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Cell-specific ablation of Hsp47 defines the collagen producing cells in the injured heart

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Short title: Celltype-specific Hsp47 deletion reveals source of fibrosis

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Abstract
Collagen production in the adult heart is thought to be regulated by the fibroblast, although cardiomyocytes and endothelial cells also express multiple collagen mRNAs. Molecular chaperones are required for procollagen biosynthesis, including heat-shock protein 47 (Hsp47). To determine the cell types critically involved in cardiac injury-induced fibrosis the Hsp47 gene was deleted in cardiomyocytes, endothelial cells or myofibroblasts. Deletion of Hsp47 from cardiomyocytes during embryonic development or adult stages, or deletion from adult endothelial cells, did not affect cardiac fibrosis after pressure overload injury. However, myofibroblast-specific ablation of Hsp47 blocked fibrosis and deposition of collagens type-I, -III and -V following pressure overload, as well as significantly reduced cardiac hypertrophy. Fibroblast-specific Hsp47 deleted mice showed lethality after myocardial infarction injury with ineffective scar formation and ventricular wall rupture. Similarly, only myofibroblast-specific deletion of Hsp47 reduced fibrosis and disease in skeletal muscle in a mouse model of muscular dystrophy. Mechanistically, deletion of Hsp47 from myofibroblasts reduced mRNA expression of fibrillar collagens and attenuated their proliferation in the heart without affecting paracrine secretory activity of these cells. The results show that myofibroblasts are the primary mediators of tissue fibrosis and scar formation in the injured adult heart, which unexpectedly affects cardiomyocyte hypertrophy.

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The extracellular matrix (ECM) in the heart is comprised of diverse structural proteins that provide a rigid but dynamic framework that mechanically supports contracting cardiomyocytes. The ECM in the adult heart is composed predominantly of collagen type-I, although other fibrillar and non-fibrillar collagens are present (1). Other critical components of the ECM in the adult heart include fibronectin, laminin, elastins, diverse proteoglycans, hyaluronan and matricellular proteins like tenascin-C, SPARC, periostin, osteopontin and CCN proteins (2, 3). The fibrillar collagens typically serve as the primary support network in the adult heart and they also likely contribute to mechanosensation as part of cardiomyocyte and fibroblast reactive signaling (4). After myocardial infarction (MI) fibrillar collagen deposition is dramatically upregulated allowing for rapid scar formation to preserve the structural integrity of the injured ventricular chamber (5). In addition, the cardiac hypertrophic response can involve induction of collagens type-I and -III along with a marked reduction of collagen degradation (6-8). While initially an adaptive response, excessive myocardial collagen deposition after pressure-overload induced hypertrophy or from a previous ischemic injury event predisposes to heart failure with diminished ventricular compliance, reduced diffusion efficiency within the tissue and maladaptive structural remodeling (8-12).

Several molecular chaperones are involved in procollagen biosynthesis and folding within the endoplasmic reticulum (ER). These include BiP/Grp78 (13), Grp94 (14), protein disulfide isomerase (PDI) (15) and heat-shock glycoprotein 47 (Hsp47) (16). Hsp47 is a stress inducible collagen-specific molecular chaperone involved in the processing and/or secretion of procollagen (17-19). Hsp47 is upregulated in various fibrotic diseases of the lung (20), liver (21), kidney (22) and heart (23). Moreover, increased expression of human Hsp47 was reported in fibrotic lesions of idiopathic pulmonary fibrosis (24), fibrotic transplanted kidney (25) and peritoneal sclerosis (26). Global Hsp47 gene-deleted mice are embryonic lethal and the embryos show deficient maturation of collagen types-I and -IV (27). Chondrocyte-specific disruption of Hsp47 causes defective endochondral bone formation and a marked reduction in collagen type-II and type-XI accumulation within the cartilage (28).

While fibroblasts are assumed to be the primary source of ECM deposition and tissue fibrosis (29, 30), other cell types are also known to generate ECM components and collagen. For example, endothelial cells produce ECM proteins including collagens (31-33), and collagen type-I, -III, and -VI synthesis was identified in cardiomyocytes (34, 35), with increased collagen1a1 mRNA during heart injury (36). Here we utilized Hsp47-\textit{loxP} targeted mice to disrupt this gene in an adult inducible manner to identify the critical cell types responsible for disease-based fibrosis and acute scar formation. Our study identifies the myofibroblast as the primary mediator of collagen generation and the fibrotic response in the adult heart after injury. Furthermore, inhibition of myofibroblast collagen production secondarily impacted the ability of the mouse heart to effectively hypertrophy following pressure overload stimulation.

Results
Hsp47 deletion attenuates collagen type-I secretion in cultured fibroblasts. To examine the role of Hsp47 in collagen secretion we first generated primary heart fibroblasts from mice with homozygous loxP-site (fl) targeted alleles for Hsp47. These primary heart fibroblasts were infected with a Cre-expressing adenovirus (AdCre) or with a β-galactosidase (Adβgal) expressing control adenovirus (Figure 1A). Immunocytochemistry 72 hrs after recombinant adenoviral infection showed nearly a complete deletion of Hsp47 protein from Hsp47<sup>fl/fl</sup> double homozygous cardiac fibroblasts with AdCre (Figure 1A). Next, Adβgal and AdCre infected Hsp47<sup>fl/fl</sup> cultured cardiac fibroblasts were stimulated for 18 hrs with a combination of ascorbic acid and transforming growth factorβ (TGFβ) to induce collagen generation. The media was collected, concentrated and assayed for collagen type-I isoforms by western blotting, which showed a near complete absence with AdCre (Figure 1B). Consistent with these findings, reduced secretion of collagen in Hsp47 deleted fibroblasts was accompanied by an increase in unfolded collagen content as detected with a collagen hybridizing peptide (CHP) that detects the unfolded collagen triple helix (39). Immunohistological analysis of collagen hybridizing peptide (CHP) showed increased accumulation of unfolded collagen content in Hsp47-deleted fibroblasts as compared to control fibroblasts 72 hours after Hsp47 deletion (Supplemental Figure 1A). Given these results we were concerned that deletion of Hsp47 might compromise the ER and general ability of fibroblasts to secrete proteins. Direct assessment of a broad array of secreted factors in cultured cardiac fibroblasts with an immune-based profiling assay showed no general defects (Supplemental Figure 2A). More specifically, deletion of Hsp47 with AdCre selectively affected the secretion of only a few factors/cytokines out of 111 examined (Supplemental Figure 2, B and C). For example, loss of Hsp47 enhanced the secretion of 11 factors/cytokines including matrix metalloproteinase 3, osteoprotegrin and pentraxin 2 (Supplemental. Figure 2D-F), while only the secretion of IL-17 was significantly reduced (Supplemental Figure 1G). Overall, these results suggest that Hsp47 specifically regulates the secretion of ECM-associated collagens in cultured cardiac fibroblasts and its deletion does not otherwise compromise the secretory function of these fibroblasts.

Cardiomyocyte-specific deletion of Hsp47 does not modulate collagen generation after injury. Global disruption of the Hsp47 gene in the mouse caused early developmental lethality just prior to embryonic day 12 (27). To investigate whether cardiomyocyte-mediated collagen deposition was involved in heart development and function, we crossed β-myosin heavy chain (βMHC) Cre-containing transgenic mice (37) with Hsp47<sup>fl/fl</sup> targeted mice (Figure 1C). Hsp47 protein was identified in the cardiomyocyte fraction from hearts of βMHC-Cre control mice by western blotting, while protein levels were reduced by more than 80% from hearts of Hsp47<sup>fl/fl-βMHC-Cre</sup> mice (Figure 1D). Hearts from Hsp47<sup>fl/fl-βMHC-Cre</sup> mice appeared otherwise without disease based on measurements employed here, suggesting that cardiomyocytes are not likely a significant source of collagen production during development. These same mice as young adults were also subjected to 4-weeks of pressure overload stimulation by transverse aortic constriction (TAC) surgery, which showed that controls (βMHC-Cre) and Hsp47<sup>fl/fl-βMHC-Cre</sup> mice displayed comparable levels of induced myocardial fibrosis (Figure 1, E and F), as well as similar cardiac function and equal induction of the hypertrophic response (Figure 1G, Supplemental
Hsp47 was also deleted from the adult heart using the tamoxifen-inducible α-myosin heavy chain (αMHC) MerCreMer (MCM) containing transgene (38) (Figure 1H). Tamoxifen was injected for 3 consecutive days 2 weeks before the TAC surgery procedure (Figure 1I). Western blotting with lysates from isolated adult cardiomyocytes from hearts of Hsp47\(^{fl/fl-αMHC-MCM}\) mice showed a greater than 90% loss of Hsp47 protein compared with control αMHC-MCM mice (Figure 1J). Similar to embryonic deletion of Hsp47 from cardiomyocytes, deletion of Hsp47 specifically from adult cardiomyocytes of mice showed no ability to reduce or otherwise alter the pressure overload-induced fibrotic response compared with αMHC-MCM controls (Figure 1, K-M, Supplemental Figure 3). Similarly, hydroxyproline assay-based quantitation of fibrosis showed no reduction after TAC with adult cardiomyocyte-specific deletion of Hsp47 (Supplemental Figure 4A). Histological quantification of using the collagen hybridizing peptide (CHP)(39) assay also showed similar accumulation of unfolded collagen content in cardiomyocyte-specific Hsp47-deleted and control hearts (Supplemental Figure 4B). In conclusion, cardiomyocyte-specific inhibition of collagen secretion had no effect on the ability of the adult heart to mount a fulminant fibrotic response following pressure overload.

Endothelial-specific deletion of Hsp47 mildly reduces collagen type-I without affecting total heart fibrosis. Endothelial cells were reported to produce ECM proteins including collagens; however, the contribution of these cells to myocardial fibrosis and progression of heart disease has not been evaluated in vivo (31-33). To investigate the endothelial cell contribution to cardiac fibrosis Hsp47\(^{fl/fl}\) mice were crossed with the endothelial cell-specific and inducible transgenic line, Tie2-CreERT2 (40) (Figure 2A). Note that this endothelial-specific Cre mouse line uses the regulatory sequences of the Tek gene, which was reported to show some degree of Cre recombinase activity in the hematopoietic lineage (41). We also crossed the Tie2-CreERT2 line with a single Rosa26-loxP-Stop-loxP-eGFP (R26\(^{eGFP}\)) reporter allele to allow Cre-dependent eGFP expression in targeted endothelial cells as previously described (40). Mice were injected with tamoxifen at 6 weeks of age, then placed on tamoxifen-laden food 48 hrs prior to surgery and continued thereafter so that deletion of Hsp47 would be ongoing as new endothelial arose during the pressure overload period (Figure 2B). Western blotting showed that eGFP-purified endothelial cells from the heart indeed expressed abundant Hsp47 protein, which was effectively deleted with the Tie2-CreERT2 transgene 4 weeks after TAC surgery (Figure 2C). Immunohistological analysis of heart sections showed a slight but significant reduction in collagen type-I staining intensity in hearts of endothelial-specific Hsp47 gene-deleted mice compared to controls (Figure 2, D and E), although collagen type-III and –V were not reduced (Figure 2, D, F and G). Additionally, picrosirius red staining (not shown) and hydroxyproline quantification showed a similar fibrotic response in both endothelial-specific Hsp47-deleted and control hearts with 4 weeks of pressure overload (Supplemental Figure 4A). Finally, the content of unfolded collagens evaluated by collagen hybridizing peptide (CHP) assay showed no difference between endothelial-specific Hsp47-deleted and control hearts (Supplemental Figure 4B). Thus, endothelial cells are not a primary cell type that mediates significant cardiac fibrosis with pressure overload.
Myofibroblast-specific Hsp47-deleted mice show reduced collagen deposition with TAC. Here we used Postn gene-targeted mice containing the same tamoxifen-inducible MCM cDNA (36) and crossed them with the Hsp47fl/fl targeted mice to delete Hsp47 in only myofibroblasts (activated fibroblasts) after cardiac injury (Figure 3A). The R26eGFP reporter allele was also included to allow tracking and isolation of all myofibroblasts from the heart (36). Mice were subjected to pressure overload for 4 weeks by TAC in the presence of a tamoxifen diet so that the inducible MCM protein continuously produced recombination in existing and newly activated fibroblasts (Figure 3B). Western blotting of lysates from eGFP-expressing FACS-sorted myofibroblasts from Hsp47fl/fl-Postn-MCM/+ R26eGFP/+ mice showed efficient deletion of Hsp47 protein compared to control Postn-MCM myofibroblasts (Figure 3C). Levels of fibrillar collagens type-I, –III and –V were assessed by immunohistochemistry (Figure 3D), which showed essentially no induction after 4 weeks of TAC with myofibroblast-specific deletion of Hsp47 compared with Postn-MCM controls (Figure 3, D-G). We also assessed TAC-induced fibrosis in picrosirius red-stained cardiac histological sections (Figure 3H) and the content of collagen type-I in whole heart ECM protein preps by western blotting in Hsp47fl/fl-Postn-MCM/+ mice, which again showed a significant reduction versus TAC-operated Postn-MCM control mice (Figure 3I). Biochemical hydroxyproline assays (Supplemental Figure 4A) and histological assays for collagen hybridizing peptide (CHP) intensity again showed that myofibroblast-specific Hsp47-deleted hearts had significantly reduced levels compared with controls hearts after 4 weeks of pressure overload (Supplemental Figure 4B). Consistent with these results, although Hsp47 is expressed in both cardiomyocytes and fibroblasts, after mechanical stretching, Hsp47 expression is induced only in fibroblasts but not in cardiomyocytes (Supplemental Figure 5A, B). Taken together, these results indicate that cardiac myofibroblasts, but not cardiomyocytes or endothelial cells, are the primary cell-type in the heart responsible for mediating the pathologic fibrotic response.

We also used Tcf21 gene-targeted mice containing the same tamoxifen-inducible MCM cDNA, which were crossed with the Hsp47fl/fl targeted mice to delete Hsp47 in only fibroblasts at baseline before cardiac injury (Supplemental Figure 6A). Mice were fed tamoxifen diet one week before surgery and then were subjected to pressure overload for 4 weeks by TAC (Supplemental Figure 6B). Levels of myocardial fibrosis were assessed by picrosirius red staining (Supplemental Figure 6, C and D), which showed a significant reduction of fibrosis with fibroblast-specific deletion of Hsp47 compared with Tcf21-MCM controls after 4 weeks of TAC injury. Taken together, these results confirm that deletion of Hsp47 in cardiac fibroblasts reduces myocardial fibrosis with pressure overload.

Loss of Hsp47 from myofibroblasts in dystrophic skeletal muscle attenuates fibrosis and enhances muscle performance. Muscular dystrophy is a longstanding chronic disease characterized by accumulation of collagen and ECM (42), although the direct involvement of the tissue resident fibroblast in skeletal muscle fibrosis is unclear. Here we used a mouse model of limb-girdle muscular dystrophy lacking the δ-sarcoglycan gene (Sgcd-
which shows continuous myofiber necrosis in skeletal muscle, inflammation and fibrosis that accumulates over time (43). As in heart, skeletal muscle cells also express select collagen mRNAs (44, 45) so it is unclear how much of disease-based fibrosis is due to myofiber collagen production versus activated fibroblasts. Here we inducibly deleted the Hsp47 gene in either myofibers or myofibroblasts of Sgcd null mice. We have previously characterized a transgene containing the MCM cDNA driven by the skeletal muscle-specific skeletal α-actin promoter (Ska-MCM) (46). Tamoxifen was given at 1 month of age with tamoxifen-laden food until harvest at 4 months of age (Figure 4, A and B). We have previously shown that the Postn-MCM allele is efficiently expressed in activated fibroblasts in injured skeletal muscle (36). Western blot analysis performed on whole muscle protein lysates from Sgcd<sup>−/−</sup> Hsp47<sup>fl/fl</sup>-Ska-MCM mice showed a greater than 80% loss of endogenous Hsp47 protein in myofibers compared with Hsp47<sup>fl/fl</sup> control mice (Figure 4C). Histological analysis of muscle disease showed that loss of Hsp47 from the myofibers did not reduce dystrophy-dependent fibrosis compared with controls (Figure 4, D-F). In contrast, deletion of Hsp47 from Sgcd<sup>−/−</sup> mice using the Postn-MCM allele showed a significant reduction in fibrosis of both the quadriceps and diaphragm (Figure 4, D-F). Mice were then challenged to run on a treadmill as an indirect measure of muscle performance. Consistent with less fibrosis, Sgcd<sup>−/−</sup> mice with Hsp47 deleted in activated fibroblasts performed significantly better compared with the appropriate controls (Figure 4G). These results confirm and extend the results observed in the heart, whereby the activated, tissue resident fibroblast is the most critical mediator of interstitial fibrosis in response to disease stimulation in striated muscle.

Reduced fibrosis in myofibroblast-specific Hsp47-deleted mice attenuates myocardial hypertrophy and increases cardiac rupture after MI injury. The mouse model of muscular dystrophy and the cardiac pressure overload surgical model represent chronic disease states whereby fibrosis slowly but progressively accumulates. In these long-term profiles the activated fibroblast was the primary mediator of the fibrotic response but it is not clear if the myofibroblast is also the primary regulator of more acute injury where scar formation must occur quickly, such as after MI. To address this question, 8 week-old mice were given tamoxifen and then MI surgery was performed, after which mice were followed for survival to monitor the success of acute scar formation (Figure 5A). Myofibroblast-specific Hsp47-deleted mice were significantly compromised in their survival in the first week following MI injury, whereas all control groups and mice with cardiomyocyte-specific deletion of Hsp47 (αMHC-MCM transgene) showed the typical low level of lethality with good overall survival and scar formation (Figure 5B). Upon post-mortem inspection, the activated fibroblast-specific Hsp47 deleted mice uniformly showed left ventricular wall rupture indicating that acute scar formation in the first week was compromised (data not shown). Thus, endogenous cardiac myofibroblasts are the primary cell type mediating both chronic disease-based fibrosis as well as acute healing and scar formation in the heart after MI injury. Since the standard MI injury induced close to 100% lethality of Hsp47<sup>fl/fl</sup> Postn<sup>MCM</sup> mice, we subjected these mice and their controls to an Ischemia reperfusion (I/R) procedure. The I/R procedure was better tolerated by Hsp47<sup>fl/fl</sup> Postn<sup>MCM</sup> mice resulting in better survival rate. Cardiac sections were then quantified for the
accumulation of cardiac fibrosis, which showed that surviving \( Hsp47^{f/f} \) \( Postn^{MCM} \) mice had a significant reduction in ventricular fibrotic area compared with \( Postn^{MCM} \) controls (Figure 5C).

As part of our analysis we also measured the cardiac hypertrophic response by gravimetric analysis after 4 weeks of TAC-induced pressure overload stimulation. The data showed that deletion of \( Hsp47 \) in cardiomyocytes or endothelial cells did not alter the otherwise robust development cardiac hypertrophy after pressure-overload stimulation, yet \( Postn \)-MCM mediated deletion of \( Hsp47 \) from myofibroblasts resulted in a significant reduction in cardiac hypertrophy versus controls (Figure 5D). Supporting this findings, myofibroblast-specific \( Hsp47 \)-deleted mice also showed reduced cardiac hypertrophy after TAC compared to control mice as assessed by echocardiography (Figure 5E). Cardiomyocyte area measured in histological sections and induction of hypertrophic marker genes were also reduced in the hearts of myofibroblast-specific \( Hsp47 \)-deleted mice after TAC compared with controls (Supplemental figures 7A-D). Additionally, we evaluated the progress of myocardial hypertrophy in these mice and their controls after a shorter 10 protocol of pressure overload, which once again showed a significant reduction in cardiac hypertrophy in myofibroblast-specific \( Hsp47 \)-deleted mice (Supplemental Figure 7E). These results suggest that a deficiency in the ability of cardiac myofibroblasts to produce collagen with ECM accumulation reduces the cardiac hypertrophic response with pressure overload stimulation (see discussion).

Systolic functional performance of the heart remained equally compromised in myofibroblast-specific \( Hsp47 \)-deleted mice compared with controls as measured by echocardiography (Figure 5F), although diastolic performance measured indirectly by tissue Doppler echocardiography indicated a lower \( E/e' \) ratio in myofibroblast-specific \( Hsp47 \)-deleted mice after TAC relative to control, suggesting that reduced fibrosis attenuated TAC-induced diastolic dysfunction (Figure 5G).

Deletion of \( Hsp47 \) reduces myofibroblasts proliferation in vivo. Deletion of \( Hsp47 \) from myofibroblasts in the hearts of mice subjected to TAC significantly reduced production of the major fibrillar collagens and the fibrotic response, although the cellular basis underlying this effect was not clear. Here we investigated the change in cardiac fibroblast dynamics over 1 week of TAC stimulation with or without \( Hsp47 \) deletion in myofibroblasts (Figure 6A). To analyze fibroblast proliferation rates, mice of the genotype \( Postn^{MCM/+} \ Hsp47^{f/f} \ R26^{eGFP/+} \) were compared to controls of the genotype \( Postn^{MCM/+} \ R26^{eGFP/+} \). Mice were also given 5-ethynyl-2′-deoxyuridine (EdU) treatment to measure proliferation, which was given in the last 4 hours before sacrifice (Figure 6A). Hearts were removed and total interstitial cells were isolated after enzymatic digestion followed by FACS to quantify total eGFP* cells normalized to CD31* endothelial cells (Figure 6, B and C). Endothelial cells expanded similarly with pressure overload stimulation in the two experimental groups with or without \( Hsp47 \) in activated cardiac fibroblasts (Figure 6D). The data show a dramatic reduction in total numbers of activated eGFP* myofibroblasts in the hearts of \( Hsp47 \)-deleted mice after 1 week of TAC compared with control mice (Figure 6, B and C). To investigate whether the reduction of total activated fibroblasts is due to a decrease of proliferation rate, histological sections were quantified for eGFP* fibroblasts and for EdU incorporation (Figure 6, E and F).
After 4 hours of EdU exposure, about 20% of eGFP+ activated fibroblasts incorporated EdU suggesting they were in cell cycle while less than 5% of the Hsp47-deleted eGFP+ myofibroblasts incorporated EdU (Figure 6, E and F). We also assessed cell death of myofibroblasts by TUNEL, which showed that myofibroblast-specific Hsp47-deletion produced a significant increase in TUNEL positive myofibroblasts compared to control (Supplemental Figure 1B).

To extend these results, primary Hsp47fl/fl cardiac fibroblasts were isolated and infected with AdCre or Adβgal for 72 hrs to evaluate cellular proliferation using (EdU) over 24 hrs. Approximately 55% of Adβgal infected fibroblasts incorporated EdU, while only ~15% of the AdCre infected fibroblasts were proliferative (Figure 6G). Thus, loss of Hsp47 from myofibroblasts in the heart compromises their proliferation with TAC stimulation, suggesting an additional contributing factor to the observed reduction in fibrosis and cardiac hypertrophy. Indeed, we have previously shown that fibroblasts normally undergo a 3-fold expansion with injury to the heart that is associated their ability to effectively generate fibrosis and a scar (47).

**Gene expression profiling defines a reduced collagen and differentiated state of Hsp47-deleted myofibroblasts.**

Here we instituted mRNA profiling in Hsp47 deleted myofibroblasts to gain insight into the molecular alterations that might underlie their reduced activity and proliferation. We first assessed mRNA levels of endogenous Col1α1, Col3α1, Col4α1 Col5α1 Col6α1 and Col11α1 from primary Hsp47fl/fl cardiac fibroblasts in culture infected with AdCre or Adβgal. Unexpectedly, the data showed a generalized and significant reduction in mRNA expression of all the collagen genes tested with Hsp47 deletion (Figure 7A). In parallel, we assessed the expression profile of a panel of ECM-related genes including fibrillin (Fbn), fibronectin (Fn), and matrix metalloproteinase 23 (Mmp23), which were also downregulated in Hsp47-deleted fibroblasts. By comparison, we observed induced expression of tenasin-C (Tnc) and matrix metalloproteinase 9 (Mmp9). Interestingly, loss of Hsp47 did not affect expression of smooth muscle α-actin (Acta2) in Hsp47-deleted versus control fibroblasts (Figure 7A). In addition, Hsp47-deleted fibroblasts displayed a diminished replicative capacity in culture which is consistent with the observed downregulated expression of zinc finger E-box binding homeobox transcription factor 1 (Zeb1) and zinc-finger transcription factor (Snail), and increased expression of the cell cycle inhibitory cyclin dependent kinase inhibitor Cdkn1a (Figure 7A).

We also performed unbiased and global mRNA profiling of FACS isolated eGFP+ activated myofibroblasts from the heart after 4 weeks of pressure overload, with and without Hsp47 deletion, as schematized in Figure 7B and 7C. Bioinformatics analysis identified modulation in genes implicated in key functional features of the fibroblast, such as ECM components, ECM modification, bone and cartilage signatures, proliferation, cell adhesion and TGFβ signaling (Supplemental Table 1). The most notable upregulated genes identified in Hsp47-deleted myofibroblasts were those underlying bone, cartilage, and tendon development or processing, followed thereafter by ECM-associated proteoglycan genes (Supplemental Table 1). Genes regulating cell adhesion and TGFβ signaling were also identified as significantly altered with Hsp47 deletion after TAC stimulation (Supplemental Table 1). Collectively the bioinformatics profile showed
that loss of Hsp47 reduced the expression of ECM-related genes, reduced expression of proliferation genes and showed mRNA signatures that are consistent with premature senescence.

In parallel, the expression profile of representative gene categories was confirmed by RT-PCR (Figure 7, D and E). Consistent with the previous data obtained in primary cardiac fibroblasts in culture, the data from in vivo isolated eGFP+ myofibroblasts showed that loss of Hsp47 caused a generalized and significant reduction in expression of mRNA levels of endogenous Col1a1, Col3a1, Fn, Fbn, and Adam12 (Figure 7E). Similarly, an induction of cartilage associated chondroadherin (Chad), leucine-rich repeat protein asporin (Aspn), tenomodulin (Tnmd), and the proteoglycans fibromodulin (Fmod) and syndecan-4 (Sdc4) was observed in Hsp47-deleted myofibroblasts, which could alter the adhesive state of these cells (Figure 7D). Modulated mRNA expression levels of cell surface receptors including integrin subunit alpha 11 (Itga11) and cadherin-7 (Cdh7) were also identified and we confirmed an induction in the mRNA levels of TGFβ signaling genes (Tgfb2 and Furin) and the gene for TGFβ-inducible glycoprotein collagen triple helix repeat-containing protein 1 (Cthrc1) that is known to reduce collagen type I mRNA (48). Finally, the expression levels of genes integral for ECM organization and maturation such as lysyl oxidase (Lox), matrix metalloproteinase 3 (Mmp3) and Postn were found to be induced in Hsp47-deleted myofibroblasts (Figure 7D).

Discussion

ECM in the adult heart is composed predominantly of fibrillar collagens in addition to matricellular proteins, proteoglycans and growth factors (2, 3). Fibrillar collagens within the ECM microenvironment serve as a critical component in supporting cardiomyocyte contraction and possibly even stretch-regulated reactive signaling (4). Collagen deposition is often upregulated in diseased hearts, which can cause an irreversible and chronic fibrotic profile that increases ventricular stiffness and reduces contractile dynamic range (49, 50). While it is accepted that myofibroblasts express and secrete collagens and other ECM proteins, so do cardiomyocytes (34, 35, 51-53) and endothelial cells (31-33). The results presented here are the first to directly demonstrate in vivo that it is the myofibroblast that serves as the primary source of collagen production in the acutely stressed or chronically diseased heart. Moreover, deletion of Hsp47 within myofibroblasts of skeletal muscle, but not within the myofibers, also reduced the extent of muscular dystrophy disease-dependent fibrosis, collectively suggesting that it is the myofibroblast that is the primary regulatory of adult disease-based tissue fibrosis in general. However, we did observe a mild, albeit significant reduction in collagen type-I expression in the heart after endothelial cell-specific deletion of Hsp47, suggesting that endothelial cells may still play a minor role in cardiac fibrosis with pressure overload stimulation. The results observed here are also consistent with past data whereby selective inhibition of key signaling pathways in myofibroblasts can disrupt the greater fibrotic response of the heart (54-57). For example, we previously showed that myofibroblast specific deletion of Smad2 and Smad3, or Tgfbr1 and Tgfbr2 reduced cardiac fibrosis in the heart during pressure overload stimulation by compromising the activity of the TGFβ signaling pathway in myofibroblasts (55).
Four weeks of pressure overload stimulation in mice typically results in reduced cardiac function, such as a reduction in ventricular fractional shortening as measured by echocardiography (Figure 5C). Interestingly, the reduction in myocardial fibrosis in myofibroblast specific Hsp47-deleted mice did not protect from cardiac decompensation versus control mice, suggesting that fibrosis in the first 4 weeks of pressure overload is not a primary inducer of decompensation. However, the reduced ECM profile associated with Hsp47 deletion in myofibroblasts did reduce the extent of cardiac hypertrophy over 4 weeks of pressure overload, suggesting that greater ECM content is needed to support fulminant hypertrophic growth of cardiomyocytes. Alternatively, it is also possible that the failure of fibroblasts to expand properly renders the heart with less fibroblast-dependent paracrine factors to support cardiomyocyte growth. Similarly, we previously observed that deletion of Tgfbr1/2 in activated fibroblasts of the mouse heart during TAC stimulation lead to a reduction in cardiomyocyte hypertrophy, and fibroblasts with Tgfbr1/2 deletion were again less active (55, 57). However, while our results do not allow us to discern the exact mechanism whereby a compromised cardiac fibrotic response renders the heart significantly less hypertrophic, we favor the interpretation that augmented ECM content and structural support are required for optimal growth of cardiomyocytes as the dominant mechanism in play. Indirect support for this hypothesis comes from cardiac development itself, whereby myocytes within the neonatal heart begin to hypertrophy towards their adult state in coordination with a switch in the cardiac ECM from fibronectin to a stiffer and more structurally supportive collagen type-I platform (58).

Unexpectedly, loss of collagen production in Hsp47-deleted myofibroblasts induced a negative feedback loop resulting in the reduction of mRNA expression of multiple collagens (Col1a1, Col2a1, Col3a1, Col4a1, Col5a1, Col6a1, and Col11a1). In parallel Hsp47-deleted myofibroblasts seemed to initiate a compensatory mechanism that includes the induction of matricellular proteins and proteoglycans such as tenascin C (Tnc), Postn, syndecan 4 (Sdc4), fibromodulin (Fmod) as well as the glycoprotein tenomodulin (Tnmd). Hsp47-deleted myofibroblasts also showed an altered differentiated state with increased expression of cartilage associated chondroadherin (Chad) and leucine-rich repeat protein asporin (Aspn) (47, 59). We have previously shown that deletion of the Postn gene, which is a matricellular protein that participates in collagen maturation, similarly alters the differentiated state of myofibroblasts from the heart and leads to an attenuated fibrotic response with injury (60). Similarly, in lung fibroblasts, knock-down of select lysyl oxidases that are required for proper extracellular collagen maturation changes the expression of select fibroblast functional genes (61). Thus, the content and relative abundance of ECM and collagen within the heart are critical regulators of fibroblast biology and ECM-related mRNA levels through some sort of feedback regulation. However, as stated above it is not clear how this regulation occurs, such as through paracrine or autocrine factors that are stored within the ECM and released as signals, or whether the structural properties of the ECM itself support fibroblast activity and differentiation through direct adhesion complexes and their inherent intracellular transducing properties.

The observations presented here suggest an important regulatory relationship between different cardiac cell types within the heart and the ECM microenvironment. Both cardiomyocytes and fibroblasts appear to
directly sense the extent of ECM construction in the heart with acute or chronic injury. Indeed, with an inability of fibroblasts to expand the ECM during pressure overload, the cardiomyocytes cannot attain the same degree of hypertrophy. At the same time newly activated fibroblasts show a defect in proliferation and an altered differentiated gene program (more senescent). Thus, while our results proved the longstanding hypothesis that it is the myofibroblast underling cardiac disease-based fibrosis in vivo, we uncovered an unanticipated regulatory feedback relationship between the collagen content in the heart and hypertrophic growth potential of cardiomyocytes and activity of fibroblasts themselves. These results suggest some obvious clinical vantage points, such as simply inhibiting collagen production or maturation in the ECM as an anti-fibrotic strategy in heart failure or muscular dystrophy.

**Methods**

*Mice.* Mice containing a genetic insertion of a tamoxifen-inducible MerCreMer (MCM) cDNA, into the *Postn* locus, or transgenic αMHC-MCM mice, were used and described previously (36, 38). *Rosa26* loxP-site-dependent reporter mice (*R26<sup>eGFP</sup>*) were previously described (62) and were purchased from the Jackson Laboratories (Stock# 012429). βMHC-Cre mice were described elsewhere (37), as were skeletal α-actin MCM transgenic mice (46). A mouse model of limb-girdle muscular dystrophy lacking the δ-sarcoglycan gene (*Sgcd<sup>−/−</sup>*) was also used (43). Tie2-CreERT2 transgenic mice were described elsewhere (40), as were loxP site-targeted *Hsp47* mice (28). PCR genotyping used the following primers: *Hsp47* forward 5′-GAGTGGGCTGAGCCCTCTCAAGAAAATCC-3′ and reverse 5′-CTTCGGTCAGGCCCAGTCTGCCAGATG-3′, which generated a 450 bp product recognizing loxP-targeted exons 6, and 350 bp WT fragments. Mice received a combination of tamoxifen-citrate chow (400 mg/kg body weight, Envigo-TD, #130860) and intra-peritoneal (i.p.) injections with pharmaceutical grade tamoxifen (75 mg/kg body weight, Sigma, #T5648) dissolved in 95% corn oil/5% ethanol. The *Postn-MCM* line was i.p. injected once 48 h before surgery and once 24 hrs after surgery, and maintained on tamoxifen-citrate chow 48 hrs before surgery for the entirety of the experiment. The Tie2-CreERT2 transgenic mouse line received 2 i.p. injections with tamoxifen 15 days before the surgical procedure followed by feeding with tamoxifen-citrate chow 48 h before surgery until the experiment was terminated. The αMHC-MCM transgenic line received 3 i.p. injections on 3 consecutive days with pharmaceutical grade tamoxifen 2 weeks before the surgical procedure. Mice in the *Sgcd<sup>−/−</sup>* background received tamoxifen in the chow beginning at 1 month of age until the experiment was terminated. All mice were in the *C57BL/6* genetic background and male mice were used throughout to reduce the total number of animals needed to achieve statistical significance and to reduce variation.

*Animal procedures.* All experimental procedures with mice were approved by the Institutional Animal Care and Use Committee of Cincinnati Children’s Medical Center, protocols IACUC 2015-0047 and 2016-0069. The number of mice used in this study reflects the minimum number needed to achieve statistical significance based
on experience and previous power analysis. Blinding was performed for some experimental procedures with mice, although blinding was not possible in every instance. Randomization of mouse groups was not performed because mice were genetically identical and often littermates, although only males were used to reduce total animal usage and to limit variability. Eight week-old mice were subjected to cardiac pressure overload by TAC surgery as described previously (55). Echocardiography was performed in a blinded fashion to assess ventricular geometry and function at 4 weeks post TAC as described before (55). Briefly, animals were anesthetized with 2% isoflurane inhalation and analyzed with a Vevo 2100 instrument equipped with 18–38 MHz transducer (VisualSonics). Fractional shortening, left ventricular end-diastolic volume, and left ventricular mass were determined from 2D M-mode echocardiograms. The effectiveness of the TAC procedure was verified by Doppler echocardiography which measured pressure gradients across the aortic constriction. Diastolic function was evaluated by tissue Doppler echocardiography presented as the E/e’ ratio that measures mitral valve inflow maximum velocity (E-wave) to posterior wall maximum tissue Doppler velocity (e’) ratio. MI was induced in mice via permanent surgical ligation of the left coronary artery (63). Briefly, 2% isoflurane anesthetized mice were subjected to a left lateral thoracotomy, the left coronary artery was isolated and occluded permanently just below the left atrium. Ischemia-Reperfusion (I/R) injury was described previously (64). Briefly, mice were anesthetized with inhaled 2% isoflurane, intubated through the mouth, and ventilated throughout the procedure. I/R injury was induced with a slipknot around the left coronary artery. After 30 min of ischemia, the slipknot was released followed by reperfusion until the mice were euthanized and the hearts harvested. A single postoperative dosage of buprenorphrine at 0.1 mg/kg was given by subcutaneous injection to reduce pain.

**Histology and immunostaining.** Isolated hearts were fixed for 4 hrs in freshly diluted 4% paraformaldehyde at 4°C and then a portion of the tissue was rinsed with PBS and cryoprotected in 30% sucrose/PBS overnight before embedding in OCT (Tissue-Tek). Another part of the tissue was washed with 70% ethanol and subjected to paraffin embedding. Afterwards, 10 μm cryosections were collected and then processed for 30 min at room temperature in a blocking solution (PBS with 5% goat serum, 2% bovine serum albumin, 0.1% Triton X-100). Collagen type-I, collagen type-III and collagen type-V primary antibodies (Abcam # ab21286, # ab7778, and #ab7046, respectively 1:100 dilution) were diluted in blocking solution and incubated on the histological sections overnight at 4 °C. The sections were then washed 3 times for 5 min each in PBS and incubated with Alexa Fluor 568-conjugated goat anti-mouse antibody (Life technologies A11031) for 2 h at room temperature at 1:400 dilutions. After washing 3 times for 5 min each, sections were stained with 4’,6-diamidino-2-phenylindole (DAPI) at a concentration of 0.1 μg/ml in water for 5 min at room temperature and mounted on slides using aqueous mounting medium (H-1400, Vector Laboratories). In some experiments, cryosections were used to visualize native eGFP fluorescence from the appropriate genotypes that contained the Rosa26-loxP-dependent reporter allele. Images were acquired with an inverted Nikon A1R confocal microscope using NIS Elements AR 4.13 software. Ten random pictures per mouse heart were taken from 5-10 sections each at
different levels of the heart for quantitative analysis. Quantification of signal intensity was done by calculating pixels numbers using with Adobe Photoshop Elements 9.

**Picrosirius red staining and hydroxyproline assessment.** Picrosirius red staining was done with a kit (Electron Microscopy Sciences, #26357-02) per manufacturer’s instructions. Picrosirius red images were captured with a Leica M165FC stereo microscope with fluorescence using a Leica DFC310 FX camera and the Leica Application Suite. Total collagen content in cultured media was analyzed by using Sirius Red Total Collagen Detection Kit (Chondrex, #9062). Hydroxyproline content in cardiac tissue was assayed as detailed earlier (65).

Collagen Hybridizing Peptide (CHP) staining was described earlier (39) and was purchased from 3Helix (#Bio300). Briefly, 4% paraformaldehyde-fixed tissue sections were blocked with 2 drops of the streptavidin and biotin reagent according to the manufacturer protocol for 15-30 minutes at 37°C in a humid chamber (Thermo Fisher Scientific, #E21390). Slides were rinsed twice with PBS for 10 min. Sections were then blocked with PBS supplemented with 0.2% Triton X-100, 5% goat serum and 1% BSA and incubated for 30 min at room temperature. A 15 μM CHP working solution was freshly prepared and warmed at 80 °C for 5 minutes, then instantly placed on ice for 15 seconds and applied to the histological sections on glass slides. The exact procedure was followed when CHP was used with cells on coverslips, however, the final concentration of company supplied reagent was 3 μM. The slides were then incubated at 4 °C overnight and subsequently washed and incubated with a secondary streptavidin-conjugated antibody that was Alexa-- Fluor 568 conjugated (Life Technologies, #S11226).

**Western blot.** Western blotting was performed as described previously (66). Briefly, protein preparations were mixed with 5x Laemmmi loading buffer and heated to 95 °C for 10 min. Equal amounts of protein were subjected to electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, #IPFL00010). The membranes were incubated with antibodies against, Hsp47 (1:000, Novus biologicals, #M16.10A1), Gapdh (1:10000 Fitzgerald, 10R-G109a) or α-tubulin (1:1000 Santa Cruz, #sc-8035). PVDF membranes were then incubated with the appropriate AlexaFluor-conjugated secondary antibodies (Thermofisher scientific, A-21057, A11367) and then visualized by using an Odyssey CLx imaging system (LI-COR Biosciences, #9140).

**Adult cardiomyocyte and interstitial heart cell isolation and ECM protein preparations.** Adult cardiomyocytes and interstitial heart cell fractions were isolated as described previously (55). Briefly, freshly beating hearts were cannulated for retrograde perfusion with modified Tyrode solution (120 mM NaCl, 14.7 mM KCl, 0.6 mM KH₂PO₄, 0.6 mM Na₂HPO₄, 1.2 mM MgSO₄, 10 mM HEPES, 4.6 mM NaHCO₃, 30 mM taurine, 5.5 mM glucose, and 10 mM butanedionemonoxime [BDM], pH 7.4), supplemented with liberaseTH (Roche, #05401151001). Hearts were then dissociated into cardiomyocytes and non-cardiomyocyte fractions and then separated by two
serial centrifugations at 10 g for 5 min at 4°C. The non-cardiomyocyte cell fraction was centrifuged at 500 g for 10 min at 4°C. Extracellular matrix protein preparations were performed as previously described (67).

**Isolation of adult cardiac fibroblasts and endothelial cells.** Cardiac ventricles were excised from mice, rinsed with cold sterile 1X Hank's Balanced Salt Solution (HBSS, Gibco, 14025092) and treated as described previously (68). Briefly, tissues were thoroughly minced with sterile fine scissors and digested in 2 ml of DMEM containing a combination of 2 mg/ml of collagenase type 4 (Worthington, #LS004188) and 0.75 mg/ml of dispase II (Roche, # 10165859001) at 37 °C for 60 min (20 minute incubation). During incubations the digesting tissue was triturated for a minute with a sterile serological pipette every 15 min and the digestion mix was incubated for 2 min to sediment before collecting the supernatants. The supernatant cell suspension containing the liberated fibroblasts were then collected in a tube containing cold DMEM supplemented with 10% Bovine Growth Serum (BGS) (GE Healthcare Life Sciences, #SH30541.03). The undigested fraction was reconstituted with 2 ml fresh digestion media and the same digestion procedure was repeated for 3 total rounds. Cell debris was eliminated by 2 serial centrifugations at 10 g for 5 min at 4 °C and the cell fraction was collected after a final centrifugation at 500 g for 10 min at 4 °C. For flow cytometry analysis, pellets were washed once with ice cold HBSS incubated for 1 min in 1 ml of red blood cell lysis buffer (Sigma, #R7757), then washed, centrifuged and resuspended in 2% BGS and 2 mM EDTA in HBSS and incubated on ice for FACS analysis using a Becton-Dickinson Aria Instrument. A fraction of this preparation was cultured in 10% BGS containing DMEM media on gelatin 0.1% coated plates for further in vitro analysis.

**Flow cytometry and cell sorting.** Flow cytometry analysis was performed on isolated cardiac interstitial cells using a Becton-Dickinson FACSCanto II running FACSDiva software with the following configuration: 405 nm laser for Alexa405, 633 nm for APC and 488 nm for eGFP. Analysis was performed using FlowJo vX (Becton-Dickinson BD). To obtain endothelial cell counts, isolated cells were stained with APC-conjugated antibodies against CD31 (eBioscience 17-0311-82) at a 1:200 dilution in 2% BGS in HBSS incubated for 30 min on ice. At the end of the incubation time cells were washed 3 times and analyzed. For sorting and analysis of lineage traced genotypes, we utilized the endogenous eGFP fluorescence expressed by the recombined R26eGFP reporter allele due to the activity of the Tie2-CreERT2 transgene and Postn-MCM allele. The count of eGFP+ cells was normalized to the number of CD31+ cells to control for the degree of variability in sample digestion and cellular isolation.

**Fibroblast cultures and treatments.** Adult heart fibroblasts were cultured in DMEM (Fisher Scientific, #SH30022FS) supplemented with 10% BGS and non-essential amino acids. Depending on the experiment, cultured fibroblasts were infected with a Cre recombinase-expressing adenovirus (AdCre) and compared with a control β-galactosidase (Adβgal) expressing adenovirus. The cells were incubated with these recombinant adenoviruses for 4 hours in serum free media, then washed and maintained in DMEM media for 72 hours.
Collagen synthesis was induced by treatment with 100 μM ascorbic acid (Sigma-Aldrich, #A5960), and/or 10 ng/ml TGFβ (R&D Systems, #101-b1-010) over 24 hours in serum free DMEM media.

**Neonatal cultures for stretching experiments.** Neonatal rat cardiomyocyte and fibroblasts were isolated from 1- to 2-day-old rat pups as described previously (69). Ventricles were placed in an isotonic salt solution composed of 116 mM NaCl, 5.4 mM KCl, 20 mM HEPES, 0.9 μM Na₂HPO₄, 5.4 mM MgSO₄, 5 mM glucose. The ventricles were then enzymatically digested using 84 U/ml collagenase type I (Worthington, # LS005273) and trypsin (Worthington, #LS003736). After isolation, cardiomyocytes were cultured overnight in M199 medium (Mediatech, #10-060-CV) supplemented with 15% FBS, penicillin/streptomycin (100 U/ml) and l-glutamine (2 mM). The following day, the medium was replaced with cardiomyocytes were cultured in 1% serum-containing M199 medium. Cardiomyocytes were seeded at a pre-adherent density of 0.5–1.0 laminin coated flexel 6 well 2.5-cm culture dishes. Neonatal rat cardiac fibroblasts were cultured as described above, and seeded on 0.01% coated gelatin Flexel plates (Flexcell International). Stretch-induced stress was performed after seeding cells on 6-well Flexcell culture plates and applying a 20% stretch to the membranes using the Flexcell vacuum device (28 mm posts, Flexcell International). Stretch oscillations were performed continuously for 24 hours at 1.0 Hz and then harvested for western blotting.

**Secretome profiling of cultured cardiac fibroblasts**
To assess the secreted factor profile of cardiac fibroblasts with or without Hsp47, cells were isolated from Hsp47-loxP mice and infected with adenoviruses expressing either Cre recombinase or β-galactosidase as described earlier. Following 24 hrs of culture in serum-free media, the media was removed, centrifuged at 14,000 g to remove cellular debris, and concentrated over Amicon Ultra-4 Centrifugal Filter Unit columns (EMD Millipore,). Samples were then analyzed using the Proteome Profiler Mouse XL Cytokine Array (R&D Systems # ARY028) per manufacturer’s instructions, except that a streptavidin-conjugated Alexa Fluor IRDye 800 (LI-COR Biosciences) was used for detection with an Odyssey CLx imaging system (LI-COR Biosciences). Quantitation was performed using LI-COR Image Studio v.3.1.4.

**RNA-sequencing and bioinformatics analysis.** RNA from eGFP+ fibroblasts was isolated with the miRNeasy Micro Kit (Qiagen, #217084). Total RNA amplification (NuGEN, 7102-32), library generation (Illumina Technologies, FC-131-1002), and cDNA fragmentation (Amplicon Tagment Mix, FC-131-1096) were described previously (55). The purified cDNA was captured on an Illumina flow cell for cluster generation. Libraries were sequenced on the Illumina HiSeq2500 instrument within the Human Genetics Department at Cincinnati Children’s Hospital. Bioinformatics analysis was carried out using AltAnalyze software (70) to identify differentially expressed genes. Gene cluster analysis of biologic pathway-based expression groupings was done using Gene Ontology Consortium enrichment analysis (71, 72). The RNA sequencing data were
deposited with the GEO database group and given accession number GSE129612. A set of modulated target genes observed in RNAseq results was validated by qRT-PCR.

Quantitative Reverse transcriptase polymerase chain reaction (qRT-PCR). RNA was isolated from FACSSorted cardiac tissue or isolated cells using a Qiashredder homogenization instrument (Qiagen, #79654) and the RNAeasy kit according to the manufacturer's instructions (Qiagen, #217084). Total RNA was reverse transcribed using random oligo-dT primers with a Verso cDNA synthesis kit (Thermofisher, AB1453) according to manufacturer's instructions. Quantitative real-time PCR was performed using Sso Advanced SYBR Green (Biorad, #6090). The \( \Delta\Delta CT \) was used to quantify the fold change of the target genes and Gapdh expression was used for normalization. The primer sets used to identify transcripts are presented in Supplemental Table 2.

EdU staining in vivo and in vitro EdU was purchased from Life Technologies (Cat#, E10187) and prepared as 5 mg/ml stock solution in PBS and i.p. injected into mice at 50 mg/kg 4 hours before sacrifice. After isolation and fixation, heart sections were stained for EdU by using Click-it Plus EDU Alexa Fluor 647 imaging kit according to the manufacturer's protocol (Thermo Fisher Scientific, #C10340). Mouse cardiac fibroblasts were isolated as previously described and treated with either an adenoviral vector encoding β-galactosidase (control) or Cre (to delete Hsp47) in 2% bovine growth serum (BGS) supplemented DMEM. Three days later, cells were incubated for 24 hours with 10 μm 5EdU before fixation in 4% paraformaldehyde for 20 minutes. Samples were washed with PBS and EdU positive cells were detected using the Click-it EdU Alexa Fluor 647 Imaging kit. Cell nuclei were detected by incubation with DAPI for 15 minutes (1:5000, Thermo Fisher Scientific #D3571,). Cells were washed and mounted with Prolong Diamond anti-fade (Thermo Fisher Scientific, #P36965). Six images were randomly taken per group and analyzed in a blinded manner using NIS-elements advanced research software (Nikon, USA).

Treadmill running. Mice were subjected to forced down-hill treadmill running using a ramping speed protocol as previously described (73). Briefly, 4 month-old mice were acclimatized to a motorized treadmill apparatus (Columbus Instrument) for 5 min at 0 m/min followed by 3 min at 6 m/min without electrical stimulation. Subsequently, mice were subjected to a forced down-hill (10 degrees) treadmill running protocol where the speed was increased in increments of 2 m/min every 3 min to a final speed of 18 m/min, during which the mice were subjected to a mild electrical shock (3 Hz) on a pad at the bottom of the treadmill to motivate their continue efforts.

Statistics. Two-way ANOVA with Bonferroni's post-hoc honestly significant difference analysis was used to determine statistical significance when comparisons were made across different Cre lines. One-way ANOVA with Tukey's post-hoc honestly significant difference analysis was used to determine statistical significance
when comparisons were made within a single Cre line. p-values < 0.05 were considered statistically significant. Averaged data are presented with standard error of the mean (S.E.M.) to indicate variability. Survival curves (Kaplan–Meier plots) were compared by log rank test. p values < 0.05 were considered statistically significant. Analyses were performed using Prism 8 software.

Study approval. Mice were observed daily and cages changed every 2 weeks by veterinary technicians at Cincinnati Children’s Hospital Medical Center. Mice were assessed for their well-being by noting adequate physical activity and food intake on a daily basis. Housing conditions and husbandry conformed to AAALAC standards and the institution's ongoing certification by this organization, as well as by the standard guidelines from the Office of Laboratory Animal Welfare (http://grants.nih.gov/grants/olaw/animal_use.htm). All animal experimentation related to this study was approved by the Office of Research Compliance and Regulatory Affairs and by the Cincinnati Children’s Hospital Institutional Animal Care and Use Committee (Protocol Number: IACUC 2016-0069, expires 11-2019). No human subjects were used.

Author contributions
HK, OK, AKJ, BDM, RJV, VP, JGB, MJB, TS, and RNL conducted experimentation. HK designed and conducted experiments, acquired and analyzed data. KN and KK generated the Hsp47-loxP-targeted mice. JDM, and HK, conceived of the study, directed the study and wrote the manuscript.

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References


**Fig 1**

- **A**
  - Adβgal (left) and AdCre (right) images with DAPI staining.
  - Hsp47+ at 100 μm.

- **B**
  - Western blot (WB) of Collagen I showing n.s. for Adβgal and AdCre.

- **C**
  - Diagram showing βMHC promoter with Cre and LoxP sites.

- **D**
  - Western blot for Hsp47 and Gapdh under βMHC-Cre and Hsp47fl/fl-βMHC-Cre conditions.

- **E**
  - βMHC-Cre (left) and Hsp47fl/fl-βMHC-Cre (right) images at 200 μm.

- **F**
  - Bar graph comparing βMHC-Cre and Hsp47fl/fl-βMHC-Cre with kDa labeling (250, 150, 100, 75, 50).

- **G**
  - Graph showing % fibrosis with Sham and TAC conditions.

- **H**
  - Schematic of αMHC promoter with MerCreMer and LoxP sites.

- **I**
  - Timeline showing Tamox, TAC, and Harvest with time points 6w, 8w, and 12w.

- **J**
  - Western blot for Hsp47 and Gapdh under αMHC-MCM and Hsp47fl/fl-αMHC-MCM conditions.

- **K**
  - Graph showing Col I intensity with αMHC-MCM and Hsp47fl/fl-αMHC-MCM conditions.

- **L**
  - Graph showing Col III intensity with αMHC-MCM and Hsp47fl/fl-αMHC-MCM conditions.

- **M**
  - Graph showing Col V intensity with αMHC-MCM and Hsp47fl/fl-αMHC-MCM conditions.
Figure 1: Cardiomyocyte-specific deletion of Hsp47 in the mouse heart does not block maladaptive fibrosis with TAC. (A) Representative immunostaining for Hsp47 (red fluorescence) in cultured cardiac fibroblasts 72 h after infection with Adβgal and AdCre. Nuclei are stained blue with DAPI. (B) Western blot showing levels of secreted collagen type-I in the culture media of heart fibroblasts 72 h after infection with either Adβgal or AdCre. White arrows show collagen isoforms. Molecular weight migration standard and sizes are also shown. Non-specific (n.s.) Ponceau staining (pink) is shown as a processing and loading control. (C) Schematic representation of breeding βMHC-Cre transgenic mice with Hsp47-loxP targeted mice. (D) Western blot analysis for Hsp47 isolated from fractionated cardiomyocytes of the 2 genotypes of mice shown. Gapdh is shown as the loading control. (E, F) Picrosirius red-stained histological heart sections and quantitation of the area of fibrosis (red) in hearts from the indicated genotypes of mice with the βMHC-Cre transgene after 4 weeks of TAC injury. Average fibrotic area ± s.e.m., n=5-8 mice in each group, *p<0.05 versus sham operated βMHC-Cre mice. P values were calculated by one-way ANOVA with Tukey's post-hoc test. Scale bar 200 µm (G) Heart-weight to body-weight (HW/BW) ratio in mice after 4 weeks of TAC. n=5-8 in each group. *p<0.05 versus βMHC-Cre sham mice. (H) Breeding scheme of αMHC-MCM transgenic mice with Hsp47-loxP-targeted mice. (I) Experimental regimen whereby mice were subjected to TAC injury or sham procedure for 4 weeks along with tamoxifen treatment by injection (vertical red arrows). (J) Western blot analysis for Hsp47 isolated from adult heart fractionated cardiomyocytes of the 2 genotypes shown. Gapdh is shown as a loading control. (K, L, M) Quantitation of collagens type I, III, and V respectively from immunohistochemical heart images from wild-type αMHC-MCM mice versus Hsp47 cardiomyocyte-specific mice as shown in Supplemental Figure 3. Ten random histological sections from each mouse heart were imaged and quantified from 5-10 mice each per group. *p<0.05 versus αMHC-MCM Sham. P values were calculated with one-way ANOVA with Tukey's post-hoc test.
Figure 2: Endothelial-specific deletion of Hsp47 in the heart. (A) Schematic of breeding tamoxifen inducible Tie2-CreERT2 transgenic mice with Hsp47-loxP-targeted mice. (B) Experimental regimen for mice subjected to TAC or a sham procedure for 4 weeks. Mice were injected at 6 weeks of age 2 times with tamoxifen and then put on tamoxifen chow prior to 8 weeks of age through harvesting at 12 weeks. (C) Western blot analysis for Hsp47 from endothelial cells isolated by FACS, from the genotypes shown. Gapdh is a loading control. (D-G) Immunohistochemical heart images stained and quantified for collagen type-I, -III, and -V from Tie2-CreERT2 transgenic mice and Hsp47 endothelial-specific-deleted mice. Mice were subjected to TAC as shown in panel "B". Quantitation shows mean intensity of immunoreactivity taken from 10 random histological sections from 5-10 mice in each group. *p<0.05 versus Tie2-CreERT2 Sham. #p<0.05 versus Tie2-CreERT2 TAC. p values were calculated with one-way ANOVA with Tukey's post-hoc test.
**Fig 3**

A. Schematic diagram showing the genetic modification of mice using the MerCreMer system and the induction of Hsp47 expression using Tamoxifen (TAC). The diagram includes a timeline indicating the time points for induction (6w, 8w, 12w) and harvest (12w).

B. Time-course of fibrosis progression following TAC treatment, with fibrosis (%) measured at 6w, 8w, and 12w.

C. Western blot analysis showing the expression of Hsp47 in Postn-MCM and Hsp47fl/fl-Postn-MCM conditions, with α-tubulin as a loading control.

D. Immunofluorescence images showing the expression of Collagen I (Col I) and Collagen III (Col III) in Sham and TAC-treated groups, with DAPI counterstaining.

E. Quantification of Col I intensity in Sham and TAC-treated groups, with statistical significance indicated by *.

F. Quantification of Col III intensity in Sham and TAC-treated groups, with statistical significance indicated by *.

G. Quantification of Collagen V (Col V) intensity in Sham and TAC-treated groups, with statistical significance indicated by *.

H. Quantification of fibrosis (%) in Sham and TAC-treated groups, with statistical significance indicated by *.

I. Western blot analysis of Collagen I expression in Sham and TAC-treated groups, showing a significant increase in collagen I expression in TAC-treated mice compared to Sham controls.
Figure 3. myofibroblast-specific deletion of Hsp47 in the heart reduces myocardial fibrosis after TAC. (A) Schematic representation of Postn-MerCreMer (MCM) targeted mice crossed with Hsp47-loxP targeted mice. (B) Experimental regimen of tamoxifen injections (red vertical arrows) and feed treatment (red horizontal line) in mice subjected to TAC for 4 weeks. (C) Western blot analysis for Hsp47 and α-tubulin from 500,000 eGFP+ cells isolated by FACS from hearts of the 2 genotypes of mice shown (R26eGFP reporter was also present). (D-G) Representative immunohistochemistry of heart tissue sections and quantitation for collagen type-I, -III and -V from hearts of Postn-MCM control mice and Hsp47 myofibroblast-specific deleted mice using the Postn-MCM allele after 4 weeks of TAC. (H) Quantitation from picrosirius red-stained histological sections in hearts from the indicated genotypes of mice after 4 weeks of TAC injury. *p<0.05 versus Postn-MCM Sham. #p<0.05 versus Postn-MCM TAC. n=5-10 mice in each group. p values were calculated using a one-way ANOVA with Tukey's post-hoc test. (I) Western blot analysis for collagen type-I from heats of sham and TAC-operated mice using cardiac extracellular matrix-specific protein preparations from the indicated genotypes of mice. The red arrows show collagen isoforms. Position of molecular weight standards (kDa) are shown on the left.
**A** Skeletal α-actin promoter (Ska) LoxP LoxP Hsp47 Postn MerCreMer OR Hsp47 E1 E2 E3 E4 E5 E6 Sgcd X Neo X

**B** Analyze Tamox. 1m Time 4m

**C** Hsp47

**D** Sgcd−/− Hsp47fl/fl Sgcd−/− Hsp47fl/fl -Ska-MCM Sgcd−/− Postn-MCM Sgcd−/− Hsp47fl/fl -Postn-MCM

**E** Quadriceps Fibrosis (%) E1 E2 X

**F** Diaphragm Fibrosis (%) Time (min)

**G** Treadmill Time (min)

Fig 4
Figure 4. myofibroblast-specific but not myofiber-specific deletion of Hsp47 in skeletal muscle reduces muscular dystrophy-dependent tissue fibrosis. (A) Schematic of the MCM cDNA driven by the human skeletal α-actin promoter (myofiber specific) or the myofibroblast-specific Postn genetic locus to delete the Hsp47 gene with tamoxifen treatment. These lines were crossed into the δ-sarcoglycan null (Sgcd⁻/⁻) background. (B) Experimental tamoxifen dosing regimen administered in the feed. (C) Western blot analysis for total Hsp47 protein using whole muscle protein lysates from the quadriceps of mice of the indicated genotypes. n = 6 mice per group. Gapdh is shown as a loading control. (D) Representative picrosirius red-stained histological sections from quadriceps and diaphragm from 4 month-old mice of the indicated genotypes. (E-F) Quantitation of fibrosis from picrosirius red-stained histological sections from quadriceps and diaphragm of 4 month-old mice of the indicated genotypes. n=7 mice in each group. (G) Average time spent running on a treadmill of 4 month-old mice of the indicated genotypes. n=6-10 in each group. Significance was determined using p values calculated by one-way ANOVA with Tukey’s post-hoc test. *p<0.05 versus Sgcd⁻/⁻-control. #p<0.05 versus Sgcd⁻/⁻ Hsp47fl/fl-Ska-MCM. The legend applies to panels E, F and G.
Figure 5. myofibroblast-specific Hsp47 deletion alters acute scar formation and the hypertrophic response. (A) Experimental scheme whereby αMHC-MerCreMer transgenic mice or Postn-MerCreMer allele-containing mice were subjected to myocardial infarction injury for 4 weeks with 2 injections (vertical red arrows) of tamoxifen treatment and then tamoxifen in the feed for 4 weeks (horizontal red arrow). (B) Kaplan-Meier plot of survival of the indicated genotypes of mice after MI injury. n=11-13 mice in each group. (C) Quantitation of fibrosis from picrosirius red-stained histological sections from hearts after 4 week of I/R injury of the indicated genotypes. n=6 mice in each group. (D) Gravimetric assessed heart weight to body weight (HW/BW) ratios in mice of the indicated genotypes after 4 weeks of TAC. n=5 sham mice, n=9-10 TAC mice in each group. *P<0.05 versus Sham; #p<0.05 versus Postn-MCM TAC. p values were calculated by two-way ANOVA and Bonferroni post-hoc test. (E, F, G) Echocardiographic assessment of ventricular (LV) calculated mass, left ventricular fractional shortening (FS%) percentage, and early mitral inflow velocity to mitral annular early diastolic velocity ratio (E/e’) in the indicated genotypes of mice after 4 weeks of TAC injury or a sham procedure. *p<0.05 versus Postn-MCM sham. *p<0.05 versus Postn-MCM TAC. p values were calculated with one-way ANOVA with Tukey's post-hoc test. Number of mice used (10-12) as shown by the dot plots.
Fig 6

A

TAC → EdU → Harvest

8w 9w

Time

B

R26eGFP; Postn-MCM

SSC-A

R26eGFP; Hsp47fl/fl-Postn-MCM

C

R26eGFP; Postn-MCM

R26eGFP; Hsp47fl/fl-Postn-MCM

E

GFP

GFP+EdU

GFP+EdU+DAPI

R26eGFP; Postn-MCM

R26eGFP; Hsp47fl/fl-Postn-MCM

100µm

100µm

D

F

EdU+/eGFP+(%) R26eGFP; Postn-MCM R26eGFP; Hsp47fl/fl-Postn-MCM

G

EdU+ cells (%) Adβgal AdCre

Hsp47fl/fl fibroblasts
Figure 6. myofibroblast-specific Hsp47 deletion reduces myofibroblasts in vivo and their proliferation. 

(A) Experimental scheme whereby mice were subjected to TAC injury for 7 days. Mice received 2 i.p injections of tamoxifen and were fed tamoxifen-laden chow 48 hrs before surgery, and then maintained on this chow until harvesting. Mice also received a single i.p EdU injection 4 hours before sacrifice at day 7 post TAC. (B) Representative flow cytometry plots of isolated eGFP+ interstitial cells (plotted as eGFP fluorescence signal on the x-axis versus side-scatter on the y-axis) from hearts of the indicated genotypes of mice, 100,000 cells are displayed in the blots. (C) The ratio of total eGFP+ myofibroblasts normalized to CD31+ cells from the hearts of the indicated genotypes of mice after 1 week of TAC. Error bars represent s.e.m. n=4 mice in each group. *p<0.05 versus Postn-MCM; R26eGFP. p values were calculated with a Student's t-test. (D) Relative number of CD31+ cells in the interstitial fractions in hearts of the indicated genotypes of mice after 1 week of TAC. (E) Representative immunohistological images of EdU+ and eGFP+ interstitial cells at the time of harvest for mice treated as shown in panel A. DAPI was used to show nuclei (blue). n=5 mice in each group. *p<0.05 versus Postn-MCM R26eGFP. (F) Quantitation of GFP positive cells that were also EdU positive in heart histological sections from mice subjected to TAC of the indicated genotype. p values were calculated with Student's t-test. (G) Quantitation of EdU-positive Hsp47flo/flo cardiac fibroblasts over 24 hrs in culture previously treated with AdCre or Adβgal infection. A total of 6 images were analyzed per group. *p<0.05, p values were calculated with Student's t-test. Data shown are the mean ±sem.
Figure 7. Hsp47 deletion in myofibroblasts reduces ECM-related gene expression and promotes an altered differentiated state. (A) Adult primary heart fibroblasts were isolated from Hsp47-loxP targeted mice infected with Adβgal (WT) or AdCre (deleted samples). Seventy-two hours after infection cells were washed and incubated in 2% serum containing DMEM media with 20 µM of ascorbic acid for 24 hours before RNA isolation. The data are real time PCR results showing the expression levels of the indicated genes. n=4 separate experiments. *p<0.05 versus Adβgal WT. (B) Schematic representation of the Postn-MCM mouse line crossed with the Hsp47-loxP site-containing gene-targeted line and the Rosa26 reporter line (R26<sup>eGFP</sup>). (C) Experimental scheme with TAC stimulation and tamoxifen with injection and laden-food. (D-E) Quantification of selected mRNAs in Hsp47-deleted eGFP<sup>+</sup> myofibroblasts isolated from hearts of Hsp47<sup>fl/fl</sup>Postn<sup>-MCM</sup>/<sup>+</sup>R26<sup>eGFP</sup>/<sup>+</sup> allele containing mice, 4 weeks after TAC injury. n=3, *P<0.5, p values were calculated with Student's t-test. Data shown are the mean ± sem.