Original Research

Effects of mesenchymal stem cells on matrix metalloproteinase synthesis in cardiac fibroblasts

Yaping Wang[1,2], Xinyang Hu[1,2], Xiaojie Xie[1,2], Aina He[1], Xianbao Liu[1,2] and Jian-an Wang[1,2]

[1]Cardiovascular Center; [2]Cardiovascular Key Lab of Zhejiang Province, Second Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang Province, 310009, P. R. China
Corresponding author: Jian-an Wang. Email: jian_an_wang@yahoo.com

Abstract

Mesenchymal stem cell (MSC) transplantation has been known to decrease matrix metalloproteinase (MMP) synthesis in myocardium after myocardial infarction (MI) and improve ventricular remodeling; however, the underlying mechanisms are unclear. This study investigated the effects of MSC on MMP synthesis in cardiac fibroblasts (CFs) through paracrine actions. CFs were cultured under hypoxic (0.5% pO_2) conditions for 24 h before co-culture with MSCs or hypoxia-preconditioned MSCs (H-MSCs) in transwell plates. CFs and MSCs/H-MSCs shared a medium with or without erythropoietin (EPO) neutralizing antibody (EPOAb) or EPO-soluble receptor (EPOsR). The results showed that protein expression and activity of MMP-2 and membrane type 1-MMP, but not MMP-9, in CFs were significantly increased in response to hypoxia and decreased after co-culture with MSCs or H-MSCs. Hypoxia up-regulated phosphorylation of extracellular signal-regulated kinase (ERK)1/2 of CFs which was down-regulated after CFs' co-culture with MSCs. Tissue inhibitors of metalloproteinases-1 (TIMP-1) in CFs was decreased after hypoxia and increased when co-cultured with MSCs or H-MSCs. Exogenous EPOAb or EPOsR partially inhibited MSCs' effect on MMP-2 expression and activity in CFs. The present findings suggested that MSCs influence MMP/TIMP expression in CFs via the ERK1/2 pathway and EPO acts as a key factor in the paracrine actions of MSCs.

Keywords: mesenchymal stem cell, cardiac fibroblast, matrix metalloproteinase, erythropoietin

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Introduction

Left ventricular (LV) remodeling after myocardial infarction (MI) is a key determinant of progression to heart failure.[1] Fundamental processes involved include extensive modifications[2] of the extracellular matrix (ECM). Cardiac fibroblast (CF) is the major non-myocyte cell type and accounts for approximately 60–70% of the cell population in the heart.[3] CFs synthesize ECM and produce matrix metalloproteinase (MMPs). MMPs maintain the balance between synthesis and degradation of connective tissue components. Increased expression of MMPs is associated with ECM remodeling. MMP-2 is the most common MMP in the myocardium and can digest type I, II and IV collagens.[4,5] Studies have shown that MMP-2 and MMP-9 are associated with ventricular remodeling.[6] They degrade gelatin and type IV collagen, and play a major role in ECM remodeling due to their capability to initiate and maintain fibrillar collagen degradation.[7] Previous studies demonstrated that knockout of MMP-2 attenuates LV remodeling, leading to progression of heart failure.[8] Tissue inhibitor of metalloproteinase (TIMP), a specific inhibitor of MMPs, participates in controlling the local activities of MMPs.[9] TIMP-1 has been shown to bind to the majority of MMPs, including MMP-2 and MMP-9.[10] Loss of TIMP-mediated inhibition has been reported in post-MI remodeling,[11] and post-injury myocardial remodeling is more extensive with TIMP-1 gene deletion.[12]

Mesenchymal stem cell (MSC) transplantation is a potential therapeutic strategy for cardiac repair and has been previously reported to improve heart function[13–15] after MI and decrease fibrosis in the heart. Mechanisms underlying ventricular remodeling improvement after MSC transplantation include angiogenesis and antiapoptotic paracrine effects on myocytes.[16–18] It has been reported that MSC transplantation reduces MMP synthesis in the myocardium. Dixon *et al.*[19] demonstrated that sheep bone marrow MSC transplantation after myocardial infarction (MI) reduced MMP expression in the myocardium. Aupperle *et al.*[20] showed that MMP levels decreased after autologous MSC transplantation in the rabbit doxorubicin cardiomyopathy model. Our previous study also found that rat MSC transplantation decreased the MMP expression of the myocardium after MI.[21] However, the exact mechanism through which MSCs affect MMP synthesis remains unknown. We hypothesized that the antifibrotic properties of MSCs

involve the regulation of MMP and TIMP production by CFs. In our study, the hypoxic environment during MI was simulated *in vitro* via simulated hypoxia using CFs. We demonstrate that MSCs exert paracrine antifibrotic effects partially by modulating MMP/TIMP expression in CFs, and erythropoietin (EPO) acts as an important paracrine factor via a p-extracellular signal-regulated kinase (ERK)/ERK pathway.

Materials and methods

Animal

Male Sprague Dawley (SD) rats were used for MSC and CF isolation. Rats were handled and maintained in accordance with the guidelines of the Animal Care and Utilization Committee of Zhejiang University (Hangzhou, China).

Cell isolation and culture

Isolation of bone marrow-derived MSCs from 80-g male SD rats was performed as described previously.[22] Bone marrow extracted from the femor and tibia was flushed with Dulbecco's modified Eagle's medium (DMEM; 10% heat-inactivated fetal-bovine serum [FBS], 100 U/mL penicillin G, and 100 μg/mL streptomycin; Gibco, Carlsbad, CA, USA). After density gradient centrifugation, the cells were gathered and re-suspended in DMEM, and then plated in culture flasks. Flasks were then washed with PBS 24 h later to leave an adherent layer of cells containing MSCs. The culture medium was changed every 3–4 days, and the cells were subcultured at 80 to 90% confluence. After 3–5 passages, MSCs were determined by fluorescence-activating cell sorting (FACS; Beckman Coulter, Fullerton, CA, USA) using fluorescein–isothiocyanate-conjugated antibodies against CD44 and CD45, and phycoerythrin-conjugated antibody against CD90.[22] All antibodies were purchased from Caltag Laboratories (Caltag, Carlsbad, CA, USA). MSCs were seeded into six-well transwell inserts with a density of 8×10^5 cells/mL prior to experiments.

Adult rat CFs were isolated as previously described with minor modifications.[23] Briefly, the left ventricles were minced and subjected to periods of incubation with 0.125% trypsin with ethylenediaminetetraacetic acid (Gibco) for digestion at 37°C by rotating at 120 rpm for 15 min. At the end of each cycle, the supernatant was collected on ice after neutralizing trypsin with FBS. The dissociated cells were centrifuged at 1500 rpm for 15 min and then re-suspended in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were allowed to attach for one hour, and then media was changed to remove weakly adherent cells, including myocytes and endothelial cells. CFs at passage 2 or 3 were used for experiments.

Simulation of hypoxia

Cells were washed with DMEM twice and then incubated at 37°C for 24 h in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA, USA) with 95% N_2/5% CO_2 and the concentration of oxygen was less than 0.5%. The control group was incubated in DMEM supplemented with 10% FBS under standard cell culture conditions (5% CO_2).

Co-culture in transwell

MSCs and CFs were cultured in two individual chambers separated by a semipermeable membrane with 3 um pores. This system allowed sharing of culture medium in the two chambers while preventing cell contact. CFs were cultured in the lower plates of transwells, and MSCs were cultured in the inserts. To identify whether erythropoietin plays a critical role in the paracrine action of MSCs on MMP production of CFs, erythropoietin-soluble receptor (EPOsR; 2.5 μg/mL; R&D System Inc, Minneapolis, MN, USA) and EPO-neutralizing antibody (EPOAb; 3 μg/mL; R&D System Inc) were separately added into the culture medium in the transwell. After 24 h of co-culture, the inserts were removed, and the culture medium of CFs was changed. Conditioned medium was collected from CFs after 24 h.

Western blot analysis

Whole-cell protein was extracted as described previously.[24] Protein concentration was determined using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of the cell protein extracts (40–60 μg/lane) were electrophoresed in 12% sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE), and transferred onto a polyvinylidene fluoride Immobilon-P membrane (Bio-Rad) using a transblot apparatus (Bio-Rad). Membranes were blocked with 5% bovine serum albumin (BSA) for one hour at room temperature followed by overnight incubation at 4°C with primary antibodies (MMP-2 and MT1-MMP: Abcam, Cambridge, MA, USA; MMP-9: Millipore Chemicon, Billerica, MA, USA; TIMP-1 and β-actin: Santa Cruz Biotechnology, Santa Cruz, CA, USA; ERK1/2 and p-ERK1/2: Cell Signaling Technology, Danvers, MA, USA). After washing with Tris-buffered saline with Tween 20, the membranes were incubated with an AP-conjugated goat antimouse IgG or goat antirabbit IgG (Santa Cruz Biotechnology) for one hour at room temperature. Protein expression signals were detected by BCIP/NBT solution (Sigma, St Louis, MO, USA) or film and analyzed by means of Image-J version 1.41 analysis software (NIH, Bethesda, MD, USA).

Zymography

Briefly, CFs were plated in six-well plates until they reached 80–90% confluence. After treatments, medium samples were collected, centrifuged at 2000 rpm for 10 min and normalized for cell protein content from cell lysates using the Bio-Rad assay.[25] Gelatinolytic activity of the supernatant was assayed under non-reducing conditions. Equal volumes of conditioned media were directly loaded onto electrophoretic gels for SDS-PAGE containing 1 mg/mL of gelatin. Gels were stained using 0.1% Coomassie Brilliant Blue R-250 and destained with 45% methanol and 10% acetic acid. All reagents for electrophoresis were purchased

from Sigma. To quantify the amount of gelatinase production, stained zymograms were scanned with an image system (Kodak, Woodbridge, CT, USA).

Reverse transcription polymerase chain reaction

Total RNA from CFs was extracted, using the TRIzol method as recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). The concentration was determined by measuring UV-absorption at 260 nm. Two micrograms of RNA samples were reverse transcribed and processed for polymerase chain reaction (PCR) reactions. All primers are listed $5' \rightarrow 3'$. Primers used were MMP-2f (AGGACAAGTGGTCCGCGTAAAG), MMP-2r (CCACTTCCGGTCATCATCGTAGT), MMP-9f (GGGAACGTATCTGGAAATTCG), MMP-9r (CAGAACCGACCCTACAAAGTTG), TIMP-1f (GACTAAGATGCTCAAAGGATTCG), MT1-MMPf (GGATACCCAATGCCCATTGGCCA), MT1-MMPr (CCATTGGGCATCCAGAAGAGAGC), TIMP-1r (ATCGCTCTGGTAGCCCTTCT), EPOf (CCGCTCCACTCCGAACACT), EPOr (GCCGGTAAGAAGGTAACAAACA), EPORf (CTGGGAGGAAGCGGCGAACT), EPORr (CGGTGGTAGCGAGGAGAT), GAPDHf (TGAAGGTCGGAGTCAACGG) and GAPDHr (TGGAAGATGGTGATGGGAT). Cycling conditions used were 2 min at 94°C, 10 s at 94°C and then 40 cycles of 10 s denaturation at 94°C and 30 s annealing at 56–64°C. The amplified DNA products were visualized on 2% agarose gels and photographed under ultraviolet light.

Statistical analysis

Each experiment was conducted at least three times. Data are presented as means ± SEM. Differences among all data were analyzed for significance by analysis of variance, with the level of statistical significance set at $P < 0.05$.

Results

Effects of MSCs on the activity of MMPs

Hypoxia treatment was used to simulate the hypoxic environment during MI. Our previous study[15] reported that hypoxia preconditioning can enhance the paracrine activities of MSCs, and cytokines like EPO, vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) were all increased with hypoxia. Leroux *et al.*[26] showed that conditioned medium from hypoxic preconditioned MSCs is more effective than conditioned medium from non-preconditioned MSCs in inducing MSC proliferation and migration. Hypoxia can influence a broad spectrum of factors, as was revealed by screening with microarrays by Ohnishi *et al.*[27] They described that many genes were upregulated when rat MSCs were cultured for 24 h in 1% pO_2 compared with normoxia. Therefore, we set up a hypoxia preconditioned MSC (H-MSC) group for co-culture with CFs. Zymography was used to evaluate the activities of MMP-2 and MMP-9. The culture medium with various treatments was collected. After co-culture, the inserts were removed and the medium of CFs was changed to eliminate interference of MMPs from MSCs. After 24 h, the medium of CFs was collected and the detection of MMP activity was determined by zymography. Results showed that hypoxia significantly increased the MMP-2 activity of CFs as compared with the control group ($P < 0.01$), but it had no significant effect on MMP-9 activity. Surprisingly, MSCs and H-MSCs diminished the effect of hypoxia on MMP-2 expression of CFs (Figure 1a). The pro-MMP was too weak to be analyzed. There were no significant differences between the effects of MSCs and H-MSCs on CFs.

Effects of MSCs on MMPs' expression

Western blot analysis was performed to evaluate the protein expression of MMP-2, MMP-9, MT1-MMP and TIMP-1. MMP-2 protein synthesis by CFs was elevated in the hypoxia group as compared with the control group ($P < 0.01$), and was decreased after co-culture with MSCs or H-MSCs ($P < 0.01$). TIMP-1 expression in CFs was down-regulated under hypoxia conditions ($P < 0.01$) and up-regulated by co-culture with MSCs or H-MSCs ($P < 0.01$) (Figure 1b). There were no significant changes in the expression of MMP-9 in the four groups. Reverse transcription PCR (RT-PCR) results showed mRNA level of MMP-2, MMP-9, MT1-MMP and TIMP-1 was in accordance with protein expression data. MT1-MMP is the major membrane MMP activating MMP-2 and was up-regulated by hypoxia and down-regulated after co-culture, showing the same tendency with MMP-2.

EPOsR and EPOAb reverse the effects of MSCs on MMP-2 and TIMP-1 synthesis by CFs after hypoxia

Our previous study[15] and others (Chen *et al.*[28]) indicated that EPO is a paracrine factor of MSCs. EPO is a myocardioprotective and antifibrotic factor that has been described as an important factor in the paracrine activities of MSCs.[29,30] Nishiya *et al.*[31] demonstrated that after MI, MMP-2 was significantly reduced in the EPO-treated groups compared with the untreated group, thereby suggesting the anti-inflammatory effects of EPO. Our RT-PCR results showed that MSCs express EPO which was increased by hypoxia and that CFs expressed EPO receptor (EPOR); yet, there was no significant difference in EPOR expression between CFs and hypoxic CFs. EPOsR and EPOAb were separately added in the culture medium of CFs to inhibit the effect of EPO secreted by MSCs or H-MSCs. Our data indicate no significant changes of MMP-2 and TIMP-1 expression in CFs after simulated hypoxia compared with that when EPO was inhibited. These data suggest that both EPOsR and EPOAb could partially inhibit the effects of MSCs and H-MSCs on MMP-2 and TIMP-1 synthesis by CFs ($P < 0.01$) after induction of hypoxia (Figures 2 and 3). Taken together, these data indicated that EPO plays a critical role in suppressing the activation of MMP-2 during this MSC paracrine action.

Mechanism underlying the effects of MSCs on MMP production by CFs

MMP synthesis and activation involve the ERK1/2 and PI3K-Akt pathways.[32,33] It is reported that hypoxia is able

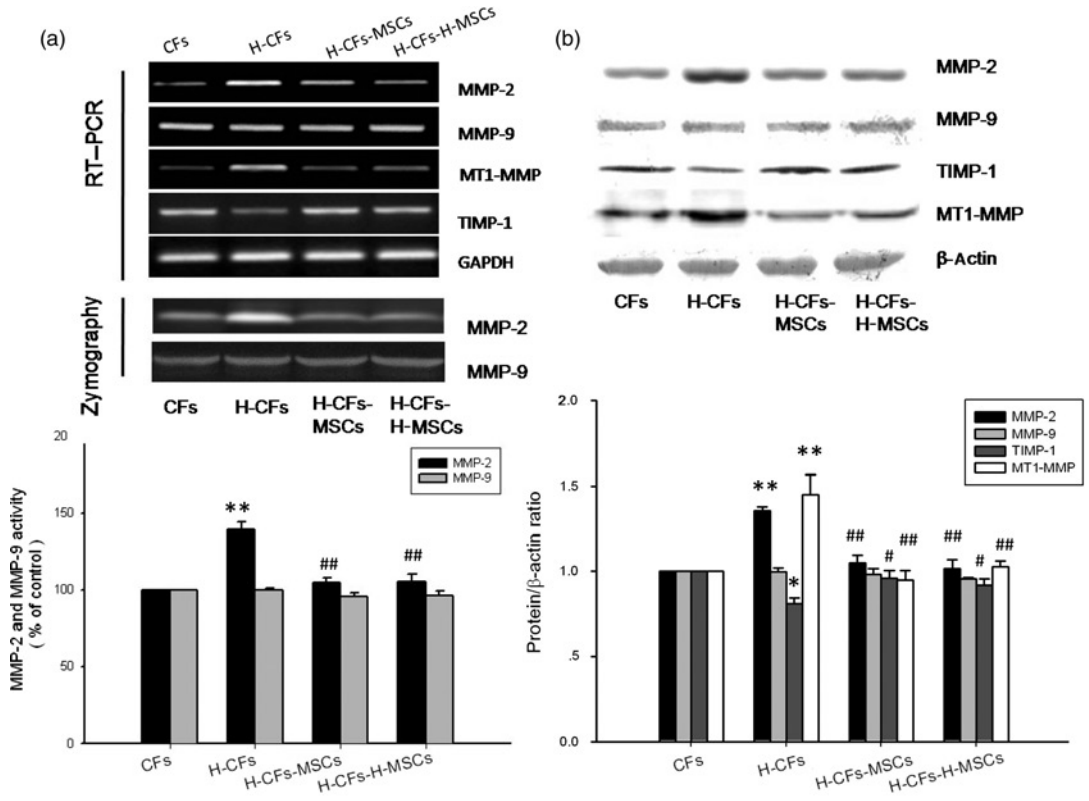

Figure 1 Effect of MSCs/H-MSCs on MMP-2, MT1-MMP, MMP-9 and TIMP-1 expression in CFs. Band intensities were quantified using ImageJ 1.41 program. (a) A representative zymogram demonstrates changes in MMP-2 and MMP-9 activities and RT–PCR shows mRNA levels of MMP-2, MMP-9, MT1-MMP and TIMP-1. Quantitative analysis of MMP-2 and MMP-9 activities in CFs, expressed as fold increase versus the control; $^{**}P < 0.01$ versus the control group, $^{\#\#}P < 0.01$ versus H-CFs group, $^{\#}P < 0.05$ versus H-CFs group (no. = 5 for each group). (b) A representative Western blot demonstrates changes in MMP-2, MT1-MMP, MMP-9 and TIMP-1 synthesis. Quantitative analysis of CF synthesis of MMP-2, MT1-MMP, MMP-9 and TIMP-1, expressed as fold increase versus the control; $^{**}P < 0.01$ versus the control group, $^{*}P < 0.05$ versus the control, $^{\#\#}P < 0.01$ versus H-CFs group, $^{\#}P < 0.05$ versus H-CFs group (no. = 5 for each group). MSC/H-MSC, mesenchymal stem cell/hypoxia-preconditioned MSC; MMP, metalloproteinase; MT1, membrane type 1; TIMP-1, tissue inhibitors of MMP-1; CF, cardiac fibroblast; H-CF, hypoxic cardiac fibroblast; RT–PCR, reverse transcriptase polymerase chain reaction

to activate the ERK pathway in several cell types,[34,35] including cardiomyocytes.[36] To explore the underlying mechanisms of the effects of MSCs on MMP-2 and TIMP-1 in CFs, the ERK1/2 and AKT pathways were examined. Phosphorylation of ERK 1/2 (p-ERK) of CFs was significantly increased by hypoxia ($P < 0.01$) and partially inhibited after CFs' co-culture with MSCs ($P < 0.01$). The ratio of p-ERK/ERK was increased in the MSCs + EPOsR group and MSCs + EPOAb as compared with the MSC group ($P < 0.01$) (Figure 4). Phosphorylation of AKT was not significantly different between groups (data not shown). These data suggest that the ability of MSCs to modulate CF MMP production through phosphorylation of the ERK1/2 pathway is not affected by hypoxia or MSC co-culture.

Discussion

LV remodeling after MI is a major determinant of progression to heart failure. MSC transplantation has shown a promising potential therapy for decreasing cardiac fibrosis, as corroborated by adequate preclinical evidence.[37] However, the underlying molecular mechanisms of this process are still unknown. In the present study, we present two main findings. First, MSCs and H-MSCs have marked paracrine-mediated effects on MMP-2 activity and expression in cultured rat CFs in response to hypoxia. Second, EPO may act as a key cytokine during this process, which is associated with the ERK1/2 pathway. These findings provide fundamental evidence for using MSC transplantation to attenuate heart failure.

MMPs have been implicated in adverse LV remodeling.[38] Cellular constituents of cardiac muscle, specifically fibroblasts, inflammatory cells, and myocytes, are known to be capable of expressing MMP-2 in response to specific stimuli.[39] Previous studies[40] suggest that MSCs regulate CF proliferation and transcriptional down-regulation of collagen I and III synthesis. Minimal studies have been done on MMP production by CFs. To address this deficit, in the current study we utilized a simulated hypoxia model of MI *in vitro* using CFs, the dominant cell type in the heart. Swiki *et al.*[41] reported that culture of rat CFs for 24 h in 1% oxygen enhanced MMP-2 synthesis five-fold. Similarly, our data indicate that expression of MMP-2, but not MMP-9, in CFs is significantly increased by hypoxia. After co-culture with MSCs or H-MSCs, only MMP-2 expression by CFs decreased compared with the hypoxia group, while there were no changes in MMP-9 expression. This finding is consistent with those of previous *in vivo* studies, where the MMP-2 level was significantly reduced

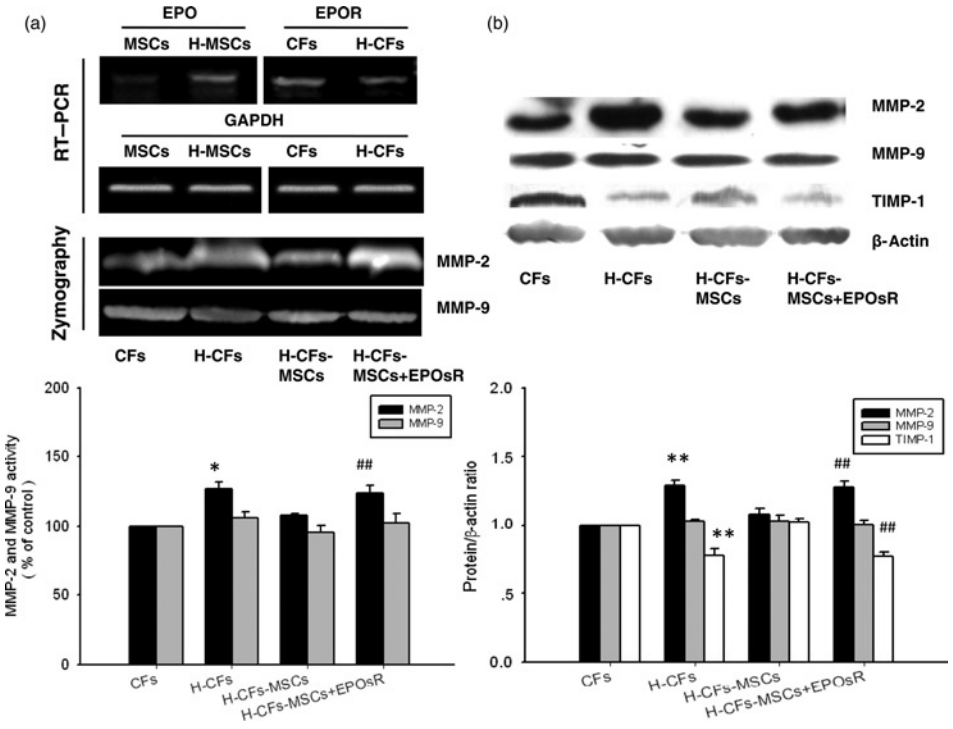

Figure 2 Effect of EPOsR on MMP expression in CFs when co-cultured with MSCs. After hypoxia for 24 h, CFs were co-cultured with MSCs in the presence or absence of EPOsR (2.5 μg/mL) for 24 h. (a) RT–PCR shows mRNA levels of EPO in MSCs and H-MSCs; EPOR in CFs and H-CFs. Zymography demonstrates changes in MMP-2 and MMP-9 activities. Quantitative analysis of CF MMP-2 and MMP-9 activities, expressed as fold increase versus the control; $^{**}P < 0.01$ versus the control group, $^{\#\#}P < 0.01$ versus the MSCs + EPOsR group (no. = 3 for each group). (b) A representative Western blot demonstrates changes in MMP-2, MMP-9 and TIMP-1 synthesis. Quantitative analysis of CF synthesis of MMP-2, MMP-9 and TIMP-1, expressed as fold increase versus the control; $^{**}P < 0.01$ versus the control group, $^{\#\#}P < 0.01$ versus the MSCs + EPOsR group (no. = 3 for each group). EPO, erythropoietin; EPOsR, EPO soluble receptor; MMP, metalloproteinase; CF, cardiac fibroblast; H-CF, hypoxic cardiac fibroblast; MSC, mesenchymal stem cell; H-MSC, hypoxia-preconditioned MSC; TIMP-1, tissue inhibitors of MMP-1

by MSC transplantation in the myocardium after MI. Most MT-MMPs are able to activate MMP-2, and MT1-MMP plays an important role in mediation of MMP-2 activity. Our results demonstrate that MT1-MMP protein expression is similar to MMP-2, which has been demonstrated in other studies. These effects of MSCs may be beneficial for the treatment of heart failure. More importantly, this study presents a possible novel mechanism for MSC transplantation.

Our data suggest that there is no significant difference between the effect of MSCs and H-MSCs on MMP-2 and TIMP-1 expression and activity of CFs. We speculate this might be due to two reasons: (1) the effects of hypoxia preconditioning of MSCs are undetectable after CF co-culture with H-MSCs for 24 h and another 24-h culture without H-MSCs for collecting the supernatant; and (2) the EPOR expression in CFs was not increased by hypoxia or co-culture with MSCs.

The effects of MSC transplantation are primarily mediated through paracrine activity. Previous studies have demonstrated that MSCs can contribute indirectly to cardiac repair by releasing a variety of factors and cytokines that exert paracrine actions on the myocardium.[42] MSCs express growth and angiogenic factors, including VEGF, Ang-1 and EPO.[15] Numerous studies have reported that EPO is myocardioprotective and antifibrotic.[43,44] Recombinant human EPO treatment attenuates ECM degradation following myocardial ischemia/reperfusion (I/R)[43] and enhances TIMP-1 synthesis in human erythroid progenitor cells.[44] Previous studies also reported that CFs express EPOR.[45] Thus, the present study sought to investigate whether the mechanisms mediating the effects of MSCs on CFs involve EPO secretion. Our RT–PCR results indicate that MSCs express EPO and CFs express EPOR as previous studies have reported. Both exogenous EPOAb and EPOsR can inhibit MSCs' effect on MMP-2 expression in CFs, suggesting that EPO acts as a key factor in the paracrine actions of MSCs.

An earlier study demonstrated that I/R acutely stimulates the phosphorylation of the ERK pathway in intact rabbit hearts.[46] George *et al.*[47] reported that hypoxia increases phosphorylation of ERK1/2 kinase in human pulmonary fibroblasts. After blocking ERK1/2 signaling in fibroblasts, cells under hypoxic conditions reduced MMP-2 secretion, indicating that hypoxia-dependent secretion of MMP-2 involves the action of ERK1/2.[48] Consistent with this, our study found that hypoxia induced phosphorylation of ERK1/2 in CFs. Co-culture with MSCs or H-MSCs also decreased CF phosphorylation of ERK1/2. Addition of either EPOAb or EPOsR to the co-culture medium counteracts this effect. These results indicate that MSCs regulate MMP production in CFs partly via the ERK1/2 pathway.

MMPs and TIMPs are the major regulators of ECM turnover. TIMPs function as important regulatory brakes by stabilizing MMP proenzymes and inhibiting the active species.[49,50] In this study, expression of TIMP-1 in CFs decreased in response to hypoxia, and slightly increased in co-culture with MSCs or H-MSCs. However, this result

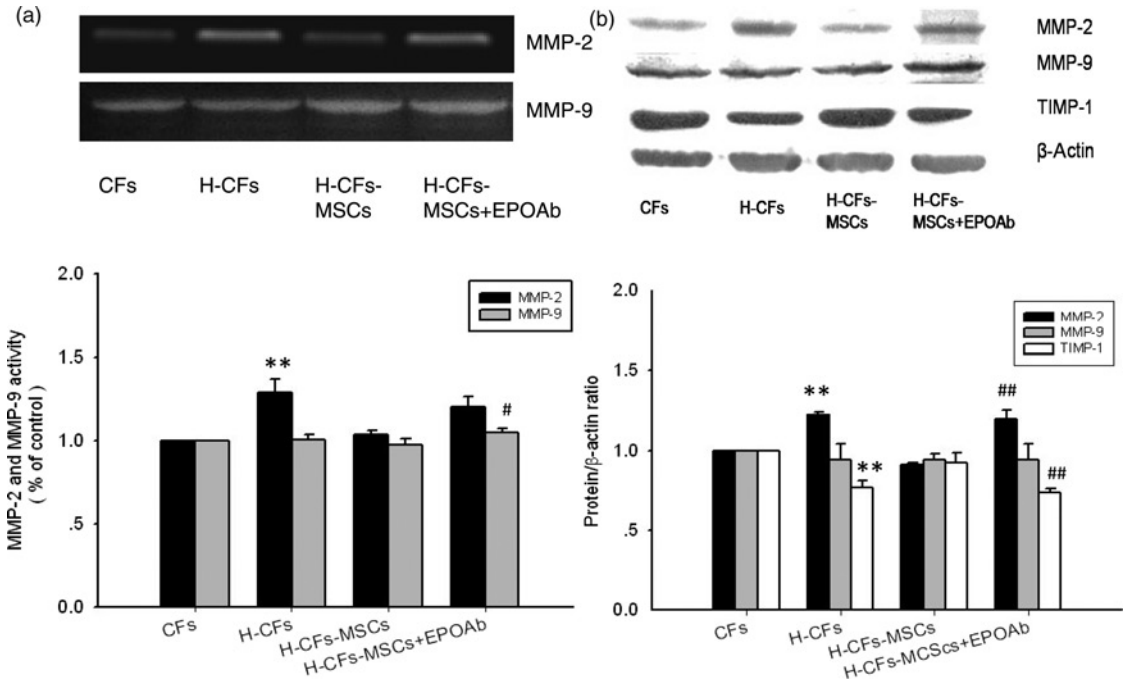

Figure 3 Effect of EPOAb on MMP-2 and MMP-9 expression in CFs when co-cultured with MSCs. After hypoxia for 24 h, CFs were co-cultured with MSCs in the presence or absence of EPOAb (3 μ/mL) for 24 h. (a) A representative zymogram demonstrates changes in MMP-2 and MMP-9 activities. Quantitative analysis of CF MMP-2 and MMP-9 activities expressed as fold increase versus the control; $^{**}P < 0.01$ versus the control group, $^{\#}P < 0.05$ versus the MSCs + EPOAb group (no. = 3 for each group). (b) A representative Western blot shows changes in MMP-2, MMP-9, and TIMP-1 synthesis. Quantitative analysis of CF synthesis of MMP-2, MMP-9, and TIMP-1, expressed as fold increase versus the control; $^{**}P < 0.01$ versus the control group, $^{\#\#}P < 0.01$ versus the MSCs + EPOAb group (no. = 3 for each group). EPOAb, erythropoietin neutralizing antibody; MMP, metalloproteinase; CF, cardiac fibroblast; MSC, MSC, mesenchymal stem cell

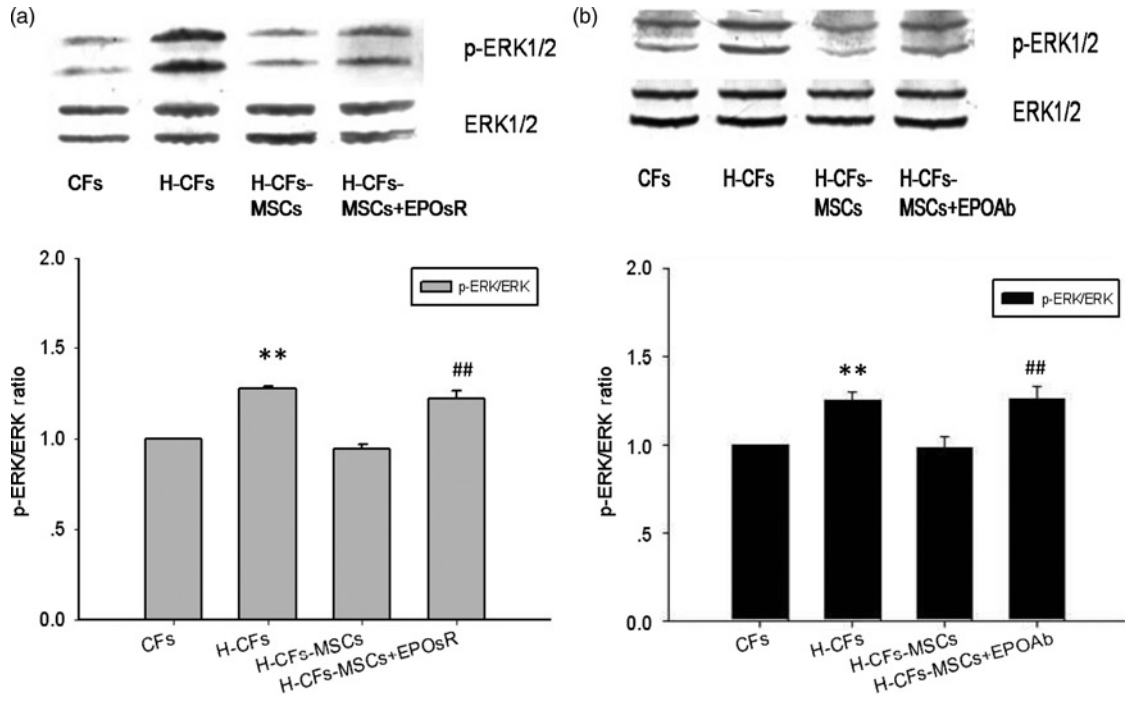

Figure 4 ERK1/2 was involved in hypoxia and EPOsR (Figure 4a) and EPOAb (Figure 4b) treatment. (a) A representative Western blot demonstrates changes in phospho-ERK1/2 in the four groups. Quantitative analysis of the relative ratio of p-ERK1/2/total ERK1/2, expressed as fold increase versus the control; $^{**}P < 0.01$ versus the control group, $^{\#\#}P < 0.01$ versus the MSCs + EPOsR group (no. = 3 for each group). (b) A representative Western blot shows changes in phospho-ERK1/2 in the four groups. Quantitative analysis of the relative ratio of p-ERK1/2/total ERK1/2, expressed as fold increase versus the control; $^{**}P < 0.01$ versus the control group, $^{\#\#}P < 0.01$ versus the MSCs + EPOAb group (no. = 4 for each group). ERK, extracellular signal-regulated kinase; EPOsR, erythropoietin soluble receptor; EPOAb, erythropoietin neutralizing antibody; MSC, mesenchymal stem cell

is inconsistent with an *in vivo* study stating that TIMP-1 significantly increased in the MI group but was decreased by MSC transplantation.[51] TIMP regulation of cell fate is a highly complex process, which is made more complex by the indirect effects of TIMP-mediated inhibition of MMP activity. Moreover, there are numerous conflicting reports on TIMP's effects on one specific cellular process. Thus, a proportionate decrease in MMP activity by TIMP up-regulation entails further investigation.

In conclusion, this study has shown that MSCs exert paracrine antifibrotic effects partially by modulating MMP/TIMP expression in CFs, which opens new perspectives for understanding the mechanisms of action of MSCs.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. YW, AH and XX conducted the experiments; YW, XH and J-aW wrote the manuscript and XL contributed data analysis.

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