Ablation of periostin inhibits post-infarction myocardial regeneration in neonatal mice mediated by the phosphatidylinositol 3 kinase/glycogen synthase kinase 3β/cyclin D1 signalling pathway

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Aims

To resolve the controversy as to whether periostin plays a role in myocardial regeneration after myocardial infarction (MI), we created a neonatal mouse model of MI to investigate the influence of periostin ablation on myocardial regeneration and clarify the underlying mechanisms.

Methods and results

Neonatal periostin-knockout mice and their wildtype littermates were subjected to MI or sham surgery. In the wildtype mice after MI, fibrosis was detectable at 3 days and fibrotic tissue was completely replaced by regenerated myocardium at 21 days. In contrast, in the knockout mice, significant fibrosis in the infarcted area was present at even 3 weeks after MI. Levels of phosphorylated-histone 3 and aurora B in the myocardium, detected by immunofluorescence and western blotting, were significantly lower in knockout than in wildtype mice at 7 days after MI. Similarly, angiogenesis was decreased in the knockout mice after MI. Expression of both the endothelial marker CD-31 and α-smooth muscle actin was markedly lower in the knockout than in wildtype mice at 7 days after MI. The knockout MI group had elevated levels of glycogen synthase kinase (GSK) 3β and decreased phosphatidylinositol 3-kinase (PI3K), phosphorylated serine/threonine protein kinase B (p-Akt), and cyclin D1, compared with the wildtype MI group. Similar effects were observed in experiments using cultured cardiomyocytes from neonatal wildtype or periostin knockout mice. Administration of SB216763, a GSK3β inhibitor, to knockout neonatal mice decreased myocardial fibrosis and increased angiogenesis in the infarcted area after MI.

Conclusion

Ablation of periostin suppresses post-infarction myocardial regeneration by inhibiting the PI3K/GSK3β/cyclin D1 signalling pathway, indicating that periostin is essential for myocardial regeneration.

Keywords

Periostin • Myocardial regeneration • Glycogen synthase kinase 3β • Cyclin D1 • Myocardial infarction

1. Introduction

While it remains under debate whether the myocardium can regenerate after ischaemic injury in the adult heart,1 neonatal mice show a capacity for myocardial regeneration after injuries such as myocardial infarction (MI) or resection of the left ventricular apex, but they lose this capacity within 7 days after birth.2–4 Recent studies have revealed that certain
relevant cytokines, proteins, physical and chemical factors, and genes may be involved in myocardial regeneration.\textsuperscript{3,5–7}

Periostin plays important roles during cardiac development and in the epithelial-mesenchymal transition.\textsuperscript{8} It was also linked to cardiovascular diseases such as dilated cardiomyopathy and MI.\textsuperscript{9,10} Several studies demonstrated function for periostin in regeneration of tissues including the myocardium.\textsuperscript{4,11,12} However, it remains controversial whether periostin can promote myocardial regeneration.\textsuperscript{13} A previous study using an adult MI model reported no significant differences in myocardial regeneration in periostin deficient or periostin transgenic mice, as compared with their corresponding wildtype strains.\textsuperscript{14} Intriguingly, using a neonatal heart resection model, a recent study demonstrated that signal transducers and activators of transcription 3 (STAT3)/periostin signalling is a critical mediator of interleukin 13 signalling in the regenerating mouse heart.\textsuperscript{8} Therefore, it may be a good way to resolve this controversy using an MI model in neonatal periostin knockout mice. We therefore used this approach to test our hypothesis that periostin is necessary for post-infarction myocardial regeneration in the neonatal heart.

In our study, we compared the myocardial regenerative capacity of neonatal periostin knockout and wildtype mice and further clarified the influence of periostin on the phosphatidylinositol 3-kinase (PI3K)/glycogen synthase kinase 3β (GSK3β)/cyclin D1 signalling pathway.

2. Methods

All procedures were performed in accordance with our institution’s guidelines for animal research that conform to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication, 8th Edition, 2011). Approval for this study was granted by our university’s ethics review board.

2.1 Neonatal mouse MI model

Periostin knockout mice (B6; 129-Postn\textsuperscript{Tm1Jmol/J}, Targeted: Null/ Knockout, Stock No: 009067. Donated by Jeffery D. Molkentin, Cincinnati Children’s Hospital) were from the Jackson Laboratory (Bar Harbor, ME, USA). The corresponding heterozygous mice were used for breeding. MI surgeries were performed on neonatal mice as described previously.\textsuperscript{4} Briefly, neonatal knockout mice and wildtype littermates, on the second day after birth, were anesthetized on ice for 3–4 min and maintained on ice during the surgical procedure. Anesthesia effectiveness was assessed based on reduced respiration. After disinfection of the incision area, the chest was opened with a horizontal incision through the muscle between the third and fourth intercostal spaces. The left coronary artery was permanently ligated with an 8-0 silk suture and the thoracic wall and skin were closed, also with an 8-0 silk suture. Sham-operated animals underwent an analogous surgical operation, but without occlusion of the coronary artery. After surgery, the skin was disinfected and the animals revived, while maintained on a thermal insulation blanket.\textsuperscript{14} Myocardial ischaemia was confirmed by an electrocardiogram ST-segment elevation after the animal was revived. Some mice were sacrificed at 1, 3, 7, or 10 days after surgery by putting them on ice for 5 min until respiration ceased. At 14 or 21 days after surgery, other mice were sacrificed by an overdose of pentobarbital sodium anesthesia (150 mg/kg intraperitoneal injection) or cervical dislocation.

In some periostin knockout mice, SB216763 (Sigma Aldrich, St. Louis, MO, USA), a GSK3β inhibitor, was administrated intraperitoneally (10 mg/kg/day, in dimethyl sulfoxide vehicle) beginning on the first day after surgery and continuing for 7 days.

2.2 Cell culture

Ventricular myocytes were isolated from neonatal periostin knockout and wildtype mice as described previously.\textsuperscript{15} The neonatal mice were killed by 2% isoflurane inhalation and cervical dislocation. Cardiomyocytes were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma Aldrich) supplemented with 10% fetal bovine serum (Equitech-Bio, Kerrville, TX, USA) for 72 h and then in serum-free DMEM for 48 h prior to use in experiments. The cells were exposed to anoxia for 3 h and reoxygenation for 24 h (AR) in the presence or absence of SB216763 (a GSK3β inhibitor, 3 μM, in dimethyl sulfoxide, added 2 h prior to AR).

2.3 Triphenyl tetrazolium chloride (TTC) staining

One day after surgery, some mice were killed, their hearts harvested and each heart cut into three pieces. MI was confirmed by staining with 1% TTC (Sigma Aldrich) at 37 °C for 20 min. Myocardial infarct size was measured using Image J Analysis software (National Institutes of Health, Bethesda, MD, USA).

2.4 Western blotting

Proteins were obtained from whole-heart homogenates, with tissue samples from three animals pooled for each biological replicate. Immunoblotting was performed with primary antibodies against GAPDH, PI3K, phosphorylated serine/threonine protein kinase B (p-Akt), Akt, p-GSK3β (1:1000, Cell Signaling Technology, Boston, MA, USA), phosphohistone3 (p-H3) (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), periostin (1:2000, Abcam, Cambridge, UK), GSK3β (1:1000, Santa Cruz Biotechnology), cyclin D1 (1:10 000, Abcam) and atrial natriuretic peptide (ANP) (1:1000, Santa Cruz Biotechnology). Samples containing equal amounts of protein per lane were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinyl difluoride membranes. The membranes were blocked with 5% bovine serum albumin (BSA) at room temperature for 2 h and then incubated overnight at 4 °C with the appropriate primary antibody. The blots were detected using a Super Signal ECL kit (Invitrogen, Carlsbad, CA, USA) in a western blotting detection system (Kodak Digital Science, Rochester, NY, USA) and quantified by densitometry using Image J Analysis software. For detection of p-Akt and p-GSK3β proteins, a stripping buffer (Thermo Fisher, Waltham, MA, USA) was used to remove conjugated antibody and the blot was then incubated with the second primary antibody. Electroblotting of each target protein and its loading control, GAPDH, was performed on the same gel, and then the gel was cut into two parts, in accordance with the molecular weight of each protein. Each gel protein was then processed separately for western blotting.

2.5 Histological examinations

Heart tissue from different groups was excised, rinsed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, embedded in paraffin and cut into 4–6 μm sections. Masson’s trichrome (Azan) staining was used to evaluate myocardial fibrosis. For immunohistochemistry (IHC), after antigen retrieval by incubation in citrate buffer (pH 6.0), sections were incubated with rabbit anti-mouse periostin or rabbit anti-mouse CD31 (Abcam) antibody overnight at 4 °C. The extent of new blood vessels was evaluated based on the density of endothelial cells using the ratio of positively stained area to gross area. To assess CD31 and periostin staining, each tissue section was scanned entirely and its staining intensity would be scored as 0 (negative), 1 (weak), 2 (medium), or 3 (strong). The extent of staining was scored as 0 (0%), 1 (1–25%),...
sections were treated with proteinase K for 20 min, incubated with the Detection Kit, TMR red (Roche, Basel, Switzerland). Heart tissues, assay. Briefly, apoptotic cells were detected with an In Situ Cell Death nucleotidyltransferase mediated (dUTP) nick-end labelling (TUNEL) 
ora B, respectively. Slides were washed 3 times in PBS and stained with 555 (1:100 dilution, Santa Cruz Biotechnology) to stain for p-H3 or aurora B. 
neutriniodyltransferase mediated (dTTP) nick-end labelling (TUNEL) assay. Briefly, apoptotic cells were detected with an In Situ Cell Death Detection Kit, TMR red (Roche, Basel, Switzerland). Heart tissues, embedded in paraffin and sectioned, were used. After deparaffinization, sections were treated with proteinase K for 20 min, incubated with the TUNEL reaction mixture or a negative control solution for 60 min at 37°C and then stained with DAPI solution for 10 min. Slides were washed twice with PBS after each step. The ratio of positive TUNEL-labelled nuclei was calculated from four different randomly selected areas, visualized by confocal microscopy.

2.6 Quantitative real-time polymerase chain reaction
Total RNA was extracted from heart tissues using Trizol reagent (Invitrogen). Real-time quantitative real-time polymerase chain reaction (qPCR) to detect mRNA for periostin, ANP and brain natriuretic peptide (BNP) and STAT3 in heart tissues was performed using a Quantitect SYBR RT-PCR kit (DRR420A, Takara, Japan). The primer sequences are shown in Supplementary material online, Table S1.

2.7 Echocardiography
Heart dimensions and cardiac function of mice at 21 days after surgery were evaluated by echocardiography using the Vevo 770 Ultrasound machine (VisualSonics, Toronto, Ontario, Canada) with a 30 M-Hz probe. After mice were anesthetized with 1.5% inhalational isoflurane, two-dimensional parasternal short-axis images of the left ventricle were obtained at the level of the papillary muscles. M-mode echocardiography was performed to evaluate internal diastolic and systolic left ventricular diameter (LVEDd, LVESd), and fractional shortening (LVFS).

2.8 Statistical analysis
Data were expressed as means ± standard error of the mean (SEM), and P < 0.05 was considered to be statistically significant. Statistical significance between two experimental groups was determined using Student’s two-tailed t-test, while comparisons of parameters among ≥3 groups with two factor levels were analyzed by two-way ANOVA, followed by Bonferroni’s correction for post hoc multiple comparisons.

3. Results
3.1 Periostin in the infarcted area was upregulated in response to MI in adult and neonatal mice
In neonatal mice, periostin mRNA was significantly upregulated at 1, 3, and 7 days after MI (Figure 1A). IHC staining revealed that periostin expression in the infarcted area peaked at 7 days and returned to baseline levels by 21 days after MI (Figure 1B and C). Periostin expression was detectable in the infarcted, border, and remote areas, but its abundance was greater in the border than in other areas (Figure 1C). In the adult mouse heart subjected to MI, periostin expression was also significantly increased (Supplementary material online, Figure S1A and B). Real-time qPCR and western blotting showed that periostin was significantly increased at 7 days after MI, compared with in the sham group (Figure 1D and E). The PCR results showed that periostin was expressed in both cardiomyocytes and fibroblasts derived from the neonatal mouse heart (Figure 1F).

3.2 Wildtype neonatal mice had the capacity to regenerate myocardium after MI
To investigate whether wildtype mice would have the capacity to regenerate myocardium after cardiac injury, we created an MI model in neonatal mice. The left coronary artery was permanently ligated, as shown in Figure 2A, and elevation of the ST segment after coronary ligation was confirmed (Figure 2A). We further verified this model with TTC staining. At 24h after MI, the infarct size was about 60% of the left ventricular area in both the periostin knockout and wildtype groups (Figure 2B). At 7 days after MI, immunofluorescence staining for p-H3 and aurora B, as well as for cell cycle entry and cytokinesis markers, was detectable in the infarcted, border, and remote areas (Figure 2C and D). These findings indicated myocardial regeneration. A time-course of Masson’s trichrome staining patterns showed significant fibrosis in the infarcted area at 1, 3, 7, 10, 14, and 17 days after MI (Figure 3F). The fibrosis had almost completely disappeared by 21 days after MI, indicating that myocardial regeneration was complete (Figure 2E).

3.3 Periostin deficiency hindered recovery of post-MI remodelling
At 7 days after surgery, there were no significant differences in the ratios of heart to body weight (HW/BW) between knockout and wildtype mice in either the sham or MI groups (Figure 3A). However, at 21 days after surgery, HW/BW in knockout MI- and sham-treated mice was significantly greater than in the corresponding wildtype groups (Figure 3A). HW/BW in both wildtype and knockout mice was not increased in response to MI (Figure 3A). At 7 days after MI, fibrosis, as detected by Masson’s trichrome staining, was similar in knockout mice and their wildtype littermates. However, at 21 days after MI, fibrosis was greater in the infarcted area of the knockout mice, while it had already completely disappeared in the wildtype mice (Figure 3B and C). This suggested that periostin deficiency suppressed myocardial regeneration after MI. TUNEL assay results showed no significant difference in apoptotic cell death between the wildtype and knockout groups at 7 days after MI (Figure 3D). Echocardiographic LVFS at 21 days after MI was significantly lower in the periostin knockout than in the wildtype group, while, in wildtype mice, there was no significant difference between sham and MI groups (Figure 3F). Echocardiographic data for left ventricular diameters are shown in Supplementary material online, Table S2.
3.4 Periostin deficiency suppressed myocardial regeneration

Western blotting results showed significantly lower levels of p-H3 in periostin knockout mice than in wildtypes at 7 days after MI (Figure 4A). Immunofluorescence staining showed that p-H3 and aurora B expression at 7 days after MI was significantly lower in knockout than in wildtype mice (Figure 4B and C), indicating involvement of periostin in myocardial regeneration after MI. In wildtype mice, qPCR and western blotting results showed, respectively, that myocardial mRNA of ANP and BNP, and ANP protein were significantly downregulated in response to MI at 7 days and ANP expression was, then, significantly upregulated at 21 days (Figure 4D and E). In periostin knockout mice, the only notable change was that ANP mRNA and protein expression were markedly upregulated at 21 days after MI, though their levels were significantly lower than in the corresponding wildtype MI group (Figure 4D and E). At 21 days after MI, ANP mRNA and protein expression were increased in hearts of both wildtype and knockout mice (Figure 4D and E). Expression of STAT3 mRNA was significantly lower in the knockout MI than in the wildtype MI group (Figure 4F).

3.5 Periostin affected the PI3K/Akt/GSK3β/cyclin D1 signalling pathway

At 7 days after MI, levels of P3K, p-Akt, Akt and cyclin D1 in wildtype mice were significantly higher than in periostin knockout mice. In contrast, levels of phosphorylated GSK3β were lower in the periostin knockout than in wildtype mice subjected to MI (Figure 5A–C). We
prepared cardiomyocytes from newborn wildtype and periostin knockout mice. When these cultured newborn mouse cardiomyocytes were treated with AR, we obtained similar results to those from the in vivo experiments (Figure 5D and E).

3.6 Periostin deficiency inhibited post-MI angiogenesis

We next investigated whether myocardial angiogenesis was affected by periostin ablation. CD31 was used to label endothelial cells, thus staining

Figure 2 Capacity for myocardial regeneration existed in neonatal mice subjected to myocardial infarction (MI). (A) Schematic diagram showing location of left coronary ligation and ST segment elevation in electrocardiogram monitoring. Scale bar = 1 mm. (B) Confirmation of MI by autopsy and TTC staining in mice 24 h after MI. Scale bar = 1 mm. The infarct size (%) was about 57% in wildtype (WT) and periostin knockout (KO) groups (t test). LV: left ventricle. (C, D) Representative images showing immunofluorescence staining for p-H3 and aurora B (arrow), in hearts from sham and MI mice obtained at 7 days after surgery and quantitative analysis. Inserts show high magnification images of p-H3 and aurora B-positive cardiomyocytes. *P < 0.01 vs. sham group, n = 8 per group (two-way ANOVA analysis). Scale bar = 50 μm (in the insert images, scale bar = 10 μm). (E) Masson’s trichrome staining of hearts obtained at 1, 3, 7, 10, 14, 17, or 21 days after MI. The lower panels show magnifications of the regions indicated by black boxes in the upper panels. Scale bar = 1 mm (upper) and 100 μm (lower).
Figure 3 Periostin deficiency resulted in post-MI cardiac fibrosis and dysfunction caused by attenuated myocardial regeneration. (A) Heart weight to body weight ratios (HW/BW), at 7 and 21 days after MI or sham surgeries in neonatal mice. *P < 0.05 compared with corresponding wildtype group, n = 5 per group (two-way ANOVA analysis). (B) Masson's trichromatic (Azan) staining of the myocardium from wildtype (WT) and periostin knockout (KO) groups, at 7 and 21 days after MI. The lower panels show magnifications of the regions indicated by black boxes in the upper panels. Scale bar = 1 mm (upper) and 100 μm (lower). (C) Semi-quantitative analysis of myocardial fibrosis in panel B. The insert images are multiple cross sections of Azan staining in hearts from mice, obtained at 7 days after MI surgery. *P < 0.01 vs. the corresponding sham group, #P < 0.05 vs. WT MI (at 21 d) group, n = 8 per group (two-way ANOVA analysis). (D) Apoptotic myocardial cell death, visualized by TUNEL staining, in knockout and wildtype mice subjected to sham and MI surgeries, after 7 d. (E) Quantitative analysis showed no differences between wildtype and knockout groups. *P < 0.01 vs. the corresponding sham group, n = 8 per group (two-way ANOVA analysis). Scale bar = 100 μm. (F) Left ventricular systolic function, quantified by left ventricle fractional shortening (LVFS) at 21 days after MI in four groups. *P < 0.05 vs. the corresponding sham group, #P < 0.01 vs. WT MI group, n = 8 per group (two-way ANOVA analysis).
Figure 4 Periostin knockout (KO) suppressed cardiomyocyte regeneration. (A) Western blots for phospho-histone 3 (p-H3) and semi-quantitative analysis of western blotting results. *P < 0.05 vs. the corresponding sham group, **P < 0.01 vs. WT MI group (n = 8 per group). Immunofluorescence for p-H3 (B) and aurora B (C) in cardiomyocytes counterstained for troponin T and the results of quantitative analysis for p-H3 and aurora B stained cells (indicated with arrows). Scale bar = 50 μm. *P < 0.05 vs. the corresponding sham groups, **P < 0.01 vs. WT MI group, n = 8 per group. (D) mRNA expression of myocardial ANP and BNP was determined by real-time qPCR in neonatal mice at 7 or 21 days after MI or sham surgeries. *P < 0.05 vs. corresponding sham group, **P < 0.01 vs. WT MI group (n = 8 per group). (E) Western blot for ANP in neonatal hearts from four groups, obtained 7 or 21 days after MI. *P < 0.05 vs. corresponding sham group. WT, wildtype; MI, myocardial infarction.
the blood vessels. At 7 days after MI, periostin knockout mice had less CD31 staining than wildtype mice (Figure 6A). To further confirm this result, we stained for a smooth muscle cell marker, smooth muscle actin (α-SMA) (Figure 6B). α-SMA staining confirmed the marked decrease in vascular area in knockout, compared with wildtype mice after MI (Figure 6B). Because CD31 and α-SMA stains can label both pre-existing and newly formed capillaries and arterioles, respectively, vascular density was compared with that in the control mice, not receiving MI, to ascertain which vessels had resulted from angiogenesis. Our findings suggested a role for periostin in post-MI angiogenesis in neonatal mice.

3.7 GSK3β inhibition in periostin knockout mice promoted myocyte regeneration and angiogenesis

We further performed rescue experiments to test whether the GSK-3β inhibitor SB216763 would improve cardiomyocyte regeneration and angiogenesis in the periostin knockout mice. SB216763 (10 mg/kg/d) was intraperitoneally injected for 7 d. Myocardial GSK-3β expression was decreased and that of cyclin D1 was increased at 7 days after MI in the SB216763 treated mice (Figure 7A). Histological immunofluorescence staining showed significantly higher levels of p-H3 and aurora B in SB216763 treated than in untreated mice at 7 days after MI (Figure 7B and C). At 21 days after MI, myocardial fibrosis was significantly lower in SB216763 treated than in untreated mice (Figure 7D). At 7 days after MI, there was a larger area of α-SMA stained vessels in SB216763 treated than in untreated mice (Figure 7E). SB216763 treatment significantly improved echocardiographic LVFS at 21 days after MI (Figure 7F; Supplementary material online, Table S3).

4. Discussion

Since the generation of periostin deficient mice, many studies examined the roles of this factor in the regeneration of various tissues including bone, heart and skin as well as in tumor growth. Based on results of studies using MI models in adult rodents, the role of periostin in
myocardial regeneration has been controversial. Kühn et al. reported that peristin released from patches placed over the infarcted area of the adult rat heart induced proliferation of differentiated cardiomyocytes and improved cardiac function, while suppressing myocardial fibrosis and hypertrophy. Cho et al. demonstrated that injection of mesenchymal stem cells overexpressing peristin into the infarcted regions of rat hearts attenuated post-MI remodelling. These findings supported involvement of peristin in promoting myocardial regeneration in the adult heart. However, Lorts et al. showed no significant difference in post-infarction myocardial regeneration between mice with modulated peristin expression (transgenic and knock out mice) and their corresponding strain-matched controls. Taniyama et al. reported that inhibition of peristin-exon 17 attenuated post-MI fibrosis in adult rats but did not affect cardiomyocyte proliferation. These reports suggested, in contrast to other findings, that peristin is not involved in post-infarction myocardial regeneration in the adult heart. Similarly, based on results obtained using neonatal rodent cardiomyocytes, the regeneration promoting ability of peristin was disputed. A recent study by White et al. indicated that the capacity of the neonatal mammalian heart for regeneration required sympathetic innervation, which might explain why peristin exerted no regenerative effect in cultured cardiomyocytes, where sympathetic activity was not a factor. It is generally believed that the capacity for cardiac regeneration is absent in the adult mammalian heart, while recent studies confirmed its existence in the neonatal mammalian heart. Accumulated evidence demonstrated that a variety of injuries could induce heart regeneration, through cardiomyocyte proliferation, in newt, zebrafish and newborn mice (review by Leone et al.). Based on such evidence, taken together, we postulated that using an in vivo neonatal heart injury model could help resolve controversies regarding the role of peristin in myocardial regeneration. Therefore we designed this study.

The regenerative model of the murine heart is controversial. Andersen et al. found no evidence of complete regeneration and questioned the utility of the apical resection model, whereas Konfinno et al. observed significantly greater scar formation following left coronary artery ligation associated with a lack of induction of cardiomyocyte proliferation. These findings contradicted substantial reports from various laboratories, demonstrating that neonatal mice have the capacity for heart regeneration in response to injuries, including resection of the apex and occlusion of the left coronary artery. There is also controversy regarding the capacity for heart regeneration in response to cryo-injury. Strungs et al. demonstrated that the apex of the heart ventricle, cryoinjured at 1 days after birth, had no visible scar and could fully regenerate myocardium, but at least two groups reported that cryo-injury or cryo-transmural infarction led to scar formation. The technical difficulties of inducing neonatal heart injury and various choices in anesthesia may have contributed to the variations in results on regeneration reported by different laboratories. Blom et al. recently characterized this model clearly with video recording and demonstrated that the MI-induced scars completely disappeared by 21 days post-injury. Peristin overexpression was proposed to promote re-entry of differentiated cardiomyocytes into the cell cycle and, consequently, contribute to myocardial repair following MI. Negative results in some studies were, therefore, not surprising because peristin may be necessary but not sufficient to induce cardiomyocyte regeneration. In neonatal mice, O’Meara et al. demonstrated that interleukin 13 induced entry of cardiomyocytes into the cell cycle and identified STAT3 and peristin as critical mediators of interleukin 13 signalling. This supports the concept that peristin can contribute to inducing regeneration but, alone, is not sufficient. In our study, we verified the regeneration-promoting potential of peristin using peristin knockout mice. We found that, in this strain of neonatal mice subjected to MI, peristin deficiency impaired the regenerative capacity of cardiomyocytes. Similar
Figure 7  GSK3β inhibitor SB216763 improved cardiomyocyte regeneration and angiogenesis in periostin knockout mice. (A) SB216763 (SB) downregulated GSK3β and upregulated cyclin D1 in periostin knockout (KO) mice at 7 days after myocardial infarction (MI). (B) Phosphorylated histone 3 (p-H3) stained cells. (C) Aurora B stained cells. (D) SB216763 attenuated myocardial fibrosis in periostin knockout neonatal mice at 21 days after MI or sham operations. Fibrotic areas were detected by Masson’s trichrome staining. Scale bar = 1 mm (upper) and 100 µm (lower). (E) α-SMA (smooth muscle actin) immunofluorescence staining was used to determine arteriolar density in periostin knockout neonatal mice at 21 days after MI or sham operations. Scale bar = 50 µm. (F) SB216763 treatment improved left ventricular fractional shortening, as determined with echocardiography. *P < 0.01 vs. the corresponding sham + V group, #P < 0.01 vs. MI + V group (n = 8 per group). V, vehicle (dimethyl sulfoxide). For all panels, two-way ANOVA analysis was used.
results were described by others using non-cardiovascular disease models, with recent studies showing that periostin promoted pancreatic exocrine regeneration and neural stem cell proliferation.\(^{44,35}\)

It was reported that plasma or myocardial ANP and BNP were elevated shortly after MI in humans and adult rodents.\(^{36,37}\) However, no reports described myocardial ANP expression in neonatal mice with MI. We found that, in neonatal mice, myocardial ANP was downregulated at 7 days after MI but was upregulated at 21 d. Schoenig et al.\(^{38}\) reported that plasma levels of the N-terminal propeptide of ANP were not increased in adult mice subjected to non-ischaemic MI for 4 weeks. In a recent report by Bielmann et al.,\(^{39}\) BNP stimulated cardiac progenitor cell proliferation and differentiation in murine hearts after birth and BNP administration induced heart regeneration. Becker et al.\(^{39}\) also demonstrated in vitro that ANP induced proliferation of neonatal murine cardiomyocytes. The potentially interesting association between periostin and natriuretic peptides should be further investigated in the future.

With regard to regeneration mechanisms of periostin, it was previously reported that PI3K, extracellular-signal-regulated kinases and STAT3/STAT6 were involved.\(^{4,40}\) Emerging evidence has shown that the GSK3\(^{\beta}\)-cyclin D1 signalling pathway is closely associated with cell proliferation and cardiovascular diseases.\(^{41-44}\) However, the role of periostin is also involved in this pathway. In our study, we found that periostin ablation led to upregulation of GSK3\(^{\beta}\) and downregulation of cyclin D1, while a GSK3\(^{\beta}\) inhibitor partially rescued the regeneration capacity of the heart after MI in the neonatal periostin knockout mice.

In adult mice with MI, whether GSK3\(^{\beta}\) is beneficial or detrimental for cardiac remodelling has been controversial.\(^{42,45-47}\) However, it is generally believed that GSK-3\(^{\beta}\) is critical for embryonic cardiomyocyte proliferation and differentiation. GSK3\(^{\beta}\) deletion induced embryonic lethality, caused by near obliteration of the ventricular cavities by proliferating cardiomyocytes. In addition, terminal cardiomyocyte differentiation was substantially blunted in embryoid bodies with GSK3\(^{\beta}\) deficiency.\(^{44,45}\) Ahmad et al.\(^{46}\) reported that cardiomyocyte-specific GSK3\(^{\alpha}\) deletion attenuated post-infarction cardiac remodelling and heart failure.\(^{46}\) These results were consistent with our observations that increased GSK3\(^{\beta}\) in periostin knockout mice impaired post-MI regeneration of the myocardium, while SB216763, a pan inhibitor of both GSK3\(^{\alpha}\) and GSK3\(^{\beta}\), improved myocyte regeneration and attenuated cardiac remodelling in post-infarcted periostin knockout mice.

The role of periostin in myocardial fibrosis in adult animals is also unclear.\(^{42,46,49}\) In our study, we focused on the role of periostin in cardiomyocyte regeneration in neonatal mice with MI. Unlike adult mammalian hearts, that respond to injury with scar formation, neonatal mouse hearts respond to MI with cardiomyocyte proliferation. We demonstrated that, in wildtype mice, myocardial fibrosis was significantly formed at 7 days after MI but was completely replaced by myocardium at 21 d, in agreement with previous studies.\(^{2,6}\) In periostin knockout mice, myocardial fibrosis in the infarcted area was still present at 21 days after MI, possibly a net result of impaired cardiomyocyte regeneration capacity, counterbalancing the anti-fibrotic effects of periostin deficiency on cardiac fibroblasts.\(^{49,51}\) In addition, other mechanisms may have also contributed to the impaired cardiomyocyte regenerative capacity in the periostin knockout mice. Periostin can affect collagen formation and recruitment of macrophages.\(^{52,53}\) Schwanekamp et al.\(^{54}\) showed that loss of periostin decreased macrophage recruitment to atherosclerotic lesions.\(^{54}\) Although periostin deficiency induced a large set of differentially expressed genes related to fibroblast function and contributed to post-MI rupture by attenuating scar (fibrosis) formation in adult mice,\(^{52}\) it was also likely to reduce macrophage recruitment. This would, in turn, inhibit myocardial regeneration and eventually lead to replacement of the infarcted myocardium with fibrotic tissue in newborn mice.\(^{24}\) Therefore, it would be worthwhile to, in future studies, investigate the contribution of macrophages to impairment of myocardial regeneration associated with periostin deficiency.

We further found that periostin ablation impaired post-MI angiogenesis, results supported by previous studies in adult animals. Kühn et al.\(^{55}\) reported that periostin improved post-MI ventricular remodelling, reduced fibrosis and increased angiogenesis.\(^{16}\) Hakuno et al.\(^{55}\) demonstrated that periostin induced angiogenesis and promoted tube formation by mobilization of endothelial cells. We noted a significant decrease of CD31 positive endothelial cells, indicating capillaries, in periostin knockout mice with MI, suggesting that periostin affected angiogenesis-associated endothelial cells. Our findings were consistent with previous reports showing that periostin promoted tube formation by mobilization of endothelial cells.\(^{55,56}\) In the postnatal heart, endothelial cells contributed to postnatal vascular development in the heart, an effect that was enhanced in response to hypoxia.\(^{57}\) This was consistent with our finding that, in wildtype mice, capillary density was higher in the infarcted areas of hearts from the MI group than in the corresponding areas of hearts from the sham group. However, it remains unclear whether periostin can affect the response of endothelial cells to ischaemia.

Although it was believed that the majority of newly formed cardiomyocytes are derived from pre-existing cardiomyocytes,\(^{2}\) there is evidence that resident non-myocytes can also be reprogrammed into cardiomyocyte-like cells by addition of Gata4, Mef2c and Tbx5, an aspect confirmed by using genetic lineage tracing and periostin-Cre R26R-lacZ mice in a murine MI model.\(^{58,59}\) It would interesting to investigate the contributions of periostin on non-myocyte derived cardiomyocyte regeneration using genetic lineage tracing.

In conclusion, our findings indicate that a lack of periostin impairs post-MI regeneration of cardiomyocytes and angiogenesis, effects mediated by the PI3K/GSK3\(^{\beta}/\text{cyclin D1 signalling pathway.}\)

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Conflict of interest**: none declared.

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