Selective Blockade of Periostin Exon 17 Preserves Cardiac Performance in Acute Myocardial Infarction

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Abstract—We previously reported that overexpression of full-length periostin, Pn-1, resulted in ventricular dilation with enhanced interstitial collagen deposition in a rat model. However, other reports have documented that the short-form splice variants Pn-2 (lacking exon 17) and Pn-4 (lacking exons 17 and 21) promoted cardiac repair by angiogenesis and prevented cardiac rupture after acute myocardial infarction. The apparently differing findings from those reports prompted us to use a neutralizing antibody to selectively inhibit Pn-1 by blockade of exon 17 in a rat acute myocardial infarction model. Administration of Pn neutralizing antibody resulted in a significant decrease in the infarcted and fibrotic areas of the myocardium, which prevented ventricular wall thinning and dilatation. The inhibition of fibrosis by Pn neutralizing antibody was associated with a significant decrease in gene expression of fibrotic markers, including collagen I, collagen III, and transforming growth factor-β1. Importantly, the number of α-smooth muscle actin–positive myofibroblasts was significantly reduced in the hearts of animals treated with Pn neutralizing antibody, whereas cardiomyocyte proliferation and angiogenesis were comparable in the IgG and neutralizing antibody groups. Moreover, the level of Pn-1 expression was significantly correlated with the severity of myocardial infarction. In addition, Pn-1, but not Pn-2 or Pn-4, inhibited fibroblast and myocyte attachment, which might account for the cell slippage observed during cardiac remodeling. Collectively, these results indicate that therapeutics that specifically inhibit Pn exon-17, via a neutralizing antibody or drug, without suppressing other periostin variants might offer a new class of medication for the treatment of acute myocardial infarction patients. (Hypertension. 2016;67:356-361. DOI: 10.1161/HYPERTENSIONAHA.115.06265.)

Key Words: extracellular matrix ■ fibrosis ■ myocardial infarction ■ myofibroblast ■ periostin

Cardiovascular diseases are still major causes of death throughout the world.1 In spite of evolutionary development of early reperfusion therapy through catheter intervention, sudden death because of acute heart failure, lethal arrhythmias, and chronic heart failure remains a clinically important issue. The main cause of heart failure after myocardial infarction (MI) is the initiation of cardiac remodeling, which leads to fibrotic scar formation and the excess deposition of extracellular matrix (ECM) proteins. However, the precise mechanisms involved in cardiac remodeling remain unclear. Periostin (Pn) is a disulfide-linked, 90-kDa cell adhesion–related protein with 4 repetitive fasciclin domains that have sequence similarity to the Drosophila protein fasciclin I, which is a protein known to be involved in neural cell–cell adhesion.2 Pn was originally identified in the cDNA library of the mouse osteoblast cell line MC3T3-E1,3 and it has been detected in developing endocardial cushions, fibroblasts,4 stimulated vascular smooth muscle cells,5 and bronchial epithelial cells undergoing epithelial-to-mesenchymal transition.6 Pn has splice variants (Figure 1A) and seems to be expressed under circumstances related to pathological fibrogenesis. In addition, Pn also functions as a matricellular protein7 and directly interacts with other ECM proteins, such as osteopontin, osteonectin, thrombospondin-1, tenascin-C, and tenascin-X. Moreover, certain integrin family proteins, such as αV/β3, αV/β5, and α4/β1, are thought to work as receptors for Pn that are related to cancer cell migration or metastasis.5,8

In the heart, Pn was shown through subtractive hybridization studies to be expressed during the process of heart failure. The expression patterns of Pn isoforms in the normal adult hearts are distinct.9–10 Our previous report described the expression of the isoform Pn-1 around the infarct area immediately after MI,11 and overexpression of this isoform leads to cardiac remodeling.12 Importantly, another early study also

Received August 10, 2015; first decision August 31, 2015; revision accepted September 18, 2015.
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The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.115.06265/-/DC1.
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© 2015 American Heart Association, Inc.
Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.115.06265

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showed that left ventricular hypertrophy and remodeling were attenuated in Pn knockout mice after MI, whereas there was no difference in cardiomyocyte content. On the contrary, Pn-4 lacking exon 17 and 21 administration protected against cardiac rapture in a Pn knockout mouse line that models AMI, suggesting that Pn-4 plays beneficial roles in pathological conditions, such as MI, whereas Pn-1 promotes the opposite effect during cardiac remodeling. Moreover, Pn-2, which lacks exon 17, induces angiogenesis and myogenesis. Therefore, we hypothesized that selective blockade of Pn-1, but not Pn-2 and -4, might offer preferable outcome after acute MI.

In this study, neutralizing antibody against Pn exon 17 was used for selective blockade of Pn-1 and tested in a rat MI model.

**Methods**

See online-only Data Supplement information for detail material and methods.

**Results**

**Expression of Pn After MI**

As shown in Figure 1A, Pn proteins are expressed as multiple spliced isoforms with different C-terminal regions. These proteins include 4 variants: a full-length form containing 23 exons (Pn-1); Pn-2, which lacks exon 17; Pn-3, which lacks exon 21; and another isoform, Pn-4, that lacks exons 17 and 21. After MI in 8-week-old male Lewis rats (n=13), all 4 isoforms were significantly increased and reached a peak at 7 days (Figure 1B). Then, the expression of the 4 isoforms gradually decreased. Corroborating the polymerase chain reaction data, in situ hybridization for Pn in a mouse MI model revealed that Pn is strongly induced in the border zone area on day 5 and that it gradually propagates into an ischemic-free wall (Figure S1 in the online-only Data Supplement). Because the protective effects of Pn-2 and -4 and the detrimental effects of Pn-1 have previously been reported, we synthesized a Pn polyclonal antibody (PnAb) designed to bind selectively to the exon 17 coded region of Pn, which is absent in Pn-2 and -4. Specificity of this antibody was confirmed by dot blot as show in Figure S2. We have reported that the antibody works as a neutralizing antibody against Pn, including isoforms containing exon 17, such as Pn-1.

**Inhibition of Infarct and Fibrosis Size by PnAb in a Rat MI Model**

The effect of the selectively neutralizing antibody against Pn exon 17 was tested in a rat MI model. The neutralizing activity of PnAb was confirmed in our previous study related to in vivo cancer progression and metastasis. Although there was no significant difference in body weight between the sham-operated group, control IgG group, and PnAb group, total infarction size was significantly decreased in the PnAb group compared with the control IgG group (Figure 2A and 2C). Total fibrosis area was significantly reduced in the PnAb group compared with the control group (Figure 2D). A significantly larger residual myocardial area in the infarct zone was clearly present in the PnAb group compared with the IgG group (Figure 2E). Although increased cardiac rupture within the first 10 days was previously reported in a Pn knockout mouse model of MI, no rupture was observed in the PnAb group in the present study. These data demonstrate that administration of PnAb significantly reduced the infarct and fibrosis areas in a rat MI model.

Inhibition of infarction size by PnAb led to better cardiac performance at 8 weeks after MI, assessed by echocardiography and Millar micropipet pressure catheter. As shown in Figure S3A and S3C, the thickness of the intraventricular septum was significantly improved in the PnAb group compared with the control IgG group, whereas there was no significant difference in posterior wall thickness. Both ejection fraction and fractional shortening were also significantly rescued by PnAb treatment. The left ventricular end-diastolic diameter and left ventricular end-systolic diameter were significantly smaller in the PnAb group. Both maximum dP/dt and minimum dP/dt was also improved by PnAb treatment. With regards to the condition of heart failure, left ventricular end-diastolic pressure was significantly elevated in the control IgG group, whereas it was significantly lower in the PnAb group. Consistent with these results, the plasma brain natriuretic peptide level was significantly increased in the IgG group, whereas it was attenuated in the PnAb group at 4 weeks postsurgery. Inhibition of MI by PnAb was also confirmed by measurement of heart size. As shown in Figure S3B, the ratio of atrium weight to body weight and the ratio of ventricle weight to body weight were significantly decreased in the PnAb group compared with the

![Figure 1](image-url)  
**Figure 1.** Gene expression of periostin (Pn) spliced isoforms. **A**, Spliced isoforms of Pn. **B**, Gene expression of Pn-1, -2, -3, and -4 after myocardial infarction (MI). The level of gene expression was quantified on days 0 (sham operation), 3, 5, 7, 14, 28, and 180 after operation by real-time polymerase chain reaction (PCR) using specific primers for Pn variants. *P < 0.05 vs Pn-1, Pn-2, Pn-3, and Pn-4 at day 0, respectively. n=3 to 5.
control IgG group. Similarly, the ratio of lung weight to body weight was also significantly lower in the PnAb group. These data indicate that PnAb treatment significantly prevented heart failure after MI.

Notably, the level of Pn-1 expression at 7 days after MI nicely correlated with the severity of MI, indicated as advanced left ventricular end-diastolic pressure, left ventricular end-diastolic diameter, left ventricular end-systolic diameter, and lower % fractional shortening (Figure S3C). Because Pn is associated with tumor growth via its angiogenetic potential, and is reported to stimulate cardiac repair after MI by increasing capillary density, we analyzed the number of capillaries in the infarcted and peri-infarcted zones.

**Angiogenesis, Myocyte Content, and Fibrotic Gene Expression**

Because Pn is associated with tumor growth via its angiogenetic potential and is reported to stimulate cardiac repair after MI by increasing capillary density, we analyzed the number of capillaries in the infarcted and peri-infarcted zones. However, there was no significant difference in capillary density between the 2 groups in all anatomic regions (Figure S4A and S4B). In addition, we counted the number of cycling cardiomyocytes by using double immunostaining because Pn-induced decreased infarct size is reported to be associated with regulation of cardiomyocyte proliferation via modulation of cell cycle reentry and the division of differentiated cardiomyocytes.

The ratio of α-sm sarcemeric actin (+) and 5-bromodeoxyuridine (+) cardiomyocytes to all α-sarcemeric actin (+) cells in 5 randomly selected positions around the peri-infarct region in each group was calculated (n=6). There was no significant difference in the ratio of cardiomyocytes in the synthetic phase (IgG: 0.30±0.34% versus PnAb: 0.37±0.27%; P=0.80). In addition, the ratio of α-sm sarcemeric actin (+) and Ki-67(+) cardiomyocytes to all α-sarcemeric actin (+) cells was calculated. There was also no significant difference between these 2 groups (IgG: 0.37±0.34% versus PnAb: 0.36±0.27%; P=0.50). However, the average diameter of cardiomyocytes around the infarcted area was significantly reduced in the IgG group compared with the PnAb group (Figure S4C), suggesting that the sustained overload observed in the IgG group was prevented by the administration of PnAb.

Thus, we focused on the role of myofibroblasts in PnAb-treated rats to clarify this mechanism because myofibroblasts appear starting several days after MI and stabilize in≈1 month as the wound heals. Importantly, at 7 days after MI, the number of α-sm smooth muscle actin–positive myofibroblasts was significantly reduced in the hearts treated with PnAb (Figure S4D). Corresponding to rapid changes in fibrosis, the expression of fibrotic marker genes, such as collagen I, collagen III, and transforming growth factor-β1 (TGF-β1), were significantly attenuated in MI rats that received PnAb treatment compared with the IgG group (Figure S4E). Overall, in vivo studies show that treatment with PnAb significantly reduced the size of MI, thereby maintaining cardiac performance, possibly through the inhibition of fibrosis.

**PnAb-Induced Mechanisms That Lead to Improvement in Cardiac Dysfunctions**

To further understand the molecular mechanisms involved in this process, we used in vitro experiments. Initially, we analyzed the expression of the Pn-1 isoform after inducing fibrosis with TGF-β1 for 48 hours. TGF-β1 significantly induced gene expression of Pn-1 by nearly 4-fold compared with the control group (Figure S5A). TGF-β1 also significantly induced the gene expression of α-sm smooth muscle actin, collagen I, and collagen III (Figure S5B–S5D). However, Pn-1 treatment stimulated TGF-β1 secretion from fibroblasts (Figure S5E), suggesting the existence of positive feedback between Pn-1 and TGF-β1. Of importance, inhibition of Pn-1 by PnAb diminished the increase in gene expression of α-smooth muscle actin, collagen I, and collagen III that was induced by TGF-β1 stimulation (Figure S5F–S5H). These data indicate that TGF-β1 significantly induces the differentiation of rat cardiac fibroblasts to a myofibroblast phenotype that is capable of producing various ECM proteins via the induction of Pn-1 expression. Moreover, PnAb treatment significantly decreased cell viability in myofibroblasts in a dose-dependent manner after 24 hours of incubation (Figure S5I; P<0.05).

The remodeling process after MI includes the thinning of the left ventricular wall and the dilation of the left ventricular chamber, mainly because of cell slippage. The disturbed cell-to-cell adhesion causes cellular slippage and seems to activate proapoptotic pathways. Thus, the attachment of fibroblasts and myocytes to dishes coated with Pn isoforms was compared. As show in Figure 3A, Pn-1 significantly reduced fibroblast attachment to the dish compared with the control, whereas...
Pn-2 significantly increased cell attachment. Similarly, Pn-1 prevented myocyte attachment and increased the LDH content in medium compared with the control (Figure 3B–3D). These observations imply that Pn-1, but not Pn-2, inhibits cell attachment and activates necrotic pathways. This mechanism might account for the attenuation of myocyte death, wall thinning, and chamber dilation induced by PnAb in the rat MI model. Finally, the ability of Pn isoforms to induce angiogenesis was compared using a Matrigel tube formation assay. Intriguingly, Pn-2 significantly increased tube formation but Pn-1 did not (Figure S6). These data also support our hypothesis that the selective blockade of Pn exon 17, which is present in Pn-1 and -3, might be an ideal therapeutic option for the treatment of heart failure after MI.

Discussion

Our previous study reported the markedly accelerated accumulation of interstitial collagen in hearts that specifically overexpressed the Pn-1 gene, which promoted heart failure. Consistent with previous result, Pn knockout mice displayed attenuated cardiac fibrosis and maintained cardiac function. However, these knockout mice also showed an increased frequency of cardiac ruptures in the earliest 10 days after MI, which was prevented by the administration of Pn-4. In addition, another artificial Pn protein (containing exons 1–16) seems to regenerate myocytes and induce angiogenesis. At this point, previous reports regarding the role of Pn are controversial, and it remains an enigma. Thus, we hypothesized that the selective blockade of Pn exon-17 would improve cardiac functions during the healing process after MI because Pn exon 17 exists in the Pn-1, but not the Pn-2 and -4 isoforms. To test this hypothesis, we synthesized a polyclonal antibody designed to specifically inhibit Pn exon 17. The present study clearly demonstrates that the specific inhibition of Pn exon 17 by a neutralizing antibody significantly decreased infarction size and improved cardiac performance after MI. These results are consistent with a previous study that used Pn knockout mice. Because Pn was ablated in the previous knockout mouse experiments, the role of Pn-1, full-length Pn, is considered to accelerate myocardial damage.

In this experiment, we first demonstrate that blockade might contribute to the prevention of fibrosis. Inhibition of fibrosis by PnAb was associated with a decrease in fibrosis-related gene expression, such as TGF-β1, and the inhibition of myofibroblast differentiation and proliferation. Second, PnAb prevented Pn-1-induced myocyte death and left a larger residual myocardium in infarcted area compared with the IgG group. Cell-to-cell slippage after MI is described to induce myocyte apoptosis, and the number of residual myocyte negatively correlate with left ventricular end-diastolic pressure. Because mechanical stretch induced by high left ventricle pressure induces Pn 1 expression mainly from myofibroblast, it is feasible assumption that PnAb might also prevent Pn-1-induced myocyte detachment, and slippage in the infarct resulted in larger residual myocyte volume. This effect may also explain that PnAb group significantly improved cardiac...
performance and left ventricle heart failure characterized by increase in brain natriuretic peptide, left ventricular end-diastolic pressure, ventricle, and lung weight.

Molecular mechanisms of Pn-1 seem to be more related to fibrosis. Pn is secreted mainly by mesenchymal cells and is highly homologous to the fasciclin domain-containing protein β-h3, which is induced by TGF-β and promotes cell adhesion and fibroblast spreading among the ECM proteins. Consistent with previous reports, the expression of Pn was induced by TGF-β1 in cardiac fibroblasts. In contrast, the secretion of TGF-β1, 2, and 3 were promoted by Pn-1. The autocrine–paracrine cycle between Pn and TGF-β1 could accelerate fibrosis after MI, causing impaired cardiac function. In addition, the differentiation to myofibroblasts, which are characterized by α-smooth muscle actin expression, from resident fibroblasts by Pn-1 could lead to fibrosis through the secretion of numerous cytokines, growth factors, and ECM proteins.

Another possible explanation is the blockade of Pn-1-induced cell slippage, which is involved in ventricular dilation and remodeling after MI. Pn family proteins are also known as adhesion-related molecules that directly interact with various matricellular proteins, such as fibronectin, osteopontin, and tenasin-C. Our present and previous studies have confirmed that the addition of Pn-1 inhibited the adhesion of cardiac fibroblasts and myocytes. Importantly, Pn is the agonist for integrin family proteins. Thus, the blockade of Pn-1 might be related to cell adhesion or migration after MI.

A neutralizing antibody against Pn exon 17 significantly decreased the size of Mls, partially by inhibiting fibrosis without increasing cardiac ruptures. The inhibition of fibrosis was associated with a decrease in fibrosis-related gene expression, which was confirmed that the addition of Pn-1 inhibited the adhesion of fibroblasts by Pn-1 could lead to fibrosis through the secretion of integrin family proteins. Thus, the blockade of Pn-1 might be related to cell adhesion or migration after MI.

Perspectives
Present study demonstrates that therapeutics that specifically inhibit Pn exon 17, via a neutralizing antibody, without suppressing other Pn variants, offers new option for the treatment of AMI. Additional study on the effect of humanized monoclonal antibody against Pn exon-17 and the timing, duration, and dose of antibody should be tested in animal model for clinical trial.

Acknowledgments
We thank the members of the Department of Clinical Gene Therapy at the Osaka University Graduate School of Medicine for their helpful discussion and technical support.

Sources of Funding
This work was partially supported by a grant-in-aid from the Organization for Pharmaceutical Safety and Research, a grant-in-aid from the Ministry of Public Health and Welfare, a grant-in-aid from Japan Promotion of Science, a START program, and a Biomedical Cluster Kansai from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government.

Disclosures
R. Morishita received honoraria, consulting fees, and funds from Novartis, Takeda, Shionogi, Astellas, Boehringer Ingelheim, Daiichi-Sankyo, and Pfizer. The other authors report no conflicts.

References

Novelty and Significance

What Is New?

• Periostin is now recognized as more than just adhesion molecule, but rather a mediator that link dynamic fibroblast–myocyte interaction or fibroblast–cancer stem cell cross talk. However, several group reports different effect of periostin on cardiac remodeling because of the lack of knowledge about periostin splice variants.
• This experiment focuses on splice variants of periostin and shows that selective blockade of periostin variants offer better outcomes in rat myocardial ischemia model.

What Is Relevant?

• Blockade of periostin exon 17 (Pn 1 or 3) by neutralizing antibody is safer than total periostin inhibition because other forms of periostin (Pn 2 and 4) contribute to angiogenesis and prevent cardiac rapture.

We believe that our finding might offer a new class of medication for the treatment of acute MI patients.

Summary

Present study demonstrates that therapeutics that specifically inhibit periostin exon-17, via a neutralizing antibody, without suppressing other periostin variants offers safe and new treatment option for acute myocardial infarction patients.
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Hypertension. 2016;67:356-361; originally published online December 7, 2015;
doi: 10.1161/HYPERTENSIONAHA.115.06265

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/67/2/356

Data Supplement (unedited) at:
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**Key Words**
periostin, myocardial infarction, fibrosis, myofibroblast, extracellular matrix

Running title: Cardiac Remodeling and Periostin

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Supplementary Methods

Rat PnAb

In order to raise the polyclonal antibody against exon 17 of rat Pn, the peptide (TKIITKLEPKIKVIQGSLQPIIKTE) was synthesized and coupled to KLH at Oriental Yeast Co., Ltd as described previously 1. The antibody was generated in immunized rabbits, and purified through an affinity column. We tested specificity of this antibody by dot plot as shown in Figure S1.

Rat Model of Myocardial Infarction

Male Lewis rats at 8 weeks of age (Charles River Laboratories, Boston, USA) underwent experimental surgery of myocardial infarction under inhalation anesthesia by 2 % isoflurane as described 2. With left intercostal thoracotomy, the left anterior descending artery (LAD) was ligated approximately 2 to 3 mm from its origin with 6-0 silk suture. Electrocardiography (AC-601G, Nihonkoden, Tokyo, Japan) was recorded simultaneously to detect ST segment changes as well as arrhythmias and the color of cardiac surface was observed to judge whether or not the operation was successfully underwent. The infarcted area was evaluated on the day of surgery and the next day to get rid of the rats with insufficient infarction. These rats were divided into two groups randomly. In Pn group (n=23), PnAb (0.2 mg/body) was administered by intravenous injection on day1, 6, 12, 18 after surgery and the same dose of IgG (R & D Systems, Minneapolis, USA) was given the remaining half rats (n=23) in the same way as a control group. Sham operated rats underwent a similar procedure without coronary ligation as sham group. MI rats were received IP injections of 50mg/kg BrdU (5-bromodeoxyuridine) on day 3, 5, 7, 9, 11, 13 to detect the cardiomyocyte in synthetic phase. After physiological and hemodynamic analysis, all rats were sacrificed 8 weeks after surgery to make sections. Importantly, animals were assigned to IgG control group and PnAb group randomly and an electric laboratory animal monitoring system (Yuasa biosystems, Japan) was used. This system consists of microchip (transponder) implanted subcutaneously to animal body and scanner/reader. Individual number of animal was identified later, therefore, researchers was able to collect data blindly.

For evaluation of myofibroblasts, we have made another MI rat groups, one was injected PnAb and the other group was injected IgG in the same way. Both groups were sacrificed at 7 days after surgery for immunohistochemical analysis. After perfused, hearts from both groups were immunostained for α-SMA (alpha smooth muscle actin), and analysis on SMA positive structures was prosecuted from 5 randomly chosen views in infarcted area and peri-infarct area of each sample.
All animal procedures were performed in accordance with the guidelines of the Institutional Animal Committee of Osaka University School of Medicine.

Physiological Studies

Cardiac function was measured under 2.0 % isoflurane anesthesia by transthoracic echocardiography using Core Vision Pro SSA-350A (Toshiba, Tokyo, Japan) as previously described 3. For hemodynamic evaluation, after rats were anesthetized in the same way, LV pressures, maximum dP/dt and minimum dP/dt were examined with Millar micro tip pressure transducer (model SPR-470, Millar Instruments Inc. Houston, USA) connected to a recorder.

Histological Analysis

Isolated perfused hearts were fixed in buffered 10% formalin, and embedded in paraffin to be sliced into horizontal 5 μm sections for hematoxylin & eosin (HE) chemical staining or Masson’s trichrome (MTC) staining. For immunohistochemistry, 5 μm cryosections were fixed in 4% paraformaldehyde and embedded in Tissue-Tek O. C. T. compound (Sakura Finetek USA, Inc. Torrance, USA). Fibrotic area, α-SMA positive structures and rat endothelial cells antibody 1 (RECA1) positive structures were measured using Image J program downloaded from the web site of National Institutes of Health (NIH, USA). The antibodies against α-SMA and RECA1 were purchased from Dako (Carpinteria, USA), and BrdU (5-bromodeoxyuridine) was purchased from Sigma, Ki-67 was from Fluorophore-conjugated secondary antibody was purchase from Dako.

In situ hybridization with Pn probe

In situ hybridization was performed as described previously 4. Sections were made at 8-μm thickness, and the cDNAs used for generation of Dig-labeled mouse riboprobes were periostin.

In vitro experiment

Rat cardiac fibroblasts (RCFs) were isolated from 1-day-old Sprague-Dawley rat heart as described previously 5,6. In short, after ventricles were minced in phosphate buffered saline (PBS, Nakarai Tesque, Kyoto, Japan), agglomerated cells were disrupted in mixed solution of Collagenase Type IA-S C9722 (Sigma-Aldrich, St. Louis, USA) and Trypsin Power 85450C (Sigma) by shaking immersed in thermostatic bath at 37°C for 15 min, fully digested through 40 μm nylon mesh strainer (BD Falcon cell strainer, Franklin Lakes, USA). Centrifuged cells in PBS and Dulbecco’s Modified Eagle’s
Medium (DMEM, Nakarai) with 10% fetal bovine serum (FBS)/penicillin-streptomycin were resuspended appropriately and cultured in collagen coated dish at 37 °C. Half an hour later, medium was changed to the DMEM with FBS/antibiotics to get rid of the floating cardiomyocytes. Subculturing for a couple of times, cardiomyocytes were completely eliminated.

Pure human cardiac fibroblasts (Cryopreserved fetal HCF) were purchased from Cell Systems Corporation, Kirkland, USA. HCFs were cultured in CSC 4Z3-500-S medium (Cell Systems) with 10% FBS / penicillin-streptomycin in standard dish at 37 °C. At passage 5, they were stimulated with 10 ng/ml transforming growth factor beta 1 (TGF-β1, Sigma) for 48 hours, 5 % CO2, 37 °C. Cells were reseeded at a concentration of 1×10^4 cells per well (96-well plate) in serum-free medium, and divided into 2 groups, one is with PnAb of 5.0 μg/ml and 1.0 μg/ml, and the other one is with control IgG at the same concentration. After 24 hours’ incubation, mitogenic activity was assessed through MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay by means of Cell Titer 96 aqueous one-solution cell proliferation assay kit (Promega, Madison, USA), which Formazan product quantity reflecting the number of living cells in culture was calculated by measuring the absorbance at 490nm with spectrophotometer Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany).

Solid-Phase Binding Assay of Cell Adhesion of Myocytes and Cardiac Fibroblasts was performed as described previously 7. Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released on cell lysis. LDH released to the culture medium by dead cells as well as LDH contained in living cells was measured with the CytoTox96 kit (Promega). This assay is based on the LDH-catalyzed oxidation of lactate to pyruvate in the presence of NAD, which is reduced to NADH. The formation of NADH is coupled to the reduction of a tetrazolium salt, measured photometrically at 490 nm on a 96-well plate reader (Molecular Devices).

Human umbilical vein endothelial cells (HUVEC) purchased from Lonza were cultured in endothelial basal medium-2 (EBM-2) (Clonetics, Walkersville, Maryland, USA) supplemented with EGM and 5 % fetal bovine serum (FBS). 9×10^4 HUVEC were plated and incubated at 37 °C for the 24 hours on a growth factor-reduced Matrigel-coated 48-well dish. HUVECs were visualized by Cellstain kit (Doujin Kagaku. Inc. Japan) and tubular length was measured by using image J soft wear.

**Quantitative Realtime PCR**

For reverse transcription, total RNA from deep frozen hearts was prepared
according to standard methods. RNA was quantified and integrity was confirmed. We used TaKaRa PCR Thermal Cycler Dice Standard (TAKARA BIO INC. Shiga, Japan) for synthesizing cDNA and Applied Biosystems 7900HT Fast (Life Technologies Corporation, Carlsbad, USA) for detection in accordance with the manufacture’s instructions. In each experiment, rat GAPDH or 18s-rRNA was amplified as a reference standard. Primer details are shown as follows.

**PCR primer list**

(1) rat periostin 1  
Sense, 5’-TAACCAAAGCTCGTGGAACC-3’  
Antisense, 5’-GTCTCCCTGAAGCAGTCTTTT-3’

(2) rat periostin 2  
Sense, 5’-CCCATGACTGTCTATAGACCT-3’  
Antisense, 5’-GTCTCCCTGAAGCAGTCTTTT-3’

(3) rat periostin 3  
Sense, 5’-TAACCAAAGCTCGTGGAACC-3’  
Antisense, 5’-CTTCTTTGCAGGTGTGTCTTT-3’

(4) rat periostin 4  
Sense, 5’-CCCATGACTGTCTATAGACCT-3’  
Antisense, 5’-CTTCTTTTGAGGTGTGTCTTT-3’

(5) rat α-SMA  
Sense, 5’-GTACCCAGGCATTGCTGACA-3’  
Antisense, 5’-GGGCCAGCTTCGTCATACTC-3’

(6) rat collagen I  
Sense, 5’-GACCAGGAATTCGGAATGGA-3’  
Antisense, 5’-TCCCAATTTTTGGCTTATT-3’

(7) rat collagen III  
Sense, 5’-TCGGAATTGCAGAGACCTGA-3’  
Antisense, 5’-CTCAGCACCAGCTCTGTCC-3’

(8) rat TGF-β1  
Sense, 5’-GAAGCCATCCGAGCAGAAG-3’  
Antisense, 5’-CCAGTGACGTCAAAAGACAG-3’

**Statistics**

For statistical analysis, the values are shown as the means ± SD. ANOVA and t-tests (unpaired, 2-tailed), followed by Bonferroni adjustment for multiple comparisons.
were used for comparing more than two groups. Sample size for in vivo study was computed to detect 1 standard deviation difference in mean of an outcome variable between 2 groups with 90 % power at 2 sided significance level of 5%. In order to allow 30 % loss of subjects due to small infarction size or unexpected death due to infection or arrhythmia, we evaluated 30 rats per group which provides 21 rats per group for the analysis.

References


S1. In situ hybridization for Pn in heart sections in mice at days 0, 3, 5, and 7 following induction of MI.
S2. Specificity of PnAb against Pn exon 17.

Specificity of PnAb against Pn exon 17 was tested by dot western blotting. 1 or 10 μg of Pn-1, -2, and -4 were blotted and detected by PnAb.
S3. Effect of PnAb on cardiac function 8 weeks after MI

A. Thinning of intraventricular septal thickness (IVST), posterior wall thickness (PWT), ejection fraction (EF), fractional shortening (FS), left ventricular end-diastolic diameter (LVDd), and left ventricular end-systolic diameter (LVDs) were measured by echocardiogram. Maximum (+) dP/dt, minimum (-) dP/dt, and left ventricular end-diastolic pressure (LVEDP) were measured by Millar microtip pressure catheter.

Plasma BNP level was measured by ELISA. *P<0.01 vs. sham. **P<0.01 vs. IgG. Numbers in parenthesis or bars indicate group sizes.

Sham = Sham-operated group without MI group (n=8), IgG = control IgG group (n=21), and PnAb = PnAb group (n=21).

B. Ratio of atrium weight to body weight, ratio of ventricle weight to body weight, and ratio of lung weight to body weight. Numbers in parenthesis or bars indicate group sizes. *P<0.05 vs. sham, **P<0.05 vs. IgG. Sham = Sham-operated group without MI group (n=8), IgG = control IgG group (n=21), and PnAb = PnAb group (n=21).

C. Pn-1 expression at day 7 after MI is significantly correlated with LVEDP, -dP/dt, LVDd, LVDs, and FS, but not +dP/dt. n=16.
S4. Effects of PnAb on angiogenesis and myofibroblasts.

A, Representative image of the fibrotic area in heart and REICA1 staining. Left panel, MTC staining; right panel, REICA1 staining. Rectangle in right panel indicates the area measured.

B, Capillary density of three anatomical areas. PIA = peri-infarct area. Outer PI (outer peri-infarct area); fibrotic area within 50 ± 10 %. Inner PI (inner peri-infarct area); fibrotic area within 90 ± 10 %. Central IA (central infarct area, literally). Scale bars, 2 mm. IgG = control IgG group, PnAb = PnAb group.

C, Average diameter of cardiomyocytes around the infarct area. *P<0.01 vs. sham. **P<0.01 vs. IgG.

D, % of α-SMA structures at 7 days after MI. *P<0.05 vs. IgG. Numbers in bars indicate group sizes. α-SMA = alpha smooth muscle actin.

E, Effects of PnAb on mRNA expression of collagen I, collagen III, and TGF-β1 in infarcted areas at 8 weeks after MI.*P<0.05 vs. sham, **P<0.05 vs. IgG. Numbers in bars indicate group sizes.
S5. In vitro analysis in fibroblasts

A-D. Effect of TGF-β1 on gene expression of Pn-1 (A), α-SMA (B), collagen I (C), and collagen III (D) at 48 hours of incubation.

E. Effect of Pn-1 on TGF-β1 production. 

F-H. Effect of PnAb on gene expression of α-SMA (F), collagen I (G), and collagen III (H) at 48 hours of incubation with TGF-1 stimulation. *P<0.05 vs. IgG. Numbers in bars indicate group sizes.

I. Cell viability. Human cardiac fibroblasts at passage 5 were stimulated with TGF-β1 for 48 hours. Cells were reseeded in serum-free medium with PnAb at 1.0 or 5.0 µg/ml or control IgG at the same concentration. After 24 hours, mitogenic activity was assessed by MTS assay. *P<0.05 vs. IgG group. **P<0.01 vs. 1 µg/ml PnAb.
S6. Effect of Pn variants on tube formation in HUVECs.

HUVECs were cultured in Matrigel with Pn variants and cultured for 24 h. Tubular lengths were then measured. Error bars indicate 95% confidential interval. *, **P<0.05 vs. CTRL, Pn-1, respectively.