Original Research

Inhibition of apoptosis by knockdown of caspase-3 with siRNA in rat bone marrow mesenchymal stem cells

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Abstract

Transplantation of bone marrow mesenchymal stem cells is a promising new strategy for the repair of infarcted cardiac tissue. However, the majority of transplanted bone marrow mesenchymal stem cells (BMSCs) die soon after transplantation, due in part to oxidative stress in the ischemic region. Oxidative stress is known to induce apoptosis through the activation of caspase-3. The aim of this study is to determine whether small interfering RNA targeting caspase-3 can inhibit the apoptosis of rat BMSCs *in vitro*. Caspase-3 siRNA expression vectors were prepared and transfected into rat BMSCs in the presence of liposomes. Western blot assay and real-time polymerase chain reaction (RT-PCR) were performed to detect caspase-3 expression. A retrovirus packaging system was employed to package 293FT cells producing caspase-3 siRNA virus, which were transfected into rat BMSCs. Those stably expressing caspase-3 siRNA were screened by Western blot assay and RT-PCR to determine caspase-3 expression levels. Stable transfection of caspase-3 siRNA significantly decreased caspase-3 protein (0.26 ± 0.001 vs. 0.42 ± 0.004, $P < 0.05$) and mRNA expression (0.19 ± 0.002 vs. 1, $P < 0.05$) in BMSCs compared to non-transfected BMSCs. Cells were incubated in H_2O_2 to induce apoptosis, which was detected by TUNEL staining, and BMSC morphology was not altered by either transient or stable transfection of caspase-3 siRNA. H_2O_2-induced apoptosis of BMSCs stably transfected with caspase-3 siRNA was dramatically reduced compared to that of normal BMSCs (11.0 ± 3.2 vs. 25.8 ± 4.2, $P < 0.05$). Caspase-3 knockdown BMSCs are thus more resistant to apoptosis than normal BMSCs, potentially increasing their survival rates under conditions that cause oxidative stress.

Keywords: Cell therapy, myocardial infarction, stable transfection, oxidative stress, BMSC

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Introduction

Transplantation of bone marrow mesenchymal stem cells (BMSCs) is a promising new strategy for the repair of infarcted cardiac tissue. While BMSCs have the potential to differentiate into cardiomyocytes, their therapeutic use is limited by short survival times after transplantation.[1] The majority of transplanted BMSCs die soon after transplantation, due in part to oxidative stress in the ischemic region.[2] Infarcted tissues are subject to oxidative stress, which is known to induce apoptosis. Increasing attention has thus been given to improving the myocardial microenvironment and attenuating inflammation following infarction. In addition, recent research has focused on molecular factors that could be used to reduce apoptosis of BMSCs during oxidative stress.

Reactive oxygen species generated during oxidative stress mediates apoptosis through a cascade of cellular events. Within this cascade, the activation of caspase-3 has been shown to be pivotal to the induction of apoptosis through several apoptosis-related signaling pathways.[3] The role of this protein in the survival of cardiac cells is indicated by the observation that high-caspase-3 expression is a major cause of myocyte apoptosis during myocardial infarction.[4] Furthermore, the inhibition of caspase-3 expression has been shown to decrease apoptosis in cardiomyocytes and BMSCs.[2,5,6] These observations raise the possibility that transplanted cells with decreased caspase-3 expression might resist apoptosis, thereby enhancing cell survival.

The present study aimed to determine whether knockdown of caspase-3 expression in BMSCs by RNA interference decreases BMSC apoptosis. RNA interference is a method developed in recent years to inhibit the synthesis of specific proteins by interfering with target gene expression.[7,8] The feasibility of using this methodology to inhibit caspase-3 expression was suggested by the successful

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knockdown of caspase-3 using siRNA in other cell lines.[6] We designed siRNA to target the caspase-3 gene and constructed a caspase-3 siRNA expression vector for transfection into BMSCs. While other studies have successfully reduced the cellular levels of caspase-3 in BMSCs through other means,[2,9,10] to our knowledge, this is the first report of direct caspase-3 expression knockdown using siRNA in BMSCs.

Apoptosis of stably transfected BMSCs in response to oxidative stress was assessed by incubating the cells in hydrogen peroxide. This reactive oxygen species is a signaling molecule produced during normal metabolic and inflammatory processes. Hydrogen peroxide is known to induce apoptosis by activating caspase-3.[11,12] This system thus serves as a model for the conditions of oxidative stress present in tissues after myocardial infarction.[2]

We demonstrate here that BMSCs stably expressing caspase-3 siRNA expressed markedly lower levels of caspase-3 mRNA and protein than controls. We also present evidence that caspase-3 knockdown decreased apoptosis in BMSCs. Our findings suggest that the viability of BMSCs could be improved by these genetic modifications.

Materials and methods

Animals

Sprague-Dawley rats (specific pathogen free) aged 2–4 months were purchased from the Animal Center of Sun Yat-sen University. The animals were raised according to the guidelines for the use and care of laboratory animals.

Main reagents and instruments

The following reagents and instruments were used in this study: DMEM (Gibco, Grand Island, NY, USA), gel image system (Shanghai Peiqing Biotech Co., Ltd, Shanghai, P.R. China), plasmid extraction kit (Beijing Tiangen Biochemical Technology Co., Ltd., Beijing, P.R. China), Trizol (TaKaRa, Shiga, Japan), primers (Invitrogen, Grand Island, NY, USA), Sybr green (TaKaRa), quantitative fluorescence thermal cycler (ABI 7300; SENSO, Germany), frozen centrifuge (HEMR), DAKO anti mouse/rabbit secondary antibody visualization system, BCA-100 protein extraction kit, electrophoresis unit (Tannon), constant temperature water tank (Shanghai Yiheng Biotech Co., Ltd., Shanghai, P.R. China), TUNEL apoptosis detection kit (Nanjing Keygen Biotech; KGA7044, Nanjing, P.R.China) and Hoechst 33258 staining kit (Nanjing Keygen Biotech; KGA211).

Design and synthesis of siRNAs and construction of caspase-3 siRNA expression vector

Three siRNAs with 19–21 bases (si-caspase3-1, si-caspase3-2, si-caspase3-3) were designed based on the caspase-3 sequence in Genbank (NM_012922). In addition, a random sequence served as a negative control (si-ncg). The siRNA sequences targeting caspase-3 were as follows: si-caspase3-1, 5′-GCACTGGAATGTCAGCTCG-3′; si-caspase-3-2, 5′-GGATAGTGTTTCTAAGGAAGA-3′; si-caspase-3-3, 5′-GCCGACTTCCTGTATGCTTAC-3′ and si-ncg, 5′-GC CAGCTTAGCACTGACTC-3′.

The siRNA sequences were introduced into the expression vector pSUPER.retro.neo to construct the plasmid pSUPERretro-egfp-neo-si-caspase-3$^{1-3}$ and si-ncg. The constructs were made by Guangzhou Langri Biotech Co., Ltd (Guangzhou, P.R. China).

Separation and identification of rat BMSCs

Whole bone marrow was isolated from rats, and BMSCs were isolated and cultured in DMEM medium. Cell growth and morphology were observed using an inverted microscope. Cells of passage 3 were seeded onto sterilized coverslips, washed three times in PBS (5 min each) and fixed in 4% formaldehyde for 30 min. After washing three times in PBS (5 min each), the cells were treated in 3% H_2O_2 at room temperature for 10 min to inactivate endogenous peroxidase and then washed again as before. Cells were then blocked in 10% goat serum for 100 min at room temperature, followed by incubation with primary antibody (CD34: 1:100; CD44: 1:100) at 4°C overnight. After washing in PBS three times (5 min each), cells were treated with DAKO rabbit anti-mouse secondary antibody at room temperature for 30 min. After washing in PBS three times (5 min each), cells were treated with DAB at room temperature for 1–10 min and observed under a microscope. After washing in ddH_2O three times (5 min each), cells were counterstained with hematoxylin, followed by treatment with 1% hydrochloric acid in ethanol. Samples were then dehydrated, transparentized and mounted with neutral gum. Representative photographs were captured under a microscope (Nikon D60) at a magnification of 400×.

Grouping and transfection of BMSCs

BMSCs were divided into five groups: normal (Group A), negative control (si-ncg, Group B), si-caspase-3-1 (Group C), si-caspase-3-2 (Group D) and si-caspase-3-3 (Group E). All BMSCs were maintained in antibiotic-free DMEM containing 10% FBS. One day before transfection, trypan blue staining was used to determine cell viability. BMSCs were seeded onto six-well plates at a density of 1.5×10^6/well, with a viability of >95%. When the cell confluence reached 90% on the next day, cells were grown in Opti-MEM (1000 μl/well) for 3 h. The plasmid (4 μg) and lipofectamine 2000 (8 μl) were each diluted into 200 μl of Opti-MEM. The solutions were incubated at room temperature for 5 min, mixed together and incubated at room temperature for 20 min. The plasmid-lipofectamine 2000 mixture was then added to each well for transfection. Twenty four hours later, cells were observed under an inverted fluorescence phase-contrast microscope (Olympus BX51), and representative photographs were captured.

Packaging with retrovirus and preparation of cells with stable expression of caspase-3 siRNA

Western blot assay and real-time polymerase chain reaction (RT-PCR) were employed to select the siRNA with the best interference efficiency. This particular siRNA was then used for packaging with retrovirus. The calcium phosphate method was employed for transfection with packaged

Table 1 Primers for RT-PCR

Gene	Direction	Sequence (5′–3′)	Primer length (bp)	Amplification length (bp)
β-actin	β-Actin-F	CCTAGGCACCAGGGTGTGAT	20	122
	β-Actin-R	TTGGTGACAATGCCGTGTTC	20	
Caspase-3	Caspase-3-F	CGATTATGCAGCAGCCTCAA	20	118
	Caspase-3-R	AGGAGATGCCACCTCTCCTT	20	

plasmids (pSuper-retro-EGFP-si-caspase-3 and pSuper-retro-EGFP) into 293FT cells, which produced sufficient quantities of viral particles. These viral particles were then used to infect BMSCs, which were cultured and screened for stable expression of caspase-3 siRNA. When cells reached nearly 100% confluence, they were digested to identify those stably expressing caspase-3 siRNA. These cells were harvested and stored at −80°C.

Detection of caspase-3 protein expression in BMSCs by Western blot assay

Protein expression was assessed at 48 h after transfection. Stably transfected cell lines were assessed using the P5 generation and were compared to the same generation of normal BMSCs. The medium was removed, and cells were rinsed twice in 1 mL of cold PBS and lysed in lysis buffer for 10 min. Lysates were harvested into centrifuge tubes, and 50 μg of protein was mixed with 5× SDS loading buffer, followed by boiling at 100°C for 5 min. Samples (20 μg of protein) were subjected to PAGE using a 5% stacking gel and 12% separating gel. Separated proteins were transferred to a nitrocellulose membrane, which was then incubated with anti-caspase-3 antibody (1:3000) at 4°C overnight. After washing in TBST three times (10 min each), the membrane was treated with HRP-conjugated swine anti-rabbit secondary antibody (1:6000) at room temperature for 120 min with continuous shaking. After washing in TBST three times (10 min each), HRP activity was visualized using an ECL kit. Films were exposed to the blots for 10 s–10 min. Membrane-bound antibodies were then removed by washing with retrieval solution, and membranes were re-treated using anti-β-actin antibody (1:3000) at 4°C overnight. A gel imaging system was employed to photograph the protein bands in the gels. The optical density of each band was determined and quantitatively analyzed using this system. The expression of target protein was normalized to that of β-actin.

Detection of caspase-3 mRNA expression in BMSCs using RT-PCR

mRNA expression was assessed at 48 h after transfection. Stably transfected cell lines were assessed using the P5 generation and were compared to the same generation of normal BMSCs. Trizol was used to extract the total RNA according to the manufacturer's instructions. The RNA was reverse-transcribed into cDNA using MMLV-RT reverse transcriptase according to the manufacturer's instructions. For real-time fluorescence quantitative PCR, the reaction mixture (20 μl) was prepared according to the manufacturer's instructions. Amplification by PCR was carried out under the following conditions: predenaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 30 s and annealing at 60°C for 35 s. The fluorescent signals were obtained at the end of each extension, and the amplification curve was determined. After 40 cycles of amplification, the conditions used were 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. The fluorescent signals were obtained during the increase in temperature from 60°C to 95°C, and the melting curve was determined. Sybr green staining was performed, and β-actin was used as an internal reference to determine the levels of caspase-3 mRNA expression (Table 1).

H_2O_2 pretreatments and detection of apoptosis by TUNEL staining

The BMSCs (1×10^5 cells) with stable expression of si-caspase3-3 siRNA were seeded on coverslips. Pretreatment with 500 μM H_2O_2 for 24 h and washed in PBS three times (5 min each). Cells were then fixed in 4% formaldehyde for 20 min and in 70% ethanol at 20°C for 30 min. After washing in PBS three times (5 min for each), cells were treated with 0.1% Triton X-100/0.1% trisodium citrate solution at room temperature for 10 min. After washing in PBS three times (5 min each), cells were treated with 3% H_2O_2 at room temperature for 10 min, washed three times in PBS (5 min each) and incubated with TdT reaction solution in dark at 37°C for 90 min in a humidified environment.

After washing in PBS twice (2 min each), Hoechst33258 staining was carried out in the dark at room temperature for 20 min. Cells were then washed in 0.5%Tween 20-PBS three times (2 min each) in the dark, followed by mounting with glycerin buffer. The representative photographs were captured under a fluorescence microscope (OlympusBX51).

Results

Culture and identification of BMSCs

The following characteristics were used to identify BMSCs: absence of CD34 and CD45 expression; presence of expression of CD29, CD44, CD71 and CD90 (markers for marrow-derived stem cells).[13] Cells not exposed to antibodies served as the negative control (Figure 1a). Most cells were negative for CD34 staining, a cell surface marker for endothelial cells (Figure 1b), but nearly 100% were positive for CD44 staining, a cell surface marker for BMSCs (Figure 1c).

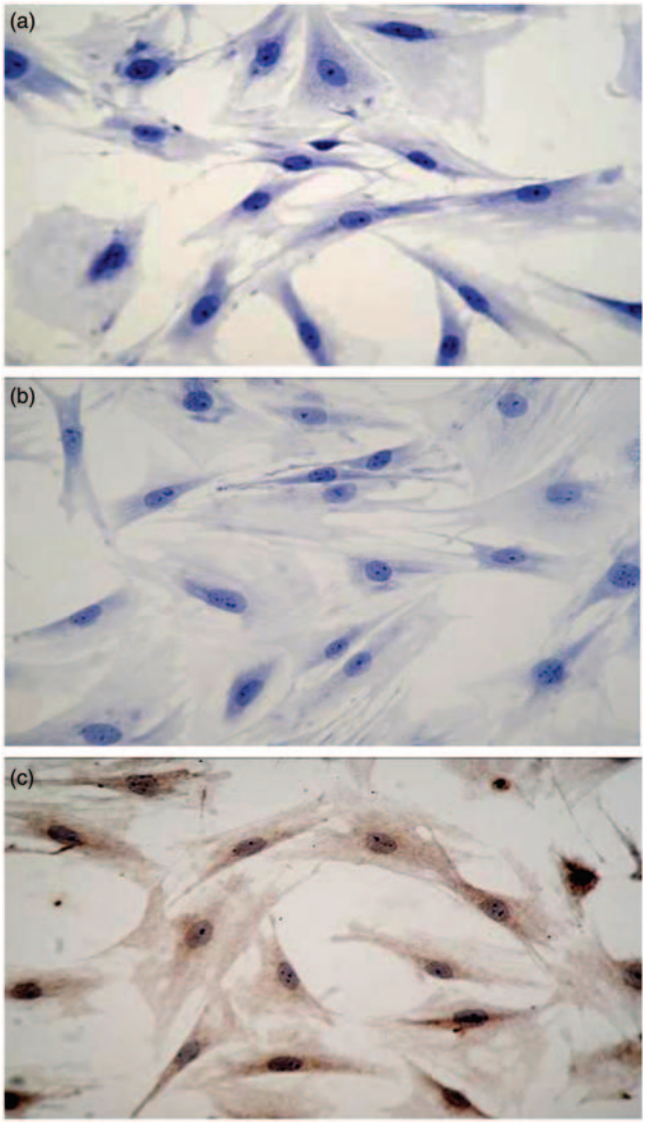

Figure 1 Culture and identification of primary rat BMSCs with staining of specific cell surface marker: (a) negative control; (b) CD34−; (c) CD44+, ×200

Efficiency of transient transfection of caspase siRNAs in BMSCs

Plasmids were transiently transfected into BMSCs, which were then observed for GFP expression using an inverted fluorescence phase-contrast microscope (Olympus BX51) (Figure 2). The transfection efficiency was high.

Expression of caspase-3 protein and mRNA in BMSCs transiently transfected with caspase-3 siRNAs

The caspase-3 protein level in transiently transfected BMSCs was detected by Western blot assay (Figure 3a). Compared to caspase-3 protein expression in control (0.14 ± 0.001) and si-ncg cells (0.18 ± 0.001), all three caspase-3 siRNAs (si-caspase-3-1, si-caspase-3-2 and si-caspase-3-3) significantly decreased caspase-3 protein expression (0.04 ± 0.001, 0.04 ± 0.001 and 0.00 ± 0.001, respectively) (Figure 3b); si-caspase-3-3 was the most effective in the knockdown of caspase-3 protein levels. Caspase-3 mRNA expression was also examined in transiently transfected BMSCs using real-time PCR. The relative expression of caspase-3 mRNA in BMSCs was significantly reduced by si-caspase-3-1 (25%), si-caspase-3-2 (34%) and si-caspase-3-3 treatment (42%) compared to control and si-ncg siRNA cells (Figure 3c).

Morphology of BMSCs with stable transfection of caspase-3 siRNA-3

Because the siRNA-3 construct most effectively knocked down caspase 3 expression, this siRNA was used to establish stable transfection of BMSCs with caspase-3 siRNA. The morphology of BMSCs stably transfected with caspase-3 siRNA-3 was similar to that of normal BMSCs (Figure 4).

Expression of caspase-3 mRNA and protein in stably transfected BMSCs

BMSC cells stably transfected with si-caspase-3-3 expressed 38% less caspase 3 protein than did control BMSCs (0.26 ± 0.001 vs. 0.42 ± 0.004, $P < 0.05$) (Figure 5a). Caspase-3 mRNA expression in si-caspase-3-3 BMSCs was decreased by about 80% that of control BMSCs (0.19 ± 0.002) ($P < 0.05$, Figure 5b).

Detection of apoptosis by TUNEL staining

TUNEL-FITC/Hoechst33258 staining was employed to distinguish apoptotic and normal cells. Photographs were captured under a fluorescence microscope (OlympusBX51) at a magnification of 250. Cells positive for both TUNEL-FITC (green fluorescence) and Hoechst33258 (blue fluorescence) were apoptotic. Normal cells were negative for TUNEL-FITC and weakly positive for Hoechst33258. Results showed that the number of apoptotic cells in cell lines with stable expression of caspase-3 siRNA was markedly lower than that of control BMSCs (11.0 ± 3.2 vs. 25.8 ± 4.2, $P < 0.05$, Figure 6).

Discussion

Transfection of rat BMSCs with capsase-3 siRNA expression vectors resulted in the expression of capsase-3 siRNA. We observed that BMSCs stably expressing capsase-3 siRNA exhibited a marked reduction in caspase-3 mRNA and protein expression. This knockdown of caspase-3 protein expression altered the normal response of these cells to stress induced by hydrogen peroxide. Our results demonstrated that hydrogen peroxide-induced apoptosis was significantly reduced in BMSCs that stably expressed caspase-3 siRNA compared to normal BMSCs.

BMSCs are attractive candidates for therapeutic use in the repair of cardiac damage, as they have multilineage differentiation potential and can differentiate into myocardium-like cells following induction *in vitro*. In addition, BMSCs are easily harvested and have low immunogenicity. Focal transplantation of BMSCs has been shown to promote angiogenesis, block ventricular remodeling and improve

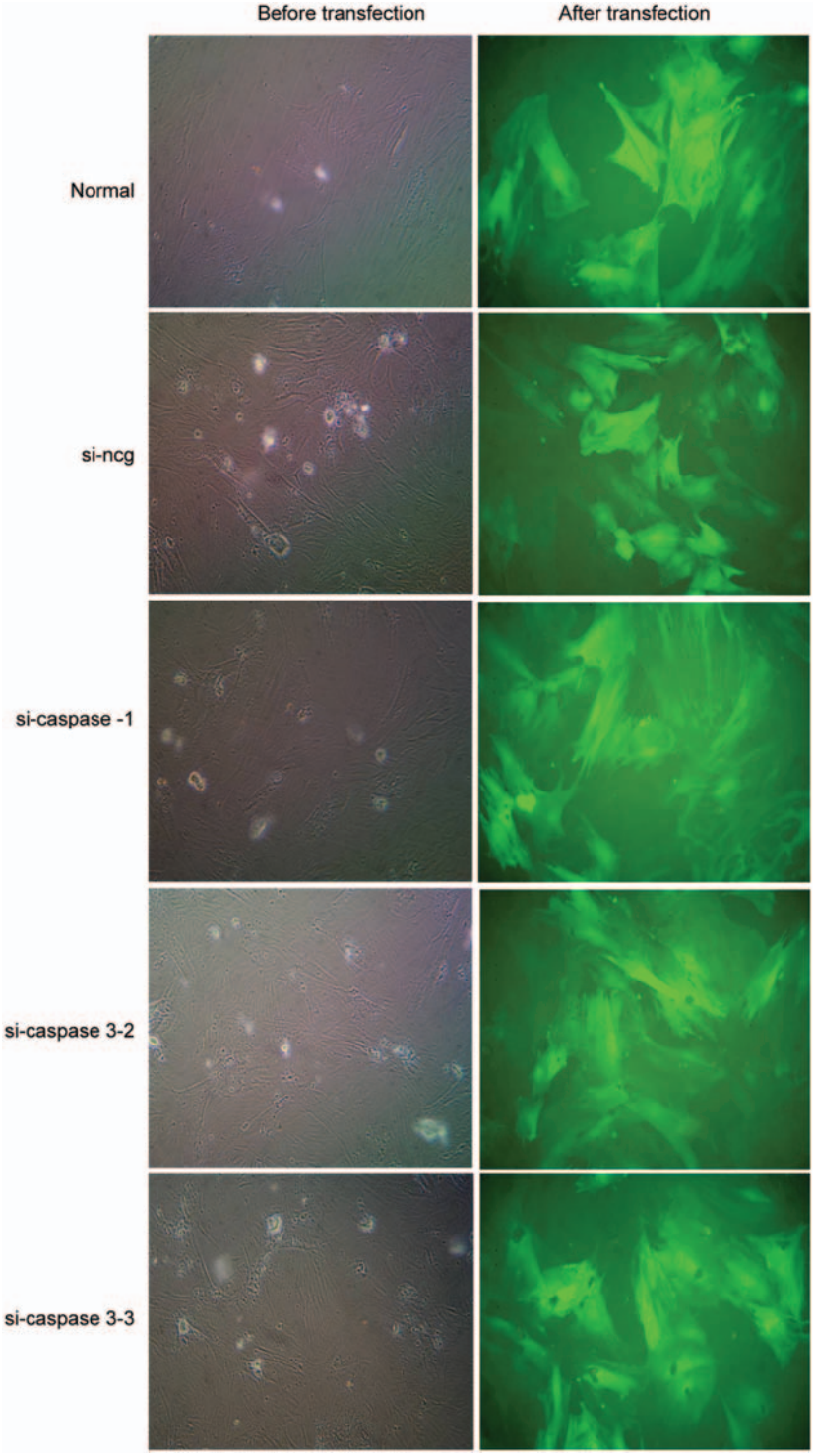

Figure 2 Transient transfection of caspase-3 siRNAs in BMSCs. The cells were observed for GFP expression under a fluorescence phase-contrast microscope after transfection. The synthesized siRNAs were transferred into BMSCs successfully (×250). Normal group, cells transfected with pSUPER.retro.neo vector only

heart function, indicating that this strategy is promising as an effective treatment for myocardial infarction.[14–16]

While transplanted BMSCs have been shown to survive in the infarct region, the long-term survival is still unsatisfactory; the majority of cells die within several days of implantation.[17,18] The survival of stem cells in the ischemic region is influenced by numerous factors, and their fate is determined by the myocardial microenvironment.[19] During ischemia/hypoxia, the infarct region fails to supply nutrients and oxygen to the transplanted cells. The death of cells

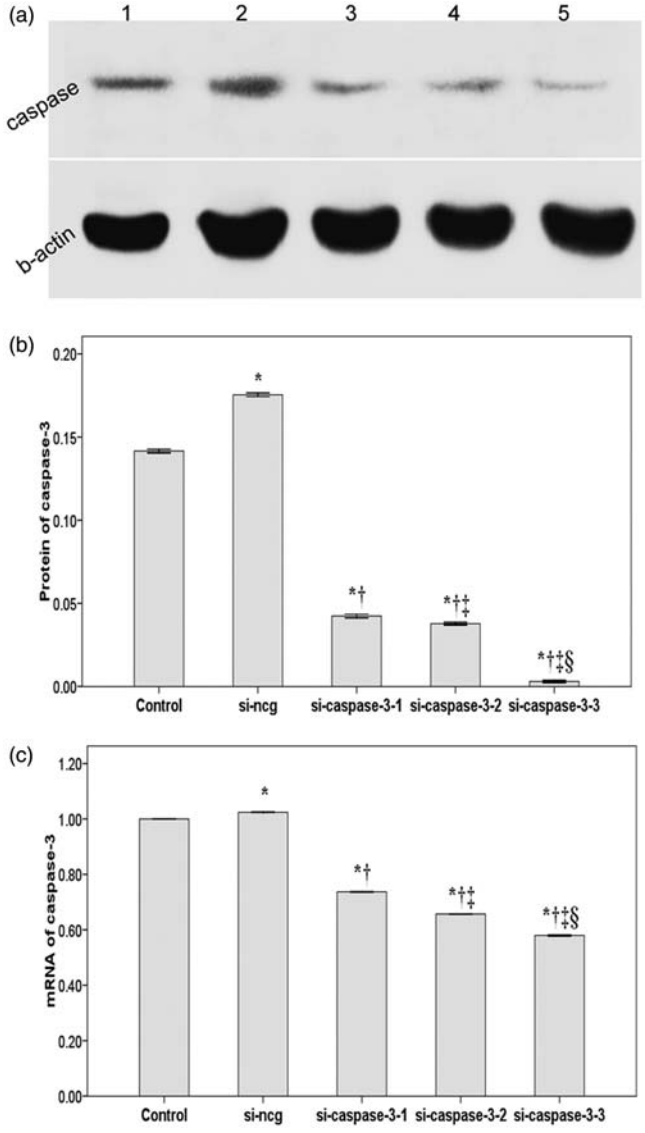

Figure 3 Knockdown of caspase-3 in BMSCs after transient transfection by western blot and real-time PCR. (a) Representative Western blot stained with antibody against caspase-3, (b) summary of caspase-3 protein level and (c) summary of real-time PCR data for caspase-3 mRNA. $^{*}P < 0.05$ comparing to Control group; $^{\dagger}P < 0.05$ comparing to si-ncg group, $^{\ddagger}P < 0.05$ comparing to si-caspase-3-1 group, $^{\S}P < 0.05$ comparing to si-caspase-3-1 group; all, after Tukey's adjustment, mean ± SD, n = 5 in each group

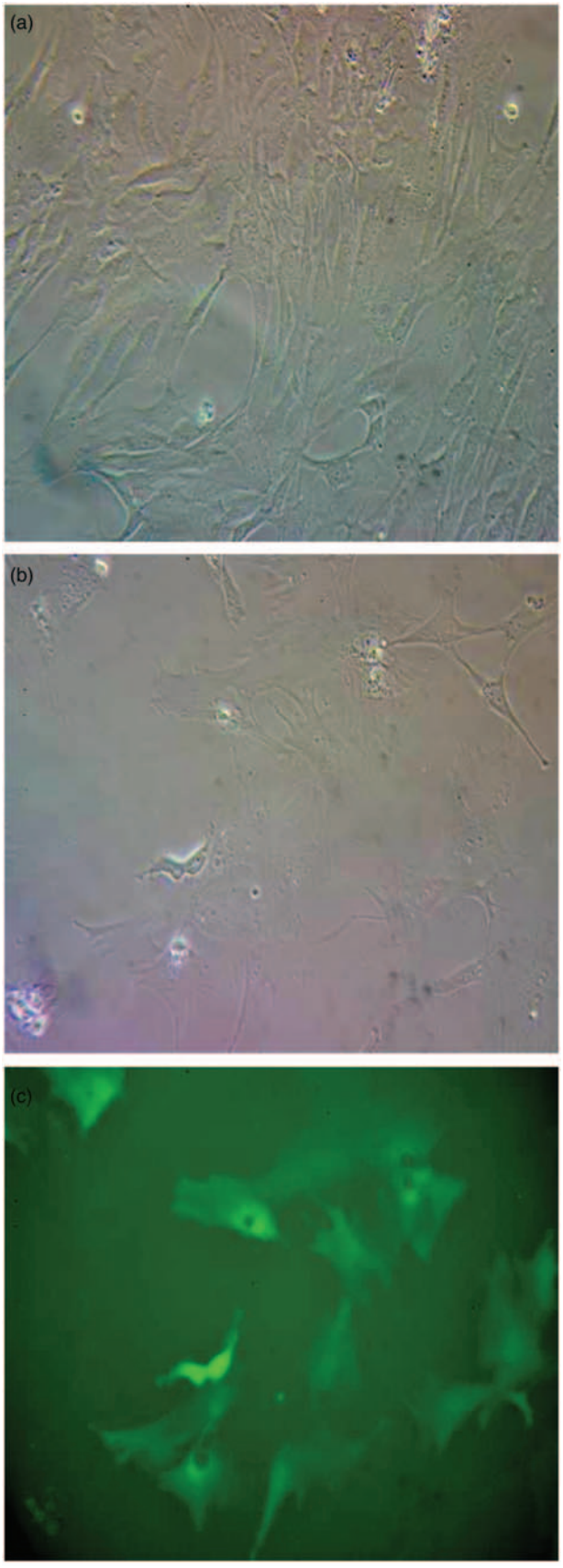

Figure 4 Morphology of BMSCs with stable transfection of caspase-3 siRNA-3. (a) Normal BMSCs under light microscope, (b) BMSCs with stable transfection of caspase-3 siRNA-3 under light microscope and (c) under fluorescent microscope

soon after transplantation is due to focal ischemia, inflammation and apoptosis.[20]

In the recent years, investigators have experimented with inflammatory chemokines, growth factors, hypoxia, drugs and gene therapy in an attempt to prolong the survival time of transplanted cells and reduce apoptosis.[20] Despite some progress, apoptosis and low-survival rates still significantly limit the application of BMSC transplantation[21] and are key obstacles to clinical transplantation of BMSCs.[1] Genetic manipulation of BMSCs is thus an attractive alternative, providing a new avenue for exploration. Several characteristics of BMSCs make them amenable to genetic manipulation. The transfection of BMSCs *in vitro* is

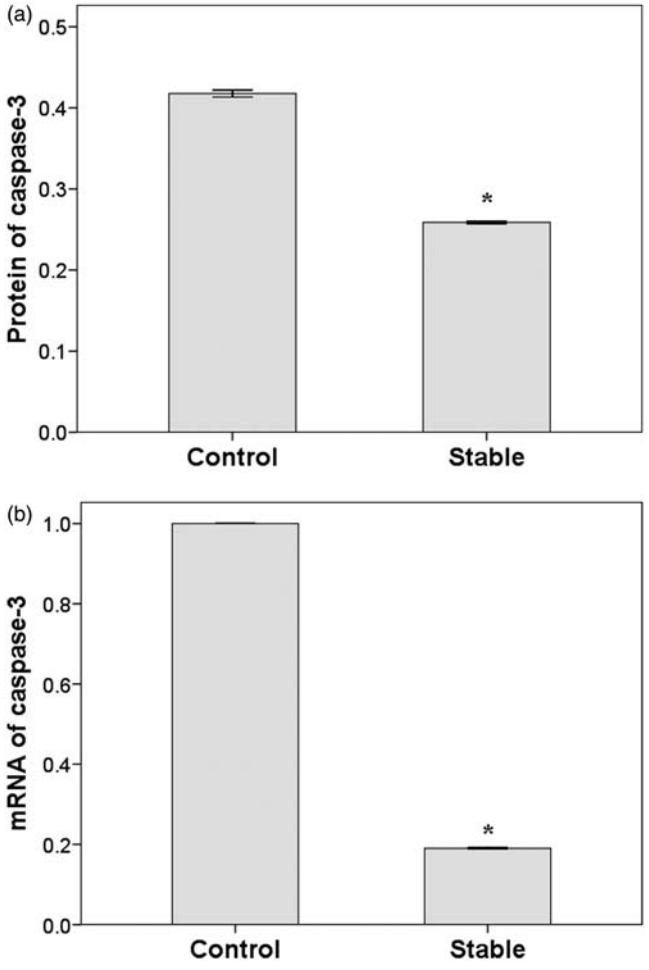

Figure 5 Detection of caspase-3 protein and mRNA expression in the BMSCs after stable transfection. (a) Western blot and (b) real-time PCR. *$P < 0.05$ compared to normal BMSCs after Tukey's adjustment; mean ± SD, n = 5 in each group

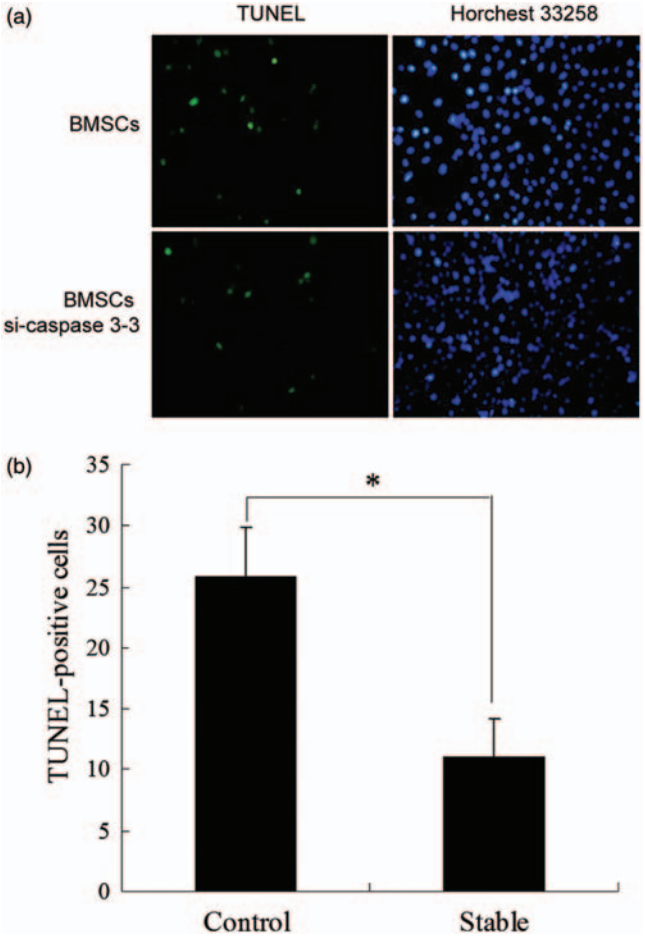

Figure 6 Apoptosis in caspase-3 knockdown BMSCs. Apoptosis was induced by pre-treatment of cells with 0.5 mM H_2O_2 for 24 h by (a) TUNEL staining and (b) apoptosis rate summary

efficient, as high as 80%. Some exogenous genes can integrate into the BMSC genomic DNA, resulting in long-term expression. Under such conditions, the proliferation and differentiation of transfected BMSCs remain unchanged, and the stable expression of target proteins can be observed at 6 months after transfection.[22] These features suggest that BMSCs could be used not only as seed cells for transplantation, but also as vectors for gene expression.[23] The transplantation of genetically altered cells has been met with success by Mangi *et al.*,[18] who observed that transplantation of MSCs overexpressing Akt into the infarcted myocardium of rats resulted in the attenuation of myocardial inflammation, collagen deposition, improvement in myocardial hypertrophy, reduction in apoptosis of transplanted MSCs, a decrease the infarct area and significant improvements in heart function.

Our study has several limitations. First, H_2O_2 (500 μM) may kill cells instantly, mainly through the non-apoptotic pathway, namely necrosis or oncosis. TUNEL is not specific for apoptosis. Second, we did not determine the efficiency of siRNA treatment, which could be determined by counterstaining transfected cells with Hoechst or DAPI. Further questions should be answered to better characterize caspase-3 knockdown BMSCs, including their proliferation, differentiation, tumorigenesis and secretion of cytokines. Our study is also limited by the *in vitro* nature of the experiments. Time-course of mRNA and protein expression level of caspase-3 should be examined too. While caspase-3 knockdown BMSCs exhibit reduced apoptosis *in vitro*, the *in vivo* function of these cells has not yet been tested. Active forms of caspase-3 may already pre-exist in transfected and control cells. As such, future *in vivo* studies are needed to address these questions.

While preliminary, the results of this study are encouraging. The genetic modification of BMSCs using siRNA was successful, producing cells that expressed markedly decreased levels of caspase-3 protein. When exposed to oxidative stress, the capsase-3 knockdown BMSCs underwent less apoptosis than normal BMSCs. These modifications thus have the potential to reduce apoptosis in BMSCs, possibly increasing their survival rates under conditions that cause oxidative stress.

Author contributions: PH: Guarantor of integrity of the entire study, study concepts, study design, definition of intellectual content, literature research, manuscript preparation, manuscript editing and manuscript review. L-bL: Definition of intellectual content, literature research, clinical studies, experimental studies, data acquisition, data analysis, statistical analysis, manuscript preparation and manuscript editing. J-lL: Clinical studies, experimental studies, data acquisition, data analysis and manuscript preparation. MW: Clinical studies and experimental studies. H-qJ: Experimental studies and data acquisition. KZ: Experimental studies and data acquisition. Y-qY: Manuscript preparation. S-rY: Guarantor of integrity of the entire study, study concepts, study design, definition of intellectual content, literature research, manuscript preparation, manuscript editing and manuscript review.

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