厚生労働科学研究費補助金
難治性疾患克服研究事業

プロスタグランジンI2合成酵素遺伝子を用いた肺動脈性肺高血圧症に対する新規治療法の開発に関する研究

平成20年度　総括研究報告書

研究代表者　福　田　恵　一

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研究要旨

本年度研究ではアデノ随伴ウイルス（ＡＡＶ）を用いたプロスタグランジン I2 合成酵素の遺伝子治療ベクターをＧＭＰレベルで大量生産する製造工程確立を目的として、共同研究企業ディナベック社を通じて、中国の本元正阻基因技術有限公司（Vector Gene Technology 社）にGLP レベルのＡＡＶ－ＰＧＩＳの作製を依頼し、工業レベルでの生産を開始した。ＡＡＶベクターは１型と２型を使用し、ベクターの作成効率、安全性・有効性を検討した。また、前臨床試験として使用するコモンマーモセットサルの原発性肺高血圧モデルを作出した。

A. 研究目的

肺動脈性肺高血圧症は若年女性を中心に発症する予後不良の疾患である。近年、プロスタグランジン I2（PGI2）、エンドセリン受容体拮抗薬、ホスホジエステラーゼV阻害薬等の薬剤が開発され、病気の進行を遷延させ予後が改善されつつあるが、後二剤の効果はさほど強いものではなく、作用が強い PGI2 は鎖骨下静脈からの持続静注療法によるため、患者の QOL は著しく制限されている。PGI2 合成酵素（PGIS）は本申請者の一人田邉忠がクローニングした遺伝子であり、PGI2 を合成できる唯一の酵素である。本研究の目的は申請者らが特許を有する PGIS を既にヒトに臨床応用され安全性が確立されているアデノ随伴ウイルスベクターに組み込み、これを用いて肺動脈性肺高血圧症を治療しようというものである。すでに申請者らは低酸素（10%酸素）負荷による肺高血圧を惹起したマウスに対して本ウイルスベクター（AAV-PGIS）を用いた遺伝子治療を行った。この方法は AAV-PGIS をマウス大腿部骨格筋に投与し、骨格筋細胞から産生される PGI2 により肺高血圧症を治療しようというもので、AAV-PGIS は投与部の骨格筋に限局して強力に発現し、その発現は長期間維持されることが示された。さらに肺微小血管の肥厚を抑制することが可能であり、肺動脈圧上昇の抑制、右室肥大の抑制が可能であること、長期予後を改善することが可能であることが示された。

本年度の研究では第一に全臨床試験に用いるためのＧＭＰレベルのＰＧＩＳ－ＡＡＶベクターを工業レベルで生産する仕組みを確立することを目的とした。そして、このＡＡＶベクターとしては、我々が従来より使用してきた生産効率がよいタイプ１型と、生産効率は落ちるが既に臨床使用されているタイプ２型を使用し、ベクターの作成効率、安全性・有効性を検討することを目的とした。

また、前臨床試験として使用するコモンマーモセットサルの原発性肺高血圧モデルを安定的に作出する方法を確立することを目的とした。

B. 研究方法

（1）GMP レベルでの AAV-PGIS の作成

AAV-PGIS の作製は既にこれまでの実験で安定的に作製できるが、これを GMP レベルで作製するための条件整備を行う。GMP レベルの大量のベクター作製はディナベック株式会社（茨城県つくば市）との共同開発を行い、中国において生産するため、中国国内で安定して GMP レベルのベクター作製する会社を調査すると共に、実際に中国に行って工場、研究所を視察する。また、依頼した会社と生産量－コストの関係を明らかにし、作成価格の交渉を行った。

（2）サルを用いた肺動脈性肺高血圧症モデルの作成

エステル型モノクロタリン（MCT-E）を用いて肺高血圧モデルを作成する。既に行った予備実験では 5 匹のコモンマーモセットサルにおいてモノクロタリンを投与したが、2 匹で肺高血圧が観察されたものの他の 3 匹では観察されなかった。これよりマーモセットではエステラーゼ活性に個体差があるため、肺高血圧症の発症に影響を与えることが明らかとなった。マーモセットサル 10 匹に MCT-E 投与後定期的に心エコーを施行し、右室肥大の進行程度を観察する。1 ヶ月および 2 ヶ月後にイソフルレンにて吸入麻酔後、人工呼吸器装着下に開胸し、肺動脈圧、右室圧、左室圧を測定する。心停止後、肺、心臓を摘出し、肺小動脈壁厚、右室重量、左室重量、心体重比等を計測する。また、右心肥大を呈した心筋の遺伝子発現（BNP 等）を解析する。

（倫理面への配慮）

遺伝子組み換え実験・遺伝子導入実験はすべて大学の遺伝子組み換え実験に関する監査委員会に申請書を提出し、認可を得ている。動物実験は大学の動物実験委員会に申請し、認可を得ている。AAV-PGIS は P2 レベルの実験室で研究が出来るため、研究上の支障となることはない。本研究（治療実験）はヒト臨床試験の前段階までであるため、大学の倫理委員会に審査請求する必要はない。健常例、肺動脈性肺高血圧症症例における採血は通常の臨床検査の余剰血液を使用するため、新たな負担を加えることはなく、特に問題となることはない。

C. 研究結果

（1）GMP レベルでの AAV-PGIS の作成

工業レベルで遺伝子治療ベクターを作成するため、共同開発企業のディナベック社と中国北京にある本元正阻基因技術有限公司（Vector Gene Technology 社）を視察に行き、工場の生産ライン、GMP レベルの製造工場を観察した。その上で、同社と GMP レベルでのベクター作成契約を行った。これまで作成していたタイプ 1 型のアデノ随伴ウイルスは作成効率が高い。これに対し生産効率は落ちるが既に臨床使用されているタイプ 2 型のウイルスベクターの両者のウイルスベクターを作成することとした。タイプ 1 型と 2 型の組織での発現効率を見るため、GFP 発現ベクターを作成した。

（2）サルを用いた肺動脈性肺高血圧症モデルの作成

モノクロタリンは肺高血圧症を惹起することが知られている植物アルカロイドであるが、これ自身は前駆物質であり、体内にはいるとエステル型に変化し、活性型になることが知られている。ヒト、ラットではこのエステル化酵素が存在するが、マウスでは存在しないためモノクロタリンは効果がない。また、イヌでは種によって差があるため一定した肺高血圧を作成することは出来ない。これに対し、サルでは予備実験によりモノクロタリンによる肺高血圧の出来方に大きな個体差があり、エステル型を使用することとした。今年度の実験ではこれにより、個体間に差が無く肺高血圧が出来るようになったが、エステル型モノクロタリンは作用が極めて強いため、投与量を減少する必要があることが明らかとなった。

D. 考察

GMP レベルの AAV ベクターの工業的生産を開始するため、中国北京の本元正阻基因技術有限公司と契約を結んだ。ヨーロッパ系のベクター作成会社では生産時間、コスト面で競争にはならない状況であった。

エステル型モノクロタリンによるコモンマーモセットを用いた肺高血圧モデルでは、個体差に寄らず肺高血圧が作成できることが明らかとなったが、イヌの投与量を元にしたエステル型モノクロタリンの投与量では致死量に近い投与量になるため、今後は投与量を慎重に決める必要があることが判明した。

E. 結論

プロスタグランジン I2 合成酵素のアデノ随伴ウイルスのGMP生産拠点を確保した。ベクター作成にはタイプ1型と2型の両者を用意し、作成効率、投与効果の評価を次年度以後に行うことにした。

前臨床試験として使用するコモンマーモセットサルの原発性肺高血圧モデルを安定的に作出する方法を確立することに成功した。

F. 健康危険情報

本年度研究は前臨床試験を行う計画であり、ヒトへの投与には現時点で至っていないため、健康危険情報に該当するものではない。

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H. 研究成果による特許権等の知的財産権の取得状況

1．テノモジュリンを有効成分とする腱断裂性疾患治療剤　発明者　福田恵一、開祐司、宿南知佐　出願人　福田恵一、開祐司、宿南知佐　(特願 2007－258357) 平成 19 年 10 月 2 日　PCT 出願　平成 20 年 10 月 2 日 (PCT/JP2008/067881)

2．医薬組成物　発明者　佐野元昭、福田恵一。出願人　学校法人慶應義塾　平成 21 年 2 月 27 日 (特願 2009－047014)

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Periostin is essential for cardiac healing after acute myocardial infarction

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Acute myocardial infarction (AMI) is a common and lethal heart disease, and the recruitment of fibroblastic cells to the infarct region is essential for the cardiac healing process. Although stiffness of the extracellular matrix in the infarct myocardium is associated with cardiac healing, the molecular mechanism of cardiac healing is not fully understood. We show that periostin, which is a matricellular protein, is important for the cardiac healing process after AMI. The expression of periostin protein was abundant in the infarct border of human and mouse hearts with AMI. We generated *periostin*$^{-/-}$ mice and found no morphologically abnormal cardiomyocyte phenotypes; however, after AMI, cardiac healing was impaired in these mice, resulting in cardiac rupture as a consequence of reduced myocardial stiffness caused by a reduced number of α smooth muscle actin–positive cells, impaired collagen fibril formation, and decreased phosphorylation of FAK. These phenotypes were rescued by gene transfer of a spliced form of periostin. Moreover, the inhibition of FAK or αv-integrin, which blocked the periostin-promoted cell migration, revealed that αv-integrin, FAK, and Akt are involved in periostin signaling. Our novel findings show the effects of periostin on recruitment of activated fibroblasts through FAK-integrin signaling and on their collagen fibril formation specific to healing after AMI.

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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

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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

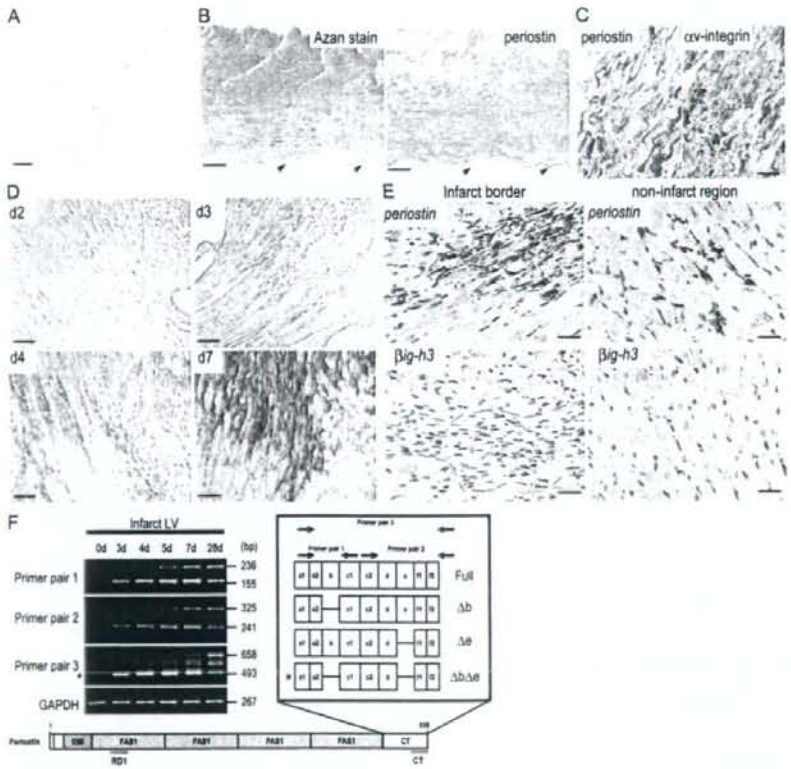

Figure 1. Periostin expression is induced after myocardial ischemia. (A–C) Detection of periostin in myocardium from human patients. LV tissue from a patient with alcoholic cirrhosis (A) and from a patient with AMI (B and C). As seen by immunostaining, periostin protein was detected (B, right) in the 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 β *ig-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 fasciclin 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. 88.85 ± 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/mm^2 in $^{+/+}$ vs. 6,913 ± 297 cells/mm^2 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 nm^2 for the $^{-/-}$ and 2,233.780 ± 25.731 nm^2 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

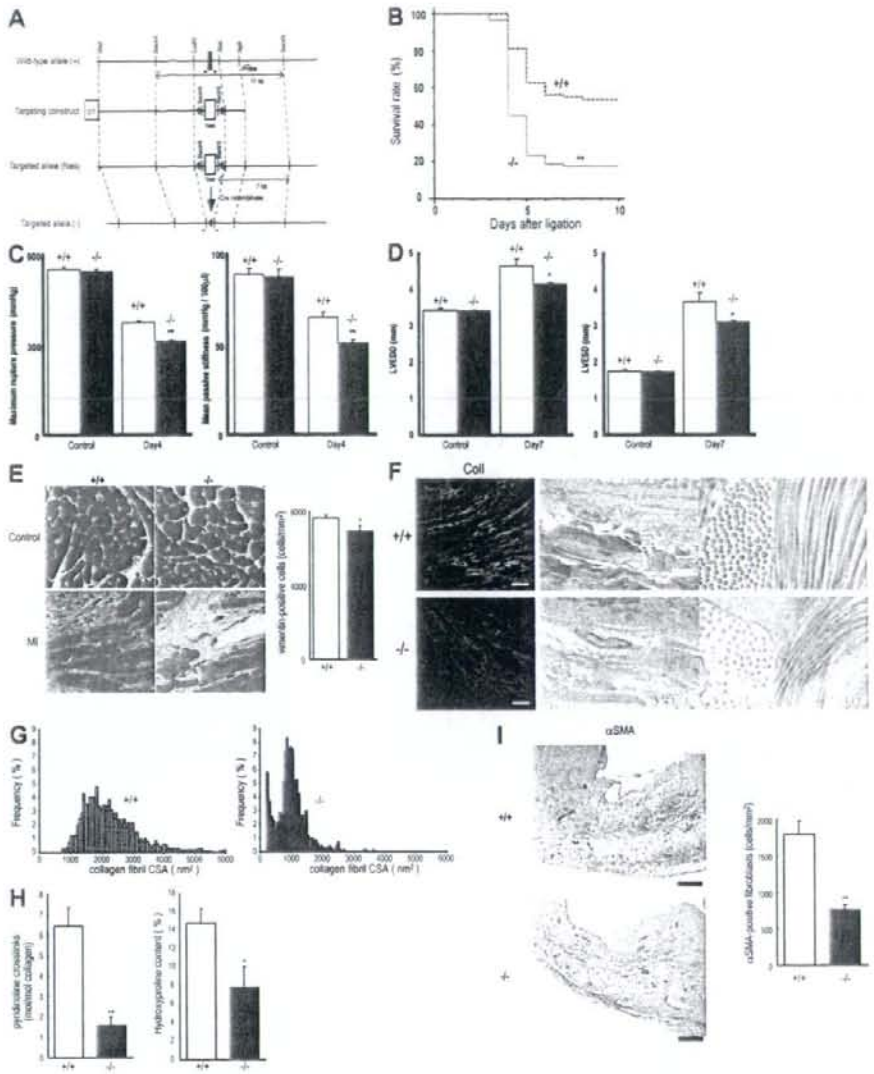

Figure 2. Cardiac rupture after AMI is caused by *periostin* disruption. (A) Schema of the targeting strategy deletes the first exon of *periostin* locus. (B) Decreased survival of *periostin*$^{-/-}$ mice ($n = 91$) compared with the survival of $^{+/+}$ mice ($n = 80$) after AMI. **, $P < 0.0001$. (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. (right) The number of αSMA-positive cells. **, $P < 0.01$, compared with $^{+/+}$ mice. Error bars represent the mean ± the SEM. Bars, 200 μm.

(1.555 ± 0.461 in $^{-/-}$, $n = 4$, vs. 6.433 ± 0.919 in $^{+/+}$, $n = 7$; $P = 0.0043$; Fig. 2 H). Moreover, the *periostin*$^{-/-}$ infarct LV tissue exhibited 52.9% less collagen content compared with the $^{+/+}$ tissue (7.832 ± 2.241% in $^{-/-}$, $n = 4$, vs. 14.795 ± 1.565% in $^{+/+}$, $n = 7$; $P = 0.0283$; Fig. 2 H). In normal heart 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/mm^2 in $^{+/+}$ vs. 758 ± 75 cells/mm^2 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 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/mm^2 in Ad-nls-LacZ–infected $^{-/-}$ mice vs. 1,535 ± 197 cells/mm^2

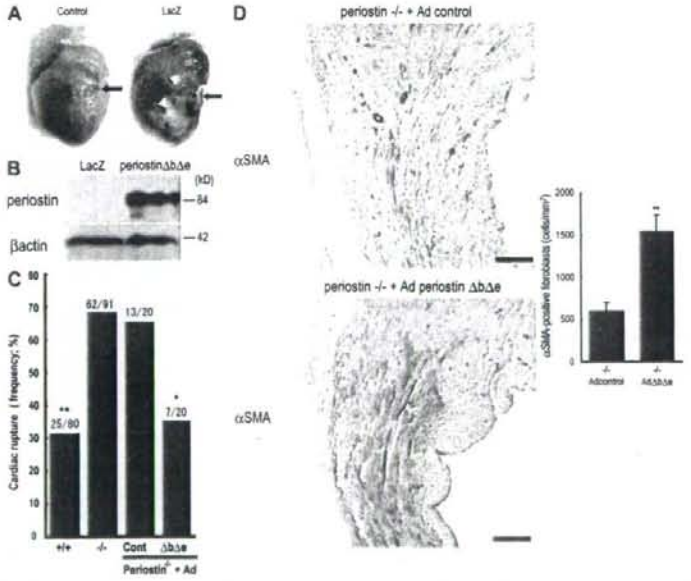

Figure 3. Adenovirus-mediated periostin ΔbΔe gene transfer prevents cardiac rupture in the *periostin*$^{-/-}$ mice. (A) Whole-mount X-gal staining 4 d after AMI showed strong expression in the border of the Ad-nls-LacZ–infected myocardial infarct (arrowheads). The arrow indicates the ligated portion. (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.

in Ad-ΔbΔe–infected $^{-/-}$ mice; $P < 0.01$; $n = 6$; Fig. 3 D). Furthermore, the Ad-ΔbΔe infection reduced the incidence of rupture frequency in *periostin*$^{-/-}$ mice (35.0%) compared with that for the Ad-nls-LacZ–treated −/− mice (65.0%; Fig. 3 C). These results demonstrate that periostin ΔbΔe was essential for in vivo recruitment of αSMA-positive fibroblasts to block rupture after AMI. As cell motility and morphology of fibroblasts are associated with the expression of the phosphorylated forms of Akt and focal adhesion kinase (FAK) (26, 27), we examined the phosphorylation of these proteins in the infarct border 5 d after AMI. The amount of phosphorylated Akt was reduced, and only a small amount of phosphorylated FAK was detected in the border of the *periostin*$^{-/-}$ infarcted 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 ΔbΔe. The presence of periostin ΔbΔe changed the cytoskeletal arrangement and motility of the cells, resulting in dynamic protrusion of their processes (Fig. 4 C). In a time-course

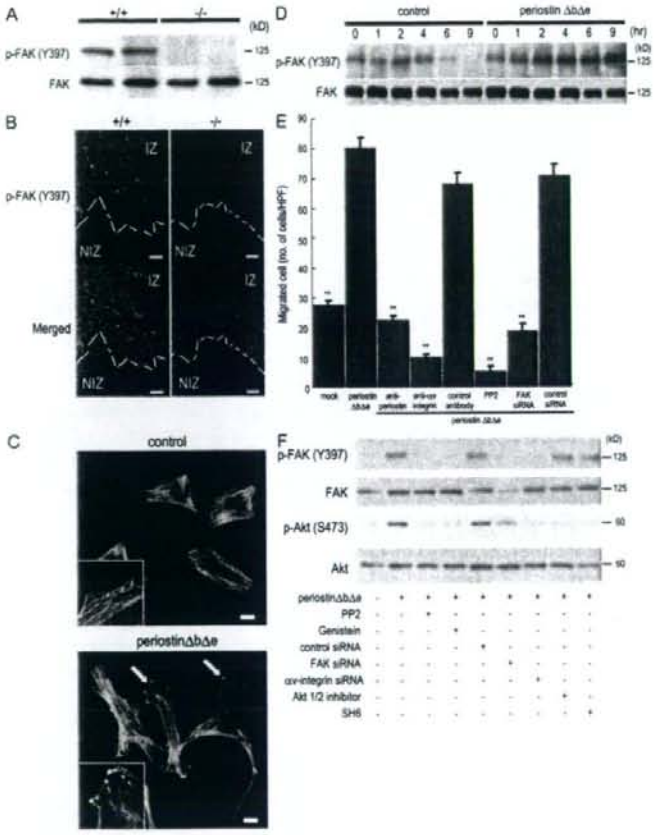

Figure 4. Periostin promotes cell migration through integrin-mediated FAK signaling. (A) Phosphorylation of FAK in infarct LV from *periostin*$^{+/+}$ mice and $^{-/-}$ mice 5 d after AMI. (B) Immunofluorescence for phosphorylated FAK (p-FAK^{Y397}) in the border of infarct LV from *periostin*$^{+/+}$ 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 ΔbΔe (C), and the p-FAK^{Y397} was examined by Western blot analysis at various times after adding periostin ΔbΔe (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 ΔbΔe, detected by an in vitro cell migration assay. Cardiac fibroblasts were significantly activated by periostin ΔbΔe, and treatment with neutralizing antibodies against periostin and αv-integrin, PP2, or FAK siRNAs reduced the cell migration. **, $P < 0.001$ vs. periostin ΔbΔe. 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 ΔbΔe with or without each siRNA or the FAK and Akt inhibitors. Bars: (B) 100 μm; (C) 20 μm.