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PRINCIPAL INVESTIGATOR: Nikolaos Frangogiannis

CONTRACTING ORGANIZATION: Albert Einstein College of Medicine

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14. ABSTRACT

Heart fibrosis and loss of blood vessels are prominent pathologic abnormalities in diabetics that lead to the development of heart failure. Moreover, reduced angiogenesis after a heart attack is responsible for defective myocardial repair in diabetic subjects. Although the negative impact of diabetes on the heart is widely appreciated, the cellular alterations and molecular signals involved in fibrosis and blood vessel loss in diabetes remain unknown. Applying genetic fate mapping tools, we have uncovered an unexpected plasticity and heterogeneity in reparative cells and identified common cellular links between angiogenesis and fibrosis. We investigate the role of these novel biological mechanisms in the pro-fibrotic and angiostatic effects of diabetes, focusing on the contribution of pericytes and endothelial cells in the cardiac tissue repair process.

15. SUBJECT TERMS
Diabetes, cardiomyopathy, heart failure, fibrosis, angiogenesis, vascular rarefaction, pericytes, endothelial cells, endothelial-to-mesenchymal transition, cellular plasticity, extracellular matrix, cell fate mapping, gene expression, signaling pathways

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Third Annual Report 09/30/2019

Page

1. Introduction……. 4

2. Keywords……. 4

3. Accomplishments……..……... 5

4. Impact……...…… 15

5. Changes/Problems...…..…… 16

6. Products…….…….…..……. 16

7. Participants & Other Collaborating Organizations…… 17

8. Special Reporting Requirements…… 20

9. Appendices…… 20

USAMRMC Proposal Number PR151029
Title: "Cellular Plasticity in the Diabetic Myocardium"
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Third Annual Report from 09/01/2018 to 08/31/2019

1. Introduction

Heart tissue fibrosis and loss of blood vessels are prominent pathologic abnormalities in diabetics and lead to the development of heart failure. Moreover, reduced angiogenesis after a heart attack is responsible for defective myocardial repair in diabetic subjects. Although the negative impact of diabetes on heart function and repair is widely appreciated, the cellular alterations and molecular signals involved in fibrosis and blood vessel loss in diabetes remain unknown. Applying genetic fate mapping tools, we have uncovered an unexpected plasticity and heterogeneity in reparative cells and identified common cellular links between angiogenesis and fibrosis. We investigate the role of these novel biological mechanisms in the pro-fibrotic and angiostatic effects of diabetes, focusing on the contribution of pericytes and endothelial cells in the cardiac tissue repair process.

2. Keywords

Diabetes, cardiomyopathy, heart failure, fibrosis, angiogenesis, vascular rarefaction, pericytes, endothelial cells, endothelial-to-mesenchymal transition, cellular plasticity, extracellular matrix, lineage tracing, cell fate mapping, gene expression profiling, signaling pathways

3. Accomplishments

Major Scientific Goals of the Project

The project has the following three major goals:

a) Determine the contribution of pericytes to the development of cardiac fibrosis in diabetic mice (**AECOM);**

b) Evaluate the role of endothelial cells in the development of cardiac fibrosis in diabetic mice (**VUMC)**;

c) Identify sex-specific molecular pathways promoting fibrosis and causing blood vessel loss in diabetic hearts (**AECOM & VUMC**).

Scientific Accomplishments of the Project

a) Contribution of pericytes to cardiac fibrosis in diabetic hearts (Work performed at (AECOM).

a1) Characterization of the cardiac pericytes.

Fibroblasts and pericytes are often considered related interstitial cell populations with common functional properties. In order to explore the distribution and function of pericytes in the myocardium, we have developed pericyte/fibroblast dual reporter mice ($NG2^{dsred}$/$PDGFR\alpha^{GFP}$ mice). Using dual immunofluorescence and flow cytometry, we demonstrated that NG2+ perivascular cells (pericytes) and PDGFRα+ fibroblasts are distinct populations in mouse hearts with minimal overlap (Figures 1, 2).

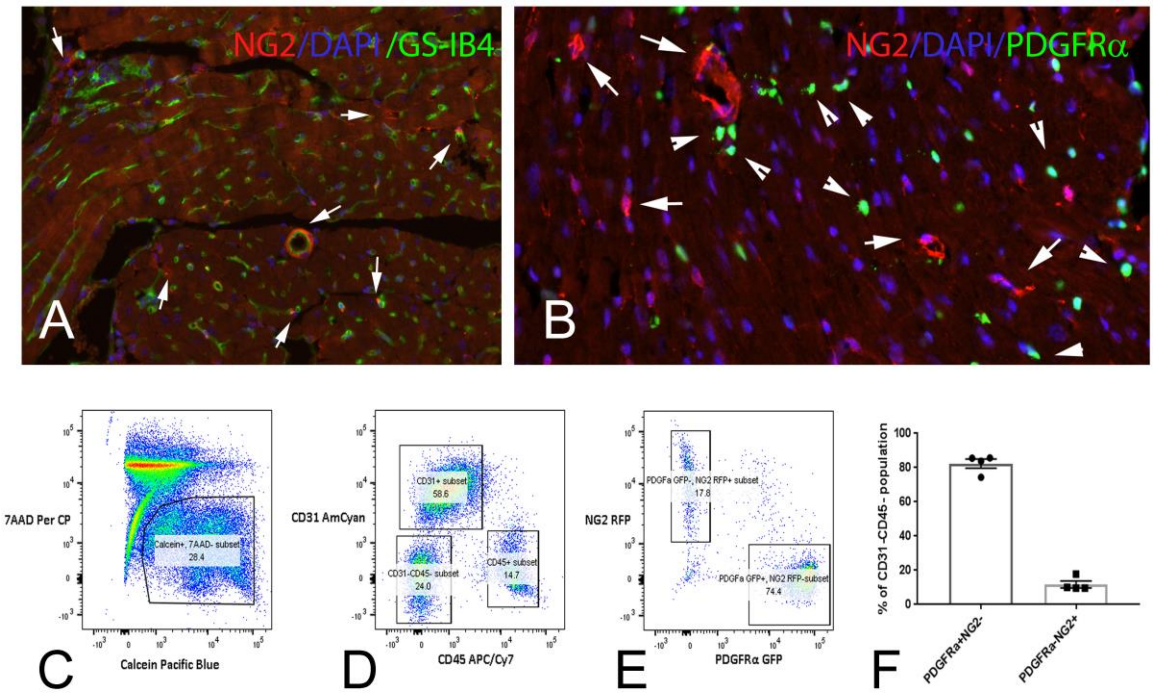


Figure 1: Fibroblasts and pericytes represent distinct populations of myocardial interstitial cells. A: NG2 is an established and reliable marker of pericytes in many tissues[29]. Because anti-NG2 antibodies are suboptimal for immunohistochemical studies, we used $NG2^{Dsred}$ pericyte reporter mice to identify pericytes in the mouse heart. Dual staining for Griffonia simplicifolia isolectin B4 (GS-IB4) and NG2 identifies abundant pericytes in the adult mouse heart as periendothelial cells (arrows). B. In order to examine possible overlap between pericyte and fibroblast populations, we generated $NG2^{Dsred}$;$PDGFR\alpha^{EGFP}$ pericyte/fibroblast dual reporter mice. In the adult mouse heart, NG2+ pericytes (arrows) and PDGFRα+ fibroblasts (arrowheads) represent distinct interstitial cell populations without significant overlap. C-F. Flow cytometric analysis of interstitial cells from $NG2^{Dsred}$;$PDGFR\alpha^{EGFP}$ pericyte/fibroblast dual reporter mice. Viable (7AAD-), and metabolically active (calcein+) cells were gated, and the CD31-CD45- cell population (non-endothelial, non-hematopoietic cells) was subgated to identify NG2+ pericytes and and PDGFRα+ fibroblasts (E) F: The percentage of interstitial cells identified as NG2+ pericytes and PDGFRα+ fibroblasts (n=4). Please note the significant population of pericytes along with a more abundant population of PDGFRα+ fibroblasts.

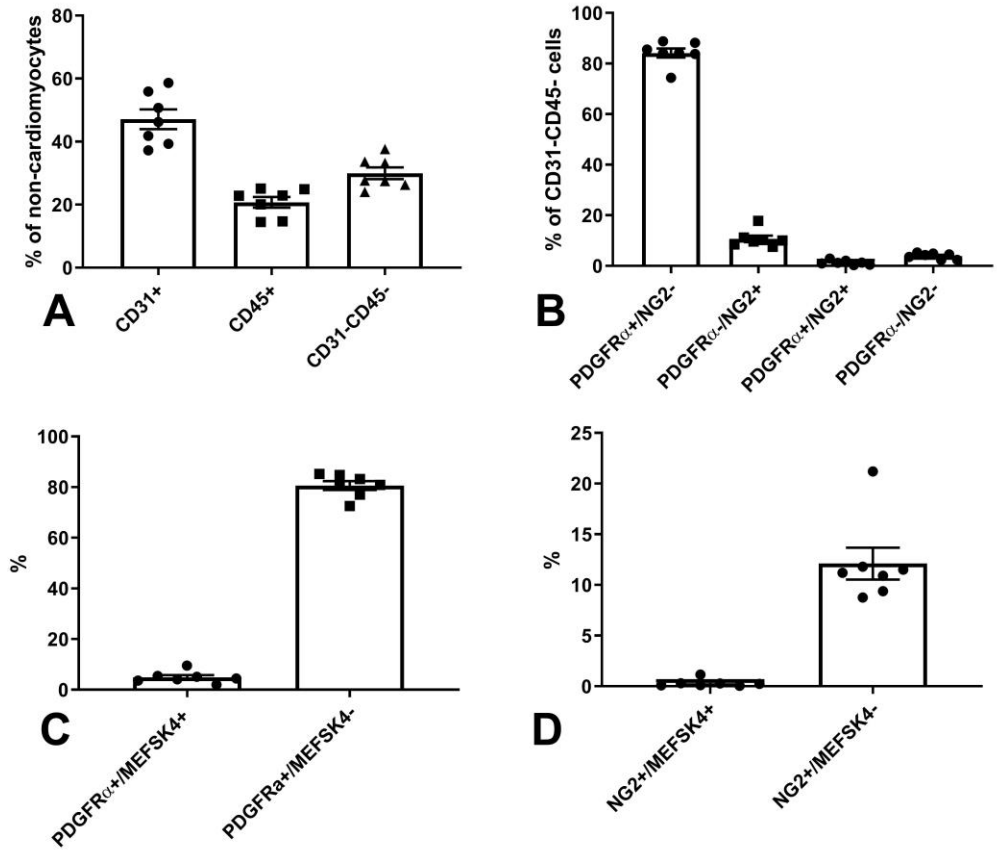


Figure 2: Flow cytometric characterization of interstitial cells in the mouse heart. A. CD31+ endothelial cells represent the most abundant population. Large populations of CD45+ hematopoietic cells, and of CD31-/CD45- interstitial cells (pericytes and fibroblasts are also identified. B. The majority of CD31-/CD45- interstitial cells are PDGFRα+/NG2- fibroblasts. A significant population of PDGFRα-/NG2+ pericytes is also identified. Double positive (NG2+/PDGFRα+) and double negative (NG2-/PDGFRα-) interstitial cells are very rare. C-D: The "fibroblast marker" MEFSK4 does not label the majority of PDGFRα+ fibroblasts (C) and NG2+ pericytes (D) in the mouse heart.

a2. PDGRFα+ fibroblasts express higher amounts of matrix genes in comparison to NG2+ pericytes.

In order to compare the transcriptomic profile of interstitial cell populations (fibroblasts and pericytes), we assessed gene expression in NG2+ pericytes and PDGFRα+ fibroblasts sorted from mouse hearts. Preliminary analysis showed that fibroblasts had much higher expression levels of structural collagens (Figure 3)

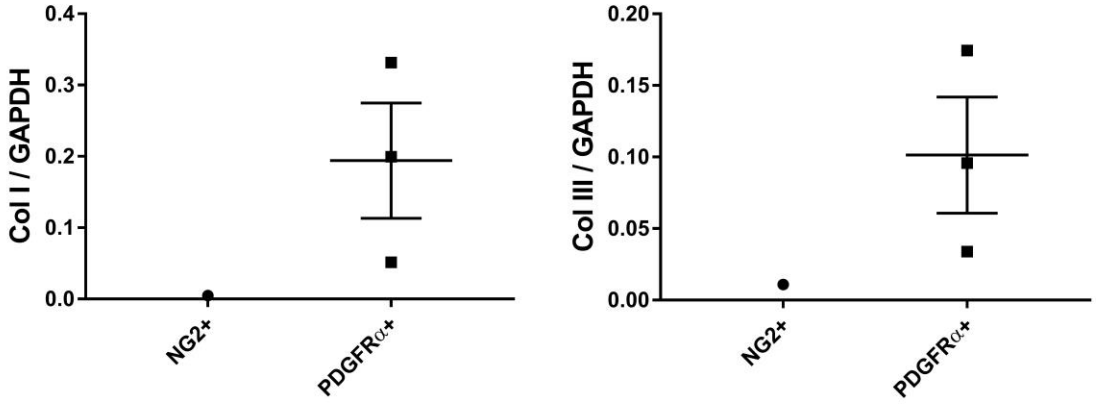


Figure 3: PDGFRα+ fibrobasts sorted from fibroblast/pericyte dual reporter mice exhibit much higher expression of collagen I and collagen III mRNA, in comparison to NG2+ pericytes.

a3. Diabetes is associated with microvascular rarefaction. Effects on pericyte density and localization.

We developed diabetic pericyte reporter mice to compare microvascular density and pericyte numbers between db/db;NG2^{dsred} mice and lean wildtype NG2^{dsred} littermates. Microvascular density was significantly lower in obese diabetic mice (Figure 4). Vascular rarefaction was associated with a trend towards reduced density of perivascular NG2+ cells (pericytes).

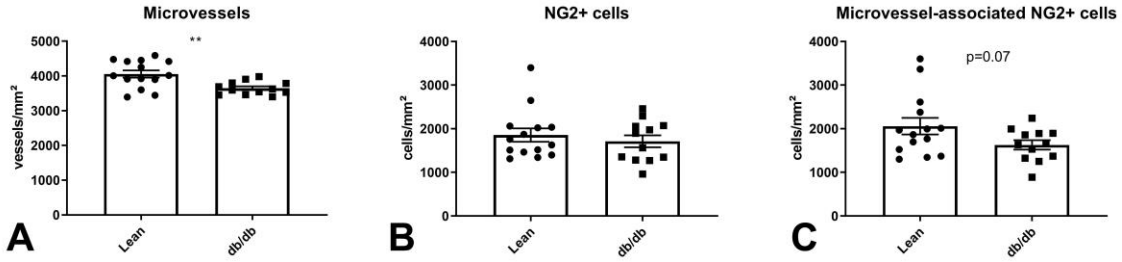


Figure 4: A. db/db mice have significantly lower microvascular density than lean wildtype littermates. Although the density of NG2+ cells was not significantly reduced (B), there was a trend towards reduced numbers of microvessel-associated NG2+ cells (C, pericytes). This finding may reflect loss of microvascular localization of NG2+ cells, due to acquisition of a fibroblast-like phenotype. This hypothesis will be tested using lineage tracing studies in inducible NG2-CreERT2 mice.

b) The role of endothelial-to-mesenchymal transition in diabetic cardiomyopathy

(Work performed at VUMC)

We are using Cre/Lox-based cell lineage tracing strategies to assess the contribution of endothelial cells to the generation of extracellular-matrix-producing cells in diabetic mouse hearts, thereby increasing interstitial fibrosis and leading to myocardial dysfunction. Specifically, we crossed the Tie1-Cre mice that express Cre recombinase in endothelial cells to the R26R*stopYFP* (Rosa-YFP) mice in order to generate double transgenic Tie1-Cre-YFP mice to uniquely label endothelial cells and their cellular deriatives. These mice were bred to each other and genotyped in order to generate Tie1-Cre-YFP mice that are homozygote for both the Cre recombinase and R26RYFP loci. Double homozygote Tie1-Cre-YFP mice were then crossed with db/+ heterozygotes to generate db/+ Tie1-Cre Rosa-YFP mice and non-diabetic Tie1-Cre Rosa-YFP siblings as controls. db/+ heterozygotes were then crossed to each other to generate db/db Tie1-Cre Rosa-YFP mice and non-diabetic Tie1-Cre Rosa-YFP siblings as controls.

Twelve hearts representing healthy control and diabetic genotypes of both sexes, displaying typical echocardiography parameters of healthy and disease phenotypes, were processed to obtain 60 sections/per heart for immunofluorescence and other histological analyses. During the last funding period, cardiac tissue sections have been analyzed by immunohistological techniques using ~15 antibodies to compare the effect of diabetes on cardiac inflammation, vascular density, vascular rarefaction, fibrosis, endothelial-to-mesenchymal (EndMT) transition and $Sca1^{+}/CD31^{-}$ pericytes with properties of cardiac progenitor cells. In the upcoming NCE period, we anticipate to complete the histological analyses and quantity differences between control and diabetic groups.

c) Molecular characterization of sex-specific DCM progression (Work performed collaboratively at VUMC and AECOM)

To determine molecular mechanisms linked to diabetic cardiomyopathy, we have isolated RNA samples from whole hearts and analyzed gene expression profiles using deep RNA sequencing. Because diabetic males and females display distinct histological and physiological abnormalities in the heart (Alex et al., 2018), we have isolated RNA samples from whole hearts of obese and diabetic *db/db* male and female mice at 3 distinct time points and established gene expression profiles by deep RNA sequencing.

Specifically, during the funding period, we have completed preparation of RNA samples from whole hearts of 2, 6 and 12 month-old male and female obese *db/db* mice and age-matched lean siblings as controls, with three mice per group for a total of 36 samples. The hearts were isolated at AECOM and shipped to VUMC were RNA samples were prepared and submitted to the VANTAGE genomics Core at VUMC for quality control. All samples had RNA Integrity Numbers above the required threshold for further processing and were sequenced using the Illumina NovaSeq6000 on paired-end-150 flow cell runs at 32 million pass-filter (PF) reads per sample. The RNA-seq data were then analyzed using Vanderbilt Technologies for Advanced Genomics Analysis and Research Design (VANGARD) resources. Aligning reads to mouse genome revealed excellent and uniform Mapping Quality, with low Zero Mapping Quality and high percentage of proper paired reads.

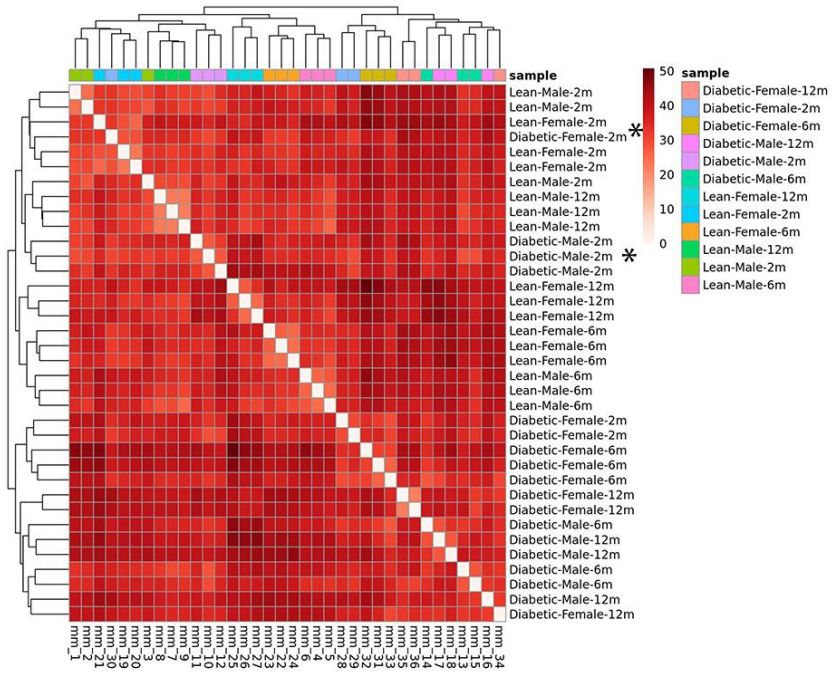


Figure 5. Clustering of all 36 RNA sequencing samples shows that most replicates have clustered together and that samples from obese diabetic mice cluster separately from lean controls. However, some diabetic samples at 2 months (marked by asterisk) are close to lean samples, indicative of fewer gene expression changes at early disease stages.

We subsequently performed clustering analysis using DESeq2. The results showed that replicate samples clustered together along disease status and time points and separately for male and female samples, providing a first indication that there are significant disease-stage- and sex- specific gene expression changes in diabetic hearts (Figure 5). Of note, month 2 samples aligned close to, or in some instances intercalated

with lean samples, likely reflecting a variability in the early onset of disease phenotypes as also seen in Echo data of young *db/db* mice.

Principal Component Analysis (PCA) of the entire gene expression dataset shows clear separation of lean from diabetic samples in Principal Component (PC) 1, suggesting that there is a core group of genes that represent a universal, i.e., time- and sex-independent molecular signature of DCM (Figure 6).

Digging deeper in the datasets, we found that there are distinct male- and female-specific molecular DCM phenotypes (Figure 6A). Furthermore, diabetes-regulated genes show clear time-dependent groups with distinct 2-, 6- and 12-month molecular phenotypes (Figure 6B). It also confirmed that a couple of the early diabetic samples clustered close to lean groups. We postulate that early gene expression changes at the onset of DCM at 2 months of age represent desease drivers that initiate pathological processes, whereas changes at later time points are characteristics of fully established DCM phenotypes (6 months) or are consequences of the ensuing HF (12 months).

We have used Partek's Gene Specific Analysis (GSA) multimodel estimation algorithm to calculate *p*-values, or the more strict false discovery rate (FDR) and fold changes to determine statistically significant ($p<0.05$ or $FDR<0.05$) gene expression changes using 1.5-fold up or down regulation as cutoff value to assemble lists of genes the expression of which is either increased or decreased at each time point either in males, females, or both. List sizes of statistically significant genes range from ~100 at 2 months to several hundred at 6 and months. To identify putative roles of common, or time-dependent and sex-dependent differentially expressed genes in diabetic hearts, we will employ Ingenuity Pathway Analysis to generate enriched pathways and processes for genes that reach statistical significance. We will also use Gene Ontology, KEGG pathways, protein-protein interrelation networks, gene sets pre-defined for related functions (e.g., chromatin remodeling), clusterprofiler to visualize functional profiles (GO and KEGG) of gene and gene clusters, MsigDB: The Molecular Signatures Database (MSigDB), and WGCNA to identify co-expression modules.

The proposed in depth RNA-seq data analysis will establish novel genes and pathways induced or suppressed in different stages of DCM specifically in males or females, or in both sexes. We expect that molecular changes that take place at all time points in both males and females will shed light on core mechanisms of the DCM pathophysiology and may provide useful biomarkers to detect the disease, as well as universal targets for future treatment strategies. On the other hand, early changes may provide clues about pathways driving disease initiation, whereas late changes might

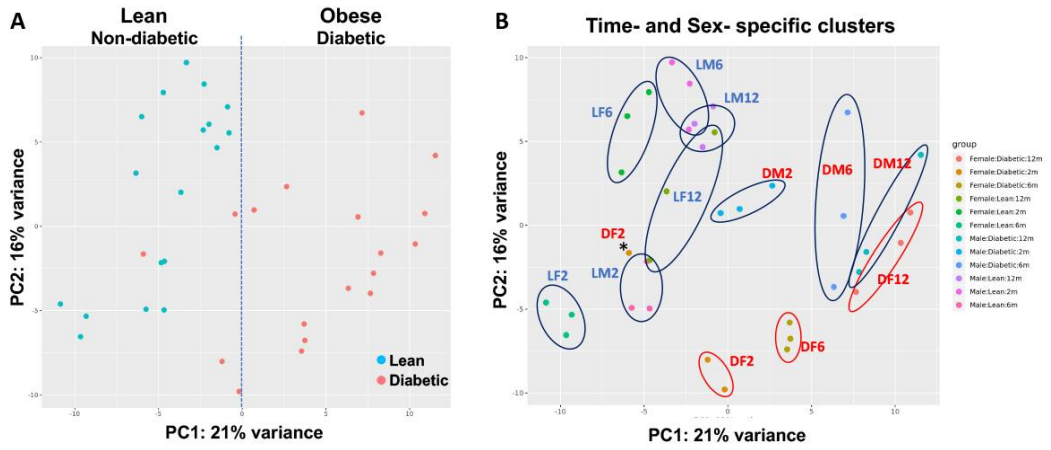


Figure 6. Principal Component Analysis (PCA). **A)** Samples from *db/db* hearts are labeled as obese/diabetic in red. Samples from lean, non-diabetic mice are labeled in blue. The results show clear separation of diabetic samples from lean controls along Principal Component 1 (PC1), which accounts for 21% of the variable gene expression among samples. **B)** Diabetic samples were color-labeled according to time point (2, 6, and 12 months) and sex (male M /female F). Diabetic samples are labeled D, and lean L. Asterisk marks the lone diabetic female month 2 (DF2) sample that clustered closer to lean samples. Samples belonging to the same time point and sex were circled for easier identification of time- and sex-specific clusters. The results show that both male and female diabetic samples become more diverse from lean samples as time progresses.

reveal mechanisms of end-stage HF in diabetes. Finally, we expect that sex-specific changes at distinct time points will provide mechanistic insights to explain the worse DCM outcomes in female patients. Taken together, these discoveries will lead to new testable hypotheses that we will explore in future approaches taking advantage of human disease databases and Electronic Health Records (EHR) such as BioVU, and mechanistic studies in animal models using available cell-specific knockout mice, CISPR/Cas9 technology to generate novel research tools, or suitable chemical agents.

Training opportunities and professional development

Cassandra Awgulewitsch is a fourth-year graduate student in the Cell and Developmental Biology Training Program who works on the project. Cassandra has received a fellowship from the NIH-sponsored T32 training grant "Program in Cardiovascular Mechanisms: Training in Investigation" (T32HL007411). Since joining our laboratory, she has published a first author review article on the role of endothelial cell plasticity in homeostasis and disease (Awgulewitsch et al., 2017), and co-authored a manuscript on the role of BMP signaling inhibition in cardiomyocyte differentiation (Bylund et al., Stem Cells & Dev., 2017). The primary training of Cassandra is laboratory work under the direct supervision of Dr. Hatzopoulos. Cassandra has performed the breeding of Tie1-Cre-YFP mice to db/+ heterozygotes to generate diabetic and non-diabetic Tie1-Cre Rosa-YFP mice for lineage tracing experiments. She has also spearheaded the RNAseq data analyses. Her training includes daily informal interactions, weekly data review in face-to-face meetings, and weekly laboratory meetings and journal clubs. This year she presented her work in a 30' presentation in the Research Exchange seminars at VU, and posters in the Cardiovascular Research Day, and Program in Developmental Biology Retreat at VU. She also attended the Basic Cardiovascular Sciences Scientific Sessions, July 29 - August 1, 2019 in Boston, MA.

David Wu is a fifth-year MD/PhD student in the Human Genetics Training Program at Vanderbilt University School of Medicine. David has completed two years of Medical School and started his PhD thesis work in our laboratory in September 2017. After completion of his thesis within the next two years, as part of the MD/PhD program, he will return for an additional two years to Vanderbilt School of Medicine to finish his medical degree. David is using the electronic medical records at VUMC that are also linked to genotyping data to search for novel associations between diabetic cardiomyopathy and genes of interest as emerge from the RNAseq studies. This year he presented his research in the Cardiovascular Research Day, and the Human Genetics Retreat at VUMC.

An essential component of the training program of both students involves participation in Journal Clubs organized weekly by the Cell & Developmental Biology Department and the Human Genetics Institute throughout the academic year and Work-

in-Progress Cardiovascular Research Seminars (CVRS). The CVRS meetings involve formal seminars by graduate students and post-doctoral fellows, giving trainees an excellent opportunity to organize their findings, develop and defend hypotheses, and refine their communication skills. There are also numerous departmental seminars each week in the VUMC campus from prominent visiting investigators, often closely related to Cassandra's scientific interests.

This year, Cassandra and David will have the opportunity to take part in local annual scientific meetings such as the Graduate Student Research Symposium (GSRS), the Program in Developmental Biology retreat and the Cardiovascular Research Day at VUMC. These meetings bring nationally recognized scientists for seminars exposing trainees to the latest in contemporary research and giving students an opportunity to present their own work to a broader audience as posters, or short oral presentations. They will also submit abstracts to large national meetings such as the American Heart Association Scientific Sessions, Keystone Symposia, etc. Finally, both students supervise the work of junior investigators in the laboratory (e.g., rotating graduate students, undergraduates and research assistants), which will help with development of leadership and mentoring skills.

How were the results disseminated to communities of interest?

Most of the findings presented in this report have been disseminated during presentations and discussions in internal work-in-progress meetings such as CVRS and teleconferences between the partnering institutions.

Plans for the next reporting period

During the next reporting period, we will complete the analysis of hearts sections obtained from male and female Tie1-Cre-ROSA-YFP. Efforts will focus on immunofluorescence and histological analysis, as well as flow cytometry characterization and RNAseq analysis of endothelial and $Sca1^{+}$ cardiac progenitor cells to assess the inflammatory phenotype of vascular endothelium, the extent of EndMT, loss of blood

vessels and the transformation of progenitor cells to pro-fibrotic cell types. Furthermore, the contribution of pericytes in fibrosis and the pathophysiology of diabetic cardiomyopathy will be assessed using the corresponding Cre lines.

Our RNAseq data show significant differences in gene activity in diabetic hearts between female and male mice. We will continue the validation of the primary RNAseq data using histological, molecular and cellular techniques. We are using bioinformatics tools to analyze in depth the assembled gene expression profiling data in order to identify genetic pathways activated or suppressed commonly or specifically in diabetic males as compared to diabetic females, thereby developing critical insights and novel hypotheses about sex-specific causes or effects of DCM. During the NCE period, we will complete gene expression analyses performed at 2, 6 and 12-month-old male and female mice in order to understand progression of the disease in both sexes, focusing on histological approaches to determine cell specificity and regional changes. These analyses will likely yield new insights in the development of diabetic cardiomyopathy and may identify new targets to treat heart disease in diabetics. Moreover, efforts will focus on preparation of the large data sets for publication and dissemination to the scientific community.

4. Impact

HFpEF is a major cause of morbidity and mortality worldwide. There is currently no effective treatment for patients with HFpEF. Although cardiac fibrosis has been implicated in the pathogenesis of HFpEF, the cellular basis for fibrotic remodeling of the ventricle is poorly understood. Metabolic diseases (such as obesity and diabetes) are associated with an increased incidence of HFpEF; however, the pathophysiological mechanisms responsible for this association remain unknown. Our experiments have established a model of HFpEF due to metabolic disease that can be used to dissect cellular mechanisms. This is of outstanding significance for pathophysiologic dissection in vivo. Our planned experiments will use lineage tracing strategies, in vivo and in vitro approaches to dissect the basis for activation of diabetic fibroblasts. The significance of the studies extends beyond the cardiovascular field, as diabetes-associated tissue fibrosis has an impact on other organs (such as the kidney and the liver).

5. Changes/Problems

No changes or problems to report

6. Products

Publications:

NG Frangogiannis. Cardiac fibrosis: cell biological mechanisms, molecular pathways and therapeutic opportunities. ***Mol Asp Med*** 2019; 65:70-99.

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D Humeres **and NG Frangogiannis**. Fibroblasts in the infarcted, remodeling and failing heart**.** ***JACC Basic Transl Res***. 2019; 4:449-467.

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A Hanna **and NG Frangogiannis**. The role of the TGF-b superfamily in myocardial infarction. ***Front Cardiovasc Med*** 2019 18 Sep, https://doi.org/10.3389/fcvm.2019.00140 (epub ahead of print).

NG Frangogiannis. Myocardial fibrosis in genetic cardiomyopathies: a cause of dysfunction or simply an epiphenomenon? ***Trends Cardiovasc Med*** 2019, in press

Wu D. H. and **A. K. Hatzopoulos**. Bone morphogenetic protein signaling in inflammation. *Exp. Biol. Med. (Maywood)*, 244: 147-156, 2019.

A. K. Hatzopoulos. Disease Models & Mechanisms in the age of Big Data. *Dis. Model Mech.*, 12: *in press*, 2019.

7. Participants & Other Collaborating Organizations (AECOM)

Participants

Name:	*Nikolaos G Frangogiannis*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Dr. Frangogiannis designed experiments, supervised the experimental work and analyzed data.*
Funding Support:	W81XWH-16-1-0563, W81XWH-16-1-062, R01 HL76246, R01 HL85440, 2P01HL078822-11, R01 HL143967.

Name:	*Linda Alex*
Project Role:	*Research fellow*
Researcher Identifier (e.g.	

ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Dr Alex is involved in generating the mouse models for identification and lineage tracing of pericytes,and performs the immunohistochemical and flow cytometry studies examining the characteristic and functional properties of pericytes.*
Funding Support:	

Name:	*Ya Su*
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Dr Ya Su is involved in breeding the diabetic animals, performs all functional studies (echocardiography, tissue Doppler imaging) and contributes to cell isolation and culture.*
Funding Support:	*R01 HL76246*

Active other support of the PI (Nikolaos Frangogiannis)

Changes in effort and grant closure since last report are marked in bold and italics.

ACTIVE

Chemokines in healing myocardial infarcts (R01 HL076246)
NIH/NHLBI
Investigator relationship: Principal Investigator
Dates of funding: 6/2005-2/2020. 0.0 calendar months
The project deals with cell-specific chemokine and cytokine actions in the infarcted myocardium. ***This project is currently under no-cost extension. A competing renewal application (FY2020) was ranked on the 12th percentile.***

Resolution of inflammation in healing myocardial infarcts (R01 HL085440)
NIH/NHLBI
Investigator relationship: Principal Investigator
Dates of funding: 12/2007-5/2021 2.7 calendar months
This project deals with signals that inhibit the innate immune responses in the infarcted heart.

Title of the project: The role of Transglutaminase-2 in ischemic and non-ischemic heart failure.
Funding agency: Department of Defense office of the Congressionally Directed Medical Research Programs (CDMRP).
Investigator relationship: Principal Investigator
Dates of funding: 9/1/2019-8/31/2022
Level of effort: 20%
Goals: This project studies the role of transglutaminase-2 in myocardial infarction and in heart failure. There is no conceptual or budgetary overlap with the current proposal.

NIH 2P01HL078822-11 9/1/2017-8/31/2021 0.6 Calendar Months
NHLBI
Protection of ischemic myocardium
Investigator relationship: Co-investigator
PI: Dr. Roberto Bolli, Project PI: Dr. Marcin Wysoczynski (University of Louisville)
The project examines the role of macrophages in cell therapy-mediated protection of the infarcted heart.

NIH 2P01HL078822-11 9/1/2017-8/31/2021 0.6 Calendar Months
NHLBI
Protection of ischemic myocardium
Investigator relationship: Co-investigator
PI: Dr Roberto Bolli, Project PI: Dr. Steve Jones (University of Louisville)
The project deals with the role of fibroblasts in cell therapy-mediated protection of the infarcted heart.

NIH R01 HL143967 4/1/2018- 2/28/2023 0.3 calendar months
NHLBI
Mechanisms of myocardial infarction-induced insulin resistance
Investigator relationship: Co-investigator
PI: Dr Partha Dutta (University of Pittsburgh).
The proposal investigates inflammatory mechanisms that mediate metabolic dysregulation following MI.

COMPLETED

Fibroblast-Cardiomyocyte Interactions in the Pressure Overloaded Myocardium (PR151134)
Department of Defense office of the Congressionally Directed Medical Research Programs (CDMRP).
Investigator relationship: Principal Investigator (Partnering PI award; co-PI: Dr Richard Kitsis, Albert Einstein College of Medicine)
Dates of funding: 9/15/2016-9/14/2019. 2.4 calendar months
The project studies interactions between fibroblasts and cardiomyocytes in the pressure-overloaded heart.

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None

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Collaboration as described above

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Editorial commentary: Myocardial fibrosis in genetic cardiomyopathies: A cause of dysfunction, or simply an epiphenomenon? ☆

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Most cardiac pathologic conditions are associated with myocardial fibrosis, expansion of the cardiac interstitium due to the deposition of extracellular matrix (ECM) proteins by activated fibroblast-like cells [1]. Because the adult mammalian heart has negligible regenerative capacity, myocardial fibrosis often reflects activation of a reparative process in response to cardiomyocyte injury. Although in most myocardial diseases, extensive fibrosis is a poor prognostic indicator, it is unclear whether this association relates to primary detrimental effects of fibrotic remodeling or simply represents an epiphenomenon, reflecting the severity of the underlying cardiomyocyte injury. It is also unknown to what extent primary fibrogenic activation of myocardial interstitial cells may occur independently of cardiomyocyte injury, acting as a direct cause of fibrosis, dysfunction and/or arrythmogenesis in human cardiomyopathy patients. These questions are particularly pertinent in understanding the significance and role of fibrosis in genetic cardiomyopathies in which significant fibrotic changes often co-exist with extensive cardiomyocyte pathology [2].

In the current issue of the journal, Eijgenraam et al. [3] provide an excellent overview of the epidemiology, morphologic characteristics, localization, and temporal course of fibrosis in genetic cardiomyopathies. The authors discuss the distribution patterns and prognostic implications of fibrotic changes in patients with hypertrophic, dilative, arrythmogenic and non-compaction cardiomyopathies. However, despite the abundance of clinical evidence, several important questions remain unanswered regarding the role of fibrosis in genetic cardiomyopathies. In patients with sarcomere gene mutations is fibrosis a significant cause of dysfunction, or does it simply reflect activation of a reparative program? Does fibrosis represent a maladaptive process that contributes to disease progression? Although in most genetic cardiomyopathies, fibrogenic activation may be triggered by paracrine signals derived from cardiomyocytes carrying mutant proteins, are there any genetic forms of "primary fibrotic cardiomyopathy" directly caused by fibroblast activation?

The role of fibrosis in hypertrophic cardiomyopathy (HCM)

Several lines of evidence support the notion that fibrosis is much more than an innocent companion in HCM, suggesting that activation of a fibrogenic program may play a key role in progression of cardiomyocyte pathology and in dysfunction. First, sarcomere-mutation carriers have high levels of circulating biomarkers of collagen synthesis, even in the absence of overt disease [4], suggesting that fibrogenic activation may precede the clinical manifestations. Second, in a mouse model carrying a mutation associated with human HCM, neutralization of the key fibrogenic mediator Transforming Growth Factor (TGF)-β attenuated fibrosis, decreased proliferation of non-cardiomyocytes, and reduced cardiac hypertrophy [5]. Third, in transgenic mice with cardiomyocyte-specific overexpression of a truncated cardiac myosin binding protein C (a mouse model that recapitulates changes observed in a relatively common human HCM mutation), myofibroblast-specific abrogation of TGF-β signaling, not only attenuated fibrosis, but also reduced cardiomyocyte hypertrophy, preserved function and prolonged lifespan [6]. Whether specific sarcomeric gene mutations or modifier genes are associated

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with a more prominent contribution of fibrotic remodeling in progression of HCM remains unknown.

Fibrosis in genetic dilated cardiomyopathy (DCM) and in arrhythmogenic right ventricular cardiomyopathy (ARVC)

Although some genetic forms of DCM are associated with severe and diffuse fibrosis [7], robust documentation of a primary role for fibrogenic activation in disease progression is lacking. In genetic mouse models of DCM, early activation of a fibrogenic program has been suggested to contribute to disease progression [8,9]. In a model of DCM caused by lamin A/C mutations, the beneficial effects of mitogen-activated protein kinase inhibitors on cardiac function were attributed, at least in part, to attenuation of cardiac fibrosis [10]. However, whether fibroblasts critically contribute to disease progression in these models has not been tested.

In ARVC patients, development of arrhythmias is typically associated with expansion and activation of fibroadipocytes, leading to deposition of ECM proteins and fatty infiltration [11]. Mutations in genes encoding desmosome proteins (such as plakophilin-2, desmoplakin and desmoglein-2) are found in many ARVC patients [12] and may trigger conversion of interstitial cells into adipocytes or activate a fibrogenic program in cardiomyocytes [13,14].

Primary cardiac fibrosis: a disease of interstitial cells?

Considering that in the majority of patients with genetic cardiomyopathies the causative mutation is unknown, it is possible that certain mutations may cause heart failure through primary activation of a fibrogenic program in cardiac interstitial cells. Myocardial fibrosis in the absence of an identifiable cause or known cardiomyocyte pathology is often found in autopsies of young patients with sudden cardiac death [15]. However, cardiomyocyte-specific gene mutations are identified in many of these patients [16], suggesting that fibrosis may not result from primary activation of interstitial fibroblasts, but may involve paracrine cardiomyocyte-derived signals [17].

Conclusions

Although fibrotic changes are commonly found in patients with genetic cardiomyopathies and have adverse prognostic implications, their causative role in disease progression, dysfunction and arrythmogenesis remains poorly documented. Although intuitive thinking suggests that deposition of ECM proteins in the interstitium may perturb cardiomyocyte function, cause conduction defects and generate arrhythmias, emerging evidence suggests protective effects of stress-activated cardiac interstitial cells that may "shield" cardiomyocytes from injury [18,19]. Future investigations will need to focus on the role of interstitial cells in the pathogenesis of cardiomyopathies caused by sarcomere gene mutations, and on identification of genetic perturbations in interstitial cells that may directly cause fibrotic remodeling and heart failure.

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Circulation

EDITORIAL

Protean Functions and Phenotypic Plasticity of Regulatory T Cells in Chronic Ischemic Heart Failure

Article, see p 206

Nikolaos G. Frangogiannis, MD

Despite abundant evidence suggesting the critical involvement of inflammation in the pathogenesis of cardiac remodeling,[1,2] targeting inflammatory cells in patients who have heart failure poses major challenges. Most inflammatory cell types exert a wide range of actions that may have context-dependent protective or detrimental functional consequences in the failing heart. Moreover, immune cells do not function as stable unidimensional effectors of a specific response, but exhibit remarkable plasticity and may undergo dramatic changes in the highly dynamic microenvironment of the failing heart.

A growing body of evidence suggests that regulatory T cells (Tregs), a subset of T lymphocytes with immunosuppressive properties, may participate in repair and remodeling of the infarcted myocardium. During the inflammatory phase of cardiac repair, Tregs infiltrate the infarcted myocardium, recruited through the activation of chemokine-dependent signals.[3] Infiltrating Tregs play an important role in repair of the infarcted heart and protect from cardiac rupture and adverse remodeling by driving activation of reparative macrophages[4] and by modulating fibroblast phenotypes.[5] The protective reparative functions of Tregs in the infarcted heart may involve systemic anti-inflammatory actions, local secretion of anti-inflammatory cytokines and growth factors, or contact-dependent effects. Although Tregs are key orchestrators of the reparative response following acute myocardial injury, their potential role in chronic ischemic heart failure has not been studied. Under the assumption that infarct Tregs maintain a stable anti-inflammatory phenotype, one would have expected that they may exert prolonged protective effects on the remodeling myocardium by attenuating the activation of macrophages and effector T cells. On that basis, it has been speculated that cell therapy with Tregs may be a promising approach for patients surviving acute myocardial infarction.

In the current issue of *Circulation*, Bansal and coworkers[6] report that, in contrast to their reparative role in myocardial infarction, Tregs in mice with chronic ischemic heart failure exhibit functional perturbations and acquire a proinflammatory phenotype, contributing to the progression of adverse remodeling. In a mouse model of nonreperfused myocardial infarction, chronic heart failure was associated with marked and prolonged expansion of Tregs in the heart, spleen, and circulating blood. During the chronic phase of cardiac remodeling, Tregs in failing hearts exhibited proinflammatory properties and impaired immunoregulatory capacity. Depletion of Tregs in mice with established postinfarction heart failure, using either a genetic approach based on a diphtheria toxin system, or treatment with an antibody to CD25, protected the heart from progressive dilation and even reversed adverse remodeling, decreasing systemic inflammation, reducing interstitial myocardial fibrosis, and attenuating capillary rarefaction.

The opinions expressed in this article are not necessarily those of the editors or of the American Heart Association.

Key Words: Editorials ■ heart failure ■ inflammation ■ lymphocytes ■ myocardial infarction ■ ventricular remodeling

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The study challenges conventional views on the phenotypic stability of Tregs following tissue injury, demonstrating that, following myocardial infarction, Tregs undergo a phenotypic transition from a reparative phenotype to a dysfunctional proinflammatory cell type that contributes to the pathogenesis of heart failure. The findings also raise several intriguing questions regarding the emerging role of Tregs in myocardial injury, repair, and remodeling.

WHICH MOLECULAR SIGNALS MEDIATE EXPANSION OF DYSFUNCTIONAL TREGS IN CHRONIC HEART FAILURE?

Traditional teachings suggest that Tregs function predominantly as immunosuppressive and anti-inflammatory cells. In contrast to these established concepts, a growing body of experimental evidence in both experimental models and in human patients suggests that, under inflammatory conditions, Tregs can be reprogrammed into T-helper cells that secrete proinflammatory cytokines.[7] In many pathological conditions, the functional in vivo role of these dysregulated, proinflammatory Tregs remains debated. The current investigation provides robust evidence, through both genetic and pharmacological approaches, supporting the notion that perturbed Treg function may be an essential contributor to chronic myocardial injury.

The molecular basis for the heart failure–associated perturbations in Treg phenotype and function is unknown (Figure). It should be emphasized that similar proinflammatory alterations have been reported in Tregs associated with a variety of inflammatory conditions, including psoriasis, inflammatory bowel disease, arthritis, and multiple sclerosis.[7] Thus, this common process of dysregulation may not be unique to heart failure, but may involve activation of inflammatory signaling cascades. Induction and prolonged secretion of proinflammatory cytokines are hallmarks of chronic heart failure; chronic cytokine stimulation may promote differentiation of Tregs into T helper 17 (Th17)-like cells with proinflammatory properties.[8] Although conversion of Tregs into other T-cell subsets has not been documented in the failing heart, several mechanisms may contribute to the acquisition of a proinflammatory phenotype in heart failure–associated Tregs. Activation of Toll-like receptor 2 signaling is also prominent in failing hearts and has been suggested to stimulate interleukin-17A expression in Tregs.[9] Activation of the renin-angiotensin-aldosterone system or adrenergic stimulation promotes proinflammatory actions in both macrophages and lymphocytes.[10,11] Whether neurohumoral stimulation of Tregs may be involved in acquisition of a dysfunctional proinflammatory phenotype remains unknown. Moreover, in the remodeling failing heart, Tregs are exposed to increased mechanical stress and are enmeshed within an interstitium enriched with a wide range of specialized extracellular matrix proteins[12] capable of modulating immune cell phenotype and function. Whether activation of mechanosensitive pathways or stimulation of matricellular actions may contribute to the perturbed phenotype of Tregs in failing hearts is unknown. Regardless of the initial activating stimulus, acquisition of a proinflammatory phenotype by heart failure Tregs may also involve perturbations of the nuclear factor-κB signaling cascade, an essential pathway for the maintenance of Treg function. It has been suggested that activation of noncanonical nuclear factor-κB signaling via accumulation of nuclear factor-κB–inducing kinase may promote Treg instability, inducing proinflammatory gene synthesis.[13]

WHICH TREG-MEDIATED SIGNALS EXACERBATE CHRONIC CARDIAC REMODELING?

Several cellular mechanisms may explain the adverse consequences of Treg perturbations on myocardial function. First, loss of immunosuppressive functions of Tregs may result in unrestrained activation of immune cells, leading to progressive cardiomyocyte death. Second, unopposed systemic and myocardial activation of effector T cells may accentuate proinflammatory signaling, promoting cytokine-driven myocardial dysfunction and activating fibrogenic cascades. Third, dysfunctional Tregs may promote a proinflammatory phenotype in macrophages, thus amplifying the consequences of perturbed Treg function on the inflammatory milieu. Fourth, as suggested by the current study, heart failure Tregs may exert contact-dependent angiostatic effects, leading to capillary rarefaction in the noninfarcted remodeling myocardium and accentuating ventricular dysfunction. Fifth, Tregs in failing hearts may acquire a matrix-degrading phenotype, leading to release of matrix fragments and disruption of prosurvival effects of the intact matrix on cardiomyocytes. Considering the relatively low myocardial density of Tregs in failing mouse hearts, a combination of direct actions of Tregs infiltrating the myocardium and effects on systemic inflammatory responses may account for the striking consequences of Treg-mediated actions in heart failure.

DOES PERTURBED TREG FUNCTION PLAY A ROLE IN THE PATHOGENESIS OF HUMAN HEART FAILURE?

Despite growing experimental evidence on the role of immune cells in the pathogenesis of heart failure in animal models, robust evidence on the phenotypic

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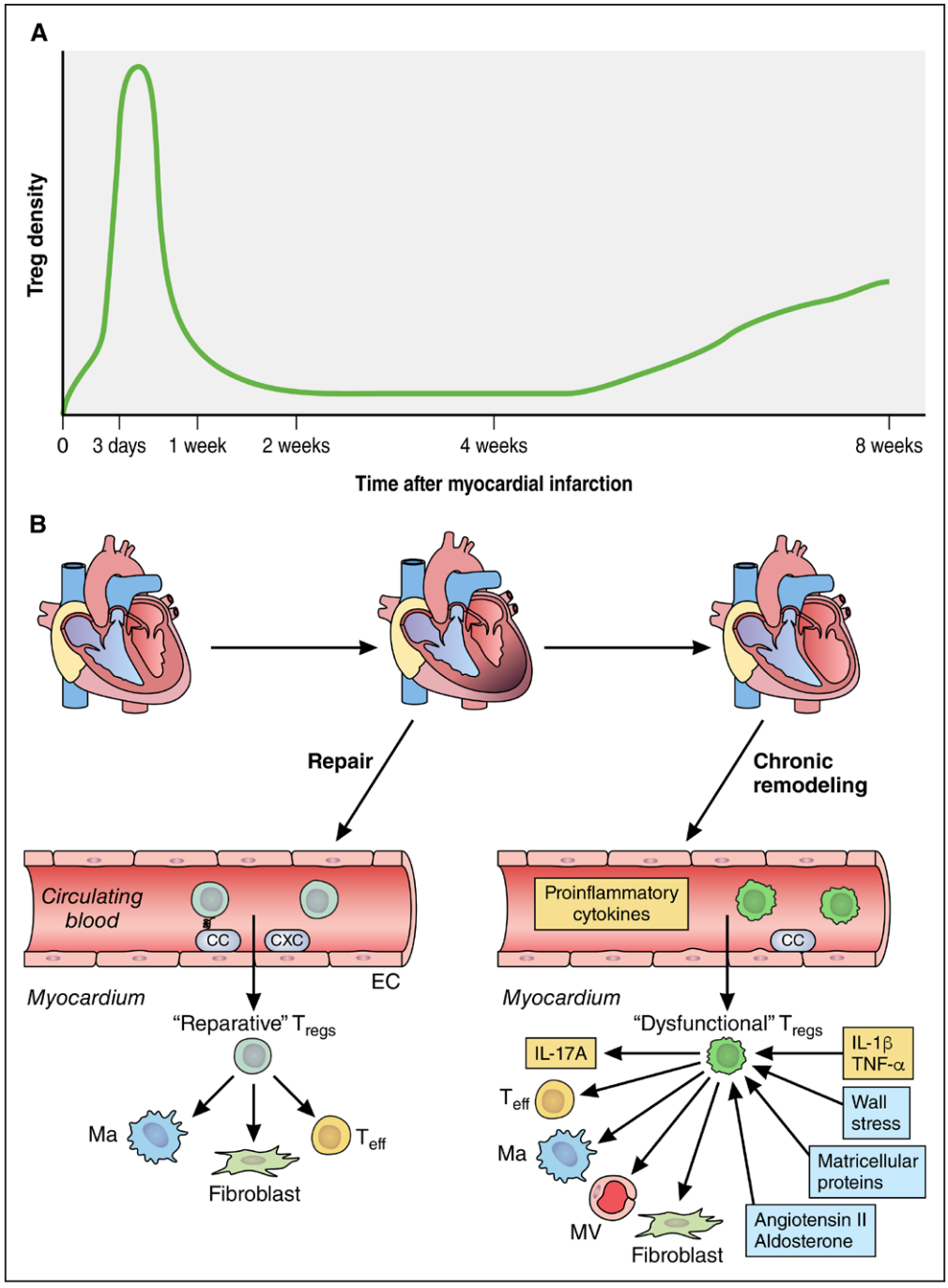


Figure. Dynamic alterations of regulatory T-cell (Treg) phenotype may play an important role in repair and remodeling of the infarcted heart. **A**, Tregs are recruited in the infarcted myocardium, exhibiting 2 waves of infiltration. The marked expansion of cardiac Tregs during the inflammatory and proliferative phase of infarct healing (3–7 days after coronary occlusion) is followed by a late, less striking increase in Treg density during the phase of chronic remodeling (8 weeks after infarction). **B**, Early recruitment of Tregs in the infarct is mediated through chemokine-dependent pathways.[3] Reparative Tregs play an important role in suppression of postinfarction inflammation and in repair of the infarcted heart by dowmodulating proinflammatory activity in macrophages (Ma) and effector T cells (Teff) and by activating fibroblasts.[4,5] During the chronic phase of cardiac remodeling, Tregs have functional perturbations, exhibiting defective immunosuppressive and anti-inflammatory activity, producing proinflammatory cytokines, and exerting fibrogenic and angiostatic actions. The molecular signals promoting expansion of dysfunctional Tregs are poorly understood, but may involve chronic stimulation with proinflammatory cytokines, neurohumoral activation, and stimulation of Toll-like receptor signaling. The study by Bansal and coworkers[6] demonstrates that dysfunctional Tregs play an important role in progression of adverse remodeling in chronic ischemic cardiomyopathy. CC indicates CC chemokine; CXC, CXC chemokine; EC, endothelial cell; MV, microvessel; IL, interleukin; and TNF, tumor necrosis factor.

changes in inflammatory cell populations in human patients with heart failure is scarce. Defective immunosuppressive function of circulating Tregs has been reported in a population of patients with heart failure; similar patterns of dysfunction were noted in subgroups with ischemic or nonischemic etiology.[14] Whether Treg dysfunction in human patients with heart failure is associated with inflammatory conversion remains unknown.

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Deep phenotyping and characterization of Treg profiles in human patients[15] are needed to explore the potential significance of Treg dysfunction in the pathophysiologically heterogeneous human heart failure populations. Such investigations may identify subsets of patients with heart failure that could be considered for experimental cell therapy by using healthy Tregs.

ARTICLE INFORMATION

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Disclosures

None.

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Macrophage Smad3 Protects the Infarcted Heart, Stimulating Phagocytosis and Regulating Inflammation

Bijun Chen, Shuaibo Huang, Ya Su, Yi-Jin Wu, Anis Hanna, Adipong Brickshawana, Jonathan Graff, Nikolaos G. Frangogiannis

Rationale: **TGF (transforming growth factor)-β is critically involved in myocardial injury, repair, and fibrosis, activating both Smad (small mothers against decapentaplegic)-dependent and non-Smad pathways. The in vivo role of TGF-β signaling in regulation of macrophage function is poorly understood. We hypothesized that in the infarcted myocardium, activation of TGF-β/Smad signaling in macrophages may regulate repair and remodeling.**

Objective: **To investigate the role of macrophage-specific TGF-β Smad3 signaling in a mouse model of myocardial infarction and to dissect the mechanisms mediating Smad-dependent modulation of macrophage function.**

Methods and Results: **TGF-βs markedly activated Smad3 in macrophages, without affecting Smad-independent pathways. Phagocytosis rapidly and directly activated macrophage Smad3, in the absence of active TGF-β release. MyS3KO (myeloid cell–specific Smad3 knockout) mice had no baseline defects but exhibited increased late mortality and accentuated dilative postmyocardial infarction remodeling. Adverse outcome in infarcted MyS3KO mice was associated with perturbations in phagocytic activity, defective transition of macrophages to an anti-inflammatory phenotype, scar expansion, and accentuated apoptosis of border zone cardiomyocytes. In vitro, Smad3 null macrophages exhibited reduced expression of genes associated with eat-me signals, such as Mfge8 (milk fat globule-epidermal growth factor factor 8), and reduced capacity to produce the anti-inflammatory mediators IL (interleukin)-10 and TGF-β1, and the angiogenic growth factor VEGF (vascular endothelial growth factor). Mfge8 partly rescued the phagocytic defect of Smad3 null macrophages, without affecting inflammatory activity. Impaired anti-inflammatory actions of Smad3 null macrophages were associated with marked attenuation of phagocytosis-induced PPAR (peroxisome proliferator-activated receptor) expression. MyS3KO mice had no significant alterations in microvascular density and interstitial fibrosis in remodeling myocardial segments.**

Conclusions: **We demonstrate that Smad3 critically regulates function of infarct macrophages, by mediating acquisition of a phagocytic phenotype and by contributing to anti-inflammatory transition. Smad3-dependent actions in macrophages protect the infarcted heart from adverse remodeling. (*Circ Res*. 2019;125:55-70. DOI: 10.1161/CIRCRESAHA.119.315069.)**

Key Words: cytokine ■ inflammation ■ macrophage ■ phagocytosis ■ transforming growth factor

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TGF (transforming growth factor)-β plays an essential role in regulation of inflammation, wound healing, and tissue remodeling by modulating phenotype and function of all cell types that participate in injury and repair. Extensive evidence suggests that various subpopulations of immune cells are major cellular targets of TGF-β and that TGF-β-mediated modulation of inflammatory cell phenotype may account for a large part of the in vivo actions of the cytokine.[1,2] In sites of injury, preformed TGF-β released from matrix-bound stores, or newly synthesized TGF-β secreted by resident cells or infiltrating leukocytes, is rapidly activated and binds to TGF-β receptors triggering downstream signaling cascades that involve a family of intracellular effectors, the Smads (small mothers against decapentaplegic),[3] or Smad-independent pathways.[4] The relative significance of Smad-dependent and non-Smad responses in mediating a TGF-β–driven biological response is dependent on the cell types involved and on a broad range of contextual microenvironmental factors.

Editorial, see p 71
In This Issue, see p 2
Meet the First Author, see p 3

Although it is widely appreciated that monocytes and macrophages are highly responsive to TGF-β stimulation and activate the Smad3 pathway in sites of injury,[5] the in vivo role of macrophage-specific TGF-β signaling in tissue injury and repair remains poorly understood. The conflicting findings of in vitro studies highlight the context-dependent actions of TGF-β. In the presence of proinflammatory cytokines or TLR (Toll-like receptor) ligands, TGF-β inhibits macrophage-derived inflammatory gene synthesis, at least in part, through pathways that involve Smad3 activation.[6–9] In contrast to these anti-inflammatory

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Novelty and Significance

What Is Known?

- Infarct macrophages are heterogeneous and exhibit dynamic phenotypic changes in response to microenvironmental stimuli.
- In the healing infarct, macrophage subsets phagocytose dead cells and matrix debris and regulate inflammatory, fibrogenic, and angiogenic responses.
- TGF (transforming growth factor)-βs are upregulated in the infarcted myocardium, and TGF-β signaling cascades may be activated in infarct macrophages.

What New Information Does This Article Contribute?

- Phagocytosis rapidly activates Smad3 (small mothers against decapentaplegic 3) signaling in macrophages in the absence of TGF-β activation.
- Macrophage Smad3 does not play a role in tissue homeostasis but protects the infarcted heart from adverse remodeling and dysfunction.
- Smad3 is implicated in macrophage phagocytosis, in part through induction of Mfge8 (milk fat globule-epidermal growth factor factor 8).
- Smad3 is implicated in anti-inflammatory transition of infarct macrophages, mediating phagocytosis-associated induction of PPARs (peroxisome proliferator-activated receptors).

In vitro, TGF-βs modulate macrophage phenotype by transducing Smad-dependent and Smad-independent cascades. The in vivo role of macrophage-specific TGF-β signaling in tissue injury and repair remains poorly understood. Macrophages infiltrate the infarcted myocardium and phagocytose dead cells and matrix debris while regulating inflammatory, fibrogenic and angiogenic responses. We report that TGF-βs rapidly stimulate Smad3 in macrophages without exerting significant effects on Smad-independent cascades. Moreover, phagocytosis rapidly activated the Smad3 pathway in macrophages in the absence of TGF-β activation. Using myeloid cell–specific Smad3 knockout mice and a wide range of in vitro models, we demonstrate protective effects of macrophage Smad3 in the infarcted heart. Macrophage-specific Smad3 acts by promoting phagocytic activation and by mediating anti-inflammatory transition of infarct macrophages. The effects of Smad3 on macrophage phagocytosis are mediated, in part, through induction of Mfge8. The anti-inflammatory actions of Smad3 may involve phagocytosis-related induction of peroxisome proliferator-activated receptors. In vitro, Smad3 is critically involved in expression of TGF-β and VEGF (vascular endothelial growth factor) by macrophages; however, in vivo, myeloid cell–specific Smad3 loss did not significantly affect myofibroblast conversion and angiogenesis. Smad3 critically regulates macrophage function, transducing signals that promote phagocytosis of dead cells and mediating anti-inflammatory transition. These actions protect from adverse remodeling.

Nonstandard Abbreviations and Acronyms

BMM	bone marrow–derived macrophage
BMP	bone morphogenetic protein
CCL	chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CXCL	chemokine (C-X-C motif) ligand
CXCR	CXC chemokine receptor
IFN	interferon
IL	interleukin
MCP	monocyte chemoattractant protein
Mertk	C-mer proto-oncogene tyrosine kinase
Mfge8	milk fat globule-epidermal growth factor factor 8
MI	myocardial infarction
MyS3KO	myeloid cell–specific Smad3 knockout
PPAR	Peroxisome proliferator-activated receptor
RBCs	red blood cells
Smad	small mothers against decapentaplegic
TGF	transforming growth factor
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
WT	wild type

actions, in the absence of proinflammatory mediators, TGF-β has been reported to promote synthesis of inflammatory cytokines and growth factors in both macrophages and in monocytes,[10] through actions that may also involve Smad3.[11] The in vivo significance of these contrasting effects remains unknown.

The heart contains a population of resident macrophages[12] that rapidly expands after injury due to the rapid recruitment of circulating monocytes, mobilized from the bone marrow or the spleen.[13–16] Release of damage-associated molecular patterns by dying cardiomyocytes induces chemokines and cytokines in resident myocardial cells and plays a critical role in recruitment of monocytes in the infarcted heart.[17–19] In the dynamic environment of the infarct, tightly regulated expression of cytokines and growth factors, and induction of matricellular proteins may modulate macrophage phenotype, resulting in sequential generation of macrophage subsets with distinct functional properties. Cell depletion experiments have suggested that infarct macrophages do not simply act as phagocytic cells but may also contribute to regulation of inflammation, angiogenesis, and fibroblast activation in the infarcted heart.[20–22] Although the TGF-β system is rapidly activated in the infarcted heart,[23] and TGF-β-mediated signaling has been reported to play an important role in regulation of fibroblast and cardiomyocyte phenotype[24–27]; the effects of TGF-β-triggered pathways on macrophage function are unknown.

In the current study, we hypothesized that TGF-β-mediated Smad3 activation in macrophages may play a critical role in cardiac repair. We report that infarct macrophages exhibit Smad3 activation in vivo and that phagocytosis directly activates Smad3 in macrophages, in the absence of TGF-β activity. Using MyS3KO (myeloid cell–specific Smad3 knockout) mice, we found that macrophage Smad3 signaling protects the infarcted heart from adverse remodeling and reduces mortality, promoting a phagocytic program and mediating anti-inflammatory transition. Smad3-dependent phagocytosis is mediated, at least in part, through induction of Mfge8 (milk fat globule-epidermal growth factor factor 8), whereas Smad3-mediated PPAR (peroxisome proliferator-activated

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receptor) upregulation may be involved in acquisition of anti-inflammatory properties.

Methods

All supporting data are available within the article and the Online Data Supplement.

MyS3KO mice were generated using the LyzM (lysozyme M) Cre driver. MyS3KO mice and Smad3 fl/fl controls underwent permanent left coronary artery occlusion protocols. In vitro, we studied the role of Smad3 in isolated bone marrow–derived macrophages (BMMs) and in macrophages or neutrophils harvested from the infarcted heart.

Detailed methodology is provided in the Online Data Supplement.

Results

TGF-βs Predominantly Activate Smad2/3 in BMMs

All 3 TGF-β isoforms were markedly upregulated in the infarcted myocardium. TGF-β1 and TGF-β2 showed early upregulation after 24 hours, whereas TGF-β3 peaked late, after 7 days of coronary occlusion (Figure 1A through 1C). In BMMs, TGF-β1, TGFβ2, or TGF-β3 rapidly stimulated Smad3 phosphorylation, without affecting total Smad3 levels (Figure 1D through 1E). BMP (bone morphogenetic protein)-2 and BMP-4 did not activate Smad3. TGF-βs also induced Smad2 phosphorylation (Figure 1F). TGF-βs, BMP-2, and BMP-4 did not affect p-JNK (c-Jun N-terminal kinase), p-Akt (protein kinase B) and p-Erk (extracellular signal-regulated kinase) levels (Figure 1G through 1I). TGF-β1, but not other TGF-βs or BMPs, triggered a modest but significant increase in p-p38 MAPK (mitogen-activated protein kinase) levels (Figure 1J). There were no additive or synergistic effects of the TGF-β isoforms on Smad3 activation (Figure 1K through 1L).

Smad3 Is Activated in a Subset of Infarct Macrophages

Using CSF1R (colony stimulating factor 1 receptor)-EGFP (enhanced green fluorescent protein) myeloid cell–reporter mice, we demonstrated a rapid increase in myeloid cell density in the infarcted myocardium peaking at 7 days postinfarction; in contrast, the number of myeloid cells in the noninfarcted myocardium did not significantly increase. Phospho-Smad3 (p-Smad3)+ myeloid cells were first identified in the infarcted myocardium 24 hours after coronary occlusion and peaked after 7 days (Online Figure I). Although in vitro, TGF-βs increased p-Smad3 expression in mouse neutrophils, dual immunofluorescence showed that no detectable p-Smad3 in infarct neutrophils, suggesting that most myeloid cells exhibiting Smad3 activation are macrophages (Online Figure II).

Phagocytosis Activates Macrophage Smad3 in a TGF-β–Independent Manner

Infarct macrophages are critically involved in phagocytosis.[21] To explore the stimulus responsible for Smad3 activation in infarct macrophages, we examined whether phagocytosis activates Smad3 in macrophages. When incubated with BMMs, aged red blood cells (RBCs) are rapidly phagocytosed, as they expose phosphatidylserines. After 30 minutes, wild-type (WT) macrophages phagocytosing RBCs exhibited markedly increased Smad3 phosphorylation (Figure 2A). Macrophages harvested from mice with global Smad3 loss served as a negative control and had no detectable Smad3 activation. Smad3 activation in WT macrophages was attenuated after 24 hours of incubation with RBCs; however, p-Smad3 levels remained higher than in control cells. To examine whether macrophage Smad3 activation is due to TGF-β release, we assessed levels active TGF-βs in the supernatant. Expression of active TGF-β2 and TGF-β3 was below detection levels. No detectable active TGF-β1 was found after 30 minutes of incubation with RBCs (Figure 2B). After 24hours of incubation, low levels of active TGF-β1 were found in both control and RBC-treated cells. Macrophages exhibiting RBC ingestion, but not macrophages without evidence of phagocytosis, had increased p-Smad3 expression (Figure 2C through 2E). To examine whether phagocytosis activates Smad3 in vivo, we performed dual staining for p-Smad3 and Mfge8, a marker of phagocytic macrophages. After 7 days of coronary occlusion, a subset of Mfge8+ cells in the infarct expressed p-Smad3 (Figure 2F). The findings are consistent with a phagocytosis-associated TGF-β-independent mechanism of Smad3 activation in macrophages.

MyS3KO Mice Exhibit No Baseline Defects and Have Normal Circulating Blood Cell Counts

Dual immunofluorescence for Smad3 and LyzM was used to document myeloid cell–specific Smad3 loss in MyS3KO mice. MyS3KO mice exhibited negligible Smad3 immunoreactivity in lung and spleen macrophages. In the infarcted myocardium, there was a marked reduction in the density of LyzM+/Smad3+ cells (Online Figure III). Adult MyS3KO mice had no significant baseline abnormalities, exhibiting comparable body weight and circulating blood cell counts with Smad3 fl/fl animals. Because macrophages have been proposed to play a role in cardiac conduction,[28] we studied the effects of myeloid cell–specific Smad3 loss on baseline cardiac function. MyS3KO mice had normal baseline heart rate, cardiac geometry, and function but showed a modest prolongation of the PR interval (Online Figure IV).

MyS3KO Mice Exhibit Increased Late Postinfarction Mortality That Is Not Caused by Cardiac Rupture

In C57Bl6J mice, significant mortality is noted 5 to 6 days after coronary occlusion, is higher in males, and is typically caused by cardiac rupture. MyS3KO and Smad3 fl/fl mice exhibited comparable survival 7 days after myocardial infarction (MI). After 28 days, MyS3KO mice had a trend towards increased mortality. Male animals in both MyS3KO and Smad3 fl/fl groups had very high mortality (MyS3KO 100%, n=23 versus Smad3 fl/fl 85.7%, n=14; P=0.61). Female MyS3KO mice had a trend towards increased mortality when compared with Smad3 fl/fl animals (P=0.06). MyS3KO mice had significantly increased late mortality (after day 6) when compared with Smad3 fl/fl mice. Deaths occurring after day 6 were not associated with evidence of cardiac rupture (Figure 3A through 3I).

Infarcted MyS3KO Mice Have Accentuated Remodeling and Worse Systolic Dysfunction

Because of the very high mortality in male MyS3KO mice (100%), functional analysis was limited to females. Seven to 28 days postinfarction, MyS3KO mice exhibited significantly increased left ventricular end-diastolic volume and left ventricular end-systolic volume, suggesting worse dilative remodeling

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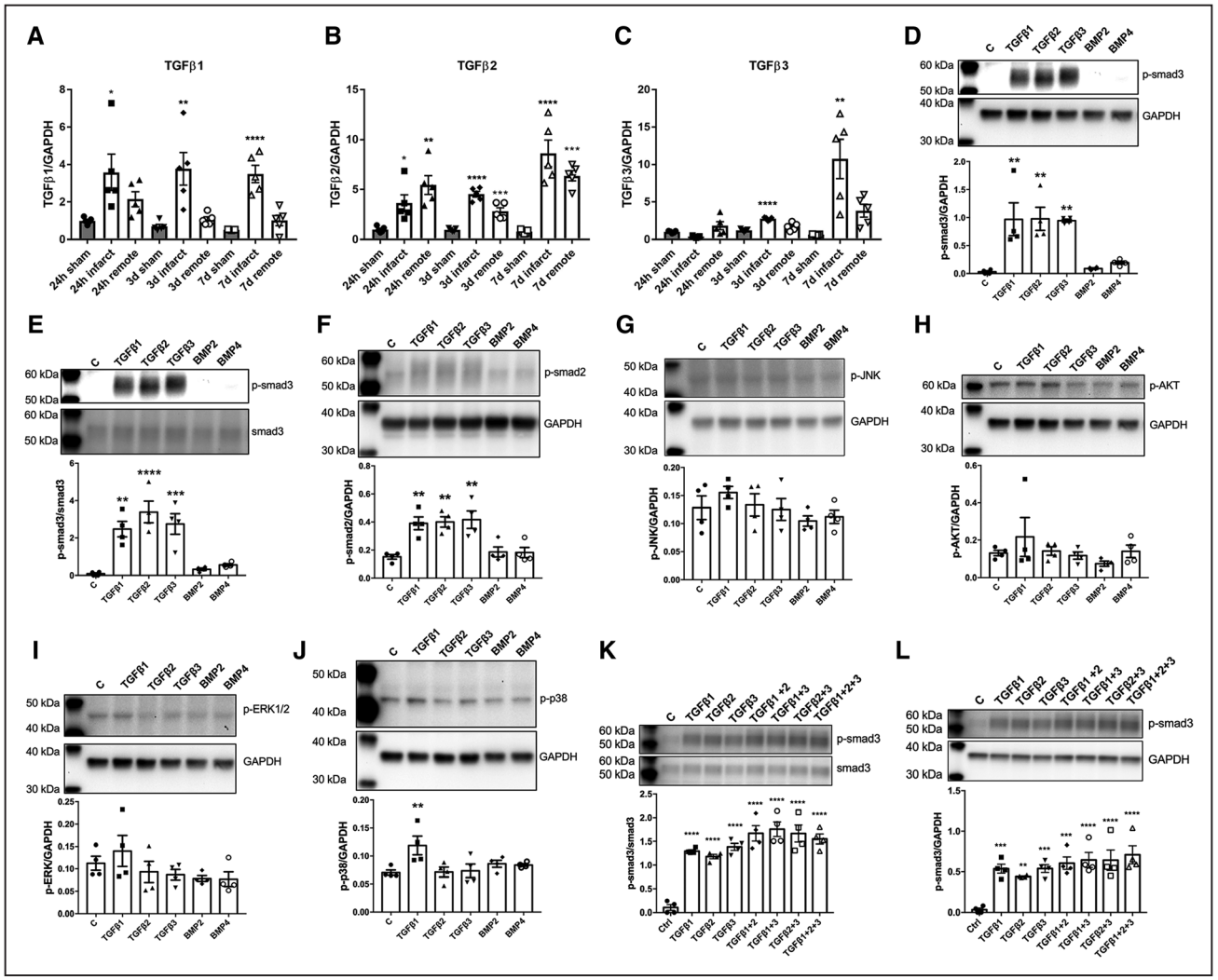


Figure 1. TGF (transforming growth factor)-βs rapidly stimulate Smad (small mothers against decapentaplegic)-dependent signaling in macrophages. **A**–**C**, Myocardial TGF-β1 (**A**), TGF-β2 (**B**), and TGF-β3 (**C**) mRNA upregulation in nonreperfused mouse infarcts. In contrast to the early upregulation of TGF-β1 and TGF-β2, TGF-β3 exhibits a late peak (**P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 vs sham; n=5/group). **D**–**E**, In vitro, TGF-β1, TGF-β2 and TGF-β3 (10 ng/mL) markedly increase levels of p-Smad3 (phospho-small mothers against decapentaplegic 3; **D**) and the p-Smad3:Smad3 ratio (**E**) in BMMs (bone marrow–derived macrophages) after 30 min of stimulation. In contrast, BMP (bone morphogenetic protein)-2 (50 ng/mL) and BMP-4 (50 ng/mL) have no significant effects. **F**, TGF-βs, but not BMPs, significantly increase p-Smad2 levels in BMMs (***P*<0.01 vs control). **G**–**I**, TGF-βs and BMPs do not significantly affect levels of p-JNK (c-Jun N-terminal kinase; **G**), p-Akt (protein kinase B; **H**) and p-Erk (extracellular signal-regulated kinase; **I**). **J**, TGF-β1, but not TGF-β2 and TGF-β3, or BMPs induces a modest increase in p-p38 MAPK (mitogen-activated protein kinase) levels. **K**–**L**, Costimulation with TGF-β1+TGF-β2, TGF-β1+TGF-β3, TGF-β2+TGF-β3, or all 3 TGF-β isoforms together, induced comparable levels of Smad3 activation with single isoform stimulation (***P*<0.01, ****P*<0.001, *****P*<0.0001 vs control). n=4/group.

(Figure 3J through 3K). Moreover, infarcted MyS3KO mice had accentuated systolic dysfunction, evidenced by significantly lower ejection fraction and fractional shortening (Figure 3L and 3M). MyS3KO mice had significantly increased heart weight and lung weight 28 days after infarction and exhibited a significant increase in cardiomyocyte size in the infarct border zone (Online Figure V). These findings suggest that myeloid cell–specific Smad3 protects the infarcted heart from adverse remodeling and the development of heart failure.

Accentuated Adverse Remodeling in MyS3KO Mice Is Associated With Late Scar Expansion

Triphenyltetrazolium chloride staining showed that MyS3KO mice and Smad3 fl/fl animals had comparable acute infarct size. Scar size 7 to 28 days after infarction was assessed through sectioning and staining of the heart from base to apex. Although scar size was comparable between groups at the 7-day time point, MyS3KO mice had significantly larger scars 28 days after infarction (Online Figure VI).

Infarcted MyS3KO Mice Exhibit Perturbed Clearance of Dead Cardiomyocytes and Neutrophils, Despite Increased Macrophage Infiltration

Nonphagocytosed dead cardiomyocytes were identified within the healing infarct, as cells with perturbed desmin distribution patterns that lacked a nucleus (due to karyolysis, Figure 4B, 4C, 4E, and 4F). Seven days after infarction, the area of nonphagocytosed cardiomyocytes was >3-fold higher in MyS3KO animals (Figure 4G). Infarcted MyS3KO mice had increased macrophage density (Figure 4H through 4L), suggesting that impaired clearance of dead cells in MyS3KO is not due to

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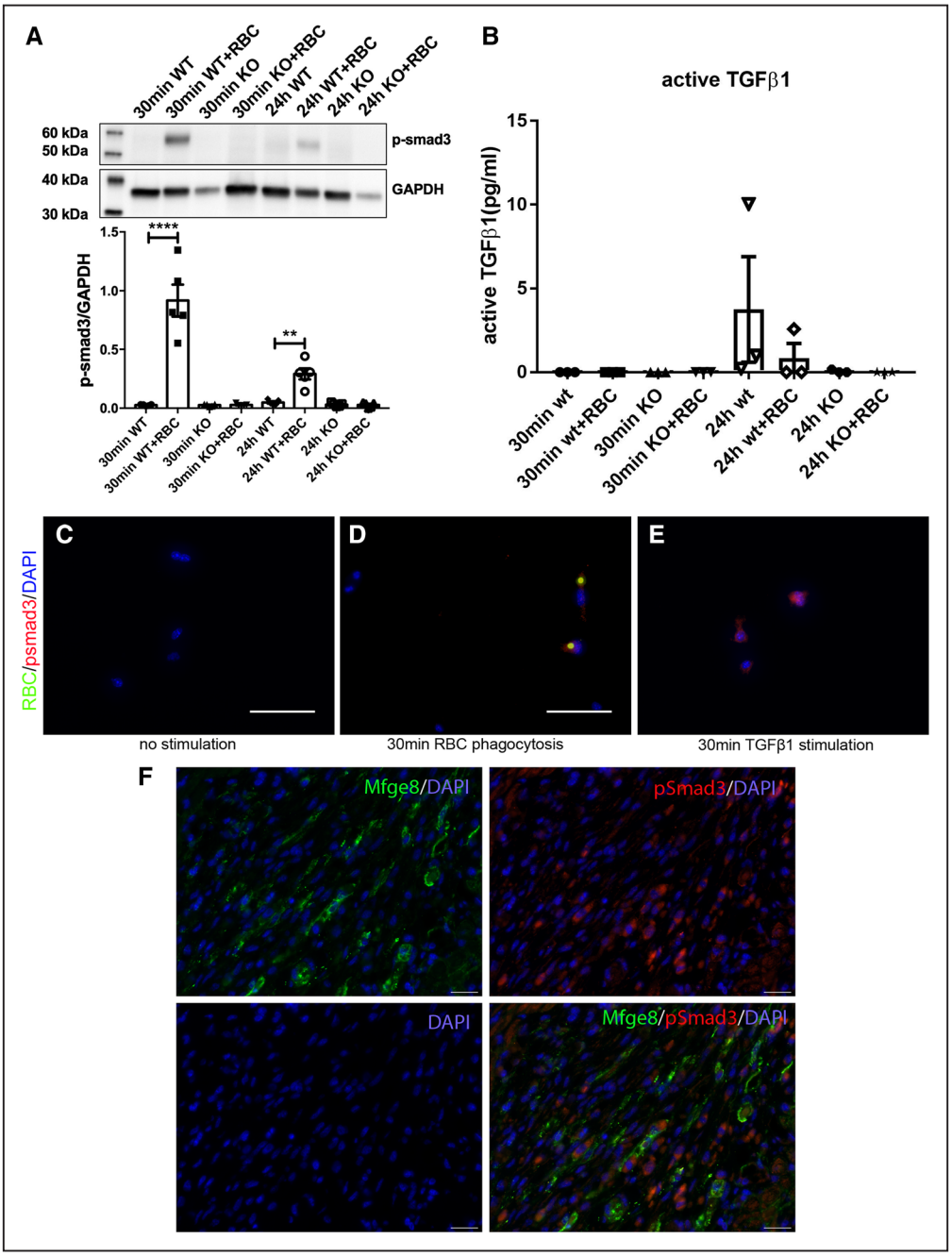


Figure 2. Phagocytosis activates Smad3 (small mothers against decapentaplegic 3) in macrophages through a TGF (transforming growth factor)-β–independent pathway. A, Aged red blood cells (RBCs) are rapidly phagocytosed by BMMs (bone marrow–derived macrophages). **A**, After 30 min of incubation with RBCs, wild-type (WT) BMMs exhibited markedly increased expression of phospho-Smad3 (p-Smad3) (****P<0.0001 vs control cells). BMMs from Smad3 knockout (KO) mice (KO, negative control), exhibited no Smad3 activation. After 24 h of incubation with RBCs, WT BMMs had persistent activation of Smad3 (**P<0.01). **B**, To examine whether the rapid activation of Smad3 is due to release of TGF-β upon RBC phagocytosis, we assessed TGF-β1 levels in the supernatant. No active TGF-β1 was detected after 30 min of incubation with RBCs. After 24 h, control WT cells and cells incubated with RBCs had low levels of active TGF-β1. **C**–**E**, Engulfment of RBCs induces p-smad3 expression in BMMs. Unstimulated BMMs have negligible p-Smad3 staining (**C**). BMMs exhibiting RBC engulfment express p-Smad3 (arrows), whereas BMMs that did not engulf RBCs have minimal p-smad3 expression (arrowheads; **D**). TGF-β1-treated BMMs served as a positive control (arrows; **E**). The findings suggest that phagocytosis may directly activate Smad3 in BMMs through a TGF-β-independent mechanism. **F**, A subset of phagocytic Mfge8 (milk fat globule-epidermal growth factor factor 8)+ cells exhibit Smad3 activation. Dual immunofluorescent staining for Mfge8 and p-Smad3 identifies a subset of p-Smad3+ cells with strong Mfge8 immunoreactivity, suggesting phagocytic activity (arrows). Scale bar =50 μm. DAPI indicates 4′,6-diamidino-2-phenylindole.

reduced recruitment of macrophages but may reflect perturbed phagocytic function. Neutrophil density was also increased in MyS3KO infarcts (Figure 4M). In vitro, infarct macrophages from MyS3KO mice exhibited impaired phagocytosis of heat stress–induced apoptotic neutrophils (Figure 4N).

Loss of Smad3 in BMMs Perturbs Phagocytic Activity

When compared with WT BMMs, Smad3 knockout macrophages exhibited reduced phagocytosis of aged RBCs in the presence or absence of TGF-β1 or TGF-β2 (Figure 5A and 5B).

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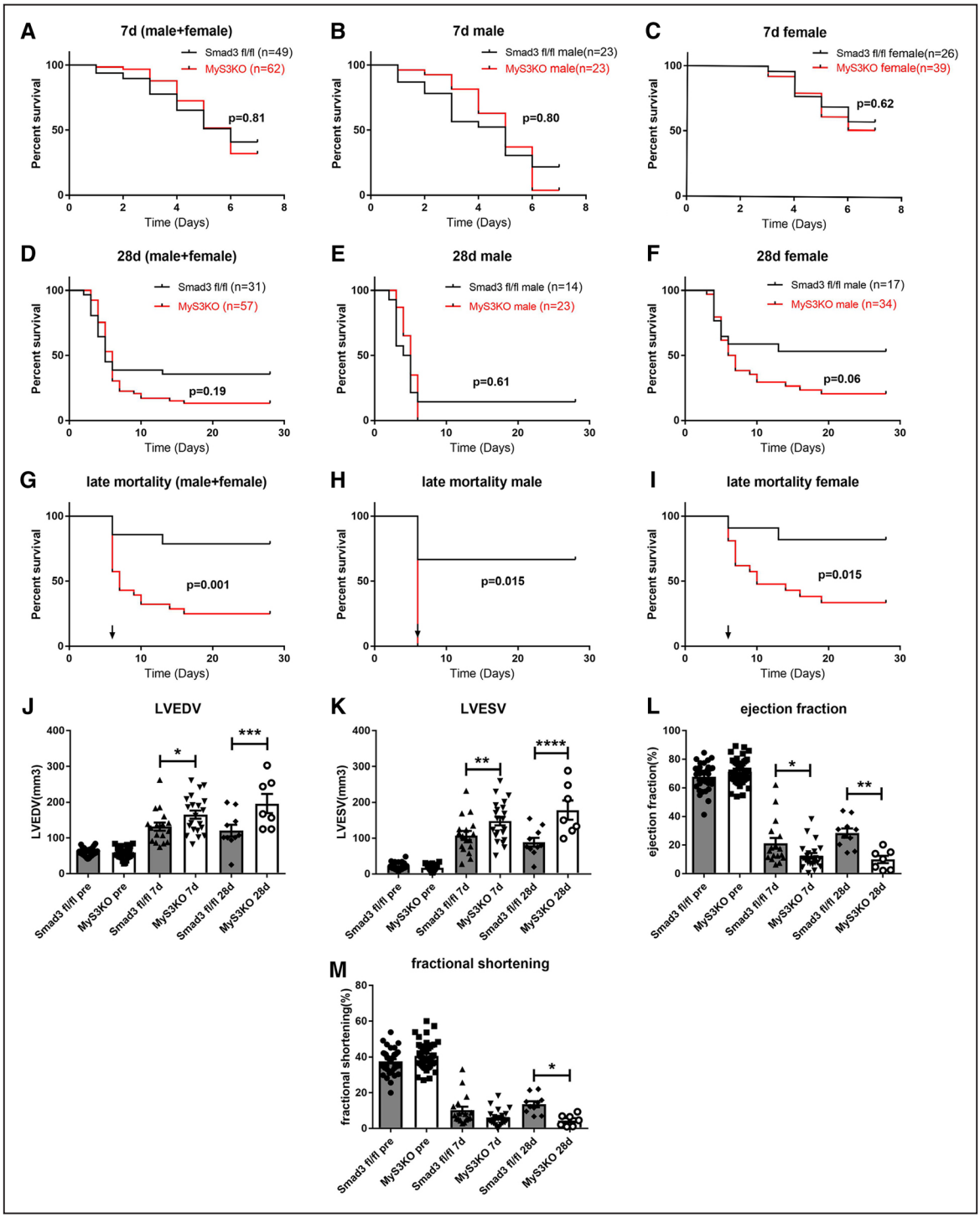


Figure 3. Smad3 (small mothers against decapentaplegic 3) loss in myeloid cells increases late mortality after myocardial infarction and is associated with dilative remodeling and dysfunction. Comparison of survival curves between Smad3 fl/fl and MyS3KO (myeloid cell–specific Smad3 knockout) mice after 7 to 28 days of permanent coronary occlusion (**A**–**F**). **A**–**C**, No significant differences in mortality were noted in the 7-d group (*P*=0.81; Smad3 fl/fl: n=49, MyS3KO: n=62). **D**–**F**, After 28 d, MyS3KO mice had a trend towards increased mortality (*P*=0.19, Smad3 fl/fl: 65%, n=31; MyS3KO: 88%, n=57; **D**). Male mice (**E**) had comparable very high mortality rates in both groups (*P*=0.61, Smad3 fl/fl: 86%, n=14, MyS3KO: 100%, n=23). Female MyS3KO mice (**F**) had a trend towards increased mortality (*P*=0.06, Smad3 fl/fl: 47%, n=17; MyS3KO: 79%, n=34). **G**–**I**, MyS3KO mice exhibited increased late mortality after the sixth day postinfarction that was not due to cardiac rupture. **J**–**M**, Echocardiographic assessment of adverse remodeling was limited to female mice because of the very high mortality in male animals. MyS3KO mice had accentuated dilative remodeling evidenced by increased left ventricular end-diastolic volume (LVEDV; **J**) and left ventricular end-systolic volume (LVESV; **K**) after 7–28 d. **L**–**M**, Moreover, MyS3KO mice had increased systolic dysfunction, showing lower ejection fraction (**L**) after 7–28 d of coronary occlusion, and reduced fractional shortening (**M**) at the 28-d time point (**P*<0.05, ***P*<0.01, *****P*<0.0001, Smad3 fl/fl n=9–17/group, MyS3KO n=7–26/group).

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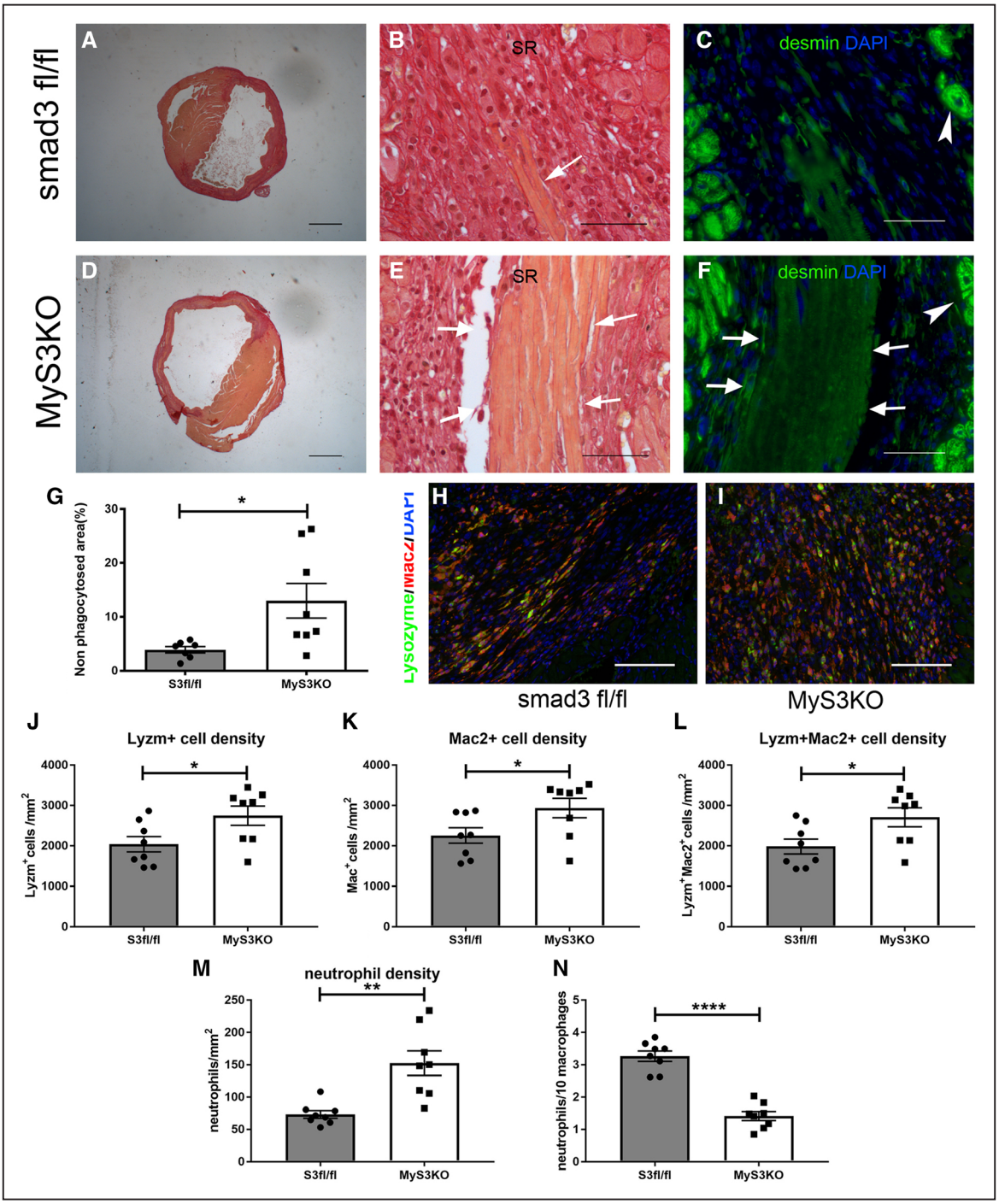


Figure 4. Myeloid cell–specific Smad3 (small mothers against decapentaplegic 3) loss perturbs phagocytosis of dead cardiomyocytes in the infarct, despite increased macrophage infiltration. Infarcted hearts were sectioned from base to apex at 300 μm partitions, 1 section from each partition was used for SR (Sirius red staining) and hematoxylin counterstaining. After 7 d of permanent occlusion, Smad3 fl/fl animals (**A–C**) show almost complete replacement of cardiomyocytes in the infarcted area with granulation tissue. **B**, Relatively small areas of nonphagocytosed cardiomyocytes (arrows) were noted in Smad3 fl/fl infarcts. These areas are comprised of dead cardiomyocytes, as suggested by the absence of hematoxylin+nuclei due to karyolysis. **C**, Desmin/DAPI (4′,6-diamidino-2-phenylindole) fluorescent staining confirms the presence of nonphagocytosed cardiomyocytes in these areas. These cells have an abnormal pattern of desmin staining (arrows) and absence of DAPI+ nuclei. In comparison, viable cardiomyocytes in the border zone exhibit preserved desmin staining patterns (**C**, arrowheads). **D–F**, In contrast, MyS3KO (myeloid cell–specific Smad3 knockout) mice have much larger areas of nonphagocytosed cardiomyocytes (**E–F**, arrows). **A** and **D**, Scale bar =1 mm. **B**, **C**, **E**, and **F**, Scale bar =50 μm. **G**, The area of nonphagocytosed cardiomyocytes is markedly increased in MyS3KO mice (*P<0.05). **H–L**, MyS3KO mice have increased influx of LyzM (lysozyme M)-expressing macrophages, Mac2 (Galectin-3)+ macrophages, and lysozyme M+/Mac2+ macrophages in the infarct (*P<0.05), suggesting that impaired phagocytosis was not due to attenuation of macrophage recruitment (n=8–9/group). Scale bar =100 μm. **M**, Infarcted MyS3KO mice exhibit increased neutrophil infiltration after 7 d of coronary occlusion (**P<0.01, n=8/group). **N**, Macrophages harvested from MyS3KO infarcts have impaired capacity to phagocytose heat stress–induced apoptotic neutrophils (****P<0.0001, n=8/group).

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Smad3 Mediates Mfge8 Synthesis by Macrophages In Vitro and In Vivo

We studied the effects of Smad3 loss on expression of phagocytosis-associated genes using a polymerase chain reaction array (Online Table I). Smad3 knockout cells had reduced expression of many genes associated with recognition of eat-me signals, or phagosome formation, including Anxa1 (Annexin A1), Axl (*AXL* receptor tyrosine kinase), Calr (calreticulin), and Pld1 (phospholipase D1; Online Figure VII). The Mertk (C-mer proto-oncogene tyrosine kinase) receptor tyrosine kinase is critical for engulfment and clearance of apoptotic cells.[29] Although Mertk mRNA expression levels were higher in Smad3 knockout cells, baseline Mertk protein levels were comparable between WT Smad3 knockout cells. TGF-β1-mediated Mertk mRNA and protein upregulation was dependent on Smad3 (Online Figure VII). Mfge8 is a secreted

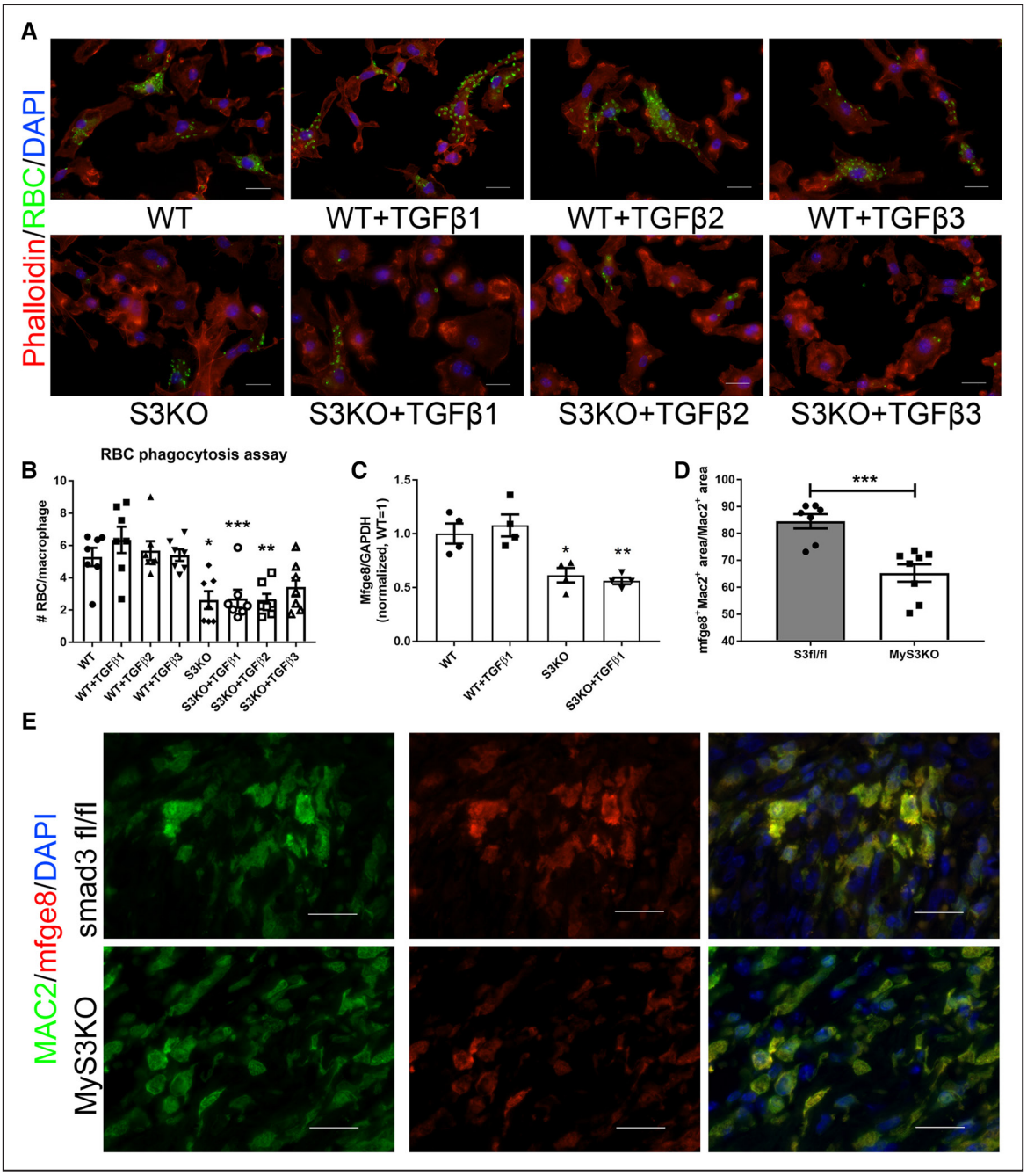


Figure 5. Smad3 (small mothers against decapentaplegic 3) loss perturbs phagocytosis of aged red blood cells (RBCs) by macrophages and is associated with reduced Mfge8 (milk fat globule-epidermal growth factor factor 8) expression. **A** and **B**, Smad3 knockout (KO) bone marrow–derived macrophages (BMMs) exhibit impaired phagocytosis of aged RBCs in the presence or absence of TGF (transforming growth factor)-βs (**P*<0.05, ***P*<0.01, ****P*<0.001, n=7/group). **C**, Smad3 KO macrophages exhibit reduced Mfge8 synthesis in the presence or absence of TGF-β1 (**P*<0.05, ***P*<0.01). **D**–**E**, Dual immunofluorescence for Mfge8 and Mac2 (Galectin-3) shows that MyS3KO (myeloid cell–specific Smad3 KO) infarcts have reduced infiltration with phagocytic Mfge8+ macrophages (7-day infarct, ****P*<0.001, n=7–8/group). Scale bar =20 μm. DAPI indicates 4′,6-diamidino-2-phenylindole; S3KO, Smad3 knockout; and WT, wild-type.

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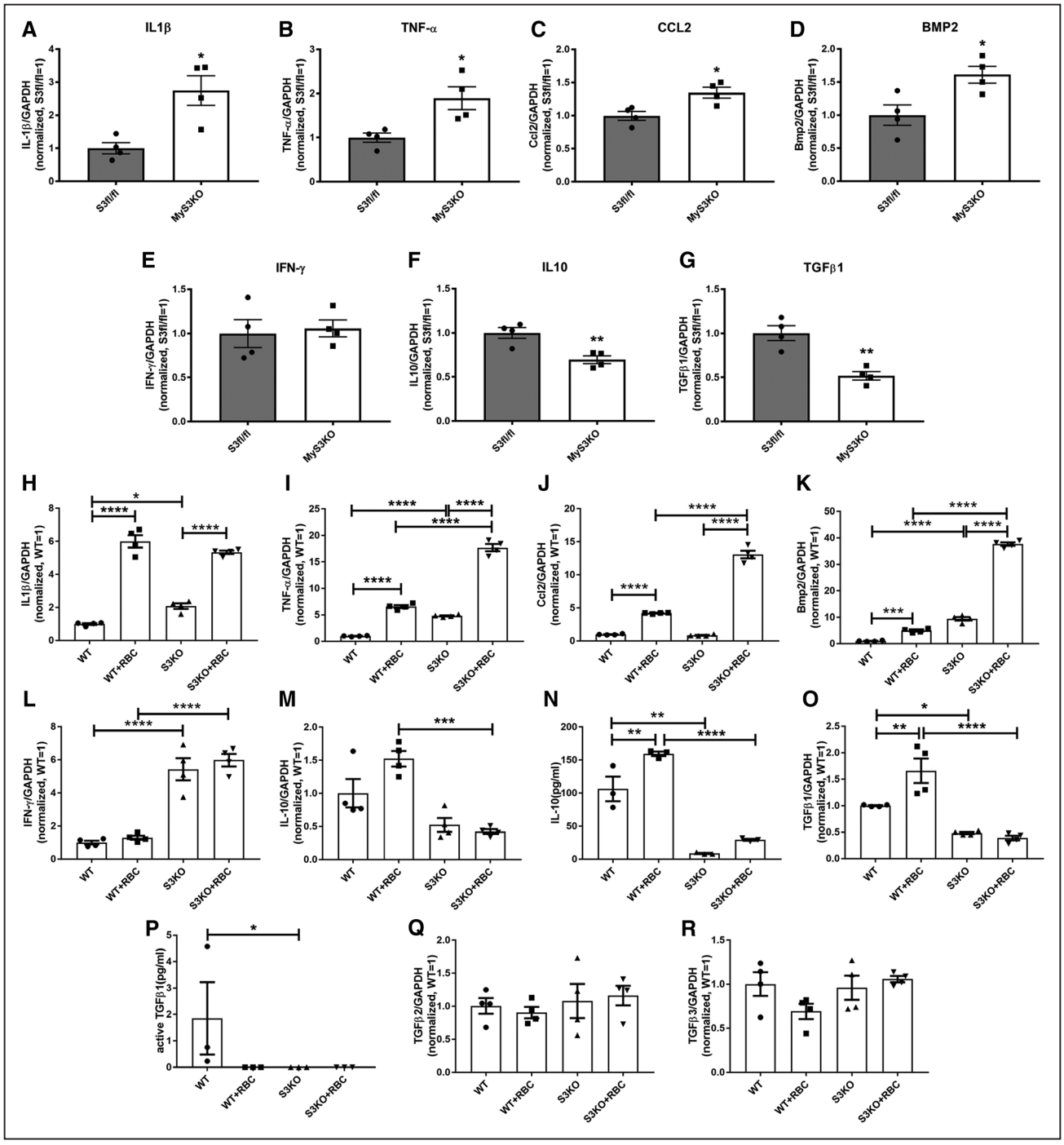


Figure 6. Smad3 (small mothers against decapentaplegic 3) mediates the anti-inflammatory properties of phagocytic macrophages. MyS3KO (myeloid cell–specific Smad3 knockout) infarct macrophages (72 h after infarction) exhibit higher gene expression of the proinflammatory cytokines IL (interleukin)-1β (**A**)TNF (tumor necrosis factor)-α (**B**), CCL (chemokine [C-C motif] ligand) 2/MCP1 (monocyte chemoattractant protein; **C**), and BMP (bone morphogenetic protein)-2 (**D**). IFN (interferon)-γ mRNA expression is comparable between groups (**E**). MyS3KO infarct macrophages have lower expression of the anti-inflammatory cytokines IL-10 (**F**) and TGF (transforming growth factor)-β1 (**G**; **P*<0.05, ***P*<0.01, n=4/group). **H**–**L**, In bone marrow–derived macrophage (BMMs) phagocytosing aged red blood cells (RBCs), Smad3 absence increased synthesis of proinflammatory cytokines and chemokines. **H**, Baseline IL-1β expression was higher in Smad3 KO cells; however phagocytic Smad3 knockout and wild-type (WT) cells had comparable IL-1β expression. After phagocytosis of aged RBCs, Smad3 KO macrophages exhibited accentuated TNF-α (**I**), CCL2/MCP1 (**J**) and BMP-2 (**K**) mRNA synthesis. **L**, Smad3 KO cells also had markedly increased expression of IFN-γ. **M**, Smad3 loss markedly attenuated synthesis of IL-10 by macrophages phagocytosing RBCs. **N**, An ELISA assay demonstrated that phagocytosis of RBCs increased IL-10 levels in the supernatant in WT cells and that Smad3 KO cells had a marked reduction in secreted IL-10. **O**, Phagocytosis of aged RBCs increased TGF-β1 mRNA synthesis in macrophages. Smad3 loss markedly attenuated TGF−β1 synthesis by macrophages. **P**, WT cells showed secretion of active TGF-β1. In contrast, active TGF-β1 levels were negligible in Smad3 KO cells. **Q**–**R**, Smad3 loss did not affect TGF-β2 and TGF-β3 mRNA synthesis by macrophages. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, n=4/group. S3KO indicates Smad3 knockout.

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glycoprotein that bridges phosphatidylserine in apoptotic cells with the vitronectin receptor on the cell surface of phagocytes, thus serving as a macrophage-derived extracellular adaptor molecule with a critical role in phagocytosis.[30] Mfge8 levels were significantly decreased in Smad3 knockout macrophages in the presence or absence of TGF-β1. In vivo, the density of Mefge8+ macrophages was significantly reduced in MyS3KO infarcts (Figure 5D and 5E).

Smad3 Mediates Transition of Infarct Macrophages to an Anti-Inflammatory Phenotype

Macrophages play a critical role in regulation of the postinfarction inflammatory response; phosphatidylserine-dependent phagocytosis of apoptotic cells by macrophages promotes synthesis of anti-inflammatory mediators. We postulated that adverse remodeling in MyS3KO mice may reflect perturbed transition of macrophages to an anti-inflammatory phenotype. Infarct macrophages harvested from MyS3KO mice exhibited accentuated expression of proinflammatory cytokines and reduced synthesis of anti-inflammatory cytokines. MyS3KO infarct macrophages had markedly increased expression of IL (interleukin)-1β, TNF (tumor necrosis factor)-α, CCL (chemokine [C-C motif] ligand) 2, and BMP-2 (Figure 6A through 6D). IFN (interferon)-γ levels were comparable between groups (Figure 6E). IL-10 and TGF-β1 levels were markedly reduced in MyS3KO infarct macrophages (Figure 6F and 6G).

Next, we studied the effects of Smad3 loss on expression of inflammation-related genes in macrophages phagocytosing aged RBCs (Online Table II). Baseline IL-1β expression was higher in Smad3 knockout cells; however, phagocytic Smad3 knockout and WT cells had comparable IL-1β expression (Figure 6H). Smad3 knockout cells exhibited markedly and significantly increased expression of the proinflammatory cytokines TNF-α , CCL2, BMP-2 and IFN-γ, (Figure 6I through 6L). Smad3 knockout macrophages had reduced IL-10 mRNA and protein synthesis on RBC phagocytosis (Figure 6M and 6N). Smad3 knockout cells also exhibited lower levels of TGF-β1 mRNA synthesis and had no upregulation of TGF-β1 transcription after phagocytosis (Figure 6O). Smad3 knockout cells secreted no detectable active TGF-β1; in contrast, WT cells had low levels of TGF-β1 activity in the supernatant (Figure 6P). The effects of Smad3 on TGF-β production were isoform-specific; TGF-β2 and TGF-β3 mRNA synthesis was not affected by loss of Smad3 (Figure 6Q and 6R).

Effects of Smad3 Loss on Macrophage-Derived Chemokine, Cytokine, and Chemokine Receptor Expression

Chemokine expression levels were profoundly affected by the absence of Smad3; however, the pattern of modulation did not consistently suggest pro or anti-inflammatory actions. Smad3 loss markedly accentuated synthesis of CCL6, CCL7, CCL9, CCL24, CX3CL1 (chemokine [C-X3-C motif] ligand 1), and PF4 (platelet factors 4)/CXCL (chemokine (C-X-C motif) ligand) 4 but downmodulated expression of CCL4, CCL5, CXCL1, and CXCL11 (Online Figure VIII). Smad3 knockout cells had reduced IL-1α and IL-1RI (interleukin 1 receptor, type1) mRNA levels after phagocytosis of aged RBCs; however, IL1Rn (interleukin 1 receptor antagonist) levels were also decreased. Expression of IL15, IL27, IL4, IL7, IL-16, IL-18, Ltb (lymphotoxin B), Osm (oncostatin M), and BMP-4 were also significantly increased in Smad3 knockout cells; however, IL-6 and MIF (macrophage migration inhibitory factor) levels were reduced (Online Figure IX). Smad3 knockout cells had markedly increased levels of the CCRs (C-C chemokine receptors) CCR2, CCR3, CCR5, and CCR10 and the CXCRs (CXC chemokine receptors) CXCR3 and CXCR5 (Online Figure X). Accentuated expression of chemokine receptors by Smad3 null cells may explain, at least in part, for the enhanced recruitment of myeloid cells in MyS3KO infarcts (Figure 4H through 4M).

Smad3 Restrains Expression of Inflammatory Genes in TGF-β-Stimulated Macrophages

Next, we examined whether Smad3 loss affects inflammatory gene synthesis in TGF-β1-stimulated macrophages (Online Table III). After stimulation with TGF-β1, Smad3 knockout macrophages had much higher expression of many inflammatory genes than WT cells, including the chemokines CCL2, CCL5, CCL7, CCL8, CCL12, CXCL9, CXCL12, the chemokine receptors CCR1, CCR5, and CXCR2 and the cytokines IFN-γ and Tnfsf11 (tumor necrosis factor [ligand] superfamily, member 11). Overall, the effects of Smad3 loss on inflammatory gene expression were much more impressive in phagocytic macrophages (Figure 6, Online Figure VIII) than in macrophages stimulated with TGF-β1 (Online Figure XI).

Mfge8 Partly Rescues the Phagocytosis Defect of Smad3 Knockout Cells

Treatment with recombinant Mfge8 partly rescued the impaired RBC phagocytosis of Smad3 knockout macrophages (Figure 7A through 7E).

Mfge8 has Modest but Significant Effects on Phagocytosis-Mediated Expression of Inflammatory Genes by Smad3 Knockout Macrophages

Mfge8 stimulates anti-inflammatory cascades.[31] Accordingly, we examined whether stimulation with recombinant Mfge8 corrected the perturbations in anti-inflammatory cytokine expression observed in Smad3 knockout macrophages. Although Mfge8 modestly, but significantly, increased IL-10 mRNA synthesis, IL-10 expression remained markedly lower in Smad3 knockout macrophages when compared with WT cells (Figure 7F). Moreover, Mfge8 had no significant effects on IL-10 protein release by WT or Smad3 knockout macrophages (Figure 7G). Effects of Mfge8 on TGF-β1 mRNA synthesis were also modest. Mfge8 significantly increased TGF-β1 mRNA synthesis in Smad3 knockout macrophages; however, TGF-β1 expression levels remained markedly lower in Smad3 knockout macrophages, when compared with WT cells (Figure 7H). Mfge8 had no significant effects on the levels of active TGF-β1 (Figure 7I). Effects of Mfge8 on IFN-γ expression in Smad3 knockout macrophages were not statistically significant (Figure 7J). Mfge8 significantly reduced BMP-2 mRNA synthesis in Smad3 knockout cells to levels comparable with WT macrophages (Figure 7K). The effects of

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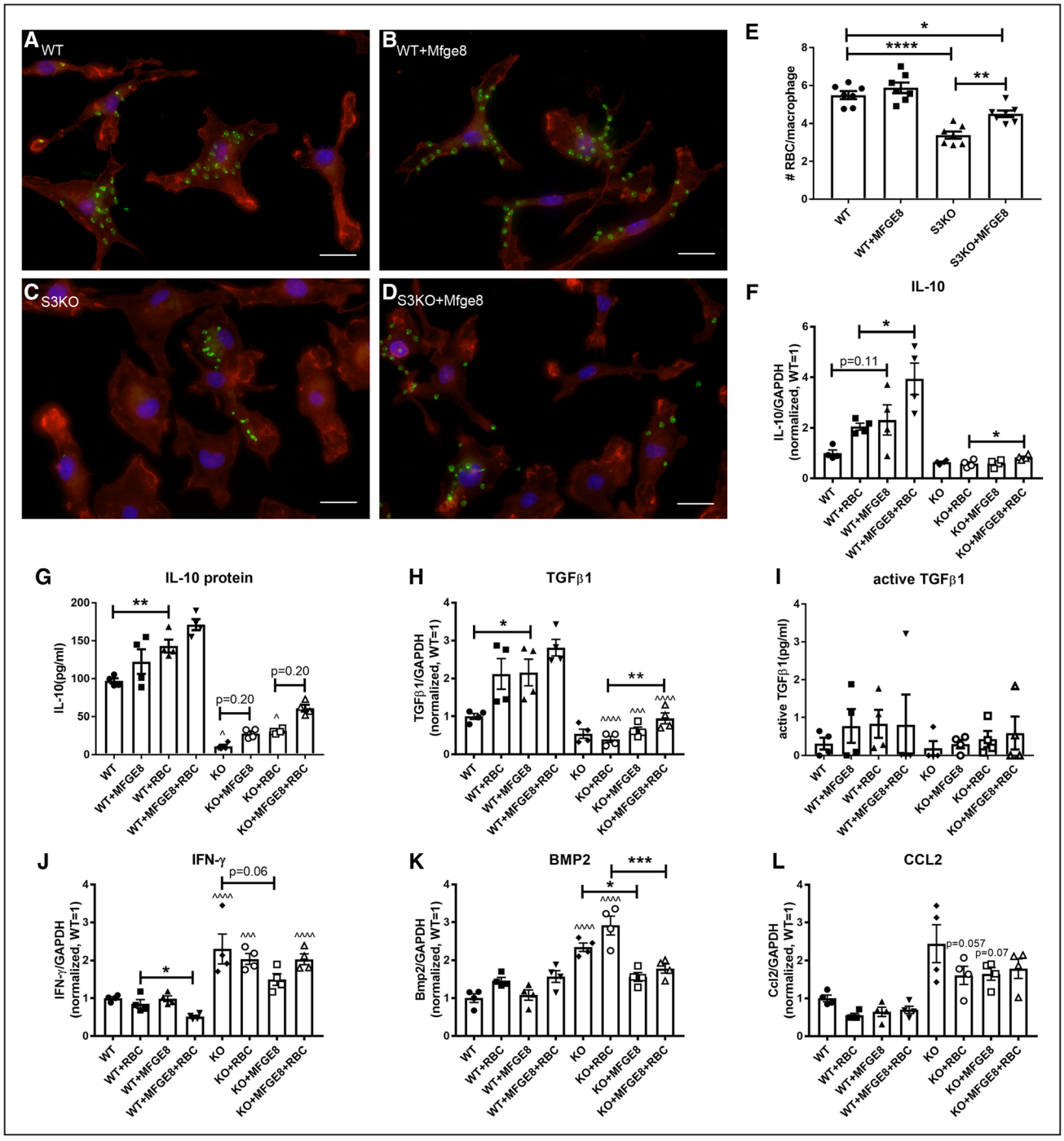


Figure 7. Perturbed phagocytosis in Smad3 (small mothers against decapentaplegic 3) knockout cells is partly rescued by Mfge8 (milk fat globule-epidermal growth factor factor 8) administration. **A–D**, Effects of Mfge8 on phagocytosis of aged red blood cells (RBCs) by wild-type (WT; **A** and **B**) and Smad3 KO (**C** and **D**) bone marrow–derived macrophage (BMMs). **E**, Smad3 KO macrophages have perturbed phagocytosis of aged RBCs that is partly rescued by Mfge8 (*P<0.05, **P<0.01, ****P<0.0001, n=7/group). Scale bar =20 μm. **F–L**, Mfge8 does not rescue the impaired anti-inflammatory phenotype of BMMs associated with Smad3 loss. **F**, Although Mfge8 treatment modestly but significantly increased IL (interleukin)-10 mRNA synthesis in Smad3 KO BMMs phagocytosing RBCs, IL-10 levels remained markedly lower in macrophages lacking Smad3, in comparison to WT cells. **G**, Mfge8 had no significant effects on IL-10 protein secretion by Smad3 KO cells. **H**, Mfge8 treatment increased TGF (transforming growth factor)-β1 mRNA synthesis in Smad3 KO BMMs phagocytosing RBCs; however, TGF-β1 mRNA levels remained markedly lower in comparison to WT cells. **I**, Mfge8 treatment did not significantly affect TGF-β1 activity in the supernatant of BMMs. **J**, Mfge8 did not affect the increased IFN (interferon)-γ expression observed in Smad3 KO cells. **K**, In contrast, Mfge8 significantly reduced BMP (bone morphogenetic protein)-2 expression in Smad3 KO cells. **L**, The effects of Mfge8 on CCL (chemokine [C-C motif] ligand) 2 expression were not significant (*P<0.05, **P<0.01, ***P<0.001, n=4; ^P<0.05, ^^P<0.01, ^^^P<0.001, ^^^^P<0.0001 vs control, n=4/group). S3KO indicates Smad3 knockout.

Mfge8 on macrophage-derived CCL2/MCP (monocyte chemoattractant protein)-1 mRNA expression did not reach statistical significance (Figure 7L). Overall, Mfge8 did not correct the perturbed anti-inflammatory profile of Smad3 knockout macrophages but modestly increased IL-10 mRNA synthesis and reduced BMP-2 expression.

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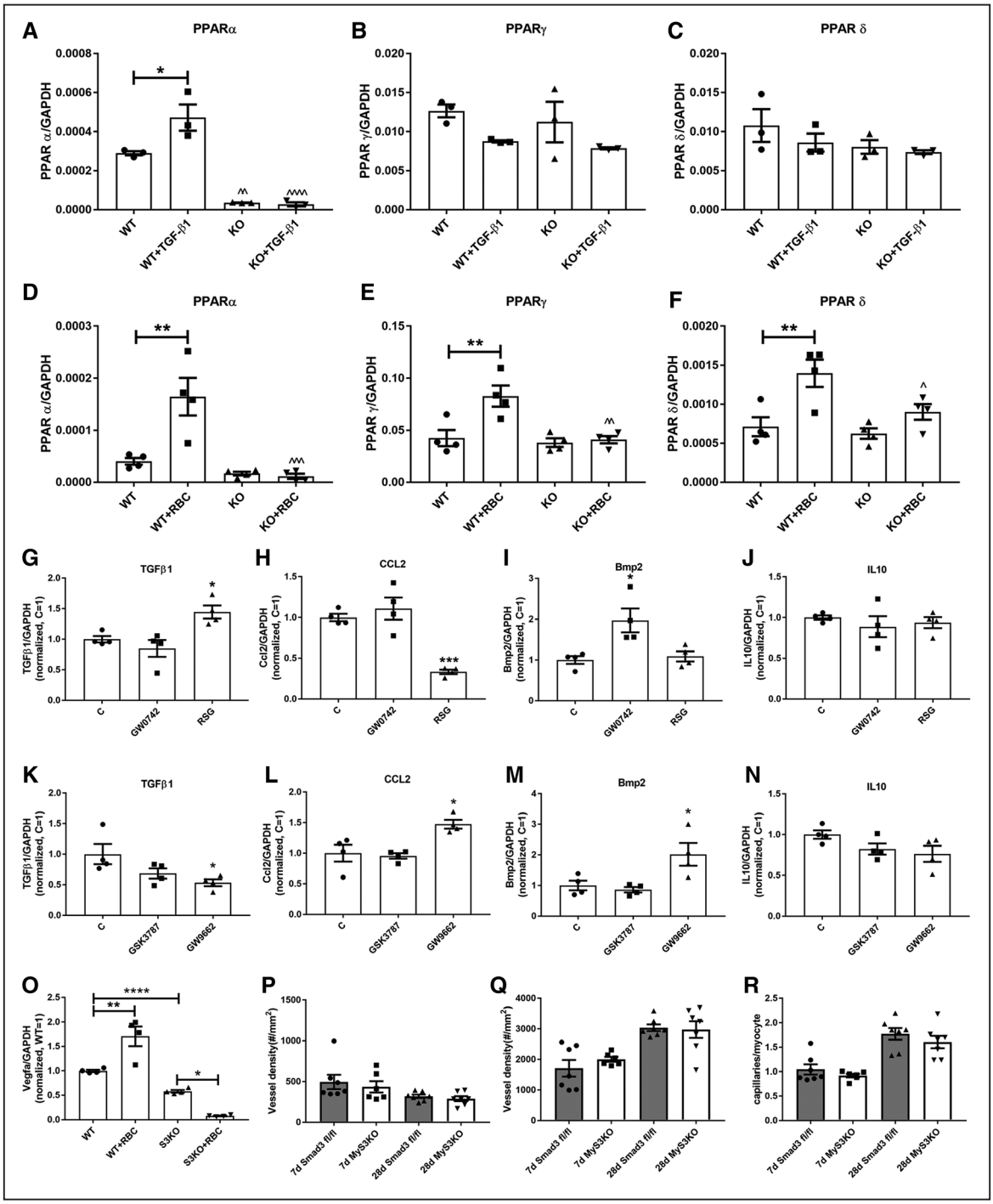


Figure 8. Attenuated PPAR (peroxisome proliferator-activated receptors) expression may be responsible for impaired anti-inflammatory transition in the absence of Smad3 (small mothers against decapentaplegic 3). **A–F**, Phagocytosis induces PPAR expression in wild-type (WT) cells in a Smad3-dependent manner. PPARα mRNA expression was much lower in Smad3 knockout (KO) cells in response to TGF (transforming growth factor)-β1 stimulation (**A**) or red blood cell (RBC) phagocytosis (**D**). TGF-β1 did not significantly affect PPARγ (**B**) and PPARδ (**C**) mRNA synthesis in both Smad3 KO and WT bone marrow–derived macrophages (BMMs). RBC phagocytosis induced PPARγ (**E**) and PPARδ (**F**) expression in WT cells. Smad3 loss attenuated PPARγ (**E**) and PPARδ (**F**) expression after phagocytosis of aged RBCs (**P*<0.05, ***P*<0.01; ^*P*<0.05, ^^*P*<0.01, ^^^*P*<0.001, ^^^^*P*<0.0001 vs WT, n=4/group). **G–N**, Pretreatment with the PPARγ agonist rosiglitazone (RSG) increased TGF-β1 (**G**) and decreased CCL (chemokine [C-C motif] ligand) 2 (**H**) mRNA synthesis in BMMs phagocytosing aged RBCs. RSG did not affect BMP (bone morphogenetic protein)-2 (**I**) and IL (interleukin)-10 (**J**) expression. Moreover, pretreatment with a PPARγ antagonist (GW9662) significantly reduced TGF-β1 (**K**), while increasing CCL2 (**L**) and BMP-2 (**M**) expression. PPARγ inhibition did not affect IL-10 expression (**N**). A PPARδ agonist (GW0742) modestly increased BMP-2 levels (**I**), without affecting TGF-β1 (**G**), CCL2 (**H**) and IL-10 (**J**) expression. A PPARδ antagonist (GSK3787) did not significantly affect TGF-β1 (**K**), CCL2 (**L**), Bmp-2 (**M**), and IL-10 (N) expression (**P*<0.05, ****P*<0.001, n=4). **O**, Smad3 loss markedly attenuates VEGF (vascular endothelial growth factor)-A expression by BMMs. Smad3 KO macrophages exhibited a marked reduction in VEGF-A mRNA synthesis in the presence or absence of aged RBCs (**P*<0.05, ***P*<0.01, *****P*<0.0001, n=4/group). **P–R**, Myeloid cell–specific Smad3 loss did not affect infarct angiogenesis. Seven to 28 d after infarction, MyS3KO (myeloid cell–specific Smad3 knockout) and Smad3 fl/fl mice controls had comparable microvascular density in the infarcted area (**P**) and in the remote remodeling myocardium (**Q**). MyS3KO mice and Smad3 fl/fl had comparable capillary-to-myocyte ratio in the remodeling myocardium (**R**; n=7/group).

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Smad3 Mediates Phagocytosis-Induced PPAR Induction

Next, we examined whether defective anti-inflammatory transition of Smad3 knockout macrophages involves dysregulation of PPARs, key regulator of macrophage function. BMMs expressed very low levels of PPARα that were significantly increased after stimulation with TGF-β1 or after RBC phagocytosis (Figure 8A through 8D). Smad3 loss markedly reduced macrophage PPARα expression. Phagocytosis of aged RBCs markedly induced macrophage PPARγ and PPARδ synthesis. PPARγ and PPARδ expression was comparable in WT and Smad3 knockout macrophages, in the presence or absence of TGF-β1 (Figure 8B and 8C). However, Smad3 loss abrogated PPARγ and PPARδ induction in response to phagocytosis (Figure 8E and 8F).

Experiments using pretreatment with PPAR agonists and antagonists suggested important effects of PPARγ on the inflammatory profile of phagocytic macrophages. The PPARγ agonist rosiglitazone induced TGF-β1 and downmodulated CCL2 synthesis, without affecting IL-10 and BMP-2 expression in macrophages phagocytosing RBCs (Figure 8G through 8J). However, stimulation with a PPARδ agonist induced BMP-2 but had no effects on CCL2 expression and no significant effects on TGF-β1 or IL-10 synthesis (Figure 8G through 8J). PPARγ antagonism attenuated TGF-β1 expression and accentuated CCL2 and BMP-2 synthesis, without affecting IL-10 levels. In contrast, pretreatment with a PPARδ inhibitor did not affect inflammatory gene synthesis by macrophages phagocytosing aged RBCs (Figure 8K through 8N). Thus, Smad3-mediated PPARγ induction in phagocytic macrophages may be involved in anti-inflammatory transition.

Smad3 Loss Markedly Attenuates Vascular Endothelial Growth Factor-A mRNA Expression in Isolated Macrophages

Macrophages may also serve as regulators of infarct angiogenesis, in part, by secreting VEGF (vascular endothelial growth factor).[32] Smad3 knockout cells had marked and consistent reductions in VEGF-A mRNA expression levels, in the presence or absence of aged RBCs and after TGF-β1 stimulation (Figure 8O, Online Table III).

MyS3KO Mice Have No Significant Defects in Infarct Angiogenesis And in Vascular Maturation

To explore the in vivo significance of the marked attenuation of VEGF expression in the absence of Smad3, we quantitatively assessed microvascular density in the infarct and in the remote remodeling myocardium using CD31 immunohistochemistry. MyS3KO and WT mice had comparable microvascular density in both infarcted and noninfarcted areas (Figure 8P through 8R, Online Figure XII). Scar maturation is associated with formation of pericyte-coated microvessels due to recruitment of vascular mural cells. Myeloid cell–specific Smad3 loss did not affect the density of coated vessels in the infarcted myocardium (Online Figure XIII).

MyS3KO Mice Have Accentuated Cardiomyocyte Apoptosis in the Remodeling Noninfarcted Myocardium

MyS3KO mice had increased density of apoptotic cardiomyocytes in remodeling myocardial segments; in contrast, numbers of apoptotic noncardiomyocytes were comparable between groups (Online Figure XIV). Thus, adverse remodeling in MyS3KO infarcts may reflect inflammation-driven accentuation of chronic cardiomyocyte apoptosis.

Myeloid Cell–Specific Smad3 Loss Does Not Affect Myofibroblast Density and Collagen Deposition

Because Smad3 null macrophages had markedly reduced synthesis of the fibrogenic mediator TGF-β1, we examined whether myeloid cell–specific Smad3 loss affects myofibroblast conversion and fibrosis in the infarcted heart. Myeloid cell–specific loss of Smad3 did not significantly affect myofibroblast density and collagen content in the infarcted and remodeling myocardium (Online Figures XV and XVI).

Loss of Smad3 Modulates Expression of Oxidative Stress Genes in Infarct Neutrophils

To examine whether MyS3KO mice had altered neutrophil phenotype and function, we assessed expression of genes associated with oxidative stress in infarct neutrophils (Online Table IV). Neutrophils from MyS3KO mice had significantly lower expression of NOX4 (NADPH [nicotinamide adenine dinucleotide phosphate] oxidase 4, a major mediator of oxidative stress), and of several antioxidant genes, such as *Gpx3*, *Gpx7*, *Prdx4*, *Sod1*, and *Sod3* (Online Figure XVII).

Discussion

Using cell-specific loss-of-function strategies, we document for the first time a crucial in vivo role for Smad3 in regulation of macrophage function. We report several novel observations: First, we show that Smad3 is activated in macrophages, not only in response to stimulation with TGF-β isoforms but also immediately after phagocytosis of phosphatidylserine-expressing cells, through a mechanism independent of TGF-β activity. Second, macrophage Smad3 does not play a role in homeostasis, but protects the infarcted heart from adverse remodeling and is critical for activation of a phagocytic program. Third, effects of Smad3 on phagocytosis are mediated, at least in part, through synthesis of Mfge8, an integrin ligand that bridges phosphatidylserine-expressing cells with the vitronectin receptor. Fourth, Smad3 is involved in the anti-inflammatory transition of phagocytic macrophages, at least in part, by mediating PPAR-γ and PPAR-δ upregulation. Fifth, although in vitro Smad3 mediates synthesis of angiogenic and fibrogenic signals (such as VEGF and TGF-β1), in the infarct myeloid cell–specific Smad3 loss does not affect angiogenesis and myofibroblast activation. Our findings identify macrophage-specific Smad3 as a key protective pathway in cardiac repair.

Activation of Smad-Dependent and Smad-Independent Cascades in Macrophages

TGF-β stimulation rapidly activates Smad2/3 signaling while also stimulating Smad-independent cascades. The relative balance of Smad-dependent and non-Smad signaling in various cell types is dependent on their TGF-β receptor expression profile (which is affected by the differentiation stage of the cell), the modulatory effects of accessory receptors (such as endoglin),[33] and on contextual microenvironmental factors,

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such as the extracellular matrix environment and the local cytokine milieu. In macrophages, TGF-β has been reported to activate both Smad pathways,[34,35] and non-Smad MAPK cascades.[36,37] Our experiments show that in macrophages all 3 TGF-β isoforms rapidly activate Smad3, but have minimal effects on activation of non-Smad pathways (Figure 1), suggesting that the effects of TGF-βs on macrophages may be predominantly mediated through Smad-dependent signaling.

Phagocytosis Triggers Smad3 Activation

Local generation of TGF-β is likely a major stimulus involved in activation of Smad3 in infarct macrophages. Our findings identified a new potential mechanism for Smad3 activation that may be independent of ligand expression. In vitro experiments demonstrated a marked rapid increase in p-Smad3 levels in macrophages phagocytosing aged RBCs in the absence of any detectable TGF-β activity in the supernatant (Figure 2). Moreover, in vivo, a subset of phagocytic Mfge8+ cells expressed p-Smad3. These observations are consistent with a novel ligand-independent mechanism for Smad3 activation. Thus, in infarct macrophages, both stimulation with TGF-βs, and TGF-β-independent phagocytosis-associated mechanisms may activate Smad3.

Macrophage Smad3 protects the Infarcted Heart by Mediating Phagocytosis of Dead Cells

Mice with myeloid cell–specific loss of Smad3 had no baseline defects but exhibited increased late mortality and worse adverse remodeling after MI (Figure 3). Adverse outcome in MyS3KO mice after MI was associated with impaired phagocytosis of dead cardiomyocytes in the infarcted area (Figure 4). Persistent presence of nonphagocytosed cardiomyocytes in the infarct zone of MyS3KO animals occurred despite a significant increase in myeloid cell infiltration, suggesting that Smad3 absence perturbs macrophage-driven phagocytic activity. In vitro, Smad3 loss significantly impaired the ability of macrophages to phagocytose PS (phosphatidylserine)-expressing aged RBCs (Figure 5). Taken together, our in vitro and in vivo studies provide the first direct evidence suggesting a role for macrophage Smad3 signaling in phagocytosis.

What is the molecular mechanism responsible for the effects of Smad3 on phagocytosis? Our findings suggest that Smad3 is critical for transcription of several macrophage genes involved in recognition of eat-me signals by professional phagocytes (Online Figure VII, Figure 5). Expression of Mfge8, a glycoprotein secreted by macrophages that bridges phosphatidylserine in apoptotic cells with the vitronectin receptor on the cell surface of phagocytes,[30] was significantly reduced in Smad3 null macrophages in vitro and in vivo (Figure 5). In vitro, incubation with recombinant Mfge8 partly rescued the defective phagocytic capacity of Smad3 null macrophages (Figure 7). Thus, Mfge8 transcription is an important target of Smad3 that increases the phagocytic activity of macrophages.

Smad3 Regulates Anti-Inflammatory Transition of Infarct Macrophages

The role of TGF-β/Smad3 signaling in regulating the inflammatory properties of monocytes and macrophages remains poorly understood. In vitro studies had produced conflicting findings, reflecting the context-dependent actions of TGF-βs. Some in vitro studies have suggested that Smad3 is critically involved in anti-inflammatory functions of macrophages. Smad3 signaling inhibits expression of the proinflammatory genes *iNOS* and *MMP12* in isolated macrophages[6,7] and suppresses synthesis of the chemokine MCP1/CCL2[8]. The effects of Smad3 were attributed to an antagonistic effect on AP (activator protein)-1 DNA protein binding and activity.[8] In contrast, several other in vitro investigations suggested that Smad3 activation may promote proinflammatory actions. TGF-β/Smad-dependent signaling induced macrophage-derived release of BAFF (B cell activating factor), a member of the TNF family that stimulates survival and proliferation of B cells.[10] Moreover, Smad3 was found to stimulate macrophage-derived synthesis of the proinflammatory cytokine IL-23.[11]

Our study demonstrates that Smad3 loss has profound effects on inflammatory gene expression in macrophages. Although our findings are not consistent with a role of Smad3 in promoting a polarized M1 versus M2 macrophage phenotype, the data suggest that Smad3 mediates transcriptional events critical for transition of phagocytic infarct macrophages to an anti-inflammatory phenotype (Figure 6). Phosphatidylserine-dependent phagocytosis of apoptotic cells by macrophages promotes synthesis and secretion of anti-inflammatory cytokines, such as TGF-β.[38] We demonstrate that Smad3 loss accentuated expression of the proinflammatory cytokines IL-1β, TNF-α, and BMP-2 in infarct macrophages and markedly reduced their capacity to produce and secrete TGF-β1 and to synthesize IL-10 mRNA and protein in response to phagocytosis. Moreover, Smad3 knockout cells also exhibited markedly increased expression of the chemokine CCL2/MCP1 and increased levels of chemokine receptors that may account for the accentuated myeloid cell infiltration in infarcted MyS3KO mice (Figure 6, Online Figure X). The proinflammatory phenotype of Smad3 deficient macrophages may reflect, at least in part, loss of phagocytosis-induced upregulation of PPAR-γ and PPAR-δ[39]

Role of Activated Macrophages in Angiogenesis and Fibrosis

Macrophage subsets have been implicated in tissue fibrosis, through secretion of fibrogenic growth factors. The markedly reduced expression of the potent fibrogenic mediator TGF-β1 in Smad3 null macrophages suggested that macrophage-specific Smad3 loss may have profound effects on myofibroblast activation. Moreover, Smad3 null macrophages had consistently low levels of VEGF expression, raising the intriguing possibility that macrophage-specific Smad3 signaling may also promote angiogenic functions. Surprisingly, our in vivo experiments showed no significant effects of macrophage-specific Smad3 loss on myofibroblast conversion, collagen deposition, infarct angiogenesis, and vascular maturation after MI (Online Figures XII, XIII, XV). These negative findings may indicate compensation by other fibrogenic, or angiogenic mediators or may reflect alternative cellular sources for these ubiquitously-expressed growth factors. However, it should be emphasized that the absence of a detectable effect of myeloid cell–specific Smad3 loss on fibrosis and angiogenesis does not necessarily exclude important Smad3-dependent actions of macrophages on myofibroblast

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or vascular cell phenotype. In the healing infarct, activation of macrophage phagocytic activity precedes any fibrogenic or angiogenic actions of macrophage subsets. Thus, the persistent presence of nonphagocytosed cardiomyocytes and the accentuated or prolonged inflammatory activity in MyS3KO infarcts may augment fibrogenic and angiogenic signaling, despite the diminished capacity of isolated Smad3 null macrophages to secrete VEGF or TGF-β1. Thus, in MI, impaired phagocytosis, as the earliest consequence of macrophage-specific Smad3 loss, may dictate the observed in vivo phenotype.

Conclusions

We report for the first time a critical role for Smad3 signaling in regulation of macrophage function in vivo and in vitro. Our findings also highlight the challenges in designing therapeutic approaches targeting the TGF-β cascade in cardiovascular disease. TGF-β–driven Smad3 activation has important effects on all cell types involved in cardiac repair. In MI, inhibition of Smad3 in cardiomyocytes may exert protective actions by attenuating apoptosis and by reducing protease-driven adverse remodeling.[25] However, any protective effects of Smad3 loss on cardiomyocytes may be outweighed by impairment of phagocytosis and anti-inflammatory cytokine production in macrophages. Thus, successful implementation of therapeutic strategies targeting the TGF-β/Smad system may require development of cell-specific interventions, or identification of well-defined temporal windows for inhibition of specific cellular functions.

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Disclosures

None.

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EDITORIAL

How do endosomal Toll-like receptors sense and extend ischaemic myocardial injury?

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This editorial refers to 'Toll-like receptor 7 deficiency promotes survival and reduces adverse left ventricular remodelling after myocardial infarction' by D.P.V. de Kleijn *et al.*, pp. 1791–1803.

Following myocardial infarction, dying cells and fragmented extracellular matrix release damage-associated molecular patterns, which activate a family of pattern recognition receptors, the Toll-like receptors (TLRs), triggering innate immune signalling pathways.[1] The TLR family is comprised of plasma membrane receptors (including TLR1, TLR2, TLR4, TLR6, and TLR10), and nucleic acid-sensing endosomal receptors, such as TLR3, TLR7, TLR8, and TLR9.[2,3] Although TLRs have evolved as a warning system against infectious agents that elicits a protective response, inappropriate or excessive activation of TLRs following tissue injury may have catastrophic consequences. In the infarcted myocardium, the cell surface TLRs, TLR2, and TLR4, have been implicated in extension of ischaemic injury, and in the pathogenesis of adverse remodelling.[4–8] In contrast, the potential role of endosomal TLRs that may respond to nucleic acids released by dying cells to activate innate immune signalling, remains poorly understood. TLR7 recognizes purine-rich single-stranded RNA (ssRNA) and is known to elicit a protective immune response to viral infection. *In vitro*, RNA released by injured cardiomyocytes has been demonstrated to induce TLR7-dependent inflammatory leucocyte activation.[9] The potential role of TLR7-dependent signalling in the pathogenesis of myocardial injury *in vivo* remains unknown.

de Kleijn *et al.*[10] studied the role of TLR7 in repair and remodelling of the infarcted heart. They found that TLR7 expression is up-regulated in the infarcted mouse heart, and in human myocardium from patients with ischaemic heart failure. In a model of non-reperfused myocardial infarction, mice with global loss of TLR7 had a decreased incidence of cardiac rupture and exhibited attenuated adverse remodelling, associated with increased recruitment of myofibroblasts and a favourable protease/antiprotease balance. Experiments using bone marrow chimaeras demonstrated that TLR7-dependent dysfunction and remodelling of the infarcted heart were mediated predominantly through effects on bone marrow-derived cells. The findings add TLR7 to the growing list of pattern recognition receptors involved in post-infarction remodelling, and suggest a potential role for extracellular ssRNA, released by dying cells, in mediating rupture, dilation and dysfunction of the infarcted heart. Moreover, the observations raise several intriguing questions regarding the identity of the ligands activating TLR7 in the infarcted heart, the cellular targets of TLR7-mediated responses, the mechanism of delivery of infarct-derived TLR7 ligands in the endosomal compartment of leucocytes, and the downstream signalling pathways responsible for the injurious actions.

How do ssRNAs access endosomal TLR7 in leucocytes infiltrating the infarct?

Due to its endosomal localization, TLR7 requires an interaction with endocytosed ssRNA to activate an innate immune signalling response.[11] Following viral infection, viral ssRNA enters the endosome via receptor-mediated endocytosis and interacts with TLR7 initiating signal transduction. One can only speculate regarding the mechanism responsible for delivery of ssRNA released by dying cardiomyocytes to immune cells infiltrating the infarcted heart (*Figure 1*). In autoimmune diseases, such as systemic lupus erythematosus, autophagic responses have been implicated in TLR7-dependent activation of inflammatory signalling.[12] Binding of nucleic acids to high-mobility group box proteins (HMGBs) has been implicated in selective activation of endosomal nucleic-acid-sensing TLRs. In healing infarcts, early release of HMGB1[13] may prime immune cells for endocytosis of ssRNAs, thus setting the stage for activation of TLR7-mediated signalling.[14,15]

Which leucocyte subpopulations are activated by TLR7?

Although the effects of TLR7 in cardiac rupture and adverse remodelling appear to involve bone marrow cells, the specific leucocyte subsets that respond to ssRNAs, activating TLR7 signalling is unknown. Constitutive expression of TLR7 has been documented in dendritic cells and B cells; lower levels of TLR7 may also be expressed in non-immune cells, including cardiomyocytes.[9] Following injury, activation of nuclear factor (NF)-κB induces TLR7 in a wide range of cell types, including macrophages.[16]

The opinions expressed in this article are not necessarily those of the Editors of *Cardiovascular Research* or of the European Society of Cardiology.

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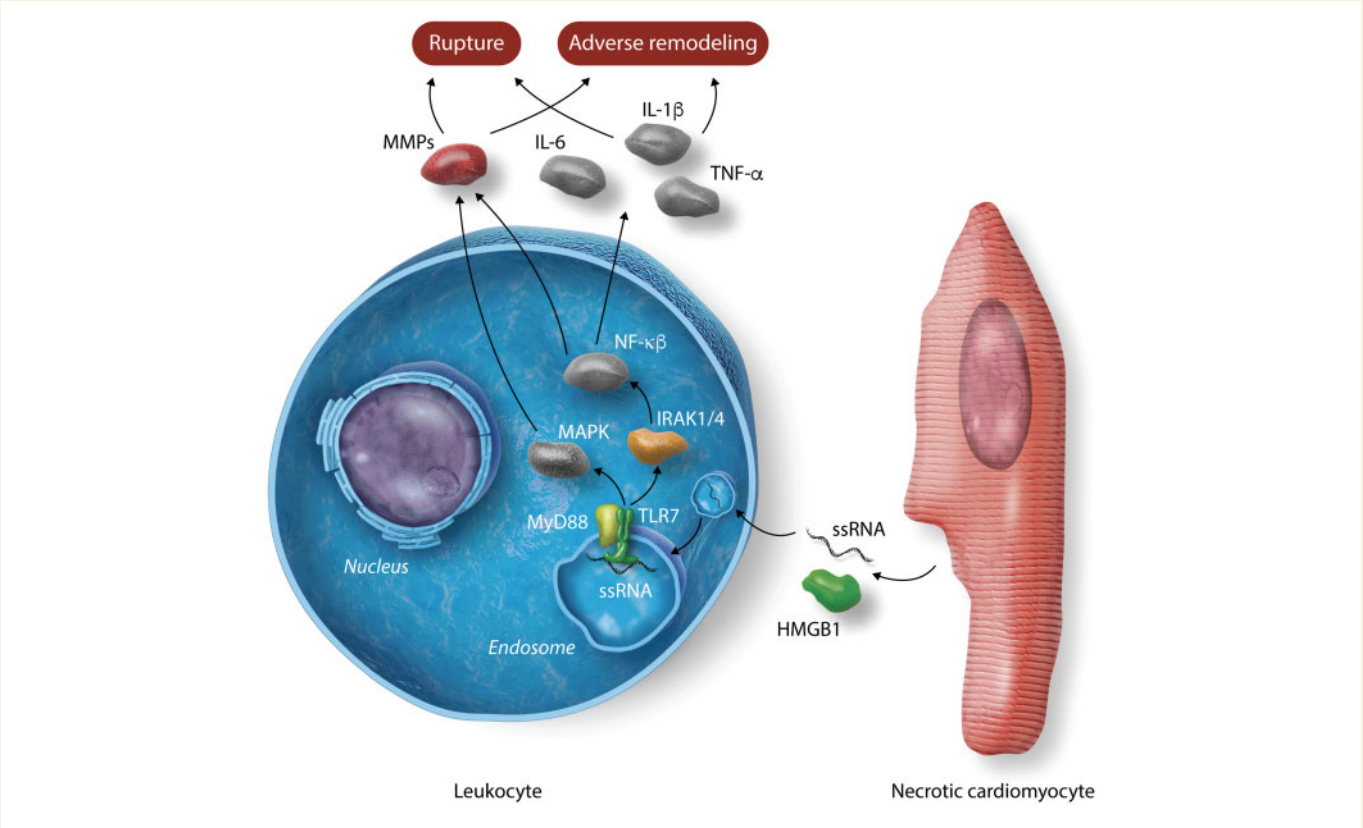


Figure 1 The role of the endosomal Toll-like receptor 7 (TLR7) in remodelling of the infarcted heart. The study by De Kleijn *et al.* suggests that endosomal TLR7 may be activated in infarct leucocytes, presumably by single-stranded RNA (ssRNA) released by dying cardiomyocytes, accentuating the immune response and promoting extracellular matrix degradation through release of matrix metalloproteinases (MMPs). Several important questions remain to be answered: (i) How does ssRNA find its way to leucocyte endosomes to activate TLR7? Receptor-mediated endocytosis and autophagic pathways may be implicated. Moreover, release of high-mobility group box protein 1 (HMGB1) following infarction may prime ssRNA for endocytosis. (ii) Which cells exhibit TLR7 activation? TLR7 is constitutively expressed by dendritic cells and B lymphocytes and is markedly induced following inflammation in macrophages and in non-immune cell types. (iii) Which downstream signals mediate the effects of TLR7 in the infarcted heart? Following interaction with ssRNA in endosomes, TLR7 dimerizes and may activate nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) through a myeloid differentiation primary response protein 88 (MyD88)-dependent pathway, thus inducing synthesis of cytokines and chemokines. TLR7-mediated MMP secretion may also play a role in mediating cardiac rupture and adverse remodelling of the infarcted heart. Additional symbols: IL, interleukin; TNF, tumour necrosis factor; IRAK, interleukin-1 receptor-associated kinase.

Thus, in the cytokine-rich environment of the infarcted heart, increased expression of TLR7 in many different cell types may account for its pro-inflammatory and matrix-degrading actions. Cell-specific loss-of-function approaches are needed to dissect the relative role of various immune cell subsets in mediating TLR7-dependent responses.

Which molecular signals are responsible for TLR7-dependent actions?

Upon interaction with ssRNA, TLR7 dimerizes and associates with myeloid differentiation primary response protein 88 (MyD88), thus initiating signal transduction, and stimulating downstream activation of mitogen-activated protein kinases (MAPKs), the NF-κB cascade, interleukin-1 receptor-associated kinases (IRAKs), and tumour necrosis factor receptor associated factors (TRAF) 3/6 (*Figure 1*). In human immune cells, TLR7 activation triggers synthesis of a wide range of pro-inflammatory cytokines, including tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6,[17] mediators with a critical role in the post-infarction inflammatory response.[18] Accentuated induction of pro-inflammatory cytokines may account for TLR7-dependent cardiac rupture and may accentuate dilative remodelling following infarction. The current study suggests that TLR7 signalling mediates MMP release in the infarcted area, thus promoting a matrix-degrading environment that may cause left ventricular rupture. TLR7-mediated MMP release may be due to cytokine-stimulated protease induction, or may reflect direct effects of TLR7 on MMP synthesis by activated leucocytes.

Conclusions

Emerging evidence suggests that endosomal TLRs (such as TLR7) do not simply serve to protect from viral infections, but may be inappropriately activated following non-viral tissue injury, accentuating inflammation. In the infarcted myocardium, activation of TLR7 signalling may have catastrophic consequences, causing rupture and precipitating heart failure.

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Further studies are needed to dissect the cellular basis for the deleterious effects of TLR7 in the infarcted heart, and to suggest a plausible mechanism for endosomal delivery of ssRNA in infarct leucocytes. If consistent and reproducible, the impressive beneficial effects of disrupting the ssRNA: TLR7 interaction suggested by the current study, may suggest TLR7 antagonism as a promising therapeutic approach for patients with myocardial infarction.

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EDITORIAL

S100A8/A9 as a therapeutic target in myocardial infarction: cellular mechanisms, molecular interactions, and translational challenges

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This editorial refers to 'Inhibition of pro-inflammatory myeloid cell responses by short-term S100A9 blockade improves cardiac function after myocardial infarction', by G. Marinkovic *et al.*, doi:10.1093/eurheartj/ehz461.

Following myocardial infarction, massive necrosis of myocardial cells releases damage-associated molecular patterns (DAMPs) that stimulate systemic inflammation, and promote recruitment of inflammatory leucocytes in the infarct zone. Infiltrating leucocytes clear the infarct of dead cells and matrix debris, and stimulate repair of the infarcted myocardium, secreting cytokines and growth factors that activate fibroblasts and vascular cells, and contribute to formation of a scar. Over the last 30 years, a large body of experimental evidence suggested that in addition to their reparative functions, pro-inflammatory cascades may also exert detrimental actions in the infarcted heart, extending ischaemic injury and accentuating adverse remodelling.[1] In experimental animal models of myocardial infarction, targeted inhibition of innate immune cascades, chemokines, cytokines, and adhesion molecules was protective, decreasing the size of the infarct, and reducing adverse dilative remodelling. Unfortunately, despite highly promising animal model evidence, clinical studies testing the effectiveness of anti-inflammatory strategies in human patients with myocardial infarction have produced disappointing results.[2]

The S100 calcium-binding proteins are abundantly expressed in sites of inflammation and serve as biomarkers reflecting disease activity in many inflammatory conditions. Some members of the family, including S100A7, S100A8, S100A9, S100A12, and S100A15, may act as DAMPs triggering or amplifying innate immune pathways following tissue injury.[3] Induction and release of S100 family members has been documented in the infarcted myocardium, and has been suggested to mediate pro-inflammatory cascades.[4,5] S100A8 and S100A9 typically exist as a S100A8/S100A9 heterodimer, are predominantly produced by myeloid cells (both neutrophils and monocytes), and are known to activate innate immune pathways through stimulation of Toll-like receptor 4 (TLR4)- and receptor for advanced glycation end-products (RAGE)-mediated cascades.[3] Although increased expression of S100A8 and S100A9 has been demonstrated both in animal models of myocardial ischaemia and in human myocardial infarction patients,[6] the potential role of S100A8/A9 as an alarmin involved in ischaemic injury, post-infarction inflammation, and cardiac remodelling has not been investigated.

In the current issue of the *European Heart Journal*, Marinkovic and co-workers[7] examined the role of S100A8/A9 in myocardial infarction in both human patients and mouse models. In patients with acute coronary syndromes, high plasma levels of S100A8/A9 during the acute event were associated with late development of systolic dysfunction and with a higher incidence of heart failure hospitalizations 1 year after the coronary event. In mouse models of reperfused and non-reperfused myocardial infarction, early short-term treatment with a small molecule inhibitor that blocks interactions between S100A9 and its receptors TLR4 and RAGE had lasting protective effects, attenuating systolic dysfunction, and increasing cardiac output for at least 3 weeks after infarction. The beneficial actions of the S100A9 inhibitor were attributed to attenuated recruitment of neutrophils and inflammatory monocytes, and to activation of a reparative phenotype in infiltrating macrophages. The findings provide important new information on the pathophysiological role of S100 proteins in mediating inflammatory injury and adverse remodelling following myocardial infarction, and suggest that S100A9 may be a novel and promising therapeutic target in patients with myocardial infarction. After three decades of frustrating translational failures in targeting inflammation following myocardial infarction, could S100A8/A9 represent the optimal inflammatory target that may allow effective and selective inhibition of injurious immune signalling in the

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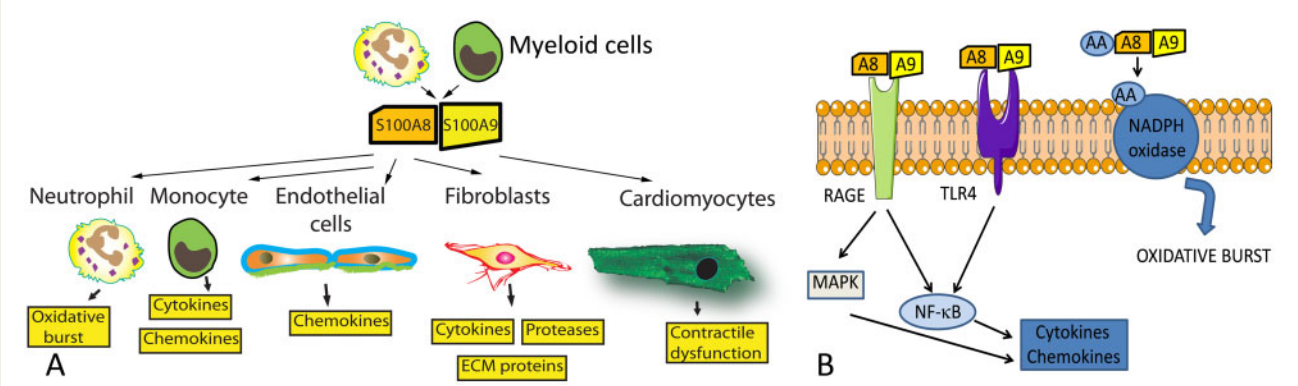


Figure 1 The cellular and molecular targets of S100A8/A9 in the infarcted heart. (*A*) Following myocardial infarction, S100A8 and S100A9 are secreted primarily from activated myeloid cells (neutrophils and monocytes). The pro-inflammatory effects of the S100A8/A9 heterodimer in the infarcted myocardium may involve: (i) activation of oxidative responses in neutrophils,; (ii) induction of pro-inflammatory cytokines and chemokines in monocytes and macrophages; (iii) chemokine induction in endothelial cells; and (iv) increased synthesis of cytokines, proteases, and extracellular matrix (ECM) proteins in fibroblasts. Moreover, S100A8/A9 may promote contractile dysfunction in cardiomyocytes. (*B*) The pro-inflammatory actions of secreted extracellular S100A8/A9 in various cell types may be mediated through activation of Toll-like receptor 4 (TLR4) and receptor for advanced glycation end-products (RAGE) signalling, leading to downstream activation of nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK). Moreover, in myeloid cells, S100A8/A9 may transfer arachidonic acid (AA) to the NADPH oxidase complex, activating the oxidative burst.

infarcted heart without perturbing repair? If so, which cellular mechanisms may explain the protective effects of S100A8/A9 inhibition?

S100A8/A9 may act both as an intracellular mediator and as a secreted signal

Predominantly expressed by myeloid cells, S100A8 and S100A9 are known to act both as intracellular effectors and as secreted alarmins. As intracellular signals, S100A8/A9 control microtubule organization, mediating migratory responses of neutrophils and mononuclear phagocytes.[8] Following inflammatory injury, increased S100A8/A9 levels in the extracellular space appear to reflect active secretion, rather than passive release from necrotic cells. S100A8 and S100A9 proteins lack a leader signal and are not secreted through the classical Golgi-associated pathway, but are actively secreted in response to cytokine stimulation via tubulin-dependent mechanisms.[9] As a secreted alarmin, the S100A8/A9 heterodimer may modulate the phenotype and function of all cells involved in cardiac repair, including leucocytes, vascular endothelial cells, cardiomyocytes, and reparative fibroblasts (*Figure 1A*).

Effects of S100A8/A9 on myeloid cells

Inflammatory activation of neutrophils and macrophages is a well-documented action of S100A8/A9 *in vitro* and *in vivo*, and may explain, at least in part, the protective actions of S100A9 blockade in the infarcted myocardium. The pro-inflammatory actions of S100A8/A9 may involve activation of several distinct mechanisms (*Figure 1B*); their relative contribution *in vivo* remains unclear. First, S100A8/A9 stimulates a pro-inflammatory programme through activation of TLR4 signalling and downstream stimulation of nuclear factor (NF)-κB signalling.[10] Secondly, effects of S100A8/A9 may also involve activation of RAGE-dependent signalling.[11] Thirdly, S100A8/A9 may activate oxidative burst in neutrophils by transferring arachidonic acid to NADPH oxidase.[12]

Effects of S100A8/A9 on endothelial cells, fibroblasts, and cardiomyocytes

In addition to its effects on immune cells, secreted S100A8/A9 may also exert important actions on vascular endothelial cells, interstitial fibroblasts, and cardiomyocytes. In healing infarcts, endothelial cells are important sources of chemokines and, through their adhesive interactions with circulating leucocytes, play a critical role in recruitment of inflammatory cells in the area of injury. S100A8/A9 can bind to proteoglycans on the endothelial surface, triggering chemokine induction,[13] and promoting leucocyte infiltration. Fibroblasts, the main matrix-producing cells in healing infarcts, also respond to S100A8/A9 stimulation, secreting cytokines, proteases, and extracellular matrix proteins.[14] Surviving cardiomyocytes in the infarct border zone may also be directly targeted by S100A8/A9 released by myeloid cells. Experiments in endotoxin-stimulated cardiomyocytes demonstrated that S100A8/A9 transduces RAGE-dependent signals that mediate contractile dysfunction.[15] In the healing infarct, the protective effects of S100A9 inhibition on systolic function may involve direct protective effects on cytokine-stimulated cardiomyocytes that may attenuate contractile dysfunction.

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S100A8/A9 as a therapeutic target in myocardial infarction

Despite the promising experimental evidence provided by Marinkovic *et al.*,[7] therapeutic targeting of S100A8/A9 in patients with myocardial infarction may pose major challenges. First, the broad cellular effects of S100A8/A9 on all cell types involved in myocardial injury and repair complicate therapeutic implementation. Inhibition of activity and function of professional phagocytes may delay clearance of apoptotic cells, leading to secondary necrosis and promoting sustained inflammation and injury. Moreover, abrogation of S100A8/A9-mediated actions on fibroblast activity may perturb reparative functions. Although no functional perturbations were noted in the current study upon S100A9 blockade, effects on the reparative response were not systematically studied. Secondly, the translational value of animal models of myocardial infarction is limited by the absence of directly translatable outcome endpoints, and by the pathophysiological heterogeneity of human post-infarction remodelling that cannot be recapitulated in animal investigations. In mouse models of myocardial infarction, injury is caused by surgical occlusion of the coronary artery; thus, any effects of the intervention on thrombotic events cannot be evaluated. On the other hand, mortality endpoints are difficult to interpret, as arrhythmic and heart failure-related deaths (the most common causes of mortality in human infarction) are relatively uncommon. Thus, conclusions regarding outcome are derived primarily through extrapolation of effects on systolic function, or geometric remodelling. Moreover, the mouse model is designed to dissect cellular and molecular mechanisms, by minimizing pathophysiological variability. For this purpose, genetically identical, sex- and age-matched young mice are studied in a well-standardized surgical model of infarctive injury. In contrast, human patient populations exhibit marked variations in the intensity and profile of inflammatory and remodelling responses following infarction. This variability is dependent not only on the size of the initial injury, but also on a wide range of factors that may affect the inflammatory response (such as age, sex, genetic differences, other concomitant conditions, the pattern of coronary disease, the use of medications, etc.). The pathophysiological heterogeneity of human myocardial infarction cannot be simulated by an animal model. It is likely that targeting inflammation may be effective only in patient subpopulations with excessive, sustained, or unrestrained inflammatory responses. Thus, successful translation of S100A8/A9 targeting in human patients may require identification of subsets of patients with exaggerated alarmin-driven immune reactions. Acute coronary syndrome patients with very high circulating levels of S100A8/A9 were identified in the current study, and may represent a promising target group for such an intervention.

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REVIEW
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The Role of the TGF-β Superfamily in Myocardial Infarction

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The members of the transforming growth factor β (TGF-β) superfamily are essential regulators of cell differentiation, phenotype and function, and have been implicated in the pathogenesis of many diseases. Myocardial infarction is associated with induction of several members of the superfamily, including TGF-β1, TGF-β2, TGF-β3, bone morphogenetic protein (BMP)-2, BMP-4, BMP-10, growth differentiation factor (GDF)-8, GDF-11 and activin A. This manuscript reviews our current knowledge on the patterns and mechanisms of regulation and activation of TGF-β superfamily members in the infarcted heart, and discusses their cellular actions and downstream signaling mechanisms. In the infarcted heart, TGF-β isoforms modulate cardiomyocyte survival and hypertrophic responses, critically regulate immune cell function, activate fibroblasts, and stimulate a matrix-preserving program. BMP subfamily members have been suggested to exert both pro- and anti-inflammatory actions and may regulate fibrosis. Members of the GDF subfamily may also modulate survival and hypertrophy of cardiomyocytes and regulate inflammation. Important actions of TGF-β superfamily members may be mediated through activation of Smad-dependent or non-Smad pathways. The critical role of TGF-β signaling cascades in cardiac repair, remodeling, fibrosis, and regeneration may suggest attractive therapeutic targets for myocardial infarction patients. However, the pleiotropic, cell-specific, and context-dependent actions of TGF-β superfamily members pose major challenges in therapeutic translation.

Keywords: TGF-β, myocardial infarction, Smad, BMP, GDF, fibrosis, inflammation

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INTRODUCTION

In human patients, myocardial infarction is typically caused by thrombotic occlusion of a coronary artery, leading to prolonged cessation of blood flow in the territory subserved by the vessel. Ischemia-induced disruption of aerobic metabolism in cardiomyocytes immediately perturbs their contractile function and ultimately results in catastrophic structural changes (1). After 20–30 min of ischemia, subendocardial cardiomyocytes in the area at risk exhibit irreversible changes. Longer ischemic intervals trigger a "wavefront" of cardiomyocyte death that ultimately involves the entire thickness of the ventricular wall supplied by the occluded coronary (2). Because the adult mammalian heart has negligible regenerative capacity, repair of the infarcted myocardium is dependent on formation of a collagen-based scar, and requires activation of a superbly orchestrated inflammatory/reparative response (3). Inflammatory chemokines and cytokines are critically involved in the reparative process, mediating activation of macrophages that clear the wound from dead cells and matrix debris, and triggering expansion of reparative cells (including fibroblasts

and vascular cells) that form granulation tissue and secrete a collagen-based matrix network. Although scar formation protects the heart from catastrophic early complications (such as cardiac rupture), infarct healing is closely intertwined with adverse remodeling, a constellation of cellular events that involve both infarcted and non-infarcted myocardium, and lead to chamber dilation, exacerbating dysfunction.

The members of the TGF-β superfamily play important roles in cell survival, differentiation, proliferation, and function and have been implicated in regulation of inflammatory and reparative responses. Following myocardial infarction, induction, and activation of endogenous TGF-β signaling pathways has been suggested to modulate injury, regulate inflammation, and orchestrate repair (4). This review manuscript discusses our current knowledge on the role of TGF-β superfamily members in injury, repair, and remodeling of the infarcted heart.

THE MEMBERS OF THE TGF-β SUPERFAMILY

In humans, the TGF-β superfamily is comprised of 33 members that can be subclassified into several subfamilies, including the TGF-βs (TGF-β1, -β2, and -β3), the bone morphogenetic proteins (BMPs), the Growth differentiation factors (GDFs), the activins, the inhibins, nodal, and anti-Mullerian hormone proteins. Although TGF-β1 remains the best-studied member of the superfamily in myocardial infarction, a growing body of evidence implicates several other members in regulation of cardiac repair and post-infarction remodeling.

THE TGF-βs

TGF-β Expression in Normal Myocardium

High constitutive expression of TGF-β is noted in both embryonic and adult mammalian hearts, and is predominantly localized in cardiomyocytes, or bound to the extracellular matrix (5). TGF-βs play an important role in cardiac development; loss of TGF-β isoforms in neonatal mice is associated with abnormal cardiac development and defective valve formation (6, 7). In normal adult mammalian hearts, TGF-β is stored in a latent form; whether low-level constitutive TGF-β activity is required to support homeostatic cardiac function remains unknown. *In vivo*, targeting Smad4 (the common intracellular effector of responses triggered by many members of the TGF-β superfamily) in adult cardiomyocytes was associated with impaired ventricular function and with perturbations in ion channel gene expression (8). Moreover, in neonatal rat cardiomyocytes cultured on fibroblast matrix under serum-free conditions, exogenous TGF-β sustains spontaneous rhythmic beating (9). However, the *in vivo* relevance of this observation is unclear.

Induction and Cellular Origin of TGF-βs in the Infarcted Myocardium

Upregulation of TGF-βs is well-documented in both mouse and large animal models of myocardial infarction (5, 10–15). In a mouse model of reperfused myocardial infarction, TGF-β1 and TGF-β2 mRNA levels peak early after 6–72 h of reperfusion; in contrast, TGF-β3 exhibits a prolonged time course and is persistently upregulated after 7 days of reperfusion (10). The distinct time course of TGF-β3 may be due to cell-specific expression patterns, or may reflect isoform-specific effects of stimuli inducing transcription of TGF-βs.

Although most myocardial cell types are capable of synthesizing and releasing significant amounts of TGF-βs; their relative contribution remains poorly defined. In a porcine model of coronary occlusion, cardiomyocytes were a major source of TGF-β (11). On the other hand, studies in mouse models of myocardial infarction suggest that infarct macrophages may be key contributors to the TGF-β response (16). Genetic disruption of the chemokine monocyte chemoattractant protein (MCP)-1/CCL2, a mediator with a crucial role in recruitment of monocytes/macrophages in inflamed tissues, was associated with reduced TGF-β2 and TGF-β3 mRNA expression following myocardial infarction, consistent with an important role for infiltrating mononuclear cells in TGF-β synthesis in the infarcted heart (17). TGF-β synthesis may mark specific subsets of infarct macrophages that respond to cytokine stimulation in the pro-inflammatory environment of the infarct (18). In addition to cardiomyocytes and macrophages, several other cell types may also secrete TGF-βs in the infarcted heart; however, robust documentation of their contribution is lacking. Platelets constitutively express significant amounts of growth factors and have been suggested to be an important source of TGF-β1 in the pressure-overloaded myocardium (19). Abundant platelets infiltrate the infarct during the early stages of repair; however, their relative role as a source of TGF-βs has not been investigated. Activated fibroblasts, vascular cells, mast cells and lymphocyte subsets can produce and secrete TGF-βs, and are found in significant numbers in infarcted hearts (20–23). However, whether they are a significant source of TGF-β isoforms following infarction remains unknown.

TGF-β Activation in the Infarcted Myocardium

In the infarcted myocardium, activated cells enrich the existing stores of latent TGF-β through *de novo* synthesis of all 3 isoforms. However, this is not sufficient for activation of TGF-β cascades. Transduction of TGF-β signals in sites of injury requires liberation of the active TGF-β dimer from the latent complexes. TGF-βs are secreted in a latent form that is comprised of the TGF-β dimer, the latency-associated peptide (LAP), which confers latency to TGF-β (24, 25), and a latent TGF-β-binding protein (LTBP), which serves to sequester the complex into the extracellular matrix (26). Extensive data from animal models suggest that myocardial infarction is associated with rapid activation of TGF-β (27), followed by stimulation of downstream Smad-dependent signaling cascades (28). However, the molecular signals that trigger TGF-β activation following infarction remain poorly understood. Several mechanisms may be involved. First, cell surface integrins may interact with the LAP, thus releasing the TGF-β dimer (29). Although *in vitro* studies have implicated αvβ5 and αvβ3 integrins in latent

TGF-β activation and in subsequent myofibroblast conversion (30); the *in vivo* significance of these interactions in the infarcted myocardium remains unknown. Second, a wide range of proteases (including serine proteases, cathepsins, matrix metalloproteinases, and cysteine proteases) have been implicated in TGF-β activation following injury. Proteases are rapidly activated following myocardial infarction (31–33); however, their potential role in activation of TGF-β in infarcted hearts remains poorly documented. Third, reactive oxygen species (ROS) are rapidly generated in ischemic hearts and may be involved in activation of TGF-β activation in the infarcted myocardium. Fourth, specialized extracellular matrix proteins (including matricellular proteins, such as thrombospondin-1), are markedly upregulated in the infarcted heart (34–36) and may play an important role in TGF-β activation. Thrombospondin-1 has been suggested to interact with the LAP, promoting release of the active TGF-β dimer from the latent complex (37). In the infarcted myocardium, TSP-1 upregulation is associated with activation of TGF-β signaling in the infarct border zone in both mouse and canine infarcts (34). Finally, an acidic environment has been suggested to promote TGF-β activation (38). Generation of lactic acid in the ischemic myocardium may reduce the pH, contributing to activation of latent TGF-β stores following infarction.

Cellular Actions of TGF-β in the Infarcted Myocardium (Figure 1)

TGF-β1, β2, and β3 are activated in the infarcted myocardium and may regulate phenotype and function of all cell types involved in cardiac injury and repair. Although the 3 TGF-β isoforms exhibit distinct patterns of regulation following infarction, little is known regarding isoform-specific actions in the infarcted heart. Most *in vitro* studies have investigated TGF-β1-mediated actions. *In vivo* experiments on the other hand, have focused on the role of TGF-β receptor-activated signaling, exploring pathways common to all three isoforms and to other members of the TGF-β superfamily.

Effects of TGF-β on Ischemic Cardiomyocytes

Early studies in isolated perfused hearts undergoing brief myocardial ischemia followed by reperfusion, suggested protective effects of TGF-β, mediated through attenuation of oxidative stress and reduced release of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α (39). *Ex vivo* and *in vitro* experiments examining the effects of TGF-β on cardiomyocyte survival have produced conflicting results. In a model of reperfused feline myocardial infarction, TGF-β1 administration reduced cardiomyocyte death, and attenuated neutrophil recruitment in the infarcted myocardium (40). It is unclear whether TGF-β-mediated protection in these studies was due to activation of direct pro-survival effects in cardiomyocytes, or reflected suppression of injurious inflammatory signaling. TGF-β1 infusion in isolated perfused hearts, protected cardiomyocytes from apoptosis through actions involving p42/p44 mitogen-activated protein kinase (MAPK) signaling during early reperfusion (41). In contrast, in rat cardiomyocytes, the pro-apoptotic effects of angiotensin II were attributed to activation of TGF-β signaling (42). Moreover, *in vitro* studies have suggested direct actions of TGF-β on cardiomyocyte function, mediated through upregulation of the laminin receptor 37/67 (43). Considering its notoriously pleiotropic and context-dependent actions, the effects of exogenous TGF-β administration are likely dose- and time-dependent, and are affected by the experimental conditions.

In vivo, mouse models of cardiomyocyte-specific TGF–β receptor disruption have been used to investigate endogenous effects of TGF–βs on cardiomyocytes (44), In a model of non-reperfused myocardial infarction, mice with cardiomyocyte-specific loss of the type 1 or type 2 TGF-β receptor were protected from death due to cardiac rupture, exhibiting marked reduction in neutrophil infiltration. The mechanism of protection remains poorly defined. It was suggested that TGF-β signaling in cardiomyocytes may suppress synthesis of cardioprotective genes, such as interleukin (IL)-33, GDF-15, and TSP-4.

TGF-β and the Post-Infarction Inflammatory Reaction

TGF-βs are critical modulators of immune cell phenotype and function (45). *In vitro*, TGF-βs can exert pro- or anti-inflammatory depending on the phenotype and the state of differentiation of the cells, and the presence or absence of other mediators. TGF-β is a potent neutrophil (46) and monocyte (47) chemoattractant; these effects may be important for recruitment of leukocytes in inflamed tissues. Despite exhibiting strong chemotactic actions for monocytes and neutrophils in single-cell assays, TGF-β may attenuate transendothelial leukocyte migration by reducing surface expression of adhesion molecules (48). Moreover, in monocytes picomolar concentrations of TGF-β stimulate synthesis of pro-inflammatory cytokines and chemokines (47, 49). In contrast to its pro-inflammatory actions in monocytes, TGF-β is known to deactivate macrophages, suppressing expression of chemokines (such as MCP-1/CCL2) and cytokines, (including IL-1β and TNF-α) (50–52). Whether TGF–β acts as a pro- or anti-inflammatory mediator *in vivo* is dependent on the context: the cell types involved, their phenotypic profile, and the cytokine environment. Thus, attribution of pro- or anti-inflammatory properties to TGF-βs should be based on documentation of robust *in vivo* effects, rather than on *in vitro* assays.

In vivo, TGF-β1 plays an essential role in immune cell homeostasis by preventing spontaneous inflammation in mammalian tissues. 50% of TGF-β1 null mice develop massive multi-organ inflammation 2–4 weeks after birth, mainly affecting the heart and lungs (53). The anti-inflammatory effects of TGF-β in normal tissues may involve suppressive actions on T cell inflammatory activity (54).

In the healing infarct, timely recruitment of inflammatory leukocytes, and subsequent activation of reparative macrophages play a crucial role in repair, but may also mediate fibrotic responses that promote adverse remodeling (3, 55, 56). TGF–β may critically regulate inflammatory leukocyte recruitment and function following myocardial infarction. *Ex vivo* studies using cardiac lymph collected following reperfused infarction as a window to the cardiac microenvironment suggested that TGF-β activity may be implicated in recruitment of

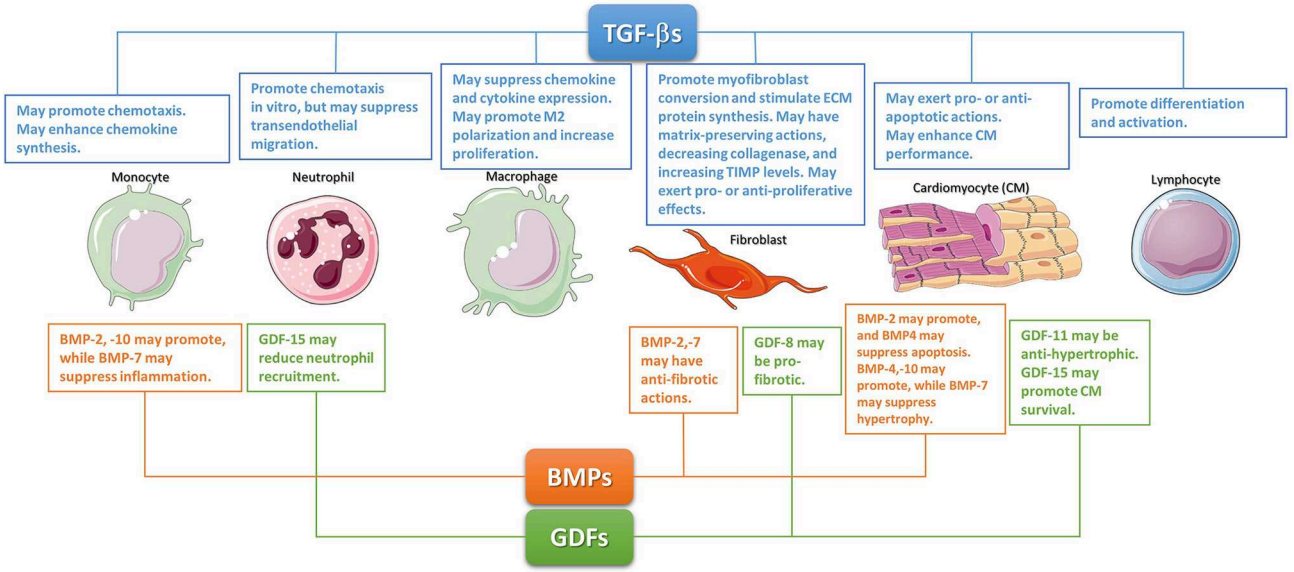


FIGURE 1 | The diverse cellular actions of the TGF-βs, BMPs, and GDFs in the infarcted myocardium. During the inflammatory phase of infarct healing, TGF-βs may regulate cardiomyocyte survival, promote monocyte chemotaxis, and modulate lymphocyte differentiation and activation. TGF-βs may be critical in modulating macrophage phenotype toward an anti-inflammatory M2 phenotype. These effects may act as a switch from inflammation to repair. The effects of BMPs and GDFs have not been systematically studied; however, several members of these subfamilies may regulate inflammation. During the reparative phase, TGF-βs are critical regulators of fibroblast activation. Although studies in cardiac fibroblasts suggested TGF-βs can have either pro- or anti- proliferative effects, TGF-βs have consistent effects on fibroblast activation, mediating myofibroblast conversion and stimulating exctracellular matrix protein synthesis. Moreover, TGF-βs also promote a matrix-preserving program by decreasing collagenase synthesis, and by upregulating tissue inhibitors of metalloproteinases (TIMPs). Members of the GDF and BMP subfamilies may also regulate phenotype and function of reparative cells. The cartoon was designed using Servier Medical Art (https://smart.servier.com/).

inflammatory monocytes in the healing infarct (27). *In vivo* experiments have suggested anti-inflammatory actions of TGF-β following infarction. Early systemic inhibition of TGF-β signaling through transfection with the extracellular domain of TβRII increased mortality in a model of non-reperfused infarction, accentuating neutrophil recruitment, and inducing expression of cytokines and chemokines (57). The specific effects of TGF-β signaling on the phenotype of immune cell subpopulations infiltrating the infarcted myocardium have not been systematically studied. TGF-β may be a critical modulator of macrophage phenotype, promoting M2 polarization (58) and suppressing expression of inflammatory cytokines and chemokines (58, 59), enhancing macrophage-colony stimulating factor (M-CSF)-induced proliferation (60), inhibiting nitrite release (61), and reducing cytotoxic activity (62). TGF-β also acts as a central mediator in T lymphocyte differentiation and activation, critically regulating phenotype and function of all subpopulations (63–65). CD4+ T cells (66) and foxp3+ regulatory T cells (Tregs) (20–22), have been implicated in regulation of repair and remodeling following myocardial infarction Whether TGF-β signaling cascades play a crucial role in differentiation and phenotypic modulation of lymphocytes in the infarcted heart remains unknown.

Effects of TGF-β on Fibroblast Phenotype and Function

During the reparative phase of cardiac repair, resident cardiac fibroblast populations become activated (67, 68), undergoing myofibroblast conversion, and secreting large amounts of structural extracellular matrix proteins (69–73). Activated infarct fibroblasts also produce cytokines, growth factors, and matricellular proteins, and participate in matrix metabolism through secretion of MMPs. TGF-β plays a crucial role in expansion of the myofibroblast population in the healing infarct and stimulates expression of extracellular matrix proteins. TGF-β potently induces expression of α-smooth muscle actin in cardiac fibroblasts, the hallmark of myofibroblast conversion (74, 75). Moreover, TGF-β significantly and consistently stimulates synthesis and secretion of extracellular matrix proteins (such as collagen I, collagen III, and fibronectin) (76, 77), and promotes a matrix-preserving phenotype by suppressing collagenase expression, and by accentuating synthesis of tissue inhibitor of metalloproteinases (TIMPs) (28, 78, 79). Whether TGF-β stimulates fibroblast proliferation in the infarcted heart is less clear. *In vitro* studies have demonstrated both proliferative and anti-proliferative effects of TGF-β on cardiac fibroblasts (77, 80). Differences in TGF–β concentration, distinct phenotypic profiles, or states of differentiation of fibroblast populations, and differences in the growth factor and extracellular matrix environment may account for the conflicting observations.

Although genetic interventions to study the *in vivo* effects of fibroblast-specific TGF-β signaling in infarcted hearts have not been performed, a large body of evidence suggests that TGF-β may play a critical role in regulation of fibroblast phenotype in healing infarcts. In a model of non-reperfused infarction, TGF-β inhibition through administration of a neutralizing antibody had

detrimental effects, accentuating chamber dilation, increasing myocardial MMP expression, and reducing collagen synthesis (81). Moreover, two independent investigations inhibiting TGF-β through gene transfer of the extracellular domain of TβRII suggested that TGF-β may play a crucial role in fibrosis of the infarcted heart (57, 82). TGF-β inhibition after the inflammatory phase of cardiac repair attenuated deposition of fibrous tissue in the infarcted region (82). The observed effects may reflect, at least in part, loss of TGF-β signaling in cardiac fibroblasts.

Effects of TGF-β on Endothelial Cell Phenotype and on Infarct Angiogenesis

Endothelial cells, the most abundant non-cardiomyocytes in the adult mammalian myocardium (83), play an important role in regulation of the post-infarction inflammatory response and in repair of the infarcted heart. During the inflammatory phase of infarct healing, endothelial cells undergo activation, producing large amounts of chemokines (84, 85), and expressing adhesion molecules. Stimulated endothelial cells interact with activated circulating leukocytes promoting their recruitment in the healing infarct (86). During the proliferative phase, angiogenic activation of endothelial cells (87) may be important for generation of neovessels that supply the healing tissue with oxygen and nutrients. Finally, during the maturation phase, endothelial cells may recruit mural cells, by producing platelet-derived growth factor-BB (88, 89); thus restraining angiogenesis, suppressing inflammation and contributing to formation of a mature scar (90).

Although both endothelial cells and pericytes are highly responsive to TGF-β (91), the role of TGF-β-mediated actions on the infarct microvasculature remains unknown. It has been suggested that suppressive effects of TGF-β on endothelial cell chemokine synthesis may contribute to suppression and resolution of the post-infarction inflammatory response (92). TGF-β may also be implicated in regulation of infarct angiogenesis and may contribute to vascular maturation by modulating interactions between endothelial cells and mural cells. It should be emphasized that TGF-β actions on endothelial cells may be either angiogenic, or angiostatic, depending on contextual factors (91, 93).

TGF-β AND CARDIAC REGENERATION

The adult mammalian myocardium has extremely limited regenerative capacity that is overwhelmed by the massive loss of cardiomyocytes following myocardial infarction. Accentuation of the regenerative capacity of the heart in order to remuscularize the infarcted myocardium is a major visionary goal in cardiovascular research. Experimental evidence suggests a broad and context-dependent role for TGF-β in cardiac regeneration. TGF-β may regulate cardiomyocyte differentiation *in vitro* (94), may be involved in endogenous remuscularization in regenerative models of cardiac injury (95), and may modulate regenerative responses to cell therapy following myocardial infarction (96). An *in vitro* study showed that TGF-β stimulation increases expression of cardiac transcription factors in murine embryonic stem cells driving cardiomyocyte differentiation (97). In contrast, in other *in vitro* experiments, a highly selective TGF-β inhibitor that promotes proteasomal degradation of TβRII enhanced differentiation of uncommitted mesoderm to cardiomyocytes (98). *In vivo* studies in fish models have supported the involvement of TGF-β in regulation of endogenous cardiac regeneration. In zebrafish, pharmacologic inhibition of TβRI abolished heart regeneration following cryoinjury (95). In contrast, a pharmacological inhibitor of the type I TGFβ receptor was found to enhance proliferation of Nkx2.5+ cardiomyoblasts (99), and improved endogenous cardiomyoblast-mediated regeneration in mice (96). TGF-β has also been found to modulate the effects of cell therapy. In a mouse model of myocardial infarction, intramyocardial implantation of TGF-β pre-programmed CD117+ stem cells was reported to promote regeneration and stimulate angiogenesis (100). The conflicting findings from various studies likely reflect the differences between experimental models and the context-dependent actions of TGF-β that typically depend on the state of differentiation of target cells. Considering that high levels of active TGF-β in the infarcted mammalian myocardium are not associated with any significant regenerative activity, it is highly unlikely that stimulation of the TGF-β cascade may be sufficient to promote remuscularization following cardiac injury. However, through its broad and pronounced effects on differentiation of many cell types, TGF-β may modulate phenotype and function of myocardial progenitor cells, thus critically regulating their regenerative capacity.

THE BMP/GDF SUBFAMILY

BMPs

BMPs are a group of evolutionarily conserved, structurally related secreted cytokines with an essential role in development and in the pathogenesis of many pathologic conditions (101). Although the name reflects the well-documented osteo-inductive properties of many members of the group, BMPs are pleiotropic and multifunctional, exerting a wide range of actions in many different cell types (102). Development of genetic loss of function models has documented the role of several BMPs in cardiac development (103, 104). Unfortunately, the potential involvement of BMPs in repair, remodeling and fibrosis of the infarcted heart remains poorly investigated.

BMP2

In vitro, BMP2 induces differentiation of cardiac progenitor cells into mature cardiomyocytes, stimulating expression of cardiac-specific genes (105). Knockout models in mice have demonstrated that BMP2 is critically involved in cardiac development (106), coordinating atrioventricular canal morphogenesis (107). Whether BMP2 plays a role in homeostatic function of the adult heart remains unclear. *In vitro*, BMP2 was found to stimulate contraction in adult rat cardiomyocytes, through activation of PI-3K (108).

Following myocardial infarction, BMP2 shows an early time course of upregulation. In a model of non-reperfused myocardial infarction, transient early induction of BMP2 was followed by increased expression of other BMPs (109). BMP2 expression

in infarcted hearts was localized in both cardiomyocytes and interstitial fibroblasts (110, 111); however, the mechanism of upregulation remains unknown. Very little is known regarding the function of endogenous BMP-2 in myocardial infarction. Accentuation of post-infarction inflammation in mice lacking the BMP inhibitor gremlin (109) may suggest important pro-inflammatory actions of BMPs. Whether neutrophils, macrophages and lymphocytes, essential effector cells of post-infarction inflammation, are major targets of BMP2 is unknown. Moreover, BMP2 may exert cytoprotective actions on cardiomyocytes. *In vitro*, BMP2 inhibits apoptosis of serum-deprived cardiomyocytes (112). In mice undergoing permanent coronary occlusion protocols, intravenous BMP-2 injection attenuated apoptosis of infarct border zone cardiomyocytes, reducing the size of the infarct (113). Although inhibitory effects of BMP-2 on fibroblast function have been reported (114), the significance of such actions in repair following myocardial infarction and in cardiac fibrosis remains unclear. Angiogenic effects of BMP2 have been documented *in vitro* and *in vivo* (115); however, the potential role of BMP2 as a regulator of neovessel formation in healing myocardial infarction has not been investigated (**Figure 1**).

BMP4

Much like BMP2, BMP4 also promotes cardiomyocyte differentiation of cardiac progenitor cells (116, 117) Defective atrioventricular septation in BMP4 KO mice suggests a critical role for BMP4 in cardiac development (118). Adult human and mouse hearts exhibit constitutive BMP4 expression that increases following myocardial infarction (109, 119). The molecular mechanisms and predominant cellular sources responsible for BMP4 upregulation following infarction remain unknown. Limited information is available on the role of BMP4 following myocardial infarction. *In vitro* BMP4 has been suggested to promote apoptosis in neonatal rat cardiomyocytes (120). *In vivo*, in a model of ischemia/reperfusion, heterozygous BMP4 +/– null mice had reduced infarct size, and attenuated cardiomyocyte apoptosis (121). BMP4 may also stimulate a hypertrophic response in cardiomyocytes of the infarct border zone or the remote remodeling myocardial areas (122). In addition to effects on cardiomyocytes, BMP4 may also modulate inflammatory, fibrogenic and angiogenic responses. *In vitro* studies using endothelial cells and macrophages have suggested both pro- and anti-inflammatory actions of BMP4 (123, 124). BMP4 also stimulates angiogenic activation of endothelial cells through a Smad1/5-mediated pathway (125). Unfortunately, experimental studies exploring the role of these BMP4-mediated actions in myocardial infarction have not been performed (**Figure 1**).

BMP6

BMP6 is critically involved in iron homeostasis. Targeted disruption of BMP6 induces rapid and massive deposition of iron in many organs, including the heart (126). Iron overload in BMP6 null mice is associated with marked reduction of hepcidin (126), a key regulator of iron transport. BMP6 is expressed in developing hearts and mice with combined BMP6 and BMP7 loss exhibit a delay in formation of the outflow tract endocardial cushions (127). Although a clinical study showed increased circulating levels of BMP6 in patients with advanced heart failure (128), information on the regulation and role of BMP6 in the infarcted myocardium is lacking.

BMP7

Although BMP7 is highly expressed in the developing myocardium (129), BMP7 loss does not affect cardiac development, and defects in BMP7 null mice are confined to the kidney and the eye (130). A systematic study of myocardial BMP expression in non-reperfused myocardial infarcts found no significant upregulation of BMP7 mRNA expression 1–21 days following coronary occlusion, but suggested trends toward reduced levels at early timepoints (109). BMP7 exerts important actions on fibroblasts, cardiomyocytes and macrophages. Anti-fibrotic effects of BMP7 have been reported in many systems and may be mediated, at least in part, through suppression of collagen synthesis by cardiac fibroblasts (131), and through inhibition of endothelial to mesenchymal transition (EndMT) (132). In cardiomyocytes, BMP7 attenuates hypertrophy by inhibiting TGF-β responses (131). In macrophages, BMP7 has been reported to promote M2 polarization (133). Considering the absence of endogenous BMP7 induction in infarcted and remodeling hearts, administration of exogenous BMP7 has been suggested as a potential strategy to attenuate fibrosis and adverse remodeling. In a rat model of non-reperfused infarction systemic administration of BMP7 was reported to reduce mortality and attenuate ventricular dysfunction (134). Although the protective effects of exogenous BMP7 were attributed to anti-fibrotic actions; dissection of the cellular targets and molecular mechanisms was not performed.

BMP10

In the developing heart, BMP10 is regulated by myocardin and plays an important role in cardiomyocyte proliferation and chamber maturation (135, 136). In the postnatal heart, BMP10 expression seems to be restricted to the right atrium (137). Experiments in a non-reperfused mouse model of myocardial infarction showed a 2-fold increase in BMP10 levels 21 days after coronary occlusion. The cellular source of BMP10 in the infarcted heart, and the basis for the delayed pattern of induction remain unknown. Moreover, the effects of endogenous BMP10 in repair and remodeling of the infarcted heart have not been investigated. *In vitro* assays demonstrated that BMP10 enhances TNF-α-driven monocyte recruitment to the vascular endothelium promoting inflammation (138). However, considering the late time course of induction in the healing infarct, pro-inflammatory effects of BMP10 are unlikely to play an important role in the infarcted heart. BMP10 has been also reported to exert pro-hypertrophic actions in cardiomyocytes through an interaction with titin-cap (Tcap) (139). A study in a rat model of myocardial infarction suggested that exogenous BMP10 administration may stimulate cardiomyocyte cell cycle reentry (140). Considering the negligible proliferative activity of cardiomyocytes in infarcted and remodeling hearts, despite increased BMP10 levels and the challenges in robust

documentation of proliferating cardiomyocytes, the significance of these findings in cardiac repair is unclear.

GDFs

Growth/differentiation factors (GDF1 to 15) are produced as inactive precursor proteins, which are then cleaved and assembled into active secreted homodimers. GDF dimers are disulfide-linked ligands that bind to a diverse array of receptors. (141, 142).

GDF8

GDF8 (also known as myostatin) is a critical negative regulator of growth and mass of skeletal and cardiac muscle, and plays an important role in energy homeostasis in adult cardiomyocytes. Cardiomyocyte-specific deletion of GDF8 in adult mice is associated with cardiomyocyte hypertrophy and heart failure, by promoting AMP-activated kinase activation (141). Following infarction, rapid upregulation of GDF8 has been reported within minutes after coronary ischemia (143). Surviving cardiomyocytes in the border zone of the infarct have been suggested as the main cellular source of GDF8 in infarcted and remodeling hearts (142). The functional role and cellular targets of GDF8 in the infarcted heart remain poorly understood. A study using a global loss-of-function model GDF8 loss may protect the infarcted heart, attenuating late dysfunction. The protective effects of GDF8 loss were attributed to attenuated fibrosis (144). The findings of this study are difficult to interpret, considering the baseline effects of myostatin loss on the myocardium, and the challenges in identifying cell-specific mechanisms. Pro-fibrotic actions of GDF8 in the myocardium may involve direct activation of fibroblasts (145–147), or effects on other cell types such as cardiomyocytes and macrophages.

GDF11

Publication of a high impact study suggesting that GDF11 levels decline with aging and that GDF11 administration may reverse age-related hypertrophy (148) (**Figure 1**) generated great interest in the potential role of this approach as therapy to reverse aging in many tissues (149). Subsequent studies have challenged the notion that GDF11 can rejuvenate the aging heart (150). Thus, additional work is necessary to settle this controversy. Although effects of GDF11 on hypertrophic responses have been suggested in models of left ventricular pressure overload (151), the regulation, cellular effects and actions of endogenous GDF11 in the infarcted myocardium have not been systematically investigated. Exogenous delivery of GDF11 was found to be protective in mouse and rat models of myocardial ischemia/reperfusion. (152, 153) Beneficial effects of GDF11 were attributed to improved angiogenesis and homing of endothelial progenitors (152), or to attenuated oxidative stress (153).

GDF15

GDF-15 is an inflammation-associated hormone with an important protective role in a wide range of infectious and inflammatory conditions (154, 155). In the mammalian myocardium, GDF15 is secreted by cardiomyocytes and may regulate body growth. In pediatric patients with heart disease, increased secretion of GDF15 negatively regulates body growth through effects on liver-derived growth hormone signaling (156). GDF15 expression is rapidly increased following myocardial ischemia and is predominantly localized in cardiomyocytes (157). Extensive experimental evidence suggests a crucial role for GDF15 in regulation of cardiomyocyte survival, post-infarction inflammation, repair, and remodeling of the infarcted heart. In a model of reperfused myocardial infarction, GDF15 exerted pro-survival actions on cardiomyocytes, reducing the size of the infarct (157). Moreover, in a model of non-reperfused infarction, GDF15 protected from cardiac rupture, restraining neutrophil recruitment through suppression of leukocyte integrin activation (155). Clinical studies have suggested that serum GDF15 levels may improve risk stratification in both STEMI and non-STEMI patients, providing important prognostic information beyond established clinical and biochemical markers (158–160) (**Figure 1**).

THE INHIBIN/ACTIVIN SUBFAMILY AND THE ROLE OF THE FOLLISTATINS

Activins are dimeric polypeptides composed of two β subunits of inhibin linked by a disulfide bridge (161). Five types of β subunits have been described (βA, βB, βC, βD, and βE); activin nomenclature is defined by the type of subunits that form the dimer. In mammals, activins A (βA/βA), B (βB/βB), AB (βA/βB), C (βC/βC), and E (βE/βE) have been isolated; however, only activins A, B and AB have been studied and are known to exhibit biological activity (162).

In a rat model of non-reperfused myocardial infarction activin βA was markedly upregulated after 2 days of coronary occlusion and remained elevated for at least 28 days. Activin A protein was predominantly localized in surviving cardiomyocytes (163). A very rapid upregulation of activin A was reported in a model of reperfused myocardial infarction, occurring as early as 30 min after coronary ischemia. Early induction of activin A was dependent on TLR4 signaling (164). Overexpression experiments *in vivo* and *in vitro* suggested that activin A may protect ischemic cardiomyocytes from apoptosis (165). Activin A may also activate fibroblasts, promoting cell proliferation and collagen synthesis through p38 and Erk MAPK pathways (166).

The activin system is regulated by follistatin and follistatin-like 3 (Fstl3), extracellular proteins that bind activins with high affinity to form inactive complexes. In a rat model of myocardial infarction, administration of exogenous follistatin was reported to attenuate inflammation (167). Endogenous Flstl3 is upregulated following myocardial infarction and attenuates the cardioprotective effects of activin A (165). Cardiomyocyte-derived Flstl3 may also exert hypertrophic actions (168) and may stimulate fibroblasts in a paracrine manner, promoting a fibrogenic phenotype (169). It should be emphasized that Flstl3 actions *in vivo* may be, at least in part, independent of activin inhibition. Activin actions may also be relevant in human ischemic heart disease. Patients with both ischemic and

non-ischemic heart failure had elevated circulating levels of activin A (163).

SIGNALING PATHWAYS ACTIVATED BY THE TGF-β SUPERFAMILY IN THE INFARCTED MYOCARDIUM (FIGURE 2)

The members of the TGF-β superfamily signal through binding to dual specificity kinase receptors (that have characteristics of both serine/threonine and tyrosine kinases) on the cell surface. Binding of a TGF-β family member assembles a heterotetrameric complex of 2 type 1 and 2 type II TGF-β receptors (170, 171). In humans, there are 7 type I receptors, termed activin-like receptor kinase (ALK)1-7, and 5 type II receptors; each member of the TGF-β superfamily signals by binding to characteristic combinations of these receptors. TGF-β isoforms transduce signaling through a single type II receptor (TβRII); in contrast, most BMPs are more promiscuous binding to three different type II receptors (ActRII, ActRIIB, and BMPRII). Binding of TGF-βs superfamily members to the type II receptor promotes oligomerization and transphoshorylates a type I receptor kinase. Activation of specific type I receptors accounts for part of the cellular specificity of TGF-β actions. For example, in most cell types TGF-βs activate the ubiquitously expressed type I receptor ALK5 (172). In endothelial cells, TGF-βs exerts distinct effects through another type I TGF-β receptor, ALK1 (173). Subsequently, activated type I receptors phosphorylate intracellular transcriptional effectors, the receptor-activated Smads (R-Smads). TGF-β-induced ALK5 activation, or activin-mediated ALK4 stimulation typically trigger phosphorylation of Smad2 and Smad3. TGF-β-induced ALK1 activation or BMP signaling generally phosphorylate Smad1, Smad5, and Smad8. Activated R-Smads form complexes with the common Smad, Smad4, and translocate to the nucleus, where they recruit co-activators or co-repression of transcriptional complexes and modulate gene transcription (174). In addition to their effects mediated through activation of Smads, the members of the TGF-β superfamily also signal through non-Smad pathways, by activating mitogen-activated protein kinase (MAPK) family responses, PI3K/Akt, focal adhesion kinase (FAK), and Rho GTPase pathways (175, 176). The multiple interactions between

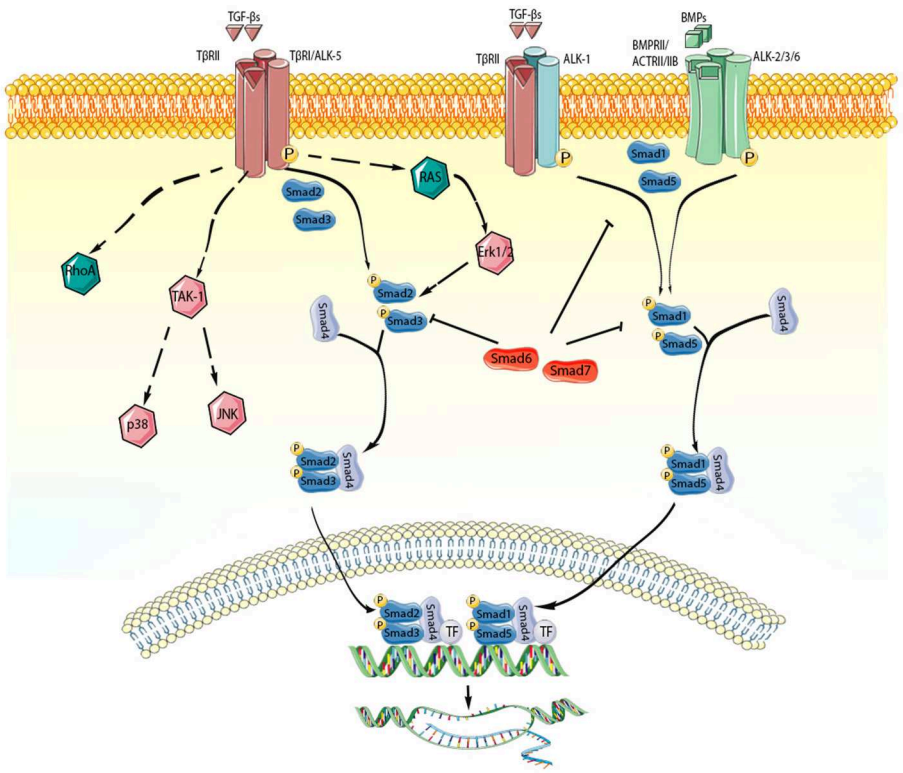


FIGURE 2 | TGF-β superfamily members act through activation of Smad-dependent and Smad-independent signaling pathways. Each member of the TGF-β superfamily signals by binding to distinct combinations of type II, and type I receptors. TGF-β dimers bind TβRII inducing transphosphorylation of the GS segments in the type I receptor (ALK-5). Activated ALK-5 phosphorylates the receptor-activated Smads (R-Smads) Smad2 and Smad3, which then form a heterotrimeric complex with the common Smad, Smad4, promoting the translocation of the Smad complex to the nucleus. Interactions between the Smad and transcriptional activators or repressors regulate transcription of target genes. In some cell types (such as endothelial cells), TGF-βs may act through the type I receptor ALK1, stimulating Smad1/5/8 signaling. BMP dimers on the other hand bind their specific type II receptors, subsequently activating type I receptors (ALK-2,-3,-6) and phosphorylating Smad1/5. Smad1 and Smad5 then bind to Smad4 and translocate to the nucleus regulating transcription. In addition, TGF-β superfamily members signal through non-canonical Smad-independent cascades. The inhibitory Smads (Smad6 and Smad7) negatively regulate TGF-β superfamily cascades. The cartoon was designed using Servier Medical Art (https://smart.servier.com/).

signaling responses of TGF-β superfamily members and other cascades (such as Wnt and Notch signaling) (177) generate additional layers of complexity that may explain, at least in part, the context-dependent actions of TGF-βs.

Smad-Dependent Signaling in Myocardial Infarction

The ALK5-Smad2/3 Cascade in the Infarcted Heart

Activation of Smad2 and Smad3 signaling is consistently noted in infarcted hearts, and is localized in border zone cardiomyocytes, fibroblasts, and immune cells (13, 28, 178–180). ALK5 inhibition attenuated Smad2 activation in healing infarcts (181). However, the relative contribution of various TGF-β superfamily members in activating Smad2 and Smad3 remains unclear. *In vivo* experiments using cell-specific knockout strategies suggested that Smad3 signaling exerts important actions in regulating cardiomyocyte, fibroblast, and macrophage function following myocardial infarction. In cardiomyocytes, Smad3 activation increased apoptosis, enhancing nitrosative stress, and increasing expression of MMPs in the remodeling myocardium (178). On the other hand, activation of Smad3 in myofibroblasts protected the infarcted heart from late rupture, and played a major role in repair of the infarct, promoting formation of organized arrays of reparative myofibroblasts in the border zone, through activation of an integrin-ROS axis (178). Macrophage-specific Smad3 activated a phagocytic program and played a major role in transition of macrophages to an anti-inflammatory phenotype (179). In contrast, Smad2 activation may have more subtle and limited effects in cardiac repair. Myofibroblast-specific loss of Smad2 was associated with a transient improvement in post-infarction remodeling, without affecting mortality, scar organization, or long-term outcome (180).

The Role of the Smad1/5/8 Cascade in Myocardial Infarction

Although the Smad1 cascade is also activated in the ischemic and infarcted heart (182–184) relatively little is known regarding its role in regulation of phenotype and function of surviving cardiomyocytes, immune cells, fibroblasts, and vascular cells. Whether Smad1 activation in infarcts is mediated predominantly through BMPs, or reflects effects of TGF-βs on cells expressing ALK1 is unknown. Cardiomyocyte-specific overexpression of Smad1was found to be protective, reducing infarct size in a model of ischemia/reperfusion, by attenuating activation of a pro-apoptotic program (182). However, the potential role of endogenous Smad1 activation in regulation of cardiac injury, repair, and remodeling has not been investigated.

Activation of Smad-Independent Pathways in the Infarcted Heart

Although the members of the TGF-β superfamily are known to activate a wide range of non-canonical cascades, (including MAPK, TAK-1, and Rho GTPase) (177, 185, 186), the relative contribution of these pathways in myocardial infarction remains unknown. *In vitro* experiments and associative data have suggested that activation of a TAK-1/p38 MAPK axis by TGF-β may be involved in hypertrophic remodeling of surviving myocardium in the infarcted heart (187). In cardiac fibroblasts, stimulation of p38 MAPK may play an important role in fibrogenic activation (188) and may mediate hypertrophy (189). However, considering the broad range of mediators that signal through MAPKs, the role of TGF-β-driven activation of p38 in the infarcted heart is unclear.

TARGETING THE TGF-β SUPERFAMILY IN MYOCARDIAL INFARCTION

Considering the critical role of TGF-β signaling cascades in tissue repair, remodeling, fibrosis and regeneration, the members of the TGF-β superfamily have been considered attractive therapeutic targets for patients with myocardial infarction (190). Unfortunately, the pleiotropic and context-dependent actions of TGF-βs, the unique and complex biology of TGF-β activation, and the complexity of the signaling cascades activated by the members of the superfamily pose major challenges in therapeutic translation. Clearly, activation of TGF-β signaling affects phenotype and function of all cells involved in cardiac repair, exerting both protective and detrimental effects. Our current knowledge of the cell-specific actions of TGF-βs *in vivo* is not sufficient to design therapy. Moreover, the distinct actions of TGF-β-mediated Smad-dependent and non-Smad pathways add a layer of complexity that further complicates therapeutic translation. The pathophysiologic heterogeneity of myocardial infarction represents another major challenge. In the clinic, patients surviving acute myocardial infarction may exhibit very different responses, which are not always dependent on the extent of initial injury. Some patients tend to develop dilative remodeling that may be due to accentuated or unrestrained inflammatory activation. Other patients may have extensive fibrosis, associated with diastolic heart failure (191). Patient-specific TGF-β responses may be critical in determining the outcome in myocardial infarction. Thus, identification of patient subpopulations with overactive TGF-β signaling through the use of carefully validated biomarkers, or imaging studies, will be essential in order to design personalized treatment strategies. In any case, targeting the TGF-β system carries significant risks, because of its critical role in homeostasis and repair and its broad effects on many different cell types (192). For example, extensive evidence suggests that disruption of the TGF-β/Smad3 axis may promote aortic aneurysm formation and rupture (193–195). Thus, attempts for clinical translation of TGF-β/Smad inhibition strategies following infarction should focus on brief therapeutic interventions, and may need to exclude vulnerable patients.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITORIAL COMMENT

Can Myocardial Fibrosis Be Reversed?*

Nikolaos G. Frangogiannis, MD

Fibrosis accompanies most cardiac pathophysiological conditions. Because the adult mammalian heart has negligible regenerative capacity, sudden massive loss of cardiomyocytes following myocardial infarction heals through formation of a collagen-based scar, leading to development of "replacement fibrosis." Several other pathophysiological perturbations, such as pressure overload, obesity, diabetes, and metabolic dysfunction, are associated with interstitial or perivascular fibrotic changes, presumably caused by primary activation of resident cardiac fibroblasts, in the absence of significant cardiomyocyte death. In a wide range of human populations, the presence and extent of cardiac fibrosis predicts adverse outcome. In both heart failure with preserved ejection fraction and in heart failure with reduced ejection fraction, the extent of cardiac fibrosis is associated with increased mortality and a higher incidence of hospitalizations (1–3). Adverse outcome in subjects with prominent fibrosis may reflect perturbations in systolic or diastolic ventricular function, or the occurrence of arrhythmias and electrical conduction defects.

Fibroblasts are the main effector cells in the pathogenesis of cardiac fibrosis (4,5). In failing hearts, neurohumoral mediators, cytokines and growth factors, and specialized extracellular matrix proteins cooperate to activate cardiac fibroblasts, promoting their conversion to activated myofibroblasts that proliferate and synthesize large amounts of extracellular matrix (ECM) proteins (6,7). Although in repair of the infarcted heart, activation of fibroblasts is transient, serving to form a scar that preserves the structural integrity of the ventricle (8), persistent or overactive fibroblast responses may lead to excessive deposition and cross-linking of ECM proteins in the cardiac interstitium, reducing cardiac compliance and perturbing propagation of the electrical impulse. Inhibition of fibrosis and restoration of normal myocardial structure and function remain a major visionary goal in the treatment of heart failure. However, to what extent established cardiac fibrosis can be reversed remains unknown.

SEE PAGE 2267

In this issue of the *Journal*, Nagaraju et al. (9) studied the phenotypic characteristics of fibroblasts harvested from failing human hearts. End-stage heart failure patients with both ischemic and dilated cardiomyopathy showed prominent fibrotic remodeling and ECM cross-linking. Most fibroblasts harvested from failing hearts exhibited a myofibroblast phenotype and expressed high levels of matricellular proteins and cross-linking enzymes. In vitro, inhibition of transforming growth factor (TGF)-β signaling converted heart failure myofibroblasts into quiescent cells, promoting depolymerization of stress fibers, reducing α-smooth muscle actin (SMA) expression, and attenuating synthesis of ECM proteins and inflammatory cytokines. The findings support the notion that activated cardiac myofibroblasts in end-stage heart failure patients retain the potential to revert to an inactive quiescent phenotype following inhibition of a single critical activating pathway. However, is this simple strategy sufficient to reverse established cardiac fibrosis in vivo?

THE PHENOTYPIC PLASTICITY OF CARDIAC FIBROBLASTS

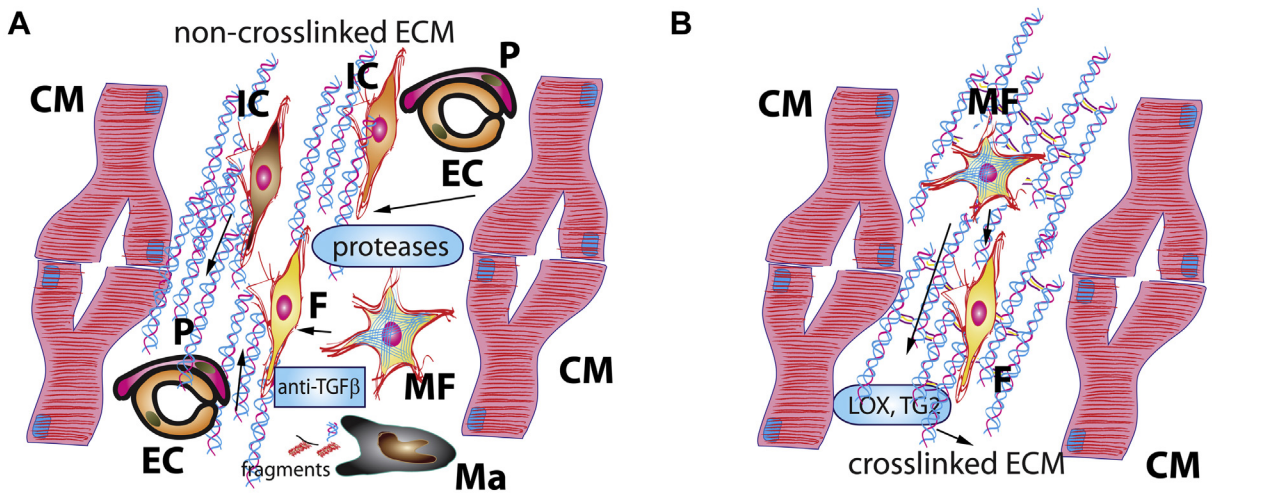
The ability of activated fibroblasts to revert into a quiescent phenotype, as noted in the current study, is not surprising. Fibroblasts exhibit remarkable phenotypic plasticity. In vitro, fibroblasts cultured in

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FIGURE 1 Is Myocardial Fibrosis Reversible?



To reverse replacement fibrosis, resorption of fibrous tissue needs to be coupled with robust myocardial regeneration. The latter is currently not feasible in adult human hearts. Reversal of interstitial fibrosis poses several challenges (see text) and may require co-operation of several different cell types **(A)**. Fibrotic foci containing large amounts of cross-linked protease-resistant extracellular matrix (ECM) (mediated through induction of lysyl oxidases [LOX] and transglutaminase-2 [TG2]) may be irreversible **(B)**. Reversal of fibrosis may require a dynamic and cellular interstitium capable of clearing excessive ECM deposits. Deactivation of myofibroblasts (MF) through inhibition of critical fibrogenic stimuli (such as transforming growth factor [TGF]-β) may be necessary to prevent progression of the fibrotic response, but may not be sufficient to reverse fibrosis. Resorption of interstitial fibrosis may also require recruitment of reparative interstitial cell subsets (IC), rapid clearance of proinflammatory matrix fragments by macrophages (Ma), and production of specialized ECM components by fibroblasts (F), endothelial cells (EC), and cardiomyocytes (CM) to restore normal cardiac architecture. P = pericyte.

plates, a high-tension environment, rapidly convert to myofibroblasts and express large amounts of ECM proteins. By contrast, when these same cells are enmeshed within the low-tension environment of a free-floating collagen pad, stress fibers disassemble, α-SMA expression is markedly reduced, and collagen synthesis is attenuated (10). In a similar manner, following myocardial infarction, fibroblast populations undergo dramatic phenotypic changes, transitioning through proinflammatory and matrix-synthetic myofibroblast phenotypes, only to become quiescent or undergo apoptosis during the maturation phase of healing (11,12). However, clearance of fibrotic changes and restoration of normal cardiac architecture and function in the failing heart require a lot more than deactivation of cardiac fibroblasts.

THE CHALLENGES OF REVERSING CARDIAC FIBROSIS

In order to improve cardiac function, reversal of cardiac fibrosis requires both localized resorption of excessive ECM and restoration of normal myocardial structure. The negligible endogenous regenerative potential of the adult mammalian heart, the biological properties of the cross-linked ECM, and the inherent proinflammatory actions of matrix fragments (which may be recognized as danger signals by resident immune cells) pose several major challenges in designing strategies aimed at reversing cardiac fibrosis (**Figure 1**). First, degradation of large areas of replacement fibrosis could be catastrophic unless accompanied by robust cardiac regeneration. Despite extensive research in the field, there are currently no effective approaches to regenerate the adult human heart. Second, cross-linked interstitial and perivascular matrix may be highly resistant to proteases. Matrix cross-linking enzymes, such as lysyl oxidases (13) and transglutaminase-2 (14), are markedly induced in remodeling and failing hearts, and may cause formation of irreversible fibrotic foci (**Figure 1B**). Third, even when locally confined to the interstitium, ECM degradation could exert potent proinflammatory actions. Matrix fragments can act as damage-associated molecular patterns, activating cytokine and chemokine-mediated inflammation. Unless rapidly cleared, these fragments may have adverse consequences on myocardial structure and function.

CO-OPERATION OF SEVERAL DISTINCT CELL TYPES MAY BE NEEDED TO REMODEL THE FIBROTIC INTERSTITIUM

In the absence of an effective regenerative approach, fibrosis may only be reversible if it is confined to the interstitium, and it is not associated with high levels of crosslinked ECM. Although deactivation of fibroblasts would be required to reverse fibrosis, TGF-β inhibition alone may not be sufficient. In experimental models of cardiac fibrosis, induction and activation of TGF-β is followed by endogenous repression of Smad-dependent signaling. Although this endogenous process may prevent expansion of fibrosis, it is clearly not sufficient for reversal. Reversing fibrosis is an active process that may require co-operation of interstitial cell subsets, immune cells, vascular cells, and cardiomyocytes (**Figure 1A**). In a study analyzing samples biopsied from patients with ischemic cardiomyopathy undergoing aortocoronary bypass surgery, we found that myocardial segments that recovered function following revascularization exhibited higher interstitial cellularity, increased levels of the matricellular protein tenascin-C (15), and accentuated inflammatory activity (16). Considering the likely requirement for resorption of interstitial fibrotic changes for functional recovery following revascularization, these findings may suggest an important role for interstitial and inflammatory cells in reverse remodeling of the failing myocardium. Cardiac interstitial cells remain poorly characterized and may include reparative subpopulations with matrix-preserving properties, capable of restoring the normal structure of the ECM. Macrophages may also be required to rapidly clear any matrix fragments generated in the remodeling interstitium, thus protecting from their proinflammatory actions. Cardiomyocyte-derived signals may also be needed to down-modulate fibroblast activity and to restore architecture by regulating matrix metabolism.

Very little is known regarding the molecular signals that may be implicated in reverse remodeling of the fibrotic heart. The absence of robust animal models to study reversal of fibrosis and the difficulties in documenting the serial changes associated with reverse remodeling pose major challenges in understanding the cell biological mechanisms. Considering the dependence of both systolic and diastolic cardiac function on preservation of interstitial architecture, and the critical role of fibroblasts in repair and remodeling of failing hearts, targeting the cardiac interstitium may hold the key for treatment of heart failure.

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Editorial

The cell biological basis for primary unloading in acute myocardial infarction

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Early initiation of mechanical circulatory support improves survival in patients with acute myocardial infarction (MI) complicated by refractory cardiogenic shock. In contrast, the potential benefits of mechanical support in MI patients without overt cardiogenic shock remain unclear. In patients with acute anterior ST elevation MI (STEMI), early mechanical support through intra-aortic balloon counterpulsation (IABC) prior to percutaneous coronary intervention (PCI) did not affect infarct size. However, 6 months after the acute event, the exploratory composite endpoint of time to death, shock or new or worsening heart failure was significantly reduced in the IABC + PCI group, raising the intriguing possibility for lasting beneficial effects of early mechanical support in patients with large anterior STEMI [1].

In the current issue of the journal, Baldetti and co-workers [2] provide an interesting and provocative perspective on the potential role of mechanical unloading performed prior to reperfusion in acute MI. This approach, termed primary unloading may exert potent cardioprotective actions. The authors review the published evidence, discuss the highly promising experimental animal studies, and propose the need for new clinical studies to test this new approach. However, the mechanistic basis for the proposed protection remains unclear. Considering the complexities of the cell biological and molecular responses in the ischemic myocardium, how can early mechanical unloading attenuate injury?

1. Effects on ischemic cardiomyocytes and on the microvasculature

Sudden coronary occlusion results in immediate cessation of aerobic metabolism, inducing rapid ATP depletion and accumulation of metabolites. Severe systolic dysfunction occurs within seconds, whereas minutes later early ultrastructural changes are noted in cardiomyocytes. These changes are initially reversible; however with coronary ischemic intervals longer than 15–20 min, an increasing number of cardiomyocytes are unable to preserve their structure and exhibit irreversible changes that lead to their death. It has been suggested that ischemia activates a "wavefront" of death, mediated through stimulation of necrotic and apoptotic pathways, ultimately leading to massive loss of cardiomyocytes in the area at risk. Lowering end-diastolic pressures and volumes through mechanical unloading reduces wall stress, and may protect vulnerable cardiomyocytes from death by limiting metabolic activity [3], or by attenuating mechanosensitive activation of apoptotic signals. Unloading-mediated activation of cytoprotective cascades that inhibit cardiomyocyte apoptosis has also been proposed [4]; however, specific mechanisms remain unclear. Preservation of calcium handling through unloading [5] may also improve contractility. Moreover, reduction in end-diastolic pressures may significantly improve coronary arteriolar flow, resulting in better perfusion of the myocardium, thus amplifying protection. These beneficial effects may significantly reduce the size of the infarct [6,7].

2. Unloading may preserve the cardiac extracellular matrix (ECM) under conditions of stress

Often overlooked, the cardiac ECM network plays a critical role in preserving myocardial structure and function, not only by providing mechanical support, but also by transducing key survival signals in cardiomyocytes. In the ischemic myocardium, rapid release and activation of matrix-degrading proteases generates matrix fragments, termed matrikines [8]. Increased ECM fragmentation has been suggested to trigger inflammation, while promoting cardiomyocyte death under conditions of stress, thus accentuating myocardial dysfunction [9]. Mechanical unloading reduces matrix metalloproteinase (MMP) expression and activity in the ischemic heart [10] and may preserve the ECM network, inhibiting ischemia-induced cell death responses and attenuating inflammatory activation. It should be emphasized that MMPs have numerous substrates in addition to ECM proteins, and may modulate levels and activity of pro-inflammatory chemokines and cytoprotective signals. For example, it has been suggested that lower MMP activity in unloaded hearts may results in decreased degradation of the "cardioprotective" chemokine CXCL12/stromal cell-derived factor (SDF)-1α, promoting cardiomyocyte survival [10]. Considering the

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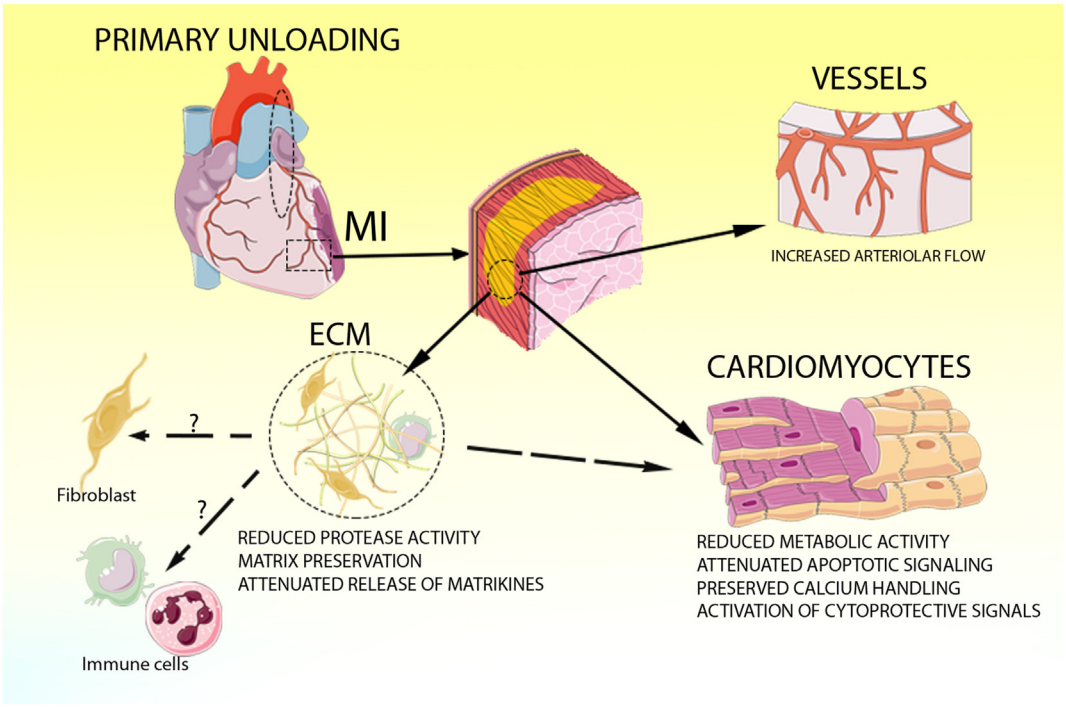


Fig. 1. Cellular mechanisms remediating the proposed protective effects of primary unloading in the infarcted heart. Cardiomyocytes, vessels, and the extracellular matrix (ECM) may be the main targets of primary unloading. Reduced end-diastolic pressures may improve coronary arteriolar flow, increasing perfusion. In addition to an increase in blood supply, unloading may also reduce demand by decreasing left ventricular wall stress, thus attenuating metabolic activity in cardiomyocytes. Unloading may reduce infarct size, by decreasing activation of mechanosensitive pro-apoptotic pathways, and by stimulating cytoprotective signals. Primary unloading may also inhibit matrix metalloproteinase (MMP) expression and activity, preserving the cardiac ECM, and reducing generation of pro-inflammatory matrix fragments (matrikines). In addition to ECM proteins, MMPs also process cytokines and chemokines. Thus, decreased MMP activity may exert protective actions by modulating the cytokine milieu in the infarcted heart. Although direct effects of unloading on resident cardiac fibroblasts and immune cells cannot be excluded, the timing and brief duration of the intervention may limit any consequences on the inflammatory and reparative response.

wide range of injurious and protective mediators processed by MMPs, the relative significance of this very specific pathway is unclear. Unloading may also act by modulating levels of matricellular proteins, macromolecules that do not play a major direct structural role, but enrich the ECM following injury and regulate signaling responses.

3. Does early unloading affect immune and reparative responses following infarction?

Mechanosensitive signaling has been implicated in activation of immune cells, and plays a critical role in stimulating both reparative and fibrogenic actions of cardiac fibroblasts. To what extent early mechanical unloading may act by modulating phenotype and function of macrophages or interstitial cells remains unclear. Effects of primary unloading on these cells may be relatively limited due to the early timing of the intervention. In the infarcted myocardium, recruitment of inflammatory leukocytes occurs in response to cardiomyocyte necrosis, whereas myofibroblast activation follows, after the peak of the inflammatory reaction. Thus, infarct macrophages and myofibroblasts are unlikely direct targets of an early and brief mechanical support intervention.

4. Conclusions

From a theoretical mechanistic perspective, primary unloading of the infarcted heart would be expected to stimulate pro-survival responses in cardiomyocytes, while improving myocardial perfusion and preserving the cardiac ECM (Fig. 1). Some of the proposed effects are supported through experimental evidence in animal models. Obviously, the current evidence is insufficient to support primary unloading as an effective adjunctive strategy in high-risk patients with acute MI. Both robust clinical studies and well-executed mechanism-oriented experimental investigations are needed to test the effectiveness of this promising new therapeutic approach.

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Declaration of Competing Interest

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MINI REVIEW

Pericytes in the infarcted heart

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Abstract

The adult mammalian heart lacks regenerative capacity and heals through activation of an inflammatory cascade that leads to the formation of a collagen-based scar. Although scar formation is important to preserve the structural integrity of the ventricle, unrestrained inflammation and excessive fibrosis have been implicated in the pathogenesis of adverse post-infarction remodeling and heart failure. Interstitial cells play a crucial role in the regulation of cardiac repair. Although recent studies have explored the role of fibroblasts and immune cells, the cardiac pericytes have been largely ignored by investigators interested in myocardial biology. This review manuscript discusses the role of pericytes in the regulation of inflammation, fibrosis and angiogenesis following myocardial infarction. During the inflammatory phase of infarct healing, pericytes may regulate microvascular permeability and may play an important role in leukocyte trafficking. Moreover, pericyte activation through Toll-like receptor-mediated pathways may stimulate cytokine and chemokine synthesis. During the proliferative phase, pericytes may be involved in angiogenesis and fibrosis. To what extent pericyte to fibroblast conversion and pericyte-mediated growth factor synthesis contribute to the myocardial fibrotic response remains unknown. During the maturation phase of infarct healing, coating of infarct neovessels with pericytes plays an important role in scar stabilization. Implementation of therapeutic approaches targeting pericytes in the infarcted and remodeling heart remains challenging, due to the lack of systematic characterization of myocardial pericytes, their phenotypic heterogeneity and the limited knowledge on their functional role.

Key Words

- pericyte
- myocardial infarction
- inflammation
- fibrosis
- angiogenesis

Introduction

Pericytes are vascular mural cells that are embedded within the microvascular basement membrane, enwrapping capillaries, venules and terminal arterioles (1, 2, 3, 4). In contrast, larger vessels, such as arteries and veins, are coated by vascular smooth muscle cells. Pericytes are ubiquitously found in all organs and contribute to vascular maturation and stability, by regulating capillary permeability and by restraining endothelial cell proliferation. In addition to their role in microvascular homeostasis, a growing body of evidence implicates pericytes in a wide range of cellular responses, including inflammation (5, 6), fibrosis (7, 8), tissue repair and regeneration (9, 10). Understanding of the role of pericytes in the pathophysiology of disease is hampered by their functional and phenotypic heterogeneity (11), the lack of specific and reliable pericyte markers and the possible overlap between pericytes and other interstitial cell populations (12).

The myocardium is perfused through a rich microvascular network; in adult mouse hearts, endothelial cells are the most abundant non-cardiomyocytes (13) Thus, it is not surprising that pericytes represent

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a significant proportion of non-endothelial, non-hematopoietic interstitial myocardial cells (14, 13). Following myocardial infarction, sudden loss of up to a billion cardiomyocytes releases danger signals, activating an intense inflammatory response that serves to clear the wound from dead cells and extracellular matrix debris (15). Because the adult mammalian heart has negligible regenerative capacity, interstitial cell populations play a critical role in repair of the infarcted heart. However, excessive, prolonged or unrestrained activation of matrix-producing interstitial cells may contribute to adverse remodeling and to the pathogenesis of post-infarction heart failure (16). Considering their abundance, phenotypic plasticity and functional diversity, pericytes may be critically involved in regulating inflammatory, fibrotic, angiogenic and reparative responses following myocardial infarction. The current review manuscript discusses our current knowledge on the role of pericytes in injury, repair and remodeling of the infarcted heart.

Pericytes in the normal myocardium

The adult mammalian heart contains abundant interstitial cells, including fibroblasts, pericytes and macrophages (13, 14). Early ultrastructural studies have identified a large population of pericytes in normal adult mammalian hearts and suggested that these cells form an incomplete layer around the microvascular endothelium (17, 18). Due to the lack of specific and reliable markers to identify pericytes, and to differentiate them from other interstitial cells, studies systematically characterizing myocardial pericyte populations are lacking. Several molecular markers have been used to identify cardiac pericytes, including the proteoglycan neural/glial2 (NG2), platelet-derived growth factor β (PDGFRβ), CD146 and CD73, α-smooth muscle actin (SMA), desmin, nestin, alkaline phosphatase and the zinc finger protein Glioma-associated oncogene protein 1 (Gli1) (8, 19). Information on the specificity and sensitivity of these markers for detection of myocardial pericytes is limited and sometimes conflicting. Methodological differences between studies, species-specific properties of pericyte populations and differences in the definition of pericytes may account for conflicting findings.

Although NG2 is expressed by cardiomyocytes in embryonic hearts (20), in the adult mammalian myocardium NG2 expression specifically labels mural cells, both pericytes and vascular smooth muscle cells (Fig. 1). In normal hearts, PDGFRβ expression also seems to be restricted to vascular mural cells (21). In contrast, α-SMA expression marks vascular smooth muscle cells and not microvascular pericytes, whereas desmin is not expressed by cardiac pericytes (19). A growing body of evidence suggests that cardiac pericytes exhibit significant heterogeneity. In rat hearts, the majority of pericytes were found to co-express PDGFRβ and NG2, while a much smaller subset (around 6%) of PDGFRβ+ cells were NG2 negative (22). Studies in mice classified pericytes on the basis of expression of the intermediate filament protein, nestin. Nestin-positive and nestin-negative NG2-expressing pericytes were noted in normal hearts; however, only nestin-expressing cells exhibited significant expansion following infarction (23).

The functional role of pericytes in cardiac homeostasis remains poorly understood. Pericytes play an important role in vascular patterning and maturation (24), restrain endothelial cell proliferation and control microvascular permeability (25). Although experiments depleting myocardial pericytes in adult animals have not been performed, several lines of evidence suggest that cardiac pericytes may support similar functions in the myocardium. First, mice lacking PDGF-B or PDGFRβ exhibit pericyte loss, associated with perturbed vascular development and formation of microaneurysms in many organs, including the heart (26). Second, administration of tyrosine kinase inhibitors (such as the anticancer agent, sunitinib) that target PDGF receptors induces cardiotoxicity, associated with pericyte loss and microvascular dysfunction (27).

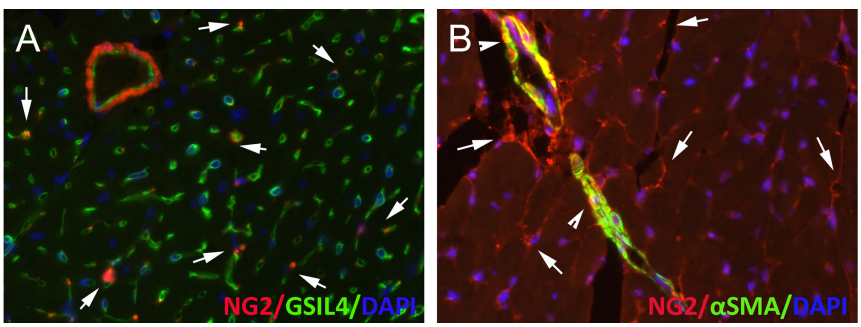


Figure 1
The adult mouse heart contains abundant pericytes. The immunofluorescence panels show myocardial sections from normal adult NG2^{Dsred} reporter mice, stained for Griffonia Simplicifolia lectin (GSIL4) to identify endothelial cells (A), and for α-SMA to label vascular smooth muscle cells (B). A significant population of NG2+/α-SMA- peri-endothelial cells is noted (arrows). Arteriolar vascular smooth muscle cells express both NG2 and α-SMA (arrowheads).

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Third, mice with endothelial cell-specific overexpression of angiopoietin-2 have marked loss of capillary-associated pericytes, associated with cardiomyopathy (28). It should be emphasized that whether pericyte loss plays a causative role in the myocardial pathology associated with PDGF-B/PDGFRβ disruption or angiopoietin overexpression was not documented. Thus, these experimental studies provide only supportive evidence on the role of cardiac pericytes in homeostasis. Fourth, conditions characterized by perturbed pericyte function, such as diabetes are associated with myocardial capillary rarefaction that may limit the functional reserve of the heart under conditions of stress (29, 30).

Pericytes in infarcted hearts

The phases of cardiac repair: from inflammation to fibrosis

In adult mammals, myocardial infarction results in sudden loss of up to a billion cardiomyocytes, overwhelming the negligible regenerative capacity of the heart. Thus, the infarcted myocardium heals through a well-orchestrated inflammatory/reparative response that ultimately leads to formation of a collagen-based scar (15, 31). The cell biological response following myocardial infarction can be divided into three distinct, but overlapping phases: the inflammatory, the proliferative and the maturation phase.

During the early *inflammatory phase*, release of danger signals from dying cardiomyocytes and protease-induced generation of matrikines, matrix fragments with pro-inflammatory properties, activates Toll-like receptor signaling pathways in cardiomyocytes, interstitial cells and immune cells and inducing cytokine and chemokine expression (32). Recruitment of abundant and diverse leukocyte populations in the infarcted heart clears the wound from dead cells and matrix fragments, while setting the stage for expansion of reparative cells through macrophage and lymphocyte-mediated secretion of growth factors (33). Induction of anti-inflammatory signals, such as TGF-βs and IL-10, not only suppresses inflammation, but also activates fibroblasts and vascular cells (34, 35, 36) leading to the proliferative phase of infarct healing. Conversion of fibroblasts to myofibroblasts is the dominant cellular event during the *proliferative phase* of infarct healing (37, 38). Activated myofibroblasts secrete both structural and matricellular extracellular matrix proteins, preserving the structural integrity of the ventricle and protecting from cardiac rupture (36, 39). The *maturation phase* follows, as myofibroblasts become quiescent (40) and a dense collagenous network comprising cross-linked collagen is formed. As the scar matures, the non-infarcted segments remodel, exhibiting hypertrophy and interstitial fibrosis and may develop worsening dysfunction. Chronic remodeling of non-infarcted segments may involve sustained neurohumoral activation and the effects of pressure and volume loads, due to the loss of contractile muscle in the infarcted area, the elevated filling pressures and the progressive chamber dilation following infarction (31).

As the heart transitions through the phases of repair and remodeling following infarction, cardiac pericytes exhibit dynamic functional and phenotypic alterations in response to the dramatic microenvironmental changes (Fig. 2). Unfortunately, myocardial biologists have ignored the pericytes; for this reason, information on their functional role in cardiac injury and repair remains extremely limited.

Pericytes in acute myocardial ischemia and during the inflammatory phase of cardiac repair

Induction of pro-inflammatory cytokines following infarction increases microvascular permeability accentuating cardiac injury. Evidence derived from experimental models of reperfused myocardial infarction suggests an important role for pericytes in regulation of capillary barriers. Although the molecular signals involved in pericyte activation remain poorly characterized, published evidence suggests an important role for nerve growth factor (NGF) and angiopoietin-2 in accentuation of pericyte-driven vascular hyperpermeability. Pro-NGF, an injury-induced cytokine, is rapidly upregulated in cardiomyocytes following myocardial ischemia and reperfusion and activates the p75 receptor in pericytes promoting microvascular dysfunction that extends the area of infarction (41). On the other hand, angiopoietin-2 is released by ischemic endothelial cells and promotes pericyte detachment, increasing microvascular permeability (42).

In addition to their effects in regulation of microvascular function, pericytes may actively participate in the post-infarction inflammatory response by producing pro-inflammatory cytokines and chemokines. Danger signals released following infarction may activate innate immune signaling cascades in pericytes promoting a pro-inflammatory phenotype. Brain and lung pericytes have been demonstrated to express TLRs and activate inflammatory cascades in response to stimulation with TLR ligands (43, 44). Stark and coworkers (45) found

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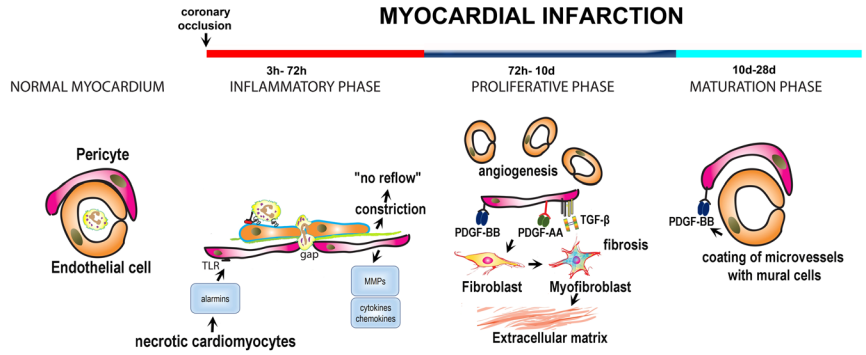


Figure 2

The role of endogenous pericytes in myocardial infarction. The adult mammalian heart lacks regenerative capacity and heals through formation of a collagen-based scar. The reparative response can be divided into three distinct, but overlapping phases: the inflammatory phase, the proliferative phase and the maturation phase. The normal myocardium contains a large population of pericytes with peri-endothelial location. During the inflammatory phase, release of alarmins from dying cells may activate TLR-mediated pathways in pericytes, stimulating cytokine and chemokine secretion. Moreover, pericytes may produce matrix metalloproteinases, thus degrading the microvascular basement membrane and stimulating an angiogenic response. Formation of gaps between pericytes in the pro-inflammatory environment of the infarct may be required for leukocyte extravasation. Pericytes also regulate microvascular function. Constriction of pericytes in the ischemic and reperfused myocardium has been implicated in the pathogenesis of 'no-reflow'. During the proliferative phase, pericytes activated by growth factors may regulate angiogenesis and fibrosis. To what extent pericyte to fibroblast conversion contributes to the expansion of myofibroblasts in healing infarcts remains unclear. Pericytes may also produce angiogenic and fibrogenic growth factors. During the maturation phase infarct neovessels recruit mural cells (both pericytes and vascular smooth muscle cells) through PDGFRβ-dependent pathways. Acquisition of a mural cell coat may be important to stabilize the infarct vasculature and to protect the infarcted heart from sustained inflammation.

that cytokine- or lipopolysaccharide-stimulated human pericytes express TLR2 and TLR4, the G-protein-coupled receptor N-formyl peptide receptor (FPR) 2, tumor necrosis factor receptor 1 (TNFR1) and the cytoplasmic receptor NLR family pyrin domain-containing 3 (NLRP3). The significance of pro-inflammatory pericyte activation in myocardial infarction remains unknown. Whether pericytes in the infarcted myocardium can respond to the release of alarmins by dying cardiomyocytes has not been investigated. Considering the well-established contributions of other cell types as sources of inflammatory cytokines and chemokines (including endothelial cells (46), fibroblasts (47), macrophages (48) and cardiomyocytes (49, 50)), the relative role of pericytes in secretion of pro-inflammatory mediators remains unclear.

Despite the absence of direct evidence in models of myocardial injury, pericytes may serve as critical regulators of leukocyte transendothelial trafficking cascades. Recruitment of neutrophils, monocytes and lymphocytes in sites of injury is driven by chemokine-dependent adhesive interactions between endothelial cells and circulating leukocytes (51). Migrating neutrophils and monocytes are found to be polarized when in close vicinity of pericytes, suggesting a role for pericyte-derived factors to drive chemotaxis of leukocytes. Pericytes may contribute to leukocyte chemotaxis by releasing chemokines and by 'instructing' leukocytes exiting through venules with pattern-recognition and motility programs (45). Intravital microscopy experiments in cremaster muscle demonstrated that cytokine-mediated pericyte activation triggers transition to an 'inflammatory phenotype', characterized by the expression of adhesion molecules and chemoattractant mediators that provide molecular cues to transmigrating leukocytes. Moreover, inflammatory activation causes shape changes in pericytes, promoting enlargement of gaps between adjacent pericytes. Thus, leukocytes use the enlarged gaps as exit points during transmigration into the injured tissue (52). Whether alterations in the morphology and adhesive properties of pericytes are involved in leukocyte trafficking in infarcted hearts remains unknown.

In addition to their role in inflammation and in leukocyte trafficking, pericytes may also play an important role in degradation of the pericellular extracellular matrix. Pericyte-mediated breakdown of the basement membrane and detachment from endothelial cells is an important early step in angiogenesis (53). Although other cardiac interstitial cells, such as fibroblasts, acquire a matrix-degrading phenotype during the early inflammatory phase of healing, producing matrix metalloproteinases in response to stimulation with IL-1β (54), whether cardiac pericytes also participate in matrix degradation remains unknown.

Pericytes have also been implicated in the pathogenesis of the 'no-reflow phenomenon'. Coronary no-reflow occurs when the myocardium fails to perfuse despite restoration of coronary flow (55). In experimental models

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of myocardial ischemia/reperfusion, endothelial injury and leukocyte plugging may cause microvessel occlusion, accentuating ischemic injury. In human patients, thrombi and atherosclerotic debris may embolize microvessels further reducing microvascular flow (56). A recently published study in a rat model of myocardial ischemia and reperfusion suggested that pericyte constriction contributes to the 'no-reflow phenomenon' by causing microvascular obstruction (22, 57). NG2-fluorescent labeling revealed that most of the blocked sites were spatially associated with pericytes. Coronary capillary lumen diameter was reduced by 37% at sites of pericyte juxtaposition upon ischemia, when compared to sham-operated hearts, suggesting a possible role for pericytes in coronary no-reflow phenomenon. Pericyte relaxation through administration of adenosine markedly increased perfusion in the ischemic and reperfused myocardium (22). Despite the limitations of the study, related to the broad effects of adenosine on many cell types, these intriguing observations support a potential role for pericyte constriction in mediating persistent reduction of microvascular flow following ischemia, despite reperfusion of the culprit vessel.

Pericytes during the proliferative phase of infarct healing: a role in fibrosis?

Transition to the proliferative phase of cardiac repair is preceded by suppression of pro-inflammatory signaling cascades (58). As pro-inflammatory signaling is attenuated, fibroblasts become activated and acquire a myofibroblast phenotype, incorporating α-SMA into cytoskeletal stress fibers and producing large amounts of extracellular matrix proteins (37, 59, 60). Myofibroblast activation is accompanied by an intense angiogenic response, presumably to support the metabolically active hypercellular wound with oxygen and nutrients (61). The potential role of pericytes in the proliferative phase of repair is poorly supported by experimental evidence. However, considering their abundance, phenotypic plasticity and responsiveness to growth factors and cytokines, pericytes may contribute to several of the cellular events associated with the proliferative phase of cardiac repair.

In fibrotic conditions involving the kidney (62), lung (63) and skin (64), pericytes have been directly implicated in myofibroblast activation and in deposition of collagenous matrix. Moreover, stimulation of pericytes with growth factors (such as PDGF-BB) has been suggested to induce pericyte-fibroblast transition in tumors (65) and in experimental models of retinopathy (66). However, evidence implicating pericytes in activation of fibrogenic pathways in the heart is lacking. Recent studies using robust lineage-tracing approaches suggest that resident fibroblast populations account for the expansion of myofibroblasts in the infarcted heart (67). Whether pericytes also contribute to cardiac fibrosis by undergoing conversion to myofibroblast-like cells remains controversial. Descriptive studies using pericyte-reporter mice suggested that, although myocardial infarction triggers expansion of a nestin-negative, NG2+ pericyte population, these cells did not exhibit collagen immunoreactivity (23). The finding was interpreted as suggestive of the absence of a role for pericytes in cardiac fibrosis. However, considering the challenges associated with the detection of collagen expression in tissue sections, the possibility that following injury pericyte-derived cells may no longer express canonical pericyte markers, and the possibility of fibrogenic effects of the pericytes that may be independent of collagen synthesis, these observations do not exclude a crucial role of pericytes in myocardial fibrosis. On the other hand, Kramann *et al*. found that a large number of myofibroblasts in remodeling and infarcted hearts are derived from Gli1+ 'pericytes' (8). The notion that pericytes may convert to myofibroblasts was also challenged by a study using lineage tracing of Tbx18+ 'pericytes', which demonstrated that these cells maintain their identity in pressure-overloaded hearts and do not significantly contribute to other lineages (68). Clearly, the lack of uniform criteria and reliable markers for defining pericytes contributes to the confusion regarding their role in myocardial fibrosis.

Pericytes during the maturation phase of cardiac repair

During the maturation phase of infarct healing, suppression of fibrogenic responses, microvessel maturation and extracellular matrix crosslinking result in the formation of a stable scar and prevent the expansion of fibrosis (39, 69). Mural cells (both pericytes and vascular smooth muscle cells) play a central role in vascular maturation, by coating infarct neovessels (70, 71), thus stabilizing the microvasculature of the scar and attenuating inflammation. Antibody inhibition experiments in a mouse model of reperfused myocardial infarction demonstrated that PDGF-BB/PDGFRβ signaling plays a crucial role in scar maturation (70). Disruption of PDGFRβ signaling perturbed coating of neovessels with mural cells and prolonged hemorrhagic and inflammatory changes in the healing infarct (70).

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Pericytes as therapeutic targets in myocardial infarction

The absence of robust data on the role of endogenous pericytes greatly limits design of therapeutic approaches targeting these cells in infarcted and remodeling hearts. Most of the evidence suggesting important roles of pericytes in inflammation and fibrosis following myocardial infarction is based on associative data. Documentation of pericyte-specific effects *in vivo* is particularly challenging; thus, therapeutic targeting of endogenous pericyte actions is heavily based on speculation. Considering their potential role in regulating microvascular function, inhibition of pericyte constriction has been suggested as a promising strategy for protection of the ischemic and reperfused myocardium from 'no-reflow' (22, 57). Whether approaches targeting endogenous pericyte-mediated actions may protect from adverse post-infarction remodeling restrain inflammation or attenuate fibrosis remains unknown.

Considering their role in angiogenesis, microvascular function, regulation of inflammation and growth factor expression profile, cell therapy with pericytes has been suggested as an attractive strategy to improve repair of the infarcted heart. Experimental studies have suggested that intramyocardial delivery of pericytes may protect the infarcted heart from adverse remodeling by exerting angiogenic and anti-apoptotic actions (72, 73, 74) through paracrine effects on cardiomyocytes, fibroblasts and vascular cells. In a mouse model of myocardial infarction, transplantation of saphenous vein-derived pericytes into the peri-infarct zone attenuated adverse remodeling and improved systolic dysfunction, by secreting angiogenic mediators (such as VEGF and angiopoietin-1) and by releasing protective microRNAs (75). Pericyte-derived miR-132 was identified a key cardioprotective factor that improved cardiac function following infarction, increasing angiogenesis and attenuating fibrosis (75). Studies in large animal models have provided additional support to the beneficial effects of pericyte cell therapy following infarction. In a blind, randomized placebo-controlled study in a swine model of reperfused myocardial infarction, transplantation of human adventitial pericytes improved capillary dysfunction and reduced fibrosis (76). However, these presumably beneficial cell biological actions of pericytes were not associated with any effects on myocardial function. It has also been suggested that human myocardial pericytes, may serve as multipotent precursors and may have limited cardiomyogenic potential (19). However, robust documentation of pericyte-driven remuscularization in vivo is lacking.

Conclusions

Despite their abundance and their potential significance in regulating a wide range of functions, including microvascular permeability, angiogenesis, inflammation, repair, and fibrosis, cardiac pericytes have been largely ignored by the cardiovascular community. There is an urgent need for studies systematically characterizing cardiac pericyte populations, identifying subsets with distinct functional properties and exploring the role of pericyte-mediated actions in cardiac injury, repair and remodeling.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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ONLINE SUPPLEMENT

Distinct roles of myofibroblast-specific Smad2 and Smad3 signaling in repair and remodeling of the infarcted heart

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Short title: Smad2 and Smad3 in infarct myofibroblasts

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SUPPLEMENTARY METHODS

Generation of mice with myofibroblast-specific loss of Smad2: In order to study the role of Smad2 in fibroblasts in the infarcted myocardium, we generated mice with loss of Smad2 in activated infarct fibroblasts (FS2KO). 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 myofibroblasts in infarcted and in pressure-overloaded hearts [4,5]. Postn-Cre mice (in a C57Bl6J background) were bred with Smad2 fl/fl mice (The Jackson Laboratory, Stock No: 022074) to generate Postn-Cre;Smad2 fl/fl animals (FS2KO) and corresponding Smad2 fl/fl controls. 55 Smad2 fl/fl mice (M, n=32, F, n=23) and 57 FS2KO animals (M, n=33, F, n=24) underwent in vivo experimentation. Material from previously published[6] mice with myofibroblast-specific loss of Smad3 (FS3KO) and corresponding Smad3 fl/fl mice undergoing infarction protocols was used to compare the effects of myofibroblast-specific Smad2 and Smad3 loss.

Mouse model of non-reperfused 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 mouse model of non-reperfused myocardial infarction was used, as previously described by our group[6]. Female and male mice, 4-6 months of age, were anesthetized using inhaled isoflurane (2%). All Smad2 fl/fl, Smad3 fl/fl, FS2KO, and FS3KO 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. To assess the time-course of activation of Smad2 and Smad3 signaling in the infarct heart, the left anterior descending (LAD) coronary artery of

C57/BL6J WT mice was permanently occluded for 24h, 3d, 7d, and 28d. To assess cardiac function and remodeling following myocardial infarction, animals underwent echocardiographic analysis at baseline and 7 days, 28 days after permanent coronary occlusion (Baseline: Smad2 fl/fl, M, n=18, F, n=19, FS2KO, M, n=16, F, n=22; 7 days, Smad2 fl/fl, M, n=19, F, n=17; FS2KO, M, n=18, F, n=19; 28 days, Smad2 fl/fl, M, n=6, F, n=11; MFS2KO, M, n=8, F, n=15). 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.

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 [7]. Parasternal long-axis (PSLAX) M-mode was used for measurement of systolic and diastolic ventricular and wall diameters. PSLAX IVS tool was used to measure and calculate the thickness of the intraventricular septum (IVS-d and IVS-s), left ventricular posterior wall (PWTh-d and PWTh-s), the left ventricular end-diastolic internal dimension (LVID-d), left ventricular end-systolic internal dimension (LVID-s), and the ratio of left ventricular mass/body weight (LVM/BW). Left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV) were measured as indicators of dilative remodeling. Left ventricular ejection fraction (LVEF= [(LVEDV－LVESV) / LVEDV] ×100%), fractional shortening (FS = [(LVID-d – LVID-s) / LVID-d] ×100%) were measured for assessment of systolic ventricular function.

Immunohistochemistry and histology: For histopathological analysis murine hearts were fixed in zinc-formalin (Z-fix; Anatech, Battle Creek, MI), and embedded in paraffin. Infarcted hearts were sectioned from base to apex at 250 μm intervals, thus reconstructing the whole heart, as previously described [8]. 20 sections (5μm thick) were cut at each level. The first section at each partition was stained with haematoxylin and eosin (H&E). Morphometric parameters were quantitatively assessed using Axiovision software. The infarcted and

non-infarcted areas were measured at each level and the volume of the infarct and of the non-infarcted remodeling myocardium at each level was calculated as: Infarct volume= infarct area X 350μm (250μm + 20 sections*5μm=350μm), and volume of non-infarcted remodeling myocardium=non-infarcted area X 350μm. The total volume of the infarcted and non-infarcted myocardium was calculated as the sum of the volumes of each partition. Scar size was measured by dividing the volume of the infarct to the total volume of the left ventricle (infarct volume+volume of non-infarcted myocardium), and was expressed as a percentage. Minimum and maximum wall thickness of both infarcted and non-infarcted left ventricular segments was measured using Axiovision software. In order to assess collagen content, 3 sections from each heart (representing the basal, mid-myocardial and apical levels) were stained with picrosirius red to label the collagen fibers. 8-10 fields from the infarcted and remote non-infarcted remodeling myocardium were scanned and the collagen-stained area was measured. Collagen content was measured in the infarct region, and in the remote remodeling myocardium using ImagePro software as the ratio of the collagen stained area to the total area and was expressed as a percentage for reach animal.

Dual immunofluorescence: In order to detect Smad2 and Smad3 activation in activated fibroblasts, we performed dual immunofluorescence for pSmad2 (using an anti-pSmad2 antibody, Invitrogen, 44-244G), or pSmad3 (anti-pSmad3 antibody, Invitrogen, PA5-37636) and α-smooth muscle actin (anti α-SMA antibody, Sigma, F3777). Activation of Smad2 or Smad3 signaling was evidenced by nuclear localization of pSmad2 or pSmad3. Myofibroblasts were identified by staining with FITC conjugated anti-α-smooth muscle actin (FITC-α-SMA) (Sigma, F3777), or by staining with an antibody to periostin (NOVUS Biologicals, AF2955) as spindle-shaped cells located outside the vascular media. Endpoints reflecting myofibroblast size, shape, alignment and polarization were assessed using ImagePro software as previously described[6]. The ratio of major to minor axis of infarct myofibroblasts was calculated to assess the myofibroblast morphology. To quatitatively assess myofibroblast alignment in the healing infarcts, angle between the long axis and the tangent of the ventricular wall (indicated by a red arrow in Figure 6) was measured. Characteristics of myofibroblast

morphology and polarization were assessed in 3-9 animals from each group, at 2 different levels. 6 fields were quantified in each animal. A total of 18 cells from each animal was assessed to perform statistical analysis. In order to compare the density of α-SMA+ myofibroblasts, myofibroblast numbers were assessed in 6 fields in each animal from 2 different levels (S2fl/fl, n=3, FS2KO, n=4, S3fl/fl, n=9, FS3KO, n=5). To document the loss of Smad2 in infarct myofibroblasts, dual staining with the Goat anti-Periostin (NOVUS Biologicals, AF2955) and Rabbit anti-Smad2 (Cell Signaling Technology, 5339) antibodies was used in the sections of S2fl/fl and FS2KO, followed by Alexa Fluor 594 (Invitrogen, A21207) and Alexa Fluor 488–conjugated secondary antibodies (Invitrogen, A21206) respectively. All sections were counterstained with DAPI. Quantitative analysis was performed by counting the number of periostin+ Smad2+ and periostin+ Smad2- cells in 5 fields from 2 different sections from each animal (S2fl/fl, n=4, FS2KO, n=5).

Isolation and stimulation of isolated cardiac fibroblasts: Fibroblasts were isolated from normal mouse (C57/BL6J) hearts as previously described [9],[10], then cultured in dishes (Corning, Cat#: 353003, Falcon® 100 mm X15 mm), or in collagen pads. For experiments examining effects of TGF-β superfamily ligands on Smad2 activation, cells plated in dishes were stimulated with TGF-β1 (10ng/ml), TGF-β2 (10ng/ml), TGF-β3 (10ng/ml), BMP2 (50ng/ml), BMP4 (50ng/ml), BMP7 (50ng/ml), Angiotensin II (50ng/ml) (all from R&D Systems) for 30 minutes. At the end of the experiment fibroblast lysates were used for protein extraction.

siRNA KD experiments were performed in fibroblasts cultured in collagen pads (to assess integrin expression), or in fibroblasts cultured in plates (to assess planar cell polarity genes). Mouse cardiac fibroblasts at passage 1 were seeded at 80% confluence (100 mm dishes) in Dulbecco's modified eagle medium (DMEM) and were either transfected with 50 nM ON-TARGET plus mouse Smad2 siRNA, Smad3 siRNA or transfected with a non-silencing control siRNA (Dharmacon) using Lipofectamine® 3000 Reagent (ThermoFisher Scientific). The ON-TARGET modification is shown to dramatically decrease the off-target effects of the siRNA. 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 (3 $\times 10^5$ cells/ml concentration). The pads were then suspended in serum free DMEM/F12 for an additional 72 h.

In additional experiments designed to assess planar cell polarity (PCP) pathway genes, fibroblasts cultured in dishes were transfected with control, Smad2 or Smad3 siRNA for 72h in DMEM/F12 with 10% FBS. Afterwards, cells were rinsed twice with DPBS, and then 1 mL Trizol solution was added in each dish for cell lysis and RNA extraction. Extracted RNA was used for PCR array analysis.

RNA extraction, qPCR and qPCR array analysis: Total RNA was extracted from cells, 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'-AGGTCGGTGTGAACGGATTTG-3', GAPDH reverse 5'-TGTAGACCATGTAGTTGAGGTCA-3', α-SMA forward 5'-GTCCCAGACATCAGGGAGTAA-3', α-SMA reverse 5'-TCGGATACTTCAGCGTCAGGA-3', Smad2 forward 5'-ATCTTGCCATTCACTCCGCC-3', Smad2 reverse 5'-TCTGAGTGGTGATGGCTTTCTC -3', Smad3 forward 5'-CACGCAGAACGTGAACACC-3', Smad3 reverse 5'-GGCAGTAGATAACGTGAGGGA-3', Itga2 forward 5'-GCACCACATTAGCATACA-3', Itga2 reverse 5'-TAGAAAGGGATACTGAAGACT-3', Itga5 forward 5'-AGTGTGAGGCTGTATATGA-3', Itga5 reverse 5'-AGAATGGCTAGGATGATGA-3', NOX2 forward 5'-ATCCATATCCGTATTGTG-3', NOX2 reverse 5'-ATCAACTGCTATCTTAGG-3', periostin forward 5'-AACCTGGATTCTGACATT -3', periostin reverse 5'-GTACATTGAAGGAATAACCAT -3'. vimentin forward 5'-CGTCCACACGCACCTACAG-3' , vimentin reverse 5'-GGGGGATGAGGAATAGAGACT-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 ΔΔCT method.

In order to assess gene expression of planar cell polarity (PCP) pathway genes, we used a PCR array. Fibroblasts cultured in DMEM/F12 with 10% FBS were transfected with siRNA for 72h and then cells were harvested for RNA extraction using Trizol. A total of 400ng RNA was reverse-transcribed into cDNA using the

RT^2 first strand kit (Qiagen, 330404). Quantitative PCR was performed using the RT^2 Profiler mouse WNT signaling pathway PCR array (PAMM-043ZA) from Qiagen according to manufacturer's protocol. The same thermal profile conditions were used for all primers sets: 95℃ for 10 minutes, 40 cycles at 95℃ for 15 seconds followed at 60℃ for 1 minute. The data obtained were exported to SABiosciences PCR array web-based template where it was analyzed using the ΔΔCT method.

Protein extraction and western blotting: Protein was extracted from cardiac fibroblasts as previously described [11]. Halt™ Phosphatase Inhibitor Cocktail (Thermo Scientific™, 78420) was used to preserve the phosphorylation state of proteins during and after cell lysis or protein extraction. Western blotting was performed using antibodies to pSmad2 [$pSpS^{465/467}$] (Cell Signaling Technology, 3108), Smad2 (Cell Signaling Technology, 5339), and GAPDH (Santa Cruz, 25778). The gels were imaged by ChemiDoc™ MP System (Bio Rad) and analyzed by Image Lab 3.0 software (Bio Rad).

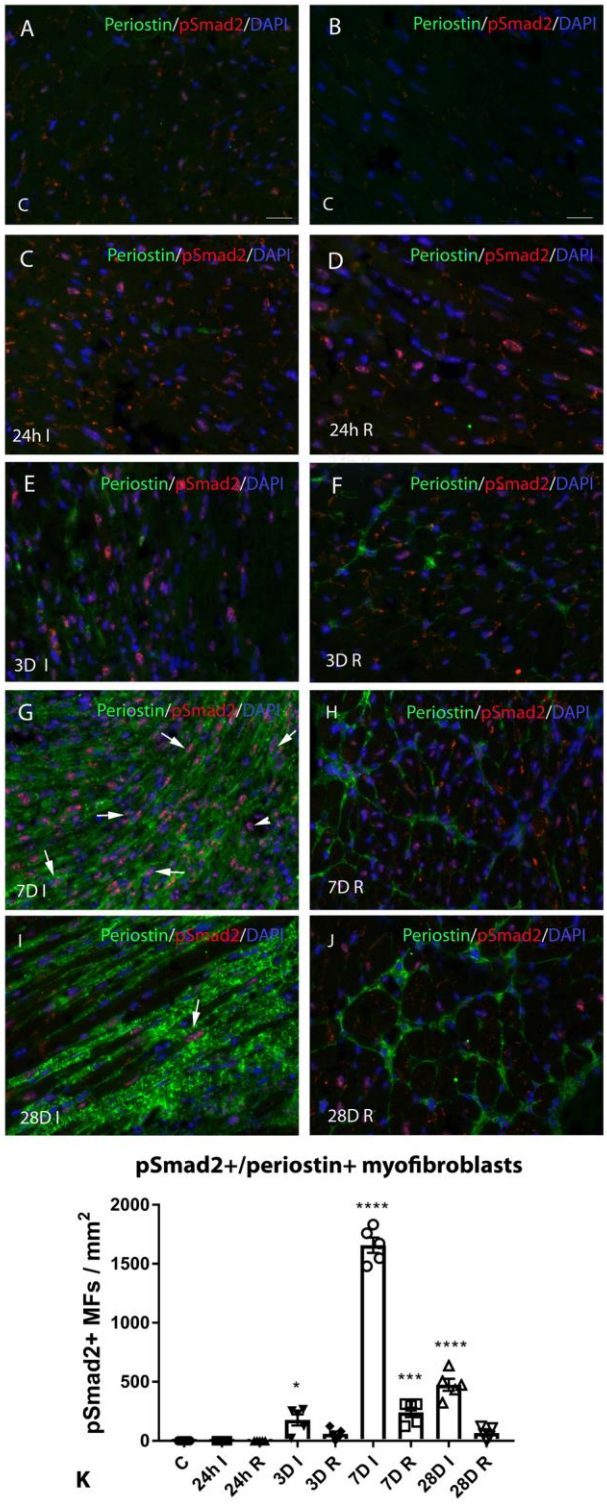
Assessment of α-SMA incorporation into the cytoskeleton of stimulated cardiac fibroblasts: To assess the effects of Smad2 or Smad3 loss on myofibroblast conversion, fibroblasts were treated with control, Smad2 or Smad3 siRNA for 72h. Specific reduction of Smad2 and Smad3 expression was documented using qPCR. Dual fluorescence with phalloidin-AF594 (Invitrogen, A12381) and anti-α-SMA-FITC-labeled antibody (Sigma, F3777) was used to assess decoration of F-actin fibers with α-SMA in fibroblasts cultured in chamber slides in the presence or absence of TGF-β1 (10ng/ml, 72h). The density of phalloidin+/α-SMA+ fibroblasts was counted in 4 chamber slides of each group. 5 fields were quantified in each chamber slide and the percentage of α-SMA+/phalloidin+ cells to the total number of phalloidin+ cells was used to quantify α-SMA incorporation into the cytoskeleton.

Fibroblast migration assay: Cardiac fibroblast migration assay was performed using colorimetric transwell system QCMTM 24-Well (Millipore Corporation, Billerica, MA), which allows cells to migrate through an 8-μm pore size polycarbonate membrane. Fibroblasts were treated with control, Smad2 or Smad3 siRNA for 72h when cells grew up to 80% confluence, followed by serum starvation for 24h prior to migration assay. Then

cells were harvested with TrypLE express (GIBCO Invitrogen Corporation, Carlsbad, CA), counted and reconstituted with serum free DMEM/F12 (GIBCO Invitrogen Corporation, Carlsbad, CA) to bring the cells to concentration of 1.0X106 cells/ml. Subsequently, 300µl fibroblasts suspensions were added into the upper chamber of each insert, and 500 µl serum free DMEM/F12, or 1% FBS DMEM/F12 with 20 ng/ml TGF-β1 were added in the lower chamber, The chambers were incubated for 6 h at 37^{o}C in 5% CO2. After incubation remaining cells/media from the top side of the insert were removed by pipetting, and the migration insert was placed into a clean well containing 400 µL of Cell Stain (crystal violet) for 20 min at room temperature. Afterwards, inserts were rinsed in distilled water. Migrated cells were visualized and photographed using an inverted microscope, and cell migration was quantified by counting cells in 5 random high power fields (40X). Dry inserts were transferred to extraction buffer and placed on rocking platform for 15 min. Optical density of dye extract was measured at 560 nm using a plate reader (BioTek).

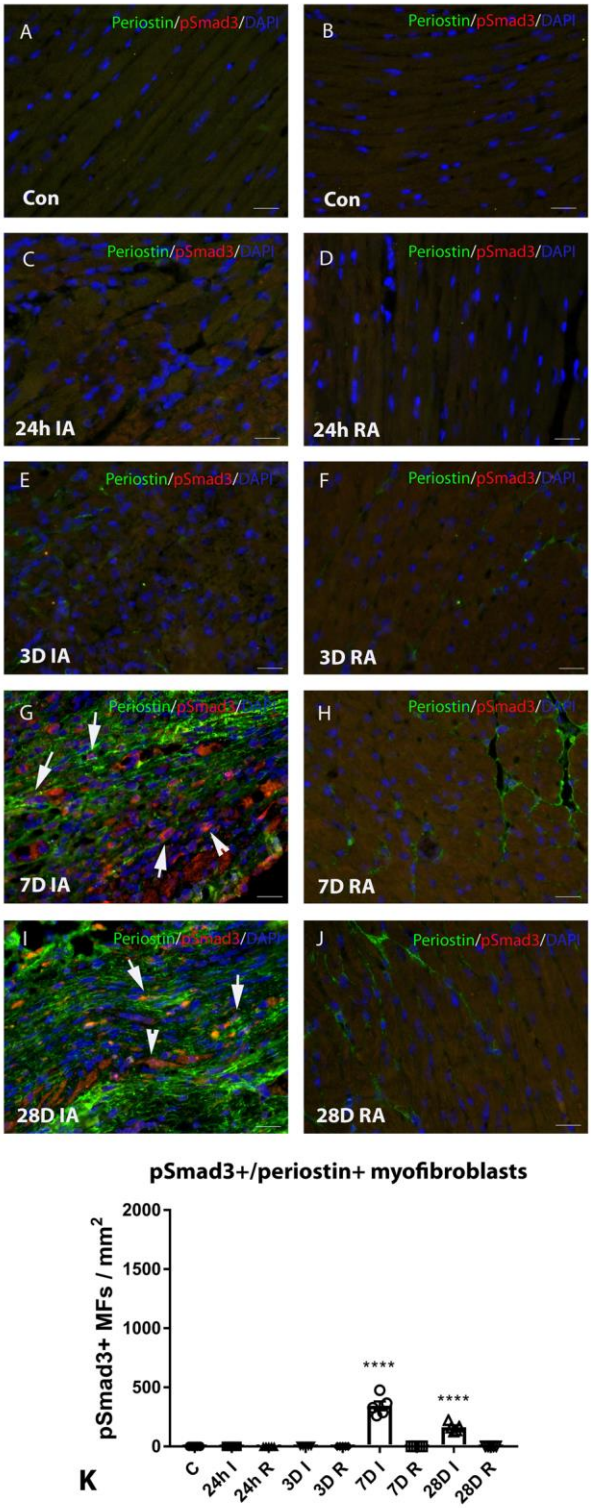
Statistics: For comparisons of two groups unpaired, 2-tailed Student's t-test using (when appropriate) Welch's correction for unequal variances was performed. The Mann-Whitney test was used for comparisons between 2 groups that did not show Gaussian distribution. For comparisons of multiple groups, 1-way 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. Survival analysis was performed using the Kaplan-Meier method. Mortality was compared using the log rank test.

SUPPLEMENTARY FIGURES:



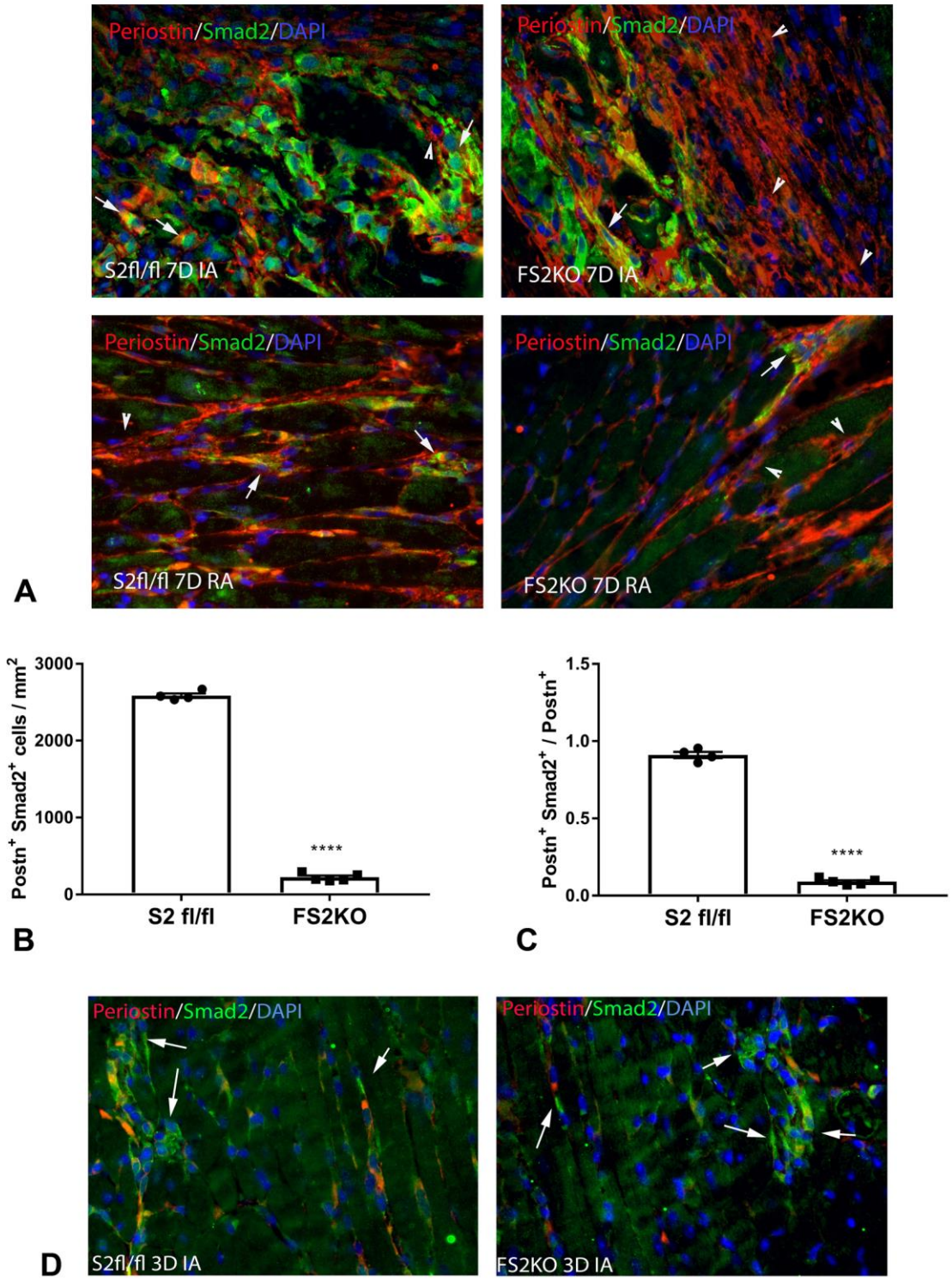
Supplemental Figure I: **Smad2 activation in periostin-expressing myofibroblasts infiltrating the infarcted myocardium.** Dual immunofluorescence for periostin and pSmad2 was performed in control hearts (A-B) and in infarcted (I) and non-infarcted remote (R) myocardial segments from mice undergoing 24h (C-D), 3-day (E-

F), 7-day (G-H) and 28-day (I-J) coronary occlusion protocols. Control (Con) hearts (A-B) showed no periostin immunoreactivity and low levels of pSmad2 expression. High levels of pSmad2 immunoreactivity were noted in abundant periostin+ myofibroblasts infiltrating the infarcted myocardium after 7 days of coronary occlusion (arrows). Periostin+/pSmad2+ cells were rare after 28 days of coronary occlusion (arrow). Please note deposition of periostin in the extracellular matrix in both infarcted (G, I) and remote remodeling segments (H, J) after 7-28 days of coronary occlusion. K: Quantitative analysis showed an early increase in the density of pSmad2+ periostin-expressing myofibroblasts after 3 days of coronary occlusion, followed by a peak after 7 days (*$p<0.05$, ***$p<0.001$, ****$p<0.0001$ vs. control, n=5/group). Increased density of pSmad2+ myofibroblasts was noted in the remodeling non-infarcted myocardium after 7 days of coronary occlusionStatistical analysis was performed using ANOVA followed by Tukey's multiple comparison test.



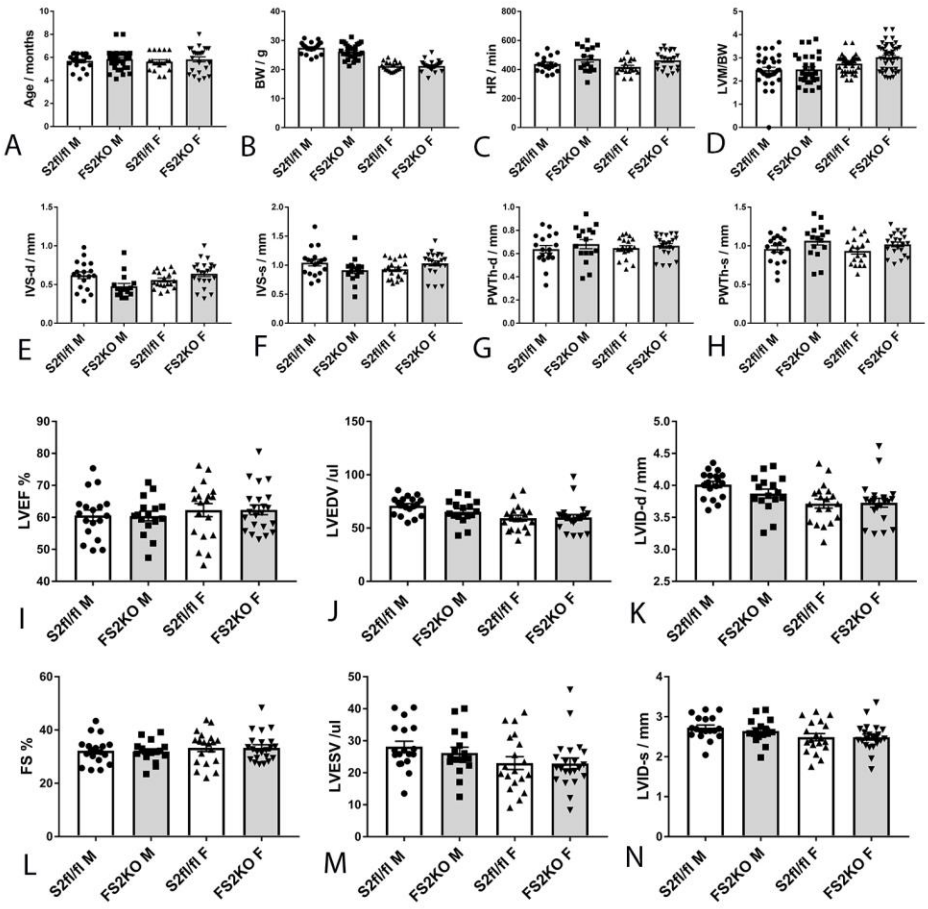
Supplemental figure II: **Smad3 activation in periostin-expressing infarct myofibroblasts**. Dual immunofluorescence for periostin and pSmad3 was performed in control hearts (A-B) and in infarcted (I) and non-infarcted remote (R) myocardial segments from mice undergoing 24h (C-D), 3-day (E-F), 7-day (G-H) and

28-day (I-J) coronary occlusion protocols. Control (Con) hearts (A-B) and infarcted heart at 24h timepoint (C-D) showed no periostin immunoreactivity or pSmad3 expression. Expression of pSmad3 was noted in periostin+ myofibroblasts infiltrating the infarcted myocardium after 7 days (G-H) and 28 days (I-J) of coronary occlusion (arrows). Please note deposition of periostin in the extracellular matrix in both infarcted (G, I) and remote remodeling segments (H, J) after 7-28 days of coronary occlusion. K: Quantitative analysis showed that Smad3 activation in periostin-expressing myofibroblasts peaked after 7 days of coronary occlusion (****$p<0.0001$ vs. control, n=5/group). Statistical analysis was performed using ANOVA followed by Tukey's multiple comparison test.



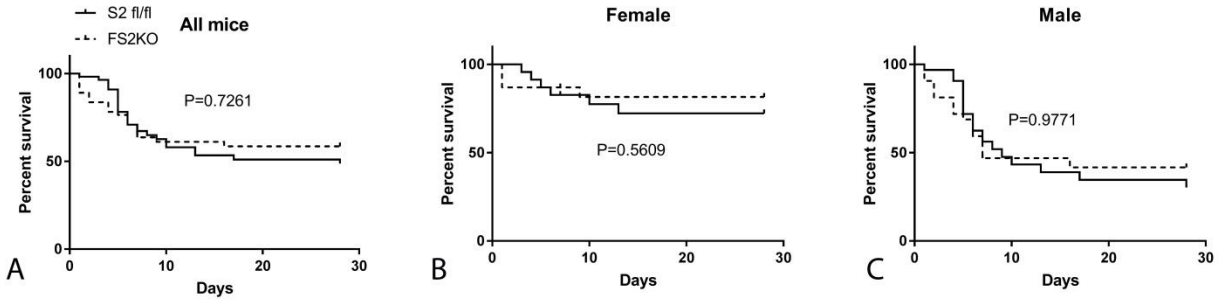
Supplemental Figure III: **FS2KO mice have reduced Smad2 expression in periostin+ myofibroblasts after 7 days of coronary occlusion**. A: In order to document myofibroblast-specific Smad2 loss, dual immunofluorescence for Smad2 and periostin was performed in Smad2 fl/fl and FS2KO mouse hearts after 7 days of coronary occlusion. Sections from the infarcted area (IA) and the remote non-infarcted area (RA) are shown. Abundant Smad2-expressing periostin+ cells were noted in Smad2 fl/fl infarcts (arrows); in contrast,

very limited Smad2 and periostin colocalization was found in FS2KO hearts (arrows). Arrowheads show the abundance of periostin+ cells that do not express Smad2 in FS2KO animals. B-C: Quantitative analysis shows that FS2KO mice had a marked reduction in the density of periostin+/Smad2+ cells (B) and a lower ratio of Smad2+ positivity in periostin-expressing myofibroblasts (C). Statistical analysis was performed using 2-tailed Student's t-test (****p<0.0001, n=4-5/group). D. After 3 days or coronary occlusion, Smad2 expression was noted in periostin-negative interstitial cells in both Smad2 fl/fl and in FS2KO mice (arrows). Some of these cells may be fibroblasts that have not yet converted to myofibroblasts and do not exhibit Smad2 loss.

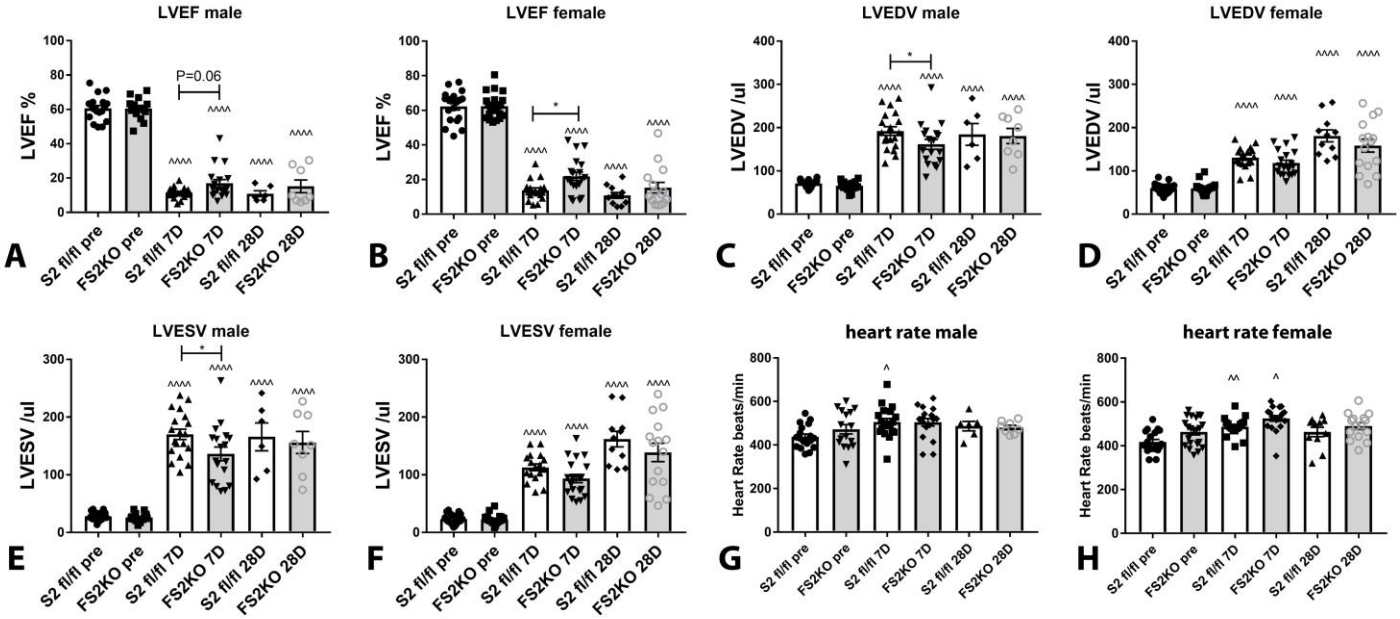


Supplemental Figure IV: **Age- and gender-matched Smad2 fl/fl and FS2KO mice had no significant differences in baseline body weight, left ventricular dimensions and function**. Echocardiographic analysis was performed in male (M) and female (F) Smad2 fl/fl and FS2KO mice at 4-6 months of age. Mean age (A) and body weight (B) was not significantly different between groups. Heart rate (C), left ventricular mass (LVM)/body weight(BW) (D), end-diastolic interventricular septum thickness (IVS-d, E), end-systolic

interventricular septum thickness (IVS-s, F), end-diastolic posterior wall thickness (PWThd, G), end-systolic posterior wall thickness (PWThs, H), left ventricular ejection fraction (LVEF, I), fractional shortening (FS, L), left ventricular end-diastolic volume (LVEDV, J), left ventricular end-systolic volume (LVESV, M), left ventricular end-diastolic internal diameter (LVID-d, K), and left ventricular end-systolic internal diameter (LVID-s, N) had no significant differences between Smad2 fl/fl and FS2KO mice, suggesting that loss of Smad2 in activated periostin+ myofibroblasts does not affect baseline cardiac geometry and function (Smad2 fl/fl, M, n=18, F, n=19, FS2KO, M, n=16, F, n=22). Statistical analysis was performed using ANOVA followed by Tukey's multiple comparison test.

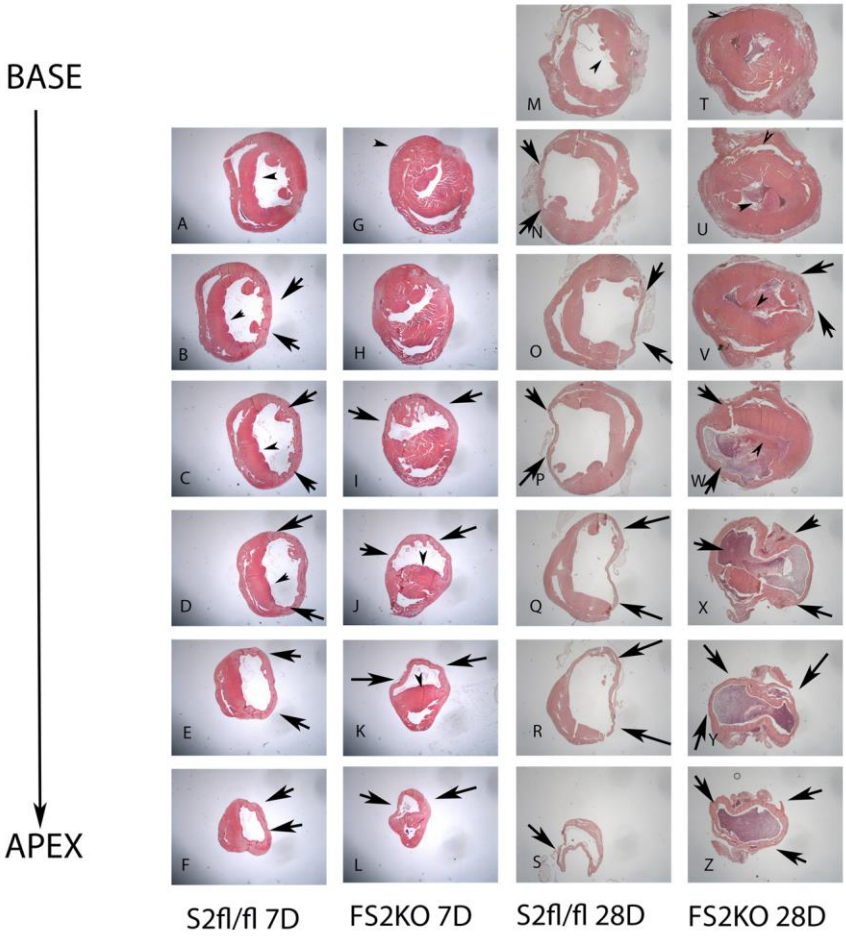


Supplemental Figure V: A-C: Female (B) and male (C) FS2KO mice and age-matched Smad2 fl/fl animals exhibited comparable mortality after 28 days of coronary occlusion. Please note the markedly higher mortality in male animals (C) (Smad2 fl/fl, M, n=32, F, n=23, FS2KO, M, n=33, F, n=24). Survival analysis was performed using the Kaplan-Meier method. Mortality was compared using the log rank test.

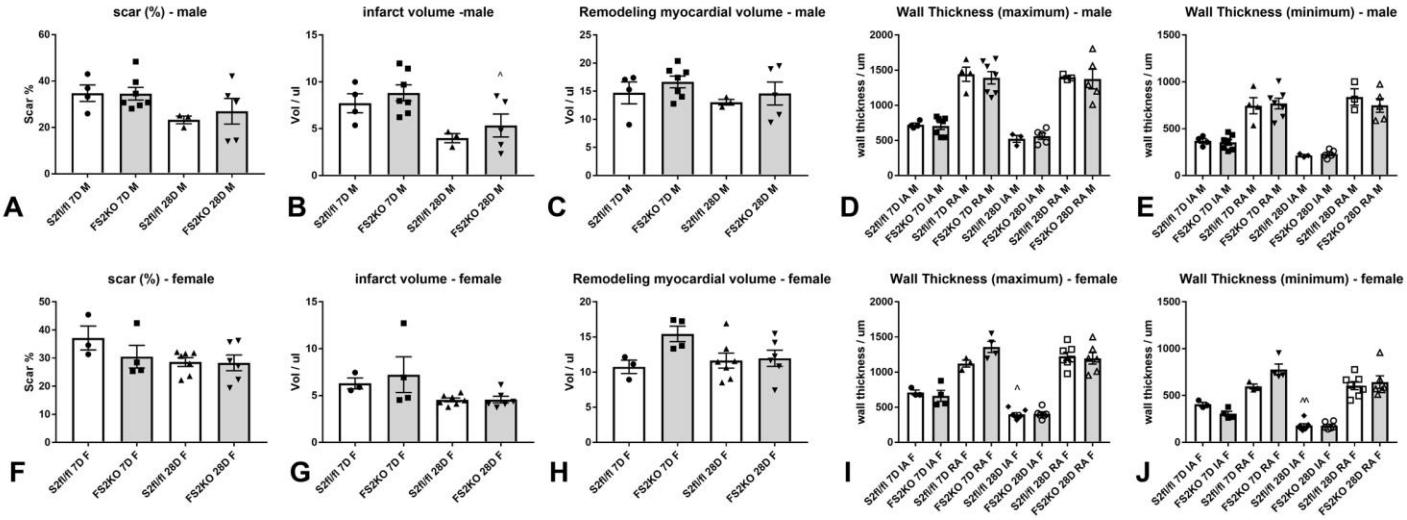


Supplemental figure VI: **Effects of myofibroblast-specific Smad2 and Smad3 loss on cardiac function following myocardial infarction in male and female animals.** Gender-specific analysis showed that female FS2KO mice had significantly better systolic function than female Smad2 fl/fl animals (B), whereas male mice exhibited only a trend towards improved function (A). After 28 days of coronary occlusion, no significant differences in ejection fraction were noted between FS2KO mice and Smad2 fl/fl animals in both male and female groups (A-B). LVEDV (C-D) and LVESV (E-F) were significantly lower in male FS2KO mice after 7 days of coronary occlusion, suggesting attenuated dilative remodeling. Female mice had no significant differences (D, F). No significant difference of heart rate was observed between Smad2 fl/fl and FS2KO mice in both genders (G-H). (*p<0.05 vs. S2fl/fl 7D, ^p<0.05, ^^p<0.01, ^^^^p<0.0001 vs. S2fl/fl pre or FS2KO pre - ANOVA followed by Tukey's multiple comparison test) , (pre-echo: Smad2 fl/fl, M, n=18, F, n=19; FS2KO, M, n=16, F, n=22; 7 days echo, Smad2 fl/fl, M, n=19, F, n=17; FS2KO, M, n=18, F, n=19; 28 days echo, Smad2 fl/fl, M, n=6, F, n=11; FS2KO, M, n=8, F, n=15).

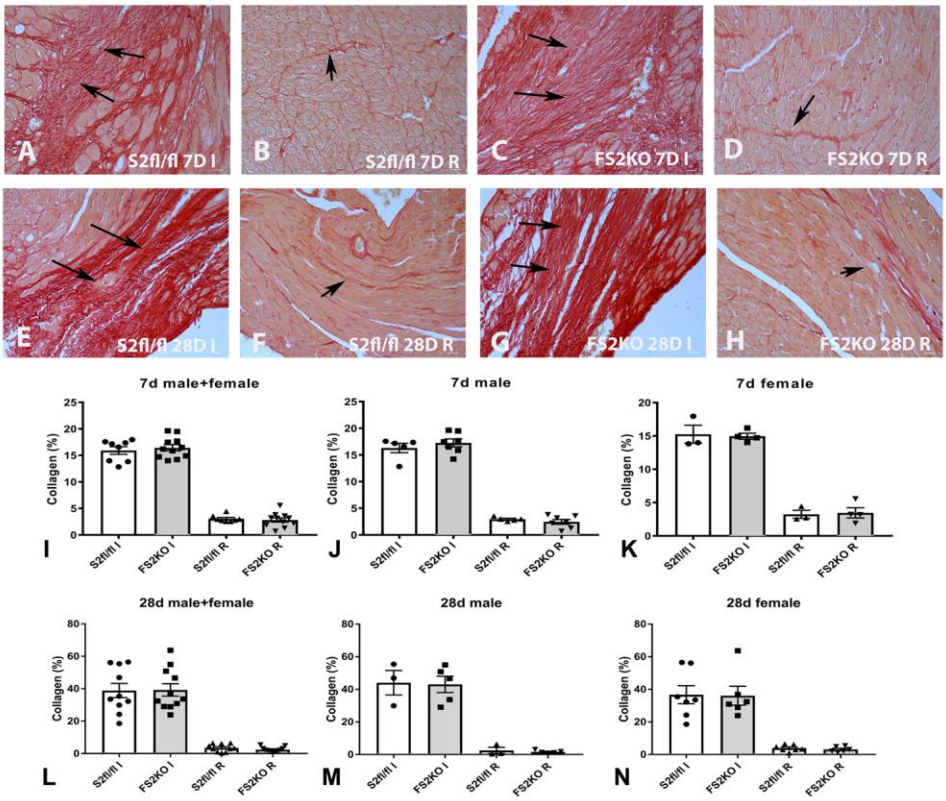
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Supplemental Figure VII: Strategy for morphometric analysis of scar remodeling in infarcted mice. In order to systematically compare morphometric parameters and scar remodeling between Smad2 fl/fl and FS2KO mice after 7-28 days of permanent coronary occlusion, the infarcted hearts were sectioned from base to apex at 250μm partitions, as previously described[6,8]. 20 sections (5μm each) were cut at each partition; the first section at each level was stained with hematoxylin/eosin (H&E). Areas of replacement fibrosis were identified at each level (arrows). Arrowheads indicate the viable remodeling myocardium. Quantitative analysis of the findings is shown in figure 4. (A-F, Smad2 fl/fl after 7 days of infarction; G-L: FS2KO after 7 days of infarction; M-S: Smad2 fl/fl after 28 days of infarction; T-Z: FS2KO after 28 days of infarction).

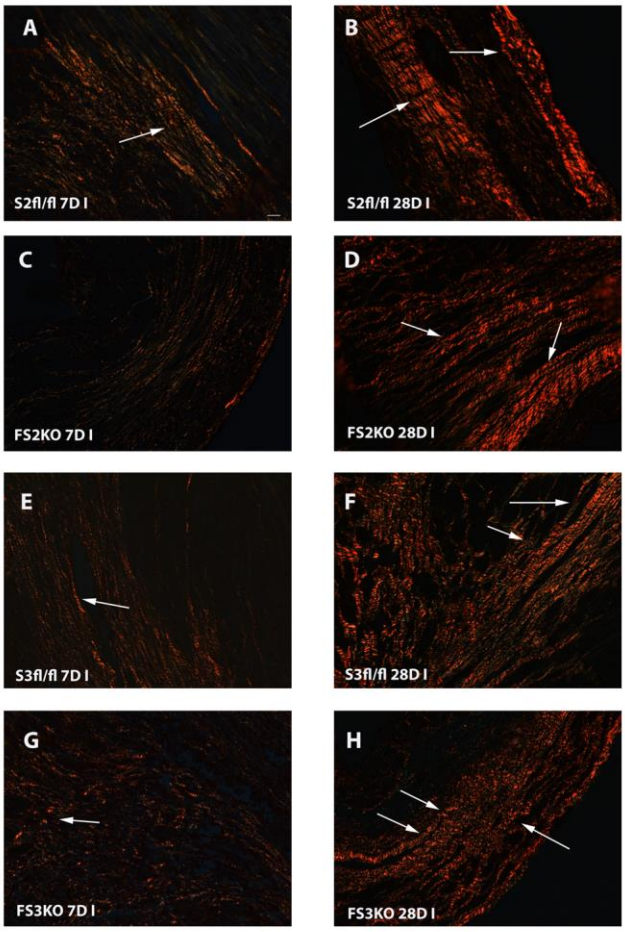


Supplemental figure VIII: **Analysis of morphometric parameters in male and female mice**. Scar size (A,F), infarct volume (B,G), the volume of the viable remodeling myocardium (C,H), and the thickness of the infarcted and non-infarcted walls (D-E, I-J) were comparable between FS2KO and Smad2 fl/fl mice in both male and female groups after 7-28 days of coronary occlusion. (^p<0.05, ^^p<0.01, vs. S2fl/fl 7D or FS2KO 7D. S2fl/fl 7D, male, n=4, female, n=3; FS2KO 7D, male, n=7, female, n=4; S2fl/fl 28D, male, n=3, female, n=7; FS2KO 28D, male, n=5, female, n=6. - ANOVA followed by Tukey's multiple comparison test).



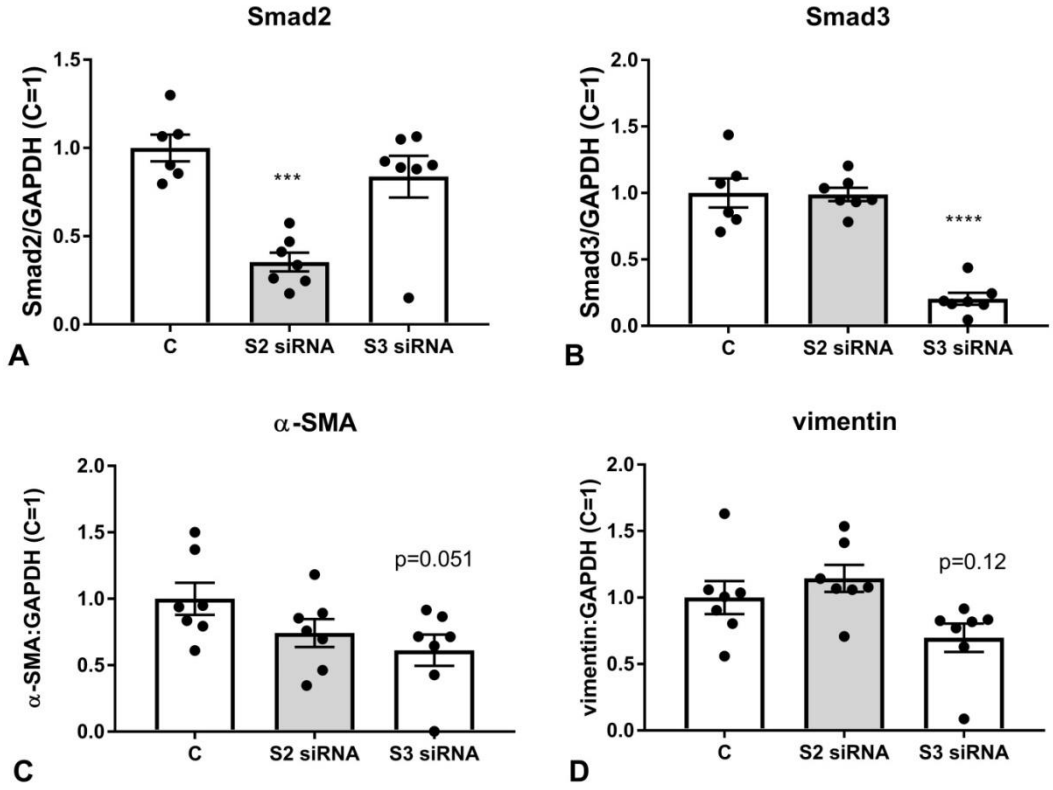
Supplemental Figure IX: FS2KO mice and Smad2 fl/fl littermates had comparable collagen content in the infarcted area (I) and in the remote remodeling myocardium (R) after 7-28 days of coronary occlusion. A-H: Collagen staining was performed using picrosirius red; the collagen-stained area was assessed

in infarcted and remodeling areas. Quantitative analysis showed comparable collagen content between Smad2 fl/fl and FS2KO animals in both infarcted and remote remodeling myocardium from male (I-K) and female (L-N) groups after 7-28 days of coronary occlusion. (S2fl/fl 7D, male, n=5, female, n=3; FS2KO 7D, male, n=7, female, n=4; S2fl/fl 28D, male, n=3, female, n=7; FS2KO 28D, male, n=5, female, n=6). Scalebar=20 μm

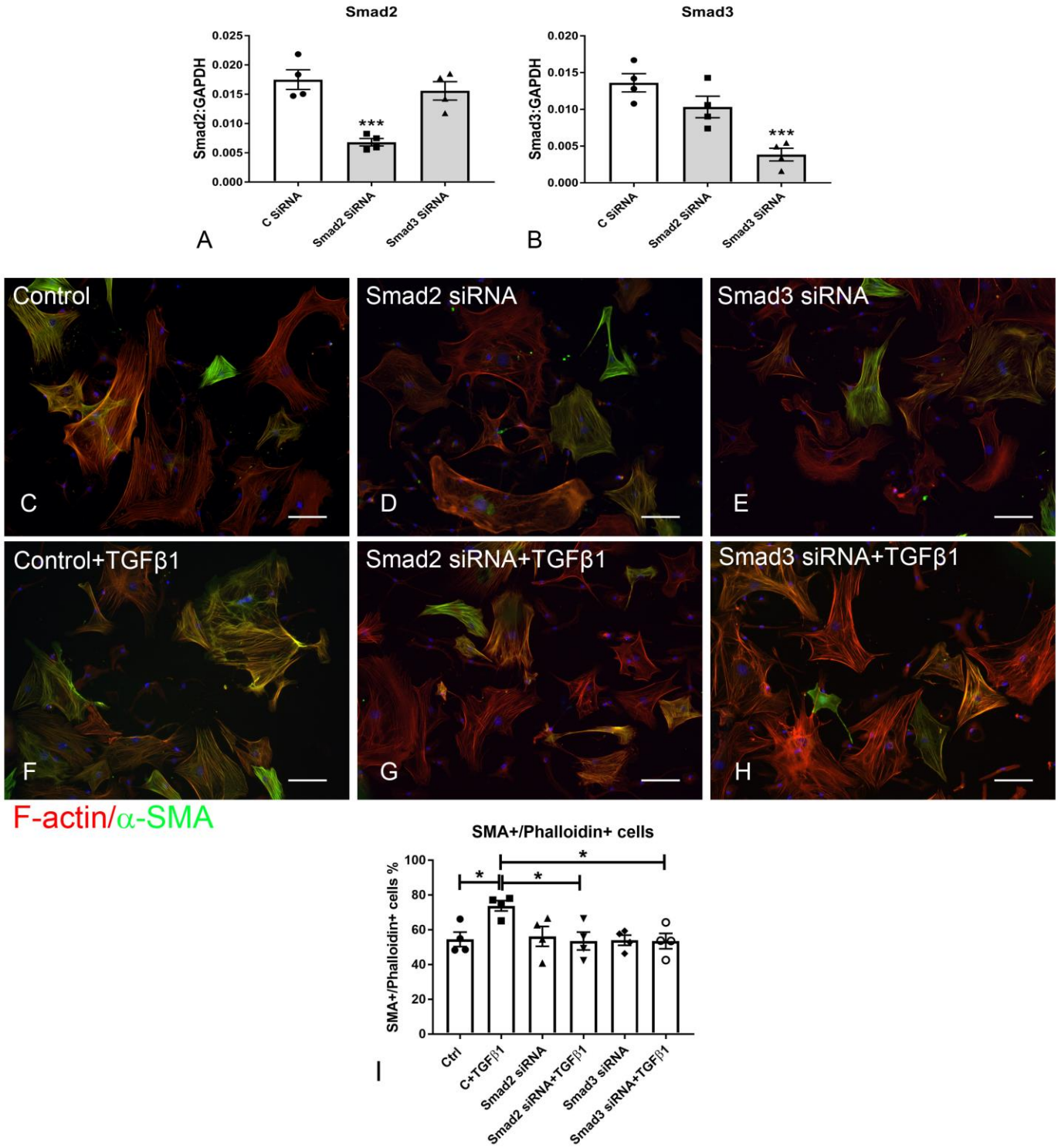


Supplemental figure X: FS3KO mice, but not FS2KO animals have perturbations of collagen fiber structure after 7 days of coronary occlusion. Sections from FS2KO, FS3KO, Smad3 fl/fl and Smad2 fl/fl infarcts were stained with with picrosirius red and viewed under polarized light microscopy. Representative images are shown. FS3KO mice had perturbed alignment of collagen fibers and evidence of collagen fragmentation in the infarct zone, when compared to the well-aligned fibers in S3 fl/fl infarcts (E-H, arrows). In contrast, FS2KO mice had a well-organized network of collagen fibers in the infarcted heart at both 7 and 28 day timepoints (A-D, arrows). Scalebar=20 μm

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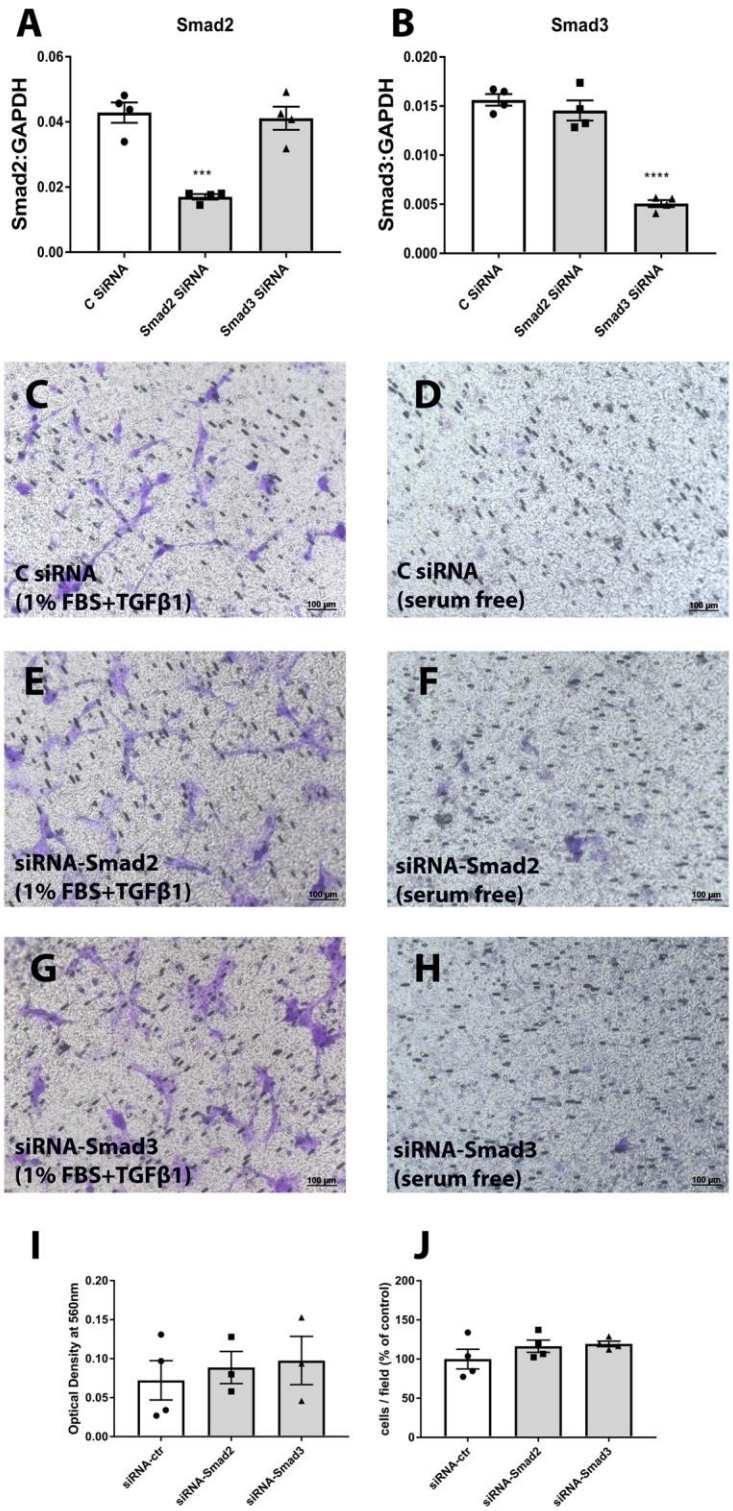


Supplemental Figure XI: Effects of Smad3 and Smad2 siRNA KD on α-SMA and vimentin gene expression in cardiac fibroblasts cultured in plates. A-B: Effectiveness and specificity of the siRNA KD strategy was assessed. Smad2, but not Smad3 siRNA KD markedly reduced Smad2 levels. B: Smad3, but not Smad2 siRNA KD decreased Smad3 expression (C, control siRNA). C: Smad3 KD cells had trends towards reduced α-SMA expression (p=0.051) and vimentin expression (p=0.12). In contrast, Smad2 KD did not affect α-SMA and vimentin levels (***p<0.001, ****p<0.0001 vs C, n=6-7/group - ANOVA followed by Tukey's multiple comparison test).



Supplemental figure XII: Smad2 or Smad3 knockdown attenuate α-SMA incorporation into stress fibers in TGF-β-stimulated fibroblasts. A-B: Effectiveness and specificity of Smad2 (A) and Smad3 (B) siRNA KD in fibroblasts was demonstrated using qPCR. Smad2 KD reduced Smad2 levels without affecting Smad3 levels, whereas Smad3 KD attenuated Smad3 synthesis without significant effects on Smad2 expression. C-H: Dual fluorescence with phalloidin-AF594 and a FITC-labeled anti-α-SMA antibody showed that Smad2 or Smad3

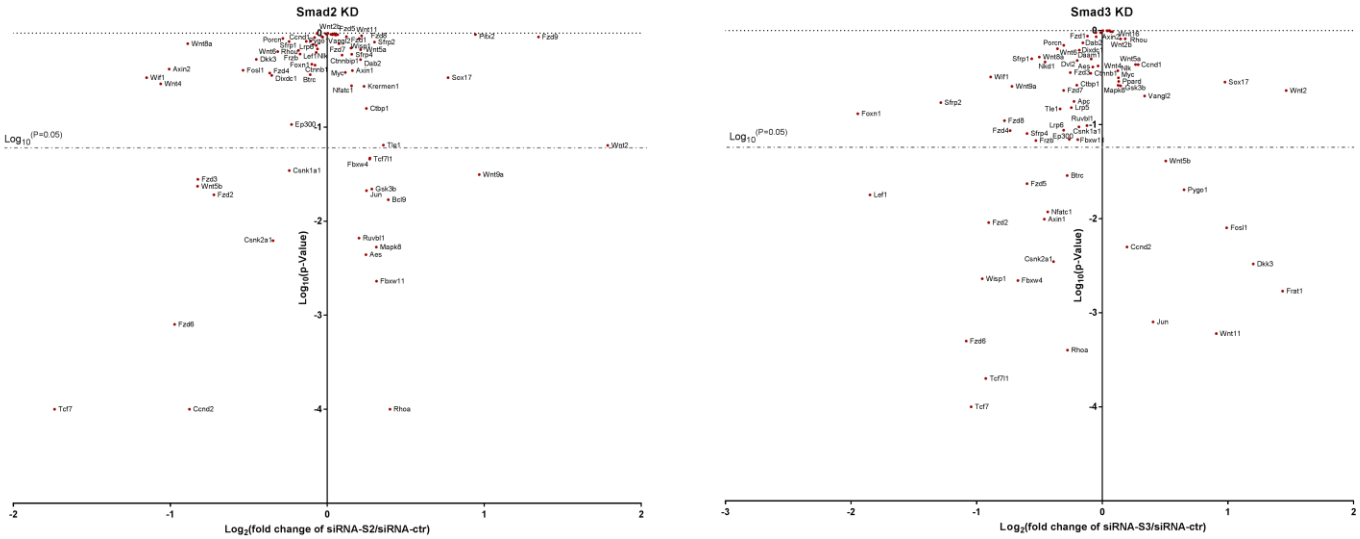
KD attenuated formation of α-SMA-decorated stress fibers in TGF-β-stimulated fibroblasts (C-H). Quantitative analysis showed that Smad2 and Smad3 KD did not affect the percentage of α-SMA+ cells at baseline (C-E, I), but significantly reduced the number of α-SMA+ cells following stimulation with TGF-β1 (F-H, I). (*$p<0.05$, n=4/group - ANOVA followed by Tukey's multiple comparison test).



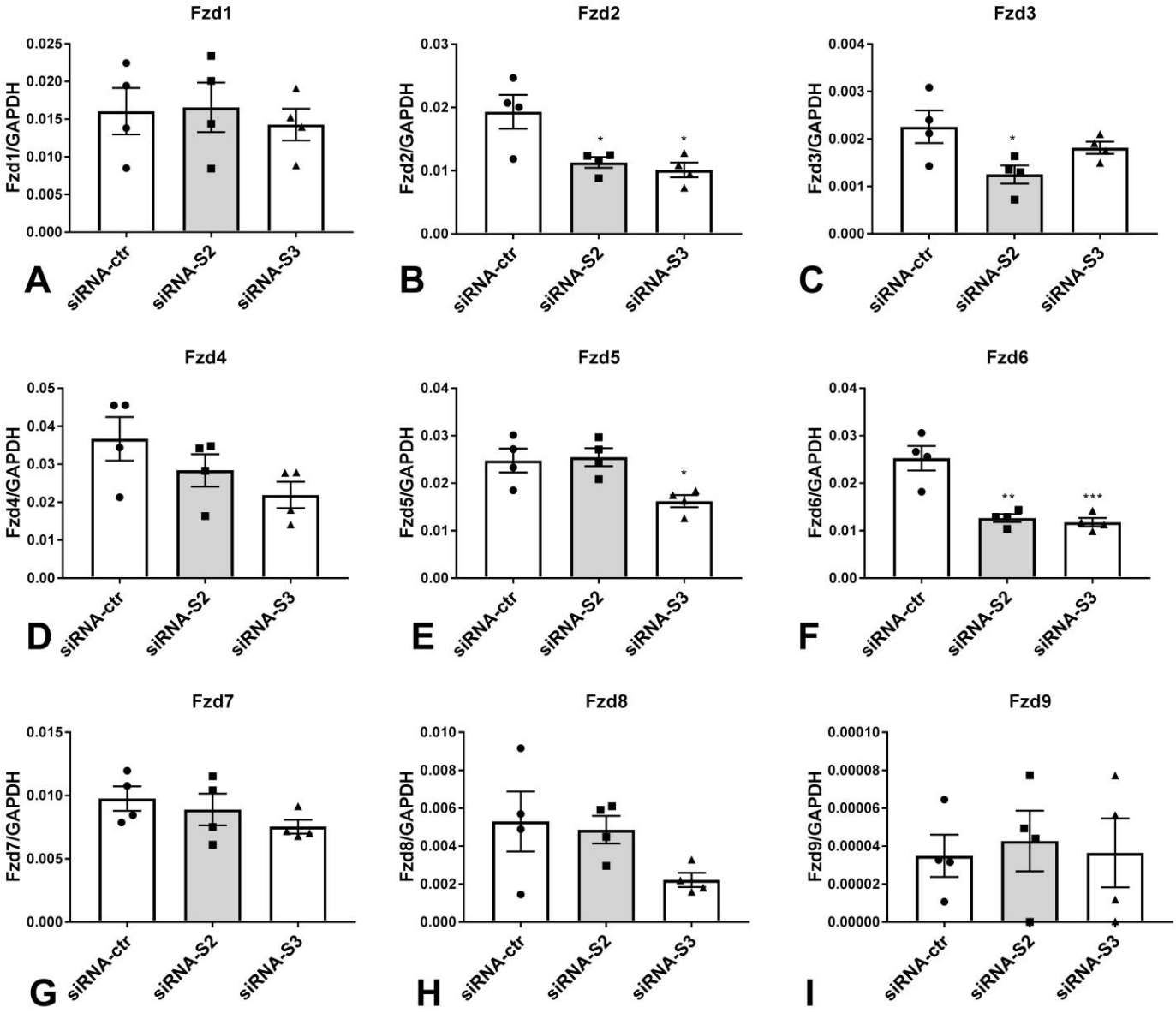
Supplemental figure XIII: Effects of Smad2 and Smad3 loss on migration of fibroblasts in response to serum and TGF-β1. qPCR was used to document Smad2 and Smad3 loss following siRNA knockdown (A-B). A transwell assay was used to study the role of Smad2 and Smad3 in fibroblast migration in response to serum and TGF-β1. Representative images of fibroblasts treated with control siRNA (C-D), Smad2 siRNA (E-F), Smad3 siRNA (G-H) that migrate towards serum free DMEM/F12 or 1% FBS DMEM/F12 +20ng/ml TGF-β1

are shown. In the serum-free chambers, there was very limited fibroblast migration (D, F, H). Stimulation with serum (1% FBS) and TGF-β1 (20ng/ml) induced significant cardiac fibroblast migration (C, E, G). Quantitative analysis demonstrated that Smad2 or Smad3 siRNA knockdown had no significant effects on fibroblast migration (I-J). (n=4/group - ANOVA followed by Tukey's multiple comparison test).

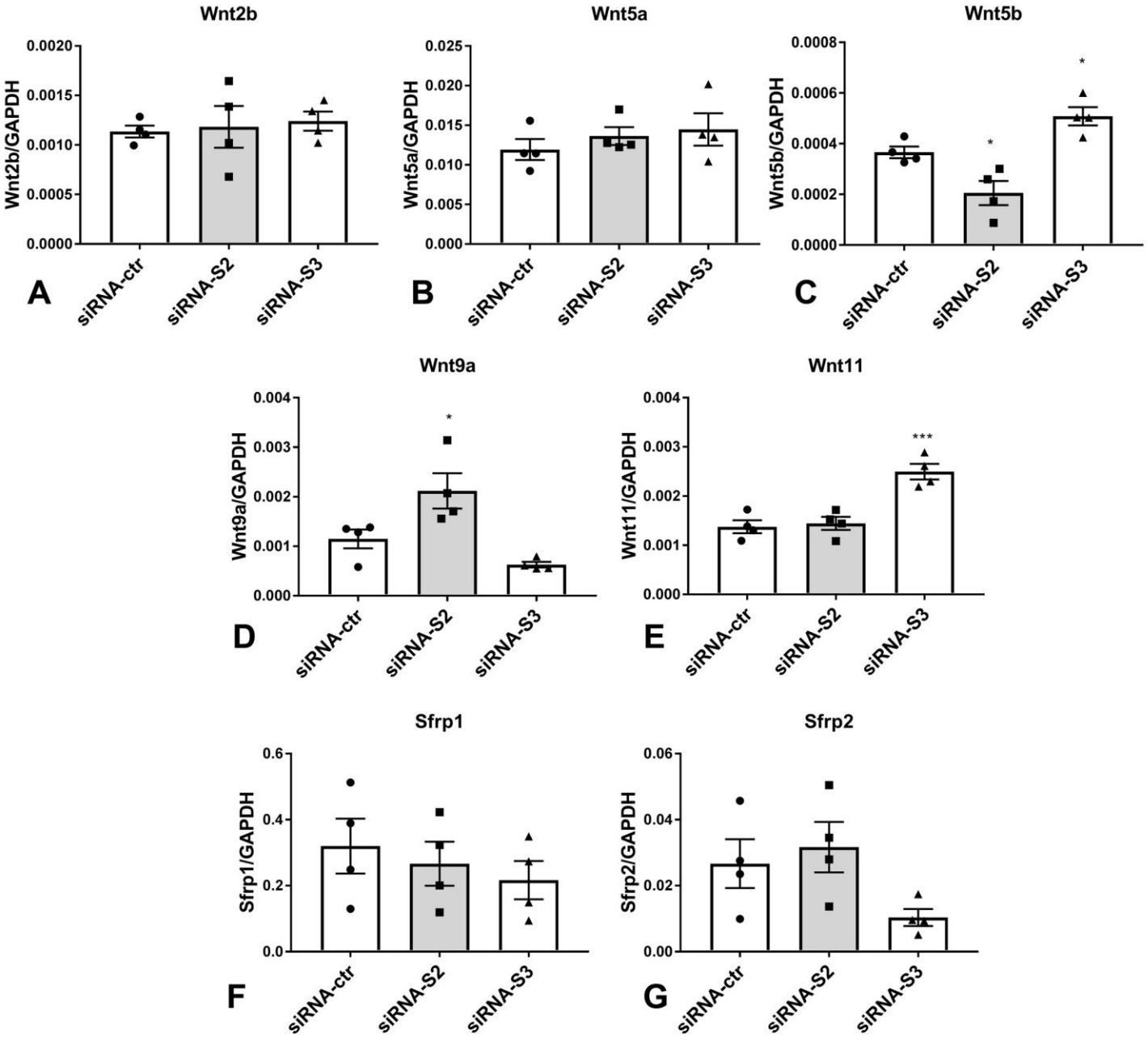
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Supplemental figure XIV: This volcano plot summarizes planar cell polarity pathways (PCP) genes expression in isolated fibroblasts treated with Smad2 siRNA (left panel) or Smad3 siRNA (right panel), compared to control siRNA. Detailed data are provided in Supplementary Table I. Statistical analysis was performed using ANOVA followed by Tukey's multiple comparison test.



Supplemental figure XV: Effects of Smad2 and Smad3 loss on frizzled (Fzd) gene expression in cardiac fibroblasts. Frizzled genes are critically involved in PCP pathway signaling; expression of Fzd2 has been observed in polarized infarct myofibroblasts[12] and may be involved in cell alignment in healing infarcts. Neither Smad2 nor Smad3 KD affected Fzd1 expression in cardiac fibroblasts (A). Fzd2 (B) and Fzd6 (F) mRNA levels were significantly reduced in both Smad2 and Smad3 KD cells. Smad2 KD reduced Fzd3 expression (C), whereas Smad3 KD decreased Fzd5 (E) levels. Expression of Fzd4 (D), Fzd7 (G), Fzd8 (H) and Fzd9 (I) was not affected by Smad3 or Smad2 loss (*$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control siRNA, n=4/group - ANOVA followed by Tukey's multiple comparison test).



Supplemental figure XVI: Effects of Smad2 and Smad3 KD on Wnt and Sfrp gene expression in cardiac fibroblasts. Wnt5b expression was significantly decreased in Smad2 KD fibroblasts, but increased in Smad3 KD cells (C). Moreover, Smad2 KD increased Wnt9a levels (D), whereas Smad3 KD accentuated Wnt11 expression (E). Expression of Wnt2b (A), Wnt5a (B) was not affected by Smad2 or Samd3 loss. Cardiac fibroblasts also expressed high levels of the secreted frizzle-related proteins Sfrp1 and Sfrp2. However, Sfrp1 (F) and Sfrp2 (G) were not affected by Smad2 or Smad3 KD (*$p<0.05$, ***$p<0.001$, n=4/group - ANOVA followed by Tukey's multiple comparison test)

SUPPLEMENTARY TABLES:

Supplemental Table 1: Effects of Smad2 and Smad3 siRNA knockdown on the expression of Wnt signaling pathway genes by cardiac fibroblasts.

Gene name	Gene symbols	Smad2 siRNA		Smad3 siRNA	
		Fold change vs. control siRNA	***P***	Fold change vs. control siRNA	***P***
Amino-terminal enhancer of split	*Aes*	1.19	0.0044†	0.95	0.4101
Adenomatosis polyposis coli	*Apc*	1.05	0.7736	0.86	0.1765
Axin1	*Axin1*	1.12	0.3998	0.73	0.0099†
Axin2	*Axin2*	0.50	0.4144	0.96	0.9518
B-cell CLL/lymphoma 9	*Bcl9*	1.31	0.0169†	0.97	0.8598
Beta-transducin repeat containing protein (b-TrCP)	*Btrc*	0.93	0.3616	0.82	0.0288†
Cyclin D1	*Ccnd1*	0.93	0.8228	1.22	0.4349
Cyclin D2	*Ccnd2*	0.54	0.0001†	1.15	0.0050†
Casein kinase 1, alpha 1	*Csnk1a1*	0.85	0.0346†	0.88	0.0946†
Casein kinase 2, alpha 1 polypeptide	*Csnk2a1*	0.79	0.0062†	0.76	0.0035†
C-terminal binding protein 1	*Ctbp1*	1.19	0.1582	0.87	0.2626

Catenin (Cadherin associated protein), beta1	*Ctnnb1*	0.95	0.457	0.94	0.3512
Catenin beta interacting protein 1	*Ctnnbip1*	1.07	0.5838	0.99	0.9496
Dishevelled associated activator of morphogenesis 1	*Daam1*	0.99	0.9799	0.94	0.4994
Disabled homolog 2 (Drosophila)	*Dab2*	1.16	0.5227	0.90	0.7409
DIX domain containing 1	*Dixdc1*	0.78	0.3555	0.88	0.6205
Dickkopf homolog 1 (Xenopus laevis)	*Dkk1*	ND	-	ND	-
Dickkopf homolog 3 (Xenopus laevis)	*Dkk3*	0.73	0.5255	2.30	0.0033†
Dishevelled, dsh homolog 1 (Dorsophila)	*Dvl1*	1.02	0.9709	1.00	0.9994
Dishevelled 2, dsh homolog (Dorsophila)	*Dvl2*	0.95	0.8992	0.87	0.4805
E1A binding protein p300	*Ep300*	0.85	0.1068	0.83	0.0697
F-box and WD-40 domain protein 11	*Fbxw11*	1.24	0.0023†	0.87	0.0693
F-box and WD-40 domain protein 4	*Fbxw4*	1.21	0.0469†	0.63	0.0022†
Fibroblast growth factor 4	*Fgf4*	ND	-	ND	-

Fos-like antigen 1	*Fosl1*	0.69	0.4024	1.99	0.0080†
Forkhead box N1	*Foxn1*	0.93	0.4672	0.26	0.1307
Frequently rearranged in advanced T-cell lymphomas	*Frat1*	1.15	0.8768	2.70	0.0017†
Frizzled-related protein	*Frzb*	0.89	0.5987	0.69	0.0677
Frizzled homolog 1 (Drosophila)	*Fzd1*	1.03	0.9885	0.92	0.8735
Frizzled homolog 2 (Drosophila)	*Fzd2*	0.61	0.0191†	0.53	0.0091†
Frizzled homolog 3 (Drosophila)	*Fzd3*	0.56	0.0279†	0.84	0.3583
Frizzled homolog 4 (Drosophila)	*Fzd4*	0.78	0.3764	0.60	0.0865
Frizzled homolog 5 (Drosophila)	*Fzd5*	1.04	0.9552	0.66	0.0236†
Frizzled homolog 6 (Drosophila)	*Fzd6*	0.51	0.0008†	0.47	0.0005†
Frizzled homolog 7 (Drosophila)	*Fzd7*	0.94	0.7573	0.81	0.2311
Frizzled homolog 8 (Drosophila)	*Fzd8*	1.16	0.9367	0.58	0.1101
Frizzled homolog 9 (Drosophila)	*Fzd9*	2.54	0.9104	1.03	0.9961
Glycogen synthase kinase 3 beta	*Gsk3b*	1.22	0.0220†	1.11	0.2588
Jun oncogene	*Jun*	1.19	0.0211†	1.32	0.0008†

Kringle containing transmembrane protein 1	*Krermen1*	1.18	0.2718	1.03	0.9971
Lymphoid enhancer binding factor 1	*Lef1*	0.95	0.6228	0.28	0.0179†
Low density lipoprotein receptor-related protein 5	*Lrp5*	0.98	0.9190	0.84	0.1516
Low density lipoprotein receptor-related protein 5	*Lrp6*	0.95	0.7395	0.81	0.0872
Mitogen-activated protein kinase 8	*Mapk8*	1.24	0.0053†	1.09	0.2618
Matrix metallopeptidase 7	*Mmp7*	ND	-	ND	-
Myelocytomatosis oncogene	*Myc*	1.08	0.3826	1.09	0.3147
Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	*Nfatc1*	1.11	0.2757	0.74	0.0118†
Naked cuticle 1 homolog (Dorsophila)	*Nkd1*	0.96	0.9934	0.73	0.4641
Nemo like kinase	*Nlk*	0.96	0.6795	1.09	0.3740
Paired-like homeodomain transcription factor 2	*Pitx2*	1.92	0.9668	1.05	0.9785
Porcupine homolog (Dorsophila)	*Porcn*	0.82	0.8785	0.81	0.6971

Peroxisome proliferator activator receptor delta	*Ppard*	1.00	0.9971	1.09	0.2880
Prickle homolog 1 (Dorsophila)	*Prickle1*	1.02	0.9617	1.06	0.9731
Pygopus 1	*Pygo1*	0.91	0.8192	1.57	0.0203†
Ras homolog gene family , member A	*Rhoa*	1.32	0.0001†	0.83	0.0004†
Ras homolog gene family , member U	*Rhou*	0.88	0.6545	1.13	0.8186
Ruvb-like protein 1	*Ruvbl1*	1.15	0.0066†	0.92	0.0977
Secreted frizzled-related protein 1	*Sfrp1*	0.84	0.8148	0.68	0.5012
Secreted frizzled-related protein 2	*Sfrp2*	1.23	0.8035	0.41	0.1715
Secreted frizzled-related protein 4	*Sfrp4*	1.11	0.5959	0.66	0.0804
SRY-box containning gene 17	*Sox17*	1.70	0.3362	1.97	0.2832
Transcription factor 7, T-cell specific	*Tcf7*	0.30	0.0001†	0.48	0.0001†
Transcription factor 7-like 1, (T-cell specific, MHG box)	*Tcf7l1*	1.21	0.0457†	0.53	0.0002†
Transducin-like enhancer of split 1, homolog of Drosophila E (spl)	*Tle1*	1.28	0.0645	0.79	0.1469
Vang-like 2 (van gogh, Dorsophila)	*Vangl2*	0.98	0.9055	1.26	0.2020

Wnt1 inhibitory factor 1	*Wif1*	0.45	0.3363	0.54	0.3219
WNT1 inducible signaling pathway protein 1	*Wisp1*	1.11	0.6937	0.52	0.0023†
Wingless-related MMTV integration site 1	*Wnt1*	ND	-	ND	-
Wingless-related MMTV integration site 10a	*Wnt10a*	ND	-	ND	-
Wingless-related MMTV integration site 11	*Wnt11*	1.09	0.9187	1.88	0.0006†
Wingless-related MMTV integration site 16	*Wnt16*	1.05	0.9640	1.04	0.9994
Wingless-related MMTV integration site 2	*Wnt2*	3.45	0.0640	2.76	0.2303
Wingless-related MMTV integration site 2b	*Wnt2b*	1.04	0.9582	1.11	0.8166
Wingless-related MMTV integration site 3	*Wnt3*	ND	-	ND	-
Wingless-related MMTV integration site 3a	*Wnt3a*	ND	-	ND	-
Wingless-related MMTV integration site 4	*Wnt4*	0.48	0.2891	0.98	0.4207
Wingless-related MMTV integration site 5a	*Wnt5a*	1.16	0.6667	1.20	0.4360
Wingless-related MMTV integration site 5b	*Wnt5b*	0.56	0.0235†	1.42	0.0411†

Wingless-related MMTV integration site 6	*Wnt6*	0.80	0.6363	0.78	0.6420
Wingless-related MMTV integration site 7a	*Wnt7a*	ND	-	ND	-
Wingless-related MMTV integration site 7b	*Wnt7b*	ND	-	ND	-
Wingless-related MMTV integration site 8a	*Wnt8a*	0.54	0.7731	0.71	0.5225
Wingless-related MMTV integration site 8b	*Wnt8b*	ND	-	ND	-
Wingless-related MMTV integration site 9a	*Wnt9a*	1.96	0.0313†	0.61	0.2553

†P value <0.05, n=4/group.

ND-Not Detected

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SPOTLIGHT REVIEW

Immune cells as targets for cardioprotection: new players and novel therapeutic opportunities

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Abstract

New therapies are required to reduce myocardial infarct (MI) size and prevent the onset of heart failure in patients presenting with acute myocardial infarction (AMI), one of the leading causes of death and disability globally. In this regard, the immune cell response to AMI, which comprises an initial pro-inflammatory reaction followed by an anti-inflammatory phase, contributes to final MI size and post-AMI remodelling [changes in left ventricular (LV) size and function]. The transition between these two phases is critical in this regard, with a persistent and severe pro-inflammatory reaction leading to adverse LV remodelling and increased propensity for developing heart failure. In this review article, we provide an overview of the immune cells involved in orchestrating the complex and dynamic inflammatory response to AMI—these include neutrophils, monocytes/macrophages, and emerging players such as dendritic cells, lymphocytes, pericardial lymphoid cells, endothelial cells, and cardiac fibroblasts. We discuss potential reasons for past failures of anti-inflammatory cardioprotective therapies, and highlight new treatment targets for modulating the immune cell response to AMI, as a potential therapeutic strategy to improve clinical outcomes in AMI patients. This article is part of a Cardiovascular Research Spotlight Issue entitled 'Cardioprotection Beyond the Cardiomyocyte', and emerged as part of the discussions of the European Union (EU)-CARDIOPROTECTION Cooperation in Science and Technology (COST) Action, CA16225.

Keywords Inflammation • Myocardial ischaemia/reperfusion injury • Acute myocardial infarction • Monocytes • Macrophages • Lymphocytes • Dendritic cells • Fibroblasts

This article is part of the Spotlight Issue on Cardioprotection Beyond the Cardiomyocyte.

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1. Introduction

Acute myocardial infarction (AMI) and the heart failure which often follows are among the leading causes of death and disability worldwide. Although short-term mortality (in-hospital and 30 days) following AMI is on the decline,[1] long-term mortality (1 year and beyond) post-AMI remains significant, and this is, in part, due to the increase in the number of patients who survive their AMI, and go onto to develop heart failure.[2,3] As such, new treatments are required to reduce myocardial infarct (MI) size, in order to preserve left ventricular (LV) function and prevent the onset of heart failure in AMI patients. In this regard, the acute inflammatory response to AMI is a key determinant of final MI size and subsequent adverse post-AMI LV remodelling, making inflammation an important target for cardioprotection.[4,5] The initial acute inflammatory response to AMI is triggered by the innate immune response to cell necrosis, which includes the release of fragments of mitochondrial DNA into the tissue that can act as danger-associated molecular patterns (DAMPs),[6] complement activation, formation of the inflammasome, and so on, and is the focus of another article in this Cardiovascular Research Spotlight Issue.[7] The innate immune response activates and triggers the accumulation of immune cells into the ischaemic myocardium, and orchestrates the initial pro-inflammatory reaction, the main purpose of which is to clear necrotic cell debris from the MI zone. The immune cell response to AMI is characterized by early infiltration of neutrophils into the MI zone from 6 to 24 h post-AMI, followed by the accumulation of pro-inflammatory monocytes and macrophages over the next 48 to 72 h, both of which contribute to the cardiomyocyte death and myocardial injury which occurs in response to myocardial ischaemia/reperfusion injury (IRI).[5] This is followed by an anti-inflammatory reparative phase (days 4–7) which is mainly driven by anti-inflammatory monocytes/macrophages and is mediated by suppression, resolution, and containment of the initial pro-inflammatory response, and which permits wound healing and scar formation to occur. Because the inflammatory response to AMI evolves over several hours to days following reperfusion, anti-inflammatory cardioprotective strategies discussed in this article are unlikely to reduce *acute* MI size (defined in this article as ≤6 h of AMI) arising from the early reperfusion injury that occurs in the first few minutes of reperfusion. As such, MI size limitation using an anti-inflammatory cardioprotective strategy would be expected to only manifest at least 6–24 h after reperfusion, and therefore mainly arises from a reduction in *early* MI size (defined in this article as ≤24 h of AMI), and reduction in *late* MI size (defined in this article as ≥24 h of AMI) from beneficial effects on infarct-remodelling. Disturbances in both the balance and transition between the initial pro-inflammatory reaction and the subsequent anti-inflammatory reparative phase can augment myocardial IRI, and worsen post-MI adverse LV remodelling thereby increasing the propensity for the development of heart failure following AMI.

In this review article, we provide an overview of the immune cells involved in orchestrating the complex and dynamic inflammatory response to AMI, which include neutrophils, monocytes/macrophages, and emerging players such as dendritic cells (DCs), lymphocytes, pericardial lymphoid cells, endothelial cells, and cardiac fibroblasts. We review ways in which the immune cell response to AMI is modulated by endogenous cardioprotective strategies such as ischaemic preconditioning (IPC), ischaemic postconditioning (IPost), and remote ischaemic conditioning (RIC). Finally, we discuss potential reasons for past failures of anti-inflammatory cardioprotective therapies, and highlight emerging targets for modulating the inflammatory response to AMI, as potential novel therapeutic strategies to improve clinical outcomes following AMI.

2. Neutrophils as targets for cardioprotection

Neutrophils are the first immune cells recruited into the ischaemic heart following AMI. Increased circulating number[8] or volume of neutrophils[9] in patients suffering an AMI positively correlates with MI size, subsequent LV function and clinical outcomes. Once recruited in the ischaemic myocardium, neutrophils maintain the initial acute pro-inflammatory response to IRI. Their rapid degradation and degranulation propagates the acute inflammatory response to neighbouring areas of the myocardium (so-called 'neutrophil-induced injury')[10] and triggers monocyte infiltration into the ischaemic tissue.[11] Interestingly, the recruitment of neutrophils into the heart after AMI demonstrates a circadian pattern, which can impact on late MI size and LV function.[12] It has been demonstrated that neutrophils can polarize macrophages towards a reparative phenotype, and thus contribute to the healing phase following AMI, highlighting a potential protective role for neutrophils.[13] Therefore, therapeutic strategies targeted to neutrophils should take into consideration the potential beneficial effects of neutrophils in post-AMI healing.

Neutrophil function following AMI can also be modulated by endogenous cardioprotective phenomena such as IPost,[14] in which brief cycles of non-lethal ischaemia and reperfusion applied at the onset of reperfusion reduced neutrophil accumulation into the MI zone.[15] However, whether the reduction in myocardial accumulation of neutrophils observed with IPost is an epiphenomenon of improved myocardial salvage or is actually required for cardioprotection is not clear. Furthermore, a clinical study demonstrated that RIC (brief cycles of non-lethal ischaemia and reperfusion applied to the upper arm) down-regulated the expression of kinin B1 and B2 receptors in neutrophils of patients undergoing cardiac surgery.[16]

In summary, neutrophils are recruited into the ischaemic heart and their rapid degradation and degranulation results in an acute pro-inflammatory response which triggers monocyte infiltration in the first few hours. Novel approaches to regulate the neutrophils are RIC or IPost which modulate the expression of kinin B1 and B2 receptors in neutrophils (*Figure 1*).

2.1 Therapeutic targeting of neutrophils for cardioprotection

A number of treatment strategies which target neutrophils have been shown to reduce early and late MI size following AMI. These include those which reduce the accumulation of neutrophils into the MI zone, and include those directed to interleukin (IL)-1 inhibition,[17] anti-nuclear factor kappa-B ligand [RANKL],[18] transient receptor potential melastatin 2 ablation,[19] plasminogen activator inhibitor-1 ablation,[20] proteasome-mediated I kappa-B alpha inhibition,[21] complement C5a,[22] and glucagon-like peptide-1[23] (see *Table 1* for details). Another group of therapies have been shown to be beneficial following AMI by inhibiting neutrophil activity, such as lipoxygenase-cyclooxygenase,[24] Urge-8,[25] CI-959,[26] Lidocaine,[27] Tetrandrine,[28] myeloperoxidase (MPO) inhibition,[29] and so on (see *Table 1* for details). There have also been neutral experimental studies targeting inflammation induced by neutrophils.[30–32] Therapeutic targeting of neutrophils to reduce early MI size in the clinical setting following AMI has proven to be very challenging. For example, clinical studies targeting CD11/CD18 subunits of the β2 integrin adhesion receptors to prevent neutrophil adhesion did not report any cardioprotective effect on early MI size following AMI[33,34] (see *Table 1* for details).

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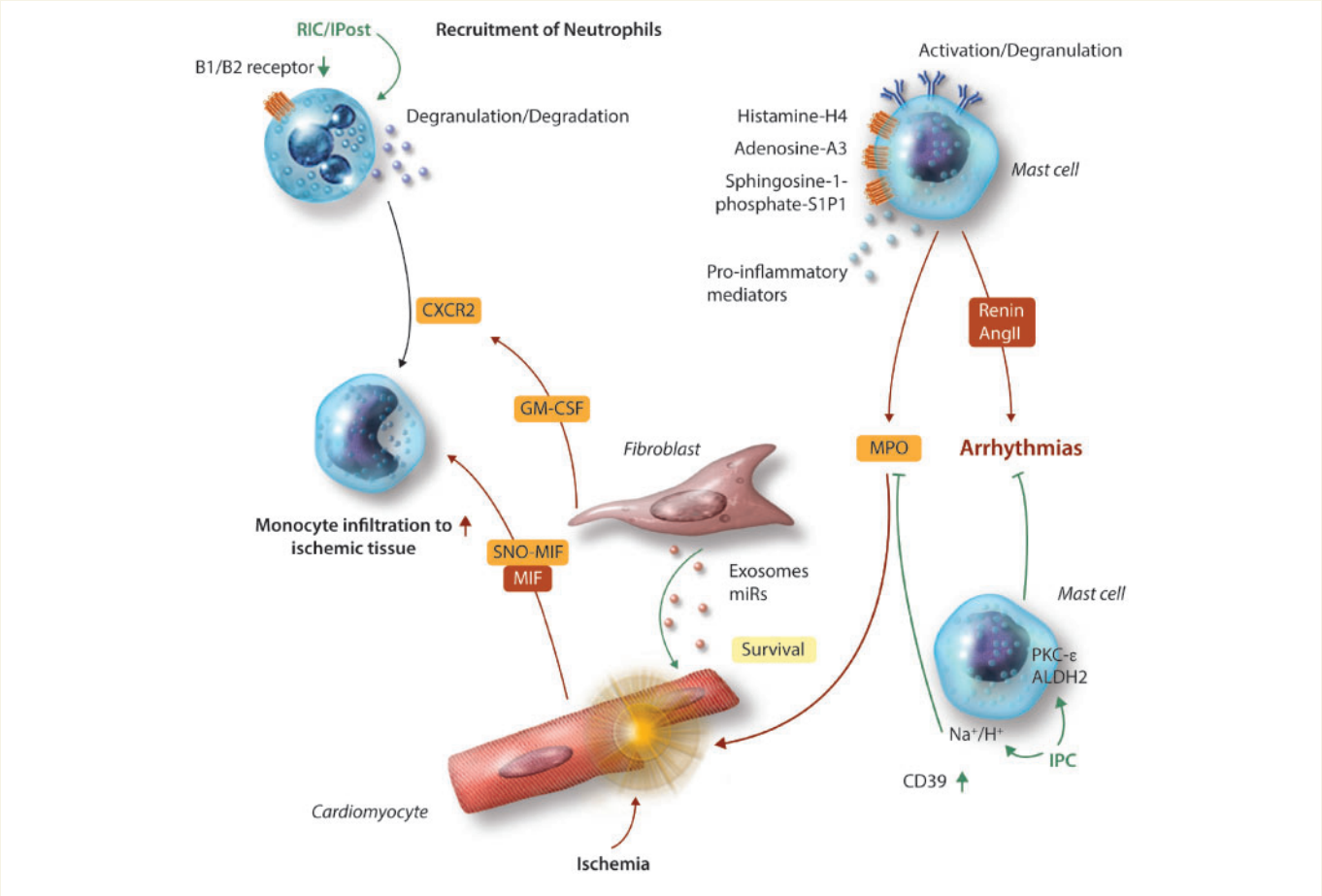


Figure 1 Immune cell changes several minutes to hours following AMI. Following AMI, neutrophils are recruited into the ischaemic heart. Their rapid degradation and degranulation result in an acute pro-inflammatory response and trigger monocyte infiltration in the first few hours. Novel approaches to regulate the neutrophils are RIC or IPost which modulate the expression of kinin B1 and B2 receptors in neutrophils. MIF is released by cardiomyocytes in response to ROS and hypoxia. MIF exerts compartmentalized and opposing effects after myocardial IRI mediated by CXCR2. At early reperfusion, fibroblast-derived GM-CSF release plays an important role in chemotactic attraction of neutrophils and monocytes into the infarcted myocardium. Mast cell activation and/or degranulation leads to the release of pro-inflammatory mediators while mast cell-derived renin induces reperfusion arrhythmias through the activation of RAS. IPC activates Gi-coupled receptors and stabilizes mast cells. The activation of Na^+/H^+ exchange attenuates MPO release and prevents post-IRI arrhythmias. Anti-RAS cardioprotection is also induced by the activation of PKC-ϵ and mitochondrial ALDH2.

In summary, although, it is well-established that neutrophils are an early contributor to the pro-inflammatory response following AMI, anti-inflammatory therapies directed towards neutrophils have failed in the clinical setting. The reasons for this failure to translate cardioprotection into the clinical setting are multiple and relate to the timing of therapy, the complexity of the inflammatory response to AMI, and the multiple players involved (see Section 9 for further discussion).

3. Monocytes and macrophages as targets for cardioprotection

Monocytes produced in the bone marrow and spleen, enter the bloodstream, and are recruited to the MI zone in the first few hours following AMI, with the spleen providing a steady source of monocytes once their reserves are depleted in the bone marrow.[35] The first population of monocytes that migrate to the site of infarction (peaking at day 3 after AMI) are the pro-inflammatory ($Ly6C^{high}$) monocytes that express tumour necrosis factor-α (TNF-α) and IL-1β, produce proteolytic enzymes, and secrete matrix metalloproteinases (MMPs), which degrades the extracellular matrix.[36] The $Ly6C^{high}$ monocytes differentiate into activated pro-inflammatory macrophages (M1 or CCR2+) that express IL-1β and TNF-α.[37] In the later stages of AMI, the $Ly\text{-}6C^{low}$ monocytes and M2 (or CCR2-) phenotypes become the predominant subtype, promote the healing response of AMI, and contribute to angiogenesis and collagen deposition to form scar tissue to replace the lost cardiomyocytes in the MI zone.[36,37] We must clarify herein that Ly6C monocyte subsets refer to those encountered in mice. Therefore, balancing the dynamic roles of monocytes/macrophages is prerequisite for optimal cardiac healing following AMI. In the clinical setting, it has been shown in AMI patients that higher levels of circulating pro-inflammatory monocytes and monocyte-platelet complexes were associated with increased myocardial injury and impaired recovery of LV function.[38,39]

Following AMI, a number of factors mediate the recruitment of $Ly6C^{high}$ monocyte migration into the MI zone including: monocyte chemoattractant protein-1 (MCP-1) production, which binds to C-C

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Table 1 Major studies investigating anti-inflammatory cardioprotective strategies targeting the immune cell response to reduce MI size and preventing adverse LV remodelling

Study	Experimental acute IRI model	Anti-inflammatory therapeutic strategy	Cardioprotective effect	Proposed mechanism(s) and translational potential
Neutrophils				
Burke *et al.*[26]	*In vivo* dogs 90 min ischaemia and 6 h reperfusion	CI-959 Before and during reperfusion	Reduction of acute MI size	Inhibiting the formation of toxic oxygen radicals by inflammatory cells
Tanaka *et al.*[147]	*In vivo* dogs 90 min ischaemia and 3 h reperfusion	Anti-CD18 Prior to ischaemia	Reduction of acute MI size	Prevents adhesion and accumulation of neutrophils. Clinical studies using this approach have been neutral (LIMIT-AMI and HALT-AMI)[33,34]
Amsterdam *et al.*[24]	*In vivo* pigs 50 min ischaemia and 3 h reperfusion	BW755C Prior to ischaemia	Reduction of acute MI size	Selective inhibition of neutrophil cytotoxic activity by inhibiting dual cyclooxygenase-lipoxygenase blocking agent without affecting neutrophil migration into injured myocardium
Vitola *et al.*[27]	*In vivo* rabbits 30 min ischaemia and 48 h reperfusion	Lidocaine First 10 min of ischaemia	Reduction of late MI size	Sodium channel blocker which inhibits several neutrophil functions
Shen *et al.*[28]	*In vivo* rats 30 min ischaemia and 1 h reperfusion	Tetrandrine Prior to ischaemia	Reduction of acute MI size	Inhibition of neutrophil priming, adhesion, and activation, and abolishment of subsequent infiltration and ROS production
Bao *et al.*[21]	*In vivo* rats 30 min ischaemia and 24 h reperfusion	PR-39 At reperfusion	Reduced late MI size	Inhibitor of proteasome-mediated I kappa-B alpha degradation and its truncated form PR-11 resulting in less neutrophil infiltration, MPO activity, and expression of ICAM-1 and VCAM-1
Kohtani *et al.*[25]	*In vivo* rats 60 min ischaemia and 24 h reperfusion	Urge-8 Prior to ischaemia and at reperfusion	Reduction of early MI size	Anti-neutrophil monoclonal antibody which inhibits chemotactic activity, superoxide production, phagocytic function, and neutrophil degranulation
Granfeldt *et al.*[15]	*In vivo* rats 30 min ischaemia and 3 h reperfusion	IPost At reperfusion	Reduction of acute MI size	Reduced accumulation of neutrophils in MI zone. No further reduction in MI size with IPost in neutrophil-depleted rats
Wu *et al.*[148]	*In vivo* mice 30 min ischaemia and 24 and 72 h reperfusion	Recombinant human IL-37 At reperfusion	Reduction of late MI size	IL-37 protected cardiomyocytes from apoptosis and suppressed the migration ability of neutrophils towards the chemokine LIX
Carbone *et al.*[18]	Mice 30 min ischaemia and 24 h reperfusion	Anti-RANKL IgG During ischaemia	Reduced late MI size and preserved LV function	Antibody to neutralization of receptor activator of nuclear factor kappa-B ligand (RANKL) reduced neutrophil infiltration, ROS and MMP-9 release.
Ali *et al.*[29]	Mice 30 min ischaemia and 48 h reperfusion	PF-1355 Post-reperfusion for 1 week	Less post-AMI LV remodelling	Pharmacological MPO inhibitor started 1 h post-AMI reduced leucocyte accumulation and prevented adverse LV remodelling.

Continued

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Table 1 *Continued*

Study	Experimental acute IRI model	Anti-inflammatory therapeutic strategy	Cardioprotective effect	Proposed mechanism(s) and translational potential
Monocytes/macrophages				
Nakano *et al.*[56]	*In vivo* mice 30 min ischaemia and 12 and 24 h reperfusion	Ibesartan At reperfusion	Reduction of early and late MI size	PPAR-γ-dependent anti-inflammatory mechanisms. Antagonizing monocyte-mediated inflammation
Zhou *et al.*[52]	*In vivo* rat 45 min ischaemia and 30 days reperfusion	Phenytoin delivered in liposomes to monocytes and macrophages at day 2 and 4 post-AMI	Reduction of late MI size and less LV fibrosis	Non-selective VGSC inhibitor inhibited pro-inflammatory monocytes and promoted differentiation of M1 to M2 macrophages
Wang *et al.*[55]	*In vivo* murine Permanent ischaemia	Topiramate After ischaemia	Reduction of late MI size and less adverse LV remodelling	GABA-A receptor agonist inhibited pro-inflammatory monocytes and promoted differentiation of M1 to M2 macrophages
Tian *et al.*[54]	*In vivo* mice 40 min ischaemia and 1 h reperfusion	BAY 60-6583 Prior to ischaemia	Reduction of acute MI size	Adenosine 2B Receptor agonist, BAY 60-6583, has anti-inflammatory effects, probably by modulating macrophages phenotype switching via a PI3K/Akt pathway
Lymphocytes				
Yang *et al.*[64]	*In vivo* mice 45 min ischaemia and 24 h reperfusion	ATL146e At reperfusion	Reduction of late MI size	A2AR agonist which inhibits $CD4^+$ T cell accumulation and activation in the reperfused heart. $CD4^+$ but not $CD8^+$ T lymphocytes contribute to acute myocardial IRI
Zouggari *et al.*[42]	*In vivo* murine Permanent ischaemia	Rituximab One hour after infarction	Less adverse LV fibrosis and remodelling	Depletion of B cells by rituximab (CD20 specific antibody) inhibited pro-inflammatory monocyte infiltration
Mast cells				
Parikh *et al.*[79]	*In vitro* rat 30 min ischaemia and 30 min reperfusion	Disodium cromoglycate During ischaemia and reperfusion	Reduction of acute MI size	DSCG stabilized mast cells and reduced MPO activation DSCG blocked cardioprotective effects of IPC
Parikh *et al.*[87]	*In vitro* rat 30 min ischaemia and 30 min reperfusion	NE Prior to ischaemia	Reduction of acute MI size	NE stabilized mast cells and reduced MPO activation
Jaggi *et al.*[78]	*In vitro* rat 30 min ischaemia and 120 min reperfusion	Ketotifen and carvedilol Compound 48/80 prior to ischaemia	Reduction of acute MI size	Ketotifen and carvedilol inhibited mast cell degranulation during ischaemia/reperfusion Compound 48/80, a mast cell granulator, was cardioprotective when administered prior to ischaemia, to degranulate mast cells prior to AMI
Cardiac fibroblasts				
Abrial *et al.*[95]	*In vivo* rats 60 min and 24 h of reperfusion	To investigate the role of CF	Late MI size reduction was observed in CF secretome treated mice compared with control	CFs participate in cardioprotection during the acute phase of ischaemia-reperfusion via a paracrine pathway involving TIMP-1

CF, cardiac fibroblast; ROS, reactive oxygen species.

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chemokine receptor type 2 (CCR2) expressed on the surface of monocytes[40,41] expression of chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-C motif) ligand 7 (CCL7), which also mediate CCR2-dependent monocyte migration.[42] Very recently it has been demonstrated that the human myocardium also contains distinct subsets of CCR2- and CCR2+ macrophages which have distinct functional properties, analogous to reparative CCR2- and inflammatory CCR2+ macrophages in the mouse heart.[43] Angiotensin II (ANG II), the concentration of which is increased in the circulation following AMI, recruit monocytes from the splenic reservoir[44] the sympathetic nervous system that stimulates the entry of haematopoietic stem cells from the bone marrow through β3-adrenergic receptor signalling, and β2-adrenergic receptors, which play a crucial role in the migration of monocytes/macrophages to MI zone.[45] The orphan nuclear hormone receptor, nuclear receptor subfamily 4, group a, member 1 (Nr4a1) is critical for limiting the migration of pro-inflammatory monocytes to the site of infarction and for increasing the differentiation of reparative macrophages, and eventually for preventing adverse cardiac remodelling.[46]

Macrophage migration inhibitory factor (MIF) plays dual roles in the setting of myocardial IRI. MIF released by cardiomyocytes in a model of IRI was shown to exert cardioprotective effects via the CD74/5′ AMP-activated protein kinase/c-Jun N-terminal kinase axis (CD74/AMPK/JNK),[47,48] and the cardioprotective effect was enhanced by S-nitrosylation of MIF.[49] In contrast, another study reported that MIF deficiency protected the heart from IRI by suppressing the pro-inflammatory response, suggesting a detrimental role for MIF following AMI.[50] The compartmentalized and opposing effects of MIF after myocardial IRI are largely mediated by C-X-C motif chemokine receptor 2 (CXCR2). MIF confers protective effects improving myocardial healing and function through CXCR2 in resident cells, however, it exerts detrimental effects on CXCR2-bearing inflammatory cells, increasing monocyte infiltration and impairing heart function.[30] During reperfusion in mice, macrophage myeloid-epithelial-reproductive tyrosine kinase (MerTK) deficiency led to decreased cardiac wound debridement, increased MI size, and depressed cardiac function, whereas blockade of CCR2-dependent monocyte infiltration into the heart reduced soluble MER (myeloid-epithelial-reproductive tyrosine kinase; i.e. soluble MER) levels post-AMI, implicating monocyte-induced MerTK cleavage as a novel contributor and therapeutic target for preventing myocardial IRI.[51] Signals and receptors involved in monocyte/macrophage recruitment/internalization are illustrated in *Figures 1–3*.

A number of therapies have been shown to protect the heart post-AMI by promoting the monocyte/macrophage phenotypic switch from pro-inflammatory to anti-inflammatory phenotypes including phenytoin, a non-selective voltage-gated sodium channel (VGSC) inhibitor,[52] BAY 60-6583, an adenosine A2B receptor ($A_{2B}R$) agonist,[53,54] and γ-aminobutyric acid (GABA) receptor antagonists such as topiramate or bicuculline[55] (see *Table 1* for details).

3.1 Therapeutic targeting of monocytes/macrophages for cardioprotection

Emerging evidence shows that anti-inflammatory strategies targeting the pro-inflammatory monocyte/macrophage subset can reduce excessive inflammation and improve cardiovascular outcomes. Angiotensin-converting enzyme (ACE) inhibitors have been shown to reduce monocyte migration from the spleen.[44] These data could explain the benefits of ACE inhibitors in patients with an early stage of AMI as demonstrated by the ability of these drugs to reduce inflammation and lower mortality.[44]

Insufficient local drug concentration in the ischaemic heart may be one factor for prior failed clinical cardioprotection studies, and nanoparticles may be used to improve delivery of cardioprotective therapies to the ischaemic heart following AMI. In this regard, it has been shown that irbesartan, an ANG II type 1 receptor blocker with a peroxisome proliferator activated receptor (PPAR-γ) agonistic effect, delivered at reperfusion using nanoparticles inhibited the recruitment of pro-inflammatory monocytes to the infarcted myocardium, and ameliorated LV remodelling at 21 days in a murine model of myocardial IRI.[56]

In summary, the monocyte/macrophage response to AMI is biphasic and therefore anti-inflammatory therapies targeting these immune cells need to take this into consideration.

4. Lymphocytes as emerging targets for cardioprotection

A role for lymphocytes in the inflammatory response to AMI has recently emerged. T lymphocytes are activated within a few days after AMI in lymph nodes draining the myocardium.[57] Analysis of transcoronary gradients from AMI patients has demonstrated early recruitment of T and B lymphocytes into myocardium within 90 min of reperfusion.[58] Animals deficient in helper T cells develop impaired healing after AMI, and this is associated with an increase in myocardial monocytes/macrophages. Additionally, very recently it has been shown that T-bet (a transcription factor that regulates the differentiation and function of immune cells) deficiency attenuates pressure overload-induced cardiac remodelling in rats, indicating that targeting T-bet in T cells may be of great importance for the treatment of heart failure.[59] Regulatory T cells (T_{reg}) are known to down-regulate the innate immune response. Indeed it has been shown that a lack of T_{reg} mirrors the phenotype of T-helper cell deficient animals after AMI. In contrast, activation of T_{reg} by superagonistic anti-CD28 monoclonal antibody administered 2 days after AMI improved healing and survival.[60] This is most likely due to differentiation effects on monocytes/macrophages that developed a 'pro-healing' phenotype after T_{reg} activation in a transforming growth factor beta 1 (TGF-β1)-dependent manner.[60] This concept is supported by another study where depletion of T_{reg} after AMI worsened LV remodelling. Moreover, T_{reg} changed the phenotype of cardiac fibroblasts with a reduced contraction of fibroblast-populated collagen pads.[61] Injection of tolerogenic DCs can modulate T_{reg}. Indeed, such DCs primed with lysate from infarcts induced a shift towards reparative macrophages via activation of T_{reg} and better healing after AMI.[62] It has been shown that some cardioprotective effects of IPC appear to depend on adequate T_{reg} function–T_{reg} depletion was associated with increased late MI size and pronounced infiltration of inflammatory cells in a rat model of IPC.[63]

Recombination activating gene 1 knockout (RAG1 KO) mice, which are deficient in lymphocytes, had significant smaller MI size when compared with wild-type animals. Adoptive transfer of $CD4^+$ T cells in RAG1-KO mice blunted the protective effect.[64] Accordingly, $CD4^+$ T cell deficient animals had smaller late MI size after AMI.[65] This effect could be reversed by an adenosine receptor agonist. Adenosine can be synthesized by CD39 and 73 from nucleotides released by tissue injury. CD39 and CD73 are expressed on lymphocytes and cardiomyocytes.[66]

Finally, B lymphocytes are also recruited to the injured myocardium after AMI. B lymphocytes produced CCL7 mediates the recruitment of $Ly6C^{high}$ monocytes. Depletion of B cells by rituximab (CD20 specific antibody) had beneficial effects after AMI.[42]

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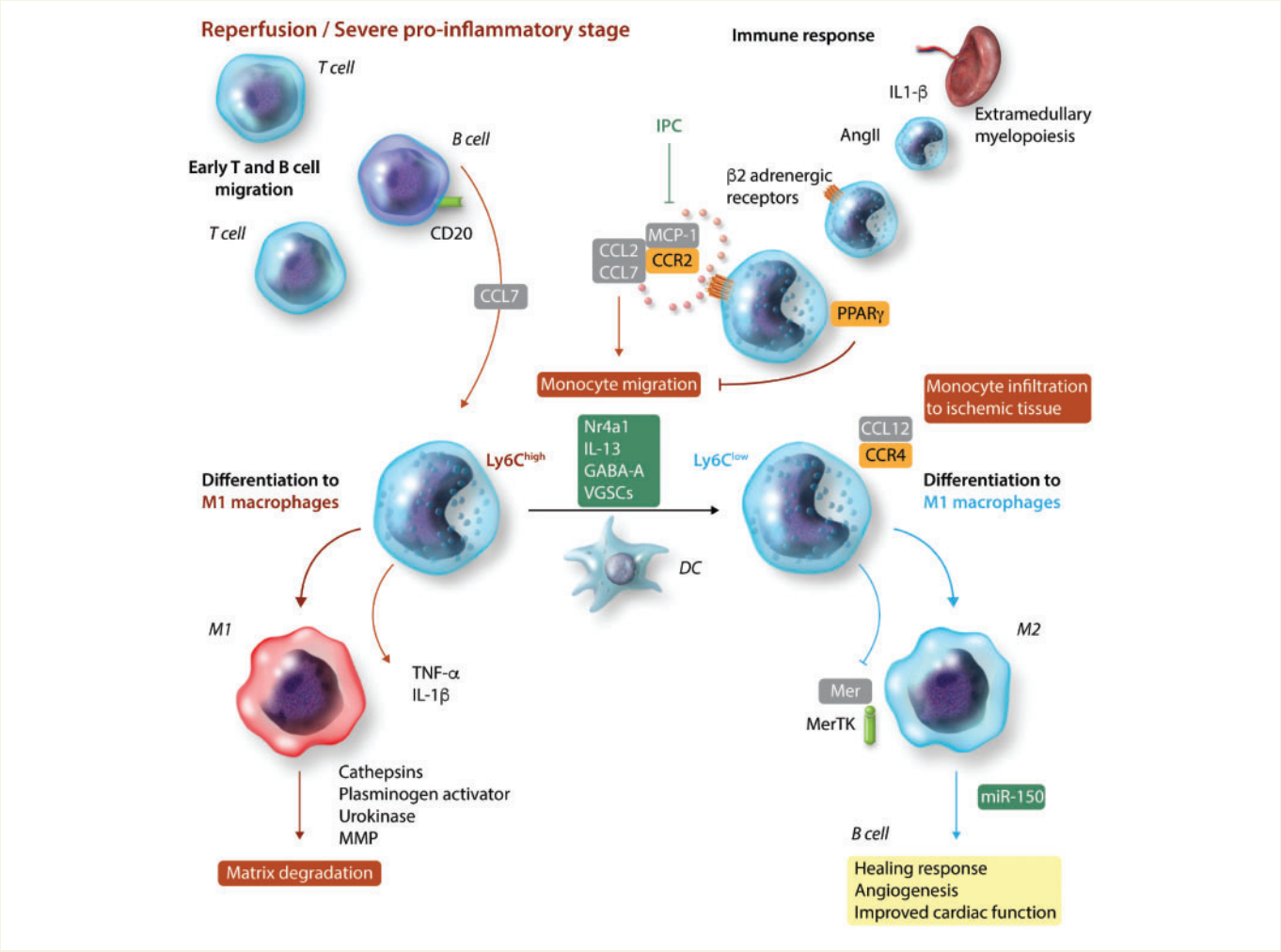


Figure 2 Immune cell changes hours to days following AMI. B and T lymphocytes are recruited early in the injured myocardium. B lymphocytes produce CCL7 which mediates the recruitment of Ly6C^{high} monocytes. The spleen provides a steady source of monocytes and they migrate to the site of myocardial injury under the regulation of IL-1β, ANG II and the binding of the chemokine ligand 2 (CCL2) to the chemokine receptor 2 (CCR2). MCP-1 and CCL7 also mediate CCR2-dependent monocyte migration. Moreover, β2-adrenergic receptors play a crucial role in the migration of monocytes/macrophages to the site of infarction following AMI. The first populations of monocytes that migrate to the site of infarction are the pro-inflammatory Ly6C^{high} monocytes which differentiate into activated pro-inflammatory macrophages (M1) that express IL-1β and TNF-α. Gradually, the Ly-6C^{low} monocytes become the predominant subtype and promote the healing response to AMI and the nuclear receptor subfamily 4, group a, member 1 (Nr4a1) is essential for Ly-6C^{low} monocyte production. IL-13, the CXCL12/CXCR4 axis, GABA-A receptor activity and VGSCs represent targets for the monocyte/macrophage phenotypic switch from pro-inflammatory to anti-inflammatory. The PPAR-γ-dependent anti-inflammatory mechanisms and monocyte-induced myeloid-epithelial-reproductive tyrosine kinase (MerTK) cleavage are considered as novel contributors and therapeutic targets for preventing IRI.

Overall, these data suggest a differential role of T cells after AMI with some T cells contributing to myocardial IRI, and others contributing to healing after AMI. The latter process seems to be antigen dependent. B cells seem to aggravate the remodelling process after AMI. The complex roles of lymphocytes to the inflammatory response to AMI, may make clinical translation difficult. A successful therapy will highly be dependent on the kind of injury and the individual time points in which lymphocytes are modified. Nevertheless, some drugs to modify a B- or T-cell response are under development and some are already in clinical use. Since lymphocytes influence other cells, their effects are multiplied moreover, lymphocyte responses are context dependent and might therefore be an ideal tool to support healing after AMI.

4.1 Pericardial lymphoid clusters as emerging targets for cardioprotection

Pericardial adipose tissue contains a high density of lymphoid clusters which serve as secondary lymphoid organs in which different types of cells such as B cells, DCs, and T cells enlarge in response to AMI.[66] In a very recent study,[67] it was demonstrated that surgical removal of murine pericardial adipose tissue, B cell depletion, and granulocyte-macrophage colony-stimulating factor blockade, reduced LV fibrosis, and preserved cardiac function following AMI in mice. The above findings highlight the major role of pericardial adipose tissue and B cells in the modulation of post-AMI outcome and indicate that activation of pericardial lymphoid clusters in response to AMI might also affect cardiac healing.

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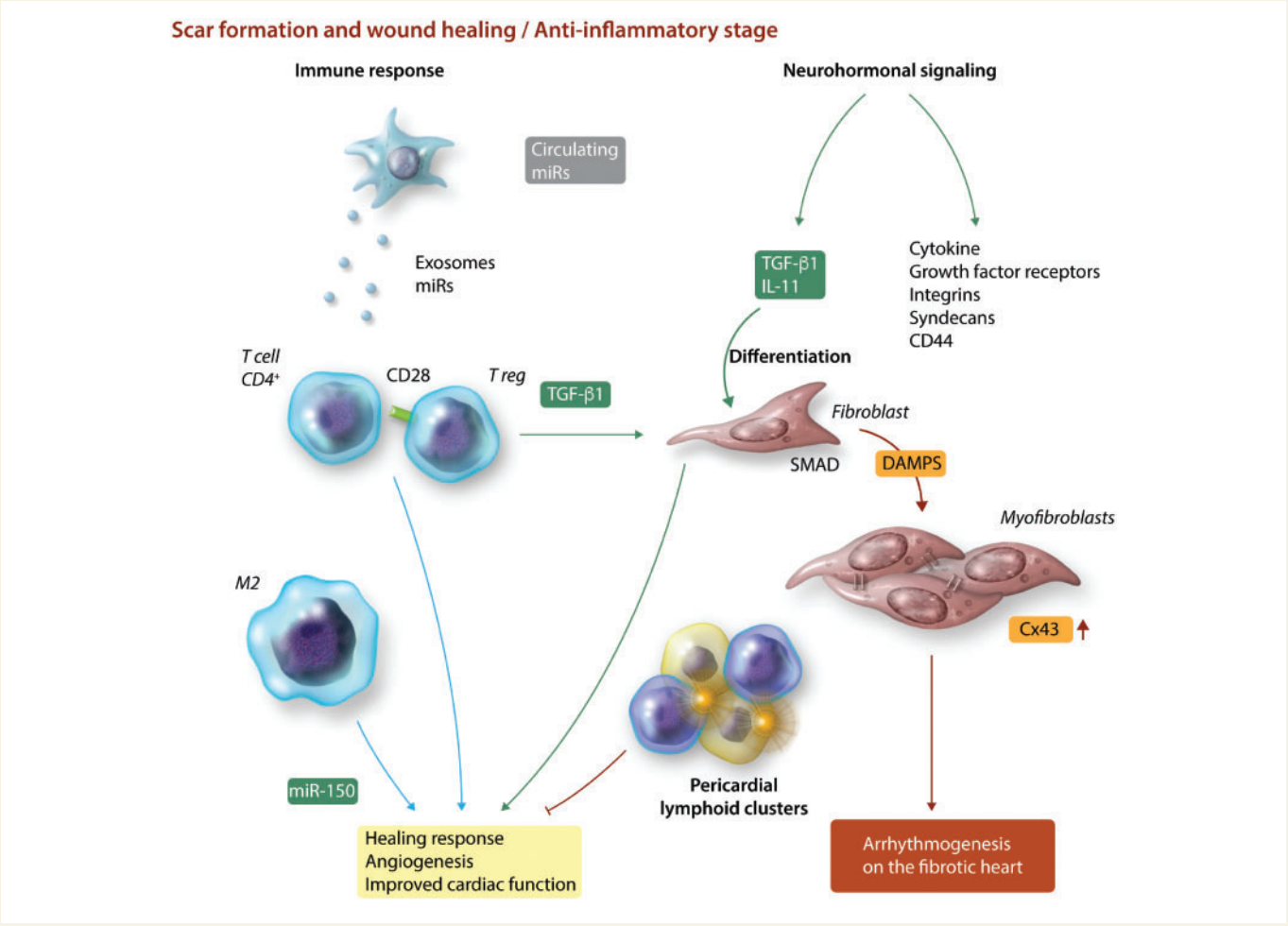


Figure 3 Immune cell changes in the healing period following AMI. Regulatory T cells (T_{reg}) undergo DC regulation and down-regulate the innate immune response. The activation of T_{reg} through CD28 is favourably associated with improved healing and survival. T_{reg} cells also determine the phenotype of cardiac fibroblasts, which affects the wound healing process. DCs might mediate the activation of $CD4^+$ T cells in the infarcted area, improving cardiac function post-AMI. There is increasing evidence that miRNAs (miRs) regulate the immune system by altering the function of DCs. DCs also produce miRs and exosomes regulating the inflammatory response after AMI. Pericardial lymphoid clusters can modulate the post-AMI outcome, indicating that activation of these clusters might affect as well cardiac healing. Cardiac fibroblasts are subjected to regulation by neurohumoral signals (ANG II and aldosterone), growth factors, such as TGF-β1 and secreted mediators that bind to fibroblast cell surface receptors (cytokine and growth factor receptors, integrins, syndecans, and CD44). These signals trigger the activation of fibrogenic programmes while TGF-β1 plays a critical role in conversion of cardiac fibroblasts into an organized scar with well-aligned myofibroblasts. However, TGF-β1 stimulation profoundly alters the electrophysiological phenotype of cardiac myofibroblasts, enhancing gap junctional coupling between myofibroblasts and cardiomyocytes by increasing connexin 43, thus contributing to arrhythmogenesis in the fibrotic heart.

5. DCs as emerging targets for cardioprotection

DCs are antigen-presenting cells that contribute to innate immunity and provide a link to the adaptive immune system.[68] In the heart, DCs are important both for promoting an immune response to specific pathogens, and for maintaining self-tolerance.[69] Myocardial IRI following AMI induces an inflammatory response which includes the migration and accumulation of DCs to the ischaemic region.[70,71] Mice depleted of DCs had worse post-MI remodelling than control mice suggesting that DCs are protective in the early pro-inflammatory response to AMI.[71] The infarcted myocardium of DC-depleted mice also displayed increased pro-inflammatory monocyte and macrophage recruitment and pro-inflammatory cytokines.[71] Interestingly, a correlation between decreased levels of DCs, increased levels of macrophages, and impaired reparative fibrosis followed by cardiac rupture, has been shown in histological samples of infarcted myocardium from patients deceased shortly after AMI.[72] This suggests a protective role of DCs against cardiac rupture.

DCs have been shown to activate cardiac-specific autoreactive $CD4^+$ T cells in a murine experimental AMI model.[73] Another study reported that exosomes secreted by DCs might mediate the activation of $CD4^+$ T cells in the infarcted area, improving cardiac function in mice post-AMI.[74] However, these findings are discordant with the data from another study showing detrimental effects of $CD4^+$ T cells in the setting of myocardial IRI.[65] It has recently been shown that administration of heart-

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specific tolerogenic DCs in post-AMI mice was beneficial to cardiac remodelling, function, and survival of infarcted mice by inducing regulatory T_{regs} that promoted a macrophage-specific repair programme from inflammatory to reparative.[62] Thus, targeting DCs could be an alternative therapeutic strategy to stimulate the beneficial action of T_{regs} and improve cardiac remodelling in post-AMI patients.

6. Resident cardiac mast cells as targets for cardioprotection

The existence of a resident population of mast cells in heart tissue has been demonstrated,[75] where they are mainly located in the adventitial lining of the coronary vasculature and within the cardiac interstitium.[76] Activation of cardiac mast cells has been shown to play an important role in AMI,[77] and may therefore present an important target for cardioprotection.

Mast cell activation and/or degranulation are noted to release pro-inflammatory mediators, and their release can be prevented by a variety of pharmacological interventions including low-dose ketotifen and carvedilol, and compound 48/80, a specific mast cell degranulating agent.[78] Similarly, disodium cromoglycate (DSCG), has been shown to stabilize mast cells and reduce myocardial injury, attenuate MPO release, and prevent post-IRI arrhythmias following AMI.[79] It should also be mentioned that dual targeting of neutrophil- and mast cell-derived proteases may represent a novel therapeutic strategy to reduce post-IRI inflammation and improve cardiac remodelling.[80]

The release of mast cell toxic aldehydes formed by lipid peroxidation, and neuropeptides such as substance P, released by juxtaposed cardiac sensory nerves, promotes mast cell degranulation and release of mast cell-derived renin.[81] Consequently, mast cell-derived renin induces reperfusion arrhythmias following myocardial IRI through the activation of the cardiac renin-angiotensin system (RAS) and locally formed ANG II that exacerbates release of norepinephrine (NE) from isolated sympathetic nerve terminals.[82–84] Activation of Gi-coupled receptors, such as histamine-H4, adenosine-A3, and sphingosinephosphate-1 receptors, all expressed at the mast cell surface, significantly reduced RAS-system activation and induced cardioprotective anti-RAS effects.

Similarly, IPC has been shown to mimic the cardioprotective effects of anti-RAS signalling. In both situations, anti-RAS cardioprotection was induced by the activation of protein kinase C epsilon (PKC-ϵ), and mitochondrial aldehyde dehydrogenase-2 (ALDH2), regulating aldehyde-induced mast cell renin release.[82–84] Furthermore, IPC also produced cardioprotection and decreased release of mast cell MPO following myocardial IRI, by activating the Na^+/H^+ exchanger and consequent degranulation of resident cardiac mast cells.[85] In addition, it has recently been proposed that ATP-induced renin release from mast cells exacerbates myocardial IRI, through the regulation of ecto-nucleoside triphosphate diphosphohydrolase 1/CD39 (CD39) and ATP availability at the mast cell surface. Thus, it has been demonstrated that overexpression of CD39 prevents ATP-induced renin release and exerts a cardioprotective effect.[86] Controversially, pharmacological preconditioning with NE produces a cardioprotective and anti-arrhythmic effect similar to IPC through degranulation of resident cardiac mast cells, reduction of lactate dehydrogenase, and MPO activation,[87] following myocardial IRI.

In summary, resident cardiac mast cells are an important detector system for initiating the pro-inflammatory response to AMI, and they are therefore potential targets for cardioprotection.

7. Cardiac fibroblasts as targets for cardioprotection

Cardiac homeostasis is supported by a network of direct and indirect interactions between cardiomyocytes and other resident myocardial cell types, such as endothelial cells, fibroblasts, pericytes and vascular smooth muscle cells, macrophages, mast cells, and epicardial adipocytes.[88,89] Because the adult mammalian myocardium has negligible regenerative capacity, healing of the injured heart following AMI is dependent on a superbly orchestrated cellular response that is driven by an inflammatory cascade, and ultimately leads to formation of a collagen-based scar.[90] As the infarct micro-environment changes, cardiac fibroblasts undergo dramatic phenotypic alterations that critically regulate repair and remodelling of the infarcted heart.[91] During the inflammatory phase of infarct healing, cardiac fibroblasts respond to DAMPs released by dying cells, and activate pro-inflammatory and matrix-degrading programmes.[92,93] Considering that several other cell types, including endothelial cells, immune cells, and ischaemic cardiomyocytes can also secrete pro-inflammatory mediators, the relative contribution of fibroblasts remains unclear. A recent study suggested that fibroblast-derived granulocyte-macrophage colony-stimulating factor (GM-CSF) release may play an important role in chemotactic attraction of neutrophils and monocytes in the infarcted myocardium.[94] It has also been suggested that during the early post-ischaemic period, fibroblasts may modulate survival pathways in cardiomyocytes, affecting their susceptibility to ischaemic death.[95,96] These effects may be mediated through secretion of soluble pro- or anti-apoptotic mediators by fibroblasts,[95] via release of exosomes containing miRNAs,[97] or through modulation of the extracellular matrix by fibroblast-derived MMPs.[98]

As professional phagocytes clear the infarct from dead cells and matrix debris, mediators repressing inflammation are released, and anti-inflammatory mononuclear cell subsets predominate.[36,99] Suppression of the inflammatory response is associated with expansion and activation of a reparative programme in cardiac fibroblasts. In the healing infarct, resident fibroblast populations are recruited to the area of necrosis, proliferate and aggregate, mediating formation of a scar.[100] Moreover, local activation of neurohumoral pathways (such as ANG II and aldosterone), and generation of growth factors, such as TGF-β1 play a critical role in conversion of cardiac fibroblasts into myofibroblasts, activated matrix-synthetic cells that incorporate contractile proteins (such as α-smooth muscle actin) in stress fibers.[101] Secreted mediators bind to fibroblast cell surface receptors (such as cytokine and growth factor receptors, integrins, syndecans, and CD44), triggering downstream signalling cascades that activate a fibrogenic programme.[102,103]

TGF-β1 has a wide range of effects on cardiac fibroblasts, mediated through activation of a series of intracellular effectors, the Smads.[104,105] Smad3-mediated induction of an integrin-mediated programme in cardiac fibroblasts has been demonstrated to play a critical role in repair of the infarcted heart, promoting formation of an organized scar with well-aligned myofibroblasts, and preserving the structural integrity of the ventricle.[105] Some of the fibrogenic effects of TGF-β1 may be mediated through activation of other fibrogenic mediators, such as IL-11.[106] Additionally to TGF-β1, *in vivo* infusion of IL-10 post-MI in mice increased fibroblast activation, and improved the LV micro-environment to diminish inflammation and facilitate cardiac wound healing.[107]

Scar maturation is associated with reduction in the population of infarct fibroblasts,[108] loss of myofibroblast phenotype, apoptosis of many activated fibroblasts,[109] and expression of specialized matrix proteins by

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surviving fibroblast subsets.[110] In contrast, in non-infarcted remodelling segments, resident fibroblasts may exhibit chronic activation in response to pressure and volume loads, contributing to adverse post-infarction remodelling.

Considering their crucial involvement in both injurious and reparative processes, targeting the fibroblasts following AMI poses major challenges. Characterization of fibroblast subpopulations with distinct functional profiles, and identification of specific fibroblast-derived mediators involved in injury, repair and adverse remodelling are needed in order to develop safe and effective therapeutic approaches.

8. Targeting long ncRNA and miRNA as a cardioprotective strategy

Non-coding RNAs (ncRNAs) are recognized as important regulators of several pathophysiological processes, including cardiovascular and inflammatory diseases, and have been investigated for their diagnostic and therapeutic potential, including in cardioprotection (reviewed in Refs.[111,112]). The many types of ncRNAs are broadly classified in small, <200 nucleotides, and long ncRNA (lncRNA), >200 nucleotides. Although there is much more information about the role of small miRNAs in the regulation of inflammatory processes after AMI, the importance of lncRNAs has been increasingly demonstrated,[113] including one particular type of lncRNA, the circular RNAs (circRNAs). MiRNAs control the metabolism of immune cells such as macrophages, T cells, B cells, and DCs,[114,115] and lncRNAs have been implicated in regulating inflammation, inflammatory factors, and macrophage activation.[116–119]

The lncRNAs ANRIL and KCNQ1OT1, were shown to be positively associated with LV dysfunction after AMI, supporting a deleterious role for these lncRNAs.[120] On the other hand, the lncRNA, autophagy-promoting factor has been suggested to be cardioprotective, because it can inhibit autophagy by sequestering one of its inducers, miR-188-3p.[121]

The circRNA, MICRA, was identified as a potential cardioprotective RNA in IRI.[122] However, these are mere associations between blood inflammatory cell levels and IRI and LV function following AMI. Mechanistic studies are therefore required to address the cardioprotective and therapeutic potential of these RNA molecules. This will likely be the aim of future research, considering that therapeutic tools based on RNA modulation are applicable in the clinic, and are the topic of active investigation.

There is increasing evidence of miRNA regulation of the immune system, including the development and function of DCs and the inflammatory response after AMI.[123,124] For instance, miR-155 is required for DCs to activate $CD4^+$ T cells.[125,126] It has been demonstrated *in vitro* that while down-regulation of miR-let-7i impaired DCs' ability to activate T cells, it improved their capability to induce T_{reg} expansion.[127] Functional experiments in mice showed that overexpression of miR-148a, miR-148b, and miR-152 inhibited DC secretion of pro-inflammatory cytokines and attenuated the expansion of $CD4^+$ T cells.[128] The up- or down-regulation of miR-23b, miR-30b, miR-99a, and miR-125a in tolerogenic DCs affected their capacity to produce ILs.[129,130] MiR-181a reduced the DC-mediated inflammatory response by oxidized low-density lipoproteins in atherosclerosis.[131] Notably, miR-181a and miR-150 play an important role in the regulation of apoptosis and the DC-mediated inflammatory response to MI.[132,133] *In vitro* overexpression of miR-181a or miR-150 suppressed cardiomyocyte apoptosis under hypoxia by attenuating the DC inflammatory response.[132] Interestingly, the cardioprotective role of miR-150 in AMI was suggested due to its regulation of monocyte migration and pro-inflammatory cytokine production in mice and human.[134] In addition, low-circulating levels of miR-150 in blood cells following an AMI were associated with inflammation, LV remodelling and poor outcome,[135] thus suggesting that this miRNA, mostly expressed in monocytes and with paracrine roles in angiogenesis,[122] could act as a cardioprotective agent in IRI. Therefore, targeting miRNAs regulating DCs may constitute another cardioprotective approach.

9. Future perspectives

A number of anti-inflammatory cardioprotective strategies, which have been shown in the experimental setting to reduce early and late MI size and prevent adverse LV remodelling, have failed in the clinical setting to show any benefit in AMI patients. The reasons for this are complex and multi-factorial, and have been extensively reviewed elsewhere[136–139] and only an overview is provided in this section.

9.1 Addressing the complex immune cell response to AMI

As highlighted in this review, the inflammatory response to AMI is hugely complex and involves a number of different immune cell types, many of which play dynamic and divergent roles by sustaining inflammation in the early phase, and/or resolving inflammation during the reparative healing phase. The natural history of the inflammatory/fibrotic response post-AMI has been illustrated in pigs indicating that there is a timely co-ordinated cellular response to myocardial IRI, and understanding the mechanisms involved in tissue repair will provide valuable information in the development of novel cardioprotective strategies.[140] The interaction of the immune cells with each other and with non-immune cells such as cardiomyocytes, endothelial cells, and fibroblasts further complicates the inflammatory response to AMI. Therefore, this needs to be taken into consideration when targeting immune cells following AMI, and requires a better understanding of the immune cell response to myocardial IRI. This may be achieved by timing the administration of anti-inflammatory cardioprotective therapies to specifically target either the initial pro-inflammatory or subsequent anti-inflammatory reparative phase, either separately or in combination. The contribution of immune cells to myocardial damage and repair in the setting of AMI at different time points, during ischaemia, early after reperfusion and during scar formation and wound healing is illustrated in *Figures* 1–3.

9.2 Multi-target approach to cardioprotection

The complex inflammatory response to AMI has many players, including the interplay between the innate immune response and subsequent immune cell infiltration into the MI zone. However, the majority of anti-inflammatory cardioprotective strategies which have failed in the clinical setting has been directed to a single component of the inflammatory response, such as a particular cytokine or a specific immune cell type, and this may have contributed to the failure of these studies. Therefore, a multi-target anti-inflammatory strategy aimed at different components of the inflammatory response to AMI may be more effective at preventing myocardial IRI. For example, one could combine the early administration of an agent which inhibits the pro-inflammatory response to AMI (e.g. pro-inflammatory monocytes) with the subsequent administration of agent which activates the anti-inflammatory reparative response to AMI (e.g. anti-inflammatory monocytes).

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However, the situation is further complicated, when one takes into consideration the other non-inflammatory components of myocardial IRI, such as the cardiomyocytes (mitochondrial dysfunction, calcium overload, and oxidative stress), coronary endothelial dysfunction and microvascular obstruction, platelet activation, and so on. As such, multi-target cardioprotective therapies should also address these different components of myocardial IRI.[141]

9.3 Preclinical rigour and reproducibility and more clinically relevant animal AMI models

The preclinical animal AMI models that have been used to test anti-inflammatory cardioprotective strategies do not represent the typical AMI patient. In most experimental studies, AMI is induced in juvenile healthy animals by occluding a healthy coronary artery, whereas in the clinical setting AMI typically occurs in middle-aged patients and is due to rupture of a coronary atherosclerotic plaque and superimposed thrombosis and inflammation.[137] In addition, more clinically relevant animal AMI models need to be used, which take into consideration co-morbidities (such as age, diabetes, hypertension, hyperlipidaemia, and so on), and co-medications (such as statins, P2Y12 inhibitors, beta-blocker), which are known to interfere with cardioprotection (reviewed in Refs.[137,142]).

Another important reason for the failed translation of cardioprotection from the bench-side to the bed-side has been the lack of rigour and reproducibility used in the preclinical testing of novel cardioprotective therapies.[143] This can be addressed by setting up robust animal AMI protocols and setting up of a network of research centres for undertaking preclinical multicentre randomized placebo-controlled small and large animal studies. This network can then be used to test carefully selected cardioprotective therapies, using standardized myocardial IRI protocols and centralized data analysis. This approach was utilized by the US-based Consortium for preclinicAl assESsment of cARdioprotective therapies (CAESAR),[144,145] and has been proposed by our EU-CARDIOPROTECTION CA16225 COST Action.[146]

In conclusion, further studies are required to determine whether targeting the immune response to myocardial IRI can improve clinical outcomes following AMI. Although a number of anti-inflammatory cardioprotective therapies have failed to reduce early and late MI size and improve clinical outcomes following AMI, the majority has been single-targeted and directed to immune cells such as neutrophils and monocytes/macrophages. Experimental studies have reported beneficial effects from targeting other components of the immune cell response to AMI, including lymphocytes, DCs, lymphocytes, and mast cells, and these have the potential to improve clinical outcomes following AMI.

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The Extracellular Matrix in Ischemic and Nonischemic Heart Failure

Nikolaos G. Frangogiannis

Abstract: **The ECM (extracellular matrix) network plays a crucial role in cardiac homeostasis, not only by providing structural support, but also by facilitating force transmission, and by transducing key signals to cardiomyocytes, vascular cells, and interstitial cells. Changes in the profile and biochemistry of the ECM may be critically implicated in the pathogenesis of both heart failure with reduced ejection fraction and heart failure with preserved ejection fraction. The patterns of molecular and biochemical ECM alterations in failing hearts are dependent on the type of underlying injury. Pressure overload triggers early activation of a matrix-synthetic program in cardiac fibroblasts, inducing myofibroblast conversion, and stimulating synthesis of both structural and matricellular ECM proteins. Expansion of the cardiac ECM may increase myocardial stiffness promoting diastolic dysfunction. Cardiomyocytes, vascular cells and immune cells, activated through mechanosensitive pathways or neurohumoral mediators may play a critical role in fibroblast activation through secretion of cytokines and growth factors. Sustained pressure overload leads to dilative remodeling and systolic dysfunction that may be mediated by changes in the interstitial protease/antiprotease balance. On the other hand, ischemic injury causes dynamic changes in the cardiac ECM that contribute to regulation of inflammation and repair and may mediate adverse cardiac remodeling. In other pathophysiologic conditions, such as volume overload, diabetes mellitus, and obesity, the cell biological effectors mediating ECM remodeling are poorly understood and the molecular links between the primary insult and the changes in the matrix environment are unknown. This review article discusses the role of ECM macromolecules in heart failure, focusing on both structural ECM proteins (such as fibrillar and nonfibrillar collagens), and specialized injury-associated matrix macromolecules (such as fibronectin and matricellular proteins). Understanding the role of the ECM in heart failure may identify therapeutic targets to reduce geometric remodeling, to attenuate cardiomyocyte dysfunction, and even to promote myocardial regeneration. (*Circ Res*. 2019;125:117-146. DOI: 10.1161/CIRCRESAHA.119.311148.)**

Key Words: collagen ■ extracellular matrix ■ fibroblasts ■ heart failure ■ inflammation

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The ECM (extracellular matrix) is a highly dynamic noncellular 3-dimensional network that is present in all tissues and plays a critical role in homeostasis and disease.[1] The ECM does not simply function as a mechanical support system that preserves tissue integrity, but also serves as a signaling hub that transduces cascades critical for cell function, and as a reservoir of growth factors that can be released following injury to modulate cell behavior and to activate a reparative program.[2] Organ homeostasis is critically dependent on the ECM network, as components of the matrix continuously interact with the cellular elements providing a stable structural support network, and coordinating cellular responses, to ensure functional integration of the cells. On the other hand, under conditions of stress, the ECM network may shield the cells from injurious stimuli, while serving as a key component in tissue repair, by orchestrating cellular responses. In many pathological conditions, expansion of the ECM network or changes in the biochemical composition of matrix proteins contribute to the pathogenesis of disease either by directly perturbing normal organ structure and function, or by transducing maladaptive signals to the cells.

The adult mammalian heart is a syncytium comprised of cardiomyocytes and interstitial cells, enmeshed within a network of ECM proteins. Most myocardial diseases are associated with expansion of the cardiac interstitium, accompanied by marked alterations in the biochemical composition of the ECM network. The myocardial ECM does not simply serve as structural scaffold that determines the mechanical properties of the myocardium. Under conditions of stress, ECM macromolecules can drive cell biological responses with an important role in ventricular dysfunction and in the pathogenesis of heart failure.

It should be emphasized that human heart failure is pathophysiologically heterogeneous. Depending on the underlying cause, several distinct pathophysiologic conditions, such as ischemia, volume and pressure overload, genetic factors, aging, and metabolic dysregulation may contribute to the pathogenesis of myocardial dysfunction. Animal model investigations

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Nonstandard Abbreviations and Acronyms

α-SMA	α-smooth muscle actin
ACE	angiotensin-converting enzyme
ADAM	a disintegrin and metalloproteinase
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AGEs	advanced glycation end products
AT1R	angiotensin II type 1 receptor
AT2R	angiotensin II type 2 receptor
BMP	bone morphogenetic protein
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CSPGs	chondroitin sulfate proteoglycans
DSPGs	dermatan sulfate proteoglycans
ECM	extracellular matrix
ED-A	extra domain A
ET-1	endothelin-1
FAK	focal adhesion kinase
FGF	fibroblast growth factor
Fn	fibronectin
HFpEF	heart failure with preserved ejection fraction
IL	interleukin
LOX	lysyl oxidase
LTBP	latent TGF-β–binding protein
MAPK	mitogen-activated protein kinase
MI	myocardial infarction
MMP	matrix metalloproteinase
MRTF	myocardin-related transcription factor
PAI-1	plasminogen activator inhibitor-1
PCOLCE-2	procollagen C-endopeptidase enhancer-2
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated protein kinase
SLRPs	small leucine-rich proteoglycans
SPARC	secreted protein acidic and rich in cysteine
TG	transglutaminase
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases
TNF	tumor necrosis factor
TRPV-4	transient receptor potential cation channel subfamily V member 4
TSP	thrombospondin
YAP	Yes-associated protein

suggest that the various pathophysiologic causes of heart failure trigger distinct ECM perturbation patterns that reflect the characteristics of the primary insult. For example, myocardial infarction (MI) rapidly activates a potent acute inflammatory response driven by necrotic cells, thus stimulating early ECM degradation. As the infarct is cleared from dead cells, suppression of inflammation and induction of growth factors favor activation of a fibrogenic program leading to formation of a scar. On the other hand, in the pressure-overloaded myocardium, mechanical stress stimulates release of neurohumoral mediators and activates mechanosensitive cascades, stimulating matrix-preserving pathways in cardiomyocytes and fibroblasts, in the absence of significant cellular necrosis. This response contrasts with the predominant disruption of the collagen network that has been described in the volume-overloaded myocardium (typically associated with valvular regurgitant lesions). This review article discusses ECM-related mechanisms associated with the most common pathophysiologic conditions that cause heart failure, while attempting to identify unifying features that may link alterations of the ECM network and cardiac dysfunction.

Normal Cardiac ECM

The cardiac ECM network is comprised of an interstitial component that surrounds all myocardial cells and forms a structural scaffold, and a pericellular component that is in close contact with certain cell types.[3] The basement membrane, a specialized form of ECM comprised of laminins, type IV collagens and proteoglycans is the predominant type of pericellular matrix and separates cardiomyocytes from the surrounding interstitial matrix.[4] Because the heart is perfused by a rich microvascular network, significant amounts of basement membrane ECM are also present in the interface between endothelial cells and pericytes. In mammalian hearts, the myocardial ECM network is organized on 3 interconnected levels: the epimysium encases the entire organ, the perimysium defines major bundles of myofibers, and the endomysium surrounds individual cardiomyocytes.[5,6] In addition to the intramyocardial (endomysial and perimysial) ECM network, significant amounts of matrix proteins are present in the adventitia of myocardial arteries and arterioles.

Early studies on the composition of the normal cardiac ECM focused predominantly on the collagens, the major structural components of the interstitial matrix.[7] Type I collagen predominantly forms thick rod-like fibers in the epimysium and perimysium and is the major structural component of the cardiac interstitium.[8,9] In contrast, type III collagen forms a fine network of fibers that is more prominent in the endomysium.[8,10,11] In addition to the fibrillar collagens, the cardiac ECM also contains glycosaminoglycans (such as hyaluronan), Fn (fibronectin), glycoproteins, and proteoglycans, and serves as a reservoir for growth factors and proteases, which are stored in the interstitium, and can be released or activated following injury. Recent proteomic studies have revealed the remarkable complexity of the cardiac ECM network in health and disease.[12–15]

The role of the myocardial ECM as a mechanical scaffold that preserves cardiac geometry and facilitates force transmission seems intuitive. However, a growing appreciation of the interactions between matrix macromolecules and myocardial cells suggests that the cardiac ECM does not simply provide structural support, but may also transduce key signals regulating cardiomyocyte function. For example, the dystrophin-glycoprotein complex serves as a molecular link between the contractile machinery of the cardiomyocytes and the surrounding ECM.[16] The transmembrane protein dystroglycan connects the cytoskeletal components of the cardiomyocytes to the ECM through interactions with the α2 chain of laminin 2,[16,17] playing an important role in cardiac homeostasis. Unfortunately, very little is known about the role of specific ECM-dependent molecular pathways in mediating the

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coordinated contraction of cardiomyocytes in normal hearts. In addition to their effects in contractile function, ECM proteins may also interact with cardiomyocytes, vascular cells, and interstitial cells to transduce survival signals.[18]

Link Between the ECM and Cardiac Dysfunction: The Significance of Cardiac Fibrosis

Although the ECM remains inconspicuous in health, it takes center stage in myocardial disease, as the cardiac interstitium undergoes dramatic and dynamic changes that have a major impact on cardiac function. The term cardiac fibrosis is used to describe the expansion of the ECM network that accompanies most forms of myocardial disease.[19] Using simple histopathologic analysis, 3 forms of cardiac fibrosis can be recognized, reflecting distinct mechanisms of fibrotic remodeling. The term replacement fibrosis describes the formation of a scar in areas of myocardial necrosis, represents the end-result of a reparative process in response to primary cardiomyocyte injury, and reflects the negligible regenerative capacity of adult mammalian cardiomyocytes. Replacement fibrosis is typically the result of MI.[20] In contrast, a wide range of injurious stimuli, including a pressure load, metabolic dysfunction, and aging may cause interstitial fibrosis, widening the endomysium and perimysium due to deposition of structural ECM proteins, or perivascular fibrosis, leading to expansion of the periadventitial collagenous area in the cardiac microvasculature. Interstitial and perivascular fibrotic lesions may not be driven by cardiomyocyte death. Whether perivascular and interstitial fibrotic changes have distinct functional consequences remains unknown. In heart failure patients, periadventitial microvascular fibrosis has been associated with reduced coronary blood flow,[21] and may perturb coronary vasodilatory responses,[22] thus exacerbating the imbalance between supply and demand under conditions of stress.[23]

Robust clinical evidence suggests a strong association between expansion of the cardiac ECM and adverse outcome in patients with heart failure. In heart failure with reduced ejection fraction patients, the severity of fibrosis predicts death and adverse cardiac events.[24] On the contrary, heart failure with preserved ejection fraction (HFpEF) patients typically exhibit expansion of the interstitial ECM network, associated with coronary microvascular rarefaction and inflammatory activation.[25,26] In subjects with HFpEF, interstitial ECM expansion is associated with increased mortality and higher hospitalization rates.[27] Although the prognostic significance of cardiac ECM deposition in heart failure is well-documented, whether worse outcome in patients with extensive fibrotic lesions is due to the adverse consequences of fibrosis, or simply reflects a more pronounced reparative response to more severe primary cardiomyocyte injury is unclear. Several mechanisms may contribute to direct adverse effects of ECM expansion and remodeling on both systolic and diastolic function. First, accentuated deposition of cross-linked collagen in the cardiac interstitium may increase myocardial stiffness, resulting in diastolic dysfunction. Second, ECM deposition may promote systolic dysfunction by perturbing the coordination of myocardial excitation-contraction coupling. Third, expansion of the periadventitial ECM may disrupt cardiomyocyte perfusion. Fourth, perturbations in the protease/antiprotease balance in the cardiac interstitium may trigger degradation of fibrillar collagens, thus disrupting ECM-dependent pathways that regulate cardiomyocyte contraction, and leading to systolic dysfunction.[28] Moreover, perturbations in the matrix network may also deprive cardiomyocytes from crucial prosurvival signals transduced by intact ECM proteins.[18,29] Fifth, generation of ECM fragments may activate proinflammatory pathways, recruiting immune cells to the interstitium and leading to cardiomyocyte dysfunction and death.[30]

The relation between perturbations in the cardiac ECM network and adverse outcome may also involve an increased incidence of arrhythmic events and conduction abnormalities. ECM deposition disrupts impulse propagation and often results in generation of reentry circuits.[31] The cellular composition of the fibrotic lesions and the ECM profile of the cardiac interstitium may play an important role in determining arrhythmogenicity in failing hearts.[32] It should be emphasized that changes in the ECM network may have an impact in geometry and function of all cardiac chambers. Changes in the right ventricular matrix may contribute to the morbidity and mortality in patients with pulmonary hypertension.[33] Atrial ECM remodeling and fibrosis are implicated in the pathogenesis and complications of atrial tachyarrhythmias.[34]

ECM Network in the Pathogenesis of Heart Failure

Heart failure is characterized by remarkable pathophysiologic heterogeneity. The patterns of ECM perturbations and the relative role of matrix macromolecules in the pathogenesis of heart failure are dependent on the type and duration of myocardial injury. Pressure overload, volume overload, ischemic injury, diabetes mellitus, obesity, and metabolic dysregulation are associated with distinct patterns of ECM alterations.

ECM in Heart Failure Associated With Pressure Overload

Cellular Effectors of ECM Remodeling in the Pressure-Overloaded Heart

Left ventricular pressure overload is the predominant pathophysiology in systemic hypertension and in conditions associated with left ventricular outflow obstruction (such as aortic stenosis) and leads to progressive development of heart failure.[35,36] In both animal models of left ventricular pressure overload, and in human patients with sustained pressure loads, early development of cardiac hypertrophy initially serves as an adaptive response that preserves cardiac output.[37] However, prolonged pressure overload triggers fibrosis and perturbs cardiomyocyte relaxation, increasing myocardial stiffness, and causing diastolic dysfunction. Persistent pressure overload eventually results in dilative remodeling and systolic dysfunction. Extensive evidence suggests that the changes in the myocardial ECM network in response to pressure overload not only increase passive stiffness by altering the mechanical properties of the ventricle, but also play a critical role in regulating inflammatory, fibrotic, and hypertrophic cellular responses. Although cardiac fibroblasts are considered essential modulators of the matrix network following pressure

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overload, several other cell types, including immune cells, vascular cells, and cardiomyocytes have been implicated in ECM remodeling either directly (by secreting proteases or antiproteases with a critical role in matrix metabolism) or indirectly (by modulating fibroblast phenotype) (Figure 1).

Fibroblasts and Myofibroblasts

Expansion and activation of fibroblasts are consistently noted in experimental models of cardiac pressure overload.[38,39] Activated fibroblasts typically undergo myofibroblast conversion, expressing contractile proteins, such as α-SMA (α-smooth muscle actin)[38] and the embryonic isoform of smooth muscle myosin heavy chain,[40] and synthesizing large amounts of structural and matricellular ECM proteins.[41] Although some investigations have suggested that endothelial cells[42] and circulating fibroblast progenitors may undergo fibroblast conversion, recent lineage tracing studies demonstrated that resident fibroblast populations are the main cellular source of activated myofibroblasts in the pressure-overloaded myocardium.[43,44] Activation of fibroblasts in response to mechanical stress likely involves several distinct mechanisms.[45] First, fibroblasts sense increased mechanical load through activation of surface integrins,[46–48] syndecans,[49] and mechanosensitive membrane channels.[50,51] Subsequent stimulation of mechanosensitive intracellular signaling cascades, such as the RhoA (Ras homolog gene family, member A)/ROCK (Rho-associated protein kinase),[52,53] FAK (focal adhesion kinase),[54–56] and MAPK (mitogen-activated protein kinase)[39] pathways may promote a fibrogenic program through activation of the transcription factor MRTF (myocardin-related transcription factor).[57,58] Second, mechanical stress has been linked with integrin-dependent activation of TGF (transforming growth factor)-β,[59] a critical mediator in myofibroblast conversion that triggers integrin synthesis in cardiac fibroblasts[60] and promotes activation of a matrix-synthetic and matrix-preserving program.[30,61] Third, pressure overload may trigger systemic and local activation of the renin-angiotensin-aldosterone system, stimulating fibroblast proliferation and ECM protein synthesis through AT1R (angiotensin II type 1 receptor) signaling. Fourth, pressure overload induces expression of fibroblast-specific microRNAs that activate MAPK signaling in fibroblasts promoting expansion of the interstitial ECM.[62] The potential link between mechanical stress and a fibrogenic microRNA profile has not been investigated. Fifth, activation of fibroblasts in the pressure-overloaded heart may require mechanosensitive activation of other cell types, including immune cells, vascular cells, and cardiomyocytes.

Immune Cells

Macrophages,[63–65] lymphocytes,[66–68] and mast cells[69,70] have been implicated in the fibrotic response in the pressure-overloaded heart (Figure 2). Although immune cells do not produce significant amounts of structural ECM proteins and do not undergo conversion to fibroblasts,[43] they may function as key effectors of ECM expansion in the pressure-overloaded myocardium by releasing fibrogenic mediators[70] and matricellular proteins,[72] or by transducing adhesion-dependent fibrogenic signals to cardiac fibroblasts.[68] Moreover, immune cell subpopulations may directly remodel the cardiac ECM by secreting MMPs (matrix metalloproteinases), or other matrix-degrading proteases.[73] What is the central mechanism that activates immune cells in the pressure-overloaded myocardium? First, macrophages and lymphocytes may respond directly to mechanical stress, activating a fibrogenic program. Mechanical stress has been suggested to stimulate T lymphocyte-driven inflammation that may activate fibrogenic signaling.[74] Macrophages may also sense mechanical cues to alter their level of activation.[75] Expression of mechanosensitive ion channels (such as TRPV [transient receptor potential cation channel subfamily V member]-4) has been observed in macrophages and may modulate their functional properties,[76] promoting a fibrogenic phenotype. Second, resident myocardial immune cells may acquire a fibrogenic profile in response to the local or systemic release of neurohumoral mediators or fibrogenic growth factors. In vivo studies have suggested that mineralocorticoid receptor signaling in myeloid cells promotes a fibrogenic macrophage phenotype,[65] that is critical for expansion of the interstitial ECM in models of hypertensive heart disease[77,78] or left ventricular pressure overload.[65] Mineralocorticoid receptor-mediated activation of lymphocytes has also been implicated in the pathogenesis of interstitial fibrosis in the pressure-overloaded heart.[79] Third, fibrosis in the pressure-overloaded heart may reflect new recruitment of fibrogenic immune cells, mediated through proinflammatory activation of the microvascular endothelium,[80,81] or through paracrine actions of cardiomyocytes.[82]

It should be noted that not all studies support the importance of inflammatory cells in pressure overload–induced heart failure. Macrophage depletion using a genetic model did not affect the progression of myocardial remodeling in mice with established pressure overload–induced heart failure.[83]

Vascular Cells

The impressive perivascular ECM deposition in pressure-overloaded hearts (Figure 2)[71] suggests that vascular cells may be important contributors to the cardiac fibrotic response. Some experimental studies have suggested that endothelial cells may contribute to ECM remodeling in the pressure-overloaded heart by undergoing endothelial to mesenchymal transition,[42] thus acquiring a fibroblast-like phenotype. However, this notion was refuted by robust lineage tracing studies[44] that reported very low numbers of endothelial-derived fibroblasts in the remodeling myocardium.[84] Endothelial cells may activate fibroblasts by secreting profibrotic mediators, such as TGF-β1, FGFs (fibroblast growth factors), or ET (endothelin)-1,[85] or by facilitating recruitment of fibrogenic immune cell subpopulations through expression of adhesion molecules and chemokines.[80]

The role of cardiac pericytes in matrix deposition and metabolism following pressure overload remains poorly documented. This is due, at least in part, to challenges in identification and characterization of cardiac pericyte populations. Pericytes may contribute to ECM remodeling through conversion to activated myofibroblasts,[86] or through secretion of fibroblast-activating growth factors.

Cardiomyocytes

Mechanical stress activates a proinflammatory and fibrogenic gene expression program in cardiomyocytes through a pathway that has been suggested to involve angiotensin II–induced

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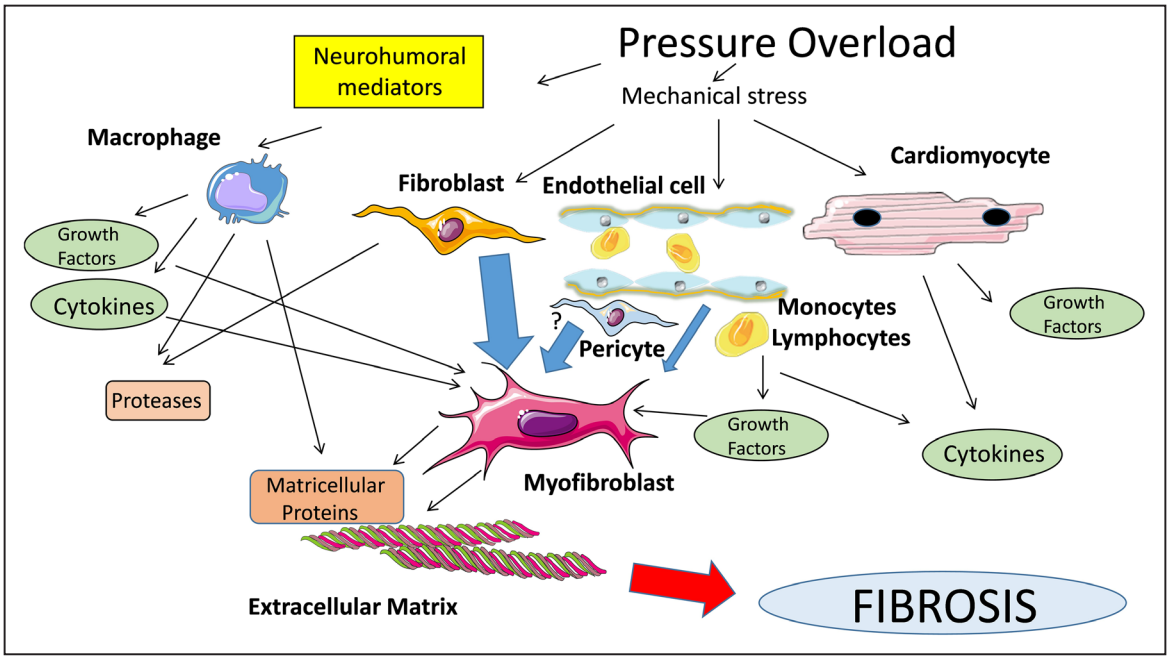


Figure 1. The cellular effectors of ECM (extracellular matrix) remodeling in the pressure-overloaded heart. Mechanical stress triggers transduction of mechanosensitive signaling cascades, activates TGF (transforming growth factor)-β, and stimulates neurohumoral mediator release (such as angiotensin, aldosterone, and norepinephrine). Fibroblasts are the main effectors of ECM expansion, producing both structural and matricellular ECM proteins. Activated myofibroblasts in the pressure-overloaded heart predominantly originate from local resident fibroblasts, other interstitial cells, such as cardiac pericytes, may also undergo myofibroblast conversion (blue arrows). Other cell types (such as endothelial cells) appear to have very limited direct contributions to the myofibroblast population in the remodeling myocardium. Cardiomyocytes, immune cells, and vascular cells are important regulators of ECM remodeling. Mechanical stress triggers synthesis of proinflammatory mediators in cardiomyocytes, promoting recruitment of lymphocytes and macrophages. Activated endothelial cells also participate in recruitment of immune cells. Macrophages and lymphocytes may promote fibroblast activation by secreting growth factors (such as TGF-βs). All myocardial cells are capable of producing proteases, involved in ECM remodeling.

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stimulation of CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II)-δ.[82,87] In a mouse model of deoxycorticosterone/salt-induced cardiac remodeling, cardiomyocyte-specific mineralocorticoid receptor signaling mediated expansion of the ECM network.[88] Growth factors released and activated in response to mechanical stress may also activate a fibrogenic profile in cardiomyocytes. In a model of left ventricular pressure overload, TGF-β receptor II signaling in cardiomyocytes was found to contribute to the development of cardiac fibrosis.[89]

ECM Proteins in the Pressure-Overloaded Myocardium

Fibrillar Collagens

Fibrillar collagens[90] constitute a subgroup within the collagen family whose members confer mechanical strength and support tissue organization in many organs. In addition to their structural role, fibrillar collagens may also interact with cell surface receptors, transducing signals. Increased deposition of fibrillar collagens by activated cardiac fibroblasts is consistently noted in pressure-overloaded hearts. Table 1 summarizes our knowledge on the expression and role of fibrillar collagens in failing hearts.

Activation of a matrix-synthetic phenotype in cardiac fibroblasts may involve neurohumoral mediators (such as angiotensin II and aldosterone), and growth factors (such as TGF-βs).[114] ACE (angiotensin-converting enzyme) inhibition, AT1R blockade, and mineralocorticoid antagonism attenuated collagen deposition in most,[115–117] but not all[118] experimental studies in models of left ventricular pressure overload. In contrast to the fibrogenic actions of the AT1R, the AT2R (angiotensin II type 2 receptor) may play an inhibitory role,[119] attenuating fibroblast proliferation, and reducing ECM deposition in models of hypertension.[120] To what extent the beneficial effects of ACE inhibition and AT1R blockade in human

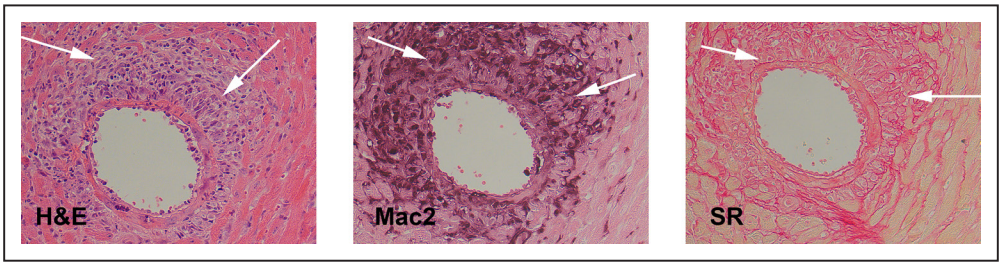


Figure 2. The link between immune cell infiltration and ECM (extracellular matrix) expansion in the pressure-overloaded heart. Serial sections from a mouse heart undergoing transverse aortic constriction for 7 d stained with hematoxylin+eosin (H&E), the macrophage marker Mac2, and Sirius red (to label collagen fibers) show periadventitial infiltration with abundant macrophages in areas of perivascular fibrosis (arrows). Original data and images reported in our published work.[71]

Table 1. Fibrillar Collagens in the Heart

Fibrillar Collagen	Cardiac Localization	Role in Cardiac Homeostasis	Changes in Hypertensive Heart Failure	Changes in Ischemic Heart Failure	Changes in Other Myocardial Conditions
Col I	Col I is the predominant fibrillar collagen in adult mammalian hearts.	Thick Col I fibers may preserve cardiac geometry and structure, and regulate compliance, while contributing to force transmission. Mice with a mutation in the proa2(I) chain have a more compliant ventricle on passive inflation.[91]	Both Col I and Col III are overexpressed in pressure-overloaded hearts.[92] Predominance of Col I was associated with increased stiffness in some human and experimental studies.[93,94] However, in SHR hearts, myocardial stiffness was not associated with changes in Col I:Col III ratio, but was rather attributed to cross-linking.[95] Moreover, in human patients with aortic stenosis, comparable induction of Col I and III was noted.[96]	In the rat model of MI, a greater increase in Col I in noninfarcted myocardium.[97] In rats undergoing infarction protocols, Col III levels seemed to increase earlier in the infarcted heart.[98,99]	In patients with DCM, increased Col I:Col III ratio may be associated with reduced myocardial compliance.[100] Inflammatory cardiomyopathy was associated with a higher proportion of Col III.[101] Conflicting findings on the effects of aging have been reported. Col I was highly expressed in neonatal hearts,[102] whereas Col III was increased with aging.[103] In other studies, aging was associated with increased number and thickness of Col I fibers.[104]
Col III	Significant amounts of Col III are found in normal hearts. The ratio of Col III:Col I is dependent on species and age.[105]	Col III forms a thin network of fibers more prominent in the endomysium. A higher Col III:Col I ratio has been associated with increased elasticity.			
Col V	Expressed in developing valves and in fetal myocardium. In adult rat hearts, Col V immunoreactivity was reported in the vascular matrix.[106]	Col V regulates collagen fibril diameter and assembly in the valves and in the left ventricular myocardium.[107]	Increased levels of Col V have been reported in pressure-overloaded rat myocardium.[108,109]	Col V immunoreactivity was reported in the peri-infarct region in a rat model of infarction.[106] Increased Col$V\alpha 2$ was found in proteomic analysis of the cardiac ECM of ischemic and reperfused porcine hearts.[15]	Autoantibodies against Col V have been suggested to play a role in antibody-mediated cardiac allograft rejection, or allograft vasculopathy.[110]
Col XI	Col XI is expressed in developing heart valves, in the supporting apparatus and in left ventricular trabeculae.[107]	Col XI regulates collagen fibril assembly predominantly in the valves.[107]	N/A	Not found to be upregulated in porcine model of I/R.[15]	N/A
Col XXIV	Col XXIV is involved in Col I fibrillogenesis in the developing bone and eye.[111] Col24a1 mRNA expression not found in mouse myocardium.[112]	N/A	N/A	Not found to be upregulated in a model of porcine I/R.[15]	N/A
Col XXVII	Col XXVII is transiently expressed in developing coronary vessels.[113] It is localized in fine fibrillar structures independent of classical collagen fibrils. In adult mice, its expression is restricted to cartilage.	N/A	N/A	N/A	N/A

DCM indicates dilated cardiomyopathy; ECM, extracellular matrix; I/R, ischemia/reperfusion; MI, myocardial infarction; N/A, no information available; and SHR, spontaneously hypertensive rats.

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patients with heart failure are due to attenuated ECM expansion remains unclear.

It has been suggested that the relative amount of collagen I versus collagen III fibers may regulate myocardial compliance in remodeling hearts. Collagen I forms thicker and stiffer fibers, whereas the finer reticular collagen III fibers are more compliant and increase elasticity. In models of hypertensive cardiac fibrosis, type I collagen exhibits more intense and prolonged upregulation than collagen III.[121] Thus, it is attractive to suggest that the predominance of type I fibers in the pressure-overloaded myocardium may mediate diastolic dysfunction. This notion is supported by some associative evidence. High levels of type I collagen was associated with increased myocardial stiffness in some human and experimental

studies.[93,94] Moreover, ACE inhibition has been suggested to reduce the myocardial type I to type III collagen ratio in models of hypertension.[121] However, other studies did not support an important role of the collagen type profile in the pathogenesis of diastolic dysfunction. In hypertensive rats, myocardial stiffness was not associated with changes in collagen I/III ratio, but was rather attributed to cross-linking.[95] Moreover, in human patients with aortic stenosis, comparable induction of collagen type I and type III was noted.[96] The technical challenges in assessment of collagen I and III levels in the myocardium may explain, at least in part, the conflicting findings.

Increased synthesis of procollagens transcripts by activated myofibroblasts is followed by secretion of soluble procollagens into the extracellular space. Once outside the cells, procollagen chains are processed, assembled into fibrils, and cross-linked. Very limited information is available on the molecular steps regulating collagen processing and maturation in pressure-overloaded hearts. Cleavage of the N- and C-terminal propeptides attached to procollagen is a critical part of collagen processing. A study in a model of left ventricular pressure overload demonstrated that the enhancer protein PCOLCE (procollagen C-endopeptidase enhancer)-2 regulates C-terminal propeptide cleavage by enhancing the activity of the C-proteinase BMP (bone morphogenetic protein)-1.[122] The matricellular protein SPARC (secreted protein acidic and rich in cysteine) also contributes to postsynthetic processing of collagen in the pressure-overloaded heart and increases diastolic stiffness.[123]

Collagen cross-linking plays an important role in regulation of geometric remodeling and dysfunction in the pressure-overloaded heart. Several cross-linking enzymes are upregulated in the remodeling myocardium, including members of the LOX (lysyl oxidase) family[124,125] and TG (transglutaminase)-2 (also known as tissue transglutaminase).[126,127] In addition to its transamidase-dependent enzymatic actions, TG-2 bind to the ECM and may also act as a signaling molecule modulating fibroblast-mediated MMP and TIMPs (tissue inhibitor of metalloproteinases) synthesis through nonenzymatic mechanisms.[128] Studies in human patients support the significance of collagen cross-linking in the pathogenesis of heart failure. In hypertensive patients with heart failure, the degree of cross-linking and not the total amount of collagen was associated with elevated filling pressures.[129] Moreover, in patients with hypertensive heart failure, increased collagen cross-linking assessed through endomyocardial biopsy was associated with a higher incidence of hospitalizations.[130] However, preservation of geometry and function in the myocardium likely requires some matrix cross-linking activity. In models of cardiac pressure, overload was associated with reduction in myocardial collagen cross-linking.[131]

Nonfibrillar Collagens

Nonfibrillar collagens do not form large fibrillar bundles, but can associate with type I and type III collagen fibrils to regulate anchoring, networking, and organization of the ECM.[132] Moreover, nonfibrillar collagens may bind to cell surface receptors, modulating cellular phenotype, or yield bioactive fragments that regulate cellular responses. On the basis of their structural properties and functions, nonfibrillar collagens are classified into 6 groups (Table 2). Unfortunately, the information available on the role of nonfibrillar collagens in remodeling of the pressure-overloaded heart is limited. Table 2 summarizes our current understanding on the expression patterns and role of nonfibrillar collagens in heart failure.

Fibrogenic growth factors (such as TGF-β) and neurohumoral mediators (such as angiotensin II) implicated in induction and secretion of fibrillar collagens are also capable of inducing synthesis of several members of the nonfibrillar collagen subfamilies (such as collagen IV, VI, and VIII).[181,182] Thus, in fibrotic pressure-overloaded hearts, deposition of collagens I and III may be accompanied by concomitant upregulation of nonfibrillar collagens that may play an important role in organization of the ECM and in activation of cardiac interstitial cells.[183,184] In vitro, collagen VI has been identified as a potent stimulus for myofibroblast conversion,[167] whereas collagen VIII may activate ECM expansion by promoting fibroblast migration and by enhancing TGF-β synthesis.[143] In vivo, loss of collagen VIII was associated with reduced infiltration of the pressure-overloaded heart with myofibroblasts and attenuated fibrosis. These antifibrotic effects were associated with increased mortality and left ventricular dilation, supporting the importance of matrix-preserving actions in protecting the heart from adverse remodeling.[143] The cellular mechanisms for the effects of nonfibrillar collagens in the remodeling heart may not be limited to fibroblast activation, but may also involve actions on cardiomyocyte survival, inflammatory cell activation, and vascular cell function.[162] Several nonfibrillar collagens can be cleaved following injury, generating bioactive fragments with important biological functions. For example, collagen IV–derived peptides (such as canstatin) have been suggested to regulate cardiomyocyte survival, fibroblast migration, and angiogenesis in failing hearts.[182] Moreover, endostatin a collagen XVIII-derived peptide is a potent endogenous inhibitor of angiogenesis[178] that may play an important role in regulation of cellular responses in failing hearts.

Specialized Matrix Proteins

Remodeling of the pressure-overloaded myocardium is associated with secretion and deposition of specialized ECM proteins, which are not part of the normal adult cardiac matrisome and do not play a primary structural role, but are induced under conditions of stress and transduce molecular signals in cardiomyocytes and interstitial cells, modulating important cellular responses. Increased expression of embryonic isoforms of cellular Fn, matricellular proteins, and extracellular proteoglycans has been extensively documented in animal models of cardiac pressure overload and in failing human hearts.[185–187] In failing and remodeling hearts, the ECM is enriched with a broad range of macromolecules that modulate growth factor and protease activity, or bind to cell surface receptors, regulating cell survival, proliferation, and gene expression. Mechanotransductive signaling, TGF-βs, and neurohumoral mediators (such as angiotensin II) are potent inducers of specialized ECM proteins in the pressure-overloaded heart.

Fibronectin

Cardiac pressure overload triggers marked upregulation of splice variants of Fn that contain the ED-A (extra domain

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Table 2. Nonfibrillar Collagens in Heart Failure

Subfamily	Member	Cardiac Localization and Role in Myocardial Homeostasis	Role in Nonischemic Heart Failure	Role in MI and in Ischemic Heart Failure
Network-forming collagens (IV, VIII, X)	IV	Col IV is a major component of the basement membrane that surrounds cardiomyocytes and contributes to the vascular structure in the myocardium. Col IV is produced by both cardiomyocytes and interstitial cells.[133] Col IV may mediate homeostatic functions in cardiomyocytes and vascular cells through activation of integrin signaling. Col IV–derived peptides (such as canstatin) have been implicated in regulation of cardiomyocyte L type Ca^{2+} channel activity.[134]	Col IV induction has been observed in many cardiomyopathic conditions[135,136] and in diabetic and aging hearts.[137,138] Collagen IV–derived peptide canstatin may regulate angiogenesis, fibroblast migration, and cardiomyocyte survival in failing hearts.[139]	Col IV is upregulated in the infarct border zone in experimental models of MI.[140]
	VIII	Col VIII is expressed transiently during cardiogenesis. Low levels of Col VIII expression are noted in normal adult hearts.[141]	In the pressure-overloaded myocardium, Col VIII expression is reduced in animals with decompensated heart failure.[142] Endogenous Col VIII has been implicated in activation of myofibroblasts and may protect the heart from dilation.[143]	N/A
	X	Col X is expressed predominantly in cartilage. Expression in valve leaflets from patients with aortic stenosis has been reported.[144]	N/A	N/A
FACITs (IX, XII, XIV, XVI, XIX, XX, XXI, XXII)	IX	Col IX is codistributed with Col II in cartilage, and eye and inner ear. Transient Cola1IX mRNA expression has been reported in developing hearts.[145]	N/A	N/A
	XII	Col XII expression has been detected in the epicardium of zebrafish.[146] Col XII mRNA expression has been reported in human hearts.[147]	N/A	In zebrafish, epicardial expression of Col XII was increased following cryoinjury.[146]
	XIV	Col XIV is highly expressed in embryonic hearts and regulates collagen fibril organization, cardiomyocyte proliferation, and fibroblast survival.[148]	N/A	Col XIV was overexpressed in reperfused porcine MI.[15].
	XVI	An immunohistochemical study suggested expression of col XVI in developing and adult mouse hearts.[149]	N/A	N/A
	XXI	Col XXI mRNA expression has been reported in adult human hearts, predominantly localized in the right atrium and ventricle.[150]	N/A	N/A
	XXII	Col XXII expression was found in the mammalian myocardium localized at insertion points of the chordae tendinae to the myocardium.[151]	N/A	N/A
MACITs (XIII, XVII, XXIII, XXV)	XIII	Col XIII, a transmembrane collagen, is expressed in the intercalated discs of the adult heart.[152] Transgenic overexpression of Col XIII caused fetal lethality, associated with myocardial defects and dysfunction.[153,154]	N/A	N/A
	XVII	Col XVII expression has been reported in developing and adult mouse hearts and is a component of hemidesmosomes.[155]	N/A	N/A
	XXIII	Col XXIII mRNA expression was found in developing mouse hearts.[156]	N/A	N/A
	XXV	Although Col XXV is predominantly expressed in the brain, weak expression in the developing heart has been suggested.[157]	N/A	N/A
Anchoring fibrils (VII)	VII	Col VII is predominantly expressed in the dermal-epidermal junction. Cardiac expression has not been systematically studied.	A subset of patients with dystrophic epidermolysis bullosa (a condition caused by Col VII mutations) exhibit dilated cardiomyopathy.[158,159]	N/A

(*Continued*)

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Table 2. Continued

Subfamily	Member	Cardiac Localization and Role in Myocardial Homeostasis	Role in Nonischemic Heart Failure	Role in MI and in Ischemic Heart Failure
Beaded filament forming collagens (VI, XXVI, XXVIII)	VI	Col VI is highly expressed in developing and adult hearts.[160,161] In adult mammals, Col VI represents ≈5% of total collagen and is broadly distributed in the endomysium and in the media and adventitia of myocardial vessels.[8] However, Col VI KO mice had no significant baseline cardiac defects[162] and patients with Bethlem myopathy, a condition associated with Col VI mutations[163] do not seem to exhibit myocardial disease.[164]	Increased Col VI deposition has been reported in many cardiomyopathic conditions.[165] Patients with DCM exhibit increased Col VI deposition, localized in the perivascular and interstitial space,[166] and within t-tubules.[161] Considering its role in myofibroblast activation,[167] Col VI may be involved in fibrosis of the pressure-overloaded heart.	Following infarction, Col VI was found to play a protective role, limiting infarct size, and attenuating adverse remodeling.[162] The protective actions may involve effects on cardiomyocyte survival and on activation of reparative myofibroblasts.[167]
	XXVIII	Col XXVIII mRNA was detected in neonatal mouse hearts, but not in adult hearts.[168]	N/A	N/A
MULTIPLEXIN (XV, XVIII)	XV	Col XV is expressed in developing and adult hearts[169,170] and plays a crucial role in cardiac homeostasis. Col XV null mice developed a cardiomyopathy, associated with increased vascular permeability, defective interstitial network formation, and perturbed organization of cardiomyocytes.[171]	Col XV loss was associated with exercise-induced myocardial injury,[172] presumably due to protective actions of Col XV on stressed cardiomyocytes and vascular cells. Pressure-overloaded mouse hearts exhibited increased Col XV deposition.[171]	N/A
	XVIII	Collagen XVIII is highly expressed in embryonic and adult hearts.[173,174] However, genetic loss of Col XVIIIa1 did not have any functional consequences in mouse hearts, despite basement membrane alterations in atrioventricular valves.[175] Proteolytic cleavage of Col XVIII within its C-terminal domain yields release of endostatin, an endogenous inhibitor of angiogenesis.	In patients with chronic heart failure, circulating endostatin levels may predict adverse outcome.[176]	Myocardial ischemia triggers endostatin release due to Col XVIII cleavage.[177] Endostatin is a potent inhibitor of angiogenesis,[178] and endostatin levels inversely correlate with collateral formation in patients with ischemic heart disease.[179] However, in a model of MI, endostatin neutralization had adverse effects suggesting protective actions of Col XVIII, presumed due to antifibrotic actions, and to inhibition of protease activity.[180]

DCM indicates dilated cardiomyopathy; FACIT, fibril-associated collagens with interrupted triple helices; KO, knockout; MACIT, membrane-associated collagens with interrupted triple helices; MI, myocardial infarction; MULTIPLEXIN, multiple triple-helix domains and interruptions; and N/A, no information available.

A; ED-A+Fn), or the extra domain B (ED-B+Fn).[188] ED-A Fn has been suggested to cooperate with TGF-β, stimulating myofibroblast conversion in vitro and in vivo.[189–191] The molecular mechanism responsible for the interaction between ED-A+Fn and TGF-β remains poorly understood. In vitro experiments suggest that ED-A+Fn may bind to LTBP (latent TGF-β–binding protein)-1, contributing to the spatial localization of activatable TGF-β in tissues.[192] On the contrary, the role of ED-B+Fn, in heart failure is unclear. ED-B deficient fibroblasts exhibit slow growth and have attenuated synthesis of ECM proteins.[193] Whether induction of ED-B+Fn in the pressure-overloaded myocardium mediates fibroblast activation has not been investigated. Polymerization of Fn following secretion plays a critical role in regulation of fibroblast phenotype and increases the tensile strength of collagen fibers.[194,195]

Matricellular Proteins

Tissue remodeling is associated with induction of a wide range of extracellular macromolecules that bind to the structural ECM, interact with growth factors and proteases, and modulate signaling responses in many cell types by binding to cell surface receptors, such as integrins and syndecans. These proteins, termed matricellular do not play a primary structural role, but serve as a molecular bridge between the ECM and the cells, acting as dynamic integrators of microenvironmental changes and as essential mediators of tissue remodeling.[185,196–198] The founding members of the matricellular family were tenascin-C, SPARC, and TSP (thrombospondin)-1; however, recognition of the complexity of matrix responses following injury resulted in rapid expansion of the family with the inclusion of several additional proteins, such as TSP-2 and TSP-4, tenascin-X, osteopontin, periostin, the fibulins,

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and the members of the CCN (connective tissue growth factor, cystein-rich protein, nephroblastoma overexpressed gene) family. In vivo studies have revealed a remarkable functional complexity of matricellular proteins, reflecting the contextual nature of their effects that depend on the various structural proteins, cytokines, and growth factors they associate with, and the cell types with which they interact in different tissues, and in various pathological conditions.

Cardiac pressure overload induces marked induction and interstitial deposition of several members of the matricellular family.[185] In the remodeling myocardium, cardiomyocytes, fibroblasts, immune cells, and vascular cells, all major cellular effectors of fibrosis, have been identified as important targets of the matricellular proteins. Table 3 summarizes our knowledge on the expression and role of the major members of the matricellular family in heart failure. Although each member has a unique repertoire of functional effects and molecular interactors, several common themes have emerged about their cell biological actions. First, several matricellular proteins have been implicated in regulation of fibroblast proliferation, survival and activation, and in myofibroblast conversion.[41,199,213,251,252,273] Thus, in pressure-overloaded hearts, matricellular proteins, such as TSP-1 (Figure 3), TSP-4, periostin, and osteopontin may act by stimulating a fibrogenic program in cardiac fibroblasts. Although most members of the family have been suggested to exert fibrogenic actions in vivo, matricellular actions that inhibit fibroblast responses, or contribute to resolution of fibrosis have also been reported.[283] Second, immune cell subpopulations are increasingly recognized as important cellular targets of matricellular proteins. For example, tenascin-C (Figure 4) may act as an important modulator of macrophage phenotype in remodeling pressure-overloaded hearts.[218,219,228] Third, some matricellular proteins may have direct actions on cardiomyocyte survival under conditions of stress.[209,215] Fourth, although the traditional definition of a matricellular protein implies absence of a structural role, several members of the matricellular family (such as SPARC, TSP-1, and TSP-2) have been implicated in collagen fibril assembly and in regulation of ECM cross-linking.[123,203,284]

The molecular targets of the matricellular proteins include a broad range of cytokines and growth factors, proteases, integrins, and nonintegrin cell surface receptors, such as CD44 and CD36. An additional layer of complexity in understanding the effects of the matricellular proteins was added by recently reported experimental findings suggesting important intracellular actions of the TSPs.[211,217] Considering the diverse functions of matricellular proteins, their multiple functional domains and their context-dependent effects, the relative contributions of specific actions in vivo remains poorly documented.

Proteoglycans

Proteoglycans are glycosylated proteins that consist of a core protein with one or more covalently attached glycosaminoglycan chains. In the mammalian heart, the profile of ECM proteoglycan composition is dependent on topography. The pericellular matrix contains predominantly heparan sulfate proteoglycans, which are typically associated with the cell surface. In areas more distant from the surface of the cells, chondroitin sulfate- and dermatan sulfate-containing proteoglycans (CSPGs [chondroitin sulfate proteoglycans] and DSPGs [dermatan sulfate proteoglycans], respectively) predominate.[285] In both human patients with heart failure and in animal models of myocardial disease, CSPGs accumulate in fibrotic regions.[286] The ECM in the pressure-overloaded heart is also enriched with a wide range of SLRPs (small leucine-rich proteoglycans), such as biglycan, decorin, fibromodulin, lumican, and osteoglycin. TGF-βs, angiotensin II, proinflammatory cytokines, and mechanotransductive signaling markedly upregulate expression of SLRPs in cardiac fibroblasts.[287–289] Once secreted in the cardiac interstitium, SLRPs bind to collagen fibrils and organize the structural ECM, but may also interact with growth factors, and cell surface receptors to transduce or modulate signaling responses. In vivo studies have implicated several SLRPs in regulation of cardiac fibrosis, remodeling, and dysfunction following pressure overload.[186] In a model of transverse aortic constriction, biglycan was found to mediate fibrosis and cardiomyocyte hypertrophy in the pressure-overloaded myocardium.[290] Other SLRPs have been implicated in negative regulation of fibrotic and hypertrophic myocardial remodeling. In hypertensive rats, decorin gene therapy attenuated fibrosis and hypertrophy and improved function by inhibiting TGF-β signaling.[291] Lumican has also been suggested to protect against systolic dysfunction in an isoproterenol infusion model, the cellular mechanism for the protective actions was not studied.[292] Osteoglycin protected the pressure-overloaded myocardium from diastolic dysfunction by attenuating inflammatory activation of macrophages and by inhibiting TGF-β–driven fibrogenic responses.[293] The combined experience from various mouse models of cardiac remodeling suggests important roles for SLRPs in regulation of fibrosis and hypertrophy; however, their functional role is dependent on the pathophysiologic context, and the cellular targets and molecular interactors remain unknown. Moreover, proteomic studies in human hearts suggested that myocardial SLRP profiles may be dependent on topography, identifying atrial-specific processed forms of decorin that may be involved in atrial fibrillation.[294]

Role of ECM Metabolism in Regulation of Geometry and Function in the Pressure-Overloaded Heart

In both experimental models of left ventricular pressure overload and in human patients with long-standing untreated hypertension or aortic stenosis, early increases in myocardial stiffness occur in the absence of systolic dysfunction, and may be followed by late dilative remodeling and systolic functional depression. Although the basis for the transition likely involves several different cellular processes, changes in the composition of the ECM may be critical in regulation of cardiac geometry and function. Regulation of the balance between proteases and antiproteases plays a crucial role in the biochemical profile of interstitial ECM proteins and has profound functional implications (Figure 5). Although only fibroblasts produce significant amounts of fibrillar collagens, the main structural components of the matrix, virtually all myocardial cell types (including cardiomyocytes, fibroblasts, immune cells, and vascular cells) can produce and activate proteolytic enzymes that process ECM proteins,[295] thus altering the geometry and the mechanical properties of the myocardium. Cardiac pressure overload is associated with induction and activation of collagenases

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Table 3. Expression, Role, and Mechanisms of Action of the Major Members of the Matricellular Family in Heart Failure

Matricellular Protein	Expression and Role in Nonischemic Cardiomyopathy	Expression and Role in MI and in Ischemic Cardiomyopathy	Proposed Mechanisms of Action
TSP-1	Expression: Transiently upregulated and deposited in the interstitial and perivascular ECM in models of LV pressure overload.[199,200] Upregulated in perivascular areas in diabetic hearts.[201]	Expression: Transiently induced in the infarct border zone.[202]	Activation of TGF-β may promote myofibroblast conversion and acquisition of a matrix-synthetic phenotype[200,202]
		Role: Protects from adverse remodeling, limiting expansion of the inflammatory infiltrate.[202]	Inhibition of MMPs[199]
	Role: Has been reported to protect the pressure-overloaded myocardium from dysfunction by stabilizing the ECM.[199]		Interaction with collagens and LOX precursors[203]
			Angiostatic effects[201]
	The TSP-1/TGF-β axis has been reported to promote fibrosis and dysfunction in the diabetic pressure-overloaded heart.[200]		Anti-inflammatory actions[202]
			TSP-1/CD47 actions may induce HDAC-3
	The TSP-1/CD47 axis has been suggested to promote heart failure in a model of pressure overload.[204]		TSP-1/CD47 regulates effects of NO on the microvasculature[205]
	Mediates capillary rarefaction in diabetic hearts.[201]		
TSP-2	Expression: Highly expressed in pressure-overloaded hearts and in human patients with cardiac hypertrophy.[206] High circulating TSP-2 are associated with adverse outcome in HFpEF patients.[207]	No published data	Inhibition of MMP synthesis and activity[208]
			Activation of Akt-mediated prosurvival pathways in cardiomyocytes[208]
	Role: Protects the pressure-overloaded heart from rupture[206] and the aging heart from dilative cardiomyopathy.[209]		
	Protective role in viral myocarditis through T reg-mediated anti-inflammatory actions.[210]		
	Protects cardiomyocytes and preserves the integrity of the cardiac ECM in doxorubicin-induced cardiomyopathy.[208]		
TSP-3	Promotes cardiomyocyte injury in response to stress.[211]	Unknown	Induction of integrins in stressed cardiomyocytes through intracellular actions that promote sarcolemmal destabilization[211]
TSP-4	Expression: Rapidly upregulated in the myocardium in response to angiotensin II-treatment, or TAC.[212]	Unknown	Suppression of ECM protein synthesis in fibroblasts and endothelial cells[213]
	Role: Protects the pressure-overloaded heart from functional decompensation, systolic dysfunction and death, and restrains hypertrophy.[214]		Activation of mechanosensitive Erk and Akt signaling in cardiomyocytes in response to stress[215]
	Transduces protective mechanosensitive signals require for augmentation of contractility in response to an acute pressure load[215] has also been suggested to inhibit fibrosis in the pressure-overloaded heart.[213,216]		Intracellular actions, augmenting ER function and protecting from injury-related ER stress[217]
Tenascin-C	Expression: Consistently upregulated and deposited in the cardiac interstitium in pressure-overloaded hearts,[71,218,219] and in myocarditis.[220] Upregulated in cardiomyocytes subjected to mechanical stress.[221] In human patients with DCM, increased serum levels of tenascin-C, and myocardial tenascin-C expression have been linked with adverse outcome.[222,223]	Expression: Highly expressed in the infarct border zone, and in remodeling myocardial areas in experimental models[224] and in human patients with myocardial scars[225] or ischemic cardiomyopathy.[226] In patients with ischemic cardiomyopathy, interstitial activity evidenced by tenascin-C deposition predicted segmental recovery following revascularization.[226]	Activation of integrin-mediated secretion of fibrogenic cytokines in macrophages[219]
			Modulation of TLR responses in macrophages[227]
			Negative regulation of inflammation[228]
	Role: Has been suggested to accelerate fibrosis, hypertrophy, and dysfunction of the pressure-overloaded heart.[218,219,231] However, in another study tenascin-C derived from bone marrow cells protected the pressure-overloaded heart from dysfunction and fibrosis by attenuating inflammation.[228]		Accentuation of fibroblast migration[229] and fibroblast activation through TLR-dependent mechanisms[230]
			Modulation of adhesive interactions between the cardiomyocytes and the ECM[224]
		Role: Has been suggested to accelerate adverse postinfarction remodeling by perturbing macrophage functions.[227]	

(*Continued*)

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Table 3. Continued

Matricellular Protein	Expression and Role in Nonischemic Cardiomyopathy	Expression and Role in MI and in Ischemic Cardiomyopathy	Proposed Mechanisms of Action
SPARC	Expression: Increased levels of myocardial SPARC are noted in experimental models of pressure overload,[123] in aging hearts.[232,233] SPARC expression was associated with fibrosis in human cardiac allografts.[234]	Expression: Upregulated in experimental models of MI.[235,236]	Contribution to postsynthetic procollagen processing[123]
		Role: Protects the infarcted heart from cardiac rupture and dysfunction by preserving the ECM network.[237]	Activation of TGF-β signaling responses in cardiac fibroblasts[237]
	Role: Mediates collagen processing and fibrosis, and promotes diastolic dysfunction in models of pressure overload.[123] Has been suggested to protect against inflammation in viral inflammation.[238]		
Osteopontin (OPN)	Expression: Marked myocardial upregulation of osteopontin is noted in experimental models of heart failure induced through pressure overload,[239] in volume overload,[240] in diabetic hearts,[241] and in hearts from mice with muscular dystrophy.[242] Myocardial osteopontin was increased in patients with end-stage dilated cardiomyopathy; levels were reduced on unloading.[243] Highly expressed in the myocardium of patients with nonischemic cardiomyopathy and associated with fibrotic lesions and hypertrophy.[244] In human patients with hypertension, osteopontin promoter variants that affect transcription were associated with diastolic function.[245]	Expression: Upregulated in myocardial infarcts and predominantly localized in activated macrophages.[246–248]	Stimulation of fibroblast proliferation through integrin activation,[249,250] and protection of fibroblasts from apoptosis[251]
		Role: Protects the infarcted heart from ventricular dilation by promoting collagen deposition.[253]	Activation of prosurvival pathways in fibroblasts through miR-21[252]
			Stimulation of myofibroblast conversion[254]
			Activation of reparative and fibrogenic macrophages through galectin-3 upregulation[248,255]
			Activation of lymphocytes[256]
			Stimulation of cardiomyocyte apoptosis via CD44[257]
	Role: Suggested to mediate fibrosis, while preventing dilation in pressure-overloaded hearts and on neurohumoral activation.[249,258–260] Prohypertrophic effects have been less consistently observed.		
	In aging hearts, osteopontin derived from adipose tissue (and not from the myocardium) was suggested to promote cardiac fibrosis and dysfunction.[261] In a mouse model of Chagas disease, OPN was implicated in cardiac inflammation, fibrosis, and dysfunction.[262] OPN mediated fibrosis in a model of viral myocarditis[263]; however, in a model of autoimmune myocarditis, osteopontin did not affect inflammation.[264]		
Periostin	Expression: Upregulated in activated myofibroblasts and deposited in the cardiac interstitium in models of cardiac pressure overload.[41,265–267] Increased myocardial expression in senescent mice,[268] in a model of volume overload,[269] and in models of cardiomyopathy induced through renal failure.[270] Negligible expression in a model of type II diabetes mellitus-related cardiomyopathy.[271] In human patients, increased periostin expression in failing hearts was reduced on unloading.[272]	Expression: Markedly upregulated in infarct myofibroblasts and deposited in the ECM following infarction.[41,273,274]	Activation and differentiation of cardiac fibroblasts, promoting migration and myofibroblast conversion[273,275,276]
			Regulation of collagen fibrillogenesis[277]
		Role: Following nonreperfused infarction, periostin is essential for cardiac repair and protects from rupture, but mediates fibrosis and adverse remodeling.[41,273]	Stimulation/activation of TGF-β signaling pathways[278]
			Modulation of MMP synthesis by many cell types, including interstitial cells and macrophages[279]
	Role: Promotes hypertrophy and fibrosis in experimental models of pressure overload.[41] Promotes dysfunction in a model of hypertensive heart disease.[266]	Has been suggested to promote cardiac regeneration in neonatal,[281] and even in adult mice.[280]	Stimulation of adult cardiomyocyte proliferation involving PI3K activation has been reported[280]
		Regenerative effects in adult animals were not supported by studies using genetic manipulation strategies.[282]	

DCM indicates dilated cardiomyopathy; ECM, extracellular matrix; ER, endoplasmic reticulum; HDAC, histone deacetylase; HFpEF, heart failure with preserved ejection fraction; LOX, lysyl oxidase; LV, left ventricular; MI, myocardial infarction; MMP, matrix metalloproteinase; OPN, osteopontin; PI3K, phosphoinositide 3-kinase; SPARC, secreted protein acidic and rich in cysteine; TAC, transverse aortic constriction; TGF, transforming growth factor; TLR, toll-like receptor; and TSP, thrombospondin.

(such as MMP-1, MMP-8, and MMP-13), gelatinases (such as MMP-2 and MMP-9), stromelysins/matrilysins, and membrane-type MMPs.[296,297] TIMPs are also upregulated in remodeling hearts and may exert matrix-preserving actions.[298,299] A large body of evidence suggests that MMP induction and activation may generate a predominantly proteolytic environment in the cardiac interstitium, leading to degradation of ECM proteins.[300] The relatively slow synthesis rate of collagen by

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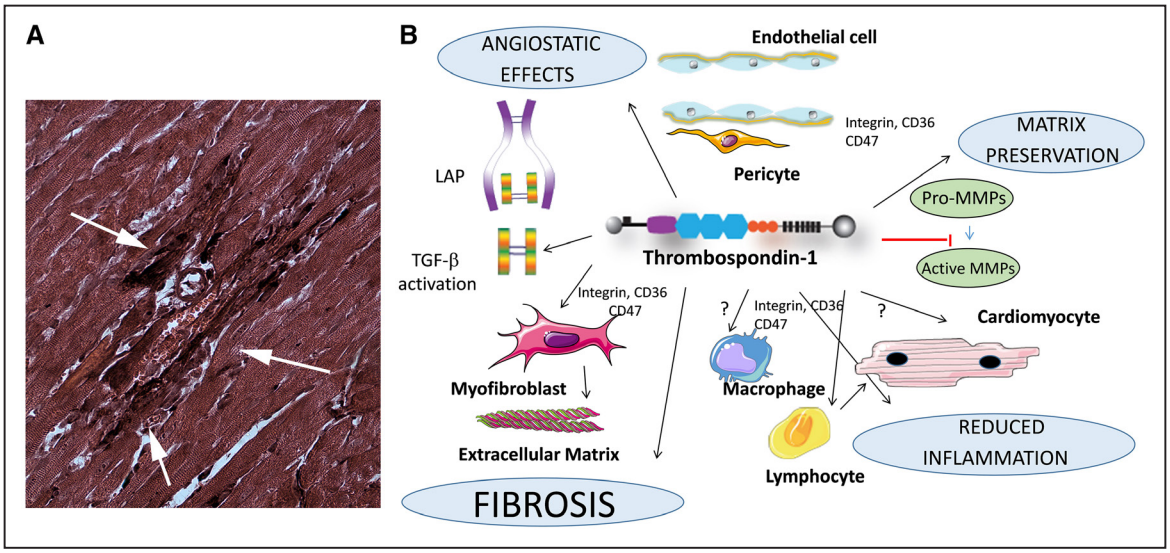


Figure 3. Expression and actions of TSP (thrombospondin)-1 in failing hearts. In a mouse model of cardiac pressure overload induced through transverse aortic constriction, TSP-1 immunoreactivity is localized predominantly in perivascular and interstitial areas (data from our published work[199]). TSP-1 overexpression in the pressure-overloaded heart may modulate inflammation, fibrosis, and matrix metabolism through several distinct molecular pathways. First, TSP-1 plays an important role in TGF (transforming growth factor)-β activation by binding to the LAP (latency-associated peptide), thus triggering release of bioactive TGF-β. Second, TSP-1 may inhibit MMP (matrix metalloproteinase) activation, promoting matrix preservation. Third, TSP-1 may exert angiostatic actions. Fourth, TSP-1 may exert anti-inflammatory actions through effects on lymphocytes and macrophages. Fifth, TSP-1 may inhibit NO production and signaling.

cardiac fibroblasts is accelerated following pressure overload, but may not be sufficient to compensate for the pronounced matrix-degrading effects of MMPs.[10,301,302] Excessive degradation of endomysial collagen may precipitate systolic dysfunction in patients with hypertensive heart failure.[303] MMP induction may be mediated through activation of proinflammatory signaling pathways. For example, TNF (tumor necrosis factor)-α induces synthesis of a wide range of MMPs in both cardiomyocytes and fibroblasts and promotes dilation of the pressure-overloaded heart by degrading the supporting ECM.[304] The proinflammatory cytokine IL (interleukin)-1β also stimulates MMP expression in cardiac fibroblasts and may exert similar actions in vivo.[305,306] Development of systolic dysfunction in the presence of excessive MMP activation in the cardiac interstitium may involve several distinct mechanisms. First, because cardiomyocytes are dependent on interactions with a preserved matrix network, loss of survival signals transduced by intact ECM proteins may promote apoptosis and contractile dysfunction of cardiomyocytes under conditions of stress. Second, ECM fragments (matrikines) generated through protease activation may induce proinflammatory cascades,[30] or activate proapoptotic pathways in cardiomyocytes. Third, several members of the MMP family have been suggested to regulate signaling cascades through effects independent of ECM processing. MMPs can process CC and CXC chemokines[307,308] and cytokines (such as TNF-α),[309] thus exerting context-dependent proinflammatory or anti-inflammatory actions. Moreover, MMPs have been implicated in TGF-β activation,[310] and may cleave transmembrane receptors, such as integrins[311] or syndecans,[312] thus modulating essential proinflammatory or fibrogenic cascades. MMPs may also act as intracellular mediators, promoting degradation of contractile proteins in cardiomyocytes, or modulating signal transduction responses in interstitial cells.[313–315] In contrast to the effects of MMP activation, overactive matrix-preserving mediators (such as induction of TIMPs) may promote deposition of structural ECM proteins, increasing myocardial stiffness, and accentuating diastolic dysfunction.[316] It should be emphasized that, much like MMPs, TIMPs can also exert actions independent of MMP activity or ECM remodeling. For example, TIMP-1 has been suggested to promote cardiac fibrosis by facilitating a CD63/β1 integrin cascade that activates Smad-dependent signaling in cardiac fibroblasts.[299]

In addition to the established role of MMPs in myocardial ECM turnover, emerging evidence implicates 2 other families of proteases, the ADAM (a disintegrin and metalloproteinase), and the ADAMTS (ADAM with thrombospondin motifs).[317,318] ADAM and ADAMTS family members can be activated in response to a wide range of stimuli and have multiple substrates. ADAMs mediate ectodomain shedding, the proteolytic cleavage of the extracellular domain of membrane-bound molecules. ADAMTS members, however, typically act as secreted proteases and have ECM protein substrates. In a model of left ventricular pressure overload, ADAM-17 expression in cardiomyocytes protected the heart from dilation, dysfunction, and fibrosis through cleavage of β1 integrin.[319] ADAMTS-2, however, was found to be upregulated in the pressure-overloaded myocardium and attenuated cardiac hypertrophy by inhibiting the PI3K/Akt axis.[320]

Effects of Volume Overload on the Cardiac ECM

Despite its important role in heart failure associated with severe valvular regurgitant lesions, and its involvement in other cardiomyopathic conditions, volume overload remains a pathophysiologic and cell biological enigma. In contrast to the marked deposition of fibrillar collagens in the interstitium of pressure-overloaded hearts, volume overload is associated with loss of interstitial collagen, prominent ECM degradation, and

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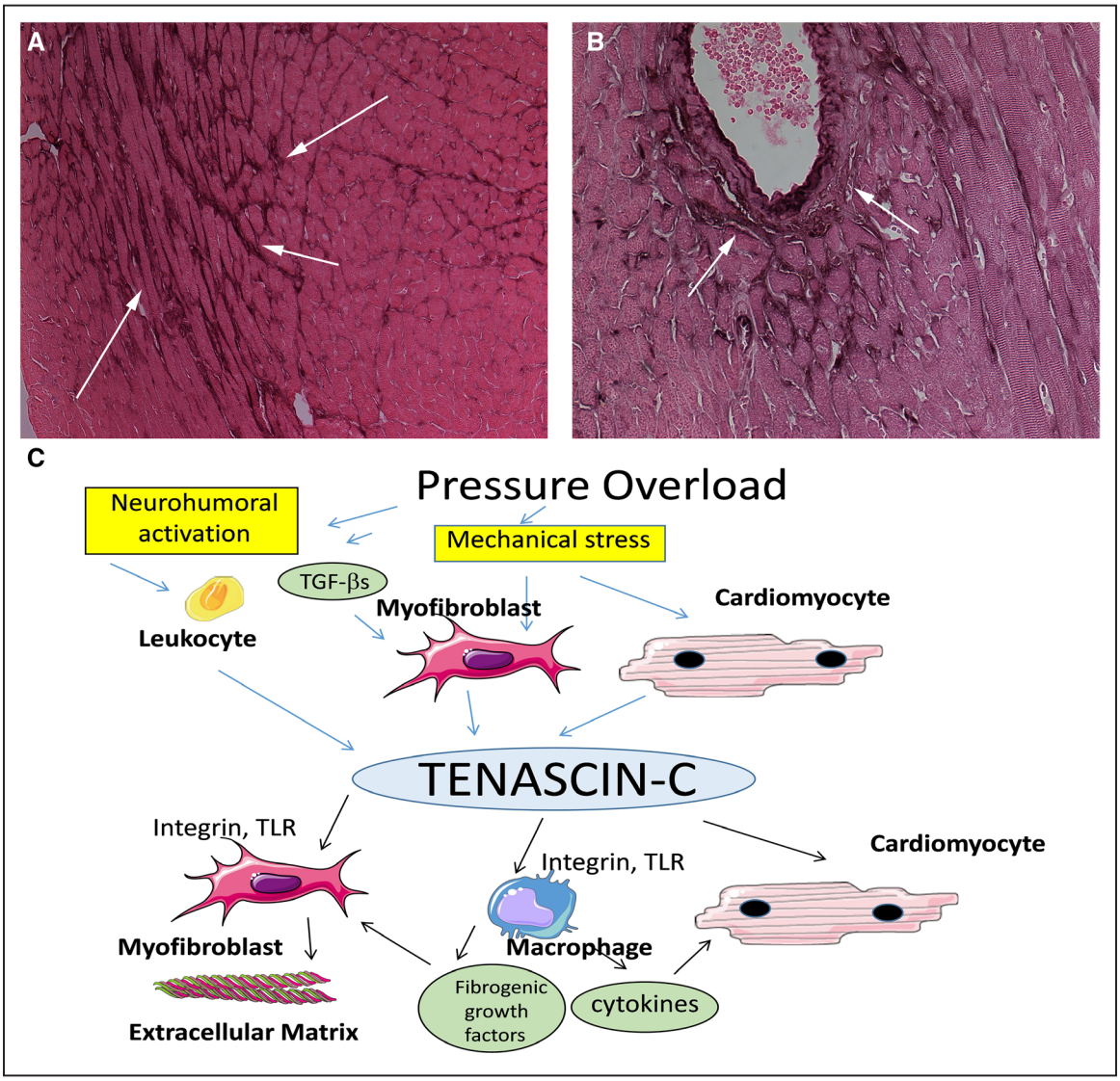


Figure 4. Expression and function of the matricellular protein tenascin-C in the failing heart. **A** and **B**, Tenascin-C immunoreactivity is localized in the interstitial and perivascular areas in a mouse model of cardiac pressure overload induced through transverse aortic constriction. Images from our own published work.[71] Tenascin-C may be produced by cardiomyocytes, myofibroblasts, and leukocytes in response to mechanical stress or neurohumoral activation. When bound to the interstitial ECM (extracellular matrix), tenascin-C may exert fibrogenic actions by directly stimulating synthesis of ECM proteins by fibroblasts, or by activating macrophages through integrin or Toll-like receptor (TLR)–dependent mechanisms. Effects of tenascin-C on cardiomyocytes remain understudied.

induction of MMPs (Figure 6).[321–323] The cellular basis for the loss of collagen and the dominant activation of proteases in volume overload–induced cardiomyopathy remains unknown. Mechanical stretch in response to volume overload may affect gene expression in both interstitial cells and cardiomyocytes. Fibroblasts from volume-overloaded hearts exhibited a hypofibrotic phenotype, characterized by reduced collagen and α-SMA synthesis.[324] It has been suggested that increased oxidative stress induced by mechanical stretch may activate an autophagic degradation of procollagen in volume-overloaded hearts.[325] Effects of volume overload on macrophages and mast cells have also been implicated.[73,326] As important sources of MMPs and proinflammatory cytokines, mast cells and macrophages may promote degradation of the interstitial ECM, leading to dilative remodeling and systolic dysfunction. Although in vitro and in vivo studies have suggested that mechanical stretch in response to volume overload may promote cardiomyocyte apoptosis[327] and alters the conformation of caveolae,[328] whether cardiomyocytes serve as important cellular effectors of ECM loss in volume-overloaded hearts has not been investigated.

Dynamic Alterations of the Cardiac ECM in the Infarcted Heart and in Chronic Ischemic Cardiomyopathy

The adult mammalian heart has negligible regenerative capacity. Thus, following MI, the heart heals through activation of an inflammatory response that clears the wound from dead cardiomyocytes and activates reparative myofibroblasts, ultimately leading to formation of a scar, predominantly comprised cross-linked collagen. Repair of the infarcted myocardium can be divided into 3 distinct but overlapping phases: the inflammatory phase, the proliferative phase, and the maturation phase.[20] Dynamic

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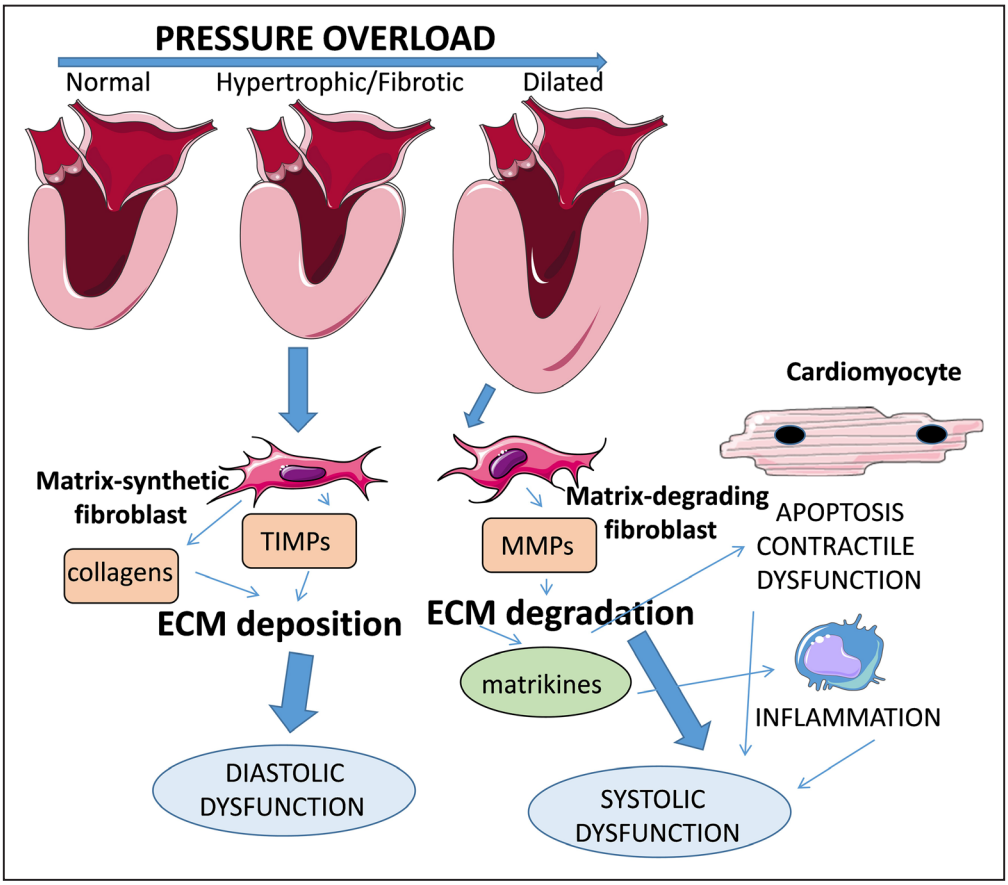


Figure 5. Sustained pressure overload triggers progressive left ventricular dilation and systolic dysfunction through changes in the protease/antiprotease balance. In the early phase, mechanical stress induces a matrix-preserving fibroblast phenotype inducing collagen synthesis and production of antiproteases (such as TIMPs [tissue inhibitor of metalloproteinase]). Accentuated ECM (extracellular matrix) deposition increases myocardial stiffness causing diastolic dysfunction. Prolonged pressure overload may be associated with transition of infiltrating fibroblasts to a matrix-degrading phenotype that produces MMPs (matrix metalloproteinases), thus causing ECM degradation and generating matrix fragments (matrikines). Matrikines may cause systolic dysfunction by promoting cardiomyocyte apoptosis and by inducing inflammation. Moreover, degradation of the ECM network may deprive cardiomyocytes from matrix-dependent signals required for preservation of function.

changes in the biochemical profile and composition of the ECM play a critical role in regulation of key cellular events in all 3 phases of infarct healing.[196,329] During the inflammatory phase, early degradation of the ECM generates matrikines that may serve as danger signals contributing to activation of an inflammatory and reparative program. Moreover, formation of a provisional fibrin-based matrix network derived from extravasated fibrinogen and plasma Fn[235] serves as a highly plastic conduit for infiltrating inflammatory cells. As macrophages clear the infarct from dead cells and matrix debris, induction of anti-inflammatory mediators suppresses inflammation and marks the transition to the proliferative phase. At this stage, activated myofibroblasts deposit both structural and matricellular ECM proteins,[98] preserving the structural integrity of the ventricle. Finally, during the maturation phase, the collagenous ECM is cross-linked and the fibroblasts become quiescent,[330] and may undergo apoptosis. The matrix-dependent molecular steps that regulate the reparative response following MI are critical for protection of the infarcted heart from cardiac rupture and from the pathogenesis of heart failure. Moreover, a large body of evidence suggests that disruption of matricellular genes exclusively or predominantly induced in the infarct zone has a major impact on the extent of adverse postinfarction remodeling.[202,237,331] These observations suggest that the characteristics and biochemical composition of the scar are a major determinant of cardiac remodeling following MI and that dysregulation of ECM-dependent responses in the healing infarct may drive postinfarction heart failure. The role of the ECM network in postinfarction cardiac remodeling has been recently reviewed.[196]

As the infarct heals, the viable noninfarcted myocardium remodels, exhibiting cardiomyocyte hypertrophy, accompanied by progressive interstitial fibrosis. Thus, although endogenous anti-inflammatory signals suppress inflammation in the infarct zone, in the viable noninfarcted segments, increased wall stress may locally activate cardiomyocytes, macrophages, and fibroblasts, triggering chronic remodeling of the ECM network.[332,333] Studies in human patients surviving acute MI suggest that high circulating levels of MMPs may predict adverse dilative remodeling, systolic dysfunction, and progression to heart failure.[334,335] Although it is tempting to hypothesize that accentuated and accelerated chamber dilation in a subset of MI patients may be caused by overactive matrix-degrading pathways, the contribution of chronic ECM remodeling in noninfarcted segments to adverse remodeling and development of postinfarction heart failure remains poorly documented. Understanding the functional consequences of specific cellular

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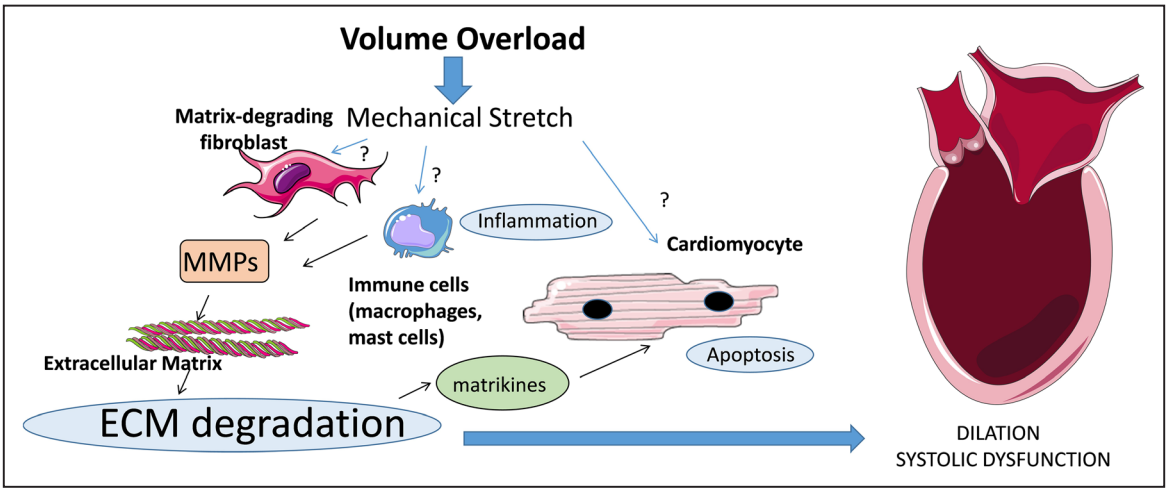


Figure 6. The enigmatic basis of ECM (extracellular matrix) degradation in the volume-overloaded heart. Experimental evidence suggests that cardiac volume overload is associated with a matrix-degrading fibroblast phenotype and with high levels of interstitial MMPs (matrix metalloproteinases). Immune cells (macrophages and mast cells) may contribute to the proteolytic environment by secreting MMPs. Persistent ECM degradation may trigger cardiomyocyte apoptosis and dysfunction and may be responsible for the dilation and systolic dysfunction typically observed in volume-overloaded hearts. The molecular links between mechanical stretch induced by volume overload and the selective activation of proteolytic pathways remain unknown.

events in remote viable myocardial segments is particularly challenging, considering that traditional loss- or gain-of-function interventions also target cells in the infarct and in the border zone that may affect cardiac hemodynamics, thus indirectly modulating responses in the noninfarcted myocardium.

Alterations in the ECM network may also contribute to the pathogenesis of chronic ischemic cardiomyopathy, in the absence of MI. Unfortunately, the effects of low flow ischemia or brief ischemic insults on the myocardial ECM remain underexplored and poorly understood. In a porcine model, reduction of coronary flow to 50% of the baseline for 90 minutes was sufficient to activate MMP-9 without affecting collagenase levels, or have effects on the structure of the cardiac ECM.[336] Moreover, in a mouse model of brief repetitive myocardial ischemia and reperfusion, deposition of the matricellular protein tenascin-C accompanied interstitial fibrosis, in the absence of a completed infarction.[337] These findings suggest that ischemic events of low duration or intensity may trigger remodeling of the cardiac ECM without causing lethal cardiomyocyte injury. Oxidative stress triggered by ischemia/reperfusion may be responsible for the ECM changes. The clinical relevance of these observations in ischemic cardiomyopathy is supported by studies in human patients. MMP-2 and MMP-9 activation were noted following reperfusion after cardioplegia in myocardial samples from patients undergoing aortocoronary bypass.[338] Moreover, patients with ischemic cardiomyopathy exhibited increased myocardial MMP-9 expression and activity, and high MMP-14 levels[339,340]; increased MMP expression was reversed following unloading through left ventricular assist device implantation.[341] Recovery of function following revascularization in ischemic cardiomyopathy may also involve the cardiac ECM. In patients undergoing aortocoronary bypass surgery for chronic ischemic cardiomyopathy, segments with functional recovery had higher levels of tenascin-C, associated with a more cellular interstitium.[226] In contrast, myocardial segments exhibiting reduced interstitial activity, evidenced by lower levels of matricellular proteins, reduced numbers of inflammatory leukocytes and more mature collagen, did not recover function following revascularization.[226,342] Thus, an active, highly cellular interstitial environment, enriched in matricellular proteins and capable of transducing growth factor responses may be required for structural and functional recovery of chronically ischemic fibrotic myocardial segments.

ECM in Diabetes Mellitus-Associated Heart Failure

Diabetes mellitus, obesity, and metabolic dysfunction are associated with progressive expansion of the interstitial or perivascular ECM in experimental animal models[271] and in human patients.[343] The cellular basis of diabetes mellitus-associated fibrosis remains poorly understood,[344] as studies exploring cell-specific actions in vivo are lacking. Oxidative stress, generation of AGEs (advanced glycation end products), neurohumoral activation, ET-1 induction, adipokine secretion, and stimulation of proinflammatory cascades in response to metabolic dysregulation may directly activate cardiac fibroblasts, promoting a matrix-synthetic phenotype. Induction of matricellular proteins, such as TSP-1, is prominent in diabetic hearts[201,345] and may contribute to activation of TGF-β signaling, directly activating cardiac fibroblasts. Despite evidence suggesting local TGF-β activation,[346] diabetes mellitus-associated cardiac fibrosis may not involve myofibroblast conversion.[271] Macrophages, cardiomyocytes, vascular endothelial cells, and pericytes may also acquire a fibrogenic program, secreting growth factors, and stimulating fibroblast-derived ECM synthesis. AGE-induced cross-linking of the collagenous ECM in vessels and myocardium may reduce vascular compliance and increase myocardial stiffness.[347]

In human patients, obesity, diabetes mellitus, and metabolic dysfunction markedly increase the risk of HFpEF, independently of the coronary artery disease.[348,349] Clinical studies have identified a distinct obesity-related phenotype of human HFpEF,[350] associated with increased plasma volume, more severe concentric left ventricular hypertrophy and greater right ventricular dilatation, despite lower plasma levels of natriuretic

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peptides.[350,351] Microvascular inflammation in obese diabetic subjects may stimulate fibrogenic signaling,[26,352] promoting ECM expansion and collagen cross-linking, and significantly contributing to diastolic dysfunction.[26] Considering the perturbations in cardiomyocyte and vascular cell phenotype and function typically associated with diabetes mellitus,[353,354] to what extent fibrogenic actions and ECM remodeling contribute to diastolic dysfunction and adverse outcome in diabetic patients remains unclear.

ECM in Age-Associated Cardiac Dysfunction

Studies in both human subjects and animal models have consistently documented perivascular and interstitial accumulation of collagen in senescent hearts.[104,355,356] Some studies have suggested that age-associated fibrosis may reflect attenuation of matrix degradation due to a reduction in MMP synthesis, rather than accentuated collagen transcription.[357–360] Increased collagen cross-linking may also contribute to fibrosis in aging hearts, by reducing the susceptibility of the interstitial ECM to protease-mediated degradation.[361]

Aging-associated fibrosis may have important functional consequences. Aging is typically associated with a decline in diastolic function and reduced exercise capacity in the absence of significant effects on systolic function at rest. Deposition of cross-linked collagen fibers may reduce myocardial compliance, contributing to the pathogenesis of HFpEF in elderly subjects. However, because aging also affects cardiomyocyte relaxation properties, the relative contribution of interstitial ECM changes in the pathogenesis of senescence-associated diastolic dysfunction remains poorly documented. Myocardial deposition of ECM proteins may also account for the increased prevalence of conduction defects and arrhythmic events in older patients.

The mechanisms responsible for aging-associated cardiac fibrosis remain poorly understood. Activation of neurohumoral pathways, chronic low-level induction of proinflammatory mediators, activation of TGF-βs and other fibrogenic mediators, reactive oxygen species generation, and downmodulation of protective antifibrotic signals have been implicated in activation of a fibrotic response in the aging heart.[362] However, the fundamental link between aging and activation of a fibrogenic program remains unclear, and most of the evidence implicating specific mediators is associative.

ECM in Genetic Cardiomyopathies

Monogenic cardiomyopathies caused by single pathogenetic mutations account for a relatively small proportion of heart failure patients.[363] It is unclear whether certain mutations can cause heart failure through primary activation of a fibrogenic program in cardiac interstitial cells. In postmortem autopsies of young patients dying from sudden cardiac death, primary cardiac fibrosis (defined as myocardial fibrosis in the absence of identifiable causes, or known cardiomyocyte pathology) is commonly found.[364] However, in many of these cases, genetic analysis identified cardiomyocyte-specific gene mutations,[365] suggesting that fibrosis may not be triggered through primary activation of interstitial cells, but may reflect actions of cardiomyocyte-derived fibrogenic signals. In a recent study, subjects with a frameshift mutation of *SEPRINE1*, the gene encoding PAI (plasminogen activator inhibitor)-1, exhibited primary cardiac fibrosis, attributed to the release of cardiomyocyte-derived TGF-β.[366] In the much more common genetic conditions associated with dilated cardiomyopathy[367] or hypertrophic cardiomyopathy,[368,369] interstitial fibrotic changes have been extensively documented and are associated with worse prognosis.[369] Transgenic mice recapitulating the human disease also exhibit substantial interstitial fibrosis.[370,371] In most monogenic cardiomyopathies, heart failure is due to primary alterations in myofilament function. Traditional concepts have suggested that interstitial cell activation and ECM perturbations may simply represent an epiphenomenon, reflecting increased hemodynamic loads, or responses to primary cardiomyocyte injury. However, recent evidence from mouse models of genetic cardiomyopathies has challenged the notion that ECM remodeling is an innocent companion. In transgenic mouse models of hypertrophic cardiomyopathy, myofibroblast-specific disruption or pharmacological inhibition of TGF-β signaling reduced myocardial fibrosis, prolonged survival, and preserved cardiac function.[372,373] These observations may suggest that activation of a fibrogenic program may exacerbate dysfunction and worsen outcome in subjects with sarcomeric mutations.

Patients with arrhythmogenic right ventricular cardiomyopathy develop arrhythmias, associated with expansion and activation of fibroadipocyte populations, ECM deposition, and fatty infiltration.[33,374–377] Experimental evidence suggests that in subsets of arrhythmogenic right ventricular cardiomyopathy patients, fibrofatty infiltration may be triggered by primary phenotypic alterations of interstitial cells. Genetic analysis has identified mutations in genes encoding desmosome proteins (including plakophilin-2, desmoplakin, and desmoglein-2) in many arrhythmogenic right ventricular cardiomyopathy patients.[378–380] In mice, loss of desmoplakin in a subset of fibroadipocyte-like interstitial cells triggered adipocyte differentiation through modulation of Wnt-dependent signaling.[381,382] To what extent these cells act by remodeling the cardiac ECM is unknown. In other cases, fibrosis may reflect activation of a fibrogenic program in cardiomyocytes.[383] Thus, the cellular basis of ECM remodeling in arrhythmogenic right ventricular cardiomyopathy remains unclear.

ECM Modulation in Heart Failure Therapeutics

Because of its important role in preserving cardiac geometry and function, its ubiquitous presence in the microenvironment of all cell types, and its crucial involvement in regulation of cellular responses in myocardial remodeling, the ECM is an attractive therapeutic target in heart failure. In fact, some of the established therapeutic approaches for heart failure patients may act, at least in part by targeting the ECM. ACE inhibition, AT1R blockade, β-adrenergic receptor antagonism, and mechanical unloading modulate deposition and metabolism of the ECM in human patients with heart failure.[341,384] Whether the effects of these interventions on the ECM are causally implicated in mediating their protective actions remains unknown. Emerging concepts suggest that targeting the cardiac ECM may hold promise to prevent adverse remodeling in both

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ischemic and nonischemic cardiomyopathy, and to attenuate diastolic dysfunction in HFpEF patients. Moreover, some experimental studies have suggested that the cardiac ECM may hold the key to the visionary goal of myocardial regeneration.

ECM Modulation to Preserve Geometry and Function of the Failing Infarcted Heart

In the remodeling infarcted heart, tight regulation of ECM deposition and cross-linking may protect from dilative remodeling and from the development of heart failure, while preventing overactive fibrosis and diastolic dysfunction. Experimental evidence suggests that an optimal balance of matrix-synthetic and matrix-degrading pathways is needed. Excessive accumulation of cross-linked collagenous ECM increases myocardial stiffness and promotes diastolic dysfunction. On the contrary, overactivation of MMP-driven proteolysis may promote dilative remodeling, causing systolic dysfunction. The major therapeutic challenge for implementation is to achieve the optimal balance of matrix metabolism for every patient at risk for postinfarction heart failure. Considering the pathophysiological heterogeneity of postinfarction remodeling, use of biomarkers and imaging strategies to measure ECM composition and metabolism is needed to stratify the patients[385] and to identify candidates for specific interventions. Individuals with overactive profibrotic responses may benefit from antifibrotic strategies targeting ECM deposition or cross-linking. On the contrary, other patients may exhibit a predominant proteolytic environment in the infarcted heart and may require strategies that restore the ECM network increasing tensile strength of the infarct. Findings suggesting age-associated defects in growth factor responses and in ECM deposition following MI[386] may suggest that in the elderly, measures to improve the reparative reserve may be more appropriate.

Targeting the Cardiac ECM in HFpEF

The association between collagen deposition and myocardial stiffness in HFpEF patients is well-established.[387] Although inhibition of cardiac fibrosis seems an attractive therapeutic approach for HFpEF patients, implementation is hampered by several concerns. First, in contrast to fibrotic conditions in other systems (such as idiopathic pulmonary fibrosis or scleroderma), cardiac fibrosis is not a primary pathological condition and often reflects activation of a reparative program in an organ that lacks effective regenerative capacity. It is difficult to establish a causative relation between ECM deposition and adverse outcome in human patients with HFpEF. Although fibrosis changes in HFpEF have clear and consistent adverse prognostic implications, these associations may represent an epiphenomenon, reflecting worse outcome in subjects with worse primary cardiomyocyte injury. Interstitial cells are heterogeneous and have a wide range of functions. The reparative actions of cardiac myofibroblasts are obvious in MI[60]; however, even in models of noninfarctive cardiac fibrosis that may recapitulate some features of human HFpEF, protective actions of cardiac myofibroblasts have been demonstrated.[30] Second, human HFpEF is pathophysiologically heterogeneous. Extensive biomarker-driven stratification may be required to identify subjects exhibiting overactive fibrotic responses that may be responsible for dysfunction and heart failure.[388] Third, the reparative functions of fibroblasts and the need to preserve and repair injury-related perturbations of the ECM network may limit chronic implementation of antifibrotic strategies. Even if patient subsets with inappropriate chronic myocardial ECM deposition are identified, the need for continuous administration of agents that attenuate fibroblast activation may carry significant risks, by abrogating essential reparative responses. Ultimately, success or failure of antifibrotic strategies in the heart will need to be tested in carefully designed clinical investigations.

Matricellular Targets in Failing Hearts

The biology of the matricellular proteins may suggest attractive strategies to modulate adverse remodeling in failing hearts. Experimental studies have demonstrated that members of the matricellular family (such as TSP-1 and SPARC) may protect the failing heart from adverse remodeling and dysfunction.[185,199,202,237] Matricellular proteins are multidomain molecules that regulate cellular responses through outside-in signaling. Thus, identification of specific domains responsible for the protective effects of matricellular proteins could lead to development of peptide-based approaches that reproduce these effects and localize protective signaling in the area of interest. Unfortunately, therapeutic implementation of such approaches is hampered by the daunting complexity and contextual actions of matricellular proteins that may be dependent on the profile of the interstitial cellular infiltrate, the composition of the ECM, and the cytokine and growth factor milieu.

Can Manipulation of the ECM Promote Cardiac Regeneration?

Despite intriguing early findings, regeneration of the adult heart remains an unfulfilled promise. In vitro studies, the role of the ECM in embryonic cardiac development, and in vivo experiments in amphibian or fish models and in neonatal mice suggest that the composition of the ECM may play a critical role in activation of a regenerative program in the myocardium. First, in vitro, ECM composition profoundly affects cell cycle entry in cardiomyocyte progenitors and in neonatal cardiomyocytes.[389,390] Second, in zebrafish, genetic ablation of matrix-producing fibroblasts or Fn loss impaired the myocardial regenerative response.[391,392] Clearly, gaining translationally relevant insights from fish models is a major challenge. Despite the abundant presence of myofibroblasts and high expression of Fn mammalian infarcts do not remusculare, suggesting that ECM- and fibroblast-dependent actions may not be sufficient to activate a regenerative program. Third, some experimental studies in mammals have suggested that matricellular macromolecules may trigger proliferation of neonatal and even adult cardiomyocytes. Agrin, a large extracellular heparan sulfate proteoglycan, has been suggested to serve as an essential component of the regenerative neonatal ECM, promoting cardiomyocyte cell cycle reentry in both neonatal and adult mice.[393] Moreover, periostin triggered cell cycle reentry in differentiated adult cardiomyocytes in an integrin-dependent manner, and when administered as an epicardial gelfoam patch following infarction, improved cardiac function, and attenuated cardiac remodeling.[280] Considering

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known biology on the challenges and complexities of myocardial regeneration, the notion that a single matrix macromolecule can remuscularize the adult mammalian heart is not plausible. For example, the idea that periostin can regenerate the heart is challenged by the abundance of periostin in the infarcted mouse myocardium in the absence of regeneration, and by the lack of regenerative capacity in infarcted periostin-overexpressing mice.[282] Despite some promising early findings, whether modulation of the ECM profile in failing hearts could be used to facilitate activation of a regenerative program remains unknown. Clearly, any attempt to remuscularize the heart would require a matrix environment that supports proliferating cardiomyocytes, or cardiomyocyte progenitors, and promotes syncytial function. Studies demonstrating the role of YAP (Yes-associated protein) signaling in neonatal heart regeneration highlight the requirement for molecular links between the ECM and the cardiac cytoskeleton.[394,395] However, a regenerative ECM profile is unlikely to be sufficient to trigger formation of new myocardium, a process that may also require activation of an endogenous regenerative program by cardiomyocytes and vascular cells.

Matrix-Driven Strategies to Engineer a Bioartificial Heart

Recent technological advances in perfusion-decellularization strategies have generated decellularized cardiac matrices that may serve as scaffold to engineer cardiac grafts.[396–398] Subsequently, the matrices can be repopulated with induced pluripotent stem cell-derived cardiomyocytes to generate structures resembling human myocardium. This technology is at an early stage. Although tissue constructs with definitive sarcomeric structure, contractile function and electrical conduction have been generated,[399] several major challenges remain that currently preclude clinical translation. Coagulation dysfunction due to incomplete re-endothelialization and subsequent activation of the coagulation system, the absence of functional valvular structures, the lack of a conduction system for transmission of electrical activity, and the potential for immunogenicity and inflammatory activation are major limiting factors of this intriguing strategy. Despite these challenging problems, this approach may represent the first step towards the generation of functional bioartificial cardiac grafts.

Conclusions

The myocardial ECM network contributes to cardiac homeostasis, not only by providing structural support, but also by facilitating force transmission and by transducing molecular signals that regulate cell phenotype and function. In failing hearts, expansion of the cardiac interstitium, induction of both structural and matricellular ECM proteins, generation of bioactive matrix fragments, and alterations in matrix biochemistry may play a critical role in regulating inflammatory, reparative, fibrotic, angiogenic, and even regenerative responses. Thus, the cardiac ECM is a key regulator of cardiac remodeling both through effects of its structural constituents on the mechanical properties of the heart and through regulation of cellular responses. Dissecting the mechanistic contributions of ECM macromolecules in cardiac remodeling is critical for understanding the pathogenetic basis of heart failure in both ischemic and nonischemic cardiomyopathy. Moreover, systematic characterization of the biochemical profile of ECM proteins in patients with myocardial disease is needed to identify perturbations associated with human heart failure.

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Disclosures

None.

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STATE-OF-THE-ART REVIEW

Fibroblasts in the Infarcted, Remodeling, and Failing Heart



Claudio Humeres, PhD, Nikolaos G. Frangogiannis, MD

HIGHLIGHTS

- **Cardiac fibroblasts become activated following injury and participate in repair and remodeling of the heart.**
- **The authors discuss the phenotypic alterations and role of fibroblasts in infarcted and failing hearts.**
- **In failing hearts, fibroblasts may deposit ECM proteins, increasing myocardial stiffness, but may also exert protective and reparative actions.**
- **Future studies will focus on characterization of the phenotypic heterogeneity of cardiac fibroblasts that may explain their functional diversity.**

SUMMARY

Expansion and activation of fibroblasts following cardiac injury is important for repair but may also contribute to fibrosis, remodeling, and dysfunction. The authors discuss the dynamic alterations of fibroblasts in failing and remodeling myocardium. Emerging concepts suggest that fibroblasts are not unidimensional cells that act exclusively by secreting extracellular matrix proteins, thus promoting fibrosis and diastolic dysfunction. In addition to their involvement in extracellular matrix expansion, activated fibroblasts may also exert protective actions, preserving the cardiac extracellular matrix, transducing survival signals to cardiomyocytes, and regulating inflammation and angiogenesis. The functional diversity of cardiac fibroblasts may reflect their phenotypic heterogeneity. (J Am Coll Cardiol Basic Trans Science 2019;4:449–67) © 2019 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Most myocardial conditions are associated with "fibrosis," expansion of the cardiac interstitium, due to deposition of extracellular matrix (ECM) proteins (1-3). In human patients with a wide variety of cardiac diseases, the extent of fibrotic changes is a strong predictor of adverse outcome. In patients with heart failure with reduced ejection fraction, and in those with heart failure with preserved ejection fraction, prominent fibrotic remodeling is associated with higher mortality, increased hospitalization rates, and an increased incidence of adverse cardiac events (4-7). Moreover, in subjects with diabetes, expansion of the myocardial interstitial space is associated with mortality and with heart failure hospitalizations (8). The association between fibrosis and poor prognosis may reflect the adverse functional consequences of ECM deposition on systolic and diastolic function or the proarrhythmic effects of fibrotic myocardial lesions. However, because the adult mammalian heart

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ABBREVIATIONS AND ACRONYMS

AT1 = angiotensin type 1

ECM = extracellular matrix

FAK = focal adhesion kinase

FGF = fibroblast growth factor

IL = interleukin

lncRNA = long noncoding ribonucleic acid

MAPK = mitogen-activated protein kinase

miRNA = micro-ribonucleic acid

MRTF = myocardin-related transcription factor

PDGF = platelet-derived growth factor

RNA = ribonucleic acid

ROCK = Rho-associated coiled-coil containing kinase

ROS = reactive oxygen species

SMA = smooth muscle actin

TGF = transforming growth factor

TRP = transient receptor potential

lacks significant endogenous regenerative capacity, cardiac fibrosis may also reflect activation of reparative mechanisms in response to primary cardiomyocyte injury. To what extent fibrotic cardiac remodeling represents a primary myocardial disease that mediates dysfunction and causes adverse outcome remains unknown.

Fibroblasts are the main effector cells of cardiac fibrosis. The adult mammalian heart contains abundant fibroblasts that expand following injury and can produce large amounts of ECM proteins. Animal model studies have identified cardiac fibroblasts both as critical reparative cells that maintain the structural integrity of the infarcted ventricle and as cellular effectors of heart failure that deposit stiff ECM in the interstitium, reducing myocardial compliance. The functional heterogeneity of fibroblast populations, their remarkable phenotypic plasticity, and the limited information on the characteristics and properties of fibroblasts in human myocardial diseases have hampered dissection of reparative and maladaptive fibroblast actions. In this review we describe the role of fibroblasts in failing and remodeling hearts. We discuss the dynamic alterations of fibroblasts in injury and repair of the infarcted heart and their role in remodeling and dysfunction of the ventricle in conditions associated with chronic heart failure.

FIBROBLASTS IN CARDIAC HOMEOSTASIS

Fibroblasts are defined and identified on the basis of functional and morphological criteria as cells of mesenchymal origin that lack a basement membrane and are involved in the formation and maintenance of connective tissues by producing a wide range of ECM proteins (9). Although several fibroblast markers have been proposed (**Table 1**), their specificity is limited. Moreover, considering that resident fibroblast populations in many tissues are heterogeneous (10) and undergo dynamic phenotypic changes following injury, identification of reliable markers that label all fibroblast subsets is a major challenge. Thus, characterization of fibroblasts typically requires the combined use of fibroblast-related markers (including ECM proteins that reflect their matrix-synthetic function) and exclusion criteria reflecting the absence of expression of endothelial, hematopoietic cell and vascular mural cell-specific proteins.

The myocardium contains a large population of resident fibroblasts enmeshed within the ECM network (11,12). For many years, fibroblasts were considered the most abundant noncardiomyocytes in the adult mammalian myocardium. A study using flow cytometry in adult mice identified 27% of myocardial cells as discoidin domain-containing receptor 2-positive fibroblasts and only 7% of the cells as $CD31^{+}$ endothelial cells (13), a finding quite surprising considering the high vascular content of the mammalian heart. In contrast, a more recent study using a combination of fibroblast reporter mouse models and cell-specific antibodies suggested that cardiac fibroblasts represent <20% of noncardiomyocytes and are greatly outnumbered by endothelial cells (which represent more than 60% of noncardiomyocytes) (14). Differences in the strategies used for cardiac cell isolation, and variability in the sensitivity and specificity of the methodological approaches used for cellular identification, may account, at least in part, for conflicting results in various investigations. Moreover, the relative numbers of various interstitial cell populations in the myocardium are also dependent on the age, sex, and species of the subjects studied. It should be emphasized that most of our knowledge on the characteristics of cardiac fibroblasts is based on studies in rodents, and relatively little is known regarding the density, phenotype, and distribution of fibroblasts in normal human hearts.

What is the function of resident fibroblasts in normal mammalian hearts? In the developing myocardium, cardiac fibroblasts have been suggested to regulate cardiomyocyte proliferation through a fibronectin/β_1-integrin-mediated pathway (15). In adult hearts, normal cardiac function may require interactions between cardiomyocytes and the surrounding ECM. Cardiac fibroblasts, enmeshed into the endomysium and perimysium, may play an important role in regulation of the synthesis and turnover of ECM components, thus preserving the structural integrity of the ventricle (16–18). Mice with global germline loss of transcription factor 21, which is essential for cardiac fibroblast development, had greatly decreased collagen levels in the cardiac interstitium and exhibited dysmorphic hearts that lacked a distinct apex (19). Although these findings are consistent with an important role of fibroblasts in cardiac development, the consequences of fibroblast depletion on cardiac homeostasis in adult mice have not been investigated.

In addition to their critical role in the formation of the cardiac ECM network, fibroblasts may also contribute to cellular communication in the cardiac

TABLE 1 Sensitivity and Specificity of Markers Used to Identify Cardiac Fibroblasts

Marker	Sensitivity	Specificity
Vimentin	Labels all fibroblasts (180,181).	Also expressed by other cells of mesenchymal origin (endothelial cells [182], vascular smooth muscle cells [183], etc.).
α-SMA	Expressed by activated myofibroblasts in fibrotic hearts (22,41,138). Not expressed by quiescent fibroblasts (137).	Also expressed by vascular mural cells.
Col1α1	Synthesis of structural collagens is a hallmark of fibroblasts in normal and remodeling hearts (42,141).	Although synthesis of structural collagens by cells other than fibroblasts has been reported, expression of Col1α1 in cardiac endothelial cells, immune cells, vascular smooth muscle cells, and pericytes is negligible when compared to fibroblasts (141). Because of labeling of the surrounding matrix, antibodies to collagens may be suboptimal for fibroblast identification. Col1α1-GFP reporter mice represent a robust tool for identification of fibroblasts in many organs, including the heart (42).
Periostin	Expressed by fibroblasts in neonatal hearts but not by fibroblasts in normal adult hearts (184). Highly expressed in activated cardiac fibroblasts after injury (185,186).	May also be expressed by subsets of vascular smooth muscle cells (187).
Fibronectin ED-A	Highly expressed by activated myofibroblasts (188).	Deposited in the matrix (189). May also colocalize with macrophages, endothelial cells, and other cell types (190,191).
PDGFRα	Highly expressed in cardiac fibroblasts in normal (41) and pressure-overloaded myocardium (141).	Although vascular smooth muscle cells have been reported to express PDGFRα, especially under conditions of stress (192), PDGFRα-GFP reporter lines seem to predominantly identify cells with fibroblast-like characteristics (193).
DDR2	High expression in cardiac fibroblasts in normal adult hearts (194), colocalizing with vimentin and col1α1 (195). May also be expressed in various subpopulations of infarct fibroblasts and myofibroblasts (196).	DDR2 expression has been reported in activated endothelial cells (197) and in stretched vascular smooth muscle cells (198). It is unclear whether this affects the specificity of DDR2 for fibroblasts in injured and remodeling hearts.
Antigen recognized by MEFSK4	The MEFSK4 antibody labels through flow cytometry almost all PDGFRα+, Col1α1+ cardiac fibroblasts (14). No antibodies are available for immunohistochemistry.	MEFSK4 has been reported to label a small subpopulation of pericytes (14).
Cluster of differentiation 90 (Thy1)	Identifies a subpopulation of fibroblasts in the normal and remodeling myocardium (14,141,199,200).	Also expressed by immune cells, lymphatic endothelial cells, and pericytes (201).
Sca1	Identifies a subpopulation (~60%) of PDGFRα+, Col1α1+ fibroblasts in the murine heart (14).	Lacks specificity. In Sca1-GFP reporter mice, Sca1 expression colocalized with endothelial and pericyte markers (202).
Tcf21	Labels the majority of fibroblast-like cells in normal myocardium (19). In infarcted and pressure-overloaded hearts, accumulation of Tcf21+ fibroblast-like cells is noted; however, according to a single report, Tcf21 may not label activated α-SMA+ myofibroblasts (203).	Relatively specific for fibroblast populations. Not expressed by immune cells (CD45+) (203), endothelial cells, and vascular smooth muscle cells (19).
FSP1	No expression in fibroblasts in the normal adult myocardium. In the infarcted and pressure-overloaded heart, there is a marked expansion of FSP1+ cells. The majority of these cells cannot be identified as α-SMA+ myofibroblasts (141,184).	Lacks specificity. The majority of FSP1+ cells in injured and remodeling hearts are endothelial cells, macrophages, and vascular smooth muscle cells (184,204).
FAP	Not expressed in normal cardiac fibroblasts (174). Labels many activated fibroblasts in infarcted rat hearts and in human myocardial samples from patients with post-infarction heart failure (205).	Specific for activated fibroblasts. However, in human failing hearts, FAP expression has been reported in small populations of inflammatory cells and endothelial cells (205).

Col1α1 = collagen 1α1; DDR2 = discoidin domain receptor 2; FAP = fibroblast-activation protein; FSP1 = fibroblast-specific protein 1; GFP = green fluorescent protein; PDGFRα = platelet-derived growth factor α; Sca1 = stem cell antigen-1; SMA = smooth muscle actin; Tcf21 = transcription factor 21.

syncytium. Given their strategic location in the interstitium, cardiac fibroblasts have been suggested to facilitate communication between myocardial layers (20). Cardiac fibroblasts express high levels of connexins (connexin-40, connexin-43, and connexin-45) and establish functional gap junctional channels with neighboring cardiomyocytes, modulating their electrophysiological properties (21). Thus, fibroblasts may act as electric couplers of myocytes from different regions that would normally be isolated by connective tissue, contributing to the synchronization of the contraction.

PHENOTYPIC CHANGES AND ROLE OF CARDIAC FIBROBLASTS IN THE INFARCTED MYOCARDIUM

Fibroblasts exhibit remarkable phenotypic plasticity and undergo dramatic alterations in their gene expression profile and functional properties in response to mechanical stress or to stimulation with soluble mediators. In vitro, cardiac fibroblasts cultured in the low-tension environment of a collagen-based pad have dendritic morphology, synthesize low levels of collagen, and have negligible

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JUNE 2019:449–67

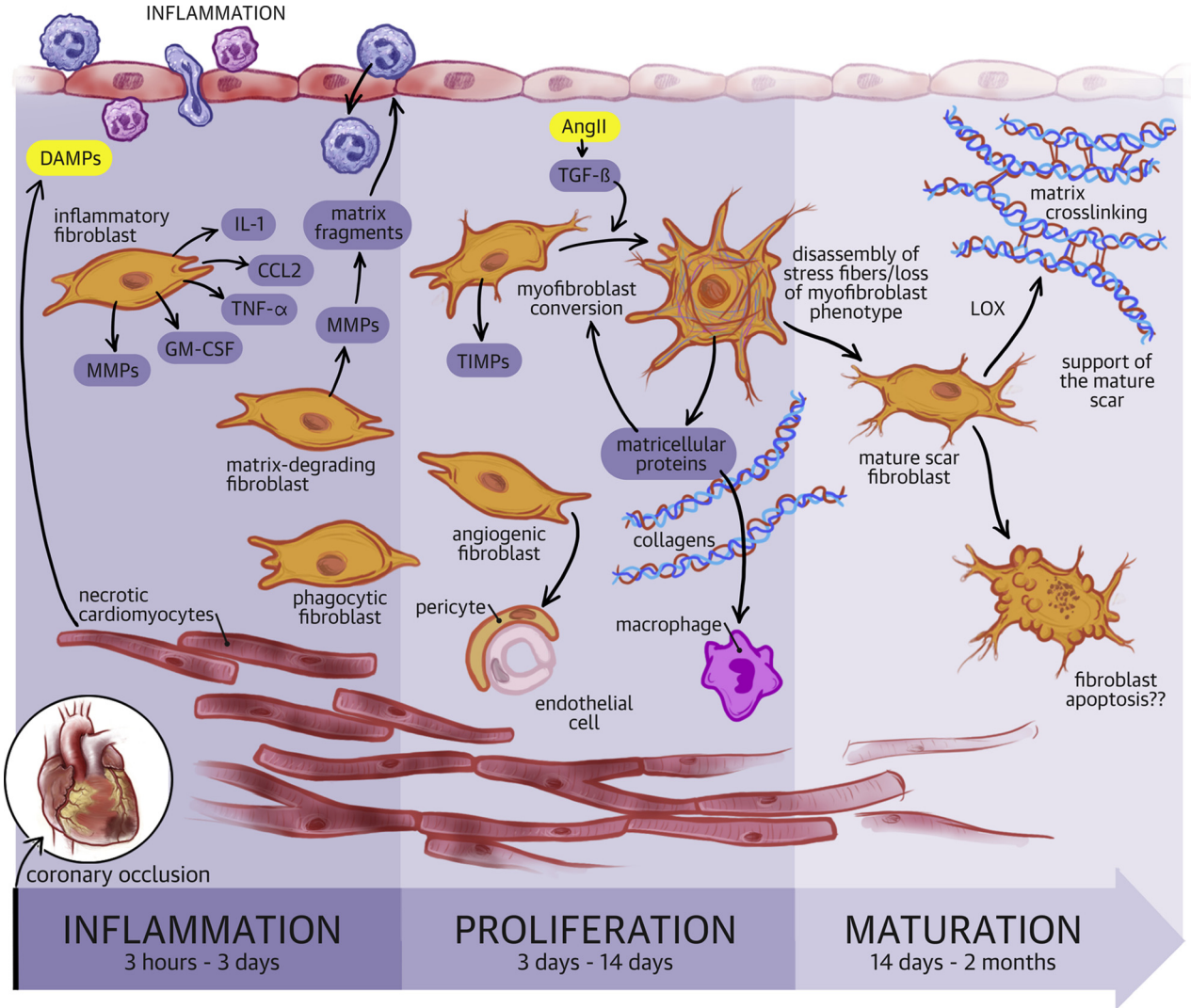
expression of myofibroblast markers, such as α-smooth muscle actin (SMA) (22). In contrast, when cultured in plates, fibroblasts undergo conversion to myofibroblasts, exhibiting activation of mechanosensitive signaling pathways that trigger incorporation of α-SMA into stress fibers and induce synthesis of ECM proteins. In vivo, cardiac fibroblasts respond to changes in their microenvironment by acquiring a wide range of phenotypic profiles, thus serving as inflammatory, matrix-synthetic, or proangiogenic cells depending on the context (**Central Illustration**).

In myocardial infarction, sudden occlusion of a coronary artery results in the death of up to 1 billion cardiomyocytes, triggering an intense inflammatory reaction (23). Because the massive loss of cardiomyocytes overwhelms the extremely limited regenerative potential of the adult mammalian heart, the infarcted myocardium heals through formation of a scar. Thus, repair of the infarcted heart is dependent on a well-orchestrated cellular response, composed of 3 distinct but overlapping phases. During the inflammatory phase, innate immune activation in response to release of damage-associated molecular patterns by dying cardiomyocytes and degraded ECM triggers cytokine and chemokine induction and recruits leukocytes that clear the infarct from necrotic and apoptotic cells and remove matrix debris (24). Macrophages phagocytosing apoptotic cells undergo transition to an anti-inflammatory phenotype, mediating suppression of inflammation and activation of a reparative program that orchestrates the proliferative phase of cardiac repair, characterized by expansion of myofibroblasts and vascular cells. The maturation phase follows and is associated with quiescence of fibroblasts, recruitment of mural cells by infarct neovessels, and formation of a crosslinked collagenous scar (25). During the 3 phases of infarct healing, cardiac fibroblasts undergo rapid phenotypic transitions from quiescence to a pro-inflammatory and matrix-degrading phenotype to a matrix-synthetic myofibroblast phenotype, only to revert to quiescence as the scar matures. Emerging evidence suggests that fibroblasts do not simply follow the changes in their microenvironment but serve as critical regulators of the cellular events in every phase of cardiac repair (26).

THE FIBROBLASTS IN THE INFLAMMATORY PHASE OF INFARCT HEALING. Fibroblasts are capable of producing large amounts of proinflammatory cytokines and chemokines in response to stimulation with reactive oxygen species (ROS), Toll-like receptor ligands, or interleukin (IL)-1β (27–29). During the early post-ischemic phase, interstitial fibroblasts may sense damage-associated molecular patterns released by dying cardiomyocytes, activating a proinflammatory program (**Figure 1**). Considering that several other cell types, including cardiomyocytes, endothelial cells, immune cells, and vascular mural cells, can also secrete proinflammatory mediators (30), the relative role of resident cardiac fibroblasts as inflammatory cells remains unclear. In vivo studies have suggested that infarct fibroblasts may exhibit activation of the NRLP3 inflammasome (31,32), thus serving as an important source of active IL-1β, a critical proinflammatory cytokine in the infarcted myocardium (33). A recent study suggested that fibroblasts may stimulate leukocyte recruitment in the infarcted myocardium by secreting large amounts of granulocyte/macrophage colony-stimulating factor (34). To what extent proinflammatory fibroblasts also contribute other chemokines or cytokines to the infarct environment remains unknown. Cytokine-activated proinflammatory fibroblasts also secrete proteases that play an important role in clearance of the infarct from matrix debris (35). Associative data have suggested that in addition to their role as proinflammatory and matrix-degrading cells, fibroblasts may protect cardiomyocytes from ischemic injury (36). The molecular signals responsible for the prosurvival actions are unclear.

THE ROLE OF FIBROBLASTS IN THE PROLIFERATIVE PHASE OF INFARCT HEALING. The potential role of infarct fibroblasts in phagocytosis and suppression of inflammation. Activation of the post-infarction inflammatory reaction is followed by rapid suppression of proinflammatory gene synthesis and subsequent resolution of the leukocytic infiltrate, marking the transition to the proliferative phase of infarct healing. Phagocytosis of apoptotic cells plays a key role in downmodulation of inflammation, stimulating release of anti-inflammatory signals, such as IL-10 and transforming growth factor (TGF)-β. To what extent fibroblasts participate in repression and resolution of post-infarction inflammation remains unknown. A recent study suggested that activated fibroblasts may serve as phagocytes, engulfing apoptotic cells from the infarct zone (37). Considering the abundance of phagocytic macrophages in the healing infarct (38), the relative contribution of fibroblasts in clearance of dead cells is unclear. Whether any phagocytic actions of fibroblasts are accompanied by secretion of IL-10 or TGF-β and by acquisition of an anti-inflammatory phenotype has not been investigated.

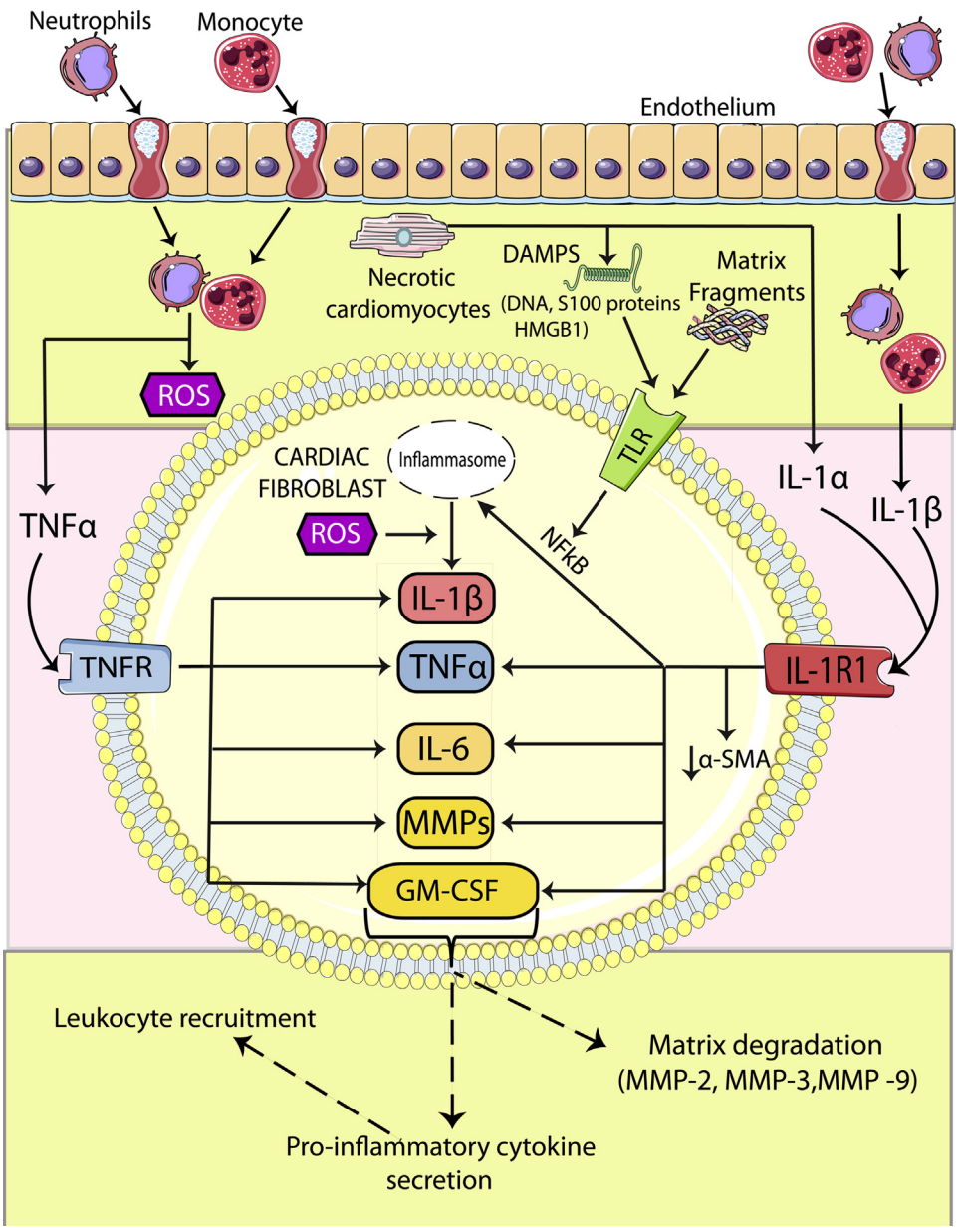
CENTRAL ILLUSTRATION **Functional Diversity of Fibroblasts in the Infarcted Myocardium**



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In the dynamic environment of the infarcted heart, cardiac fibroblasts expand, undergo phenotypic changes, and are implicated in a wide range of functions. Coronary occlusion causes death of cardiomyocytes in the area of injury. During the inflammatory phase of infarct healing, Damage-Associated Molecular Patterns (DAMPs) released by dying cells activate a pro-inflammatory phenotype in cardiac fibroblasts that secrete cytokines (such as IL-1, TNF-α, and GM-CSF), and chemokines (such as CCL2) contributing to recruitment and activation of leukocytes. Cytokine-stimulated fibroblasts also secrete matrix metalloproteinases (MMPs), promoting extracellular matrix degradation and release of pro-inflammatory matrix fragments. Some studies have suggested that infarct fibroblasts may also function as phagocytic cells; however, considering the abundance of macrophages in the healing infarct the relative contribution of "phagocytic fibroblasts" remains unclear. Clearance of the infarcted heart from dead cells stimulates anti-inflammatory signals, leading to suppression of inflammation and transition to the proliferative phase of infarct healing. Fibroblasts expand, predominantly through recruitment of resident populations and undergo myofibroblast conversion, incorporating α-SMA into cytoskeletal stress fibers. Activated myofibroblasts are the main matrix-synthetic cells in the infarcted heart and produce both structural extracellular matrix proteins and matricellular macromolecules. In addition to their contribution in matrix production, fibroblast populations may also contribute to regulation of the angiogenic response and may regulate macrophage phenotype. During scar maturation fibroblasts exhibit disassembly of α-SMA-decorated stress fibers, and may produce matrix-crosslinking enzymes such as lysyl-oxidases (LOX). Reduction of fibroblast numbers in mature scars has been suggested to involve activation of apoptosis. The molecular basis for the phenotypic transitions of cardiac fibroblasts in the phases of infarct healing remains poorly understood. The functional diversity of fibroblasts in the infarcted heart may reflect sequential activation of distinct fibroblast subpopulations, or may result from coordinated responses of the fibroblasts to the dynamic changes in their microenvironment.

FIGURE 1 Fibroblasts in the Inflammatory Phase of Infarct Healing



During the inflammatory phase of infarct healing, cardiac fibroblasts secrete proinflammatory mediators and matrix-degrading proteases. Damage-associated molecular patterns (DAMPs) released by necrotic cells and matrix fragments activate Toll-like receptor signaling in cardiac fibroblasts. Proinflammatory cytokines (such as interleukin [IL]-1β and tumor necrosis factor [TNF]-α) released by endothelial cells, immune cells, and cardiomyocytes and activation of reactive oxygen species (ROS) accentuate fibroblast inflammatory activity. IL-1/IL-1RI signaling has been suggested to reduce α-smooth muscle actin (α-SMA) expression, preventing myofibroblast conversion. Cytokines and chemokines (such as IL-1β, TNF-α, IL-6, and granulocyte/macrophage colony-stimulating factor [GM-CSF]) secreted by activated fibroblasts may contribute to the recruitment of leukocytes, whereas protease release may promote matrix degradation. Considering that several other cell types are capable of secreting inflammatory mediators, the relative contribution of fibroblasts is unclear. The cartoon was designed using Servier Medical Art (https://smart.servier.com). DNA = deoxyribonucleic acid; HMGB1 = high-mobility group protein B1; MMP = matrix metalloproteinase; TNFR = tumor necrosis factor receptor.

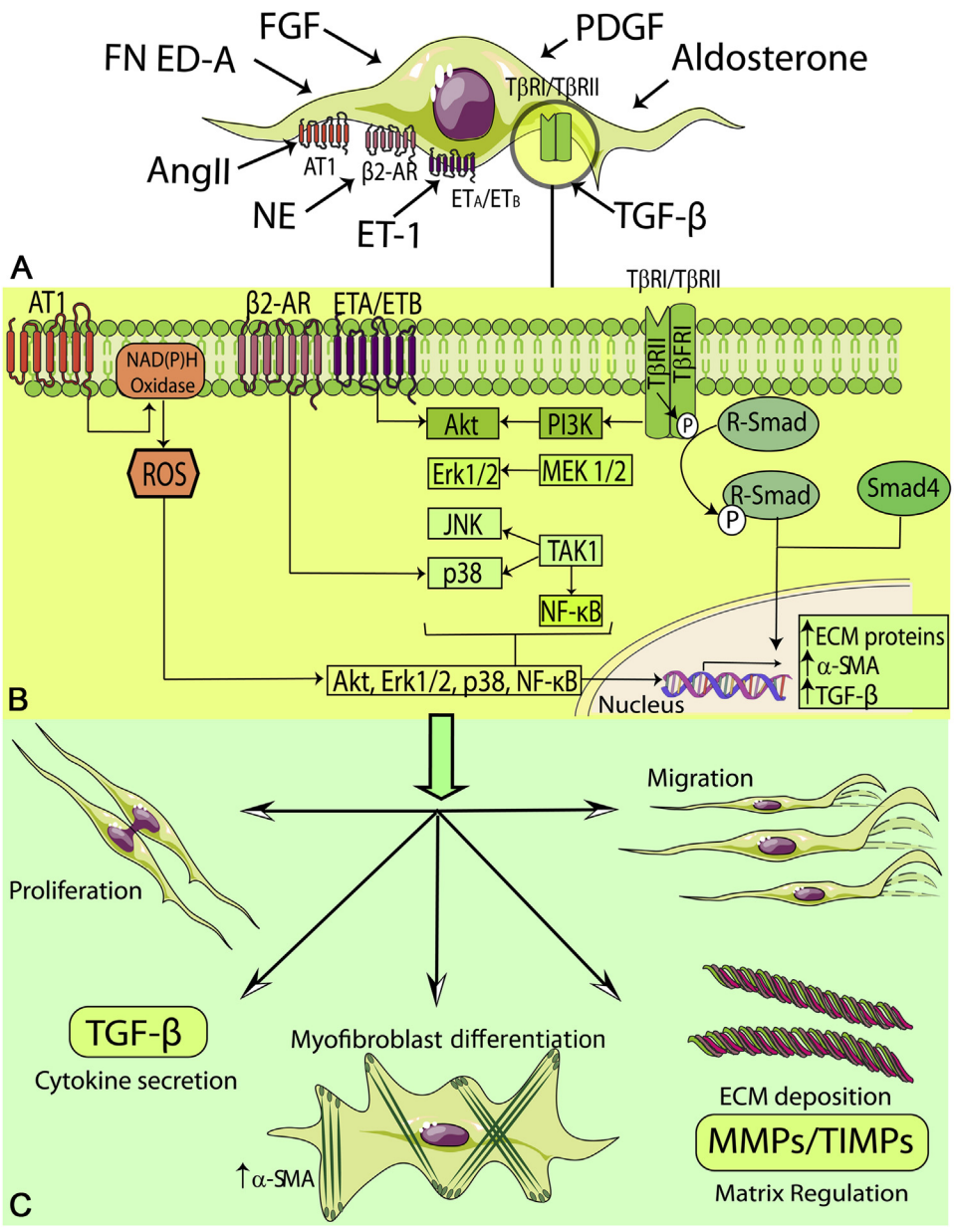
TABLE 2 Cellular Origin of Fibroblasts in Myocardial Infarction

Reference #	Main Conclusions of the Study	Strategies Used to Study the Cellular Origin of Infarct Fibroblasts	Markers Used for Fibroblast Identification
(41)	Activated fibroblasts in infarcted and remodeling hearts are derived from Tcf21^{+} tissue-resident fibroblasts. Endothelial cells, myeloid cells, and smooth muscle cells do not significantly contribute to the activated fibroblast population.	Lineage-tracing analysis using Cre drivers to study the fate of resident cardiac fibroblasts (Tcf21^{MCM}), activated myofibroblasts (Postn^{MCM}), myeloid cells (LysM^{Cre}), endothelial cells (Cdh5^{Cre}), and vascular smooth muscle cells (Myh11^{CreERT2}).	Vimentin, PDGFRα, α-SMA, FSP1
(137)	Resident Tcf21^{+} cardiac fibroblasts become activated and proliferative within 2–4 days after nonreperfused infarction, then undergo myofibroblast conversion, secreting large amounts of ECM proteins. Finally in mature scars, fibroblasts show reduced expression of α-SMA and express tendon genes.	The fate of fibroblasts was studied using 3 different lineage-tracing models: Tcf21$^{MCM/+}$ (resident cardiac fibroblasts), *Postn*-^{MCM} (activated fibroblasts), and *Acta2*-^{CreERT2} (activated myofibroblasts).	Vimentin, α-SMA
(206)	Epicardial-derived resident mesenchymal cells, not bone marrow cells, are the main source of fibroblasts in the infarcted heart.	WT1Cre mice were used for permanent genetic tracing of epicardium-derived cells. Mice reconstituted with RFP^{+} bone marrow cells were used to study bone marrow origin.	Collagen I, FSP1, DDR2, CD90, α-SMA
(207)	Following nonreperfused infarction, subsets of epicardium-derived cells differentiate into fibroblasts and smooth muscle cells.	Lineage tracing of epicardium derived cells by using inducible WT1^{CreERT2} mice.	FSP1, procollagen I, collagen III, fibronectin, α-SMA
(42)	The vast majority of activated collagen-producing fibroblasts (~96%) in nonreperfused infarcts are derived from epicardial cells. Hematopoietic, bone marrow lineages, and endothelial cells do not significantly contribute to the fibroblast population.	Lineage-tracing models to label epicardial cells (Wt1-Cre), endothelial cells (Tie2-Cre), hematopoietic cells (Vav-Cre). Transplantation with RFP^{+} bone marrow to study bone marrow origin.	Breeding with collagen 1α1-GFP reporter mice, α-SMA
(39)	Post-infarction, 35%–40% of α-SMA^{+} mesenchymal cells are derived from endothelial cells, possibly through endothelial-to-mesenchymal transition.	The endothelial cell-specific endothelial-SCL^{CreERT} mouse line was used to trace endothelial cells.	α-SMA expression, Snail, FSP1, vimentin and collagen I mRNA expression
(208)	24% of myofibroblasts in nonreperfused myocardial infarcts originate from bone marrow cells.	Transplantation with EGFP-tagged bone marrow, or bone marrow from proCol1α2 gene-driven luciferase or β-Gal reporter mice.	αSMA staining, β galactosidase activity in pro-Col1α2-driven chimeric mice
(40)	25% of vimentin^{+} fibroblasts and 57% of α-SMA^{+} myofibroblasts in nonreperfused infarcts are derived from bone marrow cells.	Transplantation with bone marrow from EGFP reporter mice to document bone marrow origin.	α-SMA, vimentin
(209)	Blood-derived cells contributed to the myofibroblast population. Treatment with G-CSF enhances recruitment of bone marrow–derived myofibroblasts.	Transplantation of GFP^{+} bone marrow.	Vimentin, α-SMA
(210)	Gli-1^{+} perivascular cells contribute to the myofibroblast population in the infarcted myocardium (approximately 60% of activated myofibroblasts are derived from Gli1^{+} cells).	Lineage tracing using Gli1^{CreERT2} mice.	Collagen I, PDGFRα, α-SMA

ECM = extracellular matrix; EGFP = enhanced green fluorescent protein; G-CSF = granulocyte-colony stimulating factor; mRNA = messenger ribonucleic acid; other abbreviations as in Table 1.

Expansion of activated fibroblasts in the infarcted myocardium. Expansion of cardiac fibroblasts and acquisition of a matrix-synthetic myofibroblast phenotype are prominent features of the proliferative phase of infarct healing. In addition to the abundant resident cardiac fibroblasts that can respond to activating signals, several other cell types have been proposed as important cellular sources for the expanding infarct myofibroblast population. Endothelial cells can undergo endothelial-to-mesenchymal transition in response to growth factor stimulation, acquiring a matrix-synthetic phenotype. Hematopoietic fibroblast progenitors can also contribute to the expansion of activated fibroblasts in injury sites. Pericytes and vascular smooth muscle cells can undergo fibroblast conversion, contributing to fibrotic responses. Over the past 10 years, studies combining bone marrow transplantation experiments, parabiosis, and lineage tracing strategies have attempted to explore the cellular origin of fibroblasts in the infarcted heart (Table 2). Although earlier investigations had suggested important contributions of endothelial cells and hematopoietic progenitors to the infarct myofibroblast population (39,40), recent studies using lineage-tracing approaches with several different Cre drivers demonstrated that resident cardiac fibroblasts are the main source for activated myofibroblasts in the infarcted heart, with much smaller contributions of endothelial and hematopoietic cells (41,42). It should be emphasized that the studies investigating the origin of infarct myofibroblasts have several limitations that may explain, at least in part, conflicting findings (43). First, the use of nonspecific fibroblast markers or Cre drivers with questionable specificity may limit the reliability of the findings. For example, some of the studies suggesting major contributions of endothelial cells to the myofibroblast population

FIGURE 2 Fibroblasts in the Proliferative Phase of Infarct Healing



During the proliferative phase of infarct healing, fibrogenic growth factors and neurohumoral mediators trigger myofibroblast conversion and stimulate fibroblast proliferation, migration, and activation. A wide range of fibrogenic mediators, induced during the proliferative phase of cardiac repair, are implicated in myofibroblast activation. Neurohumoral mediators, such as angiotensin II (AngII), aldosterone, and norepinephrine (NE), growth factors (transforming growth factor [TGF]-βs, fibroblast growth factors [FGFs], platelet-derived growth factors [PDGFs]), and specialized matrix proteins, such as ED-A fibronectin and matricellular proteins cooperate to activate intracellular signaling pathways that promote myofibroblast conversion and proliferation and modulate expression of extracellular matrix (ECM) proteins and of genes associated with matrix metabolism. The cartoon was designed using Servier Medical Art (https://smart.servier.com). AR = adrenergic receptor; ET = endothelin; MMP = matrix metalloproteinase; NF = nuclear factor; ROS = reactive oxygen species; SMA = smooth muscle actin; TIMP = tissue inhibitor of metalloproteinase.

were based on nonspecific Cre drivers (such as the Tie1-Cre line) (44). Identification of fibroblasts represents another major challenge due to the absence of specific markers (**Table 1**). Thus, in many cases, conclusions regarding conversion of other lineages into fibroblasts are based on immunofluorescence data showing expression of nonspecific markers, such as fibroblast-specific protein-1 or α-SMA (40,44).

Second, the timing of reperfusion may have dramatic effects on the fate of resident myocardial cells and on recruitment of blood-derived progenitors, thus altering the relative contribution of various cell types to the expansion and activation of fibroblasts. Early reperfusion results in accentuated and accelerated leukocyte influx and could also augment infiltration of the infarct zone with bone marrow–derived fibroblast progenitors. Prolonged coronary occlusion, in contrast, may cause ischemic death of large numbers of interstitial and vascular cells in the infarct zone, thus reducing their relative contribution to myofibroblast expansion.

Third, considering that all lineage-tracing studies were performed in mouse models, there is practically no information on the origin of myofibroblasts in human myocardial infarction.

Myofibroblast migration in the border zone of the infarct. In the healing infarct, fibroblasts undergo conversion to myofibroblasts, expressing contractile proteins, such as α-SMA and the embryonic isoform of smooth muscle myosin heavy chain, synthesizing periostin, and secreting large amounts of ECM proteins (22,45). In animal models of myocardial infarction, myofibroblasts are localized predominantly in the border zone, forming well-organized arrays (46). Fibroblast migration to the infarct border zone may be mediated by growth factors, such as TGF-β and fibroblast growth factors (FGFs) (47,48), and by proinflammatory cytokines, such as IL-1β, tumor necrosis factor-α, and cardiotrophin-1 (27,49). It has also been suggested that chemokines, such as monocyte chemoattractant protein-1/C-C motif chemokine ligand 2, may promote the migration of bone marrow–derived fibroblast progenitors in injured tissues. Considering the robust evidence documenting no significant contribution of hematopoietic cells on infarct fibroblast populations (42), the potential significance of this mechanism is unclear. C-C motif chemokine ligand 2 may contribute to fibrosis through recruitment and activation of fibrogenic monocytes and macrophages (50,51) rather than through recruitment of circulating fibroblast progenitors or modulation of fibroblast function. A recently published investigation identified a subpopulation of atypical monocytes with a critical contribution in bleomycin-induced pulmonary fibrosis (52). Whether fibrogenic monocyte subsets with distinct phenotypic profiles are recruited in remodeling hearts has not been investigated. Other members of the chemokine family, such as the CXC chemokine interferon-γ–inducible protein-10/CXCL10, may inhibit fibroblast migration, serving as an endogenous inhibitory signal that restrains the fibrotic response following injury (53,54).

Fibroblast migration is dependent on the continuous formation and disruption of adhesive interactions between fibroblast surface proteins and the surrounding cardiac ECM. Migration involves well-orchestrated activation of integrins on cardiac fibroblast cytoplasmic membrane (55), linked with the production of proteases that degrade the matrix (56) and expression of specialized matrix proteins that locally activate or transduce growth factor–mediated signals (57).

The effects of neurohumoral pathways on activation of infarct myofibroblasts. After migrating to the infarct border zone, fibroblasts acquire a proliferative matrix-synthetic phenotype through the local induction of fibrogenic mediators (**Figure 2**). Neurohumoral pathways are critically implicated in regulation of fibroblast function following myocardial infarction. Potent activation of the renin-angiotensin-aldosterone system in infarcted hearts (58) stimulates myofibroblast conversion, proliferation, and ECM protein synthesis both through direct actions, and via induction of TGF-β (59,60). The fibrogenic actions of angiotensin II are mediated predominantly through engagement of the angiotensin type 1 (AT1) receptor (61–64). In contrast, the AT2 receptor may exert inhibitory functions, suppressing fibroblast proliferation and ECM synthesis (65), and has been suggested to restrain profibrotic signaling (66). Although extensive in vivo evidence supports the profibrotic actions of AT1 signaling in experimental models of myocardial infarction (67), to what extent the prosurvival effects of angiotensin-converting enzyme inhibition and AT1 blockade in patients with acute myocardial infarction are mediated through attenuation of angiotensin-induced fibrosis remains unknown.

Both animal model studies and investigations in human patients suggest that aldosterone contributes to myocardial fibrosis (68). Mineralocorticoid receptor inhibition attenuated fibrosis in experimental models of nonreperfused myocardial infarction (69) and reduced levels of biomarkers reflecting collagen synthesis in patients with acute myocardial infarction (70). The cellular basis for these effects remains unclear. Aldosterone-mediated signaling has been

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suggested to modulate the phenotype of all cells involved in cardiac repair, driving macrophages toward a fibrogenic phenotype (71), activating T cells (72), inducing cardiomyocyte-derived fibrogenic signals (73), and directly stimulating fibroblast proliferation and collagen synthesis (74,75).

The adrenergic system is also prominently activated following myocardial infarction. Stimulation of β_2-adrenergic receptor signaling directly stimulates proliferation of cardiac fibroblasts through effects that may involve p38 mitogen-activated protein kinase (MAPK) signaling (76–78). Chronic pharmacological stimulation or transgenic overexpression of β-adrenergic receptor causes myocardial fibrosis (79); whether fibrotic remodeling is due to direct activation of fibroblasts or reflects reparative fibrosis in response to cardiomyocyte death remains unknown. Activation of G protein-coupled receptor kinase 2 in cardiac fibroblasts may transduce, at least in part, the fibrogenic actions of β-adrenergic receptors in the infarcted myocardium (80,81).

The role of TGF-βs in fibroblast activation. The fibrogenic growth factor TGF-β is a central mediator in myofibroblast conversion following myocardial infarction. All 3 TGF-β isoforms are markedly upregulated in the infarcted heart; TGF-β1 and TGF-β2 are induced earlier, whereas TGF-β3 exhibits a late peak and a prolonged time course of expression (82). Whether TGF-β isoforms play distinct roles following infarction remains unknown. Most myocardial cell types are capable of secreting TGF-β as an inactive complex bound to the latency-associated peptide (forming the small latent complex), and latent TGF-β-binding protein (forming the large latent complex). Several mediators, including ROS, cell surface integrins, proteases, and matricellular proteins (such as thrombospondin-1), have been implicated in generation of active TGF-β in the healing infarct (83–87). The active TGF-β dimer binds and sequentially transphosphorylates type II and type I TGF-β receptors, activating downstream canonical signaling pathways through receptor-activated Smad proteins (R-Smads-Smad2/3) and Smad-independent pathways (88). Both Smad-dependent and non-Smad pathways have been implicated in α-SMA and ECM protein upregulation, triggering myofibroblast conversion and activation in healing myocardial infarction (89). In the infarcted heart, activation of Smad3-dependent signaling in cardiac fibroblasts plays a crucial role in formation of well-organized fibroblast arrays in the infarct border zone by inducing integrin expression (90).

Endothelin-1. The endothelium-derived peptide endothelin-1 is a potent vasoconstrictor but has also been reported to exert fibroblast-activating effects. Endothelin-1 secreted by TGF-β- or angiotensin II-stimulated endothelial cells may stimulate fibroblast proliferation, myofibroblast conversion, and ECM synthesis through activation of the endothelin-A receptor and downstream Rac/PI3K/Akt signaling pathways (91). In vivo, cardiac-specific endothelin-1 overexpression caused myocardial fibrosis associated with biventricular systolic and diastolic dysfunction (92), whereas endothelin antagonism attenuated adverse fibrotic remodeling following myocardial infarction (93).

The role of FGFs and platelet-derived growth factors in the activation of infarct fibroblasts. FGF2 may stimulate a proliferative phenotype in infarct fibroblasts through activation of p38 MAPK and protein kinase Cδ signaling pathways (94). In vivo, FGF2-knockout mice had reduced proliferation of infarct fibroblasts, associated with decreased ECM synthesis. These defects resulted in impaired scar formation and infarct expansion. In contrast, FGF2 overexpression increased fibroblast proliferation and accentuated ECM deposition (95). PDGFs and PDGF receptors (PDGFRs) are also overexpressed in the infarcted myocardium and may play role in regulation of fibroblast function (96). Activation of PDGFRα signaling may promote fibroblast activation; in contrast, PDGFRβ actions are important for maturation of the infarct vasculature. In vitro, PDGF-AA potently stimulates cardiac fibroblast proliferation and ECM protein synthesis (97). In vivo, PDGFRα and PDGFRβ neutralization reduced collagen deposition in reperfused myocardial infarcts (96); however, PDGFRβ inhibition also prevented the recruitment of mural cells by infarct neovessels, perturbing maturation of the infarct vasculature (96).

The role of specialized ECM proteins in the regulation of infarct fibroblast phenotype. Tissue injury is associated with induction of specialized matricellular proteins that do not play a primary structural role but regulate cellular responses by transducing or modulating signaling cascades. Fibroblasts are important cellular targets of specialized ECM proteins. The ED-A domain of fibronectin plays an important role in conversion of fibroblasts into myofibroblasts (98–100). Moreover, several matricellular macromolecules, including thrombospondins (87), osteopontin (101), tenascin-C (102), secreted protein acidic and rich in cysteine (103), periostin (104), and osteoglycin (105), have been implicated in activation of myofibroblasts in healing infarcts. Nonfibrillar collagens, such as collagen VI, are also involved in the activation of a myofibroblast phenotype following myocardial infarction (106). Most

specialized matrix proteins act by binding to fibroblast surface molecules, such as integrins and syndecans, or by modulating activity of growth factors and proteases.

Intracellular molecular pathways involved in fibroblast activation. In the healing infarct, induction of fibrogenic stimuli, such as damage-associated molecular patterns, cytokines and growth factors, neurohumoral mediators, and matricellular proteins cooperate to stimulate intracellular cascades involved in myofibroblast conversion, migration, proliferation, and induction of a matrix-synthetic transcriptional program (107). Experimental studies have identified several essential intracellular pathways that contribute to fibroblast activation.

The ROS system acts as a common effector of many fibrogenic signals (108). Angiotensin II activates downstream ROS-sensitive kinases (109) and stimulates collagen synthesis through ROS generation (110). Aldosterone-induced fibroblast activation is mediated, at least in part, through oxidative stress (111). Moreover, extensive evidence suggests that ROS mediate the fibrogenic actions of TGF-β and critically regulate matrix metabolism by modulating synthesis and activity of proteases involved in ECM degradation (112).

Ca^{2+} oscillations have also been implicated in the regulation of fibroblast phenotype and function (113). Angiotensin II or TGF-β may induce fibrogenic actions, at least in part through activation of members of the transient receptor potential (TRP) family of cationic channels. In cardiac fibroblasts, the calcium channel TRPC6 has been implicated in myofibroblast conversion by activating a calcineurin–nuclear factor of activated T cells cascade (114,115).

MAPKs exhibit a broad range of functions in many different cellular responses, including cell proliferation, survival, migration, and differentiation. Both in vitro and in vivo studies suggest an important role for MAPK signaling pathways in fibroblast activation. Fibroblast-specific loss-of-function approaches suggested that activation of p38α MAPK, the major isoform expressed in cardiac fibroblasts (116), promotes myofibroblast conversion following infarction through activation of the transcription factor serum response factor and the signaling effector calcineurin (78,117). The serum response factor/myocardin-related transcription factor (MRTF) axis plays a dominant role in regulation of α-SMA transcription and subsequent myofibroblast conversion (118,119). In vivo, mice with global loss of MRTF-A had attenuated fibrosis following myocardial infarction (120). 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 (121,122).

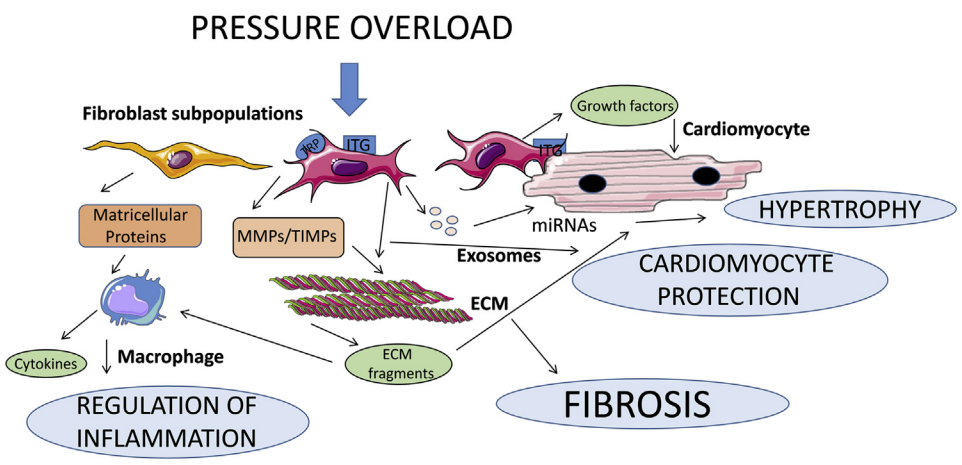
Noncoding ribonucleic acids in regulation of infarct fibroblasts. A growing body of evidence demonstrates that noncoding ribonucleic acids (RNAs), including small noncoding microRNA (miRNAs) and long noncoding RNA (lncRNA), may be implicated in the regulation of fibroblast activity in the infarcted heart (123,124). MiRNAs may act by modulating several profibrotic target pathways, including the TGF-β/Smad system, angiotensin II/MAPK signaling, the RhoA/Rho-associated coiled-coil containing kinase (ROCK) cascade, the MRTF/serum response factor axis, and the cationic channels regulating calcium responses (125). Several miRNAs, such as miR-29 and miR-101, function as negative regulators of cardiac fibroblasts; repression of these miRNAs by fibrogenic stimuli, such as TGF-β, may activate a fibrogenic program in response to infarction (126,127). Members of the miR-15 family have also been suggested to exert antifibrotic actions by inhibiting the TGF-β pathway (128). In contrast to other antifibrotic miRNAs, miR-15 is up-regulated following cardiac injury and may play a role in restraining the fibrotic response.

Other miRNAs may function as activators of the fibrogenic cascade, promoting myofibroblast conversion and activation in the infarcted heart. MiR-21 is markedly induced in infarct fibroblasts (129) and may exert fibrogenic actions by stimulating MAPK activation in cardiac fibroblasts (130) or by targeting the TGF-β cascade (131). In addition to its effects on the fibrotic response, fibroblast-derived miR-21, packaged into exosomes, may exert paracrine effects on cardiomyocyte hypertrophy and immune cell activation (132).

Evidence on the role on lncRNAs in fibroblast activation following infarction is limited (133). Wisp2 superenhancer-associated RNA, a cardiac fibroblast-enriched lncRNA, has been implicated in fibroblast proliferation, activation, and survival following myocardial infarction (134). The species specificity of lncRNAs (only 15% of mouse lncRNAs are expressed in humans and vice versa) is a major limiting factor in the use of animal models to understand their role in human diseases (135).

FIBROBLASTS IN SCAR MATURATION. In healing infarcts, secretion of structural ECM proteins by activated myofibroblasts is followed by induction of matrix crosslinking enzymes that contribute to scar maturation. As the scar matures, the density of activated myofibroblasts is dramatically reduced (45).

FIGURE 3 The Phenotypic Heterogeneity of Cardiac Fibroblast Populations May Explain Their Functional Diversity in Injured and Remodeling Hearts



In the pressure-overloaded myocardium, mechanical stress activates mechanosensitive signaling pathways in cardiac fibroblasts that may involve integrins (ITGs) and stress-activated ion channels (such as transient receptor potential [TRP] channels). Traditional views consider the fibroblasts as matrix-producing cells that secrete large amounts of fibrillar and nonfibrillar collagens, increasing extracellular matrix (ECM) deposition and promoting fibrosis and diastolic dysfunction. However, recent evidence challenges this unidimensional view of fibroblasts, suggesting that they may also play protective roles, by preserving the ECM, thus preventing generation of proinflammatory matrix fragments and by transducing prosurvival cascades in cardiomyocytes. Secretion of matricellular proteins that bind to the structural components of the ECM and modulate signaling responses and release of micro-ribonucleic acid (miRNA)–containing exosomes that may modulate cardiomyocyte responses represent major additional mechanisms implicated in fibroblast actions. The diverse effects of fibroblasts in vivo may reflect their phenotypic heterogeneity, as different fibroblast subsets may exert distinct actions. MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase.

Depletion of myofibroblasts from the mature scar may reflect apoptosis of fibroblasts (136) or loss of α-SMA expression and acquisition of a distinct fibroblast phenotype, characterized by high expression of tendon genes (137). The molecular signals responsible for apoptosis, or deactivation of scar myofibroblasts remain unknown.

CHRONIC ACTIVATION OF FIBROBLASTS IN THE REMODELING NONINFARCTED MYOCARDIUM. As the infarcted heart heals, the surviving myocardium exhibits chronic remodeling, associated with cardiomyocyte hypertrophy and interstitial fibrotic changes. Increased wall stress in noninfarcted myocardial segments may activate interstitial fibroblasts, promoting a matrix-synthetic phenotype and contributing to segmental dysfunction. Although chronic fibrotic changes have been reported in remodeling noninfarcted segments, and some studies have suggested persistence of myofibroblasts for many years in patients surviving an acute infarction (138), the relative contribution of chronic fibroblast activation in the pathogenesis of post-infarction heart failure remains unknown.

THE FIBROBLASTS IN THE PRESSURE-OVERLOADED MYOCARDIUM

Increased afterload is a common pathophysiologic companion of many cardiac pathologic conditions, including hypertensive heart disease and aortic stenosis. A pressure load imposes mechanical stress on all myocardial cells and triggers a series of molecular events leading to hypertrophic and fibrotic ventricular remodeling and ultimately heart failure (139). Expansion of resident cardiac fibroblast populations is a prominent characteristic of cardiac pressure overload (140,141) and is associated with activation of a matrix-synthetic program and subsequent deposition of collagens in interstitial and perivascular areas. Neurohumoral activation has been critically implicated in pressure overload-induced cardiac fibrosis. Angiotensin II-mediated AT1 activation mediates interstitial fibrosis in models of left ventricular pressure overload (142), through direct actions and via induction of inflammatory cytokines and growth factors. Although fibrogenic actions of proinflammatory cytokines, such as tumor necrosis

factor-α and IL-6, have been reported in pressure-overload models, whether these effects involve direct modulation of fibroblast phenotype or reflect indirect actions on macrophages or cardiomyocytes remains unknown (143,144). Moreover, TGF-β-driven activation of Smad-dependent signaling has been implicated in fibroblast activation in the pressure-overloaded heart (145).

Considering that mechanical stress is the primary insult in the pressure-overloaded myocardium, activation of mechanosensitive signaling pathways in cardiac fibroblasts may be the critical initial event involved in the pathogenesis of interstitial fibrosis. The focal adhesion-integrin complex is a primary mechanosensor in fibroblasts and transduces molecular signals that promote ECM gene transcription and myofibroblast conversion (146). Focal adhesion kinase (FAK) is a critical molecular link between mechanical stress and fibroblast activation. In vitro, FAK activation has been demonstrated to mediate mechanosensitive or growth factor–induced myofibroblast conversion (147–149). In vivo, FAK knockdown attenuated fibrotic changes in a model of cardiac pressure overload (150). However, considering the broad effects of FAK activation on cardiomyocytes and vascular and interstitial cells, the cellular basis for these effects is unclear. Evidence documenting the role of fibroblast-specific FAK activation in cardiac fibrosis is lacking.

Mechanosensitive ion channels have also been implicated in pressure overload–induced fibroblast activation (146). TRPC3 and TRPV4 have been implicated in myofibroblast conversion in response to mechanical stress or to growth factor stimulation (151,152). A recent study demonstrated a critical role for fibroblast-specific activation of the TWIK-related potassium channel in the activation of a fibrogenic response in the pressure-overloaded myocardium (153).

Mechanosensitive or neurohumoral activation of the small GTP-binding protein RhoA may also play an important role in fibroblast proliferation and activation following pressure overload, signaling through the ROCKs, ROCK1 and ROCK2 (154,155). In an experimental model of cardiac pressure overload, pharmacological inhibition of the RhoA-ROCK pathway attenuated fibrosis (156). Fibroblast-specific ROCK2 signaling has been suggested to mediate angiotensin II–mediated fibrosis, through induction of FGF2 and of the matricellular protein CCN2 (157). In addition to the direct fibrogenic actions of mechanosensitive signaling pathways, pressure overload may activate fibroblasts indirectly, through mechanical stress–induced actions on cardiomyocytes, T lymphocytes, or macrophages (158,159).

It should be emphasized that the contribution of fibroblasts in the pressure-overloaded myocardium is not limited to synthesis of ECM proteins and subsequent increase in ventricular stiffness. Activated fibroblasts may function as potent modulators of cardiomyocyte prosurvival and hypertrophic responses by secreting growth factors or through the release of miRNA-containing exosomes (160,161). Recent work suggested that TGF-β/Smad–dependent matrix-preserving actions of activated myofibroblasts prevent the generation of proinflammatory ECM fragments and play a critical role in protection of the pressure-overloaded myocardium from inflammation and systolic dysfunction (162). Thus, activated fibroblasts in the pressure-overloaded heart are not unidimensional cells that mediate fibrosis and dysfunction but may also exert protective actions preventing myocardial injury (**Figure 3**). Whether the diverse actions of fibroblasts in the remodeling myocardium are mediated through distinct fibroblast subpopulations remains unknown.

FIBROBLASTS IN THE VOLUME-OVERLOADED HEART

Conditions associated with volume overload, such as severe aortic or mitral valve regurgitation, are associated with marked ventricular dilation and progressive systolic dysfunction. Studies in experimental models of chordal rupture–induced mitral regurgitation in the dog (163,164) and of aortocaval fistula in the rat (165,166) suggest that volume overload causes unique interstitial perturbations that may contribute to adverse remodeling. In contrast to the marked increase in collagen deposition noted in pressure-overloaded hearts, the volume-overloaded myocardium exhibits a marked loss of interstitial collagen associated with increased MMP expression (163,164), reduced collagen synthesis (167), and accentuated collagen degradation (166,168). The matrix-degrading phenotype of interstitial cells in volume-overloaded hearts has been attributed to release of cardiomyocyte-derived TNF-α (165) or to down-modulation of TGF-β signaling (163). Whether these changes reflect perturbations of fibroblast phenotype and function in response to volume overload–induced stretch remains unknown.

FIBROBLAST ACTIVATION IN THE AGING AND DIABETIC HEART

Aging, diabetes, obesity, and metabolic dysfunction are associated with progressive interstitial and perivascular fibrosis that may contribute to the

pathogenesis of heart failure with preserved ejection fraction (5,169–173). In contrast to the rapid accumulation of α-SMA-expressing myofibroblasts in models of acute cardiac injury, diabetes and aging do not typically trigger myofibroblast conversion but may cause induction of a matrix-preserving program in cardiac interstitial cells (174). The cellular events and molecular mechanisms mediating fibrosis in senescent and diabetic hearts remain poorly understood. In aging hearts, fibroblast activation may involve the cooperation of several distinct pathways, including age-associated induction of the ROS system, activation of neurohumoral mediators, and stimulation of cytokine and TGF-β-mediated responses (175). In diabetic subjects, in contrast, hyperglycemia may result in accumulation of advanced glycation end-products that crosslink the cardiac ECM, while directly activating fibroblasts by triggering receptor for advanced glycation end-product-mediated signals (176) and accentuating age-associated changes. Diabetes-associated induction of matricellular proteins, such as thrombospondin-1, may also promote fibrosis by activating growth factor-dependent signaling in cardiac fibroblasts (177). Fibroblast activation in diabetic hearts may also reflect microvascular inflammation or resident macrophage stimulation and subsequent secretion of fibrogenic signals (5,178). It should be emphasized that although aging is associated with increased basal interstitial and perivascular collagen deposition, senescent animals exhibit perturbed fibroblast responses following injury, associated with blunted activation of growth factor signaling pathways (179).

CONCLUSIONS AND FUTURE DIRECTIONS

Expansion and activation of resident fibroblast populations play an important role in repair and remodeling of the injured heart and are implicated in the pathogenesis of systolic and diastolic dysfunction in chronic heart failure. Emerging evidence suggests that activated fibroblasts are not unidimensional matrix-producing cells but exhibit a wide range of phenotypes and may regulate inflammatory, hypertrophic, and prosurvival responses. Several important questions remain to be answered. Does the diversity of functional effects of cardiac fibroblasts in the remodeling heart reflect actions of distinct fibroblast subpopulations? If so, what are the phenotypic characteristics, origin, and fate of these fibroblast subsets? Does the functional pluralism of fibroblasts reflect their high responsiveness to changes in their microenvironment? Which molecular signals and environmental cues drive the dramatic phenotypic changes of cardiac fibroblasts in remodeling hearts? Do cell biological processes documented in mouse models recapitulate the phenotypic changes of fibroblasts in human hearts? Answering these questions is critical in order to design novel therapeutic approaches for patients with heart failure.

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KEY WORDS cytokines, extracellular matrix, fibroblast, infarction, remodeling

Protective Effects of Activated Myofibroblasts in the Pressure-Overloaded Myocardium Are Mediated Through Smad-Dependent Activation of a Matrix-Preserving Program

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Rationale: **The heart contains abundant interstitial and perivascular fibroblasts. Traditional views suggest that, under conditions of mechanical stress, cytokines, growth factors, and neurohumoral mediators stimulate fibroblast activation, inducing ECM (extracellular matrix) protein synthesis and promoting fibrosis and diastolic dysfunction. Members of the TGF (transforming growth factor)-β family are upregulated and activated in the remodeling myocardium and modulate phenotype and function of all myocardial cell types through activation of intracellular effector molecules, the Smads (small mothers against decapentaplegic), and through Smad-independent pathways.**

Objectives: **To examine the role of fibroblast-specific TGF-β/Smad3 signaling in the remodeling pressure-overloaded myocardium.**

Methods and Results: **We examined the effects of cell-specific Smad3 loss in activated periostin-expressing myofibroblasts using a mouse model of cardiac pressure overload, induced through transverse aortic constriction. Surprisingly, FS3KO (myofibroblast-specific Smad3 knockout) mice exhibited accelerated systolic dysfunction after pressure overload, evidenced by an early 40% reduction in ejection fraction after 7 days of transverse aortic constriction. Accelerated systolic dysfunction in pressure-overloaded FS3KO mice was associated with accentuated matrix degradation and generation of collagen-derived matrikines, accompanied by cardiomyocyte myofibrillar loss and apoptosis, and by enhanced macrophage-driven inflammation. In vitro, TGF-β1, TGF-β2, and TGF-β3 stimulated a Smad3-dependent matrix-preserving phenotype in cardiac fibroblasts, suppressing MMP (matrix metalloproteinase)-3 and MMP-8 synthesis and inducing TIMP (tissue inhibitor of metalloproteinases)-1. In vivo, administration of an MMP-8 inhibitor attenuated early systolic dysfunction in pressure-overloaded FS3KO mice, suggesting that the protective effects of activated cardiac myofibroblasts in the pressure-overloaded myocardium are, at least in part, because of suppression of MMPs and activation of a matrix-preserving program. MMP-8 stimulation induces a proinflammatory phenotype in isolated macrophages.**

Conclusions: **In the pressure-overloaded myocardium, TGF-β/Smad3-activated cardiac fibroblasts play an important protective role, preserving the ECM network, suppressing macrophage-driven inflammation, and attenuating cardiomyocyte injury. The protective actions of the myofibroblasts are mediated, at least in part, through Smad-dependent suppression of matrix-degrading proteases. (*Circ Res*. 2019;124:1214-1227. DOI: 10.1161/CIRCRESAHA.118.314438.)**

Key Words: extracellular matrix ■ fibroblasts ■ inflammation ■ macrophages ■ matrix metalloproteinases

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The adult mammalian heart contains a large pool of fibroblast-like interstitial cells that can be activated after injury to repair the myocardium but also participate in maladaptive cardiac fibrosis.[1–3] Most cardiac pathological conditions are associated with expansion of the interstitial fibroblast population. After myocardial infarction, cardiomyocyte necrosis triggers an inflammatory cascade that clears the wound from dead cells and leads to activation of reparative myofibroblasts that secrete collagens, preserving the structural integrity of the ventricle.[4,5] Genetic cardiomyopathies, valvular diseases, metabolic conditions, such as diabetes mellitus and obesity, and myocarditis are also associated with expansion and activation of fibroblasts and with increased deposition of ECM (extracellular matrix) proteins in the cardiac interstitium. In conditions associated with

Editorial, see p 1149
Meet the First Author, see p 1142

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Novelty and Significance

What Is Known?

- Cardiac pressure overload is associated with fibroblast activation and expansion of the perivascular and interstitial ECM (extracellular matrix) network.
- TGF (transforming growth factor)-β signaling cascades activate fibroblasts in vitro and in vivo.
- Activated fibroblasts are considered key effector cells in the pathogenesis of myocardial fibrosis and essential contributors to the pathophysiology of cardiac dysfunction.
- Activated fibroblasts may be therapeutic targets in patients with heart failure.

What New Information Does This Article Contribute?

- In contrast to prevailing dogma, activated cardiac myofibroblasts protect the heart from pressure overload–induced remodeling, through Smad (small mothers against decapentaplegic) 3-dependent preservation of the cardiac ECM.
- Activation of TGF-β/Smad3 signaling in cardiac myofibroblasts suppresses collagenase synthesis, reducing generation of proinflammatory matrikines and attenuating macrophage-driven inflammation.
- Nonselective targeting of activated fibroblasts in failing hearts may accentuate cardiac dysfunction under conditions of mechanical stress by abrogating key reparative signals.

TGF-β signaling is activated after tissue injury and modulates phenotype and function of all cell types involved in repair and remodeling, by stimulating Smad-dependent and Smad-independent cascades. The cell-specific actions of TGF-βs in cardiac remodeling remain poorly understood. In the heart subjected to pressure overload, activation of TGF-β/Smad cascades in fibroblasts may be involved in the pathogenesis of cardiac remodeling, fibrosis, and dysfunction. Using myofibroblast-specific Smad3 knockout mice and a wide range of in vitro models, we demonstrate protective actions of activated fibroblasts in a mouse model of cardiac pressure overload. Activation of Smad3 signaling in cardiac myofibroblasts protects the heart from pressure overload–induced systolic dysfunction, preserves cardiac extracellular matrix, prevents generation of proinflammatory matrix fragments, inhibits cardiomyocyte injury, and attenuates macrophage-driven inflammation. The protective effects of activated myofibroblasts are mediated through Smad3-dependent stimulation of a matrix-preserving program. Our findings suggest that, in contrast to traditional views suggesting deleterious effects of activated fibroblasts in failing hearts, subpopulations of the activated myofibroblasts may exert protective actions on the remodeling myocardium, by inhibiting mechanical stress-mediated extracellular matrix degradation. Thus, nonselective targeting of cardiac fibroblasts in failing hearts may abrogate key protective signals.

Nonstandard Abbreviations and Acronyms

Akt	protein kinase B
Ang II	angiotensin II
Arg1	arginase 1
BMP	bone morphogenetic protein
CCL2	C-C motif chemokine ligand 2
CCN2	connective tissue growth factor
CHP	collagen hybridizing peptide
ECM	extracellular matrix
Erk	extracellular signal-regulated kinase
FS3KO	fibroblast-specific Smad3 knockout
GDF-11	growth differentiation factor-11
iNOS	inducible NO synthase
Klf	Kruppel-like factor
KO	knockout
LV	left ventricle
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
OPN	osteopontin
PGP	proline-glycine-proline
Smad	small mothers against decapentaplegic
SPARC	secreted protein acidic and rich in cysteine
TAC	transverse aortic constriction
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
TSP	thrombospondin
VEH	vehicle
WT	wild type

cardiac pressure overload, resident fibroblast lineages proliferate and undergo activation.[6,7] Stimulation of mechanosensitive pathways activates a matrix-synthetic program in cardiac fibroblasts, leading to a progressive increase in interstitial collagen content, associated with diastolic dysfunction. However, the molecular signals responsible for fibroblast activation in the pressure-overloaded heart remain poorly understood.

Extensive evidence suggests that members of the TGF (transforming growth factor)-β superfamily critically modulate fibroblast phenotype and are involved in the pathogenesis of tissue fibrosis.[8–10] TGF-βs signal by sequentially transphosphorylating type II and type I receptors, activating downstream signaling cascades that may involve a family of intracellular effectors, the Smads (small mothers against decapentaplegic), or Smad-independent pathways.[11] Evidence derived from global loss-of-function models and in vitro experiments suggest that Smad3 plays an important role in tissue fibrosis in a wide range of pathophysiologic conditions.[12–17] In the injured and remodeling myocardium, global Smad3 loss attenuated cardiac fibrosis, reducing interstitial collagen deposition.[12,13,16,17] However, considering the broad effects of Smad3 signaling in all cell types involved in cardiac remodeling, the relative significance of fibroblast-specific Smad3 signaling remains unclear. In the current study, we tested the hypothesis that fibroblast-specific Smad3 signaling may promote fibrotic remodeling and diastolic dysfunction in the pressure-overloaded myocardium, using cell-specific loss-of-function approaches in vivo and in vitro experiments. Surprisingly, myofibroblast-specific Smad3 loss accelerated systolic dysfunction after pressure overload, accentuating matrix degradation, promoting myofibrillar loss in cardiomyocytes, and stimulating inflammatory macrophage activation. The protective effects of myofibroblast-specific Smad3

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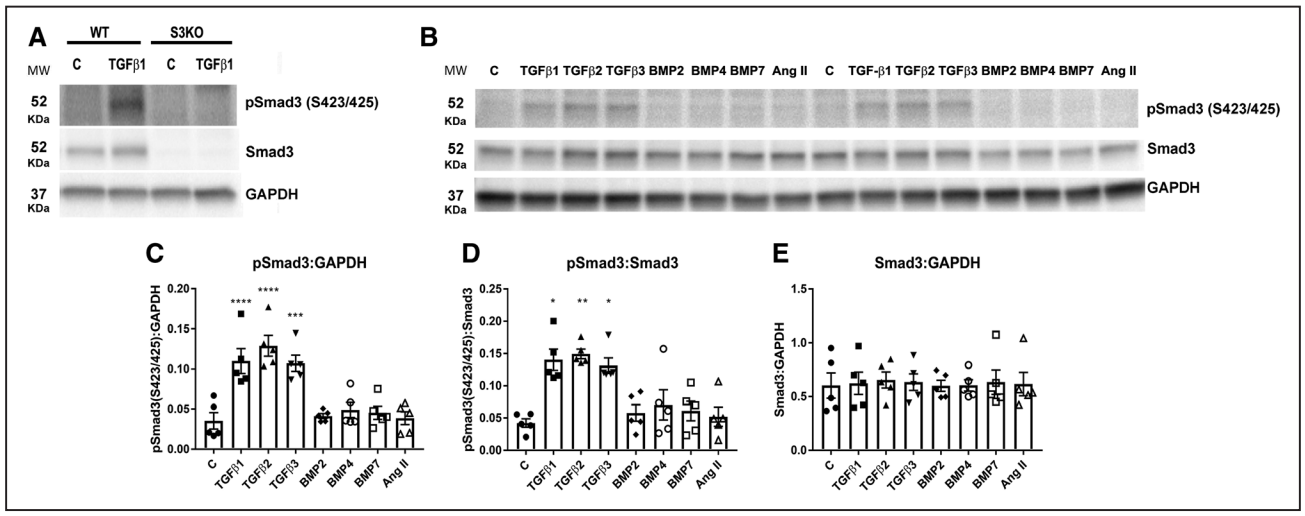


Figure 1. All 3 TGF (transforming growth factor)-β isoforms, but not Ang II (angiotensin II), BMP (bone morphogenetic protein)-2, BMP-4, and BMP-7, activate Smad (small mothers against decapentaplegic) 3 in cardiac fibroblasts. A, The specificity of the antibodies to p-Smad3 and Smad3 was validated using fibroblasts from WT (wild type) and global S3KO (Smad3 KO) hearts. **B**, Representative Western blotting experiment demonstrates that TGF-β1 (10 ng/mL), TGF-β2 (10 ng/mL), and TGF-β3 (10 ng/mL) increase Smad3 phosphorylation at the S423/S425 sites after 30 minutes of stimulation. In contrast, BMP-2 (50 ng/mL), BMP-4 (50 ng/mL), BMP-7 (50 ng/mL), and Ang II (50 ng/mL) do not activate Smad3. **C–E**, Quantitative analysis shows that TGF-βs significantly increase p-Smad3 levels (**C**) and the pSmad3:Smad3 ratio (**D**), without affecting Smad3 expression (**E**). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 vs control; n=4.

signaling are mediated through activation of a matrix-preserving program that inhibits MMP (matrix metalloproteinase)-driven ECM degradation and attenuates the proinflammatory actions of MMPs. Our findings challenge traditional views on the role of myofibroblasts as detrimental effectors of cardiac fibrosis and provide the first direct experimental evidence suggesting protective actions of activated myofibroblasts in the pressure-overloaded myocardium. TGF-β/Smad3-dependent activation of fibroblast subpopulations may afford protection in the pressure-overloaded myocardium, mediated at least, in part, through preservation of the cardiac ECM.

Methods

All supporting data are available within the article and the Online Data Supplement.

Detailed methodology is provided in the Online Data Supplement.

We generated FS3KO (myofibroblast-specific Smad3 knockout) mice, using the periostin-Cre driver. *Postn*-Cre mice were bred with Smad3 fl/fl mice[18,19] to generate FS3KO animals and corresponding Smad3 fl/fl controls. A mouse model of cardiac pressure overload induced through transverse aortic constriction (TAC) was used. In vitro experiments were performed using isolated primary cardiac fibroblasts and macrophages.

Results

TGF-β, but Not BMPs, or Ang II Activates Smad3 Signaling in Isolated Cardiac Fibroblasts

Cardiac pressure overload induces myocardial expression of TGF-β isoforms and downstream activation of TGF-β/Smad cascades.[20] To explore the signals that activate Smad3 specifically in cardiac fibroblasts, we assessed Smad3 phosphorylation in fibroblasts stimulated with members of the TGF-β superfamily or with Ang II (angiotensin II). We documented the specificity of the anti-pSmad3 antibody using Smad3 KO (knockout) cells (Figure 1A). After 30 minutes of stimulation, all 3 TGF-β isoforms significantly increased the ratio of pSmad3:Smad3 expression without significantly affecting total Smad3 protein (Figure 1B through 1E). In contrast, BMP (bone morphogenetic protein)-2, BMP-4, and BMP-7 had no effects on Smad3 phosphorylation. Ang II—a critical mediator in cardiac remodeling—has been suggested to signal by stimulating TGF-β/Smad3.[21,22] However, in cardiac fibroblasts, Ang II did not affect Smad3 phosphorylation after 30 minutes of stimulation (Figure 1B through 1E), suggesting that its reported effects on Smad3 signaling may be indirect.

Generation of Mice Lacking Smad3 in Activated Myofibroblasts

To generate FS3KO mice, we used the periostin-Cre driver, as described previously.[19] After 7 days of pressure overload, cardiac fibroblasts harvested from FS3KO mice had a marked reduction in Smad3 mRNA levels, when compared with fibroblasts from Smad3 fl/fl mice (Figure 2A). In contrast, no significant differences in Smad3 mRNA expression were noted in the spleen of FS3KO and Smad3 fl/fl animals (Figure 2B). Effective Smad3 deletion was confirmed at the protein level. Western blotting demonstrated that fibroblasts harvested from FS3KO mice had markedly lower Smad3 expression (Figure 2C and 2D). The specificity of the anti-Smad3 antibody was validated using cells from Smad3 KO mice (Figure 2C).

Myofibroblast-Specific Smad3 Loss Accelerates Systolic Dysfunction Following Pressure Overload

FS3KO and Smad3 fl/fl mice underwent TAC protocols. Three FS3KO mice and 1 Smad3 fl/fl mouse died during the perioperative period. The right:left carotid flow ratio was not different between FS3KO and Smad3 fl/fl mice (FS3KO, 8.3±0.5; Smad3 fl/fl, 8.89±0.59; *P*=nonsignificant; n=30–33 per group), reflecting comparable pressure gradients. FS3KO and Smad3 fl/fl mice exhibited comparable mortality after pressure overload (Online Figure IA). After 7 days of pressure overload, FS3KO mice had significantly higher heart

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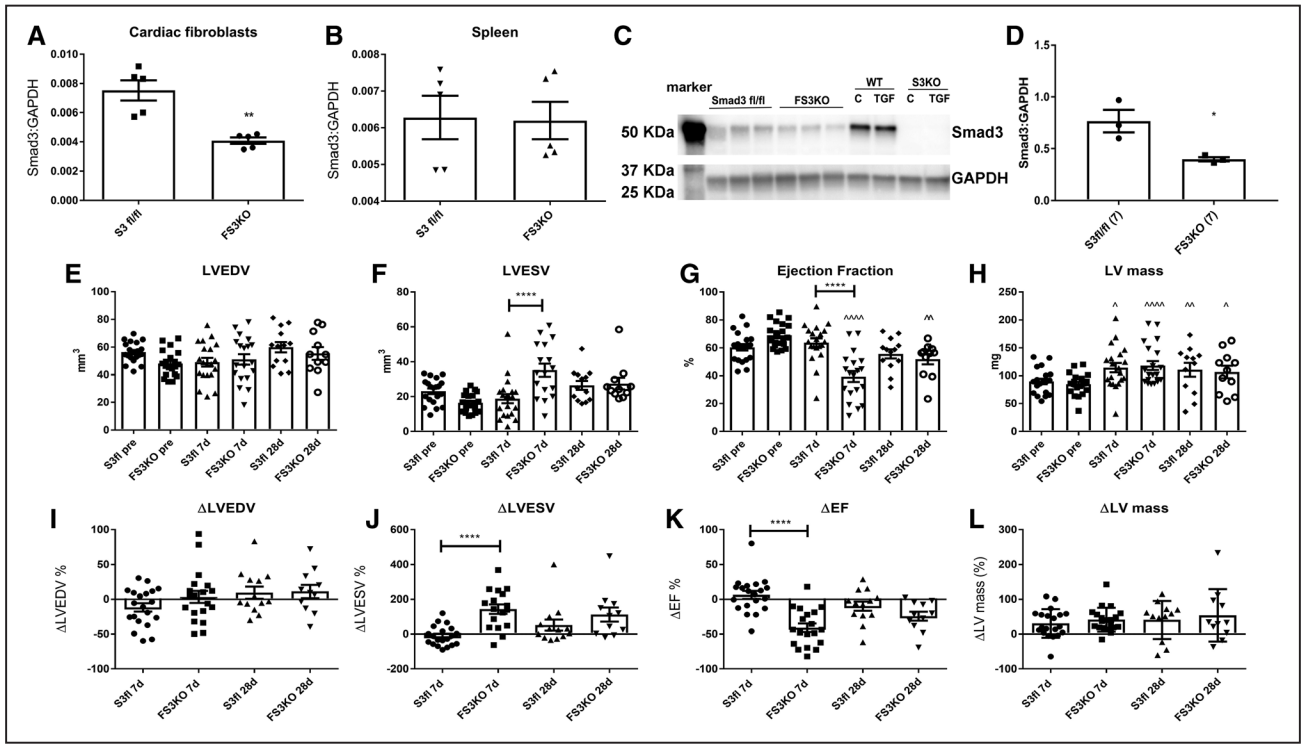


Figure 2. Fibroblast-specific Smad (small mothers against decapentaplegic) 3 loss accelerates systolic dysfunction following cardiac pressure overload. FS3KO (fibroblast-specific Smad3 knockout mice) were generated using the periostin-Cre driver. **A**–**D**, Documentation of cell-specific Smad3 knockdown in FS3KO mice. **A**, Cardiac fibroblasts isolated from pressure-overloaded hearts after 7 d of transverse aortic constriction (TAC) exhibited markedly lower Smad3 mRNA expression, when compared with Smad3 fl/fl animals (***P*<0.01; n=5 per group). In contrast, pressure-overloaded FS3KO mice and corresponding Smad3 fl/fl animals had comparable levels of Smad3 in the spleen (**B**). **C**, Western blotting showed markedly reduced Smad3 protein in fibroblasts harvested from pressure-overloaded FS3KO hearts, when compared with fibroblasts from Smad3 fl/fl hearts after 7 d of TAC (**P*<0.05; n=3 per group). Specificity of the Smad3 antibody was validated using cells from mice with global S3KO (Smad3 loss). **E**–**L**, Echocardiography was used to assess the effects of fibroblast-specific Smad3 loss on cardiac remodeling following pressure overload. **E** and **I**, FS3KO mice and Smad3 fl/fl had no statistically significant differences in left ventricular end diastolic volume (LVEDV). **E**–**L**, After 7 to 28 d of pressure overload, control Smad3 fl/fl animals exhibited left ventricular (LV) hypertrophy in the absence of significant systolic dysfunction. In contrast, FS3KO mice exhibited a marked >40% reduction in ejection fraction after 7 d of TAC (*****P*<0.0001 vs Smad3 fl/fl), suggesting accelerated systolic dysfunction (**G** and **K**). After 28 d of TAC, FS3KO and not Smad3 fl/fl mice exhibited depressed systolic function (^^*P*<0.01 vs corresponding baseline); however, there was no significant difference in ejection fraction between FS3KO and Smad3 fl/fl, reflecting late dysfunction in Smad3 fl/fl mice. **E** and **L**, The increase in LV mass was not significantly different between Smad3 fl/fl and FS3KO mice after 7 to 28 d of TAC (^*P*<0.05, ^^*P*<0.01, ^^^^*P*<0.0001 vs corresponding baseline; n=12–21 mice per group).

weight:body weight (BW) and lung weight:BW ratio than corresponding Smad3 fl/fl controls (Online Figure IB and IC), suggesting worse heart failure. However, no significant differences in heart weight:BW and lung weight:BW were noted after 28 days of pressure overload (Online Figure ID and IE). The effects of fibroblast-specific Smad3 loss on remodeling of the pressure-overloaded myocardium were assessed using echocardiography (Figure 2E through 2L). Baseline geometry and function were not significantly different between FS3KO and Smad3 fl/fl hearts. Consistent with previously published work in WT (wild type) C57/BL6J mice,[20] Smad3 fl/fl animals exhibited early development of cardiac hypertrophy in the absence of significant systolic dysfunction (Figure 2E through 2L). Surprisingly, fibroblast-specific Smad3 loss was associated with a marked (>40%) early reduction in ejection fraction after 7 days of pressure overload (change in ejection fraction in FS3KO, −41.5%±5.8, versus Smad3 fl/fl, +8.7±6%; *P*<0.0001; Figure 2G and 2K), suggesting accelerated systolic dysfunction. After 28 days of TAC, FS3KO but not Smad3 fl/fl mice had persistent systolic dysfunction (^^*P*<0.01 versus baseline); however, there was no significant difference in ejection fraction between FS3KO and Smad3 fl/fl groups, likely reflecting progressive dysfunction in control animals. The increase in left ventricular (LV) mass was comparable between Smad3 fl/fl and FS3KO animals (Figure 2H and 2L).

To assess diastolic function, we used mitral inflow Doppler and tissue Doppler imaging. No significant effects of myofibroblast-specific Smad3 loss on indicators of diastolic function were noted at baseline and after 7 to 28 days of pressure overload (Online Figure II).

Accelerated Systolic Dysfunction in FS3KO Mice Undergoing TAC Protocols Is Associated With ECM Fragmentation

To investigate the basis for accelerated dysfunction in FS3KO mice, we performed systematic histological analysis. In WT mice, TAC typically causes interstitial and perivascular fibrosis, in the absence of significant replacement fibrosis (Figure 3).[20] Surprisingly, after 7 days of pressure overload, 8 of 9 FS3KO mice exhibited evidence of replacement fibrosis (defined as >2.5% replacement of myocardium with collagen-based scar; *P*=0.015 versus Smad3 fl/fl animals; Figure 3A through 3G). Quantitative analysis showed that the increase in the area of replacement fibrosis in FS3KO mice did not reach

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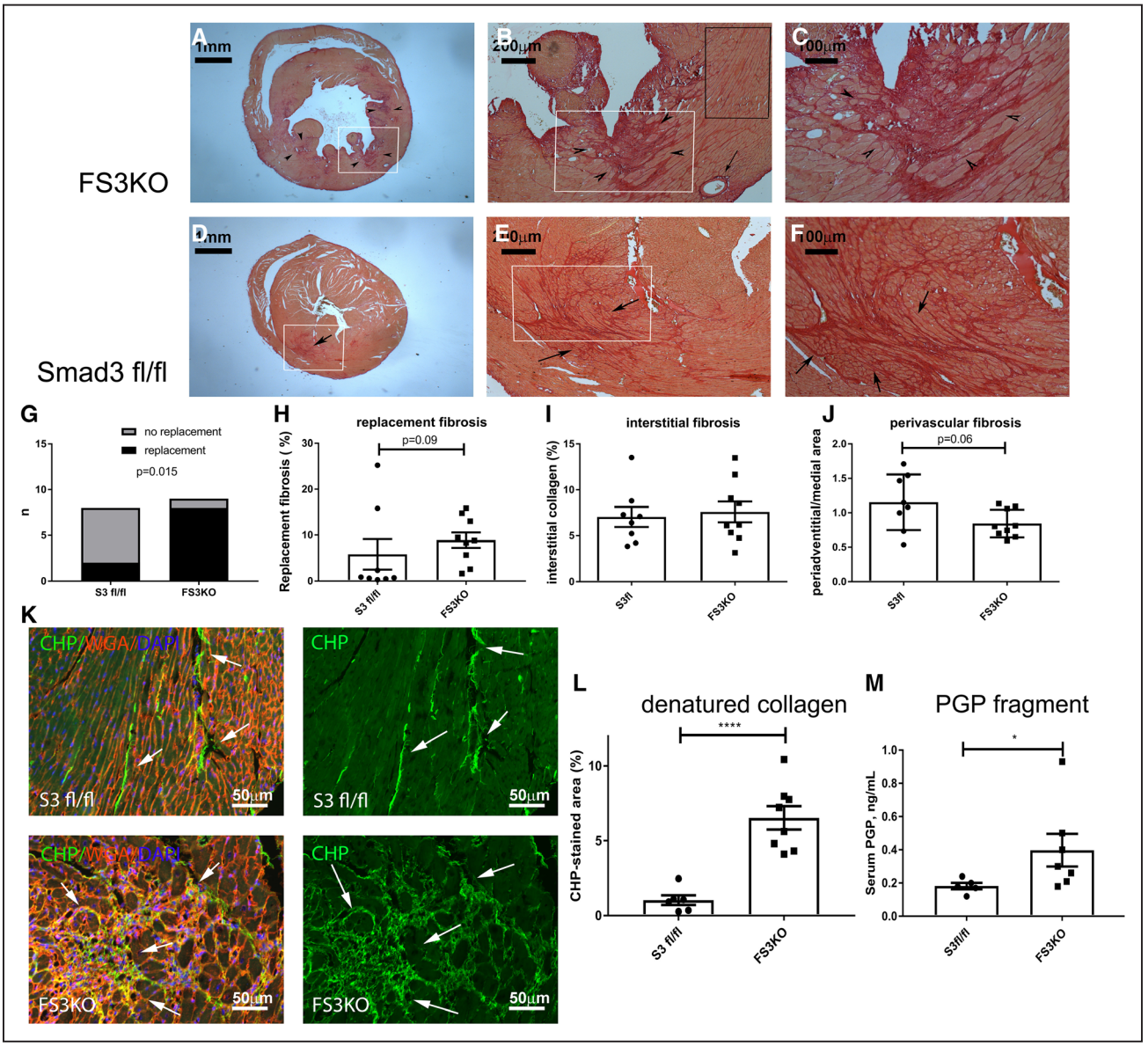


Figure 3. Fibroblast-specific Smad (small mothers against decapentaplegic) 3 loss accentuates matrix degradation in the pressure-overloaded heart, promoting release of collagen-derived matrikines. A–J, Sirius red staining was used to identify the collagen network in pressure-overloaded hearts. **A–F**, In the transverse aortic constriction (TAC) model, cardiac pressure overload is typically associated with interstitial and perivascular fibrosis (arrows), in the absence of replacement fibrosis. FS3KO (fibroblast-specific Smad3 knockout) mice exhibited foci of replacement fibrosis (arrowheads); in contrast, Smad3 fl/fl animals had predominantly interstitial and perivascular collagen deposition (arrows). **G**, Quantitative analysis of replacement fibrosis areas showed that after 7 d of TAC, 8 of 9 FS3KO mice had >2.5% of the left ventricular myocardium replaced by scar (P=0.015 vs Smad3 fl/fl mice; n=8–9 per group). **H**, The difference in the area of replacement fibrosis between pressure-overloaded FS3KO and Smad3 fl/fl mice did not reach statistical significance (P=0.09). **I**, Quantitative analysis of interstitial fibrosis was performed in areas with no evidence of replacement fibrosis (black rectangle; **B**) and showed no significant differences between Smad3 fl/fl and FS3KO animals. **J**, FS3KO and Smad3 fl/fl mice did not have a statistically significant difference in the mean ratio of periadventitial collagen-stained area:medial area—an indicator of perivascular fibrosis (P=0.06). **K–M**, Fibroblast-specific Smad3 loss accentuates matrix degradation. To study the effects of fibroblast-specific Smad3 loss on collagen fragmentation and denaturation, we used 2 distinct methods: (1) fluorescent staining with CHP (collagen hybridizing peptide), which binds only to unfolded denatured collagen fibers (**K**, arrows), and (2) mass spectrometry to assess levels of the collagen-derived matrikine PGP (Pro-Gly-Pro). FS3KO mice had extensive collagen denaturation, evidenced by a marked increase in the CHP-stained area in the pressure-overloaded cardiac interstitium after 7 d of TAC (**K**, arrows; **M**, ****P<0.0001; n=7–8 per group). M: mass spectrometry of serum samples after 7 d of TAC showed that FS3KO mice had a 2-fold increase in generation of PGP (*P<0.05; n=5 per group).

statistical significance (Figure 3H; P=0.09). Because Smad3 signaling is linked with activation of a matrix-synthetic program in fibroblasts, we also quantitatively assessed interstitial and perivascular fibrosis in myocardial areas with no evidence of replacement fibrosis. The effects of fibroblast-specific Smad3 loss on perivascular and interstitial fibrosis did not reach statistical significance after 7 days of pressure overload (Figure 3I and 3J). Next, we asked whether the detrimental effects of fibroblast-specific Smad3 loss are because of loss of matrix-preserving functions of the activated fibroblasts, leading to increased fragmentation of the cardiac ECM. We used 2 distinct strategies to study collagen breakdown. First, labeling with CHP (collagen hybridizing peptide), which specifically binds to denatured collagen,[23] was compared between Smad3

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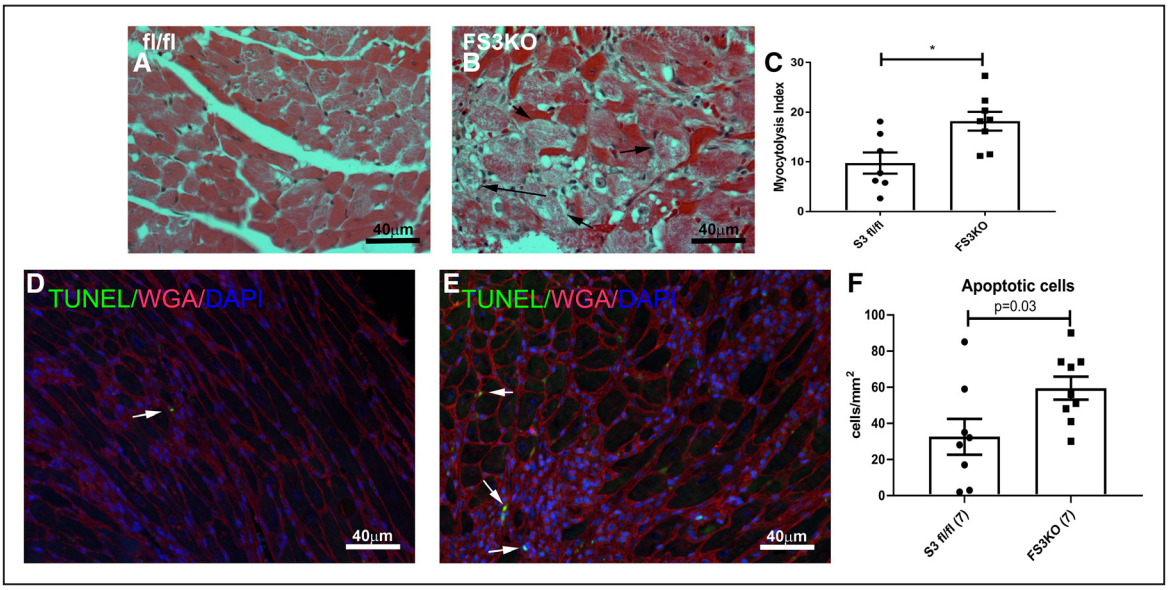


Figure 4. Fibroblast-specific Smad (small mothers against decapentaplegic) 3 loss is associated with increased cardiomyocyte injury after 7 d of transverse aortic constriction (TAC). A and **B**, Hematoxylin-eosin staining demonstrated that many cardiomyocytes in pressure-overloaded FS3KO (fibroblast-specific Smad3 knockout) mice exhibited significant myofibrillar loss (myocytolysis, arrows). **C**, Quantitative analysis showed that FS3KO animals had a significantly higher myocytolysis index (*P<0.05; n=8–9). **D** and **E**, TUNEL (terminal deoxynucleotidyl transferase [TdT] dUTP nick-end labeling) staining was used to identify apoptotic cells in pressure-overloaded hearts (arrows). Quantitative analysis showed that after 7 d of TAC, FS3KO mice had a significantly higher density of apoptotic cells (**F**), when compared with corresponding Smad3 fl/fl mice.

fl/fl and FS3KO hearts. FS3KO animals had a marked increase in the interstitial areas labeled by CHP (Figure 3K and 3L). Second, mass spectrometry demonstrated that circulating levels of the Pro-Gly-Pro peptide—a collagen-derived matrikine with proinflammatory properties[24]—were increased in FS3KO mice (Figure 3M). Thus, fibroblast-specific loss of Smad3 was associated with robust evidence of ECM degradation.

After 28 days of TAC, FS3KO mice had a trend toward increased replacement fibrosis that did not reach statistical significance (Online Figure IIIA through IIIC). Interstitial fibrosis was not different between Smad3 fl/fl and FS3KO mice (Online Figure IIID and IIIE). Fibroblast-specific Smad3 loss significantly attenuated perivascular fibrosis (Online Figure IIIF and IIIG). Western blotting analysis showed no significant effects of fibroblast-specific Smad3 loss on collagen III protein levels after 28 days of TAC (Online Figure IIIH and IIII).

Myofibroblast-Specific Smad3 Loss Accentuates Degenerative Changes in Pressure-Overloaded Cardiomyocytes

To explore the basis for early systolic dysfunction in pressure-overloaded FS3KO mice, we studied cardiomyocyte morphology. After 7 days of TAC, FS3KO mice exhibited significant myocytolysis, characterized by degenerative changes and myofibrillar loss in cardiomyocytes (Figure 4A and 4B). Quantitative analysis demonstrated that the myocytolysis index was higher in FS3KO mice (P<0.05; Figure 4C). FS3KO mice also had a higher number of TUNEL+ (terminal deoxynucleotidyl transferase [TdT] dUTP nick-end labeling) apoptotic cells in the pressure-overloaded myocardium (Figure 4D through 4F). After 28 days of TAC, the severity of myocytolysis and the number of apoptotic cells were comparable between FS3KO and Smad3 fl/fl mice (Online Figure IV). This likely reflected the progressive increase in cardiomyocyte degeneration in Smad3 fl/fl animals.

Taken together, our findings (Figures 2 through 4) suggest that myofibroblast-specific Smad3 protects the pressure-overloaded myocardium and prevents early systolic dysfunction, by preserving the cardiac ECM, thus attenuating cardiomyocyte degeneration.

Myofibroblast-Specific Smad3 Loss Accentuates Macrophage-Driven Inflammation

Because macrophage-mediated inflammation has been implicated in dysfunction of the remodeling myocardium,[25] and generation of matrix fragments may trigger macrophage recruitment, we examined the effects of myofibroblast-specific Smad3 loss on myocardial macrophage infiltration (Figure 5). After 7 to 28 days of TAC, FS3KO mice exhibited markedly higher macrophage infiltration in comparison with Smad3 fl/fl animals (Figure 5A; P<0.05). The majority of infiltrating macrophages expressed iNOS (inducible NO synthase) suggesting a proinflammatory phenotype (Figure 5B and 5D through 5G). In contrast, the density of Arg1+ (arginase 1) macrophages was comparable between groups (Figure 5C). These findings suggest that fibroblast Smad3 signaling attenuates inflammation in the pressure-overloaded heart.

Myofibroblast-Specific Smad3 Loss Accentuates MMP-8 Expression, Promoting a Matrix-Degrading Environment

Next, we explored the molecular basis for the protective effects of myofibroblast-specific Smad3 on the pressure-overloaded myocardium. Because synthesis of structural and matricellular ECM proteins is an important function of activated fibroblasts in the remodeling heart,[26] we compared the gene expression profiles of fibroblasts harvested from Smad3 fl/fl and FS3KO

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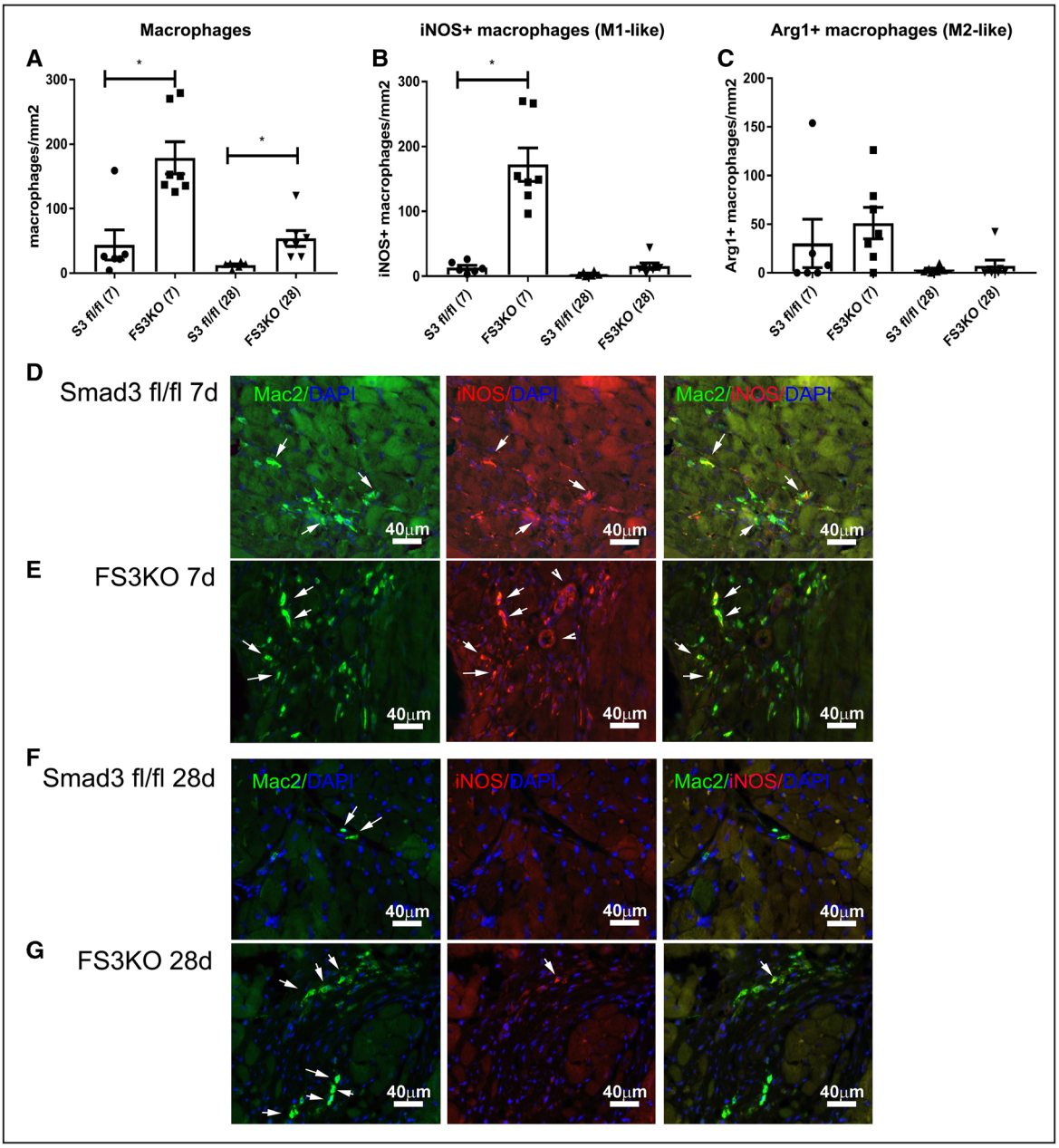


Figure 5. Fibroblast-specific Smad (small mothers against decapentaplegic) 3 loss accentuates macrophage-driven inflammation in the pressure-overloaded myocardium. A, Mac2 immunofluorescence was used to identify macrophages in the remodeling myocardium. FS3KO (fibroblast-specific Smad3 knockout) mice exhibited a marked increase in macrophage density after 7 to 28 d of transverse aortic constriction (TAC; **P*<0.05 vs Smad3 fl/fl; n=6–7 per group). **B**, Dual immunofluorescence for Mac2 and iNOS (inducible NO synthase) was used to identify proinflammatory M1-like macrophages. The density of iNOS+ macrophages was significantly higher in FS3KO mice after 7 d of TAC (**P*<0.05 vs Smad3 fl/fl). The number of proinflammatory macrophages in FS3KO mice was significantly reduced after 28 d of TAC when compared with the 7-d time point (^*P*<0.05 vs FS3KO 7 d; n=7 per group). **C**, Dual immunofluorescence for Mac2 and Arg1 (arginase 1) was used to identify anti-inflammatory M2-like macrophages. The density of Arg1+ macrophages was comparable between groups. **D**–**G**, Representative images show Mac2/iNOS dual fluorescence (arrows) in Smad3 fl/fl and FS3KO mice after 7 and 28 d of TAC.

hearts after 7 days of pressure overload. FS3KO and Smad3 fl/fl fibroblasts had comparable mRNA synthesis of type I collagen, fibronectin, TGF-β1, and of the matricellular proteins tenascin-C, TSP (thrombospondin)-1 and -2, SPARC (secreted protein acidic and rich in cysteine), OPN (osteopontin), and CCN2 (connective tissue growth factor; Online Figure V).

To examine the role of fibroblast Smad3 in regulating expression of proteases and antiproteases involved in ECM metabolism, we compared MMP and TIMP (tissue inhibitor of metalloproteinases) expression between fibroblasts harvested from pressure-overloaded FS3KO and Smad3 fl/fl mice. FS3KO fibroblasts exhibited markedly increased MMP-8 synthesis, when compared with Smad3 fl/fl cells. In contrast, MMP-2, MMP-3, MMP-9, TIMP-1, and TIMP-2 expression was comparable between groups (Figure 6A through 6F). In vivo, immunohistochemical staining showed that FS3KO mice had significantly higher density of MMP-8+ interstitial cells after 7 days of TAC (Online Figure VI).

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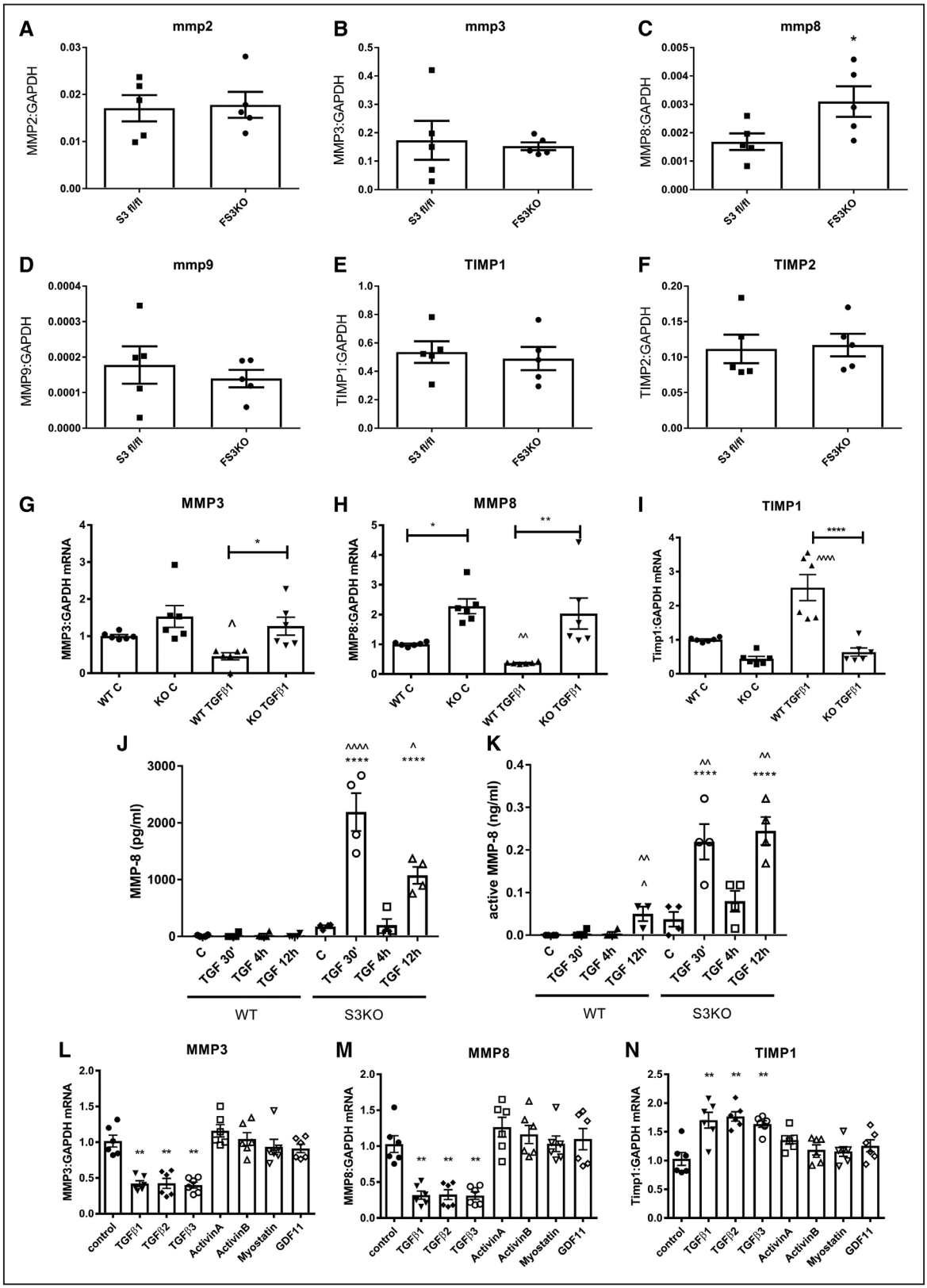


Figure 6. Smad (small mothers against decapentaplegic) 3 signaling mediates the matrix-preserving effects of TGF (transforming growth factor)-β in cardiac fibroblasts. Fibroblasts harvested from FS3KO (fibroblast-specific Smad3 knockout) and Smad3 fl/fl hearts after 7 d of transverse aortic constriction had comparable MMP (matrix metalloproteinase)-2 (**A**) and MMP-3 (**B**) mRNA expression levels but exhibited markedly elevated MMP-8 expression (**C**; *P<0.05; n=5 per group). MMP-9 (**D**), TIMP (tissue inhibitor of metalloproteinase)-1 (**E**), and TIMP-2 (**F**) expression levels were comparable between Smad3 fl/fl and FS3KO fibroblasts. **G–I**, Effects of TGF-β on MMP and TIMP expression in cardiac fibroblasts cultured in collagen pads are dependent on Smad3. In WT (wild type) cells, TGF-β1 stimulation suppressed synthesis of MMP-3 (**G**) and MMP-8 (**H**) and induced TIMP-1 (**I**) expression (**P<0.01 vs control; n=6 per group). In contrast, TGF-β1 had no significant effects on MMP-3, MMP-8, and TIMP-1 expression in Smad3 (*Continued*)

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Smad3 Mediates Matrix-Preserving Actions of TGF-β

In vitro experiments using mouse cardiac fibroblasts cultured in collagen pads demonstrated that Smad3 KO cells exhibited markedly higher baseline MMP-8 mRNA expression than WT fibroblasts and showed trends toward higher MMP-3 and lower TIMP-1 expression (Figure 6G through 6I); TGF-β1 stimulation suppressed MMP-3 and MMP-8 in WT cells but not in Smad3 KO fibroblasts (Figure 6G and 6H). Moreover, TGF-β1 induced TIMP-1 in WT cells but had no effect on TIMP-1 expression in Smad3 KO fibroblasts (Figure 6I). Next, we examined whether Smad3 loss also affects release of active MMP-8 protein by cardiac fibroblasts. WT fibroblasts secreted low amounts of MMP-8 protein, in the presence or absence of TGF-β1. In contrast, Smad3 KO cells released large amounts of MMP-8 after TGF-β1 stimulation (Figure 6J). An MMP-8 activity assay confirmed that Smad3 signaling restrains release of active MMP-8 by stimulated fibroblasts (Figure 6K). Taken together, our experiments suggest that Smad3 mediates potent matrix-preserving actions of TGF-β.

TGF-βs, but Not Activins, Myostatin, and GDF-11 Directly Stimulate a Matrix-Preserving Program

Next, we examined whether the matrix-preserving effects of TGF-β1 are shared by other members of the TGF-β superfamily, known to activate Smad3 signaling. All 3 TGF-β isoforms, but not activins, myostatin, and GDF-11 (growth differentiation factor-11), suppressed MMP-3 and MMP-8 synthesis by cardiac fibroblasts (Figure 6L and 6M). Moreover, only TGF-βs induced TIMP-1 synthesis (Figure 6N).

Effects of Non-Smad Pathways on MMP:TIMP Expression

In addition to their Smad-mediated effects, TGF-βs act through Smad-independent pathways, such as Akt (protein kinase B), p38 MAPK (mitogen-activated protein kinase), and Erk (extracellular signal-regulated kinases) MAPK. To explore the role of Smad3 in TGF-β–mediated activation of Akt, p38, and Erk, we compared responses between WT and Smad3 KO fibroblasts. TGF-β1 stimulation rapidly activated Akt, p38, and Erk in both WT and Smad3 KO fibroblasts (Online Figure VIIA through VIIF). To investigate the role of Smad-independent pathways in regulating the protease/antiprotease balance, we examined the effects of the Erk inhibitor U0126 and the p38 MAPK inhibitor SB203580. In WT cells, Erk inhibition, but not p38 MAPK inhibition, abrogated the suppressive effects of TGF-β1 on MMP-3 and MMP-8 synthesis. In contrast, in Smad3 KO cells, Erk inhibition did not affect MMP-3 and MMP-8 synthesis suggesting that the effects of Erk in mediating the suppressive effects of TGF-β1 are not independent of Smad3. In WT cells, both Erk and p38 MAPK inhibition attenuated TGF-β1–induced TIMP-1 synthesis. In contrast, in Smad3 KO cells, neither the Erk inhibitor nor the p38 MAPK inhibitor had any effects on TIMP-1 levels, suggesting that the effects of Erk and p38 MAPK on TIMP-1 expression were Smad3 dependent (Online Figure VIIG through VIII).

MMP-8 Inhibition Attenuates Systolic Dysfunction in Pressure-Overloaded FS3KO Mice

To examine whether the in vivo protective effects of fibroblast-specific Smad3 signaling are mediated through activation of a matrix-preserving program, we treated FS3KO mice and Smad3 fl/fl animals undergoing TAC protocols with an MMP-8 inhibitor.[27] Administration of the inhibitor attenuated systolic dysfunction in FS3KO animals after 7 days of TAC (Figure 7A and 7B) and reduced the increase in LV end systolic volume (Figure 7C and 7D), without exerting any significant effects on LV end diastolic volume (Figure 7E and 7F) and on LV mass (Figure 7G and 7H). Surprisingly, treatment of Smad3 fl/fl mice with the MMP-8 inhibitor worsened LV function after 7 days of TAC (Figure 7A and 7B). These findings suggest that an optimal functional response of the myocardium to a pressure load requires balanced matrix-degrading activity: both excessive MMP-8 expression (in FS3KO animals) and low levels of MMP-8 (in inhibitor-treated Smad3 fl/fl mice) suppress systolic function.

To explore the cellular mechanism for the effects of MMP-8 on the remodeling myocardium, we performed histological analysis of the myocardium in animals treated with the inhibitor or VEH (vehicle). Myocytolysis and density of apoptotic cells were not significantly different between groups (Figure 8A and 8B). However, quantitative analysis of the iNOS+ macrophages (M1 like) and Arg1+ macrophages (M2 like) showed that FS3KO mice exhibit a reduction in the M2:M1 cell ratio and that treatment with the MMP-8 inhibitor attenuated the reduction in the M2:M1 ratio (Figure 8C through 8E). In vitro, stimulation of bone marrow macrophages with recombinant MMP-8 (50 ng/mL) significantly reduced active TGF-β1 levels in the supernatant, while increasing release of the proinflammatory chemokine CCL2 (C-C motif chemokine ligand 2; Figure 8F and 8G). Thus, MMP-8 exerts direct actions on macrophages, promoting a proinflammatory phenotype.

Discussion

In patients with heart disease, myocardial fibrosis is consistently associated with adverse prognosis. In patients with heart failure with preserved ejection fraction, cardiac fibrosis was associated with increased baseline levels of biomarkers reflecting heart failure severity and with worse outcome.[28] In patients with LV systolic dysfunction, myocardial fibrosis was a powerful predictor of ventricular arrhythmic risk, in both ischemic and nonischemic cardiomyopathy groups.[29] Considering that cardiac myofibroblasts are the main source of ECM proteins in the remodeling heart, the close association between

Figure 6 Continued. KO (knockout) fibroblasts (^*P*<0.05, ^^*P*<0.01 vs corresponding control). **J** and **K**, WT cardiac fibroblasts had low levels of MMP-8 protein and activity in the supernatant, in the presence or absence of TGF-β. In contrast, Smad3 KO cells exhibited marked release of MMP-8 protein and activity 30 min and 12 h after TGF-β1 stimulation (****P*<0.0001 vs corresponding WT cells; ^*P*<0.05, ^^*P*<0.01, ^^^^*P*<0.0001 vs unstimulated cells; n=4 per group). The findings suggested that Smad3 signaling restrains release of MMP-8 protein and activity by cardiac fibroblasts. **L**–**N**, Effects of Smad3 activating members of the TGF-β superfamily on expression of MMP-3, MMP-8, and TIMP-1. Only TGF-β1, β2, and β3, and not activin-A, activin-B, myostatin, and GDF-11 (growth differentiation factor-11), suppressed MMP-3 (**L**) and MMP-8 (**M**) expression and induced TIMP-1 synthesis (**N**) in fibroblasts cultured in collagen pads (**P*<0.01 vs control; n=5 per group).

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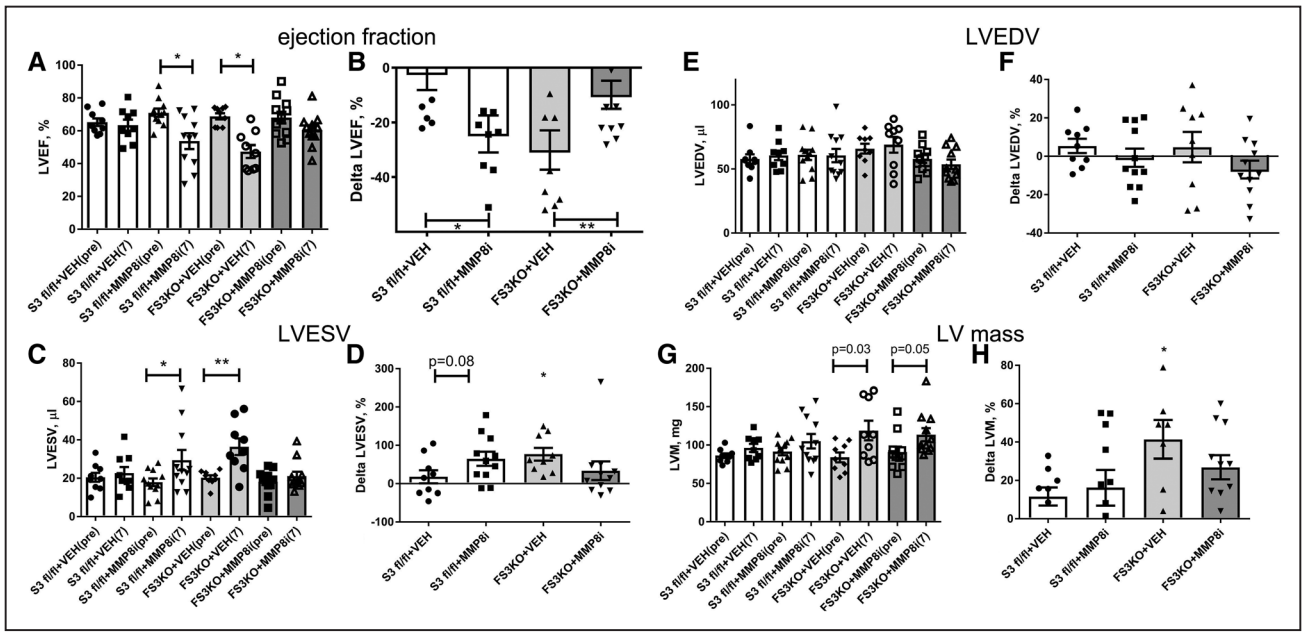


Figure 7. MMP (matrix metalloproteinase)-8 inhibition attenuates systolic dysfunction in pressure-overloaded FS3KO (fibroblast-specific Smad [small mothers against decapentaplegic] 3 knockout) mice. A and **B**, FS3KO mice exhibited significant early reduction in ejection fraction after 7 d of transverse aortic constriction (TAC; **P*<0.05 vs baseline). Administration of an MMP-8 inhibitor, but not VEH (vehicle), attenuated systolic dysfunction in FS3KO mice. Surprisingly, MMP-8 inhibition caused reduction of ejection fraction in Smad3 fl/fl mice undergoing TAC protocols, suggesting that both excessive MMP-8 (in FS3KO mice) and low MMP-8 activity (in control mice treated with inhibitor) have detrimental effects. **C** and **D**, MMP-8 inhibition also attenuated the increase in left ventricular end systolic volume (LVESV) noted in pressure-overloaded FS3KO mice. **E** and **F**, There were no significant effects of MMP-8 inhibition on LVEDV. **G** and **H**, MMP-8 inhibition did not significantly affect the effects of fibroblast-specific Smad3 loss on left ventricular mass (LVM; **P*<0.05, ***P*<0.01; n=9–13 per group).

myocardial fibrosis and adverse outcome led to a unidimensional view of cardiac myofibroblasts, as detrimental cellular effectors that mediate dysfunction in myocardial infarction and in chronic heart failure. However, this oversimplified paradigm on the role of myofibroblasts in cardiac remodeling overlooks their reparative functions. In many cardiac pathological conditions, fibroblast expansion is not the primary cause of disease but represents activation of a reparative process in response to cardiomyocyte injury. The reparative role of fibroblasts has been well documented in myocardial infarction.[5,19,30] In adult mammals, repair of the infarcted myocardium requires generation of a well-organized scar, comprised of well-aligned arrays of activated myofibroblasts that secrete a collagen-based matrix. Our current study demonstrates for the first time that the protective functions of cardiac fibroblasts are not limited to myocardial infarction but also play an important role in restraining ECM degradation, in inhibiting inflammation, and in preventing cardiomyocyte injury and systolic dysfunction in injurious responses not associated with significant cardiomyocyte necrosis. Using cell-specific approaches in vivo, we documented that in the pressure-overloaded heart, Smad-dependent myofibroblast activation protects stressed cardiomyocytes from death and attenuates macrophage-driven inflammation, by stimulating a matrix-preserving program.

Protective Functions of Myofibroblasts in the Pressure-Overloaded Myocardium Are Mediated Through Activation of a Matrix-Preserving Program

Surprisingly, loss of the critical fibrogenic effector Smad3 in activated myofibroblasts accelerated systolic dysfunction in the pressure-overloaded myocardium. Impaired systolic function in FS3KO mice was associated with accentuated collagen degradation and release of proinflammatory collagen-derived matrikines (Figure 3). Increased collagen fragmentation and denaturation in pressure-overloaded FS3KO mice was accompanied by loss of myofibrillar content in cardiomyocytes (Figure 4) and by enhanced macrophage-driven inflammation (Figure 5). Assessment of gene expression in fibroblasts isolated directly from pressure-overloaded hearts demonstrated that Smad3 absence was associated with accentuated expression of the collagenase MMP-8 (Figure 6). In vivo, administration of an MMP-8 inhibitor protected FS3KO hearts from the development of early systolic dysfunction, documenting that the matrix-degrading effects of fibroblast-specific Smad3 loss mediate systolic dysfunction. Thus, our findings suggest a novel conceptual paradigm on the role of myofibroblasts in the pressure-overloaded myocardium (Figure 8H). Activation of mechanosensitive signaling in response to a pressure load is a potent stimulus for induction and activation of MMPs.[31] TGF-β/Smad3 activation suppresses MMP synthesis and stimulates TIMP expression, playing a critical role in preservation of the cardiac ECM under conditions of mechanical stress and attenuating release of matrix fragments that may promote inflammation and accentuate cardiomyocyte injury. In the absence of Smad3, unrestrained MMP activity in response to pressure overload and subsequent release of collagen-derived matrikines may promote dysfunction.

What Are the Cellular Mechanisms of MMP-8–Driven Cardiac Dysfunction in Remodeling FS3KO Mice?

Several cellular mechanisms may explain the link between excessive MMP-8 expression and systolic dysfunction in the

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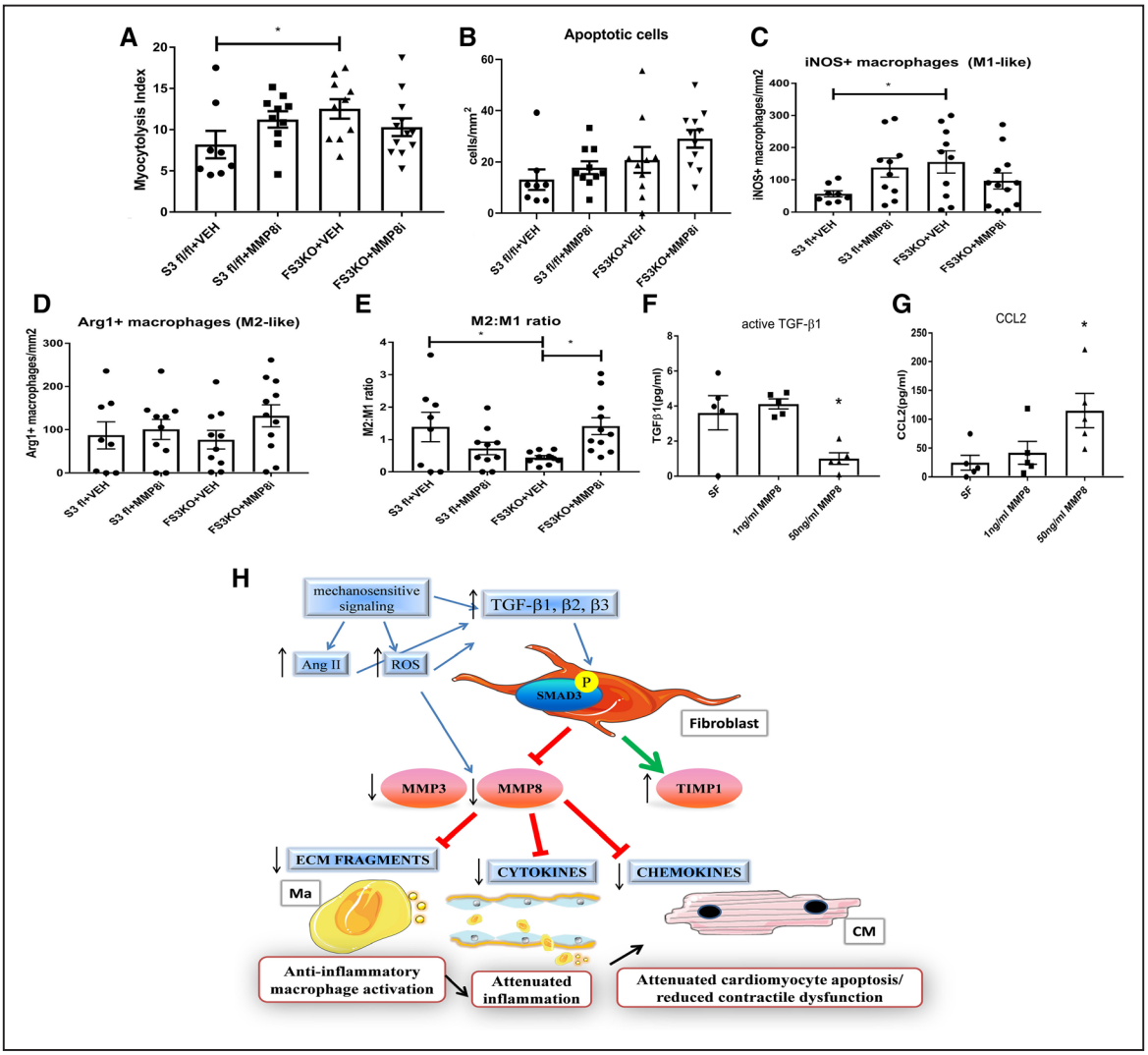


Figure 8. MMP (matrix metalloproteinase)-8 promotes a proinflammatory macrophage phenotype in vitro and in vivo. A–C, Treatment with the MMP-8 inhibitor did not improve the myocytolysis score in FS3KO (fibroblast-specific Smad [small mothers against decapentaplegic] 3 knockout) mice (**A**) and did not affect the density of apoptotic cells (**B**) in the pressure-overloaded myocardium after 7 d of transverse aortic constriction. Although the effects of MMP-8 inhibition on the density of iNOS+ (inducible NO synthase) M1-like macrophages (**C**) and Arg1+ (arginase 1) M2-like macrophages (**D**) did not reach statistical significance, administration of the inhibitor increased the ratio of M2-like to M1-like cells (**E**; **P*<0.05). In vitro, recombinant MMP-8 (50 ng/mL) markedly reduced levels of active TGF (transforming growth factor)-β1 (**F**) and increased levels of the proinflammatory chemokine CCL2 (C-C motif chemokine ligand 2; **G**) in the supernatant of isolated bone marrow macrophages (**P*<0.05; n=5 per group). **H**, Schematic cartoon illustrating the protective effects of TGF-β/Smad3-activated fibroblasts in the pressure-overloaded myocardium. Mechanical stress stimulates Ang II (angiotensin II) and generates reactive oxygen species (ROS), activating proteases. TGF-β–mediated stimulation of fibroblasts promotes a matrix-preserving program, suppressing MMP-3 and MMP-8 expression and inducing TIMP (tissue inhibitor of metalloproteinase) 1 synthesis. Reduced matrix degradation decreases release of collagen-derived matrikines and attenuates macrophage-driven inflammation protecting cardiomyocytes from death and dysfunction. Moreover, restrained MMP activity may inhibit inflammation by decreasing formation of bioactive cytokine and chemokine molecules. The cartoon was designed using Servier Medical Art (https:smart.servier.com).

pressure-overloaded heart. First, MMP-driven degradation of the pericellular ECM may deprive pressure-overloaded cardiomyocytes of critical survival signals promoting their apoptosis under conditions of mechanical stress. Second, MMP-8 is known to generate proinflammatory matrix fragments that promote chemotactic recruitment and activation of myeloid cells.[32] Our in vivo experiments demonstrated that FS3KO mice had increased levels of the proinflammatory collagen-derived matrikine PGP (proline-glycine-proline; Figure 3)—a chemotactic fragment generated through MMP-8 actions.[32,33] Third, MMP-8 may trigger proteolytic activation of cytokines and chemokines, thus stimulating inflammation. MMP-8 has been reported to exhibit TNF (tumor necrosis factor)-α converting enzyme activity, cleaving the TNF-α prodomain to generate the active cytokine.[27] Moreover, MMP-8 is implicated in CXC chemokine processing, leading to generation of potent leukocyte chemoattractants.[34,35] Our in vitro experiments demonstrated that stimulation with recombinant MMP-8 promotes a proinflammatory macrophage phenotype, increasing CCL2 release and attenuating levels of bioactive TGF-β. MMP-8–driven accentuation of inflammation may explain, at least in part, the transient effects of fibroblast-specific Smad3 loss on cardiac function, which are not associated with long-term consequences on cardiac remodeling. Thus, the detrimental

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effects of excessive MMP-8 on the remodeling myocardium may involve both activation of macrophage-driven inflammation and increased susceptibility of cardiomyocytes to stress-induced death. Moreover, considering the abundant expression of MMP-8 by neutrophils and monocytes, attenuation of dysfunction through MMP-8 inhibition in pressure-overloaded FS3KO mice may reflect actions on leukocytes and not inhibition of fibroblast-specific MMP-8 activity.

In contrast to the protective effects of MMP-8 neutralization in pressure-overloaded FS3KO animals, MMP-8 inhibition worsened cardiac function in Smad3 fl/fl mice. These effects may reflect protective actions of low amounts of MMP-8 under conditions of stress. Complete absence of MMP-8 in mice perturbed resolution of inflammation following wound healing,[34] and exacerbated inflammation, in a model of endotoxin-induced lung injury.[36] Tight regulation of MMP-8 activity may be important to protect tissues from damage under conditions of stress: both excessive MMP-8 expression and low levels of MMP-8 may have deleterious effects.

Effects of TGF-β/Smad3 Signaling on Fibroblast Phenotype In Vitro and In Vivo

Our recently published work examined the effects of fibroblast TGF-β/Smad3 signaling in myocardial infarction.[19] We demonstrated that fibroblast-specific Smad3 loss impairs repair of the infarcted heart and is associated with a high incidence of late cardiac rupture and with accentuated adverse postinfarction remodeling. The catastrophic effects of fibroblast-specific Smad3 loss were caused by perturbations in scar organization, because of reduced fibroblast integrin expression, and defective activation of downstream reparative signals. Considering the massive loss of cardiomyocytes in myocardial infarction and the negligible regenerative capacity of the adult mammalian heart, the requirement for functional activated myofibroblasts in the infarcted heart is perhaps not unexpected. The findings of the current study are much more surprising, suggesting that fibroblast-specific TGF-β/Smad3 signaling is protective in a model of nonreparative cardiac fibrosis induced by pressure overload. Thus, even in the absence of significant cardiomyocyte death, activation of fibroblasts may be required to prevent matrix fragmentation, preserving ECM structure and protecting stressed cardiomyocytes from injury.

In contrast to our observations, Khalil et al[8] recently reported that fibroblast-specific Smad3 exerts detrimental effects on the pressure-overloaded heart, mediating fibrotic remodeling. The seemingly conflicting messages of the 2 studies can be explained by differences in research design and in interpretation of the findings. First, our study examined a larger number of animals, of both genders; we have systematically assessed the functional and histological consequences of fibroblast-specific Smad3 loss at 2 different time points, after 7 and 28 days of pressure overload. In contrast, Khalil et al examined only male mice; functional analysis was performed at a late time point (12 weeks after TAC), and early effects of Smad3 loss were not studied. Second, although Khalil et al did not report data on diastolic function parameters in FS3KO animals, their findings suggested no significant effects of combined fibroblast-specific Smad2 and Smad3 loss on diastolic function. Thus, conclusions on the adverse consequences of fibroblast-specific Smad-dependent signaling were not based on functional data. Third, the studies used different approaches to delete Smad3 in cardiac fibroblasts. We have used a constitutive periostin-Cre driver; considering the absence of periostin expression in normal resident cardiac fibroblasts and the selective induction of periostin in activated injury site myofibroblasts, this approach specifically targets activated fibroblasts in the remodeling myocardium, as demonstrated by our group and other investigators. Khalil et al used an inducible periostin Mer-Cre-Mer driver and achieved almost complete Smad3 loss in fibroblasts (in contrast to a 50% reduction in our study). However, administration of tamoxifen for Cre induction and gene deletion may modulate the baseline response to pressure overload.

Functional Pluralism of Fibroblasts in the Remodeling Myocardium

Our study challenges the prevailing dogma that fibroblasts are unidimensional cells that mediate fibrosis and dysfunction. Cardiac fibroblasts are versatile cells that may acquire distinct phenotypes in response to tissue injury, exerting a wide range of inflammatory, hypertrophic, and cytoprotective actions. The functional diversity of cardiac fibroblasts may reflect, at least in part, the phenotypic heterogeneity of interstitial cell populations, which are comprised of functionally distinct subsets.[37] The diverse functions of cardiac fibroblasts have been appreciated in the dynamic environment of the infarcted heart.[26] In myocardial ischemia and reperfusion, fibroblasts have been suggested to protect cardiomyocytes from ischemic death.[38] During the early proinflammatory phase of infarct healing, fibroblasts may act as inflammatory and matrix-degrading cells, producing cytokines, chemokines, and proteases[39] and stimulating leukocyte recruitment. A recently published study suggested that fibroblast-derived GM-CSF (granulocyte-macrophage colony stimulating factor) synthesis may activate myeloid cells triggering leukocyte recruitment in the infarct.[40] Infarct fibroblasts have also been reported to participate in engulfment of apoptotic cells and in negative regulation of the postinfarction inflammatory response.[41] During the proliferative phase of infarct healing, fibroblasts undergo myofibroblast conversion and synthesize ECM proteins; formation of aligned arrays of activated myofibroblasts is critical for repair of the infarcted heart and protects from adverse dilative remodeling.[19]

In the pressure-overloaded heart, activated fibroblasts have been implicated in cardiomyocyte hypertrophy. Fibroblast-specific signaling through the zinc-containing transcription factor Klf (Kruppel-like factor)-5 was essential for adaptive hypertrophy in the pressure-overloaded heart.[42] Activation of a hypertrophic program in pressure-overloaded cardiomyocytes may be driven, at least in part, through secretion of miRNA-enriched exosomes by cardiac fibroblasts.[43]

Conclusions

Our findings document the protective functions of activated fibroblasts in the remodeling myocardium. In the pressure-overloaded heart, activation of neurohumoral and proinflammatory signals in response to mechanical stress induces expression and activation of MMPs.[44,45] Our findings suggest

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that activation of TGF-β/Smad3 signaling in cardiac myofibroblasts restrains matrix-degrading activity, preventing release of proinflammatory collagen-derived matrikines, suppressing macrophage-driven inflammation and attenuating cardiomyocyte injury (Figure 8H). Under conditions of stress, the heart is highly dependent on myofibroblast-mediated preservation of the ECM to maintain function. These observations have important therapeutic implications for patients with heart failure. Targeting activated fibroblasts in patients with heart failure may also abrogate protective actions of interstitial cells, thus exacerbating the adverse consequences of the underlying injurious process. In contrast to the primary injurious role of fibroblasts in fibrotic conditions affecting other systems, such as scleroderma[46] or idiopathic pulmonary fibrosis,[47] myocardial fibrosis often reflects a reparative/protective response to injury. In many patients with heart failure, direct inhibition of fibroblast activity may have detrimental effects. Moreover, targeting TGF-β cascades in patients with myocardial disease may carry significant risks, by interfering with important homeostatic or reparative functions. Thus, successful implementation of antifibrotic strategies in patients with myocardial fibrotic remodeling may require identification of patient subsets with excessive or dysregulated fibroblast activation.

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Disclosures

None.

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Minireview

Bone morphogenetic protein signaling in inflammation

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Impact statement

By compiling findings from recent studies, this review will garner novel insight on the dynamic and complex role of BMP signaling in diseases of inflammation, highlighting the specific roles played by both individual ligands and endogenous antagonists. Ultimately, this summary will help inform the high therapeutic value of targeting this pathway for modulating diseases of inflammation.

Abstract

Bone morphogenetic protein signaling has long been established as a crucial pathway during embryonic development. In recent years, our knowledge of the function of bone morphogenetic protein signaling has expanded dramatically beyond solely its important role in development. Today, the pathway is known to have important homeostatic functions across multiple different tissues in the adult. Even more importantly, bone morphogenetic protein signaling is now known to function as a driver of diseases in the adult spanning different organ systems. In this review, we will explore the functions of bone morphogenetic protein signaling in diseases of inflammation. Through this exploration, we will highlight the value and challenges in targeting bone morphogenetic protein signaling for therapeutic interventions.

Keywords: Atherosclerosis, myocardial infarction, vascular inflammation, anemia of inflammation, endothelial injury, inflammatory arthritis

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Introduction

Bone morphogenetic protein (BMP) ligands are part of the transforming growth factor-β (TGF-β) superfamily. As their name suggests, BMPs were first observed in the pioneering work by Urist[1] in 1965 where their activity was found to induce ectopic bone formation. Although later Urist was able to isolate the bone inducing proteins,[2] it was not until two decades later that these proteins were individually cloned and characterized.[3] Since then, BMPs have been studied extensively outside of their established role in bone and cartilage and found to possess a multitude of functions in the embryonic development of other organ systems, including blood, heart, vasculature, brain, lung, kidney, and limbs. This review will focus on the emerging role BMPs play in inflammatory processes across organ systems in the adult.

Signaling and regulatory components in the BMP pathway

Subclassification of the more than 15 known BMPs based on phylogenetic analysis of amino acid and nucleotide similarity creates particular subgroups of these ligands: BMP2/4, BMP5/6/7/8, BMP9/10, and BMP12/13/14.[4] It is important to note that BMP1 does not function like the rest of the BMP family. Although able to induce bone and cartilage development, BMP1 is not part of the TGF-β superfamily of proteins. Instead, it is a procollagen C-proteinase that functions in collage maturation.[5] The rest of the BMP family of proteins function as signaling ligands that bind to target receptors on the cell surface.

Before being secreted into the extracellular space where they become active, BMPs are first synthesized as precursor proteins in the cytoplasm. These pre-pro-peptides consist of an N-terminal signal peptide that is required for secretion, a prodomain that plays an important regulatory role for the folding of the active protein, and a C-terminal part that contains the mature peptide that is capable of binding to and activating its receptors.[6] BMP precursors form dimers in the cytoplasm that are subsequently cleaved by convertases to generate the mature BMP ligand that is being secreted into the extracellular space.

Once secreted, BMP ligands bind to their receptors on the surface of target cells to form a heterotetramic complex

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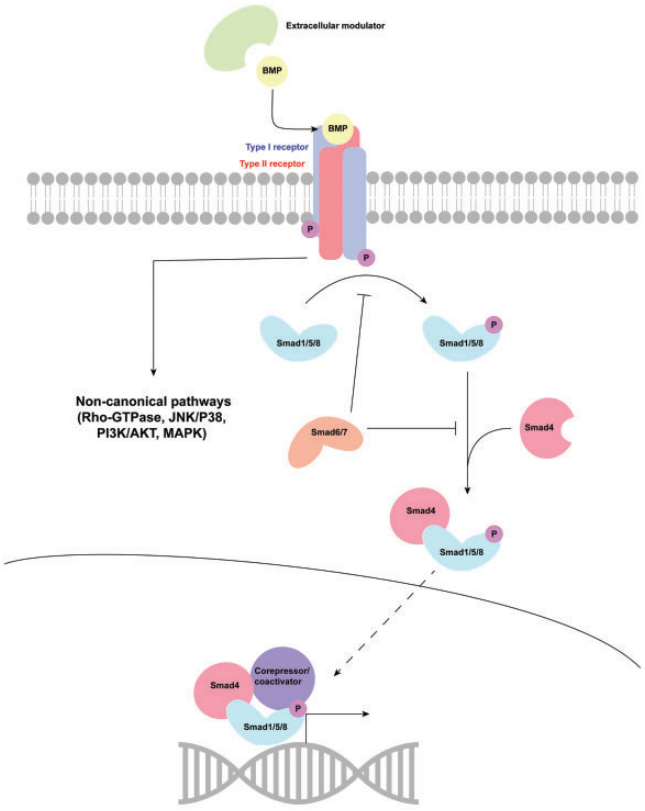


Figure 1. BMP signal transduction. Bone morphogenetic proteins transduce signals in target cells by binding to a heterotetramic complex composed of two dimers of type I and type II receptors. This allows the constitutively active type II receptor to transphosphorylate the type I receptor, causing conformational changes that lead to activation of both canonical pathways and non-canonical pathways like the Rho-GTPase, JNK/P38, PI3K/AKT, and MAPK pathways. Through the canonical pathway, ligand-receptor complex formation leads to phosphorylation of R-Smads (Smad1/5/8), which are then able to form complexes with co-Smads (Smad4). Together, the complex translocates into the nucleus where it associates with coactivators and corepressors to regulate gene expression. BMP ligands can be regulated both by extracellular modulators including protein antagonists and by intracellular modulators like inhibitory Smads (Smad6/7). Adapted from Wang et al.[45]

consisting of two dimers of type I and type II receptors[7] (Figure 1). This complex formation allows the constitutively active type II receptor to transphosphorylate the type I receptor, and activation of the type I receptor causes conformational changes that lead to phosphorylation of downstream proteins known as R-Smads (Smad1, Smad5, and Smad8). Activated, phosphorylated R-Smads in the cytoplasm subsequently form complexes with co-Smad (Smad4), and together, they translocate into the nucleus where they associate with coactivators and corepressors to regulate gene expression.[7] In addition to this so-called canonical signaling pathway, noncanonical BMP signaling pathways that are independent of Smads have also been identified. These include the Rho-GTPase, JNK/P38, and PI3K/AKT pathways in addition to various branches of the MAPK pathway.[8–10]

In addition to the diversity present in downstream signaling pathways, there also exists diversity within the interactions between BMP ligands and the membrane-bound BMP receptors. Both ligand-independent homodimerization and ligand-dependent heterodimerization of BMP type I receptors with effective signal transduction have been observed with certain type I receptor isoforms.[11] Moreover, certain heterodimeric complexes can only be assembled through binding with a specific BMP heterodimeric ligand.[12] Such complexities allow for an additional layer of signal regulation and coordination.

Adding to this complexity, BMP signaling is regulated by both intracellular and extracellular modulators. Extracellularly, BMP ligands are inhibited by proteins like Chordin, Noggin, and the Cerberus and Dan family of proteins, a protein family that includes Gremlin 2 (Grem2). While both Noggin and Grem2 possess a cysteine knot motif and form dimers,[13,14] their structure and the way they inhibit BMP ligands are very different. Noggin forms a head-to-head dimer that creates a clamp-like structure to bind to BMP dimers, inhibiting ligand activity by effectively masking the type I and type II receptor binding surfaces.[14] Grem2 forms a head-to-tail dimer,[13,15] and along its convex surface, one Grem2 monomer binds to a single monomer of the BMP dimer complex, where it interferes with the type II, but not the type I, receptor binding surface.[16] The other monomer of the Grem2 dimer is free to interact with another BMP dimer complex, and this second BMP dimer can then interact with another Grem2 dimer, creating a so-called "daisy chain" reaction. Grem2 demonstrates both a strong and broad inhibition of BMP ligands[17] and a strong specificity for the BMP subfamily of ligands within the TGF-β superfamily.[15]

Intracellularly, BMP signaling is regulated by a variety of mechanisms. Specific miRNAs can regulate expression of various components of the BMP signaling pathway,[7] whereas specific intracellular phosphatases like protein phosphatase 1 can dephosphorylate both phosphorylated R-Smads and phosphorylated type I receptors.[18,19] Moreover, a class of inhibitory Smads or I-Smad (Smad6 and Smad7) play an important role in negative feedback inhibition of BMP signaling.[20] The complexity stemming from the many downstream signal transducers, both canonical and noncanonical, and the multiple layers of regulation allow BMP signaling to coordinate and precisely modulate a wide range of biological processes.

BMP signaling is crucial for development

Since their initial discovery, BMPs have been extensively studied in embryonic development utilizing primarily mouse, zebrafish, and frog models. Such studies have found that BMP signaling is indispensable for numerous developmental processes. Deletion of BMP2, BMP4, or BMP10 is embryonically lethal,[21–23] and loss of BMP7 or BMP11 results in death shortly after birth.[24,25] Similarly, deletion of BMP-responsive receptors, BMPR1-A, ActR1A, and BMPR2[26–28] or knocking out of downstream transducers like Smad1, Smad5, Smad4, and the inhibitory Smad7 are also embryonically lethal.[29–32]

In particular, BMP4 is critical during early gastrulation as its function is required for the differentiation and formation of the mesoderm,[22,33] from which arises a number of tissues and cell types, including vascular, cardiac, skeletal,

and smooth muscles, and definitive hematopoietic stem cells (HSCs) – cells that will continue to supply blood cells into adulthood. Moreover, BMP4 is important to the function of the HSC microenvironment with deficiencies leading to reductions in both HSC function and numbers.[34]

BMP2 is necessary for further mesoderm development and is important for the proper development of the heart and the amnion and chorion.[21] In the heart, BMP2 is critical for endocardial cushion formation, development of the valves, and myocardial patterning,[35,36] whereas BMP10 is essential for cardiac growth and chamber maturation.[23] BMP signaling is also important for controlling the differentiation of cardiac progenitor cells into cardiomyocytes.[37,38] At the early stages of embryonic development, BMP signaling is critical to the proper generation of left/right axis patterning, affecting tissues including the developing heart.[39–42] Moreover, in the central nervous system, BMP signaling is important for directing neuronal differentiation and autoregulation of neurogenesis.[43,44] These and other studies have revealed the essential role BMPs play in normal development in these and many other biological systems, including reproductive and endocrine organs.[45]

Similarly, BMP antagonists also play important roles during embryonic development. In mice, Chordin is crucial for neural induction,[46] and both Noggin and Chordin have important but overlapping roles in development of the forebrain and left-right axis patterning of the developing embryo.[47] Additionally, Noggin is necessary for the development of the axial skeleton in mice.[48] Deleting Chordin leads to stillborn mice, while Noggin knockout mice die at birth.[47,48] In contrast, Grem2 is required for atrial development and cardiac laterality in zebrafish,[49] but it appears to be dispensable for mouse development.[50]

BMP signaling in inflammatory processes

Developmental studies in genetic models have cemented the critical roles of BMP signaling throughout embryogenesis. More recent studies have investigated the role of BMP signaling in adult pathophysiological conditions, revealing a broad influence on homeostasis and disease states such as pulmonary hypertension, cancer, and anemia.[51,52] Recent discussions about BMP involvement in other diseases have been reviewed elsewhere.[45,53,54] Here, we will focus on the emerging roles of BMPs as key mediators of a variety of inflammatory conditions.

BMP signaling promotes the inflammatory phenotype of endothelial cells in atherosclerosis

Atherosclerosis has long been viewed as an inflammatory disease.[55–57] Plaque formation begins with initial endothelial dysfunction that leads to accumulation of lipids in arterial intima and infiltration of immune cells. Markers of dysfunction include upregulation of cell surface molecules such as E-selectin, VCAM-1, and ICAM1, which play essential roles in the roll on, adhesion, and recruitment of immune cells.

Typically, inflammatory cytokines such as TNF-α and IL-1 induce cell adhesion receptors in endothelial cells, but they also induce expression of BMP ligands in endothelial cells.[58] Many studies have pointed out that BMPs further promote the inflammatory phenotype of endothelial cells. In particular, BMP4 is selectively expressed in endothelial cells of atherosclerotic lesions in human coronary arteries.[59] Moreover, BMP4 is upregulated in endothelial cells in response to shear stress and increases endothelial expression of membrane adhesion molecules, leading to enhanced leukocyte adhesion.[59,60] Likewise, BMP2 also upregulates expression of adhesion molecules and promotes leukocyte adhesion.[50,61] Some of these pro-inflammatory effects may be further accentuated in diabetes,[58] a known risk factor for atherosclerosis. In addition to enhancing adhesion of inflammatory cells, BMP2 also induces chemotaxis of monocytes, key immune cells implicated in the pathogenesis of atherosclerotic plaques, and impairs their differentiation into anti-inflammatory M2 macrophages.[62] The recruitment of these pathogenic inflammatory cells is further enhanced through BMP-mediated impairment of endothelial barrier function.[63]

Consequently, inhibiting BMP signaling with extracellular protein inhibitors such as the BMP-binding endothelial regulator protein BMPER and Noggin, or with small molecule chemical inhibitors such as DMH1 suppresses vascular inflammation and lessens recruitment of leukocytes.[63–67] Taken together, these studies suggest that BMP signaling promotes vascular inflammation through direct effects on the endothelium and infiltrating leukocytes and that blocking BMP signaling attenuates the inflammatory response. Thus, BMPs and downstream effectors of BMP ligands may be attractive therapeutic targets for managing inflammatory vessel diseases including atherosclerosis.

BMP signaling in vascular calcification

A clinically relevant consequence of chronic inflammation in atherosclerosis is vascular calcification. This results from a highly regulated process where non-osteoblastic cells, including vascular smooth muscle cells (VSMCs) and pericytes, differentiate into osteoblast-like cells that mineralize the vascular matrix in the tunica media through abnormal deposition of calcium phosphate.[68] Highlighting the similarities between calcification and bone formation, recent evidence suggests an important role for BMP signaling in this pathological process.

VSMCs typically exist in the tunica media as quiescent, differentiated cells, and function to maintain vascular tone through their contractile abilities. However, they are also able to enter a synthetic state characterized by proliferation and production of extracellular matrix,[69] and such a transition is accompanied by loss of cell markers associated with contractility.[70] Expressed in calcified human atherosclerotic plaques,[71] BMP2 inhibits proliferation in VSMCs[72,73] and induces loss of contractility markers once growth arrest has been established.[74]

This suggests either a role for BMP2 in VSMCs that is dependent on proliferation status or one that facilitates along a continuum in promoting the transdifferentiation process. Moreover, BMP2 also induces expression of genes in VSMCs that are crucial for osteogenic differentiation and bone formation, including Msx-2 and Runx2.[75–78]

Deficiency in these genes leads to defects in osteoblast differentiation and large defects in both endochondral and intramembranous ossification.[77,79,80] Recent studies confirm the role of Runx2 and Msx-2 in the osteogenic conversion of VSMCs.[77,81] These studies highlight the molecular similarities between the processes of vascular calcification and bone formation, and taken together, strongly posit a role for BMP signaling in vascular calcification. Moreover, studies have shown that BMP inhibition can also attenuate vascular calcification.[67,82–84]

BMP signaling in tissue fibrosis

Another consequence of chronic vascular inflammation involves induction of processes leading to extracellular matrix (ECM) protein deposition in the adjacent tissue. Under normal conditions, this process functions to repair and preserve tissue architecture and functional integrity after injury. However, pathological dysregulation of inflammation can lead to excessive deposition and an aberrant process of tissue fibrosis that impairs normal organ function. A myriad of profibrotic cytokines released by infiltrating leukocytes act to directly activate fibroblasts, upregulate production of the fibrogenic cytokine TGF-β, increase ECM protein synthesis, and reduce degradation of matrix proteins through downregulating expression of matrix metalloproteinases.[85,86]

Because of this intimate relationship between inflammation and fibrosis, canonical BMP signaling may initially play an indirect positive role through promoting the recruitment of infiltrating leukocytes by endothelial cells into the tissue and thus enhancing the magnitude of the profibrotic signal elicited by these cells. Interestingly, within the tissue itself however, BMP7, through a Smad-dependent antagonism of downstream TGF-β signaling,[87] exerts anti-fibrotic effects. While BMP7 is downregulated in pathological fibrosis of organs, its exogenous administration or overexpression is both anti-fibrotic and functionally protective in disease models of chronic kidney disease,[88–94] liver fibrosis,[95] and pulmonary fibrosis.[87,96–98] The differential roles of BMP signaling in tissue fibrosis, from enhancement at the endothelial level through leukocyte recruitment to regulation at the interstitial tissue level through antagonism of TGF-β signaling, reflect not only the specialized roles served by individual BMP ligands, but also points to a built-in regulatory element that modulates the overall effects of BMP signaling in promoting tissue fibrosis through inflammation.

BMP signaling in endothelial to mesenchymal transition

In addition to these molecular processes, endothelial to mesenchymal transition (EndMT) is a cellular process activated in response to inflammation that significantly contributes to the resulting consequences of vascular calcification and tissue fibrosis. In response to inflammatory cytokines, endothelial cells undergo a phenotypic conversion from a differentiated endothelial cell state to a more undifferentiated mesenchymal fibroblast-like cell state. During this process, these cells lose cell-to-cell adhesions and endothelial markers like CD31 and gain mesenchymal characteristics, including ECM protein production and markers like α-smooth muscle actin and fibroblast specific protein 1.[99,100] The resulting multipotent mesenchymal cell is able to differentiate and contribute to multiple different cell types to serve needed functions. Under pathological conditions, it can contribute to the fibroblast population within the tissue to help promote fibrosis in circumstances such as after acute or chronic injury,[101–104] or it can also contribute to an osteoprogenitor cell population that underly the pathophysiology of vascular calcification.[105]

The process of EndMT in the adult is well known to be activated by TGF-β signaling,[99–103] while signaling through the BMP subfamily of ligands, in particular BMP2 and BMP4, is well known to induce EndMT during embryonic development.[35,106] In the adult, these ligands likely function similarly to promote this developmentally active programming. This is supported by *in vitro* evidence demonstrating treatment of cultured endothelial cells with either BMP4 or TGF-β2 induced acquisition of mesenchymal stem cell-like features.[107] Moreover, inhibition of canonical BMP signaling by DMH1 a dorsomorphin analogue which specifically inhibits activation of Smad1/5/8,[108,109] also inhibits EndMT *in vitro*. In addition, BMP6 has also been shown to pathologically promote EndMT in a disease model of cerebral cavernous malformation both *in vitro* and in *in vivo*.[110] Interestingly, BMP7 inhibits EndMT both *in vitro* and *in vivo*.[104,111] The discrepancy can be explained by the observation that an interaction between activated Alk2 and activated Alk5 BMP receptors is necessary for EndMT.[107] Administration of either TGF-β2 or BMP4 activates both receptors to allow an interaction, whereas BMP7 solely activates Alk2, preventing the necessary Alk2-Alk5 interaction. Taken together, these findings demonstrate the important and complex role BMP signaling plays in endothelial function and disease pathology beyond the initial inflammatory response.

BMP signaling regulates the inflammatory response following cardiac ischemic injury

Inflammation is a critical component of tissue repair following acute or during chronic tissue injury. For example in the heart, the systemic inflammatory response following myocardial infarction (MI) allows infiltration of immune cells into the heart to help clear cellular debris, and this acute process is essential to proper tissue healing and repair.[112–116] Our group and others have measured BMP expression in the heart and found that BMP2 is upregulated following MI in mice.[50,117] Our results show that BMP2 is transiently induced during the inflammatory phase and promotes a pro-inflammatory phenotype in endothelial cells[50] (Figure 2). This upregulates expression of adhesion molecules and increases the number of infiltrating inflammatory cells in the heart. Moreover, we found that the acute inflammatory cytokine TNF-α upregulates BMP2 expression endothelial cells and the two work synergistically to promote the inflammatory phenotype in endothelial cells.

BMP2 also upregulates the expression of its own antagonist Grem2 (Figure 2), and consequently, Grem2 expression, but not expression of other BMP antagonists, follows

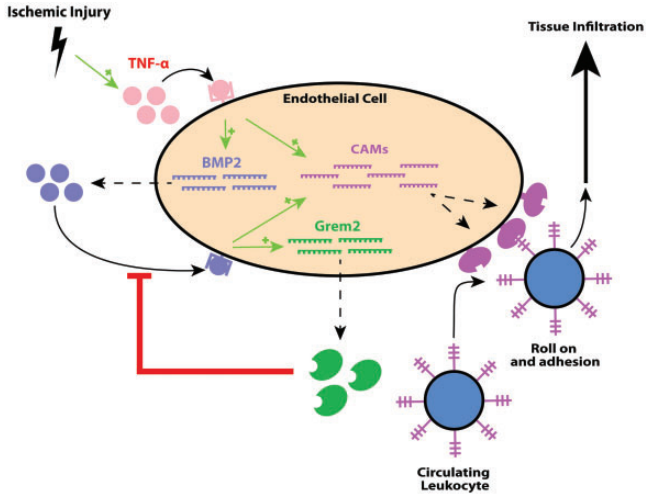


Figure 2. BMP signaling regulates the endothelial inflammatory response after ischemic injury. Ischemic injury induces local TNF-α production which promotes expression of BMP2 in endothelial cells. The two signaling proteins upregulate expression of cell adhesion molecules (CAMs), which enhances recruitment and infiltration of circulating leukocytes into the tissue. BMP2 also induces expression of its antagonist Grem2, creating a negative regulatory loop that regulates BMP activity to limit tissue inflammation.

the increasing levels of BMP2 following MI. This temporal coordination of BMP regulation is important in modulating tissue inflammation as shown by gain or loss of Grem2 function in mice. Specifically, loss of Grem2 function by homologous recombination promoted pro-inflammatory changes in endothelial cells and led to both greater levels of infiltrating inflammatory cells in the heart and worsened functional outcomes following MI.[50] Administering DMH1 rescued this pro-inflammatory phenotype. Moreover, cardiac-specific overexpression of Grem2 limited tissue inflammation and improved functional recovery.[50] These findings build upon earlier studies demonstrating the role BMPs have in promoting vascular inflammation through eliciting changes in endothelial cells that promote leukocyte recruitment. In addition, they introduce endogenous regulatory mechanisms highlighting the crucial role of endogenous BMP antagonists in limiting inflammation and the therapeutic value of regulating BMP signaling.

BMP signaling in chronic inflammatory arthritis

At the intersection of bone pathology and inflammation, studies of rheumatoid arthritis (RA) and ankylosing spondylitis (AS) offer unique opportunities to explore the role of BMPs in inflammatory processes related to a system in which they already have an established presence. The pathogenesis of both diseases involves immune-mediated inflammatory processes. RA is characterized by synovial inflammation, progressive joint damage, and bone loss, while AS is characterized by chronic enthesitis, initial bone destruction, and aberrant bone repair leading to spinal fusion and dysfunction.

BMP signaling appears to be beneficial in RA but not in AS. BMP2 and BMP6 are expressed in synovium from RA patients, and expression in patient-derived fibroblast-like synoviocytes *in vitro* becomes strongly up-regulated in response to pro-inflammatory cytokines IL-1β, TNF-α, or IL-17.[118,119] However, BMP4 expression in synoviocytes decreases in response to TNF-α,[118] aligning with clinical studies observing lower BMP4 mRNA levels in the synovium of RA patients relative to controls.[120]

The pro-inflammatory cytokines IL-17 and TNF-α induce a pro-inflammatory phenotype within synoviocytes marked by increased expression of pro-inflammatory cytokines IL-6 and GM-CSF, increased expression of the chemokine IL-8, and increased production of metalloproteinases MMP2 and MMP3. Expression and activity of these products are strongly implicated in the pathogenesis of RA.[121,122] Blocking BMP signaling with the canonical BMP inhibitor DMH-1 further augments this response and potentiates the induction of the pro-inflammatory phenotype in synoviocytes.[118] On the contrary, inducing BMP signaling with exogenous BMP6 reduced the expression of these products and interfered with the induction of a pro-inflammatory phenotype in synoviocytes.

In an *in vivo* model of AS, however, blocking BMP signaling through systemic gene transfer of the BMP antagonist Noggin slowed the initiation and progression of disease through regulating pathological bone remodeling.[123] The discrepancy in the role of BMP between these two disease states warrants further exploration and may be explained by the inherent differences between the disease pathophysiology of RA and that of AS. Moreover, it is likely that outcomes depend on cellular context, antagonistic effects of specific BMP subgroups such as BMP2/4 against BMP6/7, or differential activation of canonical versus non-canonical BMP signaling pathways. Nonetheless, these studies suggest not only an important role for BMP in well-recognized inflammatory diseases, but also in regulating inflammatory processes that drive disease.

BMP signaling in anemia of inflammation

Regulation of circulating iron is coordinated by several key proteins. As the sole exporter of intracellular iron, ferroportin functions to release intracellular iron in intestinal epithelial cells, hepatocytes, and macrophages into the circulation. An excess in circulating iron stimulates the expression of hepcidin in the liver, and releasing the protein into the circulation allows it to bind ferroportin to induce its internalization and degradation.[124] Likewise, a deficiency in iron also suppresses hepcidin expression. This negative feedback control allows for appropriate circulating iron stores, and perturbations to this system can have clinical consequences. Inflammatory cytokines such as IL-6 that are generated by a variety of diseases, including infection or autoimmune disorders, induce the hepatic expression of hepcidin, and the resulting reduction in circulating iron leads to anemia.[125]

Induction of hepcidin in response to inflammatory stimuli and, thus, iron levels is dependent on BMP signaling. During homeostasis, BMP6 is the predominant BMP ligand that functions in iron homeostasis and transduces its signal through the BMP coreceptor, hemojuvelin.[124,126,127] Expression of BMP6 in the liver is regulated by serum iron levels reflecting dietary intake,[128] and deficiency in

BMP6 leads to hepcidin deficiency and subsequent iron overload in tissues and organs.[129] It appears that other BMP ligands are unable to compensate for loss of BMP6 *in vivo* despite their ability to induce hepcidin expression in hepatocytes in vitro.[130] BMP2, however, may at least play a partial redundant role in inducing hepcidin expression. Hepcidin induction by either acute or chronic iron loading does not require BMP2, although hepcidin induction in either scenario is submaximal and relatively blunted.[131] On the other hand, intact BMP6 function is absolutely critical to hepcidin induction by acute iron loading. Additionally, inhibition of BMP6 but not BMP2/4 significantly decreases hepcidin levels and increases serum iron.[126] Moreover, BMP2 appears to be able to induce hepcidin expression independent of hemojuvelin, though at a lower efficiency.[124]

At the receptor level, the BMP type II receptors ActR2a and BMPR2 perform redundant roles in transducing signals to induce hepcidin expression. Deficiency in both receptors is required to reduce levels of hepcidin *in vivo* and is necessary to reduce BMP6-mediated induction of hepcidin expression as well as basal levels of hepcidin expression *in vitro*.[132] In addition, both of the BMP type I receptors Alk2 and Alk3 are individually necessary for hepcidin induction by BMP2 stimulation in culture and also for hepcidin induction by iron challenge *in vivo*.[133] However, when compared to a liver-specific deletion of Alk2, liver-specific deletion of Alk3 results in a much more severe iron overload phenotype and near abolishment of both basal BMP signaling and hepcidin expression. This suggests differential functions of these receptors with Alk3 individually promoting basal levels of BMP signaling and hepcidin expression, and Alk2 being a necessary component in addition to Alk3 only in the context of promoting hepcidin expression in response to BMP signal transduction and iron. This may be the result of the unique ability of Alk3 receptors to homodimerize and transduce signals for inducing hepcidin expression in the absence of ligand binding.[11] Downstream, Smad4 is also required for mediating hepcidin expression.[134]

The important roles of these BMP signaling mediators during homeostasis are also evident in response to inflammatory stimuli. Deficiencies in either BMP6 or hemojuvelin, and not in BMP2,[131] lead to impaired induction of hepcidin and hypoferremia in response to inflammation compared to normal controls,[124,129] but inflammatory induction of hepcidin persists in isolated or combinatory deficiencies of either when compared to baseline.[135] This suggests that inflammation may induce hepcidin through pathways independent of BMP signaling. Nonetheless, inhibiting BMP signaling with BMP antagonists or LDN-193189, an inhibitor of BMP type I receptor ameliorates anemia of inflammation and iron deficiency in mice.[135,136] These findings are consistent with observations that hepatocyte-specific deletion of BMP type I receptor, Alk3, attenuates phenotype of anemia of inflammation in mice.[137] Taken together, these studies suggest a pivotal role for BMP6 as a regulator of circulating iron both in homeostasis and chronic inflammatory conditions that warrants therapeutic considerations.

Conclusion and future directions

In the recent decade, our understanding of the role of BMP signaling has expanded beyond its initially discovered role in the skeletal system and outside its prominent and crucial roles in development. It is now well-recognized that BMPs are essential to a plethora of different processes throughout the body in the adult both during homeostasis and in disease states. Components of BMP signaling are prominently emerging as direct coordinators of inflammatory processes across various cell types from immune cells to endothelial and connective tissue cells. As the general role of inflammation in disease continues to develop and the intersection between immunology and other medical fields continues to grow, defining the multifaceted roles of BMP signaling in inflammation will allow greater insight into the molecular mechanisms that drive disease.

Already, recent studies have uncovered insight into the nonredundant roles of both specific BMP ligands and BMP antagonists that possess context-specific functions such as BMP6 in the regulation of circulating iron in response to inflammation, BMP2 and BMP4 in mediating inflammatory phenotype of endothelium in response to stress or tissue injury, and Grem2 in limiting post-injury tissue inflammation. This likely reflects the functional heterogeneity that exists among BMP ligands and their antagonists. Thus, special precautions should be taken regarding designing interventions targeting BMP signaling as broad inhibition of all BMP ligands to limit endothelial or tissue inflammation may also perturb iron homeostasis. With the increasing availability of small molecule BMP inhibitors and a recent pre-clinical study successfully utilizing such a compound,[138] it may be worth considering their pharmaceutical value and position to be clinically approved in the future. However, before moving forward into clinical applications, it will be important to design compounds to control specificity for the regulation of only certain BMP ligands to maximize efficacy while minimizing adverse off-target consequences.

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EDITORIAL

Disease Models & Mechanisms in the Age of Big Data

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ABSTRACT

In the decade since Disease Models & Mechanisms was launched, the emergence of Big Data as the main foundation of biological information is having a profound effect on how we do research and it has provoked some interesting questions. Is Big Data exploration replacing hypothesis-driven basic research? And, to what extent is disease modeling in the laboratory still relevant to medical research? Recent examples of synergistic approaches utilizing animal modeling and electronic medical records mining show that combining efforts between disease models and clinical datasets can uncover not only disease etiologies, but also novel molecular and cellular mechanisms linked to gene function.

KEY WORDS: Big Data, Disease modeling, GWAS, Omics

Introduction

In a series of editorial articles in the inaugural issue of Disease Models & Mechanisms (DMM), Vivian Siegel, the first Editor-in-Chief, and members of The Company of Biologists and the Editorial Board laid out how the idea for the new journal was conceived, outlined its scope and expressed their hopes and expectations for the new venture (Siegel, 2008; Freeman and St Johnston, 2008). They envisioned the launch of the new publication as a forum to foster and broaden the interactions between what they considered as two poorly connected scientific silos, biological research and animal models in one, and clinical research and medicine in the other. Much has changed since the start of DMM over ten years ago, for the relationship between biological and clinical research has greatly evolved. Although this partnership faces new challenges, as the volume, complexity and heterogeneity of scientific information continues to expand, it also promises to revolutionize both basic biological knowledge and medical care. Part of this revolution stems from the emergence of Big Data as a centerpiece of scientific discovery, offering an unprecedented opportunity to determine biological and disease mechanisms (Schüssler-Fiorenza Rose et al., 2019).

Big Data come in many different forms, for example whole-genome sequencing, RNA sequencing-based transcriptomics (bulk and single cell), proteomics, metabolomics, Genome-Wide and Phenome-Wide Association Studies (GWAS, PheWAS), and Electronic Medical or Health Records (EMR/EHR). Particularly exciting as discovery tools are EHRs that contain extensive phenotype information, which is accompanied by DNA and/or tissue biobanking and genomic information, such as the Vanderbilt University Medical Center BioVU database, the Electronic Medical Records and Genomics (eMERGE) Network, or the United Kingdom (UK) Biobank (Roden et al., 2008; Gottesman et al., 2013; Sudlow et al., 2015).

The collection, storage and mining of increasingly diverse and complex types of biological information from the general human population, or from patients with well-documented medical records – based on diagnoses organized by ICD9/10 codes and accompanied by laboratory test results, imaging and other procedures – is having a profound effect on modern-day research. This enormous data collection has thrusted humans, as the ultimate experimental model, to not only understand the pathophysiology of different diseases and to develop new therapeutic approaches, but also, rather unexpectedly, to understand basic gene function and biological mechanisms.

This emerging landscape has brought new challenges in the communication and translation of information among different disciplines, demands for new skills, and a need for innovative and sophisticated research tools. It seems that the separate research silos, which the DMM founders saw as a major impediment ten years ago, have been swept away into a vast ocean of information and Big Data, an ocean we must all learn to swim in. We can now sequence all variants in a human genome from all patients and relate them to RNA and protein levels, metabolites, or functional and phenotypic traits. Yet, how to parse, analyze and, most importantly, extract meaningful information from these datasets is not always straightforward. It is evident that Big Data depend on basic biology to understand disease mechanisms. Equally, biology needs Big Data to understand the pleiotropic effects of gene function and generate knowledge that is relevant and consequential in the current scientific climate.

Why Big Data needs biology

In the last decade, GWAS have identified numerous disease-associated loci (Visscher et al., 2017). Yet, using these links to determine disease etiology or mechanisms can be challenging. In many instances, it is hard to identify the candidate gene using GWAS because single-nucleotide polymorphisms (SNPs) are frequently found outside coding sequences in genomic regions surrounded by many genes. Current experience indicates that the closest to the SNP gene is not always the disease-causing one (Smemo et al., 2014). Another limitation may stem from the fact that GWAS typically search for specific phenotypes, potentially missing additional clinical features that share the same genetic cause. Therefore, often one has to know where and how to search in order to untangle disease-causing mechanisms, especially in complex diseases. Biological evidence in animal models may help shape and guide these searches to pinpoint the gene responsible for the phenotype, reveal the full extent of gene pathogenicity, and provide functional validation and mechanistic insights in animal models using gain- or loss-of-function approaches (Unlu et al., 2019a).

From a practical point of view, GWAS and other Big Data investigations that are based on statistical associations rely on Bonferroni corrections or similar methods to improve confidence in

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the results, often requiring extremely low *P* values to accept that an association is real. Although such strict requirements are necessary to accept statistical associations, they can also cause researchers to discard many findings, especially if the relevant cohorts are relatively small, for example in studies aiming to establish the pathogenicity of rare variants (Bastarache et al., 2018). Biological evidence in an appropriate experimental model can provide independent validation of the findings, lowering the high statistical thresholds for acceptance and opening the doors of discovery (Unlu et al., 2019).

Why biology needs Big Data

Although it might be easier to imagine why gene-disease phenotype associations, obtained by statistical means, need wet lab experimental support for validation and mechanistic insights, it is often less obvious how wet lab biology can benefit from Big Data, especially EHRs. Although the use of clinical data to assess the putative significance in human disease of a mechanism that was discovered in an animal model is expected, analyzing clinical records also has the potential to reveal new aspects of gene function that were not picked up in the laboratory.

The 'biology of real life' is more diverse and complex than 'biology in the laboratory'. Animal research is often performed in genetically identical animals (an exception is outbred zebrafish), yielding results within the context of specific gene-gene interactions, and under communal environmental conditions, thus obscuring the pathogenicity of gene malfunction in a population-at-large setting. Although such strictly controlled parameters have traditionally been necessary to perform fundamental research studies, crucial aspects in a disease pathology may also include environmental influences, behavioral patterns, lifestyle, diet, interactions with microbiota or cultural influences, each dictating to various degrees to what extent aberrant gene function contributes to disease initiation, progression or severity. These aspects cannot be easily recapitulated in the laboratory and, therefore, laboratory analyses, no matter how sophisticated, may fail to capture the full phenotypic gamut and pathogenic potential of some genes. On the other hand, these features may be uncovered in large cohorts with clinical or epidemiological data.

Most of the described phenotypes in animal models are usually based on loss-of-gene-function approaches. In human subpopulations, mutations or variants can also be gain-of-function, or exert dominant-negative effects, interfering with the activities of an entire gene family or disrupting the proper function of complex genetic networks; or, certain variants might represent partial loss of function or cause variance in gene expression in specific tissues (Müller et al., 2013; Gamazon et al., 2018; Li et al., 2019). If some of these variants cause relatively mild perturbations in gene activity they likely remain benign, except in cases when the system is stressed, for example by injury, environmental factors or a co-occurring disease like diabetes. Also, most studies in a variety of injury models are more often than not performed in young and otherwise healthy animals without comorbidities. And, although things are gradually improving thanks to current NIH guidelines, most work in the past has been carried out in male animals. Conversely, the effects of sex-differences, aging or comorbidities to disease-contributing genetic alterations might be embedded in large EHR datasets, providing a more comprehensive and complete image of gene function.

Genetic animal models are usually based on alterations in the expression and/or activity levels of a single gene. On the other hand, diseases are often the result of defects in many molecular or cellular components. Furthermore, certain genes, besides generating a recognizable phenotype in a specific organ, might also be associated with a behavioral trait or mood disorder that would likely be missed in fish or mouse models, an association that could be documented in clinical datasets. As a result, the full spectrum of clinically relevant phenotypes associated with malfunction of a gene product may not be captured in an animal model, or they may be simply overlooked because particular phenotypes are not recognized. Adding to that, as information of single-cell RNA-seq data accumulates, it appears that apparently similar cells exhibit functional and phenotypic heterogeneity that may not be evident in a controlled experimental setting, but it may be revealed in a complex population setting. It is likely some of these features will be uncovered in large human-population datasets and EHRs, which offer enormous sample sizes that are not feasible in the laboratory. Newly discovered features in humans could be then further probed for pathomechanisms, with additional tests in available animal models, or guide the generation of new models more suitable to address a specific issue.

Other important aspects of gene (mal)function that may be missed in the laboratory but can be detected in clinical datasets include long-term effects that will not manifest in animal models with short life or observational time spans. On the other hand, analyses in EHR or epidemiological studies can provide longitudinal data to complement or expand animal studies (Schüssler-Fiorenza Rose et al., 2019).

Exploring synergies

Recent elegant studies highlight the tremendous value of exploring synergies between Big Data in EHRs and biological research, especially with the advent of technologies such as CRISPR-Cas9 that have dramatically enhanced the fidelity, precision and efficacy of introducing gene variants into animal models. In one example, using BioVU and other databanks, investigators used EHR-linked genetic samples to discover gene-disease associations that were then validated in zebrafish loss-of-function models. Analysis of mutant zebrafish revealed additional phenotypes that were then verified in human patients by targeted searches among clinical phenotypes (Unlu et al., 2019). In another notable study, whole-exome sequencing in a patient with a serious lymphatic anomaly revealed a variant in *ARAF*, a gene encoding a kinase that acts upstream of the MEK/ERK signaling cascade. Modeling of the variant in human cells and zebrafish revealed overactive MEK/ERK signaling causing abnormal lymphatic morphology. The lymphatic abnormality was rescued in zebrafish with available MEK inhibitors. Treatment of the patient with a MEK inhibitor led to remodeling of the patient's lymphatic system and a remarkable improvement in his medical condition and quality of life (Li et al., 2019).

It appears that we are entering a powerful new symbiotic relationship in which Big Data depend on biological model systems to validate statistical associations, and basic biological research needs Big Data to capture the complexity of gene function outside the well-controlled environment and genetics of the laboratory. Although Big Data cannot replace hypothesis-driven basic biological studies in animal models, taking some of the early success stories into account, it is evident that better integrating the two areas of disease models and Big Data from EHRs, at the technical and personnel level, will uncover disease mechanisms and draw us nearer to fulfilling the expectations of personalized medicine. DMM is poised to play a vital role in accomplishing this goal by nurturing the emerging synergies between clinical and biological research. Off to the next decade!

Competing interests
The author declares no competing or financial interests.

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