Periostin is essential for cardiac healing after acute myocardial infarction

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Periostin, which is an extracellular matrix (ECM) molecule of the fasciclin family, acts in cell adhesion, migration, and growth in vitro (1–6). In the heart, periostin is expressed at very early stages of embryogenesis; however, it is not detected in the normal adult myocardium, except in the valves (7, 8) and in the case of various heart diseases (9–12).

The early cardiac healing process after acute myocardial infarction (AMI) can be divided into two successive phases: the inflammatory phase and the scar formation phase. In the inflammatory phase, monocytes and lymphocytes infiltrate into the necrotic myocardium, whereas in the scar formation phase, activated interstitial or circulating fibroblasts increase their motility and migrate into the lesion. The activation of TGFβ is important for regulation of this latter process. Myofibroblasts expressing α smooth muscle actin (αSMA) induced by TGFβ are specialized fibroblasts that share characteristics with smooth muscle cells (SMCs). They play an important role in wound healing by synthesizing ECM and exerting strong contraction forces to minimize wound areas (13–16). Regarding the inflammatory phase, recent knockout mouse studies indicated a positive association of inflammatory factors with cardiac rupture or dilation (17–23). However, in the scar formation phase, molecular analysis has been scant, except in respect to TGFβ. To answer two important questions for both cardiologists and basic scientists who are interested in pathological myocardial healing, i.e., what regulates formation of...
the scar phase of an ischemic injury?” and “what is the nature of the factors responsible for the ventricular healing process after AMI?,” we focused on periostin, which is a TGFβ-responding factor (1).

RESULTS AND DISCUSSION
To assess the importance of periostin in the cardiac healing process, we examined the expression of human periostin protein in the myocardial tissue of the left ventricle (LV). No expression of it was observed in the normal myocardium (Fig. 1 A), whereas immunoreactivity indicating periostin was detected in Azan-stained myocardial fibrous areas from a patient with AMI (Fig. 1, B and C), thus suggesting that periostin expression was induced in the infarct regions after AMI. In the fibrous area, strong immunoreactivity of periostin was observed around cardiac fibroblasts expressing αv-integrin, which is reported to be a receptor for periostin (Fig. 1 C) (2, 6). Next, we examined the expression of periostin in mice after AMI caused by left anterior descending artery (LAD) ligation (24). Periostin protein was not observed up to day 2, but became detectable at day 3 in the areas showing inflammatory infiltration (Fig. 1 D). This expression in the infarct LV increased significantly at day 4, and was still present at day 28 (Fig. 1 D and not depicted). To identify the cells producing periostin, we performed RNA in situ hybridization to detect periostin mRNA in the infarct LV wall of mice. Periostin mRNA was mainly expressed in fibroblasts in both the infarct and noninfarct regions after AMI (Fig. 1 E).

To confirm the periostin expression in cardiac fibroblasts, we performed RT-PCR analysis on purified cardiac cells, and these results showed the expression to be mainly in cardiac fibroblasts, but not in cardiomyocytes (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20071297/DC1). Furthermore, these fibroblasts were positive for αv-integrin, as indicated by flow cytometry using cultured cardiac cells (Fig. S1). The mRNA of βig-h3, another fasciclin family member, which is also expressed in the embryonic heart (25), was not observed in

![Figure 1. Periostin expression is induced after myocardial ischemia.](https://www.jem.org/article-pdf/207/4/296/296_296.pdf)

(A–C) Detection of periostin in myocardium from human patients. LV tissue from a patient with alcoholic cirrhosis (A) and from a patient with AMI (B and C). As seen by immunostaining, periostin protein was detected (B, right) in the myocardial area, which was shown to be fibrous by Azan staining (B, left). Arrowheads in B indicate endocardium. (C) Comparison of the expression pattern between periostin (left) and αv-integrin (right) in the fibrous area. (D–F) Periostin is up-regulated after AMI in mice. (D) Immunostaining of periostin after AMI. (E) Expression of periostin (top) and βig-h3 mRNA (bottom) in the infarct LV wall of mice was analyzed by in situ hybridization. The dashed red line shows the infarct border. (F) Expression of spliced variant forms of periostin at various times after AMI. Periostin ΔbΔe is indicated by the asterisk. Bars: (A) 25 μm; (B) 2 mm; (C–E) 50 μm.
the same regions (Fig. 1 E), thus suggesting the AMI-induced expression of fascin family molecules to be specific to periostin.

Because we previously reported that several periostin transcripts exist in human and mouse, caused by alternative splicing at a 3' site (1), we examined the expression of the splice variants in a time course experiment by RT-PCR analysis using three combinations of specific primers (Fig. 1 F). We observed four different isoforms, i.e., Δb (deletion of b domain), Δe (deletion of e domain), ΔbΔe (deletion of b and e domains), and Full (full-length), and we found that the pattern of splicing depended on the time after AMI. Interestingly, one specific spliced form, ΔbΔe (Fig. 1 F, asterisk), was dominantly found as the lowest electrophoretic band in the initial stages (3, 4, and 5 d after AMI), indicating the involvement of ΔbΔe periostin in the early healing stage of damaged tissues. By 28 d, all 4 isoforms were equally expressed. We also confirmed the expression of these isoforms at the protein level, and found the proteolytic modification of periostin during infarct healing (Fig. S1).

To investigate the role of periostin in AMI, we generated periostin−/− mice combined with Cre recombination (Fig. 2 A and Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20071297/DC1). The embryogenesis of periostin−/− mice was apparently normal; and after the birth, the mice appeared to be healthy. The observation of periostin in the developing heart prompted us to thoroughly investigate the heart structure and function in the periostin−/− mice; however, no cardiomyocyte abnormalities were found in the myocardium, valve function, pulsation, or blood pressure in the 10-wk-old mice (Fig. S2 and not depicted), which is consistent with no significant expression in the adult myocardium. We then subjected periostin−/− mice to AMI by LAD ligation. There was no significant difference in body weight or heart rate among −/−, +/−, and +/+ in the normal control condition or after the AMI (Fig. S2, Table S1, and not depicted); moreover, there was no difference in infarct size between the periostin+/+ and −/− mice after AMI (Table S1). However, the survival rate of periostin−/− mice after AMI was significantly lower than that of +/+ mice (17.58 vs. 53.76% at day 10; P < 0.0001; Fig. 2 B), whereas this rate of periostin+/− mice (55%) after AMI was similar to that of +/+ mice. The incidence of mortality in periostin−/− mice, mainly caused by cardiac rupture, which occurred within 7 d, was significantly higher (P < 0.001) than that of +/+ mice: 62/91 (68.1%) in −/− versus 25/80 (31.3%) in +/+ (Fig. 3 C), whereas this frequency of +/+ mice 6/20 (30%) was similar to that of +/+ mice. Thereafter, these survival rates reached a plateau from 8 d up to 4 wk after AMI (unpublished data). To test whether the increased rate of cardiac rupture was caused by abnormal LV wall stiffness, we analyzed the rupture threshold stiffness of the LVs of periostin−/− and +/+ mice 4 d after AMI by conducting an LV distending pressure/rupture threshold study (18). Myocardial tearing was found at the infarct border in all the ruptured LVs, and the mean of the maximum rupture pressure was significantly lower in periostin−/− mice than in +/+ mice after AMI (312.7 ± 3.2 mmHg in −/− vs. 374.3 ± 5.8 mmHg in +/+; P = 0.0008; n = 5), and the mean passive stiffness was also significantly lower in −/− mice than in +/+ mice after AMI (50.26 ± 2.13 mmHg/100 μl in −/− vs. 65.08 ± 2.55 mmHg/100 μl in +/+; P = 0.001; n = 5; Fig. 2 C). In contrast, no significant difference was observed between +/+ control noninfarct mice and periostin−/− control noninfarct mice (maximum rupture pressure was 544.0 ± 6.93 mmHg in −/− vs. 552.7 ± 7.86 mmHg in +/+; P = 0.4546; n = 5; mean passive stiffness was 87.07 ± 4.41 mmHg/100 μl in −/− vs. 88.5 ± 3.14 mmHg/100 μl in +/+; P = 0.5985; n = 5). These biomechanical data indicate that both rupture threshold and passive stiffness in the LV of the periostin−/− infarcted mice were significantly lower than those of the +/+ mice after AMI, suggesting that the periostin−/− infarct LV wall was more susceptible to cardiac rupture by mechanical stress. Although periostin deficiency did not affect heart structure, the circulatory system, or cardiac performance under physiological conditions, periostin induced in the infarct myocardium appears to play a pivotal role in the healing process after AMI.

To confirm the histomorphological stiffness of the wall in periostin−/− mice just escaping from rupture, we performed echocardiography 7 d after AMI, in addition to 1 d for heart tissue evaluation and 28 d for the analysis of chronic cardiac pathophysiology after AMI (Fig. 2 D and Table S1). Echocardiographic measurements made 7 d after AMI showed decreases in left ventricular end-diastolic dimension (LVEDD) and left ventricular end-systolic dimension (LVESD) in periostin−/− mice (n = 10), as compared with these parameters for +/+ mice (n = 15; LVEDD and LVESD values for −/− were 89.0 and 84.4%, respectively, of those for +/+). These results demonstrate that the absence of periostin attenuated ventricular remodeling after AMI. To further examine tissue stiffness histologically, we performed toluidine blue staining, immunofluorescence analysis using anti–collagen I, –fibronectin, and –vimentin antibodies, and transmission electron microscopic (TEM) observation of sections prepared from periostin+/+ and −/− mice 5 d after AMI. The results showed a lower number of cardiac fibroblasts, along with sparser pericellular ECM density in the periostin−/− mice than in the +/+ mice (Fig. 2, E and F); indeed, the number of vimentin–positive cardiac fibroblasts was decreased in the infarct region of periostin−/− mice 5 d after AMI (7,655 ± 148 cells/mm2 in +/+ vs. 6,913 ± 297 cells/mm2 in −/−; n = 6; P < 0.02; Fig. 2 C). Furthermore, reduced collagen I and fibronectin immunoreactivity was observed in the infarct border of the −/− mice (Fig. 2 F and Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20071297/DC1), and the collagen fiber cross-sectional area (CSA) in the infarct border of periostin−/− mice was significantly smaller and more uniform than that of +/+ mice 5 d after AMI (CSA of 1,014.642 ± 17.546 nm2 for the −/− and 2,233.780 ± 25.731 nm2 for the +/+; n = 6; P < 0.001, respectively; Fig. 2 G). To confirm whether periostin deficiency affected the biochemical property of collagen after AMI, we evaluated the amount of collagen (hydroxyproline concentration, percentage of tissue dry weight) and nonreducible mature cross-links (mol pyridinoline per mol collagen) in the infarct zone 4 d after AMI. We detected a significant decrease in the collagen cross-linking in the periostin−/− mice, compared with the +/+ mice.
Figure 2. Cardiac rupture after AMI is caused by periostin disruption. (A) Schema of the targeting strategy deletes the first exon of periostin locus. (B) Decreased survival of periostin−/− mice (n = 91) compared with the survival of +/+ mice (n = 80) after AMI. **, P < 0.0001. (C) Infarct LV wall stiffness was more reduced in periostin−/− mice than in +/+ mice after AMI (left). Mean passive stiffness was also significantly lower in the −/− mice than in the +/+ mice after AMI (right). Open columns, +/+; filled columns, −/−. **, P < 0.005, compared with +/+ mice. (D) Loss of periostin attenuated cardiac dilation after AMI, as shown by echocardiography. Open columns, +/+; filled columns, −/−. *, P < 0.05 compared with +/+ mice. (E) Histological analysis of heart sections from periostin−/− and +/+ mice stained with toluidine blue 5 d after AMI, showing a lower number of cardiac fibroblasts and lower ECM density in −/− mice. (right) The number of vimentin-positive cells. *, P < 0.02, compared with +/+ mice. (F) Images of the infarct border stained with anti-collagen I (left), and TEM images of infarct border, showing evidence of smaller and less abundant collagen in tissues from periostin−/− mice 5 d after AMI compared with the collagen of the +/+ infarct heart. Bar, 50 μm. (G) CSA distribution of collagen fibrils in the infarct border of +/+ and −/− mice, measured from TEM images. (H) Biochemical analysis of the collagen amount and cross-linking. *, P < 0.05; **, P < 0.01, compared with +/+ mice. (I) The number of αSMA-positive cells in the infarct area was reduced in periostin−/− mice 5 d after AMI. **, P < 0.01, compared with +/+ mice. Error bars represent the mean ± the SEM. Bars, 200 μm.
Bars, 200 in the portion. (B) Western blot analysis for Ad-4 d after AMI showed strong expression in the border of the Ad-nls-LacZ–infected myocardial infarct (arrowheads). The arrow indicates the ligated

tissues from mice of either genotype, the collagen amount was under the detection level by our methods (unpublished data), indicating that the detected collagen was newly produced after AMI. In conclusion, we observed the alterations of collagen structure in the periostin−/− mice; they were smaller and more uniform, with the decreased amount and cross-linking of collagen effecting lower stiffness. These results suggest that periostin expression contributed significantly to the amount or cross-linking of newly synthesized collagen, which is essential for the normal mechanical properties of collagen-containing tissues after MI. These findings indicate that impaired collagen fiber formation occurred in periostin−/− mice after AMI. Interestingly, although the total activity of myeloperoxidase and the numbers of Mac-3–positive inflammatory cells, ki67–positive proliferating cells, and active caspase-3–positive apoptotic cells in the infarct border were not significantly different between +/+ and −/− mice (not depicted), we observed a lower number of αSMA–positive cells in the infarct area of periostin−/− mice 5 d after AMI (1,792 ± 193 cells/mm² in +/+ vs. 758 ± 75 cells/mm² in −/−; P < 0.01; n = 6; Fig. 2 I).

However, the number of cells positive for SM1, which is a specific marker of SMCs, was not significantly different, and almost all of the αSMA–positive cells were SM1 negative (unpublished data). These results indicate that not the inflammatory cell recruitment, but rather the recruitment of cardiac fibroblasts in the infarct region, was impaired in these animals.

To determine whether the impaired cardiac healing in response to AMI could be restored by periostin directly, we performed a rescue experiment by using Ad-ΔbΔe, which is the main periostin isoform detected early after AMI. The periostin−/− mice were treated with a recombinant adenovirus expressing periostin (Ad-ΔbΔe) or with a control adenovirus (Ad-nls; nuclear localization signal-LacZ). In the control experiment, the Ad-nls-LacZ transfer was detected in the infarct border at 4 d after AMI by whole-mount X-gal staining, proving the experimental feasibility (Fig. 3 A). In periostin−/− mice infected with Ad-ΔbΔe, we first confirmed expression of transferred periostin in the infarct tissue by immunoblot and immunofluorescence analyses (Fig. 3 B and Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20071297/DC1), and then observed an increase in the area reactive with anti-αSMA antibody compared with that area of the control Ad-nls-LacZ−infected periostin−/− mice (597 ± 107 cells/mm² in Ad-nls-LacZ−infected −/− mice vs. 1,535 ± 197 cells/mm²

Figure 3. Adenovirus-mediated periostin ΔbΔe gene transfer prevents cardiac rupture in the periostin−/− mice. (A) Whole-mount X-gal staining 4 d after AMI showed strong expression in the border of the Ad-nls-LacZ–infected myocardial infarct (arrowheads). The arrow indicates the ligated portion. (B) Western blot analysis for Ad-ΔbΔe–infected periostin−/− infarct LV. (C) Infection with Ad-ΔbΔe reversed the high incidence of cardiac rupture in the periostin−/− mice to a lower level, comparable to the incidence in the +/+ mice. *, P < 0.02; **, P < 0.001, compared with control Ad-treated −/− mice. (D) Compared with the Ad-nls-LacZ–infected periostin−/− hearts, the Ad-ΔbΔe–infected hearts increased the number of αSMA–positive cells 5 d after AMI (right) the number of αSMA–positive cells. **, P < 0.01, compared with the mock infection of the −/− mice. Error bars represent the mean ± the SEM. Bars, 200 μm.
Akt was reduced, and only a small amount of phosphorylated FAK was detected in the border of the periostin+/− mice (Fig. 4, A and B, and Fig. S5).

To further investigate the role of periostin in FAK activation and cell motility, we performed immunofluorescence staining for phosphorylated-FAK and rhodamine-phalloidin staining for the actin cytoskeleton in an embryonic mesenchymal cell line, C3H10T1/2, treated or not with periostin ΔβΔε. The presence of periostin ΔβΔε changed the cytoskeletal arrangement and motility of the cells, resulting in dynamic protrusion of their processes (Fig. 4C). In a time-course

Figure 4. Periostin promotes cell migration through integrin-mediated FAK signaling. (A) Phosphorylation of FAK in infarct LV from periostin+/+ mice and −/− mice 5 d after AMI. (B) Immunofluorescence for phosphorylated FAK (p-FAK Y397) in the border of infarct LV from periostin+/+ mice and −/− mice 5 d after AMI. Merged images show an overlay of p-FAK Y397 (green) and propidium iodide–stained nuclei (red). The dotted line shows the infarct border. NIZ, noninfarct zone; IZ, infarct zone. (C and D) Promotion of cell spreading and activation of FAK phosphorylation in vitro. The morphology of starved C3H10T1/2 cells was analyzed by immunofluorescence 12 h after adding periostin ΔβΔε (C), and the p-FAK Y397 was examined by Western blot analysis at various times after adding periostin ΔβΔε (D). In C, the merged images show an overlay of p-FAK Y397 (green) and rhodamine-phalloidin (red), and the arrows point to FAK phosphorylation sites. The insets show higher magnification of the cell processes. (E) Chemotaxis of primary cardiac fibroblasts from periostin−/− mice in the absence (mock) or presence of periostin ΔβΔε, detected by an in vitro cell migration assay. Cardiac fibroblasts were significantly activated by periostin ΔβΔε, and treatment with neutralizing antibodies against periostin and αv-integrin, PP2, or FAK siRNAs reduced the cell migration. **, P < 0.001 vs. periostin ΔβΔε. Error bars represent the mean ± the SEM. (F) Periostin can stimulate FAK and Akt phosphorylation through integrin signaling. Starved C3H10T1/2 cells were incubated for 1 h with periostin ΔβΔε with or without each siRNA or the FAK and Akt inhibitors. Bars: (B) 100 μm; (C) 20 μm.
experiment, periostin ΔbΔc continuously activated the phosphorylation of FAK for 9 h after the addition of it to serum-starved cell cultures, whereas in the control, the signal had decreased by 6 h (Fig. 4 D). These results demonstrate that periostin ΔbΔc activated FAK phosphorylation and promoted formation of dynamic protrusions. Next, we tested the motility of primary cardiac fibroblasts from periostin+/− mice in the presence of periostin ΔbΔc. The result showed that periostin ΔbΔc strongly activated the cell migration of these fibroblasts (Fig. 4 E). Moreover, this migration caused by periostin ΔbΔc was significantly reduced by antibodies against either periostin or αv-integrin; by PP2, which is known as a compound that specifically inhibits adhesion-induced FAK phosphorylation (28); and by knockdown of FAK by siRNA (Fig. 4 E), suggesting that periostin ΔbΔc would activate the cell motility of their fibroblasts by FAK signaling through αv-integrin in mice subjected to AMI. Finally, we inhibited the integrin-mediated FAK pathway by using chemical compounds and siRNAs (Fig. 4 F). FAK inhibitors or siRNA down-regulated the Akt phosphorylation, and Akt inhibitors did not change FAK phosphorylation after stimulation by periostin ΔbΔc, indicating that Akt is a downstream molecule of FAK and periostin ΔbΔc. Moreover, αv-integrin siRNA treatment blocked both FAK and Akt phosphorylation after stimulation by periostin ΔbΔc. These results indicate that periostin ΔbΔc can stimulate FAK and Akt phosphorylation through αv-integrin.

We demonstrated that in the case of periostin deficiency, the collagen amount was reduced in the infarct myocardium, resulting in frequent cardiac rupture in the AMI. Our results, together with the previous findings by Norris et al. (7) on the role of periostin in collagen fibrillogenesis of skin and tendon, strongly suggest that fibrillar collagen formation, which contributes essentially to a mechanically stable scar formation, was impaired in the early stage of MI in the periostin deficiency, resulting in a high rate of cardiac rupture. Furthermore, we have found that the reduced mechanical strength, rupture of the infarct region, and repression of LV dilation in periostin deficiency were most likely caused by a reduced number of cardiac fibroblasts and by the insufficient creation of a durable collagen network caused by a lower rate of collagen synthesis and cross-linking. To reveal more about the importance of collagen production or collagen cross-linking for protection against heart rupture, after AMI, we treated mice with an inhibitor of lysyl oxidase, thus inhibiting collagen cross-linking. Interestingly, the data showed a high amount of collagen production with a larger number of vimentin-positive cells in the infarct region, resulting in effective blockage of heart rupture (unpublished data). These data suggest that periostin-stimulated migration of cardiac fibroblasts into the infarct region, the cells of which produce a high amount of collagen, is more essential than collagen cross-linking by periostin.

The expression of TGFβ was markedly up-regulated in the infarct border during the scar formation phase after AMI, and the phosphorylation of smad 2/3 was consequently increased (unpublished data), whereas there was no significant difference in the TGFβ transcription level between periostin+/+ and ΔbΔc mice; TGFβ also enhanced the periostin expression in the infarct border after AMI because anti-TGFβ antibody treatment blocked the periostin expression (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20071297/DC1). The expression of both TGFβ and periostin is up-regulated by angiotensin II and attenuated by angiotensin receptor blockers after AMI (29, 30), suggesting that periostin may play a role via angiotensin II–TGFβ signaling. The combined results on the biomechanical properties and the collagen content of the isolated infarct heart support the concept that the periostin-linked collagen fibrous skeleton is an important determinant of cardiac rupture.

The results given here indicate that periostin signals activate cell migration of cardiac fibroblasts from outside into the infarct region through FAK phosphorylation, and then the migrated cells differentiate into αSMA-positive fibroblasts, resulting in strengthening of the stiffness of the LV wall through collagen synthesis after AMI. FAK is known to be involved in tyrosine phosphorylation during integrin-mediated signaling, and this molecule plays an important role in the response of migrating cells to mechanical stress (31). Recently, FAK has been implicated as a downstream target associated with angiotensin II–stimulated cell migration (32). The mechanism underlying the periostin action of promoting the recruitment of cardiac fibroblasts followed by healing of the infarct region appears to involve activation of the FAK pathway, indicating that the periostin–induced increase in FAK phosphorylation in the infarct myocardium enhanced the motility of these fibroblasts. In contrast, three-dimensional culture studies imply that the matrix stiffness regulates cell fate by modulating integrin signaling (31, 33). Considering these accumulated results, we suggest that periostin is mainly produced by fibroblasts through angiotensin II–TGFβ signaling and may convey pathologically rapid reinforced mechanical signals to FAK-integrin signaling after AMI. The fibroblastic cells activated by these signals secrete periostin, which in turn increases their motility, contractility, and synthesis of ECM proteins, thus promoting further recruitment and activation of fibroblasts. Periostin may serve as the trigger of these feedback mechanisms in the ongoing healing processes. Additional studies to elucidate in more detail the characteristics of cardiac fibroblasts may lead to a deeper understanding of the role of periostin after AMI, as well as aid in identifying the molecular targets of therapies to augment cardiac performance and wall stiffness after AMI.

MATERIALS AND METHODS
Preparation of rabbit polyclonal antibodies against periostin. We raised polyclonal RD1 antibodies against periostin by using the peptide DNAiLSDiDIRGLESNViN, representing aa 143–158 of human periostin) for human periostin and the peptide ENLDSDiIRGLESNViN, representing aa 145–160 of mouse periostin) for mouse periostin. The antibodies were affinity-purified by using the respective immunogenic peptide.

Histology, immunostaining, and electron microscopy. Human tissue samples were obtained during autopsy and fixed in 4% neutral formalin or 20% formalin. A total of 41 cases, ranging from a fetus to an 89-yr-old patient, including 15 cases of myocardial infarction, were examined. All the cases were approved for use in research by the Ethics Committee of the...
University of Tokyo. After having been embedded in paraffin, specimens were cut at a 4-μm thickness. Hematoxylin and eosin, elasta von Gieson, and Azan staining procedures were performed. Immunohistochemistry by the ABC method was done by using an i6000 apparatus (Biogene). For histological analysis of the infarcted mice, the animals were killed at 1, 2, 3, 4, 5, 7, 14, or 28 d after surgery under anesthesia, and were perfusion fixed with 4% paraformaldehyde at physiological pressure. Fixed hearts were sectioned transversely into three equal segments from their apex to base and cryoembedded or embedded in paraffin. 4-

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SUPPLEMENTAL MATERIALS AND METHODS

Gene targeting
BAC clones for the periostin gene were isolated from a mouse C57BL6/J BAC library. Targeting strategies are shown in Fig. 2 A. Genomic organization of the periostin locus and structure of the targeting vector. The first exon and the PGK-neo cassette are depicted as a closed box and an open box, respectively. The loxP sites are depicted as closed large arrowheads. A diagram of the targeting construct designed to replace the EcoRI–XbaI fragment containing the first exon in the wild-type allele (+) with a PGK-neo cassette is shown, as is the predicted targeted allele (Neo) obtained by homologous recombination. The PGK-neo cassette was then deleted by using the Cre-loxP system. The predicted targeted allele (−) obtained by Cre recombination is also shown. Two homologous fragments of 7.3 kb (XhoI–EcoRI) and 1.2 kb (XbaI–BglII) were subcloned into the PGK-Neo–PGK-DT-A cassette. The linearized vector (50 µg) was electroporated into TT2 ES cells as previously described (1). Among the G418-resistant ES clones examined, 2 clones, #51 and #1051, were detected as homologous recombinants by PCR using a neo-specific primer, PGK–R, and a periostin genomic primer, Peri–R4. The sequences for these primers were 5′-CTAAGGGCATGCTCCAGACT-3′ and 5′-GCACCTGCTTCTTCCAAATTACAGG-3′, respectively. These clones were further verified by Southern blot analysis.

Generation of periostin-deficient mice. The aggregation method was used to generate chimeric mice (2). Germline chimeras were obtained by using the ES cell clones #51 and #1051. Chimeric mice with a high contribution of the TT2 genetic background (monitored by agouti coat color) were bred with ICR mice. The #51 chimeric mice were crossed with CAG-Cre mice (3) to excise the neo cassette, and these founder mice were then bred with C57BL/6 mice. For the studies described here, mice carrying periostin (−) alleles were backcrossed to C57BL6/J mice for at least 6 generations.

For genotyping of periostin+/− mice and periostin−/− mice, PCR with specific primers from within an intron of the periostin gene was used. The following primer pairs were used for amplification of the periostin genomic locus: sense 5′-GTCTTTACAGAAAGCAGAAGGATACT-3′ and antisense 5′-TTAAAACTCCTCAGACACAGAGACAG-3′ for detection of wild-type periostin, and sense 5′-CATGATAGCTTCTCCTTCCAGGTCTCC-3′ and antisense 5′-CTTGCAATAAGTAAAA-CAGCTTCCC-3′ for that of periostin−/−, as shown in Fig. 2 A (small arrowheads).

Genomic Southern blotting. 10 mg of genomic DNA extracted from normal or targeted ES cell clones was digested with BamHI. The digested genomic DNAs were separated on a 1% agarose gel and transferred onto a Biodyne PLUS membrane (Pall Corporation). Probe labeling and detection of chemiluminescent signals were performed by using a Gene Images Random-Prime Labeling and Detection System (GE Healthcare).

RNA in situ hybridization. Antisense and sense cRNA probes were prepared by in vitro transcription of mouse periostin cDNA (from Fas1 repeat domain 1 to domain 4) and mouse βig-h3 (full-length), by using a DIG Labeling MIX (Roche). Paraffin-embedded, paraformaldehyde-fixed sections (4 µm) were treated with 0.1 M HCl for 20 min, followed by 2×SSC for 5 min. After immersion in proteinase K buffer without enzyme, the sections were digested with 10 mg/ml proteinase K for 15 min, and then treated with 2 mg/ml glycine in PBS. After having been washed with PBS, they were treated with 0.1 M triethanolamine, pH 8.0, for 5 min and then with 0.1 M triethanolamine/0.25% acetic acid anhydride for 10 min. After that, to block endogenous peroxidase activity, we incubated the sections with 3% H2O2 for 1 h and washed them with PBS. Next, they were prehybridized for 1 h at room temperature with hybridization buffer composed of 50% formamide, 5×SSC, 10% dextran sulfate, and 250 µg/ml yeast tRNA, and subsequently incubated overnight at 58°C with hybridization buffer containing DIG-labeled antisense or sense probes (200 ng/ml). After hybridization, the sections were rinsed twice in 5×SSC for 15 min at 58°C, and incubated twice (15 min each) in RNase buffer composed of 10 mM Tris–HCl, 500 mM NaCl, and 1 mM EDTA at 37°C. To remove unhybridized RNA, we incubated the sections with 40 µg/ml RNase A in RNase buffer at 37°C for 30 min, and then sequentially washed them, once in RNase buffer at 37°C for 15 min, twice in 0.2×SSC at 58°C for 20 min, and once in 0.2×SSC for 20 min at room temperature. In situ hybridization signals were detected immunohistochemically with horseradish peroxidase–conjugated anti–DIG antibody (Roche) after blocking with 0.5% casein in TBS for 20 min. Signals were visualized with a Tyramide Signal Amplification system (PerkinElmer). The specimens were counterstained with Mayer’s hematoxylin, dehydrated, cleared, and coverslipped.

RT-PCR. To determine the expression of periostin in the infarct region after AMI, we purified mRNAs from this region 0, 1, 2, 3, 4, 5, 6, 7, 14, and 28 d after AMI and performed RT-PCR. We also analyzed mRNAs from purified cardiac fibroblasts or purified cardiomyocytes derived from the heart tissue by RT-PCR. For amplification, the following specific primers (shown in Fig. 1 F) were used: primer 1 forward (P1F), 5′-GATAAAAATACATCCAAATCAAGTGTTC-3′; primer 1

Shimazaki et al., http://www.jem.org/cgi/content/full/jem.20071297/DC1
Ligation of left coronary artery. Induction of AMI was performed as previously described (4). In brief, during anesthesia, 8-wk-old male mice were intubated and connected to a rodent ventilator (SAR-830AP; CWE Inc.). A median thoracotomy was performed, and the left anterior descending artery was identified. Afterward, a 7-0 nylon suture was passed around the artery, and subsequently tied off. Infarction was evident from discoloration of the LV. Finally, the chest wall was closed. Physiological measurements and histological and biological analyses were performed only on surviving mice. Using mid-part sections from at least 5 mice, we determined the infarct size by the previously described method (4).

Echocardiographic and hemodynamic measurements. The echocardiographic system was equipped with an 11 MHz transducer (EnVisor M2540A; Philips). Two-dimensional short-axis views of the LV were obtained. M-mode tracing was recorded through the anterior and posterior LV walls. LV diastolic and systolic internal dimensions (LVEDD and LVESD) were measured. LV percentage of fractional shortening (FS) was calculated as ((LVEDD − LVESD)/LVEDD) × 100.

LV distending pressure/rupture threshold study. Periostin+/+ and −/− mice were killed 4 d after AMI, and the infarcted hearts were harvested. The rupture threshold stiffness of LVs was determined as previously described (5).

Myeloperoxidase assay. A myeloperoxidase assay, which is an index of neutrophil infiltration in the infarct hearts, was performed as previously described (6). Infarcted hearts were weighed and frozen at −70°C, and 50 mg of the frozen tissue was sequentially homogenized in 1 ml of HTAB buffer (0.5% of HTAB and 50 mM NaHPO₄, pH 5.4), sonicated, subjected to 3 rounds of freezing/thawing, and then centrifuged at 20,000 g for 5 min. 10 µl of supernatant was transferred into a flat-bottomed 96-well plate, and 200 µl of O-dianisidine hydrochloride solution (1.76 mg/ml of O-dianisidine in 50 mM NaHPO₄, pH 5.4) was added immediately. After incubation, the absorbance was measured at 450 nm.

Cell culture. Primary adult ventricular cardiac fibroblasts were isolated from periostin+/+ and −/− mice and maintained as previously described (7). C3H10T1/2 cells were grown in DME supplemented with 10% FNS (Invitrogen). Cells were transfected with siRNA oligonucleotides at 50 nM (SMARTpool; Dharmacon) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The accession numbers of SMART pool siRNA oligonucleotides were as follows: L-041099-00 for fak and L-046779-01 for αv-integrin. SH-6 (10 µM; an Akt inhibitor), PP2 (10 µM; a Src and FAK tyrosine kinase inhibitor; Calbiochem), Akt 1/2 inhibitor (10 µM; Sigma-Aldrich), or Geneticin (10 µM; a tyrosine kinase inhibitor; Fujicco) was added 30 min before stimulation.

Western blotting. PVDF membranes were probed with antibodies against phospho-Akt (Ser 437), total Akt (Cell Signal- ing Technology), phospho-FAK (Tyr 397; Biosource), total FAK (BD Biosciences), β-actin (Sigma-Aldrich), or mouse periostin (our laboratory).

In vitro cell migration assay. Cardiac fibroblasts from 8-wk-old periostin+/− mice were harvested and washed twice in serum-free DME containing 0.1% BSA (DME-BSA). Final volumes were adjusted for all cell preparations to be 1.0 × 10⁶ cells/ml. 2.5 × 10⁴ cells were added to each upper chamber of a 24-well transwell chamber (BD Biosciences) and allowed to migrate from DME-BSA– (upper chambers) to DMEM-BSA–containing medium conditioned by periostin Δβ∆ε– or empty vector-transfected HEK293T cells (lower chambers). As inhibiting molecules, 10 µg/ml anti-periostin (R&D Systems) and 10 µg/ml anti-αv-integrin (CHEMICON International, Inc.) antibodies, 10 µM PP2, or 50 nM siRNAs were used. After incubation for 24 h, migrating cells were fixed and stained with Giemsa solution. The net number of cells that had migrated completely through the 8-µm pores was determined in 10 random high-power fields (400×) for each filter. The migration assays were performed in triplicate.

TGFβ-neutralizing antibody treatment. Wild-type male mice received intraperitoneal injections of TGFβ-neutralizing antibody (R&D Systems) once every 7 d starting at 7 wk of age. Both TGFβ 1 and 2 are known to be neutralized in vivo and in vitro by this antibody (8). We diluted the antibody in PBS and administered it at a dose of 5 mg/kg body weight. Rabbit IgG (5 mg/kg; Sigma-Aldrich) was used in a similar fashion as a negative control. Induction of AMI was performed at 8 wk of age. Mice were killed 5 d after AMI, and we used 5 mice per group for histological analysis.
REFERENCES


Shimazaki et al. | http://www.jem.org/cgi/content/full/jem.20071297/DC1

**Table S1. Echocardiographic data**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MI day 1</th>
<th>MI day 7</th>
<th>MI day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>periostin&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>periostin&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>periostin&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>periostin&lt;sup&gt;-/-&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>BW (g)</strong></td>
<td>20.4 ± 0.3</td>
<td>20.3 ± 0.3</td>
<td>20.4 ± 0.6</td>
<td>20.1 ± 0.3</td>
</tr>
<tr>
<td><strong>HR (beats/min)</strong></td>
<td>458 ± 14</td>
<td>461 ± 9</td>
<td>425 ± 14</td>
<td>397 ± 23</td>
</tr>
<tr>
<td><strong>LVEDD (mm)</strong></td>
<td>3.44 ± 0.05</td>
<td>3.41 ± 0.03</td>
<td>3.82 ± 0.06</td>
<td>4.66 ± 0.19</td>
</tr>
<tr>
<td><strong>LVESD (mm)</strong></td>
<td>1.76 ± 0.03</td>
<td>1.73 ± 0.02</td>
<td>2.88 ± 0.08</td>
<td>3.66 ± 0.24</td>
</tr>
<tr>
<td><strong>AW (mm)</strong></td>
<td>0.59 ± 0.01</td>
<td>0.58 ± 0.00</td>
<td>0.59 ± 0.03</td>
<td>0.59 ± 0.04</td>
</tr>
<tr>
<td><strong>PW (mm)</strong></td>
<td>0.60 ± 0.01</td>
<td>0.58 ± 0.00</td>
<td>0.58 ± 0.01</td>
<td>0.57 ± 0.01</td>
</tr>
<tr>
<td><strong>FS (%)</strong></td>
<td>48.9 ± 0.6</td>
<td>49.2 ± 0.3</td>
<td>24.7 ± 1.0</td>
<td>21.9 ± 2.4</td>
</tr>
<tr>
<td>Infarct size (%)</td>
<td>–</td>
<td>–</td>
<td>50.9 ± 2.4</td>
<td>45.1 ± 3.7</td>
</tr>
</tbody>
</table>

Echocardiographic parameters in the periostin<sup>+/+</sup> and periostin<sup>-/-</sup> mice under basal conditions and at 1, 7, or 28 d after LAD ligation. We demonstrated that in the case of periostin deficiency, the collagen amount was reduced in the infarct myocardium, resulting in frequent cardiac rupture in the acute phase, whereas failure in dilation and preservation of cardiac function occurred in the chronic phase of MI model. In contrast, in the chronic phase of MI, loss of periostin resulted in improvement of cardiac function. Our echocardiographic data of the chronic phase of MI are consistent with the results reported by Oka et al. (Oka, T., J. Xu, R.A. Kaiser, J. Melendez, M. Hambleton, M.A. Sargent, A. Lorts, E.W. Brunskill, G.W. Dorn, II, S.J. Conway, B.J. Aronow, J. Robbins, and J.D. Molkentin. 2007. Circ. Res. 101:313–321). Their studies, however, focused on analysis of the cardiac hypertrophy and ventricular remodeling in the chronic phase of MI, but it provided no mechanistic insight into the increase in cardiac rupture in periostin<sup>-/-</sup> mice. BW, body weight; HR, heart rate; LVEDD, left ventricle end-diastolic diameter; LVESD, left ventricle end-systolic diameter; AW, anterior wall thickness; PW, posterior wall thickness; FS, percentage of fractional shortening. aP < 0.05 versus periostin<sup>+/+</sup> with AMI.