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ORIGINAL ARTICLE

Mesenchymal stem cell transplantation attenuates cardiac fibrosis associated with isoproterenol-induced global heart failure

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Summary

We aimed to examine the ability of transplanted mesenchymal stem cells (MSCs) to attenuate cardiac fibrosis caused by global heart failure, and investigate the mechanisms that are possibly mediating this effect. Global heart failure was induced in Wistar rats by isoproterenol injection. Four weeks later, MSCs were transplanted by intramyocardial injection, while control groups were treated by injection of cell culture medium alone. Four weeks after transplantation, heart function was assessed, and histologic and molecular analyses conducted. Compared with the medium-treated group, MSC transplantation significantly decreased the expression of collagens I and III, and matrix metalloproteinase 2 and 9, but heart function was improved in MSC-treated animals. In addition, expression of antifibrotic factor, hepatocyte growth factor (HGF), was detected in cultured MSCs, suggesting a possible mechanism underlying antifibrotic effects. Importantly, HGF expression levels were higher in MSC-treated hearts, compared with medium-treated hearts. Therefore, we could conclude that MSC transplantation can attenuate myocardial fibrosis in a rat model of global heart failure, and this may be at least partially mediated by paracrine signaling from MSCs via antifibrotic factors such as HGF.

Introduction

Cardiac fibrosis is a major factor in the progression of heart failure. Prevention of cardiac fibrosis is a critical goal in the treatment of heart failure [1,2]. Previous studies have showed that cell transplantation could attenuate cardiac fibrosis of the regionally impaired myocardium, such as that prevailing after a myocardial infarction [3,4]. The effect of attenuating cardiac fibrosis on global heart failure is not well known.

Subcutaneous administration of isoproterenol (ISO) in rats can produce diffuse myocardial cell death and myocardial fibrosis, leading to progressive global heart failure, while maintaining an intact coronary vasculature [5,6]. The effectiveness of reperfusion therapy and potential confounding effects of an abnormal blood supply underscore the importance of studying ventricular fibrosis arising in the presence of an intact vascular system [6]. For these reasons, the ISO-treated rat heart is an excellent model system for investigations into ventricular fibrosis and global heart failure.

Mesenchymal stem cells (MSCs) are undifferentiated cells that are capable of clonally differentiating into osteoblasts, adipocytes, chondrocytes, and cardiac myocytes [7–9]. The multi-lineage potential of MSCs, their ability to escape detection by the host immune system upon transplantation, and the relative ease of expansion in culture make MSCs a promising source of stem cells for transplantation [8,10].

In this article, we investigate whether MSC transplantation can improve cardiac function following ISO-induced global heart failure in rats by preventing cardiac fibrosis. Interestingly, MSC transplantation-based treatment of murine hindlimb ischemia has been shown to improve

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blood flow and limb function by stimulating vascular endothelial growth factor (VEGF) production [11]. We therefore predicted that paracrine signaling also participated in the effects observed following MSC transplantation into ISO-treated rat hearts.

Materials and methods

Animals and experimental model

Male Wistar rats (180–200 g) were obtained from Harbin Medical University Laboratorial Animal Center. All experimental procedures were approved by the Care of Experimental Animals Committee of Harbin Medical University, China.

Isoproterenol-induced global heart failure was induced as described, with minor modifications [6]. Briefly, ISO (170 mg/kg in 0.5 ml saline; Sigma, Saint Louis, MO, USA) was subcutaneously injected into rats every day for four consecutive days. Sham-treated rats were injected subcutaneously with 0.5 ml saline alone.

Isolation and transplantation of MSCs

Mesenchymal stem cells were prepared according to previously described methods [12]. Briefly, femurs and tibias were dissected from Wistar rats, and bone marrow cells were isolated by flushing cavities with phosphate-buffered saline (PBS). Bone marrow cells were plated in DMEM/F12 (Gibico, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibico, Carlsbad, CA, USA). Two days after plating, non adherent cells were removed, and adherent cells were propagated for three to four passages prior to transplantation. Four weeks after the final ISO injection, a total of 3×10^6 MSCs in 150 μl DMEM/F12, or 150 μl DMEM/F12 alone, were injected into the myocardium in four places.

Indentification of MSCs

Cultured MSCs were analyzed by fluorescence-activated cell sorting (FACS) as described [13]. Cells were trypsinized and incubated for 15 min with 10 μg/ml antibodies in PBS per 1×10^6 cells at room temperature in the dark. Antibodies used in this study were the hematopoietic progenitor marker phycoerythrin (PE)-conjugated mouse anti-rat CD34 (Santa Cruz, CA, USA), the pan-leukocyte marker fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD45 (Caltag; Burlingame, CA, USA), and the MSC marker PE-conjugated anti-rat CD29 (Biolegend; San Diego, CA, USA). The antibodies of CD45 and CD29 were added together into one tube, and CD34 was added into the other tube separately. Quantitative analyses were performed by using a flow cytometer (BD, Rochville, MD, USA).

For osteogenic differentiation, MSCs were incubated with the osteogenic induction medium consisting of DMEM, 10% FBS, 10^{-7} M dexamethasone, 10 mM glycerophosphate disodium, and 50 μg/ml ascorbic acid for 4 weeks. Calcium deposits were demonstrated by von Kossa staining [14]. For adipogenic differentiation, cells were exposed to the adipogenic induction medium consisting of 10^{-6} M dexamethasone, 100 μg/ml 3-isobutyl-1-methylxanthine, 50 μM indomethacin, and 10 μg/ml insulin for 3 weeks. Adipogenic differentiation was conducted by oil red O staining [15].

Echocardiographic studies

Animals were examined by echocardiography 4 weeks after the final ISO injection and again 4 weeks after transplantation. Two-dimensional, targeted M-mode tracings were obtained at the level of the papillary muscles with an echocardiographic system equipped with a 12-MHz transducer (HP 5500; Hewlett-Packard, Palo Atto, CA, USA). The left ventricular end-systolic dimension (LVDs, mm), the left ventricular end-diastolic dimension (LVDd, mm), and the ejection fraction (EF, %) were measured during three heartbeats, and values averaged. Fractional shortening (FS, %) was defined as (LVDd − LVDs)/LVDd × 100.

Histologic analysis

Three rats were used to examine whether transplanted MSCs differentiated into cardiomyocytes. Prior to transplantation, MSCs were fluorescently labeled with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI), following manufacturer's instructions. Four weeks after transplantation, the hearts were dissected and embedded in optimum cutting temperature (OCT) compound, snap-frozen in liquid nitrogen, and cut into sections. Immunofluorescent staining for cardiac markers was performed using monoclonal mouse anti-desmin, anti-cardiac troponin T. FITC-conjugated immunoglobulin G antibody was used as a secondary antibody.

To detect fibrosis in cardiac muscle, the hearts ($n = 6$ from each group) were excised above the origin of the great vessels. Myocardium was then fixed in 10% formalin, cut transversely, embedded in paraffin, and stained with hematoxylin and eosin (H & E) and Masson's trichrome. The size of fibrotic areas was quantified as follows. Transverse sections were randomly obtained from two levels, and 10 randomly selected fields per section were analyzed. After each field was scanned with a digital image analyzer, the area of the collagenous fraction was calculated as the sum of all areas containing connective tissue divided by the total area of the image.

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Table 1. Primer sequences used.

Primer	Direction	Sequence (5′–3′)	Tm (°C)	No. cycles	Size (bp)
Collagen I	Forward	TGCCGTGACCTCAAGATGTG	64	25	462
	Reverse	CACAAGCGTGCTGTAGGTGA			
Collagen III	Forward	CGAGGTGACAGAGGTGAAAGA	64	29	336
	Reverse	AACCCAGTATTCTCCGCTCTT			
MMP-2	Forward	GGCCATGCCATGGGGCTGGA	64	28	762
	Reverse	CCAGTCTGATTTGATGCTTC			
MMP-9	Forward	AACCCTGGTCACCGGACTTC	55	30	235
	Reverse	CACCCGGTTGTGGAAACTCAC			
HGF	Forward	CTGTCACCATCCCCTATG	55	30	337
	Reverse	CTGCCTCCTTTACCAATG			
β-actin	Forward	ATATCGCTGCGCTCGTCGTC	64	23	760
	Reverse	GCATCGGAACCGCTCATTGC			

MMP, matrix metalloproteinase; HGF, hepatocyte growth factor.
Cycling parameters: 5 min at 95 °C, 23–30 cycles of 30 s at 95 °C, 40 s at 55–64 °C, and 45 s at 72 °C, and a final extension at 72 °C for 5 min.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from cardiac muscle tissue or cultured MSCs using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Medison, WI, USA) and oligo (dT) primers according to the manufacturer's instructions. Primer sequences and reaction conditions are listed in Table 1. The constitutively expressed gene β-actin was used as an internal control for the amount of input cDNA. An amount of 5 μl of each PCR product was run on a 1.5% agarose gels. Densitometry was applied to quantify amplicon amounts from cardiac tissue.

Western blotting

To identify the protein expression of MMP-2, MMP-9 and hepatocyte growth factor (HGF), Western blotting was performed. Protein extract (60 μg) was subject to electrophoresis on 8% SDS polyacrylamide gel and then transferred to PVDF membrane. After being blocked, the membrane was incubated with primary antibody (1:200 for MMP-2, MMP-9, HGF and β-actin) for 2 h and for an additional hour in secondary alkaline phosphatase-conjugated secondary antibody (1:1000). The membrane was then developed with the use of the NBT/BCIP kit (Beyotime, Nantong, Jiangsu, China), and measured by densitometry. Western blotting analysis with a rabbit polyclonal antibody raised against β-actin was used as a protein loading control.

Statistical analysis

All values are expressed as mean values ± standard error. Differences between echocardiographic examination before and after transplantation were evaluated by a pairwise *t*-test. Comparisons of parameters among groups were analyzed using the one-way analysis of variance (ANOVA). Differences were considered significant if $P < 0.05$.

Results

Morphology and characterization of MSCs

Mesenchymal stem cells were collected from rat femurs and tibias and expanded in culture. Three to four days after initial plating, presumptive MSCs appeared fibroblast-like in morphology. MSCs formed a confluent monolayer within 10–14 days after the initial plating. MSCs were characterized by immunofluorescent staining with antibodies that recognize the hematopoietic progenitor marker CD34, the pan-leukocyte marker CD45, and the MSC marker CD29, followed by FACS analysis. CD29 was detected in 96.84% of cultured MSCs, while CD45 was detected in 18.07% of MSCs, and CD34 was detected in 1.57% of MSCs (Fig. 1a). These observations are consistent with isolation of a highly purified MSC population. In addition, the results of oil red O staining and von Kossa staining also showed that MSCs had the ability to differentiate into adipocytes and osteocytes (Fig. 1b and c).

Effects of MSC transplantation on heart function

Four weeks after ISO injection, rats exhibited increased LVDs and decreased EF and FS values when compared with the sham rats, consistent with ISO-dependent induction of heart failure. Rats were then treated by injection of either MSCs or culture medium alone. Four weeks after MSC transplantation, LVDs were significantly smaller ($P < 0.05$), while FS ($P < 0.05$) and EF ($P = 0.051$)

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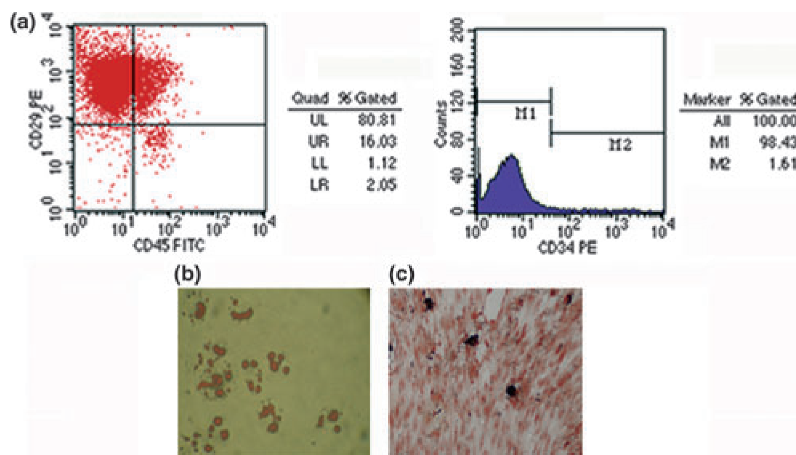
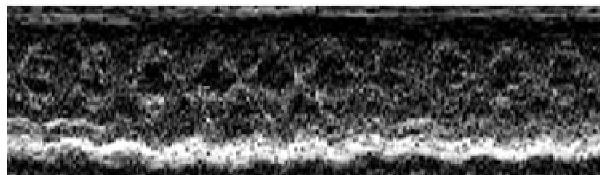
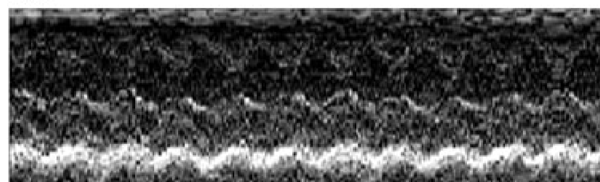


Figure 1 (a) Flow cytometric analysis of adherent, spindle-shaped mesenchymal stem cells (MSCs) (passage three or four). Most cultured MSCs expressed CD29. In contrast, the majority of MSCs were CD34 and CD45 negative. (b) Adipogenic induction, oil red O staining. MSCs developed some lipid droplets. (c) Osteogenic induction, von Kossa staining. Mineralized matrix was formed in MSCs.

Sham



Cell



Medium

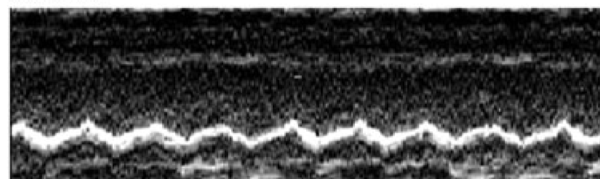


Figure 2 Representative echocardiographic images showing poor movement in the medium-treated group, and improvement of cardiac contractility in the mesenchymal stem cells-treated group.

values were significantly larger than in the medium-treated group (Fig. 2, Table 2). In addition, there were no changes in any variables between the value at baseline and post-treatment in the medium-treated group, however, there were significant increases in EF (a pairwise *t*-test, $P < 0.01$) and FS (a pairwise *t*-test, $P < 0.01$) and decrease in LVDs (a pairwise *t*-test, $P < 0.01$) 4 weeks after MSC transplantation in the cell-treated group (Table 2).These results suggest that MSC transplantation can significantly improve heart function.

Table 2. Echocardiographic assessment of cardiac function.

Variable	Medium-treated (*n* = 8)	MSC-treated (*n* = 7)	Sham (*n* = 8)
Baseline (4 weeks after ISO injection)			
LVDd (mm)	5.25 ± 0.62	5.46 ± 0.40	5.12 ± 0.76
LVDs (mm)	3.56 ± 0.41*	3.79 ± 0.37†	2.96 ± 0.46
FS (%)	32.18 ± 3.32†	28.23 ± 6.32†	42.24 ± 2.18
EF (%)	66.70 ± 4.5†	60.29 ± 10.10†	79.04 ± 2.28
4 weeks after transplantation			*P* (med versus cell)
LVDd (mm)	5.65 ± 0.38	5.05 ± 0.79	0.08
LVDs (mm)	3.83 ± 0.69	2.91 ± 0.54‡	<0.05
FS (%)	32.38 ± 10.22	42.09 ± 6.53‡	<0.05
EF (%)	65.40 ± 12.33	78.51 ± 6.78‡	0.051

MSC, mesenchymal stem cells; LVDs, left ventricular end-systolic dimension; LVDd, left ventricular end-diastolic dimension; EF, ejection fraction; FS, fractional shortening; ISO, isoproterenol.
Data are mean ± SD.
*$P < 0.05$ versus sham group; †$P < 0.01$ versus sham group; ‡$P < 0.01$ versus baseline. Differences between echocardiographic examination before and after transplantation were evaluated by a pairwise *t*-test. Comparisons of parameters among groups were analyzed using ANOVA.

Myogenesis of transplanted MSCs *in vivo*

To observe the myogenesis of MSCs *in vivo*, the nuclei of MSCs were labeled with DAPI. Four weeks after ISO treatment, DAPI-labeled MSCs were transplanted into rats. As shown in Fig. 3, immunofluorescence demonstrated that transplanted MSCs were not positive for the cardiac troponin T and desmin.

Effect of MSC transplantation on myocardial fibrosis

Next, we investigated the extent of myocardial fibrosis by Masson's trichrome staining. In rats undergoing heart failure, a modest increase in myocardial fibrosis was detected. However, MSC transplantation appeared

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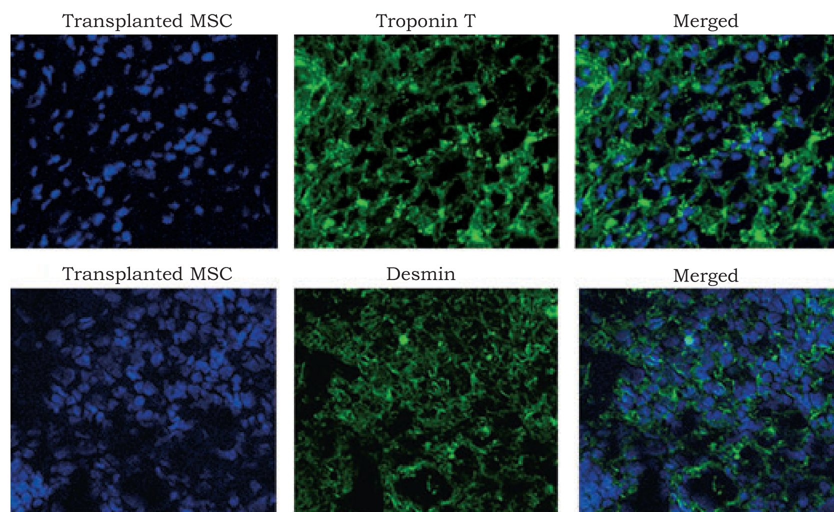
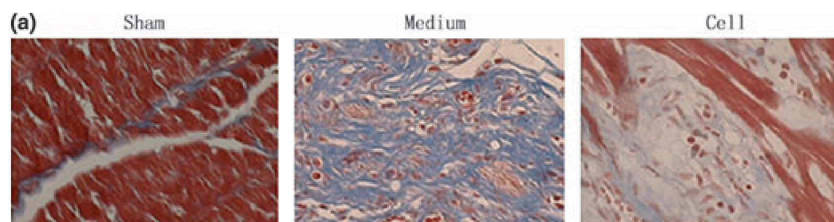


Figure 3 Differentiation of transplanted mesenchymal stem cells (MSCs) *in vivo*. Engrafted MSCs were negative for cardiac troponin T and desmin. Magnification 400×.



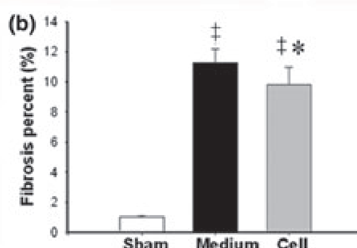


Figure 4 (a) Representative myocardial sections showed markedly decreased cardiac fibrosis after mesenchymal stem cells (MSC) transplantation, 400×. (b) Quantitative analysis of collagen fraction showing that MSC transplantation significantly decreased cardiac fibrosis, $n = 6$.

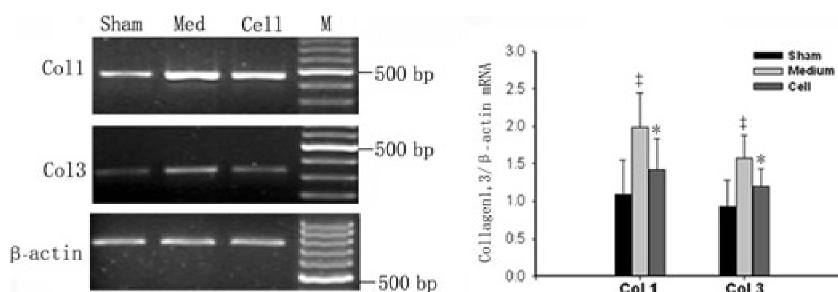


Figure 5 Semiquantitative reverse transcription-polymerase chain reaction analysis of mRNA levels. Expression levels of collagen I (col1) and collagen III (col3) were significantly decreased in the mesenchymal stem cells-treated group compared with the medium-treated group. ‡$P < 0.01$ versus sham group; *$P < 0.05$ versus medium group. Sham group, $n = 5$; cell-treated group, $n = 6$; medium-treated group, $n = 6$.

to significantly attenuate myocardial fibrosis (Fig. 4a). Quantitative analysis demonstrated a reduced collagen fraction in the MSC-treated group, compared with the medium-treated group: 9.8 ± 1.2 vs. 11.3 ± 0.9 (ANOVA, $P < 0.05$, Fig. 4b). In addition, RT-PCR analysis confirmed that MSC treatment markedly decreased the expression of collagens I (ANOVA, $P < 0.05$) and III mRNA (ANOVA, $P < 0.05$), compared with the medium-treated group (Fig. 5). These results suggest that transplantation of MSCs can significantly decrease the

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development of myocardial fibrosis following ISO-induced global heart failure.

Effect of MSC transplantation on MMP expression

We, thereafter, investigated changes in levels of MMP-2 and MMP-9 by RT-PCR and Western blotting. The results showed that both MMP-2 mRNA ($P < 0.05$) and MMP-9 mRNA ($P < 0.05$) were significantly increased in the medium-treated group compared with the sham-treated group. Interestingly, however, the increases in MMP-2 mRNA ($P < 0.05$) and MMP-9 mRNA ($P < 0.05$) levels were attenuated markedly by MSC transplantation (Fig. 6a). In addition, MSC transplantation had significant lower MMP-2 ($P < 0.01$) and MMP-9 ($P < 0.01$) protein levels, compared with the medium-treated group (Fig. 6b).

Paracrine signaling by MSCs

Hepatocyte growth factor is an important antifibrotic factor. Expression of HGF mRNA was detected in cultured MSCs by RT-PCR (Fig. 7a). Next, we examined the expression of HGF in the myocardium. Similarly, RT-PCR analysis showed that the downregulation of HGF expression in the myocardium of the medium-treated group was significantly enhanced in the MSC-treated group ($P < 0.05$, Fig. 7b). And Western blotting analysis also showed that HGF protein level in the MSC-treated

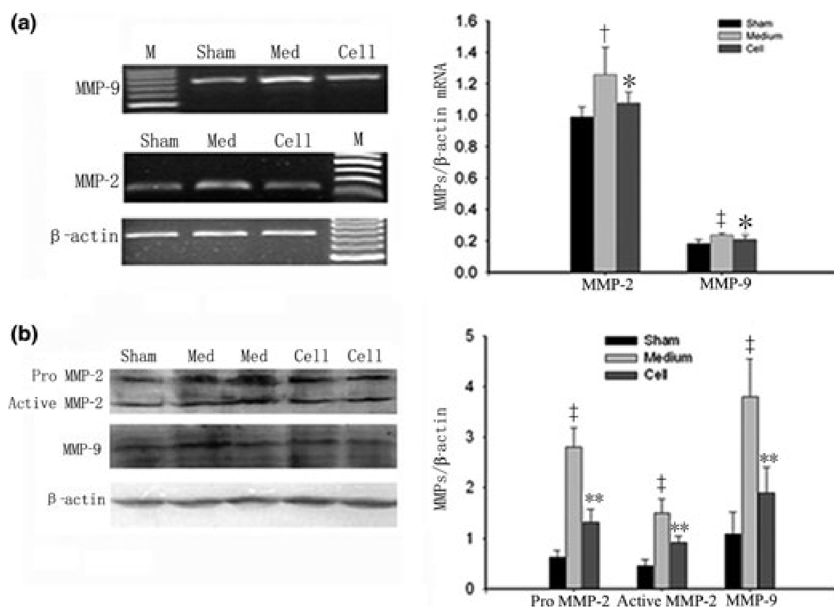

Figure 6 (a) Semiquantitative reverse transcription-polymerase chain reaction analysis of mRNA levels. Expression levels of matrix metalloproteinase MMP-2 and MMP-9 were significantly decreased in the mesenchymal stem cells-treated group compared with the medium-treated group. (b) Western blotting analysis of protein levels. † $P < 0.05$ versus sham group, ‡$P < 0.01$ versus sham group; *$P < 0.05$ versus medium group. Sham group, $n = 5$; cell-treated group, $n = 6$; medium-treated group, $n = 6$.

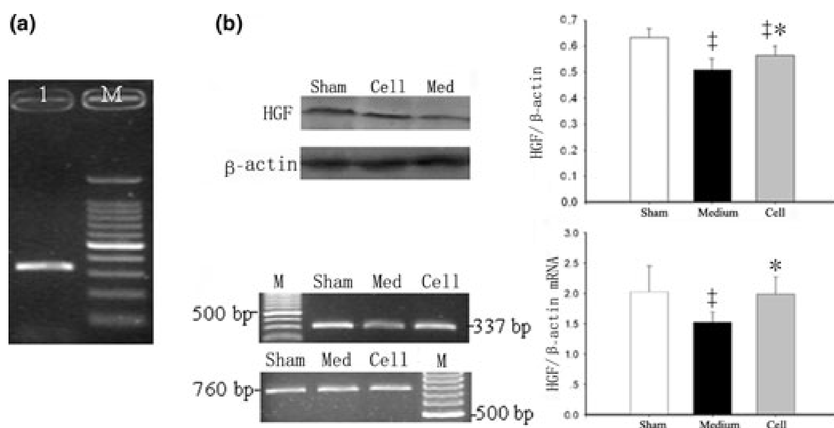

Figure 7 (a) Reverse transcription-polymerase chain reaction analysis of hepatocyte growth factor (HGF) expression in mesenchymal stem cells. Lane 1, HGF (337 bp); lane M, 100 bp marker. (b) Cell transplantation improved the expression of HGF. ‡$P < 0.01$ versus sham group; *$P < 0.05$ versus medium group. Sham group, $n = 5$; cell-treated group, $n = 6$; medium-treated group, $n = 6$.

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group increased more than that in the medium-treated group ($P < 0.05$, Fig. 7b).

Discussion

In this article we showed evidence that [1] MSC transplantation improved cardiac function and attenuated cardiac fibrosis associated with ISO-induced global heart failure, and [2] MSCs exerted an antifibrotic effect, probably by acting in a paracrine manner.

Previous cell transplantation studies have focused on myocardial infarction and ongoing myocardial damage (as in the Syrian hamster cardiomyopathy or viral myocarditis models) [12,16,17]. However, our model of global myocardial damage induced by ISO is time-dependent progressive heart failure, providing the advantages of leaving the vasculature intact and having not continued insult. It may thus more accurately mirror the natural processes of heart failure [18]. In addition, an intact coronary circulation makes this model suitable for investigating cardiac fibrosis.

In this study, MSCs were engrafted into the myocardium in a rat model of heart failure. Four weeks after transplantation, the transplanted MSCs were negative for cardiac troponin T and desmin. These results suggest that MSCs do not differentiate into cardiomyocytes in the myocardium. However, our results showed that MSC transplantation improved cardiac function, as indicated by a significant decrease in LVDs and increases in EF and FS. Thus, the improvement in cardiac function may not be attributable to regeneration. Other mechanism may be participating in the improvement of heart function.

Isoproterenol was reported to induce heart failure and diffuse myocardial fibrosis 4 weeks after injection [6,19]. Furthermore, it is well known that there are many patients of old myocardial infarction and dilated cardiomyopathy, and that myocardial fibrosis have already formed in these patients [20]. Therefore, in order to investigate whether cell transplantation can inhibit the progress of myocardial fibrosis of these patients, we chose the interval of 4 weeks after ISO injection to initiate MSC transplantation. Importantly, our results showed that compared with the medium-treated group, the cell-treated group had a significant increase in heart function and decreases in collagenous fraction and collagen gene expression. Therefore, MSC transplantation may inhibit the fibrosis of ISO-induced heart failure, leading to the improvement of heart function.

Matrix metalloproteinase (MMPs) play an important role in ventricular remodeling by degrading extracellular matrix (ECM). Inhibition of MMP activities prevents progressive left ventricular remodeling and improves heart function in an animal model of heart failure [21]. Regardless of the inciting cause, such as myocardial infarction (MI), there appear to be increased expression of MMPs in the initial phase and the final phase of heart failure, leading to marked ventricular dilatation and fibrosis [22,23]. In patients of dilated cardiomyopathy (DCM), increased expression of MMPs is associated with decrease of cardiac function [24]. Similarly, in this study 8 weeks after ISO injection, there was a significant decrease in heart function associated with marked increases in the expression of MMP-2 and -9 in the medium-treated rats. Furthermore, our results demonstrated that compared with the medium-treated rats, 4 weeks after transplantation the cell-treated rats had significantly decreased the expression of MMP-2 and -9. We therefore propose that MSC transplantation can decrease the expression of MMP-2 and -9, resulting in decrease in normal collagen degradation and improvement of heart function. In addition, we also can conclude that the impact of MSC transplantation on MMPs is not instantaneous but persistent for at least 4 weeks, supporting the role of MSC transplantation.

Ventricular remodeling may be involved in both systolic and diastolic dysfunction of the failing heart. In the failing heart, normal collagens are degraded by increased levels of MMPs, and fibrous interstitial deposits of poorly cross-linked collagens are synthesized. This, in turn, may lead to myocardial fibrosis, dilation of the ventricles, and cardiac dysfunction [23,25]. Preserving collagen homeostasis is likely able to ameliorate heart failure. Our results showed that MSC transplantation inhibited the expression of collagen and MMPs. Taken together, we can conclude MSC transplantation, performed when fibrosis have already formed, can further inhibit the degradation of normal collagen and the formation of poorly cross-linked collagens, resulting in attenuation of cardiac fibrosis and improvement of heart function.

Next, we aimed to investigate mechanisms mediating the antifibrotic effects of MSCs. We showed that cultured MSCs express HGF, raising the possibility that transplanted MSCs secrete HGF *in vivo*. HGF is a growth factor that has been shown to exert antifibrotic, antiapoptotic, and angiogenic effects [26–28]. Previous studies have shown that HGF levels in the myocardium of cardiomyopathic rats were significantly decreased compared with those in normal hearts, and this was attributable to repression of angiotensin II and transforming growth factor-β [26,27]. Transfection of an HGF expression vector into the myocardium of these rats significantly decreased the size of the fibrotic area [26]. Consistent with previous studies, our study also shows lower levels of HGF expression in ISO-induced failing hearts than in sham hearts. Importantly, we also show *in vivo* that transplantation of MSCs increased the expression both of HGF mRNA and HGF protein in the

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myocardium. These observations therefore suggest that MSC-derived HGF may participate in the amelioration of myocardial fibrosis. MSCs can secrete several cytokines. Further studies will be required to detect other cytokines which may also mediate the antifibrotic effect.

In conclusion, MSC transplantation can improve heart function following ISO-induced global heart failure by attenuating ventricular remodeling. The beneficial effects of MSC transplantation may be, at least in part, because of the actions of secreted HGF.

Authorship

LLL: designed and performed study, collected data and wrote the paper. YZ and YL: designed study and provided fund. BY and ZZG: designed study. YX and SDZ: performed study.

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