CIRCULATIONAHA.117.030742.

Wang YS, Li SH, Guo J, Mithc A, Wu J, Sun L, Davis K, Weisel RD, Li RK. Role of miR-145 in cardiac myofibroblast differentiation. J Mol Cell Cardiol. 2014;66:94–105. DOI: 10.1016/j.yjmcc.2013.08.007.

Huang S, Chen B, Su Y, Alex L, Humeres C, Shinde AV, Conway SJ, Frangogiannis GN. Distinct roles of myofibroblast-specific Smad2 and Smad3 signaling in repair and remodeling of the infarcted heart. J Mol Cell Cardiol. 2019;132:84–97. DOI: 10.1016/j.yjmcc.2019.05.006.

Xiao Q, Chen Z, Jin X, Mao R, Chen Z. The many postures of noncanonical Wnt signaling in development and diseases. Biomed Pharmacother. 2017;93:359–369. DOI: 10.1016/j.biopha.2017.06.061.

Abdolmaleki F, Abdapour-Yazdi H, Hayat SMG, Gheibi N, Johnston TP, Saebekhar A. Wnt network: a brief review of pathways and multifunctional components. Crit Rev Eukaryot Gene Exp. 2020;30:1–18. DOI: 10.1615/CritRevEukaryotGeneExp.2019025774.

Kikuchi R, Nakamakara K, MacLauchlan S, Ngo D-M, Shimizu I, Fuster JJ, Katanasaka Y, Yoshida S, Qiu Y, Yamaguchi TP, et al. An antiangiogenic isoform of VEGF-A contributes to impaired vascularization in peripheral artery disease. Nat Med. 2014;20:1484–1471. DOI: 10.1038/nm.3703.

Blankestin WM, Essers-Janssen YP, Verluyen MJ, Daemen MJ, Snits JF. A homologue of drosophila tissue polarity gene frizzled is expressed in postmitotic neurons and promote neurite outgrowth. J Vis Exp. 2015;64:1235–1248.

Chavkin et al. 2000;24:271–274. DOI: 10.1038/73488.

Laaksonen M, Elenius K. Receptor tyrosine kinase profiling of ischemic cardiomyopathy. J Mol Cell Cardiol. 2007;39:2139–2149. DOI: 10.1016/j.yjmcc.2010.04.002.

Winter CG, Wang B, Ballew A, Royou A, Karess R, Axelrod JD, Luo L. Wnt5a control of fibroblast phenotype after myocardial infarction. J Mol Cell Cardiol. 2008;320:365–369. DOI: 10.1126/science.1153671.

Huang S, Chen B, Su Y, Alex L, Humeres C, Shinde AV, Conway SJ, Frangogiannis GN. Distinct roles of myofibroblast-specific Smad2 and Smad3 signaling in repair and remodeling of the infarcted heart. J Mol Cell Cardiol. 2019;132:84–97. DOI: 10.1016/j.yjmcc.2019.05.006.

Leinholts L, Peters J, Krull S, Heilig L, Vogler M, Levay M, van Belle GJ, Ridley AJ, Lutz S, Katsoyannis DM, et al. Hypoxia suppresses myofibroblast differentiation by changing RhoA activity. J Cell Sci. 2019;132:jcs232230. DOI: 10.1242/jcs.232230.

Khalil H, Kanisicak O, Prasad V, Correll RN, Xu F, Schips T, Vagnozzi RJ, Liu R, Huyhn T, Lee SJ, et al. Fibroblast-specific TGF-beta-Smad3 signaling underlies cardiac fibrosis. J Clin Investig. 2017;127:3770–3783.

Winter CG, Wang B, Ballew A, Royou A, Karess R, Axelrod JD, Luo L. Drosophila Hpo-associated kinase (Drok) links frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. Cell. 2001;105:81–91. DOI: 10.1016/S0092-8674(01)00298-7.

Gong Y, Mo C, Fraser SE. Planar cell polarity signaling controls cell division orientation during zebrafish gastrulation. Nature. 2004;430:689–693. DOI: 10.1038/nature02796.

Dempsey D, Fuchs E, Planar polarization in embryonic epidermis orchestrates global asymmetric morphogenesis of hair follicles. Nat Cell Biol. 2008;10:1257–1268. DOI: 10.1038/ncb1784.

Witze ES, Litman ES, Argast GM, Moon RT, Ahn NG. Wnt5a control of cell polarity and directional movement by polarized redistribution of adhesion receptors. Science. 2008;320:365–369. DOI: 10.1126/science.115250.
82. Lee J, Andreeva A, Sipe CW, Liu L, Cheng A, Lu X. PTK7 regulates myosin II activity to orient planar polarity in the mammalian auditory epithelium. *Curr Biol.* 2012;22:956–966. DOI: 10.1016/j.cub.2012.03.068.

83. Li X, Yamagata K, Nishita M, Endo M, Arfian N, Rikita K, Tanaka Y, Minami Y. Activation of Wnt5a-Ror2 signaling associated with epithelial-to-mesenchymal transition of tubular epithelial cells during renal fibrosis. *Genes Cells.* 2013;18:608–619. DOI: 10.1111/gtc.12064.

84. Takahashi D, Suzuki H, Kakei Y, Yamakoshi K, Minami Y, Komori T, Nishita M. Expression of Ror2 associated with fibrosis of the submandibular gland. *Cell Struct Funct.* 2017;42:159–167. DOI: 10.1247/csf.17019.

85. Li C, Smith SM, Peinado N, Gao F, Li W, Lee MK, Zhou B, Bellusci S, Pryhuber GS, Ho H-Y, et al. WNT5A-ROR signaling is essential for alveologenesis. *Cells.* 2020;9:384. DOI: 10.3390/cells9020384.

86. Wilson DH, Jarman EJ, Mellin RP, Wilson ML, Waddell SH, Tsokkou P, Younger NT, Raven A, Bhalla SR, Noll ATR, et al. Non-canonical Wnt signalling regulates scarring in biliary disease via the planar cell polarity receptors. *Nat Commun.* 2020;11:445. DOI: 10.1038/s41467-020-14283-3.

87. Doll S, Dreßen M, Geyer PE, Itzhak DN, Braun C, Doppler SA, Meier F, Deutsch M-A, Lahm H, Lange R, et al. Region and cell-type resolved quantitative proteomic map of the human heart. *Nat Commun.* 2017;8:1469. DOI: 10.1038/s41467-017-01747-2.

88. Kardys I, Knetsch AM, Bleumink GS, Deckers JW, Hofman A, Stricker BH, Witteman JC. C-reactive protein and risk of heart failure. The Rotterdam Study. *Am Heart J.* 2006;152:514–520. DOI: 10.1016/j.ahj.2006.02.023.

89. Kalogeropoulos A, Georgiopoulou V, Psaty BM, Rodondi N, Smith AL, Harrison DG, Liu Y, Hofmann U, Bauer DC, Newman AB, et al. Inflammatory markers and incident heart failure risk in older adults: the Health ABC (Health, Aging, and Body Composition) study. *J Am Coll Cardiol.* 2010;55:2129–2137. DOI: 10.1016/j.jacc.2009.12.045.

90. Everett BM, Cornel JH, Lainscak M, Anker SD, Abbate A, Thuren T, Libby P, Glynn RJ, Ridker PM. Anti-inflammatory therapy with canakinumab for the prevention of hospitalization for heart failure. *Circulation.* 2019;139:1289–1299. DOI: 10.1161/CIRCULATIONAHA.118.038010.

91. Abbate A, Toldo S, Marchetti C, Kron J, Van Tassell BW, Dinarello CA. Interleukin-1 and the inflammasome as therapeutic targets in cardiovascular disease. *Circ Res.* 2020;126:1260–1280. DOI: 10.1161/CIRCRESAHA.120.315937.

92. Van Tassell BW, Canada J, Carbone S, Trankle C, Buckley L, Oddi Erdle C, Abouzaid NA, Dixon D, Kadarly A, Christopher S, et al. Interleukin-1 blockade in recently decompensated systolic heart failure: results from REDHART (Recently Decompensated Heart Failure Anakinra Response Trial). *Circ Heart Fail.* 2017;10:e004373. DOI: 10.1161/CIRCHEARTF AILURE.117.004373.
Data S1. RNA sequencing results. See Excel file.

Data S2. GO term analysis of RNA sequencing. See Excel file.
| Gene   | Forward Primer                  | Reverse Primer                  | Ref. |
|--------|---------------------------------|---------------------------------|------|
| Fsp1   | CTTCCTCTCTCTTTGGTCTGGTC         | TTTGTGGAAGGGTGACAA              | 53   |
| Acta2  | ACTCTCTCTCCAGCCATTTCA           | ATAGGTGTTTCTGGATGC              | 53   |
| Postn  | AAGCTGCAGGAAGACAAG              | TCAAATCTGCAGCTTTCAAG            | 53   |
| Ror1   | AGTTCTCATCATGCGATCC             | CTTTGTCACGAAAGAAGTA             | n.a. |
| Ror2   | CAGACGGCAATCCTGCCT             | GATGACCCCTCGTGCTTT              | n.a. |
| Slug   | CATTCCTTGTGTCTGCAAG             | CAGTGAAGGCAAGAAGAAG             | 53   |
| Snail  | CTTGTGTCAGCACGCGT              | AGGAGATGGACCTTCACCA             | 53   |
| Ptk7   | TGACACTCTTTAGTGTTT             | GGACGCTTTGTTAGTGT              | n.a. |
| Prickle| TGCTCAGGAGATCCAGTCC            | CTCTCTTTAACAGTACG               | 54   |
| Vangl2 | TGCTCATGTGCTTTGGCT             | GGAGCTCAGAAGAAACTTC             | 55   |
| Il6    | GCTACCAAAACCTGGATATAATCAGGA    | CCAGGTAGCTATGCTACTCCAGAA        | 56   |
| Tnfa   | CGGAGTCCGGGCGAG                | GCTGGTAGAGAATGGATGAA            | 56   |
| Il1b   | TGACAGTGATGAAGGAGCTCTGGTC     | TGGAAGCAAGACACCTCTACCT          | 56   |
| Ccl2   | CAGCGAGATGCTTTAACGC            | GCCTACTCATTTGGAACATCTCC         | 56   |
| CD31   | GAGCCCAATCAGTTTGTAGTT         | TCCCTCTGGCTTTCTGCGTAGCT         | 57   |
| CD45   | GGGTTGTGTTGTGTGCTGGTT         | CGGAGAACACAGTTAGCG             | 57   |
| Actb   | AGAGGGAAATCGTGCGTGAC          | CAATAGTGATGACCTGGC             | 57   |
Figure S1. Endothelial cells, leukocytes, and fibroblasts were isolated 7 days after Sham or TAC surgery, and RNA expression of relevant genes was quantified (bars represent mean ± SD, sample size = 4 per group).
Figure S2. A) Cardiac fibroblasts were isolated by langendorff apparatus and identified by flow cytometry using the gating strategy outlined. B) Ror2 expression in the fibroblast population was determined after TAC or Sham surgery (Antibody isotype control staining as a control), with Mean Fluorescence Intensity (MFI) reported.
Figure S3. Primary fibroblast cells from cardiac tissue were imaged at different time points while plated on tissue culture plastic to visualize early fibroblast activation.
Figure S4. Ror1/2 receptor protein levels were quantified in primary murine cardiac fibroblasts at Day 0 and Day 9 post-isolation by western blot (n = 3 samples shown, overall protein loading was adjusted for β-actin levels).
Figure S5. Echocardiography measurements of functional cardiac parameters were quantified in Ror1/2/fl/fl (WT) and Ror1/2/fl/fl + Ubc-CreER\textsuperscript{T2} mice (DKO) 3 months after tamoxifen-induced DNA recombination: A) Fractional shortening, B) Posterior wall thickness at end diastole, C) Left ventricular diameter at end systole, and D) Left ventricular diameter at end diastole (bars represent mean ± SEM, sample size = 4 per group).
Figure S6. RNA expression of A) Ror1 and B) Ror2 was quantified in control and Ror1/2-KO fibroblasts after control or TGF-β1 treatment. C) Acta2 fluorescent intensity of immunofluorescent stained control and Ror1/2-KO fibroblasts after control or TGF-β1 treatment was quantified (bars represent mean ± SD, sample size = 3 per group).
Figure S7. Tissues from control and transgenic Ror1/2 double knockout mice 3 days post-TAC were isolated and imaged: A) Heart, and B) Lungs.