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14. ABSTRACT

Prevailing dogma suggests that fibroblasts exert detrimental actions on the pressure-overloaded heart, by secreting large amounts of extracellular matrix proteins that reduce myocardial compliance and promote diastolic dysfunction. Our exciting preliminary data challenge this concept, demonstrating that activated fibroblasts may protect cardiomyocytes in the pressure-overloaded heart. Fibroblast-specific loss of Smad3 accelerates systolic dysfunction following pressure overload, increasing cardiomyocyte apoptosis and leading to replacement fibrosis. We hypothesize that in the hostile environment of the pressure-overloaded heart, TGF-β-activated fibroblasts serve a protective role, by directly inducing a pro-survival program in cardiomyocytes exposed to mechanical stress, by secreting cardioprotective mediators, or by depositing specialized extracellular matrix proteins that prevent cardiomyocyte anoikis. This novel hypothesis will be tested using in vivo approaches and in vitro co-culture experiments.

15. SUBJECT TERMS
fibroblast, cardiomyocyte, apoptosis, TGF-beta, extracellular matrix, matrix metalloproteinases, mechanical stress, fibrosis, contractile dysfunction.

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Second Annual Report from 09/15/2017 to 09/14/2018

1. Introduction

Fibrosis is prominent in hypertensive heart disease and has been implicated in the pathogenesis of heart failure. Fibroblasts, the most abundant cardiac interstitial cells, become activated in response to pressure overload, differentiating into myofibroblasts, and secreting large amounts of extracellular matrix proteins into the interstitial space. Traditional concepts suggest that, as the main cellular effectors of fibrosis, cardiac fibroblasts exert detrimental actions, increasing myocardial stiffness and promoting both diastolic and systolic dysfunction. This notion is based primarily on associative evidence. Based on exciting new data, we propose a new concept on the role of the fibroblasts in cardiac remodeling. We suggest that, in the hostile environment of the pressure overloaded heart, activated fibroblasts play a crucial role in protecting cardiomyocytes from death. This collaborative proposal will identify key pathways responsible for fibroblast-mediated cardiomyocyte protection.

2. Keywords

Cardiomyocyte, fibroblast, cytokine, TGF-beta, cell death, apoptosis, extracellular matrix, matrix metalloproteinases, collagen.

3. Accomplishments

Major Scientific Goals of the Project

The project has the following three major goals:

a) Investigate the role of fibroblast-specific activation of Smad-dependent signaling in fibrosis, remodeling and dysfunction of the pressure overloaded myocardium (Dr Frangogiannis laboratory)**.**

b) Examine the in vivo mechanisms responsible for the protective effects of fibroblasts in the pressure-overloaded heart (Dr Frangogiannis and Dr Kitsis laboratories);

c) Identify novel fibroblast-derived mediators and downstream cardiomyocyte-specific intracellular pathways through unbiased screening (Dr Kitsis and Dr Frangogiannis laboratories).

Scientific Accomplishments of the Project

Work performed in the Frangogiannis laboratory:

A. Role of fibroblast-specific Smad3 signaling in remodeling of the pressure-overloaded heart.

A large part of the findings on the phenotype of fibroblast-specific Smad3 KO mice following pressure overload was presented in the first progress report (October 2017). During the second year, this work was submitted for publication. We are currently working on the revisions. The revised version of the manuscript will be submitted within the next few weeks.

We have also published a high-impact manuscript on the role of fibroblast Smad3 signaling in repair and remodeling of the infarcted heart (Kong et al Circulation 2018). Our study demonstrated that fibroblast Smad3 signaling is critical for repair of the infarcted heart, protecting the myocardium from cardiac rupture and adverse remodeling.

The effects of Smad3 on fibroblasts are crucial for formation of an organize scar (Figure 1), and involve activation of an integrin-dependent reactive oxygen species pathway.

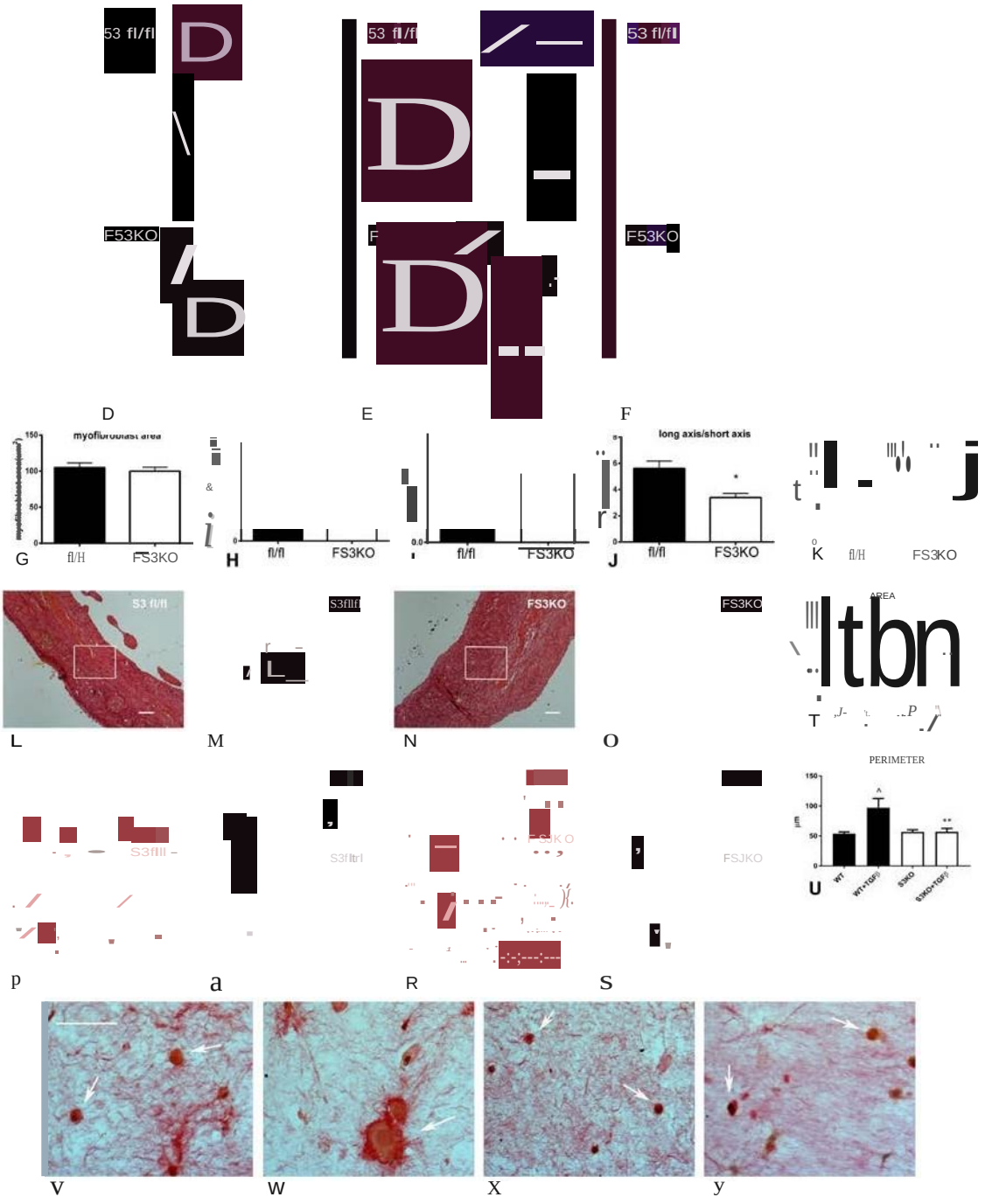

Figure 1: Fibroblast-specific Smad3 loss perturbs scar organization and attenuates TGF-13 mediated changes in cell geometry. A-C. a.-SMA staining of healing myocardial infarcts (7 days) shows that, in Smad3fi1l mice, a.-SMA+ myofibroblasts are spindle-shaped cells, localized in highly organized arrays (arrows, B-C). D-F. In contrast, distribution of a.-SMA+ myofibroblasts in FS3KO mouse infarcts is disorganized and chaotic, as the cells

have a rounded morphology and are malaligned (arrows, E-F). Scalebar=30μm. Although mean area of the myofibroblasts was comparable between groups (G), cell perimeter (H), perimeter:area ratio (I) and the long axis:short axis ratio (J) were lower in FS3KO animals (*p<0.05, n=5), reflecting the altered geometry of the cells. K. In order to quantitatively assess cell alignment in the healing infarct, we measured the angle between the long axis of the cell and the tangent of the ventricular wall (indicated by an arrow in panels A and D). The alignment angle was markedly higher in FS3KO mice, reflecting cellular malalignment in the absence of Smad3 (**p<0.01 vs fl/fl, n=5). Perturbed cellular alignment would be expected to reduce the tractional forces exerted by the cells, resulting in impaired scar contraction. L-S: Both light microscopy (L, N, P, R) and polarized light microscopy (M, O, Q, S) in sirius red-stained sections showed that while Smad3 fl/fl animals had aligned collagen fibers (P-Q, arrows), FS3KO animals exhibited areas of matrix disorganization (R-S, arrows). Scalebar=40μm. T-Y. Smad3 loss abrogated the effects of TGF-β1 on fibroblast shape. Fibroblasts from WT (V-W) and Smad3 KO mice (S3KO, X-Y) were cultured in collagen pads in the presence (W, Y) or absence (V, X) of TGF-β1. Sections of fibroblast-populated collagen pads were stained with sirius red/hematoxylin to identify fibroblasts (arrows) and the extracellular matrix. Scalebar=25μm. TGF-β1 stimulation increased cell area and perimeter in WT, but not in S3KO fibroblasts (T-U). (^p<0.05 vs. WT, **p<0.01 vs. corresponding WT, n=6-7).

In, contrast, cardiomyocyte-specific Smad3 signaling has detrimental actions, promoting adverse remodeling. A pdf file containing the full manuscript is provided in the appendix. The schematic cartoon shown below (Figure 2) summarizes the major observations of the study

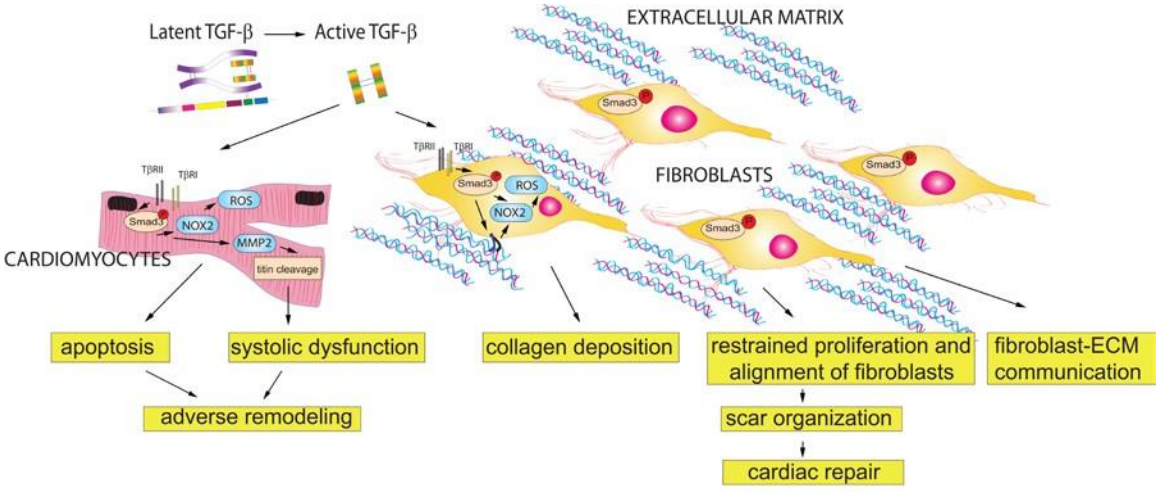

Figure 2: Cell-specific actions of Smad3 regulate post-infarction remodeling. Generation of active TGF-β in the infarcted myocardium triggers Smad3 activation in

fibroblasts and cardiomyocytes. Our study investigates for the first time the role of fibroblast and cardiomyocyte-specific Smad3 signaling in the infarcted myocardium. Smad3 activation in cardiac fibroblasts restrains cell proliferation and controls alignment of fibroblasts in the infarct and formation of an organized collagen-based scar, preventing late cardiac rupture and adverse dilative remodeling. Our findings suggest that Smad3-dependent activation of a novel integrin (ITG)-NOX2 axis may stimulate extracellular matrix (ECM) protein deposition and organization in the infarcted heart. In contrast, activation of Smad3 signaling in cardiomyocytes has deleterious effects, promoting cardiomyocyte apoptosis and enhancing adverse remodeling and dysfunction. The effects of Smad3 on cardiomyocytes may also involve activation of a NOX2-mediated ROS-dependent axis. However, in cardiomyocytes, oxidative stress may promote cell death and accentuate MMP2 expression. Overactive MMP2 may cause adverse remodeling through its effects on the ECM, and may exacerbate systolic dysfunction by targeting proteins involved in sarcomere function, such as titin.

B. Role of fibroblast-specific Smad2 signaling in remodeling of the pressure-overloaded heart.

During the 2nd year of funding we have generated and extensively characterized mice with fibroblast-specific loss of Smad2. At baseline these animals exhibit no abnormalities. These animals were subjected to myocardial infarction protocols. Our findings suggest that, in contrast to Smad3, fibroblast-specific Smad2 does not play an important role in repair of the infarcted heart:

B1. All 3 TGF-b isoforms activate Smad2 in mouse cardiac fibroblasts

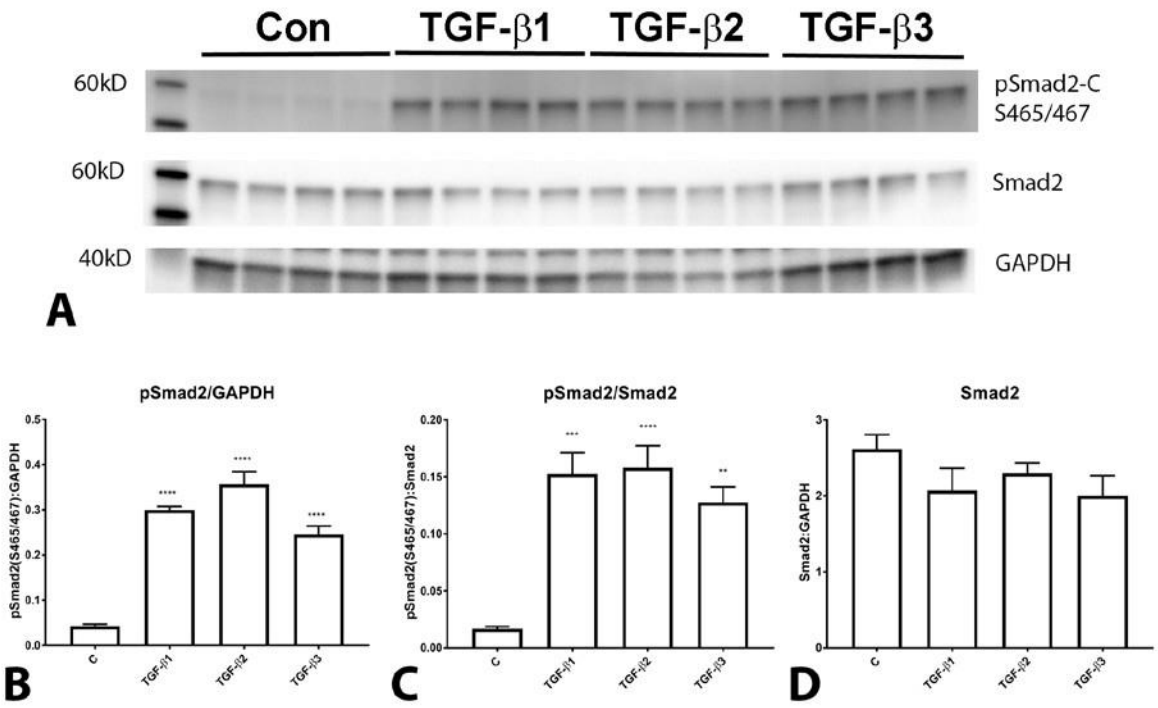

Figure 3: A. TGF-b1, TGFb2 and TGFb3 (10 ng/ml) stimulation rapidly activate Smad2 signaling in cardiac fibroblasts after 30 min of stimulation. Expression of phosphorylated Smad2 (pSmad2) (B) and the p-Smad2:Smad2 ratio (C) were markedly increased in stimulated cells. In contrast, total Smad2 levels (D) were not affected.

2. Smad2 activation in infarct fibroblasts.

Dual immunofluorescence for periostin (a matricellular protein induced in activated myofibroblasts and p-Smad2 demonstrates activation of Smad2 signalingin infarct myofibroblasts, 7 days after infarction.

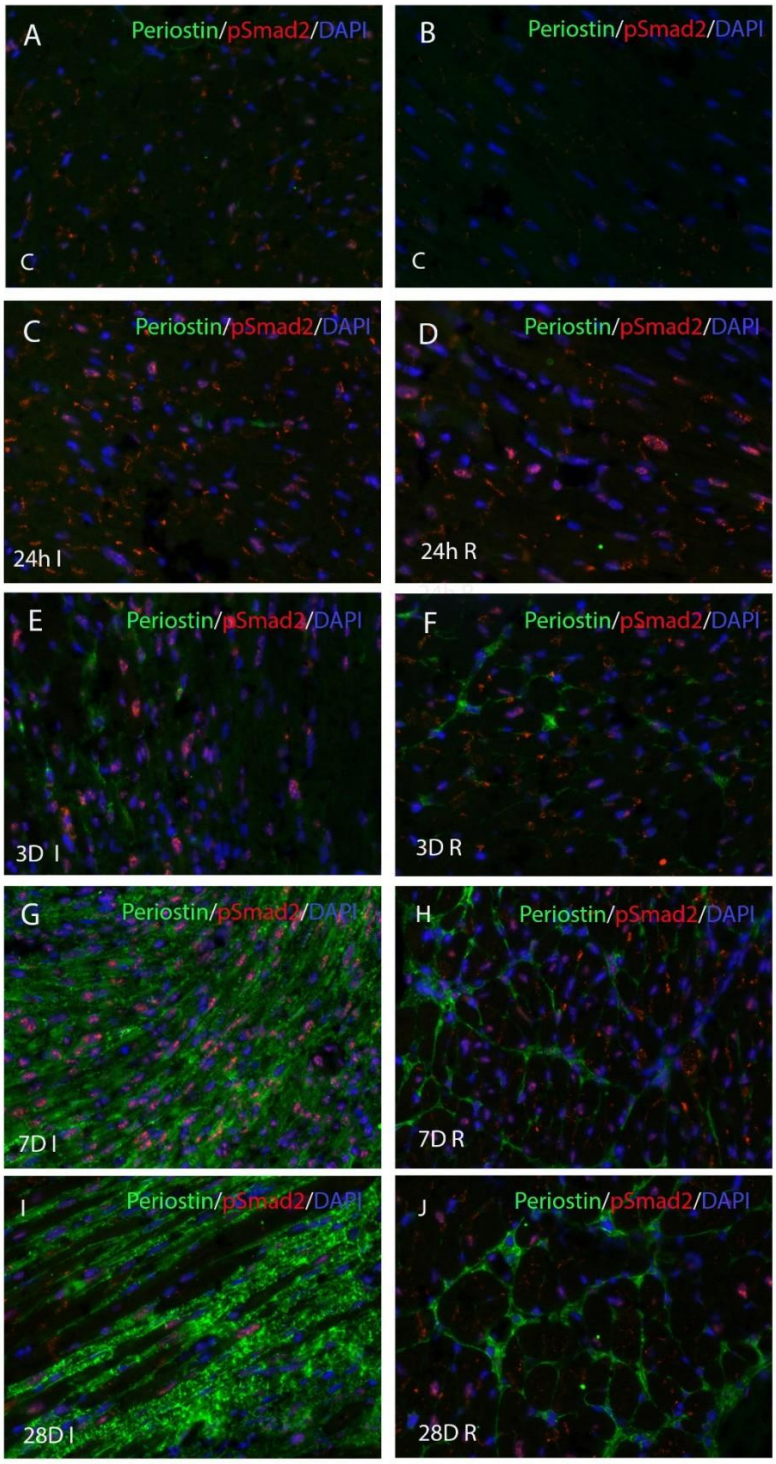

Figure 4: Dual immunofluorescence for periostin and p-Smad2 demonstrated nuclear localization of p-Smad2 in periostin-expressing cells, peaking 7 days after infarction.

Fibroblast-specific Smad2 loss did not affect mortality following myocardial infarction.

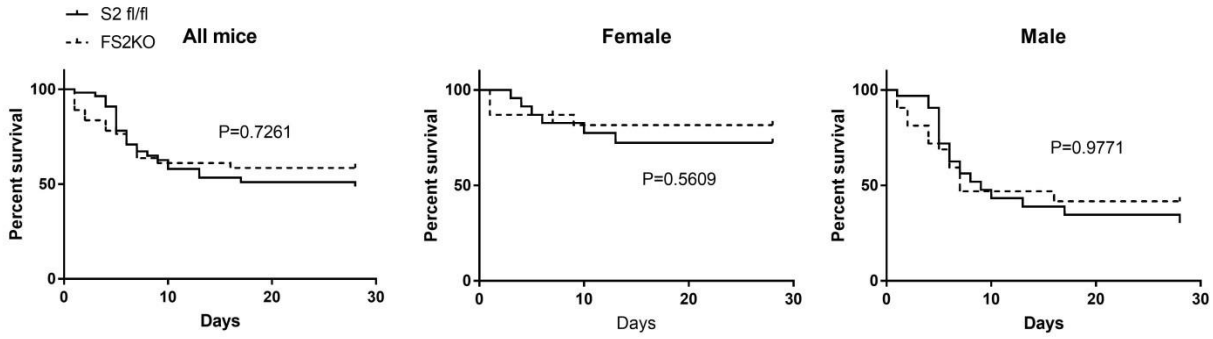

Figure 5: Survival curves show that fibroblast-specific Smad2 loss does not affect mortality following myocardial infarction.

Transient protection of fibroblast-specific Smad2 KO cells from post-infarction systolic dysfunction.

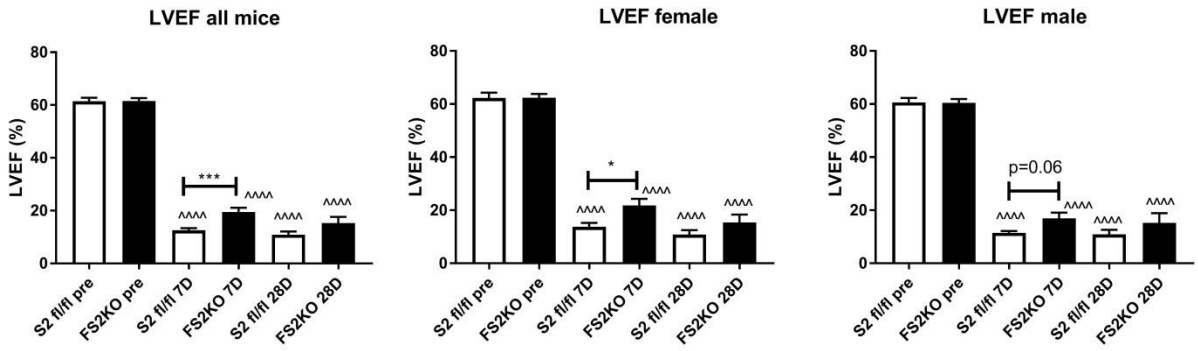

Figure 6: Fibroblast-specific Smad2 KO mice exhibit a transient improvement from left ventricular dysfunction following myocardial infarction, showing a significantly higher ejection fraction than corresponding Smad2 fl/fl controls at the 7-day timepoint. In contrast, systolic function is comparable between groups at the 28 day timepoint.

Work performed in the Kitsis laboratory

In contrast to the traditional concept in which the major action of cardiac fibroblasts in the pressure overloaded heart is to secrete extracellular matrix proteins that contribute to diastolic dysfunction, the central hypothesis of this project is that cardiac fibroblasts in this pathological context also secrete factors that protect cardiomyocytes against dysfunction and death. This hypothesis is supported by our preliminary data suggesting that a TGF-β- and SMAD2/3-dependent program operates within cardiac fibroblasts to inhibit the deaths of cardiomyocytes – and the cardiac fibroblast themselves – and, thereby, preserve systolic function. In last year's progress report, we described improved systems that we had established – including adult murine cardiomyocytes and human iPS cell-derived cardiomyocytes – to assess the effects of various components of the cardiac fibroblast

secretome on these cardiomyocytes functions, and these studies are in progress. In addition, we described the beginnings of some very exciting findings concerning the role of chaperone-mediated autophagy (CMA) in the cardiomyocytes and cardiac fibroblast compartments, the relevance of CMA being that it is activated by pressure overload and TGF-β and, as will be shown below, dramatically impacts cardiac function.

Since the studies described here are the first to define the role of CMA in the heart, we will first provide some background information. Autophagy, a cellular process in which macromolecules and organelles are trafficked to lysosomes for degradation, is activated by a wide variety of cellular stresses and serves to transiently attenuate the impact of these stresses on cellular dysfunction until homeostasis can be re-established. Several autophagy programs have been described. The most studied is macroautophagy, in which proteins, lipids, and organelles are transported to lysosomes in double-membrane vesicles called autophagosomes. In contrast, CMA is a distinct process that differs from macroautophagy in several key respects. First, the substrates of CMA are limited to proteins only – and specifically to proteins that reside in the cytosol. Second, these translocate to lysosomes during CMA in complex with Hsc70 and co-chaperones - rather than in autophagosomes. Third, in contrast to macroautophagy, CMA is a selective process with substrates bearing a specific recognition motif (KFERQ and variants thereof). Fourth, uptake of CMA substrates at the lysosome is mediated by Lysosomal Associated Membrane Protein 2A (LAMP2A), which is necessary, specific, and rate-limiting for CMA.

Data

1. Pressure overload induces CMA in the heart *in vivo*

   To test whether pressure overload activates CMA in the heart, we subjected mice to transverse aortic constriction (TAC), which results in a ~70 mm Hg pressure gradient in our hands. We observed induction of CMA as assessed by increases in LAMP2A protein (Fig 1A, B) and by lysosomal localization of a fluorescent reporter protein (dendra) fused to a CMA recognition motif that has been transgenically expressed in all cells of the mouse (Fig 1C). Note that time course of induction of CMA is much slower than that of macroautophagy, which occurs within hours – suggesting that CMA and macroautophagy serve different functions in the heart. Much of the induction of CMA in these micrographs appears to be occurring in cardiomyocytes. But, some is occurring in cardiac fibroblasts as evidenced by puncta remaining with Lamp2A is knocked out only in cardiomyocytes (Fig 1C; also knockouts discussed in Fig. 2). Since cardiac fibroblasts are smaller and more difficult to recognize cells, we are now carrying out analogous studies using mice in which the fibroblasts have been genetically marked with a second fluorophore.

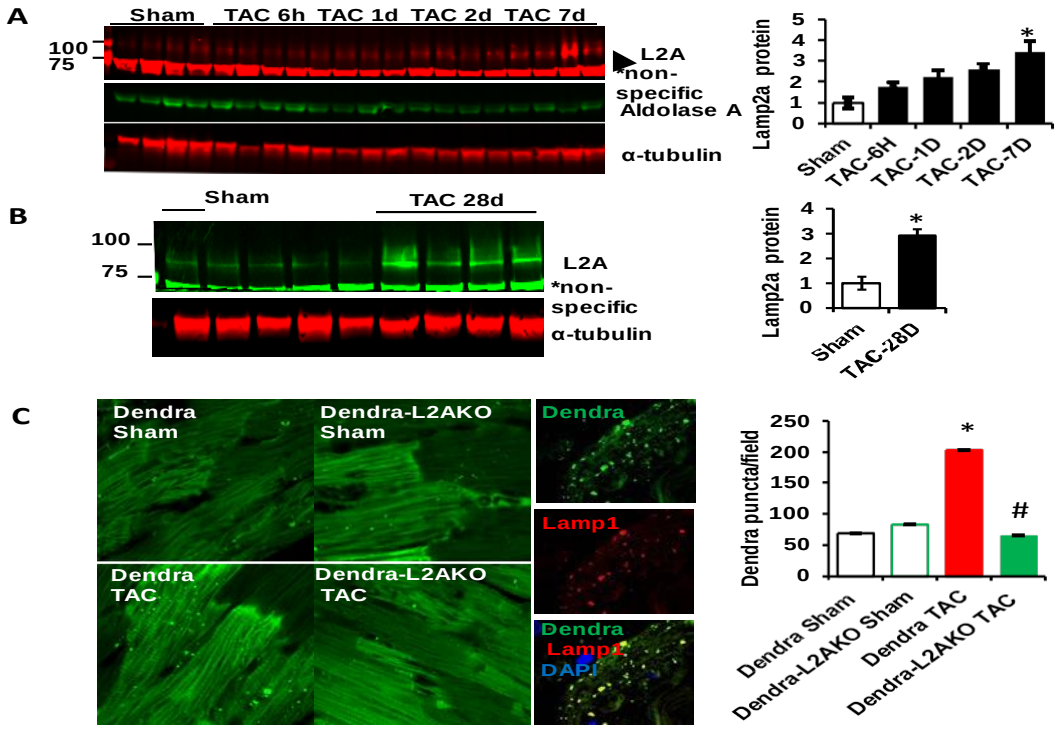

Fig 1. TAC-induced activation of CMA in the heart. A and B. Immunoblotting and quantification showing time-dependent increases in levels of LAMP2A and parallel decreases in those of aldolase A, an endogenous CMA substrate. C. Repressive images and quantification of a fluorescent CMA reporter. Note that in mice with cardiomyocyte-specific deletion of *Lamp2a*, some fluorescent puncta remain in non-myocytes. Note also co-localization of fluorescent puncta with LAMP1, an endogenous lyososomal protein Error bars indicate SD. *, # $P < 0.05$.

2. <u>Inactivation of CMA in cardiomyocytes or cardiac fibroblasts by cell type-specific deletion of *Lamp2a*.</u>

We wish to understand the role of CMA in both cardiomyocytes and fibroblasts. Since LAMP2A is necessary, specific, and rate-limiting for CMA, we can disable CMA by deleting *Lamp2a* separately in each of those compartments. To accomplish this in cardiomyocytes, we used a tamoxifen-activatable Cre recombinase whose expression was driven transgenically using Myh6 (α-myosin heavy chain) promoter and 5' untranslated sequences (active in most cardiomyocytes and highly cardiomyocyte-specific) (Fig 2A, left). In contrast, we deleted *Lamp2a* in cardiac fibroblasts using a tamoxifen-activatable Cre that had been knocked into the endogenous Tcf21 locus (active in ~80% of cardiac fibroblasts but also in other fibroblastic populations) (Fig 2C). Cardiomyocyte-specific deletion reduced LAMP2A levels ~60% as assessed in whole heart tissue (Fig 1A, right). But, this is explained by the much higher LAMP2A levels in the cardiac fibroblast - versus cardiomyocyte – compartment, as evidenced by

analysis of LAMP2A levels in isolated cardiomyocytes in which expression was decreased >95% (data not shown). Deletion of *Lamp2a* did not result in any changes in the expression of *Lamp2b*, *Lamp2c*, or *Lamp1* (Fig 2B). Analysis of LAMP2A protein levels resulting from deletion of *Lamp2a* in cardiac fibroblasts is in progress, but we know that deletion was successful as evidenced by Cre-mediated excision of a loxP-stop-loxP cassette preceding a reporter gene encoding the tdTomato fluorescent protein that had been knocked into the *Rosa26* locus of the same mice (Fig 2D). The red signal marks cardiac fibroblasts in which *Lamp2a* was deleted.

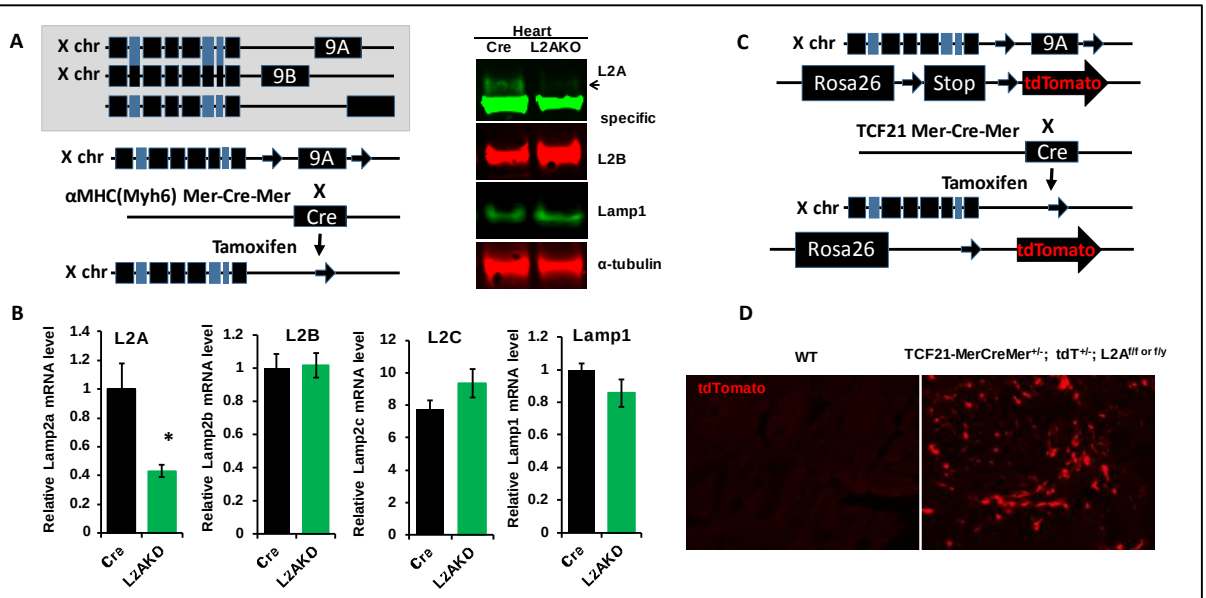

Fig 2. Generation and analysis of cardiomyocytes-specific and cardiac fibroblast-specific *Lamp2a* knockout mice. A. Left: Genetic schema for cardiomyocyte-specific knockout. *Lamp2* is on the X chromosome and exon 9A (specific for LAMP2A) was targeted. Right: Immunoblot shows reduction of LAMP2A in whole heart tissue. The residual LAMP2A remaining is in non-myocytes, as demonstrated by immunoblotting of lysates from isolated cardiomyocytes in which 95% of the LAMP2A was eliminated (not shown). B. Quantitative RT-PCR analysis of transcript levels of *Lamp 2a*, *Lamp2b*, *Lamp2c*, and *Lamp1* (a separate gene) in mice with cardiomyocyte-specific deletion of *Lamp2a* and controls. C. Genetic schema for fibroblast-specific *Lamp2a* knockout. Cre-mediated recombination also activates a *Rosa-26*-tdTomato reporter in the same cells. D. Representative cardiac sections from *TCF21*^{MerCreMer}; *Rosa26*-tdTomato; *Lamp2a*$^{f/f\ or\ f/y}$ and wild-type (WT) mice treated with tamoxifen and showing red stain of cardiac fibroblasts resulting from tdTomato expression in fibroblasts in which Cre-mediated excision took place.

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3. <u>Cardiomyocyte-specific deletion of LAMP2A paradoxically attenuates – rather than worsens – pressure overload-induced systolic dysfunction</u>

Having inactivated CMA selectively in cardiomyocytes and fibroblasts, we next set to determine the phenotype. We report here the phenotype of the cardiomyocyte-specific *Lamp2a* knockouts. Analysis of the fibroblast-specific knockouts is in progress. While cardiomyocyte-specific deletion of *Lamp2a* resulted in no detectable effects on cardiac structure, histology, or function by echocardiography

(not shown), we discovered a striking phenotype when mice were subjected TAC. We would have predicted that loss of CMA in cardiomyocytes would have exacerbated TAC-induced cardiac dysfunction. In fact, we observed the opposite. Loss of CMA in cardiomyocytes *attenuated* TAC-induced mortality and systolic dysfunction (Fig 3). We have now repeated this experiment in 50-80 mice in each group.

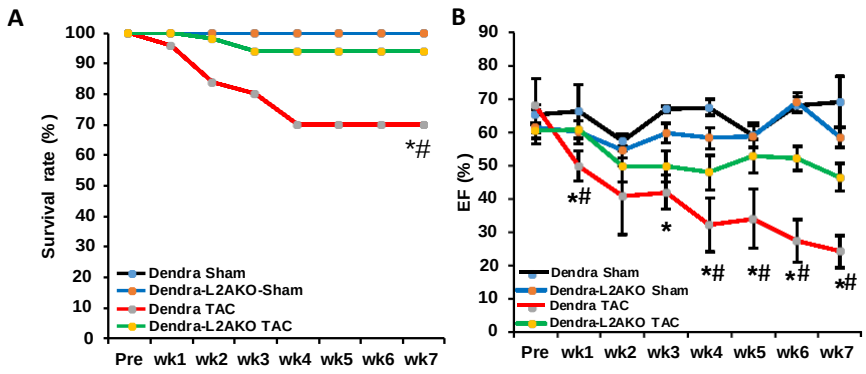

Fig 3. Cardiomyocyte-specific deletion of *Lamp2a* paradoxically protects hearts against TAC. A. Survival rates. B. Cardiac systolic function by echocardiography. Dendra refers to mice containing the CMA reporter in which *Lamp2a* is wild type. Dendra-L2AKO refers to mice containing the CMA reporter in which *Lamp2a* has been deleted in cardiomyocytes. In panel A, * $P \leq 0.05$ Dendra TAC versus Dendra Sham, # $P \leq 0.05$ Dendra TAC versus Dendra-L2AKO TAC. In panel B, * $P \leq 0.05$ Dendra TAC versus Dendra Sham, # $P \leq 0.05$ Dendra TAC versus Dendra-L2AKO TAC.

4. <u>Mitochondria derived from cardiomyocytes disabled for CMA exhibit increased mitochondrial respiration and ATP production</u>

We next wished to understand he mechanism for the resistance to TAC-induced cardiac dysfunction resulting from cardiomyocytes-specific inactivation of CMA. After pursuing multiple possibilities, we reasoned that cellular energetics is critical for cardiomyocyte function, and examined the function of cardiac mitochondria derived from cardiomyocytes-specific *Lamp2a* knockout mice. Most of these mitochondria come from cardiomyocytes because these cells are larger than fibroblasts and contain a higher concentration of these organelles. Using an oxygen electrode, we observed significant increases in rates of oxygen consumption, ATP production, and ATP content in cardiac mitochondria isolated from cardiomyocytes-specific *Lamp2A* knockout mice at both baseline and following TAC (Fig 4A, B).

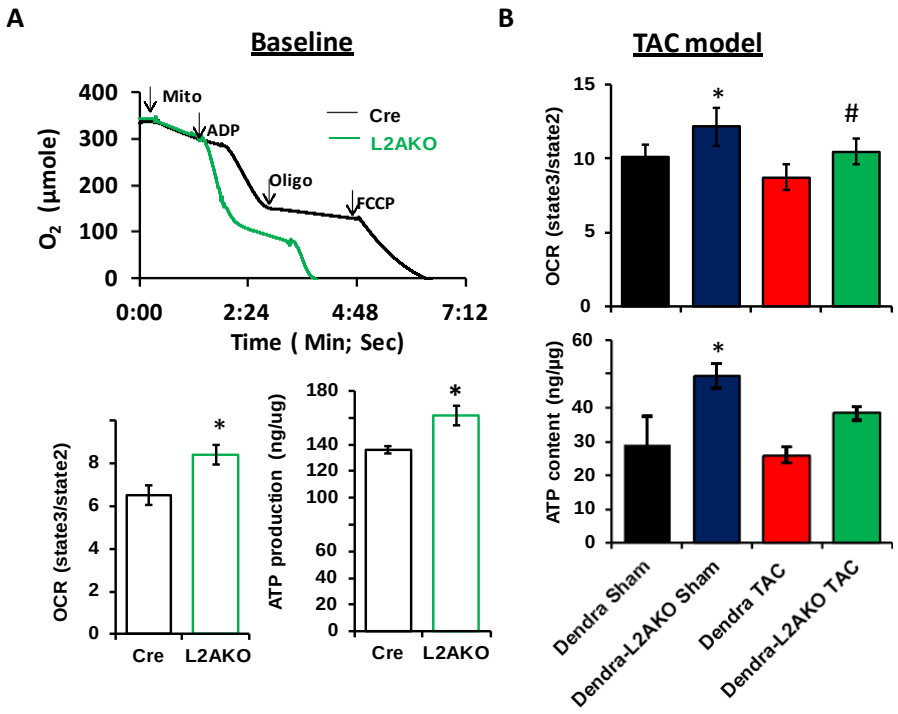

Fig 4. Cardiac mitochondrial function studies at baseline (A) and under TAC conditions (B). A. Top: Representative oxygen consumption curves in cardiac mitochondria from cardiomyocyte-specific Lamp2a knockout mice and controls. Bottom left: State 3 ADP-stimulated/state 2 ADP-limited) rates of oxygen consumption. Bottom, right: ATP production. B. Analogous studies comparing sham-operated and TAC mice. Dendra refers to mice containing the CMA reporter in which *Lamp2a* is wild type. Dendra-L2AKO refers to mice containing the CMA reporter in which *Lamp2a* has been deleted in cardiomyocytes. * P ≤0.05 L2AKO versus control under basal conditions. # P ≤ 0.05 Dendra TAC versus Dendra-L2AKO TAC (N=40-50).

5. Mitochondrial proteomic analysis reveals increases in ATP5L and ATP5J, components of the mitochondrial F0-F1 ATP synthase, resulting from inhibition of CMA in cardiomyocytes.

   To determine the molecular basis for the augmented oxygen consumption and ATP synthesis in cardiac mitochondria obtained from the cardiomyocyte-specific

Lamp2a knockout mice, we performed quantitative mitochondrial proteomics. This was done in the basal state and also under starvation conditions to activate CMA. As it turns out, major changes were observed even under basal conditions. Among multiple increases and decreases in proteins that reside at mitochondria were relatively modest increases (~1.5-fold) in the abundances of two proteins in the mitochondrial F0-F1 ATP synthase: ATP5L and ATP5J (Fig 5A). These increases were confirmed by quantitative immunoblotting (Fig 5B, top).

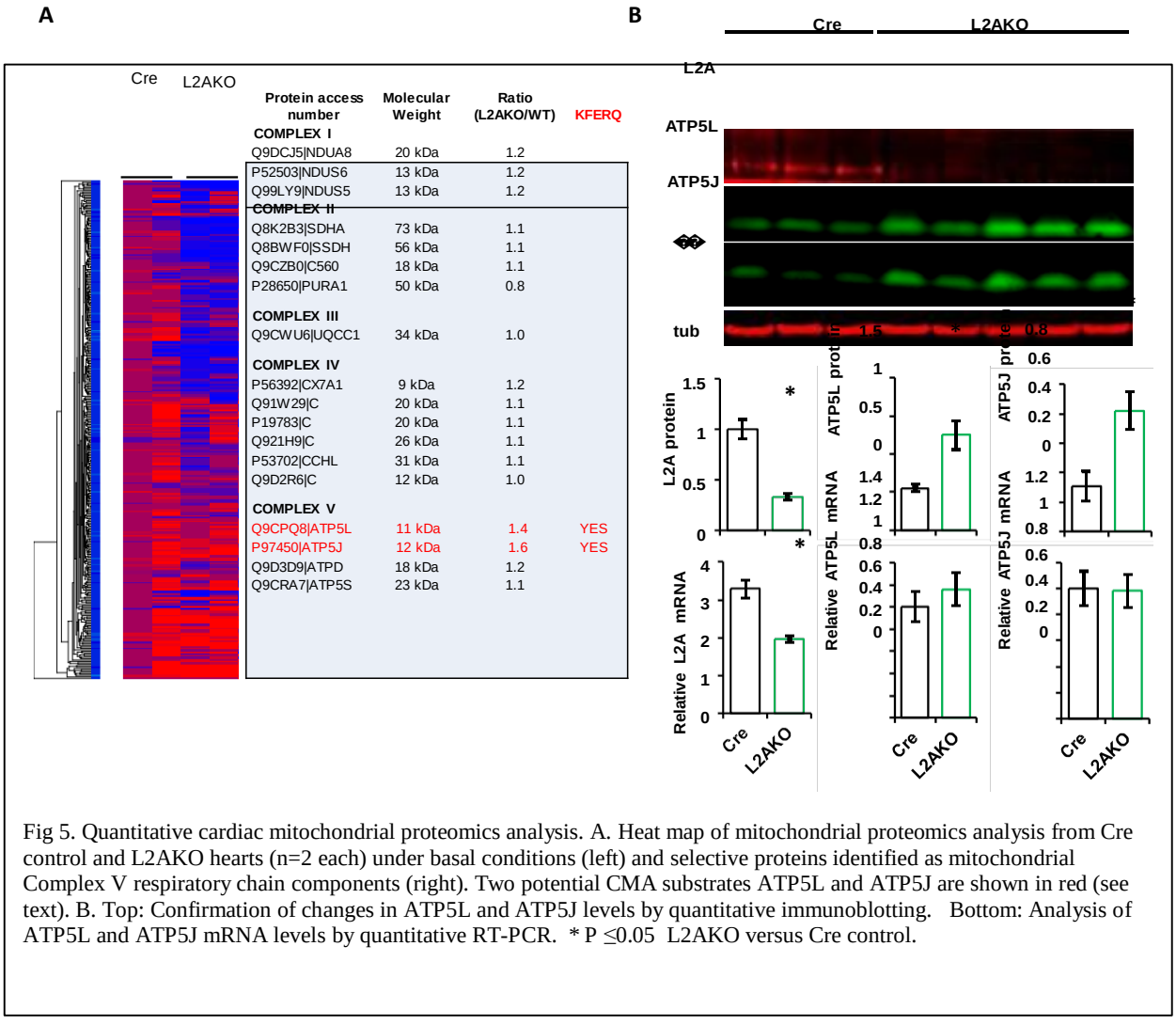

Fig 5. Quantitative cardiac mitochondrial proteomics analysis. A. Heat map of mitochondrial proteomics analysis from Cre control and L2AKO hearts (n=2 each) under basal conditions (left) and selective proteins identified as mitochondrial Complex V respiratory chain components (right). Two potential CMA substrates ATP5L and ATP5J are shown in red (see text). B. Top: Confirmation of changes in ATP5L and ATP5J levels by quantitative immunoblotting. Bottom: Analysis of ATP5L and ATP5J mRNA levels by quantitative RT-PCR. * P ≤0.05 L2AKO versus Cre control.

6. <u>ATP5L and ATP5J are CMA substrates</u>.

Both ATP5L and ATP5J contain potential CMA recognition motifs (Fig 5A). Moreover, both proteins are nuclear encoded – meaning that they have to pass through the cytoplasm on their way to the mitochondria. These two

characteristics mean that they may be CMA substrates. In further support of the notion that increases in their abundances is attributable to inactivation of CMA, levels of the mRNAs encoding each of these proteins were unchanged in hearts with cardiomyocytes-specific deletion of *Lamp2a* (Fig B, bottom). To formally test whether ATP5L and ATP5J are CMA substrates, we tested whetherATP5L and ATP5J interact with Hsc70 in a manner dependent on their CMA recognition motifs. This, in fact, proved to be the case (Fig 6A-C). Next, we tested whether ATP5L and ATP5J localize at lysosomes under conditions in which CMA is activated and only when LAMP2A is present. This also was demonstrated (Fig 6D). Therefore, ATP5L and ATP5J meet criteria as direct CMA substrates and increases in their abundances when CMA is in activated reflect this rather than an indirect effect of CMA (e.g. at the transcriptional level).

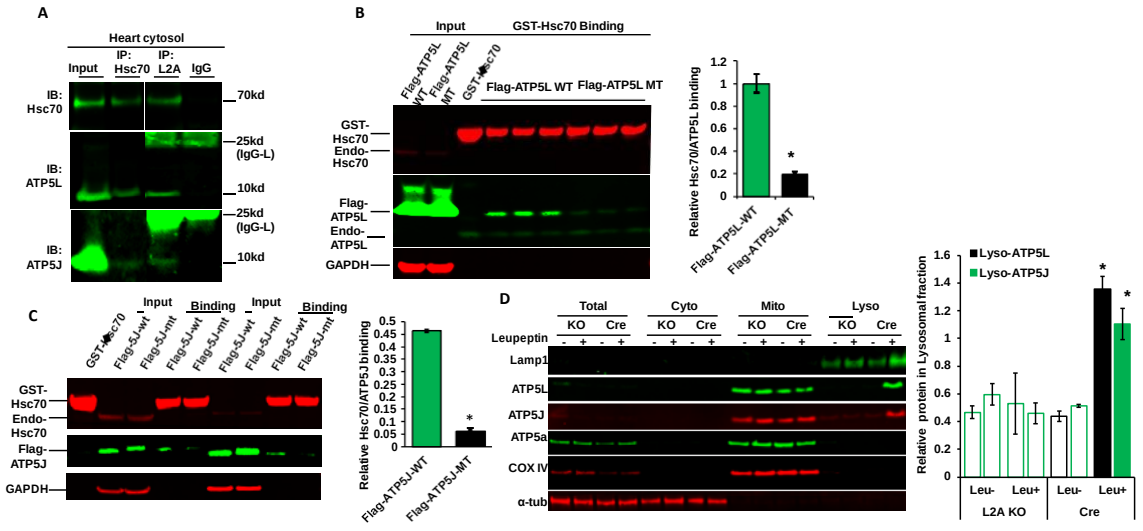

Fig 6. ATP5L and ATP5J are CMA substrates. A. Co-immunoprecipitations of heart cytosol showing that ATP5L and ATP5J interact with Hsc70. B, C. GST pulldowns showing that ATP5L (B) and ATP5J (C) interact with Hsc70 in a manner dependent on their CMA targeting motifs. Mut indicates mutation of these motifs. D. Immunoblots showing localization of ATP5L and ATP5J at lysosomes only when LAMP2A (specific for CMA) is present. KO indicates Lamp2a deleted in cardiomyocytes. Cre indicates control. N =3. * $P \leq 0.05$ Lamp2a KO versus control.

7. Increases in ATP5L and ATP5J account for the augmented mitochondrial oxygen consumption and ATP production resulting from inhibition of CMA

Next, since the levels of multiple mitochondrial proteins change in cells inactivated for CMA, we addressed whether the increases in ATP5L and ATP5J could account for the observed enhanced mitochondrial oxygen consumption and ATP production. We first used *wild type* and *Lamp2a*-null mouse embryonic fibroblasts (MEFs) to study this. Most importantly, we found that knockdown of either ATP5L or ATP5J reversed the increases mitochondrial oxygen consumption and ATP production resulting from inactivation of CMA in Lamp2a-null MEFs (Fig

7B, D). In addition, overexpression of ATP5L or ATP5J in wild type MEFs – to levels observed in *Lamp2a*-null MEFs – was sufficient to augment mitochondrial oxygen consumption and ATP synthesis (Fig 7A, B). Finally, even the acute incubation of isolated cardiac mitochondria with recombinant ATP5L in a cuvette

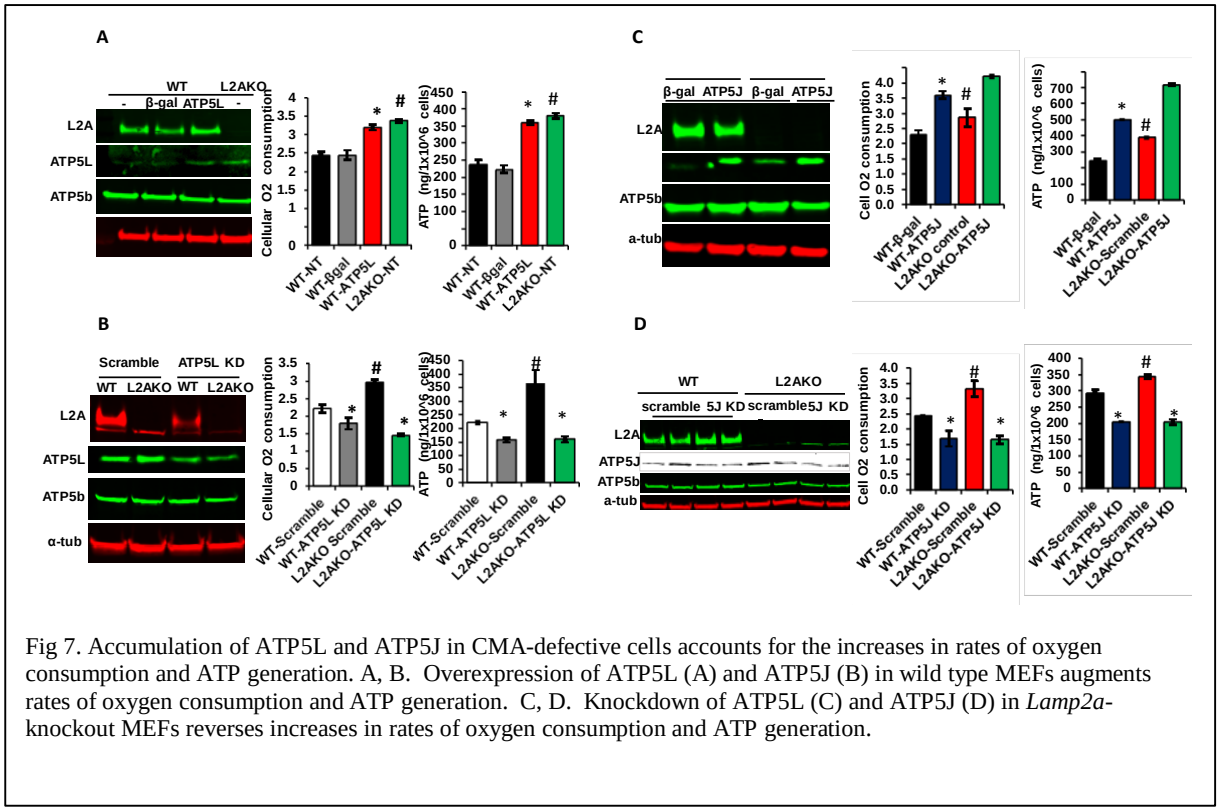

Fig 7. Accumulation of ATP5L and ATP5J in CMA-defective cells accounts for the increases in rates of oxygen consumption and ATP generation. A, B. Overexpression of ATP5L (A) and ATP5J (B) in wild type MEFs augments rates of oxygen consumption and ATP generation. C, D. Knockdown of ATP5L (C) and ATP5J (D) in *Lamp2a*-knockout MEFs reverses increases in rates of oxygen consumption and ATP generation.

was sufficient to augment these parameters (not shown). Thus, increases in ATP5L and ATP5J are sufficient to account for the increased mitochondrial function in cells in which CMA has been inactivated.

8. <u>Increases in mitochondrial respiration and ATP production in cardiomyocytes disabled for CMA reflect increased mitochondrial supercomplex formation</u>

Next, since the mitochondrial F0-F1 ATPase complex (complex V) contains 33 proteins, we next examined how increases in the stoichiometry of two of these proteins, ATP5L and ATP5J, could influence mitochondrial respiration and ATP synthesis. In fact, very little is known about these proteins. ATP5L (also called protein g) resides in the membrane portion of the F0-F1 ATPase and may mediate dimerization of this complex. ATP5J (also called subunit F6 or coupling factor 6), on the other hand, resides in the stalk (F1 portion) of the complex and may mediate the interaction between the F0 and F1. Blue native gel analysis of mitochondrial complexes demonstrated that mitochondria from hearts with cardiomyocytes-specific *Lamp2a* deletion exhibit increased Complex V supercomplex formation (Fig 8). While the precise mechanism by which supercomplexes facilitate electron transport in the respiratory chain are poorly

understood, these biochemical data suggest that increases in ATP synthesis rates reflect augmentation of respiratory chain function.

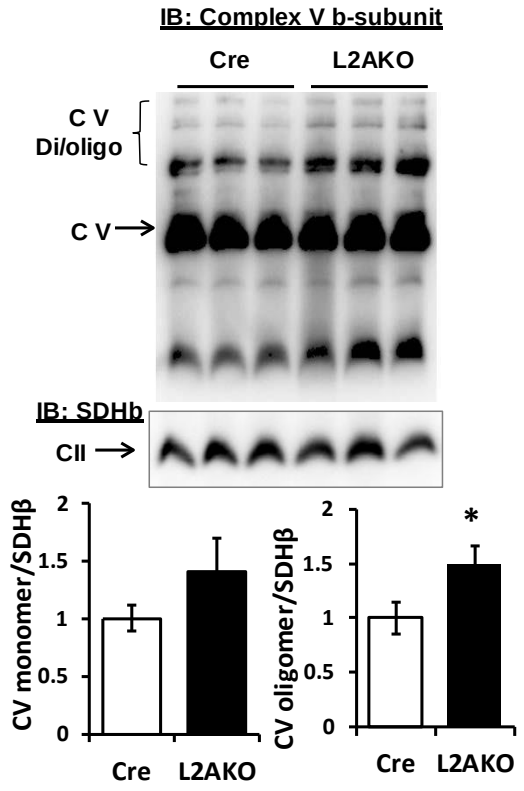

Fig 8. Heart mitochondrial complex V supercomplexes formation. Cardiac mitochondria were studied by blue native polyacrylamide gel electrophoresis with immunoblotting for ATP5b (Complex V) and succinate dehydrogenase (SDHβ, Complex II). Abundance of Complex V monomers and oligomers were normalized to levels of SDHβ. N=3-4. * P ≤0.05.

9. Augmentation of CMA in cardiomyocytes decreases ATP5L and ATP5J levels, mitochondrial respiration, and ATP production

Thus far, we have shown that inactivation of CMA in cardiomyocytes through deletion of *Lamp2a* augments mitochondrial function by increasing levels of ATP5L and ATP5J, components of the mitochondrial F0-F1 ATPase, thereby maintaining cardiac function in the face of pressure overload. We also showed that pressure overload activates CMA in wild type hearts. We next tested whether activation of CMA in cardiomyocytes is sufficient by itself to cause cardiac systolic dysfunction. We used intravenous administration of a cardiotropic adeno-associated virus expressing *Lamp2a* under the control of the cardiomyocyte-specific troponin T promoter to overexpress Lamp2a in the cardiomyocytes of wild type mice *in vivo*. Mild overexpression of *Lamp2a* (Fig 9A) resulted in the opposite of deletion: increased cardiomyocyte CMA as evidenced by the CMA reporter (Fig 9B), decreased ATP5L and ATP5J levels (Fig

9C), decreased mitochondrial oxygen consumption and ATP production (Fig 9D), decreased Complex V supercomplex formation (Fig 9E), and decreased systolic function (Fig 9F).

Thus, in aggregate, these loss- and gain of function studies show that the net effect of CMA in cardiomyocytes is to suppress mitochondrial function and ATP production through depletion of ATP5L and ATP5J. Further, they reveal a previously unrecognized pathway in cardiomyocytes that contributes to pressure overload-induced cardiac dysfunction.

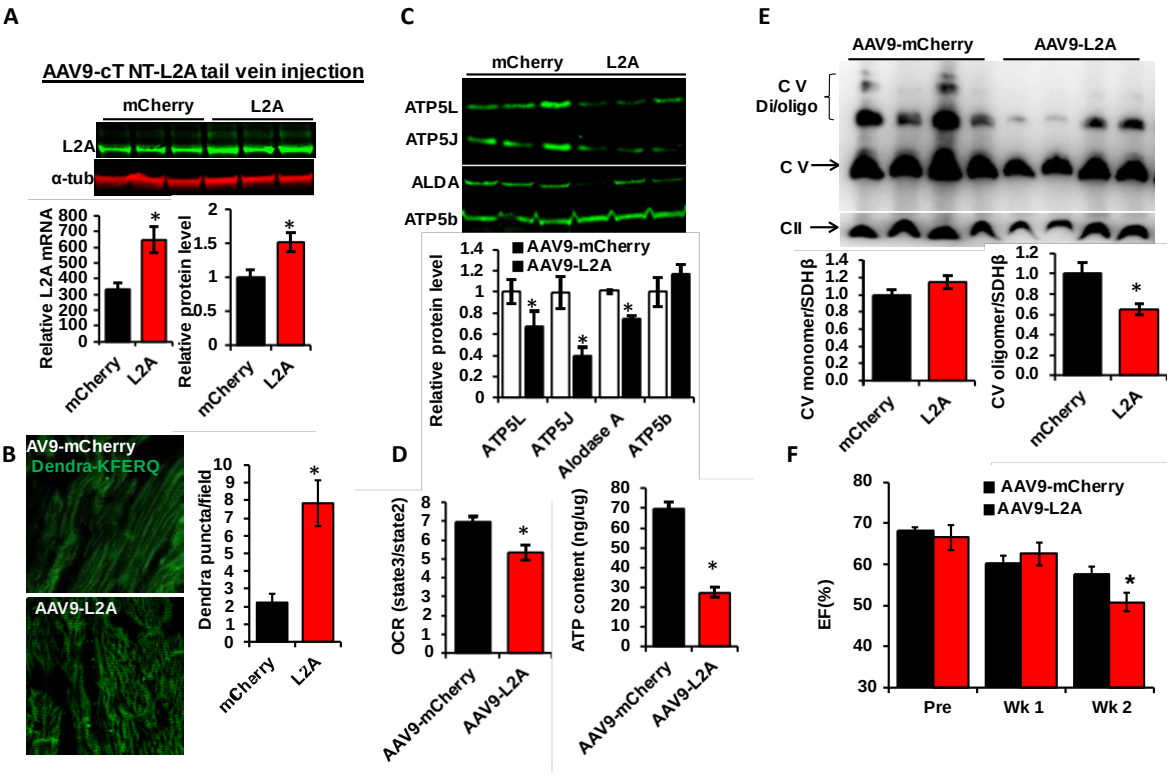

Fig 9. Gain of CMA function in the heart induces cardiomyopathy. Dendra mice were injected by tail vein with AAV9-cTNT-mCherry (control) or AAV9-cTNT-L2A (n=4-6). Hearts were harvest at week 2 post injection and subjected to analysis. A. L2A protein and mRNA expression. B. CMA reporter analysis as previously described. C. Immunoblotting for the indicated proteins in cardiac homogenates. D. Oxygen consumption rates (OCR) and ATP content in isolated cardiac mitochondria. E. Complex V supercomplex abundance as described in Fig 8. F. Systolic function at various time points by echocardiography. * $P \leq 0.05$ AAV9-L2A versus AAV9-mCherry.

We are now performing analogous studies using the cardiac fibroblast-specific *Lamp2a* knockout mice we have generated to determine the function of CMA in these cells. Since the biology of cardiac fibroblasts differs markedly from cardiomyocytes, we are not necessarily expecting to find the same "energetics/mitochondrial" function for CMA in the fibroblasts. Rather, we are examining effects of CMA on functions specific to fibroblasts including the assumption of an inflammatory phenotype and differentiation into myofibroblasts among others. Finally, we will examine the upstream signaling that activates CMA in each cell type. One possibility is that pressure overload activates CMA

in each cell type in parallel. However, a second not-mutually exclusively possibility is that secreted factors from cardiac fibroblasts modulate both CMA and cell survival in cardiomyocytes, a possibility we also intend to follow-up using the cardiac fibroblast secretome data now being generated.

Training opportunities and professional development

This proposal provides training and opportunities for professional development for two young scientists: Dr Ilaria Russo (Frangogiannis lab), and Dr Jose Corbalan (Kitsis lab) Dr Russo is a post-doctoral fellow who recently completed her PhD thesis work on the role of the fibrotic response in cardiac dysfunction. Dr Russo has generated and characterized the FS3KO mice and has identified novel protective actions of fibroblasts in the pressure-overloaded heart. This project provides a unique training opportunity for Dr Russo, combining in vitro and in vivo studies to reveal novel actions of fibroblasts in myocardial disease. Dr Corbalan began his postdoctoral fellowship with the Kitsis lab in December, 2016. His training had been mainly in physiology. He came to the laboratory to learn molecular approaches including biochemistry, molecular cellular biology, and genetically manipulated mice. He is employing all of these approaches in the cardiac fibroblast/cardiomyocyte cell death experiments and CMA/fibroblast projects. Dr. Corbalan aspires to a career as an independent scientist in academia.

How were the results disseminated to communities of interest?

Some of the data described above have been published (see products and appendix). The manuscript reporting the effects of Smad3 on fibroblast phenotype and function in the pressure-overloaded myocardium (Russo et al.) is currently in the final stages of revision. The work on the role of Smad2 in fibroblast function is in preparation. The concepts explored in this proposal have significantly contributed to development of our paradigm on the pathogenesis of cardiac fibrosis and are discussed in a series of invited review manuscripts and editorials (please see “products”).

Plans for the next reporting period

During the next reporting period, we will complete the work examining the role of Smad2 in fibroblast signaling, and we will systematically investigate the basis for the distinct actions of Smad2 and Smad3. Over the next year we will also pursue our studies characterizing the fibroblast secretome. Unfortunately, the first experiments attempting to

study the secretome showed substantial variability. We have optimized and standardized our fibroblast preparations, and we plan to repeat the proteomic studies, in order to identify novel fibroblast-derived mediators that may modulate cardiomyocyte survival responses. We will also complete CMA studies involving the cardiac fibroblast-specific *Lamp2a* knockout mice.

4. Impact

The current concepts regarding the role of fibroblasts in the remodeling heart are unidimensional, suggesting that fibroblasts simply serve as matrix-producing cells and that inhibition of cardiac fibrosis is an important therapeutic target. The proposed have transformative potential in the field. Based on robust data, we suggest that the functions of cardiac fibroblasts extend beyond their capacity to produce and secrete extracellular matrix proteins. Cardiac fibroblast populations may play an important role in protection of cardiomyocytes under conditions of stress. Our studies will identify specific fibroblast-derived signals that may modulate cardiomyocyte survival and function in the pressure-overloaded heart. This is of outstanding significance for therapeutic design. We propose that therapeutic strategies targeting fibrosis in heart failure need to take into account the protective effects of fibroblasts. Non-specific inhibition of fibroblast function may abrogate important protective signals, thus extending injury. On the other hand, identification of fibroblast-derived mediators that prevent cardiomyocyte death may suggest new therapeutic approaches in heart failure.

5. Changes/Problems

No changes or problems to report

6. Products

Publications:

AV Shinde, and NG Frangogiannis. Tissue transglutaminase in the pathogenesis of heart failure. *Cell Death Differentiation* 2018; 25(3): 453-456.

P Kong, A Shinde, Y Su, I Russo, B Chen, A Saxena, SJ Conway, JM Graff, and NG Frangogiannis. Opposing actions of fibroblast and cardiomyocyte Smad3 signaling in the infarcted myocardium. *Circulation* 2018 Feb 13;137(7):707-724.

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7. Participants & Other Collaborating Organizations (AECOM)

Participants

Name:	*Richard N. Kitsis*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Dr. Kitsis designed experiments, supervised the experimental work and analyzed data.*
Funding Support:	*W81XWH-16-1-0564, NIH R01 HL128071, NIH R01 HL130861, NIH R01 HL138475, NIH R01HL123543, AHA 18SRG34280018, Fondation Leducq*

Name:	*Jessica Mar*
Project Role:	*Co-investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*

Contribution to Project:	*Dr. Mar has been involved with informatics aspects of the project.*
Funding Support:	

Name:	*J. Jose Corbalan*
Project Role:	*Postdoctoral Research Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	*Dr. Corbalan is working on the CMA studies and the analysis of the effects of secreted products from cardiac fibroblasts on cardiomyocytes.*
Funding Support:	W81XWH-16-1-0564

Name:	*Min Zheng*
Project Role:	*Research Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	*Dr. Zheng is involved in preparation of cardiomyocytes, cardiac fibroblasts, and performs the TAC procedures.*
Funding Support:	W81XWH-16-1-0564

Active other support of the PI (Richard N. Kitsis)

NIH R01 HL128071 04/01/15-02/28/19
Linking cell death and mitochondrial quality control mechanisms in heart disease
PIs: Richard N. Kitsis and Gerald W. Dorn II (MPI)
This project explores the bidirectional antagonism between Bax and Parkin to define mechanistic connections between cell death and mitophagy and the integrated roles these processes play in cardiac disease. No overlap with the current project.

NIH R01 HL130861 01/15/16-12/31/19
A new molecular pathway for diabetic cardiomyopathy
PIs: Richard N. Kitsis and Jun Yoshioka (MPI)
This project defines a novel pathway by which hyperglycemia induces cardiomyocyte death in the transition from diastolic dysfunction to systolic failure in diabetic cardiomyopathy. No overlap with the current project.

NIH R01 HL138475 07/15/17-06/30/21
Chaperone-mediated autophagy in normal cardiac biology and heart failure
PI: Richard N. Kitsis
This project explores the relationships between chaperone-mediated autophagy and mitophagy (mediated by macroautophagy) in the heart. No overlap with the current project.

NIH R01HL123543 08/07/15-05/31/19
Regulation of cardiac death and energy metabolism by MCL-1
PI: Joseph T. Opferman
Co-Investigator: Richard N. Kitsis
This application defines separable functions for MCL-1 in inhibiting cell death and promoting metabolism when localized to the outer versus inner mitochondrial membranes respectively. No overlap with the current project.

AHA 18SRG34280018 07/01/18-06/30/20
Creating novel drugs to protect the heart against cancer therapies
PIs: Richard N. Kitsis and Evripidis Gavathiotis
This project employs structural, chemical, and molecular approaches to design and optimize small molecule allosteric BAX inhibitors to reduce cardiac damage from cancer therapies. No overlap with the current project.

Fondation Leducq TNE 01/01/16-12/31/20
Modulating autophagy to treat CV disease
PIs: Junichi Sadoshima and Luca Scorrano
Project Leader: Richard N. Kitsis
This network seeks to understand the roles of autophagy in heart and vascular disease and to develop small molecules to manipulate these processes. No overlap with the current project.

COMPLETED
None

PENDING
NIH T32HL144456 07/01/19-06/20/24 0.9 calendar months
Mechanisms of Cardiovascular Disease
PIs: Richard N. Kitsis and Nicholas E. Sibinga (MPI)
This is a pre- and postdoctoral training grant. No overlap with the current project.

OVERLAP
None

Collaborating Organization

Collaborating PI: Dr. Nikolaos Frangogiannis, Albert Einstein College of Medicine

Collaboration as described below

Organization Name	Albert Einstein College of Medicine	
Location of Organization	Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx NY 10461	Collaborating PI: Dr. Nikolaos Frangogiannis
Partner's Contribution	Dr. Frangogiannis is an expert in the biology of cardiac fibroblasts. This collaborative proposal combines expertise in cell death and fibroblast biology to investigate the effects of activated fibroblasts in cardiomyocyte survival and dysfunction under conditions of stress.	
Financial Support		
Facilities		
Collaboration	Collaboration between the PIs as outlined above	
Personal exchanges		
Other		

Dr. Frangogiannis will submit his own report. Both reports contain the same description of the accomplishments; contributions of each laboratory to the reported accomplishments are clearly indicated.

8. Special Reporting Requirements

N/A

9. Appendices (contain pdf files of publications supported by this award).

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Cell Death & Differentiation

COMMENT

Tissue transglutaminase in the pathogenesis of heart failure

Arti V. Shinde[1] · Nikolaos G. Frangogiannis[1]

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First described in the 1950s, tissue tranglutaminase (tTG, transglutaminase 2), is a unique multifunctional member of the transglutaminase family with effects that extend beyond its enzymatic actions. A recently published study demonstrates an important role for endogenous tTG in the pathogenesis of pressure overload-induced heart failure, suggesting crosslinking actions that promote diastolic dysfunction, and protective matrix-preserving effects that prevent chamber dilation [1]. The functions of tTG in the failing heart may involve both enzymatic and non-enzymatic effects with several different cellular targets and a wide range of molecular interactions.

Ca^{2+}-dependent transamidation, the best characterized enzymatic function of tTG, modifies proteins by crosslinking their reactive carboxamide side chains to primary amines [2]. tTG also exerts enzymatic functions that do not require Ca^{2+}, acting as a GTPase, protein kinase or protein disulfide isomerase, and participates in non-enzymatic interactions with many different proteins, serving adapter and signaling functions both within and outside the cells [3]. In vitro studies have implicated tTG in a wide range of cellular functions, including cell survival, adhesion and migration, cell growth, proliferation, and differentiation [4]. The absence of significant baseline phenotypic abnormalities in mice with global loss of tTG [5] demonstrated that, despite its broad repertoire of presumed cellular functions, tTG does not play a crucial role in maintaining tissue homeostasis. In contrast, a growing body of evidence suggests that tTG is overexpressed and activated following tissue injury, and may regulate pathophysiologic responses.

tTG in heart disease

tTG is constitutively expressed in normal mammalian myocardium, and is upregulated in injured and failing hearts [6]. In a recently published study [1], we provided the first evidence suggesting an important role for tissue transglutaminase in the pathogenesis of heart failure. We used a mouse model of transverse aortic constriction that recapitulates the cardiomyopathy of pressure overload, one of the most common pathophysiologic conditions associated with heart failure in human patients. In the pressure-overloaded heart, early development of cardiac hypertrophy and fibrosis increases cardiac stiffness causing diastolic ventricular dysfunction, despite preserved systolic function. At a later stage, persistent pressure overload leads to progressive ventricular dilation, decompensation, and development of systolic heart failure. tTG was the only member of the transglutaminase family that is upregulated in the pressure-overloaded mouse myocardium. In remodeling hearts, tTG was localized in cardiomyocytes, macrophages, and interstitial cells, and was deposited in the extracellular matrix. Experiments in tTG knockout mice suggested that endogenous tTG plays no significant role in cardiac homeostasis, but participates in cardiac remodeling following pressure overload, exerting both detrimental and protective actions. tTG promoted collagen crosslinking, increasing chamber stiffness following pressure overload, but also protected the remodeling heart from dilation, preventing excessive matrix metalloproteinase activation, and restraining fibroblast proliferation. These observations highlight the functional complexity of tTG, but also generate several important questions:

Which stimuli induce tTG expression and secretion in the remodeling myocardium?

Cardiac pressure overload activates mechanosensitive signaling pathways in all myocardial cells. In response to biomechanical stress, cardiomyocytes activate integrins, transducing downstream mitogen-activated protein kinase

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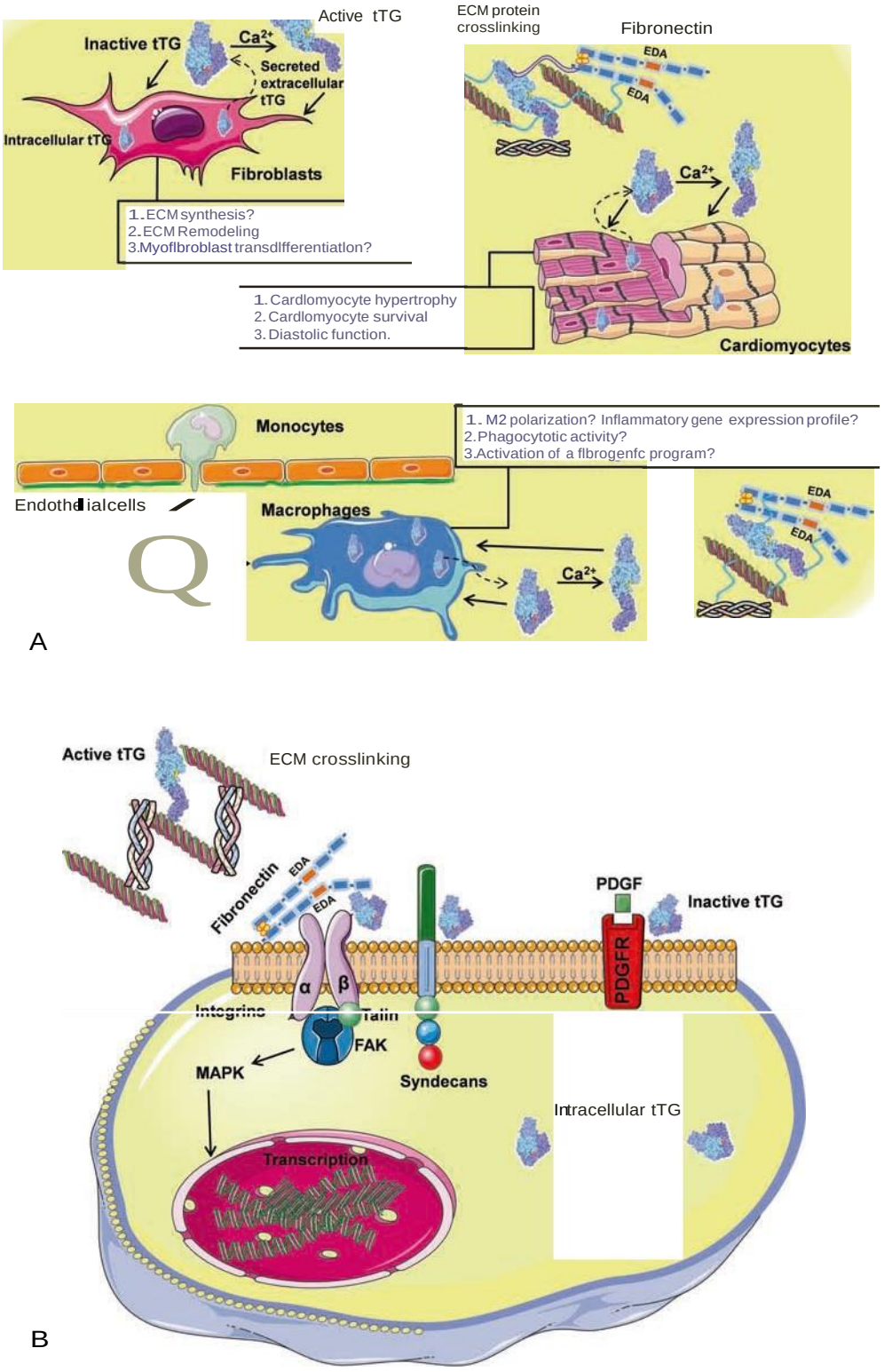

cascades, that may be involved in induction of tTG transcription [7]. Mechanosensitive signaling may also stimulate (fG transcription by locally activating transfonning growth factor (TGF)-through integrin-dependent actions [8], and through induction ofTGF- -activating matricellular proteins. Our findings suggested that TGF-upregulates

Fig. 1 The cellular and molecular targets of tTG in the failing heart. a In the pressure-overloaded myocardium, tTG is upregulated and secreted in the interstitium. Activated tTG plays a role in extracellular matrix (ECM) crosslinking, promoting diastolic dysfunction. Both enzymatically active and inactive tTG may exert effects on cardiomyocytes, fibroblasts, and macrophages by regulating intracellular signaling cascades, or by modulating outside-in signaling. In fibroblasts, tTG may modulate ECM synthesis and remodeling and may play role in myofibroblast activation. In cardiomyocytes, tTG may promote hypertrophic response, may modulate cell survival in response to stress and may play a role in diastolic dysfunction. Polarized M2 macrophages express tTG. tTG may be implicated in phagocytotic function of macrophages and may play a role in regulating inflammatory gene synthesis and macrophage-driven fibroblast activation. b tTG may interact with a wide range of proteins in the cell surface, cytoplasm, or ECM. Active tTG may promote ECM crosslinking. tTG may also modulate signaling responses in a transamidase-independent manner. tTG may facilitate fibronectin-activated integrin signaling transducing a focal adhesion kinase (FAK) cascade, may bridge integrins with growth factor receptors (such as platelet-derived growth factor receptor/PDGFR), and may activate syndecan-mediated responses. Additional symbol: MAPK, mitogen-activated protein kinase

tTG expression in both cardiac fibroblasts and macrophages through distinct signaling pathways and may play an important role in tTG induction in vivo.

What are the main cellular targets of tTG in the failing heart?

Considering its ubiquitous expression, its secretion and deposition in the activated extracellular matrix, and its broad functional repertoire, tTG may have a wide range of cellular targets in heart failure (Fig. 1). Published studies suggest important (and sometimes conflicting) effects of tTG on all cell types involved in cardiac remodeling. In cardiomyocytes, tTG overexpression activated cycloxygenase-2 signaling promoting cell death and inducing ventricular dysfunction [9]. In contrast, loss-of-function experiments suggested that tTG may protect cardiomyocytes from ischemic death [10]. tTG expression in fibroblasts promotes cell adhesion [11], regulates fibroblast migration, stimulates fibronectin synthesis and has been implicated in organization of the fibroblast-derived extracellular matrix network [12]. tTG is also expressed in macrophages, and has been implicated in recognition and phagocytosis of apoptotic cells by professional phagocytes [13]. A recent study demonstrated that tTG is a consistent and preserved marker of polarized M2 macrophages in humans and mice [14]; however, whether tTG regulates macrophage polarization in vivo remains unknown.

In our study, use of a mouse line with global loss of tTG precludes conclusions regarding the in vivo role of specific cellular responses in mediating the effects of tTG loss. Thus, our experiments did not explain the cellular basis for the strikingly increased mortality in tTG null mice undergoing pressure overload protocols. Accentuated dilative remodeling due to loss of protective matrix-preserving effects may be in part responsible for increased mortality in pressure-overloaded tTG knockout animals; however, the severity of systolic dysfunction could not account for the observed effect on survival. Whether endogenous tTG protects the myocardium from arrhythmogenesis or defective impulse conduction is unknown.

On the other hand, the observed effects on matrix remodeling and fibrosis may reflect important actions of tTG on fibroblasts, or effects on the fibrogenic phenotype of immune cells (such as macrophages). Our in vitro findings suggested that tTG restrains fibroblast proliferative capacity and promotes a matrix-preserving program in cardiac fibroblasts by inducing tissue inhibitor of metalloproteinases (TIMP)1 synthesis.

Do the in vivo effects of tTG involve enzymatic effects

Although tTG expression is markedly induced in the remodeling myocardium, whether increased protein levels are associated with accentuated enzymatic activity remains unclear. In normal tissues, tTG activity is tightly regulated. In the cytoplasm, low Ca^{2+} concentrations prevent intracellular transamidation activity, and GTP/GDP act as allosteric inhibitors, reducing accessibility of the catalytic domain, and preventing inappropriate intracellular tTG activation. In the pressure-overloaded heart, increased calcium concentrations may co-operate with pro-inflammatory cytokines, triggering intracellular tTG activation. In the cardiac interstitium, high levels of Ca^{2+} may stimulate tTG-mediated transamidase activity, promoting crosslinking of extracellular matrix proteins and leading to formation of a protease-resistant matrix. The crosslinking actions of tTG may also serve to localize and activate TGF-β, further accentuating fibrosis. However, interstitial activation of tTG may be transient due to release of mediators that inactivate tTG, such as reactive oxygen and nitric oxide [15]. Thus, the relative significance of enzymatic tTG actions in the pathogenesis of cardiac remodeling is not known.

What is the in vivo significance of non-enzymatic functions of tTG?

Non-enzymatic functions of tTG may play an important role in activation of cardiac interstitial cells and in the pathogenesis of myocardial fibrosis. Through binding with both

fibronectin and heparin sulfate proteoglycans, tTG may function as a molecular bridge between the matrix and the cells, activating syndecan-4-mediated signaling [16]. tTG may also co-operate with fibronectin to trigger integrin activation and downstream stimulation of Focal adhesion kinase signaling; these effects are transamidase-independent [12]. Moreover, cell surface tTG may bridge β1 integrins with growth factor receptors [17]. In our study, matrix-bound tTG exerted matricellular effects on fibroblasts inducing TIMP1 expression in a transamidase-independent manner. The matricellular actions of tTG may be particularly important in vivo as they do not require persistent enzymatic activation.

Future directions

Our recently published findings suggest that tTG is a critical stress-induced signal in the failing heart, exerting both profibrotic effects that increase ventricular stiffness and protective actions that preserve extracellular matrix integrity, preventing chamber dilation. Considering the potential translational significance of tTG-mediated actions in heart failure, study of the molecular basis for the effects of tTG is an important priority. Future experiments should focus on several different directions. First, dissection of the enzymatic and non-enzymatic actions of tTG in the remodeling myocardium and identification of the main substrates is important in order to design safe and effective therapeutic strategies. Second, investigation of the cellular targets of tTG in the remodeling heart is needed in order to identify specific cellular responses that could be targeted in human patients. Third, characterization of the molecular signals interacting with tTG and understanding of their role in mediating specific cellular responses can provide critical information on specific mechanisms of action with direct translational implications. Fourth, systematic analysis of changes in myocardial tTG expression in human failing hearts may provide insights into the role of tTG in specific heart failure subpopulations. Considering the heterogeneity of human heart failure, identification of patients with prominent fibrosis and excessive activation of myocardial tTG-mediated matrix crosslinking may mark a subpopulation that may benefit from attenuation of enzymatic tTG actions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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Circulation

ORIGINAL RESEARCH ARTICLE

Opposing Actions of Fibroblast and Cardiomyocyte Smad3 Signaling in the Infarcted Myocardium

BACKGROUND: Transforming growth factor–βs regulate a wide range of cellular responses by activating Smad-dependent and Smad-independent cascades. In the infarcted heart, Smad3 signaling is activated in both cardiomyocytes and interstitial cells. We hypothesized that cell-specific actions of Smad3 regulate repair and remodeling in the infarcted myocardium.

METHODS: To dissect cell-specific Smad3 actions in myocardial infarction, we generated mice with Smad3 loss in activated fibroblasts or cardiomyocytes. Cardiac function was assessed after reperfused or nonreperfused infarction using echocardiography. The effects of cell-specific Smad3 loss on the infarcted heart were studied using histological studies, assessment of protein, and gene expression levels. In vitro, we studied Smad-dependent and Smad-independent actions in isolated cardiac fibroblasts.

RESULTS: Mice with fibroblast-specific Smad3 loss had accentuated adverse remodeling after reperfused infarction and exhibited an increased incidence of late rupture after nonreperfused infarction. The consequences of fibroblast-specific Smad3 loss were not a result of effects on acute infarct size but were associated with unrestrained fibroblast proliferation, impaired scar remodeling, reduced fibroblast-derived collagen synthesis, and perturbed alignment of myofibroblast arrays in the infarct. Polarized light microscopy in Sirius red–stained sections demonstrated that the changes in fibroblast morphology were associated with perturbed organization of the collagenous matrix in the infarcted area. In contrast, α-smooth muscle actin expression by infarct myofibroblasts was not affected by Smad3 loss. Smad3 critically regulated fibroblast function, activating integrin-mediated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase–2 (NOX-2) expression. Smad3 loss in cardiomyocytes attenuated remodeling and dysfunction after infarction. Cardiomyocyte-specific Smad3 loss did not affect acute infarct size but was associated with attenuated cardiomyocyte apoptosis in the remodeling myocardium, accompanied by decreased myocardial NOX-2 levels, reduced nitrosative stress, and lower matrix metalloproteinase–2 expression.

CONCLUSIONS: In healing myocardial infarction, myofibroblast- and cardiomyocyte-specific activation of Smad3 has contrasting functional outcomes that may involve activation of an integrin/reactive oxygen axis.

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Key Words: cardiomyocyte □ fibroblast □ heart failure □ remodeling □ SMAD

Sources of Funding, see page 723

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Clinical Perspective

What Is New?

- Our study demonstrates for the first time cell-specific effects of Smad3 signaling in the infarcted myocardium.
- Smad3 activation in myofibroblasts plays a critical role in repair after myocardial infarction, restraining fibroblast proliferation and contributing to scar organization by stimulating integrin synthesis.
- Smad3 signaling in cardiomyocytes does not affect acute ischemic injury but triggers nitrosative stress and induces matrix metalloproteinase expression in the remodeling myocardium, promoting cardiomyocyte death and contributing to systolic dysfunction.

What Are the Clinical Implications?

- Implementation of therapeutic strategies targeting transforming growth factor–β cascades in patients with myocardial infarction has been hampered by the pleiotropic and context-dependent actions of the cytokine.
- Using genetic tools with in vivo and in vitro experiments, we demonstrated the cellular specificity of transforming growth factor–β/Smad3—dependent actions that stimulate distinct cellular responses in fibroblasts and cardiomyocytes.
- Therapeutic targeting of transforming growth factor–β in pathological conditions is likely to interfere with both detrimental and beneficial actions.
- Design of interventions with specific cellular targets is needed for the development of safe and effective therapies.

Transforming growth factor (TGF)–βs are highly pleiotropic mediators with critical roles in regulating cellular phenotype and function in embryonic development, tissue homeostasis, and disease. Normal tissues contain stores of latent TGF-β bound to the extracellular matrix through its association with a large binding protein, the latent TGF-β binding protein. Tissue injury is associated with marked induction of TGF-β isoforms and activation of TGF-β signaling cascades. Parenchymal cells, extravasated leukocytes, and platelets synthesize and release large amounts of TGF-β in the injury site. Reactive oxygen species, proteases, matricellular proteins, and integrins cooperate to trigger the release of bioactive TGF-β from the latent stores. Subsequent binding of the active TGF-β dimer to the type II TGF-β receptor, followed by transphosphorylation of the type I receptor, triggers the TGF-β signaling response. The cellular effects of TGF-β are mediated through a canonical pathway involving a series of intracellular effectors, the Smads, or through activation of noncanonical signaling cascades. Activation of TGF-β signaling induces phosphorylation of the receptor-activated Smads, Smad2 and Smad3, which can form heteromeric complexes with the common Smad, Smad4. These complexes are transported to the nucleus, where they regulate gene transcription. TGF−β receptors and Smads are ubiquitously expressed by all cell types. Thus, all cells are responsive to the actions of TGF-β.

Cardiac injury is associated with the marked induction of TGF-β and activation of TGF-β cascades.[1,2] Our laboratory and other investigators have documented activation of Smad2 and Smad3 signaling in the infarcted myocardium,[3,4] localized in both cardiomyocytes and interstitial cells.[5] In isolated cardiac fibroblasts, Smad3 signaling accentuates myofibroblast transdifferentiation and stimulates a matrix-preserving program.[5] In a model of reperfused infarction, global loss of Smad3 attenuated remodeling after infarction.[4] However, considering the ubiquitous expression of Smad3 in all cell types, the cell biological basis for the actions of Smad3 in the infarcted heart remains unknown.

Our study dissects the cell-specific actions of Smad3 signaling in the infarcted myocardium by developing and studying mice with cell-specific loss of Smad3 in activated fibroblasts and cardiomyocytes. It is surprising that fibroblast-specific loss of Smad3 worsened remodeling after infarction, resulting in accentuated chamber dilation. The deleterious consequences of fibroblast-specific Smad3 loss reflected unrestrained fibroblast proliferation, defective scar remodeling, and perturbed organization of myofibroblast arrays in the border zone. Smad3 signaling regulated fibroblast function, activating integrin-mediated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX)–2 expression. In contrast, cardiomyocyte-specific loss of Smad3 protected the infarcted heart from dysfunction after infarction. The protective effects of cardiomyocyte-specific Smad3 loss were associated with attenuated cardiomyocyte apoptosis in remodeling myocardium and accompanied by decreased NOX2 levels, reduced nitrosative stress, and decreased matrix metalloproteinase (MMP)–2 expression.

METHODS

Detailed methods are described in the online-only Data Supplement

Relevant data, methods, and study materials will be made available on request.

Generation of Mice With Myofibroblast and Cardiomyocyte-Specific Smad3 Loss

We generated mice with Smad3 loss in activated infarct myofibroblasts (FS3KO) by breeding Smad3$^{fl/fl}$ mice[6] with transgenic mice in which Cre recombinase is driven by a 3.9-kb mouse *Postn* promoter.[7] Endogenous periostin, which is encoded by

Postn, is not expressed in cardiomyocytes, vascular cells, hematopoietic cells, or quiescent cardiac fibroblasts[8] but is upregulated in injury site fibroblasts in infarcted hearts.[9] To generate mice with cardiomyocyte-specific loss of Smad3 (CMS3KO), Smad3$^{fl/fl}$ mice were crossed with α-myosin heavy chain (MHC) MHC-Cre-transgenic animals.[10]

Mouse Models of Myocardial Infarction

Animal studies were approved by the Institutional Animal Care and Use Committee at Albert Einstein College of Medicine and conform with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. A closed-chest model of reperfused myocardial infarction and a model of nonreperfused infarction were used, as previously described.[11] Female and male mice, 2 to 4 months of age, were anesthetized using inhaled isoflurane; 75 Smad3$^{fl/fl}$ mice, 73 FS3KO mice, and 36 CMS3KO mice underwent in vivo experimentation.

Acute Infarct Size Assessment

Infarct size was assessed using Evans blue–triphenyltetrazolium chloride staining.

Echocardiography

Echocardiography was performed before instrumentation and after 7 days and 28 days of reperfusion using the Vevo 2100 system (VisualSonics).

Immunohistochemistry and Histology

For histopathologic analysis, murine hearts were fixed in zinc-formalin and embedded in paraffin.

Isolation and Culture of Cardiac Fibroblasts

Fibroblasts were isolated from normal wild-type (WT) and Smad3 knockout (KO) mouse hearts as previously described.[4]

Isolation of Fibroblasts and Myeloid Cells From Infarcted Hearts

Macrophages and fibroblasts were isolated from infarcted hearts for RNA extraction as previously described.[9]

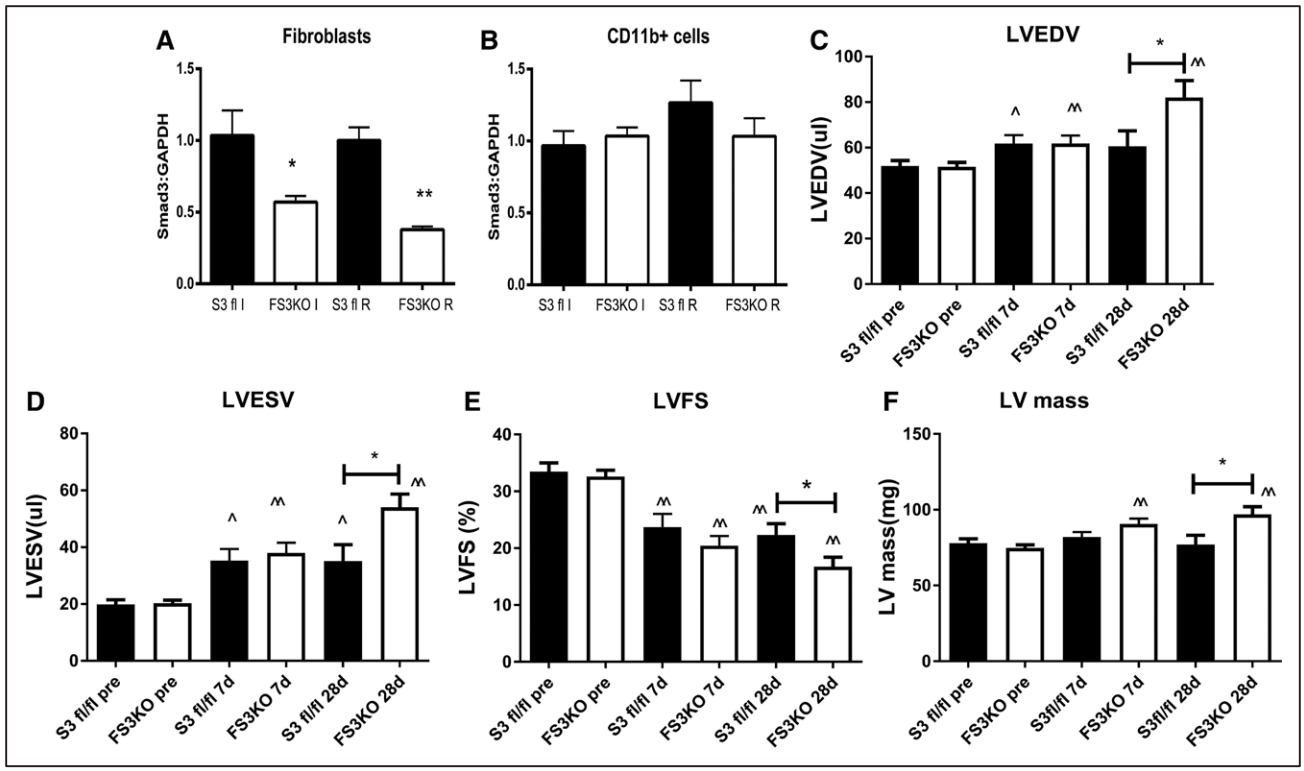

Figure 1. Fibroblast-specific Smad3 disruption accentuates adverse remodeling following reperfused myocardial infarction.

A and B, Smad3$^{fl/fl}$Postn-Cre mice (FS3KO) exhibited reduced Smad3 expression in fibroblasts (**A**) but not in CD11b^{+} myeloid cells (**B**) harvested from the infarcted (I) or remodeling myocardium (R) after 1 hour of ischemia and 7 days of reperfusion. (*P<0.05, **P<0.01 versus corresponding Smad3$^{fl/fl;}$ n=5/group). **C**–**F,** Fibroblast-specific Smad3 loss increased adverse remodeling and worsened systolic dysfunction after 1 hour of ischemia and 28 days of reperfusion. In the model of reperfused myocardial infarction, FS3KO mice exhibited increased LVEDV (**C**) and LVESV (**D**), reduced LV fractional shortening (**E**), and increased LV mass (**F**) in comparison with Smad3$^{fl/fl}$ controls after 28 days of reperfusion (*P<0.05, ^P<0.05, ^^P<0.01 versus corresponding baseline values; n=11/group). FS3KO indicates fibroblast-specific Smad3 knockout mice; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVFS, left ventricular fractional shortening; LV mass, left ventricular mass; and S3, Smad3.

Collagen Pad Contraction Assay

A collagen pad contraction assay was performed as previously described.[11]

RNA Extraction and Quantitative Polymerase Chain Reaction

Gene expression was assessed using quantitative polymerase chain reaction.

Protein Extraction and Western Blotting

Western blotting using protein extracted from hearts or cardiac fibroblasts was performed.

Statistical Analysis

Data are presented as mean±SEM. For comparisons of 2 groups, the unpaired, 2-tailed Student's *t* test using (when appropriate) Welch's correction for unequal variances was performed. The Mann-Whitney test was used for comparisons between 2 groups that did not show Gaussian distribution. For comparisons of multiple groups, 1-way analysis of variance was performed, followed by Tukey's multiple comparison test. The Kruskal-Wallis test, followed by Dunn's multiple comparison posttest, was used when ≥1 groups did not show Gaussian distribution. The paired *t* test was used for comparisons of functional data within the same group. Survival analysis was performed using the Kaplan-Meier method. Mortality was compared using the log rank test.

RESULTS

FS3KO Mice Have Normal Baseline Cardiac Geometry and Function

To generate mice lacking Smad3 in infarct myofibroblasts, we used a transgenic mouse line in which Cre is driven by the mouse *Postn* promoter.[7] Periostin is not expressed by quiescent fibroblasts in the adult mouse heart but is markedly and specifically upregulated in infarct myofibroblasts. Postn-Cre mice have been used for fibroblast-specific targeting in models of cardiac injury and remodeling.[12,13] Smad3$^{fl/fl}$Postn-Cre mice (FS3KO) had normal baseline cardiac geometry and function. At 4 months of age, FS3KO animals and Smad3$^{fl/fl}$ controls had comparable left ventricular volume, ejection fraction, wall thickness, and left ventricular mass (Figure I in the online-only Data Supplement). To examine the efficacy of Cre-mediated deletion in infarct fibroblasts, we assessed Smad3 levels in fibroblasts and CD11b$^+$ myeloid cells harvested immediately from the infarcted and noninfarcted myocardium to avoid effects of culture conditions on cellular phenotype. FS3KO mice had markedly reduced Smad3 expression in fibroblasts isolated from the infarct and the remodeling noninfarcted myocardium (Figure 1A). FS3KO and Smad3$^{fl/fl}$ mice exhibited comparable Smad3 expression in CD11b$^+$ myeloid cells harvested from the infarct and noninfarcted remodeling heart (Figure 1B), indicating fibroblast-specific Smad3 targeting.

Smad3 Loss in Activated Fibroblasts Accentuates Dilative Remodeling in Both Reperfused and Nonreperfused Myocardial Infarction

Both FS3KO and Smad3$^{fl/fl}$ mice subjected to reperfused infarction had low mortality; however, FS3KO mice exhibited worse remodeling. After 28 days of reperfusion, FS3KO animals had increased left ventricular end-diastolic volume and left ventricular end-systolic volume (Figure 1C and 1D), suggesting accentuated dilative remodeling. Moreover, fibroblast-specific Smad3 loss worsened systolic dysfunction (Figure 1E) and augmented hypertrophy after reperfused infarction (Figure 1F and Figure II in the online-only Data Supplement).

In contrast to the negligible mortality associated with reperfused infarction, mice undergoing nonreperfused infarction protocols exhibit ≈50% mortality during the first week after coronary occlusion primarily because of cardiac rupture. Statistical analysis showed that FS3KO mice had a trend toward higher mortality (percent survival at 28 days: Smad3$^{fl/fl}$, 37.0% versus FS3KO, 18.7%, P=0.16, n=42–46). Although all deaths in Smad3$^{fl/fl}$ mice occurred during the first 5 days after coronary occlusion, FS3KO animals continued to exhibit mortality after the first 5 days. Mortality analysis after day 6 after occlusion showed that FS3KO mice had significantly increased late mortality (P=0.007), reflecting a high incidence of late cardiac rupture (Figure 2A and 2B). FS3KO animals surviving nonreperfused infarction exhibited trends toward increased dilative remodeling and worse systolic dysfunction (Figure 2C through 2F).

Fibroblast-Specific Smad3 Loss Does Not Affect Acute Infarct Size But Is Associated With Larger Scars, Increased Myofibroblast Density, and Accentuated Myofibroblast Proliferation

Next, we examined whether worse remodeling after infarction in FS3KO mice was caused by accentuated acute cardiomyocyte injury. Fibroblast-specific Smad3 loss did not affect acute infarct size (Figure 3A through 3C). Despite comparable acute injury, FS3KO mice had significantly larger scars after 7 to 28 days of reperfusion (Figure 3D through 3H). Increased scar size in FS3KO mice was associated with higher myofibroblast density in the infarcted region (Figure 3I through 3M). We have previously demonstrated that in vitro, Smad3 mediates the antiproliferative effects

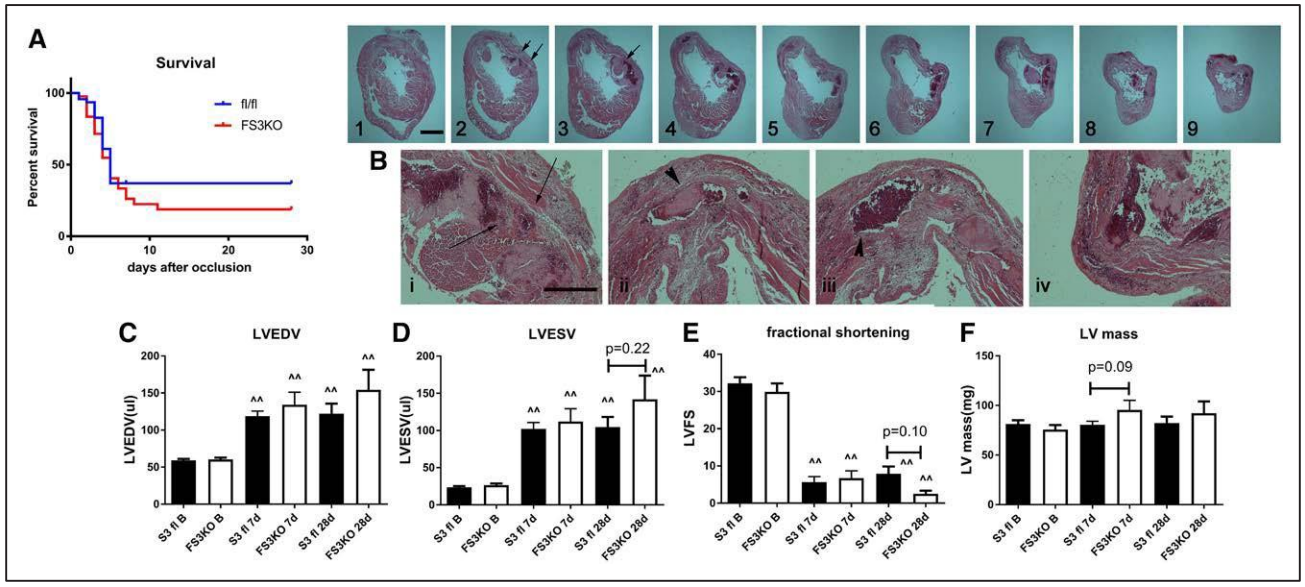

Figure 2. Smad3$^{fl/fl}$Postn-Cre (FS3KO) mice exhibit increased late rupture-related mortality after nonreperfused myocardial infarction.

A, In contrast to mice undergoing reperfused infarction protocols (that exhibit low mortality rates), nonreperfused infarction is associated with a ≈50% mortality in WT mice predominantly because of cardiac rupture. Control Smad3$^{fl/fl}$ mice had a 37.0% survival rate compared with 18.7% for FS3KO animals (P=0.16, n=42–46 mice). In Smad3$^{fl/fl}$ animals, all deaths occurred during the first 5 days after coronary occlusion. Comparison of survival curves for late deaths (after the sixth day) suggested that FS3KO mice had increased late mortality (P=0.007). **B,** Systematic histological analysis of the heart of an FS3KO mouse that died 11 days after coronary occlusion shows H&E staining performed at 9 different levels, sectioned at 250-μm partitions (1–9). The site of rupture is shown (arrows), filled with clot (scale bar=0.5 mm). Higher magnification images (Bi-iv) identify the site of rupture (arrows). Please note the presence of a dilated vascular structure within the healing scar (arrowhead) (scale bar=200 μm). **C**–**F,** Echocardiographic analysis showed that surviving FS3KO mice had comparable LVEDV (**C**) and trends toward higher LVESV (**D**), lower fractional shortening (**E**), and increased LV mass (**F**) (Smad3$^{fl/fl}$: 7 d, n=16; 28 d, n=9; FS3KO: 7 d, n=10; 28 d, n=4; ^^P<0.01 versus corresponding baseline values [**B**]). FS3KO indicates fibroblast-specific Smad3 knockout mice; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVFS, left ventricular fractional shortening; LV mass, left ventricular mass; and S3, Smad3.

of TGF-β in cardiac fibroblasts.[5] Accordingly, we examined whether increased myofibroblast density in FS3KO mice is a result of increased proliferative capacity of infiltrating fibroblasts. Dual immunofluorescence for α-smooth muscle actin (SMA) and ki-67 showed that FS3KO mice had a trend toward increased density of proliferating cells and significantly higher numbers of proliferating myofibroblasts (Figure III in the online-only Data Supplement). Taken together, the findings suggest that fibroblast-specific Smad3 loss may accentuate fibroblast proliferation while perturbing contraction and remodeling of the healing scar.

Effects of Fibroblast-Specific Smad3 Loss on Collagen Deposition in the Infarcted and Remodeling Myocardium

Sirius red staining followed by polarized light microscopy was used to investigate the effects of fibroblast-specific Smad3 loss on collagen deposition in the infarcted and remodeling myocardium. When visualized under polarized microscopy, thicker cross-linked fibers show orange or red birefringence, whereas thinner fibers appear green (Figure IVA through IVP in the online-only Data Supplement). Quantitative analysis showed no significant effects of fibroblast-specific Smad3 loss on collagen deposition in the infarct and remote remodeling myocardium after 7 days of reperfusion. However, after 28 days of reperfusion, FS3KO infarcts had lower levels of thinner green fibers in the infarct zone and higher amounts of green fibers in the remote remodeling myocardium. These findings may reflect the accentuated adverse remodeling in FS3KO animals (Figure IV in the online-only Data Supplement).

Smad3 Loss Impairs Contraction of Fibroblast-Populated Collagen Pads

In healing wounds, transdifferentiated myofibroblasts mediate scar contraction.[14] To explore the role of fibroblast Smad3 in fibroblast-mediated collagen contraction, we used an in vitro assay, in which fibroblasts harvested from the mouse heart populate and contract free-floating collagen pads.[5] First, we examined the effects of

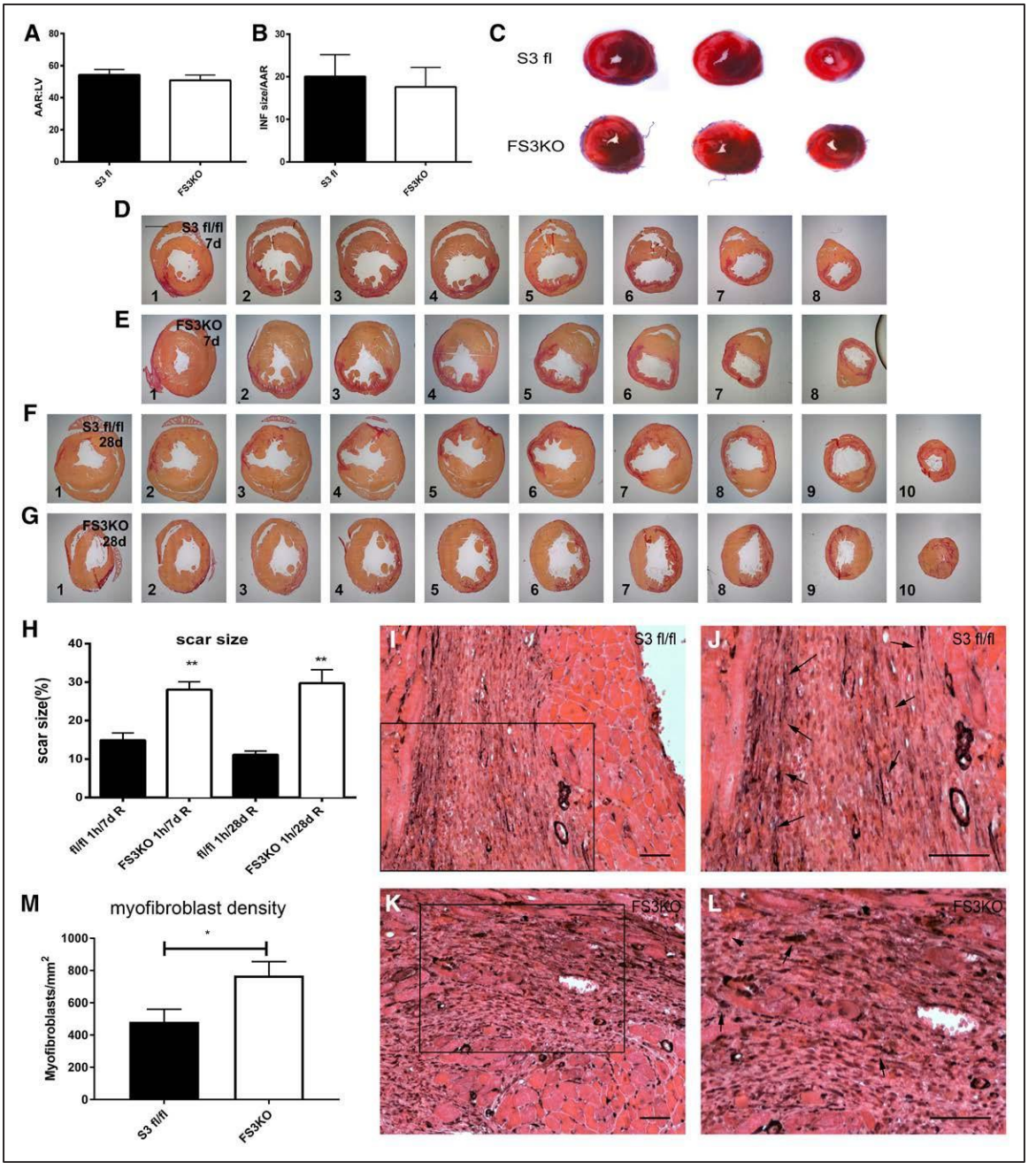

Figure 3. Fibroblast-specific loss of Smad3 does not affect acute infarct size but is associated with larger scars and increased myofibroblast density.

A–**C,** Smad3$^{fl/fl}$Postn-Cre (FS3KO) and Smad3$^{fl/fl}$ mice had comparable area at risk (AAR) (**A**) and infarct size (IS):AAR (**B**) (*P*=NS, n=10–11/group). **D**–**H,** However, despite comparable acute cardiomyocyte injury, FS3KO animals had significantly larger scars after 28 days of reperfusion. Scar size was assessed by sectioning the entire heart from base to apex and by staining for Sirius red the first section from each 300-μm partition. **D**–**G,** Representative Sirius red–stained sections from Smad3$^{fl/fl}$ mouse and an FS3KO animal after 7 days of reperfusion (**D** and **E**, respectively) and after 28 days of reperfusion (**F** and **G**, respectively) (scale bar=1 mm). **H,** Despite similar segmental distribution of the infarct, the size of the collagenous scar was larger in FS3KO animals after 7 and 28 days of reperfusion (****P<0.01 versus Smad3$^{fl/fl}$, n=7–10/group). Fibroblast-specific Smad3 loss impaired contraction and remodeling of the scar. **I**–**L,** α-SMA immunohistochemistry was used to identify myofibroblasts in the infarct border zone after 7 days of reperfusion as α-SMA^{+} cells located outside the vascular media (arrows) (scale bar=60 μm). **M,** Quantitative analysis showed that FS3KO mice had higher myofibroblast density (*P<0.05, n=9/group).

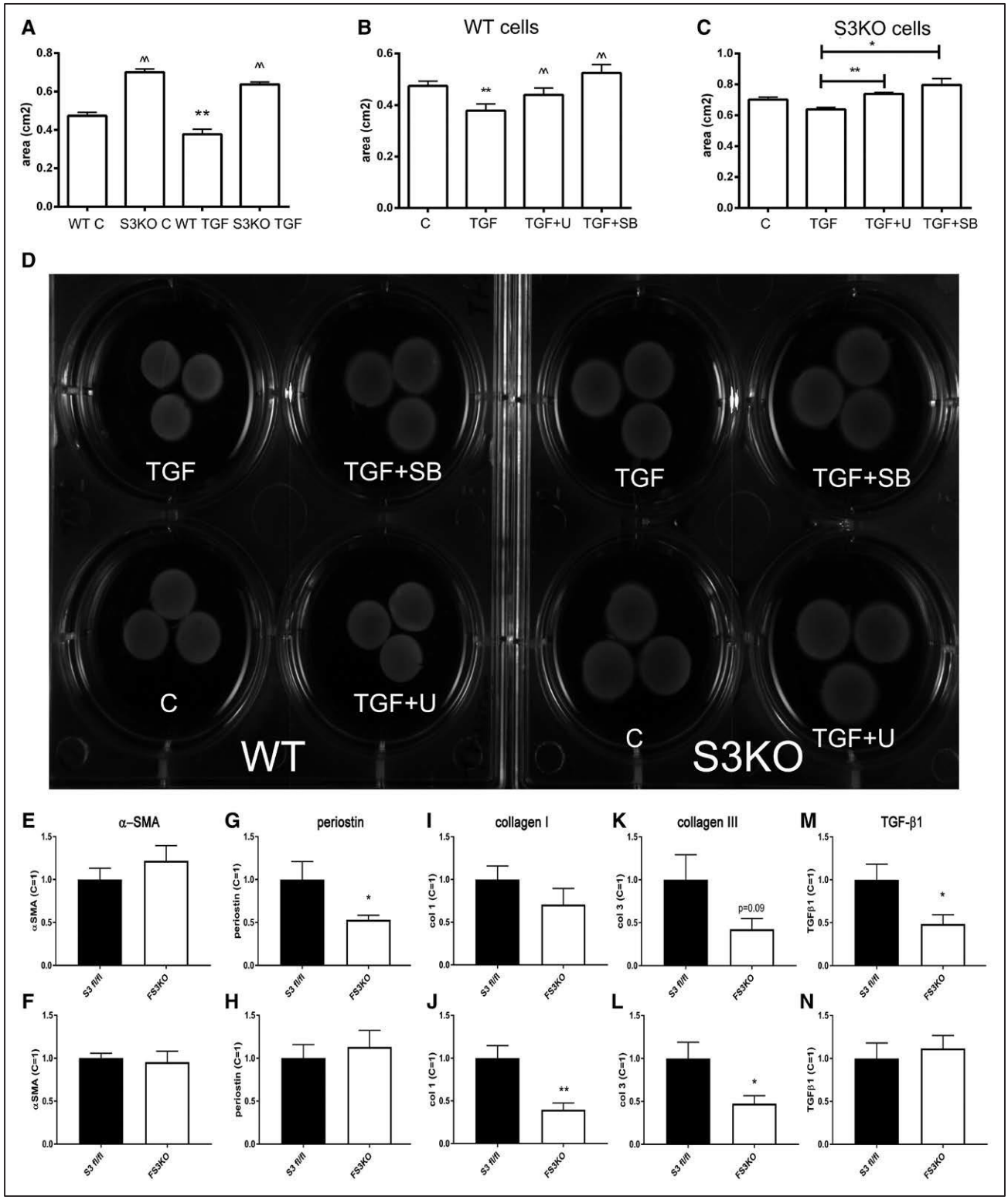

Figure 4. Smad-dependent and Smad-independent pathways mediate fibroblast-induced collagen pad contraction. **A,** Smad3 knockout (KO) fibroblasts exhibited impaired capacity to contract collagen pads (^^*P*<0.01 versus wild-type [WT], n=6). Transforming growth factor (TGF)–β1 increases contraction in WT cells (*******P*<0.01, n=6) but has no significant effects on Smad3 KO cells. **B,** Both Erk inhibition (with U0126) and p38 mitogen-activated protein kinase (MAPK) inhibition (with SB203580) attenuated TGF-β–induced pad contraction (^^*P*<0.01 versus TGF, n=6), **C,** Incubation with U0126 or SB203580 reduced pad contraction in TGF-β–stimulated Smad3 KO cells, suggesting that Erk and p38 MAPK actions are independent of Smad3 (**P*<0.05, *******P*<0.01 versus TGF; n=6). **D,** Representative experiments illustrate the findings. **E**–**N,** Comparison of gene expression in infarct fibroblasts harvested from Smad3$^{fl/fl}$ and Smad3$^{fl/fl}$Postn-Cre (FS3KO) mice after 3 days (*Continued*)

known Smad3 activators on fibroblast-induced pad contraction. Only TGF-β1, TGF-β2, and TGF-β3 increased fibroblast-mediated pad contraction. In contrast, activins A and B, myostatin, and growth differentiation factor–11 had no effects (Figure V in the online-only Data Supplement). Then we examined the role of Smad3 in contraction of fibroblast-populated collagen pads. We obtained Smad3 KO (S3KO) cardiac fibroblasts from mice with global Smad3 loss.[5] S3KO cells had impaired capacity to contract collagen pads at baseline and on stimulation with TGF-β1 (Figure 4A and 4D). To investigate the potential role of Smad-independent pathways, we performed Erk and p38 mitogen-activated protein kinase (MAPK) inhibition experiments. Coincubation with the Erk inhibitor U0126 or the p38 MAPK inhibitor SB203580 significantly attenuated TGF-β1–mediated pad contraction in both WT and S3KO cells (Figure 4B and 4C), suggesting that gel contraction involved both Smad3-dependent and Smad-independent actions.

Effects of Fibroblast-Specific Smad3 Loss on Synthesis of α-SMA and Extracellular Matrix Proteins

Because α-SMA induction and incorporation in the cytoskeleton are implicated in contractile function of myofibroblasts,[15] we hypothesized that perturbed collagen pad contraction by S3KO fibroblasts in vitro and impaired scar remodeling in vivo may be a result of reduced α-SMA expression levels. To examine whether impaired scar contraction in FS3KO mice was a result of attenuated myofibroblast conversion, we compared α-SMA expression levels in infarct myofibroblasts harvested from $S3^{fl/fl}$ and FS3KO mice after 3 and 7 days of reperfusion. $S3^{fl/fl}$ and FS3KO fibroblasts showed comparable levels of α-SMA expression after 3 days (Figure 4E) and 7 days (Figure 4F) of reperfusion. Comparison of extracellular matrix protein expression levels showed that FS3KO cells had lower periostin expression after 3 days of reperfusion and reduced collagen I and III synthesis after 7 days of reperfusion (Figure 4G through 4L). Expression levels of TGF-β1 were lower in infarct fibroblasts harvested at the 3-day time point (Figure 4M and 4N).

Because ED-A fibronectin is critically involved in myofibroblast conversion and activation in response to TGF-β, we examined whether fibroblast-specific Smad3 loss affects fibronectin transcription and localization in the healing infarct. Quantitative polymerase chain reaction analysis showed that fibroblasts harvested from infarcted FS3KO mice after 7 days of reperfusion had significantly lower fibronectin transcription. Moreover, dual immunofluorescence studies showed that FS3KO mice had attenuated ED-A fibronectin immunoreactivity in areas exhibiting myofibroblast infiltration (Figure VI in the online-only Data Supplement).

Next, we examined the effects of Smad3 loss on α-SMA expression by activated cardiac fibroblasts in vitro using 2 different models: cardiac fibroblasts cultured in the high-tension environment of the culture plate and cells cultured in the low-tension environment of the pad. As we have recently demonstrated,[16] high-tension fibroblasts expressed 70- to 80-fold higher α-SMA mRNA levels than low-tension pad fibroblasts (Figure VII in the online-only Data Supplement). Immunofluorescence showed that although high-tension fibroblasts exhibit a myofibroblast phenotype, associated with intense cytoskeletal α-SMA staining, low-tension pad fibroblasts have a dendritic morphology and express low levels of punctate α-SMA staining (Figure VIIB in the online-only Data Supplement). In contrast to the reduction in α-SMA levels noted in high-tension Smad3 KO fibroblasts, Smad3 loss did not affect the low levels of α-SMA expressed by low-tension pad cells (Figure VIIA through VIIC in the online-only Data Supplement). Thus, impaired collagen contraction in pads populated with S3KO cells and perturbed scar contraction in FS3KO mice (Figures 3 and 4) cannot be attributed to attenuated α-SMA expression.

Fibroblast-Specific Smad3 Loss Perturbs Scar Organization

To identify fundamental alterations responsible for defective contractile activity in the absence of Smad3, we compared the morphological characteristics of infarct myofibroblasts between $Smad3^{fl/fl}$ and FS3KO animals. In the infarcted myocardium, myofibroblasts are organized in arrays and exhibit alignment along the direction of the ventricular wall. Fibroblast-specific Smad3 loss resulted in defective organization of the healing scar, associated with perturbed alignment of infarct myofibroblasts that exhibited a more rounded shape and chaotic orientation (Figure 5A through 5F). Quantitative analysis demonstrated that infarct myofibroblasts in FS3KO mice had a comparable mean cell area (Figure 5G) but a smaller perimeter (Figure 5H), lower perimeter/area ratio (Figure 5I), and reduced long axis/short axis ratio (Figure 5J). To compare the alignment of fibroblasts in the healing infarct, we measured the angle between the long axis of the fibroblast and the tangent of the ventricular wall. In FS3KO infarcts, the mean angle

Figure 4 Continued. (**E**, **G**, **I**, **K**, **M**) and 7 days (**F**, **H**, **J**, **L**, **N**) of reperfusion. Fibroblast-specific Smad3 loss did not affect α-SMA mRNA expression (**E** and **F**). FS3KO fibroblasts exhibited lower periostin (**G**) and TGF-β1 (**M**) expression after 3 days of reperfusion and lower levels of collagen I and III mRNA (**J** and **L**) after 7 days of reperfusion (*P<0.05, **P<0.01 versus corresponding $Smad3^{fl/fl}$; n=5–6).

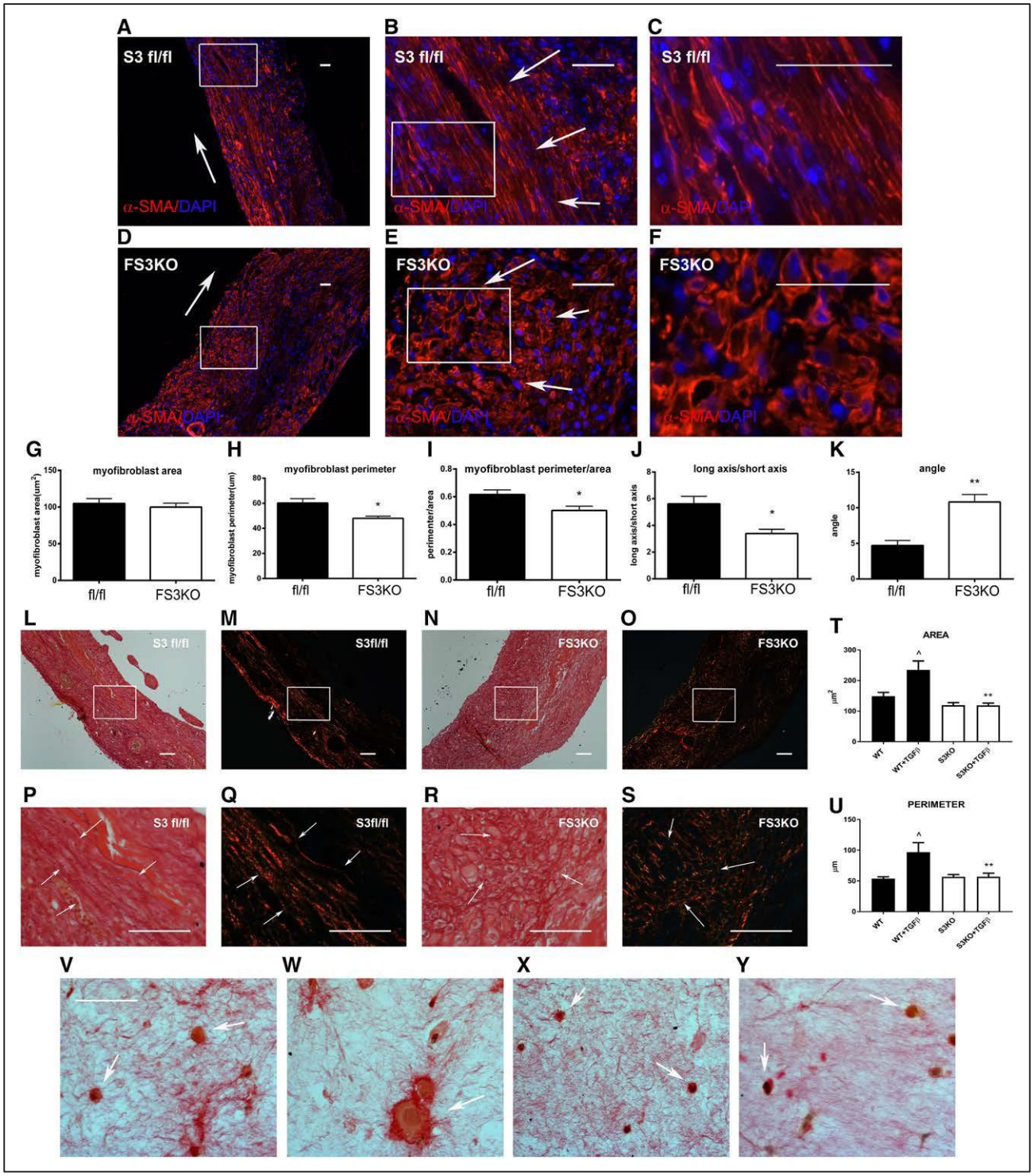

Figure 5. Fibroblast-specific Smad3 loss perturbs scar organization and attenuates transforming growth factor (TGF)–β—mediated changes in cell geometry.

A–**C,** α-SMA staining of healing myocardial infarcts (7 days) shows that, in Smad3$^{fl/fl}$ mice, α-SMA^{+} myofibroblasts are spindle-shaped cells, localized in highly organized arrays (arrows, **B** and **C**). **D**–**F,** In contrast, distribution of α-SMA^{+} myofibroblasts in Smad3$^{fl/fl}$Postn-Cre (FS3KO) mouse infarcts is disorganized and chaotic because the cells have a rounded morphology and are misaligned (arrows, **E** and **F**) (scale bar=30 μm). Although the mean area of the myofibroblasts was comparable between groups (**G**), the cell perimeter (**H**), perimeter/area ratio (**I**), and long axis/short axis ratio (**J**) were lower in FS3KO animals (*P<0.05, n=5), reflecting the altered geometry of the cells. **K,** To quantitatively assess cell alignment in the healing infarct, we measured the angle between the long axis of the cell and the tangent of the ventricular wall (indicated by an arrow in **A** and **D**). The alignment angle was markedly higher in FS3KO mice, reflecting cellular malalignment in the absence (*Continued*)

was significantly higher than in Smad3$^{fl/fl}$ animals (Figure 5K), indicating that fibroblast-specific Smad3 loss resulted in the misalignment of infarct myofibroblasts. Polarized light microscopy of Sirius red–stained sections showed that FS3KO mice also exhibited perturbations in the alignment of collagen fibers (Figure 5L through 5S). In vitro experiments showed that Smad3 loss abrogated the increase in cell area and perimeter induced by TGF-β1 stimulation in cardiac fibroblasts cultured in collagen pads (Figure 5T through 5Y).

Smad3 Mediates α2, α5, and β3 Integrin Expression in Cardiac Fibroblasts

Misalignment of myofibroblasts in FS3KO infarcts suggests impaired interactions between cells and the extracellular matrix. We hypothesized that Smad3 loss may perturb scar organization by altering the expression of integrins, surface proteins that mediate cell–matrix interactions. Accordingly, we examined the effects of Smad3 loss on integrin expression in pad fibroblasts. S3KO fibroblasts had significantly lower baseline expression of α2, α5, and β3 integrin (Figure 6A through 6C). Moreover, TGF-β1 stimulation markedly upregulated α2, α5, and β3 integrin expression in WT cells but not in S3KO fibroblasts (Figure 6A through 6C). In contrast, Smad3 loss did not affect fibroblast α1 and β1 integrin expression (Figure 6D and 6E). To investigate the role of Smad-independent pathways in the regulation of integrin synthesis, we examined the effects of Erk and p38 MAPK inhibition (Figure VIII in the online-only Data Supplement). Erk inhibition attenuated TGF-β1–mediated β3, α2, and α5 integrin mRNA expression but had no effects on β1 and α1 integrin synthesis. p38 MAPK inhibition attenuated α5 integrin expression without affecting synthesis of the other integrins. Erk and p38 MAPK inhibition did not affect integrin expression in Smad3 KO cells, suggesting that the effects of Erk and p38 in WT cells are not independent of Smad3.

α2 and α5 Integrin Mediate Contraction in Fibroblast-Populated Pads

Next we examined whether integrin blockade affects fibroblast function. In the collagen pad assay, α2 and α5 integrin blockade markedly impaired collagen contraction mediated by both WT and S3KO cells (Figure 6F), demonstrating the critical role of integrin signaling in pad contraction.

Smad3-Mediated Integrin Activation Induces NOX2 Transcription in Cardiac Fibroblasts

Reactive oxygen species generation is critical for fibroblast function;[17] activation of integrin signaling stimulates an oxidative response.[18] Accordingly, we examined the role of integrin signaling in the expression of genes associated with reactive oxygen generation and scavenging in cardiac fibroblasts. In WT cells, α5 but not α2 blockade markedly reduced NOX2 expression levels (Figure 6G) but had no effects on superoxide dismutase (SOD)–1, SOD2, and glutathione reductase levels (Figure 6H through 6J). S3KO cells exhibited markedly lower baseline expression of NOX2 (Figure 6G) and significantly higher expression of SOD1 (Figure 6H) but comparable SOD2 and glutathione reductase levels. In contrast to its effects on WT cells, α5 blockade did not affect NOX2 expression in S3KO cells (Figure 6G), suggesting that NOX2 transcription in cardiac fibroblasts is dependent on Smad3-mediated α5 integrin activation.

Integrin α5 Is Not Sufficient for Contraction of Fibroblast-Populated Collagen Pads

Next we performed overexpression experiments to investigate whether defective function of Smad3-null fibroblasts is caused by attenuated α5 integrin synthesis (Figure 6K). Although α5 integrin overexpression increased pad contraction in WT cells, no significant effects were noted in pads populated with Smad3 KO cells, indicating that α5 expression is not sufficient to restore defective function in Smad3 KO fibroblasts (Figure 6L).

Cardiomyocyte-Specific Smad3 Loss Attenuates Adverse Remodeling After Myocardial Infarction

In contrast to the detrimental effects of fibroblast-specific loss of Smad3 (Figure 1), mice with global loss of

Figure 5 Continued. of Smad3 (**P<0.01 versus *fl/fl*, n=5). Perturbed cellular alignment would be expected to reduce the tractional forces exerted by the cells, resulting in impaired scar contraction. **L**–**S,** Both light microscopy (**L**, **N**, **P**, **R**) and polarized light microscopy (**M**, **O**, **Q**, **S**) in Sirius red–stained sections showed that although Smad3$^{fl/fl}$ animals had aligned collagen fibers (**P**–**Q**, arrows), FS3KO animals exhibited areas of matrix disorganization (**R** and **S**, arrows) (scale bar=40 μm). **T**–**Y,** Smad3 loss abrogated the effects of TGF-β1 on fibroblast shape. Fibroblasts from wild-type (WT) (**V** and **W**) and Smad3 knockout (KO) mice (S3KO, **X** and **Y**) were cultured in collagen pads in the presence (**W** and **Y**) or absence (**V** and **X**) of TGF-β1. Sections of fibroblast-populated collagen pads were stained with Sirius red/hematoxylin to identify fibroblasts (arrows) and the extracellular matrix (scale bar=25 μm). TGF-β1 stimulation increased cell area and perimeter in WT but not in S3KO fibroblasts (**T** and **U**) (^P<0.05 versus WT, **P<0.01 versus corresponding WT, n=6–7).

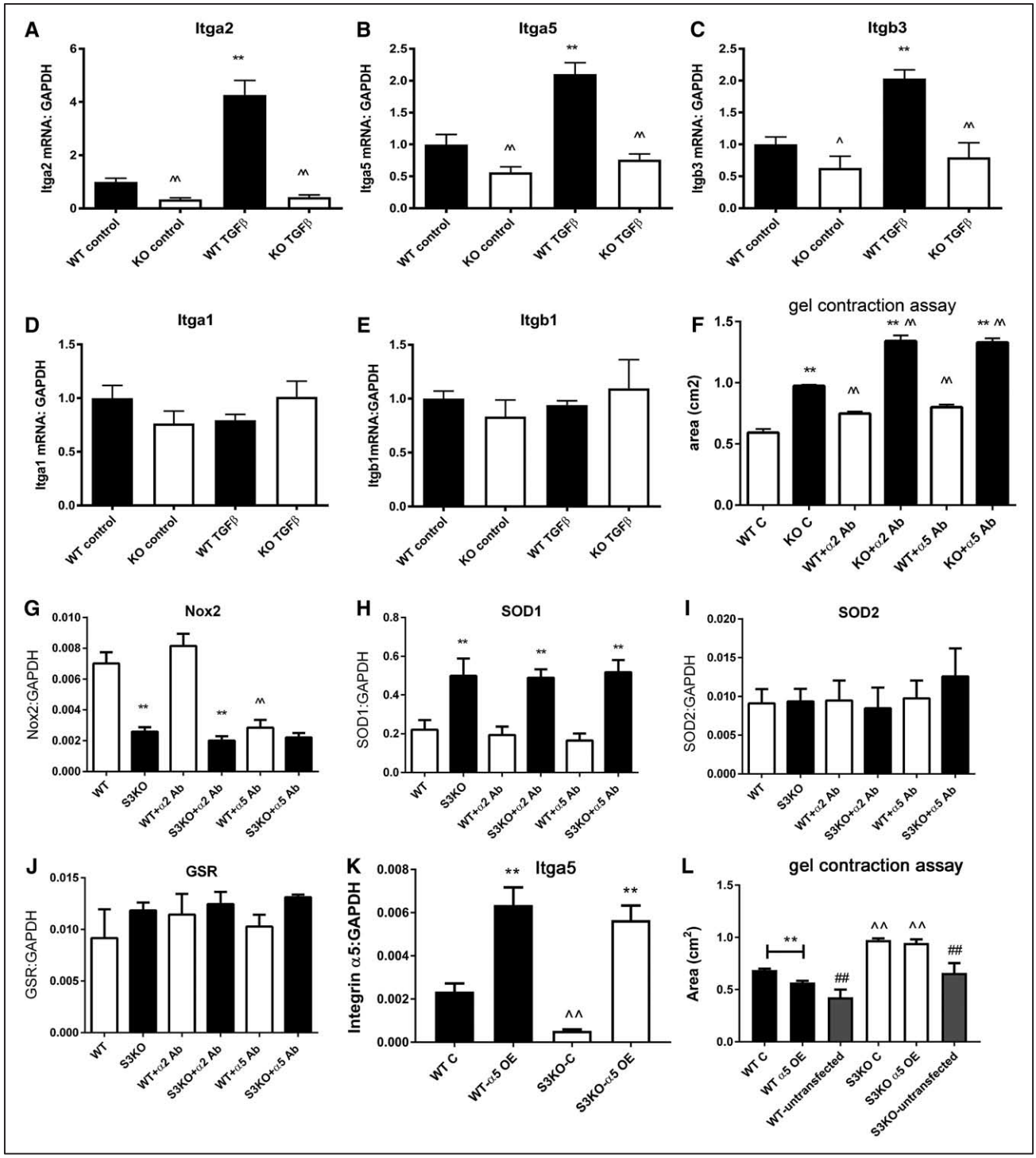

Figure 6. Smad3 loss markedly reduces expression of α2, α5, and β3 integrins in cardiac fibroblasts. **A**–**C,** In comparison with wild-type (WT) cells, Smad3 knockout (KO) cardiac fibroblasts had significantly lower baseline expression of α2 (**A**, Itga2), α5 (**B**, Itga5), and β3 (**C**, Itgb3) mRNA. Transforming growth factor (TGF)–β1 stimulation markedly induced α2, α5, and β3 expression in cardiac fibroblasts in WT cells (**P<0.01 versus WT control, n=6) but not in Smad3 KO cells. α2, α5, and β3 integrin expression levels were significantly lower in Smad3 KO cells in the presence or absence of TGF-β1 (^P<0.05, ^^P<0.01, n=6). **D** and **E,** In contrast, Smad3 loss did not affect α1 (**D**, Itga1) and β1 (**E**, Itgb1) integrin expression (expression normalized to **C**; WT=1). **F**–**J,** Integrins mediate fibroblast activation, inducing expression of NOX2. **F**, α2 and α5 integrin mediate pad contraction in both WT and Smad3 KO cells. α2 or α5 integrin blockade reduces contraction of collagen pads populated with WT or Smad3 KO fibroblasts (^^P<0.01 versus corresponding control, n=6). KO cells exhibit impaired capacity to contract gels in comparison with WT cells (**P<0.01). **G**, α5 blockade attenuates NOX2 mRNA synthesis in WT fibroblasts (^^P<0.01 versus WT control, n=3). In contrast, α2 blockade does not affect NOX2 expression. (*Continued*)

Smad3 exhibit attenuated remodeling.[4] Thus, Smad3 signaling in other cell types involved in cardiac injury and repair may exert detrimental actions on the infarcted heart. Because TGF-β signaling and the Smad3 pathway have been implicated in the regulation of cardiomyocyte function and survival in vitro,[19] we examined the in vivo role of cardiomyocyte-specific Smad3 signaling after myocardial infarction. Smad3$^{fl/fl}$α-myosin heavy chain (MHC)-Cre (CMS3KO) mice were healthy and exhibited normal baseline cardiac geometry and function at 4 months of age (Figure I in the online-only Data Supplement). mRNA analysis showed that when compared with Smad3$^{fl/fl}$ animals, CMS3KO mice had significantly lower Smad3 levels (Figure IXA in the online-only Data Supplement). Dual fluorescence combining Smad3 immunohistochemistry and wheat germ agglutinin lectin histochemistry (to outline cardiomyocytes) showed that CMS3KO mice had reduced Smad3 immunoreactivity in cardiomyocytes (Figure IXB in the online-only Data Supplement). Echocardiographic analysis showed that, when compared with Smad3$^{fl/fl}$ controls, CMS3KO mice had reduced left ventricular dilation, attenuated systolic dysfunction, and decreased hypertrophic remodeling (Figure 7 and Figure X in the online-only Data Supplement) after reperfused infarction.

The Protective Effects of Cardiomyocyte-Specific Smad3 Loss Are Not Caused by a Reduction in Acute Infarct Size

Because attenuated remodeling in the model of reperfused infarction may reflect reduced acute cardiomyocyte injury, we examined the effects of cardiomyocyte-specific Smad3 loss on the size of the infarct. CMS3KO and Smad3$^{fl/fl}$ controls had a comparable area at risk and infarct size/area at risk ratio (Figure XIA through XIC in the online-only Data Supplement). Moreover, cardiomyocyte-specific loss of Smad3 did not significantly affect scar size after 7 to 28 days of reperfusion (Figure XID in the online-only Data Supplement). Quantitative analysis of Sirius red–stained sections visualized under polarized light microscopy demonstrated that CMS3KO mice had reduced deposition of thinner green collagen fibers in the remote remodeling myocardium after 7 days of reperfusion. After 28 days of reperfusion, CMS3KO mice had significantly increased deposition of thicker red collagen fibers in the infarct zone and marked reductions in deposition of orange and green collagen fibers in the remote remodeling myocardium (Figure XIE through XIX in the online-only Data Supplement).

Effects of Cardiomyocyte-Specific Smad3 Loss on Cardiomyocyte Apoptosis After Myocardial Infarction

Next we examined whether protection from adverse remodeling in cardiomyocyte-specific Smad3 KO mice is caused by attenuated cardiomyocyte apoptosis. Dual labeling for terminal deoxynucleotidyl transferase dUTP nick end labeling and wheat germ agglutinin was performed at 2 different time points after 2 and 7 days of reperfusion. No significant difference occurred in the density of apoptotic cardiomyocytes in the infarcted area after 48 hours of reperfusion. After 7 days of reperfusion, cardiomyocyte-specific loss of Smad3 was associated with a modest but significant attenuation in the number of apoptotic cardiomyocytes in the viable remodeling myocardium (Figure 8A and 8B).

CMS3KO Mice Have Reduced NOX2 Expression and Attenuated Nitrosative Stress in the Remodeling Myocardium

Oxidative and nitrosative stress are implicated in cardiomyocyte apoptosis.[20] Because our findings in fibroblasts suggested that Smad3 may regulate fibroblast function stimulating NOX2 expression, we hypothesize that the proapoptotic effects of cardiomyocyte Smad3 may also involve modulation of genes involved in reactive oxygen species generation and scavenging. Quantitative polymerase chain reaction analysis demonstrated that after 7 days of reperfusion, CMS3KO mice had significantly reduced NOX2 levels in the noninfarcted remodeling myocardium (Figure 8C) but comparable NOX4 and SOD1 expression (Figure 8D and 8E). No significant differences were noted in the NOX2, NOX4, and SOD1 levels between infarcted segments. Moreover, cardiomyocyte-specific Smad3 loss also reduced nitrosative

Figure 6 Continued. Smad3 KO cells exhibited markedly lower expression of NOX2 (**P<0.01 versus WT). In contrast to its effects on WT cells, α5 blockade did not affect NOX2 expression in Smad3 KO cells, suggesting that NOX2 transcription in cardiac fibroblasts is dependent on Smad3-mediated α5 integrin activation. **H,** Smad3 KO cells also had a higher expression of superoxide dismutase (SOD)–1 (**P<0.01 versus corresponding WT). However, SOD1 levels were not affected by integrin inhibition. **I** and **J**, SOD2 and GSR expression was not affected by Smad3 loss or integrin blockade. **K** and **L**, α5 integrin overexpression does not reverse the contraction defect in Smad3 KO cells. **K**, α5 integrin overexpression in WT and Smad3 KO cells resulted in markedly increased α5 integrin expression (**P<0.01 versus corresponding controls, ^^P<0.01 versus WT C, n=6). **L**, Although α5 integrin overexpression (OE) increased contraction in WT cells, no effects on pad contraction were noted in Smad3 KO cells, indicating that α5 integrin is not sufficient to correct the contraction defect. Please note that untransfected cells exhibit increased contraction, suggesting that transfection modestly but significantly reduces contraction in fibroblast-populated pads (##P<0.01 versus corresponding cells transfected with control plasmid).

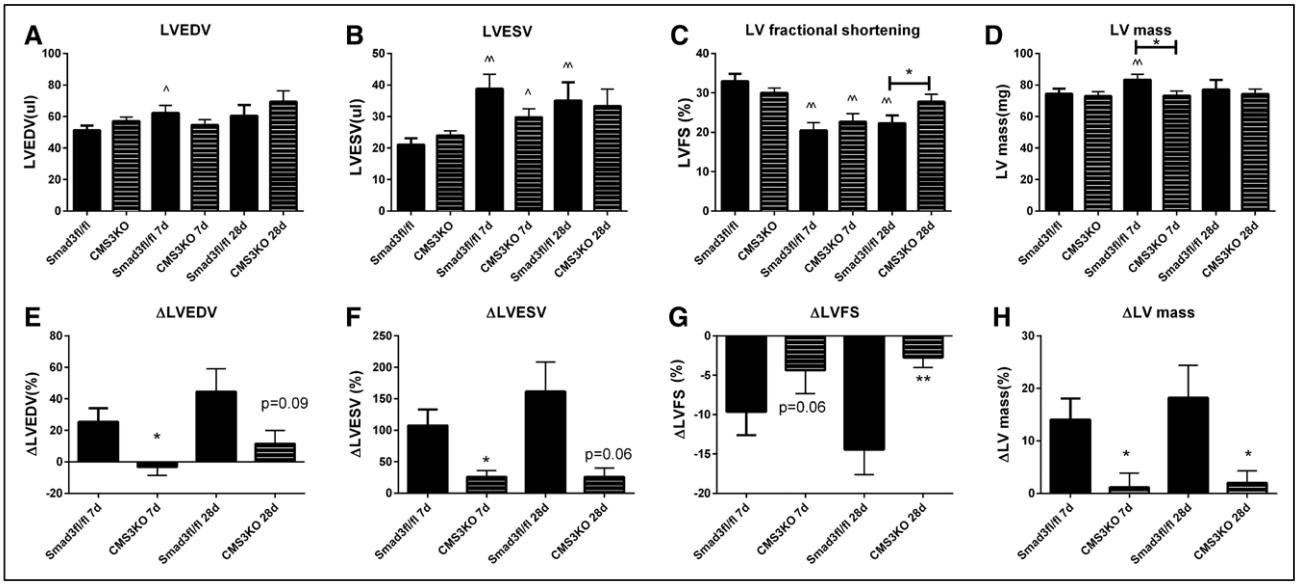

Figure 7. Cardiomyocyte-specific Smad3 loss attenuates systolic dysfunction after infarction and reduces hypertrophic remodeling.

A–**C,** Although LVEDV (**A**) and LVESV (**B**) were comparable between CMS3KO and Smad3$^{fl/fl}$ animals, cardiomyocyte-specific Smad3 loss attenuated systolic dysfunction (28 days) (**C**). **D,** LV mass was lower in CMS3KO mice after 7 days of reperfusion (*P<0.05, n=10–18/group; ^P<0.05, ^^P<0.01 versus corresponding baseline values). **E** and **F,** CMS3KO mice exhibited significantly lower ΔLVEDV and ΔLVESV after 7 days and trends toward reduced ΔLVEDV and ΔLVESV after 28 days of reperfusion, reflecting attenuated dilative remodeling. **G,** ΔLVFS was significantly lower in CMS3KO animals after 28 days, reflecting decreased systolic dysfunction (**P<0.01). **H,** ΔLVmass was markedly attenuated at both time points (*P<0.05).

stress after myocardial infarction. Western blotting demonstrated that CMS3KO animals had markedly reduced 3-nitrotyrosine levels in the noninfarcted remodeling myocardium and the infarcted area (Figure 8F and 8G).

CMS3KO Mice Have Reduced MMP2 Levels in the Infarct and in the Noninfarcted Remodeling Myocardium

Induction and activation of MMPs play important roles in adverse remodeling of the infarcted heart.[21,22] Because TGF-β signaling pathways are involved in the regulation of MMPs,[23,24] we examined whether cardiomyocyte-specific Smad3 loss affects MMP2 expression. CMS3KO mice had markedly lower levels of latent and active (cleaved) MMP2 in the infarcted segment and in the noninfarcted remodeling myocardium (Figure 8H through 8J).

DISCUSSION

We report for the first time that cell-specific activation of Smad3 signaling in cardiomyocytes and cardiac myofibroblasts differentially regulates repair and remodeling of the infarcted heart. Using cell-specific Smad3 KO mice and in vitro studies, we demonstrate that fibroblast-specific Smad3 signaling is crucial for scar organization, mediating the formation of aligned myofibroblast arrays in the infarct border zone. Smad3 restrains fibroblast proliferation and stimulates integrin-dependent fibroblast activation and NOX2 transcription. In contrast, Smad3 actions in cardiomyocytes are detrimental, accentuating adverse remodeling and worsening systolic dysfunction after myocardial infarction. Cardiomyocyte-specific Smad3 does not act by increasing acute infarct size but accentuates NOX2 transcription, augments nitrosative stress, and increases MMP2 expression in noninfarcted remodeling myocardial segments. These actions may stimulate cardiomyocyte apoptosis and trigger MMP-dependent degradation of contractile proteins, thus promoting dysfunction. Our findings dissect the cell-specific actions of TGF-β–mediated Smad signaling in injury and repair and highlight the crucial role of activated cardiac myofibroblasts in protecting the infarcted heart (Figure 9).

Myofibroblast Activation in Repair and Remodeling of the Infarcted Heart

The adult mammalian heart has negligible endogenous regenerative capacity. After myocardial infarction, sudden death of a large number of cardiomyocytes triggers an inflammatory response that clears the wound of dead cells and activates fibroblasts and vascular cells, resulting in the formation of granulation tissue. During the early hours after reperfused myocardial infarction, the abundant cardiac fibroblasts that reside in the ischemic myocardium respond to the proinflammatory environment of

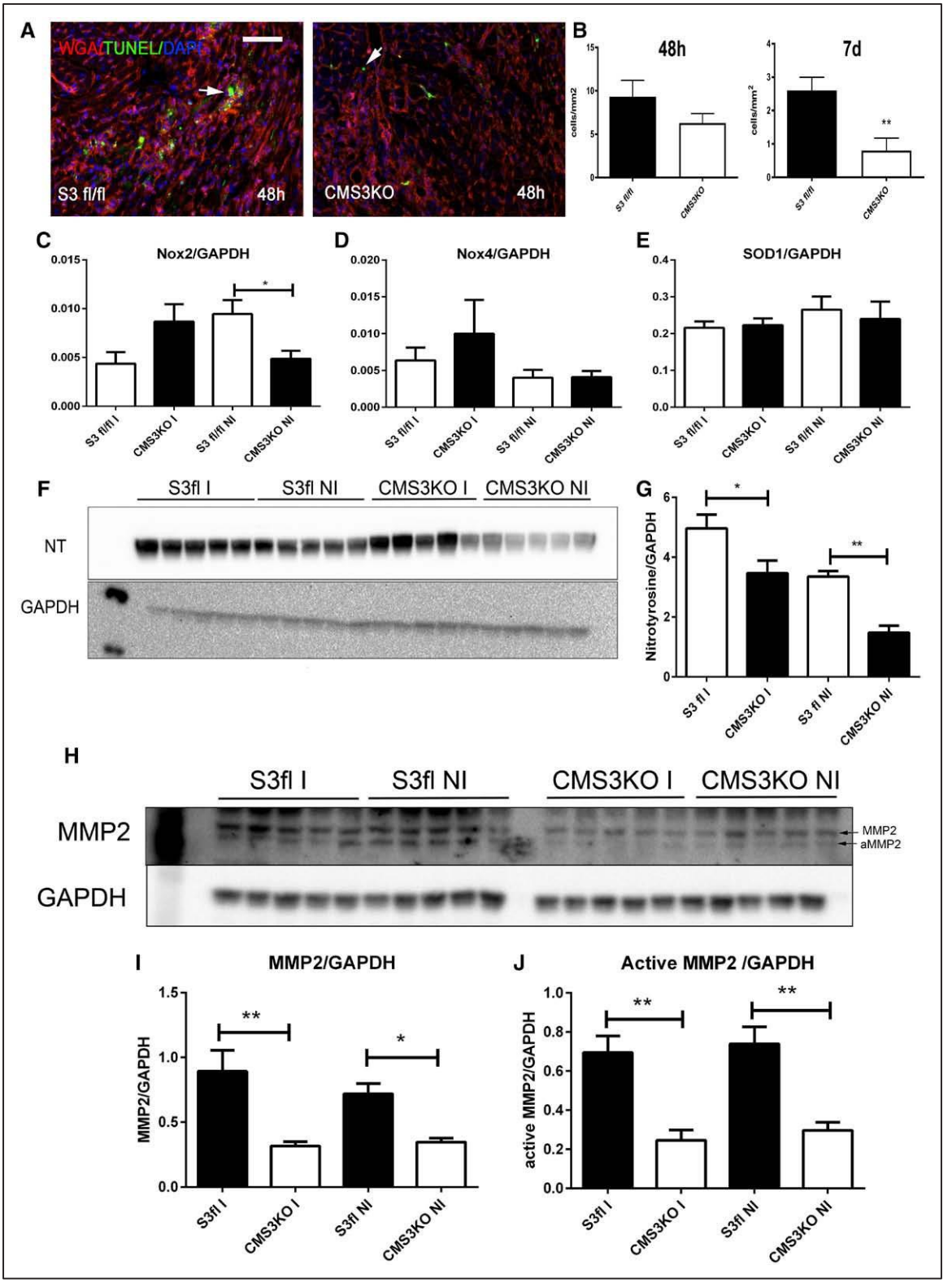

Figure 8. Cardiomyocyte-specific Smad3 loss attenuated cardiomyocyte apoptosis in the viable remodeling myocardium, reduced NOX2 expression, and decreased nitrosative stress and matrix metalloproteinase–2 (MMP2) expression.

A and **B**, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)/Wheat Germ Agglutinin (WGA) dual staining was used to identify apoptotic cardiomyocytes in the infarcted myocardium after 48 hours of reperfusion and in the viable remodeling myocardium after 7 days of reperfusion (scale bar=50 μm). No statistically significant difference in the density of apoptotic cardiomyocytes was noted after 48 hours of reperfusion (*P*=0.21, n=6–7/group). After 7 days of (*Continued*)

the infarct by releasing cytokines and chemokines and by secreting MMPs.[25] Induction of interleukin-1β in the infarct zone inhibits myofibroblast conversion,[25] preventing premature matrix deposition until the wound is cleared of dead cells and matrix debris. Debridement of the wound and phagocytosis of apoptotic cells by professional macrophages are associated with activation of inhibitory cascades that suppress inflammation while stimulating the reparative properties of fibroblasts. During the proliferative phase of infarct healing, the cardiac fibroblast population is enriched through the proliferation of resident fibroblasts via endothelial to mesenchymal transition and through recruitment of circulating fibroblast progenitors.[26,27] Fibroblasts in the healing infarct acquire a matrix-synthetic myofibroblast phenotype[28] and form highly organized arrays in the infarct border zone.[29] Experimental studies have suggested that activated cardiac myofibroblasts may serve reparative functions[30] but may also promote diastolic dysfunction by secreting extracellular matrix proteins in the cardiac interstitium[4] and stimulating cardiomyocyte hypertrophy.[31] Whether reparative and profibrotic fibroblast functions are mediated through distinct molecular pathways remains unknown.

Fibroblast-Specific Smad3 Signaling Protects the Infarcted Heart From Adverse Remodeling, Playing a Critical Role in Activation and Topographical Organization of the Fibroblast-Based Scar

TGF-β is induced and activated in the healing infarct[1,32] and is ideally suited to act as a key regulator of the myofibroblast phenotype. In vitro, TGF-β activates myofibroblast conversion and promotes a matrix-synthetic phenotype,[33] exerting actions mediated through Smad-dependent and Smad-independent pathways.[5,34,35] Our findings demonstrate for the first time that cell-specific activation of Smad3 signaling in infarct myofibroblasts is of critical significance for cardiac repair. Myofibroblast-specific loss of Smad3 accentuates adverse remodeling and dysfunction in a model of reperfused myocardial infarction and increases mortality, causing late rupture in a model of nonreperfused infarction (Figures 1 through 3). The deleterious effects of myofibroblast-specific Smad3 loss are not caused by effects on the size of the acute infarct but involve disruption of key reparative functions that regulate scar organization and remodeling.

Our in vivo and in vitro findings suggest that fibroblast Smad3 signaling mediates formation of an organized scar by restraining fibroblast proliferation and stimulating integrin-dependent fibroblast activation, fibroblast-derived collagen synthesis, and subsequent formation of well-aligned arrays of myofibroblasts in the infarct border zone (Figure 9). The phenotypic and functional alterations of Smad3-null infarct myofibroblasts are associated with perturbed organization of the collagen fibers in the healing scar (Figure 5). Smad3 loss in fibroblasts does not affect the expression of α-SMA by activated infarct myofibroblasts in vivo (Figure 4) but is associated with a hyperproliferative fibroblast phenotype and perturbed contraction of the collagenous matrix both in vitro and in vivo (Figures 3 and 4). Defective contraction of the scar in mice with fibroblast-specific loss of Smad3 may accentuate adverse remodeling by increasing the length of the infarcted segment, thus promoting chamber dilation. Optimal force transmission in the healing infarct is dependent on the presence of well-aligned arrays of fibroblasts in the infarct border zone. In contrast, chaotic orientation of infarct myofibroblasts in the absence of Smad3 may result in perturbed wound contraction and adverse ventricular remodeling.

Smad3 is critically involved in the regulation of fibroblast–matrix interactions by inducing the expression of integrins, the molecular anchors that bridge the cells to the extracellular matrix.[18,36,37] Smad3 loss in fibroblasts markedly reduces the synthesis of α2, α5, and β3 integrins (Figure 6), disrupting interactions between cells and the extracellular matrix, which are critical for fibroblast function and wound contraction. The role of integrin-dependent actions in mediating Smad-dependent profibrotic actions has been recently suggested in a model of renal fibrosis.[38] Our findings suggest that the loss of α2 or α5 integrin–dependent signaling inhibits fibroblast-mediated contraction of the collagenous matrix (Figure 6F) and perturbs fibroblast organization in the healing scar. Integrin-mediated adhesion triggers an oxidative response (Figure 6G) and may regulate actin polymerization[18,39] and extracellular matrix remodeling[40] in cardiac fibroblasts. However, α5 integrin overexpression in Smad3 KO fibroblasts was not sufficient to induce contraction in pads populated with Smad3 KO cells (Figure 6L), suggesting that restoration of fibroblast function may require cooperation of additional molecular signals. A growing body of evidence suggests that Smads interact with other intracellular effectors, such

Figure 8 Continued. reperfusion, cardiomyocyte-specific Smad3 loss attenuated cardiomyocyte apoptosis in the viable remodeling myocardium (**P<0.01 versus S3$^{fl/fl}$, n=6). **C**, CMS3KO mice had reduced NOX2 mRNA expression in the noninfarcted remodeling myocardium (NI) after 7 days of reperfusion (*P<0.05, n=5–9/group). **D** and **E,** NOX4 (**D**) and superoxide dismutase (SOD)–1 (**E**) levels were comparable between CMS3KO and Smad3$^{fl/fl}$. NOX2, NOX4, and SOD1 expression in infarcted segments (I) was comparable between groups (**C**–**E**). **F** and **G,** CMS3KO mice exhibited reduced 3-nitrotyrosine levels in infarcted (I) and noninfarcted (NI) areas, reflecting attenuated nitrosative stress (*P<0.05, **P<0.01, n=5/group). **H**–**J,** Moreover, CMS3KO animals had decreased levels of total and active MMP2 (aMMP2) (*P<0.05, **P<0.01, n=8/group).

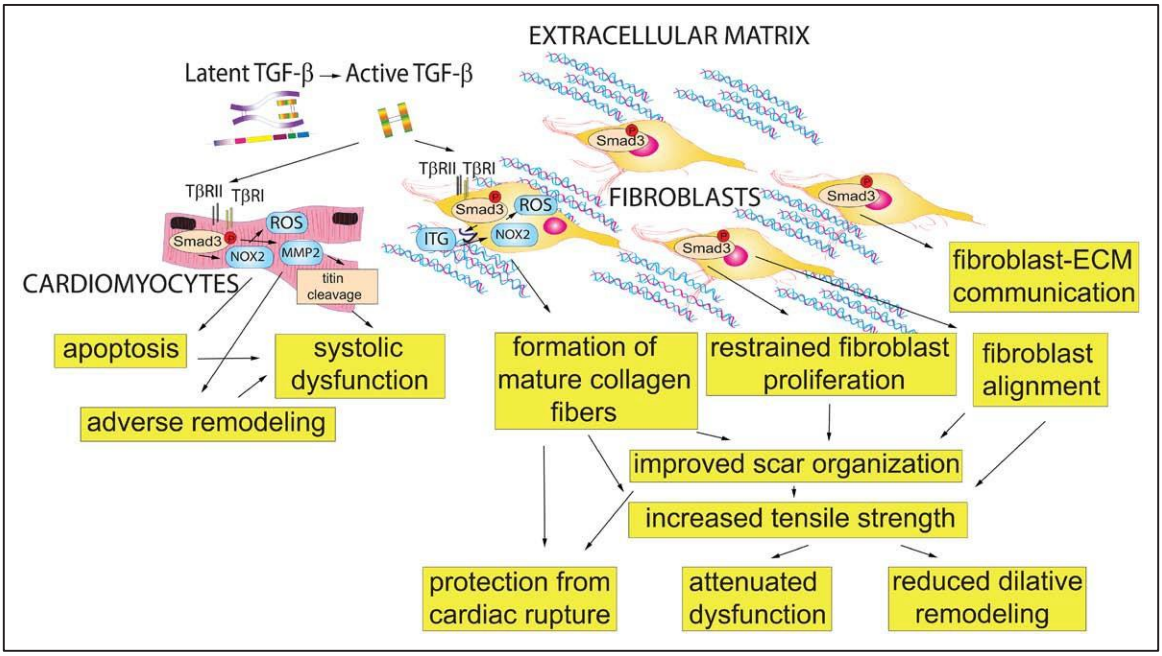

Figure 9. Schematic illustration of the novel findings of the study.
Generation of active transforming growth factor (TGF)–β in the infarcted myocardium triggers Smad3 activation in fibroblasts and cardiomyocytes. Our study investigates for the first time the role of fibroblast and cardiomyocyte-specific Smad3 signal- ing in the infarcted myocardium. Smad3 activation in cardiac fibroblasts restrains cell proliferation and controls alignment of fibroblasts in the infarct and the formation of an organized collagen-based scar, preventing late cardiac rupture and adverse dilative remodeling. Our findings suggest that Smad3-dependent activation of a novel integrin (ITG)-NOX2 axis may stimulate extracellular matrix (ECM) protein deposition and organization in the infarcted heart. In contrast, activation of Smad3 signaling in cardiomyocytes has deleterious effects, promoting cardiomyocyte apoptosis and enhancing adverse remodeling and dysfunction. The effects of Smad3 on cardiomyocytes may also involve activation of a NOX2-mediated reactive oxygen species (ROS)-dependent axis. However, in cardiomyocytes, oxidative stress may promote cell death and accentuate MMP2 expression. Overactive MMP2 may cause adverse remodeling through its effects on the ECM and may exacerbate systolic dysfunction by targeting proteins involved in sarcomere function, such as titin.

as signal transducer and activator of transcription 3, to modulate the fibroblast phenotype.[41]

Cardiomyocyte Smad3 Signaling Accentuates Cardiac Remodeling

Cardiomyocytes in the infarct border zone and remodeling myocardium also exhibit activation of TGF-β/Smad-dependent signaling[4] and are highly responsive to the effects of TGF-β. In vitro experiments have suggested both proapoptotic and antiapoptotic effects of TGF-β on cardiomyocytes,[42,43] highlighting the context-dependent actions of the cytokine. In vivo studies using conditionally targeted mice suggested that, in the infarcted myocardium, cardiomyocyte-specific TGF-β signaling suppresses the expression of protective cytokines, enhancing inflammatory leukocyte infiltration and causing cardiac rupture.[44] Our study demonstrates that, in contrast to the critical reparative role of fibroblast Smad3, cardiomyocyte-specific Smad3 signaling is implicated in chronic adverse remodeling of the ventricle. Cardiomyocyte-specific loss of Smad3 had no effect on baseline cardiac geometry and function but attenuated adverse remodeling and dysfunction after reperfused myocardial infarction (Figure 7). The protective actions of cardiomyocyte Smad3 disruption were not caused by effects on the size of the infarct but reflected attenuated cardiomyocyte apoptosis in the noninfarcted remodeling segments, associated with reduced NOX2 expression levels, decreased nitrosative stress, and significantly lower MMP2 levels (Figure 8). Attenuated MMP2 expression in CMS3KO infarcts is associated with the formation of a scar rich in thick collagen fibers that may increase tensile strength, preventing dilation and adverse remodeling of the ventricle. However, the effects of MMP2 may not be limited to remodeling of the extracellular matrix network. In cardiomyocytes, increased oxidative and nitrosative stress mediates cardiomyocyte apoptosis[45] and may also augment MMP expression.[46] Myocardial MMP2 expression after ischemic injury is known to induce cardiac dysfunction through effects independent of extracellular matrix proteolysis.[47] In the ischemic and reperfused myocardium, MMP2 cleaves titin, promoting systolic dysfunction.[48] Thus, the distinct effects of fibroblast- and cardiomyocyte-specific Smad3 responses on the infarcted heart may reflect the consequences of

cell-specific activation of a Smad-dependent oxidative response. In fibroblasts, integrin-mediated oxidative activity may be important for cardiac repair. In contrast, in cardiomyocytes, Smad-dependent oxidative stress may induce dysfunction by stimulating MMP-mediated degradation of contractile proteins (Figure 9).

Targeting the TGF-β/Smad System in Cardiac Remodeling

Despite its critical involvement in the pathogenesis of cardiac remodeling,[44,49] TGF-β remains a challenging therapeutic target. A recently published study showed that in the pressure-overloaded heart, fibroblast-specific Smad3 signaling critically contributes to the fibrotic process,[50] suggesting that interventions targeting Smad-dependent signaling may be effective in patients with chronic heart failure by attenuating fibrosis. In contrast, our findings highlight the reparative function of fibroblasts in infarctive myocardial injury. Because the adult mammalian heart lacks regenerative capacity, after myocardial infarction, activated fibroblasts serve a critical reparative role, preserving the structural integrity of the ventricle and protecting from adverse remodeling. Moreover, the contrasting functional consequences of fibroblast and cardiomyocyte-specific Smad3 loss in the infarcted heart illustrate the cellular specificity of TGF-β/Smad3–dependent actions. Because most cell types are highly responsive to the effects of TGF-β family members, therapeutic targeting of TGF-β in pathological conditions is likely to interfere with both detrimental and beneficial actions. The complexity of TGF-β signaling, involving both Smad-dependent and Smad-independent actions, further complicates the design of therapeutic interventions. Dissection of cell-specific actions in vivo using genetic tools and design of interventions with specific cellular targets are needed for the development of safe and effective therapies.

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Disclosures

None.

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Opposing Actions of Fibroblast and Cardiomyocyte Smad3 Signaling in the Infarcted Myocardium

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SUPPLEMENTAL MATERIAL

Opposing actions of fibroblast and cardiomyocyte Smad3 signaling in the infarcted myocardium

Kong et al. Cell-specific actions of Smad3 in infarction

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SUPPLEMENTAL METHODS

Generation of mice with myofibroblast and cardiomyocyte-specific loss of Smad3: In order to study the role of Smad3 in fibroblasts in the infarcted myocardium, we generated mice with loss of Smad3 in activated infarct myofibroblasts (FS3KO). We used a transgenic mouse line in which Cre recombinase was driven by a 3.9-kb mouse *Postn* promoter [1,2]. Periostin, which is encoded by *Postn*, is not expressed in cardiomyocytes, vascular cells, hematopoietic cells or quiescent cardiac fibroblasts [3, 4], but is upregulated in injury site-fibroblasts in infarcted and in pressure-overloaded hearts [4,5]. Postn-Cre mice were bred with Smad3 fl/fl mice[6] to generate Postn-Cre;Smad3 fl/fl animals and corresponding Smad3 fl/fl controls. In order to generate mice with cardiomyocyte-specific loss of Smad3 (CMS3KO), Smad3 fl/fl mice were crossed with α-MHC-Cre transgenic animals [7]. 75 Smad3 fl/fl mice, 73 FS3KO mice and 36 CMS3KO mice underwent in vivo experimentation.

Mouse models of myocardial infarction: Animal studies were approved by the Institutional Animal Care and Use Committee at Albert Einstein College of Medicine and conform with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. A closed-chest model of reperfused myocardial infarction and a model of non-reperfused infarction were used, as previously described by our group [8,9,10,11]. Female and male mice, 2-4 months of age, were anesthetized using inhaled isoflurane (4% for induction, 2% for maintenance). Smad3 fl/fl, FS3KO, CMS3KO mice used for in vivo experiments were from our own colonies. For analgesia, buprenorphine (0.05-0.2 mg/kg s.c) was administered at the time of surgery and q12h thereafter for 2 days. Additional doses of analgesics were given if the animals appeared to be experiencing pain (based on criteria such as immobility and failure to eat). Intraoperatively, heart rate, respiratory rate and electrocardiogram were continuously monitored and the depth of anesthesia was assessed using the toe pinch method. The left anterior descending coronary artery was occluded for 1h then reperfused for 7d, or 28d. To assess cardiac function and remodeling following myocardial infarction, animals underwent baseline and pre-sacrifice echocardiographic analysis (ischemia/reperfusion: Smad3 fl/fl 7 days, n=19; FS3KO 7 days, n=26; CMS3KO 7d, n=16; Smad3 fl/fl 28 days, n=9; FS3KO 28 days KO n=10; CMS3KO 28 days, n=10 - permanent occlusion model: Smad3 fl/fl 7 days, n=16; FS3KO, n=10; Smad3 fl/fl 28 days, n=9; FS3KO

28 days, n=4). At the end of the experiment, euthanasia was performed using 2% inhaled isoflurane followed by cervical dislocation. Early euthanasia was performed with the following criteria, indicating suffering of the animal: weight loss>20%, vocalization, dehiscent wound, hypothermia, clinical signs of heart failure (cyanosis, dyspnea, tachypnea), lack of movement, hunched back, ruffled coat, lack of food or water ingestion.

Acute infarct size assessment: To assess the size of acute infarcts, the Evans blue-TTC staining method was used as previously described [12], [13]. Smad3 fl/fl mice (n=11), FS3KO (n=10) and CMS3KO animals (n=9) underwent left anterior descending (LAD) coronary ischemia and reperfusion protocols and the heart was harvested after 24h of reperfusion. The aorta was cannulated, the LAD was reoccluded and 1% Evans Blue was perfused into the aorta. Subsequently the heart was sectioned from based to apex into 1mm sections. The sections were stained with 1.5% TTC. After TTC staining the viable myocardium is brick red, whereas the infarct appears pale white. All sections were imaged. The area at risk (AAR) and the area of infarction was assessed planimetrically using Image Pro software. The total volume of the AAR and the infarct area were calculated and expressed as a percentage of the total volume of the ventricle. AAR, infarct size and the infarct size/AAR ratio were compared between FS3KO, CMS3KO and Smad3 fl/fl mice.

Echocardiography: Echocardiographic studies were performed before instrumentation, 7 and 28 days after coronary occlusion using the Vevo 2100 system (VisualSonics. Toronto ON), as previously described [14]. Long-axis B-mode was used to assess the geometric characteristics of the left ventricle after myocardial infarction. Short-axis M-mode was used for measurement of systolic and diastolic ventricular and wall diameters. The left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), LVESV, and LVEDV were measured as indicators of dilative remodeling. Left ventricular mass (LV mass) was measured as an indicator of hypertrophic remodeling. Fractional shortening (FS = [LVEDD - LVESD] ×100/LVEDD) was calculated for assessment of systolic ventricular function. The percent change in these parameters after infarction was quantitatively assessed using the following formulas: ΔLVEDD=(LVEDD 7 days or 28 days-LVEDD pre)×100/LVEDD pre, ΔLVESD=(LVESD 7 days or 28 days-LVESD pre)×100/LVESD pre, ΔFS=(FS

pre-FS 7 days or 28 days)×100/FS pre, ΔLVESV=(LVESV 7 days or 28 days-LVESV pre)×100/LVESV pre, ΔLVEDV=(LVEDV 7 days or 28 days-LVEDV pre)×100/LVEDV pre.

Immunohistochemistry and histology: For histopathological analysis murine hearts were fixed in zinc-formalin (Z-fix; Anatech, Battle Creek, MI), and embedded in paraffin. Sections were cut at 5 μm. In order to identify myofibroblasts in the infarct, sections were stained with an anti-α-SMA antibody (Sigma, St. Louis, MO) as previously described [11]. Myofibroblasts were identified as spindle-shaped α-SMA-positive cells located outside the vascular media. Smad3 immunofluorescence was performed using an anti-Smad3 antibody (Novus Biologicals). Endpoints reflecting myofibroblast size, shape and polarization were assessed using ImagePro software. Picrosirius red staining was used to label the collagen-based scar and collagen content was quantitatively assessed in the infarct region, peri-infarct area (<250μm from the area of replacement fibrosis) and remote remodeling myocardium using ImagePro software. Infarcted hearts were sectioned from base to apex at 250 μm intervals, thus reconstructing the whole heart, as previously described [9]. One section from each interval was stained for collagen. Scar size was measured by quantitating the scar area in relation to the total left ventricular area.

Identification of apoptotic cardiomyocytes cells in infarcted hearts was performed using the DeadEnd™ Fluorometric TUNEL System (Promega, Cat# G3250) and WGA staining (to visualize cardiomyocytes by labeling cell membranes and the surrounding extracellular matrix)[15]. Two different timepoints were studied: 1h ischemia 48 h reperfusion to assess apoptosis in cardiomyocytes in the ischemia area, and 1h of ischemia/7 days of reperfusion to assess apoptosis in the viable remodeling myocardium (n=7/group). Cardiomyocyte size in the non-infarcted remodeling myocardium was assessed using WGA-stained sections in order to measure the area of 50 cardiomyocytes cut in cross-section from each mouse, followed by assessment of mean cardiomyocyte area, as previously described[9]. Proliferating cells were identified using immunohistochemical staining with a rat anti-mouse ki67 antibody (eBioscience) as previously described[16]. Proliferating myofibroblasts were identified using dual fluorescence for ki67 and α-SMA, as spindle shaped α-SMA+ cells located outside the vascular

media. Quantitative analysis was performed by counting the number of proliferating cells in 12 fields from 3 different sections from each animal.

Dual immunofluorescence: F**o**r studies examining the localization of cellular FN, dual immunofluorescence combining EDA FN/α-SMA was performed. Myofibroblasts were identified by staining with a rabbit antibody to α- smooth muscle actin (α-SMA) (Novus Biological, NB600-531) (1:100) as spindle-shaped cells located outside the vascular media. Purified anti-EDA mouse monoclonal DH1 antibody (MAB1940, EMD Millipore) was used to localize EDA Fibronectin (1:200) using a M.O.M Basic Kit (BMK-2202, Vector Laboratories).

Polarized light microscopy for assessment of collagen content: Polarized light microscopy was used to assess collagen deposition, as previously described by Whittaker et al. [17]. Briefly, paraffin sections (5 µ thick) were stained using picrosirius red. Circularly polarized images were obtained using Axio Imager M2 for polarized light microscopy (Zeiss). When the collagen fibers are stained with picrosirius red and viewed with polarized light, depending on the thickness of the collagen fibers, the hues range from green to yellow to orange to red. The respective proportions of different hues were assessed using Image J software. The 8-bit hue images contain 256 colors and we distinguished different colors based on visible hues. We used the following hue definitions; red 2-9 and 230-256, orange 10-38, yellow 39-51, green 52-128. The number of pixels within each hue range was expressed as a percentage of the total number of collagen pixels, which in turn was expressed as a percentage of the total number of pixels in the image. For quantitation, 7-10 mice from each group (S3 fl/fl, FS3KO and CMS3KO) from 7 d and 28d I/R were studied. Infarct was assessed at 3-5 different levels for each mouse and at least 16-25 different fields from the infarct zone and 6-8 fields from the remote areas per mouse were used to quantitate polarized collagen.

Isolation and culture of cardiac fibroblasts: Fibroblasts were isolated from normal mouse hearts as previously described [18],[13], then cultured in collagen pads. Smad3 -/- mice (from our own colony) [19],[15] were used to harvest cardiac fibroblasts along with corresponding C57BL6J littermates. Cells were stimulated with TGF-β1 (10ng/nl) for 24h.

Isolation of fibroblasts and myeloid cells from infarcted hearts: Macrophages and fibroblasts were isolated from infarcted hearts for RNA extraction as previously described [4]. Briefly, single cell suspensions were obtained from infarcted hearts. Cells were reconstituted with MACS buffer ((Miltenyi Biotec)) and total cell numbers were determined with trypan Blue. 10^7 cells in 90ul MACS buffer were incubated with 10ul CD11b microbeads (Miltenyi Biotec cat. 130-049-601) at 4°C for 15 min; and then washed once and centrifuged. Resuspended cells went through a MACS Column (Miltenyi Biotec) set in a MACS Seperator (Miltenyi Biotec). The magnetically labeled CD11b+ cells were retained on the column. Approximately 2 ml of MACS buffer was applied onto the column. Cells were flushed out by firmly pushing the plunger and collected into a tube. Unlabeled cells that passed through were collected and washed once with PBS as the CD11b- fraction was plated on cell culture plates; adherent cells (predominantly fibroblasts) were harvested for RNA isolation. Gene expression in fibroblasts harvested from Smad3 fl/fl and FS3KO mice was compared after 3 and 7 days of reperfusion (n=6/group)

Collagen pad contraction assay: WT and Smad3 KO cardiac fibroblasts were were cultured to passage 2 and serum-starved overnight (16 hrs). Collagen matrix was prepared by diluting a stock solution of rat 3.0 mg/ml collagen I (GIBCO Invitrogen Corporation, Carlsbad, CA) with 2x DMEM and distilled water for a final concentration of 1 mg/ml collagen. Cell suspensions were mixed with collagen solution to achieve the final $3*10^5$ cells/ml concentration. Subsequently, 500 µl of this suspension was aliquoted to a 24-well culture plate (BD Falcon, San Jose, CA) and allowed to polymerize at 37ºC for 30 min. Following polymerization, the pads were released from the wells, transferred to 6-well culture plate (BD Falcon, San Jose, CA) and cultured in 0% FCS DMEM/F12 or in presence of TGF-β1 (10ng/ml), TGF-β2 (10ng/ml), TGF–β3 (10ng/ml), activin A (50ng/ml), activin B (50ng/ml), myostatin (100ng/ml), or GDF11 (10ng/ml) (all from R&D systems) for 24 h. At 0 h and after 24 h the pictures of the plates were taken in flatbed scanner and the area of each pad was measured using Image Pro software. In order to study the role of non-Smad pathways, WT and Smad3 KO cardiac fibroblasts were pre-treated with the Erk inhibitor U0126 (Sigma, 10µM) in the presence or absence of

TGF-β1. In order to investigate the role of p38 MAPK, cells were pretreated with SB203580 (Sigma, 20μM). For integrin inhibition experiments, anti-α2- integrin (BD Bioscience, 15μg/ml) and anti-α5-integrin (eBioscience, 15μg/ml) antibodies were added to cell suspension and incubated for 10 min before mixing with collagen.

Collagen pads from WT cardiac fibroblasts with (n=6) or without TGF-β1 (10ng/ml) (n=6) and S3KO cardiac fibroblasts with (n=7) or without TGF-β1 (10ng/ml) (n=7) were fixed in zinc-formalin and embedded in paraffin for histological analysis. The pads were cut in 5 μm sections and then stained with Picrosirius red and counterstained in hematoxylin to identify the fibroblasts and to quantitate their density and cell area. At least 125 random fibroblasts were selected from each pad and their cell dimensions (Area, perimeter) were measured using Axiovision software (Zeiss). In additional experiments, mRNA was harvested from the collagen pads to assess integrin expression levels.

Overexpression experiments: Mouse cardiac fibroblasts at passage 1 were seeded at 80% confluence (10 cm dishes) in complete medium and were either transfected with 2.5 ng of integrin α5 cDNA (Origene™ Technologies) or transfected with a control entry vector using Lipofectamine®3000 Reagent (ThermoFisher Scientific). The cells were returned to a 5% CO2 incubator and allowed to recover for 24 h. After 24h, the cells were harvested using TrypLE ™ Express reagent, counted and populated on collagen pads ($3x10^5$ cells/ml concentration). The pads were either suspended in serum free DMEM/F12 or stimulated with media containing 10% serum or 10 ng/ml TGF-β for 72 h after which the pads were imaged using Bio-Rad ChemiDoc Imager and contraction was assessed using Image J software. The pads were then processed for RNA extraction and subsequent qPCR analyses.

RNA extraction and qPCR: Total RNA was extracted from cells and mouse hearts, cDNA was amplified using the SsoFast EvaGreen Supermix reagent and the C1000 thermal cycler apparatus from Bio-Rad following the manufacturer's recommendations. The following primer pairs were used: GAPDH forward 5'-AACGACCCCTTCATTGACCT-3', GAPDH reverse 5'-CACCAGTAGACTCCACGACA-3', α-SMA

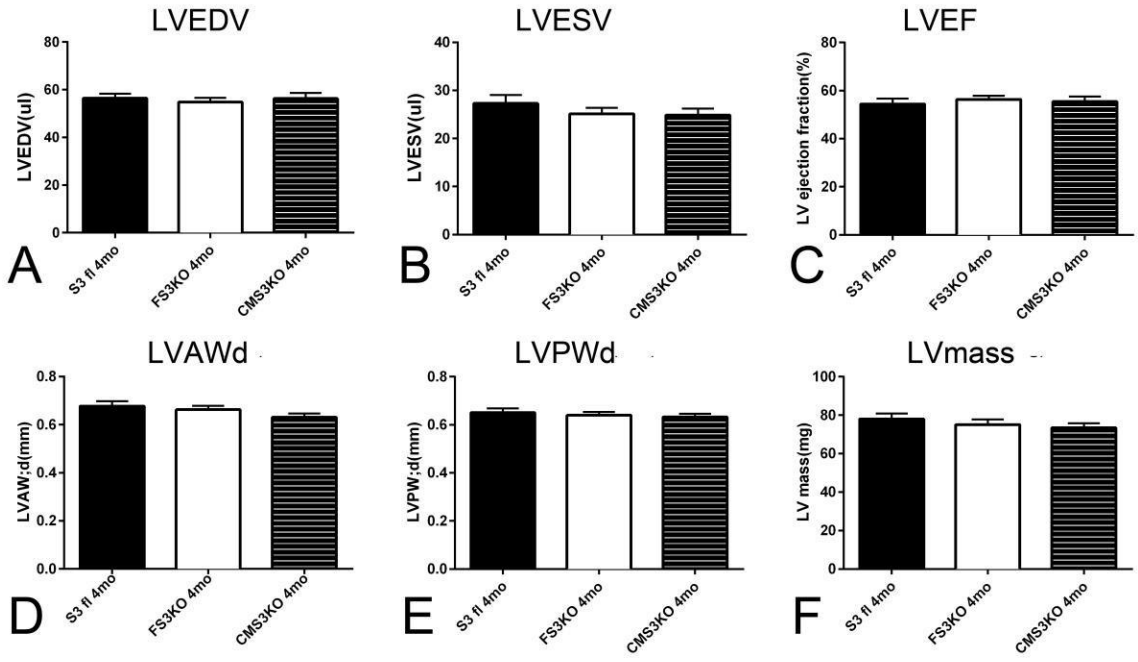
forward 5’-ATCAGCGCCTCCAGTTCCTT-3’, α-SMA reverse 5’-TCTCACCTAACAGAAACACAA-3’, Smad3 forward 5’-CACAGCCACCATGAATTACG-3’, Smad3 reverse 5’-TGGAGGTAGAACTGGCGTCT-3’, Itga2 forward 5’-GCACCACATTAGCATACA-3’, Itga2 reverse 5’-TAGAAAGGGATACTGAAGACT-3’, Itga5 forward 5’-AGTGTGAGGCTGTATATGA-3’, Itga5 reverse 5’-AGAATGGCTAGGATGATGA-3’, Itgb1 forward 5’-GATGAAGTGAACAGTGAAGA-3’, Itgb1 reverse 5’-ACAGACACATTCTCCATTG-3’, Itga1 forward 5’-AAGAAGAATCGTCCGTATAAG-3’, Itga1 reverse 5’-TAT GCT GTG CTG AGA GTA-3’, Itgb3 forward 5’—CCTTCACCAATATCACCTAC-3’. Itgb3 reverse 5’-CACATACTGACATTCTTCCA-3’, NOX1 forward 5'-GTGTCTTGCTTGATAATCTTC-3', NOX-1 reverse 5'-TTGGTCGTTCTATGTTGTT-3', NOX2 forward 5'-ATCCATATCCGTATTGTG-3', NOX2 reverse 5'-ATCAACTGCTATCTTAGG-3, NOX4 forward 5'-GTAGACTGAACTGTGGAGAC-3', NOX4 reverse 5'-ACATCAAGCCTGGACAAG-3', SOD1 forward 5'-GGACAAATTACAGGATTA-3', SOD1 reverse 5'-TTCTTAGAGTGAGGATTA-3', SOD2 forward 5'-ATTGCTCTTGATTGAACA-3', SOD2 reverse 5'-TTACAGACTCTCCTACAG-3', GSR forward 5'-AAGTTAGTGTACCTGATGGAT-3', GSR reverse 5'-GCTGGAGGATTCTGAGTT-3'; periostin forward 5'-CCTGGATTCTGACATTCGCA -3', periostin reverse 5'- CCATGCCGTGTTTCAGGTCC -3', collagen I forward 5'- GATACTTGAAGAATATGAAC-3', collagen I reverse 5'- AATGCTGAATCTAATGAA-3', collagen III forward 5'- TACTCATTCACCAGCATA-3', collagen III reverse 5'-GTATAGTCTTCAGGTCTCA-3', fibronectin forward 5’-GAAGACAGGACCAATGAA-3’, fibronectin reverse 5’-ATAGACACTGACTTCGTATT-3’, TGF-β1 forward 5'- TATTTAAGAACACCCACT-3', TGF-β1 reverse 5'- ATAGTCCTGAATAATTTGAG-3'. The housekeeping gene GAPDH was used as internal control. The qPCR procedure was repeated three times in independent runs; gene expression levels were calculated using the $\Delta\Delta C_T$ method.

Protein extraction and western blotting: Protein was extracted from hearts or cardiac fibroblasts as previously described [19]. Western blotting was performed using antibodies to MMP2 (from R&D systems), 3-nitrotyrosine

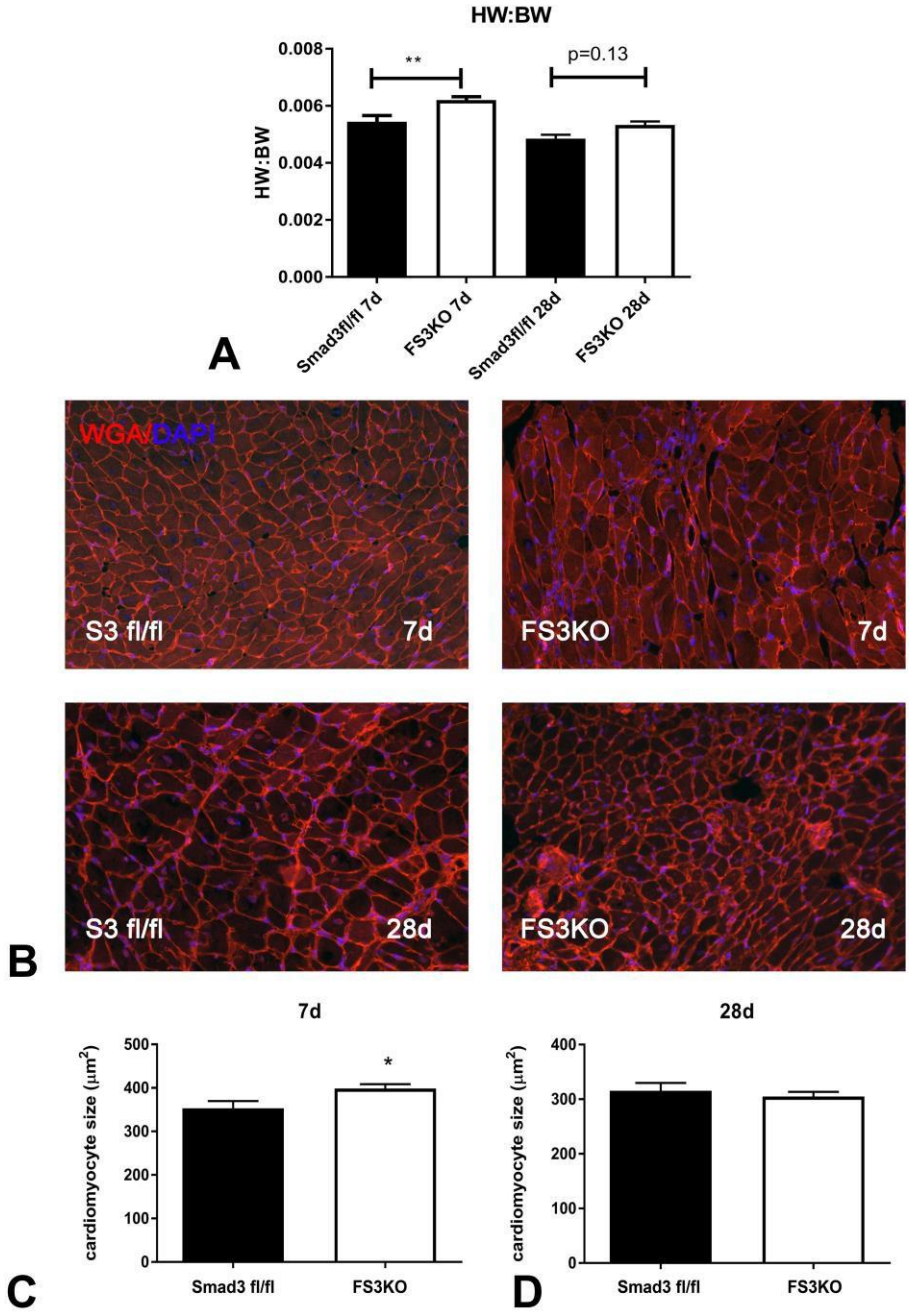
and GAPDH (from Santa Cruz), as previously described[19]. The gels were imaged by ChemiDoc™ MP System (Bio Rad) and analyzed by Image Lab 3.0 software (Bio Rad).

Statistics: For comparisons of two groups unpaired, 2-tailed Student's t-test using (when appropriate) Welch's correction for unequal variances was performed. The Mann-Whitney test was used for comparisons between 2 groups that did not show Gaussian distribution. For comparisons of multiple groups, 1-way ANOVA was performed followed by Tukey's multiple comparison test. The Kruskall-Wallis test, followed by Dunn's multiple comparison post-test was used when one or more groups did not show Gaussian distribution. Paired t-test was used for comparisons of functional data within the same group. Survival analysis was performed using the Kaplan-Meier method. Mortality was compared using the log rank test.

SUPPLEMENTAL FIGURES AND FIGURE LEGENDS:

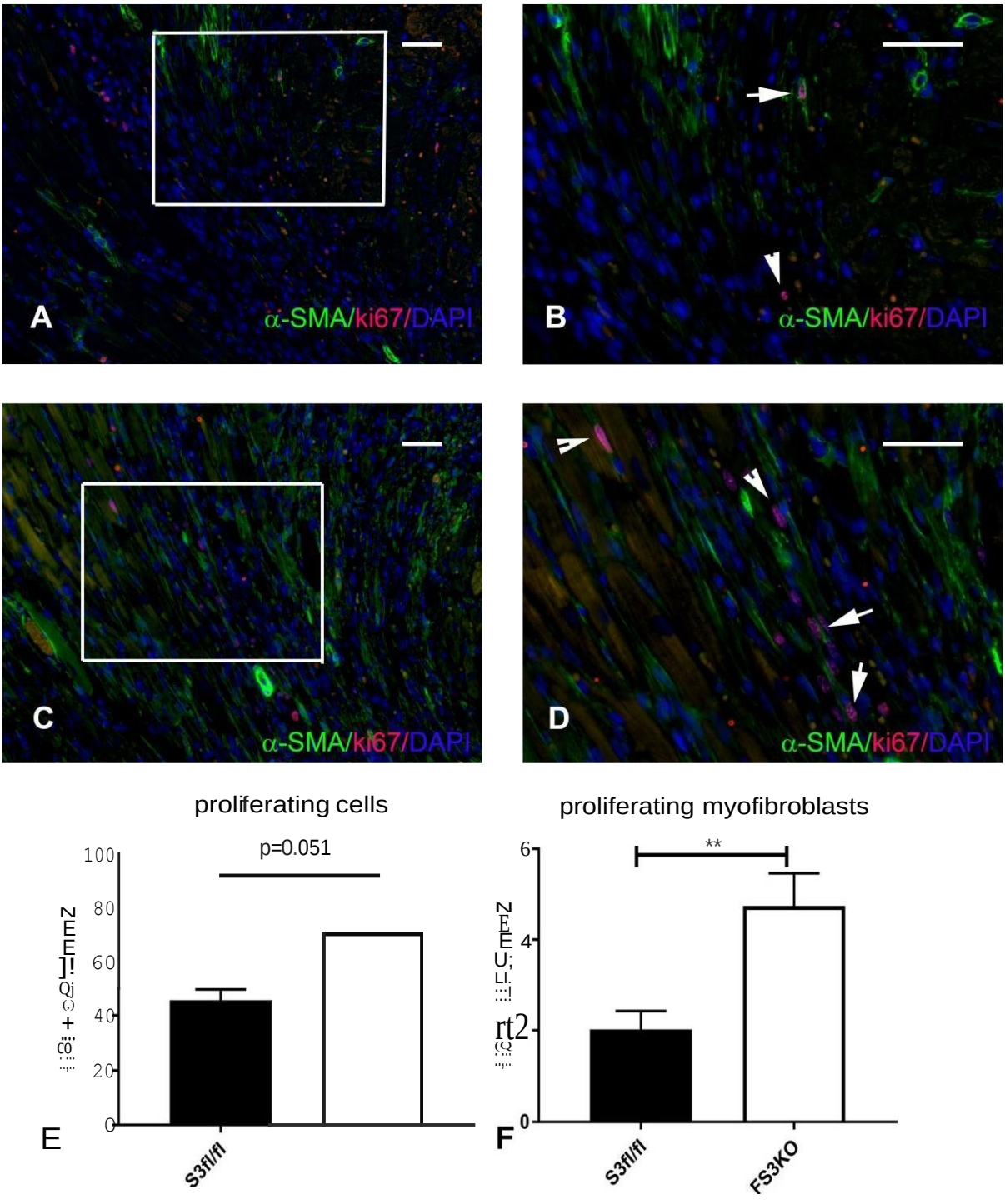


Supplemental figure 1: Mice with cell-specific Smad3 disruption in activated periostin+ fibroblasts, or in cardiomyocytes exhibit no differences in baseline cardiac function and geometry, when compared with Smad3 fl/fl controls. At 4 months of age, mice with fibroblast-specific Smad3 loss (FS3KO), animals with cardiomyocyte specific Smad3 loss (CMS3KO) and corresponding Smad3 fl/fl controls (S3 fl) had comparable left ventricular end-diastolic volume (A- LVEDV), left ventricular end-systolic volume (B- LVESV), left ventricular ejection fraction (C- LVEF), end-diastolic left ventricular anterior wall thickness (D- LVAWd), end-diastolic left ventricular posterior wall thickness (E- LVPWd) and left ventricular mass (F- LV mass) (n=10-19/group).



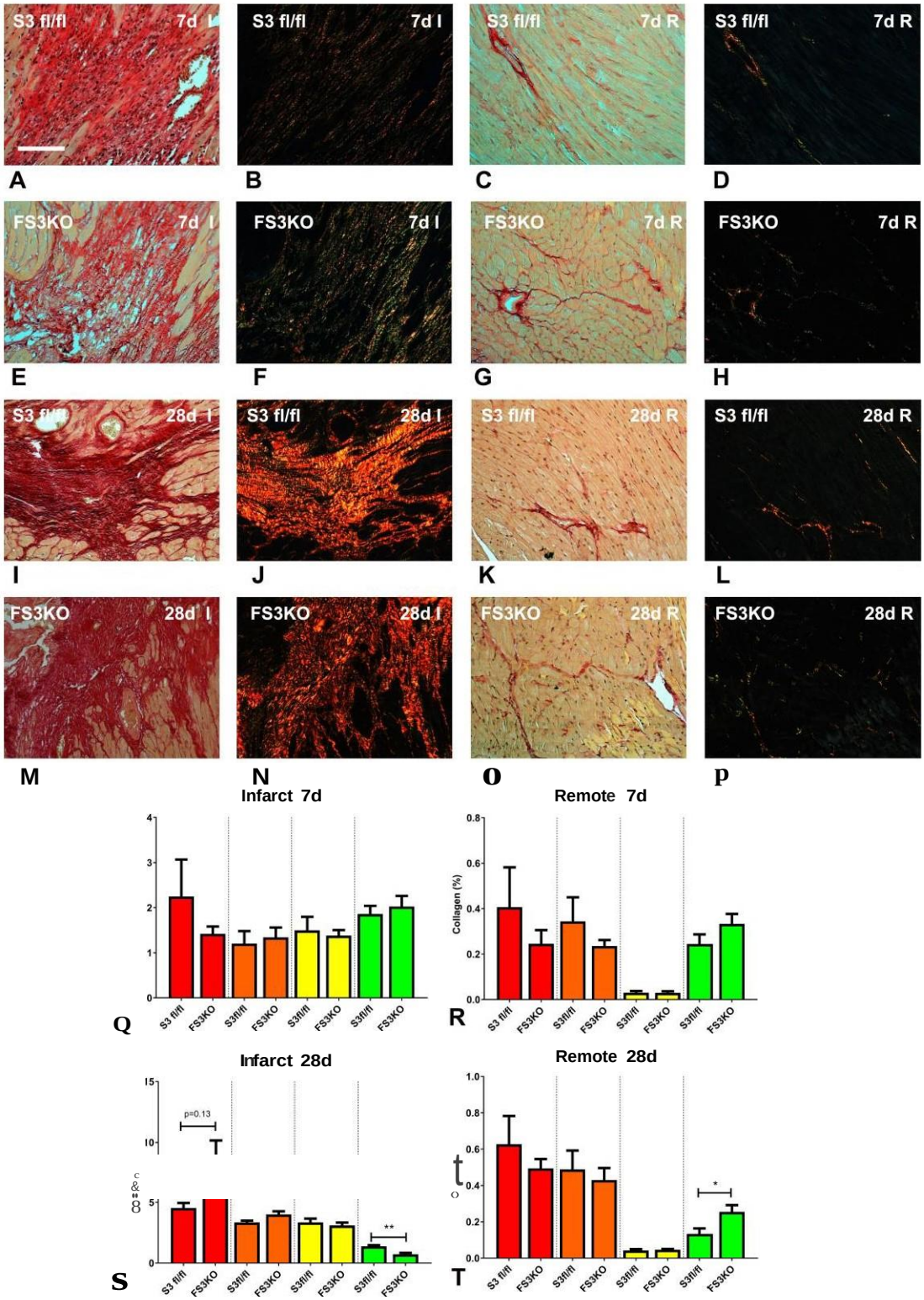
Supplemental figure 2: FS3KO mice exhibit accentuated cardiomyocyte hypertrophy in the viable remodeling myocardium after 1 h of ischemia and 7 days of reperfusion. A: The heart weight to body weight (HW:BW) ratio was significantly higher in FS3KO mice after 7 days of reperfusion (**p<0.01 vs Smad3 fl/fl). At the 28 day timepoint, there was a trend towards increased HW:BW ratio in FS3KO mice. B-D: In order to investigate the effects of fibroblast-specific Smad3 loss on cardiomyocyte hypertrophy, we performed WGA lectin immunofluorescence (B). Quantitative analysis showed that after 7 days of reperfusion, cardiomyocyte size was

higher in FS3KO animals in comparison to corresponding Smad3 fllfl controls (C). No significant differences in cardiomyocyte size were observed at the 28 day timepoint (*p<0.05, n=9-10/group).



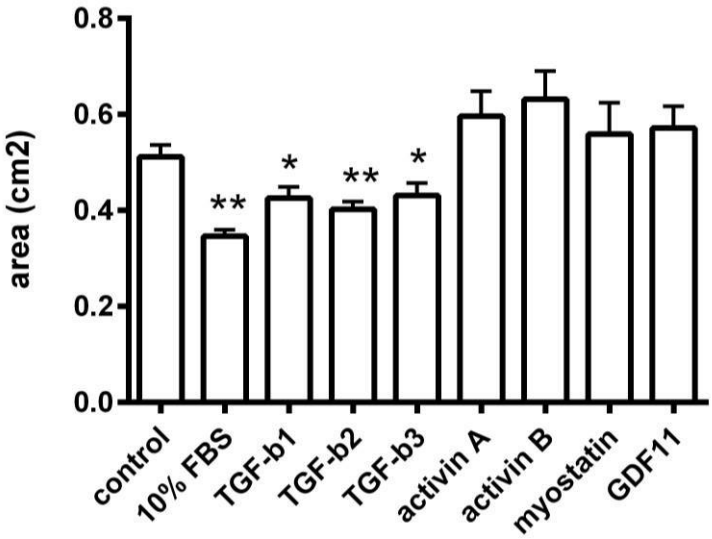
Supplemental figure 3: A-D. Dual fluorescence for a-SMA and ki-67 was used to identify proliferating myofibroblasts (arrows) in Smad3 fllfl (A, B) and in FS3KO (C, D) infarcts after 7 days of reperfusion. Scalebar=40)-Ull. E-F: When compared to infarcted Smad3 fl/fl mice, FS3KO animals had a trend towards

increased density of proliferating cells (p=0.051, n=9/group) and increased density of proliferating myofibroblasts (MFs) (**p<O.Ol, n=9/group).

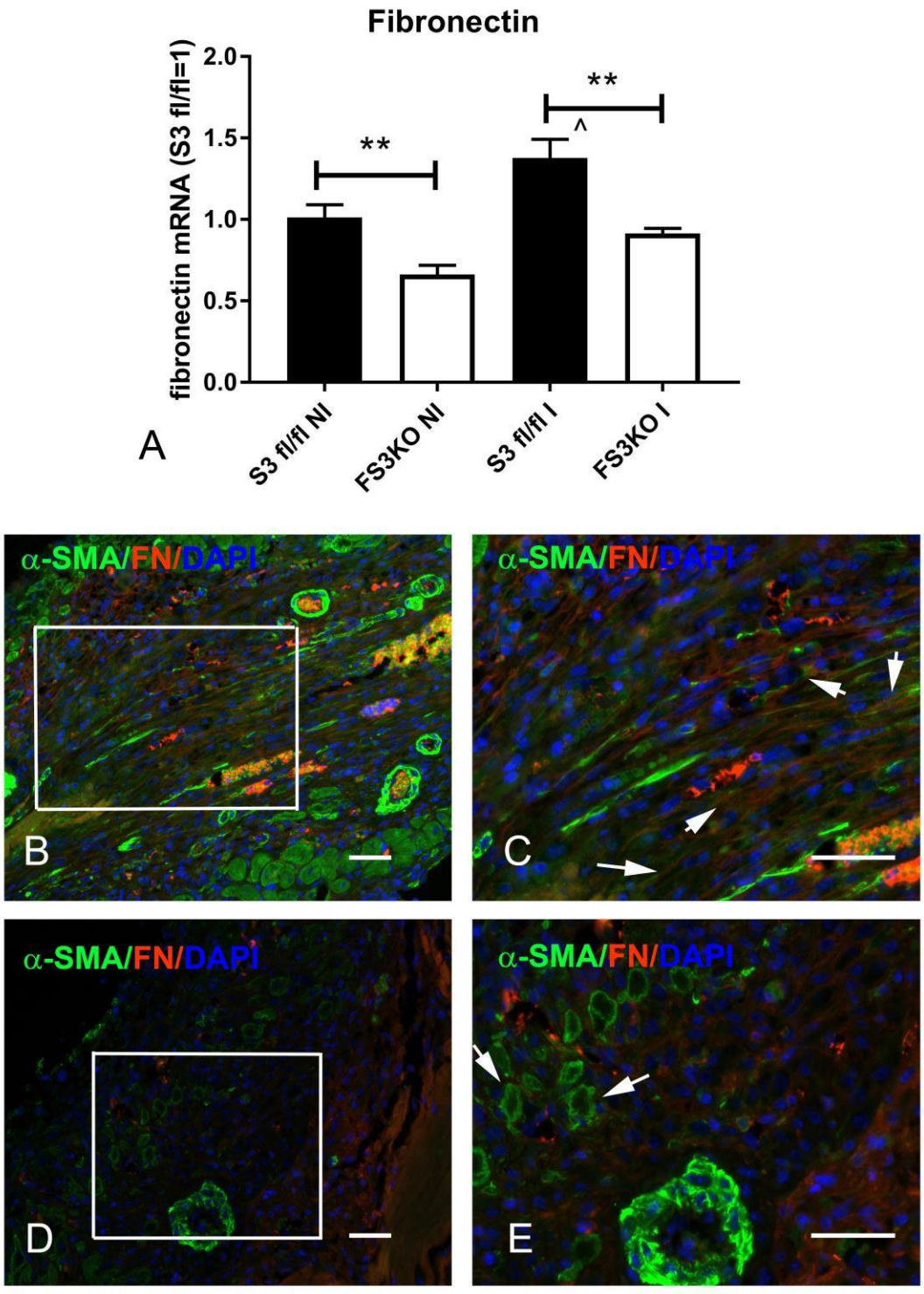


Supplemental figure 4: Effects of fibroblast-specific Smad3 loss on collagen content following myocardial infarction. Light microscopy and polarized light microscopy were used to identify collagen fibers in

Smad3 fl/fl mice (A-D, I-L) and in FS3KO animals (E-H, M-P) after 7 days (A-H) or 28 days (I-P) of reperfusion in the infarct zone (I) and in the remote remodeling myocardium (R). Scalebar=60μm. When the collagen fibers are stained with picrosirius red and viewed with polarized light, thicker fibers exhibit red-orange birefringence, whereas thinner fibers appear green or yellow. Q-T: Quantitative analysis showed no significant effects of fibroblast-specific Smad3 loss on collagen deposition in the infarct (Q) and remote remodeling myocardium (R) after 7 days of reperfusion. However, after 28 days of reperfusion, FS3KO infarcts had lower levels of thinner green fibers in the infarct zone (S, **p<0.01) and higher amounts of green fibers in the remote remodeling myocardium (T, *p<0.05). These findings may reflect the accentuated adverse remodeling in FS3KO animals (n=7-10/group; quantitative analysis based on at least 18-25 fields from each infarcted mouse).

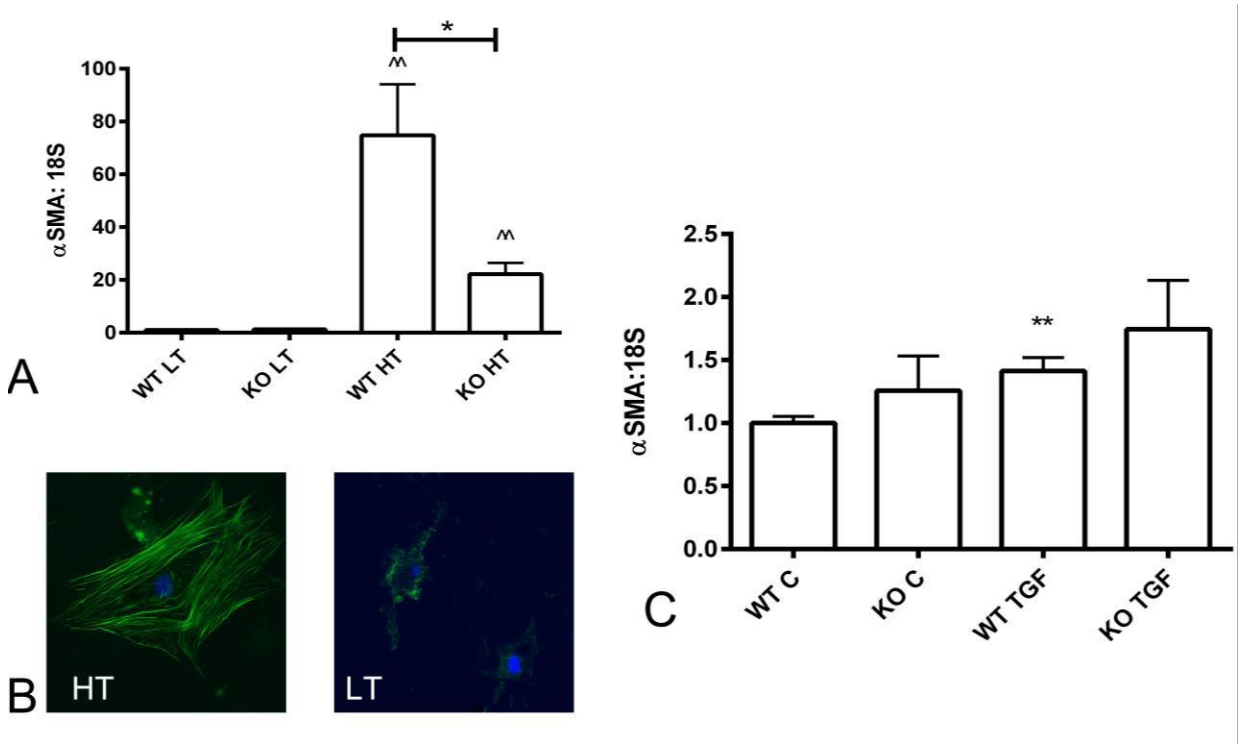


Supplemental figure 5: TGF-β1, β2 and β3, stimulate contractile capacity of fibroblasts in collagen pads. In contrast, other members of the TGF-β family known to activate Smad3 (activins A and B, myostatin and GDF11) do not induce gel contraction (*p<0.05, **p<0.01 vs. control, n=6). Concentrations: TGF-β1 (10ng/ml), TGF-β2 (10ng/ml), TGF–β3 (10ng/ml), activin A (50ng/ml), activin B (50ng/ml), myostatin (100ng/ml), and GDF11 (10ng/ml).

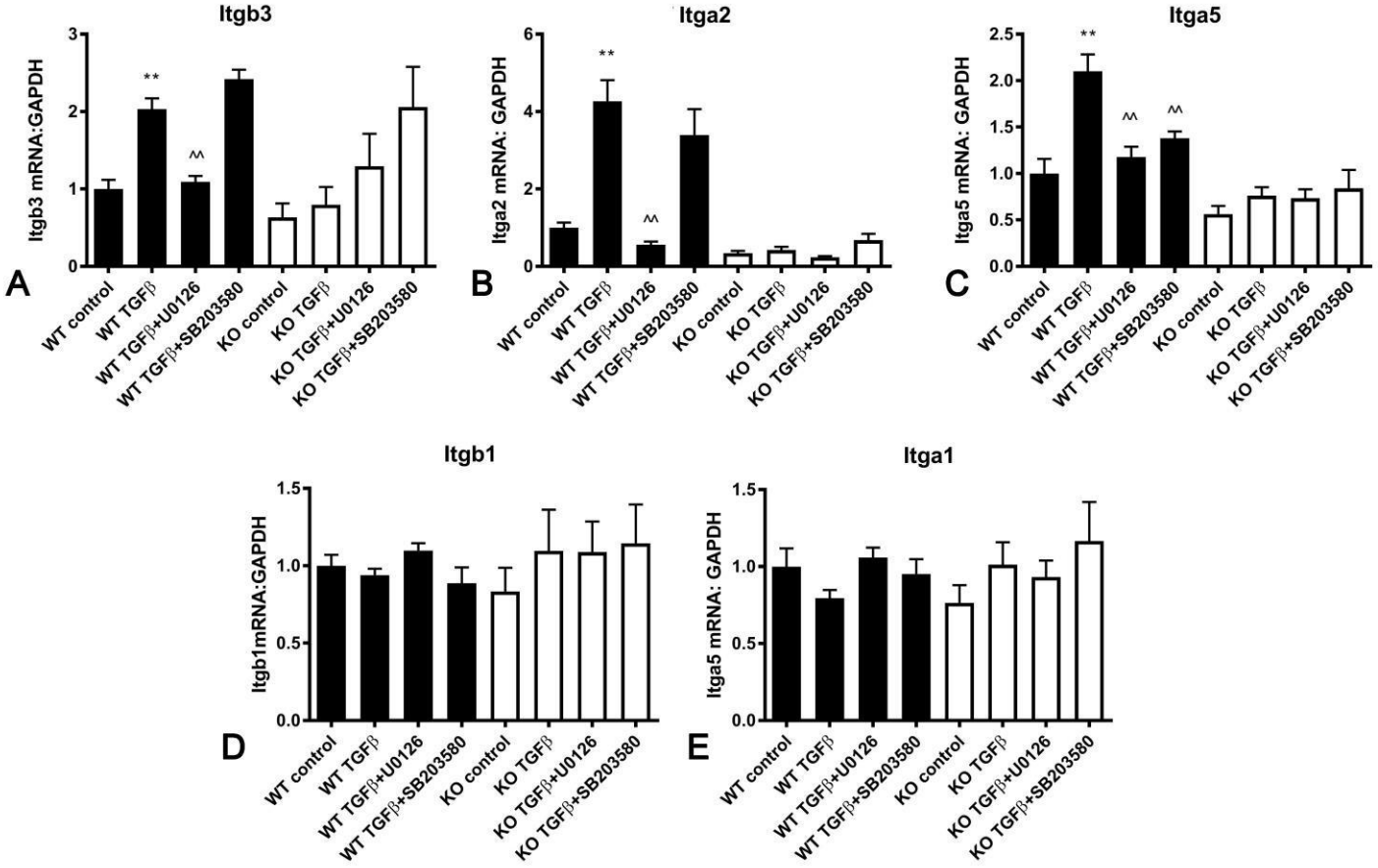


Supplemental figure 6: Smad3 loss reduces fibronectin synthesis in infarct fibroblasts and attenuates ED-A fibronectin localization in the infarcted myocardium. A. qPCR analysis showed that fibroblasts harvested from the infarct zone (I) or the non-infarcted myocardium (NI) of FS3KO mice after 7 days of reperfusion had lower fibronectin mRNA expression that corresponding cells from Smad3 fl/fl animals (**$p<0.01$ vs. corresponding Smad3 fl/fl, n=5-6; ^$p<0.05$ vs. corresponding cells from non-infarcted myocardium). B-E: Dual

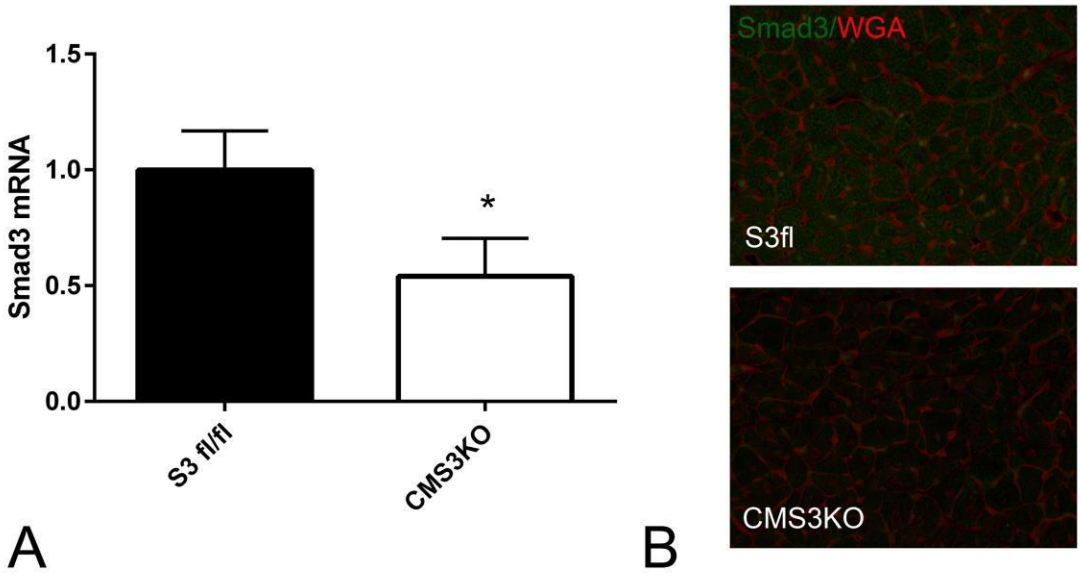
immunofluorescence combines staining for α-SMA (green) to label myofibroblasts as spindle-shaped cells located outside the vascular media, and ED-A fibronectin (red). In Smad3 fl/fl animals (B-C), ED-A fibronectin was localized in the area of myofibroblast infiltration (arrows). D-E: FS3KO mice showed attenuated ED-A fibronectin immunoreactivity in the area populated by α-SMA+ cells (E, arrows).



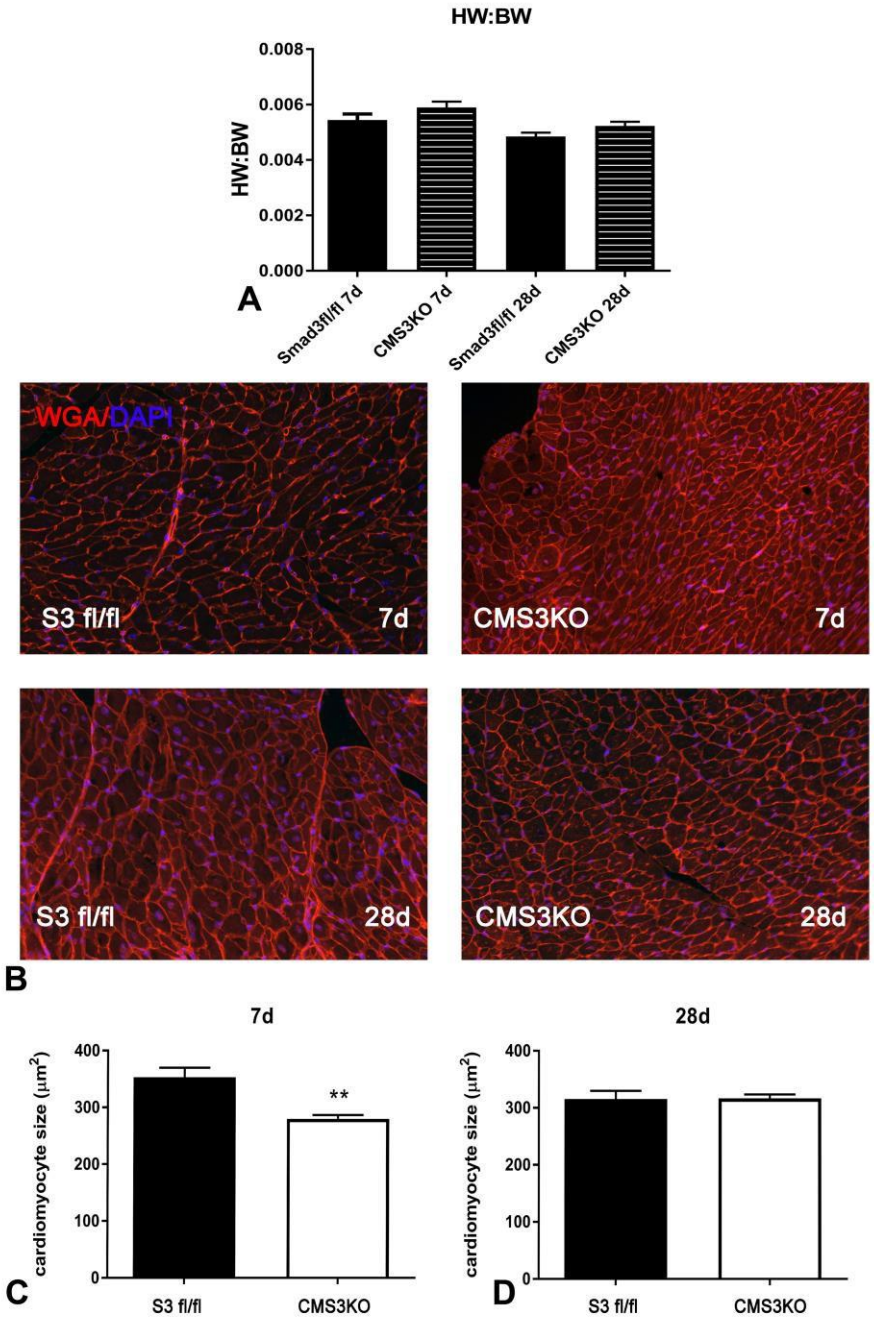
Supplemental figure 7: Impaired contraction in the absence of Smad3 is not due to attenuated expression of the contractile protein α-SMA. Fibroblasts cultured in the high tension (HT) environment of the cultured plate become myofibroblasts, expressing large amounts of α-SMA (A, B). In contrast, fibroblasts cultured in the low tension (LT) environment of the collagen pad exhibit very low levels of α-SMA expression (A, B). qPCR analysis showed that WT HT fibroblasts have 70-fold higher α-SMA mRNA expression compared to LT fibroblasts (A, ^^$p<0.01$, n=6). Smad3 loss reduced α-SMA expression in HT fibroblasts (*$p<0.05$ vs. WT HT), but had no significant effects on the very low levels of α-SMA expression in LT fibroblasts. B. α-SMA immunofluorescence demonstrated that HT mouse cardiac fibroblasts are large myofibroblast-like cells with incorporation of α-SMA in the cytoskeleton (A, HT). In contrast, mouse cardiac fibroblasts cultured in collagen pads exhibit modest punctate staining for α-SMA (B, LT). Although Smad3 loss markedly reduces α-SMA expression in HT fibroblasts (A, *$p<0.05$ vs. WT HT), in LT cells Smad3 deletion does not affect (the very low) α-SMA levels in Smad3 KO cells, in the presence or absence of TGF-β (C; p=NS n=6) (**$p<0.01$ vs. WT C, n=6).



Supplemental figure 8: The role of Smad-independent pathways on integrin mRNA expression in cardiac fibroblasts. A. TGF-β1 induces β3 integrin (Itgb3) expression in WT (**p<0.01 vs. WT control), but not in Smad3 KO fibroblasts. Erk, but not p38 MAPK inhibition abrogates TGF-β-induced β3 integrin upregulation (^^p<0.01 vs. WT TGF-β) in WT, but not in Smad3 KO cells, indicating that the effect of Erk is not independent of Smad3. B. TGF–β1 markedly upregulates α2 integrin (Itga2) synthesis (**p<0.01 vs. WT control), but not in Smad3 KO fibroblasts. Erk, but not p38 MAPK inhibition abrogates TGF-β-induced α2 integrin upregulation (^^p<0.01 vs. WT TGF-β) in WT, but not in Smad3 KO cells, indicating that the effect of Erk is not independent of Smad3. C. TGF-β1 induces α5 integrin (Itga5) expression in WT but not in Smad3 KO fibroblasts. Erk or p38 MAPK inhibition abrogates TGF-β1-mediated α5 integrin upregulation (^^p<0.01 vs. WT TGF-β) in WT, but not in Smad3 KO cells, indicating that these effects are not independent of Smad3. D-E. Expression of β1 (Itgb1) and α1 (Itga1) integrin was not modulated by TGF-β1 and was not affected by Erk or p38 MAPK inhibition.

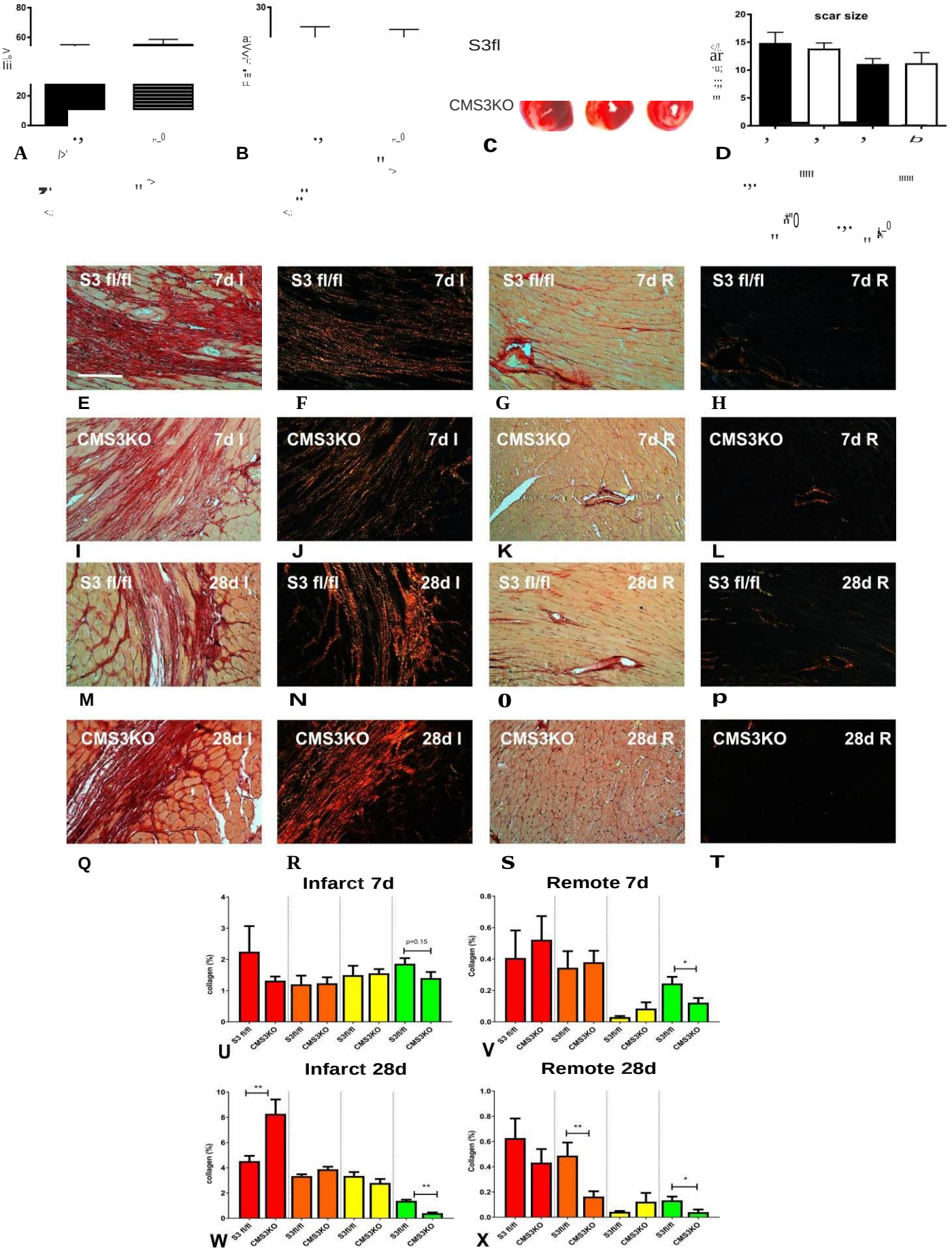


Supplemental figure 9: Generation of mice with cardiomyocyte-specific loss of Smad3. A: qPCR analysis shows marked reduction in Smad3 mRNA levels in CMS3KO hearts when compared with Smad3 fl/fl myocardium (*$p<0.05$, n=5). B: Immunofluorescence demonstrated markedly reduced Smad3 immunoreactivity in cardiomyocytes of CMS3KO animals. WGA lectin fluorescence serves to outline the cardiomyocytes.



Supplemental figure 10: CMS3KO mice exhibit attenuated cardiomyocyte hypertrophy in the viable remodeling myocardium after 1h of ischemia and 7 days of reperfusion. A. The HW:BW ratio was not significantly different between Smad3 fl/fl and CMS3KO animals after 7-28 days of reperfusion. B In order to assess effects of cardiomyocyte-specific loss of Smad3 on cardiomyocyte hypertrophy, WGA lectin fluorescence was performed (B). Quantitative analysis demonstrated that CMS3KO animals had attenuated

cardiomyocyte hypertrophy after 7 days of reperfusion (C). Cardiomyocyte size was not different between groups after 28 days of reperfusion (D) (**p<0.01 vs. Smad3 fl/fl, n=9-10/group).


A
B
S3fl
CMS3KO
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scar size
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S3 fl/fl 7d I
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G
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H
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I
CMS3KO 7d I
J
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K
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L
S3 fl/fl 28d I
M
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N
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0
S3 fl/fl 28d R
p
CMS3KO 28d I
Q
CMS3KO 28d I
R
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S
CMS3KO 28d R
T
Infarct 7d
collagen (%)
p=0.15
U
Remote 7d
Collagen (%)
V
Infarct 28d
collagen (%)
W
Remote 28d
Collagen (%)
X
S3 fl/fl
CMS3KO

Supplemental figure 11: A-D: Attenuated remodeling and ameliorated function in infarcted CMS3KO mice is not due to reduced acute infarct size (p=NS, n=10-11). A-C. TTC/Evans Blue staining showed that the area at risk and infarct size:area at risk were comparable between Smad3 fl/fl and CMS3KO mice. D. Cardiomyocyte-specific loss of Smad3 did not affect scar size after 7-28 days of reperfusion. E-T: Light microscopy and polarized light microscopy were used to identify collagen fibers in Smad3 fl/fl mice (E-H, M-P) and in CMS3KO animals (I-L, Q-T) after 7 days (E-L) or 28 days (M-T) of reperfusion in the infarct zone (I) and in the remote remodeling myocardium (R). Scalebar=75μm. When the collagen fibers are stained with picrosirius red and viewed with polarized light, thicker fibers exhibit red-orange birefringence, whereas thinner fibers appear green or yellow. U-X: When compared with Smad3 fl/fl animals, CMS3KO mice had significantly lower content of green fibers in the remote remodeling myocardium after 7 days of reperfusion (*$p<0.05$). After 28 days of reperfusion, CMS3KO mice had significantly increased deposition of thicker red collagen fibers in the infarct zone (**$p<0.01$), and marked reductions in deposition of orange and green collagen fibers in the remote remodeling myocardium (n=7-10/group).

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REVIEW | *Guidelines in Cardiovascular Research*

Guidelines for experimental models of myocardial ischemia and infarction

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Lindsey ML, Bolli R, Canty JM Jr, Du XJ, Frangogiannis NG, Frantz S, Gourdie RG, Holmes JW, Jones SP, Kloner RA, Lefer DJ, Liao R, Murphy E, Ping P, Przyklenk K, Recchia FA, Schwartz Longacre L, Ripplinger CM, Van Eyk JE, Heusch G. Guidelines for experimental models of myocardial ischemia and infarction. *Am J Physiol Heart Circ Physiol* 314: H812–H838, 2018. First published January 12, 2018; doi:10.1152/ajpheart.00335.2017.—Myocardial infarction is a prevalent major cardiovascular event that arises from myocardial ischemia with or without reperfusion, and basic and translational research is needed to better understand its underlying mechanisms and consequences for cardiac structure and function. Ischemia underlies a broad range of clinical scenarios ranging from angina to hibernation to permanent occlusion, and while reperfusion is mandatory for salvage from ischemic injury, reperfusion also inflicts injury on its own. In this consensus statement, we present recommendations for animal models of myocardial ischemia and infarction. With increasing awareness of the need for rigor and reproducibility in designing and performing scientific research to ensure validation of results, the goal of this review is to provide best practice information regarding myocardial ischemia-reperfusion and infarction models.

Listen to this article's corresponding podcast at ajpheart.podbean.com/e/guidelines-for-experimental-models-of-myocardial-ischemia-and-infarction/.

animal models; cardiac remodeling; heart failure; myocardial infarction; reperfusion; rigor and reproducibility

INTRODUCTION

Ischemia occurs when blood flow to the myocardium is reduced (129). Ischemia of prolonged duration induces myocardial infarction (MI), and MI is a common cause of heart failure (295). Ischemic cardiomyopathy is the most common cause of heart failure and can arise from remodeling after an acute ST segment elevation myocardial infarction (STEMI) from multiple small nontransmural infarctions or from chronic repetitive ischemia in the absence of infarction (15). Ischemia can range in its extent from low flow to total coronary occlusion, can be of short to long duration, can be successfully reversed by reperfusion in a timely manner or not reperfused at all, and can induce injury or provide cardioprotection. Likewise, there is a diverse variety of animal models to address each type of ischemia within this spectrum. Figure 1 shows the range of models that reflect the scale of ischemia and variety of models available to better understand how the heart responds to ischemia and the mechanisms whereby the heart can either adapt to ischemia or progress to failure.

Experimental models of myocardial ischemia serve two nearly opposing aims, both worthy of investigation. The first aim is to provide better mechanistic insight that cannot be obtained from a clinical situation. To achieve this aim, experimental studies may be reductionist with low direct applicability to the clinical situation (e.g., when using temporally induced cell specific over- or underexpression of a gene). The second aim is to provide mechanistic insight from an experimental study for translation to the clinical situation, and for this aim experimental models must replicate the clinical setting as closely as possible (127).

For cardiovascular science to continue advancing, experimental results should be reproducible and replicable, and rigorous experimental design is a fundamental element of reproducibility. Reproducibility refers to results that can be repeated by multiple scientists and is a means of validation across laboratories. Rigor refers to robust and unbiased experimental design, methodology, analysis, interpretation, and reporting of results. With increasing awareness by journals and granting agencies of the need for reproducibility and rigor in designing and performing scientific research in preclinical studies, the goal of this consensus article is to provide best practice information regarding myocardial ischemia and infarction models. The strengths and limitations of the different models are discussed, with a summary shown in Table 1. We also address ways to incorporate Animals in Research: Reporting In Vivo Experiments (ARRIVE) guidelines and similar standard operating procedures (168). The extensive reference list provided also serves as a resource for researchers new to the field.

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IN VITRO AND EX VIVO MODELS

Myocyte Cell Culture

Model rationale. Isolated fresh or cultured cardiomyocytes can be used as a powerful in vitro model of ischemia-reperfusion (I/R), whereby ischemia is simulated with hypoxia and reperfusion with reoxygenation (H/R). This system allows precise control of the cellular and extracellular environment, notably the specific impact of hypoxia and reoxygenation on cardiomyocytes without confounding influences of other cell types (e.g., fibroblasts, endothelial cells, inflammatory/immune cells, and platelets) or circulating factors (e.g., hormones, neurotransmitters, and cytokines).

Variables measured. The most common use of this model system is for in vitro testing of specific factors proposed to be involved in I/R injury or cardioprotection (31, 166, 227, 240, 244, 330). After H/R, cultured cardiomyocytes undergo apoptosis, accompanied by cytochrome *c* release and caspase activation or necrosis (200, 296). Thus, assays of cell viability are often performed to assess the role of a particular genetic or pharmacological intervention in exacerbating or protecting the cell from H/R-induced cell death. Viability may be measured with a variety of assays, including lactate dehydrogenase (LDH) release or propidium iodide exclusion as an indicator of membrane integrity (24, 31, 58). Apoptosis is assessed with TUNEL or annexin V staining (24, 58, 166). Mitochondrial damage, including disruption of mitochondrial membrane potential, is also a key component of cellular injury after H/R and may be assessed using fluorescent dyes, such as tetramethylrhodamine methyl ester (TMRM). The loss of mitochondrial membrane potential causes TMRM to leak from the mitochondria, decreasing fluorescence (24, 31). In addition, reactive oxygen species (ROS) have been implicated in H/R injury (275); thus, ROS production is another common assessment (24, 58, 124, 125, 227).

More detailed analyses of cardiomyocyte responses to H/R include assessments of morphology, contractile function (i.e., cell shortening), intracellular Ca^{2+} handling, and action potentials (124, 166, 207). Contractile function is an important but often overlooked variable in H/R assays, since contraction requires ~70% of total energy utilization within a myocyte (182). Many H/R studies use quiescent myocytes; however, markedly impaired recovery of myocyte function and increased cell death result when cells are stimulated to contract throughout the H/R protocol (207). For all variables assessed, technical replicates on the same sample should be performed to establish the variability of the measurement technique. Biological replicates often include measurements on plates or myocytes from the same heart/harvest. If these are to be treated as independent samples, *n* values for both the plate/myocyte number as well as heart/harvest number should be fully reported.

There is currently no standardized protocol for H/R in cultured cardiomyocytes, but cardiomyocyte source and H/R conditions must be carefully considered. Theoretically, freshly isolated adult cardiomyocytes are the ideal gold standard for H/R experiments (166, 207, 244), although neonatal cardiomy-

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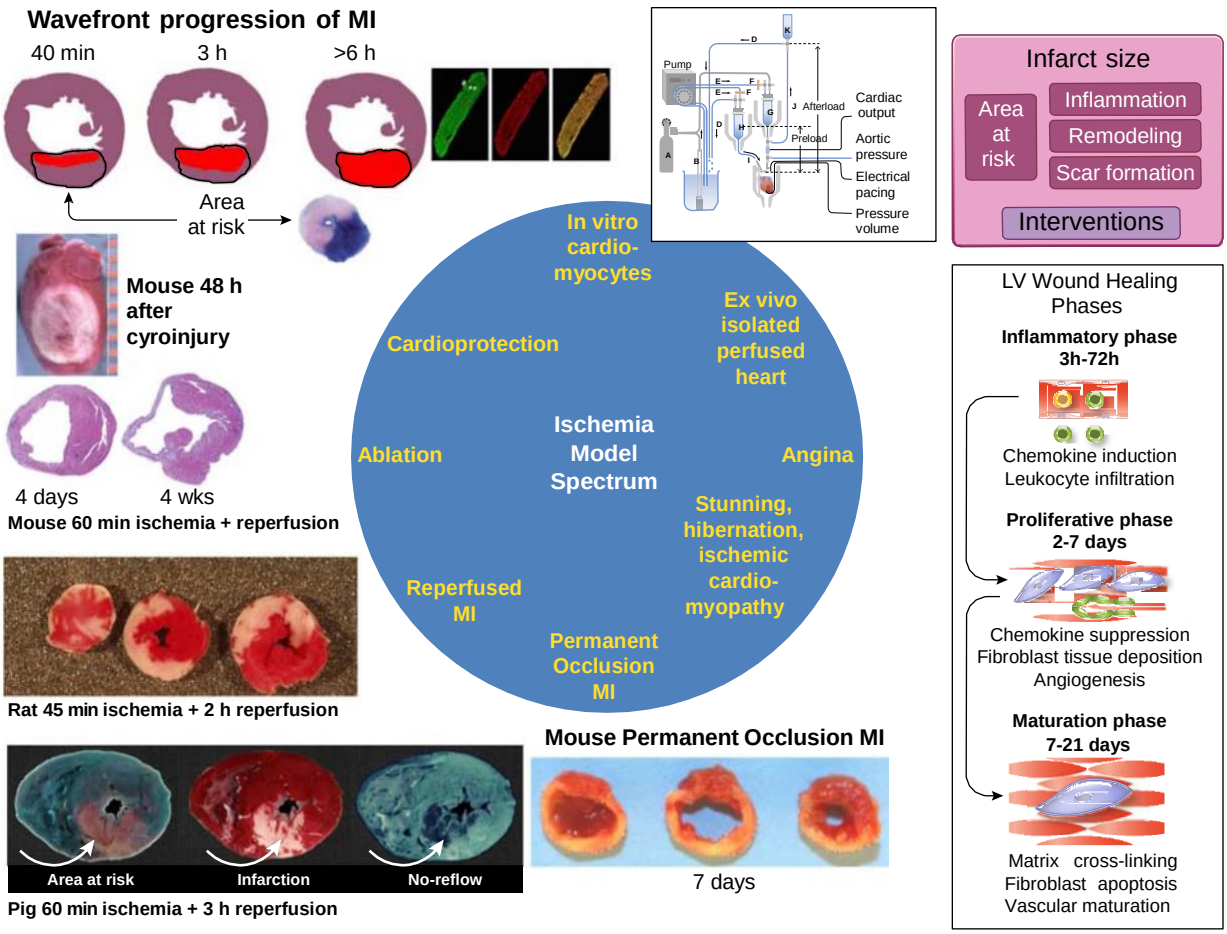

Fig. 1. The spectrum of ischemia encapsulates in vitro, ex vivo, and in vivo models of ischemia that range from transient to prolonged in duration with acute to chronic consequences. The pig section (*bottom left*) is modified from Heusch (126). MI, myocardial infarction; LV, left ventricular;

ocytes (58, 227, 296), cardiac progenitor cells (12), induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) (24, 31), and various cell lines such as H9c2 and HL-1 have been used (330). Of these, neonatal cardiomyocytes are most commonly used, due to their relative ease of isolation and robust viability for several days in culture; however, neonatal cardiomyocytes are more resistant to hypoxia than adult myocytes (236, 255), and the mechanisms of this resistance remain incompletely resolved, which may limit interpretation of such results when designing more translational studies (235). Adult myocytes are preferred, because ischemic heart disease almost exclusively occurs in adults, and fresh isolation eliminates the potential confounding factor of phenotypic transformation in culture. The caveat is that adult cardiomyocytes are more difficult to isolate and do not survive long in culture, impeding longer H/R protocols or those requiring pre-H/R transfection. Therefore, many investigators turn to neonatal cardiomyocytes or cell lines (e.g., H9c2 or HL-1) if genetic modifications are necessary, in which case these genetically engineered cells may be an important complement to in vivo or adult cardiomyocyte studies. In addition, adult myocytes do not form monolayers as seen with neonatal cells, limiting their use for studies on gap junctions and electrical conductivity. Thus, authors should carefully consider experimental end points in the context of overall study design, because either neonatal or adult cell sources will be the best choice depending on the question posed. For both neonatal and adult primary cells, isolation protocols must assure for a cardiomyocyte-enriched population (i.e., free of fibroblasts and other cell types). For neonatal cells, the differential attachment technique is often used, whereas gravity separation is typical for adult cardiomyocytes (161, 287). Regardless of the isolation and enrichment method, manual or automated cell counting, expression of cardiomyocyte-specific markers, and visualization or quantification of T-tubular structure should be performed to verify purity and phenotype. Use of iPSC-CMs may overcome some of these primary cell limitations, but the response of iPSC-CMs to H/R has not yet been fully characterized and may depend on their maturation state (256).

The most common in vitro conditions used to simulate in vivo ischemia are anoxia (<1% O_2, 5% CO_2, 94+% N_2) and complete substrate depletion (serum-free, glucose-free medium). There are many variations to the protocol with additional conditions that more closely mimic the ischemic heart, such as partial hypoxia, partial or no substrate depletion, hyperkalemia, acidosis, and use of electrical stimulation (207). The cell type needs to be carefully considered when determining optimal control and ischemic conditions. Neonatal cardiomyocytes and cell lines (H9c2 and HL-1) favor glu-

Table 1. *Comparison of different approaches with strengths and limitations for each method*

Approach	End-Point Measurements	Strengths	Limitations and Pitfalls
In vitro cardiomyocytes	Cell viability (live-dead assay) Type of cell death (i.e., apoptosis)	High throughput Isolate effects of hypoxia/reoxygenation on cardiomyocytes without other cell types or circulating factors	Reductionist Cardiomyocyte viability may not predict changes in infarct size in vivo Adult cardiomyocyte culture technically challenging
Isolated perfused hearts	Infarct size per area at risk Left ventricular function Assessment of cardiac troponin I as a secondary cardiac injury index	Relatively easy and reproducible Can study ischemia and reperfusion Accurate measure of infarct size Ample sample for biochemistry Compatible with NMR studies Capacity for high throughput Neurohormonal factor independent Eliminates confounding effect of intervention on systemic blood vessels or circulating factors	Tissue edema May not fully represent the in vivo response Glucose as the sole substrate Limited stability Excessive coronary flow Reductionist
Angina	Regional flow and function Metabolism, morphology, molecular biology, nerve activity, rhythm	Close to clinical situation	Technically complex; time and cost intensive
Hibernation/ stunning	Regional flow and function Metabolism, morphology, molecular biology, rhythm	Close to clinical situation	Technically complex; time and cost intensive
Permanent occlusion MI	Inflammation, wound healing, scar formation, remote region myocytes, electrical activity	In the era of percutaneous coronary intervention, ~15–25% patients are not successfully reperfused in a timely manner (53, 104) Robust remodeling response; large effect size	Does not reflect the reperfused MI patient response
Ischemia-reperfusion MI	Inflammation, wound healing, scar formation, myocyte viability, electrical activity	Close to clinical scenario	More technically challenging surgery Reperfusion injury can expand area of damage
Ablation	Inflammation, wound healing, scar formation, myocyte electrical activity	Geometrically defined lesion Infarct size/location independent of coronary anatomy	Nonischemic lethal injury Mechanisms of cell death different from ischemia
Cardioprotection	Infarct size per area at risk Left ventricular geometry and function; no reflow; circulating biomarkers such as cardiac troponin I	Mouse, rat, rabbit, and pig: models of low collateral flow (measurement of regional myocardial blood flow not required) Rat and rabbit: reliable infarct production; relatively high survival rate Pig: mimics humans with low collateral flow Dog: large amount of historical data; shows effect of intervention in the setting of variable collateral flow; mimics humans with high collateral flow	Mouse: small size; substantial variability requiring large *n* values Rat, rabbit, pig, and dog: not high throughput; higher cost Rabbit, pig, and dog: potential for lethal arrhythmias Dog: variability in collateral perfusion (regional myocardial blood flow must be measured)

MI, myocardial infarction.

cose metabolism and under control conditions are often cultured in hyperglycemic medium, which is known to induce ROS production and cell death in adult cardiomyocytes. Furthermore, neonatal cardiomyocytes are insulin resistant and supraphysiological concentrations of insulin are required to increase glucose uptake in these cells compared with adult cardiomyocytes (201). Thus, studies focusing on metabolism with H/R and/or metabolic pathology need to carefully consider the cellular environment in both control and ischemic conditions. In addition to the cellular environment, the duration of hypoxia and reoxygenation is also an important consideration, as myocyte viability not only depends on the duration of hypoxia but also the duration of reoxygenation (166, 244).

To summarize, the major strengths and limitations of studies using isolated cardiomyocytes are shown in Table 1. The major strength of H/R experiments in cultured cardiomyocytes is the ability to control precisely the cellular and extracellular environment, each factor present in ischemic conditions (e.g., hypoxia, metabolic inhibition, or acidosis) can be tested alone and in combination to determine individual contributions to cellular injury. Even with the most carefully designed experimental protocol, in vitro conditions can never fully recapitulate the full spectrum of I/R injury in vivo. Thus, although in vitro experiments can be mechanistically informative and identify new targets for intervention, it is imperative that the results are later validated in an appropriate intact animal model. Nonetheless, even if there is a discrepancy between in vivo I/R and in vitro H/R experiments, important insight is to be gained from parallel studies. As an example, angiopoietin-like protein 4 (ANGPTL4) reduces infarct size after I/R in vivo but does not prevent cardiomyocyte death in vitro (90). These findings indicate that other cell types (e.g., endothelial cells, fibroblasts, or immune cells) are key to the cardioprotective effect of ANGPTL4. Likewise, factors that prevent myocyte cell death in culture may not reduce infarct size in vivo, suggesting that the cardioprotective effect may be outweighed by noncardiomyocyte factors.

Isolated Perfused Hearts

Model rationale. The isolated perfused heart is a convenient and reproducible model to test mechanisms of myocardial injury and cardioprotection (14, 192). The heart is

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removed from the animal and perfused, typically with a physiological saline solution such as Krebs-Henseleit buffer. For screening drugs or interventions for protective properties, this model is ideal, because the isolated perfused heart is studied independently of circulating factors or neuroendocrine inputs from other organs but retains the function, composition, and architecture of the intact heart. This approach is also easily amenable to biochemistry or imaging studies in a nuclear magnetic resonance (NMR) magnet, which can provide useful information to decipher mechanisms of cardioprotection.

Perfused hearts can be studied in a working heart mode or in a nonworking Langendorff mode. In the Langendorff mode, the perfusate enters the coronary arteries to perfuse and oxygenate the heart, which continues to beat for several hours (292). Heart rate and left ventricular (LV) developed pressure are measured with a fluid-filled balloon placed in the cavity of the LV and connected to a pressure transducer as indexes of cardiac function (physiology). The heart can be perfused at constant pressure, in which case the flow rate can vary, or flow can be set with a pump, in which case the perfusion pressure can vary. In the Langendorff mode, the heart does not pump against a gradient and does not perform external work (226). In the working heart mode, the perfusate enters the atrium at a filling pressure set by the operator, and the heart pumps perfusate against a hydrostatic pressure set to different levels (30). A heart model performing external work is technically more challenging, particularly with smaller hearts.

In a model of global ischemia, perfusate flow to the entire heart is stopped, whereas in a regional ischemic model, a suture is tied around a single coronary artery for occlusion. After the ischemic period (typically 20–40 min for rodent models), perfusion is restarted and the heart will usually beat and develop a lower LV developed pressure than at baseline, reflecting postischemic contractile dysfunction or stunning. Contractile dysfunction is a measure of ischemic injury but is not synonymous with cell death. Both contractile dysfunction and cell death often result from the same mechanisms. It is therefore important not to infer that protection against contractile dysfunction is the same as protection against infarct size.

Variables measured. To measure cell death or infarct size, it is necessary to reperfuse the heart for a sufficient duration (at least 60–120 min) to wash out reductive equivalents (79, 271). Triphenyltetrazolium chloride (TTC) is then added to the perfusate or hearts are cut into transverse slices and incubated in TTC solution. TTC is a dye that stains viable myocardium red due to a formazan reaction with NADH and NADPH, which are washed out from irreversibly injured myocardium (81, 172), whereas necrotic tissue remains unstained and thus appears white. Necrotic tissue area is normalized to the total ventricular area (global ischemia) or the ischemic area at risk for infarction (regional ischemia). For regional ischemia preparations, the area at risk is measured after coronary reocclusion and staining of the nonischemic myocardium with a dye such as Evans blue.

The susceptibility of the heart to arrhythmias during I/R is readily assessed through recording of an electrocardiogram (313). Updated guidelines exist for the quantification of such arrhythmias (56). The isolated heart is amenable to monitoring of intracellular ions by optical methods using fluorescent indicators (where the signal originates from a thin layer of epicardial cells) or by NMR spectroscopy (where the signal is a global average from the whole heart). Intracellular Na^+ and Ca^{2+} concentrations have been monitored and intracellular H^+ concentration (i.e., intracellular pH) has been estimated in isolated rodent hearts perfused and subjected to I/R within the vertical bore of the NMR magnet (288). Intracellular high-energy phosphate (ATP and creatine phosphate) have also been monitored by this method, and recent developments using hyperpolarized substrates now also allow real-time analysis of metabolic flux through distinct pathways (167).

The rate of occurrence of cell death is determined by the work that the heart performs at the time it becomes ischemic. When global flow is stopped completely, the heart will continue to beat for a short period of time and continue to consume ATP. Reducing work at the start of ischemia is cardioprotective, and this is the basis of cardioplegic solutions. Therefore, it is important to assure that work is similar between control and experimental hearts, which means that heart rate and temperature must be controlled and held constant in the different treatment groups (281, 292). Due to the lack of neurohumoral factor influences on the perfused heart, heart rate is typically lower than in an intact animal, and it can be controlled by pacing. A slight (<1°C) difference in temperature can result in a large difference of infarct size. Temperature is usually measured by a probe in the heart and controlled by immersing the heart in a fluid bath.

In the absence of blood, the reduction in the oxygen-carrying ability of the saline perfusate results in edema and an increase in flow rate. Because the mouse has a high heart rate, it is likely that oxygen delivery is on the edge of oxygen demand under baseline perfusion conditions. Furthermore, Krebs-Henseleit buffer typically contains only glucose as a substrate, whereas the heart normally uses fatty acids as its prime substrate. Fatty acids can be given as substrates but require the addition of a vehicle such as BSA and also require specialized methods for oxygenating the buffer. An advantage of the perfused heart model is that it allows one to examine the impact of different substrates either alone or in combinations. The effect of differences associated with perfusion with long-chain versus short-chain fatty acids also can be studied. Ex vivo hearts can be readily perfused with radioactive- or stable isotope-labeled substrates, allowing evaluation of substrate selection and metabolism (167, 211, 245).

The no-reflow phenomenon (179) can also impact infarct size and its measurement in isolated hearts. During ischemia and early reflow, the heart goes into contracture, which restricts flow to the subendocardium. The severity of no reflow depends on the severity of ischemic injury and can vary between control and protected hearts. In isolated hearts, no-reflow issues can be reduced by deflating the balloon in the LV for a few minutes right at the start of reperfusion (94).

To summarize, the major strengths and limitations of the isolated perfused heart are shown in Table 1. In consideration for all of the above factors, it is imperative that control and treated hearts are studied under identical conditions. Although ischemic pre- and postconditioning were originally described in an in vivo dog model, much of the information about the molecular signaling pathways responsible for protection was established in perfused heart models (128).

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IN VIVO MODELS

Chronic Coronary Artery Disease

Coronary stenosis and stress-induced myocardial ischemia: model rationale and variables measured. Reversible episodes of ischemia can lead to contractile dysfunction in the absence of significant myocyte necrosis (42). Because ischemic entities are frequently encountered in clinical practice, the task of understanding their pathophysiology and testing therapeutic interventions has stimulated the development of animal models in which acute and chronic adaptations to ischemia and the ensuing functional recovery can be evaluated over time. Most of these entities are consequences of either brief or chronic episodes of ischemia, and the models developed to study them will be discussed separately below.

Chronic stable angina is a clinical condition whereby a patient has one or more coronary stenoses that have largely compromised or even exhausted autoregulatory coronary reserve. Frequently, these limitations are partially compensated by collateral blood flow from adjacent less-compromised coronary arteries such that myocardial blood flow and contractile function remain normal at rest. Stress situations such as exercise, emotions, or pain, however, can precipitate acute myocardial ischemia with or without typical chest pain. Chronic stable angina in patients does not usually inflict global myocardial ischemia but is a regional event. The acute precipitation of myocardial ischemia requires an in vivo model where an acute coronary stenosis can be produced to reduce coronary blood flow. Alternatively, a stable stenosis must be created where blood flow is maintained at baseline but acute ischemia is elicited, e.g., by pacing or adrenergic activation in anesthetized animals or by exercise in conscious animals (9, 93, 131).

To reflect the regional character of chronic stable angina, regional myocardial blood flow and regional contractile function must be measured. The standard approach to monitor regional blood flow is to use microspheres (142), which have traditionally been labeled with radioactive isotopes (66) and, more recently, nonradioactive colored dyes or fluorescent dyes (107, 184). Analysis of regional myocardial blood flow during acute ischemia reveals an inability of perfusion to increase distal to a stenosis compared with normal remote myocardium (33, 39). As coronary vasodilator reserve is exhausted, there is a major redistribution of blood flow away from the ischemic region toward the nonischemic myocardium where metabolic vasodilation prevails. In addition, there is a transmural blood flow redistribution from the ischemic subendocardium toward the subepicardium (93).

The gold standard for experimental regional contractile function measurements is sonomicrometry (265). Simultaneous measurements of regional myocardial blood flow and regional contractile function provide a means to determine the quantitative relationship between regional blood flow (as a surrogate for oxygen/energy supply) and regional contractile function (as a surrogate for oxygen/energy demand). The relation between regional contractile function and subendocardial perfusion (flow-function relation) demonstrates close coupling during steady-state ischemia at rest as well as over a wide range of cardiac workloads (33, 35, 37, 91, 309). During steady-state acute myocardial ischemia, there appears to be no imbalance between supply (blood flow) and demand (function); rather, there is a matched reduction in both parameters (129, 130). Such matching between regional blood flow and contractile function also persists during major changes in heart rate, when both blood flow and contractile function are normalized for a single cardiac cycle (92). This matching can persist for several hours and contribute to the maintenance of myocardial viability and full recovery of contractile function after eventual reperfusion (212). More specifically, the hallmarks of short-term myocardial hibernation are a perfusion-contraction match (259), together with metabolic signs of adaptation to ischemia (210, 267) and the potential to recruit an inotropic reserve in the dysfunctional myocardium (267). Also, all pharmacological interventions to attenuate acute myocardial ischemia (e.g., by nitrates, β-blockers, Ca^{2+} antagonists, or their combinations) operate along a fixed flow-function relationship (213–215). The two major mechanisms that precipitate myocardial ischemia and must therefore be pharmacologically addressed are tachycardia (112, 113) and coronary vasoconstriction (134, 273).

Studies evaluating brief total coronary occlusions can be conducted in a variety of species. In contrast, to study coronary artery stenosis, the animal under study must be large enough that coronary artery instrumentation along with regional flow and function measurements is feasible (i.e., in dogs and pigs that can be instrumented with a hydraulic occluder on the coronary artery). Acutely anesthetized animals have provided insight into short-term coronary flow regulation over minutes to hours but cannot evaluate the effects of chronic coronary stenosis on long-term microvascular remodeling and collateral growth over days to weeks (285). Acute experiments have the advantage that sequential myocardial biopsies can be taken for the analysis of metabolic and molecular analyses (101, 174, 210, 283). Microdialysis probes can be implanted to evaluate interstitial mediators (209, 270), and the activity of the cardiac innervation can be measured (132).

A significant experimental challenge is maintaining a fixed degree of coronary artery narrowing throughout a study using a hydraulic occluder. This limitation can by circumvented by perfusing the coronary artery at constant pressure from a reservoir, controlling flow with an extracorporeal pump or perfusion of the region of interest with an extracorporeal pressure control system (38). A major limitation of studies in acutely anesthetized animals are the substantial confounding effects of anesthesia and neurohormonal activation on hemodynamics and flow, which alter coronary autoregulation and produce varying degrees of coronary vasodilation and vasoconstriction that modulate autoregulatory responses (33, 36, 39). The limitations of acute studies can be circumvented by studying conscious, chronically instrumented animals. While strict control of hydraulic occluders and coronary collaterals stimulated by repetitive ischemia and chronic stenosis at first seems a limitation, these factors can be capitalized upon by provoking coronary collateral development to the point where the artery can be totally occluded without reducing resting myocardial perfusion. This is typically accomplished in dogs using ameroid occluders, which are hygroscopic and gradually swell to produce a progressive stenosis resulting in a total occlusion within 3–4 wk.

Collateral blood vessel growth can also be stimulated in dogs by repetitive brief coronary occlusions using a hydraulic occluder (303, 320). Once collaterals are developed, variability

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in the hydraulic stenosis severity is no longer a problem, and intervention effects on stress-induced ischemia can be studied under multiple conditions. While pigs can also develop collateral-dependent myocardium after ameroid occluder placement (260), collaterals grow more slowly than in dogs, and pigs frequently develop a subendocardial infarction (230). The admixture of infarcted and normal myocardium greatly complicates measurements of perfusion and function. Infarction can largely be circumvented by employing a fixed diameter stenosis on the coronary artery of farm-bred swine, resulting in a much more severe limitation of subendocardial flow reserve than in dogs, and there is usually contractile dysfunction at rest (74, 77). While this is an extremely useful model to study chronic vascular adaptations and interventions to promote angiogenesis, alterations in myocardial physiology can complicate the interpretation of flow changes. A major drawback of chronic large animal models of coronary stenosis and collateral-dependent myocardium is their expense and the labor-intensive nature of the animal care and handling. The guinea pig has a very well-developed collateral circulation that prevents infarction from occurring following occlusion of a single main coronary artery; to obtain infarction, multiple coronary arteries need to be ligated (216). Thus, guinea pigs are not suitable for in vivo ligation studies but can be used for heart perfusion with global ischemia experiments. These issues have motivated studies to assess flow regulation using repetitive coronary occlusions in rats and mice, including genetically altered animals (303).

Coronary microembolization: model rationale and variables measured. Subclinical atherosclerotic plaque rupture or erosion that does not result in complete thrombotic occlusion of the coronary artery but leaves a residual blood flow into the distal coronary microcirculation occurs spontaneously, with or without clinical symptoms. Coronary microembolization is also induced iatrogenically during percutaneous coronary interventions. Atherosclerotic debris from the culprit lesion, together with thrombotic material, soluble vasoconstrictors, as well as thrombogenic and inflammatory substances, is washed into the coronary microcirculation where it causes microvascular obstruction with resulting patchy microinfarcts and an inflammatory reaction (135, 173). The inflammatory response includes increased expression of tumor necrosis factor-ex associated with profound contractile dysfunction and upregulation of signal transduction pathways involving nitric oxide, sphingosine, and ROS, which contribute to impaired excitation-contraction coupling (32, 299). Repetitive coronary microembolization can result in global LV dysfunction and, even in the absence of overt infarction, in heart failure (261). Coronary microembolization can be simulated experimentally by intracoronary infusion of inert particles of various diameter (67) and also by intracoronary infusion of autologous microthrombi (191). When the target under study is ischemic heart failure, repeated coronary microembolization can be used in both small and large animal models. When the target under study is a spontaneous or periprocedural minor infarction, the animal must be large enough such that regional myocardial measurements of flow, contractile function, metabolism, and morphology are possible (i.e., dog or pig models are preferable).

The major strengths and limitations of angina models are shown in Table 1.

Stunning, Hibernation, and Ischemic Cardiomyopathy

Stunning: model rationale and variables measured. When ischemia caused by a total coronary occlusion is brief (e.g., as may be experienced from coronary vasospasm), regional contractile dysfunction persists for hours after reperfusion but then completely normalizes within 24 h. This phenomenon was first demonstrated after a 15-min circumflex coronary artery occlusion in chronically instrumented dogs, was subsequently called stunned myocardium, and is common in patients with acute coronary syndrome (17, 19, 143). Most investigators assume that the complete normalization of function, lack of evidence of infarction by TTC staining, and lack of sarcolemmal disruption on electron microscopy indicate that no cardiomyocyte death is associated with stunning. While pathological evidence of myocyte necrosis is indeed absent, TUNEL staining performed 1 h after reperfusion demonstrates that programmed cell death or myocyte apoptosis develops in rare isolated cardiac myocytes and circulating cardiac troponin I is increased (314). Thus, while there is no evidence of infarction in stunned myocardium, regional myocyte loss can develop when stunning becomes repetitive.

Because the essence of stunned myocardium consists of relatively rapid (24–48 h) reversibility of contractile dysfunction in the absence of TTC or pathological evidence of infarction, most studies use chronic large animal models in which serial measurements of function can be performed. In addition, regional ischemia is the preferred model to allow assessment of the remote nonischemic regions of the heart as an internal control. While stunned myocardium occurs after demand-induced ischemia distal to a coronary stenosis (144), most studies have used transient total coronary occlusion. Animals are instrumented with a hydraulic occluder to produce brief ischemia 1–2 wk after recovery from surgical instrumentation.

To assess regional function, most studies have used sonomicrometry for direct measurements of subendocardial segment length shortening or wall thickening. Recent studies have used transient occlusion of the left anterior descending coronary artery (LAD) using a balloon angioplasty catheter in closed-chest sedated animals where regional function can be assessed with imaging approaches such as echocardiography (314). The latter approach circumvents the need for chronic surgical instrumentation through a prior thoracotomy. Echocardiography can also be employed to assess stunning in mice chronically instrumented with an occluder to produce transient ischemia (63).

The dog (20, 143), pig (291, 314), and rabbit (18) are the most commonly used species to study myocardial hibernation. Pigs and rabbits offer the advantage of having little or no collateral circulation, so that the severity of the ischemic insult and of the subsequent contractile dysfunction are more uniform. In contrast, dogs exhibit a highly variable degree of collateral circulation resulting in widely different degrees of myocardial stunning (20). There are also species differences in the time course of recovery despite similar occlusion durations (277). Many studies have also used open-chest animal models, although the severity of myocardial stunning in these preparations is significantly exacerbated versus conscious animal models (21, 304). Most experimental approaches to assess stunning are quite straightforward, although ventricular fibrillation can develop. This is more common in swine as opposed to canine

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models, because pigs have little innate coronary collateral flow (164). Because myocardial function assessed using segment shortening and wall thickening is load dependent, it is important to ensure that heart rate, systolic blood pressure, and LV end-diastolic pressure remain reasonably constant over the time frame of the measurements. An advantage of chronic models using regional ischemia is that each animal can potentially serve as its own control; hence, it is possible to use the same animals to study the effects of pharmacological interventions on physiological end points.

Short-term hibernation: model rationale and variables measured. A prolonged episode of moderately severe ischemia can be sustained for a period of hours in the absence of pathological evidence of infarction. This phenomenon is termed short-term hibernation (139, 212). An approximate 50% reduction in perfusion leads to reduced function and perfusion-contraction matching, which largely prevents irreversible myocyte injury. If the heart is reperfused within a few hours, contractile dysfunction persists in a fashion similar to stunned myocardium but with a more protracted time course of recovery (i.e., lasting in the timeframe of days rather than hours) as is typically seen with stunning after a brief total occlusion. This in part appears to relate to reversible myofibrillar disassembly and myolysis in the absence of sarcolemmal disruption (279). Unfortunately, when the initial adaptive response to moderate ischemia in short-term hibernation is present for longer than 12 h, progressive myocardial necrosis begins to develop, resulting in some degree of myocardial infarction usually confined to the subendocardium (48, 185, 269). While imposition of acute moderate ischemia was initially proposed as a mechanism of chronic hibernating myocardium, the development of progressive infarction when flow reductions last longer than 12 h leads to a pathological entity with myofibrillar disassembly and cardiac biomarker release that can no longer be defined as hibernation but, rather, is more in line with subendocardial infarction (48, 279).

Studies of short-term hibernation usually require closed-chest animal models, although considerable insight about adjustments between flow and function has been gleaned from open-chest studies of myocardial metabolism (137, 210, 239). The latter studies usually use a cannulated branch of the left coronary artery perfused at constant pressure. Closed-chest animal studies usually employ chronically instrumented dogs or pigs. In these studies, a hydraulic occluder is placed around a coronary to reduce flow or coronary pressure to a fixed level, which is released after several hours.

Chronic hibernation and stunning: model rationale and variables measured. While both stunning and short-term hibernation are characterized by complete functional recovery, chronic contractile dysfunction can develop when recurrent ischemia develops before functional normalization (21). Chronic contractile dysfunction from repetitive ischemia develops in the absence of histological infarction, and both hibernation and stunning involve the loss of myocytes via apoptosis in a fashion similar to what happens after brief episodes of ischemia (193, 314). Unlike stunning, which was initially an experimental observation that later became associated with multiple clinical correlates, chronic hibernating myocardium was first characterized in patients with chronic ischemic heart disease displaying regional contractile dysfunction in the absence of manifest ischemia (23, 139). Only later were animal models used to identify cellular and molecular mechanisms responsible for the adaptive responses to chronic ischemia (77).

While it was originally controversial whether or not flow was reduced or normal at rest (34), it is now clear that chronic repetitive ischemia initially results in contractile dysfunction with normal resting flow or chronic stunning (73, 278). When this situation persists, the reduction in function leads to a secondary reduction in regional energy utilization accompanied by reduction in resting flow (76). Thus, the reduction in resting flow characteristic of chronic hibernating myocardium is a result, rather than cause, of regional dysfunction.

In contrast to models of short-term ischemia, animal models of hibernating myocardium are based on chronic coronary stenoses that frequently progress to total occlusion and collateral-dependent myocardium. In studies using ameroid occluders that gradually swell to produce chronic stenosis, dogs usually do not develop contractile dysfunction at rest but can do so when preexisting epicardial collaterals are ligated at the time of instrumentation (36). Swine ameroid occluder models frequently have contractile dysfunction in collateral-dependent myocardium, and this is usually associated with some degree of subendocardial infarction (230).

A more consistent model of hibernating myocardium can be produced by instrumenting juvenile swine with a fixed diameter stenosis (1.5-mm diameter) on the proximal LAD (73, 77). As the animals grow over the subsequent 3 mo, there is a slowly progressive limitation in coronary flow reserve, because the LAD stenosis limits maximal myocardial perfusion, while the mass of myocardium distal to the stenosis increases in parallel with cardiac growth. As a result, there is a more prolonged and gradual stimulus for coronary collateral development so that LAD occlusion almost always develops in the absence of infarction.

After 3 mo, regional contractile dysfunction with mild reductions of resting flow in the absence of infarction is consistently manifest and is similar to the changes seen in humans with hibernating myocardium caused by a chronic LAD occlusion (308). Serial studies of this animal model have demonstrated that the heart progressively adapts from a state of contractile dysfunction with normal resting flow (chronic stunning) to a state where resting flow decreases, consistent with hibernating myocardium (41). Such chronic hibernation is associated with a downregulation in mitochondrial metabolism and regional myocyte hypertrophy that maintains myocardial wall thickness constant in the setting of regional apoptosis-induced myocyte loss.

Over longer periods of time (up to 6 mo) the adaptive response of hibernating myocardium persists unchanged (75), and the downregulation in metabolism and upregulation of proteins involved in cellular survival and cytoprotection prevent cell death and, hence, further myocyte loss (62). While infarction does not develop in this model, revascularization only partially reverses myocardial dysfunction and does so over a much longer time frame than seen with either myocardial stunning or short-term hibernation (237). Chronic contractile dysfunction in the absence of infarction can also be induced using a hydraulic occluder to produce an acute stenosis in chronically instrumented animals. Chronic stunning can develop in swine subjected to daily episodes of short-term hibernation (169). A more rapid transition from chronic stunning to

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hibernating myocardium than the one observed in the fixed diameter stenosis model can be achieved by acutely imposing a critical stenosis on the LAD (301). The latter model can develop reductions in flow with regional contractile dysfunction after 2 wk of a stenosis sufficient to prevent reactive hyperemia.

The fixed diameter chronic stenosis model is advantageous in that hibernating myocardium develops reproducibly in a predictable time frame. A limitation of the fixed stenosis porcine model is that it requires cardiac growth to produce a progressive physiological impairment in maximum myocardial perfusion, and the 3- or 4-mo period required to develop hibernating myocardium is viewed as cost prohibitive. This model has so far only been studied in juvenile farm bred swine and may produce variable results if cardiac growth is attenuated by limiting feeding. It is not clear whether the model can be effected in purpose-bred swine and, particularly, in miniswine, where growth rates are substantially attenuated. An additional disadvantage is that the chronic stenosis model is associated with a high rate of spontaneous ventricular fibrillation (40). This has provided insight into the mechanisms of sudden cardiac arrest in chronic coronary disease but reduced the success of studying chronic adaptations to ischemia in survivors. Finally, because of the long duration of the studies in the presence of animal growth, it is not feasible to chronically instrument animals. Nevertheless, it is feasible to use telemetry to assess chronically LV pressure and arrhythmias in untethered conscious animals (242).

Ischemic cardiomyopathy: model rationale and variables measured. Ischemic cardiomyopathy is the underlying cause of LV dysfunction in two out of every three patients with heart failure (105). Ischemic cardiomyopathy in humans can arise from LV remodeling after a large myocardial infarction but, more commonly, is the result of extensive multivessel coronary artery disease with modest amounts of diffuse fibrosis and patchy infarction in multiple coronary artery distributions (15). Along these lines, preclinical studies have established that chronic coronary artery stenosis can induce significant myocyte loss with modest global replacement fibrosis that leads to global LV dysfunction and varying degrees of congestive heart failure when the area at risk is large. Conceptually, the stenosis does not limit blood flow at rest. Rather, by reducing maximal perfusion in response to stress, it sets the stage for repetitive episodes of subendocardial ischemia. A key feature of all animal models of ischemic cardiomyopathy is that the myocardium at risk of repetitive ischemia represents a large portion of the LV (>70% of LV mass). This has been achieved using stenosis of the left main coronary artery in rodents or multivessel coronary artery stenoses in large animals. As a result of the large area at risk, myocyte cell death arises from both ischemia and myocyte stretch and slippage from increased LV end-diastolic pressure (possibly also reflecting transient ischemia).

In rats, ischemic cardiomyopathy can be induced by producing a fixed coronary stenosis of ~50−60% diameter reduction on the proximal left coronary artery, which causes variable degrees of LV dysfunction (44, 45). While replacement fibrosis occurs in these animals, it is patchy and modest, only increasing twofold over control for an average of <10% of LV cross-sectional area. Interestingly, the degree of LV dysfunction is primarily related to myocyte cell loss (necrosis and apoptosis) and the elevation in LV end-diastolic pressure related to fibrosis. A similar model of ischemic cardiomyopathy has also been obtained in mice (189). While rodent models afford the ability to perform higher throughput studies and use transgenic animals to study molecular mechanisms, they have relatively high surgical and postoperative mortality. In addition, there is considerable variability in physiological outcomes, such that frequently animals are retrospectively categorized into mild, moderate, and severe heart failure groups. Reproducibility of ischemic cardiomyopathy models, therefore, is indeed a concern.

While left main coronary stenosis is not feasible in large animals, multivessel coronary stenoses can produce a large ischemic risk area and recapitulate ischemic cardiomyopathy. When fixed diameter occluders are placed on both the proximal LAD and circumflex arteries in growing farm-bred swine, LV ejection fraction declines with elevated resting LV end-diastolic pressure (74), consistent with compensated LV dysfunction and no overt evidence of heart failure. These animals also exhibit primary myocyte loss with only an approximately twofold increase in extracellular matrix accumulation. A similar condition has been produced using multivessel ameroid occluders in dogs (80). Aside from requiring survival surgery, the major disadvantage of these approaches arises from the development of sudden cardiac arrest, which in swine is related to both ventricular fibrillation and to a lesser extent bradyarrhythmias. In mice, a state of ischemic cardiomyopathy can be induced using repetitive brief coronary occlusions, and this model is associated with substantial but reversible fibrosis of the myocardial region subjected to repetitive ischemia (63).

Noninvasive imaging. Noninvasive cardiac imaging technologies such as echocardiography, magnetic resonance imaging (MRI), and computed tomography can measure regional and global contractile function and are increasingly available for preclinical studies, particularly in larger animals. NMR spectroscopy can provide information on cardiac energetics (114). More sophisticated imaging technologies such as positron emission tomography can measure regional myocardial perfusion and regional myocardial metabolism and sympathetic innervation and are increasingly used in preclinical studies (77, 187, 268). MRI can serially measure myocardial perfusion (264) and can provide reliable measurements of infarct size and microvascular obstruction. MRI-derived edema, however, is time dependent and sensitive to cardioprotective interventions (141, 148). Therefore, MRI-derived edema can be used to stratify an ischemic/reperfused myocardial region for protocol assignment but not for quantitative normalization of infarct size to area at risk.

To summarize, the major strengths and limitations of stunning, hibernation, and ischemic cardiomyopathy models are shown in Table 1.

Myocardial Infarction Models: Permanent Coronary Artery Occlusion with Nonreperfused and Reperfused Myocardial Infarction

MI: general considerations. Coronary occlusion causes immediate cessation of aerobic metabolism in the ischemic myocardium, leading to rapid ATP depletion and metabolite accumulation and resulting in severe systolic dysfunction within seconds (86). If the duration of the ischemic insult is <15 min

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in larger mammals such as dog and pig, restoration of flow reverses the early ischemic cardiomyocyte changes (transient mitochondrial swelling or glycogen depletion) and all cardiomyocytes in the ischemic area can survive (158). Longer periods of ischemia cause death of an increasing number of cardiomyocytes. A 20- to 30-min interval of severe ischemia is sufficient to induce irreversible changes in some cardiomyocytes of the subendocardial area, inducing sarcolemmal disruption and striking perturbations in mitochondrial architecture, such as ultrastructural evidence of amorphous matrix densities and severe mitochondrial swelling (156). These early ultrastructural alterations mark cardiomyocytes that cannot be salvaged and will ultimately die in the infarct environment (157).

Experimental studies in the canine model of MI demonstrate a transmural heterogeneity in the myocardial response to ischemia, suggesting that the subendocardium, where myocardial oxygen demand is greatest, is more susceptible to ischemic injury than the midmyocardium and subepicardium (2). Thus, the prevailing paradigm suggests a wavefront of cardiomyocyte death that progresses from the more susceptible subendocardium to the less vulnerable subepicardium as the duration of the ischemic insult increases (159, 252). Experimental studies in large animal models have demonstrated that ischemic myocardium cannot be salvaged by reperfusion after 6 h of coronary occlusion (251). The increased vulnerability of subendocardial regions to coronary occlusion may reflect a greater reduction of the subendocardial blood flow due to transmural differences in vascularization (2, 25) and extravascular compression (68, 286). The wavefront concept of ischemia developing into infarction was derived from experimental studies in dogs, where a substantial coronary collateral circulation influences the time course of cardiomyocyte necrosis (86).

The major species difference in the MI response lies in the temporal and spatial kinetics of events and differences due to myocardial size. In mice, durations of coronary occlusion exceeding 60–90 min are considered irreversible, and inflammation and wound healing processes are accelerated (64, 88, 221, 222). In mouse and rat models, reperfused infarcts are typically midmyocardial, and subepicardial and subendocardial regions are relatively spared (50, 69, 325). Studies in a sheep model of reperfused infarction also suggest that the midmyocardium may be most vulnerable to ischemic injury; in contrast, the subendocardium is relatively resistant (263). The pig model of coronary occlusion-reperfusion comes closest to human STEMI in its temporal and spatial development, but other models are nevertheless useful to study fundamental mechanisms of MI (140).

MI: technical considerations. Extensive protocols providing technical details for performing permanent occlusion MI and reperfused MI in mice and rats are available (221, 222, 228, 317, 327). While MI is most commonly performed in rodent models, protocols in other animal models are also available (151, 183, 218, 331). For mice, the quality of open-chest surgery to induce coronary occlusion directly impacts study outcomes (152, 221, 222). Minimizing the size of the thoracotomy and limiting bleeding by entering the thorax through intercostal muscles are recommended.

Biomarkers that have been used to evaluate the presence of MI include cardiac troponins and creatine kinase, and plasma proteins such as macrophage migration inhibitory factor can also be measured as indices of injury (47, 55). Infarct size is widely measured as a key variable for testing genetic or therapeutic intervention efficacy, and infarct size measurements taken serially at both early and late time points can evaluate the extent of infarct expansion (22). Echocardiography can also be used for infarct sizing, with the caveat that echocardiography does not distinguish between reversible ischemic dysfunction (stunning) and irreversible loss of function and therefore a secondary method is needed for confirmation of infarct size at early time points. For more details on measuring cardiac function in mice, the reader is advised to consult the article *Guidelines for measuring cardiac physiology in mice* (196).

Permanent occlusion MI: model rationale and variables measured. Permanent coronary occlusion is a relevant animal model of acute STEMI reflective of patients who, due to contraindications or logistic issues, do not receive timely or successful reperfusion (53, 104). Permanent coronary occlusion yields acute ST segment elevation infarction with robust myocardial inflammation and long-term remodeling, thus providing a large effect size that reduces the sample size needed to detect differences between groups. Infarction assessed in the first 1–14 days after coronary ligation is histologically characterized by coagulation band necrosis with a fulminant inflammatory infiltrate in the infarct and border zone regions. Infarction is geometrically and physiologically characterized by wall thinning, increases in LV dimensions and volumes, and decreases in fractional shortening and ejection fraction.

Changes that occur over the first week provide information on myocyte cell death and infarct development, inflammation and leukocyte physiology, extracellular matrix (ECM) turnover and fibroblast activation, and the role of endothelial cells in neovascularization (83, 154, 165, 194, 205). Chronic evaluation at time points 4–8 wk post-MI provides information on long-term remodeling. Whether the infarct region or remote region is the focus of investigation depends on the question asked. Examining the infarct region provides details on active inflammation and scar formation, while examining the remote region provides details on still-viable myocytes within the myocardium and remote inflammatory and ECM processes.

Perioperative and postoperative survival should be assessed, and the time point of delineation between these two phases should be defined. For some laboratories, the perioperative phase includes the time until the animal recovers and becomes ambulatory (usually within 1–3 h for mice). For other laboratories, the perioperative phase includes the first 24 h after surgery. Perioperative death within 24 h post-MI in mice is usually due to surgical errors (or very large infarct sizes), and, in established laboratories, the 24 h surgical mortality rate due to technical issues is <10%. In the permanent occlusion MI model in mice, postoperative death (deaths at >24 h time point) typically occurs during *days 3–7* post-MI and is due to rupture, acute heart failure, or arrhythmias (59, 98, 233). Autopsy is strongly recommended for all mice that die prematurely, to evaluate early deaths due to technical issues and later deaths due to complications of MI. Seven-day postoperative mortality rates are ~10–25% (75–90% survival) for female young mice and 50–70% (30–50% survival) for male young mice (47, 61, 89, 98, 152, 170, 195, 202, 206, 234, 310–312, 319, 323). Immediate survival from the surgery can also be affected by baseline characteristics such as obesity, diabetes, or high levels of circulating inflammatory cells, which, in turn,

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determine the response to anesthesia and surgery (60, 123, 202, 203). While there is no difference in infarct tolerance between young and middle-aged mice (323), older mice may survive better than younger mice (202, 319).

For permanent occlusion MI models, infarct size must be measured in fresh LV slices at the time of necropsy by TTC staining and typically ranges from 30% to 60% of the total LV (47, 48, 61, 89, 98, 152, 195, 202, 206, 223, 310–312, 319, 323). The method for calculating infarct size varies across laboratories. Some laboratories use area calculations, other laboratories measure length in the midmyocardium, and other laboratories measure and average lengths in the subendocardium and subepicardium. There is no need to use Evans blue for area at risk assessment in permanent coronary occlusion models that pass the point from ischemia to infarction, as the entire area at risk is infarcted. It is important that MI surgical success is confirmed and that the initial infarct injury is comparable across groups, to assess remodeling differences at later stages. In mice, ligating the coronary artery at the same anatomical location across groups is important; 1 mm distal to the left atrium is the recommended site to generate large infarcts (35–60% of total LV). Failure to induce MI can occur, usually due to missing the coronary artery during the ligation step. Monitoring the electrocardiogram for ST segment elevation during the procedure reduces this possibility. Echocardiography at 3 h after coronary occlusion can be used to exclude animals with excessively small or large MI before randomizing groups (153, 155, 195). Late gadolinium-enhanced MRI is also useful for selecting animals with consistent infarct sizes (262). When assessing effects of treatments initiated post-MI, it is important to show that infarct size is not different between groups before treatment. Plasma sampling at 24 h post-MI can be used to assay cardiac biomarkers, such as troponins and inflammatory cytokines, with the caveat that these measurements can indicate presence or absence of infarct and not extent of injury. After coronary occlusion, care should be taken in performing these assessments to minimize disturbing animals at times when cardiac rupture may be triggered by stress, particularly at *days 3–7* post-MI in untreated controls (96, 98). Small infarcts may reflect technical issues in missing the coronary artery, resulting in damage from the suture rather than reflecting the intended myocardial ischemia and infarction. Infarct sizes <30% are typically excluded. If included, small and large infarcts may need to be grouped separately to reduce possible type II statistical errors.

Cardiac wound healing and remodeling, typically assessed days to weeks post-MI, can be examined using a wide variety of approaches, including echocardiography, histology, biochemistry, and cell biology (5, 217, 328). Serial measurements of cardiac geometry and function by echocardiography are useful for defining phenotypes. Cardiac dimensions vary depending on heart rate and depth of anesthesia, and these parameters must be carefully controlled and matched across groups. Cardiac functional reserve can be assessed by measuring the contractile response to inotropic drugs or volume overload. Cardiac MRI and hemodynamic assessment by pressure-volume catheterization are other ways to measure cardiac physiology parameters. It is feasible to quantify infarct size noninvasively and serially by using cardiac MRI (181). Hemodynamic evaluation in mice is a terminal procedure, which prevents its use in serial assessments.

Hematoxylin and eosin staining provides information on areas of necrosis and inflammation, while picrosirius red staining provides information on total collagen accumulation both in the scar and remote regions (316). Immunohistochemistry for neutrophils, macrophages, lymphocytes, fibroblasts, and endothelial cells provides information on the extent of inflammation, scar formation, and neovascularization. Isolating individual cell types and assessment of ex vivo phenotypes in culture can further aid in understanding mechanisms. Studies have revealed that inflammation evoked by acute myocardial infarction also occurs systemically and that the spleen and liver are important sources of cells and factors that influence LV remodeling (71, 72, 95, 116, 198, 199, 293).

I/R MI: model rationale and variables measured. Implementation of myocardial reperfusion strategies has significantly reduced mortality in acute STEMI. Reperfusion has contributed to the growing pool of patients who survive the acute event and are at risk for adverse remodeling and subsequent development of heart failure (133, 136). In addition to salvaging cardiomyocytes, reperfusion has profound effects on cellular events responsible for repair and remodeling.

Although timely reperfusion is essential to salvage viable cardiomyocytes from ischemic death, extensive preclinical and clinical evidence suggests that reperfusion itself causes injury (119, 121, 147). Reperfusion-induced arrhythmias and myocardial stunning are self-limited and reversible forms of reperfusion injury, while microvascular obstruction and lethal cardiomyocyte injury are irreversible and extend damage, thus contributing to adverse outcomes following MI (13, 126, 177, 241, 318). In patients, no reflow during reperfusion may be exacerbated due to the generation of microemboli composed of atherosclerotic debris and thrombi during percutaneous coronary interventions (135, 253).

MI both with or without reperfusion shares many of the same technical guidelines, and this information is provided above. The one technical difference is whether the ligation is removed at 45–60 min after the occlusion to reperfuse the myocardium. Similar to permanent occlusion MI, studies investigating the inflammatory and reparative response following MI with reperfusion need to take into account the dynamic sequence of cellular events involved in repair. Common measurements shared by the two MI models are shown in Table 2. For studies aimed at investigating acute myocardial injury using a reperfusion strategy, the duration of coronary occlusion needs to be sufficient for the induction of significant MI but not overly prolonged to cause irreversible injury in the entire area at risk. From the cell physiology perspective, the reparative response after MI can be divided into three distinct but overlapping phases: inflammation, proliferation, and maturation (26, 65). In the infarcted myocardium, dying cardiomyocytes release damage-associated molecular patterns and induce cytokines and chemokines to recruit leukocytes into the infarcted region, thus triggering an intense inflammatory reaction that serves to clear the infarct from dead cells and ECM debris, while initiating a reparative response (84). Early reperfusion after irreversible cardiomyocyte injury accelerates and accentuates the inflammatory reaction and has profound effects on the pathological features of the infarct. Microvascular hyperpermeability is evident in the myocardium with acute I/R (97). Rapid extravasation of blood cells through the hyperpermeable vessels may result in hemorrhagic changes (98, 178). Influx of

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Table 2. *Common output measurements for in vivo MI and MI/reperfusion studies*

Measurement	Information Provided
Infarct size	Infarct size (MI)
	Infarct size per area at risk (MI/reperfusion)
	Initial ischemic stimulus
	Final area of damage
	Effect of therapy or intervention
Plasma biomarkers	Ischemia: creatine kinase, troponins
	Inflammation: cytokines and chemokines
	Scar formation: growth factors and the ECM
	Neovascularization: angiogenic factors
Left ventricular physiology (echocardiography, MRI, positron emission tomography imaging)	Geometry and function: dimensions, wall thickness, left ventricular dimensions and volumes, fractional shortening, ejection fraction, remodeling index
	Electrophysiological function: PR, QRS, and QT intervals/morphology; spontaneous and inducible arrhythmias
Inflammation	Immunohistochemistry and immunoblot analysis for cell numbers and inflammatory protein expression
	Flow cytometry analysis of the digested myocardium for individual cell phenotypes
	Gene expression
	Systemic and circulating inflammation
ECM scar	Picrosirius red for collagen deposition
	Immunohistochemistry and immunoblot analysis for ECM proteins and cross-linking enzymes
	Gene expression
	Scar strength assessment
Neovascularization	Blood vessel numbers
	Vessel type and quality
Microvascular damage	Microvascular plugging
	Hyperpermeability/edema
	Hemorrhage

MI, myocardial infarction; ECM, extracellular matrix; MRI, magnetic resonance imaging.

phagocytotic macrophages is accelerated, resulting in more rapid removal of dead cardiomyocytes compared with permanent occlusion MI. In reperfused infarcts, dying cardiomyocytes often exhibit large contraction bands, comprised of hypercontracted sarcomeres. Subsarcolemmal blebs and granular mitochondrial densities, which are already present in irreversibly injured cardiomyocytes before restoration of blood flow, become more prominent upon reperfusion.

Phagocytosis of dead cells by activated macrophages results in the activation of endogenous anti-inflammatory pathways, ultimately leading to resolution of the inflammatory infiltrate. Suppression of inflammation is followed by recruitment of activated myofibroblasts that deposit large amounts of ECM proteins and by activation of angiogenesis (145). As the scar matures, fibroblasts become quiescent and infarct neovessels acquire a coat of mural cells (332). Compared with large mammals, rodents exhibit an accelerated time course of infiltration with inflammatory and reparative cells (64).

Leukocyte infiltration during the inflammatory phase of infarct healing and myofibroblast activation and accumulation during the proliferative phase are predominantly localized in the infarct region and border zone (87, 122, 280). During scar maturation, the cellular content in the infarcted region is reduced. At the same time, however, the number of activated macrophages and fibroblasts in the remote remodeling myocardium increases. Therefore, study of inflammatory and reparative cell infiltration and assessment of ECM protein deposition should include systematic assessment of each end point in the infarcted region, the peri-infarct area, and the remote remodeling myocardium.

Sympathetic nerves are damaged by permanent coronary occlusion but can regenerate after injury (220). In the setting of chronic MI, regional hyperinnervation around the infarcted region has been observed, and activation of cardiac sympathetic nerves is important in triggering ventricular arrhythmias, and such proarrhythmic action is dependent on the extent of infarction (1, 70, 315, 326). In contrast, after I/R, chondroitin sulfate proteoglycans prevent reinnervation (99, 100). Thus, the model selected for sympathetic nerve evaluation should be taken into consideration and depends on what question is being asked.

MI: intervention considerations. The effects of interventions on post-MI remodeling can be studied using both nonreperfused MI and reperfused MI/R models (11, 219, 232, 294, 322). Typically, nonreperfused MI yields accentuated dilative remodeling and exacerbated dysfunction compared with a reperfused infarct involving the same vascular territory, reflecting a combination of more extensive infarct and less effective repair. In the reperfused MI/R model, the effects of genetic or pharmacologic interventions implemented early after reperfusion may reflect differences in the extent of acute cardiomyocyte injury rather than differences in wound healing responses. With permanent occlusion MI (assuming a standardized area at risk) or very late reperfusion models, differences in geometry and function of the remodeling heart are independent of acute cardiomyocyte injury and reflect effects on inflammatory, reparative, or fibrotic cascades. In the presence of an occluded coronary artery, the delivery of systemically administered pharmacologic agents to the infarcted region of large animal models may be dependent on formation of collaterals.

While the development of genetically targeted animals (mice, rats, and rabbits) resulted in an explosion of studies dissecting cell biological mechanisms and molecular pathways, large animal models are considered closer to the clinical situation for translational studies to test safety and effective-

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ness. Optimal study of molecular, cellular, and LV functional end points and interpretation of the findings require understanding of the underlying pathophysiology. Assessment of infarct size is typically the primary end point for investigations examining the mechanisms of cardioprotection. Assessment of chamber dimensions using echocardiography or MRI is crucial to study the progression of adverse remodeling. Systolic and diastolic cardiac geometry and function can be assessed noninvasively using echocardiography (including Doppler ultrasound and speckle tracking), MRI, and hemodynamic assessment. Mechanistic dissection of specific pathways may require inclusion of additional cell physiology and molecular or proteomic end points. In experimental models of MI, understanding the time course of the cellular and molecular events is critical for optimal study design. The effects of varying ischemic intervals on survival and activation of noncardiomyocyte cellular and acellular (e.g., ECM) compartments are poorly understood. Longer coronary occlusion times have distinct effects on cardiac repair, by extending infarct size and by influencing susceptible noncardiomyocyte populations, such as endothelial cells, fibroblasts, pericytes, and immune cells (85). Most studies characterizing responses to myocardial injury have so far been performed in healthy young animals. Comorbid conditions, such as aging, diabetes, and metabolic dysfunction, affect the pattern of ischemic injury and modify the time course and qualitative characteristics of the inflammatory and reparative responses (27, 106, 202, 238, 298, 319, 323). These comorbidities are relevant in the clinical context and must be considered in translation of experimental findings to the clinic.

To summarize, the major strengths and limitations of the nonreperfused and reperfused MI models are shown in Table 1.

Ablation

Model rationale and variables measured. The primary advantages of ablative injury techniques such as cryo-, thermal-, and radio-frequency ablation are rigid and reproducible control over the size, shape, and location of the region of damage. With such methods, a wound can be stamped on the target myocardial tissue with consistent dimensions, shape, and transmural depth. Because the size of the damaged region is independent of animal-to-animal variations in coronary anatomy (223), the resultant ablation scar is also more reproducible than ligation-induced injury (52, 160, 307), aiding studies of long-term structural and functional remodeling and providing better power to detect the effects of an experimental drug or cell therapy. Infarct location can thus be controlled independently from coronary anatomy and infarct transmurality can be controlled (52, 160, 290, 305, 307). There are, however, important differences in the modes of cell death in ablative vs. occlusion injuries. For example, cryoinjury results in necrosis due to the generation of ice crystals and disruption of the cell membrane rather than direct ischemia. Furthermore, ablative injuries are typically generated from the epicardial surface inward, whereas ischemic infarcts tend to be propagated outward from the inner myocardial layers (52, 160).

Unlike MI or MI with reperfusion, cryoinjury kills all (or nearly all) cells within the core of the damaged region and creates distinct wound margins. Thus, a number of studies have used cryoinjury to avoid confounding effects of resident surviving cells when testing stem cell and other related therapies (6, 7, 258, 302). Ablation procedures typically apply a cooled/heated probe to either the epicardial or endocardial surface of the heart. The extent and depth of the lesion depend on both the temperature of the probe and the time it remains in contact with the tissue; damage can be extended by generating multiple adjacent lesions or by repeat application at the same location. Because these physical factors are central to injury formation, investigators should report probe size and material, temperature, method and duration of preheating/cooling, precise anatomic location and duration of probe application, and interlesion time and number of lesions (if applicable). Cryoablation has been used to generate reproducible wounds and scar tissue for the study of myocardial injury response in various species including dogs (160, 171, 297), rabbits (6, 7, 302), rats (49, 82, 149, 190), and mice (109, 204, 257, 289, 305, 307). In mice, survival rate after cryoinjury was nearly twice that of permanent coronary ligation over an 8-wk period (307), whereas dysfunction was similar. Lower mortality may be a consequence of smaller infarct size. Ablative methodologies have also been used in nonmammalian species such as zebrafish, to probe the response of cardiac electrical properties to injury, regeneration and scar formation (43, 46, 108). The ability to destroy all cells within the cryoinfarct has provided interesting clues regarding regeneration of fetal myocardium following injury. In neonatal mice, mechanical or ischemic injuries to the ventricular apex typically trigger regeneration, producing recovery of myocardial structure and function without scarring (243, 276). Nontransmural cryoinfarcts in neonatal mice similarly heal with minimal evidence of scarring and full functional recovery with ongoing postnatal growth, while injuries spanning the full thickness of the ventricular wall do not regenerate muscle (57). Because neonatal mice can regenerate myocardium during the first postnatal week, models of myocardial ischemia in neonatal mice may be used to identify pathways involved in cardiac regeneration (8, 208).

As with coronary ligation models, evaluation of LV geometry and function with echocardiography and assessment of electrophysiological remodeling and arrhythmia risk are routinely performed in cryoinjury models. Given the early time course and different mechanisms of necrotic injury in cryoinjury versus ligation models, cryoinjury studies are typically more focused on long-term myocardial regeneration or mechanical/electrophysiological remodeling rather than mechanisms of acute postinjury cell death, inflammation, and scar formation (52, 160). Ablation procedures are now used commonly in clinical electrophysiology, and it is therefore not surprising that a number of experimental electrophysiology studies have taken advantage of geometric control provided by this model (54, 231). Cryoinjury and ischemic injury differ in their transmural localization and the amount of surviving myocardium (52, 109, 257, 258, 305). Finally, methods for ablative targeting of cardiac neural tissues have proven useful in studies of the role of autonomic inputs in normal and arrhythmic hearts (254).

To summarize, the major strengths and limitations of the cryoinjury model are shown in Table 1.

Cardioprotection

Model rationale and variables measured. An intervention that is cardioprotective is broadly defined as serving to protect

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the heart (https://www.merriam-webster.com/dictionary/cardioprotective), thereby in theory encompassing all of the aforementioned aspects of cardiac damage and dysfunction. To date, the only clinically established cardioprotective intervention is early reperfusion (119, 133). The archetypal additive cardioprotective intervention is, without question, ischemic conditioning, encompassing the phenomena of ischemic preconditioning, postconditioning, and remote conditioning (118, 175, 224, 247, 248, 329). Despite differences in the timing of the protective stimulus (with preconditioning applied in a prophylactic manner and postconditioning administered at the time of reperfusion) and the site of the protective trigger (either locally, or, for remote conditioning, in a tissue or organ distant from the at-risk myocardium), all three forms of conditioning share a common theme: there is overwhelming agreement that ischemic preconditioning, postconditioning, and remote conditioning render the heart resistant to lethal I/R-induced injury (78, 118, 128, 163, 247).

This consensus with regard to ischemic conditioning and cardioprotection is, however, a notable exception in the field. Indeed, for the vast majority of the innumerable cardioprotective strategies that have been investigated, the current preclinical literature on the topic of cardioprotection is fraught with controversies and a lack of reproducibility among investigators and laboratories (163, 188). The ensuing confusion in the field may be attributed in part to two confounding factors: an overly broad use of the term cardioprotection in some studies, together with false positive and false negative outcomes derived from protocols executed in a suboptimal manner (162, 163, 188).

In some instances, a broad and suitably framed definition of cardioprotection incorporating, for example, endothelial integrity and vascular function, is appropriate (126, 225). A generally acknowledged and more narrowly focused hallmark of cardioprotection is defined as an agent or intervention that, when administered in the setting of ischemia/reperfusion, augments myocardial salvage and reduces myocardial infarct size beyond that achieved by reperfusion alone (128). Accordingly, for our purposes, we focus on cardiomyocyte viability and define cardioprotection as a strategy that attenuates cardiomyocyte death. Cardiomyocyte viability can be assessed in a full spectrum of models, ranging from cardiomyocytes in culture to isolated buffer-perfused hearts to in vivo studies in rodents (mice and rats) or larger animals (including rabbits, dogs, pigs, sheep, and, in a small number of studies, primates). There is no ideal model that completely mirrors the clinical scenario to fully ensure absolute translational relevance. Rather, each model has merits and disadvantages (as shown in Table 3).

The overwhelming strength of the mouse species is the availability of genetically modified strains to elucidate molecular mechanisms once candidate cardioprotective strategies have been identified, a benefit that is balanced by inherent variability and resultant requirement for large sample sizes. An additional problem of the mouse is the atypical geometry of nontransmural infarcts, in which the subendocardium is spared from death by diffusion of oxygen from the LV cavity and occupies an inordinate proportion of the total LV wall thickness. In all rodents, heart rate is much higher and therefore infarct development is much faster than in larger mammals and humans. Studies conducted in large animals (in particular, the pig) are considered to have the greatest potential for preclinical

Table 3. *Recommendations for cardioprotection studies*

Model	Gold Standard Primary End Point	Required Covariates	Potential Secondary End points	Advantages	Limitations
Cultured cardiomyocytes	Cell viability (live-dead assay)	None	Types of cell death (i.e., apoptosis), mitochondrial function; ROS production	Capacity for high throughput	Reductionist
Isolated buffer-perfused hearts (mouse, rat, and rabbit)	Infarct size (TTC)	For models of regional ischemia: area at risk	Measures of LV function and coronary flow; cTn as a secondary index of cardiac injury	Throughput higher than in vivo; eliminates confounding effect of intervention on systemic blood vessels	Reductionist
Mouse	Infarct size (TTC)	Area at risk; hemodynamics	Measures of LV function; measures of no reflow; CK or cTn as secondary indexes of cardiac injury	Availability of genetically modified strains; model of low collateral flow (measurement of RMBF not required)	Small size; differences between strains substantial variability requiring large *n* values
Rat and rabbit	Infarct size (TTC)	Area at risk; hemodynamics	Measures of LV function; measures of no reflow; CK or cTn as secondary index of cardiac injury	Reliable infarct production; relatively high survival rate in experienced hands; commercial availability of strains that have comorbidities; model of low collateral flow (measurement of RMBF not required)	Not high throughput
Dog	Infarct size (TTC)	Collateral blood flow; area at risk; hemodynamics	Measures of LV function; measures of no reflow; CK or cTn as secondary indexes of cardiac injury	Large amount of historical data; shows effect of intervention in the setting of variable collateral flow; mimics humans with high collateral flow	High cost; variability in collateral perfusion (RMBF must be measured); potential for lethal arrhythmias; not high throughput
Pig	Infarct size (TTC)	Area at risk; hemodynamics	Measures of LV function; measures of no reflow; CK or cTn as secondary indexes of cardiac injury	Model of low collateral flow (measurement of RMBF not required); mimics humans with low collateral flow	High cost; high incidence of lethal arrhythmias; not high throughput

TTC, triphenyltetrazolium chloride; LV, left ventricular; CK, creatine kinase; cTn, cardiac troponin; RMBF, regional myocardial blood flow.

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relevance (103, 140). This advantage is accompanied by substantial costs incurred and, particularly in pigs, the well-documented high incidence of lethal ventricular arrhythmias (282).

In all studies focused on cardioprotection, the primary end point must be a quantitative assessment of cardiomyocyte viability. In cell culture models, these data may be obtained using a live-dead assay such as trypan blue exclusion, propidium iodide exclusion, or other commercially available assays. In intact hearts, including isolated buffer-perfused hearts and all in vivo models, the gold standard end point is myocardial infarct size by TTC staining and, at later time points, histopathologic analysis (79, 81). For models involving reperfusion, the area at risk must be quantified and infarct size must be expressed as a proportion of the risk region. With global rather than regional ischemia/reperfusion, the entire heart is rendered at risk and thus infarct size is appropriately expressed as a proportion of the total ventricular area. The duration of ischemia must be sufficient to cause significant infarction but not complete death of at-risk cardiomyocytes in the control cohort. That is, if the duration of ischemia is selected such that infarct size in controls is either inordinately small or excessively large, the scope for salvage and probability of achieving cardioprotection with any intervention is negligible. Irrespective of the model used, the protocol must involve I/R (or, in cell culture models, H/R) rather than ischemia (or hypoxia) alone. This requirement reflects the fact that even the most powerful and well-established cardioprotective strategies such as ischemic preconditioning simply delay, rather than prevent, the progression to cardiomyocyte death and infarction (224, 324). The duration of reperfusion must be sufficient to allow for the accurate and unambiguous delineation of necrotic and viable myocardium. This is of particular importance when infarct size is quantified using TTC staining: a minimum of 1–2 h of reperfusion is considered mandatory in rodent hearts, while longer periods of at least 3 h are standard in large animal models (283).

Attention must be paid to essential covariates and possible cofounders. Important considerations for all in vivo models include body temperature, the choice of anesthetics and analgesics, and changes in the determinants of myocardial oxygen supply and demand, all of which are well recognized to have profound effects on infarct size (111, 247). Particular care must be taken to avoid the possibility of inadvertent preconditioning: examples include triggering a protective phenotype by unintentionally subjecting the myocardium to brief periods of hypoxia or ischemia during surgical preparation, or intentionally imposing a period of ischemia in an effort to identify the extent of the at-risk myocardium (180). Additional covariates and confounders are model specific. In rodents, age, sex, and strain of the animals have all been implicated or identified to influence myocardial infarct size (10, 111, 306) Circadian variation, the time of day at which experiments are performed, may also be important (16, 28, 110, 266). The canine model is known for its variability in the magnitude of collateral blood flow. Accordingly, when using this model, measurement of regional myocardial blood flow during coronary artery occlusion and incorporation of collateral flow as a covariate in the analysis of infarct size are mandatory (64, 284).

Finally, the overwhelming majority of studies conducted to date have assessed the efficacy of candidate cardioprotective strategies using healthy, juvenile, or adult animals. There is evidence that the infarct-sparing effect of these purportedly protective interventions may be lost or attenuated in the setting of clinically relevant comorbidities, including aging, type 1 and type 2 diabetes, hypercholesterolemia, and hypertension, and may be influenced by diet or exercise (3, 4, 51, 78, 115, 117, 146, 150, 186, 197, 246, 249, 321). Accordingly, once proof of principle is established, it is imperative that promising cardioprotective therapies be reevaluated, adhering to the essential elements of rigor described above, in comorbid models (127). All protocols must include concurrent and appropriate control cohorts. For example, when potential cardioprotective drugs are evaluated, controls must receive matched volumes of vehicle administered in an identical manner.

THE ISSUE OF TRANSLATION: TOWARD A RANDOMIZED CONTROLLED STUDY OR TRIAL DESIGN

There is currently no established intervention, aside from timely reperfusion, that limits damage to hearts of patients experiencing myocardial ischemia to the extent that clinical outcome is improved (133, 138). Discussions of prior failures and hope for future successes have been reviewed elsewhere (120, 127, 176). Several elements missing from preclinical studies of infarct size reduction have been identified and include absence of critical investigator blinding, statistical weaknesses (underpowered studies), and insufficient methodological detail. These deficiencies explain in part the failure to translate preclinical results into effective infarct-sparing treatments in patients. Thus, many have questioned the reproducibility of interventions to protect from MI (i.e., reduce infarct size).

Much of the lack of reproducibility has been ascribed to limited or lacking scientific rigor (29, 250). In response to these and other concerns, the United States National Institutes of Health now includes explicit requirements for applicants to show, and reviewers to evaluate, the level of scientific rigor in grant applications. Whether suboptimal rigor fully explains and underlies the reproducibility crisis is unclear (162). Nonetheless, advocating for reproducibility and scientific rigor is welcome.

Appropriate statistical issues should be considered before initiating an infarct-sparing intervention. Investigators should know the standard deviation of their primary, prespecified end point, such as infarct size, chamber dimension, or ejection fraction. A power analysis for the primary end point will determine and justify the number of subjects to be enrolled in each group. This may not be feasible when investigating an entirely innovative strategy as there may be no basis for an estimation of the expected effect size. The standard deviation of the investigator's most recent blinded study can be used to determine group size (229). The choice of statistical analyses must be appropriate for the study design (300). For two-group studies, *t*-tests (or nonparametric equivalent) may be used; for protocols involving multiple cohorts, ANOVA (or nonparametric equivalent) is mandatory. Analysis of covariance, assessing the effects including variations in risk region and collateral blood flow, may also be applied.

Blinded assignment of animals and randomization to control or treated groups is mandatory whenever possible. Incorporation of randomization with blinding is an easy and logical

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approach. For testing classical drug-based interventions or when comparing mutant mice, individuals responsible for blinding can use block randomization and label tubes or mice with a simple unique alphanumeric code. The surgeon performing the protocol should have no knowledge of the intervention or genotype. We recommend that the same surgeon perform all surgeries; if multiple surgeons are used, equal numbers from all groups should be matched across the surgeon pool. It is imperative that neither the individual analyzing infarct size, nor the individual performing any other secondary analyses, knows the intervention or genotype until all data are compiled. If the potential for excluding animals exists, this should be done based on previously declared exclusion and inclusion criteria, and all decisions should be made before disclosure of group assignment; details of such exclusions should be made clear in any publications. Table 4 shows ARRIVE guidelines for manuscript submission, modified to focus on ischemia studies.

The Consortium for Preclinical Assessment of Cardioprotective Therapies (CAESAR) endeavored to address the primary issues related to reproducibility in cardioprotection studies (163). Performing the same protocol at multiple sites and in multiple species was extraordinarily challenging; the most notable were challenges in identifying the underlying explanations for differences in infarct size (or other variables) between centers while they were implementing consonant protocols. Interestingly, there were several instances of mice being ordered simultaneously from the same vendor, only to have significantly different body weights at the time of study (despite being fed the same chow). To be clear, the weight differences were relatively small but on occasion were significantly different for reasons unknown. It is likely that small differences, such as this, could theoretically affect the results (or the perception of not being able to reproduce studies) of published studies. During the CAESAR experience, numerous seemingly extraneous factors were considered, such as differences in municipal water source, room temperature, relative humidity, type of lighting, traffic in the room, and other seemingly innocuous details that may or may not affect the responsiveness of mice to an infarct-sparing regimen. All of these variables reflect inherent challenges in performing in vivo studies.

More important than slight differences in body weight or other such ancillary factors were initial challenges in generating equivalent infarct sizes at different institutions, despite using the same protocol. Such occasional variations emphasize the unequivocal requirement in all protocols for concurrent and appropriate controls (see *Cardioprotection*).

OVERALL DISCUSSION AND CONCLUSIONS

As highlighted throughout these guidelines, ischemia and I/R have multiple consequences that show temporal variation in terms of both incidence and influence on outcomes and may be model dependent. Examples range from acute effects (including biochemical perturbations in cardiomyocytes and other cardiac cell types, disruption in cardiac conduction and development of arrhythmias, contractile dysfunction, abnormalities in endothelial, and vascular reactivity) to longer term outcomes (such as cardiomyocyte death, microvascular obstruction and no-reflow, scar healing, and LV remodeling) and, ultimately, major adverse cardiac events, including heart failure and death (136). Figure 1 shows the diversity in models available to assess ischemia across its spectrum, and Tables 4 and 5 show general recommendations for rigor and reproducibility in ischemia studies.

While writing these guidelines, the authors discussed whether an algorithm to define the choice of model for a given scientific question would be helpful. The consensus was that the topic of myocardial ischemia and infarction and the many clinical manifestations of coronary artery disease resulting in and from myocardial ischemia or infarction are so broad and so complex that we find ourselves unable to provide an algorithm that truly covers all potential scientific approaches. Indeed, such an algorithm may be used by regulatory and funding agencies to actually limit research in the field, which would be counterproductive to the goals of this document.

The approach used will vary depending on the questions being addressed; as such, all of the approaches described above may be considered good and a gold standard if appropriate to address the target hypothesis. In vitro studies using isolated cardiomyocytes or even isolated organelles (e.g., mitochondria) are well suited to identify single molecular targets of injury and protection (102). Isolated, buffer-perfused heart

Table 4. *Checklist of considerations for rigor and reproducibility, modified from the ARRIVE Guidelines (168)*

Item	Details
Ethical statements	Institutional Animal Care and Use Committee approval, *Guide for the Care and Use of Laboratory Animals*, welfare assessments and interventions
Animal description, housing, husbandry	Species, strain, source, age (mean and range), sex, genotypes, body weight (mean and range), health/immune status, housing type (specific pathogen free), cage type, bedding material, number of cage companions, light-dark cycle, room temperature and humidity, food type, food and water access
Study design	Define model used; define groups; matching, randomization, and blinding protocols; order of treatment and assessment of groups; method to confirm model success; inclusion/exclusion criteria (e.g., lower limit for infarct size); daily monitor to record time and cause of death; define timing of perioperative and postoperative phases for survival analysis, flow chart for complex designs
Experimental procedures	Define area examined (e.g., infarct, remote, both regions); positive and negative controls; for drugs: formulation, dose, site, and route of administration; anesthesia and analgesic use and pain monitoring; surgical procedure details and monitoring records (e.g., electrocardiogram, heart rate, and anesthesia depth); method of euthanasia; time of day performed
Sample sizes	Number of animals used per group for each experiment; sample size calculation; number of independent replicates for cell culture studies
Variables measured	Primary and secondary end points, list any animals or samples removed from analysis with reason
Statistics	Methods used for each analysis; test for assumptions

ARRIVE, Animals in Research: Reporting In Vivo Experiments.

Table 5. *Recommendations for ischemia studies*

Common	Experimental design should follow ARRIVE guidelines (see Table 4)
Cardiomyocytes	When comparing groups, geometry and function end points by echocardiography assessment may not change. This does not necessarily indicate no effect of the intervention. Control groups can be shared across studies to reduce animal use, as long as the samples are collected within the same timeframe, under identical conditions, and details are provided in the methods. Previously collected historical controls should be avoided or clearly indicated. For time-course studies, sham surgery can be replaced by *day 0* negative controls if minimally invasive procedures are used, which greatly reduces animal use. Use to discern direct cardiomyocyte effects and responses
Isolated perfused hearts	Use to discern cardiac effects and responses
Angina, stunning, hibernation, and ischemic cardiomyopathy	Use to reflect a particular clinical scenario
MI	Use nonreperfused or reperfused MI to study repair and remodeling Use nonreperfused MI to test interventions in a robust remodeling model and to test interventions in a model clinically relevant to the nonreperfused patient Use reperfused MI to test interventions in a model clinically relevant to the reperfused patient Use reperfused MI to study cardioprotection Essential to measure infarct size for nonreperfused MI and infarct size and area at risk for reperfused MI
Ablation	Use to control size, shape, or location Use to achieve maximal and uniform cell death in the target region Use to investigate mechanisms of action of corresponding to clinical ablation technique While not suited to study MI pathophysiology, is well suited to study repair and regeneration Essential to quantify transmural extent of damage, to assess transmural variation Essential to recognize that transmural extent of the lesion may evolve over time Essential to standardize experimental protocol (e.g., probe temperature and contact time) to achieve consistent lesions
Cardioprotection	Use to evaluate potentially protective strategies in the ischemia-reperfusion model Essential to measure infarct size and area at risk

ARRIVE, Animals in Research: Reporting In Vivo Experiments; MI, myocardial infarction.

models can be used to study the acute biochemical and functional mechanisms of myocardial I/R injury and cardioprotection. Due to the need for stable stenosis and the required spatial resolution of regional myocardial blood flow and contractile function measurements, large animal preparations are recommended as models for the clinical manifestations of chronic stable angina and coronary microembolization. In vivo preparations are required to study more long-term effects of myocardial I/R and respective therapeutic interventions. We distinguish between permanent occlusion MI and reperfused MI models and also highlight measurements in common. Permanent coronary occlusion MI and reperfused MI models are both well suited to study repair and remodeling. I/R models are mandatory to study cardioprotection, and the ischemia must be of sufficient severity and duration to cause some infarction. Cryo-/thermoinjury is not suited to study the pathophysiology of MI but well suited to study repair and regeneration processes.

Prospective planning of study design, i.e., randomization for appropriate control versus treatment, blinding of investigators (as much as possible for a given experimental protocol and the subsequent data analysis), and adequate statistics, is mandatory for reproducibility of all experimental models of myocardial I/R and infarction. As larger data sets are being acquired, consideration for how to harness big data should be given (272, 274). Compiling databases to incorporate results from across studies and across laboratories will provide a means to use epidemiological approaches or big data tools to validate published findings, generate novel hypotheses, and assess individual variability in response to ischemia.

In conclusion, these guidelines provide recommendations to help the investigator plan and execute a full range of studies involving myocardial ischemia and infarction.

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DISCLAIMERS

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health, American Heart Association, United States Department of Defense, United States Veterans Administration, National Health and Medical Research Council, or German Research Foundation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.L.L. and G.H. conceived and designed research; M.L.L., R.G.G., K.P., C.M.R., and G.H. prepared figures; M.L.L., R.B., J.M.C., X.-J.D., N.G.F., S.F., R.G.G., J.W.H., S.P.J., R.K., D.J.L., R.L., E.M., P.P., K.P., F.A.R., L.S.L., C.M.R., J.E.V.E., and G.H. drafted manuscript; M.L.L., R.B., J.M.C., X.-J.D., N.G.F., S.F., R.G.G., J.W.H., S.P.J., R.K., D.J.L., R.L., E.M., P.P., K.P., F.A.R., L.S.L., C.M.R., J.E.V.E., and G.H. edited and revised manuscript; M.L.L., R.B., J.M.C., X.-J.D., N.G.F., S.F., R.G.G., J.W.H., S.P.J., R.K., D.J.L., R.L., E.M., P.P., K.P., F.A.R., L.S.L., C.M.R., J.E.V.E., and G.H. approved final version of manuscript.

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REVIEW ARTICLE

Anti-inflammatory therapies in myocardial infarction: failures, hopes and challenges

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In the infarcted heart, the damage-associated molecular pattern proteins released by necrotic cells trigger both myocardial and systemic inflammatory responses. Induction of chemokines and cytokines and up-regulation of endothelial adhesion molecules mediate leukocyte recruitment in the infarcted myocardium. Inflammatory cells clear the infarct of dead cells and matrix debris and activate repair by myofibroblasts and vascular cells, but may also contribute to adverse fibrotic remodelling of viable segments, accentuate cardiomyocyte apoptosis and exert arrhythmogenic actions. Excessive, prolonged and dysregulated inflammation has been implicated in the pathogenesis of complications and may be involved in the development of heart failure following infarction. Studies in animal models of myocardial infarction (MI) have suggested the effectiveness of pharmacological interventions targeting the inflammatory response. This article provides a brief overview of the cell biology of the post-infarction inflammatory response and discusses the use of pharmacological interventions targeting inflammation following infarction. Therapy with broad anti-inflammatory and immunomodulatory agents may also inhibit important repair pathways, thus exerting detrimental actions in patients with MI. Extensive experimental evidence suggests that targeting specific inflammatory signals, such as the complement cascade, chemokines, cytokines, proteases, selectins and leukocyte integrins, may hold promise. However, clinical translation has proved challenging. Targeting IL-1 may benefit patients with exaggerated post-MI inflammatory responses following infarction, not only by attenuating adverse remodelling but also by stabilizing the atherosclerotic plaque and by inhibiting arrhythmia generation. Identification of the therapeutic window for specific interventions and pathophysiological stratification of MI patients using inflammatory biomarkers and imaging strategies are critical for optimal therapeutic design.

Abbreviations

11β-HSD, 11-β hydroxysteroid dehydrogenase; ACS, acute coronary syndrome; CRP, C-reactive protein; ECM, extracellular matrix; GR, glucocorticoid receptor; iNKT, invariant natural killer T cells; IRAK, IL-1 receptor associated kinase; MI, myocardial infarction; MR, mineralocorticoid receptor; NSAIDs, nonsteroidal anti-inflammatory drugs; PCI, percutaneous coronary intervention; SDF, stromal cell-derived factor; STEMI, ST elevation myocardial infarction; Tregs, regulatory T cells; α-SMA, α-smooth muscle actin

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Introduction

Myocardial infarction (MI) is a major cause of morbidity and mortality worldwide. Implementation of reperfusion strategies in patients presenting with ST elevation MI (STEMI) has significantly reduced acute mortality. However, this remarkable therapeutic success resulted in an expansion of the pool of patients who, while surviving the acute event, remain at risk for development of heart failure. The pathogenesis of heart failure following MI is intricately linked with repair and remodelling of the infarcted heart. The term 'post-infarction ventricular remodelling' describes a constellation of cellular, molecular and proteomic changes in both infarcted and non-infarcted myocardium that ultimately result in chamber dilation, hypertrophy of viable segments and progressive myocardial dysfunction. In human patients, dilative remodelling of the ventricle is associated with higher mortality and increased incidence of ventricular arrhythmias. The severity of adverse post-infarction remodelling is dependent on the size of the infarct but is also affected by the qualitative characteristics of cardiac repair and by the profile of cellular and molecular alterations in the viable myocardium.

Extensive experimental evidence suggests that MI is intricately associated with activation of an inflammatory reaction (Frangogiannis, 2014a). Inflammatory mediators are directly involved in the pathogenesis of the vulnerable plaque, leading to occlusion of the coronary vessel and subsequent necrosis of the myocardial territory served by the vessel. Cardiomyocyte necrosis triggers both a systemic inflammatory response, mobilizing bone marrow-derived immune cells, and a local reaction, leading to recruitment of circulating inflammatory cells that serve to clear the infarct from dead cells and matrix debris. Although leukocyte subsets play an important role in repair of the infarcted heart, prolonged activation of inflammatory pathways is involved in chronic adverse remodelling of the ventricle. Despite an impressive growth in our understanding of the role of inflammation in the pathogenesis of coronary occlusion and in the pathophysiology of cardiac repair remodelling and fibrosis, development of therapeutic strategies targeting inflammatory signals in patients with MI poses major challenges. This review provides a brief overview of the role of inflammatory cascades in injury, repair and remodelling of the infarcted heart, describes the long history of failed attempts to attenuate post-ischaemic dysfunction and to reduce adverse remodelling by targeting inflammation, and discusses promising new therapeutic approaches and the challenges of clinical implementation.

The role of inflammation in plaque rupture

In patients, ruptured atherosclerotic plaques are responsible for the majority of cases of fatal MI (Davies and Thomas, 1984). Both systemic inflammation and local activation of macrophage-driven inflammatory signalling in the microenvironment of the plaque have been implicated in the pathogenesis of plaque rupture (Crea and Libby, 2017). Induction of chemokines, such as CCL2 and fractalkine/CX3CL1, mediate recruitment of macrophages in atherosclerotic plaques (Gu et al., 1998; Lesnik et al., 2003). The diverse phenotypic profiles of macrophages critically regulate progression, evolution and even regression of the atherosclerotic process (Mantovani et al., 2009; Rahman et al., 2017). Angiotensin II (Schieffer et al., 2000), oxidized LDL (Xu et al., 1999), CD40 signalling (Mach et al., 1997) and pro-inflammatory cytokines stimulate macrophage-derived expression of proteases (including MMPs and cathepsins), degrading the extracellular matrix (ECM) of the fibrous cap (Shah et al., 1995) and promoting plaque fissuring. Moreover, local release of cholesterol crystals activates the inflammasome, generating active IL-1β and triggering pro-inflammatory signalling (Freigang et al., 2011). In addition to the direct actions of pro-inflammatory mediators on macrophage phenotype, mast cell degranulation, dysregulation of T cell subsets, B-cell-derived cytokine synthesis and stimulation of vascular cells in the plaque environment have also been implicated in activation of inflammatory macrophages in atherosclerotic plaques (Kaartinen et al., 1996; Mazzolai et al., 2004; Tay et al., 2016; Sage and Mallat, 2017; Tabas and Lichtman, 2017).

Activation of the post-infarction inflammatory response

Prolonged coronary occlusion leads to death of the cardiomyocytes in the tissues served by the vessel, triggering activation of an intense inflammatory reaction. The post-infarction inflammatory response can be divided in three phases: the alarm phase characterized by release of damage-associated molecular pattern (DAMP) proteins that stimulate innate immune pathways; the leukocyte mobilization phase, marked by recruitment of neutrophils, monocytes and lymphocytes in the infarcted area; and the resolution phase, associated with suppression of pro-inflammatory signalling and clearance of the leukocyte infiltrate (Figure 1).

During the alarm phase, necrotic cardiomyocytes release danger signals (such as high mobility group box-1, heat shock proteins, adenosine, extracellular RNA, and IL-1α) that stimulate innate immune signalling (Andrassy et al., 2008; Chen et al., 2014; Lugrin et al., 2015). Generation of ECM fragments also contributes to the intense inflammatory reaction in the infarcted area (Huebener et al., 2008). Stimulation of innate immune responses following infarction involves effects of alarmins on Toll-like receptor and receptor for advanced glycation end-products-dependent pathways in leukocytes, vascular cells and fibroblasts triggering transcription of pro-inflammatory cytokines and chemokines (Arslan et al., 2011a; Zhang et al., 2015). Activation of the complement cascade also contributes to the post-infarction inflammatory response (Hill and Ward, 1971; De Hoog et al., 2014). Post-infarction inflammation not only serves to clear dead cells and matrix debris from the infarcted tissue but also sets the stage for repair of the infarcted area. In addition to activation of a local myocardial inflammatory response, infarction also triggers systemic inflammation, stimulating release of bone marrow-derived leukocytes. In mouse models of MI, the spleen has also been suggested as an

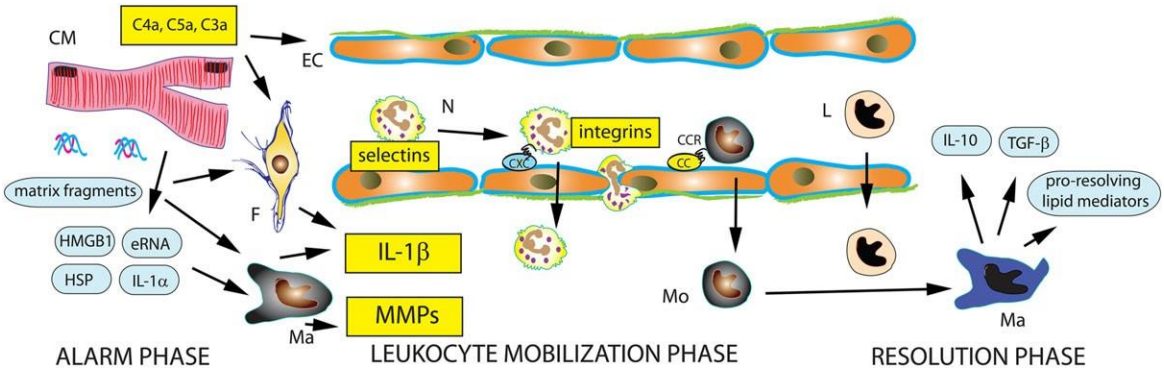

Figure 1

The inflammatory response following MI can be divided into three phases: the alarm phase, the leukocyte mobilization phase and the resolution phase. Necrotic cardiomyocytes (CM) release alarmins (heat shock proteins [HSP], high mobility group box 1 [HMGB1], extracellular RNA/eRNA, IL-1α and other danger signals) that activate innate immune signalling pathways. ECM fragments also trigger inflammatory signalling. Induction of pro-inflammatory cytokines, such as IL-1, and chemokines mediates recruitment of neutrophils (N) and pro-inflammatory monocytes (Mo) through interactions with endothelial cells (EC) that involve selectins and integrins. Clearance of dead cells and matrix debris from the infarct triggers transition to the resolution phase. Anti-inflammatory lymphocyte (L) and macrophage (Ma) subsets release mediators that suppress pro-inflammatory signalling, such as IL-10, TGF-β and pro-resolving lipid mediators. Experimental studies suggest that inhibition of the complement cascade, IL-1β antagonism, CCL2 inhibition, selectin and leukocyte integrin neutralization may be promising therapeutic strategies for patients with MI. F, fibroblast.

important contributor of inflammatory leukocytes (Swirski et al., 2009). Although the relative role of the cardio-splenic axis in human MI remains unclear, clinical investigations have suggested that patients with acute coronary syndromes (ACS) have increased splenic metabolic activity and that activation of the spleen independently predicts cardiovascular events (Emami et al., 2015).

The cytokines and chemokines

Induction of pro-inflammatory cytokines is a hallmark of the post-infarction inflammatory response. Early release of TNF-α (Frangogiannis et al., 1998) triggers a cytokine cascade that mediates recruitment of leukocytes in the infarcted myocardium. Activation of the inflammasome platform in fibroblasts, cardiomyocytes and immune cells (Kawaguchi et al., 2011; Mezzaroma et al., 2011) stimulates release of active IL-1β, a critical mediator in regulation of cardiac inflammation and repair. IL-1 signalling stimulates chemokine synthesis and promotes leukocyte infiltration in the infarcted myocardium (Bujak et al., 2008). Cardiac fibroblasts also respond to IL-1, by acquiring a pro-inflammatory and matrix-degrading phenotype and by secreting cytokines, chemokines and MMPs. Moreover, IL-1 delays myofibroblast conversion, suppressing synthesis of α-smooth muscle actin (α-SMA) (Saxena et al., 2013). The effects of IL-1 on cardiac fibroblasts may serve to prevent premature acquisition of a matrix-synthetic phenotype, until the infarct is cleared of dead cells and matrix debris.

Chemokines are also markedly up-regulated in the infarcted heart and have been demonstrated to mediate leukocyte recruitment. Induction of both CXC and CC chemokines has been consistently demonstrated in experimental models of MI. CXC chemokines containing the ELR motif (Glu-Leu-Arg), such as CXCL8/IL-8, have been implicated in neutrophil recruitment (Ivey et al., 1995). On the other hand, members of the CC chemokine subfamily, such as CCL2 and CCL7, mediate recruitment of pro-inflammatory monocytes (Dewald et al., 2005; Nahrendorf et al., 2007; Zouggari et al., 2013). Some members of the chemokine family may have effects on non-haematopoietic cells, such as cardiomyocytes, fibroblasts and vascular cells. The CXC chemokine SDF-1/CXCL12 may recruit progenitor cells with angiogenic potential (Liehn et al., 2011), contributing to neovascularization of the scar, and may stimulate pro-survival cascades in ischaemic cardiomyocytes (Aiuti et al., 1997; Askari et al., 2003). The CXC chemokine IP-10/CXCL10 is markedly up-regulated in experimental models of MI and may suppress fibrosis by inhibiting growth factor-mediated fibroblast migration (Bujak et al., 2009; Saxena et al., 2014a).

Recruitment of leukocytes

Chemokines and cytokines play a critical role in recruitment of inflammatory leukocytes in the infarcted myocardium. Cytokine-mediated induction of adhesion molecules in endothelial cells and integrin activation in leukocytes trigger adhesive interactions, ultimately leading to neutrophil, monocyte and lymphocyte extravasation in the infarcted area (Yamazaki et al., 1993; Frangogiannis, 2014a). Leukocyte subpopulations have been suggested to play important roles in both injurious and repair processes following MI. Early studies suggested that infiltrating neutrophils may extend ischaemic injury by exerting cytotoxic effects on viable cardiomyocytes in the infarct border zone (Entman et al., 1992). On the other hand, neutrophils have been suggested to orchestrate repair of the infarcted heart by modulating macrophage phenotype (Horckmans et al., 2017).

The macrophages

Monocytes recruited to the infarct region differentiate into macrophages and phagocytose dead cells and matrix debris, while secreting cytokines and growth factors that orchestrate repair. Clearance of apoptotic cells by professional phagocytes, a process known as efferocytosis (Wan et al., 2013), triggers cascades that suppress inflammation and promote activation of reparative mesenchymal cells. Ingestion of apoptotic cells is associated with release of anti-inflammatory cytokines, such as IL-10 and TGF-β (Huynh et al., 2002), suppressing inflammation and activating a fibrogenic and matrix-preserving programme. Several lines of evidence suggest crucial protective actions of macrophages in cardiac repair. First, macrophage depletion increased adverse remodelling in infarcted mice (van Amerongen et al., 2007). Second, in experimental models, macrophages played a crucial role in preventing mural thrombus formation following MI (Ben-Mordechai et al., 2013; Frantz et al., 2013). Third, generation of alternatively activated macrophages exhibiting an M2-like phenotype is critical to protect the infarcted heart from cardiac rupture (Shiraishi et al., 2016). Transition of macrophages into an anti-inflammatory phenotype may also require activation of intracellular inhibitory cascades that restrain the immune response, such as expression of IL-1 receptor associated kinase (IRAK)-M, an inhibitory member of the IRAK family that suppresses innate immune signalling (Chen et al., 2012). It has been suggested that therapeutic activation of the reparative properties of macrophages through administration of IL-4 may exert protective actions in acute MI (Shintani et al., 2017). However, such therapeutic approaches need to be cautiously implemented, considering the known pro-fibrotic actions of IL-4 in the remodelling heart (Peng et al., 2015).

The lymphocytes

Early infiltration of the infarcted heart with lymphocyte subsets has been extensively documented in experimental models of MI (Frangogiannis et al., 2000a; Yan et al., 2013). Moreover, clinical studies have suggested that effector T cells may be trapped in the coronary microcirculation following reperfusion of the infarcted area and may contribute to the pathogenesis of microvascular obstruction, extending ischaemic cardiomyocyte injury (Boag et al., 2015). Early recruitment of lymphocyte subpopulations to the infarcted myocardium has been suggested to stimulate neutrophil and monocyte infiltration. B cells have been demonstrated to promote mobilization of pro-inflammatory monocytes, thus playing a central role in activation of the inflammatory cascade (Zouggari et al., 2013). $CD4^{-}\gamma\delta$T-cells have been implicated in neutrophil and macrophage infiltration and may promote adverse remodelling following MI (Yan et al., 2012). It should be emphasized that other lymphocyte subsets, such as regulatory T cells (Tregs), CD4+ helper T cells and invariant natural killer T (iNKT) cells, may have important repair functions following MI, negatively regulating inflammation, and activating mesenchymal and angiogenic cell populations to limit adverse remodelling (Dobaczewski et al., 2010; Hofmann et al., 2012; Sobirin et al., 2012; Weirather et al., 2014; Saxena et al., 2014b).

Negative regulation of the post-infarction inflammatory response

Although macrophages are key effector cells in suppression of the post-infarction inflammatory response, several other cell types may contribute to downmodulation of pro-inflammatory signalling. Anti-inflammatory lymphocyte subsets, such as Tregs (Dobaczewski et al., 2010; Weirather et al., 2014; Saxena et al., 2014b), iNKT cells (Sobirin et al., 2012) and dendritic cells (Anzai et al., 2012) have been identified as important sources of anti-inflammatory cytokines in the healing infarct. Surviving cardiomyocytes in the infarct border zone may also limit and restrain inflammation by secreting mediators that recruit and activate regulatory and reparative macrophages (Lorchner et al., 2015). Acquisition of an anti-inflammatory phenotype by vascular cells may also contribute to negative regulation of post-infarction inflammation. Members of the TGF-β family may inhibit adhesion molecule expression by endothelial cells and leukocytes, inhibiting leukocyte-endothelial cell interactions and preventing uncontrolled leukocyte recruitment (Kempf et al., 2011). Recruitment of mural cells by infarct neovessels may serve to suppress endothelial pro-inflammatory activation (Zymek et al., 2006). Thus, timely suppression and spatial containment of the inflammatory response following infarction is dependent on activation of a wide range of molecular signals with actions on several different cell types. In animal models, defects in these regulatory mechanisms result in unrestrained, prolonged or expanded inflammation, leading to accentuated cardiac remodelling and worse dysfunction following infarction. In patients, defective negative regulation of the post-infarction inflammatory response may be involved in the pathogenesis of adverse remodelling and heart failure in patients surviving an acute MI (Frangogiannis, 2014a).

Myofibroblast activation

Because the adult mammalian heart has negligible regenerative capacity, repair of the infarcted myocardium is dependent on fibroblast activation and subsequent formation of a collagen-based scar. Perturbations in fibroblast activation and in the profile of ECM proteins in the infarcted and remodelling myocardium can be associated with increased dysfunction and adverse remodelling (Frangogiannis et al., 2005; Kong et al., 2017). During the proliferative phase of infarct healing, the cardiac fibroblast population markedly expands (Frangogiannis et al., 2000b). Activated myofibroblasts form organized arrays in the infarct border zone (Blankesteijn et al., 1997). These cells incorporate into their cytoskeleton contractile proteins (such as α-SMA and the embryonal isoform of smooth muscle myosin) (Willems et al., 1994; Frangogiannis et al., 2000b; Shinde et al., 2017) but do not express markers of mature vascular smooth muscle cells, such as the SM1 and SM2 isoforms of smooth muscle myosin heavy

chain (Frangogiannis et al., 2000b). Infarct myofibroblasts are predominantly derived from epicardium-derived fibroblast populations (Ruiz-Villalba et al., 2015; Kanisicak et al., 2016). During the proliferative phase of cardiac repair, suppression of pro-inflammatory signals, activation of TGF-β cascades and deposition of specialized matrix proteins, such as ED-A fibronectin (Arslan et al., 2011b) and matricellular proteins (Frangogiannis, 2017a), trigger conversion of interstitial fibroblasts into myofibroblasts.

Activated myofibroblasts have been identified as the main source of ECM proteins in the healing infarct (Cleutjens et al., 1995). In addition to their matrix synthetic capacity, activated infarct fibroblasts may also contribute to phagocytosis of dead cells (Nakaya et al., 2017) and may secrete mediators that modulate cardiomyocyte survival (Abrial et al., 2014) or mediate activation of immune cells (Anzai et al., 2017). Whether distinct subpopulations are responsible for the functional pleiotropy of infarct myofibroblasts remains unknown. Excessive fibroblast activation may lead to expansion of the fibrotic area, increasing myocardial stiffness and promoting diastolic dysfunction. The potential involvement of negative regulatory mechanisms that restrain fibrogenic signals, in the prevention of uncontrolled fibrosis following MI, has not been investigated.

Inflammation in the remodelling myocardium

In the presence of a large infarction, massive loss of contractile myocardium is associated with activation of an inflammatory response in remote remodelling myocardial segments, accompanied by progressive interstitial fibrosis (Sager et al., 2016b). Several mechanisms may contribute to inflammatory activation in the remodelling myocardium. First, volume and pressure loads, related to dilation of the chamber following infarction and to the elevation of filling pressures. Mechanical stress in the remodelling myocardium may locally activate macrophages stimulating their proliferation and promoting a fibrogenic environment (Sager et al., 2016b). Second, defective suppression or impaired spatial containment of the inflammatory response in the infarct border zone may lead to prolonged activation of inflammatory pathways or expansion of the inflammatory infiltrate to viable segments (Frangogiannis et al., 2005). Third, an immune-mediated response triggered by poorly defined antigens may mediate chronic inflammation in the remodelling myocardium (Ismahil et al., 2014). In human ischaemic heart failure, subpopulations of patients may exhibit dysregulated inflammatory responses that may contribute to the pathogenesis of the cardiomyopathy.

The rationale for targeting inflammation after MI

The critical role of inflammation in all aspects of the myocardial response to injury suggests that targeting inflammatory signals may hold promise to reduce mortality and prevent heart failure in patients surviving an acute MI. The cell biological basis supporting therapeutic interventions that modulate the post-MI inflammatory cascade may involve several distinct beneficial actions. First, attenuation of inflammation during the early post-ischaemic inflammatory phase may prevent leukocyte-mediated cardiomyocyte injury in surviving cardiomyocytes of the border zone. Second, inhibition of inappropriate late activation of pro-inflammatory signalling may protect cardiomyocytes in the remodelling area from chronic apoptosis. Third, attenuation of inflammation may restrain protease activation, increasing the tensile strength of the healing scar and preventing adverse remodelling. Fourth, suppression of inflammation-driven fibrogenic signalling may protect the heart from dysregulated fibrotic remodelling. Fifth, selective activation of chemokine-dependent recruitment of progenitor cells into the area of infarction may promote angiogenesis and even contribute to the ultimate goal of myocardial regeneration. Sixth, pro-inflammatory signalling has been linked to ventricular arrhythmias; thus, attenuation of inflammation may have direct anti-arrhythmic actions. Finally, anti-inflammatory approaches may prevent plaque rupture reducing the incidence of recurrent coronary events.

It should be emphasized that some of the protective effects of certain established therapeutic approaches in patients with MI, such as angiotensin converting enzyme inhibition (Leuschner et al., 2010), angiotensin receptor blockade (Kohno et al., 2008), mineralocorticoid receptor (MR) inhibition (Fraccarollo et al., 2008), β-adrenoceptor blockade (Garcia-Prieto et al., 2017) and administration of statins (Zhang et al., 2005), may involve direct modulation of inflammation. For example, leukocyte-specific β_2-adrenoceptor signalling has been reported to mediate leukocyte recruitment in the infarcted heart (Grisanti et al., 2016), and a recent study suggested that the infarct-limiting effects of β-blockade with metoprolol in a mouse model of MI were lost following neutrophil depletion or through genetic knockdown of genes associated with platelet:neutrophil interactions (Garcia-Prieto et al., 2017). However, considering the broad effects of neurohumoral pathways on both cardiomyocyte and non-cardiomyocyte populations, the relative contribution of inflammatory cell modulation remains unclear.

Early attempts to inhibit inflammation were primarily focused on the use of broad anti-inflammatory strategies, such as glucocorticoids. These approaches were often associated with adverse consequences. Over the last 30 years, progress in fundamental immunology and better understanding of cardiac pathophysiology led to implementation of new therapeutic strategies targeting specific inflammatory pathways. Despite the increasing sophistication of the approaches, therapeutic implementation of inflammatory targets in patients with MI remains challenging, in part due to the remarkable pathophysiological heterogeneity of the human condition.

Broad anti-inflammatory interventions

Glucocorticoids

Due to the ubiquitous expression of the glucocorticoid receptor (GR) in all nucleated cells, glucocorticoids have a wide range of effects on many different cell types and are

potent regulators of the inflammatory response (Cain and Cidlowski, 2017). During the alarm phase, glucocorticoids attenuate responses to danger signals, suppressing production of inflammatory mediators. Glucocorticoids also markedly reduce leukocyte infiltration into the tissues, by decreasing chemokine expression and by suppressing adhesive interactions between leukocytes and endothelial cells. During the resolution phase, glucocorticoids exert a wide range of actions on both inflammatory and reparative cells. Glucocorticoids are known to promote clearance of apoptotic cells (Liu et al., 1999) and direct macrophages towards an anti-inflammatory phenotype (Snyder and Unanue, 1982). In addition to their effects on immune cells, glucocorticoids also inhibit repair, by reducing fibroblast-derived collagen synthesis and by inhibiting the angiogenic response. It should be emphasized that the effects of glucocorticoids are not limited to activation of GR signalling. Glucocorticoids also bind with high affinity to the MRs (Arriza et al., 1987) and may also act by activating transcription-independent non-classical pathways, involving cell surface receptors (Samarasinghe et al., 2012). Stimulation of MR signalling mediates pro-inflammatory macrophage activation in vitro and in vivo (Usher et al., 2010), whereas activation of endogenous myeloid cell-specific GR signalling has been suggested to mediate reparative pathways (Galuppo et al., 2017). The relative role of GR and MR activation in the response to glucocorticoid treatment is likely to depend on the specific agent used, the dose and on the cell biological context.

Numerous experimental studies have examined the effects of glucocorticoids in experimental models of MI (Table 1). Unfortunately, most of the studies were performed in models of non-reperfused MI, limiting the value of the conclusions in the current era of myocardial reperfusion. Although the effects are dependent on the agent used, the dose and duration of treatment, several studies have suggested that glucocorticoids may protect the infarcted myocardium, reducing cardiomyocyte necrosis and apoptosis (Libby et al., 1973; Xu et al., 2011). The basis for these protective actions is unclear, especially considering the use of permanent coronary occlusion models that would be expected to cause death of most cardiomyocytes in the area at risk. Other in vivo studies suggested that high-dose corticosteroid therapy impairs clearance of dead cells from the infarct and may disrupt fibroblast function (Kloner et al., 1978), leading to formation of thinner scars. These effects would be expected to be detrimental in repair of the infarcted heart and may precipitate adverse remodelling. Clinical studies on the use of corticosteroids in patients with MI have produced conflicting results (Table 2). Although some studies reported protective effects, in other studies, significant concerns were raised regarding the safety of the approach (Roberts et al., 1976; Giugliano et al., 2003). Considering their broad actions on all cell types involved in cardiac injury and repair and their effects on several molecular cascades, glucocorticoids cause a wide range of adverse effects and are unattractive therapeutic options for patients with MI. However, understanding the cell-specific actions of GR activation may suggest more targeted approaches with therapeutic potential. Moreover, recent insights into the pathways involved in tissue-specific intracellular metabolism of glucocorticoids have suggested novel therapeutic directions (Gray et al., 2017). The enzyme 11-β hydroxysteroid dehydrogenase (11-β-HSD) catalyses intracellular regeneration of glucocorticoids from inert metabolites. In a mouse model, global deletion of 11-β-HSD1, the more widely distributed isoform of the enzyme, promoted angiogenesis and attenuated infarct expansion following MI (White et al., 2016), suggesting that endogenous glucocorticoid regeneration may inhibit repair and exacerbate remodelling following MI. Thus, approaches inhibiting 11-β-HSD1 may be of therapeutic value to prevent development of heart failure following MI.

Nonsteroidal anti-inflammatory drugs (NSAIDs)

NSAIDs (including aspirin) have broad anti-inflammatory actions as their effects are mediated through inhibition of COX, the rate limiting enzyme in prostaglandin synthesis. There are two major isoforms of COX, the constitutively expressed COX-1 and COX-2, which is not found in normal tissues but is induced by inflammation, ischaemia and stress. Non-selective NSAIDs, introduced in the 1950s, inhibit both COX isoforms. Inhibition of COX-1-induced prostaglandins in the gastric mucosa by traditional NSAIDs is often associated with gastrointestinal toxicity, including peptic ulcer disease. Thus, selective COX-2 inhibitors were introduced in the late 1990s in an attempt to develop anti-inflammatory strategies without the risk of gastrointestinal side effects (Boulakh and Gislason, 2016).

Aspirin acts through non-competitive, irreversible acetylation of COX. In nucleated cells, the ability of the cells to synthesize COX-1 and COX-2 de novo allows recovery of prostaglandin synthesis despite inhibition by aspirin. In contrast, in platelets, thromboxane A_2 production is dependent on preformed COX-1. Thus, irreversible binding of aspirin to platelet COX-1 results in inhibition of platelet aggregation for the entire life of the platelet and mediates aspirin's potent cardioprotective actions by reducing the incidence of new cardiovascular events (ISIS-2 Collaborative Group, 1988). In contrast, because the NSAIDs, apart from aspirin, competitively and reversibly inhibit COX, they do not cause sustained inhibition of platelet aggregation and do not provide long-term protection from atherothrombotic cardiovascular events (Vonkeman and van de Laar, 2010).

Although some early studies in animal models suggested that both non-selective non-aspirin NSAIDs and selective COX-2 inhibitors may have protective effects following MI (Lefer and Polansky, 1979), by attenuating adverse remodelling, by reducing cardiomyocyte apoptosis and by increasing arteriolar density (Abbate et al., 2006; Straino et al., 2007), other experimental studies demonstrated detrimental effects on infarct healing, resulting in scar thinning (Brown Jr et al., 1983; Hammerman et al., 1984a; Hammerman et al., 1983b) and accentuated systolic dysfunction (Table 3) (Timmers et al., 2007). Moreover, genetic loss-of-function studies demonstrated protective effects for endogenous COX-2 in myocardial ischaemia/reperfusion models (Camitta et al., 2001; Bolli et al., 2002).

In the clinical setting, a large body of evidence suggests that exposure to NSAIDs increases risk of cardiovascular events in patients with known cardiovascular disease. In post-MI patients, administration of NSAIDs is associated with

Table 1

Effects of glucocorticoids in experimental models of MI

Model	Animal	Agent, dose and duration of treatment	Major findings	Ref.
Non-reperfused MI	Cat	Methylprednisolone (MP, 30 mg·kg^{-1}, i.v.) 30 min prior or 60 min following occlusion	Both pretreatment and post-occlusion administration reduced myocardial injury, assessed through reduction of ST segment elevation.	(Spath Jr et al., 1974)
Non-reperfused MI	Dog	Group1: Hydrocortisone (HC, 50 mg·kg^{-1}, i.v.) 30 min after occlusion, followed by supplementary dose of 25 mg·kg^{-1} 12 h after occlusion; Group2: HC (50 mg·kg^{-1}, i.v.) 6 h after occlusion, followed by supplementary dose of 25 mg·kg^{-1} 12 h after occlusion	HC reduced infarct size and attenuated cardiomyocyte necrosis, even when administered 6 h after occlusion.	(Libby et al., 1973)
Non-reperfused MI	Rat	MP (50 mg·kg^{-1} i.v.) 5 min after occlusion, followed by (50 mg·kg^{-1} i.m.) 3, 6 and 24 h after occlusion	MP treatment was associated with reduced collagen deposition, delayed inflammation and repair, and persistent presence of 'mummified' cardiomyocytes (cells with preservation of striations and sarcolemmal membrane that exhibited nuclear degeneration).	(Kloner et al., 1978)
Non-reperfused MI	Rat	1. HC: 50 mg·kg^{-1} i.v. 5 min after occlusion; 2. Single-dose MP: 50 mg·kg^{-1} i.v. 5 min after occlusion; 3. Multiple dose MP: 50 mg·kg^{-1} i.v. 5 min after occlusion, followed by 50 mg·kg^{-1} i.m. at 3, 6 and 24 h	1. Based on histological analysis and measurements of creatine kinase (CK) activity, glucocorticoids salvaged injured myocardium (by 15% in the HC group, 21% in MP single dose group, and 21% in the MP multiple dose group. 2. Multiple dose MP caused infarct thinning	(Maclean et al., 1978)
Non-reperfused MI	Cat	Dexamethasone (Dx), 8 mg·kg^{-1} i.v., 30 min prior to or 60 min following occlusion	1. Dx pre- or post- administration significantly attenuated the increase in plasma CPK activity. 2. Dx had no effects on the haemodynamic response within the first 5 h following MI.	(Spath and Lefer, 1975)
Non-reperfused MI	Dog	Group1: high dose MP (50 mg·kg^{-1} i.v). 15 min and 3, 24 and 48 h after occlusion; Group2: low dose MP (30 mg·kg^{-1} i.v). 15 min after occlusion	1. Low dose MP reduced the size of the infarct. 2. The high dose MP protocol (but not low dose MP) caused infarct thinning and worsened systolic dysfunction without affecting collagen content.	(Hammerman et al., 1983a)
Non-reperfused MI	Dog	MP, 7.5 mg·kg^{-1}, i.v. twice daily for 7 days after occlusion	MP reduced infarct size and attenuated compensatory hypertrophy without affecting haemodynamics.	(Slutsky and Murray, 1985)
Non-reperfused MI	Rat	MP (50 mg·kg^{-1}, i.v.) immediately after occlusion, followed by 50 mg·kg^{-1}, i.p., q6 h for 3 days	MP decreased collagen content in the infarcted heart.	(Vivaldi et al., 1987)

continues

Table 1
(Continued)

Model	Animal	Agent, dose and duration of treatment	Major findings	Ref.
Non-reperfused MI	Mouse	Dx (20 mg·kg^{-1}, i.p.) 20 h prior to occlusion	Dx treatment reduced infarct size, attenuating cardiomyocyte apoptosis.	(Xu et al., 2011)
Non-reperfused MI	Rat	MP (5 mg·kg^{-1}, i.p.) starting 7 days post-MI and continued to 21 day after occlusion	MP did not affect mortality or infarct size, but attenuated hypertrophic remodelling and significantly increased capillary density.	(Van Kerckhoven et al., 2004)
Reperfused MI	Dog	MP single dose (50 mg·kg^{-1}, i.v.) after occlusion	MP did not affect infarct size and haemodynamic variables.	(Genth et al., 1982)
Reperfused MI	Dog	MP (30 mg·kg^{-1}, i.v.) after occlusion;	MP increased myocardial blood flow during ischaemia, but had no effect on blood flow after reperfusion.	(da-Luz et al., 1982)
Ischaemia and reperfusion in vivo	Rabbit	low dose prednisolone (5 mg·kg^{-1}/24 h i.m.) or high dose prednisolone (10 mg·kg^{-1} /24 h i.m.) protocols	Infarct healing was significantly delayed in both low and high dose groups. However, infarct thinning was not affected.	(Shizukuda et al., 1991)

a high risk of death and reinfarction and an increased risk of hospitalization due to heart failure (Gislason et al., 2006; Brophy et al., 2007). Several mechanisms may explain the detrimental effects of NSAIDs in patients with MI. First, COX-2 inhibition may promote pro-thrombotic events by inhibiting generation of prostacyclin. Second, loss of renal actions of COX-2 may elevate blood pressure and promote heart failure decompensation. Third, COX-2 inhibition may block atheroprotective actions, thus accelerating atherosclerosis (Egan et al., 2004). Fourth, COX-2 targeting may inhibit important cardioprotective actions and exert pro-arrhythmic effects. Fifth, COX inhibition may disrupt important reparative functions, leading to formation of a defective scar (Hammerman et al., 1984b). Considering the abundant clinical evidence suggesting detrimental effects, all non-aspirin NSAIDs should be avoided in patients with established cardiovascular disease (Schmidt et al., 2016).

Immunomodulatory strategies

Few studies have tested the effects of non-specific immunomodulation and immunosuppression in MI. In animal models, early administration of low-dose immunosuppressive agents has been reported to exert beneficial actions. However, clinical studies have provided disappointing results. In a rat model of reperfused MI, cyclophosphamide administration attenuated leukocyte infiltration and reduced ventricular dysfunction (Zhu et al., 2008). Methotrexate treatment was also found to exert protective effects on the ischaemic and reperfused myocardium in both large animal (Asanuma et al., 2004) and rodent models (Maranhao et al., 2017). However, in a small clinical trial, methotrexate administration to patients with STEMI did not affect acute infarct size and worsened systolic dysfunction 3 months after the acute event (Moreira et al., 2017). Immune modulation with intravenous immunoglobulin also failed to reduce infarct size and attenuate adverse remodelling in STEMI patients (Gullestad et al., 2013).

Cyclosporine, another potent immunosuppressive agent, has attracted interest as a therapeutic agent for patients with MI, because of its effects as a cyclophilin B inhibitor. It has been proposed that cyclophilin B inhibition may inhibit opening of the mitochondrial permeability transition pore, thus protecting ischaemic cardiomyocytes from death. In addition to its protective actions on cardiomyocytes, cyclosporine may also reduce inflammation in the infarcted heart (Squadrito et al., 1999). Despite promising early results in pilot studies, suggesting reduced infarct size in STEMI patients treated with cyclosporine (Piot et al., 2008), a large randomized double-blind controlled trial showed no beneficial effects of a bolus dose of cyclosporine in STEMI patients undergoing percutaneous coronary intervention (PCI) (Cung et al., 2015).

The failures of broad anti-inflammatory inhibition with corticosteroids and NSAIDs and advances in understanding the biology of the inflammatory response led to the development of specific inflammatory targets following MI.

Targeted anti-inflammatory interventions

Experimental studies have identified crucial molecular signals mediating the inflammatory response following MI. Therapeutic interventions in animal models suggested that

Table 2

Effects of glucocorticoid treatment in patients with MI

Type of study	Number of patients	Agent, dose and duration	Major findings	Ref.
Double blind clinical trial	132	oral prednisone (starting dose: 30 mg·day^{-1}), for 12 days	No difference in acute mortality. No difference in rhythm and conduction disturbance, and cardiac rupture.	(Sievers et al., 1964)
Prospective cohort study	446	Hydrocortisone (HC), 500 mg i.v. for the first 4 days	HC significantly reduced mortality. There were no differences in the incidence of acute complications (acute heart failure, shock, cardiac arrhythmia, infections).	(Barzilai et al., 1972)
Prospective cohort study	39	Methylprednisolone (MP): 3 g, 7–12 h following rise of serum CPK	MP treatment reduced infarct size.	(Morrison et al., 1975)
Prospective cohort study	66	MP (i.v.): 1. Single 2.0 g dose 7–25 h following initial rise of CPK; 2. Two 2.0 g doses, 3–12 h apart	Reduction of infarct size and mortality in both MP treatment groups.	(Morrison et al., 1976)
Prospective cohort study	44	1. Single dose: MP, 30 mg·kg^{-1}, i.v., 7 h from first CPK elevation; 2. Multiple dose: MP, 30 mg·kg^{-1}, i.v. starting after 7 h from first CPK elevation, every 6 h, for 48 h	Neither single dose nor multiple dose affected haemodynamics. Multiple dose MP (but not single dose) extended infarct size, increased ventricular dysrhythmias, and caused hyperglycaemia.	(Roberts et al., 1976)
Prospective cohort study	29	MP 30 mg·kg^{-1}, i.v. 7 h and 10 h from onset of symptoms	High doses of MP given early in the course of MI have neither deleterious nor beneficial effects.	(Peters et al., 1978)
Prospective cohort study	10	MP, 2.0 g i.v. single dose, average of 13 h from onset of chest pain	MP administration had no short-term protective effects and worsened haemodynamics.	(Heikkila and Nieminen, 1978)
Prospective cohort study	45	Methylprednisolone, single dose 25 mg·kg^{-1}, i.v. within 4 h after onset of chest pain	MP delayed cardiomyocyte injury and may stabilize lysosomal membranes during acute myocardial ischaemia.	(Welman et al., 1979)
Prospective cohort study	42	MP, 30 mg·kg^{-1}, i.v four doses, q6 h.	Early short-term high-dose MP had no effects on infarct size, dysrhythmias, complications, or left ventricular function 2 weeks after infarction.	(Bush et al., 1980)
Prospective cohort study	28	MP (30 mg·kg^{-1}), i.v. two doses, 2.5 h apart	MP administration in AMI patients had no effect on survival, infarct size and metabolic parameters, but increased cardiac output and reduced systemic vascular resistance.	(Henning et al., 1981)
Retrospective cohort study	1746	N/A	Previous or inpatient corticosteroid use did not affect the incidence of cardiac rupture, or non-rupture related mortality in MI patients.	(Dellborg et al., 1985)

continues

Table 2
(Continued)

Type of study	Number of patients	Agent, dose and duration	Major findings	Ref.
Retrospective study	41	N/A	This uncontrolled retrospective study suggested that use of anti-inflammatory agents (glucocorticoids and NSAIDs) before and after MI may be associated with a high incidence of cardiac rupture.	(Silverman and Pfeifer, 1987)
Double blind, randomized trial (RCT)	1118	Group 1: early MP: 30 mg·kg^{-1}, i.v. within 6 h of chest pain; repeated administration 3 h later; Group 2: late MP (30 mg·kg^{-1}, i.v.) 6–12 h from onset of chest pain; repeated administration 3 h later	Late treatment (6–12 h) with MP reduced mortality without affecting cardiac rupture, early malignant ventricular arrhythmias or other adverse cardiac events. Late MP treatment reduced 28 day and 6 month mortality in patients with inferior/posterior infarction, but not in anterior MI. Early treatment had no effects.	(Metz et al., 1986) (The Solu-Medrol Sterile Powder AMI Study Group, 1986)
RCT	40	MP, 2.0 g, i.v. within 6 h, repeated same dose 3 h later	MP infusion had no effects on mortality, death from cardiac rupture, peak cardiac injury enzymes, arrhythmias, haemodynamics and 6 months hospitalization rates.	(Madias and Hood Jr, 1982)

targeted inhibition of specific inflammatory signals may protect the infarcted heart from acute injury and prevent adverse remodelling following MI. Despite promising results in animal models, therapeutic implementation of inflammatory targets in patients with MI has been challenging (Table 4).

Targeting the complement cascade

Activation of the complement cascade is a critical part of the innate immune response following MI and has been suggested to extend ischaemic injury (Yasojima et al., 1998). In experimental studies, complement inhibition strategies have consistently reduced the size of the infarct and improved function in both rodent and large animal models of reperfused MI (Vakeva et al., 1998; Pischke et al., 2017). Unfortunately, clinical studies have been disappointing. Approaches targeting the complement system, an upstream activator of the innate immune response, were equally disappointing. In the Assessment of Pexelizumab in Acute Myocardial infarction clinical trial, treatment of STEMI patients with the anti-C5 antibody pexelizumab did not affect 30 day mortality and the composite endpoint of death, cardiogenic shock and congestive heart failure (Armstrong et al., 2007).

Targeting C-reactive protein (CRP)

Inflammatory injury is associated with release of pentraxins (such as CRP), prototypical acute phase proteins involved in host defense (Deban et al., 2011). In an experimental model of non-reperfused infarction, CRP injection accentuated cardiomyocyte injury by activating the complement cascade, whereas (somewhat predictably) administration of low MW inhibitors of CRP blocked the deleterious effects of CRP (Pepys et al., 2006). Despite these early promising findings, enthusiasm regarding the potential value of CRP inhibition in patients with MI is dampened by conflicting findings on the effects of CRP in atherosclerosis and thrombosis. Although, in apolipoprotein-E null mice, treatment with human native CRP accelerated atherosclerotic disease (Schwedler et al., 2005), in other studies, transgenic overexpression of human CRP had no effects on atherosclerosis, thrombosis and inflammation (Hirschfield et al., 2005; Tennent et al., 2008) or even delayed plaque formation (Kovacs et al., 2007). The conflicting in vivo effects of CRP may be explained by the contextually regulated formation of CRP isoforms with distinct functional properties. CRP is known to undergo dissociation from a native pentameric form (pCRP) to potently pro-inflammatory monomeric subunits (mCRP) that may serve to localize the inflammatory response (Eisenhardt et al., 2009). Inhibition of CRP dissociation has been suggested as a promising anti-inflammatory strategy in MI.

Integrins and selectins

Selectins and leukocyte integrins are critically implicated in leukocyte extravasation in the infarcted myocardium (Figure 1). Numerous experimental studies demonstrated that neutralizing antibodies targeting members of the integrin and selectin families reduced the size of the infarct in myocardial ischaemia/reperfusion models (Simpson et al., 1988; Ma et al., 1991; Aversano et al., 1995; Arai et al., 1996; Christia and Frangogiannis, 2013). More recently, a

Table 3

Effects of NSAIDs in experimental models of MI

Model	Species	Agents dose duration delivery method	Major findings	Ref.
Reperfused MI	Dog	Group1: BW755C (inhibitor of both lipoxygenase and COX, 10 mg·kg^{-1}, i.v.) 30 min after reperfusion Group2: Indomethacin (5 mg·kg^{-1}, i.v.) 10 min before reperfusion	BW755C treatment reduced infarct size and decreased the incidence of arrhythmias, attenuating leukocyte infiltration. Treatment with indomethacin did not affect infarct size and leukocyte migration into the ischaemic myocardium.	(Mullane et al., 1984)
Non reperfused MI	Dog	Indomethacin (10 mg·kg^{-1}, i.v.) at 15 min and 3 h after occlusion	Indomethacin caused infarct expansion and scar thinning.	(Hammerman et al., 1984b)
Non reperfused MI	Cat	Ibuprofen (12.5 mg·kg^{-1}, i.v.) 0 and 2.5 h after occlusion	Ibuprofen attenuated myocardial injury without affecting haemodynamics during the early stage of infarction.	(Lefer and Polansky, 1979)
Non reperfused MI	Rat	Aspirin (25 mg·kg^{-1}·day^{-1}, i.p.) 2 days before occlusion until the end of the experiment	Aspirin significantly attenuated interstitial and perivascular fibrosis in the spared myocardium, without affecting wound healing, compensatory hypertrophy and LV dysfunction.	(Kalkman et al., 1995)
Non reperfused MI	Mouse	Aspirin (120 mg·kg^{-1}·day^{-1}, subcutaneously) after occlusion for 4 weeks	High dose aspirin did not affect post-infarct cardiac remodelling and cardiac dysfunction, but attenuated pro-inflammatory cytokine levels.	(Adamek et al., 2007)
Non-reperfused MI	Pig	Celecoxib (COX-2i; 400 mg p.o. bid) after occlusion until end of protocol	Celecoxib increased mortality, promoted infarct thinning, LV dilatation, and accentuated systolic dysfunction.	(Timmers et al., 2007)
Non-reperfused MI	Rat	Parecoxib (COX-2i, 0.75 mg·kg^{-1} i.p.) 24 h after occlusion, daily for 5 days	Parecoxib ameliorated remodelling, reducing peri-infarct apoptosis and preserving arteriolar density. Parecoxib did not affect mortality, infarct size and plasma inflammatory cytokines.	(Straino et al., 2007)
Non-reperfused MI	Mouse	Parecoxib (COX-2i, 0.75 mg·kg^{-1}, i.p.) 24 h after surgery, daily for 5 days	Parecoxib treatment significantly reduced apoptosis in the peri-infarct region; No difference of mortality on day 7.	(Abbate et al., 2006)
Reperfused and non-reperfused MI	Mouse	Parecoxib (COX2i, 0.75 mg·kg^{-1}·day^{-1}, i.p.) for 7 days	Parecoxib treatment reduced mortality and attenuated apoptosis in non-reperfused infarcts, but had no effects in reperfused infarction.	(Salloum et al., 2009)

continues

Table 3
(Continued)

Model	Species	Agents dose duration delivery method	Major findings	Ref.
Non-reperfused MI	Rat	Group 1: DFU (COX2i, 5 mg·kg^{-1}·day^{-1}, p.o.); Group 2: low dose aspirin (1 mg·kg^{-1}·day^{-1}, p.o.); Group 3: high dose aspirin (25 mg·kg^{-1}·day^{-1}, p.o.) 30 min prior to occlusion for 3 months	DFU treatment significantly reduced left ventricular end-diastolic pressure, reduced infarct size and improved cardiac contractility without affecting mortality. Aspirin had no effects on cardiac function.	(Saito et al., 2004)
Non-reperfused MI	Mouse	NS-398 (COX-2i, 3 mg·kg^{-1}·day^{-1}, p.o.) 48 h after occlusion for 2 weeks; Rofecoxib (COX2i, 15 mg·kg^{-1}·day^{-1}, p.o.), 48 h after occlusion for 2 weeks	NS-398 attenuated adverse remodelling and dysfunction following MI without affecting infarct size. Both COX2i reduced cardiac hypertrophy, collagen production, and TGF-β expression in the infarcted heart.	(LaPointe et al., 2004)
Non-reperfused MI	Dog	Piroxicam, (1 mg·kg^{-1} i.v.) 15 min, 3 h after occlusion	Piroxicam caused moderate scar thinning without perturbing regional function.	(Hammerman et al., 1984a)

nanoparticle-based strategy silencing five key adhesion molecules was reported to preserve function in the infarcted myocardium (Sager et al., 2016a). Protection of the myocardium was presumably due to attenuation of leukocyte-mediated cardiomyocyte injury. Unfortunately, clinical trials did not confirm the impressive protective effects of anti-adhesion molecule approaches observed in experimental studies. Anti-CD11/CD18 and anti-CD18 integrin targeting failed to reduce infarct size in STEMI patients (Baran et al., 2001; Rusnak et al., 2001; Faxon et al., 2002) (Table 4). On the other hand, administration of the P-selectin inhibitor inclacumab in patients with ACS reduced cardiomyocyte injury but did not affect clinical outcome (Tardif et al., 2013; Seropian et al., 2014).

Chemokines as therapeutic targets

Approaches targeting chemokines involved in recruitment of pro-inflammatory leukocytes have shown promising results in experimental animal models. Anti-CCL2 therapy reduced mortality, attenuated chamber dilation and improved systolic function in a model of non-reperfused infarction (Hayashidani et al., 2003). Inhibition of CCL5 also exerted protective effects, improving cardiac function and attenuating fibrotic remodelling (Montecucco et al., 2012). Silencing the chemokine receptor CCR2, the main receptor for CCL2, that mediates recruitment of pro-inflammatory monocytes in sites of inflammation, was reported to have beneficial effects not only in the infarcted heart but also in the composition of atherosclerotic plaques and in metabolic dysfunction (Leuschner et al., 2011). However, it should be emphasized that broad targeting of the effects of CC chemokines may also have detrimental actions. Chemokine-mediated signalling is important for recruitment of leukocyte subsets with anti-inflammatory properties and may be involved in activation of a programme of repair. In a mouse model of reperfused MI, genetic disruption of CCR5 was associated with accentuated dilative remodelling, presumably due to impaired recruitment of anti-inflammatory monocyte subsets and of Tregs (Dobaczewski et al., 2010). Thus, recruitment of specific leukocyte subsets through chemokine-chemokine receptor interactions may be critical for repression and resolution of post-infarction inflammation.

Administration of the chemokines that recruit reparative cells, such as CXCL12, may also hold therapeutic promise. Therapy with CXCL12 reduced infarct size and accentuated angiogenesis in experimental models of MI, attenuating systolic dysfunction and improving left ventricular mechanics (Hu et al., 2007; Saxena et al., 2008; Macarthur Jr et al., 2014; MacArthur Jr et al., 2013). Although these experimental findings seem promising, clinical translation is challenging. Loss-of-function studies have suggested important pro-inflammatory actions of CXCL12 in the infarcted heart (Proulx et al., 2007; Jujo et al., 2010) raising concerns that administration of this chemokine may also have injurious effects, accentuating or prolonging inflammatory cascades. Clinical evidence remains extremely limited, as the effects of CXCL12 therapy in MI has not been studied. However, in a Phase II clinical trial in patients with high-risk ischaemic cardiomyopathy, CXCL12 gene therapy was safe but did not meet the primary endpoint for functional improvement (Chung et al., 2015).

Targeting the cytokines

Although a growing body of experimental evidence suggests that pro-inflammatory cytokines may be promising therapeutic targets in patients with MI, their pleiotropic and multifunctional effects, and their involvement in both

Table 4

Targeted anti-inflammatory and immunomodulatory therapies in patients with MI

Type of study	Number of patients	Agent, dose and duration	Major findings	Ref.
Anti-integrin				
RCT: FESTIVAL	88	Rovelizumab (anti-CD11/18, also known as LeukArrest or Hu23F2G) Low dose: (0.3 mg·kg^{-1}) High dose: (1.0 mg·kg^{-1}) i.v. after coronary angiography	Hu23F2G was well tolerated, with no increase in adverse events, including infections. Single-photon emission computed tomographic (SPECT) imaging showed no significant effects of anti-CD11/CD18 treatment on myocardial infarct size in STEMI patients.	(Rusnak et al., 2001)
RCT: HALT-MI	420	Rovelizumab (anti-CD11/18) Low dose: (0.3 mg·kg^{-1}) High dose: (1.0 mg·kg^{-1}) i.v. before coronary angioplasty	Treatment with anti-CD11/CD18 did not affect infarct size in STEMI patients who underwent primary angioplasty.	(Faxon et al., 2002)
RCT: LIMIT-AMI	394	Anti-CD18 (i.v. bolus of 0.5 or 2.0 mg·kg^{-1}, before commencing recombinant tissue plasminogen activator (rtPA)	No significant effects on coronary blood flow, infarct size, or the rate of ECG ST-segment elevation resolution STEMI patients treated with rtPA.	(Baran et al., 2001)
Anti-Selectin				
RCT: SELECT-ACS	544	Inclacumab (anti-P-Selectin), single infusion 5 or 20 mg·kg^{-1}, 1–12 h before PCI	Inclacumab at a dose of 20 mg·kg^{-1} appeared to reduce myocardial damage after PCI in non-STEMI patients, without significant difference in adverse events.	(Tardif et al., 2013)
IL-1 inhibition				
Pilot Study: VCU-ART	10	Anakinra (IL-1RN) 100 mg·day^{-1} subcutaneously for 14 days	IL-1 blockade with anakinra was safe and ameliorated LV remodelling in STEMI patients.	(Abbate et al., 2010)
Pilot Study: VCU-ART2	30	Anakinra (IL-1RN) 100 mg·day^{-1} subcutaneously for 14 days	Anakinra blunted the acute inflammatory response in STEMI patients, without showing benefits of LV remodelling or function.	(Abbate et al., 2013)
RCT: MRC-ILA Heart Study	182	Anakinra (IL-1RN) 100 mg·day^{-1} subcutaneously for 14 days	Following 14 day treatment of IL-1RN, inflammatory markers were reduced; In patients with NSTE-ACS, IL-1RN treatment significantly increased major adverse cardiac events at 1 year, but not at day 30 or 3 months.	(Morton et al., 2015)
RCT: CANTOS	10 061	Canakinumab (anti-IL-1β) at three different doses: 50, 150 and 300 mg	In patients with previous MI and an hs-CRP level ≥ 2 mg·L^{-1}, canakinumab (150 mg) significantly reduced hs-CRP,	(Ridker et al., 2017)

continues

Table 4

(Continued)

Type of study	Number of patients	Agent, dose and duration	Major findings	Ref.
		subcutaneously, once every 3 months	but increased the incidence of fatal infection and sepsis. The 300 mg dose had similar effects, but the multiple statistical comparisons yielded non-significant differences in comparison to the placebo group. The reduced rate of recurrent cardiovascular events in treated patients was independent of lipid level lowering.	
Anti-complement				
Pilot study	31	Complement 1 inhibitor (loading dose 50 or 100 $U \cdot kg^{-1}$ 6 h after MI, followed by continuous infusion 1.25 or 2 $U \cdot kg^{-1} \cdot h^{-1}$ for 48 h)	In MI patients who received early thrombolytic therapy, C1 inhibitor treatment reduced troponin T and creatine kinase-MB levels, without causing adverse effects.	(de Zwaan et al., 2002)
RCT: COMPLY	943	Pexelizumab (anti-C5) bolus (2 $mg \cdot kg^{-1}$), or pexelizumab bolus (2 $mg \cdot kg^{-1}$) followed by pexelizumab infusion (0.05 $mg \cdot kg^{-1} \cdot h^{-1}$) for 20 h	In STEMI patients receiving fibrinolysis, adjunct treatment with pexelizumab neither reduced infarct size nor improved clinical outcomes.	(Mahaffey et al., 2003)
RCT: COMMA	960	Pexelizumab bolus (2 $mg \cdot kg^{-1}$), or pexelizumab bolus (2 $mg \cdot kg^{-1}$) followed by pexelizumab infusion (0.05 $mg \cdot kg^{-1} \cdot h^{-1}$) for 20 h	In STEMI patients undergoing PCI, adjunct treatment with pexelizumab had no measurable effect on infarct size or on the composite of 90 day death, new or worsening heart failure, shock and stroke. 90 day mortality was significantly reduced in pexelizumab bolus plus infusion.	(Granger et al., 2003)
RCT: APEX-AMI	5745	Pexelizumab prior to PCI, i.v. bolus 2 $mg \cdot kg^{-1}$, followed by 0.05 $mg \cdot kg^{-1} \cdot h^{-1}$ infusion over the subsequent 24 h	In patients treated with primary PCI for STEMI, adjunct pexelizumab treatment showed no significant effect on mortality or the composite endpoint of death, cardiogenic shock, and heart failure at day 30 or day 90.	(Armstrong et al., 2007)
IL-6 antagonism				
RCT	117	Tocilizumab (IL-6Ra, single dose 280 mg, i.v.) prior to coronary angiography	Tocilizumab attenuated the inflammatory response and PCI-related troponin T (TnT) release in NSTEMI patients. Tocilizumab did not affect coronary flow reserve during hospitalization or after 6 months.	PMID: (Kleveland et al., 2016) (Holte et al., 2017)

continues

Table 4
(Continued)

Type of study	Number of patients	Agent, dose and duration	Major findings	Ref.
TNF-α antagonism				
RCT	26	Etanercept (TNF-α antagonist) at a single intravenous dose of 10 mg	Following acute MI, etanercept reduced systemic inflammation but increased platelet activation without affecting peripheral vasomotor or fibrinolytic function.	(Padfield et al., 2013)
Proteinase inhibition				
RCT: TIPTOP	429	Doxycycline (non-selective MMP inhibitor, 100 mg p.o.) immediately after primary PCI and then twice daily for 7 days	In patients with a first STEMI and LV dysfunction treated with primary PCI, a timely short-term treatment with doxycycline significantly reduced adverse LV remodelling and decreased infarct size assessed through SPECT.	(Cerisano et al., 2014)
RCT: PREMIER	253	PG-116800 (MMP inhibitor), 200 mg oral dose taken twice daily for 90 days	MMPs inhibition with PG-116800 after MI failed to reduce LV remodelling or improve clinical outcomes in patients with STEMI.	(Hudson et al., 2006)
Pilot clinical trial	10	Prolastin C, human plasma-derived alpha1 antitrypsin (AAT), single infusion of 60 mg·kg^{-1}, within 12 h of revascularization	A single administration of Prolastin C in patients with STEMI is well tolerated and is associated with a blunted acute inflammatory response.	(Abbate et al., 2015b)

injurious and reparative responses pose major therapeutic challenges. Recent experimental and clinical studies have suggested that the IL-1 system may represent a promising therapeutic target in patients with MI (Saxena et al., 2016). Safe and effective strategies for IL-1 inhibition are extensively used treatment of patients with inflammatory arthritides or auto-inflammatory syndromes. Anakinra is a non-glycosylated recombinant form of IL-1 receptor antagonist that binds to the type I IL-1 receptor but does not activate a signalling response, thus functioning as a competitive IL-1α/IL-1β inhibitor. On the other hand, anti-IL-1β antibodies (such as canakinumab) selectively target IL-β-mediated responses. In most experimental MI studies, inhibition of the IL-1 system with anakinra or anti-IL-1β antibodies exerted protective effects, reducing chamber dilation and improving dysfunction (Abbate et al., 2008; Toldo et al., 2013). Early evidence from clinical studies has also produced promising results. Pilot studies demonstrated that anakinra can be safely administered as a 2 week course in STEMI patients and may attenuate adverse remodelling, while protecting from the development of post-infarction heart failure (Abbate et al., 2010; Abbate et al., 2013; Abbate et al., 2015a). In the recently reported Canakinumab Antiinflammatory Thrombosis Outcome Study trial (Ridker et al., 2017), IL-1β inhibition in high-risk patients with atherosclerotic disease attenuated inflammation and reduced cardiovascular events. In patients treated with 150 mg of canakinumab, the primary endpoint (the composite of MI, nonfatal stroke and cardiovascular death) was significantly lower. The beneficial effects of canakinumab were modest: to avoid one primary endpoint event, 156 patients had to be treated for 1 year. Moreover, the canakinumab group exhibited a very low, but significantly higher than placebo, death rate due to infection. Despite these issues and the concerns regarding the very high cost of the antibody, this landmark clinical trial supports the case for targeted anti-cytokine therapy in selected patients with MI. An emerging body of evidence suggesting that IL-1-mediated inflammation accentuates adverse remodelling post-MI (Bujak et al., 2008) and may promote arrhythmia generation (Monnerat et al., 2016; De Jesus et al., 2017) further strengthens the rationale for targeting IL-1 in high-risk subpopulations of MI patients, suggesting that protection may not be limited to reduction of new atherothrombotic events.

The TGF-β system

Despite the critical involvement of TGF-β signalling in cardiac injury and repair (Frangogiannis, 2017b), targeting TGF-β following MI poses several major challenges. First, TGF-βs are known to modulate phenotype and function of

all cell types involved in cardiac injury and repair. Thus, TGF-β inhibition would be expected to affect both injurious and protective actions. The effects of TGF-β inhibition may be dependent on the timing of the intervention. Thus, early neutralization of TGF-β may block anti-inflammatory signalling in macrophages, accentuating inflammation and increasing the incidence of cardiac rupture, whereas late suppression may attenuate pro-fibrotic signals, improving diastolic function (Ikeuchi et al., 2004). Second, because TGF-β is involved in preservation of cardiac and vascular homeostasis, TGF-β inhibition following MI may carry significant risks, promoting aneurysmal rupture in vulnerable patients (Engebretsen et al., 2014; Frangogiannis, 2014b; Biernacka et al., 2015). Third, the complex biology of TGF-β signalling further complicates design of therapeutic strategies. TGF-β signals through intracellular effectors, the Smads and through activation of non-Smad pathways (such as p38 MAPK, Erk MAPK, and JNK). Design of effective therapeutic strategies requires understanding of the relative role of Smad-dependent and Smad-independent signalling in vivo (Bujak et al., 2007; Rainer et al., 2014). In the infarcted heart, experimental studies have suggested distinct effects of Smad-dependent signalling in cardiomyocytes and fibroblasts (Kong et al., 2017). Moreover, non-Smad pathways may also contribute to activation of interstitial cells towards a fibrogenic phenotype (Molkentin et al., 2017). Dissection of cell-specific responses to TGF-β and understanding of the temporal sequence of its cellular actions in the infarcted heart are needed to design safe and effective therapeutic approaches.

Targeting the MMP system

MMPs are involved in post-MI repair and remodelling, not only by critically regulating ECM metabolism but also by processing inflammatory mediators, such as chemokines and cytokines (Fingleton, 2017; Frangogiannis, 2017a). Genetic deletion of MMP2 and MMP9 has been shown to attenuate post-MI ventricular dilation and to protect from cardiac rupture in mouse models of non-reperfused MI (Ducharme et al., 2000; Matsumura et al., 2005). However, pharmacological inhibition of MMPs in animal models of MI has produced conflicting results, depending on the timing of the intervention, the inhibition profile of the agent used and the experimental model. Early treatment with doxycycline, a non-selective MMP inhibitor, attenuated adverse remodelling in a rat model of non-reperfused infarction (Villarreal et al., 2003). In contrast, early inhibition of MMP9 delayed resolution of inflammation and worsened dysfunction in a mouse model of permanent coronary occlusion (Iyer et al., 2016). In clinical studies, no consistent beneficial effects of MMP inhibition have been reported. In the TIPTOP trial, administration of doxycycline (100 mg p.o. bid for 7 days) in patients with STEMI and left ventricular dysfunction reduced the size of the infarct and attenuated cardiac remodelling (Cerisano et al., 2014). In contrast, in the PREMIER trial, administration of an MMP inhibitor with high affinity for MMP2, MMP3, MMP8, MMP9, MMP13 and MMP14 did not improve clinical outcomes and left ventricular remodelling in STEMI patients (Hudson et al., 2006).

Challenges in targeting inflammation following MI

The diverse roles of inflammatory cascades in injury and repair

The critical involvement of inflammation in both injury and repair of the infarcted heart complicates attempts to target inflammatory signals in patients with MI (Saxena et al., 2016). Inflammatory pathways have been implicated in extending cardiomyocyte death and in triggering matrix degradation but also play a critical role in clearance of dead cells from the infarct and in formation of a scar that preserves the structural integrity of the ventricle. Moreover, inflammatory mediators have been implicated in recruitment of progenitor cells involved in infarct angiogenesis (Taghavi and George, 2013). Thus, inhibition of an inflammatory signal involved in early injury may also inhibit a crucial repair response. Design of therapeutic strategies targeting inflammation in patients with MI needs to take into account important temporal and spatial considerations. There is ample evidence to suggest that prolonged or expanded pro-inflammatory signalling may accentuate adverse remodelling by activating proteases that degrade the cardiac ECM, by transducing pro-apoptotic responses in cardiomyocytes and by promoting fibrogenic signalling in the viable non-infarcted myocardium (Chen et al., 2012; Frangogiannis et al., 2005). It is likely that following MI, there is a therapeutic window of opportunity for safe and effective targeting of specific inflammatory signals. Understanding the time course of the cellular actions of specific inflammatory signals is critical for optimal design of therapeutic strategies.

The pathophysiological heterogeneity of human post-infarction remodelling

The remarkable pathophysiological heterogeneity in human patients surviving MI further complicates therapeutic implementation of promising targets. The extent of adverse post-MI remodelling is only partly dependent on the size of the infarct. Differences in susceptibility to adverse remodelling between patients may be explained by age and gender, genetic substrate, the presence or absence of concomitant conditions, the pattern of atherosclerotic disease, administration of medications and other poorly understood factors. Certain subpopulations of patients may have defective mechanisms for negative regulation of inflammation, thus exhibiting prolonged or expanded inflammatory responses. Others may have accentuated fibrotic reactions. Pathophysiological stratification of the patients on the basis of their biochemical profile, clinical characteristics and functional responses may identify patients with overactive post-infarction inflammatory responses that may benefit from targeted anti-inflammatory strategies (Frangogiannis, 2014a). Clinical and experimental studies suggest that certain patient subpopulations, such as diabetics, may exhibit dysregulated inflammatory reactions following MI that may be responsible for accentuated remodelling and worse dysfunction. Patients with diabetes have a high incidence of diastolic dysfunction following MI, despite a smaller infarct size and comparable systolic dysfunction (Stone et al., 1989). In experimental models, diabetes and obesity are associated with

cardiomyocyte hypertrophy and interstitial fibrosis. These changes may reflect exaggerated angiotensin-mediated responses and increased TGF-β/Smad signalling (Biernacka et al., 2015). Targeting fibrogenic mediators may be a promising therapeutic strategy in these patients. On the other hand, other patients may exhibit prolonged activation of pro-inflammatory signals. These patients may benefit from strategies targeting critical inflammatory cascades, such as IL-1. Biomarkers (Seropian et al., 2016) and imaging approaches (Wollenweber et al., 2014; Nahrendorf et al., 2015) may be used to assess inflammatory activation in these patients, in order to design personalized therapeutic approaches.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017a,b,c,d,e).

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Conflict of interest

The authors declare no conflicts of interest.

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The Cellular Origin of Activated Fibroblasts in the Infarcted and Remodeling Myocardium

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Inflammation is intricately associated with fibrosis. Macrophages, lymphocytes, and mast cells infiltrating injured tissues can acquire a fibrogenic phenotype, activating local fibroblast populations, and leading to increased deposition of extracellular matrix proteins. In addition, some experimental studies have indicated a more direct link between inflammation and fibrosis, suggesting that leukocyte subpopulations may be capable of fibroblast conversion. The notion that fibroblasts infiltrating injured tissues may derive from circulating cells was proposed >150 years ago[1] and has remained an area of controversy ever since. In 1994, Bucala et al[2] coined the term fibrocyte to describe a circulating, bone marrow (BM)–derived cell with features of both fibroblasts and monocytes, which has the ability to adopt a mesenchymal phenotype and contributes to scar formation. Fibrocytes are characterized through the combined expression of hematopoietic and progenitor cell markers (such as CD45 and CD34) and the production of structural extracellular matrix proteins, such as collagen. Fibrocytes represent a small fraction of circulating leukocytes in normal subjects, but are mobilized in response to inflammation, and have been reported to infiltrate tissues in patients with fibrotic diseases.

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Several studies have identified fibroblasts of BM origin in injured and failing hearts.[3] However, recent investigations using lineage tracing approaches suggested that populations of intracardiac cells (resident fibroblasts, pericytes, or epicardial-derived interstitial cells) are the predominant cellular source of activated fibroblasts in infarcted and remodeling myocardium[4–8] (Figure). Considering the conflicting experimental evidence, the relative contribution of circulating progenitors in the marked expansion of fibroblasts observed after myocardial injury remains unclear.

In the current issue of the journal, Moore-Morris et al[10] used a combination of lineage tracing and BM transplantation strategies to explore the contribution of BM lineages to the population of activated fibroblasts in nonreperfused mouse infarcts. Lineage tracing studies showed that practically no infarct fibroblasts were derived from Vav+ hematopoietic cells. Experiments using chimeric mice with collagen1α1-GFP (green fluorescent protein)-expressing BM cells confirmed the absence of BM-derived fibroblasts in the healing infarct. Ninety-six percent of infarct fibroblasts were derived from WT1+ epicardial cells, whereas endothelial cells contributed a small proportion (≈4%). The findings provide strong support to a growing consensus on the origin of fibroblasts in myocardial disease, suggesting that the majority of fibroblasts in the remodeling heart originate from intracardiac populations, and not from leukocytes, or vascular endothelial cells.

Hard Evidence on the Contribution of BM-Derived Fibroblasts in Cardiac Remodeling

In many published studies, the notion that BM-derived fibroblasts contribute to cardiac fibrosis is based solely on immunohistochemical data showing expression of hematopoietic cell markers by collagen-producing cells. Considering the questionable specificity of many immunohistochemical methods, this is not sufficient. Chimeric mice with genetically labeled BM cells represent a powerful tool to study the relative role of BM-derived fibroblasts in myocardial disease. Lineage tracing studies can provide important complementary information by investigating the role of specific hematopoietic or myeloid cell lineages. Considering the lack of specific and reliable fibroblast markers, documentation of fibroblast conversion is a major challenge and often limits the conclusions of the studies. Vimentin is often used as a fibroblast marker but lacks specificity, as it labels all mesenchymally derived cells. Localization of α-smooth muscle actin (α-SMA) in interstitial cells is a useful marker of myofibroblast conversion, but may also label pericytes, or vascular smooth muscle cells. Despite concerns regarding its specificity,[4] expression of collagen is a reliable and functionally relevant marker of the fibroblast phenotype, reflecting the fibrogenic capacity of the cell. Online Table I summarizes the findings of published in vivo studies that used robust BM transplantation or lineage tracing approaches, coupled with appropriate strategies for fibroblast identification, to explore the role of BM-derived cells in myocardial fibrosis. Investigations in models of myocardial infarction have produced conflicting results. Although 2 independent studies using BM chimeras[3,9] suggested that ≈24% of infarct fibroblasts are derived from the BM, other investigations using similar BM transplantation protocols found insignificant numbers of BM-derived fibroblasts in the infarct.[5,11]

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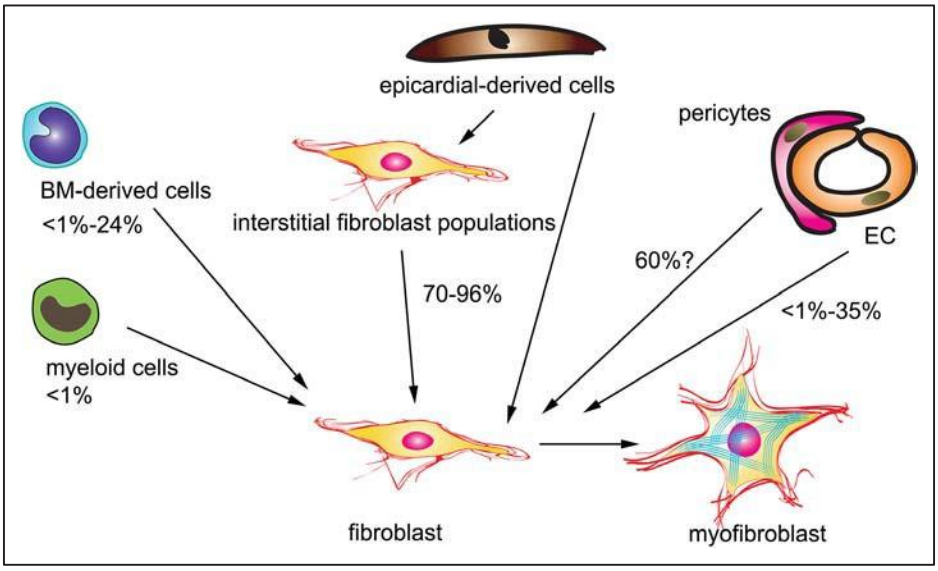

Figure. Schematic cartoon illustrating the origin of activated fibroblasts in the infarcted myocardium. Although 2 independent studies using bone marrow (BM) transplantation strategies suggested that up to a quarter of infarct fibroblasts may be derived from the BM,[3,9] the bulk of the evidence suggests low numbers of BM-derived fibroblasts in the infarcted myocardium.[4,5,10,11] Lineage tracing strategies documented that intracardiac interstitial cells (Tcf21+fibroblasts or epicardial-derived interstitial cells) are the predominant source of infarct fibroblasts.[4,5,10] Although a study investigating the contribution of Gli-1+cells in tissue fibrosis suggested that 60% of infarct myofibroblasts are derived from perivascular cells,[8] the role of the pericytes (and the potential overlap between fibroblast and pericyte populations) remains understudied. In some studies, endothelial cells (EC) were found to be a major source of infarct fibroblasts,[12] whereas other investigations showed very low numbers of endothelial-derived fibroblasts in the infarct.[4,10] Conflicting findings reflect the use of different approaches to trace the cells and the absence of reliable and specific markers to identify fibroblasts.

Moreover, lineage tracing experiments found no evidence of myeloid cell–derived fibroblasts in healing myocardial infarction.[4] Using 2 independent strategies to trace BM-derived cells, and a well-characterized collagen1α1-GFP reporter line to identify fibroblasts, the current study is sufficiently robust to shape our cell biological paradigm on the origin of fibroblasts in cardiac repair. By comparison, previously published observations only used BM transplantation strategies to trace BM-derived cells and identified fibroblasts through the expression of less-specific markers, such as α-SMA or vimentin. However, are the findings sufficiently conclusive to exclude any contribution of BM-derived fibroblasts in cardiac repair, remodeling, and fibrosis?

Relative Contribution of BM-Derived Fibroblasts May Depend on the Pathophysiological Context

Various types of cardiac injury have profoundly different effects on the cellular composition of the myocardium. In myocardial infarction, the timing of reperfusion may have dramatic effects on the fate of resident myocardial cells and on leukocyte recruitment, thus, altering the relative contributions of various cell types to the expansion and activation of fibroblasts. Early reperfusion is associated with marked induction of chemokines and with accentuated and accelerated influx of leukocytes and may also augment recruitment of BM-derived fibroblasts. On the other hand, prolonged ischemic insults may result in death of vascular and interstitial cells in the infarcted region, thus, reducing their relative contribution to the expansion of myofibroblasts. In their absence, recruitment of interstitial cells from noninfarcted areas may be required to expand the fibroblast population.

Other mechanisms of myocardial injury may selectively activate specific cell types, thus, altering the profile of fibroblast progenitor cells. For example, in myocarditis, induction of an intense inflammatory response may be associated with chemokine-driven recruitment of abundant BM-derived fibroblasts.[13] In the current study, the surprising presence of BM-derived fibroblasts around the surgical instrumentation site is independent of myocardial infarction and highlights the distinct cell biological responses to different types of myocardial injury. Considering that both infarction and instrumentation injury are driven by cellular necrosis and are associated with inflammation, the selective recruitment of BM-derived fibroblasts in the suture area is surprising and has no obvious mechanistic explanation.

It should also be emphasized that our knowledge on the origin of fibroblasts in cardiac injury is based almost exclusively on studies using mouse models. Information in human patients with heart disease is scarce. Sex-mismatched cardiac transplantation provides a unique opportunity to explore the potential role of circulating cells in myocardial fibrosis. A small study using endomyocardial biopsies of 7 male patients who had received a female allograft suggested that the majority of fibroblast-like cells in fibrotic regions were Y chromosome negative,[14] providing further support to the intracardiac origin of fibroblasts. However, these findings do not exclude a significant contribution of BM-derived fibroblasts in myocardial infarction or in myocarditis, as immunosuppressive therapy after transplantation may inhibit recruitment of BM-derived cells.

Does the Cellular Origin of Fibroblasts Have Functional Implications?

Experimental studies have suggested that populations of intracardiac interstitial fibroblasts, perivascular cells, and (to a lesser extent) endothelial cells may contribute to the expansion of fibroblasts in infarcted and remodeling hearts. It is tempting to hypothesize that fibroblasts originating from different cellular sources may have distinct functional properties, thus, explaining the wide range of functions of fibroblast populations in injury sites. Unfortunately, evidence supporting this notion is lacking. Although classification of fibroblast-like cells into subpopulations with different functional properties seems attractive and may explain the wide range of reparative and detrimental properties of fibroblasts in injured hearts,[15] whether distinct fibroblast profiles can be identified in vivo remains unclear.

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Disclosures

None.

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Key Words: Editorials □ extracellular matrix □ fibroblast □ inflammation □ leukocyte □ myocardial infarction

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ONLINE SUPPLEMENT

The cellular origin of activated fibroblasts in the infarcted and remodeling myocardium

Short title: Cellular origin of infarct fibroblasts

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Supplemental table I: Contribution of bone marrow (BM)-derived fibroblasts in experimental models of cardiac fibrosis

Model	Ref	Contribution of BM-derived fibroblasts	Approaches used to trace BM-derived cells.	Markers used for fibroblast labeling	Contribution of non-BM derived fibroblasts
Non-reperfused MI (mouse)	[1]	21% of infarct myofibroblasts originate from the BM. Moreover, a 4-fold increase in βGal activity is noted in pro-Col1α2-chimeric mice	Transplantation with EGFP-tagged BM, or BM from proCol1α2 gene-driven luciferase or βGal reporter mice.	α-SMA, β galactosidase activity in pro-Col1α2-driven chimeric mice	Not studied.
Non-reperfused MI (mouse)	[2]	24% of vimentin+ fibroblasts, 57% of αSMA+ myofibroblasts are derived from the BM.	Transplantation with BM from EGFP reporter mice	α-SMA, vimentin	Not studied.
Non-reperfused MI (mouse)	[3]	BM cells are a minor contributor to infarct myofibroblasts.	Transplantation with RFP+ BM.	α-SMA, CD90, collagen 1, DDR2	Epicardium-derived WT1+ cells are major contributors to activated fibroblasts following MI.
Non-reperfused MI (mouse)	[4]	<1% of activated myofibroblasts are derived from lysozyme-M+ myeloid cells.	Lineage tracing of lysozyme M+ cells was used to study the contribution of myeloid cell-derived fibroblasts.	Periostin, α-SMA, vimentin, PDGFRα.	70% of activated fibroblasts were derived from Tcf21+ cardiac fibroblasts.
Non-reperfused MI (mouse)	[5]	Infarct fibroblasts are not derived from BM cells.	Vav-Cre-RosatdT/+; Collagen1α1-GFP+/- mice, mice were used to trace hematopoietic cells, and BM transplantation with RFP-labeled BM, or BM from Collagen1α1-GFP mice.	Collagen 1α1 reporter line, α-SMA	Activated fibroblasts (over 96%) are derived from epicardial (WT1 Cre) lineage and not from endothelial (Tie 2 Cre)
Reperfused MI (1h ischemia/3-7 days reperfusion (rat)	[6]	No α-SMA+ or vimentin+ cells of BM origin are noted in the infarct at 3 days post-MI. After 7 days, few vimentin+ cells originate from the BM.	eGFP-tagged BM from transgenic mice was transplanted to nude rats.	αSMA, vimentin	Not studied.

Brief repetitive ischemia/reperfusion in the mouse.	[7]	A population of BM-derived α-SMA+/collagen 1+ cells is identified in the interstitium.	Transplantation with Rosa26-lacZ BM.	α-SMA,collagen1	Not studied
Autoimmune myocarditis	[8]	>60% of α-SMA+, collagen+, fibronectin+ fibroblasts are derived from the BM.	Transplantation with EGFP-tagged BM.	α-SMA, collagen, fibronectin	Not studied.
Left ventricular pressure overload (transverse aortic constriction in mice)	[9]	No evidence of BM-derived fibroblasts on the basis of BM transplantation, and parabiosis experiments. Fibroblasts are not derived from Vav+ hematopoietic cells.	BM transplantation, parabiosis-mediated blood chimerism between WT and GFP-labelled transgenic mice, fate mapping of Vav+ hematopoietic cells.	CD90, vimentin, DDR2, PDGFRα, Collagen1	The majority of activated fibroblasts in remodeling myocardium is derived from epicardial cells, and a small proportion from endothelial lineage.
Left ventricular pressure overload (mouse)	[10]	A population of BM-derived α-SMA+/vimentin+ cells is found in perivascular fibrotic areas.	Transplantation with EGFP+ BM	α-SMA, vimentin	Not studied
Left ventricular pressure overload (mouse)	[11]	BM-derived cells do not contribute to the collagen-expressing fibroblast population	Collagen1α1GFP/ Vav Cre-Rosa tdT transgenic mice	Collagen1α1 GFP reporter line	The majority of activated fibroblasts is derived from resident fibroblast populations (~80%) and a smaller proportion from endothelial cells.
Genetic mouse model of cardiomyopathy (cardiomyocyte-specific overexpression of Mst1)	[12]	17% of fibroblasts in cardiomyopathic hearts were derived from BM.	Transplantation of GFP+ BM in cardiomyocyte-specific Mst1 overexpressing mice	ollagen1, α-SMA, imentin	Not studied.
Diabetic cardiomyopathy (streptozotocin-induced type 1 diabetes in mice)	[13]	A 2.5 fold increase in BM-derived α-SMA+ interstitial cells was noted in the diabetic myocardium	Transplantation with EGFP+ BM.	α-SMA+ interstitial cells	Not studied.

Ref,, reference; MI, myocardial infarction; BM, bone marrow; α-SMA, α-smooth muscle actin

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Current Opinion in Pharmacology

Cell therapy for peripheral artery disease

Nikolaos G Frangogiannis

Patients with severe peripheral artery disease (PAD) who are not candidates for revascularization have poor prognosis. Cell therapy using peripheral blood-derived or bone marrow-derived mononuclear cells, mesenchymal stem cells, or marker-specific subsets of bone marrow cells with angiogenic properties may hold promise for no-option PAD patients. Injected cells may exert beneficial actions by enhancing local angiogenesis (either through maturation of endothelial progenitors, or through secretion of angiogenic mediators), or by transducing cytoprotective signals that preserve tissue structure. Despite extensive research, robust clinical evidence supporting the use of cell therapy in patients with critical limb ischemia is lacking. Larger, well-designed placebo-controlled clinical trials did not support the positive results of smaller less rigorous studies. There is a need for high-quality clinical studies to test the effectiveness of cell therapy in PAD patients. Moreover, fundamental cell biological studies are needed to identify the optimal cell types, and to develop strategies that may enhance homing, survival and effectiveness of the injected cells.

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Introduction

Lower extremity peripheral artery disease (PAD) is a major health burden, representing the third-leading cause of cardiovascular morbidity related to atherosclerotic disease after coronary disease and stroke. The prevalence of PAD rises sharply with age, affecting almost 20% of the US population at the age of 80 [1,2]. Epidemiologic studies have highlighted the global impact of the disease, suggesting dramatic recent increases in PAD prevalence in low and middle-income countries, and supporting the notion that we are faced with a global PAD pandemic, affecting more than 200 million men and women in both high-income countries and in the developing world [3]. Considering the mortality, morbidity and disability associated with PAD, there is an urgent need to develop new therapeutic strategies in order to prevent development and progression of the disease, and to treat life-threatening or limb-threatening complications. Experimental studies and early stage clinical trials have suggested that cell therapy may be a promising new approach for patients with PAD [4]. The current review manuscript discusses the potential role of cell therapy approaches in the treatment of PAD.

The pathophysiologic basis of PAD

The clinical manifestations of PAD reflect the consequences of a mismatch between blood supply and demand [5,6]. The typical symptom of PAD is intermittent claudication, a characteristic squeezing leg pain associated with walking and relieved by rest. In normal subjects, exercise is associated with marked increases in peripheral artery blood flow and limb oxygen uptake, driven by increased metabolic demand. In contrast, in PAD patients, fixed stenotic lesions in peripheral arteries limit blood flow, reducing the supply of the affected territory and leading to ischemia. Although the main cause of supply and demand disequilibrium in PAD patients is structural, excessive vascular tone due to activation of neurohumoral pathways, or impaired vasodilatory responses due to endothelial dysfunction may increase vascular resistance, further limiting blood flow in the extremity [7].

Repetitive limb ischemia followed by reperfusion causes mitochondrial dysfunction in skeletal myocytes and triggers generation of reactive oxygen species (ROS), leading to chronic structural changes in the skeletal muscle. ROS-driven apoptosis of skeletal myocytes leads to a reduction in skeletal muscle mass and is accompanied by fatty infiltration, impaired peripheral nerve function and fibrosis [8,6,9,10]. These pathologic alterations are associated with chronic skeletal muscle dysfunction and significant functional impairment. In a subset of patients, chronic ischemia follows an aggressive clinical course that culminates in the development of rest pain and significant tissue loss, a condition termed critical limb ischemia (CLI). Traditional treatment strategies in patients with CLI are focused on surgical bypass or endovascular interventions, aimed at restoring perfusion to prevent amputation of the affected limb [11]. However, a significant percentage of CLI patients do not have revascularization

options; these patients have poor prognosis and often require amputation.

Cell therapy as a therapeutic approach in PAD

Considering the limited treatment options for patients with severe PAD, the rationale for cell therapy approaches is sound. In patients with severe atherosclerotic disease of the native arterial circulation, administration of cell populations capable of activating an angiogenic program may result in formation of neovessels, improving perfusion of the affected limb. Increased blood supply may prevent ischemic episodes and may even contribute to restoration of normal skeletal muscle structure. It should be emphasized that any beneficial effects of cell therapy in PAD may not be necessarily due to incorporation of the cells into the vascular network, but may involve paracrine effects mediated through secretion of angiogenic mediators. Cell therapy may also activate yet unidentified cytoprotective and regenerative pathways that may improve limb function through effects independent of neovessel formation.

A growing body of experimental and clinical evidence suggests that cell-based therapy may hold promise in patients with severe PAD. Experimental investigations have used models of hindlimb ischemia to study the effectiveness of cell therapy approaches in promoting angiogenesis and in attenuating skeletal muscle injury. On the other hand most clinical studies investigating the effectiveness of cell therapy in patients with CLI are small phase I or II clinical trials. Considering the variable approaches used by different groups, the wide range of cell types used, and the absence of standardized protocols for characterization of the cells and for evaluation of clinical outcome, there is substantial uncertainty regarding the effectiveness of various cell types in PAD patients.

The therapeutic potential of endothelial progenitor cells (EPCs)

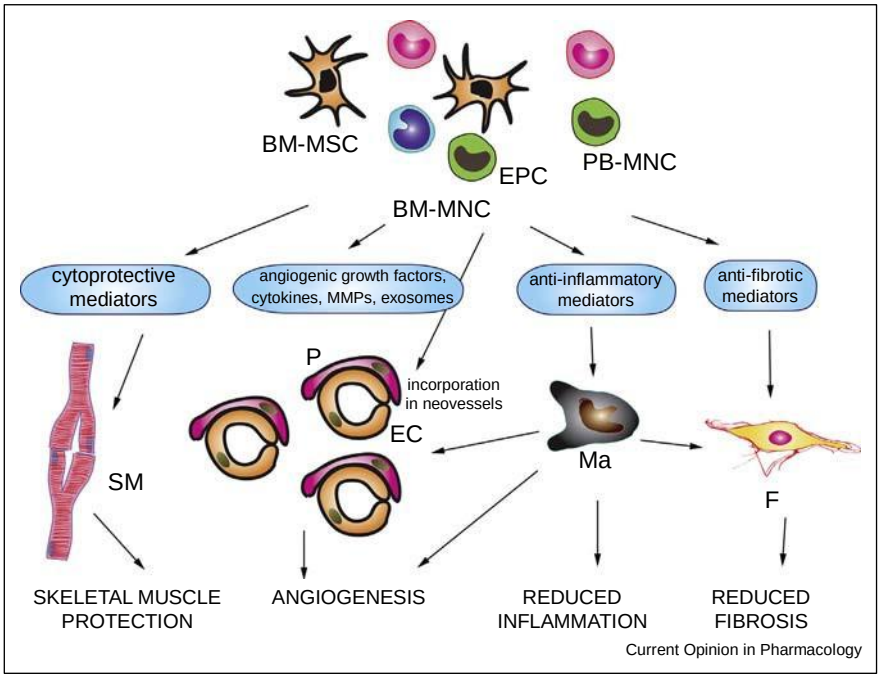
The identification of EPCs, as bone marrow-derived progenitors, that home to sites of injury and may contribute to angiogenesis [12] provided a strong rationale for the use of cell therapy in PAD patients. It should be noted that, despite progress in understanding the mechanistic basis of the angiogenic response, the contribution of blood-derived progenitors in neovessel formation following injury remains controversial. In a mouse model of hindlimb ischemia, both marrow-derived and non-marrow derived endothelial progenitor populations have been implicated in formation of neovessels [13]. Despite the recent use of lineage tracing approaches in mouse models, the origin of neovascular endothelial cells in sites of injury remains controversial. Studies in the ischemic myocardium suggested a significant contribution of mesenchymal cells that undergo conversion into endothelial cells through a p53-dependent mechanism [14]. In contrast, other investigations suggested that practically all neovessels in the injured myocardium are derived from pre-existing endothelial cells, and not through lineage transdifferentiation [15]. It is plausible that the relative contributions of various cellular sources in the angiogenic response may be dependent on the pathophysiologic context and on the site of injury. Unfortunately, lineage tracing studies investigating the cellular origin of angiogenic vascular cells in ischemic skeletal muscle have not been performed.

Regardless of the origin of endogenous angiogenic endothelial cells in the ischemic limb, local injection of circulating endothelial progenitors would be expected to enrich the ischemic site with a pool of angiogenic cells, promoting neovessel formation and improving function. To achieve this goal, several different approaches have been used, injecting unselected or marker-specific mononuclear cells from the bone marrow, or the peripheral blood. These populations may contain a subset of bona fide endothelial progenitors that incorporate to the vascular network forming new vessels, and other cell types that may contribute to the angiogenic process by secreting cytokines, angiogenic growth factors, matrix metalloproteinases, matricellular proteins, or miRNA-containing exosomes (Figure 1) [16•,17,18].

Bone marrow-derived mononuclear cells (BM-MNC) and peripheral blood-derived mononuclear cells (PB-MNC) in the treatment of PAD

Unselected mononuclear cells harvested, derived either from the bone marrow or the peripheral blood, represent a mixture of monocytes, non-hematopoietic stromal cells (including mesenchymal stem cells), and EPCs and have been used in both experimental models of limb ischemia and in patients with PAD. In the TACT (Therapeutic Angiogenesis using Cell Transplantation) study, injection with autologous BM-MNCs in the gastrocnemius of the ischemic limb reduced rest pain and increased transcutaneous oxygen pressure in patients with CLI; improvement was sustained for at least 24 weeks [19]. Over the last few years, several additional clinical trials suggested that intramuscular or intra-arterial injections of BM-MNCs or PB-MNCs in patients with CLI are safe, and may reduce rest pain and improve ulcer healing (Table 1) [20,21]. In many studies, improved clinical outcome was associated with objective evidence of enhanced perfusion. However, in most studies, effects on amputation rates did not reach statistical significance. Some of the larger, more rigorous, and well-designed studies failed to support the beneficial effects [22•], suggested by smaller nonplacebo controlled investigations [20]. The conflicting findings may reflect the clinical improvement observed in placebo-treated patients [22•], and emphasize the importance of rigorous design, large population size and accurate blinding in order to test the

Figure 1



Cell biological mechanisms responsible for the protective effects of cell therapy in peripheral artery disease. Most clinical studies have tested the effects of intramuscular or intra-arterial injections of bone marrow (BM) or peripheral blood-derived mononuclear cells (BM-MNC and PB-MNC respectively), marker-specific mononuclear cells, or bone marrow-derived mesenchymal stem cells (BM-MSC). These cells contain subsets of endothelial progenitor cells (EPCs) that may promote angiogenesis by incorporating into the vascular system. Injected cells may also act through paracrine actions by secreting angiogenic cytokines, growth factors, matrix metalloproteinases and mi-RNA-containing exosomes. Moreover, cell therapy may protect ischemic skeletal myocytes (SM), modulate inflammatory activity and inhibit fibrogenic pathways, preserving structure and function. Additional symbols: Ma, macrophage; F, fibroblast; EC, endothelial cell; P, pericyte.

effectiveness of therapy. The optimal cell type, concentration of cells and route of delivery remain unclear. Moreover the mechanistic basis for any protective effects remains obscure. Prolonged survival of the injected cells in the hostile environment of the ischemic limb has not been documented. Thus, it is unclear whether any benefit related to cell therapy is due to direct involvement of the cells in angiogenesis, or reflects paracrine effects that may include indirect stimulation of angiogenic pathways, modulation of inflammatory cascades, or cytoprotective actions on the ischemic limb.

Administration of marker-specific cells

Considering the functional and phenotypic heterogeneity of MNCs, identification of specific MNC subsets with angiogenic or cytoprotective properties represents a rational approach for further development of cell therapy strategies for PAD patients. Evidence suggests that CD34 may mark a subset of cells with angiogenic potential; a subset of CD34+ cells may be capable of differentiation to mature endothelial cells. In a randomized controlled pilot study, intramuscular injection of Granulocyte-Colony Stimulating Factor (G-CSF)-mobilized CD34+ cells in patients with CLI was safe and was associated with trends towards reduced amputation rates [23]. Strategies using multicellular subsets of bone marrow-derived cells have also been tested. Ixmyelocel-T is an expanded population of bone marrow cells comprised predominantly of CD90+ mesenchymal cells and a subset of bone marrow macrophages. In a randomized phase 2 trial in patients with CLI, treatment with Ixmyelocel-T was safe, but did not significantly affect major amputation rates [24]. Expression of high levels of the cytosolic enzyme aldehyde dehydrogenase (ALDH) has also been used to select a subset of angiogenic progenitors derived from bone marrow cells. In a randomized double-blind phase 2 clinical trial, intramuscular injection of cells with high ALDH activity did not improve peak walking time and perfusion in patients with claudication [25•].

Mesenchymal stem cells (MSC)

Because of their potential for transdifferentiation and their potent effects in modulation of cell survival, inflammation and angiogenesis, MSCs are promising candidates for PAD cell therapy. A randomized controlled trial showed that infusion of autologous bone marrow-derived MSCs (BM-MSC) improved symptoms, accelerated ulcer healing and accentuated collateral blood vessel growth in diabetic patients with CLI [21]. Moreover, administration of allogeneic bone marrow-derived MSCs from healthy donors in patients with end-stage PAD was safe and well-

Table 1

Findings of randomized controlled trials examining the effects of cell therapy in patients with Critical Limb Ischemia (CLI)

Type of cell therapy	Patient population	Follow-up	Main findings	Ref.
Autologous BM-MNCs (im). Autologous PB-MNCs (im).	Patients (84% males, 69% diabetic) with chronic limb ischemia, rest pain, non-healing ulcers (22%), gangrene (40%) without revascularization options. Group A: 25 patients with unilateral ischemia receiving BM-MNCs (vs. saline infusion in the less ischemic limb). Group B: 22 patients with bilateral ischemia, receiving BM-MNCs (in one limb) vs. PB-MNCs (in the other).	24 w–3 y	Cell therapy increased ankle-brachial index (ABI), reduced rest pain, improved walking time, and increased transcutaneous oxygen pressure (TcO_2). Increases in ABI were higher in patients receiving BM-MNCs (vs. PB-MNCs). Follow-up suggested sustained improvement in leg pain and ulcer size for at least 2y.	[19]
Autologous G-CSF-mobilized PB-MNC (im)	28 diabetic patients (cell therapy 14, control 14) Gender: 18M, 10W. Mean age: 71 y	3 mo	Cell therapy reduced leg pain, reduced amputation rate (AR) (treatment: 0%, control: 20%), improved ulcer healing, increased perfusion, improved angiographic scores, and increased ABI.	[20]
Autologous G-CSF-mobilized PB-MNC (im)	40 diabetic patients (cell therapy 20, control 20). Gender: 29M, 11W. Mean age: 71.4 y	12 w	Cell therapy reduced pain, improved Fontaine score, improved ulcer healing, and increased ABI and TcO_2. No significant effects on AR (treatment: 15%, control: 25%).	[38]
Autologous G-CSF mobilized PB-MNCs (im)	21 diabetic patients (control 14, cell therapy 7) Mean age: 64 y	3 mo	Cell therapy improved walking ability, reduced AR (treatment: 0%, control: 50%), improved blood flow and increased ABI. There was a trend towards a reduction in ulcer size.	[39]
Autologous CD34+ cells (im, low and high dose protocols)	28 patients (control 12, low dose 7, high dose 9; 54% diabetic) Gender: 19M, 9F Mean age: 66 y.	12 mo	Cell therapy was safe and well-tolerated. There was a trend towards a reduction in AR (control: 75%, low dose: 43%, high dose: 22%)	[23]
Autologous expanded blood-derived 'angiogenic cell precursors' (VesCell)	20 patients (control 10, cell therapy 10, 60% diabetic) Gender: 13M, 7F Mean age: 61.8 y.	3 mo–2 y	Cell therapy reduced AR (major amputations in the treatment group: 0% at 3 mo and 30% at 2 y and in the control group 60% at 3 mo and 2 y), increased ABI at 3 mo and 2 y, and increased TcO_2.	[40]
Autologous CD133+ cells sorted from G-CSF-mobilized PB-MNCs	10 patients (2:1 randomization) Gender: 8M, 2W. Mean age: control 85 y, treatment 65 y.	12 mo	Subject enrollment was suspended due to a high rate of mobilization failure. Cell therapy was associated with non-significant trends towards lower AR (control: 66%, treatment: 14%), reduced walking impairment and improved quality of life	[41]
Autologous BM-MNCs (im)	25 patients (treatment group n = 13, control n = 12). Age: treatment 62y, control 68 y.	1 mo	Cell therapy improved rest pain, and increased ABI and TcO_2. There were no reports of amputations in control or treatment groups.	[42]
Autologous bone marrow aspirate concentrate (im)	96 patients Cell therapy: n = 42 (36M, 6F), Control: n = 54 (42M, 12F) Age: treatment 66 y, control 64 y. Diabetes: treatment 88.1%, control 98.2%.	120 d	Cell therapy reduced rest pain, decreased AR (treatment 21%, control: 44%), and increased ABI and TcO_2. The bone marrow concentrate of patients who failed therapy exhibited lymphopenia.	[43]
Autologous bone marrow aspirate concentrate (im)	48 patients. Treatment n = 34 (23M, 11F), control n = 14 (9M, 5F). Age: treatment 72.5 y, control 65.7 y. Diabetes: treatment 52.9%, control 42.8%.	6 mo	Cell therapy was well tolerated. There were trends towards reduced AR (treatment: 17.6%, control: 28.6%), reduced pain, increased ABI and better quality of life in the cell therapy group.	[44,45]
Autologous BM-MNCs (im) Autologous BM-MSCs (im)	41 diabetic patients, mean age: 64 y BM-MNC group (n = 21 limbs, males: 42%), BM-MSC group (n = 20 limbs, males: 39%), Normal saline (n = 41 limbs).	24 w	Administration of BM-MNCs and BM-MSCs was well-tolerated. Cell therapy reduced AR (0% in BMMSC and BM-MNC group, vs. 16.2% in control group) and improved rest pain. Ulcer healing was accelerated in the BM-MSC group in comparison with BM-MNC patients.	[21]

Table 1 (Continued)

Type of cell therapy	Patient population	Follow-up	Main findings	Ref.
Autologous BM-MNC (ia)	39 patients (treatment n = 19, control n = 20) Age: treatment 64.4 y, control 64.5 y. Diabetes: treatment 53%, control 48%. Male: treatment 84%, control 62%.	3 mo	Cell therapy did not significantly affect AR (treatment 21%, control 5%) and did not significantly increase ABI. However, cell therapy improved ulcer healing and reduced rest pain.	[46]
Autologous BM-MNCs (im)+ VEGF gene therapy	32 non-diabetic patients. Treatment: n = 16, 11M 5W, mean age 66.8 y. Control: n = 16, 10M, 6W, mean age 68.3 y.	3 mo	No statistically significant difference in AR between groups (treatment: 25%, control: 50%). Only patients enrolled in the treatment group exhibited increased ABI (75% of treated patients) and ulcer healing (69% of treated patients).	[47]
Autologous BM-MNCs (im)	58 patients BM-MNC: (n = 29, 22M, 7F), Placebo: (n = 29, 23M, 6F). Diabetes: treatment 44.8%, control 41.4%. Mean age: treatment 61 y, control 63 y.	6 mo	Cell therapy improved rest pain and ulcer healing and increased ABI. There was no significant difference in the rate of major amputations (treatment 10%, control 17%).	[48]
Autologous expanded BM-MNCs, containing CD90+ cells and a subset of macrophages (Ixmyelocel-T)	72 patients Treatment: n = 48, 34M 14F. Control: n = 24 14M 10F. Diabetes: treatment 44%, control 63%. Age: treatment 69.2 y, control 67.3 y.	12 mo	Cell therapy was well-tolerated, but did not affect AR (control: 25%, treatment 21%).	[24]
Repetitive (3 times in a 3-week interval) autologous BM-MNC (ia)	160 patients. Treatment: n = 81 (57M, 24F), control: n = 79, (51M 28F). Age: treatment 69 y, control 65 y. Diabetes: treatment 36%, control 39%.	6 mo	Cell therapy had no effects on AR (control 13%, treatment 19%), rest pain, ABI and TcO_2.	[22•]
Autologous BM-MNC (im)	38 patients (treatment n = 18, placebo n = 20). Diabetes: treatment 55%, control 35%.	6–12 mo	Cell therapy has no effects on AR (placebo: 26%, treatment: 18%). Cell therapy and control groups had comparable improvements in rest pain and in TcO_2.	[49]
Allogeneic expanded BM-MSC derived from healthy donors (im)	20 patients. MSC group (n = 10), placebo (n = 10) Patients had either atherosclerotic disease or thrombangitis obliterans	6 mo	Cell therapy was safe and increased ABI. There were no significant effects on AR.	[26]
Allogeneic expanded BM-MSC derived from healthy donors (high and low dose groups — im)	90 patients with CLI due to thrombangitis obliterans. Control (n = 18), low dose (n = 36), high dose (n = 36). Age range: 38–42 y.	6 mo	AR was comparable between groups. High dose BM-MSCs reduced rest pain and improved ulcer healing. Both low and high-dose groups exhibited improved quality of life scores.	[50]

CLI, critical limb ischemia; Ref., Reference; G-CSF, granulocyte-colony stimulating factor; AR, amputation rate; ABI, ankle-brachial index; TcO_2, transcutaneous oxygen pressure; PB-MNC, peripheral blood-derived mononuclear cells; BM-MNC, bone marrow-derived mononuclear cells; BM-MSC, bone marrow-derived mesenchymal stem cells; im, intramuscular; ia, intra-arterial; M, men; W, women; mo, months; w, weeks; d, days; y, years.

tolerated, but did not have significant effects on amputation rates [26].

Appraisal of the clinical evidence on the effects of cell therapy in PAD

A recent meta-analysis of randomized, nonrandomized and noncontrolled studies for treatment of PAD suggested that although cell therapy did not affect all-cause mortality, it may have significantly improved the chances of amputation-free survival and ameliorated endpoints related to limb perfusion, pain and functional capacity in comparison with control treatment [27•]. However, efficacy of cell therapy on all endpoints was no longer significant in placebo-controlled studies and disappeared in randomized controlled trials with a low risk of bias [27•]. Thus, there is currently no robust evidence to support the effectiveness of cell therapy in patients with PAD.

PAD patients who are not candidates for revascularization strategies due to high risk, unfavorable vascular involvement, or failed endovascular approaches (no-option patients) represent a major therapeutic challenge and have a poor prognosis. It has been argued that, because there is no alternative to amputation in patients with end-stage CLI, cell therapy should be administered if available, even in the absence of robust evidence to support effects on amputation-free survival [27•,4]. However, implementation of expensive treatment strategies with minimal or no benefit to the affected patient population is of limited value, and may be harmful by reallocating healthcare resources, depriving other patient populations from highly effective therapies.

What are the reasons for the limited success of cell therapy in PAD patients? First, design of cell therapy approaches has

been based predominantly on empiricism, and much less on sound cell biological insights. The lack of experimental animal models that recapitulate the pathophysiology of human PAD greatly complicates design of new strategies and limits our ability to test their effectiveness. Experimental evidence, when available, is often based on studies performed in young healthy animals that may not be relevant to the human PAD populations, typically comprised of older subjects with a high prevalence of diabetes, smoking and chronic atherosclerotic disease. Second, BM-MNCs and PB-MNCs typically used for treatment are highly heterogeneous, and may contain several different subsets with a wide range of effects. Specific subpopulations with protective angiogenic properties need to be defined. Third, the use of autologous cells in patients with comorbid conditions (such as diabetes) that may perturb their reparative and angiogenic properties may greatly limit effectiveness. Fourth, the fate of the cells following injection is unclear. Survival of the cells in the hostile environment of the ischemic limb may be limited; persistence of the cells in ischemic regions has not been consistently documented. Fifth, human PAD is characterized by anatomical heterogeneity, resulting in marked regional differences in the severity of ischemia and tissue damage. Refined administration strategies taking into account the regional distribution of ischemic lesions in PAD patients may be required to improve effectiveness.

There is little doubt that cell therapy in PAD treatment has a bright future. To achieve the full potential of this highly promising strategy, there is a need for a concerted effort to advance our knowledge on the fundamental cellular mechanisms of angiogenesis, while investing in robust clinical studies to test the most promising strategies in PAD patients.

Conclusions and future directions

The need for randomized double-blind placebo controlled studies to document any effects of cell therapy approaches in PAD patients cannot be overemphasized. The improvement observed in placebo-treated patients in PAD clinical trials [22•,28] emphasizes the need for rigorously designed and well-controlled studies in order to derive robust conclusions. Moreover, interpretation of the findings of clinical trials is dependent on introduction of endpoints for assessment of the cell biological consequences of the strategy. Assessment of cell homing and survival, and quantitative analysis of the effects of therapy on the vasculature in the ischemic area can provide critical information to understand the basis for success or failure, and to identify patient subpopulations with favorable responses.

Most importantly, we need to introduce new cell biological concepts in the design of cell therapy approaches. Dissection of the cell biological mechanisms of angiogenesis is critical to design an effective cell therapy approach for PAD patients. Understanding the phenotypic profile, properties, and mobilization mechanisms of endothelial progenitor populations and of mononuclear cell subsets with angiogenic properties is needed to define optimal cell types for therapy. Moreover, treatment with mediators that improve mobilization, homing and survival of endogenous progenitor cells may be useful to maximize benefit of cell therapy.

Chemokines are a family of chemotactic cytokines with an important role in leukocyte trafficking following ischemia [29]. Several members of the chemokine family play essential roles in mobilization and migration of endogenous EPCs and may regulate their recruitment in ischemic sites [30,31]. Accentuation of chemokine signaling may be a promising strategy to enhance infiltration of the ischemic tissue with angiogenic cells. The CXC chemokine Stromal cell-derived factor (SDF)-1/CXCL12 is a key regulator of bone marrow cell mobilization and is critically involved in recruitment of progenitor cells in ischemic tissues [32,33]. It has been suggested that in certain pathologic conditions, such as diabetes, hyperglycemia-mediated downmodulation of chemokine receptor expression in EPCs and other progenitor cells may reduce their homing in sites of injury, resulting in defective angiogenesis and impaired reparative responses. A recent study demonstrated that manipulation of EPCs to increase expression of the chemokine receptor CXCR7 (one of the receptors that mediate CXCL12 actions) improved outcome in a model of limb ischemia in diabetic mice, enhancing the angiogenic function of the cells [34•]. Other members of the chemokine family may act indirectly, increasing the angiogenic capacity of MNCs. The CX3C chemokine Fractalkine/CX3CL1 has been suggested to increase angiogenic potential of bone marrow-derived macrophages by accentuating expression of platelet factor-4/CXCL4 [35]. Pre-treatment of MNCs with mediators inducing an angiogenic program may stimulate their therapeutic potential in PAD. Moreover, genetic manipulations activating a pro-survival program in EPCs [36] or incorporation of matrix substrates that prolong survival and promote differentiation [37] may accentuate the beneficial actions of cell therapy.

Conflict of interest statement

Nothing declared.

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Current Opinion in Physiology

Cell biological mechanisms in regulation of the post-infarction inflammatory response

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Inflammation plays a crucial role in cardiac repair, but may also extend ischemic injury and contribute to post-infarction remodeling. This review manuscript discusses recent advances in our understanding of the cell biology of the post-infarction inflammatory response. Recently published studies demonstrated that the functional repertoire of inflammatory and reparative cells may extend beyond the roles suggested by traditional teachings. Neutrophils may play an important role in cardiac repair by driving macrophages toward a reparative phenotype. Subsets of activated fibroblasts have been implicated in protection of ischemic cardiomyocytes, in phagocytosis of apoptotic cells, and in regulation of inflammation. Dissection of the cellular effectors of cardiac repair is critical in order to develop new therapeutic strategies for patients with acute myocardial infarction.

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Introduction

Implementation of early reperfusion strategies significantly reduced acute mortality in patients with myocardial infarction. However, this important therapeutic success resulted in expansion of the pool of patients who while surviving the acute event, exhibit adverse cardiac remodeling, and are susceptible to development of chronic heart failure. Following myocardial infarction, sudden death of up to a billion cardiomyocytes triggers an inflammatory response that plays a crucial role in repair of the infarcted heart, but is also implicated in the pathogenesis of adverse ventricular remodeling [1]. Excessive, prolonged, or dysregulated inflammatory responses following myocardial infarction are associated with accentuated dilation, infarct expansion and increased fibrosis and may worsen ventricular dysfunction [2]. Despite a growing understanding of the molecular signals regulating the post-infarction inflammatory response, therapeutic targeting of inflammatory mediators in patients with myocardial infarction has proved challenging [3]. This manuscript will discuss recent advances that contributed to our understanding of the role of inflammatory pathways in cardiac injury, repair and remodeling following myocardial infarction.

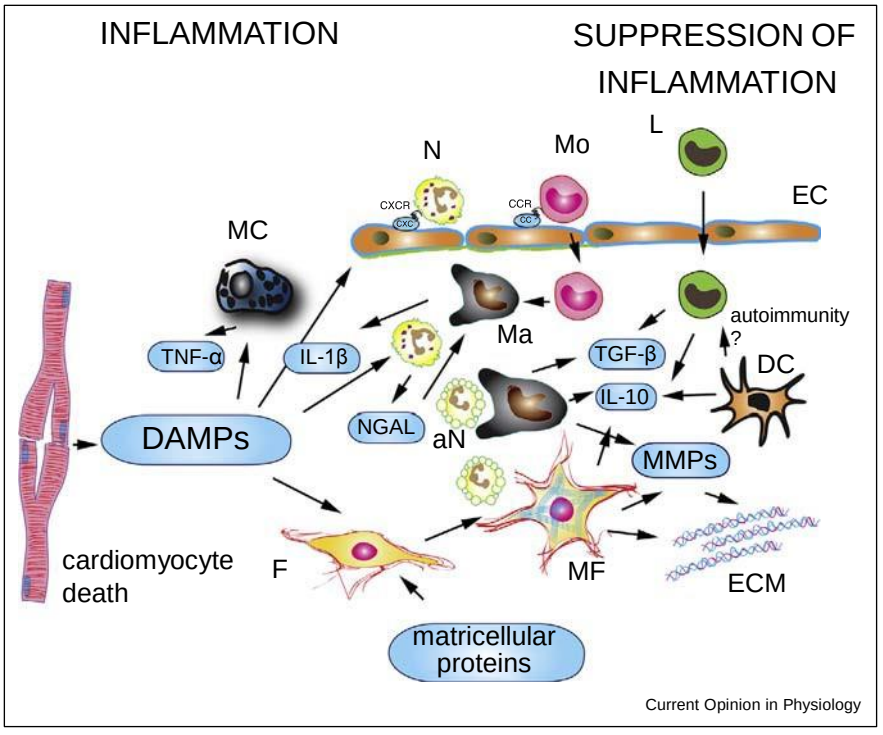
Activation of the post-infarction inflammatory response

Release of damage-associated molecular patterns (DAMPs) by dying cells represents the key molecular link between cardiomyocyte death and activation of the post-infarction inflammatory response. Experimental studies have suggested that a wide range of danger signals, such as high mobility group box-1 (HMGB1) [4,5], Interleukin (IL)-1a [6], and extracellular RNAs [7] are released by dying cells and activate innate immune pathways. Extracellular matrix protein fragments are rapidly generated in the infarcted heart and may also activate inflammatory cascades, linking injury of the interstitial matrix with the inflammatory response [8]. Recently published work has added several mediators to the list of alarmins, capable of activating inflammatory signaling following myocardial infarction. Mitochondrial DNA is released following cardiac injury and may activate innate immune pathways extending cardiomyocyte injury [9]. Fragments of sarcomeric proteins are generated following infarction and may also activate inflammatory cascades [10]. DAMPs have a wide range of likely cellular targets, including resident cardiac macrophages and mast cells, vascular endothelial cells, fibroblasts, and infiltrating leukocytes (Figure 1). The cellular specificity of the alarmins released in the infarcted myocardium has not been investigated.

Endothelial cells

Endothelial cells are the most abundant non-cardiomyocytes in the adult mouse myocardium mouse myocardium [11] and represent a major source of pro-inflammatory chemokines [12,13] following myocardial infarction. Moreover, induction of adhesion molecules on the endothelial surface plays an important role in recruitment of inflammatory leukocytes in the healing infarct. The signals responsible for inflammatory activation of endothelial cells in the infarcted myocardium remain poorly understood. Recently published studies have suggested that in the infarcted myocardium, natriuretic peptides released by

Figure 1



The cell biology of the inflammatory response following myocardial infarction. Dying cardiomyocytes release damage-associated molecular patterns (DAMPs), activating inflammatory signaling in resident macrophages (Ma), mast cells (MC) and fibroblasts (F) and in infiltrating leukocytes. Secretion of pro-inflammatory cytokines (such as IL-1b and TNF-a) stimulates expression of CXC and CC chemokines in endothelial cells (EC), promoting recruitment of neutrophils (N), monocytes (Mo) and lymphocytes (L). In addition to their pro-inflammatory actions, neutrophils may also trigger polarization of reparative macrophages. Phagocytotic macrophages clear the infarct from dead cells (including apoptotic neutrophils, aN) and acquire anti-inflammatory properties, releasing TGF-b and IL-10. Recently published experimental work suggests that myofibroblasts (MF) may also play a role in phagocytosis of dead cells and in negative regulation of the inflammatory reaction. Dendritic cells (DC) are also activated in the infarcted myocardium and have been suggested to inhibit pro-inflammatory signaling by secreting IL-10. Activation of autoimmune pathways by dendritic cells has been suggested, but remains poorly documented in myocardial infarction. Matricellular proteins play an important role in regulating inflammatory and reparative responses in the infarcted myocardium. Matrix metalloproteinases (MMPs) secreted by hematopoietic cells and fibroblasts are involved in extracellular matrix metabolism and regulate inflammation by modulating activity of inflammatory mediators.

cardiomyocytes, may activate an inflammatory phenotype in endothelial cells, promoting leukocyte recruitment [14]. Moreover, activation of forkhead box protein O4 (FoxO4), a transcription factor involved in regulation of many cellular pathways has also been implicated in inflammatory activation of endothelial cells following myocardial infarction [15•]. The upstream signals responsible for FoxO4 activation in the endothelium have not been identified.

Neutrophils

Myeloid cells are rapidly recruited in the infarcted myocardium and have been implicated in clearance of dead cells, repair, fibrosis, and remodeling of the infarcted heart [16]. Abundant neutrophils infiltrate the infarcted myocardium in response to induction of chemotactic signals, including chemokines, complement and leukotrienes. Although early investigations demonstrated that neutrophils may exert cytotoxic effects on viable cardiomyocytes in the infarct border zone extending ischemic injury [17], current concepts suggest that neutrophils may also have reparative functions, by regulating recruitment and activation of mononuclear cells. Secretory products of neutrophils and platelets have been suggested to act synergistically in driving monocyte recruitment in the infarcted heart [18]. A recent study showed that neutrophil depletion using infusion of an Ly6G-specific monoclonal antibody did not affect infarct size but caused progressive deterioration in cardiac function following myocardial infarction. The beneficial effects of neutrophils were presumed due to macrophage polarization toward a reparative phenotype and were mediated, at least in part, through secretion of neutrophil gelatinase-associated lipocalin (NGAL), a key regulator of macrophage function [19•]. It should be emphasized that neutrophil depletion studies in experimental models of myocardial infarction have produced conflicting results [20,21,19•], likely reflecting differences in the effectiveness and specificity of various approaches and the wide range of functions of neutrophils in injured tissues. In addition to transducing reparative signals, neutrophils

also secrete injurious mediators, such as myeloperoxidase, implicated in the pathogenesis of adverse remodeling and arrhythmogenesis following myocardial infarction [22]. Moreover, neutrophils infiltrating the infarcted heart may exhibit phenotypic heterogeneity. A recent study identified abundant early recruitment of pro-inflammatory N1 neutrophils in the infarcted heart, followed by late infiltration with N2 cells [23]. Activation of Toll-like receptor (TLR)4 signaling was implicated in N1 polarization, suggesting that DAMPs released in the infarct may stimulate a pro-inflammatory phenotype in neutrophils.

Macrophages in cardiac repair, remodeling and fibrosis

The adult mammalian heart contains a relatively small population of resident macrophages [24,25]; the role of these cells in cardiac homeostasis remains unclear. Recent studies in the mouse suggested that cardiac macrophages may play an important role in facilitating cardiac conduction [26•]; the significance of these observations in function of the human conduction system remains unknown. Following myocardial infarction, chemokine-driven recruitment of monocytes [27] results in marked expansion of the macrophage population in the infarcted region. Infarct macrophages exhibit phenotypic and functional heterogeneity and orchestrate the inflammatory and reparative response. Efferocytosis of apoptotic cardiomyocytes by macrophages suppresses expression of pro-inflammatory mediators and may drive resolution of inflammation following myocardial infarction [28]. Activation of negative regulators of the innate immune response, such as Interleukin receptor-associated kinase (IRAK)-M in macrophage subsets, suppresses inflammation and attenuates protease activation in the infarcted heart [29]. A subpopulation of alternatively activated M2-like macrophages activates a reparative program in fibroblasts, promoting repair of the infarcted heart [30]. Macrophages may also serve as an important source of Vascular endothelial growth factor (VEGF)-A in the infarcted heart, stimulating repair through activation of infarct angiogenesis [31•].

Which signals activate myeloid cells in the infarcted myocardium?

Myeloid cells respond to the dynamic changes in their microenvironment and acquire a wide range of functional phenotypes. Growth factors and cytokines, neurohumoral mediators, and components of the extracellular matrix network play an important role in modulating myeloid cell function. During the early pro-inflammatory phase of infarct healing, IL-1 promotes a pro-inflammatory macrophage phenotype inducing chemokine synthesis and protease activation [32]. Members of the bone morphogenetic protein family (BMP) may also stimulate pro-inflammatory signaling [33], whereas release of TGF-b by macrophages ingesting apoptotic cells may suppress inflammatory gene synthesis, marking the transition to the reparative phase. Maintenance and proliferation of macrophage populations in the infarcted myocardium and acquisition of an M2-like phenotype require activation of CSF-1 signaling cascades [34•,35]. Neurohumoral pathways and mechanosensitive signaling have been suggested as important modulators of macrophage activation [36,37]. Adrenergic signaling cascades may critically modulate leukocyte function following myocardial infarction. b2 adrenergic receptor signaling in leukocytes was found to be important for recruitment of leukocytes into the infarcted myocardium, and for repair following infarction [38,39•]. On the other hand, the b1 adrenergic receptor cascade has been suggested to exert detrimental effects, by promoting neutrophil-mediated injury following myocardial infarction [40]. Considering the wide range of cell types targeted by neurohumoral mediators, the relative significance of leukocyte-specific effects of catecholamines or angiotensin II in regulation of post-infarction injury and repair remains unknown.

Lymphocytes

Lymphocyte subpopulations are rapidly recruited in the infarcted myocardium and may downmodulate post-infarction inflammation by secreting inhibitory cytokines, such as IL-10 [41,42]. A growing body of evidence implicates regulatory T cells (Tregs) in suppression of post-infarction inflammation [43]. Although relatively small numbers of regulatory T cells (Tregs) infiltrate the infarcted myocardium, these cells seem to have important effects on macrophage and fibroblast phenotype [44,45]. A recent study suggested that epicardial activation of YAP and TAZ, two core Hippo pathway effectors, may suppress post-infarction inflammation and fibrosis by inducing Interferon (IFN)-g and by promoting recruitment of Tregs [46]. The anti-fibrotic effects of IFN-g may involve upregulation of the anti-fibrotic chemokine CXCL10/IFN-g-inducible protein (IP)-10 [47].

Mast cells and dendritic cells

Early studies in a canine model of reperfused myocardial infarction demonstrated that in ischemic myocardial segments, cardiac mast cells degranulate, releasing histamine, mast cell-specific proteases and a wide range of cytokines and growth factors [48–50]. In comparison to large animals, mice have much lower numbers of cardiac mast cells and exhibit modest mast cell infiltration following myocardial infarction [51,52]. A recent study in a mouse model of myocardial infarction demonstrated that cardiac mast cells may be protective, enhancing cardiomyocyte contractility following myocardial infarction. Mast cell-mediated preservation of cardiomyocyte function was attributed to tryptase secretion and subsequent activation of protease-activated receptor (PAR)-2, leading to reduction in protein kinase A (PKA) activity and modulation of myofilament protein phosphorylation [53•]. Considering the wide range of mediators secreted by mast cells, and their diverse actions on many cell types involved in cardiac remodeling, the vivo role of this specific pathway remains unclear.

Dendritic cells are also activated following myocardial infarction; however, their role in regulation of post-infarction inflammation remains unclear. Depletion experiments in a mouse model of myocardial infarction suggested that dendritic cells may downmodulate post-infarction inflammation by secreting anti-inflammatory mediators, such as IL-10 [54]. In contrast, a recent study suggested that dendritic cells undergo activation following infarction and may prime cardiac-specific autoreactive CD4+ T cells [55]. However, the potential involvement of autoimmunity in extending or exacerbating injury following infarction has not been documented.

The cardiac fibroblasts as regulators of inflammation

The adult mammalian heart contains a significant population of resident fibroblast-like cells [11]. Following infarction, fibroblasts undergo myofibroblast conversion, express contractile proteins such as a-smooth muscle actin (a-SMA) and secrete large amounts of extracellular matrix proteins, serving as the main matrix-secreting cells in the healing scar [56–58]. Recent evidence suggests that infarct fibroblasts may have a diverse range of functions beyond matrix synthesis. During the early inflammatory phase of cardiac repair, fibroblasts may secrete inflammatory mediators and proteases, or modulate cardiomyocyte survival in the ischemic heart [59,60]. It has also been suggested that myofibroblasts may exhibit phagocytotic properties and, much like macrophages, may negatively regulate the inflammatory response as they engulf dead cells [61•]. Experiments in a mouse model of myocardial infarction suggested that expression of milk fat globule epidermal growth factor 8 (MFG-E8) may mediate engulfment of apoptotic cells, leading to acquisition of an anti-inflammatory myofibroblast phenotype. Quantitation of TUNEL+ signals in a-SMA+ myofibroblasts and in CD68+ macrophages suggested that the number of apoptotic cells engulfed by myofibroblasts in the infarcted heart was about 40% of that engulfed by macrophages. Fibroblast-specific loss-of-function approaches are needed to document the impact of the contribution of fibroblast-mediated phagocytosis in healing infarcts.

Targeting inflammation in myocardial infarction

Despite extensive experimental evidence suggesting that targeting the inflammatory cascade may be effective in attenuating injury following infarction and in preventing adverse remodeling and heart failure, clinical translation has proved challenging [62–64]. Clearly, broad non-selective inhibition of post-infarction inflammation can be detrimental by perturbing the reparative response. The risks of broad immunomodulatory approaches are illustrated by recent observations showing that methotrexate administration in patients with ST elevation myocardial infarction (STEMI) worsened cardiac function [65]. However, several selective approaches targeting specific well-documented inflammatory signals appear to hold promise for clinical translation.

Considering the robust experimental evidence suggesting a crucial role for IL-1 signaling in dilative remodeling and dysfunction following myocardial infarction [66], targeting IL-1 represents a promising therapeutic approach for patients with myocardial infarction [67]. The promising effects of anakinra administration in early pilot studies in patients with myocardial infarction [68], and the effectiveness of IL-1b inhibition in attenuating inflammation and in reducing cardiovascular events in high-risk patients with atherosclerotic disease in the recently reported Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS) [69,70] offer a rare air of optimism in a field marred by disappointments. Recent evidence suggesting that IL-1-driven inflammation may be implicated in arrhythmogenesis [71,72] may further strengthen the rationale for IL-1 inhibition in selected subpopulations of patients with myocardial infarction.

In addition to IL-1, several additional inflammatory targets have been suggested in patients with myocardial infarction. In human patients undergoing primary percutaneous coronary intervention (PCI) for acute myocardial infarction, intracoronary nitrite treatment decreased inflammatory activation of leukocytes; these effects were associated with attenuated microvascular obstruction and with a reduction in infarct size [73]. IL-6 inhibition through administration of the IL-6 receptor antagonist tocilizumab attenuated the systemic inflammatory response and reduced cardiomyocyte injury in patients with non-STEMI undergoing PCI [74]. In human patients with non-STEMI undergoing PCI, administration of the P-selectin antagonist inclacumab reduced myocardial injury [75]. Finally, administration of high dose v-3 fatty acids in patients with acute myocardial infarction reduced adverse ventricular remodeling; the association of benefit with decreased levels of circulating inflammatory biomarkers may suggest that the protective effects may reflect attenuation of inflammation [76].

Conclusions

After decades of research in the field of myocardial inflammation, we may be closer than ever to therapeutic translation. Strategies targeting inflammatory cascades may exert beneficial actions in patients with myocardial infarction. Emerging evidence suggests that modulation of inflammation may mediate any protective effects of cell therapy with mesenchymal stem cells or with cardiosphere-derived cells [77,78]. Moreover, inflammatory cells may hold the key to the visionary goal of cardiac regeneration [79]. A lot remains to be done in order to advance the clinical implementation of strategies targeting inflammatory signals. First, understanding the cell biological mechanisms of myocardial inflammation, repair and fibrosis is crucial in order to design sound therapeutic

strategies [3,80]. Second, identification of patient subpopulations with specific perturbations in inflammatory response is needed. Considering the pathophysiologic heterogeneity of myocardial infarction in the clinical context, identification of patients with excessive, prolonged or dysregulated inflammatory responses is critical in order to define patient subsets likely to benefit from targeted anti-inflammatory interventions.

Conflicts of interest

None.

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Editorial

Galectin-3 in the fibrotic response: Cellular targets and molecular mechanisms

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Galectin-3 is a β-galactoside-binding animal lectin that functions in nuclear, cytoplasmic and extracellular compartments, and has been implicated in regulation of inflammatory, angiogenic, and fibrogenic responses [1]. Galectin-3 expression is markedly increased in injured and remodeling tissues, predominantly expressed by activated macrophages, but also localized in other cell types (such as fibroblasts and vascular cells), and in the extracellular matrix. Studies in animal models and human patients demonstrated that galectin-3 is consistently upregulated in failing and remodeling hearts, reflecting, at least in part, the expansion and activation of cardiac macrophages [2,3]. A potential role for galectin-3 as a prognostic biomarker in patients with chronic heart failure is suggested by an association between serum galectin-3 levels and mortality [4]. Whether endogenous galectin-3 plays a causative role in myocardial remodeling and fibrosis remains debated. Some studies have suggested a crucial role for galectin-3 in fibrosis and dysfunction of the pressure-overloaded heart [5], whereas other investigations using similar mouse models suggested that galectin-3 induction in failing hearts may be redundant [3]. Although the in vivo role of endogenous galectin-3 in various pathophysiologic conditions may be dictated by contextual factors, there is little doubt that at the cellular level, galectin-3 stimulation regulates macrophage phenotype and activates fibroblasts, promoting a fibrogenic program. However, the mechanisms responsible for these cellular effects remain poorly understood.

In the current issue of the journal, Ibarrola and co-workers [6] explore the molecular basis for the fibrogenic actions of galectin-3 in human cardiac fibroblasts. Using isobaric tags for relative and absolute quantitation (i-TRAQ) proteomic analysis, the authors identified fumarate hydratase (FH), an enzyme involved in conversion of fumarate to L-malate, as a novel molecular target of galectin-3. Stimulation of human cardiac fibroblasts with galectin-3 downregulated FH expression, increasing fumarate levels, and accentuating oxidative stress. On the other hand, galectin-3 knockdown increased FH expression by cardiac fibroblasts, reducing fumarate production. In a rat model of cardiac pressure overload, galectin-3 inhibition attenuated oxidative and nitrosative stress. Moreover, in patients with critical aortic stenosis, myocardial FH expression was reduced and was inversely correlated with galectin-3 expression.

The study demonstrates that downmodulation of FH may be an important mechanism of galectin-3-driven fibroblast activation, leading to accentuated oxidative stress, and subsequent activation of a pro-inflammatory and fibrogenic program. The significance of this pathway may extend beyond the pathogenesis of cardiac fibrosis, as the proposed link between galectin-3 and oxidative stress may explain pro-inflammatory actions of the molecule and effects in other cell types, such as endothelial and immune cells. However, considering the wide range of molecular targets of galectin-3, what is the relative significance of FH modulation in regulating tissue remodeling and fibrosis in vivo? The current study does not address this question. Experiments assessing FH expression levels in galectin-3 null animals subjected to injury, and studies examining whether fibroblast-specific FH disruption abrogates any anti-fibrotic effects of galectin-3 loss are needed to document the in vivo role of this pathway.

1. The cellular and molecular targets of galectin-3 in inflammation and fibrosis

A large body of cell biological evidence suggests that galectin-3 may promote fibrosis predominantly through effects on macrophages and fibroblasts (Fig. 1). Supplemental Table 1 summarizes published evidence on the molecular signals that may mediate galectin-3-driven tissue fibrosis. Galectin-3 stimulation has been suggested to promote alternative macrophage activation by binding to CD98 [7]. Secretion of fibrogenic cytokines by alternatively-activated macrophages may stimulate myofibroblast conversion, triggering extracellular matrix protein synthesis. Several in vitro studies have suggested direct effects of galectin-3 on fibroblasts; the in vivo significance of these direct actions in the pathogenesis of fibrosis has not been documented. Galectin-3 potently stimulates fibroblast proliferation through interactions with cell surface glycoconjugates, and downstream activation of mitogenic signaling cascades. Moreover, galectin-3 has been suggested to mediate transforming growth factor (TGF)-β-driven myofibroblast activation, possibly by regulating TGF-β receptor function [8]. Such effects may be mediated through binding of secreted galectin-3 to surface

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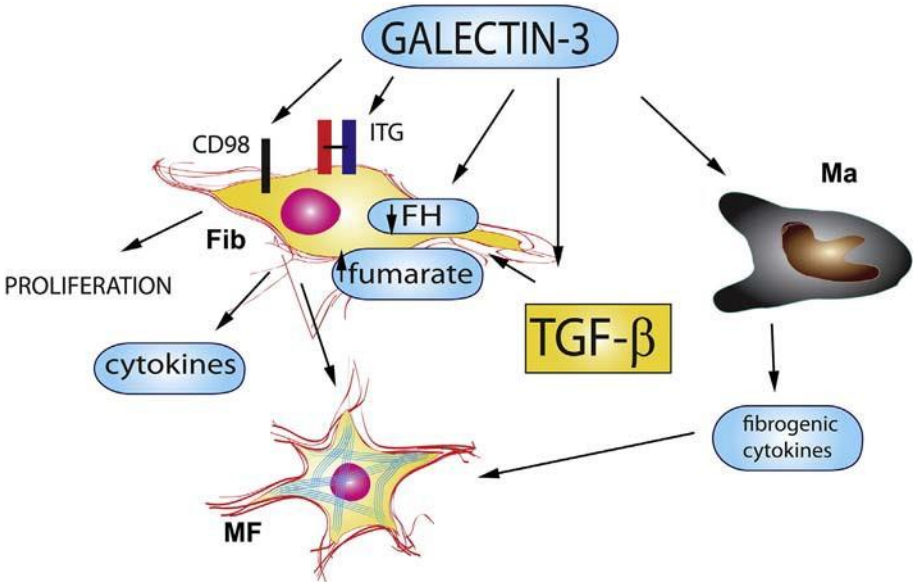

Fig. 1. Schematic cartoon illustrating the fibrogenic actions of galectin-3. Galectin-3 may induce alternative activation of macrophages (Ma), promoting secretion of fibrogenic cytokines. Moreover, secreted galectin-3 may have direct effects on fibroblasts (Fib), by potentiating TGF-β actions, by activating CD98, and by transducing integrin-mediating cascades. In vitro, galectin-3 has been suggested to bind to surface glycoproteins, promoting fibroblast proliferation, and enhancing TGF-β-mediated myofibroblast (MF) conversion. Ibarrola et al. [6] propose that galectin-3 may activate fibroblasts, by downmodulating fumarate hydratase (FH), thus enhancing fumarate levels and accentuating oxidative stress.

glycoproteins and downstream activation of kinase cascades, or through transduction of integrin-dependent signaling. It has also been suggested that galectin-3 may act as a matricellular protein, binding to components of the extracellular matrix, and to cell surface receptors, and modulating signaling responses.

2. Is FH a potential therapeutic target?

The current study suggests that restoring FH levels in order to reduce fumarate accumulation in failing hearts may hold promise as a therapeutic strategy in heart failure [6]. Clearly, direct evidence supporting this notion is lacking, as the role of FH in regulation of fibroblast activity in remodeling hearts has not been investigated. Moreover, approaches attempting to reduce fumarate levels in the injured and remodeling myocardium may have detrimental effects by abrogating key cytoprotective pathways in cardiomyocytes. In vivo studies demonstrated that cardiac-specific FH loss protects from ischemic injury by stabilizing the transcriptional activator nuclear factor erythroid 2 (NFE2)-related factor (Nrf)2 [9]. The cell-specific effects of stress-induced signaling responses in the remodeling myocardium [10] greatly complicate therapeutic translation in patients with heart failure.

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Disclosures

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2018.01.128.

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Pharmacologic inhibition of the enzymatic effects of tissue transglutaminase reduces cardiac fibrosis and attenuates cardiomyocyte hypertrophy following pressure overload

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Pharmacologic inhibition of the enzymatic effects of tissue transglutaminase reduces cardiac fibrosis and attenuates cardiomyocyte hypertrophy following pressure overload

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ABSTRACT:

Tissue transglutaminase (tTG) is a multifunctional protein with a wide range of enzymatic and non-enzymatic functions. We have recently demonstrated that tTG expression is upregulated in the pressure-overloaded myocardium and exerts fibrogenic actions promoting diastolic dysfunction, while preventing chamber dilation. Our current investigation dissects the *in vivo* and *in vitro* roles of the enzymatic effects of tTG on fibrotic remodeling in pressure-overloaded myocardium. Using a mouse model of transverse aortic constriction, we demonstrated perivascular and interstitial tTG activation in the remodeling pressure-overloaded heart. tTG inhibition through administration of the selective small molecule tTG inhibitor ERW1041E attenuated left ventricular diastolic dysfunction and reduced cardiomyocyte hypertrophy and interstitial fibrosis in the pressure-overloaded heart, without affecting chamber dimensions and ejection fraction. *In vivo*, tTG inhibition markedly reduced myocardial collagen mRNA and protein levels and attenuated transcription of fibrosis-associated genes. In contrast, addition of exogenous recombinant tTG to fibroblast-populated collagen pads had no significant effects on collagen transcription, and instead increased synthesis of matrix metalloproteinase (MMP)3 and tissue inhibitor of metalloproteinases (TIMP)1 through transamidase-independent actions. However, enzymatic effects of matrix-bound tTG increased the thickness of pericellular collagen in fibroblast-populated pads. tTG exerts distinct enzymatic and non-enzymatic functions in the remodeling pressure-overloaded heart. The enzymatic effects of tTG are fibrogenic and promote diastolic dysfunction, but do not directly modulate the pro-fibrotic transcriptional program of fibroblasts. Targeting transamidase-dependent actions of tTG may be a promising therapeutic strategy in patients with heart failure and fibrosis-associated diastolic dysfunction.

Keywords: tissue transglutaminase, cardiac fibrosis, extracellular matrix, fibroblast

1. INTRODUCTION:

Tissue transglutaminase (tTG, transglutaminase 2), the most ubiquitous and best-studied member of the transglutaminase family, is a multifunctional protein with a wide range of enzymatic and non-enzymatic functions [1],[2]. As a transglutaminase, tTG is capable of mediating Ca^{2+}-dependent isopeptide bond formation, in which free amines (from protein-bound lysine residues, or small molecules such as serotonin) are covalently linked to glutamine residues of an acceptor protein [3]. Transamidase-dependent actions of tTG have been implicated in the function, stability and immunogenicity of its substrate proteins, such as collagen and fibronectin [4]. In addition to its enzymatic functions, tTG has been suggested to exert a wide range of non-enzymatic effects. Following tissue injury, tTG is secreted to the extracellular matrix (ECM), where it binds with high affinity to fibronectin and heparin sulfate proteoglycans (HSPGs) [5]. Matrix-bound tTG may serve as a bridging protein that activates syndecan-4 signaling – in a transamidase-independent manner [6], or may function as a co-receptor for integrin-mediated cell adhesion [7],[8],[9]. The relative contributions of the enzymatic and non-enzymatic functions of tTG to cellular responses *in vivo* remain unknown.

Cardiac pressure overload activates a hypertrophic and fibrogenic program in the myocardium leading to the development of heart failure [10],[11]. Using a mouse model of transverse aortic constriction (TAC), we have recently demonstrated that tTG is the only member of the transglutaminase family that is upregulated in the pressure-overloaded myocardium [12]. Using mice with global loss of tTG, we found that tTG promotes diastolic dysfunction, while protecting the pressure-overloaded heart from dilation and systolic dysfunction [12]. In addition to its effects on collagen crosslinking, tTG also restrained fibroblast proliferation and attenuated

activation of matrix metalloproteinase (MMP)2 [12]. Whether the in vivo functions of tTG in the remodeling myocardium are mediated through enzymatic effects, or through transamidase-independent actions remains unknown. In this study, we dissected the role of enzymatic tTG actions in the remodeling myocardium, by treating mice undergoing pressure-overload protocols with ERW1041E, a specific, irreversible pharmacological inhibitor of enzymatically-active tTG[13]. Our findings provide the first direct evidence of tTG activation in the pressure-overloaded myocardium and demonstrate that enzymatic actions of tTG are involved in activation of a fibrogenic program and in the pathogenesis of diastolic dysfunction in the pressure-overloaded heart. Moreover, our *in vitro* experiments demonstrate that stimulation with matrix-bound, recombinant tTG does not affect collagen transcription in cardiac fibroblasts, but induces MMP3 and TIMP1 synthesis through non-enzymatic actions. The fibrogenic enzymatic *in vivo* actions of tTG may reflect effects on collagen processing.

2. METHODS:

2.1. Animal Protocols: Animal experiments were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee. Male and female, 3-4 month-old wildtype (WT) mice on a C57BL/6J background (purchased from Jackson labs) underwent TAC protocols to induce pressure overload, as previously described [14],[10],[15]. Mice were anesthetized with inhaled isoflurane. Aortic banding was achieved by creating a constriction between the right innominate and left carotid arteries. The degree of pressure overload was assessed by measuring right-to-left carotid artery flow velocity ratio after constricting the transverse aorta. Mice with a very low (<4) or a very high (>15) carotid flow

ratio were excluded from further analysis. In order to investigate the role of enzymatic tTG-mediated actions, age- and gender-matched male and female wild type C57BL/6J mice treated with either a small molecule tTG inhibitor or vehicle control underwent TAC protocols (n= 25-26 per group) for 28 days. As a control, a sham operation without aortic constriction was performed on gender and age-matched mice treated with either a small molecule tTG inhibitor (n=12) or vehicle control (n=12). ERW1041E ((S)-Quinolin-3-ylmethyl 2-((((S)-3-Bromo-4, 5-dihydroisoxazol-5-yl)methyl)carbamoyl)pyrrolidine-1-carboxylate) is a specific, irreversible small molecule inhibitor of tTG that pharmacologically blocks activated tTG *in vivo*. This compound was synthesized as described previously [16]. The efficacy and duration of blockade of tTG activity have been already determined and published [16]. ERW1041E solution was formulated as follows: 10 mg/mL ERW1041E was reconstituted in 90% ultrapure water, 10% DMSO (biotechnology, endotoxin free grade), 2% Tween-80 detergent, and 2.5% (m/v) 2-hydroxypropyl beta-cyclodextrin and then it was filter sterilized. This solution was freshly prepared before every injection to ensure there were no solubility issues or degradation. Control animals were injected with the vehicle without ERW1041E. Both the inhibitor and vehicle-treated group animals from TAC and sham groups underwent echocardiography at baseline and after 28 days of TAC or sham surgery. Based on the estimated $t_{1/2}$ of tTG inhibition by ERW1041E (12±1 hr.) [16], a twice daily dose of 25 mg/kg was administered to the mice intraperitoneally for 28 days following TAC, with the first IP injection administered on the day of TAC. At the end of the experiment, mice were euthanized, the hearts were harvested and the basal 2/3 were fixed in Z-fix (Anatech Ltd, Fisher Scientific) and processed for paraffin histology. The apical 1/3 was snap frozen and used for protein extraction.

2.2. Echocardiography and strain rate imaging: Short axis M-mode echocardiography was performed prior to instrumentation and before the end of each experiment, after 28 days of TAC or sham surgery, using the Vevo 2100 system (VisualSonics, Toronto ON), as previously described [17]. The following parameters were assessed as indicators of function and remodeling: left ventricular end-diastolic volume (LVEDV), ejection fraction, and left ventricular mass (LV mass). Speckle tracking echocardiography was performed at baseline and after 7-28 days of pressure overload in order to study the effects of the tTG inhibitor on radial strain rates using parasternal long axis views. Analysis was performed using the VevoStrain package (VisualSonics).

2.3. *In vivo* transglutaminase assay and quantitation of transglutaminase activity: 5-biotinamidopentylamine (5BP) is a synthetic substrate of tTG that can be used to probe tTG activity *in vivo* [18]. 5-BP·HCl was synthesized as previously described [16] and was used to localize the *in vivo* transglutaminase activity of tTG in the pressure-overloaded heart and to assess the effectiveness of the tTG inhibitor. Age- and gender-matched male and female wild type C57BL/6J mice, treated with either a tTG inhibitor ERW1041E or vehicle control, underwent 14 days of TAC (n=6 per group, 3 males and 3 females). Solutions were formulated just prior to the experiment and used the same day for each mouse experiment. 5BP was dissolved in PBS (20 mg/mL) and was then filtered through a disposable 0.2 µm sterile polyvinylidenefluoride (PVDF) membrane filter prior to injection. Ninety minutes prior to sacrifice, mice were injected with 5BP (100 mg/kg i.p.). To ensure that 5BP incorporation depended on the tTG activity, ERW1041E (25 mg/kg i.p) or vehicle control was contemporaneously administered. The 5BP injection was repeated at 60 and 30 mins before the mice were sacrificed. Subsequently, the heart was dissected into two segments. The basal two-

third portion of the heart was directly embedded in optimal cutting temperature (OCT) (Tissue-Plus O.C.T compound, Fisher Scientific), frozen in -80°C, and used for assessment of enzymatic activity. The apical one-third segment was used for RNA extraction.

Frozen mouse tissue cryomolds were transferred to a cryostat equilibrated to −20 °C. Tissue blocks were removed from the cryomolds, cut into 10 µm sections, and transferred to Superfrost Plus slides (ThermoFisher Scientific). Tissue sections were stored at −80 °C and used for assessment of enzymatic activity using a peroxidase-based method. Sections were fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature and thoroughly washed with PBS. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 15 mins followed by thorough washing with PBS. The 5BP signal was amplified using the Vectastain Elite ABC Peroxidase Kit (PK-6100, Vector Labs). DAB (3, 3'-diaminobenzidine) was used to detect peroxidase (SK-4100). The slides were then rinsed in distilled water and counterstained either with Hematoxylin or Eosin. Coverslips were then mounted using Cytoseal XYL (Richard Allen Scientific) mounting medium. Images were then obtained at 200X on Zeiss Axio Imager M2.

For quantitation of transglutaminase activity, stained slides from both the ERW1041E-treated and vehicle-treated control groups were analyzed by light microscopy. 8 fields (200X) from each mouse (n=5/group) were scored individually for intensity and extent of staining. For intensity of staining, the following scale was used: no staining: 0, low intensity of staining: 1, moderate intensity of staining: 2, strong intensity of staining: 3. For extent of staining: no staining: 0, staining in <25% of the section:1, staining in 25-75% of the section: 2, staining in >75% of the section: 3. Then the average of the intensity and extent of staining was used as a measure of tTG activity in each field.

2.4. Assessment of cardiomyocyte size: Cardiomyocytes were outlined using wheat germ agglutinin (WGA) histochemistry, as previously described, using an Alexa Fluor® 594 Conjugate (Life Technologies) (dilution 1:100) [19]. Cardiomyocyte size was quantified using AxioVision LE 4.8 software (Zeiss). A total of 80 cells from ten fields from three non-adjacent stained sections at 3 different levels were used for analysis (n=13-14 hearts/group). Cardiomyocyte area was expressed in μm^2.

2.5. Cardiac fibroblast isolation, culture, and RNA extraction: Cardiac fibroblasts were isolated from C57BL/6J animals using enzymatic digestion as previously described [20] and were cultured in DMEM/F12 (GIBCO Invitrogen Corporation, Carlsbad, CA) with 10% Fetal Calf Serum (FCS). Cells were serum-starved at passage 2 for 16h and subsequently used in collagen pad assays. Total RNA was isolated from the collagen pads using GeneJET RNA Purification Kit (ThermoFisher Scientific) and the RNA obtained was used for quantitative PCR.

2.6. Stimulation of cardiac fibroblasts populating collagen pads with recombinant and inactive tTG: Cardiac fibroblasts isolated from both male and female adult wild-type C57BL/6J mice and tTG global knockout mice in a C57BL/6J background (from our colony) [21], [12] were cultured to passage 2 and serum-starved overnight (16 h). Collagen matrix was prepared on ice by diluting a stock solution of rat collagen I (3.0 mg/mL, GIBCO Invitrogen Corporation, Carlsbad, CA) with 2X MEM and distilled water to a final concentration of 1 mg/ml collagen. Cell suspensions in 2X MEM were mixed with collagen solution to achieve the final concentration of $3x10^5$ cells/ml. Subsequently, 500 µl of this suspension was aliquoted to a 24-well culture plate (BD Falcon, San Jose, CA) and allowed to polymerize at 37ºC for 30 min. Following polymerization, pads were released from wells, transferred to 6-well culture plate (BD

Falcon, San Jose, CA) and cultured in 0% FCS DMEM/F12 for 24 h. After incubation, pads were used either for RNA extraction, or for histological processing, as previously described [22]. For experiments examining the effects of tTG on fibroblast phenotype, enzymatically active recombinant mouse tTG (T040, Zedira GmbH), or the C277S active site null mutant of human recombinant tTG (T018, Zedira GmbH) was incorporated into collagen pads at a concentration of 50 µg/ml (pads used for RNA analysis: n=12-16/group for WT cells, n=4-6/group for tTG KO cells, pads used for histological analysis: n=10-13 pads/group for WT cells, n=6-7 pads for tTG KO cells).

In additional experiments examining effects of tTG on fibroblast phenotype, three site-specific inhibitors of tTG were used: Boc-DON (B003, Zedira GmbH), which is a potent cell impermeable irreversible site-specific inhibitor [23], Z-DON (616467, Calbiochem), a potent cell-permeable peptide-based irreversible site-specific inhibitor [23], and TAMRA-DON (R002, Zedira GmbH), a fluorescent blocker of tTG [24]. These inhibitors were added at a final concentration of 100 µm to 0% FCS DMEM/F12 media in which collagen pads prepared as described above were cultured for 24 h, following which the pads were used for RNA extraction (5-9 pads/group).

2.7. Quantitative real-time PCR: Isolated total RNA from mouse hearts or collagen pads was reverse transcribed to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad) following the manufacturer's guidelines. Quantitative PCR was performed using the SsoFast™ EvaGreen® Supermix (Bio-Rad) method on the CFX384™ Real-Time PCR Detection System (Bio-Rad). Primers were synthesized by Integrated DNA Technologies. The following sets of primers were used in the study: TIMP1 forward GCCTGAACACTGTCTACTT reverse TTGCTGCTGTCTGATAGTT; TIMP2 forward CTTCTGTCTCTTAATGTC reverse

CTGTCTATATTGTTAGTGAT; MMP2 forward TCCGCTGCATCCAGACTT, reverse GGTCCTGGCAATCCCTTTGTATA; MMP3 forward ATTTGGGTTTCTCTACTT, reverse GAAGAACTATAAGCATCAG; MMP8 forward TTAGGATGAGCCATAAGT, reverse TTGCTTGGTCTCTTCTAT; MMP9 forward TCTTACATTGGAGAACAC , reverse GAAGGAAGAACCAACATT; collagen I forward GATACTTGAAGAATATGAAC, reverse AATGCTGAATCTAATGAA; collagen III forward TACTCATTCACCAGCATA, reverse GTATAGTCTTCAGGTCTCA; GAPDH forward AACGACCCCTTCATTGACCT, reverse CACCAGTAGACTCCACGACA.

2.8. Protein extraction and western blotting. The apical 1/3 of the hearts undergoing 28-day TAC protocols were used for protein extraction. Protein was isolated using T-PER tissue protein extraction reagent (78510, ThermoFisher), 10% protease inhibitor cocktail (Roche), 10% phosphatase inhibitor cocktail (Roche). Protein concentrations were determined and proteins (25 μg) in denaturing conditions were subjected to SDS-PAGE (4%-20%) and then electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad). The blots were then blocked with 5% BSA dissolved in Tris-buffered saline containing 0.1% Tween 20 (TTBS) either overnight or 2 h at room temperature, washed three times with TTBS for 5 min each and incubated overnight at 4°C with specific primary antibodies anti-tTG (ab109200, Abcam, 1:2000 dilution), anti-Collagen III (ab7778 Abcam; 1:1000 dilution), anti-MMP2 (ab37150, Abcam 1:1000 dilution), in TTBS containing 3% BSA. On the next day, membranes were washed (3 times for 5 min each) with TTBS and incubated for 1h at room temperature with horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody (1:5000 dilution) in TTBS containing 2% nonfat dry milk. Protein bands were visualized by enhanced chemiluminescence using Super Signal West Pico or Femto reagents (Pierce). Signal intensity was measured with a Bio-Rad Imaging

Station. Membranes were then stripped and reprobed with GAPDH antibody to verify equal loading and densitometric analysis was performed using Image J software.

In order to study the time course of tTG protein expression in pressure-overloaded hearts, we used myocardial protein samples from C57BL/6J mice undergoing 3-28 day TAC protocols and from corresponding sham-operated controls (n=4-7/group) [12]. The specificity of the tTG antibody was validated using protein samples from tTG KO mice and from cardiac tTG KO fibroblasts.

2.9. Sirius red staining and polarized light microscopy for assessment of collagen content.

Polarized light microscopy was used to assess collagen deposition, as previously described [25], [26]. Briefly, paraffin sections (5 μm thick) were stained using sirius red. Circularly polarized images were obtained using Axio Imager M2 for polarized light microscopy (Zeiss). When the collagen fibers are stained with sirius red and viewed with polarized light, depending on the thickness of the collagen fibers, the hues range from green to yellow to orange to red. The respective proportions of different hues were assessed using ImageJ software. The 8-bit hue images contain 256 colors, and we distinguished different colors based on visible hues. We used the following hue definitions; red 2-9 and 230-256, orange 10-38, yellow 39-51, green 52-128. The number of pixels within each hue range was expressed as a percentage of the total number of collagen pixels, which in turn was expressed as a percentage of the total number of pixels in the image. At least 15 different fields from three non-adjacent stained sections per mouse at 3 different levels were obtained and analyzed per heart sample (n=13-14 hearts/group).

In a separate experiment, enzymatic actions of tTG on collagen processing and structure were studied using collagen pads which incorporated rtTG and itTG, as described above. Polarized light microscopy was used to visualize the pericellular "halo" of orange/red thick

fibers around fibroblasts embedded in the collagen pad. This halo represents new synthesis and/or processing of collagen by the cells. Using AxioVision LE4.8 software (Zeiss), the pericellular collagen area was traced and quantitatively assessed. 15 cells per WT (n=10-13 pads/condition) or tTG KO pad (n=6-7 pads/condition) were used for quantitation.

2.10. Statistical analysis: Comparisons between more than 2 groups were performed using one-way ANOVA followed by t-test corrected for multiple comparisons (Sidak's test). For non-Gaussian distributions, non-parametric ANOVA was used (Kruskal-Wallis). Comparisons between 2 groups were performed using unpaired t-test, or the Mann-Whitney U test (for non-parametric distributions). Mortality was compared using the log rank test. Data were expressed as mean±SEM. Statistical significance was set at p=0.05.

3. RESULTS:

3.1. Upregulation of tTG in the pressure-overloaded myocardium.

We have previously demonstrated that cardiac pressure overload is associated with myocardial tTG mRNA upregulation [12]. In the current study, we used western blotting experiments to assess expression of tTG protein in the pressure-overloaded heart in WT C57Bl6J mice. Myocardial tTG protein levels were significantly increased after 3 days of TAC in comparison to corresponding shams (Supplemental figure 1A, C). Samples from tTG KO sham hearts and from tTG KO fibroblasts served as a negative control, exhibiting absence of tTG (Supplemental figure 1A). After 28 days of TAC, there was a trend towards increased tTG levels (Supplemental figure 1B, D).

3.2. Activation of tTG in the pressure-overloaded myocardium is attenuated by infusion of the tTG inhibitor ERW1041E.

Next, we used 5-BP, an established tTG activity probe[18], to localize tTG activity in the pressure overloaded myocardium and to assess the effectiveness of the tTG inhibitor. Negligible tTG activity was found in sham hearts (Figure 1A, D, G, J). After 14 days of pressure overload, tTG activity was localized in the perivascular areas (Figure 1B, E), in cardiomyocytes (Figure 1H) and in the cardiac interstitium (Figure 1K). Treatment with the tTG inhibitor ERW1041E markedly attenuated tTG activity in the pressure overloaded heart (Figure 1C, F, I, L). Quantitative analysis suggested intense activation of tTG in the pressure-overloaded myocardium that was markedly reduced after treatment with the tTG inhibitor ERW1041E (***$p<0.001$, Figure 1M).

3.3. Treatment with tTG inhibitor attenuates development of left ventricular hypertrophy without affecting chamber dimensions and ejection fraction following cardiac pressure overload.

TAC induced a marked increase in the R:L carotid flow ratio in comparison to sham-operated mice (Supplemental figure 2A). In pressure-overloaded mice, the carotid flow ratio was comparable between vehicle- and inhibitor-treated animals (ERW), suggesting comparable pressure loads between groups (Supplemental figure 2A). Two vehicle-treated mice had very high R:L carotid flow ratios (>15), indicating an extremely high pressure load, and were excluded from further analysis. Mortality following TAC was not affected by tTG inhibition (Supplemental figure 2B). The survival rate at 28 days was 84% for vehicle-treated mice and 73.1% for inhibitor-treated animals (p=NS, n=25-26/group). Vehicle-treated controls and inhibitor-treated animals had no significant differences in body weight (BW) at baseline and

after 28 days of TAC (Figure 2A). Pressure overload markedly increased the heart weight to body weight ratio (HW:BW) in both vehicle- and ERW-treated animals. ERW1041E-treated animals exhibited a trend towards reduced HW:BW after 28 days of TAC (Figure 2B). In contrast, the lung weight:body weight ratio (LW:BW) was comparable between vehicle and ERW-treated animals (Figure 2C). Echocardiographic analysis showed that tTG inhibition did not significantly affect LVEDV (Figure 2D) following pressure overload. Ejection fraction was significantly increased in sham animals, likely reflecting a hyperdynamic circulation due to the daily injections. Cardiac pressure overload was associated with a significantly reduced ejection fraction in both vehicle and ERW-treated groups, in comparison to shams (Figure 2E, G). However, tTG inhibition did not significantly affect ejection fraction in sham or pressure-overloaded animals. Cardiac pressure overload was associated with a significantly increased echocardiographically-derived LV mass in vehicle-treated, but not in ERW-treated animals (Figure 2F, H), suggesting that tTG inhibition attenuated cardiac hypertrophy. In contrast, ERW administration had no significant effects on LV mass in sham animals.

3.4. Gender-specific effects of tTG inhibition in cardiac remodeling.

Next, we examined whether tTG inhibition exerts distinct effects on cardiac function and remodeling in male and female mice. Male and female pressure-overloaded mice receiving vehicle or ERW had comparable R:L carotid flow ratio (Supplemental figure 2C-D). Treatment with the tTG inhibitor did not significantly affect BW in either male or female animals (Supplemental figure 3A-B). ERW-treated male mice had a trend towards decreased HW:BW ratio than vehicle-treated controls; in contrast, in female mice the reduction in HW:BW in inhibitor-treated animals did not reach statistical significance (Supplemental figure 3C-D).

Gender-specific analysis of the echocardiographic data showed no significant effects of the inhibitor on LVEDV or ejection fraction (Supplemental figure 3E-H). When treated with vehicle, both male and female mice undergoing TAC exhibited a significant increase in LV mass in comparison to baseline values. In contrast, no significant increase in LV mass was noted in male or female inhibitor-treated animals undergoing TAC. After 28 days of pressure overload, ERW-treated female mice had significantly lower LV mass than corresponding vehicle-treated controls (Supplemental figure 3I-J).

3.5. tTG inhibition attenuates diastolic dysfunction in the pressure overloaded myocardium.

Speckle tracking echocardiography demonstrated that male mice treated with tTG inhibitor had preserved systolic strain rate after 28 days of pressure overload, suggesting improved systolic function (Figure 3A-C). In contrast, no significant effects were noted in female mice (Figure 3B). In order to assess the effects of tTG inhibition on diastolic dysfunction, we measured diastolic strain rate. When compared with gender-matched vehicle-treated animals, mice treated with tTG inhibitor exhibited higher diastolic strain rate, suggesting preserved diastolic function (Figure 3D-F).

3.6. tTG inhibition attenuates cardiomyocyte hypertrophy and reduces collagen deposition in the pressure-overloaded heart.

WGA lectin histochemistry was used to assess cardiomyocyte size in the pressure-overloaded myocardium (Figure 4A-B). Treatment with the tTG inhibitor significantly reduced cardiomyocyte size after 28 days of TAC (Figure 4C). Gender-specific analysis showed a trend towards larger cardiomyocyte size in male animals, when compared with female mice (male: $371.3\mu m^2 \pm 9.5$ n=8, vs. female $324\mu m^2 \pm 24.3$, n=5, p=0.057). Although treatment with the

inhibitor had more impressive effects in male mice (reduction in cardiomyocyte size: 31% in males vs. 20% in females), tTG inhibition significantly attenuated cardiomyocyte hypertrophy in both male and female animals (Supplemental figure 4A-B). Sirius red staining was used to label collagen fibers in the remodeling myocardium (Figure 4D-E). Quantitative analysis demonstrated that tTG inhibition attenuated collagen deposition in the pressure-overloaded myocardium after 28 days of TAC (Figure 4F). Gender-specific analysis demonstrated that although the extent of fibrosis following TAC was comparable between male and female animals (p=NS), the tTG inhibitor significantly attenuated collagen deposition only in male mice (*$p<0.05$). Female mice had a trend towards reduced collagen content, when treated with the tTG inhibitor ($p=0.10$, Supplemental figure 4C-D).

3.7. tTG inhibition reduces collagen thickness in the pressure-overloaded myocardium.

Sirius red staining followed by polarized light microscopy was used to investigate the effects of tTG inhibition on collagen structure. When visualized under polarized microscopy, thicker cross-linked fibers show orange or red birefringence, whereas thinner fibers appear green (Figure 5A-H). tTG inhibition significantly reduced deposition of both thin and thick collagen fibers in the pressure overloaded myocardium after 28 days of TAC (Figure 5I).

3.8. tTG inhibition reduces collagen and TIMP transcription in the pressure-overloaded myocardium.

The global attenuation of both thin and thick collagen fibers by tTG inhibition suggested that the *in vivo* enzymatic actions of tTG may extend beyond its known effects on collagen cross-linking. Accordingly, we investigated the *in vivo* effects of tTG inhibition on transcription of

genes associated with matrix synthesis and metabolism. Mice treated with ERW1041E exhibited attenuated myocardial levels of type I and type III collagen mRNA after 14 days of TAC (Figure 6A-B). Moreover, tTG inhibition modestly, but significantly, reduced MMP2 mRNA expression (Figure 6C), but had no statistically significant effects on MMP3 (Figure 6D), MMP8 (Figure 6E) and MMP9 (Figure 6F) synthesis. Animals treated with tTG inhibitor had markedly reduced expression of TIMP1 and TIMP2 mRNA (Figure 6G-H). Western blotting studies showed that inhibitor-treated animals had a significant reduction in collagen III (Figure 6I-J) and MMP2 protein levels (Figure 6K-L), when compared with vehicle-treated mice. These findings suggest that in vivo, tTG inhibition reduces ECM protein synthesis and attenuates expression of genes associated with matrix metabolism.

3.9. The *in vitro* effects of exogenous tTG on expression of genes associated with matrix synthesis and metabolism are predominantly non-enzymatic.

Our *in vivo* findings suggested that tTG activity is localized in the interstitial and perivascular space following pressure overload, and that enzymatic actions of tTG mediate synthesis of fibrosis-associated genes and collagen deposition. Accordingly, we used an *in vitro* model of cardiac fibroblasts populating collagen pads to examine whether extracellular tTG activates a fibrogenic program in fibroblasts through enzymatic actions. In order to dissect enzymatic and non-enzymatic actions of tTG on fibroblast gene expression, we used 2 independent strategies: a) we compared the effects of recombinant tTG (rtTG) and inactive tTG (bearing a C277S mutation that renders the active site catalytically inactive, itTG) and b) we examined the effects of 3 different tTG inhibitors on tTG-mediated modulation of fibroblast genes. Neither rtTG nor itTG affected collagen I or III expression in cardiac fibroblasts

populating collagen pads (Figure 7A-B). Both rtTG and itTG markedly induced MMP3 expression, suggesting that this effect was mediated through non-enzymatic actions (Figure 7C). In contrast, MMP8 mRNA synthesis was not affected by stimulation with rtTG or itTG (Figure 7D). rtTG induced a 4.0 fold increase in TIMP1 mRNA levels through actions that were independent of its enzymatic activity (Figure 7E). Because stimulated fibroblasts are known to produce and secrete tTG, we examined the effects of rtTG and itTG on gene expression by tTG KO cells. rtTG and itTG induced MMP3 and TIMP1 mRNA synthesis in both WT and tTG KO cells, suggesting that these actions are independent of endogenous cell-derived tTG, and reflect non-enzymatic effects of matrix-bound tTG on the fibroblasts (Figure 7F-J). Moreover, incubation with 3 different tTG inhibitors (Z-DON, Boc-DON and TAMRA-DON) did not affect rtTG-induced gene expression (Figure 7K-O), further supporting the notion that the effects of rtTG are non-enzymatic. The absence of enzymatic actions of tTG on fibroblast gene expression in vitro suggest that the *in vivo* observations indicating profound effects of the tTG inhibitor on transcription of fibrosis-associated genes (Figure 6) may result from actions on other cell types (such as macrophages, cardiomyocytes, mast cells, or vascular cells), or may reflect enzymatic effects of tTG on collagen processing.

3.10. Recombinant tTG increases pericellular collagen deposition in collagen pads through enzymatic actions

In order to examine whether enzymatic actions of tTG in vivo reflect effects on collagen processing, we studied the effects of rtTG and itTG on collagen structure in fibroblast-populated collagen pads. When visualized under polarized microscopy, fibroblasts in collagen pads show a pericellular "halo" of orange/red thick fibers, reflecting new synthesis and/or processing of

collagen by the cells (Figure 8A-F). In contrast to itTG, rtTG markedly increased the thickness of the pericellular collagen in both WT and tTG KO cells (Figure 8A-F). Quantitative analysis showed that the area of pericellular collagen was markedly increased by rtTG stimulation, but was not affected by itTG, suggesting that matrix-bound tTG increases deposition of collagen fibers through enzymatic actions (Figure 8G).

4. DISCUSSION:

We report for the first time that the enzymatic functions of tTG mediate fibrosis and diastolic dysfunction in the pressure-overloaded myocardium. Using a mouse model of cardiac pressure overload induced through TAC, and *in vitro* experiments investigating the effects of active and inactive tTG on isolated cardiac fibroblasts, we found that: a) tTG activity is localized in the interstitial and perivascular areas of the remodeling myocardium, b) inhibition of the enzymatic actions of tTG with ERW1041E, a specific small molecule inhibitor, attenuates diastolic dysfunction following pressure overload, c) the protective effects of tTG inhibition are associated with decreased cardiomyocyte hypertrophy, attenuated interstitial fibrosis, and reduced thickness of collagen fibers, d) although enzymatic inhibition of tTG in vivo attenuates expression of collagen and other fibrosis-associated genes, the *in vitro* effects of tTG on cardiac fibroblast gene expression are transamidase-independent, e) enzymatic actions of matrix-bound tTG promote collagen deposition in fibroblasts populating collagen pads. Our observations highlight the complexity of the in vivo effects of tTG, and the distinct effects of enzymatic and non-enzymatic actions.

4.1. Activation of tTG in the remodeling myocardium.

Our findings demonstrate that tTG is upregulated and activated in the remodeling pressure-overloaded myocardium (Fig 1, Supplemental figure 1). Although tTG is constitutively expressed in isolated cardiomyocytes [27] and in adult mammalian hearts (Supplemental figure 1) [28], minimal tTG activity was noted in normal mouse myocardium (Figure 1A). In mammalian tissues, tTG activity is tightly regulated to prevent spontaneous or excessive activation and subsequent intracellular cross-linking. Low Ca^{2+} concentrations prevent intracellular transamidation activity. Moreover, intracellular GTP/GDP act as allosteric inhibitors of tTG, inducing a closed conformation, reducing accessibility of the catalytic domain, and preventing inappropriate intracellular tTG activation [29],[30],[31]. In the pressure-overloaded myocardium, tTG activity is present in the interstitial and perivascular space (Figure 1B, E, H, K), but the mechanisms of secretion remain unknown. In the extracellular space, high levels of Ca2+ may contribute to activation of tTG-mediated crosslinking activity. Induction of inflammatory stimuli, such as Toll-like receptor signaling cascades and pro-inflammatory cytokines, and upregulation of the antioxidant protein thioredoxin [32] may also promote extracellular tTG activation [33],[13],[34].

4.2. Enzymatic and non-enzymatic functions of tTG in the pressure-overloaded myocardium.

Perturbations in transglutaminase-mediated ECM cross-linking have been previously associated with the development of cardiomyopathy in senescent mice lacking the matricellular protein thrombospondin (TSP)-2 [35]. Recently, we showed that tTG KO mice exhibit increased mortality following pressure overload, associated with chamber dilation and increased MMP2 activity, but are protected from diastolic dysfunction, and have attenuated collagen crosslinking [12]. The effects of tTG may be due to enzymatic or non-enzymatic functions. In order to dissect

the role of tTG-dependent transamidation in the pressure-overloaded myocardium, we treated mice undergoing TAC protocols with the specific tTG small molecule inhibitor ERW1041E. Effective tTG inhibition was documented *in vivo* (Figure 1). In contrast to the increased mortality and dilation associated with global loss of tTG, enzymatic inhibition exerted protective actions, attenuating cardiomyocyte hypertrophy, interstitial fibrosis and diastolic dysfunction (Figures 3-5). Our findings suggest that transamidase-dependent tTG actions are involved in fibrotic and hypertrophic remodeling of the pressure-overloaded heart (Figure 9), but do not contribute to protection from chamber dilation and death.

4.3. The cellular basis for the fibrogenic actions of tTG.

Attenuated collagen deposition in mice treated with the tTG inhibitor is associated with marked reductions in myocardial collagen mRNA and protein levels (Figure 6). In order to examine whether attenuated collagen synthesis reflects direct effects of tTG on fibroblast-derived collagen expression, we performed *in vitro* experiments investigating the effects of tTG on isolated cardiac fibroblasts. Surprisingly, tTG did not affect collagen expression by cardiac fibroblasts (Figure 7A-B), suggesting that the observed in vivo effects of tTG inhibition may reflect indirect actions on other cell types. tTG is known to modulate the phenotype of macrophages, cardiomyocytes, vascular cells and mast cells. *In vitro* and *in vivo* studies have suggested that tTG is implicated in macrophage-dependent efferocytosis, and may regulate transition of macrophages to an anti-inflammatory phenotype upon ingestion of apoptotic cells [36],[37],[38]. Moreover, recent investigations in human and mouse tissues have demonstrated that tTG is a conserved marker for M2 macrophages [39]. Whether tTG plays a role in acquisition of a fibrogenic phenotype by macrophages remains unknown. Moreover, tTG has been implicated in mast cell-mediated macrophage activation [40]. Considering the important

role of mast cells as a source of cytokines and growth factors in the injured and remodeling myocardium [41], and their involvement as cellular effectors of cardiac fibrosis [42], tTG may exert fibrogenic actions by orchestrating interactions between inflammatory cells in the remodeling heart. In cardiomyocytes, tTG has been implicated as a mediator of hypertrophic and pro-survival responses [27],[43]. Endothelial tTG has been suggested to regulate inflammatory activation, and may also promote angiogenic responses [44] by regulating Vascular Endothelial Growth Factor (VEGF) signaling [45], [46], [47]. Whether the pro-fibrotic enzymatic effects of tTG are due to activation of a fibrogenic program in cardiomyocytes and vascular cells has not been investigated.

4.4. Transamidase-independent effects of tTG modulate transcription of genes associated with matrix remodeling in cardiac fibroblasts, whereas enzymatic actions may regulate collagen processing.

Our *in vitro* experiments demonstrated that recombinant tTG induces expression of genes associated with matrix metabolism in isolated cardiac fibroblasts, inducing a 10-fold increase in MMP3 and a 4-fold increase in TIMP1 expression (Figure 7). We used 2 independent strategies to examine whether the effects of tTG on fibroblast gene expression are mediated through enzymatic actions. First, recombinant tTG and enzymatically inactive mutant tTG (itTG) had similar effects on MMP3 and TIMP1 synthesis (Figure 7). Second, incubation with 3 different tTG inhibitors did not affect tTG-mediated gene expression. Both approaches suggested that the effects of tTG on fibroblast gene expression are non-enzymatic. In contrast, stimulation with recombinant active, but not with inactive tTG increased pericellular collagen deposition in fibroblast-populated pads (Figure 8), suggesting that enzymatic effects of tTG may be implicated in pericellular collagen processing. Experiments using tTG KO cells demonstrated that both the

enzymatic and transamidase-independent effects of extracellular, matrix-bound tTG are independent of endogenous tTG expression (Figure 7-8).

Obviously, our study has significant limitations. First, the in vitro model of fibroblast-populated pads does not recapitulate the complex in vivo environment, in which tTG may exert a wide range of actions on all cell types involved in cardiac remodeling. Second, collagen deposition in fibrotic tissues is a complex process. Large amounts of pro-collagen undergo intracellular degradation in fibroblasts before secretion and deposition in the ECM. Thus, mRNA levels do not necessarily correlate with collagen deposition. Moreover, net accumulation of collagen is dependent on post-translational modifications, the presence of other cross-linking enzymes, and on the balance between proteases and anti-proteases in the interstitial space [48], [49]. Our previously reported effects of global tTG loss on lysyl-oxidase levels in the pressure-overloaded myocardium [12] highlight the complex in vivo interactions between proteins involved in ECM regulation.

4.5. The enzymatic effects of tTG: a therapeutic opportunity for patients with heart failure with preserved ejection fraction (HFpEF)?

HFpEF currently accounts for the majority of heart failure cases and its prevalence continues to rise [50]. Interstitial fibrosis is commonly found in patients with HFpEF and is associated with increased left ventricular stiffness [51],[52],[53]. Inhibition of enzymes that crosslink the ECM, such as the members of the lysyl oxidase family, reduces cardiac fibrosis, attenuating diastolic dysfunction in experimental models of fibrotic cardiac remodeling that recapitulate aspects of HFpEF [54],[55].[56]. Our findings suggest that although non-enzymatic actions of tTG transduce protective signals that preserve myocardial geometry in the remodeling heart [12],[57] the enzymatic effects of tTG contribute to fibrosis and diastolic dysfunction

(Figure 9). Considering the availability of effective and selective tTG inhibitors [58],[59], targeting tTG-dependent transamidation may be a promising therapeutic strategy for subsets of HFpEF patients with a prominent fibrotic phenotype.

4.6. Conclusions

tTG is a ubiquitously expressed, multifunctional protein with an important role in regulating inflammation, repair and tissue remodeling. tTG is upregulated and activated in the remodeling pressure-overloaded myocardium and exerts fibrogenic actions dependent on transamidase activity, and protective non-enzymatic actions that regulate matrix metabolism and MMP activity [12]. The significant effects of pharmacologic inhibition of the enzymatic actions of tTG on diastolic dysfunction in mice may suggest a promising new therapeutic approach for patients with HFpEF. Clinical studies are needed in order to assess myocardial tTG expression and activity in various subpopulations of heart failure patients, and to identify subjects with excessive deposition of crosslinked ECM. Moreover, considering the broad functional repertoire of tTG, its wide distribution in myocardial cells and in the interstitial ECM, and the diversity of tTG interactions with ECM proteins, growth factors, integrins and syndecans, there is a need to dissect the *in vivo* mechanisms of tTG actions, focusing on cell biological targets, molecular interactors and the relative significance of enzymatic and non-enzymatic effects.

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6. CONFLICTS: None

FIGURE LEGENDS:

Figure 1: tTG activation in the pressure-overloaded myocardium is markedly attenuated following daily injection of the specific small molecule inhibitor ERW1041E (ERW). 5-biotinamidopentylamine (5BP), a synthetic substrate of tTG, was used as a probe for tTG activity in tissues. When administered systemically, 5BP is covalently attached to extracellular matrix proteins in tissues in which tTG is enzymatically active. 5BP injections were performed in mice undergoing TAC protocols and sham controls. 5BP was visualized in frozen sections using a biotin-avidin-peroxidase method and was developed with DAB+nickel (black). Representative images from sham (A, D, G, J), TAC (B, E, H, K) and ERW-treated TAC animals (C, F, I, L) are shown. Sham hearts showed negligible tTG activity (A, D, G, J). After 14 days of TAC, intense staining, reflecting tTG activation was noted in areas of perivascular fibrosis (B, E, arrows), in the interstitium (H, K, arrows) and in cardiomyocytes. Treatment with ERW markedly attenuated tTG activity (C, F, I. L). M: Semiquantitive analysis showed marked reduction in tTG activity in ERW-treated animals after 14 days of TAC (***$p<0.001$, n=5/group). Scalebar=50µm.

Figure 2: Effects of tTG inhibition on cardiac geometry and function following pressure overload. A. tTG inhibition had no significant effects on body weight (BW) in sham or pressure-overloaded mice. B. Cardiac pressure overload significantly increased the heart weight to body weight ratio (HW:BW) after 28 days of TAC (^^^^$p<0.0001$ vs. sham, n=12-19/group). There was a trend towards a lower HW:BW in animals treated with tTG inhibitor ($p=0.08$, n=19/group). C: The lung weight to body weight ratio (LW:BW) was comparable between groups. D: Echocardiographic analysis showed that treatment with the tTG inhibitor did not affect LVEDV after 28 days of TAC. E: Ejection fraction was increased in sham animals

receiving vehicle or ERW, likely reflecting a hyperdynamic circulation due to the stress of daily injection protocols (^p<0.05, ^^p<0.01 vs. corresponding baseline). Ejection fraction was significantly lower in both vehicle and ERW-treated groups after 28 days of TAC (****p<0.0001 vs. corresponding sham); however, treatment with the inhibitor did not affect ejection fraction. F: TAC significantly increased LV mass in vehicle-treated controls (^p<0.05), but not in inhibitor-treated animals. G. Treatment with the inhibitor did not affect the reduction in ejection fraction (ΔLVef) following pressure overload (**p<0.01, ***p<0.001 vs. corresponding sham). H: Vehicle-treated mice exhibited a 25% increase in LV mass (ΔLVmass) in comparison to baseline levels (*p<0.05 vs. sham). In contrast, the increase in LV mass in ERW-treated mice did not reach statistical significance (p=NS vs. sham), suggesting that tTG inhibition attenuates cardiac hypertrophy (n=12-19/group).

Figure 3: tTG inhibition attenuates diastolic dysfunction in the pressure-overloaded myocardium. A: Speckle tracking echocardiography demonstrated that in comparison to vehicle-treated pressure overloaded animals (V), mice treated with the tTG inhibitor (ERW) had higher systolic strain rate (SSR) after 28 days of TAC. The effects of the inhibitor were noted in male (C), but not in female animals (B). D: tTG inhibition preserved diastolic strain rate (DSR) after 28 days of TAC, suggesting attenuated diastolic dysfunction. E-F: The protective effects of the inhibitor were noted in both female and male animals (*p<0.05, **p<0.01, n=14-15/group; male: n=5-9/group; female: n=5-10/group).

Figure 4: tTG inhibition attenuates cardiomyocyte hypertrophy and reduced fibrosis in the pressure-overloaded myocardium. A-C. Sections stained for WGA lectin were used to assess

cardiomyocyte size. 80 cells from 10 fields from 3 non-adjacent sections from 3 different levels were used for analysis. When compared to vehicle-treated controls (A), inhibitor-treated animals (B) had a significant reduction in cardiomyocyte size (C) after 28 days of TAC. D-F: Sirius red (SR) staining was used to label collagen. In comparison to vehicle-treated animals (D), inhibitor-treated animals (E) had significantly reduced collagen content (F). (**p<0.01 vs. vehicle (V)-treated, n=13-14/group). Scalebar=50µm.

Figure 5: tTG inhibition reduces thickness of collagen fibers in the pressure-overloaded myocardium. Polarized light microscopy in sirius red-stained sections was used to assess the morphological characteristics of collagen fibers. Panels A-D show areas of perivascular fibrosis, whereas panels E-H show interstitial fibrosis (A, C, E, G: light microscopy; B, D, F, H: polarized microscopy). After 28 days of TAC, vehicle treated animals showed extensive perivascular (A, B) and interstitial (E, F) fibrosis with many fibers exhibiting red birefringence, suggestive of increased thickness. Treatment with the tTG inhibitor attenuated deposition of both thicker (red) and thinner (yellow, green) fibers in perivascular (C, D) and interstitial (G, H) areas. I: Quantitative analysis showed that tTG inhibition (ERW) markedly reduced deposition of fibers with red, orange, yellow and green birefringence (***p<0.001 vs. vehicle-treated (V), n=13-14). Scalebar=50µm.

Figure 6: tTG inhibition attenuates expression of collagens and genes associated with matrix metabolism in the pressure-overloaded myocardium. qPCR showed that inhibitor treatment reduced collagen I (A) and collagen III mRNA (B) levels. Treatment with the inhibitor also reduced MMP2 levels (C), but had no statistically significant effects on MMP3, MMP8 and

MMP9 expression. TIMP1 and TIMP2 levels were markedly reduced in inhibitor-treated animals (*p<0.05, **p<0.01, n=6/group). Western blotting showed that treatment with the tTG inhibitor markedly reduced myocardial collagen III (I-J) and MMP2 (K-L) protein expression levels following 28 days of pressure overload. (**p<0.01 vs. vehicle-treated, n=8-15/group).

Figure 7: **Transamidase-independent actions of tTG modulate fibroblast gene expression**. A-J: qPCR was used to assess the effects of recombinant tTG (rtTG) and inactive mutant tTG (itTG) on gene expression in WT or tTG KO cardiac fibroblasts populating collagen pads. In WT cells, rtTG and itTG had no effects on collagen I (A) and III (B) transcription. C: Both rtTG and itTG markedly induced synthesis of MMP3, suggesting a non-enzymatic effect. D: rtTG and itTG had no significant effects on MMP8 expression. E: Both itTG and rtTG upregulated TIMP1 expression by cardiac fibroblasts, suggesting non-enzymatic actions (***p<0.001, ****p<0.0001 vs. control, n=12-16/group). F-J: In order to examine whether these effects are independent of endogenous tTG, we used tTG KO cells. rtTG and itTG did not significantly affect collagen I (F), and collagen III (G) mRNA levels in tTG KO cells, but (much like in WT cells) markedly induced MMP3 (H), and TIMP1 (J) synthesis. rtTG had a modest, but significant effect on MMP8 expression by tTG KO cells (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. control, n=4-6/group). K-O: We examined the effects of 3 different tTG inhibitors (Z-DON, Boc-DON and TAMRA-DON) on rtTG-mediated fibroblast gene expression. The inhibitors did not affect collagen I (K), collagen III (L) and MMP8 (N) mRNA expression. Moreover, rtTG-mediated induction of MMP3 (M) and TIMP1 (O) was not affected by enzymatic inhibition (*p<0.05, **p<0.01, ***p<0.001 vs. control, n=5-9/group).

Figure 8: **The effects of tTG on pericellular collagen deposition in fibroblast-populated collagen pads are dependent on transamidase activity.** Collagen pads populated with WT (A, C, E) or tTG KO cardiac fibroblasts (B, D, F) were stimulated with rtTG (C, D) or itTG (E, F) for 24h. Paraffin-embedded sections of the pads were stained with sirius red and visualized under polarized light (A-F). Intracellular procollagen localized in cardiac fibroblasts is dark red under light microscopy, but is not visualized under polarized light (C – large arrowheads). Polarized light microscopy identifies the green fibers of the collagen pad (C – small arrowheads), and also labels the pericellular network of thicker orange/red collagen fibers (C- small arrows), that reflects processing of collagen fibers by the cells. Images are representative of 5 independent experiments. G: Quantitative analysis of the pericellular collagen area showed that rtTG (C), but not itTG (E) increased the area of pericellular collagen in both WT (C, E) and tTG KO (D, F) cells (^p<0.05, ^^p<0.01, ^^^p<0.001 vs. corresponding control; ***p<0.001 vs corresponding tTG KO, n=90-150 cells from 6-10 different pads from each condition). The findings demonstrate that matrix-bound extracellular tTG promotes collagen deposition and organization through transamidase-independent actions. Scalebar=20μm.

Figure 9: Schematic cartoon illustrating the enzymatic and non-enzymatic actions of tTG in the pressure-overloaded myocardium. tTG is upregulated, secreted and activated in the pressure-overloaded myocardium and exerts a wide range of enzymatic and non-enzymatic actions in cardiomyocytes and fibroblasts. *In vivo*, the enzymatic effects of tTG promote cardiomyocyte hypertrophy, fibrosis and diastolic dysfunction. *In vitro*, extracellular matrix-bound tTG induces synthesis of fibrosis-associated genes (such as MMP3 and TIMP1) through a transamidase-independent mechanism. These effects may involve binding of inactive tTG to

fibronectin and facilitation of integrin or syndecan-mediated signaling pathways, or stimulation of growth factor receptor (GFR)-dependent cascades. Matrix-bound tTG also acts through enzymatic actions that may involve extracellular matrix protein crosslinking, or effects on collagen processing. Additional symbols: MAPK, mitogen-activated protein kinase; FAK, focal adhesion kinase.

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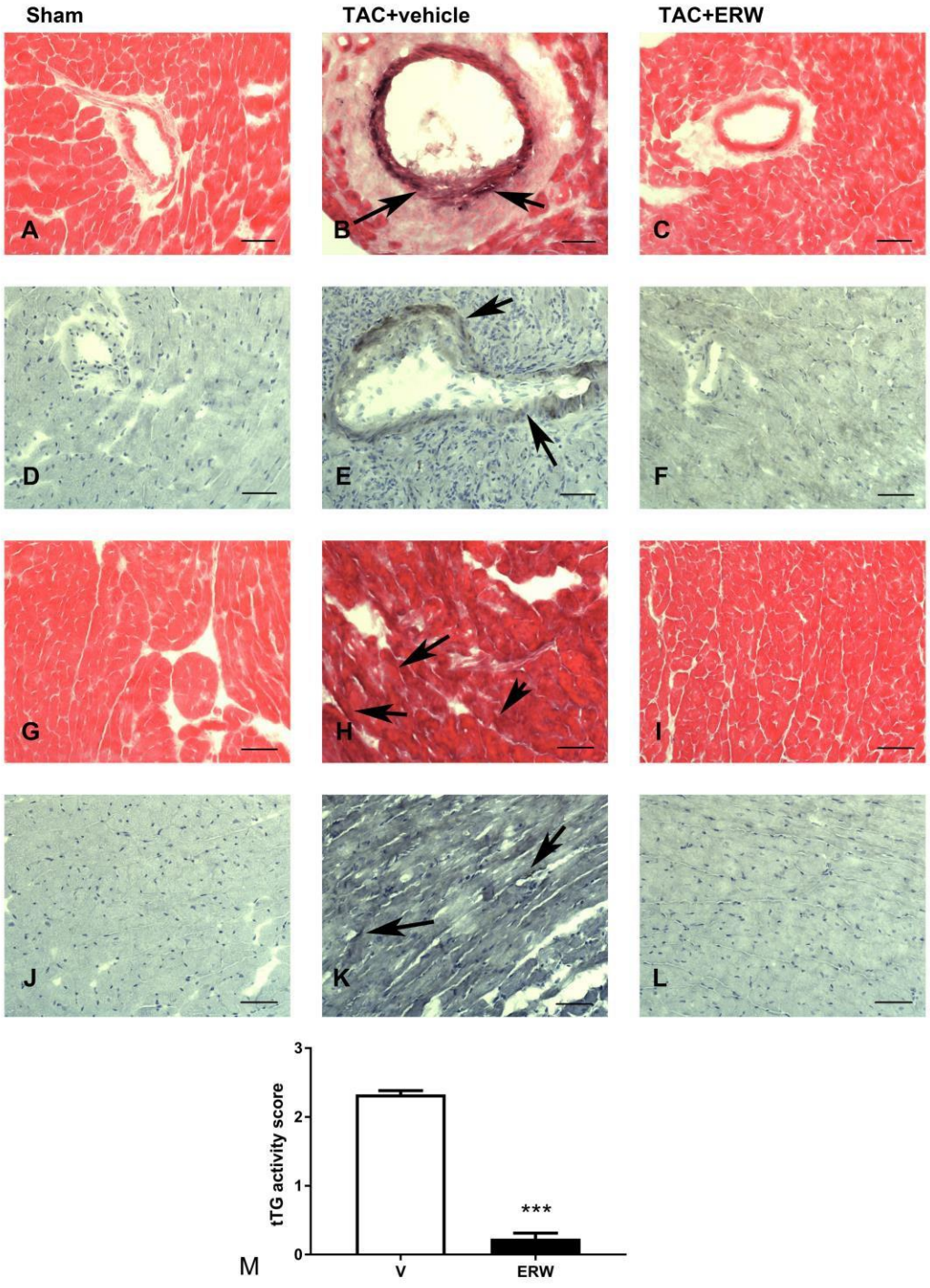

Figure 1

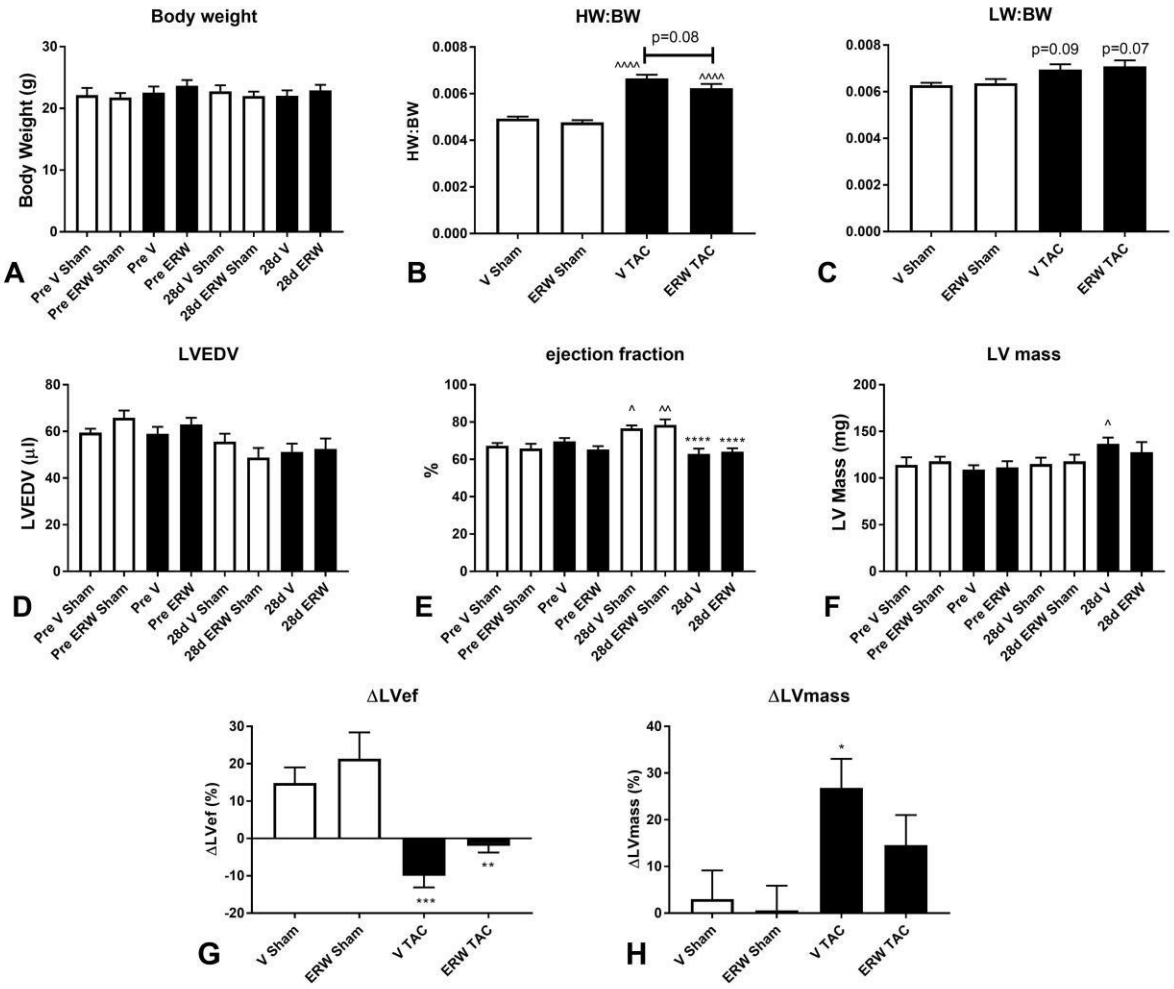

Figure 2

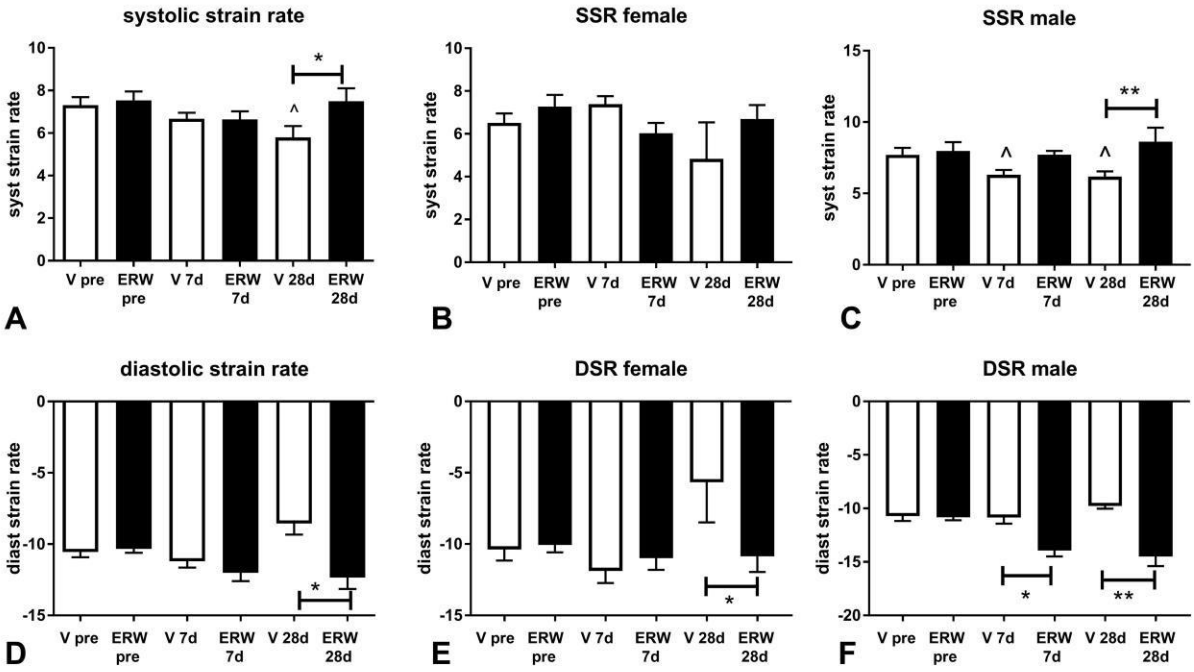

Figure 3

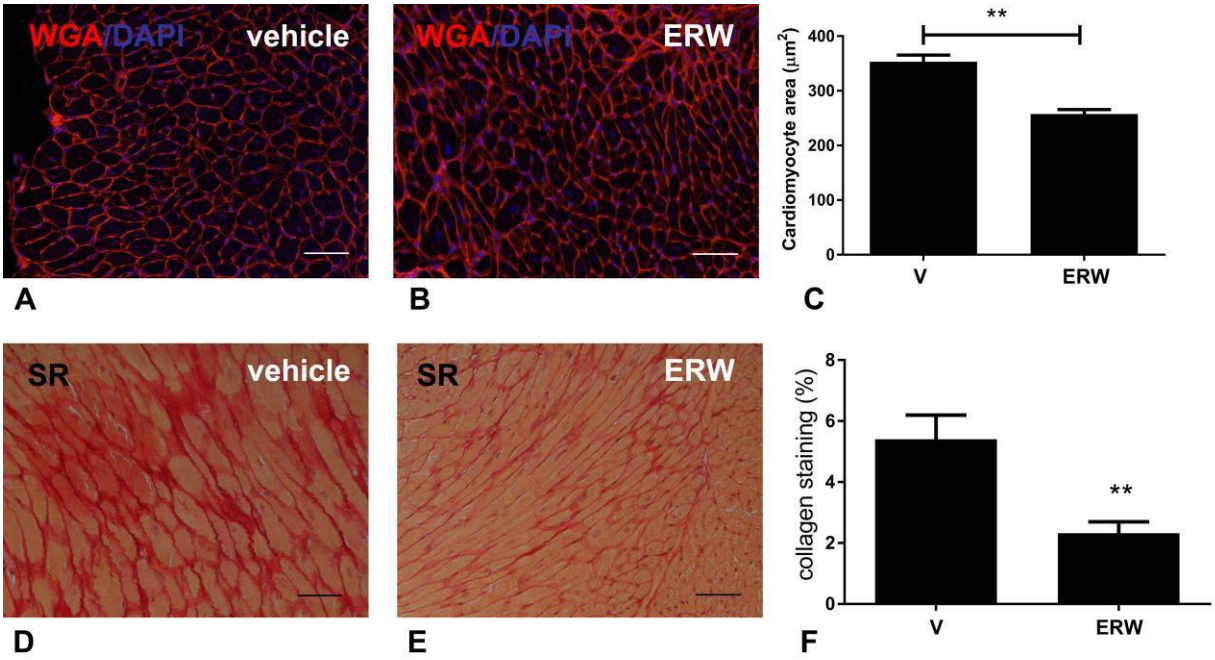

Figure 4

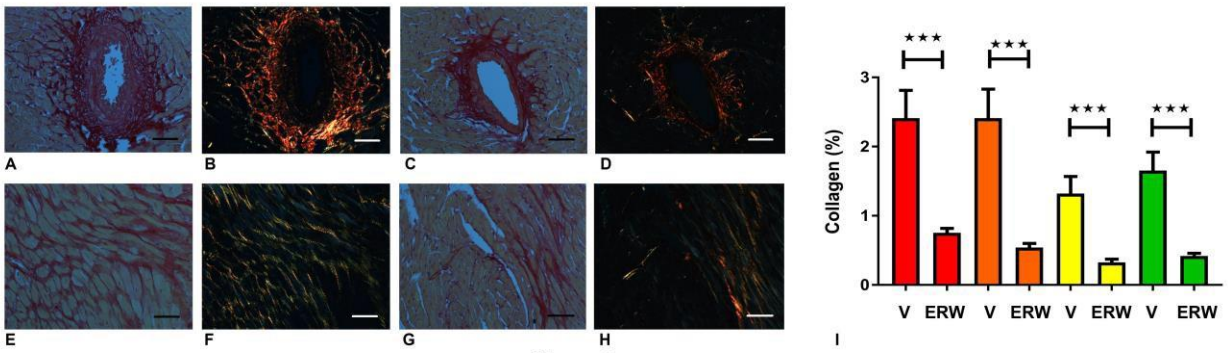

Figure 5

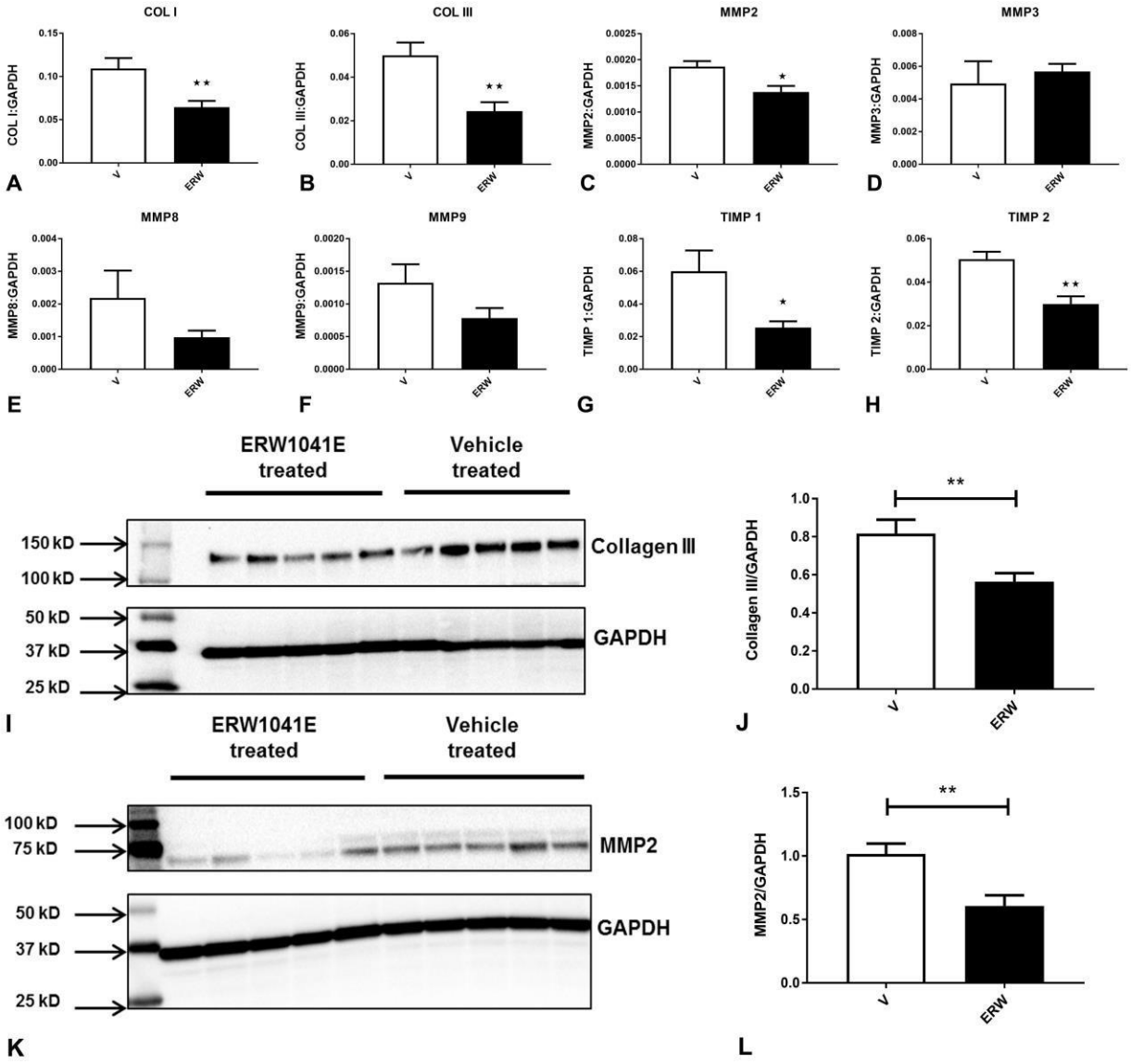

Figure 6

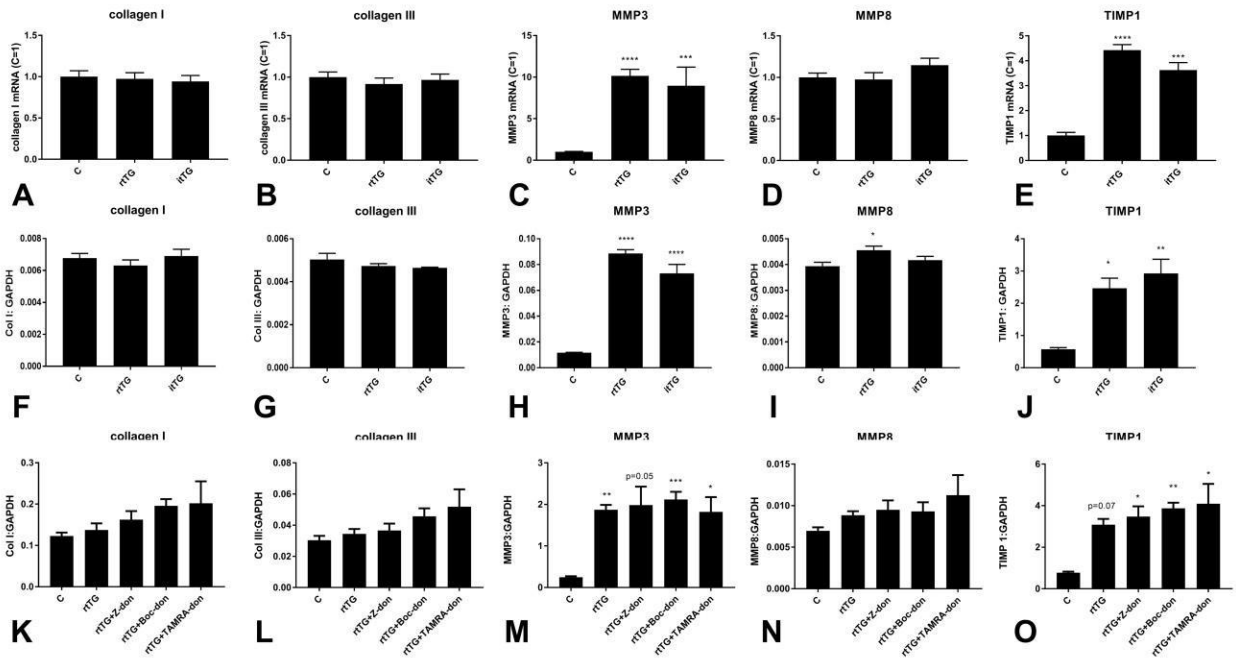

Figure 7

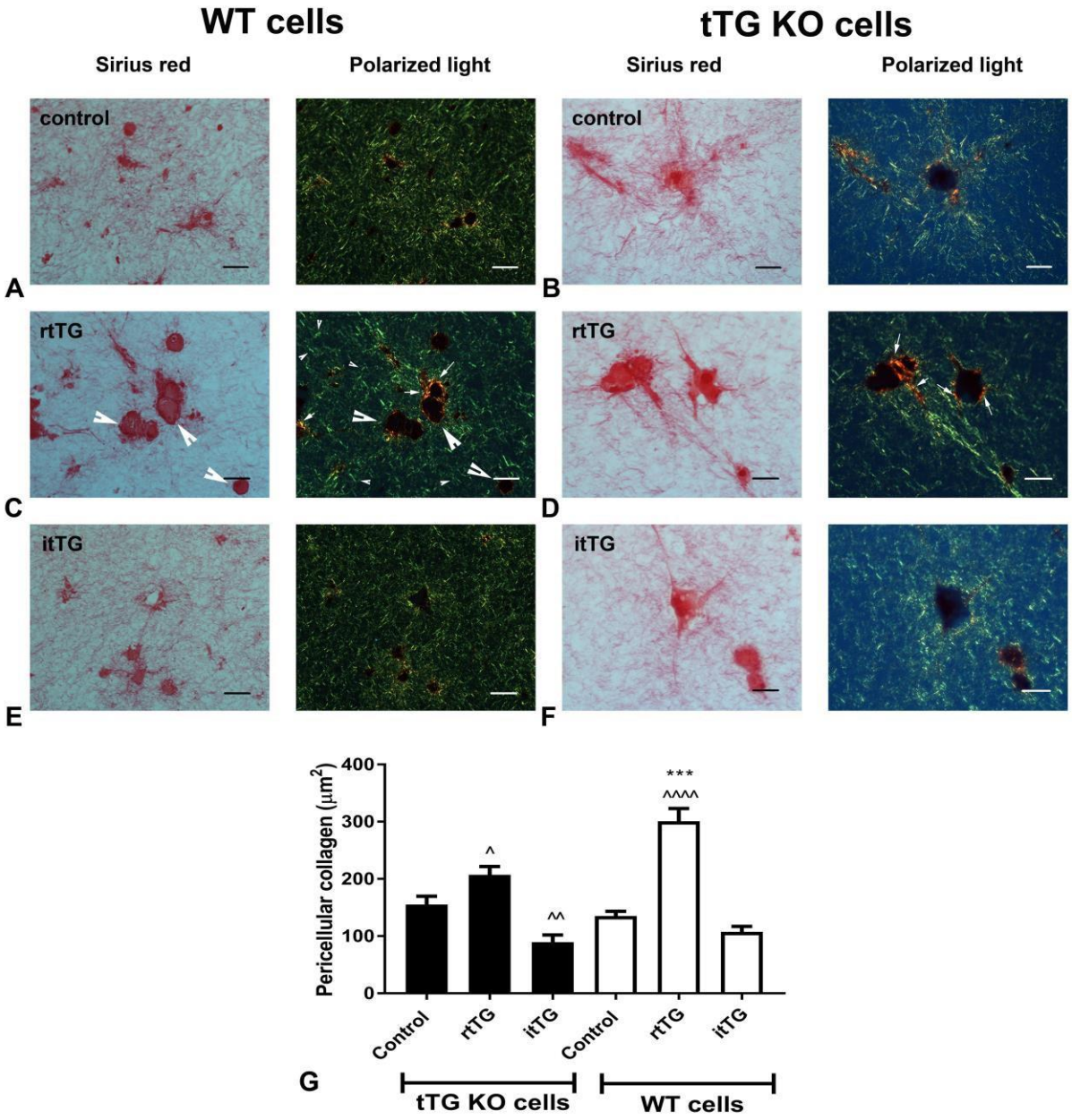

Figure 8

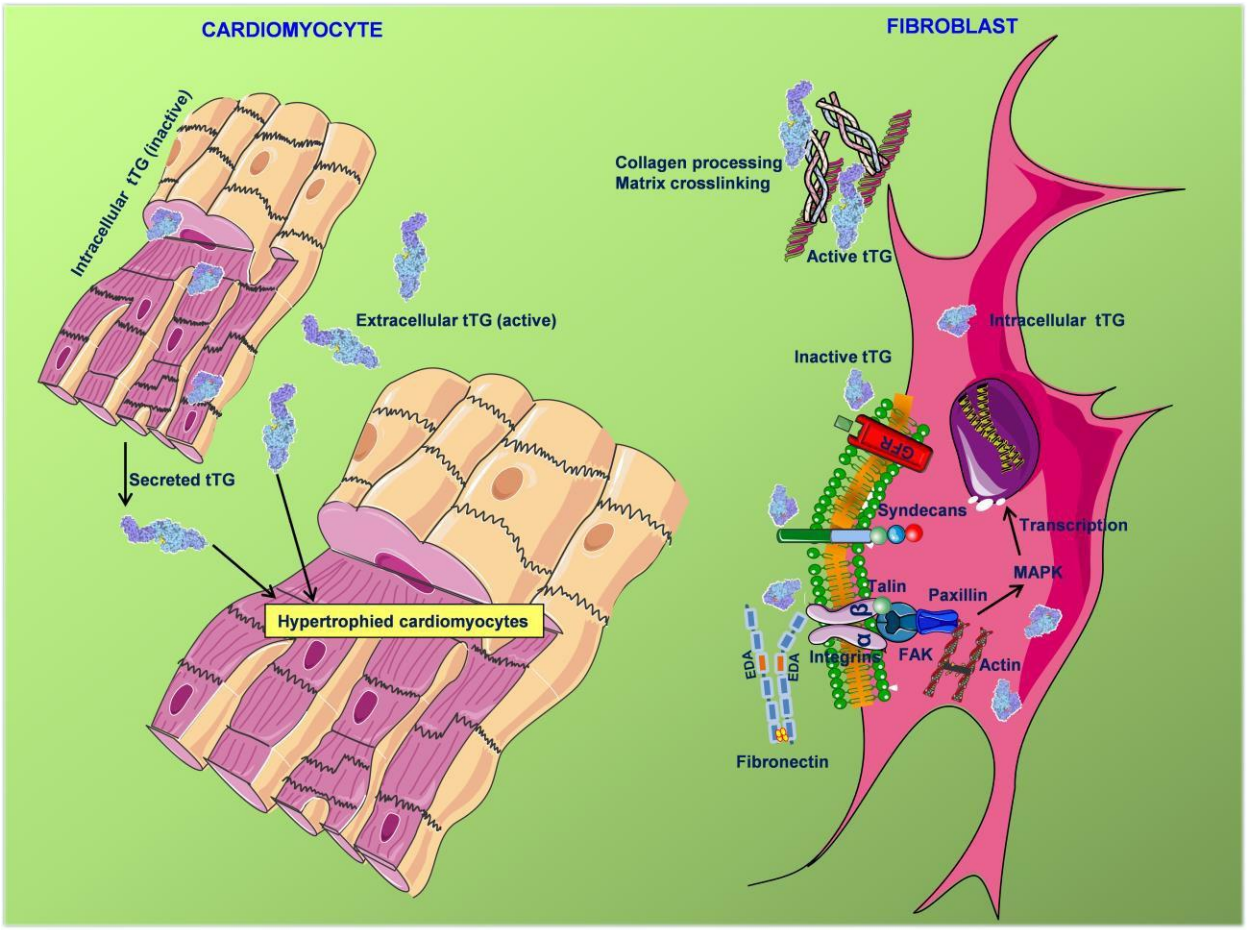

Figure 9

HIGHLIGHTS:

- Tissue transglutaminase (tTG) is activated in the pressure -overloaded myocardium.
- tTG inhibition attenuates diastolic dysfunction, and reduces hypertrophy and fibrosis.
- In vivo, inhibition of the enzymatic actions of tTG markedly reduces collagen synthesis.
- In vitro, recombinant tTG modulates MMP and TIMP mRNA through non-enzymatic actions.
- Enzymatic actions of tTG increase pericellular collagen thickness and may be invol ved in collagen processing.

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EDITORIAL FOCUS

Galectin-3 in the pathogenesis of heart failure: a causative mediator or simply a biomarker?

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First described over three and a half decades ago, galectin-3 has gone by many names and is found in many tissue types. Encoded by a single gene, *LGALS3*, galectin-3 is a 30-kDa -galactoside-binding lectin predominantly located in the cytoplasm and the extracellular space. Galectin-3 expression is markedly upregulated in injured and remodeling tissues and is predominantly expressed by infiltrating macrophages and a subset of activated fibroblasts and vascular cells, serving as a marker of inflammation and fibrosis. Because of its consistent association with tissue injury, regardless of the affected organ or etiology, galectin-3 has been extensively studied as a biomarker in a vast array of diseases, with one review literally describing it as a biomarker for everything from A, asthma, to Z, zoster-related pain, including atherosclerosis, liver and kidney fibrosis, cutaneous inflammation, and cancer (7). Galectin-3 expression is increased in the failing and remodeling myocardium, and circulating galectin-3 has been suggested as a prognostic biomarker in patients with heart failure (9). Although galectin-3 has been implicated in the regulation of inflammation, angiogenesis, and fibrosis in many tissues, whether it plays a crucial role in the pathogenesis of adverse cardiac remodeling in failing hearts remains controversial. Robust documentation of a causative role for endogenous galectin-3 in the pathogenesis of myocardial fibrosis, hypertrophy, and dysfunction could identify a promising new therapeutic target for patients with heart failure.

To explore the role of galectin-3 in heart failure, in a recently published article in the *American Journal of Physiology-Heart and Circulatory Physiology*, Nguyen et al. (6) used both genetic and pharmacological loss of function approaches in a mouse $_2$-adrenoceptor overexpression model of cardiomyopathy (6). In agreement with previous studies, the authors observed a robust progressive increase in galectin-3 mRNA and protein levels during the 6 mo in which the mice developed cardiomyopathy. However, in contrast to other studies in heart failure models, pharmacological inhibition of galectin-3 with *N*-acetyllactosamine (*N*-Lac; 5 $mg \cdot kg^{-1} \cdot day^{-1}$ ip, 3 times/wk for 3 wk) or with modified citrus pectin (MCP) and genetic removal of the galectin-3 gene in a knockout mouse did not prevent cardiomyopathy. Specifically, the authors observed no improvement in fibrosis, dysfunction, or remodeling by echocardiography or in inflammatory and fibrotic gene profiles (although *N*-Lac alone had a mild suppressive effect on a handful of profibrotic genes). As both the pharmacological and genetic inhibition occurred before the development of the cardiomyopathy, the findings suggest that galectin-3 loss was unable to prevent, much less reverse, dysfunction in this model.

These negative results contribute to an ongoing controversy in the field regarding the role of endogenous galectin-3 in cardiac remodeling and the potential benefits of galectin-3 inhibition in heart failure. Several experimental studies in models of heart failure have suggested that galectin-3 loss may be beneficial, although Nguyen et al. (6) are not alone in seeing little to no advantage. Table 1 shows the major studies that examine the effect of genetic and/or pharmacological galectin-3 inhibition in clinically relevant experimental models of heart failure. The study by Yu et al. (10) used pharmacological inhibition of galectin-3 with *N*-Lac at the same dose and timing as used by Nguyen et al. (although for 1 wk longer) and genetic loss of function experiments in mice subjected to cardiac pressure overload through transverse aortic constriction (TAC). Both studies used only this single dose of *N*-Lac, so a full dose-response curve is still absent. This particular inhibitor works by binding to the carbohydrate recognition domain that is responsible for binding to -galactosides, so if galectin-3 has signaling effects through other domains, they may not be affected. Yu et al. found that in mice that underwent TAC protocols and in hypertensive transgenic Ren2 rats, galectin-3 inhibition was beneficial, reducing fibrosis and improving function. Furthermore, in both mouse and rat models of aldosterone-induced cardiac fibrosis, galectin-3 loss attenuated fibrotic changes and reduced cardiac dysfunction (2, 4).

In contrast, Frunza et al. (3) found no significant effects of global genetic galectin-3 loss on survival, cardiac fibrosis, and dysfunction after pressure overload. Although the pressure overload protocol was similar to the Yu et al. (10) study and the same galectin-3 knockout mouse line was used, several differences in experimental design (such as sex and age of the mice studied, analysis of different time points, and differences in the intensity of the pressure load) may explain the conflicting observations. Further complicating the issue, Frunza et al. discovered galectin-3 expression in atrial myocytes at baseline and in a subset of ventricular myocytes after pressure overload in a mosaic pattern specifically adjacent to areas of fibrosis. The significance and potential role of galectin-3 expression by cardiomyocytes were not explored in this study and remain

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Table 1. *Studies investigating the role of galectin-3 in clinically relevant models of heart failure*

Experimental Models	Sex and Sample Size	Strategy Used	Functional Effects of Galectin-3 Loss	Histological and Molecular Effects	Reference
1) Mouse model of angiotensin II infusion *2*) Mouse model of pressure overload induced through TAC (end points studied after 28 days) *3*) Ren2 rat model of heart failure	Male WT and galectin-3 KO mice (n = 5–12 mice/group); male Ren2 rats (n = 5–12 rats/group)	*1*) Pharmacological inhibition with *N*-acetyllactosamine (5 mg/kg ip 3 times/wk) *2*) Genetic loss (global galectin-3 KO mice)	Genetic loss of galectin-3 attenuated systolic and diastolic dysfunction in both angiotensin II infusion and TAC models; similar protective effects were noted in Ren2 rats that received galectin-3 inhibitor	Both genetic disruption and pharmacological inhibition attenuated cardiac fibrosis	10
1) Rat and mouse models of experimental hyperaldosteronism (aldosterone/salt-induced cardiac fibrosis) *2*) SHRs	Male Wistar rats (n = 9–10 rats/group); male SHRs (n = 8 rats/group); male WT and galectin-3 KO mice (n = 7 mice/group)	*1*) Pharmacological inhibition with oral MCP (100 $mg \cdot kg^{-1} \cdot day^{-1}$) *2*) Genetic loss (galectin-3 KO mice)	Effects on cardiac function were not studied	Galectin-3 inhibition reduced inflammation and attenuated fibrosis in hyperaldosteronism models and in SHRs; genetic loss of galectin-3 attenuated inflammation in the hyperaldosteronism model	4
Rat and mouse models of experimental hyperaldosteronism (aldosterone/salt-induced cardiac fibrosis)	Male Wistar rats (n = 9–10 rats/group); male WT and galectin-3 KO mice (n = 7 mice/group)	*1*) Pharmacological inhibition with oral MCP (100 $mg \cdot kg^{-1} \cdot day^{-1}$) *2*) Genetic disruption (galectin-3 KO mice)	Galectin-3 inhibition attenuated the hypertensive response and reduced cardiac hypertrophy and diastolic dysfunction	Both galectin-3 inhibition and genetic loss attenuated renal and cardiac fibrosis in response to aldosterone	2
Rat model of obesity-related cardiomyopathy induced through administration of a high-fat diet (6 wk)	Male Wistar rats (n = 8 rats/group)	Oral MCP (100 $mg \cdot kg^{-1} \cdot day^{-1}$)	Galectin-3 inhibition had no significant effects on left ventricular wall thickness, chamber dimensions, or systolic function; diastolic function was not studied	Galectin-3 inhibition did not affect cardiomyocyte hypertrophy but attenuated myocardial inflammation and fibrosis and reduced oxidative stress	5
Mouse model of pressure overload induced through TAC (end points studied after 7, 28, and 56 days of TAC)	Male and female WT and galectin-3 KO mice (C57BL/6J strain, n = 10−20 mice/group)	Galectin-3 KO mice	Galectin-3 loss did not affect survival or systolic or diastolic function but was associated with accelerated hypertrophy; female galectin-3 KO mice had delayed chamber dilation	Galectin-3 loss did not affect fibrosis and cardiomyocyte hypertrophy in the pressure-overloaded heart	3
Mouse model of isoproterenol-induced heart failure	Male mice (n = 10−12 mice/group)	Oral MCP (100 $mg \cdot kg^{-1} \cdot day^{-1}$)	Galectin-3 inhibition attenuated systolic dysfunction and dilative remodeling in isoproterenol-treated animals	Galectin-3 inhibition reduced cardiomyocyte hypertrophy, diminished inflammation, and decreased interstitial fibrosis	8
Rat model of supravalvular aortic constriction (end points studied after 6 wk)	Male adult Wistar rats (n = 7 rats/group)	Oral MCP (100 $mg \cdot kg^{-1} \cdot day^{-1}$)	Galectin-3 inhibition abrogated the reduction in left ventricular end-diastolic diameter observed in pressure-overloaded hearts without affecting wall thickness and ejection fraction	Galectin-3 inhibition attenuated cardiomyocyte hypertrophy, decreased fibrosis, diminished inflammatory gene expression, and attenuated the increase in cardiac brain natriuretic peptide and atrial natriuretic peptide levels	1

KO, knockout; MCP, modified citrus pectin; SHRs, spontaneously hypertensive rats; TAC, transverse aortic constriction; WT, wild type.

unclear. In broad agreement with other studies, however, Frunza et al. also observed a robust increase in macrophage galectin-3 expression in the TAC model.

What is the basis for conflicting findings in studies that have examined the role of galectin-3 in heart failure? In some cases, contrasting conclusions between studies reflect different interpretations of experimental findings. For example, in two studies, the effects of galectin-3 inhibition on cardiac remodeling are supported exclusively by histological data, despite the absence of significant differences in functional end points (1, 5). In other cases, differences in protocol standardization, the timing of disease progression in various models, challenges related to dosing, delivery, and off-target effects of pharmacologic inhibitors, and sex-specific actions may explain disparate findings. Inconsistencies between studies using different models of heart failure may reflect context-dependent actions of galectin-3. The cell biological basis for heart failure in each model may be a major determinant of the relative significance of galectin-3. Considering its well-documented effects on fibroblast and immune cell phenotype, galectin-3 may play more important roles in heart failure associated with prominent inflammation and fibrosis. In contrast, the effects of galectin-3 may be less prominent in the genetic model of cardiomyocyte-specific β_2-adrenergic receptor overexpression used by Nguyen et al. (6).

It should be emphasized that negative studies, such as that by Nguyen et al. (6), are important for revealing the dynamic topography of a subject. Unfortunately, studies with negative findings often remain unpublished. Not surprisingly, investigators with negative data cannot attract funding and are often discouraged from pursuing their research. These patterns of funding and publication create a bias against negative studies, leading to selective publication of positive investigations that can develop an oversimplified view of a field. This typically creates an overly optimistic perspective of the potential impact of new therapies, often followed by painful translational challenges and failures. Publication of all rigorous investigations, both positive and negative, is critical to paint a more realistic translational landscape.

Human heart failure exhibits remarkable pathophysiological heterogeneity and cannot be recapitulated by a single animal model. Just as one animal study is not sufficient to predict the effectiveness of a therapeutic approach in human patients, the article by Nguyen et al. (6) does not mean that galectin-3 inhibition will not work. What it does, however, is to document that in a cardiomyopathy caused by an exaggerated β_2-adrenergic response, galectin-3 is not critical for dysfunction, adverse remodeling, and progression of disease. From a translational perspective, this type of information is valuable for identification of pathophysiologically distinct patient subpopulations that may be unlikely to respond to galectin-3 inhibition. Clinical trials rarely get do-overs if the patient population originally targeted does not respond significantly. Qualifying (negative) studies, those that find the circumstances where an approach does not work, are critical for identifying which therapeutic strategies deserve further development and which ones should be abandoned.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.A.K. and N.G.F. drafted manuscript; J.A.K. and N.G.F. edited and revised manuscript; J.A.K. and N.G.F. approved final version of manuscript.

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EDITORIAL COMMENT

The Role of Macrophages in Nonischemic Heart Failure*

Bijun Chen, MD, PhD, Nikolaos G. Frangogiannis, MD

The mammalian heart contains a population of resident macrophages that expands in response to injury through the recruitment of circulating monocytes (1). After myocardial infarction, the sudden death of a large number of cardiomyocytes releases danger signals, mobilizing monocytes in the bone marrow and possibly also in extramedullary sites, such as the spleen. Induction of CC chemokines, such as CC motif chemokine ligand 2, in the infarcted myocardium recruits abundant monocytes that express the chemokine receptor CCR2, and exhibit a proinflammatory phenotype (2,3). $CCR2^{b}$ monocytes and macrophages play an important role in the clearance of necrotic cardiomyocytes and in cardiac repair after infarction, but also contribute to the pathogenesis of adverse cardiac remodeling (2). Although macrophages have been extensively implicated in the reparative and remodeling responses to myocardial infarction, their role in chronic nonischemic heart failure remains unclear. In mouse models of heart failure associated with left ventricular pressure overload, and in heart failure with preserved ejection fraction patients, the density of myocardial macrophages is significantly increased (4,5). However, the mechanisms of recruitment and activation of cardiac macrophages in response to chronic pressure overload and their functional role in the development of heart failure are poorly understood.

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In this issue of JACC: Basic to Translational Science, Patel et al. (6) demonstrate an important role for $CCR2^{b}$ macrophages in the pathogenesis of fibrosis, hypertrophy, and cardiac dysfunction in the pressure-overloaded heart. In a mouse model of transverse aortic constriction, a significant increase in circulating $Ly6C^{hi}CCR2^{b}$ monocytes was associated with a marked expansion of myocardial $CCR2^{b}$ macrophages, driven by cardiac induction of CC chemokines. Blocking CCR2 through early administration of the small molecule CCR2 antagonist RS-504393, or through treatment with the anti-CCR2 monoclonal antibody MC21, inhibited myocardial infiltration with $CCR2^{b}$ macrophages, abrogated the expansion of T cells in heart-draining mediastinal lymph nodes, and reduced left ventricular hypertrophy after 1 week of pressure overload. Moreover, 4 weeks after transverse aortic constriction, MC21-treated mice exhibited significantly attenuated systolic dysfunction and profoundly reduced cardiac interstitial fibrosis, in comparison to immunoglobulin G-treated animals.

The study adds to a growing body of evidence suggesting that chemokine-driven, macrophage-mediated inflammation plays a critical role in the pathogenesis of pressure overload-induced heart failure. The findings not only support the significance of $CCR2^{b}$ macrophages as cellular effectors of myocardial fibrosis, hypertrophy, and dysfunction in nonischemic heart failure, but also open new research directions by raising several important questions.

WHAT IS THE STIMULUS FOR THE EXPANSION OF MACROPHAGES IN PRESSURE-OVERLOADED HEARTS?

In conditions associated with extensive cellular necrosis, the release of danger signals mobilizes bone

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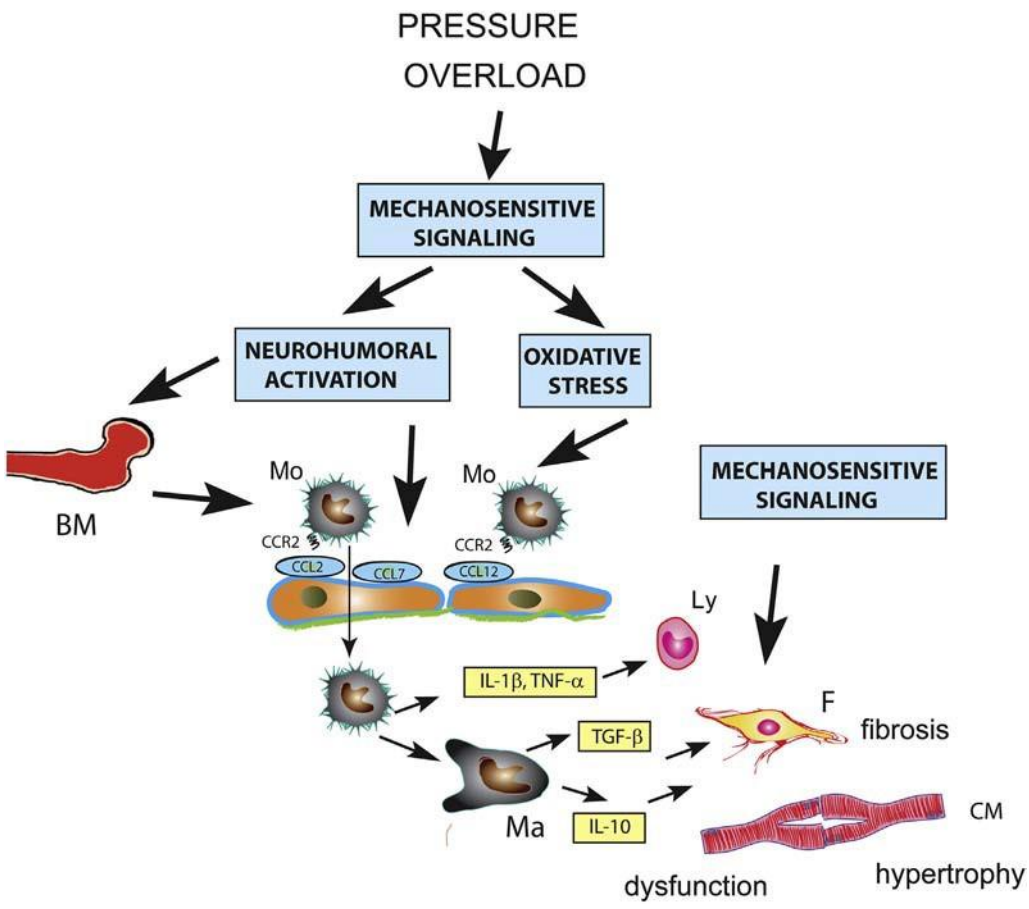
FIGURE 1 Expansion and Activation of Macrophages in the Pressure-Overloaded Myocardium



Pressure overload activates neurohumoral pathways and increases oxidative stress, mobilizing bone marrow (BM)-derived monocytes (Mo). Angiotensin II- and reactive oxygen species-triggered induction of chemokines in the pressure-overloaded myocardium mediates recruitment of monocytes expressing CCR2. $CCR2^{b}$ monocytes secrete proinflammatory cytokines (such as IL-1b and tumor necrosis factor-a) and may activate lymphocytes (Ly), causing dysfunction of the remodeling heart. Monocytes may differentiate into macrophages (Ma) that secrete transforming growth factor-bs and IL-10, activating fibroblasts (F) and promoting cardiomyocyte (CM) hypertrophy.

marrow monocytes and triggers intense chemokine-driven inflammation (7). However, in the pressure-overloaded myocardium, cardiomyocyte necrosis is limited, and the molecular link between mechanical stress and monocyte mobilization is unclear. Several mechanisms may explain the observed induction of chemokines and subsequent infiltration of the pressure-overloaded myocardium with proinflammatory macrophages (Figure 1). First, activation of neurohumoral cascades may mobilize bone marrow and splenic monocytes and may upregulate inflammatory signaling in the myocardium. Angiotensin II is known to activate proinflammatory signaling in both vascular cells and monocytes, acting at least in part through chemokine induction (8). Second, increased oxidative stress in the pressure overloaded myocardium, triggered through neurohumoral activation or via activation of mechanosensitive signaling pathways, may promote chemokine-mediated recruitment of inflammatory cells (9). Third, mechanosensitive signaling may exert direct proinflammatory effects on resident myocardial cells (including cardiomyocytes, fibroblasts, and vascular cells), triggering local induction of cytokines and chemokines that mediate recruitment of monocytes (10). Thus, cellular necrosis is not necessary for the activation of an inflammatory program in the remodeling myocardium.

DO THE ACTIONS OF THE CC CHEMOKINE/CCR2 AXIS INVOLVE EXCLUSIVELY EFFECTS ON MACROPHAGES?

The authors suggest that the protective effects of CCR2 blockade may involve actions on monocytes and macrophages. Although this is plausible, it should be emphasized that CCR2 expression has also been reported in many other cell types, including lymphocyte subsets, fibroblasts, and vascular cells. Several in vitro studies have suggested direct effects of CCR2 ligands on fibroblast phenotype and gene expression (11); however, evidence supporting the notion that chemokine-driven myocardial fibrosis is mediated through direct actions on cardiac fibroblasts is lacking (12). The crucial role of lymphocyte subpopulations in cardiac fibrosis (13), and the involvement of the CC motif chemokine ligand 2/CCR2 axis in regulation of lymphocyte responses (14) suggest that the effects of CCR2 blockade may be mediated at least in part, through the modulation of lymphocyte recruitment and activation.

WHAT IS THE MOLECULAR BASIS FOR THE EFFECTS OF CCR2^{D} MACROPHAGES IN THE PRESSURE-OVERLOADED HEART?

Macrophages exhibit remarkable functional heterogeneity and are capable of expressing a wide range of genes. After tissue injury, dynamic microenvironmental changes induce dramatic alterations in the phenotypic characteristics of macrophages. In the infarcted myocardium, early release of danger signals induces a proinflammatory and phagocytotic macrophage phenotype. The ingestion of apoptotic cells has been proposed to trigger a regulatory phenotype in macrophages, inducing expression of anti-inflammatory mediators (such as interleukin [IL]-10 and transforming growth factor-b). Fibrogenic and angiogenic macrophage subsets have also been identified and may play an important role in repair of the infarcted heart. Moreover, macrophages may also contribute to extracellular matrix remodeling by producing matrix metalloproteinases and their inhibitors.

The molecular signals that may mediate the profibrotic and hypertrophic actions of macrophages in the pressure-overloaded myocardium remain unknown. The current study suggests that macrophage-driven T-cell expansion may play an important role in adverse remodeling; the specific proinflammatory cytokines that may mediate lymphocyte activation are unclear. The direct effects of macrophage-derived mediators on fibroblast activation may also be involved. A recent study suggested that IL-10 secreted by myeloid cells may be an important fibrogenic signal in experimental models of cardiac fibrosis (5). Macrophage-derived secretion of transforming growth factor-b family members may also contribute to activation of a fibrogenic program. Macrophages may also promote fibrosis by releasing tissue inhibitors of metalloproteinases, thus exerting potent matrix-preserving actions (15). Considering the wide range of mediators secreted by macrophages, it is unlikely that the profibrotic actions of CCR2^{b} cells are mediated through the release of a single molecular signal.

IS CHEMOKINE-DRIVEN INFLAMMATION A THERAPEUTIC TARGET IN HUMAN HEART FAILURE?

The protective effects of CCR2 blockade reported in the current study may have direct therapeutic implications for patients with heart failure. On the basis of these experimental observations, it could be proposed that CCR2 inhibition may attenuate myocardial dysfunction and inhibit fibrosis in patients with heart failure with a prominent pressure overload pathophysiology. It should be emphasized that, although this approach may seem to be promising, therapeutic translation in human heart failure poses major challenges. Human heart failure is pathophysiologically heterogeneous and cannot be recapitulated by a single animal model. Successful translation may require the identification of patient subpopulations with a prominent inflammatory response that may benefit from inhibition of the chemokine axis. Moreover, the need for chronic treatment generates major concerns regarding the risk profile of the approach. Chronic administration of an anti-inflammatory agent may carry significant risks by inhibiting reparative and protective effects of inflammation. CCR2 signaling has been implicated in the activation of the angiogenic process (16); thus, inhibition of the CC chemokine/CCR2 axis may also perturb angiogenic responses, necessary to preserve perfusion in subjects with coexisting ischemic heart disease. Studies aimed at understanding the involvement of cardiac macrophages in human patients are critical to explore the potential value of chemokine targeting in heart failure.

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Editorial

Targeting mitochondrial calcium transport in myocardial infarction

Contraction of each cardiomyocyte is dependent on the generation of a ventricular action potential by ion currents. At the organ level, normal ventricular function requires coordinated propagation of an electrical impulse in the myocardium. Many myocardial diseases perturb generation or conduction of the electrical impulse, thereby causing an increased incidence of silent or life-threatening arrhythmias. Ventricular tachyarrhythmias represent a potentially fatal complication after myocardial infarction. In the early stages of myocardial ischemia, sudden cessation of blood flow results in ATP depletion, acidosis, and dramatic ionic alterations, thus favoring arrhythmogenesis.[1,2] Moreover, adrenergic activation enhances the automaticity of ischemic Purkinje fibers, thus contributing to a highly arrhythmogenic substrate. As the infarct heals, structural remodeling of the ventricle and development of fibrosis contribute to arrhythmia generation by disrupting impulse propagation and by promoting re-entry. Despite the high clinical significance of ischemic arrhythmic events, the underlying mechanistic basis remains poorly understood and specific pharmacologic treatment options are limited.

A increasing body of evidence suggests an important role for preserved mitochondrial function in maintaining normal electrical activity of the heart.[3,4] In adult cardiomyocytes, mitochondria occupy more than 30% of the cellular volume and are necessary to generate the massive amounts of adenosine triphosphate (ATP) required for contraction. Mitochondria also generate reactive oxygen species (ROS) and contribute to the regulation of the ionic equilibrium in cardiomyocytes. Myocardial ischemia perturbs all major mitochondrial functions, thereby disrupting ATP generation and increasing production of ROS. ATP depletion profoundly affects the electrical activity of cardiomyocytes by altering ionic fluxes. Moreover, the accumulation of ROS may induce oxidative changes in mitochondrial ion channels, further accentuating the electrophysiological disturbance. Despite their potential significance in arrhythmogenesis, the role of mitochondria as a potential therapeutic target in ischemia-associated arrhythmias remains underappreciated.

In the current issue of the journal, Sventzouri and co-workers[5] from Dr Malliaras' laboratory tested the hypothesis that targeting mitochondrial calcium handling may have protective effects following myocardial infarction. Using a translationally relevant porcine model of reperfused myocardial infarction, the authors examined the effects of pharmacologic inhibition of the mitochondrial Na^{+}/Ca^{2+} exchanger (mNCX), the main mitochondrial channel responsible for Ca^{2+} efflux. Intracoronary infusion of the mNCX inhibitor CGP-37157 before the onset of ischemia, or 5 minutes before reperfusion, was well tolerated but had no significant effects on the size of the infarct and the no-reflow area. However, treatment before coronary occlusion protected the animals against ventricular arrhythmias, thus suppressing the incidence of events and reducing the number of defibrillations required for cardioversion. On the other hand, infusion of the mNCX inhibitor at the end of coronary occlusion had no effects on arrhythmic events (in part, due to the absence of ventricular arrhythmias in the control group) but accelerated the resolution of ST-segment changes. The findings provide interesting but preliminary large animal model-based evidence in support of the potential therapeutic effectiveness of mNCX inhibition in myocardial infarction. Moreover, the observations open important new directions for future research and raise several intriguing questions.

1. The role of the mNCX in mitochondrial calcium handling

There is broad consensus regarding the central role of intramitochondrial $Ca2+$ in cardiac physiology and pathophysiology. Mitochondrial $Ca2+$ regulates energy production, has a major effect on whole cell $Ca2+$ signaling, and plays a critical role in cell survival.[6e8] Two major pathways are involved in the regulation of mitochondrial $Ca2+$ transport (Fig. 1). The mitochondrial $Ca2+$ uniporter (mCU) controls $Ca2+$ influx,[9] whereas the mNCX is an electrogenic $3Na+:Ca2+$ exchanger[10] that functions as the main channel involved in $Ca2+$ extrusion. It should be noted that a reverse mode of function has been described for mNCX under conditions of metabolic inhibition and may be important in disease states, extruding $Na+$ in exchange for $Ca2+$ influx.[11] The significance of the reverse mode in myocardial ischemia has not been studied.

2. Is there sufficient evidence to support protective effects of mNCX inhibition in myocardial ischemia?

Most of our information on the role of mitochondrial $Ca2+$ transporters in the ischemic heart is derived from investigations that used pharmacologic inhibitors. Unfortunately, these agents lack specificity. Diltiazem has been suggested to exert protective actions on the ischemic heart[12]; however, its effects are not limited to mNCX inhibition and may be predominantly mediated through the modulation of sarcolemmal L-type $Ca2+$ channels. On the other hand, CGP-37157, the clonazepam derivative used to inhibit the mNCX in the current study, is considered relatively specific but has been associated with spontaneous $Ca2+$ oscillations when used in isolated rat cardiomyocytes.[12] Although the current investigation represents an important step in the attempt toward clinical

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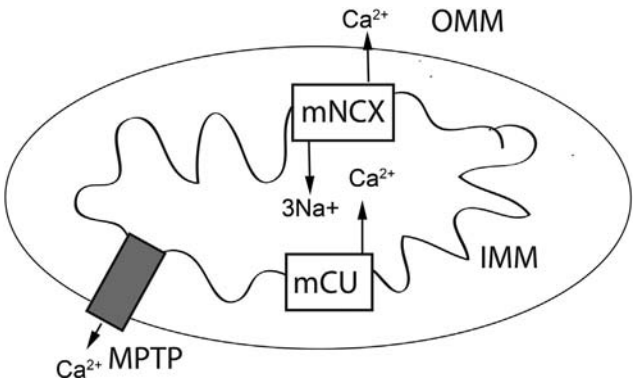

Fig. 1. Calcium transport in the mitochondria of cardiomyocytes is regulated by the mitochondrial calcium uniporter (mCU, influx) and the mitochondrial $Na^{þ}/Ca^{2þ}$ exchanger (mNCX, efflux). The mitochondrial permeability transition pore (MPTP) remains closed under physiological conditions but can act a nonselective $Ca^{2þ}$ efflux pathway when open. Perurbations in intramitochondrial $Ca^{2þ}$ dynamics are involved in cardiomyocyte death, dysfunction, and in the generation of arrhythmias (see text).

translation, the evidence provided to support the effectiveness of the agent is rather preliminary. Antiarrhythmic effects could be documented only in animals treated with the mNCX inhibitor before coronary occlusion, a protocol of relatively limited practical therapeutic value. The protective effects of the inhibitor in pigs treated before reperfusion were limited to accelerated resolution of ST segments, an endpoint that is commonly studied to assess the effectiveness of reperfusion strategies in human patients,[13] but is of unclear pathophysiological significance in animal model investigations. A systematic study of ventricular and electrophysiological function was not been performed. Moreover, the exclusive focus of the study on a single time point (2 h after reperfusion) further limits conclusions regarding the protective actions of this approach. Additional studies will be needed to examine whether treatment with the mNCX inhibitor represents a viable therapeutic approach in myocardial ischemia and infarction.

A recently published study[14] generated a conditional loss of function model to investigate the role of the mNCX in the myocardium. Cardiomyocyte-specific deletion of mNCX in adult mice was associated with lethality due to fulminant heart failure. The phenotype was attributed to calcium overload-induced necrotic cell death. Moreover, cardiac-specific overexpression of mNCX protected against cardiomyocyte death and heart failure in a mouse model of reperfused myocardial infarction.[14] These findings demonstrated an important role for mNCX in cardiomyocyte homeostasis, survival, and function and may suggest that augmenting Ca2þ efflux may be protective following myocardial ischemia. The conflicting findings between the genetic study in rodents[14] and the large animal investigation by Sventzouri et al.[5] raise important concerns regarding the potential therapeutic role of mNCX targeting. How can we explain the contrasting observations? First, the protective effects of CGP-37157 treatment may be due to actions independent of mNCX inhibition. In contrast to the specificity of genetic loss-and gain-of-function strategies, pharmacological approaches are remarkably nonselective; yet unidentified actions of CGP-37157 may account for the protective effects. Second, the findings may reflect the requirement for baseline levels of mNCX function to maintain cardiac homeostasis. Complete abolition of the exchanger in cardiomyocytes (achieved through genetic targeting in mice) may be catastrophic; however, partial mNCX inhibition under ischemic conditions (presumably achieved upon administration of the pharmacological inhibitor in the porcine model) may be protective, hence exerting anti-arrhythmic actions. Third, species-specific effects and the limitations of the infarction models used may contribute to the contrasting observations. Clinically relevant large animal models of myocardial infarction exhibit a profile of postinfarction arrythmogenesis comparable to the human disease process and provide essential information for therapeutic translation. Translational research requires a combination of animal model strategies: mouse models are critical to dissect the cell biological mechanisms, whereas large animal models contribute clinically relevant insights.

3. What is the mechanistic basis for the protective effects of mNCX inhibition?

Considering the broad effects of mitochondrial $Ca^{2þ}$ on cardiomyocyte survival, function, and ionic equilibrium, any protective effects of mNCX inhibition following myocardial ischemia may involve several potential cellular mechanisms. First, restoration of mitochondrial Ca2þ dynamics may reduce cardiomyocyte death by modulating the function of the mitochondrial permeability transition pore (MPTP), a protein with a critical role in regulation of cell survival. Unfortunately, the current study did not systematically examine potential effects of the mNCX inhibitor on cardiomyocyte death. The absence of significant effects on acute infarct size may reflect an underpowered study design, or analysis at a very early time point that is not sufficient to accurately assess cardiomyocyte death. Second, a favorable intramitochondrial Ca2þ environment may enhance ROS scavenging, thus protecting from the pro-arrhythmic effects of oxidative stress. Third, inhibition of the mNCX may stabilize the intracellular ionic dynamics in ischemic cardiomyocytes, thereby exerting direct anti-arrhythmic effects by preventing prolongation of the action potential.

4. Could mNCX inhibition be effective in other myocardial diseases?

The pro-arrhythmic effects of mitochondrial dysfunction are not limited to the setting of acute myocardial ischemia.[15] In a guinea pig model of heart failure and sudden cardiac death induced through combined left ventricular pressure overload and þ-adrenergic stimulation, treatment with the mNCX inhibitor CGP-37157 was reported to reduce mortality, thereby preventing fatal ventricular fibrillation and protecting from decompensated heart failure.[16] Whether mNCX inhibition affords antiarrhythmic protection in translationally relevant large animal models of heart failure remains unknown.

5. The challenges of clinical translation

Promising early evidence suggests potential protective actions of mNCX inhibitors in animal models of acute myocardial infarction[5] and chronic heart failure.[16] On the other hand, emerging concepts derived from genetic studies in mice[14] suggest an important role for mNCX in cardiac homeostasis and function. The basis for the conflicting messages is unclear. Further progress in the field will require the acquisition of mechanistic insights with rodent models and characterization of the pharmacological inhibitors to understand the basis for their protective effects. Despite their high cost and descriptive nature, well-executed large animal investigations, like the one performed by Sventzouri and co-workers,[5] are urgently needed to evaluate the effectiveness of promising new antiarrhythmic approaches in the infarcted and remodeling heart.

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Disclosures

None.

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RESEARCH ARTICLE | *Integrative Cardiovascular Physiology and Pathophysiology*

Characterization of a mouse model of obesity-related fibrotic cardiomyopathy that recapitulates features of human heart failure with preserved ejection fraction

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Alex L, Russo I, Holoborodko V, Frangogiannis NG. Characterization of a mouse model of obesity-related fibrotic cardiomyopathy that recapitulates features of human heart failure with preserved ejection fraction. *Am J Physiol Heart Circ Physiol* 315: H934–H949, 2018. First published July 13, 2018; doi:10.1152/ajpheart.00238.2018.—Heart failure with preserved ejection fraction (HFpEF) is caused, or exacerbated by, a wide range of extracardiac conditions. Diabetes, obesity, and metabolic dysfunction are associated with a unique HFpEF phenotype, characterized by inflammation, cardiac fibrosis, and microvascular dysfunction. Development of new therapies for HFpEF is hampered by the absence of reliable animal models. The leptin-resistant *db*/*db* mouse has been extensively studied as a model of diabetes-associated cardiomyopathy; however, data on the functional and morphological alterations in *db*/*db* hearts are conflicting. In the present study, we report a systematic characterization of the cardiac phenotype in *db*/*db* mice, focusing on the time course of functional and histopathological alterations and on the identification of sex-specific cellular events. Although both male and female *db*/*db* mice developed severe obesity, increased adiposity, and hyperglycemia, female mice had more impressive weight gain and exhibited a modest but significant increase in blood pressure. *db*/*db* mice had hypertrophic ventricular remodeling and diastolic dysfunction with preserved ejection fraction; the increase in left ventricular mass was accentuated in female mice. Histological analysis showed that both male and female *db*/*db* mice had cardiomyocyte hypertrophy and interstitial fibrosis, associated with marked thickening of the perimysial collagen, and expansion of the periarteriolar collagen network, in the absence of replacement fibrosis. In vivo and in vitro experiments showed that fibrotic changes in *db*/*db* hearts were associated with increased collagen synthesis by cardiac fibroblasts, in the absence of periostin, a-smooth muscle actin, or fibroblast activation protein overexpression. Male *db*/*db* mice exhibited microvascular rarefaction. In conclusion, the *db*/*db* mouse model recapitulates functional and histological features of human HFpEF associated with metabolic dysfunction. Development of fibrosis in *db*/*db* hearts, in the absence of myofibroblast conversion, suggests that metabolic dysfunction may activate an alternative profibrotic pathway associated with accentuated extracellular matrix protein synthesis.

NEW & NOTEWORTHY We provide a systematic analysis of the sex-specific functional and structural myocardial alterations in *db*/*db* mice. Obese diabetic C57BL6J *db*/*db* mice exhibit diastolic dysfunction with preserved ejection fraction, associated with cardiomyocyte hypertrophy, interstitial/perivascular fibrosis, and microvascular rarefaction, thus recapitulating aspects of human obesity-related heart failure with preserved ejection fraction. Myocardial fibrosis in *db*/*db* mice is associated with a matrix-producing fibroblast phenotype, in the absence of myofibroblast conversion, suggesting an alternative mechanism of activation.

diabetes; diastolic dysfunction; fibroblast; fibrosis; hypertrophy

INTRODUCTION

Approximately 50% of patients with heart failure (HF) in the community have preserved ejection fraction (HFpEF). Because of the absence of effective treatment strategies, HFpEF is associated with a high mortality, a low quality of life, and a high incidence of hospitalizations (13). Development of new therapies for patients with HFpEF is hampered by the remarkable pathophysiological heterogeneity of the syndrome, which is caused or exacerbated by a wide range of cardiac, or extracardiac, comorbidities (16) and by the absence of reliable animal models that recapitulate the human condition (51). Obesity, diabetes, and metabolic dysfunction markedly increase the risk of HFpEF, independent of the occurrence of coronary artery disease (27, 38). A growing body of evidence supports the existence of a distinct obesity-associated phenotype of human HFpEF (37). Patients with "obese HFpEF" exhibit increased plasma volume, more concentric left ventricular (LV) hypertrophy, and greater right ventricular dilatation, despite lower plasma levels of natriuretic peptides (5, 37). However, the cell biological basis and molecular mechanisms responsible for the unique HFpEF phenotype associated with obesity remain unknown.

To date, treatment strategies for HFpEF are largely restricted to symptomatic relief (1). Development of new therapeutics is impeded by the lack of understanding of the cellular and molecular basis of HFpEF; our limited knowledge is often attributed to the absence of translationally relevant animal models of the disease. Considering the heterogeneity of human HFpEF and its relation with a range of comorbid conditions, designing an animal model that mimics the broad spectrum of pathophysiologies underlying the human condition is practically impossible. Instead, our focus should be to systematically characterize and understand models that recapitulate specific pathophysiological aspects of human HFpEF (42, 51). Rodent models of cardiomyopathy associated with diabetes, obesity, and metabolic dysfunction have been extensively used to

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investigate the mechanisms of HF associated with metabolic disease. Interpretation of the findings is often challenging because of differences in the observed phenotypes and is further hampered by the unclear relations between the functional changes observed in the animal model and the human pathological condition.

The *db*/*db* mouse has a point mutation in the gene encoding the leptin receptor, resulting in a protein with a truncated cytoplasmic domain that is functionally inactive (10). As a result, *db*/*db* mice are resistant to the central effects of leptin and develop a voracious appetite at an early age, leading to severe obesity and the development of overt diabetes. Although obesity, diabetes, and metabolic dysregulation in *db*/*db* mice have been associated with significant alterations in myocardial structure and function (11, 29, 46, 56), the findings are conflicting. Although some studies have reported that *db*/*db* mice exhibit a significant reduction in ejection fraction (53, 56), other investigations have suggested that *db*/*db* mice may have an early improvement in systolic function (2). In contrast, numerous other studies have shown evidence of diastolic dysfunction associated with preserved systolic function (21, 22), whereas some investigations reported no significant functional and structural differences between *db*/*db* mice and lean age-matched control mice (28). The age and strain of the experimental animals, sex-specific effects, and different methods used for functional analysis may account for the contrasting observations.

In the present investigation, we provide a systematic analysis of the functional and structural myocardial alterations in *db*/*db* mice. Our findings suggest that *db*/*db* mice in the C57BL6J background exhibit diastolic dysfunction with preserved ejection fraction, associated with cardiomyocyte hypertrophy, interstitial/perivascular fibrosis, and microvascular rarefaction, thus recapitulating aspects of human obesity-related HFpEF. Sex-specific analysis suggested that female *db*/*db* mice exhibit modest arterial hypertension, associated with accentuated hypertrophic remodeling, whereas male mice have exaggerated microvascular loss. Myocardial fibrosis in *db*/*db* mice was associated with activation of a matrix-producing fibroblast phenotype, in the absence of myofibroblast conversion, suggesting that activation of diabetic fibroblasts may involve an alternative mechanism, distinct from the cellular events leading to fibrosis in infarcted and pressure-overloaded hearts.

MATERIALS AND METHODS

Animals. Animal experiments were approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine and the Animal Protocol Review Committee of Baylor College of Medicine. We used $Lepr^{dm/+}$ on a C57BL6J background (*db*/+) and wild-type (WT) C57BL6J animals from our own colonies (originally purchased from Jackson Laboratories, Bar Harbor, ME) (4, 18). Genotyping was performed using standard protocols. Mice were fed a regular chow diet (LabDiet 5001).

Assessment of body fat content. Body composition (body fat mass) of WT and *db*/*db* mice was quantitatively assessed at 2 and 6 mo of age using nuclear magnetic resonance (EchoMRI Whole Body Composition Analyzer, Echo Medical System, Houston, TX) at the Albert Einstein College of Medicine Diabetes Research Center, Animal Physiology Core. In a separate group of WT and *db*/*db* animals, fat tissue mass was measured at 4 mo of age by dual-energy X-ray absorptiometry using the PIXImus Densitometer (Lunar, Madison, WI). Each mouse was anesthetized in a chamber for the entire procedure (5% isoflurane-95% oxygen). Once anesthetized, each mouse was placed in the densitometer in the prone position, with the limbs and tail extended. Data were analyzed with PIXImus software (2.0, GE/Lunar).

Assessment of plasma glucose. Animals used for the assessment of plasma glucose were euthanized at 2 or 6 mo of age, and blood was collected by aortic puncture in EDTA anticoagulant-coated tubes. Plasma was extracted by centrifugation at 850 *g* for 15 min at 4°C and stored at −80°C. Mice were fasted for 12 h before blood collection. Glucose levels were measured using a glucometer (One Touch Ultra, LifeScan, Milpitas, CA).

Echocardiography. For echocardiographic analysis, WT and *db*/*db* mice were imaged at 2 and 6 mo of age (29–81 mice/group). The procedures used complied with guidelines for measuring cardiac physiology in mice (34). Mice were initially anesthetized in a chamber (2% isoflurane-95% oxygen at 0.8 l/min) and afterward were supinely placed on a heating pad at 37°C on maintenance anesthesia (1.5% isoflurane). Echocardiographic assessment was performed using a Vevo770 ultrasound (Visualsonics, Toronto, ON, Canada) with a real-time microvisualization transducer (RMVB710B, 12–38 MHz at a frame rate of 110–120 frames/s) applied parasternally to the shaved chest wall. Images were taken in the parasternal long-axis, parasternal short-axis, and short-axis M-mode (SAMM). SAMM images were acquired by vertically placing the M-mode cursor at the parasternal short-axis view when both papillary muscles were visualized. Views and data were exported for offline calculation using dedicated Vevo 770 quantification software (Vevo 770 version 3.0.0). Images from SAMM were used to measure LV end-diastolic volume (LVEDV), LV mass, and ejection fraction. To compare the remodeling response between groups, we measured the global "remodeling index" (remodeling index $\propto EDV^{1/3}/t$, where t is time), an indicator of maladaptive hypertrophic remodeling that has been used for risk stratification in patients with hypertension (17). The echocardiographic offline analysis was performed by a sonographer blinded to the study groups. Echocardiographic parameters were not normalized to body weight.

Doppler echocardiography and tissue Doppler imaging. WT and *db*/*db* mice underwent Doppler echocardiography and tissue Doppler imaging (TDI) at 2 and 6 mo of age (14–35 mice/group). Images from the apical four-chamber view were acquired to assess LV filling and diastolic function. Transmitral LV inflow velocities were measured by pulsed-wave Doppler. Peak early E wave (*E*) and late A wave (*A*) filling velocities and the *E*-to-*A* ratio (*E*/*A*) were measured. TDI was obtained by placing a 1.0-mm sample volume at the medial annulus of the mitral valve. Analysis was performed for the early (e') and late (a') diastolic velocity. The mitral inflow *E* velocity-to-tissue Doppler e' wave velocity ratio (E/e') and tissue Doppler early e' velocity-to-tissue Doppler late a' velocity ratio were calculated to assess diastolic function. All Doppler spectra were recorded for three to five cardiac cycles at a sweep speed of 100 mm/s. The color Doppler preset was at a Nyquist limit of 0.44 m/s. The echocardiographic offline analysis was performed by a sonographer blinded to the study groups.

Immunohistochemistry and histology. For histopathological analysis, mice were euthanized at 6 or 12 mo of age, and hearts were fixed in Z-fix (Anatech, Battle Creek, MI) and embedded in paraffin (8 mice/group, 4 male and 4 female mice). Sequential 5-μm sections were cut by microtomy. Collagen fibers were identified by picrosirius red staining using protocols established in our laboratory (6). Endomysial collagen was semiquantitatively assessed in 10 different ×200 fields from each mouse using the following scale: *0*, normal endomysial collagen pattern; *1*, increased endomysial collagen deposition involving <50% of the field; and *2*, increased endomysial collagen deposition involving >50% of the field. The mean score was calculated for each mouse. Perimysial collagen thickness was assessed by measuring the average thickness of each perimysial strand by averaging measurements at three different points (which included the thickest and thinnest dimensions and a third random point). To assess

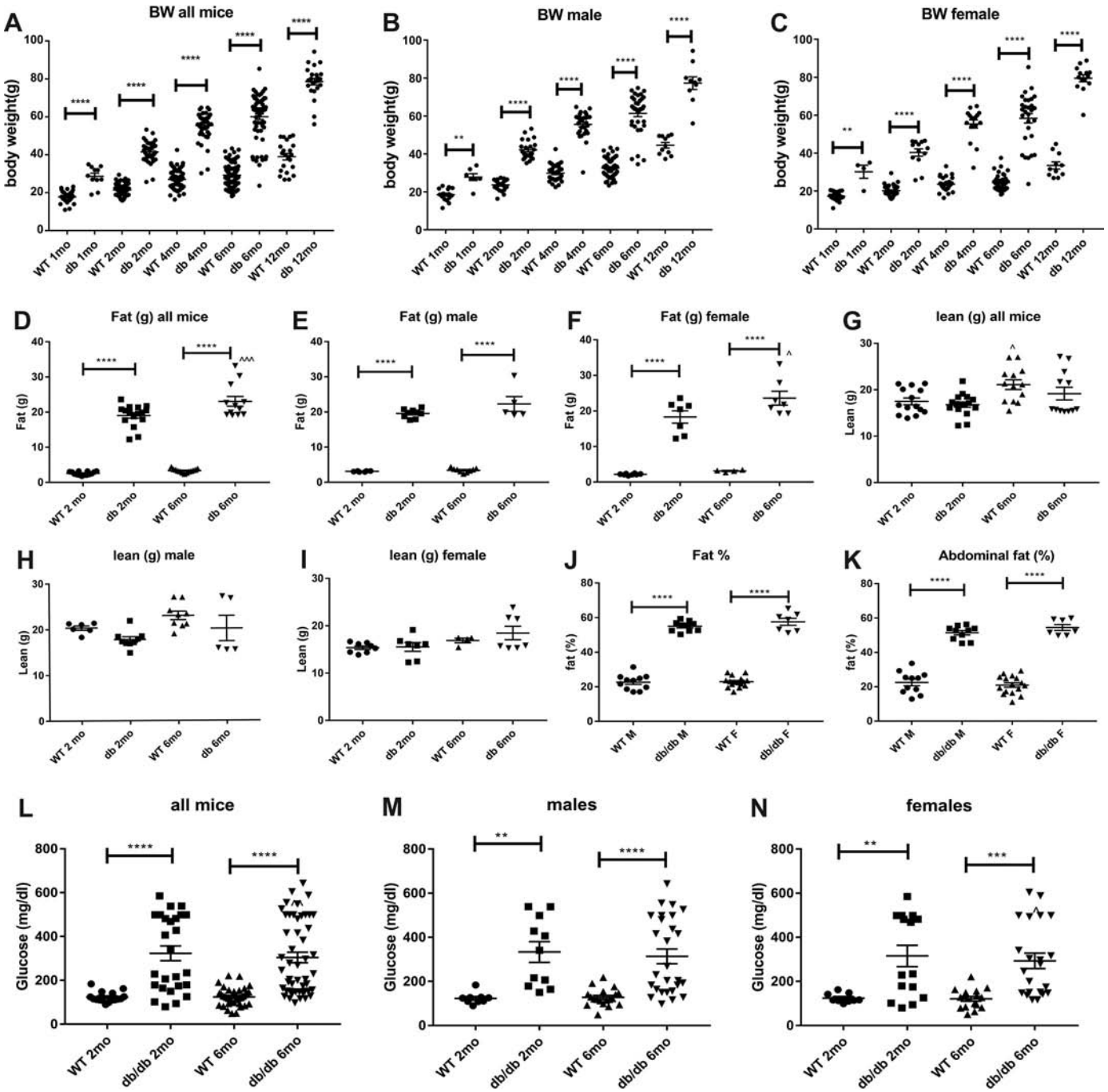


Fig. 1. Both male and female *db*/*db* (db) mice in a C57BL6J background exhibit severe obesity, increased adiposity, and overt diabetes. *A–C*: body weight (BW) was markedly higher in *db*/*db* mice compared with age-matched wild-type (WT) control mice. Compared with age-matched WT mice, female *db*/*db* mice had a more impressive increase in body weight than male mice (female *db*/*db* mice: 2.38-fold higher body weight than age-matched WT mice at 6 mo of age and 2.37-fold higher at 12 mo vs. male *db*/*db* mice: 1.88-fold at 6 mo and 1.74-fold at 12 mo of age). *D–F*: magnetic resonance imaging showed a marked increase in body fat weight in both male and female *db*/*db* mice at 2 and 6 mo of age. *G–I*: in contrast, lean mass was comparable between age- and sex-matched WT and *db*/*db* mice. *J* and *K*: dual-energy X-ray absorptiometry performed at 4 mo of age also showed that *db*/*db* mice had a marked increase in total (*G*) and abdominal (*H*) fat content. *L–N*: although there was significant variability, both male (*M*) and female (*N*) *db*/*db* animals exhibited a marked increase in fasting plasma glucose levels at 2 and 6 mo of age. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, and $^{\wedge}P < 0.05$ vs. corresponding 2-mo-old mice; $^{\wedge\wedge\wedge}P < 0.001$ vs. corresponding 2-mo-old mice. Body weight sample size: WT mice: 1 mo n = 37, 2 mo n = 63, 4 mo n = 58, 6 mo n = 91, 12 mo n = 22; *db*/*db* mice: 1 mo n = 11, 2 mo n = 35, 4 mo n = 45, 6 mo n = 68, 12 mo n = 24. Male mice: WT 1 mo n = 16, 2 mo n = 23, 4 mo n = 32, 6 mo n = 53, 12 mo n = 11; *db*/*db*: 1 mo n = 7, 2 mo n = 22, 4 mo n = 29, 6 mo n = 38, 12 mo n = 10. Female mice: WT 1 mo n = 21, 2 mo n = 40, 4 mo n = 31, 6 mo n = 38, 12 mo n = 11; *db*/*db*: 1 mo n = 4, 2 mo n = 13, 4 mo n = 16, 6 mo n = 30, 12 mo n = 14. Magnetic resonance imaging fat content: n = 12–16 mice/group, n = 5–9 male mice/group, n = 4–8 female mice/group. Dual-energy X-ray absorptiometry: n = 7–16/group. Fasting plasma glucose: n = 18–49 mice/group, n = 9–27 male mice/group, and n = 9–22 female mice/group.

the perimysial collagen thickness for each mouse, measurements for at least 10 perimysial strands were averaged. To assess periarteriolar collagen deposition, at least 50 arterioles from each group were identified in picrosirius red-stained sections and selected for analysis only if the ratio of the major to minor axes was <3:1 (to exclude vessels sectioned longitudinally). The medial area and adventitial collagenous area were quantitatively assessed for each vessel using Axiovision software.

To identify cardiac arterioles and to label myofibroblasts, immunofluorescence for a-smooth muscle actin (a-SMA) was performed using FITC-conjugated anti-a-SMA antibody (F3777, mouse monoclonal antibody, 1:100, Sigma). To further investigate myofibroblast activation in the *db*/*db* myocardium, immunohistochemistry for periostin (no. 92460, rabbit polyclonal antibody, 1:200, Abcam) and fibroblast activation protein (FAP) was performed (no. 53066, rabbit polyclonal antibody, 1:200, Abcam). Sections from infarcted mouse hearts after 7 days of permanent coronary occlusion (49) were used as a positive control. Substitution of primary antibodies with corresponding species IgG in blocking buffer was used as a negative control, and no background staining was noted. Staining was performed using a peroxidase-based technique with the Vectastain ELITE kit (Vector Laboratories). After antigen retrieval with citrate buffer, sections were treated with 3% hydrogen peroxide to inhibit endogenous peroxidase activity and blocked with 10% serum to block nonspecific protein binding. Peroxidase activity was detected using diaminobenzidine with nickel.

To perform collagen immunofluorescence, freshly harvested hearts of WT and *db*/*db* mice (8 animals/group, 4 male and 4 female mice) were embedded in OCT compound (no. 4585, Fisher HealthCare) and frozen in 2-methylbutane (Millipore) kept in dry ice. Cryosections (7 11m) were cut, acetone fixed, and then probed for collagen I (no. 21286, rabbit polyclonal antibody, 1:500, Abcam).

Griffonia Simplicifolia-I lectin histochemistry and assessment of microvascular density. To identify microvessels in WT and *db*/*db* hearts, we used histochemical staining for *Griffonia Simplicifolia* (GSL)-I lectin (DL1207, Vector) as previously described (44). Assessment of microvascular density was performed for each heart by counting the number of GSL-I lectin-positive profiles in at least 10 fields for each mouse at X200 magnification.

Wheat germ agglutinin lectin histochemistry and quantification of cardiomyocyte size. Cardiomyocyte size was assessed using staining with Alexa Fluor 488-conjugated wheat germ agglutinin (WGA) lectin (W11261, Invitrogen) to label cardiomyocyte membranes (12, 20). For each mouse, the area of 50 cardiomyocytes from subendocardial or subepicardial regions cut in cross section was measured. Cardiomyocytes were identified from five different random subendocardial or subepicardial fields, scanned from two different sections for each mouse. The mean of these cardiomyocyte cross-sectional areas was calculated for each mouse.

Blood pressure measurements. Invasive blood pressure measurements were performed with a commercial pressure-volume analysis system (Scisense) as previously described (4). Mice undergoing blood pressure measurement were anesthetized with isoflurane and allowed to rest for 10 min to stabilize blood pressure. The right carotid artery was exposed by blunt dissection and tied in three spots 3 mm apart with 6-0 silk suture. Distal suture was tied permanently with two knots, whereas mid and proximal sutures were tied with one knot. With the use of fine scissors, a small incision was made between the distal and mid suture. A 1.2-Fr pressure-volume catheter (Scisense) was inserted and advanced until the tip of the catheter passed midsuture, which was tightened to secure the catheter. Subsequently, the proximal suture was removed, and the catheter was advanced to the aortic arch and secured with a suture. The catheter was left in place for 10 min to allow blood pressure equilibration. Pressure tracings were recorded for 5 min and analyzed to derive average systolic blood pressure (SBP) and diastolic blood pressure (DBP).

Invasive assessment of LV end-diastolic pressure. LV pressure was recorded in WT and *db*/*db* mice as previously described (12). The right carotid artery was cannulated with a modified RADI PressureWire catheter (RADI Medical Systems, Upsala, Sweden). Subsequently, the catheter was advanced in the ascending aorta and

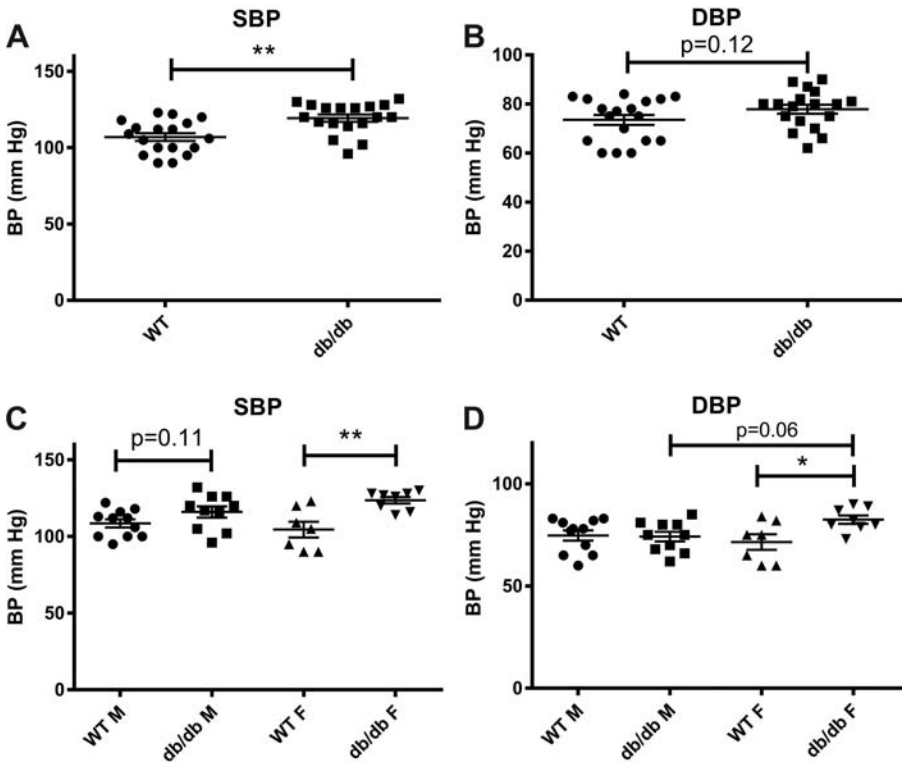


Fig. 2. Female *db*/*db* mice have a modest but significant elevation in systolic blood pressure (SBP) and diastolic blood pressure (DBP). *A* and *B*: at 4 mo of age, obese diabetic *db*/*db* mice had a significant elevation in SBP and a trend toward higher DBP. *C* and *D*: sex-specific analysis showed that male *db*/*db* mice had a trend toward increased SBP (*C*) and no significant difference in DBP (*D*) compared with lean WT control mice. Female *db*/*db* mice had significantly increased SBP (*C*) and DBP (*D*) compared with age-matched WT control mice (n = 10–11 male mice/group, n = 7–8 female mice/group, n = 18 male + female mice/group). *P < 0.05 and **P < 0.01. BP, blood pressure.

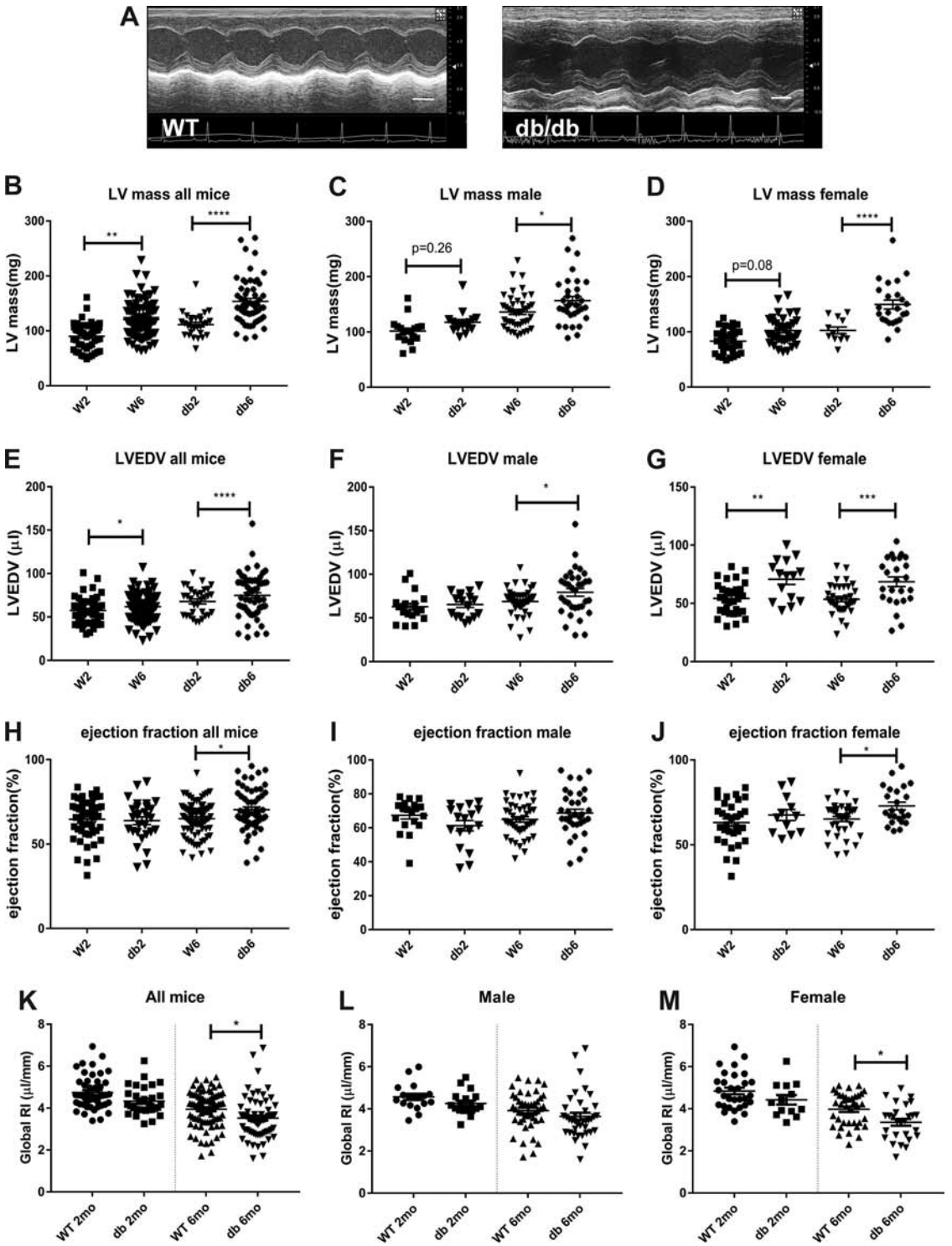


Fig. 3. Obese diabetic *db*/*db* mice exhibit cardiac remodeling with preserved ejection fraction. *A*: echocardiographic imaging was performed in wild-type (WT) and *db*/*db* mice. Representative images show evidence of cardiac remodeling in *db*/*db* mice at 6 mo of age compared with lean WT control mice (scale bar = 2 mm). *B*: *db*/*db* mice had significantly higher left ventricular (LV) mass at 2 and 6 mo of age. *C* and *D*: both male and female *db*/*db* mice had increased LV mass at 6 mo of age. However, the increase in LV mass was exaggerated in female *db*/*db* mice. At 6 mo of age, male *db*/*db* mice had a 15.1% higher LV mass than age-matched WT mice, whereas female *db*/*db* animals at the same age exhibited a 46.4% increase. *E*: LV end-diastolic volume (LVEDV) was increased in *db*/*db* mice. *F* and *G*: female mice had earlier ventricular dilation than male mice. *H–J*: obese diabetic *db*/*db* mice exhibited slightly but significantly higher ejection fraction at 6 mo of age than age-matched WT control mice. *K–M*: the global remodeling index was assessed to compare maladaptive hypertrophic remodeling between groups. *K*: at 6 mo of age, *db*/*db* mice had a lower remodeling index than corresponding lean WT control mice. *L* and *M*: female but not male *db*/*db* mice had a significant reduction in the global remodeling index, indicating accentuated hypertrophic remodeling (n = 19–44 male mice/group, n = 12–37 female mice/group, n = 29–81 male + female mice/group). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

then in the LV (41). LV end-diastolic pressure (LVEDP) was calculated from the LV pressure signal.

Fibroblast isolation. Mouse fibroblasts from WT and *db*/*db* hearts were isolated as previously described (49) by enzymatic digestion and cultured in DMEM-F-12 (GIBCO Invitrogen, Carlsbad, CA) with 10% FCS. At *passage 3*, cardiac fibroblasts were serum starved for 24 h, harvested, and suspended in 1X serum-free MEM (GIBCO Invitrogen). Cells were stimulated with transforming growth factor (TGF)-\3$_1$ (10 ng/ml) for 4 h. At the end of the experiment, cell lysates were used for RNA extraction using standard protocols.

mRNA extraction and quantitative PCR. Isolated total RNA from cardiac fibroblasts was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's guidelines. Quantitative PCR was performed using the SYBR green (Bio-Rad) method on an iQ5 Real-Time PCR Detection System (Bio-Rad). Collagen type I and type III mRNA levels were assessed using the following primers: collagen type I, forward 5=-GTATGCTTGATCT-GTATCTG-3= and reverse: 5=-CGACTCCTACATCTTCTG-3=; and collagen type III, forward 5=-CCTTGGTCAGTCCTATGAG-3= and reverse 5=-CAGGAGCAGGTGTAGAAG-3=.

Statistical analysis. For comparisons of two groups, an unpaired two-tailed Student's *t*-test using (when appropriate) Welch's correction for unequal variances was performed. The Mann-Whitney test was used for comparisons between two groups that did not show Gaussian distribution. For comparisons of multiple groups, one-way ANOVA was performed followed by Sidak's multiple-comparison test. The Kruskal-Wallis test, followed by Dunn's multiple-comparison posttest, was used when one or more groups did not show Gaussian distribution. Data are expressed as means ± SE. Statistical significance was set at 0.05.

RESULTS

db/db mice exhibit severe obesity, high adiposity, and hyperglycemia. db/*db* mice in a C57BL6J background exhibited rapid weight gain when fed a regular chow diet. Body weight was significantly higher in both male and female *db*/*db* mice compared with age- and sex-matched control C57BL6J mice (Fig. 1, *A*–*C*). Compared with age-matched WT mice, female *db*/*db* mice had a more impressive increase in weight than male mice (fold increase in mean body weight vs. WT mice, female mice: 2.38-fold at 6 mo of age and 2.37-fold at 12 mo and male mice: 1.88-fold at 6 mo and 1.74-fold at 12 mo). Both magnetic resonance imaging (Fig. 1, *D*–*I*) and dual-energy X-ray absorptiometry (Fig. 1, *J* and *K*) showed a marked increase in adiposity and in abdominal fat content in both male and female *db*/*db* animals compared with age-matched WT control animals (Fig. 1, *D*–*F*, *J*, and *K*). In contrast, lean weight was comparable between groups (Fig. 1, *G*–*I*). Plasma glucose levels were markedly increased in both male and female *db*/*db* mice at 2 and 6 mo of age compared with age-matched WT control mice (Fig. 1, *L*–*N*).

Female but not male db/db mice exhibit mild hypertension. Compared with age-matched WT animals, 4-mo-old *db*/*db* mice exhibited a modest but significant elevation of SBP and a trend toward increased DBP (Fig. 2, *A* and *B*). Both SBP and DBP were significantly increased in female *db*/*db* mice. In contrast, male *db*/*db* animals had a trend toward increased SBP and comparable DBP with WT control animals (Fig. 2, *C* and *D*).

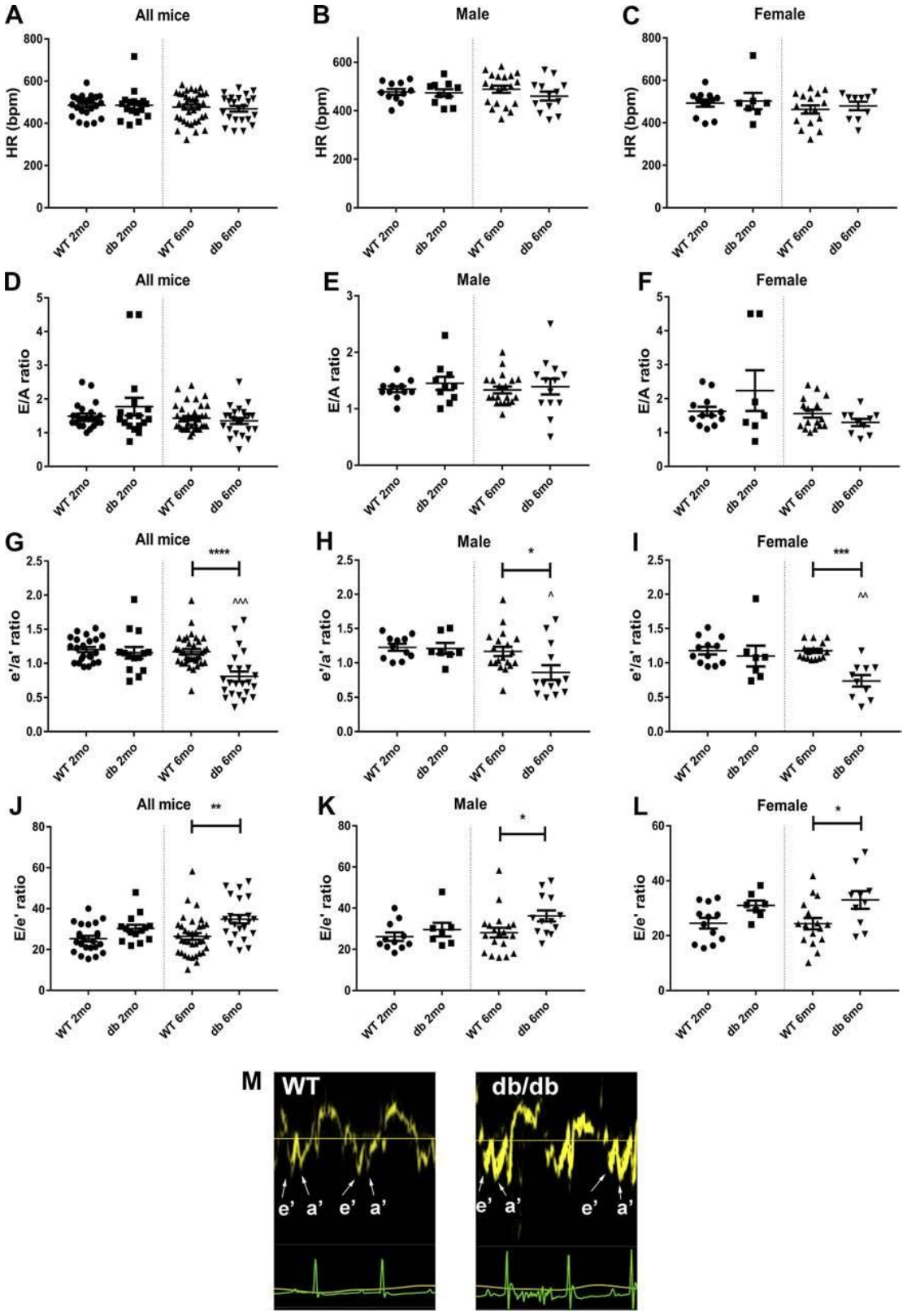
db/db mice exhibit cardiac remodeling in the absence of systolic dysfunction. Compared with age-matched WT mice, *db*/*db* mice exhibited significantly higher LV mass (Fig. 3, *A* and *B*) at 2 and 6 mo of age accompanied by increased LVEDV (Fig. 3*E*). At 6 mo of age, ejection fraction was slightly but significantly higher in *db*/*db* mice (Fig. 3*H*). To compare the remodeling response between groups, we measured the remodeling index, an indicator of maladaptive hypertrophic remodeling that has been used for risk stratification in patients with hypertension. At 6 mo of age, *db*/*db* mice had a significantly lower remodeling index compared with lean WT mice, indicating accentuated hypertrophic remodeling (Fig. 3*J*).

Cardiac remodeling is accentuated in female db/db mice. Sex-specific analysis demonstrated that cardiac remodeling was exaggerated in female *db*/*db* mice. Six-month-old male *db*/*db* mice had a 15.1% increase in LV mass compared with age-matched WT mice, whereas female *db*/*db* mice at the same age exhibited a 46.4% increase (Fig. 3, *C* and *D*). The obesity-associated increase in LVEDV was also more impressive in female mice (Fig. 3, *F* and *G*). At 6 mo of age, female *db*/*db* mice had slightly higher ejection fraction than WT mice. In contrast, no significant difference in ejection fraction was noted in male animals (Fig. 3, *I* and *J*). The remodeling index was significantly reduced in female but not male mice at 6 mo of age (Fig. 3, *L* and *M*).

TDI suggests that both male and female db/db mice have evidence of diastolic dysfunction. Mitral inflow Doppler echocardiography and TDI were used to assess diastolic function. At 2 and 6 mo of age, male and female *db*/*db* mice had comparable heart rates with age- and sex-matched WT control mice (Fig. 4, *A*–*C*). Mitral inflow Doppler experiments showed no significant differences in the *E*/*A* ratio between WT and *db*/*db* mice (Fig. 4, *D*–*F*). TDI showed that at 6 mo of age, *db*/*db* mice had a significant reduction in the *e*=/*a*= ratio compared with WT control mice (Fig. 4*G*). The *e*=/*a*= ratio was significantly reduced in both male (by 26.3%) and female (by 37.1%) animals (Fig. 4, *H* and *I*). The *E*/*e*= ratio was significantly increased in *db*/*db* mice at 6 mo of age (Fig. 4, *J*–*L*). Male mice exhibited a statistically significant increase of the ratio by 28.8% ($P < 0.05$), whereas female mice had a 35.6% increase ($P < 0.05$). The representative images in Fig. 4*M* show the changes in the *e*=/*a*= ratio in *db*/*db* mice at 6 mo of age.

Female db/db mice have elevated LVEDP. At 6 mo of age, *db*/*db* mice had higher LVEDP than age-matched WT mice

Fig. 4. Tissue Doppler imaging suggests that *db*/*db* mice develop diastolic dysfunction. *A*–*C*: both male and female *db*/*db* mice and age-matched wild-type (WT) control mice had comparable heart rates. *D*–*F*: mitral inflow Doppler showed no significant differences in the *E*-to-*A* ratio between groups. *G*–*I*: tissue Doppler imaging showed that the *e*=-to-*a*= ratio was significantly reduced in both male and female *db*/*db* mice at 6 mo of age. *J*–*L*: the *E*-to-*e*= ratio was significantly increased in both male and female *db*/*db* mice at 6 mo of age. *M*: representative images of tissue Doppler tracings showing the changes in the *e*=-to-*a*= ratio in 6-mo-old *db*/*db* mice compared with age-matched lean control mice. These findings suggest that *db*/*db* mice develop diastolic dysfunction. Tissue Doppler imaging may be more sensitive than mitral inflow Doppler in detecting changes in diastolic function in mice (n = 7–19 male mice/group, n = 7–16 female mice/group, n = 14–35 male + female mice/group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ vs. corresponding lean mice; ^$P < 0.05$, ^^$P < 0.01$, and ^^^$P < 0.0001$ vs. corresponding 2-mo-old mice.



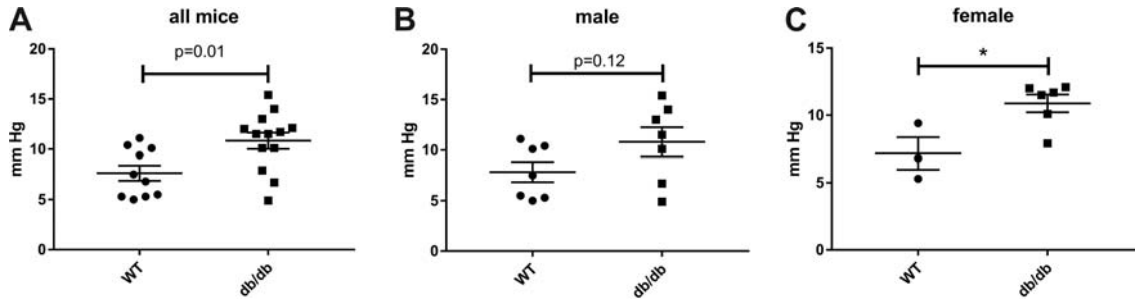


Fig. 5. Obese diabetic *db*/*db* mice had significantly higher left ventricular end-diastolic pressure (LVEDP) than lean wild-type (WT) mice at 6 mo of age. A: *db*/*db* mice had significantly higher LVEDP than age-matched lean WT mice. *B* and *C*: sex-specific analysis showed that male *db*/*db* mice had a trend toward increased LVEDP (*B*), whereas female mice had significantly higher LVEDP (*C*; n = 6–7 male mice/group, n = 3–7 female mice/group, n = 10–13 male + female mice/group). *$P < 0.05$.

(Fig. 5*A*). Compared with sex-matched WT mice, male *db*/*db* mice exhibited a trend toward increased LVEDP (Fig. 5*B*). Female *db*/*db* mice had significantly higher LVEDP than corresponding WT mice ($P < 0.05$; Fig. 5*C*).

Both male and female db/db mice exhibit cardiomyocyte hypertrophy. WGA histochemistry was used to assess cardiomyocyte size in WT and *db*/*db* mice (Fig. 6*A*). At 6 and 12 mo of age, both male and female *db*/*db* mice had markedly larger cardiomyocytes than age-matched WT mice (Fig. 6, *B–D*). To examine the effects of obesity on the noncardiomyocyte compartment, we assessed the density of interstitial cells in WT and *db*/*db* mice. No significant differences in interstitial cellularity were noted between the groups at 6 and 12 mo of age (Fig. 6, *E–G*).

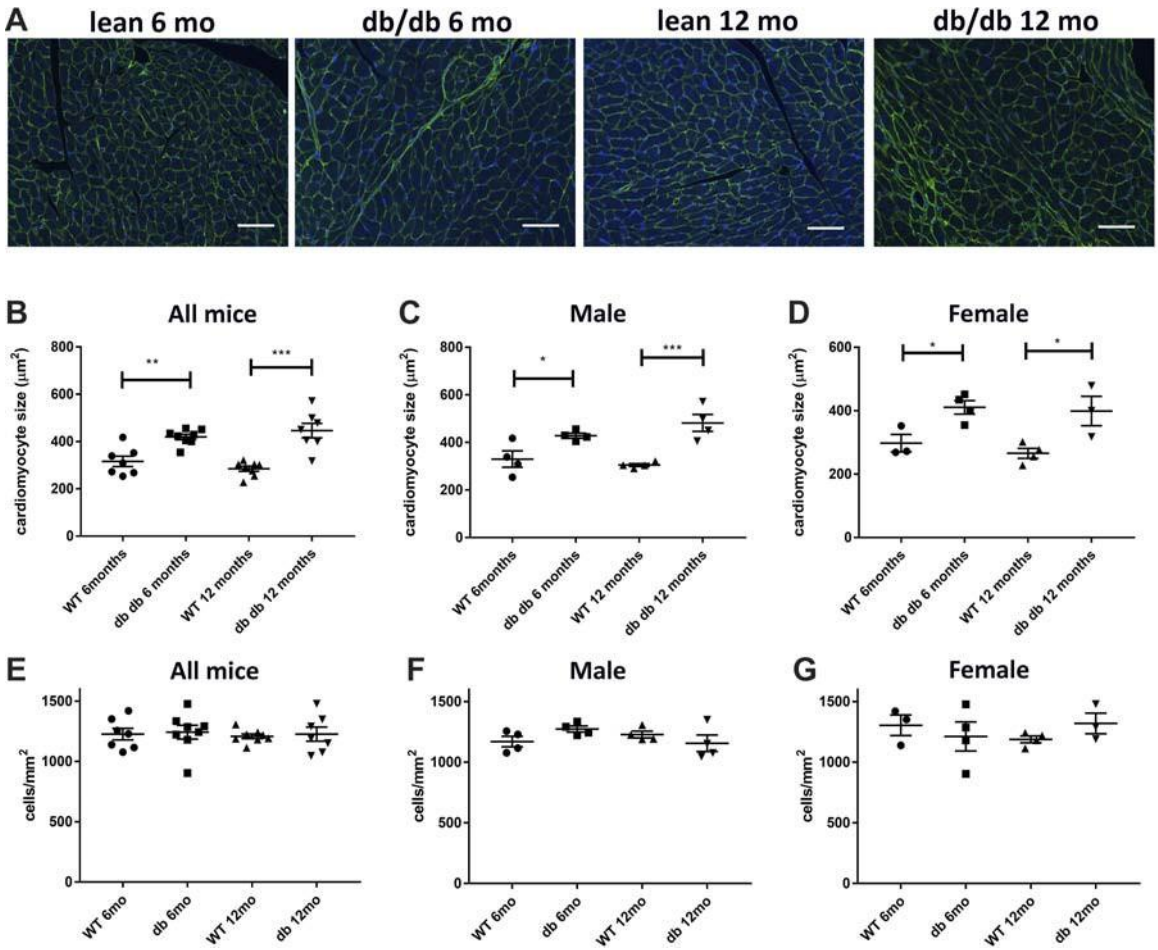


Fig. 6. Obese diabetic *db*/*db* mice exhibit a marked increase in cardiomyocyte size without a significant increase in the density of interstitial cells. *A*: wheat germ agglutinin (WGA) lectin histochemistry was used to quantitatively assess cardiomyocyte size. DAPI staining was used for the quantification of interstitial cell density. *B–D*: both male and female *db*/*db* mice had a marked increase in cardiomyocyte size at 2 and 6 mo of age. *E–G*: interstitial cell density was comparable between groups (n = 4 male mice/group, n = 4 female mice/group, n = 8 male + female mice/group). Scale bar = 50 μm. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

db/db mice have increased perimysial collagen thickness and a higher endomysial collagen content. Endomysial collagen content and thickness of perimysial collagen were assessed in picrosirius red-stained sections (Fig. 7, *A* and *B*). Compared with age-matched WT mice, *db*/*db* mice had higher endomysial collagen content at 6 mo of age but not at 12 mo of age (Fig. 7*C*). Sex-specific analysis showed no significant differences in endomysial collagen content between sex-matched *db*/*db* and WT animals (Fig. 7, *D* and *E*). Compared with WT mice, *db*/*db* mice had markedly higher perimysial collagen thickness at 6 and 12 mo of age (Fig. 7*F*). Whereas male *db*/*db* mice had significantly higher perimysial collagen thickness at both time points studied, the difference in female mice reached statistical significance only for the 6-mo time point (Fig. 7, *G* and *H*).

Coronary arterioles in db/db mice exhibit expansion of the media, associated with an increase in the area of perivascular collagen. Cardiac fibrosis is typically associated with expansion of the perivascular adventitial collagen. We used picrosirius red-stained sections to quantitatively assess the adventitial collagen network in WT and *db*/*db* mice (Fig. 8, *A* and *B*). Quantitative analysis showed that the area of adventitial col-

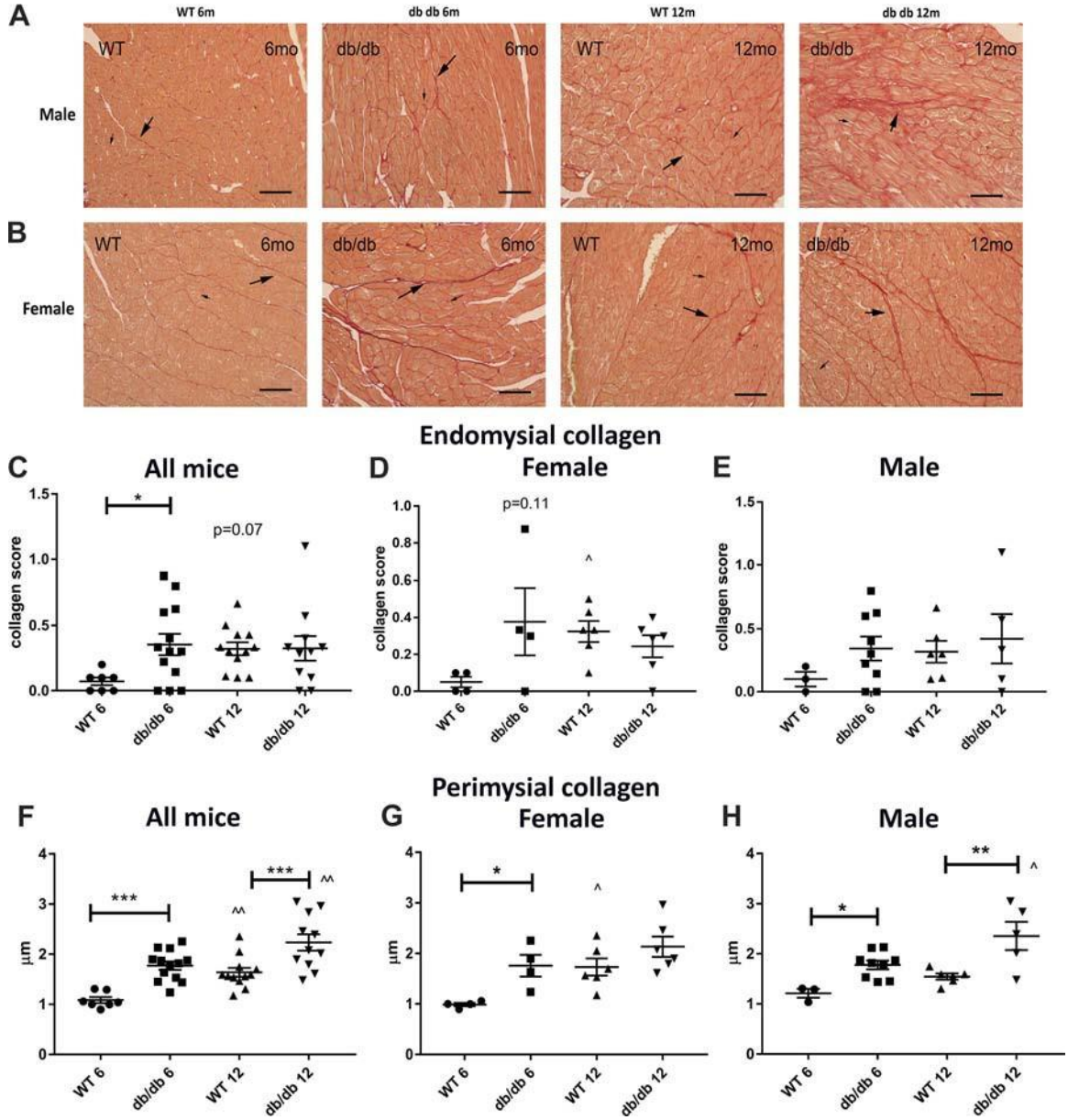


Fig. 7. Obese diabetic *db*/*db* mice do not have replacement fibrosis but exhibit thickening of the perimysial collagen network and increased endomysial collagen. *A* and *B*: picrosirius red staining was used to identify perimysial collagen fibers that form the sheath that groups cardiomyocytes into bundles (long arrows) and endomysial collagen fibers surrounding each individual cardiomyocyte (short arrows). There was no evidence of replacement fibrosis in any of the *db*/*db* hearts. *C–E*: semiquantitative analysis showed that *db*/*db* mice have accentuated endomysial collagen at 6 mo of age. The increased endomysial collagen score in male or female mice did not reach statistical significance. *F–H*: perimysial collagen thickness was markedly increased in *db*/*db* mice at 6 and 12 mo of age. Both female and male mice had increased perimysial collagen thickness at 6 mo of age (n = 4 male mice/group, n = 4 female mice/group, n = 8 male + female mice/group). Scale bar = 50 μm. *P < 0.05, **P < 0.01, ***P < 0.001, ^P < 0.05, and ^^P < 0.01 vs. the corresponding 6-mo group.

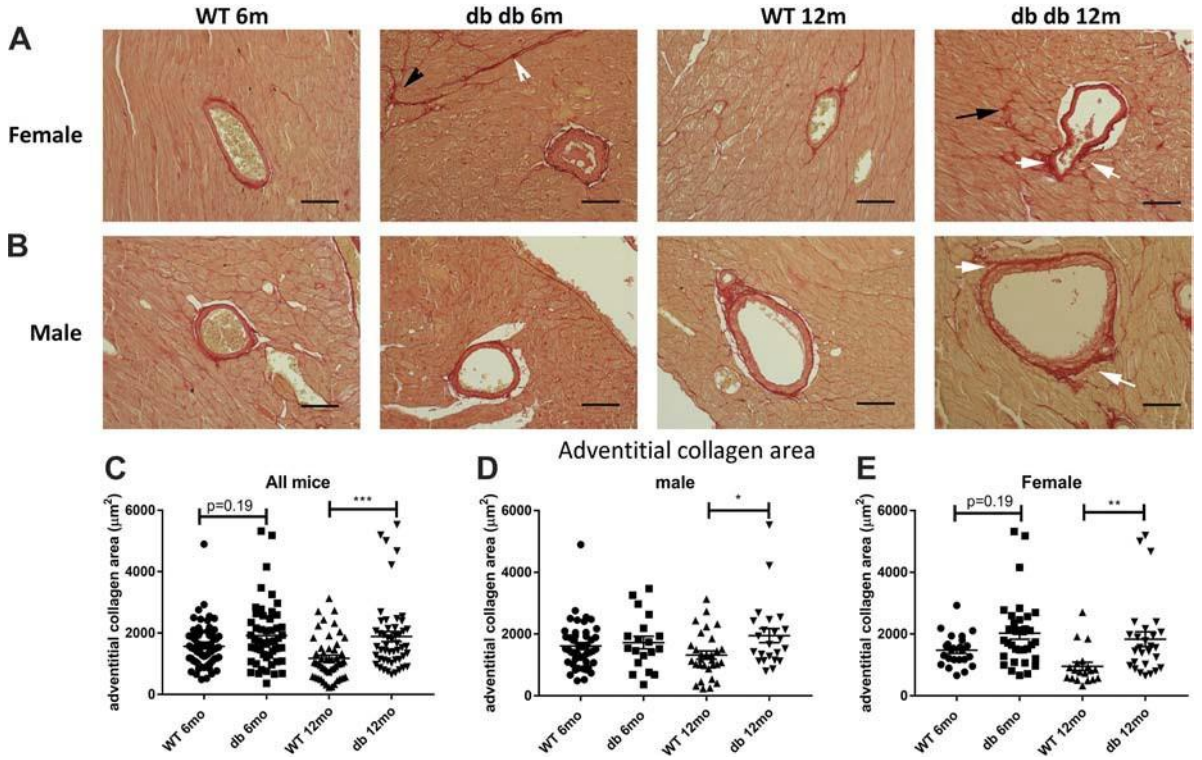


Fig. 8. *db*/*db* mice exhibit expansion of the periadventitial collagen network in coronary arterioles. *A* and *B*: periarteriolar collagen was identified using picrosirius red staining in male and female wild-type (WT) and *db*/*db* mouse hearts (white arrows). Please note the increased perimysial thickness (arrowheads) and the accentuated deposition of endomysial collagen (black arrow) in *db*/*db* mouse hearts (quantified in Fig. 7). *C*: quantitative analysis showed that at 12 mo of age, the periadventitial collagen area was higher in arterioles of *db*/*db* mice than in the corresponding vessels of WT mice. *D* and *E*: both male (*D*) and female (*E*) animals exhibited expansion of the periadventitial collagen area (n = 19–45 vessels/group for male mice, n = 19–35 vessels/group for female mice, n = 50–70 vessels/group for male + female mice). Scale bar = 50 μm. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

lagen was significantly increased in *db*/*db* mice at 12 mo of age (Fig. 8*C*). Although both male and female mice had increased adventitial collagen content, adventitial expansion was more marked in female mice (Fig. 8, *D* and *E*). Arterioles in *db*/*db* mice had a higher mean medial area than in age-matched WT mice (Fig. 9*A*). Arteriolar medial area was significantly increased in female *db*/*db* mice; in contrast, male mice showed a trend toward medial expansion (Fig. 9, *B* and *C*). Immunofluorescence for a-SMA showed the hypertrophy of arteriolar media in *db*/*db* hearts (Fig. 9, *D* and *E*).

Fibroblasts in db/db mice do not undergo myofibroblast conversion. In the fibrotic myocardium, deposition of collagen is typically associated with myofibroblast conversion, since activated fibroblasts incorporate contractile proteins, such as a-SMA, in the cytoskeleton and express matricellular proteins, such as periostin (14, 30). Moreover, in infarcted and remodeling hearts, fibroblasts express activation markers, such as FAP (50). In contrast to the robust expression of FAP in fibroblast-like cells infiltrating the healing infarct, WT and *db*/*db* mouse hearts showed negligible FAP immunoreactivity (Fig. 10, *A–C*). Moreover, fibroblasts in *db*/*db* hearts had no expression of the myofibroblast conversion markers periostin (Fig. 10, *D–F*) and a-SMA (Fig. 10, *G–I*).

db/db mice exhibit expansion of the population of collagen-producing cells. Increased synthesis of structural extracellular matrix proteins, such as collagens, is an indicator of fibroblast activation. We performed immunohistochemical staining for collagen type I using cryosections to compare the density of collagen-producing fibroblasts in WT and *db*/*db* hearts (Fig. 11, *A* and *B*). Fibroblasts were identified as collagen-immunoreactive cells. *db*/*db* hearts exhibited a trend toward higher number of collagen-positive fibroblasts ($P = 0.05$; Fig. 11*C*). Sex-specific analysis did not reveal significant differences in the density of collagen-positive cells between male and female animals (Fig. 11*D*).

Isolated cardiac fibroblasts from db/db mice have increased baseline expression of collagen but exhibit blunted responses to TGF-β1 stimulation. In vitro, cardiac fibroblasts harvested from *db*/*db* mice had significantly higher collagen type I and type III mRNA expression compared with fibroblasts from WT mice (Fig. 11, *E* and *F*). TGF-β1 stimulation for 4 h induced collagen mRNA synthesis in WT but not in *db*/*db* fibroblasts (Fig. 11, *E* and *F*).

Male db/db mice exhibit significant microvascular rarefaction. Microvascular density was quantitatively assessed in WT and *db*/*db* hearts using histochemical staining for GSL-I (Fig. 12, *A* and *B*). *db*/*db* mice had trends toward reduced microvascular density at 6 and 12 mo of age (Fig. 12*C*). Sex-specific analysis showed a significant reduction in microvascular density in male (Fig. 12*E*) but not female *db*/*db* mice (Fig. 12*D*) compared with age-matched WT control mice.

DISCUSSION

Our study characterized the sex-specific functional, structural, and histopathological alterations in the *db*/*db* mouse

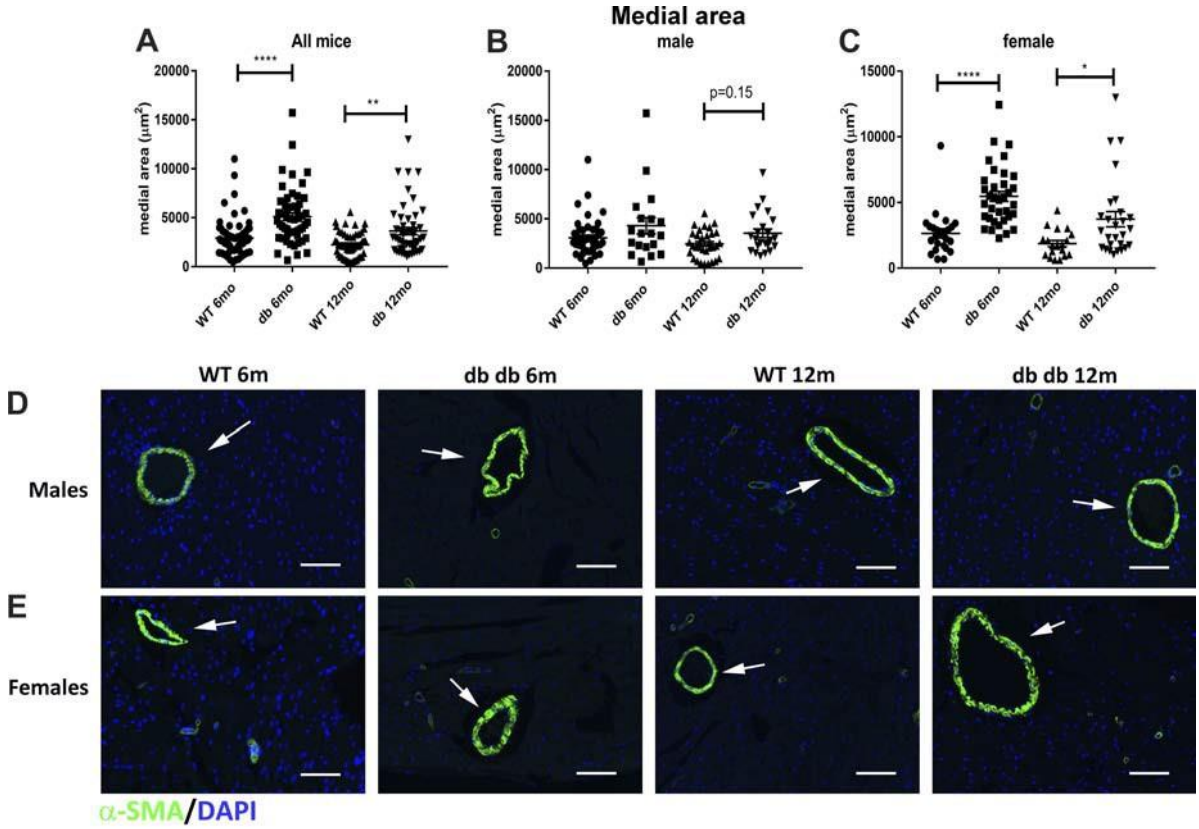


Fig. 9. Female *db*/*db* mice show significant hypertrophy of the coronary arteriolar media. *A*: quantitative analysis of the picrosirius red-stained sections (shown in Fig. 8) suggested that *db*/*db* mice had a significantly higher mean arteriolar area compared with wild-type (WT) mice at 6 and 12 mo of age. *B*: male mice had a trend toward increased arteriolar area at 12 mo of age. *C*: female mice had significantly higher arteriolar area at both 6- and 12-mo time points. *D* and *E*: a-smooth muscle actin (a-SMA) immunofluorescence showed hypertrophy of the arteriolar media (arrows) in female *db*/*db* mice (n = 19–45 vessels/group for male mice, n = 19–35 vessels/group for female mice, n = 50–70 vessels/group for male + female mice). Scale bar = 50 μm. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$.

model of type 2 diabetes, obesity, and metabolic dysfunction. Here, we report that *1*) hyperglycemia and markedly increased adiposity in C57BL6J *db*/*db* mice are associated with increases in LV mass and with diastolic dysfunction, in the absence of a significant reduction in ejection fraction; *2*) the functional changes in *db*/*db* mice are associated with cardiomyocyte hypertrophy, perimysial collagen thickening, mild endomysial and perivascular fibrosis, and vascular rarefaction; *3*) fibrosis in *db*/*db* mice is not associated with generation of myofibroblast-like cells but involves acquisition of a matrix-synthetic phenotype by interstitial fibroblasts in vivo and in vitro; and *4*) sex is a major determinant of the functional and structural alterations in *db*/*db* mice. Female *db*/*db* animals exhibit accentuated weight gain, hypertension, and exaggerated increases in LV mass, accompanied by worse diastolic dysfunction and early expansion of the perivascular collagen network, whereas male mice have accelerated microvascular rarefaction. Our findings highlight the sex-specific aspects of the cardiomyopathy associated with metabolic disease and show that the *db*/*db* mouse recapitulates important features of human HFpEF found in patients with diabetes, obesity, and metabolic dysfunction.

The contribution of metabolic dysregulation in the pathogenesis of a distinct clinical phenotype of human HFpEF. In human patients, HFpEF involves a wide range of pathophysiological mechanisms that result in a spectrum of clinical phenotypes (32, 47). It has been suggested that, in contrast to HF with reduced ejection fraction, which is predominantly driven by cardiomyocyte loss, HFpEF reflects a myocardial response to extracardiac comorbidities (such as hypertension, diabetes, obesity, dyslipidemia, and renal dysfunction) that may stimulate myocardial microvascular inflammation, fibrosis, and cardiomyocyte hypertrophy, ultimately reducing compliance of the cardiac muscle (39, 47). Studies that have examined endomyocardial biopsy samples showed that patients with HFpEF have significant interstitial fibrosis, accompanied by infiltration with inflammatory leukocytes (54). Diabetes and obesity may accentuate inflammatory and fibrogenic signaling, leading to exaggerated interstitial remodeling in patients with HFpEF with metabolic dysfunction (8, 45). This notion is supported by the following clinical evidence: circulating levels of biomarkers reflecting inflammation and fibrosis, such as galectin-3 and COOH-terminal telopeptide of collagen type I, were higher in patients with diabetes versus nondiabetic patients with HFpEF (33). Whether the inflammatory activation and fibrogenic stimulation in diabetic hearts play a direct role in the pathogenesis of diastolic dysfunction remains unknown. Dissection of the cell biological events and molecular signals requires systematic characterization of experimental models of diabetic cardiomyopathy that recapitulate features of human HFpEF.

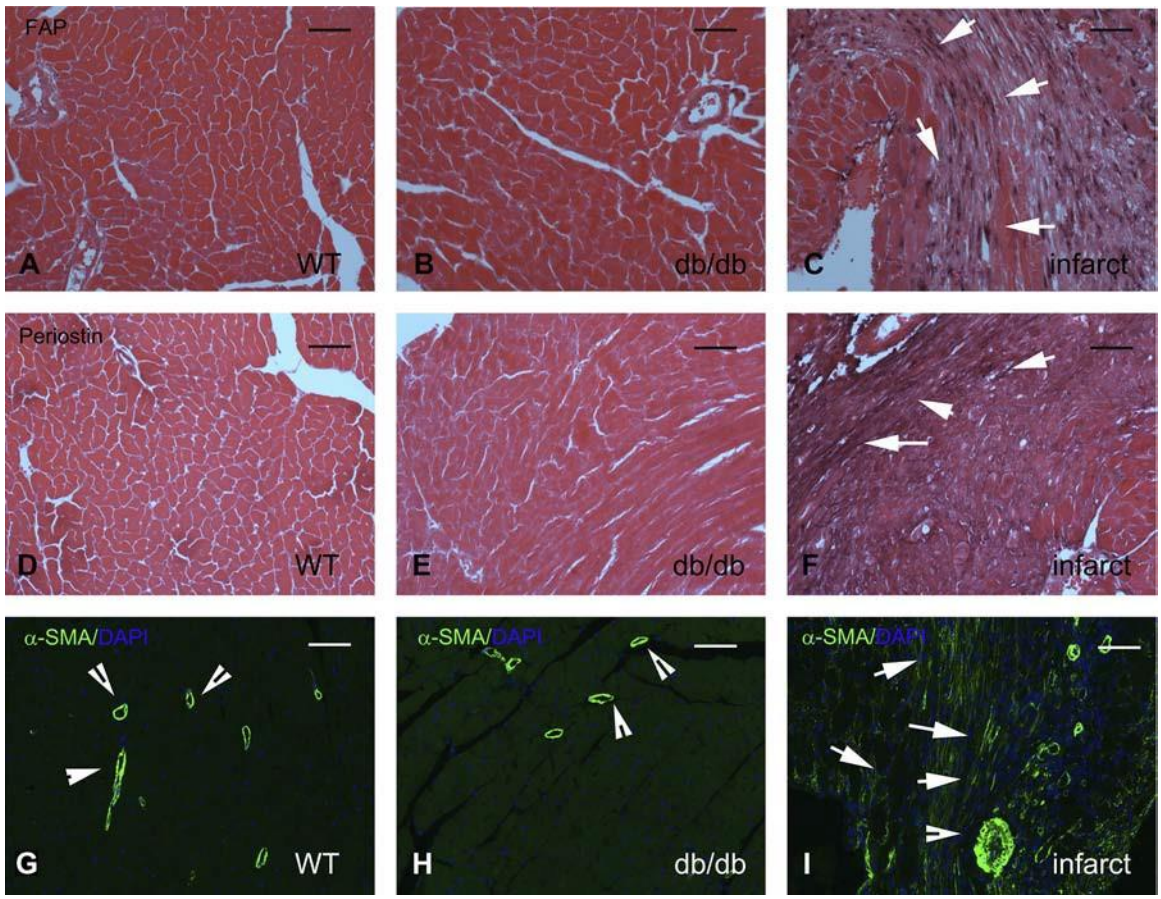


Fig. 10. Increased collagen deposition in *db*/*db* hearts is not associated with myofibroblast conversion. *A–C*: immunohistochemical staining for fibroblast activation protein (FAP), a marker for activated fibroblasts, in lean wild-type (WT; *A*), uninjured diabetic (*B*), and infarcted WT C57BL6J (*C*) mouse hearts. No FAP^+ cells were noted in WT or diabetic myocardium. In contrast, abundant FAP^+ fibroblasts infiltrated the infarcted myocardium 7 days after coronary occlusion (arrows in *C*). Images were counterstained with eosin. *D–F*: periostin staining in lean WT (*D*), uninjured diabetic (*E*), and infarcted WT C57BL6J (*F*) mouse hearts. In injury sites and in fibrotic tissues, activated myofibroblasts typically exhibited periostin expression. *E*: please note the complete absence of periostin immunoreactivity in *db*/*db* hearts. In contrast, infarcted hearts (*F*) exhibited periostin expression in activated myofibroblasts and in the surrounding extracellular matrix (arrows). *G–I*: a-smooth muscle actin (a-SMA) immunofluorescence was used to identify activated myofibroblasts as spindle-shaped immunoreactive cells located outside the vascular media. In uninjured WT (*G*) and *db*/*db* (*H*) hearts, a-SMA was exclusively localized in the arteriolar media (arrowheads). *I*: please note the abundant a-SMA-expressing myofibroblasts in the infarcted myocardium (arrows). Images show sections from 6-mo-old-mice representative of at least 4 different animals/group. Scale bar = 50 μm.

The db/db mouse as a model of metabolic disease-associated HFpEF. Although the *db*/*db* mouse has been extensively studied to investigate the cellular basis and molecular mechanisms of diabetic cardiomyopathy, reported findings on the cardiac functional phenotype and structural characteristics of these animals are conflicting. Some studies have reported that, compared with lean controls, *db*/*db* mice have a significantly reduced ejection fraction, associated with cardiomyocyte apoptosis (3, 40, 48). In contrast, numerous other studies have suggested that *db*/*db* mice have preserved ejection fraction but develop premature diastolic dysfunction as they age (4, 18, 21, 22, 25, 43). Some investigations found no functional or structural differences between *db*/*db* mice and age-matched control mice (28). Several factors may explain the conflicting observations. First, genetic background is an important determinant of phenotype in *db*/*db* mice. C57BL/KsJ mice are more susceptible to the development of diabetes than C57BL6J mice in both genetic and streptozotocin-induced models (31) and may have accelerated cardiac involvement. Comparison of the cardiac phenotype in different strains of *db*/*db* mice has not been performed. Second, the female sex is associated with a greater predisposition for HFpEF in human patients (19) and with accentuated diastolic dysfunction in mouse models of diabetes and obesity (9). Many published investigations that have studied *db*/*db* mice were limited to the study of male animals; moreover, in the studies that have included both male and female animals, no sex-specific analysis was performed. Third, diet plays an important role in the severity of metabolic dysfunction in mouse models of obesity and may be a major factor driving development of complications. Differences in the composition of the diet used in various studies may explain, at least in part, conflicting observations. Fourth, the time points examined are different in various studies. Conflicting conclusions may reflect observations performed at different time points, capturing changes at different stages of the cardiomyopathic process in *db*/*db* mice. Finally, methodological differences in functional assessment of the ventricle between studies may affect the sensitivity and specificity of documentation of myocardial dysfunction.

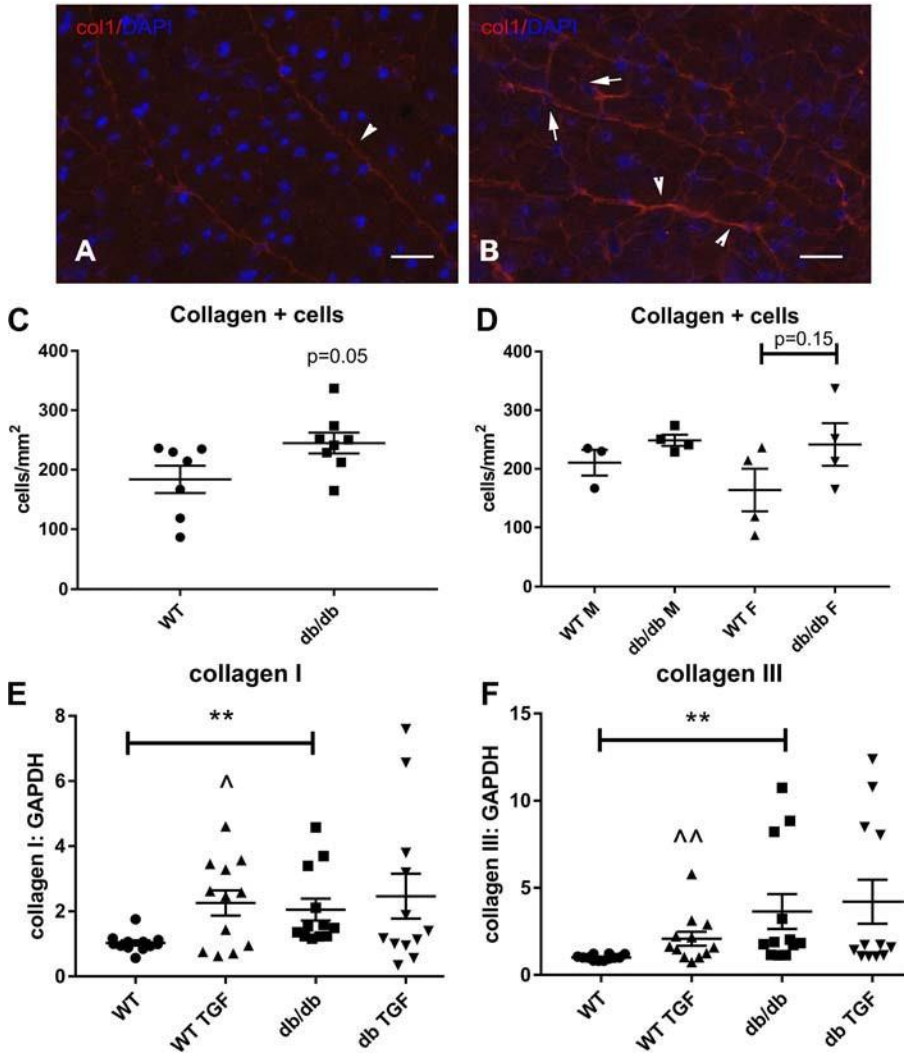


Fig. 11. Fibroblasts in *db*/*db* hearts show increased collagen synthesis. *A* and *B*: cryosections from 6-mo-old lean wild-type (WT) and *db*/*db* mouse hearts were stained with an anticollagen type I antibody. DAPI counterstaining was used to identify collagen type I-expressing interstitial cells. *C*: *db*/*db* hearts had a higher density of collagen type I-expressing interstitial cells that did not reach statistical significance (n = 8 hearts/group, P = 0.05). *D*: sex-specific analysis showed a trend toward higher density of collagen type I-expressing cells in female *db*/*db* hearts (n = 4 hearts/group). *E* and *F*: in vitro, cardiac fibroblasts harvested from 4-mo-old *db*/*db* mice had a 2.0- to 3.0-fold increase in baseline collagen type I (*E*) and type III (*F*) mRNA expression compared with fibroblasts from WT hearts. Activated fibroblasts from *db*/*db* hearts were less responsive to transforming growth factor (TGF)-β_1 stimulation. TGF-β_1 (10 ng/ml) stimulation for 4 h stimulated collagen type I and type III mRNA synthesis in WT cells but did not significantly increase expression of collagens in *db*/*db* fibroblasts (n = 7–8/group). Scale bar = 25 μm. $^{**}P < 0.01$, $^{\wedge}P < 0.05$, and $^{\wedge\wedge}P < 0.01$ vs. corresponding unstimulated cells. F, female; M, male.

Our study analyzed data from large populations of male and female WT and *db*/*db* mice in a C57BL6J background from our own colony. Our findings suggest that *db*/*db* mice in the C57BL6J background exhibit both functional and histopathological features of human HFpEF associated with metabolic dysfunction. *db*/*db* mice have evidence of diastolic dysfunction based on both TDI and hemodynamic assessment, in the absence of a significant reduction in ejection fraction (Figs. 3–5). The increased LV filling pressures observed in our present study (Fig. 5) may explain the left atrial remodeling previously reported in *db*/*db* mice (23). Assessment of structural parameters demonstrated that *db*/*db* animals had predominant hypertrophic LV remodeling, associated with a reduction in the global remodeling index (Fig. 3). Increases in LV mass and in LVEDP were accentuated in female *db*/*db* animals, highlighting the importance of gender in the cardiac phenotype in models of metabolic dysfunction. The increased susceptibility of female *db*/*db* mice may be related, at least in part, to the more impressive weight gain observed in female mice (Fig. 1) and to an exaggerated pressure load resulting from a modest but significant sex-specific increase in systemic blood pressure (Fig. 2).

Histopathological myocardial changes in db/db mice: cardiomyocyte hypertrophy, interstitial fibrosis, and vascular rarefaction. Functional changes in *db*/*db* mice were associated with histopathological abnormalities. Cardiomyocyte size was markedly and consistently increased in both male and female *db*/*db* animals (Fig. 6). Although *db*/*db* hearts had no evidence of replacement fibrosis, they exhibited marked thickening of the perimysial collagen network, mild endomysial fibrosis associated with an increased density of interstitial collagen-expressing cells, and expansion of the collagenous adventitia of myocardial arterioles (Figs. 7 and 8).

Microvascular rarefaction has been previously documented in both human diabetic hearts and in animal models of diabetic cardiomyopathy (24, 59). Our study demonstrated a significant reduction in microvascular density in *db*/*db* mice, showing that capillary loss is more pronounced in male mice (Fig. 12). The cellular basis for microvascular loss in diabetic hearts is likely multifactorial and may involve pericyte loss, endothelial cell apoptosis, and diabetes-induced alterations in the composition of the extracellular matrix, leading to the induction and deposition of angiostatic matricellular proteins (15, 18).

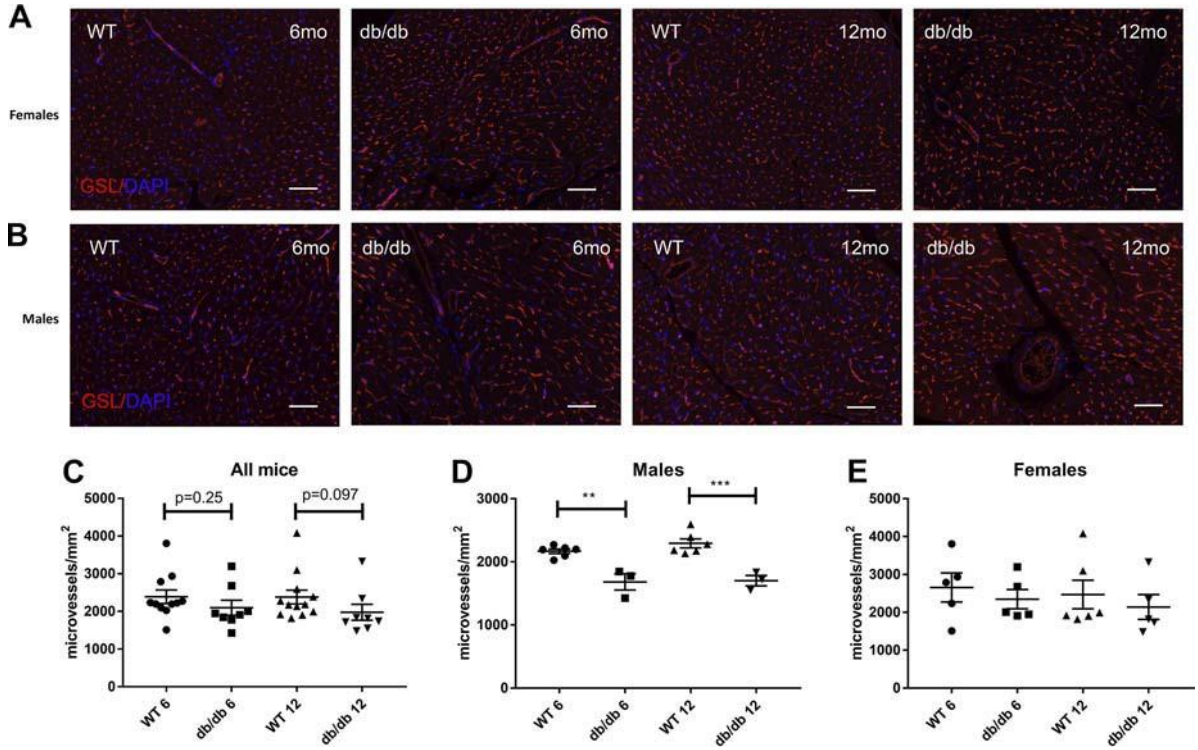


Fig. 12. Male *db*/*db* mice had microvascular rarefaction. *A* and *B*: microvascular density was assessed in *db*/*db* and lean wild-type (WT) mouse hearts using *Griffonia simplicifolia* (GSL)-I lectin staining. *C*: *db*/*db* mice had a trend toward reduced microvascular density at 6 and 12 mo of age. *D* and *E*: although female *db*/*db* mice had comparable microvascular density with age-matched WT control mice, male mice exhibited a markedly lower microvascular density (n = 4 male mice/group, n = 4 female mice/group, n = 8 male + female mice/group). Scale bar = 50 μm. $^{**}P < 0.01$ and $^{***}P < 0.001$.

Fibrosis in db/db mice does not involve myofibroblast conversion but is associated with increased fibroblast-derived collagen synthesis. We demonstrate that, in contrast to the marked expansion of α-SMA-positive/periostin-positive interstitial cells observed in the infarcted myocardium (49, 55), *db*/*db* hearts do not contain significant numbers of α-SMA-positive myofibroblasts and have negligible expression of the matricellular protein periostin and the fibroblast activation marker FAP (Fig. 10). However, despite the absence of indicators of myofibroblast transdifferentiation in the *db*/*db* myocardium, cardiac fibroblasts harvested from *db*/*db* hearts exhibited a 2.0- to 3.0-fold increase in baseline collagen type I and type III mRNA synthesis (Fig. 11). This finding suggests that, in the diabetic heart, fibrotic remodeling does not require conversion of fibroblasts into myofibroblasts but may involve an alternative activation pathway that stimulates synthesis of structural extracellular matrix proteins, without inducing expression of contractile proteins, such as α-SMA.

The relative contribution of collagen type I and type III in the cardiac matrix network has been implicated in the regulation of myocardial compliance. Collagen type I forms thicker and stiffer fibers, whereas the finer reticular collagen type III fibers are more compliant. In patients with dilated cardiomyopathy, expansion of collagen type I fibers has been associated with reduced ventricular compliance (36). Whether diastolic dysfunction in diabetes, obesity, and metabolic dysfunction is due at least in part to perturbations of the collagen type I-to-type III ratio has not been investigated.

Cardiac fibrosis in mouse models of obesity and type 2 diabetes. Fibrotic changes have been also described in other models of obesity and type 2 diabetes. Studies in leptin-deficient *ob*/*ob* mice have produced conflicting results. Although some studies have reported pericoronary fibrosis in *ob*/*ob* mice (57), other investigations found no significant fibrotic changes in the *ob*/*ob* myocardium (52). Documentation of cardiac fibrosis in mouse models of diet-induced metabolic dysfunction is dependent on the type and duration of the diet used, the sex and strain of the mice, and the sensitivity of the methodology used to assess fibrotic changes. Administration of a high-fat diet for 8–16 mo caused a significant increase in myocardial collagen type I and type III levels in male C57BL6J mice (7). High-fat diets with a high content of simple carbohydrates may accelerate the development of fibrosis. Male C57BL/6 mice fed a high-fat diet that was also rich in simple carbohydrates for 6 mo exhibited increased deposition of cross-linked collagen, associated with diastolic dysfunction (58). Male C57BL6 mice fed a high-fat diet containing high-fructose corn syrup exhibited myocardial fibrosis after 16 wk of feeding (26). Female mice may be more susceptible to fibrosis and diastolic dysfunction when fed a high-fat/high-fructose diet, exhibiting accentuated collagen type I deposition and increased myocardial stiffness after 8 wk of feeding (35).

Conclusions. Our study shows that the *db*/*db* mouse in the C57BL6J background can serve as a pathophysiologically relevant model of human HFpEF associated with metabolic dysfunction. Much like in human patients with diabetes and obesity-associated HFpEF, *db*/*db* mice exhibit evidence of

diastolic dysfunction with preserved ejection fraction, associated with cardiomyocyte hypertrophy, interstitial fibrosis, and microvascular rarefaction. Interpretation of studies using *db*/*db* mice requires careful consideration of sex-specific effects. The absence of myofibroblast conversion in *db*/*db* hearts highlights the distinct mechanisms of fibroblast activation in the repair of the infarcted heart and in metabolic dysfunction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.A. and N.G.F. conceived and designed research; L.A., I.R., and V.H. performed experiments; L.A., I.R., V.H., and N.G.F. analyzed data; L.A., I.R., V.H., and N.G.F. interpreted results of experiments; L.A., I.R., and N.G.F. prepared figures; L.A. and N.G.F. drafted manuscript; L.A., I.R., and N.G.F. edited and revised manuscript; L.A., I.R., V.H., and N.G.F. approved final version of manuscript.

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Cardiac fibrosis: Cell biological mechanisms, molecular pathways and therapeutic opportunities

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Extracellular matrix

ABSTRACT

Cardiac fibrosis is a common pathophysiologic companion of most myocardial diseases, and is associated with systolic and diastolic dysfunction, arrhythmogenesis, and adverse outcome. Because the adult mammalian heart has negligible regenerative capacity, death of a large number of cardiomyocytes results in reparative fibrosis, a process that is critical for preservation of the structural integrity of the infarcted ventricle. On the other hand, pathophysiologic stimuli, such as pressure overload, volume overload, metabolic dysfunction, and aging may cause interstitial and perivascular fibrosis in the absence of infarction. Activated myofibroblasts are the main effector cells in cardiac fibrosis; their expansion following myocardial injury is primarily driven through activation of resident interstitial cell populations. Several other cell types, including cardiomyocytes, endothelial cells, pericytes, macrophages, lymphocytes and mast cells may contribute to the fibrotic process, by producing proteases that participate in matrix metabolism, by secreting fibrogenic mediators and matricellular proteins, or by exerting contact-dependent actions on fibroblast phenotype. The mechanisms of induction of fibrogenic signals are dependent on the type of primary myocardial injury. Activation of neurohumoral pathways stimulates fibroblasts both directly, and through effects on immune cell populations. Cytokines and growth factors, such as Tumor Necrosis Factor-α, Interleukin (IL)-1, IL-10, chemokines, members of the Transforming Growth Factor-β family, IL-11, and Platelet-Derived Growth Factors are secreted in the cardiac interstitium and play distinct roles in activating specific aspects of the fibrotic response. Secreted fibrogenic mediators and matricellular proteins bind to cell surface receptors in fibroblasts, such as cytokine receptors, integrins, syndecans and CD44, and transduce intracellular signaling cascades that regulate genes involved in synthesis, processing and metabolism of the extracellular matrix. Endogenous pathways involved in negative regulation of fibrosis are critical for cardiac repair and may protect the myocardium from excessive fibrogenic responses. Due to the reparative nature of many forms of cardiac fibrosis, targeting fibrotic remodeling following myocardial injury poses major challenges. Development of effective therapies will require careful dissection of the cell biological mechanisms, study of the functional consequences of fibrotic changes on the myocardium, and identification of heart failure patient subsets with overactive fibrotic responses.

1. Introduction

Cardiac fibrosis, the expansion of the cardiac interstitium due to net accumulation of extracellular matrix (ECM) proteins is a common pathophysiologic companion of most myocardial diseases (Berk et al., 2007), (Kong et al., 2014). Although, in both human patients and in experimental models of heart disease, the extent of fibrotic remodeling is closely associated with adverse outcome, myocardial fibrosis is not necessarily the primary cause of dysfunction. In many cases, cardiac fibrosis is the result of a reparative process that is activated in response to cardiomyocyte injury. Adult mammalian hearts have negligible regenerative capacity; thus, loss of a significant number of cardiomyocytes triggers a reparative program, leading to formation of fibrous tissue. For example, in acute myocardial infarction, sudden death of up to a billion cardiomyocytes initiates an intense inflammatory reaction, ultimately leading to replacement of dead myocardium with a collagen-based scar (Frangogiannis, 2012b). After a transmural infarction, the fibrous scar is critical to protect the heart from rupture, by preserving the structural integrity of the ventricle and by protecting from dilative remodeling. Many other pathophysiologic conditions cause much more insidious forms of interstitial or perivascular fibrosis, often in the absence of significant cardiomyocyte replacement. Left ventricular pressure overload, typically caused by systemic hypertension or aortic stenosis, results in progressive interstitial

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and perivascular fibrosis, associated with markedly reduced myocardial compliance (Berk et al., 2007). Conditions associated with volume overload, such as valvular regurgitant lesions are also associated with a form of myocardial interstitial fibrosis that is characterized by disproportionately large amounts of non-collagenous matrix (Borer et al., 2002). Aging and metabolic perturbations, such as diabetes and obesity trigger an interstitial myocardial fibrotic response that is typically associated with increased myocardial stiffness and the development of heart failure with preserved ejection fraction (HFpEF) (Biernacka and Frangogiannis, 2011), (Russo and Frangogiannis, 2016), (Cavalera et al., 2014). Emerging evidence suggests that cardiac fibrosis associated with genetic cardiomyopathies may not be simply an epiphenomenon reflecting cardiomyocyte injury, as suggested by traditional teachings, but may be directly involved in the pathogenesis of myocardial dysfunction. For example, in patients with sarcomeric gene mutations that cause hypertrophic cardiomyopathy, a profibrotic state has been reported to precede the development of left ventricular hypertrophy (Ho et al., 2010). Although activation of cardiac fibroblasts is a common cell biological event in all myocardial fibrotic conditions, disease-specific pathophysiologic perturbations may trigger distinct molecular patterns of fibroblast activation that modulate the composition of the interstitial ECM, and profoundly affect the functional properties of cardiomyocytes.

This manuscript reviews the cell biological basis of cardiac fibrosis and discusses the molecular cascades mediating the fibrotic myocardial response. Considering the close association between myocardial fibrosis, cardiac dysfunction and adverse outcome, understanding the pathogenesis of fibrotic myocardial remodeling is crucial in order to identify new therapeutic targets for patients with heart disease.

2. The relation between cardiac fibrosis, dysfunction and adverse clinical outcome

In human patients with a wide range of myocardial conditions, cardiac fibrosis is a strong predictor of adverse outcome. In heart failure with reduced ejection fraction (HFrEF) patients, severe fibrosis documented with endomyocardial biopsy was found to predict death and adverse cardiac events (Aoki et al., 2011). On the other hand, HFpEF patients exhibit prominent interstitial myocardial fibrosis, associated with coronary microvascular rarefaction and inflammatory activation (Mohammed et al., 2015), (Paulus and Tschope, 2013). In HFpEF patients, focal myocardial fibrosis assessed by Magnetic Resonance Imaging (MRI) is associated with high mortality and increased hospitalization rates (Kato et al., 2015). In diabetic subjects, fibrotic expansion of the cardiac interstitium assessed through MRI is also associated with adverse outcome (Wong et al., 2014). Whether the prognostic significance of cardiac fibrosis in human patients with heart failure is due to direct adverse effects of the fibrotic response, or simply reflects a more pronounced reparative response to accentuated cardiomyocyte injury is unclear. However, several lines of evidence support the notion that fibrosis may have adverse consequences on myocardial function that may result in worse clinical outcome.

Normal ventricular systolic and diastolic function requires preservation of the myocardial architecture. Disturbance of the balance between synthesis and degradation of myocardial interstitial ECM proteins results in profound functional abnormalities. Increased deposition of interstitial collagen in the perimysial and endomysial space is typically associated with a stiffer ventricle and results in diastolic dysfunction. Perturbations of the interstitial collagen network in the fibrotic heart may also cause systolic dysfunction through several distinct mechanisms. First, fibrosis may disrupt the co-ordination of myocardial excitation-contraction coupling. Second, perivascular fibrosis may trigger microvascular dysfunction, perturbing cardiomyocyte perfusion under conditions of stress. Third, interstitial collagen deposition in fibrotic regions may stimulate protease-dependent pathways that lead to degradation of fibrillar collagens, thus disrupting the link between the matrix and the sarcomeric contractile apparatus, and leading to dysfunction (Iwanaga et al., 2002). ECM fragmentation may also deprive cardiomyocytes from key matrix-dependent molecular signals that promote cell survival, resulting in “anoikis”, a form of cell death caused by the loss of cell-matrix interactions (Michel, 2003). Interactions between cardiomyocytes and an intact ECM may also be required to preserve contractile function (Wang et al., 2006). Fourth, in certain cardiac fibrotic conditions, expansion and activation of interstitial immune cells, or acquisition of an inflammatory phenotype by activated fibroblasts may result in secretion of mediators that suppress cardiomyocyte function.

In addition to its potential role in cardiac dysfunction, cardiac fibrosis may also be critically involved in the pathogenesis of dysrhythmias, by perturbing conduction of the electrical impulse, resulting in subsequent generation of re-entry circuits (Khan and Sheppard, 2006). The ECM composition and cellular content of the cardiac interstitium may play an important role in determining arrhythmogenicity in a fibrotic heart. Evidence derived from rodent models suggests that infarct myofibroblasts are electrically coupled with border zone cardiomyocytes; however, their voltage responses exhibit slower kinetics, reflecting lower electrical conduction across heterocellular junctions (Rubart et al., 2018). On the other hand, deposition of ECM in fibrotic regions with a low cellular content may be associated with marked disruption of impulse propagation.

The arrhythmogenic effects of myocardial fibrosis are not limited to the left ventricle. Right ventricular fibrosis may be implicated in the pathogenesis of arrhythmias in chronic pulmonary hypertension (Umar et al., 2012), (Frangogiannis, 2017b) and in patients with arrhythmogenic right ventricular cardiomyopathy (ARVC) (Hamilton, 2009). On the other hand, fibrotic atrial remodeling is often associated with elevated atrial pressures due to ventricular dysfunction and predisposes to initiation and maintenance of atrial fibrillation (Thomas and Abhayaratna, 2017), (Tanaka et al., 2007), (Nattel, 2016), (Hanif et al., 2017). The presence of severe atrial fibrosis in patients with atrial fibrillation has adverse prognostic implications. In a retrospective study, patients with atrial fibrillation and severe atrial fibrosis assessed through late gadolinium enhancement cardiac MRI had a higher incidence of major cardiovascular and cerebrovascular events, primarily driven by stroke and transient ischemic attacks (TIA) (King et al., 2017). Whether there is a direct link between atrial fibrosis and the predisposition to thrombotic events remains unclear. The findings may simply reflect an increased likelihood of thrombosis in subjects with very low atrial flow velocities, due to severe fibrosis-related atrial dysfunction.

3. The mammalian cardiac interstitium

The adult mammalian ventricular myocardium is comprised of layers of tightly coupled cardiomyocytes and a highly cellular interstitium that is defined by an intricate network of ECM proteins. The cardiac ECM is comprised predominantly of fibrillary collagens (Fig. 1) and does not only serve as a scaffold for the cellular elements, but is also important for transmission of the contractile force. In normal adult mammalian hearts, type I collagen accounts for about 85% of total myocardial collagen and is primarily associated with thick fibers that confer tensile strength. In comparison, type III collagen represents about 10% of the total myocardial collagen, typically forms thin fibers, and maintains the elasticity of the cardiac ECM (Jugdutt, 2003), (Weber, 1989). The cardiac ECM also contains glycosaminoglycans, glycoproteins and proteoglycans, and contains stored latent growth factors and proteases, that can be activated in response to injury triggering a reparative response. From a morphological perspective, the cardiac ECM can be subdivided into epimysial, perimysial and endomysial constituents (Weber, 1989). The epimysium is located on the endocardial and epicardial surfaces and provides support for endothelial and mesothelial cells. The perimysium groups muscle fibers

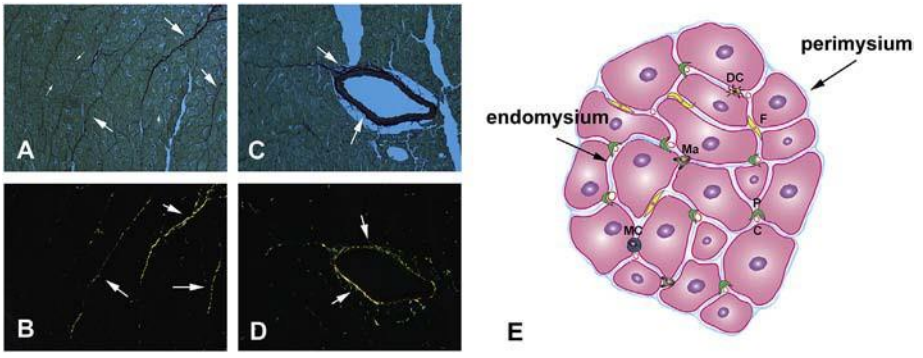

Fig. 1. The collagenous ECM in normal mammalian myocardium. Representative images show collagen fibers in 6-month old mouse hearts using picrosirius red-stained sections visualized under light microscopy (A, C), or polarized microscopy (B, D) A–B: Perimysium collagen fibers (large arrows) group muscle fibers into bundles, whereas the endomysium (small arrows) arises from the perimysium and surrounds individual cardiomyocytes. C–D: Peri-adventitial collagen fibers are located around vessels. E. The cardiac interstitium contains a wide variety of cells. The mammalian heart has a rich network of capillaries (C); thus, endothelial cells are the most abundant non-cardiomyocytes in adult mouse hearts. Large populations of resident fibroblasts (F) and pericytes (P) are also noted. The heart also contains smaller populations of immune cells, including macrophages (Ma), mast cells (MC), and dendritic cells (DC).

into bundles, whereas the endomysium arises from the perimysium and surrounds individual cardiomyocytes (Berk et al., 2007), (Shirwany and Weber, 2006).

Vascular endothelial cells are the most abundant non-cardiomyocytes in adult mouse hearts, reflecting the high microvascular density of the myocardium (Pinto et al., 2016). The cardiac interstitium also contains a large number of resident fibroblasts, pericytes and smooth muscle cells, and smaller populations of macrophages, mast cells, dendritic cells and lymphocytes (Gersch et al., 2002). The relative numbers of various cell types are dependent on the species and strain studied, and on the age of the animal. Intuitively, considering the high metabolic needs of cardiomyocytes, vascular cells are critical to ensure reliable blood supply that responds to the rapidly changing needs of the organ. The role of other interstitial cell subsets in cardiac homeostasis is less clear. Cardiac fibroblasts are enmeshed in the endomysial and perimysial ECM and may play an important role in preservation of the structural integrity of the matrix network (Eghbali et al., 1989), regulating collagen turnover, a process that requires ongoing synthesis and degradation of ECM proteins (Brown et al., 2005), (Spinale, 2007). Cardiac fibroblast subpopulations may also support cardiomyocyte survival and function. In the developing myocardium, cardiac fibroblasts have been suggested to regulate cardiomyocyte proliferation through a fibronectin/β1-integrin-mediated pathway (Ieda et al., 2009). During the neonatal period, elevated left ventricular pressures as the heart transitions from the fetal to the neonatal circulation are associated with expansion and transient activation of the cardiac fibroblast population (Banerjee et al., 2007), (Kong et al., 2013a). In young adult hearts, resident cardiac fibroblasts have a low level of activity, but can be readily activated in response to a wide range of injurious stimuli.

4. The histopathological types of cardiac fibrosis

Using histopathological criteria, three distinct forms of cardiac fibrosis can be recognized (Fig. 2). Replacement fibrosis reflects the generation of fibrous scar tissue in areas of cardiomyocyte necrosis, and represents the predominant pathology in myocardial infarction (Frangogiannis, 2015b). The term "interstitial fibrosis" is used to describe the widening of the endomysium and perimysium, due to deposition of structural ECM proteins. Expansion of the peri-adventitial collagenous area in the cardiac microvasculature is called "perivascular fibrosis". These distinctions have pathophysiologic and functional implications. Replacement fibrosis represents the end-result of a reparative response to primary necrotic injury of cardiomyocytes, and is typically associated with systolic ventricular dysfunction. In contrast, other injurious stimuli (such as mechanical stress due to pressure overload, myocardial inflammation, or metabolic dysregulation) may activate pro-fibrotic pathways in the absence of cell death, resulting in perivascular and/or interstitial fibrotic changes. These fibrotic alterations are typically associated with diastolic dysfunction.

Additional criteria could be used to characterize various types of myocardial fibrotic lesions, based on their cellular content, the relative contribution of inflammatory cells, the level of fibroblast activation, and the amount of cross-linked ECM proteins. For example, in human patients with ischemic cardiomyopathy, interstitial fibrotic lesions with a higher cellular content, increased inflammatory activity, and higher levels of matricellular proteins (suggesting an active fibrotic process) were associated with an increased likelihood of recovery after aorto-coronary bypass surgery (Frangogiannis et al., 2002a), (Frangogiannis et al., 2002b). Unfortunately, imaging tools are currently extremely limited in providing information on the cellular composition and molecular profile of fibrotic lesions in human hearts.

5. The cellular effectors of cardiac fibrosis

Fibroblast transdifferentiation into secretory and contractile cells, termed myofibroblasts, is a key cellular event that drives the fibrotic response in many different conditions associated with heart failure. Activated myofibroblasts are the main source of structural ECM proteins in fibrotic hearts (Cleutjens et al., 1995), and can also contribute to the regulation of matrix remodeling by producing proteases, such as matrix metalloproteinases (MMPs), and their inhibitors. Several other cell types (including macrophages, mast cells, lymphocytes, vascular endothelial cells, and pericytes) are implicated in fibrotic remodeling of the heart, either by secreting fibrogenic mediators and matricellular proteins, or in some cases, by undergoing conversion to activated myofibroblasts. Cardiomyocytes are also critical contributors to the myocardial fibrotic response. In myocardial infarction, cardiomyocyte death is the initial stimulus that triggers fibrosis through the release of alarmins and downstream activation of inflammation-dependent fibrogenic signaling. In other pathophysiologic conditions associated with heart failure, a variety of injurious stimuli, including mechanical stress, metabolic dysfunction, or inflammatory cytokines may induce a fibrogenic program in cardiomyocytes leading to activation of fibroblasts. The relative contribution of the various cell types in the myocardial fibrotic response is dependent on the underlying cause of cardiac injury.

5.1. Fibroblasts and myofibroblasts

5.1.1. Definition and functional properties of fibroblasts and myofibroblasts

Fibroblasts are cells of mesenchymal origin that populate connective tissues and produce significant amounts of structural ECM proteins. Due to the absence of specific and reliable markers, fibroblasts are typically identified on the basis of their location (enmeshed within connective tissues), morphological characteristics (flat or elongated cells that lack a basement membrane), and function (production of structural ECM proteins). The dynamic changes in the phenotypic and functional characteristics of fibroblasts in vivo and in vitro further

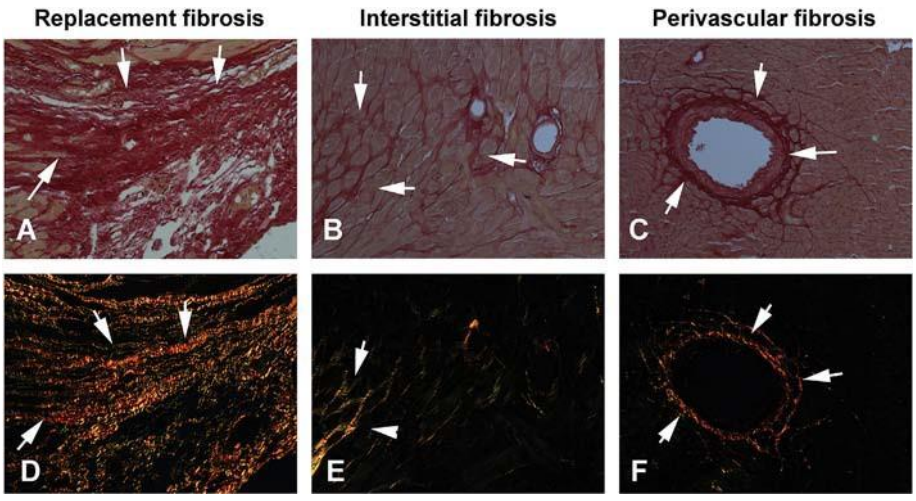

Fig. 2. Histopathologic types of cardiac fibrosis. Images show sirius red-stained section from mouse models of myocardial infarction (1 h ischemia/28 days reperfusion - A, D) and left ventricular pressure overload induced through transverse aortic constriction (28 days of pressure overload - B-C, E-F). Sections are visualized under light microscopy (A–C) or under polarized microscopy (D–F). A, D: Myocardial infarction is associated with sudden death of a large number of cardiomyocytes. Because the adult mammalian heart has negligible regenerative capacity, the infarcted heart heals through formation of a collagen-based scar (arrows - replacement fibrosis). B-C, E–F: Left ventricular pressure overload results in expansion of the endomysial and perimysial collagen network (B-C: interstitial fibrosis – arrows) and in deposition of collagen around vessels (E–F: perivascular fibrosis – arrows).

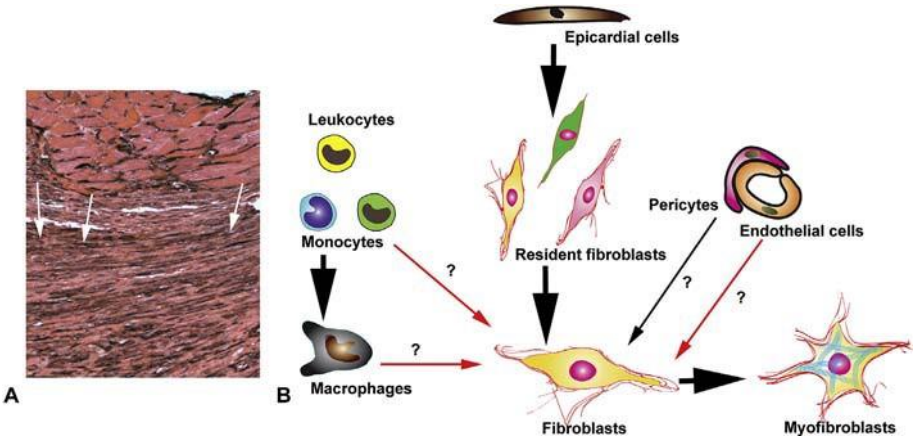

Fig. 3. Origin of activated myofibroblasts in fibrotic hearts. A. Immunohistochemical staining for α-smooth muscle actin (α-SMA) shows abundant myofibroblasts (arrows), organized in aligned arrays, infiltrating the border zone of a myocardial infarct (mouse model of reperfused infarction – 1 h ischemia/7 days reperfusion, counterstained with eosin). B. Although early evidence suggested that circulating leukocyte subsets and endothelial cells may account for a significant percentage of activated myofibroblasts in infarcted and remodeling hearts, recent investigations combining lineage tracing approaches, bone marrow chimeras and parabiosis experiments suggested that the vast majority of myofibroblasts in fibrotic mouse hearts are derived from resident populations. The role of pericytes remains unclear, as potential overlap between fibroblast and pericyte populations has not been adequately addressed. Leukocytes and endothelial cells appear to provide minor contributions. Resident fibroblast populations are heterogeneous; whether specific fibroblast subpopulations selectively expand following injury remains unknown. Moreover, the relative significance of various cell types may be dependent on the type of myocardial injury (see section 5.1.2.).

challenge efforts to define these poorly characterized cells (Shinde et al., 2017b). In injury sites, fibroblasts become activated and undergo conversion to myofibroblasts, cells that combine the presence of an extensive endoplasmic reticulum, a feature of synthetically active fibroblasts (Hinz, 2007), (Hinz, 2010), with the expression of contractile proteins, such as α-smooth muscle actin (α-SMA), and the de novo synthesis of matricellular proteins, such as periostin. Although α-SMA is useful to identify myofibroblasts in injured tissues (Fig. 3), its expression is not a requirement for the myofibroblast phenotype. At an early stage of conversion, myofibroblast-like cells may lack α-SMA expression, but may exhibit stress fibers rich in cytoplasmic actins and are termed “proto-myofibroblasts” (Hinz et al., 2007).

5.1.2. The origin of myofibroblasts in the fibrotic myocardium

Activated myofibroblasts accumulate in the myocardium in a wide range of pathologic conditions, including myocardial infarction (Willems et al., 1994), myocarditis (Blyszczuk et al., 2017), cardiac pressure or volume overload (Leslie et al., 1991), (Wang et al., 2003), and alcoholic cardiomyopathy (Law et al., 2012). Over the last 10 years, several groups have combined lineage tracing strategies, bone marrow transplantation studies, and parabiosis experiments to explore the cellular sources of activated myofibroblasts in the fibrotic myocardium (Aisagbonhi et al., 2011), (Zeisberg et al., 2007), (Mollmann et al., 2006), (Ali et al., 2014), (Moore-Morris et al., 2014), (Kanisicak et al., 2016) (Fig. 3). Although early investigations have suggested major contributions of endothelial cells (Aisagbonhi et al., 2011), (Zeisberg et al., 2007) and hematopoietic fibroblast progenitors (Mollmann et al., 2006), most recent studies combining lineage tracing experiments with several distinct Cre drivers suggest that subpopulations of resident cardiac fibroblasts are the main source for activated myofibroblasts in infarcted and remodeling hearts (Ali et al., 2014), (Moore-Morris et al., 2014), (Kanisicak et al., 2016), (Shinde and Frangogiannis, 2017), (Moore-Morris et al., 2018). The role of cardiac pericytes is less clear, as these cells remain poorly characterized, and may overlap with resident fibroblast subpopulations.

It should be emphasized that several major limitations of studies investigating the cellular origin of activated fibroblasts preclude absolute conclusions regarding the contributions of various cell types (Alex and Frangogiannis, 2018). First, specific markers for fibroblasts are lacking; identification of fibroblasts and fibroblast-like cells is typically based on myofibroblast conversion, or on evidence suggesting ECM protein expression (Kong et al., 2013a). In some studies, use of non-specific fibroblast markers may have produced unreliable results. For example, the use of fibroblast-specific protein (FSP)-1 to identify fibroblasts in some studies has been criticized due to the lack of specificity of this marker, that is also expressed by macrophages and vascular cells (Kong et al., 2013a). Second, use of relatively non-specific Cre drivers to trace endothelial cells or leukocyte subsets may challenge interpretation of the findings. Third, the relative contributions of various cell types may depend on the type of myocardial injury and on the fate of resident interstitial cell populations. For example, resident cardiac fibroblasts in the infarct zone may be less susceptible than

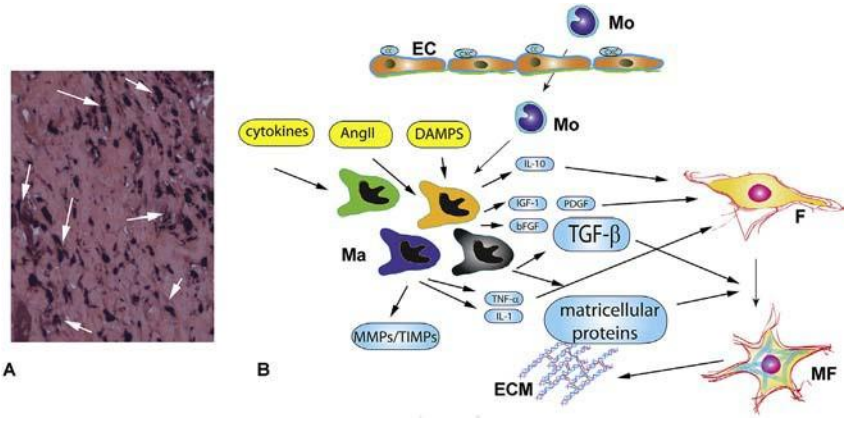

Fig. 4. Fibrogenic actions of macrophages in the remodeling myocardium. A. Immunohistochemical staining for the macrophage marker Mac2 shows abundant macrophages in an area of replacement fibrosis in a mouse model of reperfused myocardial infarction (arrows – 1 h ischemia/7 days reperfusion, counterstained with eosin. B. Schematic cartoon illustrates the mechanisms of macrophage activation in fibrotic hearts and the macrophage-derived mediators that may contribute to fibrotic remodeling. Macrophage (Ma) expansion is mediated through chemokine-dependent recruitment of circulating monocytes (Mo). Depending on the type of myocardial injury, macrophages are activated through effects of Damage-Associated Molecular Patterns (DAMPs), neurohumoral activation (AngII, angiotensin II) or cytokine secretion. Functionally distinct subsets of macrophages may contribute to the fibrotic response through secretion of a wide range of mediators, including pro-inflammatory cytokines (such as TNF-α and IL-1), anti-inflammatory and fibrogenic cytokines (such as IL-10 and TGF-β), or growth factors (such as IGF-1, FGFs and PDGFs). Macrophages also secrete matricellular proteins and may directly contribute to extracellular matrix (ECM) remodeling by producing matrix metalloproteinases (MMPs) and their inhibitors. Macrophage-derived fibrogenic mediators may play a critical role in conversion of fibroblasts (F) into myofibroblasts (MF) and may promote a proliferative and matrix-synthetic fibroblast phenotype.

cardiomyocytes and may survive, if the ischemic territory is rapidly reperfused. In contrast, prolonged ischemic insults in non-reperfused infarction may result in death of interstitial cells in the infarcted area along with neighboring cardiomyocytes, thus reducing their relative contribution to the expansion of the myofibroblast population. In their absence, recruitment of interstitial cells from non-infarcted areas, or activation of other cell types may be required to expand the fibroblast population. Moreover, various non-ischemic mechanisms of myocardial injury may selectively activate specific cell types, thus altering the profile of fibroblast progenitor cells. For example, in myocarditis, induction of an intense inflammatory response may be associated with chemokine-driven recruitment of circulating fibroblast progenitors (Kania et al., 2009) that may significantly contribute to the myofibroblast population.

5.1.3. Mechanisms involved in myofibroblast activation in fibrotic hearts

Myofibroblast activation in the infarcted and remodeling myocardium requires the co-operation of growth factors and specialized matrix proteins, which signal through cell surface receptors to activate intracellular signaling pathways that lead to synthesis of contractile proteins (such as α-SMA) and transcription of matrix macromolecules (Shinde and Frangogiannis, 2017). Macrophages, mast cells and lymphocytes infiltrating the remodeling heart play an important role in fibroblast activation by secreting a wide range of bioactive mediators, including cytokines (such as Transforming Growth Factor (TGF)-β1 and IL-10) and matricellular proteins (Carlson et al., 2017), (Nevers et al., 2015), (Saxena et al., 2014b), (Shiraishi et al., 2016), (Frangogiannis, 2012a). Stimulation of injury-site cardiomyocytes and vascular cells may also activate molecular cascades that modulate fibroblast behavior (Sassi et al., 2014).

Neurohumoral, cytokine-mediated and matricellular pathways have been critically implicated in activation of myofibroblasts in a wide range of cardiac injury models. Activation of the renin-angiotensin-aldosterone (RAAS) system stimulates fibroblast proliferation and ECM protein synthesis in the infarcted and remodeling myocardium, through activation of the angiotensin type 1 receptor (AT1), or through mineralocorticoid receptor signaling (Ju et al., 1997), (van den Borne et al., 2009). Clinical investigations support this concept demonstrating that administration of an aldosterone antagonist reduces the levels of circulating markers of collagen synthesis in patients with myocardial infarction and in subjects with HFrEF or HFpEF (Hayashi et al., 2003), (Pandey et al., 2015), (Zannad et al., 2000). Moreover, AT1 blockade significantly reduced indicators associated with myocardial fibrosis in patients with hypertensive heart disease (Ciulla et al., 2004). TGF−β also plays a crucial role in activation of fibroblasts in the remodeling myocardium, acting through pathways involving a family of intracellular effectors, the Smads, or through Smad-independent cascades. In addition to members of the TGF-β superfamily, a wide range of growth factors and immune cell-derived fibrogenic mediators have been implicated in the pathogenesis of cardiac fibrosis. Specific pathways involved in activation of fibroblasts in the remodeling heart are discussed in section 7.

5.2. Immune cells in cardiac fibrosis

5.2.1. The macrophages

Monocytes and macrophages play a critical role in regulation of fibrotic responses in many different tissues (Wynn and Vannella, 2016). The normal adult mammalian myocardium contains a relatively small population of resident macrophages (Epelman et al., 2014), (Heidt et al., 2014), (Mylonas et al., 2015), (Gersch et al., 2002) that has been suggested to play a role in cardiac homeostasis by facilitating atrioventricular conduction (Hulsmans et al., 2017). Following injury, resident cardiac macrophages which are derived from embryonic yolk sac cells are replaced by an abundant population of monocyte-derived macrophages (Epelman et al., 2014), (Heidt et al., 2014), recruited through activation of chemokine-dependent pathways (Frangogiannis et al., 2007), (Dewald et al., 2005). Macrophages in injured hearts are highly heterogeneous and exhibit functional and phenotypic versatility that enables them to participate in a wide range of processes, including regulation of inflammation, fibrosis, matrix remodeling, angiogenesis and regeneration (Honold and Nahrendorf, 2018), (Chen and Frangogiannis, 2017), (Frangogiannis, 2015a), (de Couto et al., 2015). Thus, subsets of activated macrophages may regulate fibrosis by serving as a major source of cytokines and growth factors with fibrogenic properties, by secreting proteases that participate in matrix remodeling, and by producing matricellular proteins (Fig. 4). It has also been suggested that a subset of myeloid cells may contribute directly to the fibrotic process through conversion to activated fibroblasts.

5.2.1.1. Do monocytes and macrophages serve as a source of myofibroblasts in fibrotic hearts?. Several investigations using bone marrow transplantation strategies to generate chimeric mice with labeled bone marrow cells have suggested that at least a subset of activated fibroblasts infiltrating fibrotic hearts may be of hematopoietic origin (Mollmann et al., 2006), (Szardien et al., 2012), (Chu et al., 2010). The identity of the circulating fibroblast progenitor cells remains obscure: these cells may represent leukocyte subsets capable of fibroblast transdifferentiation with similarities to the $CD14^{+}$ "fibrocytes" identified in human subjects (Bucala et al., 1994). Several recent

investigations using a combination of lineage tracing strategies and bone marrow transplantation experiments challenged the direct contribution of circulating progenitors, suggesting that bone marrow lineages do not undergo fibroblast conversion in infarcted and pressure-overloaded hearts (Kanisicak et al., 2016), (Moore-Morris et al., 2018), (Ali et al., 2014). Thus, macrophage-driven fibrosis likely requires induction and release of a wide range of fibrogenic mediators by activated macrophage subsets.

5.2.1.2. Macrophage subpopulations as sources of fibrogenic mediators. Monocytes and macrophages exhibit remarkable heterogeneity and phenotypic plasticity and are known to respond to microenvironmental cues by modulating synthesis of cytokines and growth factors (Mantovani et al., 2013). Macrophage subsets can produce large amounts of pro-fibrotic growth factors, such as TGF-β (Assoian et al., 1987), platelet-derived growth factors (PDGFs) (Osorniovargas et al., 1990), insulin-like growth factor (IGF)-1 (Tonkin et al., 2015) and fibroblast growth factors (FGFs) (Phan et al., 1987). Traditional concepts based on in vitro experiments have documented 2 distinct macrophage polarization states. Classically activated M1 macrophages, induced by Interferon (IFN)-γ, either by itself or in combination with Tumor Necrosis Factor (TNF)-α, or Granulocyte Macrophage-Colony Simulation Factor (GM-CSF), express pro-inflammatory cytokines and activate reactive oxygen species (ROS)-dependent cascades. In contrast, M2 macrophages, induced by Interleukin (IL)-4 or IL-13, express high amounts of IL-10 and have been suggested to participate in resolution of inflammation, angiogenesis and tissue repair (Mantovani et al., 2005; Shiraishi et al., 2016). Due to the complexity of the in vivo context, this oversimplified model of macrophage polarization is of limited value in understanding the contributions of macrophages in cardiac fibrotic conditions. Cardiac injury is likely to trigger generation of multiple macrophage subpopulations with nuanced phenotypes that may be implicated in regulation of inflammatory, angiogenic, or fibrogenic actions. Although differentiation of M2-like macrophages in the myocardium has been associated with the development of cardiac fibrosis (Yang et al., 2012), (Falkenham et al., 2015), and both IL-10 (Hulsmans et al., 2018) and TGF-βs have been suggested as important macrophage-derived fibrogenic signals, whether macrophage-driven fibrosis is dependent on a specific secreted mediator remains unknown.

5.2.1.3. Which stimuli activate a fibrogenic phenotype in cardiac macrophages?. The mediators that may activate a pro-fibrotic macrophage phenotype are poorly understood, and are likely dependent on the underlying cause of cardiac fibrosis. In infarcted hearts, release of damage-associated molecular patterns (DAMPs) activates Toll-like receptor (TLR) signaling in macrophages, thus triggering an inflammatory reaction that may lead to fibroblast activation (Wang et al., 2017). Fibrogenic neurohumoral signals such as angiotensin II and aldosterone are activated in many types of cardiac injury and may act, at least in part, by stimulating expression of fibrogenic mediators by macrophages (Usher et al., 2010), (Rickard et al., 2009). Pro-inflammatory cytokines (such as IL-1 and TNF-α), secreted in many cardiac fibrotic conditions may promote a fibrogenic macrophage phenotype by inducing transcription of IL-10, PDGFs, or members of the TGF-β superfamily. Moreover, the phagocytic function of macrophages may stimulate a fibrotic response, by inducing release of fibroblast-activating signals, such as TGF-βs (Huynh et al., 2002).

5.2.1.4. Are monocytes and macrophages essential for cardiac fibrotic responses?. Although cardiac injury is associated with recruitment of abundant macrophages, most macrophage-derived fibrogenic mediators can also be produced by other cell types. Thus, whether macrophages are essential effectors in fibrotic cardiac remodeling, or simply contribute to specific aspects of cardiac fibrosis has been questioned. A growing body of in vivo evidence suggests that macrophages are required for fibrotic responses in both myocardial infarction and cardiac pressure overload. In a mouse model of cryoinjury, macrophage depletion attenuated collagen deposition, perturbing repair of the infarcted myocardium (van Amerongen et al., 2007). In a model of salt-induced hypertension, macrophage depletion attenuated cardiac fibrosis; however, a surprising reduction in blood pressure in macrophage-depleted animals may have contributed to the observed cardiac phenotype (Kain et al., 2016). In a mouse model of ischemic non-infarctive cardiac fibrosis due to brief repetitive ischemic insults followed by reperfusion, chemokine-mediated recruitment of macrophages was crucial for the development of interstitial fibrosis (Frangogiannis et al., 2007). Moreover, in experimental models of hypertensive fibrotic remodeling, angiotensin II and mineralocorticoids were suggested to mediate their pro-fibrotic actions, at least in part, through activation of macrophage responses (Tokuda et al., 2004), (Sun et al., 2002), (Rickard et al., 2009), (Usher et al., 2010).

5.2.1.5. The potential role of macrophages in inhibition and resolution of cardiac fibrosis. Most published investigations have focused on the pro-fibrotic actions of macrophages. Considering their abundance and persistence in fibrotic lesions, their dynamic phenotype, and their ability to expand their functional repertoire by expressing proteases that degrade the ECM, certain macrophage subsets may contribute to resorption and resolution of fibrosis. In a model of hepatic fibrosis, Ly6Clo macrophages expressed high levels of MMPs and were suggested to play a role in regression of the fibrotic response (Fallowfield et al., 2007), (Ramachandran et al., 2012). Additional anti-fibrotic effects of macrophages may include suppression of fibroblast activation through secretion of mediators that deactivate fibroblasts, or through phagocytic removal of apoptotic myofibroblasts. Direct evidence supporting macrophage-dependent anti-fibrotic mechanisms in the remodeling myocardium is lacking, and subpopulations of putative "anti-fibrotic macrophages" have not been characterized.

5.2.2. The mast cells

The adult mammalian myocardium contains a small resident mast cell population; cardiac mast cells are more abundant in large mammals (such as dogs) than in mice (Sperr et al., 1994), (Frangogiannis et al., 1999). Regardless of etiology, cardiac fibrosis is associated with expansion of the myocardial mast cell population (Frangogiannis et al., 1998b), (Somasundaram et al., 2005), (Patella et al., 1998), (Shiota et al., 2003), (Wei et al., 2003), (Luitel et al., 2017). The mediators triggering accumulation of mast cells in fibrotic myocardial areas are poorly understood. Stem cell factor (SCF), a growth factor involved in recruitment of mast cell progenitors and in differentiation and growth of mature mast cells, is significantly induced in infarcted hearts and may contribute to the local expansion of cardiac mast cells (Frangogiannis et al., 1998b). A recent investigation suggested that mast cell progenitors infiltrating the infarcted myocardium may originate from the adipose tissue (Ngkelo et al., 2016).

Experimental evidence suggests a critical role for mast cell expansion in the pathogenesis of cardiac fibrosis (Levi-Schaffer and Rubinchik, 1994), (Levick et al., 2012), (Levick and Widiapradja, 2018). In a mouse model of left ventricular pressure overload, mast cell deficient mice had attenuated perivascular fibrosis, associated with reduced progression to decompensated heart failure (Hara et al., 2002). Following pressure overload, mast cells also accumulated in the atria and were involved in the development of atrial fibrosis and in the pathogenesis of atrial fibrillation through expression of PDGF-A (Liao et al., 2010). In a rat model of hypertension, pharmacologic mast cell stabilization attenuated fibrotic cardiac remodeling, by preventing infiltration of the myocardium with macrophages (Levick et al., 2009). In a model of fibrotic cardiomyopathy due to transgenic TNF-α overexpression, interactions between mast cells and fibroblasts were implicated in the development of cardiac fibrosis (Zhang et al., 2012). The potent pro-fibrotic actions of mast cell-derived mediators on the

myocardium are supported by findings demonstrating diastolic left ventricular dysfunction in many patients with systemic mast cell activation disorders (Kolck et al., 2007).

How do mast cells trigger fibrosis? Mast cells are known to store a wide range of pre-formed fibrogenic mediators in their granules, and can synthesize de novo fibroblast-activating cytokines and growth factors upon stimulation. Degranulation of mast cells following injury may be triggered by complement activation, ROS generation, adenosine, or cytokine stimulation, and may result in release of significant amounts of fibrogenic mediators that may trigger or amplify the fibrotic response. Several mast cell-derived mediators have been implicated in fibroblast activation, including TNF-α (Frangogiannis et al., 1998a), TGF-β (Shiota et al., 2003), IL-4 (Kanellakis et al., 2012), and PDGFs (Nazari et al., 2016). However, these fibrogenic mediators are also synthesized by many other cell types involved in cardiac fibrosis, including macrophages, lymphocytes, vascular cells and cardiomyocytes; thus, the relative contribution of mast cells is unclear. Due to their restricted localization to mast cells, histamine and the mast cell-specific proteases tryptase and chymase may represent a unique contribution of mast cells to the fibrotic process (de Almeida et al., 2002).

Chymase can generate angiotensin II (Urata et al., 1990b), and may represent the most important mast cell-derived mediator in cardiac fibrosis. It has been suggested that in failing hearts over 75% of cardiac-specific angiotensin II may be derived from activation of the Angiotensin Converting Enzyme (ACE)-independent chymase pathway (Urata et al., 1990a). This pathway is not affected by ACE inhibitors and may constitute a potential mechanism for the progression of cardiac fibrosis despite ACE inhibition. Moreover, chymase may also participate in the fibrotic response through activation of MMPs (Fang et al., 1997), (Stewart et al., 2003). In vivo studies in both rodent and large animal models of cardiac fibrosis support the importance of mast cell chymase in fibrotic cardiac remodeling, suggesting a promising therapeutic strategy. In a dog model of tachycardia-induced heart failure chymase inhibition decreased fibrosis and attenuated diastolic dysfunction (Matsumoto et al., 2003). In a porcine model of reperfused infarction chymase antagonism reduced cardiac fibrosis and decreased MMP expression (Oyamada et al., 2011). In a rat model of non-reperfused myocardial infarction, chymase inhibition attenuated interstitial fibrosis and protected from the development of diastolic dysfunction (Kanemitsu et al., 2006). Tryptase, the most abundant secretory product of the human mast cells, is also a potent activator of fibroblasts, stimulating proliferation (Ruoss et al., 1991) and inducing collagen I synthesis (Cairns and Walls, 1997) through the protease-activated receptor (PAR)-2, and downstream activation of Erk MAPK signaling (McLarty et al., 2011). Despite abundant in vitro evidence supporting fibrogenic actions of tryptase and the marked expansion of tryptase-positive mast cells in the fibrotic heart (Somasundaram et al., 2005), (Frangogiannis et al., 1998b), studies examining the potential in vivo role of tryptase in fibrotic cardiac remodeling have not been performed.

Although the bulk of the evidence suggests that mast cell-derived mediators promote fibrous tissue deposition, some experimental studies have suggested anti-fibrotic effects of mast cells (Joseph et al., 2005). The basis for such protective actions remains unclear and may reflect indirect actions of mast cell-derived mediators on cardiomyocyte survival, or growth factor expression profile function (Ngkelo et al., 2016). Much like macrophages, mast cells may respond to microenvironmental cues by altering their growth factor and protease expression profile, thus a transition from a pro-fibrotic to an anti-fibrotic mast cell phenotype cannot be excluded.

5.2.3. Lymphocytes and cardiac fibrosis

A growing body of evidence suggests an important role for T lymphocyte subpopulations in mediating cardiac fibrosis in both myocardial infarction and in non-ischemic heart failure (Nevers et al., 2015), (Laroumanie et al., 2014), (Li et al., 2017), (Bansal et al., 2017), (Ramos et al., 2016), (Wei, 2011), (Abdullah and Jin, 2018). In the infarcted heart, T cells may be recruited through activation of chemokine-dependent pathways (Dobaczewski et al., 2010c). The mechanism for T cell recruitment and activation in the pressure-overloaded myocardium is unclear, but may involve mechanical stress-induced activation of neurohumoral pathways (Li et al., 2017), (Amador et al., 2014), and subsequent induction of adhesion molecules in the microvascular endothelium (Salvador et al., 2016), leading to lymphocyte infiltration. Several cellular mechanisms have been proposed to explain the role of T lymphocytes in cardiac fibrosis. First, certain T cell subsets may act directly by activating fibroblasts. In a model of left ventricular pressure overload, direct adhesive interactions between activated Th1 cells and cardiac fibroblasts have been suggested to trigger fibroblast-derived TGF-β synthesis that may stimulate fibroblast to myofibroblast conversion (Nevers et al., 2017). Moreover, expansion of Th2 cells has been reported in fibrotic hearts (Duerrschmid et al., 2015), (Cieslik et al., 2011) and may be associated with upregulation of the pro-fibrotic cytokines IL-4 and IL-13, both potent inducers of fibroblast-derived collagen synthesis. Th17 cells also infiltrate the fibrotic myocardium and have been suggested to play a role in development of cardiac fibrosis in autoimmune myocarditis (Baldeviano et al., 2010). Second, T lymphocytes may exert fibrogenic actions by activating a fibrogenic program in macrophages. Third, pro-fibrotic actions of T lymphocytes may reflect effects on cardiomyocyte survival, ultimately leading to replacement of dying cells with fibrous tissue (Kallikourdis et al., 2017).

Whether certain T cell subpopulations may negatively regulate fibrosis remains unknown. A growing body of evidence suggests that cell therapy with regulatory T cells (Tregs) may attenuate myocardial fibrosis in experimental models of hypertensive heart disease (Kanellakis et al., 2011), angiotensin-induced cardiomyopathy (Kvakan et al., 2009) and myocardial infarction (Tang et al., 2012). Tregs may act by reducing the fibrogenic activity of macrophages (Weirather et al., 2014), by modulating fibroblast phenotype (Saxena et al., 2014b), or through secretion of yet unidentified anti-fibrotic signals. It should be emphasized that Tregs can also secrete fibrogenic mediators, such as TGF-β. Thus, their role in regulation of fibrotic response may be contextual and dependent on the balance between pro- and anti-fibrotic cellular actions.

5.2.4. The role of granulocytes in cardiac fibrosis

Neutrophils are prominently associated with acute myocardial inflammatory responses and contribute to tissue repair by modulating macrophage phenotype (Horckmans et al., 2017), but are rapidly cleared from sites of injury. Thus, the contribution of neutrophils to chronic myocardial fibrosis may be limited to the early phase of fibroblast activation. A recent study suggested an interesting neutrophil-dependent mechanism that may contribute to age-associated myocardial fibrosis. In senescent hearts, ROS activation in neutrophils may induce neutrophil extracellular trap (NET) formation (NETosis) through a process mediated by activation of the enzyme peptidylarginine deiminase 4 (PAD4). In vivo experiments suggested that PAD4-dependent NET formation may mediate interstitial fibrotic changes, triggering left ventricular diastolic dysfunction (Martinod et al., 2017).

Due to their low numbers and limited presence in injured myocardium, eosinophils and basophils are not considered important cellular effectors in most cardiac fibrotic conditions. Eosinophilic myocarditis, a rare inflammatory disease found in a subset of patients with hypereosinophilic syndromes, is associated with progression to a fibrotic stage, characterized by extensive endomyocardial scarring (Cheung et al., 2017). Whether these fibrotic changes are due to eosinophil-mediated fibroblast activation, or simply reflect a response to cardiomyocyte death is unknown. It should be noted that in experimental mouse models of autoimmune myocarditis, eosinophil depletion did not affect the development of fibrosis (Diny et al., 2017). A single study suggested that basophils may play a role in development of myocardial fibrosis in a mouse model of cardiac allograft vasculopathy, serving as the main source of the pro-fibrotic cytokine IL-4 (Schiechl

et al., 2016).

5.3. The pro-fibrotic effects of platelets

Platelets form a hemostatic plug during the early stages of the reparative response following myocardial infarction (Frangogiannis, 2014) and may be an important source of cytokines and growth factors that may directly activate fibroblasts, and/or promote a fibrogenic phenotype in macrophages and/or lymphocytes. However, their relative contribution to the myocardial fibrotic response remains poorly understood. Recently published studies suggested that platelet-derived TGF-β1 may play an important role in the pathogenesis of cardiac fibrosis in mouse models of left ventricular pressure overload (Meyer et al., 2012) and protease-inhibitor induced cardiomyopathy (Laurence et al., 2017). Considering the multiple likely cellular sources of TGF-βs in the remodeling myocardium (including cardiomyocytes, macrophages, fibroblasts and vascular cells), the significance of platelet-derived TGF-β may be context-dependent.

5.4. The vascular endothelial cells as effectors of cardiac fibrosis

The high prevalence of perivascular fibrosis in animal models of heart disease (Xia et al., 2009) and in human patients with cardiomyopathic conditions (d'Amati and Factor, 1996) may suggest the involvement of vascular cells in fibroblast activation. Following myocardial injury, vascular endothelial cells may contribute to fibrotic cardiac remodeling through several distinct mechanisms. First, induction of adhesion molecules, such as Intercellular adhesion molecule (ICAM)-1 in stimulated microvascular endothelial cells may play an important role in recruitment of fibrogenic macrophages and lymphocytes in both infarcted and pressure-overloaded hearts (Salvador et al., 2016). Second, endothelial cells may be an important source of pro-fibrotic mediators, such as TGF-β1, FGFs, or endothelin (ET)-1. Endothelial cell-derived ET-1 has been implicated in the development of myocardial fibrosis in a model of angiotensin-induced cardiomyopathy (Adiarto et al., 2012) and in diabetes (Widyantoro et al., 2010). Third, pro-inflammatory cytokines and chemokines released by endothelial cells may be implicated in the fibrotic response by contributing to recruitment and activation of pro-fibrotic leukocytes. Fourth, several experimental studies have suggested that following myocardial injury, endothelial cells may undergo endothelial to mesenchymal transition (EndMT) (Zeisberg et al., 2007), (Aisagbonhi et al., 2011) thus directly contributing to the expansion of the fibroblast pool in cardiac fibrosis. However, robust lineage tracing studies in both infarction and pressure overload models (Kanisicak et al., 2016), (Ali et al., 2014) reported very low numbers of endothelial-derived fibroblasts in the remodeling myocardium, suggesting a limited role for EndMT in the cardiac fibrotic response (Li et al., 2018).

In addition to their pro-fibrotic actions, endothelial cells could also serve as negative regulators of fibrosis, secreting mediators that deactivate cardiac fibroblasts. Evidence supporting an anti-fibrotic endothelial cell phenotype in vivo remains limited. Expression of Hypoxia Inducible Factor (HIF)-1 in endothelial cells has been suggested to exert anti-fibrotic actions in the pressure-overloaded myocardium, mediated at least in part through suppression of TGF-β signaling (Wei et al., 2012). Moreover, endothelial cells may serve as an important source of the CXC chemokine Interferon-γ-inducible Protein (IP)-10/CXCL10, a mediator that inhibits fibroblast migration in the infarcted myocardium, exerting anti-fibrotic actions (Frangogiannis et al., 2001), (Bujak et al., 2009).

5.5. The vascular mural cells in cardiac fibrosis

The heart contains a large population of resident pericytes that enwrap the rich cardiac microvasculature. Despite their broad range of potential functions in perfusion, angiogenesis, inflammation, fibrosis and regeneration (Chen et al., 2015), (O'Farrell and Attwell, 2014), (Birbrair et al., 2014), (Avolio and Madeddu, 2016), pericytes have remained understudied in cardiovascular disease. Lineage tracing studies suggested that Gli-1+ perivascular progenitor cells provide a major contribution to the population of activated myofibroblasts in remodeling hearts (Kramann et al., 2015). Moreover, pericytes may participate in the fibrotic response by secreting inflammatory mediators and fibroblast-activating growth factors. The molecular signals responsible for pericyte activation in fibrotic hearts remain unknown.

5.6. The cardiomyocytes

Under conditions of stress, cardiomyocytes can produce and secrete a wide range of inflammatory and pro-fibrotic mediators. Neurohumoral and growth factor-mediated pathways may play an important role in activation of cardiomyocyte-derived fibrogenic signals. In a mouse model of deoxycorticosterone/salt-mediated cardiac fibrosis, cardiomyocyte-specific mineralocorticoid receptor signaling mediated the fibrotic response (Rickard et al., 2012). Moreover, in a model of cardiac pressure overload, TGF-β receptor II signaling in cardiomyocytes was also found to contribute to the development of cardiac fibrosis (Koitabashi et al., 2011). In aging hearts, IGF-1 signaling in cardiomyocytes was implicated in the development of interstitial fibrosis (Ock et al., 2016). In the infarcted myocardium, release of DAMPs by necrotic cardiomyocytes is an important early stimulus triggering an inflammation-driven fibrotic response (Frangogiannis, 2008), (Prabhu and Frangogiannis, 2016). Thus, focal replacement fibrosis may result from cardiomyocyte death, rather than reflecting direct actions of cardiomyocyte-derived fibrogenic signals. Whether cardiomyocytes can also release anti-fibrotic signals is less convincingly established. Genetic manipulations of cardiomyocytes to overexpress the angiotensin II type 2 receptor (AT2), or the muscle-specific ubiquitin ligase atrogin-1 inhibited pressure overload-induced or aging-associated cardiac fibrosis respectively (Kurisu et al., 2003), (Mota et al., 2018). Moreover, expression of plasminogen activator inhibitor (PAI)-1 by cardiomyocytes has been suggested to protect the myocardium from fibrotic remodeling by attenuating TGF-β synthesis (Flevaris et al., 2017). Whether such effects are due to modulation of the fibrogenic mediator expression profile of cardiomyocytes, or simply reflect reduced cardiomyocyte death remains unknown.

6. ECM composition of the fibrotic heart determines the mechanical properties of the ventricle and regulates cellular function

Increased deposition of ECM proteins is the hallmark of the fibrotic process and has a major impact on cardiac diastolic and systolic function. However, the role of the cardiac ECM is not limited to structural support and to the transmission of mechanical force. ECM proteins play an active role in modulating cellular responses by transducing signals through direct binding to cell surface receptors, or by modulating cytokine- and growth factor-mediated signaling (Frangogiannis, 2017a; Herum et al., 2017). In fibrotic hearts, the cardiac ECM is enriched with a wide range of matricellular proteins, secreted macromolecules which are typically not part of the normal ECM and do not play a prominent structural role, but are induced following injury and participate in regulation of cellular signaling. As the heart remodels, the dynamic alterations in the composition of the ECM modulate phenotype and function of cardiomyocytes, fibroblasts, immune cells and vascular cells and may facilitate fibrotic remodeling by transducing key fibrogenic signals. The extent and time course of these alterations are dependent on the underlying etiology of fibrosis. In myocardial infarction, activation of an intense inflammatory reaction triggers degradation of the native cardiac ECM, resulting in generation of matrix fragments, followed by deposition of a fibrin- and fibronectin-based provisional network and by the deposition of newly-synthesized matricellular

proteins (Dobaczewski et al., 2010b), (Frangogiannis, 2012a). In models of chronic heart failure, acute ECM changes are less dramatic, leading to progressive, but prolonged deposition of specialized ECM macromolecules.

6.1. The fibrillar collagens

Regardless of the underlying cause, cardiac fibrosis is associated with increased synthesis of both type I and type III collagen (Cleutjens et al., 1995), (Weber, 1989). It has been suggested that the relative contribution of collagen I and III fibers may regulate myocardial compliance. Collagen I forms thicker and stiffer fibers, whereas the finer reticular collagen III fibers are more compliant. In models of hypertensive cardiac fibrosis and in the infarcted myocardium, type I collagen exhibits more intense and prolonged upregulation than collagen III (Mukherjee and Sen, 1993), (Whittaker et al., 1989). In patients with dilated cardiomyopathy expansion of collagen I fibers have been associated with reduced ventricular compliance (Marijianowski et al., 1995). In contrast, in patients with ischemic cardiomyopathy the ratio of collagen I:collagen III synthesis was decreased (Mukherjee and Sen, 1991), highlighting the context-dependent nature of collagen type induction. Activated myofibroblasts are the main cellular sources of collagens in the fibrotic heart; once outside the cell procollagen chains are processed, assembled into fibrils and cross-linked. Cross-linking enzymes, including members of the lysyl-oxidase (LOX) family (Zibadi et al., 2009), (Adam et al., 2011), (El Hajj et al., 2016), (Lopez et al., 2010b), (Yang et al., 2016) and transglutaminase-2 (TG2, tissue transglutaminase) (Shinde et al., 2017a), (Shinde and Frangogiannis, 2018) have been implicated in ECM crosslinking in fibrotic hearts. In addition to its enzymatic matrix cross-linking actions, matrix-bound TG2 also exerts transamidase-independent actions, modulating fibroblast-mediated MMP and TIMP synthesis (Shinde et al., 2018). Although excessive collagen cross-linking contributes to the development of diastolic dysfunction in the fibrotic heart (Lopez et al., 2012), a certain level of cross-linking activity may be required to preserve the integrity of the cardiac ECM and to prevent chamber dilation (Woodiwiss et al., 2001).

6.2. Non-fibrillar collagens

In addition to the deposition of fibrillar collagens, cardiac fibrosis in cardiomyopathic conditions is associated with increased myocardial levels of non-fibrillar collagens, including collagen IV, VI and VIII (Gil-Cayuela et al., 2016). Growth factors implicated in induction and secretion of fibrillary collagens (such as TGF-β) are also capable of inducing synthesis of non-fibrillar collagens. The role of non-fibrillar collagens in regulation of the fibrotic response is poorly understood. In vitro, non-fibrillar collagens have profound effects on fibroblast phenotype and function. Collagen VI potently stimulates myofibroblast transdifferentiation (Naugle et al., 2006), whereas collagen VIII promotes fibroblast migration and enhances TGF-β synthesis (Skrbic et al., 2015). In vivo, collagen VI disruption reduced fibrosis and attenuated dysfunction in an experimental model of myocardial infarction (Luther et al., 2012). Moreover, collagen VIII loss was associated with reduced infiltration of the pressure-overloaded heart with myofibroblasts and attenuated fibrosis; these effects were associated with increased mortality and left ventricular dilation (Skrbic et al., 2015). The cellular basis for the effects of non-fibrillar collagens remains unknown. In addition to their actions on fibroblasts, non-fibrillar collagens also modulate cardiomyocyte survival under conditions of stress and may exert important actions on inflammatory and vascular cells (Luther et al., 2012). Robust evidence using confocal and super-resolution fluorescence microscopy localized collagen IV and VI in the lumen of the t-tubules in patients with heart failure, suggesting a potential link between alterations in the fibroblast-derived ECM and the microstructural and functional characteristics of cardiomyocytes (Crossman et al., 2017).

6.3. Fibrin and fibronectin: components of the provisional matrix that regulate fibroblast phenotype and function

Increased permeability of the microvasculature in the injured myocardium results in extravasation of fibrinogen and plasma fibronectin, leading to formation of provisional matrix network (Dobaczewski et al., 2006) that serves as a dynamic conduit for inflammatory and mesenchymal cells. This network is most prominent in infarcted hearts. Fibrin and fibronectin facilitate fibroblast and leukocyte migration (Flick et al., 2004) and stimulate fibroblast proliferation (Rybarczyk et al., 2003) and activation through interactions that involve cell surface molecules, such as integrins (Greiling and Clark, 1997) and syndecans (Lin et al., 2005). In healing infarcts, lysis of the plasma-derived provisional matrix by granulation tissue cells is followed by generation of an organized cell-derived "second order" provisional matrix that contains cellular fibronectin and hyaluronan (Welch et al., 1990). Components of the provisional matrix are critically implicated in myofibroblast conversion. The ED-A variant of cellular fibronectin co-operates with TGF-β stimulating acquisition of the myofibroblast phenotype (Arslan et al., 2011), (Serini et al., 1998), (Hinz et al., 2007). The molecular basis for the ED-A-driven myofibroblast conversion remains poorly understood. In vitro experiments suggest that ED-A fibronectin binds to Latent TGF-β—Binding Protein (LTBP)-1, contributing to the spatial localization of activatable TGF-β in tissues (Klingberg et al., 2018). In vivo evidence demonstrated that ED-A fibronectin is consistently upregulated in the infarcted and pressure-overloaded heart and in models of chronic cardiac rejection associated with cardiac fibrosis (Knowlton et al., 1992), (Samuel et al., 1991), (Villarreal and Dillmann, 1992), (Franz et al., 2010). In healing infarcts ED-A fibronectin has been implicated in myofibroblast conversion (Arslan et al., 2011; Santiago et al., 2010). Fibronectin polymerization is a critical regulator of ECM organization and stability (Sottile and Hocking, 2002), and has been directly implicated in myofibroblast activation in the infarcted heart (Valiente-Alandi et al., 2018).

6.4. The matricellular proteins

Cardiac remodeling, regardless of etiology, is associated with induction and interstitial deposition of matricellular proteins (Frangogiannis, 2012a). Matricellular proteins bind to components of the structural matrix and to cell surface receptors (such as integrins and syndecans), and function as "molecular bridges" between the ECM and the cells, transducing or modulating cytokine and growth factor responses (Bornstein, 2009). The family includes the thrombospondins (TSPs), the tenascins-C and -X, osteopontin, SPARC (secreted protein acidic and rich in cysteine), periostin, and the members of the CCN family; new members and proteins that exhibit some matricellular functions are being increasingly recognized. In the fibrotic myocardium, cardiomyocytes, fibroblasts, immune cells (including macrophages and lymphocytes), and vascular cells, the major cellular effectors of fibrosis, have been identified as important targets of the matricellular proteins. Important actions of matricellular proteins include regulation of myofibroblast conversion, activation, and survival (Xia et al., 2011), (Zohar et al., 2004), (Oka et al., 2007), (Shimazaki et al., 2008) activation of growth factors (such as TGF-β) (Adams and Lawler, 2004), modulation of protease activation (Rodriguez-Manzaneque et al., 2001), (Hogg, 1994), (Schroen et al., 2004) regulation of angiogenesis (Gonzalez-Quesada et al., 2013), activation of cardiac macrophages and other immune cell subpopulations (Shimojo et al., 2015), and effects on cardiomyocyte survival and function (Cingolani et al., 2011), (Swinnen et al., 2009). Although most members of the family appear to exert fibrogenic actions in vivo, matricellular actions that inhibit the fibrotic response or contribute to resolution of fibrosis have also been reported (Jeong et al., 2016).

6.5. Extracellular proteoglycans

Proteoglycans are glycosylated proteins that consist of a protein core with covalently attached glycosaminoglycan (GAG) chains. In most tissues, the regional profile of ECM proteoglycans is dependent on topography. Heparan sulfate proteoglycans (HSPGs) are typically associated with the cell surface, or the pericellular matrix. As we move away from the cells, chondroitin- and dermatan sulfate-containing proteoglycans (CSPGs and DSPGs) predominate (Iozzo and Schaefer, 2015). In both human patients with heart failure and in animal models of myocardial disease, CSPGs accumulate in fibrotic regions (Zhao et al., 2018); their upregulation may be induced by growth factors, such as TGF-β. The normal and fibrotic cardiac ECM also contains a wide range of small leucine rich proteoglycans (SLRPs), such as decorin, fibromodulin, lumican and osteoglycin. SLRPs bind to collagen fibrils and organize the structural ECM, but may also interact with growth factors, receptor tyrosine kinases, or TLRs to transduce or modulate signaling responses. Following cardiac injury, induction of SLRPs has been implicated in regulation of the fibrotic response (Christensen et al., 2018), (Engebretsen et al., 2013). Biglycan was found to be critically involved in organization of the structural ECM following myocardial infarction (Westermann et al., 2008), but was also reported to mediate fibrosis and dysfunction in the pressure-overloaded myocardium (Beetz et al., 2016). Lumican has been suggested to protect against cardiac fibrosis and dysfunction in aging and isoproterenol infusion models (Chen et al., 2017b). On the other hand, decorin gene therapy exerted anti-fibrotic actions in hypertensive rats by neutralizing TGF−β-mediated responses (Yan et al., 2009). In a model of reparative infarctive fibrosis, osteoglycin protected from dilation and dysfunction, by contributing to the assembly of the collagenous ECM network (Van Aelst et al., 2015). The combined experience from various mouse models of cardiac remodeling suggests important roles for SLRPs in regulation of myocardial fibrosis; however, their functional role is dependent on the pathophysiologic context, and the cellular targets remain unknown.

6.6. Modulation of the cardiac ECM by proteolytic enzymes

Both interstitial cells and cardiomyocytes can produce and activate proteolytic enzymes that process ECM proteins (Borg et al., 1997), altering the mechanical properties of the myocardium. Induction and activation of collagenases (such as MMP1, MMP8, and MMP13), gelatinases (such as MMP2 and MMP9), stromelysins/matrilysins and membrane-type MMPs has been extensively reported in experimental models of cardiac fibrosis (DeLeon-Pennell et al., 2017), (Goldsmith et al., 2013). Inhibitors of metalloproteinases, such as TIMP1 and TIMP3 are also upregulated in fibrotic hearts and may exert matrix-preserving actions (Takawale et al., 2017a), (Takawale et al., 2017b). Regulation of the protease/antiprotease balance plays a critical role in the biochemical profile of ECM proteins in the cardiac interstitium and has profound functional implications. Overactive matrix-preserving pathways (associated with induction of TIMPs) may promote deposition of structural ECM proteins, increasing myocardial stiffness and accentuating diastolic dysfunction. On the other hand, MMP induction and activation may generate a predominantly proteolytic environment in the cardiac interstitium, leading to degradation of ECM proteins (Spinale et al., 2013), and subsequent loss of important pro-survival signals transduced by the intact matrix. Thus, MMP activation and matrix degradation may promote cardiomyocyte apoptosis, or perturb contractile function in the fibrotic heart. The complexity of MMP biology in the remodeling heart is further increased by the wide range of ECM-independent actions of MMPs that may modulate the inflammatory signaling cascade and regulate cell surface receptor-mediated signaling. Members of the MMP family have been reported to process CC and CXC chemokines (McQuibban et al., 2002), (McQuibban et al., 2001) and cytokines (such as TNF−α) (Lee et al., 2014), thus exerting context-dependent pro- or anti-inflammatory actions. Moreover, MMPs have been implicated in activation of the potent fibrogenic mediator TGF-β (D'Angelo et al., 2001), and may cleave integrins (Deryugina et al., 2002), syndecans (Endo et al., 2003), or other transmembrane receptors thus modulating essential fibrogenic cascades. Actions of MMPs in the intracellular compartment have also been reported and may promote degradation of contractile proteins in cardiomyocytes, or modulate signal transduction responses in interstitial cells (Jobin et al., 2017), (Sawicki et al., 2005), (de Castro Bras et al., 2014). The relative contribution of the diverse proteolytic and non-enzymatic actions of MMPs in the extracellular and intracellular compartments in regulating in vivo responses remains unclear.

7. Molecular pathways implicated in cardiac fibrosis

Although similar molecular pathways may mediate the different forms of cardiac fibrosis, their relative significance may be dependent on the underlying cause of the fibrotic reaction. In response to various types of cardiac injury, neurohumoral pathways, inflammatory signals, fibrogenic growth factors, and matricellular macromolecules co-operate to activate fibroblast cell surface receptors, thus transducing intracellular signaling cascades that lead to transcription of ECM genes and modulation of proteins associated with matrix remodeling.

7.1. Neurohumoral pathways

7.1.1. The renin-angiotensin-aldosterone system (RAAS)

Activation of the RAAS is consistently found in remodeling and fibrotic hearts, regardless of the underlying etiology. Surviving cardiomyocytes, infiltrating macrophages and fibroblasts produce renin and angiotensin converting enzyme (ACE) in the remodeling myocardium, molecules necessary for generation of angiotensin II (Weber et al., 2012), (Hokimoto et al., 1996). Locally released angiotensin II serves as a potent activating stimulus for cardiac fibroblasts both through direct actions and through TGF-β-mediated effects (Kagami et al., 1994). In vitro studies have demonstrated that angiotensin II stimulates cardiac fibroblast proliferation and enhances their collagen-synthetic activity through AT1 receptor-dependent interactions (Schorb et al., 1993) (Sadoshima and Izumo, 1993), (Crabos et al., 1994). In contrast, AT2 signaling has been suggested to inhibit AT1-mediated actions, suppressing fibroblast proliferation and matrix synthesis (Ohkubo et al., 1997), and thus serving as a negative regulator of angiotensin II-mediated pro-fibrotic responses (Kurisu et al., 2003). In vivo, extensive evidence supports the pro-fibrotic actions of AT1 signaling. AT1 blockade significantly reduced interstitial fibrosis in models of myocardial infarction (Schieffer et al., 1994) and left ventricular pressure overload (Regan et al., 1997). The beneficial effects of ACE inhibition and AT1 blockade in patients with chronic heart failure or acute myocardial infarction may be due, at least in part, to inhibition of angiotensin-induced fibrogenic actions.

Aldosterone is also capable of inducing fibrotic changes in the myocardium (Lijnen and Petrov, 2000), as suggested by experimental animal studies and by the development of reactive myocardial fibrosis in patients with adrenal adenomas (Campbell et al., 1992). In vitro studies and experiments using cell-specific mineralocorticoid receptor knockout mice subjected to pressure overload protocols suggested that fibroblasts, cardiomyocytes, myeloid cells, lymphocytes and vascular cells are important cellular targets of aldosterone in the remodeling heart. Aldosterone-mediated signaling drives macrophages towards a fibrogenic phenotype (Rickard et al., 2009), activates T cells (Li et al., 2017), induces cardiomyocyte-derived fibrogenic signals (Rickard et al., 2012), and directly stimulates fibroblasts, triggering proliferation (Neumann et al., 2002) and accentuating collagen synthesis (Brilla et al., 1994). Although aldosterone is known to stimulate cytokine and chemokine expression by endothelial cells (Sun et al., 2002), endothelial cell-specific mineralocorticoid loss did not affect

inflammation and fibrosis in a model of cardiac pressure overload (Salvador et al., 2017). To what extent the protective effects of mineralocorticoid antagonism in patients with heart failure (Pitt et al., 1999) are due to anti-fibrotic actions remains unknown.

7.1.2. Adrenergic signaling

Chronic administration of β-adrenergic receptor (AR) agonists, and transgenic overexpression of β2-ARs trigger fibrotic cardiac remodeling (Nguyen et al., 2015), that is caused, at least in part, by cardiomyocyte death and subsequent reparative fibrosis (Benjamin et al., 1989), but may also involve direct activation of fibroblasts, or stimulation of a cytokine cascade and recruitment of macrophages (Xiao et al., 2018). In vitro and in vivo studies have documented activating effects of β-AR signaling on cardiac fibroblasts that may be mediated in part through p38 MAPK signaling (Molkentin et al., 2017). Moreover, β-AR signaling regulates cytokine expression by macrophages (Noh et al., 2017) and induces synthesis of growth factors by cardiomyocytes (Nuamnaichati et al., 2018). The relative role of β1-and β2-AR responses in regulating the fibrotic response is unknown. Although in some experimental animal models of heart failure, β-AR blockade attenuated cardiac fibrosis (Le et al., 2013), such effects may represent an epiphenomenon reflecting reduced cardiomyocyte death, and the significance of anti-fibrotic actions in mediating protection is unclear. On the other hand, recent evidence suggests that β3-AR signaling in cardiomyocytes may protect the heart in a model of pressure overload-induced cardiac fibrosis. The anti-fibrotic effects were presumed due to downmodulation of the oxidative stress-dependent expression of the matricellular protein CCN2 by cardiomyocytes (Hermida et al., 2018).

In the injured and remodeling myocardium, adrenergic stimulation induces conformational changes in G protein βγ subunits, ultimately resulting in activation of G protein-coupled receptor kinase 2 (GRK2). GRK2 activation in cardiac fibroblasts has been reported to exert important fibrogenic actions in experimental models of reperfused myocardial infarction (Woodall et al., 2016), (Travers et al., 2017). The specific fibrogenic signals activated by GRK2 remain poorly characterized.

7.1.3. Endothelin (ET)-1

Both in vitro and in vivo studies suggest that ET-1, is a potent fibrogenic mediator that may act downstream of cytokines and neurohumoral mediators (Leask, 2010), serving as a link between inflammation and fibrosis (Alvarez et al., 2011). Both TGF-β and angiotensin II are capable of inducing ET-1 in various cell types (Shiwen et al., 2007); ET-1 is secreted in failing human hearts (Tsutamoto et al., 2000) and is upregulated in experimental models of hypertensive and age-associated cardiac fibrosis (Yamamoto et al., 2000a), (Wang et al., 2015). In vitro, ET-1 enhances cardiac fibroblast proliferation (Piacentini et al., 2000), promotes matrix protein synthesis, decreases collagenase activity (Guarda et al., 1993), and induces an apoptosis-resistant fibroblast phenotype (Kulasekaran et al., 2009). In vivo, cardiac-specific overexpression of ET-1 induced myocardial fibrosis associated with biventricular systolic and diastolic dysfunction (Mueller et al., 2011). Moreover, ET-1 antagonism attenuated fibrotic myocardial remodeling in animal models of hypertensive, and reparative cardiac fibrosis (Ammarguellat et al., 2001), (Mulder et al., 1997). Whether the fibrogenic effects of ET-1 are due to direct fibroblast activation, or reflect actions on other cell types, such as cardiomyocytes (Ceylan-Isik et al., 2013) or vascular cells remains unknown.

7.2. Inflammatory cascades

Chronic inflammation is associated with recruitment of fibrogenic macrophages and lymphocytes, and with induction and secretion of cytokines and growth factors that directly stimulate fibroblast expansion and activation. Moreover, persistent myocardial inflammation may cause cardiomyocyte death, leading to development of replacement fibrosis. Several members of the cytokine and chemokine families have been implicated in fibrotic remodeling of the injured myocardium. The relative contribution of pro-inflammatory signals in the pathogenesis of cardiac fibrosis in human patients with heart failure is likely dependent on the underlying pathophysiologic condition.

7.2.1. Chemokines

Chemokines are a family of chemotactic cytokines with a critical role in leukocyte trafficking (Rollins, 1997). Depending on the number of aminoacids between their first 2 cysteine residues, chemokines can be subdivided into CC, CXC, CX3C, and XC subfamilies. Several members of the chemokine family have been implicated in regulation of fibrotic responses, either through recruitment of pro-fibrotic leukocyte subsets, or through direct actions on fibroblasts (Dobaczewski and Frangogiannis, 2009). The CC chemokine CCL2/Monocyte Chemoattractant Protein (MCP)-1 is the best-studied chemokine in heart disease (Xia and Frangogiannis, 2007), and has been suggested to mediate interstitial fibrosis in experimental models of ischemic, inflammatory and pressure-overload induced cardiomyopathy (Frangogiannis et al., 2007), (Koyanagi et al., 2000), (Kuwahara et al., 2004). In a transgenic mouse model, cardiac-specific CCL2 overexpression is associated with inflammation-driven fibrosis (Kolattukudy et al., 1998). In the remodeling myocardium, CCL2 upregulation in microvascular endothelial cells and in cardiac macrophages is triggered through activation of neurohumoral pathways, or through induction of pro-inflammatory cytokines (Tokuda et al., 2004). CCL2 may mediate pro-fibrotic effects through several distinct mechanisms. First, activation of the CCL2/CCR2 axis results in recruitment of abundant macrophages in the injured myocardium, resulting in expansion of the pool of cells that can secrete fibrogenic mediators, such as TGF-β and Fibroblast Growth Factors (Frangogiannis et al., 2007). Second, in addition to its effects on monocyte recruitment, CCL2 may also contribute to fibrogenic activation of macrophages, by inducing TGF-β1 synthesis (Sakai et al., 2006) and by accentuating synthesis of matricellular proteins, such as osteopontin (Dewald et al., 2005). Third, in some studies, CCL2 has been suggested to directly promote fibroblast activation. In vitro, CCL2 stimulation enhanced portal fibroblast proliferation and myofibroblast differentiation (Kruglov et al., 2006), upregulated collagen and TGF-β1 expression by rat pulmonary fibroblasts (Gharaee-Kermani et al., 1996), and stimulated synthesis of MMP1 and TIMP-1 by human cutaneous fibroblasts (Yamamoto et al., 2000b). However, in isolated cardiac fibroblasts, CCL2 had no significant effects on MMP expression (Frangogiannis et al., 2007). Fourth, associative studies have suggested that CCL2 may promote cardiac fibrosis through recruitment of fibroblast progenitors (Quan et al., 2004), (Moore et al., 2005), (Haudek et al., 2006), (Haudek et al., 2010). Considering, the growing body of evidence suggesting that most activated fibroblasts in the fibrotic myocardium are derived from resident populations (Moore-Morris et al., 2018), (Moore-Morris et al., 2014), the relative contribution of chemokine-derived recruitment of circulating fibroblast progenitors is unclear.

The potential role of other inducible members of the chemokine family in the pathogenesis of cardiac fibrosis remains poorly understood. Considering the involvement of specific chemokine-chemokine receptor interactions in recruitment of monocyte and lymphocyte subsets, several chemokines may be involved in regulation of the fibrotic response in the remodeling myocardium by mediating infiltration of fibrogenic subsets of inflammatory cells. A recent study suggested that the CXC chemokine CXCL1 may contribute to the development of angiotensin-induced cardiac fibrosis through recruitment of a fibrogenic monocyte subpopulation (Wang et al., 2018).

Some members of the chemokine family may be involved in negative regulation of fibrosis. Anti-fibrotic effects of chemokines may be mediated through recruitment of, yet unidentified leukocyte subsets that contribute to resolution of the fibrotic response, or through direct de-activating effects on cardiac fibroblasts. In a model of reperfused

myocardial infarction, the CXC chemokine CXCL10/IP-10 has been identified as an anti-fibrotic mediator (Frangogiannis et al., 2001), (Bujak et al., 2009). The inhibitory effects of CXCL10 on the fibrotic response were associated with attenuation of growth-factor-induced fibroblast migration (Bujak et al., 2009), and were not mediated through the main CXCL10 receptor, CXCR3, but involved interactions with proteoglycans (Saxena et al., 2014a).

7.2.2. The pro-inflammatory cytokines in cardiac fibrosis: TNF-α, IL-1, and IL-6

Expression of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, is consistently induced following cardiac injury, and often precedes fibrotic remodeling (Torre-Amione et al., 1996), (Habib et al., 1996), (Francis et al., 1998), (Plenz et al., 1998), (Feng et al., 2017). In patients with dilated cardiomyopathy, myocardial expression of IL-6 and TNF-α is associated with collagen deposition and MMP transcription (Sivakumar et al., 2008), and serum TNF-α and IL-6 levels correlate with markers of collagen turnover (Timonen et al., 2008). These associative findings suggest a possible link between cytokine activation and ECM remodeling. Extensive in vivo and in vitro evidence suggests that pro-inflammatory cytokines potently regulate cardiac fibroblast phenotype and gene expression (Siwik and Colucci, 2004). Their effects on cardiac fibroblasts do not involve direct activation of a matrix-synthetic phenotype, but induction and release of proteases that may contribute to ECM degradation (Siwik et al., 2000). In vitro, TNF-α, IL-1β and IL-6 decrease collagen synthesis in isolated cardiac fibroblasts and increase MMP expression and activity (Bujak et al., 2008a), (Saxena et al., 2013), while reducing synthesis of MMP inhibitors (Li et al., 2002). Both IL-1α and IL-1β inhibit conversion of fibroblasts into myofibroblasts (Saxena et al., 2013), (Bronnum et al., 2013), (van Nieuwenhoven et al., 2013). IL-1β is also known to exert potent anti-proliferative effects on cardiac fibroblasts (Palmer et al., 1995), altering expression of fibroblast cyclins and cyclin-dependent kinases (Koudssi et al., 1998). Moreover, IL-1β, and (to a lesser extent) TNF-α, stimulate concentration-dependent increases in cardiac fibroblast migration (Mitchell et al., 2007).

It should be emphasized that, in addition to their direct actions on cardiac fibroblasts, pro-inflammatory cytokines modulate the fibrotic response by inducing expression of fibrogenic mediators. In vivo, the effects of pro-inflammatory cytokines in the fibrotic process are context-dependent. Extensive evidence suggests that TNF-α promotes cardiac fibrosis, triggering a predominant matrix-degrading phenotype. Transgenic mice with cardiac-specific overexpression of TNF-α develop heart failure (Bryant et al., 1998) associated with increased collagen synthesis, deposition and denaturation, and significantly enhanced MMP2 and MMP9 activity (Li et al., 2000). Fibrotic remodeling of the TNF-α overexpressing heart is associated with increased expression of TGF-βs (Sivasubramanian et al., 2001) and has been suggested to involve interactions between fibroblasts and mast cells (Zhang et al., 2011). On the other hand, global genetic loss of TNF-α reduced perivascular and interstitial fibrosis in models of cardiac pressure overload induced through aortic banding, or angiotensin II infusion (Sriramula and Francis, 2015), (Sun et al., 2007). The pro-fibrotic effects of TNF signaling in the myocardium appear to be due to interactions involving the type 1 TNF receptor (TNFR1) (Duerrschmid et al., 2013); in contrast TNFR2 signaling may reduce fibrosis (Hamid et al., 2009). Whether the effects of endogenous TNF signaling are mediated through actions on cardiac fibroblasts, or involve activation of other cell types remains unknown. On the other hand, the in vivo effects of IL-1 on the myocardial fibrotic response seem to be less consistent. In an experimental model of reperfused myocardial infarction, global loss of IL-1 signaling attenuated adverse fibrotic remodeling (Bujak et al., 2008a). Moreover, in a model of viral myocarditis local IL-1 antagonism through overexpression of IL-1 receptor antagonist (IL-1Ra) reduced fibrosis (Lim et al., 2002). In both cases, attenuation of the fibrotic response was associated with marked suppression of inflammation. In contrast, in a model of radiation-induced cardiomyopathy, genetic or pharmacologic IL-1 disruption failed to affect fibrosis, despite protection from functional depression (Mezzaroma et al., 2015). Studies on the role of IL-6 in cardiac fibrosis have produced conflicting data. Global loss of IL-6 attenuated cardiac fibrosis and dysfunction in models of left ventricular pressure overload (Zhao et al., 2016), (Gonzalez et al., 2015) and diabetic cardiomyopathy (Zhang et al., 2016). In contrast, another study using a model of pressure overload induced through transverse aortic constriction showed no effects of germline IL-6 loss on cardiac fibrosis and ECM protein deposition (Lai et al., 2012). Whether any pro-fibrotic actions of IL-6 in vivo are due to direct activating effects of the cytokine on cardiac fibroblasts, or reflect actions on other cell types remains unknown.

In both reparative fibrosis, and in pathophysiologic conditions associated with interstitial and perivascular fibrosis, endogenous pathways that inhibit and restrain cytokine-driven inflammation may play an important role in protection from excessive and uncontrolled fibrotic responses. Endogenous inhibitory cascades activated in fibroblasts, immune cells and vascular cells may include expression of decoy receptors (such as IL-1RII) (Saxena et al., 2013), downmodulation of adhesion molecules and induction of intracellular signals that inhibit pro-inflammatory TLR/IL-1 signaling, such as Interleukin receptor-associated kinase (IRAK)-M (Chen et al., 2012). Signals inhibiting cytokine-mediated activation of cardiac fibroblasts remain poorly understood. Expression of IRAK-M by cardiac fibroblasts attenuated their matrix-degrading capacity, but also appeared to enhance myofibroblast conversion (Saxena et al., 2015). Negative regulation of TNF signaling in fibroblasts, driven by mammalian sterile 20-like kinase 1 (Mst1) has been suggested to protect the pressure-overloaded myocardium from fibrosis and dysfunction (Del Re et al., 2010).

7.2.3. IL-4, IL-10 and IL-13

IL-4, IL-10 and IL-13 exert anti-inflammatory actions in many cell types, but have also been implicated in the pathogenesis of tissue fibrosis (Gieseck et al., 2018). IL-10 is markedly upregulated following cardiac injury and is predominantly expressed by T cells and by a subset of macrophages (Frangogiannis et al., 2000). In vivo studies in experimental models of left ventricular pressure overload suggest that IL-10 may contribute to the development of cardiac fibrosis (Hulsmans et al., 2018), (Verma et al., 2017). The cellular targets of IL-10 are poorly defined. In vitro, IL-10 had no significant effects on fibroblast gene expression profile (Zymek et al., 2007). The anti-fibrotic effects of IL-10 may be predominantly mediated through actions on the cytokine expression profile of macrophages and lymphocytes. IL-10-mediated upregulation of TIMP-1 synthesis in macrophages (Frangogiannis et al., 2000) may also contribute to its matrix-preserving functions.

IL-4 and IL-13 may also exert pro-fibrotic actions on the remodeling myocardium through effects on cardiac fibroblasts and on macrophages. In vitro, IL-4 stimulation induces collagen synthesis in cardiac fibroblasts through activation of STAT6 (Peng et al., 2015). In vivo, both genetic loss-of-function (Peng et al., 2015) and pharmacologic inhibition (Kanellakis et al., 2012) of IL-4 have been reported to attenuate cardiac fibrosis in experimental models of pressure overload. Limited experimental evidence suggests a role for IL-13 in regulation of cardiac fibrosis. Increased expression of IL-13 has been documented in fibrotic senescent mouse hearts (Cieslik et al., 2011). Experiments in a chronic transplant rejection model suggested that IL-13 may contribute to allograft fibrosis by inducing TGF-β1 synthesis (Brunner et al., 2013). In the injured and remodeling myocardium, IL-13 may act predominantly by modulating macrophage phenotype (Cihakova et al., 2008), (Hofmann et al., 2014). It should be emphasized that the in vivo effects of IL-4, IL-10 and IL-13 in the myocardial fibrotic response may be dependent on the balance between their anti-inflammatory and pro-fibrotic actions. Suppression of pro-inflammatory cytokine expression by these immunomodulatory cytokines may inhibit downstream activation of fibrogenic pathways and, depending on the context, may

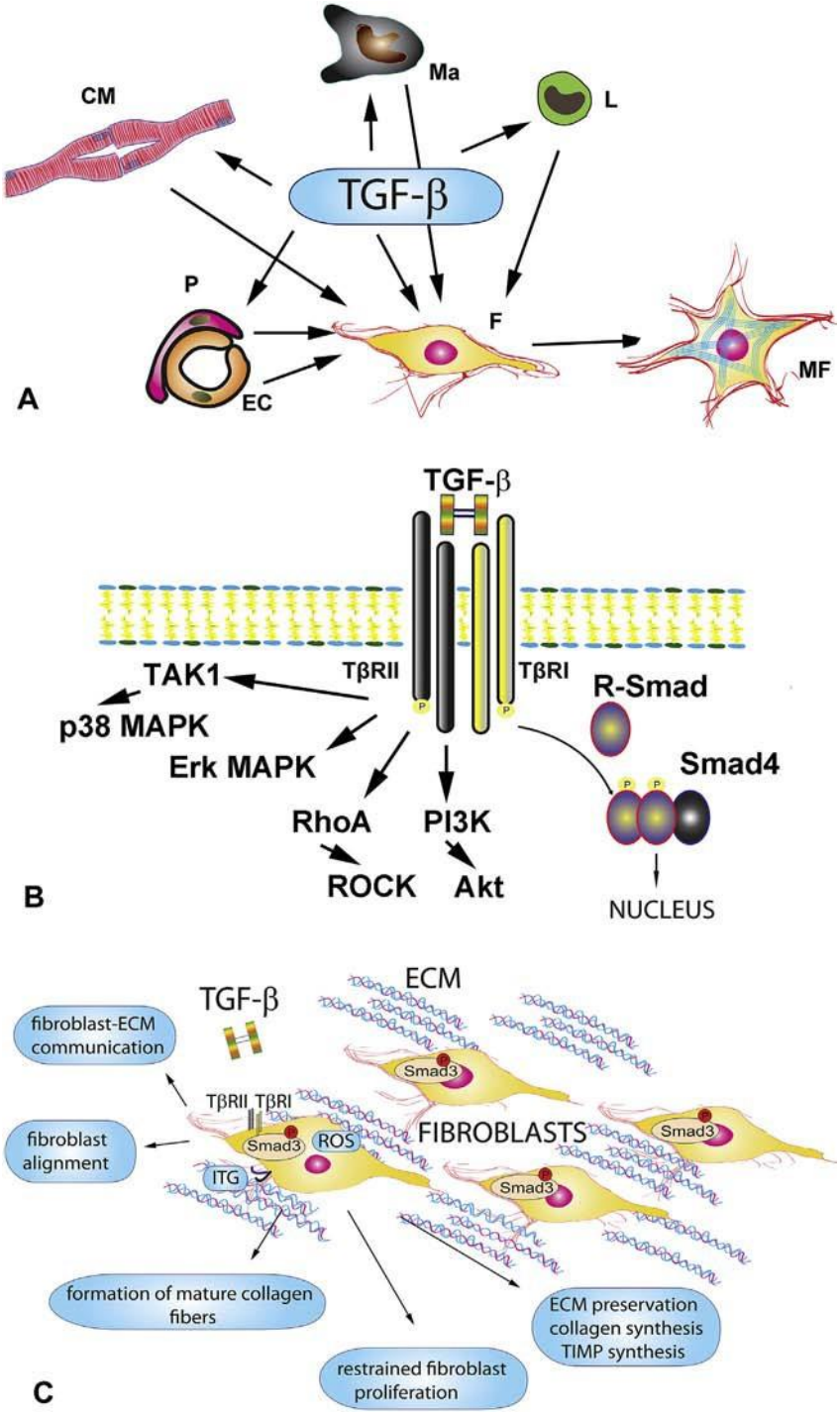

Fig. 5. The role of TGF-β in cardiac fibrosis. A. TGF-β modulates phenotype and function of all cells involved in myocardial fibrosis. Although direct actions on fibroblast (F) to myofibroblast (MF) conversion and on myofibroblast activation are likely important, fibrogenic actions of TGF-βs may also involve effects on macrophage (Ma) phenotype, lymphocyte (L) differentiation and function, and cardiomyocyte (CM) survival and gene expression. Moreover, TGF-β may promote pericyte (P) to fibroblast conversion and endothelial (EC) to mesenchymal transdifferentiation (EndMT), while stimulating expression of fibrosis-associated genes by vascular cells. B. TGF-βs modulate cell phenotype by activating both Smad-dependent and non-Smad pathways. C. The effects of TGF-β/Smad3 signaling in cardiac fibroblasts. Recent studies using cell-specific loss-of-function approaches demonstrated that, following myocardial infarction, activation of Smad3 is critical for formation of organized arrays of myofibroblasts (Kong et al., 2018). Absence of Smad3 in fibroblasts perturbed repair of the infarcted heart leading to an increased incidence of late cardiac rupture and adverse dilative remodeling. These observations highlight the reparative functions of activated fibroblasts in the infarcted heart. The effects of Smad3 were mediated through activation of an integrin-ROS axis.

outweigh their pro-fibrotic actions.

7.2.4. The TGF-β superfamily

The TGF-b superfamily is comprised of several subgroups of proteins, including the TGF-βs, the bone morphogenetic proteins (BMP)s, growth differentiation factors (GDF)s, and the activin/inhibin family, that share common downstream signaling pathways. Our knowledge on the role of TGF-β superfamily members is limited to the actions of TGF-βs; understanding of the potential role of other members is limited.

7.2.4.1. TGF-βs in cardiac fibrosis. TGF-β, the best characterized fibrogenic growth factor (Biernacka et al., 2011), is markedly and consistently activated in animal models of cardiac remodeling and fibrosis (Dobaczewski et al., 2011), (Frangogiannis, 2017c) and in fibrotic human hearts (Pauschinger et al., 1999), (Li et al., 1997). In mammals, TGF-β is found in three isoforms (TGF-β1, 2 and 3) (Schiller et al., 2004) that signal through the same cell surface receptors and share common cellular targets, but exhibit distinct patterns of expression. Information on the in vivo functions of each TGF-β isoform is lacking; most of our knowledge is limited to TGF-β1, or relates to the effects of activation of common downstream signaling pathways.

The adult mammalian myocardium contains stores of TGF-β as a latent complex that is unable to associate with its receptors. Following cardiac injury, the extracellular concentration of TGF-β activity is markedly increased, both through de novo synthesis and secretion of

TGF-β by macrophages, fibroblasts, platelets, vascular cells and cardiomyocytes (Dobaczewski et al., 2011), (Dewald et al., 2004), and (most importantly) through release of bioactive protein from the latent stores. Generation of active TGF-β involves several different molecular signals, including proteases, such as plasmin, MMP2 and MMP9 (Annes et al., 2003), (Ignotz and Massague, 1986), (Rifkin et al., 1999), matricellular macromolecules (Xia et al., 2011), (Frangogiannis et al., 2005) and ROS generation (Barcellos-Hoff et al., 1994). The relative contribution of specific TGF-β activating signals in the fibrotic heart remains unknown.

A large body of in vivo evidence suggests that TGF−β exerts profibrotic actions in the myocardium. Myocardial overexpression of TGF-β1 was associated with ventricular fibrosis, characterized by accentuated collagen deposition and inhibition of interstitial collagenases (Seeland et al., 2002), (Rosenkranz et al., 2002). Constitutive expression of an activated mutant TGF-β protein also induced myocardial fibrosis (Accornero et al., 2015). On the other hand, transgenic mice with a large proportion of constitutively active TGF-β1 in the heart (due to a mutation that blocks covalent tethering of the TGF-β1 latent complex to the ECM) exhibited only atrial fibrosis (Nakajima et al., 2000). Whether this phenotype reflects increased responsiveness of atrial fibroblasts to the fibrogenic effects of TGF-β remains unknown. Loss-of-function approaches using several distinct experimental models suggested the involvement of endogenous TGF-β in the pathogenesis of fibrotic ventricular remodeling. Heterozygous TGF-β1 ± deficient mice exhibited attenuated age-associated fibrosis (Brooks and Conrad, 2000), whereas. TGF-β blockade prevented myocardial fibrosis in a rat model of cardiac pressure overload (Kuwahara et al., 2002). A recent study demonstrated that genetic conditional deletion of the type I and type II TGF-β receptors (TβRI, or TβRII) in fibroblasts attenuated cardiac fibrosis in a model of left ventricular pressure overload (Khalil et al., 2017), suggesting that fibroblasts are major targets of TGF-βs under conditions of stress. In vitro, TGF-β stimulation induces myofibroblast transdifferentiation (Desmouliere et al., 1993), enhances ECM protein synthesis, increases expression of integrins (Kong et al., 2018), and exerts potent matrix-preserving actions on cardiac fibroblasts, through induction of protease inhibitors, such as Plasminogen Activator Inhibitor (PAI)-1 and TIMP1 (Schiller et al., 2004). Although it has been suggested that TGF-β-mediated fibrosis may require downstream release of the matricellular protein CCN2 (Mori et al., 1999), experiments using genetic loss-of-function approaches have challenged the in vivo significance of CCN2 in mediating pro-fibrotic actions driven by an overactive TGF-β system (Accornero et al., 2015). The effects of TGF-β in regulation of cardiac fibrosis may also involve other cell types, including cardiomyocytes, macrophages, lymphocytes and vascular cells (Fig. 5).

Which pro-fibrotic signaling cascades mediate the in vivo effects of TGF-β? In vitro and in vivo evidence suggests an important role for both canonical Smad2/3 pathways and non-canonical Smad-independent signaling. In vitro, Smad3 signaling mediates, at least in part, the TGF-β-induced upregulation of ECM protein synthesis, integrin transcription, and α-SMA expression (Bujak et al., 2007), (Dobaczewski et al., 2010a), (Kong et al., 2018). It should be emphasized that activation of Smad3 signaling in fibroblasts restrains cell proliferation, generating activated matrix-synthetic myofibroblasts (Bujak et al., 2007), (Dobaczewski et al., 2010a), (Kong et al., 2018). Moreover, Smad3 activation in infarct myofibroblasts is critical for the formation of well-aligned arrays of activated myofibroblasts that preserve the structural integrity of the infarcted ventricle through activation of an integrin-ROS axis (Kong et al., 2018) (Fig. 5). On the other hand, activation of p38 MAPK in fibroblasts also stimulates myofibroblast conversion through signals involving the transcription factor and the signaling effector calcineurin (Molkentin et al., 2017). MAPK activation occurs in response to many different stimuli; thus, the relative role of TGF-β in activation of common kinase pathways is unclear.

A recently published study suggested that the multifunctional cytokine IL-11 may be a critical downstream effector of TGF-β-driven cardiac fibrosis (Schafer et al., 2017). In vitro, IL-11 neutralization attenuated the fibroblast-activating effects of TGF-β1. The fibrogenic actions of IL-11 were post-transcriptional and involved activation of ERK signaling. In vivo, infusion or overexpression of IL-11 induced cardiac fibrosis and global loss of IL-11Ra attenuated fibrotic remodeling of the pressure-overloaded heart. It should be emphasized that considering the multiple likely cellular targets of IL-11, the potential role of in vivo actions specifically targeting fibroblasts remains poorly defined. Despite the use of recombinant human IL-11 for prevention of chemotherapy-induced thrombocytopenia and in bone marrow failure syndromes, side effects related to development of fibrosis have not yet been reported.

Negative regulation of endogenous TGF-β signaling may play an important role in restraining cardiac fibrosis. The mechanisms for endogenous inhibition of TGF-β cascades remain underexplored. Cleavage and release of a soluble form of the accessory TGF-β receptor endoglin has been suggested to act as a negative regulator of TGF-β signaling in heart failure (Kapur et al., 2012). Expression of the TGF-β pseudo-receptor BAMBI (Bone Morphogenetic Protein/BMP and activin membrane-bound inhibitor) in the pressure-overloaded heart may down-modulate TGF-β signaling, attenuating its profibrotic actions (Villar et al., 2013). Moreover, a recent study suggested that delta-like homologue-1 (Dlk-1), a non-canonical Notch ligand, may restrain TGF-β signaling in cardiac fibroblasts, inhibiting myofibroblast conversion (Rodriguez et al., 2018). Because prevention of uncontrolled TGF-β responses in the remodeling myocardium is crucial to preserve structure and function following injury, multiple distinct pathways may co-operate for negative regulation of the TGF-β system (Itoh & ten Dijke, 2007).

7.2.4.2. BMPs, activins, GDFs and follistatins. Information on the role of other TGF-β superfamily members in fibrotic cardiac remodeling is limited. Members of the BMP family are markedly upregulated in experimental models of reparative cardiac fibrosis. In the infarcted heart, BMP2 induction is followed by upregulation of BMP4, BMP6 and BMP10 (Sanders et al., 2016). Although pro-inflammatory effects of BMP2 in the infarcted myocardium have been suggested, the in vivo role of BMP signaling in regulation of the fibrotic response has not been systematically investigated. Cardiomyocyte-specific loss of the BMP type 1 receptor activing receptor-like kinase (ALK)2 attenuated cardiac fibrosis in a model of angiotensin II infusion (Shahid et al., 2016); whether these effects are due to abolition of a paracrine fibrogenic signal, or reflect attenuated cardiomyocyte death is unclear. Exogenous administration of BMP7 was found to attenuate cardiac fibrosis in experimental models of pressure overload and diabetic cardiomyopathy (Merino et al., 2016), (Urbina and Singla, 2014). The anti-fibrotic actions of BMP7 are presumed due to attenuation of TGF-β driven fibroblast-activating responses (Zeisberg et al., 2003).

In vitro studies have demonstrated that activin A promotes cardiac fibroblast proliferation and stimulates myofibroblast conversion (Hu et al., 2016). Partial loss of ALK4, a major receptor for activins, was reported to attenuate fibrosis and reduce dysfunction in a mouse model of pressure overload (Li et al., 2016); however, the cellular basis for these effects remains unclear. Circulating GDF-15 levels have been associated with cardiac fibrosis in patients with heart failure (Lok et al., 2012); however, whether this association reflects a causative role remains unknown. The common receptors and downstream signaling pathways shared by several members of the TGF-β superfamily hamper dissection of their specific functions and in vivo effects.

Members of the follistatin family function as extracellular inhibitors of TGF-β superfamily ligands, by binding with specific agonists and by disrupting their interactions with the corresponding receptors. Emerging evidence suggests that follistatins may play a role in regulation of myocardial fibrosis through actions that may be independent of their neutralizing effects, but may involve direct activation of signaling cascades in cardiac fibroblasts. In vitro, cardiomyocyte-derived follistatin-like 3 has been reported to activate cardiac fibroblasts (Panse

et al., 2012). Moreover, in an experimental model of myocardial infarction, Follistatin-like 1 promoted activation of a reparative fibroblast phenotype through Erk1/2 signaling, protecting the infarcted heart from cardiac rupture (Maruyama et al., 2016).

7.2.5. The PDGFs

The PDGF family is comprised of homo- or hetero-dimeric growth factors (including PDGF-AA, -BB, AB, CC and DD) that signal through two different receptors: PDGFR-α and PDGFR-β. PDGF isoforms and PDGFRs are overexpressed in fibrotic cardiac conditions (Zymek et al., 2006); however, the multiple cellular targets of PDGFs and their pleiotropic actions have hampered understanding of their in vivo role. In vitro, PDGF-AA potently stimulates cardiac fibroblast proliferation and ECM protein synthesis (Simm et al., 1998). In vivo, cardiac overexpression of PDGF-A caused a severe fibrotic cardiomyopathy leading to heart failure-related mortality, whereas PDGF-B overexpression resulted in local fibrotic changes associated with moderate cardiac hypertrophy (Gallini et al., 2016). In a model of chronic allograft rejection adenoviral-mediated delivery of PDGF-A, -C and –D, but not PDGF-B, accelerated cardiac fibrosis enhancing TGF-β expression (Tuuminen et al., 2009). Loss-of-function approaches suggested an important role for PDGFR signaling in myocardial fibrotic responses. PDGFR-α and PDGFR-β neutralization reduced collagen deposition in reperfused myocardial infarcts (Zymek et al., 2006); however, PDGFR-β inhibition also prevented mural cell recruitment by infarct neovessels suppressing vascular maturation (Zymek et al., 2006). In pressure-overloaded hearts, PDGFR-α neutralization attenuated atrial fibrosis and reduced the incidence of atrial fibrillation (Liao et al., 2010). Whether these effects are due to direct inhibition of PDGFR-driven fibroblast activation, or reflect actions on other cell types, such as cardiomyocytes or pericytes remains unclear.

7.2.6. The galectins

Galectins are a family of β-galactoside-binding lectins, involved in a broad range of functions, including cell adhesion and migration, cell proliferation, inflammatory and immune responses (Johannes et al., 2018). Emerging evidence suggests a role for galectin-3 in the pathogenesis of tissue fibrosis. In fibrotic myocardial lesions, galectin-3 is markedly upregulated and is predominantly expressed by activated macrophages, but is also localized in subsets of cardiomyocytes and fibroblasts (Frunza et al., 2016). Moreover, in heart failure patients, galectin-3 is associated with fibrotic cardiac remodeling and predicts adverse outcome (de Boer et al.), (Filipe et al., 2015). Extensive cell biological evidence suggests that galectin-3 stimulates fibrogenic activation in both macrophages and fibroblasts (MacKinnon et al., 2008), (Frangogiannis, 2018). Secreted galectin-3 may bind to glycoproteins on the surface of fibroblasts, potently stimulating proliferative responses and modulating TGF-β receptor expression and responsiveness (Mackinnon et al., 2012). Moreover, galectin-3 may act as a matricellular protein, binding to ECM components and to cell surface receptors and transducing, or modulating fibrogenic signaling cascades. Despite its potent fibrogenic actions in vitro, the in vivo role of endogenous galectin-3 in cardiac fibrosis remains debated. Some investigations using knockout mice have suggested a crucial role for galectin-3 in fibrosis and dysfunction of the remodeling heart (Yu et al., 2013), (Martinez-Martinez et al., 2015); in contrast, other studies using similar loss-of-function approaches reported no significant effects of galectin-3 in fibrotic cardiac remodeling (Frunza et al., 2016), (Nguyen et al., 2018). Other galectins, such as galectin-1 and galectin-7, have been suggested to regulate fibrotic responses in other organs by modulating growth factor responses (Lim et al., 2014), (Inagaki et al., 2008). Although galectin-1 has been implicated in regulation of the inflammatory response following myocardial infarction (Seropian et al., 2013), its potential role in cardiac fibrosis has not been investigated.

7.3. Surface molecules in cardiac fibrosis

Following myocardial injury, myocardial cells sense changes in their microenvironment through surface molecules that, upon activation, transduce intracellular cascades. Soluble mediators released in injury sites, such as alarmins, cytokines, growth factors, and neurohumoral mediators stimulate specific fibroblast cell surface receptors that may induce a pro-inflammatory, migratory, matrix-degrading, or matrix-secreting program. In addition, fibroblasts sense changes in ECM composition and stiffness by activating promiscuous integrin or proteoglycan receptors that initiate or modulate signaling cascades.

7.3.1. Integrins

Integrins are transmembrane receptors that bridge the cells with the surrounding ECM, thus transducing downstream signals in response to changes in the cellular microenvironment. Although integrin signaling cascades have been implicated in all forms of cardiac fibrosis (Chen et al., 2016), (Leask, 2013b), the complexity of the integrin system and the broad involvement of integrins in modulating phenotype of macrophages, fibroblasts, cardiomyocytes and vascular cells have challenged dissection of their cell biological actions. Integrin β1 is upregulated in activated fibroblasts infiltrating the infarcted myocardium (Sun et al., 2003), and has been implicated in fibrogenic activation of fibroblasts in pressure-overloaded hearts (Takawale et al., 2017b). TGF-β induces integrin expression in cardiac fibroblasts through Smad3-dependent signaling (Kong et al., 2018). Pro-fibrotic actions of integrins may also involve immune or vascular cells. Fibrogenic macrophage activation of αvβ3 integrin by the matricellular protein tenascin-C has been implicated in the pathogenesis of cardiac fibrosis, in a model of angiotensin II infusion (Shimojo et al., 2015). In a model of angiotensin-mediated cardiac remodeling, αv integrin expression in perivascular PDGFRβ+ cells has been implicated in the development of fibrosis (Murray et al., 2017). Whether the effects of αv integrin are directly mediated through conversion of perivascular cells to fibroblasts, or involve paracrine secretion of fibrogenic mediators is unknown.

7.3.2. Cell surface proteoglycans

The syndecans are the best studied cell surface proteoglycans in cardiac fibrosis. Although traditionally viewed as transmembrane molecules that contribute to signal transduction, syndecans can also translocate to the nucleus, and have been found to undergo proteolysis, thus releasing their ectodomain as a soluble HSPG. Studies using loss-of-function models have documented important roles for syndecan-1 and -4 in the pathogenesis of cardiac fibrosis. Syndecan-1 expression is increased following myocardial infarction and plays a critical role in reparative fibrosis, protecting the ventricle from rupture and dilative remodeling (Vanhoutte et al., 2007). In contrast, in a model of angiotensin II-induced fibrosis, syndecan-1 mediated fibroblast activation promoting dysfunction (Schellings et al., 2010). The contrasting effects of syndecan-1 in models of reparative fibrosis and pressure overload-induced remodeling reflect the context-dependent reparative and maladaptive effects of fibroblast activation in cardiac injury. Syndecan-4 has also been implicated in activation of cardiac myofibroblasts in infarcted (Matsui et al., 2011) and in pressure-overloaded hearts (Herum et al., 2015). The fibrogenic effects of the syndecans have been suggested to involve regulation of TGF-β signaling (Schellings et al., 2010), (Chen et al., 2004); however, potential ligands and specific syndecan-dependent molecular signals that may be involved in cardiac fibroblast activation remain poorly characterized. It should be emphasized that syndecan-mediated regulation of cardiac fibrosis may also involve effects on immune cell recruitment and function (Lipphardt et al., 2018), (Strand et al., 2015), or actions on cardiomyocyte survival (Echtermeyer et al., 2011).

7.3.3. CD44

CD44 is a broadly-expressed transmembrane glycoprotein that has

been implicated in a wide range of cellular functions, including cell adhesion, migration, proliferation and differentiation (Senbanjo and Chellaiah, 2017). Hyaluronan and the matricellular protein osteopontin bind to CD44 and transduce intracellular cascades that modulate immune cell and fibroblast function. Experimental studies in a model of reparative infarctive fibrosis (Huebener et al., 2008), and in a model of angiotensin II-induced cardiomyopathy (Yang et al., 2018) suggested that CD44 transduces activating signals in cardiac fibroblasts, triggering a proliferative response and promoting activation of TGF-β cascades. Moreover, CD44 signaling may regulate cardiac fibrosis by modulating inflammatory activity in macrophages and lymphocytes (Huebener et al., 2008). Alternative splicing of CD44 results in generation of isoforms with distinct pro- or anti-fibrotic properties (Midgley et al., 2017), contributing additional layers of complexity to the role of this multifunctional molecule.

7.4. Intracellular molecular pathways involved in the pathogenesis of cardiac fibrosis

In most cardiac fibrotic conditions, activation of fibroblasts involves the cooperation of several distinct but interacting intracellular signaling cascades. DAMPs released by dying cardiomyocytes, inflammatory cytokines and growth factors secreted by leukocytes, neurohumoral mediators, matricellular proteins and integrin-driven mechanosensitive pathways trigger a wide range of interacting intracellular cascades that may mediate fibroblast to myofibroblast conversion, migration, proliferation and induction of a matrix-synthetic transcriptional program (Roche et al., 2015). Experimental studies in animal models of cardiac fibrosis have identified several essential intracellular pathways that contribute to fibroblast activation. Their relative significance in the fibrotic response is likely dependent on the specific pathophysiologic context.

7.4.1. Oxidative stress

Oxidative stress critically regulates fibroblast phenotype and function and plays an important role in tissue fibrosis (Grosche et al., 2018). ROS generation is consistently noted in many cardiac pathophysiologic conditions, is triggered by a wide range of cytokines and growth factors, and may represent a common link between the various forms of cardiac stress and fibrosis. Cytokine-driven induction of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases plays a critical role in ROS generation and may mediate the fibrotic response (Kong et al., 2018), (Cucoranu et al., 2005) A recently published study identified the fibroblast-enriched endoplasmic reticulum protein thioredoxin domain containing protein 5 (TXNDC5) as a crucial activator of ROS that mediates myocardial fibrosis in a model of isoproterenol-induced cardiac injury (Shih et al., 2018).

Once generated in cardiac fibroblasts, ROS regulate the quantity and quality of the interstitial ECM matrix by modulating both matrix protein expression and degradation. Both matrix-preserving and matrix-degrading effects of ROS have been reported. In vitro, many fibroblast-activating effects of growth factors and neurohumoral signals have been attributed to activation of the ROS system (Cheng et al., 2003). The effects of angiotensin II appear to be in part dependent on ROS. Angiotensin II activates downstream ROS-sensitive kinases with a critical role in fibrotic remodeling of the heart (Ohtsu et al., 2005). In cardiac fibroblasts, angiotensin II-stimulated collagen production is mediated through ROS generation (Lijnen et al., 2006). Experiments in a rat model demonstrated that the profibrotic actions of aldosterone infusion may be mediated at least in part through ROS generation (Iglarz et al., 2004). A large body of evidence suggests that ROS-dependent mechanisms may mediate the fibrogenic actions of TGF-β. Moreover, ROS may be involved in generation of active TGF-β in the cardiac interstitium (Barcellos-Hoff and Dix, 1996) through interactions with the latency-associated peptide (LAP). ROS critically regulate synthesis and activity of proteases involved in ECM degradation and remodeling (Siwik et al., 2001). Inflammatory cytokine-mediated activation of MAPKs and stress-responsive protein kinases is redox sensitive, and activates transcription factors such as activator protein (AP)-1 and Nuclear factor (NF)-κB leading to enhanced MMP transcription (Siwik and Colucci, 2004).

The oxidative response is tightly regulated through endogenous antioxidant enzymes. Several in vivo studies have demonstrated that accentuation of antioxidant signals attenuates fibrosis under conditions of myocardial stress. Mice that overexpress catalase targeted to mitochondria are resistant to cardiac hypertrophy and fibrosis after angiotensin infusion (Dai et al., 2011). Moreover, in a model of fibrotic ischemic cardiomyopathy due to brief repetitive myocardial ischemia and reperfusion, Extracellular Superoxide Dismutase (EC-SOD) overexpression attenuated interstitial fibrosis, reducing chemokine generation (Dewald et al., 2003). It should be emphasized that the fibrogenic actions of ROS are not limited to cardiac fibroblasts, but may also involve activation of immune cells.

7.4.2. Ionic changes in cardiac fibrosis

Ca^{2+} oscillations have been broadly implicated in both the contractile function of myofibroblasts and in regulation of ECM synthesis (Mukherjee et al., 2015), (Janssen et al., 2015), (Follonier Castella et al., 2010), (Godbout et al., 2013). Descriptive studies have characterized the dynamics of calcium responses to various fibrogenic agonists in isolated cardiac fibroblasts; however, in vivo evidence documenting the role of Ca^{2+} in myocardial fibrosis is scarce. Recent studies have revealed that profibrotic mediators, such as angiotensin II or TGF-β may activate members of the Transient Receptor Potential (TRP) family of cationic channels. In cardiac fibroblasts, TRPC6 is induced through TGF-β-mediated Smad-independent signaling and has been implicated in myofibroblast conversion by activating a calcineurin-Nuclear Factor of Activated T cells (NFAT) cascade (Nishida et al., 2007), (Davis et al., 2012). TRPM7 and TRPV4 may also be involved in myofibroblast transdifferentiation in response to mechanosensitive stimuli that may activate the TGF-β cascade (Du et al., 2010), (Adapala et al., 2013).

7.4.3. Focal adhesion kinase (FAK) in cardiac fibrosis

FAK activation may be a critical molecular link between mechanical stress and cardiac fibroblast activation. In vitro, FAK activation mediates mechanosensitive, or growth factor-induced conversion of fibroblasts into myofibroblasts (Leask, 2013a), (Zhang et al., 2013), (Chan et al., 2009). In vivo, pharmacologic inhibition of FAK has been reported to attenuate cardiac fibrosis in a model of myocardial infarction (Fan et al., 2015; Zhang et al., 2017a). Moreover, FAK siRNA knockdown reduced fibrotic changes in a model of cardiac pressure overload (Clemente et al., 2007). The cellular basis for these effects is unclear. Considering the broad effects of FAK activation on cardiomyocytes, vascular and interstitial cells (Cheng et al., 2011), (Hakim et al., 2009) FAK-mediated fibrosis may reflect actions on several different cell types. In vivo studies documenting the role of fibroblast-specific FAK in mechanosensitive or growth factor-mediated activation are lacking.

7.4.4. The Ras homologue gene family, member A (RhoA)/Rho-associated coiled-coil containing kinases (ROCK) pathway

Activation of the small GTP-binding protein RhoA plays an important role in regulation of cell survival, proliferation, migration and differentiation through actions on the actin cytoskeleton (Loirand et al., 2013). Mechanosensitive or ligand-mediated stimulation of tyrosine kinase and G-protein-coupled receptors recruits Rho guanine nucleotide-exchange factors (RhoGEFs), leading to activation of GTP-bound RhoA. Subsequently RhoA signals through the Rho-associated coiled-coil containing kinases (ROCKs), ROCK1 and ROCK2. A substantial body of evidence suggests an important role for the RhoA/ROCK pathway in the pathogenesis of cardiac fibrosis (Shimizu and Liao, 2016). The mechanisms responsible for RhoA/ROCK activation in

fibrotic hearts remain poorly understood. In vitro experiments have suggested that p63RhoGEF may represent an important molecular link between angiotensin II and activation of the RhoA axis (Ongherth et al., 2015). In vivo, pharmacologic inhibition of the RhoA-ROCK pathway attenuated fibrosis in experimental models of cardiac pressure overload (Phrommintikul et al., 2008). The cellular basis for the fibrogenic actions of RhoA remains unclear. Experiments in a model of cardiac pressure overload suggested that cardiomyocyte-specific RhoA signaling triggers fibrogenic pathways, while exerting protective actions on cardiomyocytes (Lauriol et al., 2014). The cardiomyocyte-derived signals that may trigger fibroblast activation are not known. Moreover, global hemizygous ROCK1 ± mice (Rikitake et al., 2005) and homozygous ROCK1 null animals (Haudek et al., 2009) were reported to exhibit attenuated cardiac fibrosis in experimental models of left ventricular pressure overload and ischemic cardiomyopathy. The relative role of ROCK1 and ROCK2 in mediating fibroblast activation in response to growth factor stimulation or mechanical stress remains poorly defined. A recent study suggested that fibroblast-specific ROCK2 signaling may contribute to angiotensin II-mediated fibrosis, presumably through CCN2 and FGF2 induction (Shimizu et al., 2017).

7.4.5. MAPKs

MAPKs are serine threonine protein kinases with a broad range of functions in many different cellular processes, including cell proliferation, differentiation, migration, apoptosis and inflammatory activation. In mammalian species, MAPKs include c-Jun NH2 terminal kinase (JNK), p38 MAPK and extracellular signal-regulated kinase (ERK); each MAPK exits in several isoforms with distinct cellular localization and different functions (Kim and Choi, 2015). Following myocardial injury, MAPKs are activated by many different extracellular stimuli, including cytokines, growth factors, neurohumoral mediators, matricellular proteins, and mechanical stress. Both in vitro studies and in vivo evidence suggest an important role for MAPK signaling pathways in activation of cardiac fibroblasts. Recent in vivo studies using fibroblast-specific loss-of-function approaches demonstrated that activation of p38α MAPK, the major isoform expressed in cardiac fibroblasts (Sinfield et al., 2013), promotes myofibroblast conversion upon ischemic injury or neurohumoral stimulation through signals involving the transcription factor serum response factor (SRF) and the signaling effector calcineurin (Molkentin et al., 2017), (Bageghni et al., 2018). Much less is known regarding the role of ERK and JNK in mediating fibroblast responses in the remodeling myocardium. Although in vitro ERK activation triggers cardiac fibroblast proliferation (Hu et al., 2016), and in a model of Marfan syndrome cardiomyopathy, ERK activation was prominent in non-cardiomyocytes (Rouf et al., 2017), the role of fibroblast-specific ERK signaling in fibrotic hearts has not been systematically investigated.

7.4.6. The Wnt/β-catenin axis

The Wnt family is comprised of a diverse group of glycoproteins, that can be secreted following injury (Aisagbonhi et al., 2011), and can bind to frizzled family receptors, transducing signaling cascades that modulate a wide range of cellular responses (Wang et al., 2014). Secretion of Wnt proteins is triggered by growth factors, such as TGF-β (Blyszczuk et al., 2017), and requires post-translational modification by the acyltransferase Porcupine (Porcn), a process that supplies a single fatty acid adduct necessary for exit of Wnt proteins from the endoplasmic reticulum and transport to the plasma membrane. Binding of secreted Wnt ligands to their receptors (members of the frizzled family) initiates signaling responses through canonical and non-canonical pathways. The canonical Wnt signaling pathway involves accumulation of the protein β-catenin in the cytoplasm, followed by nuclear translocation and interactions with transcriptional co-activators that regulate gene expression. Wnt proteins may also activate β-catenin-independent non-canonical "calcium" and "planar cell polarity" pathways.

Several lines of evidence suggest that Wnt proteins mediate cardiac fibrosis. In vitro, members of the Wnt family activate cardiac fibroblasts triggering myofibroblast conversion and promoting cytokine synthesis (Abraityte et al., 2017), (Carthy et al., 2011). Administration of a small molecule inhibitor that disables Porcn, thus preventing acylation and subsequent secretion of Wnt proteins, attenuated fibrosis in a model of myocardial infarction (Moon et al., 2017). The pathways responsible for the fibrogenic actions of Wnt proteins remain poorly understood. Several studies implicate the canonical β-catenin-dependent pathway in fibroblast activation (Duan et al., 2012), (Xiang et al., 2017). In a mouse model of cardiac pressure overload, fibroblast-specific activation of β-catenin mediated both fibrosis and cardiomyocyte hypertrophy (Xiang et al., 2017). The molecular basis for the β-catenin-dependent pro-hypertrophic effects of activated fibroblasts is unknown. Evidence supporting a role for non-canonical Wnt signaling in cardiac remodeling is much more limited (Meyer et al., 2017). It should be emphasized that Wnt proteins have a broad range of cellular targets modulating responses in cardiomyocytes, vascular cells and immune cells (Meyer et al., 2017), (Paik et al., 2015). Thus Wnt-mediated regulation of fibrosis may not necessarily involve direct effects on cardiac fibroblasts.

7.4.7. The myocardin-related transcription factor (MRTF)/Serum response factor (SRF) axis

SRF is a ubiquitously expressed transcription factor that binds and activates promoters harboring the DNA element CArG box (Norman et al., 1988). Interactions between SRF and members of the myocardin family of transcriptional co-activators have been implicated in stimulation of transcription of genes encoding smooth muscle cell contractile proteins. In vascular and visceral smooth muscle cells, myocardin/SRF interactions are responsible for constitutive induction of contractile proteins, such α-SMA/Acta2. In fibroblasts, on the other hand, the SRF/MRTF axis has been suggested to play a dominant role in regulation of α-SMA transcription and subsequent myofibroblast conversion, through interactions that may involve Smad3 and the Rho/ROCK system (Tomasek et al., 2005), (Lighthouse and Small, 2016). In vivo studies showed that mice with global loss of MRTF-A had attenuated fibrosis following myocardial infarction and in response to angiotensin II infusion (Small et al., 2010). Whether these observations reflect abrogation of MRTF-dependent effects on fibroblasts remains unclear, considering that MRTF-A may also modulate cardiomyocyte and vascular cell phenotype and function (Weng et al., 2015), (Trembley et al., 2018).

7.5. Non-coding RNAs in cardiac fibrosis

A growing body of evidence implicates noncoding RNA transcripts, including microRNAs (miRNAs) and long non coding RNAs (lncRNAs) in the pathogenesis of cardiac fibrosis (Creemers and van Rooij, 2016), (Thum, 2014). miRNAs may act by targeting many different fibrogenic cascades including the TGF-β/Smad system, angiotensin II/MAPK signaling, the RhoA/ROCK cascade, the MRTF/SRF axis and the cationic channels regulating calcium responses (Piccoli et al., 2016). Moreover, certain miRNAs can be loaded into exosomes, secreted in the cardiac interstitium and taken up by other cell types, modulating cardiomyocyte function or immune cell activation (Bang et al., 2014). Although several in vivo studies have suggested that increased abundance of miRNAs in fibroblasts may play a role in the pathogenesis of cardiac fibrotic responses, our understanding of the in vivo actions of miRNAs is hampered by their broad range of molecular targets, and by the complexity of their effects on many different cell types. In vivo data on the effects of various miRNAs in cardiac fibrosis are sometimes contradictory. In a model of cardiac pressure overload, miR-21 has been suggested to activate MAPK signaling pathways in fibroblasts, thus promoting a fibrotic response (Thum et al., 2008). In contrast, other studies suggested that fibrosis in response to pressure overload is not

affected by the absence of miR-21 (Patrick et al., 2010).

A growing list of miRNAs has been implicated in negative regulation of cardiac fibrosis. In a model of myocardial infarction, miR-29 downregulation was implicated in fibroblast activation, triggering derepression of ECM genes (van Rooij et al., 2008). The signals responsible for suppression of miR-29 following injury are not known. Members of the miR-15 family have also been suggested to play an antifibrotic role, by counteracting TGF-β-mediated actions (Tijsen et al., 2014). miR-101 was also found to inhibit interstitial fibrosis following myocardial infarction through effects that may involve inhibition of a c-fos/TGF-β1 axis (Pan et al., 2012).

Information on the potential role of lncRNAs in myocardial fibrosis is limited (Jiang and Zhang, 2017). Recent studies have identified maternally expressed gene 3 (Meg3) (Piccoli et al., 2017) and Wisp2 super-enhancer associated RNA (Wisper) (Micheletti et al., 2017), as cardiac fibroblast-enriched lncRNAs that regulate myocardial fibrosis. Meg3 was implicated in regulation of MMP2 expression (Piccoli et al., 2017). Wisper on the other hand had broad effects in regulation of fibroblast gene expression programs, involved in ECM deposition, cell differentiation, proliferation and survival (Micheletti et al., 2017). Whether lncRNAs are involved in the pathogenesis of human conditions associated with cardiac fibrosis remains unknown. Correlation studies using cardiac samples from patients with ischemic cardiomyopathy suggested a strong association between expression of lncRNAs and ECM genes (Huang et al., 2016).

8. Epigenetic regulation of cardiac fibrosis

Epigenetic regulation of fibroblast gene expression through modifications in nucleosomal chromatin has been implicated in the pathogenesis of fibrosis in many tissues. Post-translational modifications of histones, such as acetylation and methylation of lysines in the amino-terminal tail, have been extensively studied and have profound consequences on expression of fibrosis-associated genes (Zhang et al., 2017b), (Schuetze et al., 2014), (Tao et al., 2018). Histone lysine acetylation is reversible: lysine residues are acetylated through the actions of histone/lysine acetyltransferases (HATs) and are deacetylated by a family of enzymes, the histone deacetylases (HDACs) (Seto and Yoshida, 2014). Studies examining the role of HATs in fibrotic remodeling of the heart are lacking. In contrast, a growing body of in vivo evidence using small molecule inhibitors supports an important role for HDAC activity in the pathogenesis of myocardial fibrosis (Stratton and McKinsey, 2016), (Williams et al., 2014), (Nural-Guvener et al., 2014). HDAC inhibition has been demonstrated to protect from diastolic dysfunction in mouse models of cardiac aging or hypertension by reducing fibroblast activation and by attenuating perturbations of myofibril relaxation in cardiomyocytes (Jeong et al., 2018), (Blakeslee et al., 2017). The cellular targets and molecular cascades responsible for the anti-fibrotic effects of HDAC inhibitors remain poorly understood; however, effects of HDAC inhibition on fibroblast activation and proliferation have been implicated (Schuetze et al., 2017). In vitro and in vivo evidence suggested that HDAC-driven modulation of miRNA expression may be an important mechanism in regulation of fibrosis in the pressure-overloaded myocardium (Renaud et al., 2015).

9. Negative regulation of cardiac fibrosis

Our understanding of the molecular signals that negatively regulate myocardial fibrotic responses is limited. In healing infarcts, suppression of pro-fibrotic signaling pathways may be critical for quiescence of activated fibroblasts after a scar is formed, thus preventing uncontrolled fibrosis (Prabhu and Frangogiannis, 2016). As the scar matures, many myofibroblasts become apoptotic (Takemura et al., 1998); others survive (Fu et al., 2018) but become quiescent, exhibiting lower levels of ECM protein synthesis. Deactivation of myofibroblasts may involve removal of fibrogenic mediators from the infarct, clearance of matricellular proteins, and suppressive effects of a cross-linked ECM network. Moreover, endogenous anti-fibrotic pathways may be triggered by fibrogenic signals, serving as active suppressors of fibrosis that protect the myocardium from excessive or prolonged fibroblast activation. Relatively few studies have identified endogenous mediators that inhibit fibroblast activation in cardiac fibrosis. A complex network of signals has been implicated in negative regulation of TGF-β signaling (Zeglinski et al., 2015), (Miyazawa and Miyazono, 2017); however, the role of these endogenous inhibitors in regulation of cardiac fibrosis is unclear. The apelin pathway has been suggested as a negative regulator of angiotensin II-mediated fibrotic remodeling (Zhang et al., 2017c). A recent study identified the cytoskeletal protein Ckap4 as a novel inducible marker of fibroblast activation that restrains expression of fibrosis-associated genes (Gladka et al., 2018). Activation of a senescence program, involving p53 and p16-mediated pathways, has also been implicated in negative regulation of cardiac fibrosis in both experimental models and in fibrotic human hearts (Meyer et al., 2016). As discussed above, a growing body of evidence suggests that certain miRNAs, including miR-29, miR-15 and miR-101 may attenuate fibroblast activation (van Rooij et al., 2008), (Tijsen et al., 2014), (Pan et al., 2012); however, their pattern of downregulation following cardiac injury does not support their role as endogenous inducible anti-fibrotic mechanisms.

10. Targeting the fibrotic response in myocardial disease

Implementation of anti-fibrotic strategies has been proposed as a promising therapeutic approach for patients with heart failure or myocardial infarction. However, the rationale for these approaches remains poorly developed. It should be emphasized that cardiac fibrosis is not a single disease entity, but rather a common pathologic abnormality that accompanies most myocardial diseases and often represents a reparative response. In human subjects, there are no myocardial diseases with a well-documented primary fibrotic cause. Because the adult mammalian heart lacks regenerative capacity, myocardial fibrosis is often reparative, reflecting a response to primary cardiomyocyte injury. Even in conditions associated with predominant interstitial and perivascular fibrosis, in the absence of significant cardiomyocyte death (such as diabetes and obesity), the relative contribution of fibrosis in dysfunction and adverse outcome is unclear. Thus, recommendations regarding the therapeutic promise of anti-fibrotic strategies in patients with heart disease should take into account the distinct cellular alterations that occur with each type of myocardial injury and their implications on myocardial function.

10.1. Is myocardial fibrosis reversible?

Whether established cardiac fibrosis is reversible is a key question that needs to be answered in order to design effective therapy. Reversibility of cardiac fibrosis is likely dependent on the etiology and extent of disease, the age of the fibrotic lesions, and the amount of protease-resistant cross-linked ECM. Clearly, established replacement fibrosis in response to a large myocardial infarction is irreversible. Regression of myocardial scars requires extensive myocardial regeneration, a major visionary goal of modern cardiovascular research. In contrast, in experimental models of interstitial or perivascular fibrosis, fibrotic changes may be reversible. In an experimental mouse model of ischemic interstitial fibrosis due to brief repetitive ischemia and reperfusion in the absence of myocardial infarction, discontinuation of the ischemic insults resulted in reversal of fibrosis (Dewald et al., 2003). Moreover, experimental studies in rat models have suggested that hypertensive fibrosis may be reversible upon treatment with ACE inhibitors (Brilla et al., 1996). Evidence from clinical investigations remains inconclusive. In a small clinical study, 35 patients with hypertension and left ventricular hypertrophy had significant regression of fibrosis (assessed through endomyocardial biopsy) and attenuated

diastolic dysfunction after a 6-month course of lisinopril (Brilla et al., 2000). In contrast, fibrosis may not be reversible in patients with advanced heart failure. Despite robust unloading in patients with end-stage heart failure treated with Left ventricular assist device (LVAD) support, the extent of cardiac fibrosis was not affected (Farris et al., 2017). Moreover, patients with severe aortic stenosis showed no reversal of myocardial fibrosis 9 months after aortic valve replacement (Weidemann et al., 2009).

The mechanisms responsible for reversal of established fibrotic myocardial disease remain unknown. Clearance of collagen and other matrix proteins from the fibrotic heart likely requires activation of proteases. Whether specific subpopulations of "anti-fibrotic" macrophages and lymphocytes are involved in resolution of fibrotic lesions remains unknown. Moreover, the functional characteristics and molecular profile associated with a pro-regression phenotype in cardiac fibroblasts have not been investigated. In a clinical investigation, studying patients with ischemic cardiomyopathy undergoing aortocoronary bypass, recovery of function after revascularization was predominantly noted in myocardial segments with a higher cellular content, increased numbers of macrophages, higher levels of matricellular proteins, and lower collagen content (Frangogiannis et al., 2002b), (Frangogiannis et al., 2002a). These observations may suggest that high interstitial cellularity may be needed for resorption and resolution of fibrotic lesions.

10.2. Targeting fibrotic cardiac remodeling in various pathophysiologic conditions

10.2.1. Myocardial infarction

Acute myocardial infarction leads to sudden death of up to a billion cardiomyocytes overwhelming the limited regenerative capacity of the heart. As a result, repair of the infarcted myocardium and preservation of the structural integrity of the ventricle is dependent on activation of fibroblasts and on formation of a collagen-based scar (Frangogiannis, 2015b), (Prabhu and Frangogiannis, 2016). Following myocardial infarction, myofibroblast conversion and activation of a matrix-synthetic program are essential for cardiac repair. Abrogation of fibroblast function would be expected to have catastrophic consequences, promoting cardiac rupture and accentuating dilative remodeling due to a marked reduction in the tensile strength of the scar. On the other hand, excessive, prolonged or expanded fibrotic responses following myocardial infarction may markedly reduce ventricular compliance, leading to increased diastolic dysfunction. No single animal model can recapitulate the spectrum of functional phenotypes observed in human post-infarction heart failure. Thus, decisions regarding therapeutic strategies for patients surviving myocardial infarction require pathophysiological stratification, on the basis of relevant biomarkers (such as indicators of collagen synthesis and degradation or galectin-3) (Lopez et al., 2010a), (Perea et al., 2016) or imaging studies (Hervas et al., 2016) that reflect ECM synthesis, or inflammatory activation. Subjects with evidence of excessive ECM deposition may benefit from brief administration of an anti-fibrotic agent. On the other hand, patients with accentuated and prolonged inflammatory activation that is often associated with dilative remodeling and systolic dysfunction may be candidates for targeted anti-inflammatory therapy, such as IL-1 inhibition, or CCL2 antagonism (Frangogiannis, 2014), (Huang and Frangogiannis, 2018).

10.2.2. Targeting fibrosis in chronic heart failure associated with pressure overload

The pathophysiologic consequences of left ventricular pressure overload play a dominant role in the pathogenesis of the fibrotic cardiomyopathy associated with HFpEF. In animal models, pressure overload induces early hypertrophy, fibrosis and diastolic dysfunction, followed by decompensation, dilative cardiomyopathy and the development of systolic dysfunction (Xia et al., 2009). A similar time course of geometric and functional changes would be expected in patients with untreated malignant hypertension or uncorrected critical aortic stenosis. A growing body of evidence suggests an important role for inflammatory activation in the pathogenesis of fibrosis and dysfunction following pressure overload (Salvador et al., 2016), (Sun et al., 2007), (Tokuda et al., 2004). Although due to the absence of substantial cellular necrosis, the inflammatory response in the pressure-overloaded myocardium does not exhibit the intense acute activation noted in infarcted hearts (Xia et al., 2009), (Nicoletti et al., 1996), the prolonged stimulation of pro-inflammatory signals may promote fibrosis by inducing recruitment of fibrogenic macrophages and lymphocytes. It should be noted that not all studies support the importance of inflammatory cells in pressure overload-induced fibrosis. Experiments using the macrophage Fas-induced apoptosis (MAFIA) transgenic mouse model suggested that macrophage depletion does not affect the remodeling response in mice undergoing pressure overload protocols (Patel et al., 2017).

Regardless of the relative contributions of inflammatory leukocytes, activation of fibroblasts towards a matrix-synthetic phenotype is critical for fibrotic remodeling of the pressure-overloaded myocardium. Mechanosensitive pathways, neurohumoral mediators (such as angiotensin II), fibrogenic cytokines and growth factors (such as TGF-β and IL-10), and matricellular proteins co-operate to activate interstitial fibroblasts, increasing deposition of ECM proteins (Schellings et al., 2004), (Frangogiannis, 2012a). Although targeting fibroblast activation seems a promising therapeutic strategy in the fibrotic cardiomyopathy associated with pressure overload, several concerns hamper clinical implementation. First, the contribution of interstitial fibrosis in human patients with heart failure is unclear. Although fibrotic changes in heart failure patients have adverse prognostic implications, this may represent an epiphenomenon, reflecting worse outcome in subjects with worse cardiomyocyte injury. Second, even in subsets of patients with inappropriate or excessive fibrosis, the need for continuous administration of agents that attenuate fibroblast activation may carry risks by abrogating essential reparative or protective responses. Third, although animal models of left ventricular pressure overload are extremely valuable to gain pathophysiologic insights, their value in therapeutic translation is extremely limited, due to the pathophysiologic complexity of human conditions. Ultimately, success or failure of anti-fibrotic strategies will need to be tested in carefully-designed clinical investigations.

10.2.3. Fibrosis-related targets in conditions associated with volume overload

Despite the prominent role of volume overload in the cardiomyopathy associated with severe valvular regurgitant lesions, and its involvement in post-infarction remodeling, its pathophysiologic consequences on the myocardium remain enigmatic. ECM degradation has been reported to be the hallmark of the interstitial response to volume overload. In contrast to the net collagen deposition noted in pressure-overloaded hearts, volume overload is associated with marked loss of interstitial collagen (Zheng et al., 2009), associated with induction of MMPs (Zheng et al., 2009), (Nagatomo et al., 2000). The basis for the dominant activation of proteases in volume overload-induced cardiomyopathy remains unknown. Direct effects of a volume load on fibroblast gene expression, or modulation of the phenotypic characteristics of immune cells, such as macrophages and mast cells may be implicated (Levick et al., 2012), (Janicki et al., 2006). As important sources of MMPs and pro-inflammatory cytokines, mast cells and macrophages may stimulate ECM degradation, leading to the prominent dilative remodeling that characterizes the cardiomyopathy associated with volume overload. The volume overload-activated signals that may drive interstitial cells towards a matrix-degrading phenotype are not known.

10.2.4. Targeting aging-associated cardiac fibrosis

Aging is associated with both ventricular and atrial fibrosis. In the

absence of concomitant pathologic conditions, normal aging does not induce systolic dysfunction but promotes ECM deposition in the cardiac interstitium, progressively increasing ventricular stiffness (Biernacka and Frangogiannis, 2011). Some descriptive studies have suggested that aging-associated fibrosis may be due to reduced matrix degradation, rather than to increased collagen synthesis (Robert et al., 1997). Fibrotic remodeling of the aging heart may involve the co-operation of several distinct pathways, including generation of ROS, neurohumoral signaling, TGF-β-mediated responses, and induction of inflammatory chemokines (Chen and Frangogiannis, 2010). However, our understanding of the molecular signals involved in age-associated fibrosis is based, for the most part, on associative evidence. The relative contribution of cardiomyocyte-mediated paracrine factors, macrophage activation, and direct fibroblast stimulation remains unclear. It should be emphasized that, although aging is associated with basal interstitial and perivascular collagen deposition, following injury, senescent animals exhibit impaired scar formation, at least in mouse models (Bujak et al., 2008b). Perturbed repair was attributed to age-associated impairment in responsiveness of senescent fibroblasts to growth factors (Bujak et al., 2008b). Although age-related cardiac fibrosis may be implicated in the increased prevalence of HFpEF in elderly subjects, the uncertainties regarding the underlying cellular mechanisms and the chronicity of the process make anti-fibrotic strategies an unattractive option for this patient population.

10.2.5. Fibrosis as a therapeutic target in diabetes, obesity and metabolic dysfunction

Diabetes, obesity and metabolic dysfunction are typically associated with progressive interstitial or perivascular fibrosis in experimental animal models (Cavalera et al., 2014), (Russo and Frangogiannis, 2016), (Biernacka et al., 2015), (Sorop et al., 2018), (Chen et al., 2017a). In human patients, obesity, diabetes and metabolic dysfunction markedly increase the risk of HFpEF, independently of the occurrence of coronary artery disease (Kenchaiah et al., 2002), (Packer and Kitzman, 2018). Emerging clinical evidence suggests a distinct obesity-related phenotype of human HFpEF (Obokata et al., 2017), associated with increased plasma volume, more concentric left ventricular hypertrophy and greater right ventricular dilatation, despite lower plasma levels of natriuretic peptides (Obokata et al., 2017), (Buckley et al., 2018). Fibrosis may significantly contribute to the pathogenesis of diastolic dysfunction in obese and diabetic patients (Paulus and Tschope, 2013); however, the mechanisms responsible for increased interstitial collagen deposition are not known. It has been suggested that microvascular inflammation may play an important role in activation of fibrogenic signaling in subjects with metabolic dysfunction (Paulus and Tschope, 2013), (Packer, 2018). Moreover, in diabetic patients, hyperglycemia may directly activate a fibrogenic response, leading to accumulation of advanced glycation end-products (AGEs) that crosslink the cardiac ECM, while transducing ROS-dependent fibrogenic signals, or triggering receptor for AGE (RAGE)-mediated fibroblast activation (Zhao et al., 2014). Neurohumoral activation, endothelin-1, adipokine secretion, and increased ROS generation may stimulate myocardial expression of pro-inflammatory cytokines and chemokines, leading to recruitment of leukocyte subpopulations with fibrogenic properties in the cardiac interstitium. Induction of matricellular proteins is prominent in diabetic tissues (Gonzalez-Quesada et al., 2013), (Kong et al., 2013b) and may contribute to activation of transforming growth factor-β signaling, directly activating a matrix-synthetic program in cardiac fibroblasts. To what extent fibrogenic actions contribute to diastolic dysfunction and adverse outcome in diabetics remains unclear. Targeting fibrosis in diabetic subjects may have beneficial effects on function of other organs affected by diabetes-associated fibrogenic activation, such as the kidney. Because most pathways involved in cardiac fibrosis are important in regulation of tissue repair, prolonged treatment with certain anti-fibrotic mediators may be undesirable. Thus, implementation of effective therapeutic strategies may require identification of molecular signals or cellular effectors specifically involved in non-reparative fibrotic responses.

11. Conclusions

Myocardial fibrosis is associated with ventricular dysfunction and arrhythmias, and is an adverse prognostic indicator for patients with heart failure. Despite the clear association between cardiac fibrosis and adverse outcome, important questions regarding the pathogenesis, role, and consequences of myocardial fibrotic lesions remain to be answered. Considering the absence of regenerative capacity in the adult mammalian heart, to what extent does human myocardial fibrosis represent a reparative response to cardiomyocyte injury? Is primary activation of a myocardial fibrotic process a significant cause of dysfunction in human patients? Considering the co-existence of cardiomyocyte and interstitial alterations in most myocardial conditions, what is the relative contribution of fibrotic lesions on cardiac dysfunction? In view of the heterogeneity of interstitial cell populations, are there subsets of fibroblasts with unique functional properties? Answers to these key questions are urgently needed in order to dissect the cell biology of myocardial fibrosis, to provide a clear rationale for anti-fibrotic strategies in patients with heart disease, and to identify patient subpopulations that may benefit from such interventions.

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Appendix A. Supplementary data

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News

Aging, cardiac repair and Smad3

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The adult mammalian myocardium lacks endogenous regenerative capacity; thus, following myocardial infarction, the heart heals through formation of a collagen-based scar. Cardiac repair is dependent on a superbly orchestrated inflammatory cascade that sequentially recruits inflammatory cells, fibroblasts and vascular cells in the infarct zone. Expansion of fibroblast populations in the infarcted myocardium and conversion into activated myofibroblasts play a critical role in infarct healing, maintaining the structural integrity of the ventricle and preventing cardiac rupture. However, exaggerated myofibroblast activation may promote excessive deposition of extracellular matrix proteins in the infarct border zone and in the viable remodeling myocardium, precipitating heart failure [1]. Members of the Transforming Growth Factor (TGF)-β superfamily have been implicated in activation of fibroblasts in healing and remodeling tissues. TGF-βs act by transducing signaling cascades mediated through a series of intracellular effectors the Smads, or through Smad-independent pathways. Smad2/3 signaling is activated in all cell types involved in cardiac repair [2, 3], and may modulate inflammatory, reparative and remodeling responses.

Cell-specific effects of Smad3 signaling in the infarcted myocardium

In a recently published study, we generated cell-specific Smad3 knockout mice, in order to investigate the role of Smad3 signaling in regulating fibroblast and cardiomyocyte function following myocardial infarction [4]. We found that cardiomyocyte Smad3 signaling has no effects on cardiac homeostasis, but promotes cardiomyocyte apoptosis and accentuates dilative remodeling, enhancing matrix metalloproteinase expression, and increasing nitrosative stress following myocardial infarction. In contrast, Smad3 signaling in activated infarct myofibroblasts is protective, restraining fibroblast proliferation and contributing to scar organization by stimulating integrin-dependent interactions between the fibroblasts and the extracellular matrix In the infarcted myocardium, myofibroblasts are organized in arrays, exhibiting alignment along the direction of the ventricular wall. Myofibroblast-specific loss of Smad3 perturbs alignment of myofibroblast arrays in the infarct, leading to formation of a disorganized scar. Disturbances in scar formation in myofibroblast-specific Smad3 knockout mice are associated with anincreased incidence of cardiac rupture and with accentuated dilative remodeling. These findings highlight the crucial reparative role of activated myofibroblasts following myocardial infarction. Moreover, our observations may have major implications in understanding the basis for worse outcome and accentuated post-infarction remodeling in senescent subjects.

The role of TGF-β/Smad3 signaling in repair of the senescent heart

Senescent hearts exhibit a modest baseline expansion of the cardiac interstitium, associated with increased collagen deposition, and worse diastolic function [5. On the other hand, older subjects exhibit impaired reparative responses with a major impact on their prognosis following myocardial injury. Elderly patients have an increased incidence of post-infarction heart failure and accentuated adverse remodeling that cannot be explained by larger infarcts. Using a mouse model of reperfused myocardial infarction, we have previously demonstrated that senescent animals (>24 months of age) exhibit worse adverse remodeling following myocardial infarction, when compared with young mice (3-4 months of age). Age-related adverse post-infarction remodeling is associated with a delayed reparative response, and with markedly reduced collagen deposition in the scar. In vitro, cardiac fibroblasts isolated from senescent hearts have impaired responses to TGF-β stimulation, exhibiting attenuated activation of Smad-dependent signaling [6]. Our recent findings on the crucial reparative role of the Smad3 pathway in cardiac fibroblasts [4], suggest that defective repair in senescent mice may be due, at least in part, to perturbed activation of TGF-β/Smad signaling.

What is the basis for attenuated reparative Smad-dependent responses in senescent fibroblasts?

Several distinct mechanisms may explain the perturbed reparative response of senescent cardiac fibroblasts to TGF-β (Figure 1). First, aging may be associated with marked changes in the cellular composition of the cardiac interstitium, leading to selective expansion of cells with low responsiveness to growth factors [7]. Published evidence suggests that defective responses of senescent cardiac fibroblasts are not limited to TGF-β, but may also involve other activating mediators, such as angiotensin II [8. Second, fibroblasts in senescent hearts

may exhibit lower levels of TGF-β receptors, or activation of pseudoreceptors such as BAMBI (BMP and activin membrane-bound inhibitor) that silence TGF-β signaling. Third, senescent fibroblasts may exhibit activation of phosphatases that dephosphorylate Smads, or TGF-β-driven induction of inhibitory Smads (such as Smad7), that inhibit TGF-β signaling responses. The chronic low-level activation of the TGF-β system in senescent hearts may induce baseline expression of endogenous inhibitors of the Smad cascade that attenuate Smad2/3 stimulation in response to acute injury.

Increasing the reparative reserve of the senescent heart

Taken together, our studies suggest that adverse outcome in senescent subjects surviving myocardial infarction may involve defective TGF-β/Smad3-dependent fibroblast activation. Blunted responses of senescent fibroblasts to growth factors may result in formation of a disorganized scar following infarction, reducing tensile strength and accentuating adverse remodeling and systolic dysfunction (Figure 1). This intriguing hypothesis has not yet been tested in vivo. However, if true, this mechanism of age-associated dysfunction may suggest new strategies to improve outcome following cardiac injury in elderly subjects, by implementing strategies that enhance fibroblast activity and promote repair. Brief and cautious administration of growth factors, along with the injection of biomaterials, or cell therapy with healthy reparative fibroblasts may represent effective new strategies for prevention of post-infarction heart failure in elderly patients surviving myocardial infarction.

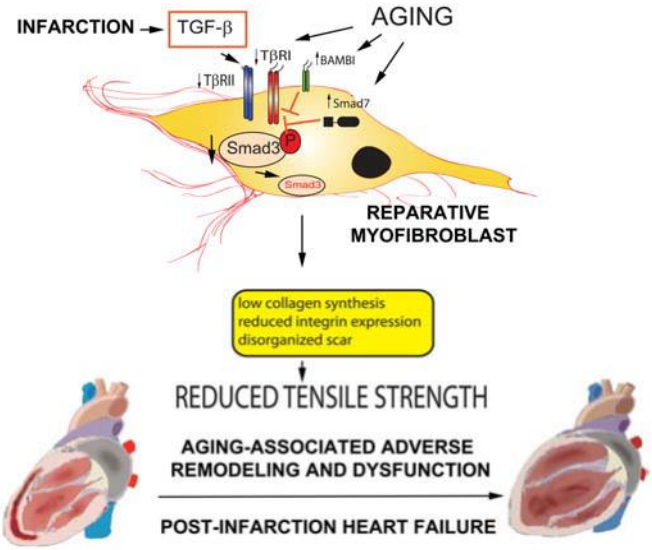

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Figure 1. Age-related accentuation of adverse post-infarction remodeling may be due to an impaired response of reparative fibroblasts to TGF-β. Our observations suggest that: a) myofibroblast-specific loss of Smad3 disrupts scar organization, increasing adverse remodeling following myocardial infarction, b) senescent hearts exhibit impaired infarct healing, accompanied by decreased collagen deposition, and c) fibroblasts harvested from senescent hearts exhibit blunted Smad2/3 activation in response to TGF-β stimulation. Taken together these observations suggest the intriguing hypothesis that age-associated impairment in TGF-β/Smad signaling in reparative fibroblasts may reduce tensile strength of the healing scar, accentuating adverse remodeling following infarction and causing heart failure. Several mechanisms may account for reduced TGF−β responses in senescent cardiac fibroblasts. Age-associated alterations of the expression of signaling TGF-β receptors (TβRI and TβRII) and pseudoreceptors (such as BAMBI) may modulate the response to TGF-β. Activation of phosphatases or induction of inhibitory Smad7, such as Smad7 may attenuate Smad2/3 activation in response to TGF-β. Because aging is associated with baseline activation of a fibrogenic program (that may involve low level increase in basal TGF-β activity), TGF-β-driven induction of endogenous signals that inhibit Smad-dependent signaling may be responsible for blunted responses to the sudden burst in TGF-β activity observed following cardiac injury.

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RCAN1–Calcineurin Axis and the Set-Point for Myocardial Damage During Ischemia-Reperfusion

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RCAN1 (regulator of calcineurin 1) is an endogenous inhibitor of the Ca^{2+}-activated protein phosphatase calcineurin. It has been known for some time that deletion of RCAN1 in the mouse exacerbates ischemia/reperfusion-induced infarction in heart[1] and brain.[2] In this issue of *Circulation Research*, Parra et al[3] elucidate an underlying mechanism.

Article, see p e20

Calcineurin is a widely expressed serine/threonine phosphatase consisting of 1 catalytic and 1 regulatory subunit.[4] This enzyme is activated by binding of Ca^{2+} to EF-hand motifs in the regulatory subunit and by calmodulin binding to the catalytic subunit. Calcineurin exerts pleiotropic effects including T-lymphocyte activation, neurite outgrowth, heart valve formation, skeletal myocyte differentiation, and cardiac and skeletal muscle hypertrophy. These effects are attributable primarily to calcineurin-mediated dephosphorylation of transcription factors (eg, NFAT [nuclear factor of activated T cells][5,6]) resulting in their nuclear translocation and activation of various gene expression programs. Calcineurin, however, also acts on other substrates including structural proteins, receptors, channels, and signaling molecules.[7]

RCAN1, encoded by a gene in the Down syndrome critical region 1 on human chromosome 21, is enriched in striated muscle and brain.[4] RCAN1 inhibits calcineurin through direct binding to its catalytic subunit. As might be expected from the actions of calcineurin, cardiomyocyte-specific overexpression of RCAN1 in mice inhibits cardiac hypertrophy elicited by pathological or physiological stimuli.[8] Less expected are effects of RCAN1 on ischemia/reperfusion injury. Cardiomyocyte-specific transgenic overexpression ameliorates myocardial damage in vivo, whereas the opposite is observed in mice with generalized RCAN1 knockout.[1] Moreover, pharmacological inhibition of calcineurin reverses this RCAN1-deficient phenotype showing that these effects of RCAN1 depletion are mediated by unleashing of calcineurin.

So, what mechanisms connect RCAN1–calcineurin signaling with myocardial ischemia/reperfusion injury (Figure)? Parra et al postulated that mitochondria may be involved. One molecule known to link calcineurin with mitochondria is DRP1 (dynamin-related protein 1)—a GTPase involved in mitochondrial fission. Calcineurin-mediated dephosphorylation of serine 637 in human DRP1 triggers DRP1 translocation from cytosol to mitochondria, where it promotes outer mitochondrial membrane constriction events involved in mitochondrial fission.[9] In fact, the investigators observed that RCAN1 depletion induces mitochondrial fragmentation in a variety of cell types, including mouse embryonic fibroblasts and primary neonatal and adult rodent cardiomyocytes—an effect that was reversed by pharmacological inhibition of calcineurin or DRP1. Moreover, DRP1-mediated mitochondrial fission may not be the only mechanism responsible for mitochondrial fragmentation because RCAN1 knockdown also results in selective decreases in OPA1 (optic atrophy 1) and, under some experimental conditions, MFN2 (mitofusin 2)—proteins that mediate mitochondrial fusion. Although the mechanisms linking RCAN1 with OPA1 and MFN2 are not known, these observations suggest that RCAN1 maintains mitochondrial connectivity through coordinate inhibition of fission and promotion of fusion.

The relationship between mitochondrial dynamics (fission and fusion) and cell death is poorly understood. BAX (BCL-2 [B-cell leukemia/lymphoma 2]–associated X) is a BCL-2 protein that mediates permeabilization of the outer mitochondrial membrane and cytochrome c release during apoptosis. Although complex interactions have been reported between DRP1 and BAX,[10] the role of mitochondrial fission in apoptosis remains unclear.[11] An additional wrinkle is that other studies suggest that promotion of mitochondrial connectivity (whether by increasing fusion or decreasing fission) sensitizes cells to Ca^{2+}-induced opening of the permeability transition pore (mPTP) on the inner mitochondrial membrane and necrotic cell death[12]—arguably of more relevance in myocardial ischemia–reperfusion injury than apoptosis.

To gain insights beyond mitochondrial dynamics, Parra et al chose to examine the role of Ca^{2+} itself in the death process and made an interesting observation: RCAN1-deficient cardiomyocytes exhibit a deficit in mitochondrial Ca^{2+} uptake and concomitant elevation of cytosolic Ca^{2+} concentrations. The mechanism by which RCAN1 depletion dampens mitochondrial Ca^{2+} uptake is not clear but may reflect observed decreases in polarization across the inner mitochondrial

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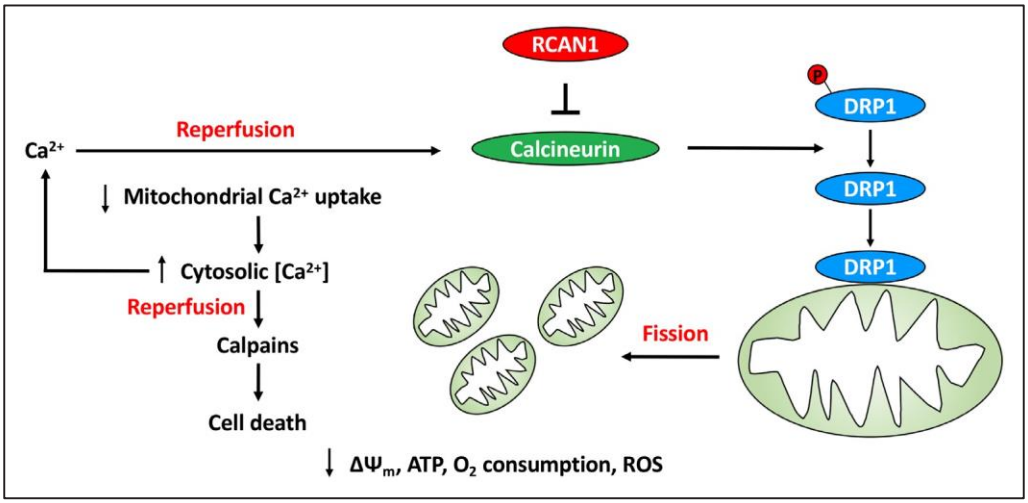

Figure. RCAN1 (regulator of calcineurin 1) modulates tissue damage during myocardial ischemia–reperfusion. Depletion of RCAN1 disinhibits the Ca^{2+}-activated protein phosphatase calcineurin resulting in calcineurin-mediated dephosphorylation of serine 637 in human DRP1 (dynamin-related protein 1). This stimulates the translocation of DRP1 from cytosol to the outer mitochondrial membrane where it promotes mitochondrial fission. Depletion of RCAN1 may also promote mitochondrial fragmentation through decreases in mitochondrial fusion proteins OPA1 (optic atrophy 1) and MFN2 (mitofusin 2) (not shown). Fragmented mitochondria manifest decreased Ca^{2+} uptake, which may reflect their loss of electric potential difference across the inner mitochondrial membrane ($\Delta\psi_m$) or perhaps a more direct action of calcineurin on the mitochondrial Ca^{2+} import machinery. Impaired uptake of Ca^{2+} results in elevation of cytosolic Ca^{2+} concentrations, thereby predisposing to the activation of calpains—Ca^{2+}-activated proteases. Calpains cleave signaling and structural proteins to induce cell death. Activation of calcineurin and calpains in this schema takes place during reperfusion because the activities of these enzymes are inhibited in the acidic environment of ischemia.

membrane ($\Delta\psi_m$)—a driving force in the movement of Ca^{2+} into the mitochondrial matrix through the mitochondrial calcium uniporter.[13] This begs the question, however, as to why $\Delta\psi_m$ is decreased in RCAN1-depleted cardiomyocytes. One possibility is that decreases in $\Delta\psi_m$ reflect merely the poor health of the fragmented mitochondria, which also exhibit decreased ATP levels. Another possibility is that RCAN1 deficiency somehow causes a primary defect in mitochondrial Ca^{2+} uptake which, in turn, results in decreased catabolism of nutrients. In fact, lower rates of oxygen consumption and ROS (reactive oxygen species) production were observed in RCAN1-depleted cells. Further study will be needed to test whether the RCAN1–calcineurin axis directly regulates mitochondrial Ca^{2+} uptake.

Although decreased mitochondrial Ca^{2+} uptake could compromise substrate oxidation in mitochondria, it would be expected to be protective against Ca^{2+}-induced opening of the mPTP and necrosis. What then would account for the increased sensitivity to cell death in RCAN1-deficient cardiomyocytes? Given the relative increases in cytosolic Ca^{2+}, the investigators considered Ca^{2+}-dependent mechanisms that may be operating in this cellular compartment. In fact, they observed activation of calpains—Ca^{2+}-stimulated proteases whose substrates include signaling and structural molecules involved in apoptotic and necrotic cell death.[14,15] Importantly, combined knockdown of calpains 1 or 2 or pharmacological inhibition of these proteases reversed the increased sensitization to simulated ischemia–reperfusion-induced cell death resulting from RCAN1 knockdown.

Thus, in essence, what RCAN1 deficiency seems to be doing is to shift the dominant cell death mechanism during ischemia–reperfusion away from the mPTP and toward cytosolic calpain activation. The augmentation of cell killing resulting from this shift may reflect the fact that high Ca^{2+} concentrations are less well tolerated in the cytosol than in the mitochondrial matrix.

The investigators also explored the effects of increasing RCAN1 levels. High levels of RCAN1 overexpression from adenoviral-mediated transduction of cardiomyocytes resulted in hyperconnected mitochondria with augmented rates of oxygen consumption and ROS levels. Moreover, in keeping with the cardioprotection afforded by transgenic overexpression in cardiomyocytes in vivo, RCAN1 overexpression ameliorated cardiomyocyte death elicited by simulated ischemia–reperfusion. Similar findings were obtained with more mild overexpression of RCAN1 as observed in cardiomyocytes derived from trisomic 21 human-induced pluripotent stem cells compared with cardiomyocytes derived the same induced pluripotent stem cells in the disomic state (the extra chromosome 21 being spontaneously lost with passage in culture). Importantly, the phenotype in the trisomic cells is attributable specifically to RCAN1—rather than the extra copy of multiple other genes on chromosome 21—because it is reversed by RCAN1 knockdown. Moreover, these findings suggest that excess RCAN1 is an important factor in the high degrees of oxidative stress present in the cells of patients with Down syndrome.

In summary, this study establishes the importance of RCAN1 in maintaining a connected mitochondrial network. In addition, it delineates a critical mechanism by which the RCAN1–calcineurin axis regulates the susceptibility of the myocardium to damage from ischemia–reperfusion (Figure). Future work is needed to elucidate the precise molecular connections by which calcineurin impacts mitochondrial Ca^{2+} uptake. In addition, the existence of this mechanism does not preclude the possibility that other calcineurin substrates contribute to the set point of

tissue damage resulting from ischemia–reperfusion. Finally, the possibility that this mechanism also operates during ischemia–reperfusion in the brain merits further investigation.

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Disclosures

None.

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COMMENTARY

PDCD5 says no to NO

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Blood vessels are more than mere conduits, and their dynamic function is regulated to a large extent by their inner layer, the endothelium. Endothelial cell-derived nitric oxide (NO), a product of endothelial NO synthase (eNOS), is important for normal endothelial function and vascular health. Programmed cell death 5 (PDCD5) is a regulator of multiple cellular processes through its interactions with other proteins. In PNAS, Lee et al. (1) describe a novel mechanism by which PDCD5 alters vascular function by reducing NO production.

Integrated function of the cardiovascular system is essential for the maintenance of homeostasis in complex multicellular organisms. Mutations that affect components of this system frequently result in embryonic lethality (2, 3), and maladaptive changes during adulthood lead to cardiovascular disease, the number one cause of death worldwide (4, 5). The endothelium functions as a selective barrier and controls vascular tone, hemostasis and thrombosis, inflammation, and redox balance (6). Endothelial cells regulate these functions in part by producing NO, which can diffuse across cell membranes to work as a paracrine factor on neighboring smooth muscle cells and circulating leukocytes and platelets. Impaired NO synthesis or availability contributes significantly to vascular diseases, including hypertension and atherosclerosis (6, 7).

Endothelial cells produce NO from L-arginine mainly through the activity of eNOS, although other isoforms may be involved (7). Regulation of eNOS activity is complex and occurs at transcriptional, posttranscriptional, and posttranslational levels. Posttranslational modifications such as phosphorylation, acylation, and nitrosylation control eNOS subcellular localization and activity (8). For instance, phosphorylation of human eNOS at Ser-617, Ser-635, and Ser-1177 is stimulatory, while phosphorylation at Ser-116 or Thr-495 is inhibitory (9). A myriad of extracellular signals including shear stress, acetylcholine, bradykinin, histamine, and vascular endothelial growth factor (VEGF) stimulate NO production through distinct kinases that converge on eNOS (7, 8), but the regulatory mechanisms and differential contributions of these pathways have not been fully elucidated. One important kinase is Akt, which phosphorylates eNOS at Ser-1177 to enhance its activity in a calcium-independent manner (10).

PDCD5 is a ubiquitously expressed cytosolic and nuclear protein. It facilitates p53-dependent apoptosis through its interaction with mouse double minute 2 homolog (MDM2) (11) and may function as a tumor suppressor. PDCD5 has also been reported to regulate other cellular processes [e.g., autophagy (12)], although the relevant mechanisms are less defined. PDCD5 binds multiple cellular proteins, including histone deacetylase 3 (HDAC3) (13). One function of HDAC3 in leukemia cells is to bind to and deacetylate Akt, thereby facilitating Akt phosphorylation and activation (14). Lee and coworkers (13) have previously shown that binding of PDCD5 to HDAC3 displaces p53 and promotes HDAC3 degradation in cancer cells. However, previous work defining a role for PDCD5 in the vascular system has been limited to a study showing that it is induced by cerebral ischemia/reperfusion and contributes to infarction and vascular permeability (15).

Lee et al. (1) explore the role of PDCD5 in regulating the HDAC3–Akt interaction in the context of endothelial function. In vitro studies demonstrated that loss of PDCD5 in endothelial cells increases the interaction of HDAC3 with Akt—tested by coimmunoprecipitation and proximity ligation assays—augmenting levels of phosphorylated forms of Akt and eNOS, and NO production. Functionally, loss of PDCD5 in endothelial cells was shown to increase microvessel sprouting in an in vitro aortic ring assay (1). Conversely, overexpression of PDCD5 in VEGF-stimulated endothelial cells decreased the HDAC3–Akt interaction, levels of phosphorylated eNOS and Akt, NO production, and endothelial tube formation in vitro (1). None of these effects were observed when a mutant form of PDCD5, defective for binding HDAC3, was overexpressed (1), demonstrating that PDCD5 binding to HDAC3 is critical. These

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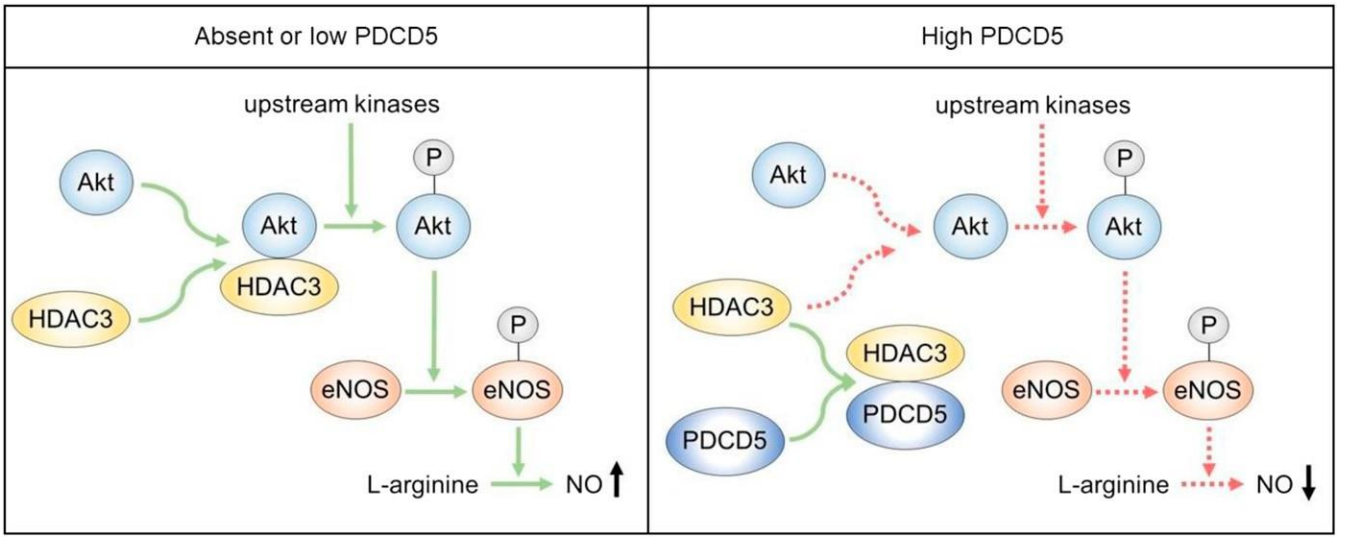

Fig. 1. Programmed cell death 5 (PDCD5)-dependent inhibition of endothelial nitric oxide synthase (eNOS). In endothelial cells in culture, loss of PDCD5 increases both the interaction of the kinase Akt with histone deacetylase 3 (HDAC3) and phosphorylation of Akt. This in turn enhances Akt-dependent phosphorylation and activation of eNOS, and subsequent nitric oxide (NO) production from L-arginine. Conversely, increased expression of PDCD5 disrupts the interaction of HDAC3 with Akt and decreases the levels of phosphorylated Akt and eNOS, reducing NO production. Red dotted arrows indicate processes that are restricted.

observations suggest that PDCD5 interacts with HDAC3 and disrupts HDAC3–Akt binding, which, in turn, decreases Akt phosphorylation and Akt-dependent activation of eNOS in endothelial cells (Fig. 1). Lee et al. further evaluate the importance of HDAC3 in this pathway using genetic knockdown and pharmacologic inhibition. Decreases in HDAC3 abundance or function in VEGF-stimulated endothelial cells impaired Akt and eNOS phosphorylation, NO production, and microvessel sprouting from aortic rings (1), consistent with the proposed model. These mechanistic studies focus on presumed cytoplasmic events but do not exclude contributions from changes that may stem from loss of PDCD5 or HDAC3 in the nucleus (11).

To explore this mechanism in vivo, Lee et al. generated mice with endothelial-specific deletion of PDCD5 and used partial carotid artery ligation and administration of the Paigen diet to induce vascular remodeling. Deletion of PDCD5 in endothelial cells markedly reduced neointimal growth compared with wild-type mice studied in the same protocol (1). Moreover, mice with endothelial cell-specific deletion of PDCD5 exhibited increased endothelial expression of phosphorylated Akt (1), consistent with their model. Caveats include that the PDCD5–HDAC3 and HDAC3–Akt interactions and levels of HDAC3 and phosphorylated eNOS were not assessed in the in vivo context; and that loss of PDCD5 in the hematopoietic lineage may also have contributed to the observed effects because the Tie2-Cre deletor used in these studies may also be active in those cells (16). Despite these limitations, the in vivo findings are interesting and constitute a rationale to study PDCD5-dependent inhibition of Akt and eNOS in endothelial cells in clinically relevant models of vascular disease, including atherosclerosis and hypertension.

PDCD5 can be detected in serum and other fluids (17, 18), and Lee et al. studied human serum samples from a high-risk cohort to test whether serum levels correlate with adverse cardiovascular conditions. They found an inverse relationship between levels of PDCD5 and those of nitrite/nitrate (stable end products of NO) (1). Moreover, circulating PDCD5 levels were higher in males and in patients with hypertension, diabetes mellitus, left ventricular hypertrophy, higher coronary calcium scores, and lower HDL levels (1). These results are provocative, although the predictive power of serum PDCD5 levels will require further study. These correlations also raise interesting biological questions: Given its ubiquitous expression (19), what are the sources of circulating PDCD5? Specifically, do levels of PDCD5 in serum reflect endothelial dysfunction? Conversely, is PDCD5 in the blood able to act on and change the phenotype of endothelial cells? Some clues to this last question are provided by pertinent in vitro studies in Lee et al. showing that PDCD5 is detected in media conditioned by human endothelial cells, and addition of recombinant human PDCD5 directly to cell culture media reduces the levels of phosphorylated forms of Akt and eNOS in endothelial cells. Finally, is PDCD5—in the blood and/or in cells—a potential target for therapeutic intervention? This possibility is exciting, although the fact that PDCD5 impacts other cellular processes (11–13, 15) also needs to be considered.

This study adds a new and likely complex PDCD5-based angle to our understanding of mechanisms that affect eNOS activity, endothelial dysfunction, and cardiovascular disease. Assessing the significance and specificity of this regulatory pathway in biology and disease is an important goal for future studies.

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