Syndecan-4 Prevents Cardiac Rupture and Dysfunction After Myocardial Infarction

Yutaka Matsui, Masahiro Ikesue, Keiko Danzaki, Junko Morimoto, Mami Sato, Shinya Tanaka, Tetsuhito Kojima, Hiroyuki Tsutsui, Toshimitsu Uede

**Rationale:** Syndecan-4 (Syn4), a cell-surface heparan sulfate proteoglycan, has been detected in the infarct region after myocardial infarction (MI), but its functional significance has not been elucidated.

**Objective:** We examined whether and how Syn4 regulates the cardiac healing process after MI.

**Methods and Results:** Although the heart in Syn4-deficient (Syn4−/−) mice was morphologically and functionally normal, Syn4−/− mice exhibited impaired heart function and increased mortality rate as a result of cardiac ruptures after MI. Cardiac ruptures in Syn4−/− mice were associated with reduced inflammatory reaction and impaired granulation tissue formation during the early phase of MI, as evidenced by reduced numbers of leukocytes, fibroblasts, myofibroblasts, macrophages, and capillary vessels, along with reduced extracellular matrix protein deposition in the infarct region after MI. Transforming growth factor-β1-dependent cell signaling was preserved, whereas cell migration, fibronectin-induced cell signaling, and differentiation into myofibroblasts were defective in Syn4−/− cardiac fibroblasts. We also found that Syn4 was involved in basic fibroblast growth factor-dependent endothelial cell signaling, cell proliferation, and tube formation. Finally, overexpression of the shed form of Syn4 before MI creation led to an increase in mortality due to cardiac rupture via its action as a dominant-negative inhibitor of endogenous Syn4 signaling, which suggested a protective role of Syn4 signaling in MI.

**Conclusions:** These results suggest that Syn4 plays an important role in the inflammatory response and granulation tissue formation, thereby preventing cardiac rupture and dysfunction after MI. (*Circ Res. 2011;108:1328-1339.)*

**Key Words:** myocardial infarction | cardiac rupture | endothelial cells | extracellular matrix | fibroblasts

After myocardial infarction (MI), the left ventricle (LV) undergoes a continuum of molecular, cellular, and extracellular responses that result in LV wall thinning, dilatation, and dysfunction. Improper cardiac healing after MI can lead to cardiac rupture or maladaptive cardiac remodeling such as further LV dilatation and dysfunction.1

The cardiac healing process after MI can be divided into 4 phases: (1) death of cardiac myocytes; (2) the inflammatory phase, as evidenced by monocyte and lymphocyte infiltration into the necrotic myocardium; (3) formation of granulation tissue, characterized by the presence of fibroblasts, macrophages, myofibroblasts, new blood vessels, and extracellular matrix (ECM) proteins; and (4) the formation of scar, which is characterized by acellular and cross-linked collagen-rich regions.2 Although the precise molecular mechanisms involved in the cardiac healing process have not been fully elucidated, these successive phases are strictly coordinated by the interaction of cells with the surrounding ECM proteins and growth factors.1

Heparan sulfate proteoglycans exist at the cell surface or as a component of the ECM. Syndecans are the major cell-surface heparan sulfate proteoglycans and function as coreceptors and reservoirs for ECM proteins, growth factors, and chemokines through their heparan sulfate chains, thereby regulating inflammation, wound healing, and tissue remodeling.3,4 Syndecan-4 (Syn4) is a member of the syndecan family and is an important constituent of host defense mechanisms, because it acts as a regulatory receptor that monitors dynamic changes in ECM composition during tissue injury.5,6 Importantly, Syn4-deficient (Syn4−/−) mice exhibited delayed wound repair and impaired angiogenesis in granulation tissue after skin excisional wounding.7 Previously, Syn4 has been...
detected in the infarct region after MI. Therefore, we hypothesized that Syn4 plays an important role in the cardiac healing process after MI. The objectives of the present study were to determine whether Syn4 is important for the cardiac healing process after MI and, if so, to clarify the molecular mechanisms by which Syn4 mediates the cardiac healing process after MI.

Methods
The experimental protocol is depicted in Figure 1A. A detailed description of the methods and materials is available in the Online Data Supplement at http://circres.ahajournals.org.

Mouse Model of MI
MI was created as described previously. In brief, mice were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). Mice were subjected to permanent occlusion of the left coronary artery, followed by recovery for 4 or 28 days.

Ultrasound Cardiographic Analysis and Hemodynamic Measurement
Mice were anesthetized with an intraperitoneal injection of 2.5% tribromoethanol (12 μL/g body weight). Transthoracic ultrasound cardiography was performed as described previously. Two-dimensional targeted M-mode tracings were recorded at a paper speed of 50 mm/s. Hemodynamic measurement was performed with a 1.4F micromanometer-tipped catheter (Millar Instruments, Houston, TX) as described previously.

Histological Examination
All morphometric analyses were performed in a standard manner on 5 transverse sections of each heart. Tissue sections were stained with

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>Syn4</td>
<td>syndecan-4</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>AdSyn4</td>
<td>adenovirus harboring mouse Syn4</td>
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<tr>
<td>AdSyn4Ig</td>
<td>adenovirus harboring an extracellular domain of mouse Syn4 fused with an Fc portion of human IgG1</td>
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<td>FAK</td>
<td>focal adhesion kinase</td>
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Figure 1. Syn4 expression is upregulated after MI. A, Experimental protocol after MI by permanent occlusion of the left coronary artery. B, Syn4 gene expression in the infarct region of sham-operated and infarcted WT mice on the indicated days after MI. *P<0.05 against sham-operated WT mice. n>4 per group. C, Serum levels of Syn4 in WT mice after MI. *P<0.05 against sham-operated WT mice. n>3 per group. D, Representative microphotographs of Syn4 expression in infarcted hearts. The infarct region (middle) and remote region (right) of WT mice at day 3 after MI and the sham-operated normal heart of WT mice (left) were immunostained with anti-Syn4 antibody. Scale bars, 50 μm.
hematoxylin and eosin and Masson’s trichrome or used for immunohistochemistry as described previously.10

Cell Culture
Primary cardiac fibroblasts were isolated from 8-week-old WT and Syn4−/− mice as described previously.11 All experiments were performed on second- or third-passage cells.

Immunoblotting
Immunoblotting was performed as described previously.10 Lysates of cultured cardiac fibroblasts or human umbilical vein endothelial cells and tissue lysates of infarcted hearts were obtained.

Gene Expression Analysis
RNA was isolated from sham-operated or infarcted tissue, and real-time quantitative polymerase chain reaction was performed as described previously.10 Some of the specific primers used in the present study are shown in Online Table I.

Immunofluorescence
Immunofluorescence was performed as described previously.10 Cardiac fibroblasts were plated on fibronectin precoated chamber slides, fixed, and incubated with rhodamine-conjugated phalloidin and a specific antibody for vinculin or α-smooth muscle actin (α-SMA).

Matrigel Tube Formation Assay
Matrigel tube formation assay was performed as described previously10 with some modifications.

Construction of Adenoviruses
Recombinant adenoviruses were constructed, propagated, and titrated as described previously.10 We generated adenoviruses that harbored either mouse Syn4 (AdSyn4) or an extracellular domain of mouse Syn4 fused with an Fc portion of human IgG1 (AdSyn4Hg).

Results
Upregulation of Syn4 at the Infarction Site
To assess the importance of Syn4 in the cardiac healing process, we first examined the kinetics of Syn4 gene expression in the infarct region of the heart of WT mice after MI. Syn4 expression was elevated in the infarct region compared with sham-operated heart. Syn4 expression reached a peak at day 1 and gradually decreased but persisted up to day 14 after MI (Figure 1B). Similar to the gene expression kinetics, serum Syn4 protein levels were elevated immediately after MI (Figure 1C). Immunohistologically, Syn4 protein was barely detected in the sham-operated heart. In contrast, Syn4 was clearly upregulated at the infarct site, where strong inflammatory reactions were present, but not at a remote region (Figure 1D; Online Figure I). To identify the cells that expressed Syn4, we performed immunohistochemical studies in the infarcted heart. Syn4 colocalized with vimentin-positive fibroblasts, F4/80-positive macrophages, and CD45-positive leukocytes in the infarct region of the heart at day 3 after MI (Online Figure II). At day 1 after MI, when Syn4 gene expression peaked, it is reasonable to speculate that the infiltrating inflammatory cells were neutrophils or monocytes. We found that normal splenic neutrophils and monocytes expressed Syn4 (data not shown), which suggests that neutrophils in the infarct region of the heart may also express Syn4. These results suggest that Syn4 was expressed by the majority, if not all, of the fibroblasts, macrophages, and leukocytes in the infarct region and may play a pivotal role in the cardiac healing process after MI.

Absence of Syn4 Leads to LV Rupture After MI
We then subjected WT and Syn4−/− mice to MI by permanent occlusion of the left coronary artery, and survival analysis was performed after MI. Forty (60.7%) of 66 WT mice but only 15 (26.8%) of 56 Syn4−/− mice survived until day 28 after MI, which indicates that Syn4 deficiency led to increased mortality rates after MI (P<0.01; Figure 2A). The cause of death was mainly cardiac rupture, the incidence of which was higher in Syn4−/− mice than in WT mice (Figure 2B). There was no significant difference in mortality due to congestive heart failure or arrhythmia between these mice (Figure 2C). In addition, there was no significant difference in the acute infarct size between WT and Syn4−/− mice 24 hours after MI (Online Figure III).

Syn4−/− Mice Exhibit Cardiac Dysfunction After MI
Results from survival studies showed that the majority of deaths occurred within 6 days after MI in WT and Syn4−/− mice (Figure 2A), which suggests that critical events in cardiac structure occurred in the early phase after MI. Therefore, we examined cardiac function at days 4 and 28, in the early and the remodeling phases of MI, respectively. Ultrasound cardiographic and hemodynamic measurements were performed in the surviving mice at days 4 and 28 after MI, and the results are shown in Tables 1 and 2, respectively. The heart functions of sham-operated Syn4−/− mice were similar to those in sham-operated WT mice.

As expected, LV dilatation and dysfunction occurred at days 4 and 28 in WT mice after MI, as judged by ultrasound cardiography studies (Table I). More specifically, LV end-diastolic diameter and LV end-systolic diameter were increased markedly in WT mice at days 4 and 28 after MI, concomitant with a significant decrease in percent fractional shortening compared with sham-operated WT mice. Importantly, Syn4−/− mice showed more deterioration in LV systolic function, as judged by percent fractional shortening, than WT mice at days 4 and 28 after MI. Furthermore, LV end-diastolic and end-systolic diameters were increased significantly in Syn4−/− mice compared with WT mice at day 28 after MI, which reflects severe LV dilatation and cardiac remodeling in Syn4−/− mice.

Hemodynamic studies (Table 2) showed that LV overload and dysfunction occurred in WT mice after MI, as expected. Specifically, at days 4 and 28 after MI, increased LV end-diastolic pressure and decreased LV +dP/dt and LV −dP/dt were detected in WT mice compared with sham-operated WT mice. Again, LV overload and dysfunction were greater in Syn4−/− mice than in WT mice at days 4 and 28 after MI. LV systolic function was more severely impaired in Syn4−/− mice than in WT mice, because LV +dP/dt was reduced in Syn4−/− mice compared with WT mice at days 4 and 28 after MI.
and 28 after MI. LV diastolic function was also more severely impaired in Syn4−/− mice than in WT mice, because LVEDD was significantly reduced in Syn4−/− mice compared with WT mice. In addition, LV end-diastolic pressure was higher in Syn4−/− mice than in WT mice at days 4 and 28 after MI.

We next performed morphometric analysis by staining heart sections with Masson’s trichrome. At day 28, the infarcted area was replaced with fibrous tissues with little inflammatory cell infiltration in WT and Syn4−/− mice; however, infarct size at day 28 after MI was significantly larger in Syn4−/− mice than in WT mice (Online Figure IV). Taken together, these results suggest that Syn4 plays a protective role in cardiac dysfunction, dilatation, and remodeling after MI.

**Granulation Tissue Formation and Inflammatory Response Are Impaired in Syn4−/− Mice After MI**

Because cardiac dysfunction was unveiled within 4 days after MI in Syn4−/− mice (Tables 1 and 2), we examined the molecular basis of cardiac rupture and dysfunction by histological analysis of the heart in Syn4−/− mice at day 4 after MI. We performed hematoxylin-and-eosin staining and CD31 immunostaining and confirmed that sham-operated Syn4−/− mice were morphologically normal and that capillary density was not different from WT mice at baseline (data not shown).

![Image of Figure 2: Increased mortality rates due to LV rupture and impaired granulation tissue formation after MI in Syn4−/− mice. A, Kaplan-Meier survival curves of WT (n=66) and Syn4−/− (n=56) mice after MI. The difference between groups was tested by the log-rank test. B, Mortality of WT and Syn4−/− mice from ventricular rupture after MI. *P<0.05. C, Mortality of WT and Syn4−/− mice from congestive heart failure (CHF) or arrhythmia after MI. N.S. indicates no significant difference between 2 groups. Heart sections from infarcted WT and Syn4−/− mice at day 4 after MI were immunostained with anti-vimentin, α-SMA, and CD31 antibodies. Quantitative histological analyses of vimentin-positive fibroblasts (D), α-SMA-positive myofibroblasts (E), and CD31-positive capillary vessels (F) in the infarct regions of WT and Syn4−/− mice. *P<0.05. n=7 per group.](https://source.scrib-journals.org/)

**Table 1. Ultrasound Cardiographic Analysis of WT and Syn4−/− Mice After MI**

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<th></th>
<th>Sham</th>
<th>WT (n=22)</th>
<th>Syn4−/− (n=6)</th>
<th>At Day 4 After MI</th>
<th>WT (n=13)</th>
<th>Syn4−/− (n=10)</th>
<th>At Day 28 After MI</th>
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<th>Syn4−/− (n=13)</th>
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<td>%FS</td>
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<td>30.5±1.1</td>
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<td>14.9±0.8†</td>
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<td>9.7±1.1*†</td>
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**WT** indicates wild type; **Syn4−/−**, syndecan 4–deficient mice; **MI**, myocardial infarction; **n**, No. of mice; **HR**, heart rate; **LVEDD**, left ventricular end-diastolic diameter; **LVESD**, left ventricular end-systolic diameter; **DSEPt**, diastolic septal wall thickness; **DPWt**, diastolic posterior wall thickness; and **%FS**, percent fractional shortening.

Values are mean±SE.

*P<0.05 vs sham-operated WT; †P<0.05 vs infarcted WT at day 4 after MI; ‡P<0.05 vs infarcted WT at day 28 after MI.
and Online Figure V). However, we found that the numbers of vimentin- or fibroblast-activating protein–positive fibroblasts, α-SMA–positive myofibroblasts, CD31-positive capillary vessels, and F4/80-positive macrophages were reduced in Syn4−/− mice compared with WT mice at day 4 after MI (Figures 2D–2F; Online Figure VI). To further confirm these findings, we also evaluated the gene expression of α-SMA, CD31, and CD68. In the infarcted hearts of WT mice, gene expression of α-SMA, CD31, and CD68 was upregulated at day 4 after MI compared with sham-operated WT mice, but the expression of these genes was reduced in Syn4−/− mice compared with WT mice (Online Figure VII). Representative photomicrographs are shown in Figure 3 and Online Figure VI. Interstitial fibrosis, as evidenced by blue staining with

Table 2. Hemodynamic Parameters of WT and Syn4−/− Mice After MI

<table>
<thead>
<tr>
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<th>Sham</th>
<th>At Day 4 After MI</th>
<th>At Day 28 After MI</th>
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<td></td>
<td>WT (n=18)</td>
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<td>480±33</td>
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<td>LVP, mm Hg</td>
<td>94±2</td>
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<td>93±2</td>
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<tr>
<td>LVEDP, mm Hg</td>
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<td>11.8±1.1*</td>
</tr>
<tr>
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<td>7907±595</td>
<td>6826±225*</td>
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<tr>
<td>LV −dp/dt, mm Hg/s</td>
<td>7298±311</td>
<td>7385±581</td>
<td>6492±224*</td>
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</table>

WT indicates wild type; Syn4−/−, syndecan 4–deficient mice; MI, myocardial infarction; n, No. of mice; HR, heart rate; LVP, left ventricular pressure; LVEDP, left ventricular end-diastolic pressure; and LV, left ventricular.

Values are mean±SE.

*P<0.05 vs sham operated WT; †P<0.05 vs infarcted WT at day 4 after MI; ‡P<0.05 vs infarcted WT at day 28 after MI.

Figure 3. Impaired granulation tissue formation in Syn4−/− mice after MI. Heart sections from infarcted WT and Syn4−/− mice at day 4 after MI were stained with hematoxylin and eosin (HE) and immunostained with anti-vimentin, α-SMA, and CD31 antibodies. Representative microphotographs are shown. Scale bars, 100 μm.
Masson’s trichrome, was reduced in the infarct region of Syn4\(^{-/-}\) mice compared with WT mice after MI (data not shown). Supporting this notion, immunoblot analysis of infarcted hearts revealed reduced protein expression of immature collagen I (130 kDa) and mature collagen I (70 kDa) in Syn4\(^{-/-}\) mice compared with WT mice (Figures 4A and 4B). These results indicate that the formation of granulation tissue, which is composed of fibroblasts, myofibroblasts, capillary vessels, macrophages, and ECM proteins, was impaired in Syn4\(^{-/-}\) mice after MI. Because ECM proteins play a critical role in the preservation of ventricular integrity and prevention of cardiac rupture, it appears that impaired granulation tissue formation accounts for cardiac rupture and dysfunction after MI in Syn4\(^{-/-}\) mice.

Given that inflammation is a major determinant of the myocardial healing process and that Syn4 has been shown to be a modulator of inflammation, we also examined whether deficiency of Syn4 affected cardiac inflammation after MI by quantifying inflammatory cells. The number of CD45-positive leukocytes within the infarcted myocardium was analyzed in WT and Syn4\(^{-/-}\) mice after MI. As shown in Online Figure VIII, the number of CD45-positive leukocytes was significantly decreased in Syn4\(^{-/-}\) mice compared with WT mice at day 3 after MI. Although the precise role of CD45-positive leukocytes in the cardiac healing process after MI is currently unknown, the reduced inflammatory response could lead to the impairment of the formation of granulation tissue and thereby to impaired cardiac healing process in Syn4\(^{-/-}\) mice after MI.

**Reduced Gene Expression of Cytokines, Chemokines, ECM Proteins, and Proteinases in Syn4\(^{-/-}\) Mice After MI**

To elucidate the molecular mechanisms that account for impaired formation of granulation tissue in Syn4\(^{-/-}\) mice after MI, we examined the gene expression of cytokines,
chemokines, and proteinases that were involved in the cardiac healing process after MI. We found that most genes analyzed in WT mice were clearly upregulated within 2 days and peaked 4 days after MI compared with sham-operated WT mice (Online Figure IX). Therefore, we compared the expression of these genes in WT and Syn4−/− mice at day 4 after MI (Figures 4C–4E). The expression levels of tumor necrosis factor-α, monocyte chemotactic protein-1, and matrix metalloproteinase (MMP)-9 genes were lower in Syn4−/− mice than in WT mice, whereas the expression of MMP-2 was not reduced in Syn4−/− mice (Figure 4C). We also evaluated the gene expression of transforming growth factor-β1 (TGF-β1) and ECM proteins that are implicated in the differentiation of fibroblasts into myofibroblasts or ECM synthesis. Gene expression of TGF-β1, collagen I, and collagen III, but not fibronectin, was reduced in Syn4−/−/infarcts compared with WT infarcts (Figure 4D). In addition, we examined the gene expression of basic fibroblast growth factor (bFGF), fibroblast growth factor receptor-1, stromal cell–derived factor-1, and CXCR4 (C-X-C chemokine receptor type 4) because the bFGF/fibroblast growth factor receptor-1 and stromal cell–derived factor-1/CXCR4 signaling pathways play important roles in cardiac healing after MI. We found that the gene expression of fibroblast growth factor receptor-1 and CXCR4, but not bFGF or stromal cell–derived factor-1, was reduced in Syn4−/−/infarcts compared with WT infarcts (Figure 4E).

We further examined whether the Syn4 deficiency might result in compensatory changes in expression of other syndecans. Transcript levels of syndecan-1 (Syn1), syndecan-2 (Syn2), and syndecan-3 (Syn3) were determined in Syn4−/− and WT hearts at day 4 after MI and in sham-operated hearts (Online Figure X). There was no significant difference in mRNA expression of Syn1, Syn2, and Syn3 between WT and Syn4−/− mice at baseline. Transcript levels of Syn1, Syn2, and Syn3 were increased significantly in WT and Syn4−/−/infarcts 4 days after MI compared with sham-operated hearts, concordant with previous findings. Importantly, Syn4 deficiency led to the decreased expression of Syn3, but not Syn1 or Syn2, compared with WT mice at day 4 after MI. Although the precise role of Syn3 in the process of cardiac healing after MI is currently unknown, it is possible that decreased expression of Syn3 might account for the increased rupture rate and LV dysfunction observed in Syn4−/− mice after MI. Thus, the expression levels of several genes that have been implicated in the cardiac healing process after MI were reduced in infarcted Syn4−/− mice compared with infarcted WT mice.

Cardiac Fibroblast Function Is Impaired in Syn4−/− Mice

Because fibroblasts are important components of granulation tissues, and the number of cardiac fibroblasts was reduced in the infarct region of Syn4−/− mice in vivo (Figures 2D and 3; Online Figure VI), we investigated the effect of Syn4 on various functional aspects of cardiac fibroblasts. Syn4-deficient fibroblasts exhibited reduced migration activity compared with fibroblasts from WT mice in an in vitro wound-healing assay (Figure 5A). Next, because the differentiation of fibroblasts into α-SMA–positive myofibroblasts is regulated by TGF-β1, we determined whether this process was impaired in Syn4−/− mice. Syn4 did not affect TGF-β1–dependent signaling, as defined by phosphorylated SMAD2 or TGF-β1–induced myofibroblast differentiation, or collagen gel contraction (Online Figure XI). Interestingly, we found that Syn4-deficient cardiac fibroblasts had less stress fiber formation, as defined by phalloidin–positive staining, than WT cardiac fibroblasts in response to fibronectin, an important ECM component (Figure 5B, top panel). More importantly, Syn4-deficient cardiac fibroblasts failed to form the focal adhesion complexes (as defined by positive phalloidin and vinculin staining, indicated by arrowheads in Figure 5B) with which actin stress fibers were associated, whereas WT cardiac fibroblasts exhibited the mature focal adhesions associated with actin stress fibers (Figure 5B, arrows). Fibroblast differentiation into myofibroblasts is regulated by ECM proteins such as fibronectin. WT cardiac fibroblasts plated on fibronectin exhibited well-developed α-SMA–positive stress fibers; however, the percentage of α-SMA–positive cells in Syn4−/− cardiac fibroblasts was reduced (Figure 5C). These results suggest that Syn4 is critical for fibronectin-induced formation of stress fibers, maturation of focal adhesions, and differentiation of cardiac fibroblasts into myofibroblasts.

Because cell motility and stress fiber formation are associated with activation of focal adhesion kinase (FAK), Akt, and the small G protein RhoA, we examined the phosphorylation status of these proteins and RhoA activity in cardiac fibroblasts. After fibronectin stimulation, WT cardiac fibroblasts showed increased phosphorylation of FAK and Akt and increased activity of RhoA, whereas the activation of FAK, Akt, and RhoA was reduced in Syn4−/− cardiac fibroblasts (Figures 5D and 5E). To further confirm the importance of Syn4 in fibronectin-mediated kinase activation and RhoA activity and myofibroblast differentiation, Syn4-deficient cardiac fibroblasts were transduced with an adenovirus vector that contained either Syn4 gene or control LacZ gene and were stimulated with fibronectin. Reexpression of Syn4 rescued fibronectin-induced kinase activation and RhoA activity, as well as differentiation of fibroblasts to α-SMA–positive myofibroblasts (Online Figure XII). These in vitro findings were consistent with our in vivo data in which FAK phosphorylation was reduced in the infarcted hearts of Syn4−/− mice compared with the infarcted hearts of WT mice (Figures 4A and 4B). These results suggest that ECM proteins such as fibronectin regulate FAK, Akt, and RhoA activation of cardiac fibroblasts and contribute to increased motility and myofibroblast differentiation and that Syn4 is required for these processes.

bFGF-Induced Endothelial Cell Viability, Proliferation, and Tube Formation Are Impaired by Syn4 Knockdown

It is known that angiogenesis is the critical component of wound healing, and CD31–positive capillary vessels were reduced in Syn4−/− mice after MI (Figure 2F). Thus, we examined whether knockdown of Syn4 affected endothelial cell function using human umbilical vein endothelial cells.
Because Syn4 works as a coreceptor for bFGF via its heparan sulfate chains, and bFGF is one of the critical endothelial cell growth factors, we examined whether Syn4 played a role in bFGF-induced cell proliferation and tube formation. Human umbilical vein endothelial cells were transfected with Syn4 small interfering RNA or control scrambled small interfering RNA and stimulated with bFGF. Stimulation of endothelial cells by bFGF resulted in activated extracellular signal-regulated kinase, and this bFGF-induced extracellular signal-regulated kinase phosphorylation was suppressed by Syn4 knockdown (Figure 6A). Moreover, cell viability and bFGF-induced cell proliferation were impaired by Syn4 knockdown (Figure 6B), and Syn4 knockdown markedly impaired bFGF-induced tube formation (Figure 6C). These data indicate that Syn4 is involved in cell viability, bFGF-induced cell proliferation, and tube formation in endothelial cells, thereby mediating the process of cardiac wound healing via angiogenesis.

Overexpression of an Extracellular Domain of Syn4 Augments Cardiac Rupture After MI

Finally, to address the critical role of Syn4 in cardiac rupture and dysfunction after MI, a replication-deficient adenovirus that harbored a fusion protein of an extracellular domain of mouse Syn4 fused with an Fc portion of human immunoglobulin IgG1 (AdSyn4Ig) or control AdLacZ (adenovirus harboring β-galactosidase) was injected into WT mice 1 day before MI. Serum levels of Syn4Ig were approximately 330 μg/mL at day 7 after inoculation with AdSyn4Ig, which was consistent with the previous report (Online Figure XIII).30 Overexpression of Syn4Ig resulted in an increased mortality rate due to cardiac rupture and impairment of heart function at day 14 after MI compared with AdLacZ-treated mice (Online Figure XIII and data not shown). Cardiac ruptures in WT mice treated with AdSyn4Ig were associated with impaired granulation tissue formation during the early phase of MI, as evidenced by reduced numbers of myofibroblasts and impaired fibroblast migration and fibronectin-induced cell signaling in Syn4−/− mice. A, Migration of cardiac fibroblasts was assessed with the scratch wound migration assay. The number of migrated cells was counted 22 hours after the scratch. HPF indicates high-power field. *P<0.05. Data represent the mean of 3 independent experiments. B, Formation of stress fibers and focal adhesion plaques in response to fibronectin. WT and Syn4−/− cardiac fibroblasts were stained with rhodamine-phalloidin (top; red) or anti-vinculin antibodies followed by fluorescein isothiocyanate-conjugated anti-mouse antibody (middle; green). Merged images are also shown (bottom; yellow). Arrows indicate mature focal adhesions associated with stress fibers, and arrowheads indicate focal complexes at the cell periphery. Scale bars, 10 μm. C, FN-induced fibroblast differentiation into α-SMA-positive myofibroblasts. WT and Syn4−/− cardiac fibroblasts were plated on FN-precoated chamber slides and incubated for 24 hours. Cells were stained with mouse anti-α-SMA antibody. Representative microphotographs are shown (left). Scale bars, 10 μm. Quantitative analysis of α-SMA-positive cells (right). *P<0.05. Data represent the mean of 3 independent experiments. D, Activation of FAK and Akt in cardiac fibroblasts in response to FN. Cell lysates were prepared and subjected to immunoblotting analysis with antibodies specific for the phosphorylated form of FAK (p-FAK), total FAK (t-FAK), phosphorylated Akt (p-Akt), total Akt (t-Akt), and GAPDH. Representative blots are shown (left). Quantitative analysis of p-FAK/t-FAK and p-Akt/t-Akt ratios (right). *P<0.05. Data are from 4 independent experiments. E, Activation of Rho kinase in cardiac fibroblasts in response to FN. WT and Syn4−/− cardiac fibroblasts were plated on FN-precoated dishes. Cell lysates were prepared and subjected to an ELISA-based RhoA activation assay biochemistry kit. Relative RhoA activities are shown. *P<0.05. Data are from 4 independent experiments.
capillary vessels in the infarct region (Online Figure XIV), which suggests that the shed form of Syn4 acted as a dominant-negative inhibitor of endogenous Syn4 signaling.

**Discussion**

The major findings of the present study were as follows: (1) Syn4−/− mice exhibited impaired heart function and increased mortality rate after MI as a result of cardiac rupture. (2) Cardiac rupture in Syn4−/− mice was associated with a reduced inflammatory response and impaired granulation tissue formation, as evidenced by the reduced number of leukocytes, fibroblasts, myofibroblasts, macrophages, and capillary vessels, and reduced ECM protein deposition in the infarct region during the early phase of MI. (3) Cell migration and fibronectin-induced cell signaling and myofibroblast differentiation were defective in Syn4−/− cardiac fibroblasts. (4) Syn4 is involved in bFGF-dependent endothelial cell signaling, cell proliferation, and tube formation. (5) Prophylactic treatment of MI mice with AdSyn4G1g led to the enhanced incidence of ventricular ruptures associated with the impairment of granulation tissue formation.

Cardiac rupture is an acute fatal complication that occurs after MI and accounts for 1% to 6% of deaths.31 Many studies have focused on the mechanistic insights by which cardiac rupture occurs after MI using gene-targeted mice (Online Table II). In those studies, the main causes of cardiac ruptures were reported to be reduced, inadequate, and/or disrupted ECM protein deposition, consistent with the present finding that Syn4−/− mice had reduced expression of collagen I and III (Figures 4A, 4B, and 4D) and a disorganized and immature collagen matrix (data not shown). Because ECM proteins are mainly produced by fibroblasts and myofibroblasts, we hypothesized that alteration of the number and function of fibroblasts and myofibroblasts could result in the cardiac rupture–prone phenotype. In fact, we found that Syn4−/− mice had reduced numbers of fibroblasts and myofibroblasts in the infarct region in vivo (Figures 2D and 2E) and that Syn4-deficient fibroblasts were defective in migration (Figure 5A), consistent with the notion that Syn4 directly regulates some of the basic fibroblast functions.32

It is known that ECM proteins such as fibronectin regulate wound-healing processes, and Syn4 is reported to be a coreceptor for fibronectin.26,32 In this regard, we demonstrated several important roles of Syn4 in fibronectin-induced fibroblast functions. First, fibronectin-induced α-SMA–positive stress fiber formation was impaired in the absence of Syn4 (Figures 5B and 5C), which is consistent with previous reports.26 Second, fibronectin-induced activation of FAK, Akt, and RhoA, all of which are implicated in cell migration and stress fiber formation,27–29 was impaired in the absence of
Syn4 (Figures 5D and 5E). Nevertheless, reduced activation of FAK, Akt, and RhoA in Syn4-deficient fibroblasts was rescued by transduction of the Syn4 gene (Online Figure XII). Syn4 deficiency itself did not reduce the expression of fibronectin gene (Figure 4D) but affected fibronectin-induced cell signaling. More importantly, Syn4 did not affect TGF-β1–dependent SMAD signaling, TGF-β1–induced myofibroblast differentiation, or collagen gel contraction (Online Figure XI); however, TGF-β1 gene expression was reduced in the infarcted heart of Syn4−/− mice after MI (Figure 4D). F4/80-positive macrophages, a major source of TGF-β1, were reduced in infarcted hearts of Syn4−/− mice (Online Figure VI, B and D), which may account for the reduced TGF-β1 expression. Because TGF-β1 plays an important role in the inhibition of cardiac rupture by stimulating ECM synthesis,23 Syn4 might also be involved in maintaining fibroblast and myofibroblast function by providing tissue microenvironments that are favorable for TGF-β production, thus preventing cardiac rupture after MI.

The gene expression level of a key matrix-degrading enzyme, MMP-2,15 was not increased in Syn4−/− mice compared with WT mice after MI (Figure 4C), which suggests that augmented degradation of ECM proteins is not a critical determinant of the cardiac rupture seen in Syn4−/− mice. Another important finding is that Syn4 contributed to angiogenesis after MI (Figures 2F and 3). Because angiogenesis is important for granulation tissue formation,33 reduced angiogenesis in Syn4−/− mice could partly explain impaired granulation tissue formation. Syn4 acts as a coreceptor for bFGF, a major regulator of angiogenesis.43 In the present study, we clarified the mechanism by which Syn4 regulates angiogenesis. We found that Syn4 was required for the bFGF-induced activation of extracellular signal-regulated kinase, cell proliferation, and tube formation in human endothelial cells (Figure 6). In addition, gene expression of the bFGF receptor, fibroblast growth factor receptor-1, was reduced in the infarct region of Syn4−/− mice in vivo (Figure 4E), which suggests that reduced angiogenesis in Syn4−/− mice is due to impaired bFGF/fibroblast growth factor receptor-1 signaling. We also found that expression levels of the tumor necrosis factor-α, monocyte chemotactic protein-1, and MMP-9 genes, all of which function in angiogenesis, were reduced in the infarct region of Syn4−/− mice (Figure 4C). Furthermore, CXCR4 gene expression was also reduced in the infarct region of Syn4−/− mice (Figure 4E). CXCR4-positive endothelial progenitor cells play a critical role in angiogenesis in the ischemic tissue,24 which suggests that reduced gene expression of CXCR4 in Syn4−/− mice leads to impaired angiogenesis. Consistent with this hypothesis, it was shown that Syn4 is a signaling molecule in the stromal cell–derived factor-1/CXCR4 pathway.34

In the present study, we showed that the inflammatory response was impaired in Syn4−/− mice at day 3 after MI (Online Figure VIII). Inflammation is thought to be a major determinant of the myocardial healing process.13 There are many reports showing that an excessive inflammatory response leads to cardiac rupture through the enhanced activation of MMP and the degradation of ECM components after MI.17,35,36 On the other hand, it has been suggested that an optimal inflammatory reaction, including leukocyte infiltration after MI, is also required for the phagocytic removal of dead cells, the release of growth factors, the formation of granulation tissues, and the transition of the infarcted myocardium into reparative fibrosis.37 For example, senescent mice showed suppressed inflammation, delayed granulation tissue formation, and reduced collagen deposition, which led to cardiac dilation, remodeling, and dysfunction after MI.38 Consistent treatment with dalcetrapib, a low-molecular-weight heparin, was shown to result in the delayed recruitment of inflammatory cells, reduced formation of granulation tissue, and increased cardiac rupture after MI.39 Therefore, although an excessive inflammatory response causes cardiac rupture, we believe that a reduced inflammatory response could impair the formation of granulation tissue and abrogate the proper reparative process of interstitial collagens, thereby leading to cardiac rupture in Syn4−/− mice after MI. However, further detailed studies are needed to examine the precise role of inflammation in the cardiac healing process of Syn4−/− mice after MI.

We also found that cardiac function was impaired in Syn4−/− mice after MI (Tables 1 and 2). Syn4 may critically regulate cardiac function in many ways. Impaired angiogenesis may lead to cardiac dysfunction in Syn4−/− mice.40 Myofibroblasts in the infarct area may aid global contractility of the LV in collaboration with the surviving myocytes.41 Moreover, cardiac fibroblasts play an essential role in cardioprotection by producing paracrine factors.42 Thus, reduced numbers of myofibroblasts and fibroblasts may explain the impaired cardiac function in Syn4−/− mice after MI.

To further address the role of Syn4 in cardiac wound healing after MI, we overexpressed soluble forms of Syn4 that consisted of an extracellular domain of Syn4 fused with an Fc portion of human IgG1 by AdSyn4Ig before the creation of MI. Overexpression of Syn4Ig led to a significantly increased mortality rate at day 14 after MI, mainly due to cardiac rupture (Online Figure XIII). This was associated with impaired granulation tissue formation during the early phase of MI, as evidenced by reduced numbers of myofibroblasts and capillary vessels in the infarct region after MI (Online Figure XIV). The present data strongly suggest that the shed form of Syn4 acted as a dominant-negative inhibitor of endogenous Syn4 signaling. A similar dominant-negative function was reported previously in the shed form of the Syn1 extracellular domain.43 Therefore, if we expect to achieve a therapeutic application of Syn4 gene therapy on cardiac rupture and dysfunction after MI, we may have to express the full-length form of Syn4, which ensures Syn4 signaling.

Finally, it is known that many factors, such as age, gender, and strain difference, might affect the rate of cardiac rupture after MI.31,38,44 In this regard, we examined whether the effect of Syn4 deficiency on cardiac rupture after MI was dependent on gender. Although female Syn4−/− mice exhibited a somewhat reduced mortality rate compared with male Syn4−/− mice, there was no significant difference. Nevertheless, female Syn4−/− mice exhibited an increased mortality rate compared with female WT mice (data not shown), which suggests that the effect of Syn4 deficiency overcomes the gender effect. However, further studies are necessary to...
examine whether age and strain of mice affect the incidence of cardiac rupture in Syn4−/− mice. In the present study, MI was created by a permanent coronary artery occlusion; however, the majority of patients with acute coronary disease now receive reperfusion therapy such as thrombolytics and angioplasty. Therefore, an MI model induced by permanent coronary artery occlusion does not properly represent the majority of patients with acute coronary disease and appears to be somewhat anachronistic.45 Cardiac injury protocols affect the cardiac phenotypes, including rupture rates and cytokines,47,48 the role of Syn4 in the cardiac healing process after ischemia and reperfusion involves complex processes mediated by oxidative stress, apoptosis, inflammation, and cytokines,47,48 the role of Syn4 in the cardiac healing process after ischemia and reperfusion should be examined in future studies.

In conclusion, the present study showed that Syn4 plays an important role in the inflammatory response and in granulation tissue formation, thereby preventing cardiac rupture and dysfunction after MI. Manipulation of Syn4 or Syn4-dependent signaling pathways may be a therapeutic approach for cardiac healing after MI.

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Disclosures

None.

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**Novelty and Significance**

**What Is Known?**

- Syndecan-4 (Syn4), a cell-surface heparan sulfate proteoglycan, functions as a co-receptor and reservoir for extracellular matrix proteins, growth factors, and chemokines, thereby regulating inflammation, wound healing, and tissue remodeling.
- Syn4 has been detected in the infarct region after myocardial infarction (MI).

**What New Information Does This Article Contribute?**

- Syn4 plays an important role in the formation of granulation tissue, thereby protecting against cardiac rupture and dysfunction after MI.
- Syn4-deficient cardiac fibroblasts are defective with respect to cell migration and fibronectin-induced cell signaling and differentiation to myofibroblasts.
- Syn4 is involved in endothelial cell signaling, proliferation, and tube formation that is dependent on basic fibroblast growth factor.

One critical event regulating the cardiac healing process after MI is a coordinated interaction between cells and their surrounding extracellular matrix. Heparan sulfate proteoglycans (of which Syn-4 is a member) are an important component of extracellular matrix because they are able to interact with various functional molecules that are known to influence inflammation, wound healing, and tissue remodeling. Although Syn4 has been shown to be present in the infarct region after MI, this is the first study to elucidate its role in cardiac healing in this setting, showing that it provides protection from cardiac rupture and dysfunction. We have clarified the molecular pathways that account for impaired granular tissue formation in Syn4-deficient mice. Specifically, Syn4 is involved in signaling by fibronectin-dependent cardiac fibroblasts and basic fibroblast growth factor-dependent endothelial cells, which suggests that Syn4 acts as a co-receptor for fibronectin and growth factors and regulates granulation tissue formation and the cardiac healing process after MI. The present data further suggest that manipulation of Syn4 or the Syn4-dependent signaling pathway may provide a therapeutic option to promote cardiac healing after MI.
Syndecan-4 Prevents Cardiac Rupture and Dysfunction After Myocardial Infarction
Yutaka Matsui, Masahiro Ikesue, Keiko Danzaki, Junko Morimoto, Mami Sato, Shinya Tanaka, Tetsuhito Kojima, Hiroyuki Tsutsui and Toshimitsu Uede

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Supplemental Material

Supplemental Material and Methods

Mice
All animal experiments were conducted in accordance with the guidelines of an institutional committee at Hokkaido University. C57BL/6 mice were obtained from Japan SLC. Syndecan-4-deficient (Syn4−/−) mice, backcrossed >10 times into the C57BL/6 background, were obtained from the Center for Animal Resources and Development 1 and maintained under specific pathogen-free conditions. Eight- to twelve-week-old male Syn4−/− (n = 123) and age-matched male WT (n = 152) mice on a C57BL/6 background were used in this study. In addition, eight- to twelve-week-old female Syn4−/− (n = 12) and age-matched female WT (n = 12) mice on a C57BL/6 background for gender effect study and eight- to twelve-week-old WT mice (n = 38) on a C57BL/6 background for gene transfer study were used in this study.

Mouse model of MI
Acute myocardial infarction (MI) was created as described previously 2. In brief, mice were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). A rodent ventilator (model 683; Harvard Apparatus Inc) was used with oxygen during the surgical procedure. The pericardial sac was opened, and the left anterior descending branch of the coronary artery (LAD) was visualized and ligated approximately 1.0 - 2.0 mm from the tip of the normally positioned left auricle.

Assessment of acute infarct size
Assessment of acute infarct size in vivo was performed as described previously 2. In brief, mice were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). A rodent ventilator (model 683; Harvard Apparatus Inc) was used with oxygen during the surgical procedure. The pericardial sac was opened, and the LAD was visualized. Ischemia was achieved by ligating the LAD using an 8-0 nylon suture approximately 1.0 - 2.0 mm below the border between left atrium and LV. After 24 hours, mice were reanesthetized and intubated, and the chest was opened. After arresting the heart at the diastolic phase by KCl injection, the ascending aorta was canulated and perfused with saline to wash out blood. To demarcate the ischemic area at risk (AAR), alcian blue dye (1%) was injected into LV cavities. Hearts were excised, and LV was sliced into 1-mm thick cross sections. The heart sections were then incubated with a 1% triphenyltetrazolium chloride solution at 37°C for 10 min. The infarct area (pale), the AAR (not blue), and the total LV area from both sides of each section were measured using Adobe
Photoshop (Adobe Systems Inc.), and the values obtained were averaged. AAR/LV and infarct size/AAR were expressed as a percentage.

**Ultrasound cardiographic analysis and hemodynamic measurement**

Transthoracic ultrasound cardiography (UCG) was performed as described previously. Mice were anesthetized with an intraperitoneal injection of 2.5% tribromoethanol (12μL/g body weight). Two-dimensional targeted M-mode tracings were recorded at a paper speed of 50 mm/s. Left ventricular (LV) end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), diastolic septal wall thickness (DSEP WT), and diastolic posterior wall thickness (DPW WT) were measured. Percent fractional shortening (%FS) was calculated as %FS = \([(LVEDD - LVESD)/LVEDD]\) × 100 to estimate the cardiac systolic function. Hemodynamic measurement was performed using a 1.4-F micromanometer-tipped catheter (Millar Instruments, Houston, TX, USA) as described. Mice were anesthetized with an intraperitoneal injection of 2.5% tribromoethanol (12μL/g body weight).

**Survival analysis**

Survival analysis was performed in WT and Syn4/− mice. During the study period of 28 days, cages were inspected everyday for dead mice. All dead mice were examined for the presence of MI and either pleural effusion or blood clot; the cause of death was classified as either congestive heart failure/arrhythmia or cardiac rupture, respectively. In another experiment, survival analysis was performed in WT mice treated with AdLacZ or AdSyn4Ig. AdLacZ or AdSyn4Ig was injected intravenously 1 day before LAD ligation. During the study period of 14 days, cages were inspected everyday for dead mice. All dead mice were examined for the presence of MI and either pleural effusion or blood clot; the cause of death was classified as either congestive heart failure/arrhythmia or cardiac rupture, respectively.

**Histological examination**

All morphometric analyses were performed in a standard manner on five transverse sections of each heart. Tissue sections were stained with hematoxylin and eosin (HE) and Masson’s trichrome (MT) or used for immunohistochemistry. Immunohistochemical studies were performed as previously described. To amplify the specific signals of the antigen-antibody reaction, we applied a two-step immunohistochemistry procedure with high sensitivity based on labeled polymers. Rat monoclonal antibodies specific for CD45 (BD Biosciences) and F4/80 (AbD serotec, UK), a mouse monoclonal antibody specific for vimentin (Sigma-Aldrich, Inc., MO, USA), and rabbit polyclonal antibodies specific for Syn4 (IBL), CD31 (ab28364, Abcam), and fibroblast activation protein (FAP) (ab53066, Abcam) were diluted 1/50, 1/200, 1/500, 1/100,
1/200, and 1/200 in 10% goat serum in PBS, respectively. The primary monoclonal antibody specific for α-smooth muscle actin (α-SMA) (clone 1A4; Sigma) was biotinylated using the Biotin Labeling Kit-NH₂ (Dojindo Molecular Technologies Inc, MD, USA) and diluted to 5 μg/ml. To count the number of vimentin-, α-SMA-, CD31-, F4/80-, and FAP-positive cells, at least 6 fields derived from each of five transverse sections of the infarct region were quantified. For infarct size analysis at day 28 after MI, the boundary lengths of the infarcted and noninfarcted epicardial and endocardial surfaces were traced after MT staining. Infarct size was calculated as the sum of the endocardial and epicardial scar length divided by the sum of the LV endocardial and epicardial circumferences of all the sections 5.

Cell culture
Primary cardiac fibroblasts were isolated from 8-week-old male WT and Syn4⁻/⁻ mice 6. The heart was cut into pieces. These pieces were treated with 0.1% type II collagenase (Worthington Biochemical) in PBS at 37°C by vigorous stirring. After washing, fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C for 4 hours in a humidified chamber. Attached cells were used as cardiac fibroblasts. All experiments were performed on second or third passage cells. Cells were maintained in DMEM supplemented with 10% FBS and incubated at 37°C in a humidified chamber. Human umbilical vein endothelial cells (HUVECs) (Lonza Cologne AG, Switzerland) were cultured in endothelial growth medium-2 (Takara Bio Inc. Japan).

siRNA transfection
The siRNA sequences were as follows: Syn4 siRNA 5’-AAGGCCGAUACUUCUCCGGAG-3’ and Control scramble siRNA 5’-AAGGCUCUCCGGAGCGAUACU-3’. HUVECs were transfected with 50 nmol of siRNA, using Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, 5 x 10⁵ cells were seeded into each well of a 6 cm dish plate the day before transfection. Culture medium was changed 6 hours after transfection and the cells were further incubated at 37°C for 42 hours.

ELISA
The serum level of mouse Syn4 was measured using an ELISA system, which was established using 10 μg/ml of rabbit anti-mouse Syn4 antibody (IBL) as the capture antibody and 3 μg/ml of biotinylated anti-HS antibody (Seikagaku Kogyo) as the detection antibody 7. A purified fusion protein of mouse Syn4 extracellular domain with human IgG1 Fc portion was used as a standard. The serum level of Syn4Ig was measured using a human IgG-Fc ELISA system (GenWay Biotech, Inc., CA), as previously described 8.
**Immunoblotting**

Immunoblotting was performed as previously described. WT and Syn4−/− cardiac fibroblasts (1 × 10⁶ cells) were plated on FN pre-coated dishes (30 µg/ml) and incubated for 3 hours. Cardiac fibroblasts were lysed in lysis buffer containing protease inhibitor cocktail tablets. HUVECs (1 × 10⁶ cells) transfected with either siRNASyn4 or control scramble siRNA were stimulated with bFGF (10 ng/ml) for 15 min. HUVECs were lysed in lysis buffer containing protease inhibitor cocktail tablets. Lysates of cultured cardiac fibroblasts or HUVECs and tissue lysates of infarcted hearts were obtained by centrifugation at 15,000 rpm for 10 min to remove cell debris. Protein concentration was determined using the BCA Protein Assay Kit (Thermo scientific, Rockford, IL, USA). Protein lysates were separated by SDS-PAGE using a 10–12% gel, and proteins were transferred to nitrocellulose membranes. Membranes were incubated with the following primary antibodies: anti-phospho- Y397- and total-FAK, phospho-S473- and total-Akt, phospho-T202/Y204 and total-ERK, phospho-S465/467 and total-SMAD2 (Cell Signaling Technology), collagen-I (Millipore), and GAPDH (clone 6C5, EMD Chemicals Inc., Darmstadt, Germany), followed by horseradish peroxidase-conjugated secondary antibodies. Membranes were developed using an enhanced chemiluminescence detection system (GE Healthcare UK Ltd., Buckinghamshire, England). GAPDH was used as a loading control. Densitometric analysis was performed using Scion Image software (Scion).

**Gene expression analysis**

RNA was isolated from sham-operated or infarcted tissue using the TRIzol (Invitrogen) reagent and was stored at -80°C. RNA was reverse transcribed into complementary DNA with a first-strand cDNA synthesis kit (GE Healthcare Biosciences), and real-time quantitative PCR was performed using the LightCycler FastStart DNA Master SYBR Green I system (Roche Diagnostics). Some of the specific primers used in this study are shown in supplemental Table 1. The mRNA expression level was calculated by the calibration curve method using LightCycler software version 3. Data were normalized to GAPDH.

**Flow cytometric analysis**

Spleen was aseptically removed from normal mice and cell suspensions were prepared by passage through a 200-gauge stainless steel mesh. Then cells were washed twice in RPMI 1640 medium with 10% fetal bovine serum. They were incubated with a mixture of PE-labeled Syn4 (clone KY/8.2, BD Pharmingen) and fluorescein isothiocyanate conjugated (FITC)-labeled Mac1 (BD Pharmingen), Gr1(BD Pharmingen), or CD14 (BD Pharmingen). Cells were stained with FITC-labeled rat IgG or PE-labeled rat IgG as control. Dead cells were excluded by 7-AAD Viability
Dye (BD Pharmingen). FITC-positive cells were gated, and expression of Syn4 was examined by PE staining. All analyses were performed on a FACSCalibur flow cytometer (BD Biosciences) with FlowJo software (Tree Star) as previously described.9

**Cell viability assay**
HUVECs (1 × 10<sup>6</sup> cells) were suspended in DMEM with 10% FBS and seeded onto 6 cm dishes. After 24 hours, the cells were incubated in DMEM with 2% FBS and transfected with siRNA against Syn4 or control scramble RNA. After 42 hours, cells were stimulated with or without bFGF (10 ng/ml, WAKO) for 48 hours. The adherent cells were washed, harvested with trypsin, and the number of viable cells was counted. Dead cells were distinguished from viable cells by a dye-exclusion test using trypan blue staining.

**Scratch wound migration assay**
Cardiac fibroblast cultures were cultured for 2 days in DMEM with 10% FBS followed by an overnight culture DMEM with 2% FBS. The in vitro scratch wound migration assay was performed as described previously.10 The number of migrated cells was counted immediately and 22 hours after the scratch.

**Cell migration assay**
Cardiac fibroblast cell migration toward 5% FBS was measured as described previously.11 Six fields were counted via light microscopy and averaged for each condition studied.

**Immunofluorescence**
Cardiac fibroblasts (2 × 10<sup>4</sup>) were plated on fibronectin (FN) (30 μg/ml) pre-coated Lab-Tek II chamber slides and incubated for 3 or 24 hours. In another experiment, cardiac fibroblasts (2 × 10<sup>4</sup>) were plated on gelatin pre-coated Lab-Tek II chamber slides and treated with or without human transforming growth factor-β1 (TGF-β1) (10 ng/ml, R&D Systems) for 24 hours. After incubation, samples were fixed in 2% paraformaldehyde followed by permeabilization with 0.1% TritonX-100 and were incubated for 30 min with rhodamine-conjugated phalloidin and a specific antibody for anti-vinculin (diluted 1/400; clone hVIN-1; Sigma) or anti-α-SMA (diluted 1/250; clone 1A4; Sigma). Samples were incubated for 30 min with FITC-conjugated anti-mouse IgG (diluted 1/100; Jackson ImmunoResearch Laboratories, Inc.) and embedded with Vectashield Mounting Medium with DAPI (Vector Laboratories). For the negative control, purified mouse IgG (diluted 5 μg/ml) was used as a substitute for primary antibody. In another experiment, transverse heart sections were stained with rat monoclonal antibodies specific for CD45 (BD Biosciences), F4/80 (AbD serotec), a mouse monoclonal antibody specific for vimentin (Sigma-
Aldrich), or rabbit polyclonal antibodies specific for Syn4 (IBL). These antibodies were diluted 1/50, 1/200, 1/500, and 1/200 in 10% goat serum in PBS, respectively. Mouse and rabbit IgG was used as isotype control. Alexa Fluor 546 goat anti-rat IgG (1/400) for CD45 and F4/80, Alexa Fluor 594 goat anti-mouse IgM (1/400) for vimentin, and Alexa Fluor 488 goat anti-rabbit IgG (1/400) for Syn4 were used as secondary antibodies. Samples were mounted using SlowFade Light Antifade Kit (Molecular Probes), and the fluorescence was observed under a confocal laser microscope (FV1000-D, OLYMPUS).

**Collagen gel contraction assay**

Collagen gel contraction assay was performed using Cellmatrix Type I-A (NITTA GELATIN NA INC., Japan) as described previously. In brief, cardiac fibroblasts (2 × 10⁴) with or without human transforming growth factor-β1 (TGF-β1) (10 ng/ml, R&D Systems) were dissolved in 85% of collagen solution, 10% of 10 × MEM and 5% sodium bicarbonate (7.5% solution) to yield three gels of 2 ml volume containing 5 x 10⁵ fibroblasts. The gel solution (2 ml per well of a six-well culture plate) was allowed to polymerize at 37°C for 30 minutes. Immediately after polymerization, 2 ml of DMEM containing 10% FCS was added to each well; gels were detached from the well and left free floating in the medium. After 72 hours, gel contraction was quantified by measuring the area of the floating gels.

**Matrigel tube formation assay**

HUVECs were suspended in DMEM with 10% FBS and seeded onto 6-cm dishes. After 24 hours, the cells were incubated in DMEM with 2% FBS and transfected with siRNA against Syn4 or control scramble RNA. After 42 hours, cells were harvested, counted, and suspended in DMEM with 2% FBS with or without bFGF (10 ng/ml) and plated on extracellular matrix basement (Matrigel™; Beckton Dickinson). After 24 hours, cells were stained with fluorescence and immediately imaged. The percentage of cell-covered area to total area was measured.

**Construction of adenoviruses**

Recombinant adenoviruses were constructed, propagated, purified, and titrated as described. We generated replication-defective human adenovirus type 5 (devoid of E1) harboring mouse Syn4 (AdSyn4) and the extracellular domain of mouse Syn4 fused with Fc portion of human IgG1 (AdSyn4Ig). Adenovirus harboring β-galactosidase (Ad-LacZ) was used as a control. Cardiac fibroblasts were transduced with AdSyn4 or AdLacZ at 10 MOI. After incubation for 24 hours, cells were washed, incubated with serum-free DMEM for further 24 hours, and used for the experiments.
**Adenovirus-mediated gene transfer in vivo**
The virus purification method used, involving cesium chloride ultracentrifugation, was described previously[^4]. 1 day before LAD ligation, a volume of 200 μl containing 1.0 × 10⁹ PFU of AdLacZ or AdSyn4Ig virus was injected into a tail vein of male WT mice.

**RhoA activity assay**
WT and Syn4⁻/⁻ cardiac fibroblasts (5 × 10⁵ cells) were plated on FN pre-coated dishes (30 μg/ml) and incubated for 3 hours. To measure RhoA activity, cardiac fibroblasts were lysed in lysis buffer containing protease inhibitor cocktail tablets. The lysates were centrifuged at 15,000 rpm for 10 min to remove cell debris, and protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). An equal amount of protein lysate was used, and RhoA activity was measured using an ELISA-based RhoA Activation Assay Biochem Kit (G-LISA; Cytoskeleton, Denver, CO) according to the manufacturer’s instructions.

**Statistical analysis**
Statistical analyses to detect differences between two groups were performed based on the Student’s t test. A value of p < 0.05 was considered significant. Statistical analyses between more than three groups were performed by 1-way ANOVA. Differences of means among groups were evaluated using the Fisher’s project least-significant difference post test procedure for group data, and a probability value of p < 0.05 was considered significant. Data are expressed as the mean ± SEM.
Supplemental Figure III

A

Area at risk (% of LV)

WT    Syn4−/−
N.S.

B

Infarct size (% of AAR)

WT    Syn4−/−
N.S.
Supplemental Figure IV

A

WT

Syn4−/−

MT

500μm

B

Infarct area (%)

WT

Syn4−/−

*
Supplemental Figure VI
Supplemental Figure VII
Supplemental Figure IX
Supplemental Figure X
Supplemental Figure XI
Supplemental Figure XIV

A

α-SMA

AdLacZ

AdSyn4Ig

50μm

B

CD31

AdLacZ

AdSyn4Ig

50μm

C

α-SMA-positive myofibroblasts (cells/mm²)

AdLacZ

AdSyn4Ig

D

Capillary density (vessels/mm²)

AdLacZ

AdSyn4Ig

*
Supplemental Figure Legends

Supplemental Figure I. Upregulation of Syn4 at the infarction site
(A) Representative immunofluorescent microphotographs of Syn4 expression in hearts. The sham operated heart of Syn4−/− mice (left) or WT mice (middle) and the infarct region (right) of WT mice at day 3 after MI were immunostained with anti-Syn4 antibody. Scale bars indicate 50 μm. (B) Syn4 gene expression of the sham operated heart of Syn4−/− mice (left) or WT mice (middle) and the infarct region (right) of WT mice at day 3 after MI. Expression levels were normalized to GAPDH. *p < 0.05. n > 5 per group.

Supplemental Figure II. Syn4 was expressed by the vimentin-positive fibroblasts, F4/80-positive macrophages, and CD45-positive leukocytes in the infarct region of the heart at day 3 after MI
Expression of Syn4 and vimentin (A), F4/80 (B), and CD45 (C) was examined in the infarct region of the heart from WT mice by immunohistochemistry at day 3 after MI. Representative immunohistochemical images and merged images are shown. Note that significant numbers of Syn4 positive cells co-localized with vimentin-positive fibroblasts, F4/80-positive macrophages, and CD45-positive leukocytes in the infarct region of the heart at day 3 after MI. Scale bars indicate 50 μm.

Supplemental Figure III. Acute infarct size between WT and Syn4−/− mice 24 hours after MI
Initial area at risk (AAR; % of LV) (A) and initial infarct size (% of AAR) (B) were assessed by Evans blue dye and triphenyltetrazolium chloride staining 24 hours after MI. N.S. denotes no significant difference between WT and Syn4−/− mice. n > 6 per group.

Supplemental Figure IV. Histological analysis of heart sections from infarcted WT and Syn4−/− mice at day 28 after MI
(A) Representative microphotographs of sections from infarcted hearts of WT and Syn4−/− mice stained with MT. Scale bars indicate 500 μm. (B) Infarct area (expressed as % of total area) at day 28 after MI was analyzed. *p < 0.05. n = 6 per group.

Supplemental Figure V. Number of CD31 positive capillary vessels was not decreased in Syn4−/− mice at baseline
Heart sections from WT and Syn4−/− mice after sham operation were immunostained with anti-CD31 antibody. (A) Representative microphotographs are shown. Scale bars indicate 50 μm. (B) Quantitative histological analyses of CD31-positive capillary vessels in the heart of WT and
Syn4−/− mice. N.S. denotes no significant difference between WT and Syn4−/− mice. n = 4 per group.

Supplemental Figure VI. Number of FAP-positive fibroblasts and F4/80-positive macrophages was decreased in Syn4−/− mice at day 4 after MI
Heart sections from infarcted WT and Syn4−/− mice at day 4 after MI were immunostained with anti-FAP antibody (A and C) or anti-F4/80 antibody (B and D). (A and B) Representative microphotographs are shown. Scale bars indicate 50 μm. (C and D) Quantitative histological analyses of FAP-positive fibroblasts and F4/80-positive macrophages in the heart of WT and Syn4−/− mice. *p < 0.05. n = 6 per group.

Supplemental Figure VII. Gene expression of α-SMA, CD31, and CD68 of infarcted hearts at day 4 after MI
Gene expression of α-SMA (A), CD31 (B), and CD68 (C) in the infarct regions of WT and Syn4−/− mice at day 4 after MI was evaluated by real time PCR. Expression levels were normalized to GAPDH. *p < 0.05. n = 10 in sham-operated WT mice, 5 in sham-operated Syn4−/− mice, 15 in infarcted WT mice, and 9 in infarcted Syn4−/− mice.

Supplemental Figure VIII. Number of CD45-positive leukocytes was decreased at day 3 after MI in Syn4−/− mice
Heart sections from infarcted WT and Syn4−/− mice at day 3 after MI were immunostained with anti-CD45 antibody. (A) Representative microphotographs are shown. Scale bars indicate 50 μm. (B) Quantitative histological analyses of CD45-positive leukocytes in the heart of WT and Syn4−/− mice at day 3 after MI. *p < 0.05. n = 4 per group.

Supplemental Figure IX. Kinetic analysis of gene expression from sham-operated and infarcted WT mice
(A) TNF-α, MCP-1, and MMP-2/9, (B) TGF-β1, collagen-I/III, and FN, and (C) bFGF/FGFR-1 and SDF-1/CXCR4. Total RNA was extracted from the infarct region of sham-operated and infarcted WT mice at days 2, 4, and 14 after MI and was subjected to quantitative real-time PCR. Expression levels were normalized to GAPDH. *p < 0.05 against sham-operated WT mice. n = 6 per group.

Supplemental Figure X. Syndecans gene expression analysis of infarcted hearts
Analyses of in vivo syndecans gene expression in the hearts. Total RNA was extracted from the infract region of sham-operated and infarcted WT and Syn4−/− mice at day 4 after MI and was
subjected to real-time PCR. (A) Syndecan-1, (B) Syndecan-2, and (C) Syndecan-3. Expression levels were normalized to GAPDH. *p < 0.05. N.S. denotes no significant difference. n = 5 in sham-operated WT mice, 5 in sham-operated Syn4−/− mice, 7 in infarcted WT mice, and 5 in infarcted Syn4−/− mice.

Supplemental Figure XI. Intact TGF-β1-mediated cell signaling in Syn4 deficient cardiac fibroblasts

(A) Analysis of TGF-β1-induced SMAD2 activation. Cardiac fibroblasts from WT and Syn4−/− mice were cultured, serum starved for 24 h, and treated with or without TGF-β1 (10 ng/ml) for 24 hours. Cell extracts were prepared, and equal amounts of lysates were subjected to immunoblotting analysis with anti-p-SMAD2 and anti-t-SMAD2 antibodies and horseradish peroxidase-conjugated secondary antibodies. Data are representative of 3 independent experiments. (B) WT and Syn4−/− cardiac fibroblasts were cultured, serum starved for 24 hours, and treated with TGF-β1 (10 ng/ml) for 24 hours. Cells were fixed in paraformaldehyde and stained with a mouse anti-α-SMA antibody and a fluorescein isothiocyanate-conjugated anti-mouse antibody. Representative microphotographs are shown (left). Scale bars indicate 10 μm. α-SMA-positive and total cells were counted to generate percentage values. Quantitative analysis of % α-SMA-positive cells (right). *p < 0.05. N.S. denotes no significant difference. Data were obtained from 3 independent experiments. (C) Collagen gel contraction assay was performed using WT and Syn4−/− cardiac fibroblasts (2 × 10⁴) with or without TGF-β1 (10 ng/ml). The gel was polymerized and detached from the well. After 72 hours, gel contraction was quantified by measuring the area of the floating gels. Representative photographs are shown (left). Quantitative analysis of % gel area (right). *p < 0.05. N.S. denotes no significant difference. Data were obtained from 3 independent experiments.

Supplemental Figure XII. Re-expression of Syn4 rescued FN-induced FAK, Akt, and RhoA activation and myofibroblast differentiation in Syn4 deficient cardiac fibroblasts

WT or Syn4−/− cardiac fibroblasts transduced with AdSyn4 or AdLacZ at 10 MOI were cultured for 24 hours, washed, and further incubated with serum free DMEM for 24 hours. Cardiac fibroblasts (5 × 10⁵) were plated on FN pre-coated (30 µg/ml) culture dishes and incubated for 3 hours. (A) Cell extracts were prepared and equal amounts of lysate were subjected to immunoblotting analysis with antibodies specific for p-FAK, t-FAK, p-Akt, t-Akt, and GAPDH and horseradish peroxidase-conjugated secondary antibodies. Lysates were also used for RhoA activity ELISA assay in B. Representative blots are shown (left). Quantitative analysis of p-FAK/t-FAK ratio and p-Akt/t-Akt ratio (right). *p < 0.05. Data are from 4 independent experiments. (B) Quantitative analysis of RhoA activity is shown. *p < 0.05. Data are from 4 independent experiments. (C)
Some of cells prepared in (A) were also used for immunostaining. Cardiac fibroblasts ($2 \times 10^4$) were plated on FN pre-coated (30 µg/ml) Lab-Tek II chamber slides and incubated for 3 h. Cells were fixed with paraformaldehyde and stained with a mouse anti-α-SMA antibody and a fluorescein isothiocyanate-conjugated anti-mouse antibody. α-SMA-positive and total cells were counted to calculate the percentage values. *p < 0.05. Data are from 3 independent experiments.

Supplemental Figure XIII. Increased mortality rates after MI due to left ventricular rupture in mice treated with AdSyn4Ig

(A) Serum concentrations of Syn4Ig after gene transfer. Expression of Syn4Ig was analyzed in the sera at different times after gene transfer, using ELISA The maximum concentration was obtained at day 3 after the administration of AdSyn4Ig. The serum Syn4Ig concentration decreased, but was still maintained around 330 µg/ml at day 7 after MI. However, the serum Syn4Ig concentration was almost under the detection level at day 14. (B) Kaplan-Meier survival curves of mice treated with AdLacZ (n = 16) or AdSyn4Ig (n = 14) mice after MI. The difference between groups was tested by the log-rank test. (C) Mortality of mice treated with AdLacZ (n = 16) or AdSyn4Ig (n = 14) from cardiac rupture after MI.

Supplemental Figure XIV. Impaired granulation tissue formation after MI in mice treated with AdSyn4Ig

Heart sections from infarcted mice treated with AdLacZ or AdSyn4Ig at day 4 after MI were immunostained with anti-α-SMA (A) and CD31 (B) antibodies. Representative microphotographs are shown. Scale bars indicate 50 µm. Quantitative histological analyses of α-SMA-positive myofibroblasts (C) and CD31-positive capillary vessels (D) in the infarct regions of mice treated with AdLacZ or AdSyn4Ig. *p < 0.05. n = 4 per group.
### Online Table I. Sequences of primers used for real time PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’)</th>
<th>Reverse Primer (3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>FW 5’-ACCACAGTCCATGCCCATCAC-3’</td>
<td>RV 5’-TCCACCACCCTGTGGCTGTA-3’</td>
</tr>
<tr>
<td>FGFR-1</td>
<td>FW 5’-GCGATGTGCTTTTTTGAGT-3’</td>
<td>RV 5’-TCCATTCGTAGACCCCTCTTC-3’</td>
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<tr>
<td>TGF-β1</td>
<td>FW 5’-TGGACCGCAACACGCGATCTATGAGAACC-3’</td>
<td>RV 5’-TGGACCGCAACGCGATCTATGAGAACC-3’</td>
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<tr>
<td>CXCR4</td>
<td>FW 5’-GCAAGTCCAGGGAGGTTGTG-3’</td>
<td>RV 5’-CCAAAGGTAAGCTGTCCAAGGA-3’</td>
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<tr>
<td>TNF-α</td>
<td>FW 5’-ATGGATGCTTCTCTCTCT-3’</td>
<td>RV 5’-TCCCTGGGAAGCTGCTTCC-3’</td>
</tr>
<tr>
<td>MMP-2</td>
<td>FW 5’-AGATCTTCTTCTTCTTTCAGGACCGTT-3’</td>
<td>RV 5’-TCCCTGGGAAGCTGCTTCC-3’</td>
</tr>
<tr>
<td>MMP-9</td>
<td>FW 5’-CAACACACACACACTGAAAC-3’</td>
<td>RV 5’-TCCCTGGGAAGCTGCTTCC-3’</td>
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<tr>
<td>MCP-1</td>
<td>FW 5’-CCCAAGAGGAGTGGGTGC-3’</td>
<td>RV 5’-TCCCTGGGAAGCTGCTTCC-3’</td>
</tr>
<tr>
<td>SDF-1</td>
<td>FW 5’-GAGCCAACGTCAGCTACTTG-3’</td>
<td>RV 5’-TCCCTGGGAAGCTGCTTCC-3’</td>
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<tr>
<td>bFGF</td>
<td>FW 5’-TTCCCAAGGCACACTTCA-3’</td>
<td>RV 5’-TCCCTGGGAAGCTGCTTCC-3’</td>
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<td>Syn4</td>
<td>FW 5’-CAGGGCAGCAACACATTTGA-3’</td>
<td>RV 5’-TCCCTGGGAAGCTGCTTCC-3’</td>
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<tr>
<td>Syn2</td>
<td>FW 5’-GTGGGTGATCGGCTTCT-3’</td>
<td>RV 5’-TCCCTGGGAAGCTGCTTCC-3’</td>
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</table>

**FW, forward primer; RV, reverse primer.**
**Online Table II. Gene-targeted mice susceptible to cardiac rupture after MI**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Cause of rupture</th>
<th>Heart function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II type 2 receptor KO</td>
<td>Reduced fibrosis and collagen gene expression</td>
<td>→</td>
<td>Circulation. 2002;106:2244.</td>
</tr>
<tr>
<td>Factor XIII KO</td>
<td>Reduced number of macrophages and collagen gene expression</td>
<td>→</td>
<td>Circulation. 2006;113:1196.</td>
</tr>
<tr>
<td>Class A macrophage scavenger receptor KO</td>
<td>Loosely distributed collagen fibers and the retention of unprocessed necrotic myocardium</td>
<td>→</td>
<td>Circulation. 2007;115:1904.</td>
</tr>
<tr>
<td>Muscle-specific RING-finger 3 KO</td>
<td>Accumulated FHL2 and gamma filamin</td>
<td>↓</td>
<td>Proc Natl Acad Sci USA. 2007;104:4377.</td>
</tr>
</tbody>
</table>

KO, knockout mice; α-SMA, alpha-smooth muscle actin; ECM, extracellular matrix; →, not altered; ↓, declined; ↑, improved. In all studies, heart function in KO mice was compared with that in wild type mice.