Original Paper

Gremlin2 Regulates the Differentiation and Function of Cardiac Progenitor Cells via the Notch Signaling Pathway

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Key Words

Cardiac progenitor cells (CPCs) • CPC differentiation • Myocardial infarction • Cardiac Function

Abstract

Background/Aims: The transplantation of cardiac progenitor cells (CPCs) improves neovascularization and left ventricular function after myocardial infarction (MI). The bone morphogenetic protein antagonist Gremlin 2 (Grem2) is required for early cardiac development and cardiomyocyte differentiation. The present study examined the role of Grem2 in CPC differentiation and cardiac repair. **Methods:** To determine the role of Grem 2 during CPC differentiation, c-Kit⁺ CPCs were cultured in differentiation medium for different times, and Grem2, Notch1 and Jagged1 expression was determined by RT-PCR and western blotting. Short hairpin RNA was used to silence Grem2 expression, and the expression of cardiomyocyte surface markers was assessed by RT-PCR and immunofluorescence staining. *In vivo* experiments were performed in a mouse model of left anterior descending coronary artery ligation-induced MI. **Results:** CPC differentiation upregulated Grem2 expression and activated the Notch1 pathway. Grem2 knockdown inhibited cardiomyocyte differentiation, and this effect was similar to that of Notch1 pathway inhibition *in vitro*. Jagged1 overexpression rescued the effects of Grem2 silencing. *In vivo*, Grem2 silencing abolished the protective effects of CPC injection on cardiac fibrosis and function. **Conclusions:** Grem2 regulates CPC cardiac differentiation by modulating Notch1 signaling. Grem2 enhances the protective effect of CPCs on heart function in a mouse model of MI, suggesting its potential as the therapeutic protein for cardiac repair.

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Introduction

Cardiovascular diseases are the leading cause of death worldwide [1]. Ischemic disease is the most common cause of heart failure, the prevalence of which is increasing at an accelerated rate. Despite significant advances in therapeutic options for cardiovascular diseases, the prognosis of patients with heart failure remains poor, with a 5-year mortality of 50% [1]. Stem cell therapy has gained increasing attention for its potential in the regeneration of myocardial tissues [2]. Cardiac progenitor cells (CPCs), which are found in the fetal and adult heart of many mammals, are resident cardiac stem cells with the capacity for self-renewal and differentiation into myogenic, vascular endothelial, and smooth muscle lineages *in vitro* [3]. CPCs are divided into c-kit, Sca-1, and Isl-1 cells according to the expression of surface markers. Although present in small numbers endogenously, injected CPCs have shown the capacity to regenerate ischemic/infarcted myocardium in research animals as well as in human trials, and they are therefore of interest for their use in cardiac repair [2, 4-6].

Gremlin 2 (Grem2) is an antagonist of bone morphogenetic proteins (BMP), which belong to the transforming growth factor β superfamily of ligands and play important roles during cardiac development [7]. BMP signaling is induced in response to ischemic injury and has been implicated in cardiomyocyte apoptosis during ischemia/reperfusion [8, 9]. Grem2 is necessary for cardiac development and cardiomyocyte differentiation, and Grem2 expression is induced in response to cardiac injury [10]. BMP signaling involves binding to a type II receptor leading to the phosphorylation of SMAD proteins and the formation of a complex that translocates to the nucleus to regulate the transcription of target genes [11]. Factors that modulate SMAD signaling regulate the Notch pathway, an evolutionarily conserved system with important roles in cellular differentiation, and crosstalk between the Notch and BMP pathways regulates differentiation and organ development. Notch receptors interact with their ligands (Delta-like and Jagged), leading to the cleavage of the Notch intracellular domain and its translocation to the nucleus, where it regulates the expression of different target genes [12]. Notch signaling thus regulates cell fate and plays a role in vascular development.

In the present study, we examined the role of Grem2 in the differentiation of CPCs *in vitro* and explored the underlying regulatory mechanisms. The involvement of Grem2 in CPC differentiation *in vivo* and its effect on myocardial fibrosis and cardiac function after CPC transplantation was examined in a mouse model of myocardial infarction (MI).

Materials and Methods

Cardiac progenitor cell isolation

c-Kit⁺ CPCs were isolated from 8-week-old male C57BL/6 mice as previously described [13]. Primary cell culture was performed for 3 days in CPC-maintenance medium (DMEM/F12-K 1:1, 20% ES cell qualified FBS, 10 ng/mL bFGF, 20 ng/mL EGF, 100U LIF, and 1 $\times$ insulin-transferrin-selenium [ITS]) and differentiated at passage 3. At this passage the cells were positive for c-Kit and CD29 (Fig. 1A). The characteristics of isolated c-Kit⁺ CPCs were determined by flow cytometry in a BD FACSCanto II flow cytometer using BD FACSDiva software (Becton Dickinson, San Jose, CA, USA).

CPC differentiation

For CPC differentiation cells were seeded at a density of 25000 cells/cm² in CPC-maintenance medium and after 24 h, the medium was replaced with CPC-differentiation medium (advanced DMEM/F12, 0.2% w/v BSA, 2 mM L-glutamine, 1 $\times$ ITS, and 250 μ mol/L ascorbic acid). The medium was changed every 2 days.

Short hairpin RNA (shRNA) and plasmid transfection

Mouse Gremlin 2 shRNA and control shRNA plasmids were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For transfection, c-Kit⁺ CPCs at passage 2 were seeded into 6-well plates at a density

Fig. 1. Upregulation of Gremlin 2 and activation of Notch1 signaling in differentiated CPCs. (A) c-Kit and CD29 expression in passage 3 CPCs was determined by flow cytometry. At this passage the cells were positive for c-Kit and CD29. CPCs were incubated in differentiation medium for the indicated times. The mRNA (B) and protein (C) levels of Gremlin 2 (Grem2) were determined by RT-PCR and western blotting. The expression of Notch1, Notch2, Jagged1, and Jagged2 was assessed by RT-PCR (D). (E) Representative western blots showing the levels of the full-length (FL) Notch1, the Notch1 intracellular domain (Notch1 ICD), and Jagged1 in differentiated CPCs. β -actin was used as the loading control. All experiments were repeated independently three times. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

of 5×10^4 cells/well in CPC maintenance medium (2 mL). After 24 h, transfection complex [1 μ g of vector DNA (10 μ L) diluted in OptiMem serum free medium (Invitrogen, Carlsbad, CA) (90 μ L) plus Lipofectamine 2000 and 20 min incubation] was added to the cells and incubated for 24 h. Cells were trypsinized, seeded into 75 cm² flasks, and selected with puromycin. Stable cell lines were obtained at passage 3. Gremlin 2 expression was confirmed by western blotting and RT-PCR.

Mouse Jagged1 ORF clone, mouse Gremlin 2 ORF clone and pCMV6-Entry empty plasmids were obtained from OriGene (Rockville, MD, USA). Smad2 or Smad3 siRNA and non-targeting siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For inhibitor studies, CPCs were stimulated with the γ secretase inhibitor DAPT (10 μ M, Sigma).

Real-time RT-PCR

Total RNA was extracted with the Trizol reagent (Invitrogen) according to the manufacturer's instructions, reverse transcribed, and amplified using a QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) and the ABI PRISM 7900 quantitative PCR system (Applied Biosystems, Foster City, CA, USA). β -actin was included as an internal control. Each sample was measured in triplicate. Primers used for RT-PCR are listed in Table 1.

Western blotting

Cells were lysed in RIPA buffer supplemented with protease inhibitors (Roche), centrifuged, and the supernatants were collected for analysis. Protein content was quantified using the BCA Protein Assay (Pierce, Rockford, IL, USA). Aliquots containing 30 μ g of protein per lane were separated in 4–12% gels and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking in 5% nonfat milk in PBS, membranes were incubated with rabbit polyclonal antibodies against Grem2, Jagged1, Hes1, β -actin (Santa Cruz Biotechnology, Santa, CA, USA), and full-length Notch, Notch1 ICD, Gata4, and cardiac Troponin-I (cTnI), p-Smad1/5, p-Smad2, p-Smad3, Smad1, Smad2, Smad3 (Abcam, Cambridge, MA, USA) overnight at

4°C. Bands were visualized by incubation with HRP-conjugated secondary antibodies and the enhanced chemiluminescence system (Pierce, Rockford, IL, USA).

Immunofluorescence staining

For immunofluorescence staining, cells were fixed with 2% paraformaldehyde and incubated with primary antibodies against

Mef2c and cTnI overnight at 4°C followed by Alexa-Fluor conjugated secondary antibodies (Invitrogen) at 1:500 dilution for 1 h at room temperature. Nuclei were stained by DAPI.

Flow cytometry analysis

Cell surface marker detection was performed as described previously [14, 15]. For CPC differentiation, the induced cells were collected and incubated with Mef2c or cTnT (Abcam) and FITC-conjugated secondary antibody respectively for 1 hour at room temperature. Finally, the cells were washed twice with PBS, resuspended in PBS and fixed 4% paraformaldehyde for 20 min. Mef2c or cTnT expression rate was detected by flow cytometry analysis.

Animal experimental protocols

All experiments were approved by the Institutional Animal Research Committee of Baotou Central hospital, Inner Mongolia, China, and were performed in conformance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). All mice were randomized into treatment and sham-operated groups.

MI and cell injection were performed in 12-week-old male C57BL/6J mice. Mice underwent thoracotomy under general anesthesia with 2.5% sevoflurane, and MI was induced by ligation of the left anterior descending coronary artery. For cell injection, 10⁵ CPCs from each group suspended in 21 µl PBS or vehicle were injected into three sites around the infarct border zone. After injection, the chest was closed and animals were allowed to recover.

Cardiac function measurement by echocardiography

Cardiac function was monitored by echocardiography 1 day before the ligation (baseline) and at 2 and 4 weeks after surgery using the Vevo 770 (VisualSonics, Toronto, ON, Canada) with a 30 MHz transducer. M-mode tracings were used to measure LV wall thickness, and end-systolic and end-diastolic diameter. The percentage fractional shortening (%FS) and ejection fraction (%EF) were calculated as described previously [16].

Histology

For histological analyses, mice were sacrificed 4 weeks after MI and the hearts were fixed overnight at 4°C in 10% neutral buffered formalin, followed by 30% sucrose for 24 h at 4°C. The heart was cut into 0.5 mm sections, which were paraffin embedded and cut into 10 micron slices. Sections were stained with Masson's trichrome to assess fibrosis. The stained sections were imaged using light microscopy, and photographed. Percent fibrosis was determined using ImageJ software to quantify blue (fibrotic) versus nonblue (nonfibrotic) pixels.

Statistical analysis

All data are expressed as the mean ± SD. Differences were analyzed using the *t*-test or analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. Significance was set at *p* < 0.05.

Table 1. Primers for RT-PCR

Primer	Sense	Antisense	Product size
Gremlin 2	AGAGTGACTGGTGCAAGACG	TGATTGGGAAAGGTGGGTCG	234bp
Notch1	TGACATCGATGACTGCCGC	TCAGGTGTGTTGTTCTCGCA	240bp
Notch2	GCTGGGTTGGTGCAACTGT	TTCCTTGGTTTCAGACACGGG	183bp
Jagged1	CTGCTTGAATGGGGGTCAC	GGTACGGCAGTGGTCTTTCA	223bp
Jagged2	GTATATGTGGCCCTCAGGG	GAGGCAGTCGTTGGGATTGA	227bp
Nkx2-5	ATTTTACCCGGGAGCCTACG	ACAGGTACCGCTGTTGCTTG	218bp
Gata4	ACCCTGGAAGACACCCCAAT	CTTAATGAGGGCCGTTTGA	179bp
Mef2c	CTGTTCCACCTCCAGCTT	ATCAGACCGCTGTGTTACC	196bp
cTnI	GTTGCCAGCCTTTGGAGTTG	AAACTTGCCACGGAGGTCAT	182bp
β-actin	CCCCTGAACCCTAAGGCCA	ATGGCGTGAGGAGAGCATA	200bp

Results

Gremlin2 is upregulated and Notch signaling is activated during the differentiation of CPCs

Differentiation of c-Kit⁺ CPCs was induced by incubation in differentiation medium for the indicated times. The expression level of Gremlin2 increased in a time-dependent manner, reaching peak levels (approximately 10-fold upregulation) on day 7 and decreasing thereafter, as determined by RT-PCR and western blotting (Fig. 1B and C). The role of the Notch pathway in CPC differentiation was analyzed by RT-PCR and western blotting. As shown in Fig. 1D, incubation in differentiation medium significantly downregulated Notch1 at 7 and 14 days of differentiation, with a concomitant significant upregulation of the Notch ligand Jagged1. Changes in Notch2 expression were not significant, and Jagged2 was significantly downregulated on day 14. Western blot analysis showed that the cleaved active form of Notch1 (Notch1 ICD) and the Jagged1 protein were markedly upregulated on days 7 and 14, whereas full-length Notch1 was downregulated (Fig. 1E). These data indicated that Gremlin2 and the Notch1 pathway may be involved in the differentiation of CPCs into cardiomyocytes.

Gremlin2 silencing inhibited the differentiation of CPCs into cardiomyocytes by inhibiting Notch1 signaling

To investigate the role of Gremlin2 in CPC differentiation, Gremlin2 knockdown and control CPCs were incubated in differentiation medium for the indicated times. The upregulation of Gremlin2 expression induced during differentiation was abolished in Gremlin2 shRNA treated

Fig. 2. Gremlin2 knockdown inhibited CPC differentiation. CPCs expressing Gremlin2 shRNA or control shRNA were differentiated for the indicated times. The mRNA (A) and protein (B) levels of Gremlin2 were determined by RT-PCR and western blotting. (C) RT-PCR analysis of the cardiac markers Nkx2.5, Gata4, Mef2c, and cTroponin-I (cTnI) in differentiated CPCs. (D) CPCs expressing Gremlin2 shRNA or control shRNA were cultured for 7 days in CPC-differentiation medium. The cells were subsequently stained with Mef2c or cTnI antibodies. DAPI was used to stain nuclei. Scale bar, 100 μ m. The number of positive cells is shown in the histogram. (E) CPCs expressing Gremlin2 shRNA or control shRNA were cultured in CPC-differentiation medium for 0, 7 and 14 days. The protein levels of Gata4 and cTnI were detected by western blotting. β -actin was used as the loading control. * $P < 0.05$, ** $P < 0.01$.

CPCs compared with control shRNA-treated CPCs at the mRNA and protein levels (Fig. 2A and B). RT-PCR showed that the expression of the cardiac markers *Nkx2.5*, *Gata4*, *Mef2c*, and *cTnI* was upregulated during differentiation in control CPCs, and this effect was inhibited by *Grem2* knockdown (Fig. 2C). To validate the qPCR experiments, *c-Kit*⁺ CPCs were cultured for 7 days in CPC differentiation media and then stained for two cardiac markers, *Mef2c* and *cTnI*. Differentiated control CPCs showed robust staining for *Mef2c* and *cTnI*, while the number of *Mef2c* and *cTnI* positive cells was severely reduced in differentiated *Grem2* knockdown CPCs (Fig. 2D). Western blot analysis of the markers *Gata4* and *cTnI* showed that the differentiation-induced upregulation of *Gata4* and *cTnI* was suppressed by *Grem2* knockdown in CPCs (Fig. 2E). These results indicated that *Grem2* may play a key role in the differentiation of CPCs into cardiomyocytes.

Grem2 mediated CPC differentiation involves the Jagged1-Notch1 pathway

RT-PCR showed that *Grem2* knockdown significantly upregulated the mRNA levels of *Notch1* (Fig. 3A) and downregulated those of *Jagged1* during CPC differentiation (Fig. 3B). Similar results were obtained by western blot analysis, which showed that *Grem2* shRNA markedly inhibited the differentiation-induced upregulation of *Notch1* ICD and *Jagged1* and the downregulation of full length *Notch1* (Fig. 3C), suggesting that loss of *Grem2* inhibited the activation of the *Jagged1-Notch1* pathway during CPC differentiation. The activation of *Notch* signaling was blocked in CPCs with the γ -secretase inhibitor DAPT. RT-PCR demonstrated that the differentiation medium-induced upregulation of cardiac

Fig. 3. The Notch1 pathway is involved in Gremlin 2-mediated CPC differentiation. CPCs expressing *Grem2* shRNA or control shRNA were differentiated for the indicated times. The mRNA levels of *Notch1* (A) and *Jagged1* (B) were determined by RT-PCR. * $P < 0.05$, ** $P < 0.01$. (C) Representative Western blots showing the levels of the full-length (FL) *Notch1*, the *Notch1* intracellular domain (*Notch1*ICD), and *Jagged1* in differentiated CPCs. β -actin was used as the loading control. (D) CPCs were treated with DAPT (10 μ M) or *Grem2* shRNA and transfected with *Jagged1*-overexpressing plasmids. Treated cells were cultured in CPC-differentiation medium for 0 and 7 days. The mRNA expression of the cardiac markers *Nkx2.5*, *Gata4*, *Mef2c*, and *cTroponin-I* (*cTnI*) was determined by RT-PCR. ** $P < 0.01$. (E) At 7 days, the percentage of CPCs positive for *Mef2c* and *cTnI* was determined by flow cytometry. ** $P < 0.01$. The histogram was obtained from a single experiment that is representative of three independent experiments. (F) The protein levels of *Gata4* and *cTnI* were detected by Western blotting. β -actin was used as the loading control.

Fig. 4. Grem2 activates the Jagged1-Notch1 pathway via controlling Smad1/5 and Smad2/3 signaling balance during the differentiation of CPCs (A) The Smad phosphorylation was determined in differentiated CPCs with Grem2 knockdown or Grem2 overexpression at 0, 7 and 14 days. Representative western blots showing the levels of the phospho (p)-Smad1/5, p-Smad2, p-Smad3, Smad1, and Smad2 in differentiated CPCs. (B) The control (C) or Grem2 overexpression (G)-CPCs

were transfected with non-targeting (NT) siRNA, Smad2, Smad3 or Smad2/3 siRNA, the induction of Jagged1, the expression of Hes1 as the downstream gene of Notch signaling and the cardiac markers Gata4 and cTnI were detected by western blotting after 7 days of culture in cardiac differentiation medium. β -actin was used as the loading control. All experiments were repeated independently three times.

markers such as Nkx2.5, Gata4, Mef2c, and cTnI at 7 days was significantly reduced by DAPT treatment and Grem2 knockdown, whereas Jagged1 overexpression rescued the inhibitory effect of Grem2 knockdown on cardiac marker expression (Fig. 3D). The number c-Kit⁺ CPCs positive for Mef2C or cTnI was assessed by flow cytometry after 7 days of culture in cardiac differentiation medium. Approximately 80% of wild-type c-Kit⁺ CPCs were positive for either Mef2C or cTnI following differentiation, whereas DAPT treatment or Grem2 silencing significantly reduced the number of Mef2C or cTnI positive c-Kit⁺ CPCs (Fig. 3E) Jagged1 overexpression rescued the inhibitory effect of Grem2 knockdown (Fig. 3E). These results were confirmed by western blotting for Gata4 and cTnI (Fig. 3F). Taken together, these results suggested that the Jagged1-Notch1 pathway plays a role in Grem2-mediated CPC differentiation.

Grem2 activates the Jagged1-Notch1 pathway by controlling Smad1/5 and Smad2/3 signaling balance during the differentiation of CPCs

Based on the fact that Grem2 as a BMP antagonist, we hypothesized that Grem2 might influence BMP-mediated signaling and downstream Smad phosphorylation. To examine this possibility, Smad phosphorylation in differentiated CPCs was assessed at 0, 7, and 14 days. Grem2 knockdown significantly enhanced the phosphorylation of Smad1/5, whereas the phosphorylation of Smad2/3 was impaired; Grem2 overexpression reduced the phosphorylation of Smad1/5 and increased the phosphorylation of Smad2/3 (Fig. 4A).

To determine the role of Smad signaling on Jagged1-Notch1 induction, we used siRNA to target individual Smads in CPCs. As shown in Fig. 4B, the induction of Jagged1 was decreased in Grem2 overexpressing-CPCs transfected with Smad3 siRNA after 7 days of culture in cardiac differentiation medium. By contrast, siRNA for Smad2 had no detectable effect when used alone, however, siRNA for Smad2 potentiated the inhibitory effects of Smad3 siRNA on Jagged1 induction. Moreover, the expression of Hes1 as the downstream gene of Notch signaling and the cardiac markers Gata4 and cTnI was also detected by western blotting. Grem2 overexpression upregulated the expression of Hes1, Gata4 and cTnI. Smad3 siRNA downregulated Hes1, Gata4 and cTnI at control and Grem2 overexpressing-CPCs. Smad2 siRNA alone had no detectable effect; however, a combination of the siRNAs potentiated the inhibitory effects. These results suggested that Grem2 controls Smad1/5 and Smad2/3 signaling balance to regulate the Jagged1-Notch1 pathway, which plays a role in the differentiation of CPCs.

Fig. 5. Gremlin 2 knockdown inhibited the protective effect of CPC transplantation *in vivo* after MI (A) Masson trichrome staining was used to determine the fibrotic areas at 4 weeks after CPC transplantation (N=6 per group). Representative images and quantification are shown. Scale bars, 100µm. ** P<0.01, # P<0.05. (B) Ejection fraction (EF) and fractional shortening (FS) were monitored by echocardiography 1 day before MI and at 2 and 4 weeks after

MI and CPC/vehicle injection (N=6 per group). * P<0.05 vs. MI-Vehicle; # P<0.05 vs. MI-Ctr shRNA-CPC. ** P<0.05, ** P<0.05 vs. MI-Vehicle; # P<0.05 vs. MI-Ctr ShRNA-CPC.

Loss of Grem2 abolished the protective effect of CPC transplantation *in vivo* after MI

To evaluate the potential effect of Grem2 on CPC differentiation *in vivo*, control shRNA- or Grem2 shRNA-expressing CPCs were injected into the infarct border zone in an MI mouse model. After 4 weeks after CPC transplantation, Masson's trichrome staining showed that fibrosis was significantly reduced in animals injected with control shRNA CPCs compared with that in animals injected with vehicle, and Grem2 knockdown inhibited the protective effect of CPCs on injured cardiac tissue (Fig. 5A). Cardiac function was monitored by echocardiography 1 day before MI and at 2 and 4 weeks after MI and CPC/vehicle injection. Mice injected with CPCs expressing control shRNA showed significantly greater ejection fraction (EF) and fractional shortening (FS) at 2 weeks after MI than those injected with vehicle, whereas the increased EF and FS were abolished in mice injected with CPCs expressing Grem 2 shRNA (Fig. 5B). At 4 weeks after MI, no significant differences were detected among the three groups (Fig. 5B).

Taken together, these data indicated that Grem2 is involved in CPC differentiation into cardiomyocytes *in vivo* and in the protective effect of CPC transplantation in the heart.

Discussion

The failure of the human heart to repair or replace myocardial tissues after ischemic injury partially underlies the significant morbidity and mortality of cardiac diseases worldwide [17]. In myocardial infarction, the myocardium damaged by hypoxic injury is replaced by noncontractile scar tissue, potentially leading to ventricular remodeling and heart failure. Therefore, stem cell therapy has received increasing attention for its potential as a strategy for cardiac repair and regeneration of functional cardiomyocytes. Here, we elucidated a potential mechanism regulating the differentiation of CPCs into cardiomyocytes involving the BMP antagonist Grem2 and the Notch1/Jagged signaling pathway.

The results of the present study showed that Grem2 was significantly upregulated in CPCs induced to differentiate into cardiomyocytes, and knockdown of Grem2 inhibited cardiomyocyte differentiation. Grem2 is used experimentally to generate cardiomyocytes from

mouse embryonic stem cells (ESCs) [18] and BMP-4 inhibits cardiomyocyte differentiation from mouse ESCs [19]. Grem2 was previously shown to induce the differentiation of embryonic stem cells into a cardiomyocyte lineage via the activation of the c-Jun N-terminal kinase pathway [20]. In their study, Tanwar et al. demonstrated that Grem2 promoted the differentiation of stem cells into cardiomyocytes with atrial characteristics, and suggested a mechanism involving the Grem2 mediated downregulation of the transcription factor Hey2, which is a downstream target of Notch.

In the present study, the upregulation of Grem2 during CPC differentiation occurred in parallel with the activation of Notch1 signaling. Furthermore, Grem2 silencing inhibited cardiomyocyte differentiation and Notch activation, and the Notch signaling inhibitor DAPT suppressed differentiation. This effect was rescued by Jagged overexpression, confirming the involvement of the Notch1/Jagged pathway in the effect of Grem2 on CPC differentiation. Notch signaling plays a role in cardiovascular development by promoting cardiac cell proliferation and differentiation (Tanwar 2014). Notch1 activation favors the differentiation of CPCs into a myocyte lineage and cardiomyogenesis [21]. The activation of Notch1/Jagged1 signaling promotes the differentiation of mesenchymal stem cells into cardiomyocytes, and this effect is suppressed by the γ -secretase inhibitor DAPT, which blocks Notch signaling [22]. The Notch signaling pathway plays an important role in the regulation of cardiomyocyte proliferation and thus is important for cardiac regeneration after injury [23]. The role of Notch receptors as regulators of cardiovascular development and homeostasis has been reported extensively, and Notch1 and Jagged1 in particular have been shown to play important roles in vasculogenesis [24]. Furthermore, Notch1 has been shown to have a protective effect in the heart by preventing cardiomyocyte apoptosis, recruiting CPCs, promoting neovascularization and inducing differentiation. Grem2, as a BMP antagonist, might influence BMP-mediated signaling and downstream Smad phosphorylation. The results of the present study showed that Grem2 controls Smad1/5 and Smad2/3 signaling balance. The Smad2/3 signaling mediated the induction of Jagged1, Hes1 (the downstream gene of Notch signalling) in Grem2-mediated CPC differentiation. Our present results are therefore important because they shed light on the crosstalk between the BMP and Notch pathways and provide a potential mechanism underlying the effect of Grem2 on modulating the differentiation of CPCs

Transplantation of CPCs into the heart promotes cardiac repair, decreases left ventricular dysfunction, and promotes LV remodeling in animal models of MI, although the underlying mechanisms remain unclear [25]. The effect of transplanted CPCs on ameliorating cardiac dysfunction after MI was suggested to be mediated by cardiomyocyte differentiation [26]. However, studies have also indicated that the beneficial effects of transplanted CPCs are not mediated by differentiation into cardiomyocytes but rather by paracrine effects [25, 27, 28]. The lineage tracing was performed in mice, but the role of cKit+ cells might be different in larger mammals. However, it remains possible that cell therapy approaches with cKit+ cells in humans after myocardial infarction might still be beneficial, such as through a proposed paracrine effect of the transplanted cells [28, 29]. Our present *in vivo* results showed that Grem2 silencing abolished the protective effects of CPC injection on fibrosis and cardiac function in a mouse model of left anterior descending coronary artery ligation-induced MI, verifying its role in cardiac protection. However, the fate of transplanted CPCs injected into the heart is difficult to trace, and future studies should be aimed at further exploring the mechanisms underlying the effect of Grem2 in our *in vivo* model.

In conclusion, the present study showed that Grem2 plays a role in the differentiation of CPCs into a cardiomyocyte lineage through the modulation of the Notch signaling pathway. Our *in vitro* results demonstrated that Grem2 promotes cardiomyocyte differentiation by activating Notch1/Jagged1 signaling. *In vivo* experiments in a mouse model of MI showed that Grem2 can promote the differentiation of CPCs and enhance their protective effect on heart function, suggesting its potential as therapeutic protein for cardiac repair.

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Disclosure Statement

The authors declare to have no conflict of interests.

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