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Original Article

Bone marrow-derived mesenchymal stem cells attenuate ischemia-reperfusion injury of rat renal tubular epithelial cells through expression of HGF

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Abstract: Transplantation of mesenchymal stem cells (MSCs) has been proven to be effective in attenuation of ischemia-reperfusion injury (IRI), which was related to the paracrine effects of MSCs. In this study, we used hepatocyte growth factor (HGF) siRNA to knock down the HGF expression of bone marrow-derived mesenchymal stem cells (BM-MSCs); Transwell chambers and 6-well plates were then used to build up non-contact co-culture systems of BM-MSCs and IRI NRK cells. Real-Time PCR and Western blot were used to determine the mRNA and protein expression of HGF, high mobility group box 1 (HMGB1), interleukin-1 beta (IL-1β) and tumor necrosis factor-α (TNF-α), respectively; MTT array was used to determine the proliferation rate of BM-MSCs and NRK cells. Our results showed that, on the one hand, co-culture with IRI NRK cells can significantly accelerate BM-MSCs proliferation and HGF expression when compared to BM-MSCs alone ($P<0.05$); On the other hand, IRI NRK cells proliferation and inflammatory factors expression were all improved by co-culture with both wild type BM-MSCs and BM-MSCs transfected with HGF siRNA when compared with IRI NRK cells alone ($P<0.05$); While the wild type BM-MSCs showed more capability in promoting IRI NRK cells proliferation and inhibiting inflammatory factors expression, which means that, the IRI protection role of BM-MSCs was partially resulted from HGF secretion.

Keywords: Ischemia-reperfusion injury, BM-MSCs, hepatocyte growth factor, paracrine effects

Introduction

Renal ischemia-reperfusion injury is a common clinical pathological process occurred in the kidney surgeries such as kidney transplant and nephrolithotomy, which is characterized by restriction of blood supply to the kidney followed by restoration of blood flow and re-oxygenation. Lots of evidences have revealed that IRI can resulting in ischemic acute kidney injury, influencing the function of transplanted kidney, eventually reducing the number of patients of long-term surviving [1, 2]. Considering the critical role of IRI playing in the kidney operation, scientists have never stopped looking for a satisfactory solution to overcome it, ischemic preconditioning [3, 4], drug therapy [5-7], and MSCs transplantation [8, 9] are 3 hotspots regarding IRI treatment.

MSCs are type of cells with potential of self-renewal and multi-directional differentiation, which exist in many different kinds of tissues and organs such as bone marrow, liver, umbilical cord and placenta. Recently, series studies have demonstrated that both autologous and allogeneic MSCs transplantation were safe and capable of improving kidney damage resulted from IRI, making it a promising option for IRI treatment [10, 11].

Better understanding the underlying mechanisms will likely shed light on how MSCs exert their therapeutic effects of accelerating the recovery of renal IRI. Wise AF et al found that BM-MSCs can home to injured kidneys and promote repair [12]. Besides, evidences also suggested that MSCs had the ability to differentiate into renal tubular epithelial cells [13, 14].

Table 1. Primer Sequences Used for Real-time PCR

Gene		Primers (5'-3')
HGF	For:	TCCAGAGGTACGCTACGAAGTC
	Rev:	CGGTGTGGTGTCTGCTGATC
HMGB1	For:	TCTGTTCTGAGTACCGCCCAAA
	Rev:	ATCCGCAGCAGTGTTGTTCCA
IL-1β	For:	GTTTCCCTCCCTGCCTCTGACA
	Rev:	TCGACAATGCTGCCTCGTGAC
TNF-α	For:	TGCCTCAGCCTCTTCTCATT
	Rev:	GCTTGGTGGTTTGCTACGAC
GAPDH	For:	CTCCCATTCCTCCACCTTTG
	Rev:	CCACCACCTTGTTGCTGTAG

HGF, Hepatocyte growth factor; HMGB1, High mobility group box 1; IL-1β, Interleukin-1 beta; TNF-α, Tumor necrosis factor-α; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

Most importantly, studies have shown or suggested that MSCs can secrete a variety of cytokines, such as vascular endothelial cell growth factor (VEGF), hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1), those factors mediate beneficial paracrine effects and may greatly contribute to IRI repair [15-17]. In this study, we try to explore the potential paracrine effects of MSCs on IRI cells by transfected BM-MSCs with HGF siRNA.

Materials and methods

Experimental cell line and animals

The rat proximal tubular epithelial cell line NRK-52E (Hereinafter referred to as: NRK) was purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). 4-5 weeks old male Sprague-Dawley (SD) rats, SPF grade, weighing 80-100 g, were provided by the Laboratory Animal Center of Southern Medical University. Animals were housed in a well ventilated room at temperature 28-30°C under controlled light cycles (12 hr light: dark) with free access to standard chow and water. All animal experiments were approved by the Animal Care Committee of Guangzhou Medical University.

Separation, cultivation and identification of rats BM-MSCs

After 7 days of adaption, SD rats were killed by cervical dislocation and disinfected with 75% alcohol. Femurs were cut off from the back limbs followed by removing its two epiphyseal ends; after then, marrow was washed out by phosphate buffer. Percoll discontinuous density gradient centrifugation was used to collect mononuclear cells. Collected cells were cultured with DMEM-LG medium (Invitrogen, CA, USA) which contained 100 g/L Hyclone fetal bovine serum (Hyclone, Utah, US).

Identification of passage 3 MSCs was performed according the method provided by Mosna F et al [18].

HGF siRNA transfection

HGF siRNA (Sense: 5'-GAUCCAGCACUGAAGAUAATT-3'; Anti-Sense: 5'-UUAUCUUCAGUGCTGAUCTG-3') and negative control siRNA (Refer to: NC siRNA; Sense: 5'-UUCUCCGAACGUGUCACGU-3'; Anti-Sense: 5'-ACGUGACACGUUCGGAGAA-3') were designed and synthesized by Santa Cruz Biotechnology, Inc. (Texas, USA). To determine the transfection efficacy of HGF siRNA, passage 3 MSCs were transfected with nothing (Negative control group), transfection reagent (Mock group), NC siRNA (NC siRNA group) or HGF siRNA (HGF siRNA group) using lipofectamine™ 2000 reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Cell morphology was observed by an inverted fluorescence microscope. After 24 h transfection, mRNA levels of HGF were determined by Real-Time PCR method. Briefly, total RNA was isolated using Trizol (Takara, Japan) and reverse transcribed into cDNA using the M-MLV Reverse Transcriptase kit (Promega, Beijing, China) as instructed. Real-time PCR amplifications were carried out using the ABI 7500 system (Applied Biosystems, CA, USA). PCR primers of HGF and GAPDH (See **Table 1**) were synthesized by Sangon Biotech (Shanghai) Co., Ltd (Shanghai, China). The PCR was performed with once pre-degeneration (95°C for 1 min), followed by 45 cycles repeating (95°C melting for 10 s, 60°C annealing for 15 s, 72°C extension for 45 s). mRNA amount of HGF was normalized by GAPDH. The relative expression level of HGF was calculated using the $2^{-\Delta\Delta Ct}$ method as reported [19].

Establishment of in vitro ischemia-reperfusion model in NRK cells

In vitro I/R model was induced by adapting the method of Zhao WY et al [20]. Briefly, NRK cells were cultured with DMEM-LG complete medi-

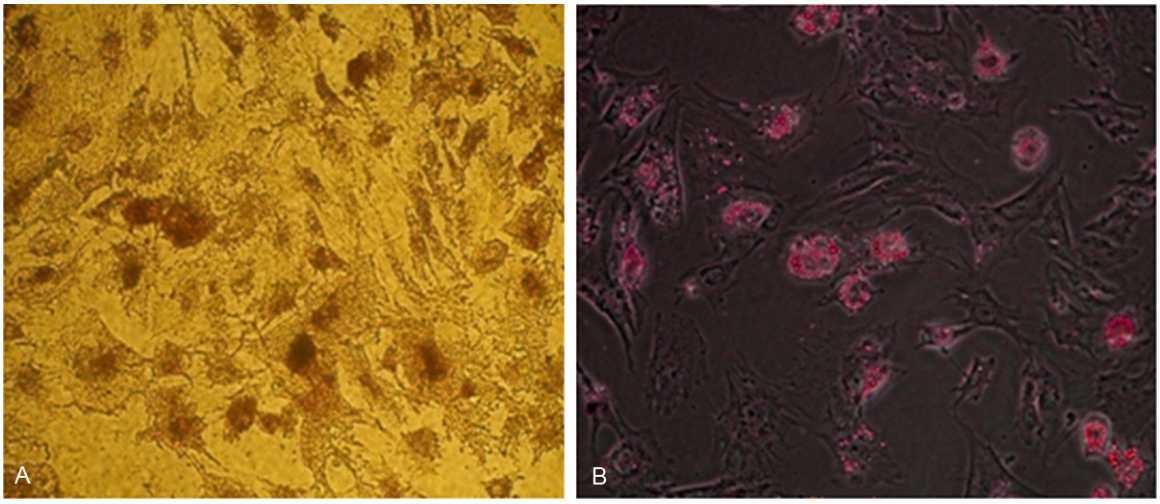


Figure 1. Identification of rats BM-MSCs. A. Alizarin Red staining after 14 days induction, major nodules together with calcium salt deposition were observed (200×); B. After 14 days induction, Oil Red O staining showed adipogenic differentiation characterized by red cytosolic fat droplets (200×).

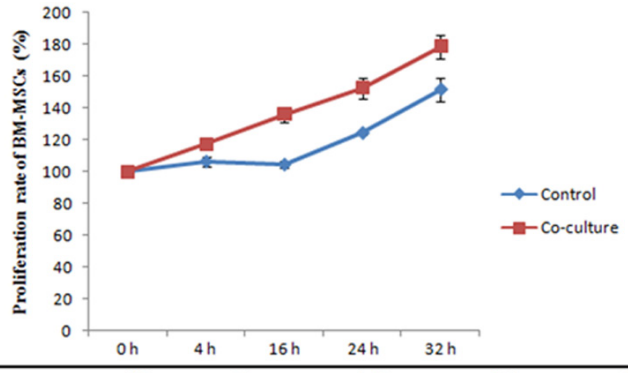
um under normoxic conditions and transferred to serum-free medium when cells under logarithmic phase of growth. After 24 h culturing, approximately 90% medium was discarded. Cells were then put back into an incubator for 1 h anaerobic cultivation followed by 1 h normoxic cultivation.

Determination of the effects of IRI on BM-MSCs proliferation and HGF secretion

To determinate the effects of IRI on BM-MSCs proliferation and HGF secretion, we used transwell chamber (Corning Inc., NY, USA) and 6-well plate (Corning Inc., NY, USA) to build up non-contact co-culture systems of BM-MSCs and ischemia-reperfusion injured NRK cells (referred to IRI NRK cells). Two groups were set up based on the seeded cells in the top/bottom chambers. For the control group, only 1.5×10^5 passage 3 BM-MSCs were seeded in the top chamber; For the Co-culture group, 1.5×10^5 passage 3 BM-MSCs were seeded in the top chamber, 1.5×10^5 IRI NRK cells were seeded in the bottom chamber. After 4 h, 16 h, 24 h and 32 h of co-culture, cells in the top chambers were collected and suspended. 100 μl suspensions were used to perform MTT array to determinate the cells proliferation rate. Briefly, 10 μl MTT agent (5 mg/ml) was added to the suspension; After an additional 4 h culture under 37°C, 150 μl of DMSO was added and the optical density (OA) of suspension was measured at 490 nm in a Microplate reader (Molecular Devices, CA, USA). Cell proliferation rate of BM-MSCs was calculated based on the following equation. Proliferation rate (%) = OD of test point/OD of beginning ×100%. After 48 h of co-culture, cells in the top chambers were collected, mRNA levels of HGF were determined by the above-mentioned RT-PCR method. Protein levels of HGF were determined by Western blot. Briefly, Anti-bodies against HGF (Abcam, USA, 1:1000) was used to probe the membranes, followed by incubation with HRP-conjugated secondary antibody (Kirkegaard & Perry Laboratories, Inc., MD, USA). GAPDH (Abmart, China, 1:300) was used for normalization. The density of each reactive band was quantified using Quantity One software (Bio-RAD Laboratories, CA, USA). All assays were performed in triplicate.

Determination of the paracrine effects of MSCs on NRK cells and inflammatory factors

To determinate the paracrine effects of MSCs on NRK cells proliferation and inflammatory factors expression, we also used transwell chamber (Corning Inc., NY, USA) and 6-well plate (Corning Inc., NY, USA) to build up non-contact co-culture systems of BM-MSCs and IRI NRK cells. Three groups were created based on the seeded cells in the top/bottom chambers. For group A, only 1.5×10^5 IRI NRK cells were seeded in the top chamber; For group B,



	0 h	4 h	16 h	24 h	32 h
Control group	100±0	106.49±3.0	104.56±2.05	124.32±0.8	151.67±7.36
Co-culture group	100±0	117.39±3.60	136.08±4.70	152.55±6.60	178.38±7.49
P value	-	0.016	0.000	0.017	0.012

Figure 2. IRI NRK cells can promote BM-MSCs proliferation. Control group: BM-MSCs alone; Co-culture group: BM-MSCs co-cultured with IRI NRK cells. After 4 h, 16 h, 24 h and 32 h of co-culture, the BM-MSCs proliferation rate of the Co-culture group was significantly higher than that of the Control group (P<0.05).

1.5×10^5 IRI NRK cells were seeded in the top chamber, 1.5×10^5 passage 3 MSCs transfected with HGF siRNA were seeded in the bottom chamber; For group C, 1.5×10^5 IRI NRK cells were seeded in the top chamber, 1.5×10^5 passage 3 BM-MSCs were seeded in the bottom chamber. After 4 h, 16 h, 24 h and 32 h of co-culture, cells in the top chambers were collected. Cells proliferation rate was determined based on the above-mentioned MTT method. After 48 h of co-culture, mRNA and protein levels of HMGB1, IL-1β and TNF-α were determined by the above-mentioned method of RT-PCR and Western blot, respectively. PCR primers of all analyzed genes were synthesized by Sangon Biotech (Shanghai) Co., Ltd and showed in **Table 1**. Anti-bodies against HMGB1 (Abcam, USA, 1:2000), IL-1β (Abcam, USA, 1:300) and TNF-α (Abcam, USA, 1:300) were used in the Western blot analysis. All assays were performed in triplicate.

Results

Identification of rats BM-MSCs

Differentiation into osteocytes: At passage 3, Most BM-MSCs were under adherent growth conditions when osteogenesis induced liquid was added. After 7 days culture, cells were gathered into clusters with little calcium salt deposition was found. Another 7 days later, nodous cells were occurred, together with lots of calcium salt deposition. Major red cell nodules were observed after alizarin red staining (**Figure 1A**).

Differentiation into adipocytes: Bright vacuole was formed within the cells at 3 days after adipogenic culture medium was added, which was increased over time. After 14 days culture, vacuoles were stained red by Oil Red O (**Figure 1B**).

Flow cytometry results showed that the percentage of $CD90^+$ cells, $CD105^+$ cells, $CD45^+$ cells were 96.9%, 95.7%, and 1.1%, respectively (Figures were not shown).

Effect of HGF siRNA on HGF expression in BM-MSCs

After 24 h transfection, inverted fluorescence microscope demonstrated that the majority transfected cells were under good condition. The ratio of transfection in BM-MSCs was greater than 70%. RT-PCR results showed that the relative mRNA expression level of HGF in the Negative control group, Mock group, NC siRNA group and HGF siRNA group was 1.0 ±0.37, 0.99±0.17, 0.97±0.13, and 0.58±0.04, respectively.

IRI- NRK cells can promote BM-MSCs proliferation and HGF secretion

MTT results showed that, after 4 h, 16 h, 24 h and 32 h of culture, BM-MSCs proliferation rate of the Control group was 106.49±3.0, 104.56±2.05, 124.32±0.8 and 151.67±7.36, respectively; Corresponding data of the Co-culture group was 117.39±3.60, 136.08±4.70, 152.55±6.60 and 178.38±7.49, respectively. When compared with the Control group, the BM-MSCs proliferation rate of the Co-culture group was significantly increased at the time of each test (P<0.05) (**Figure 2**), which means that

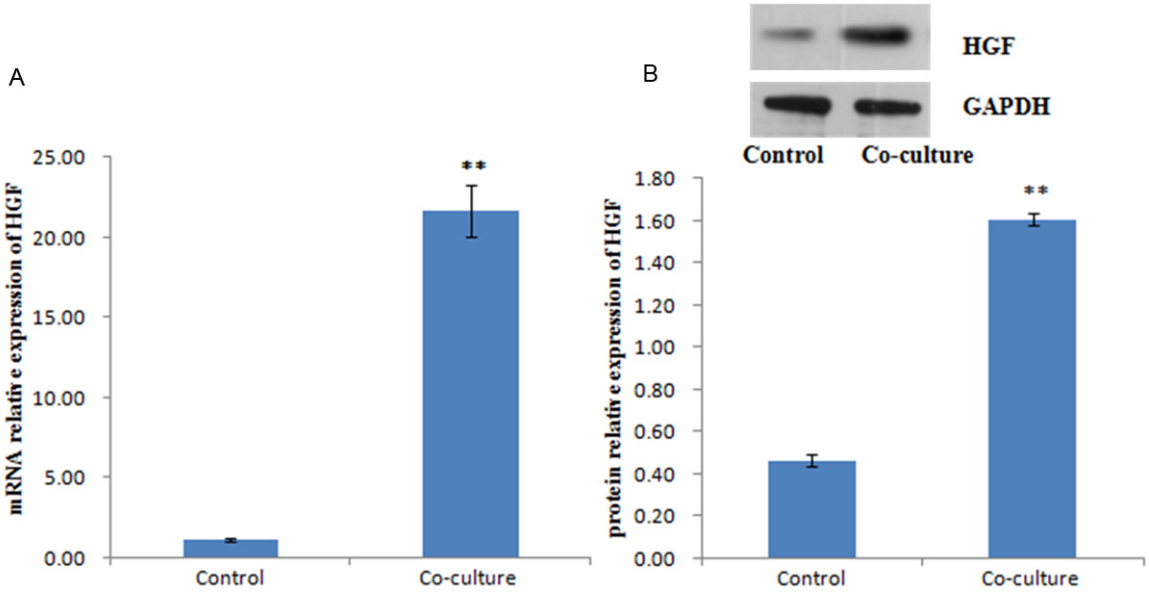
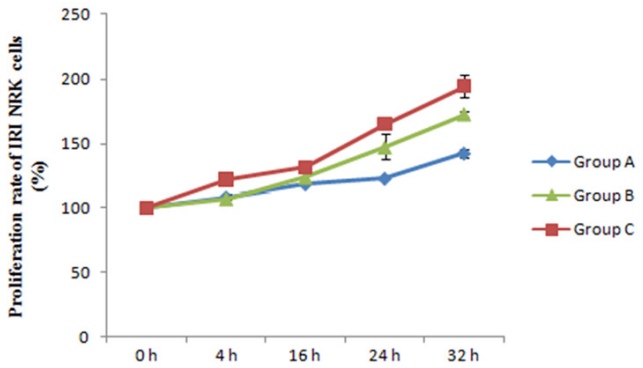


Figure 3. IRI NRK cells can promote BM-MSCs expression of HGF. A. After 48 h of co-culture, the mRNA relative expression of HGF of the Co-culture group was significantly higher than that of the Control group (21.66±1.58 vs. 1.14±0.14, P<0.01); B. After 48 h of co-culture, the protein relative expression of HGF of the Co-culture group was significantly higher than that of the Control group (1.61±0.03 vs. 0.47±0.03, P<0.01). **indicated that when compared with the Control group, P<0.01.



	0 h	4 h	16 h	24 h	32 h
Group A	100±0	108.23±2.52	118.59±0.92	122.8±0.55	142.44±3.18
Group B	100±0	107.01±3.73	123.75±3.95	147.39±9.61*	172.36±2.17*
Group C	100±0	112.18±3.27*	131.49±4.88**	164.87±2.85**	194.25±8.76**

Figure 4. Both wild type BM-MSCs and BM-MSCs transfected with HGF siRNA can promote IRI NRK cells proliferation. Group A: IRI NRK cells alone; Group B: IRI NRK cells co-cultured with BM-MSCs transfected with HGF siRNA; Group C: IRI NRK cells co-cultured with wild type BM-MSCs. *Indicated that when compared with the group A, P<0.05; # indicated that when compared with the group B, P<0.05.

co-culture with IRI NRK cells can promote BM-MSCs proliferation.

RT-PCR and Western blot results showed that, after 48 h of culture, mRNA and protein expression levels of HGF in BM-MSCs of the Co-culture group were all significantly increased when compared with the Control group (21.66±1.58 vs. 1.14±0.14, P<0.05; 1.61±0.03 vs. 0.47±0.03, P<0.05), which means that, co-culture with IRI NRK cells can promote BM-MSCs secretion of HGF (**Figure 3**).

HGF secreted by BM-MSCs can promote IRI NRK cells proliferation and inhibit inflammatory factors expression

MTT results showed that, after 4 h, 16 h, 24 h and 32 h of co-culture, IRI NRK cells proliferation rate of the group A was 108.23±2.52, 118.59±0.92, 122.8±0.55 and

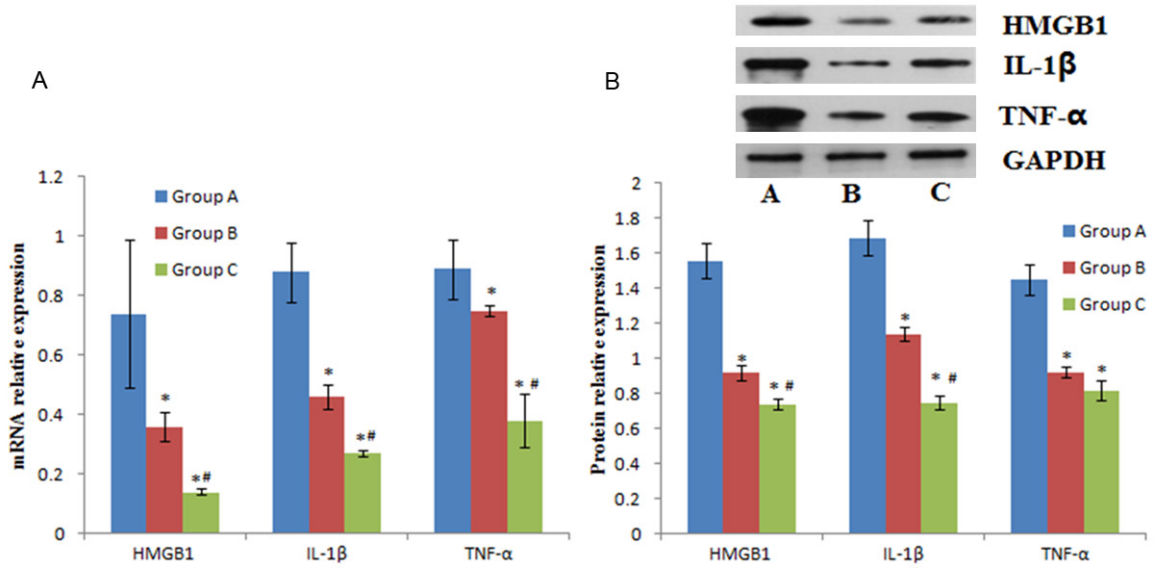


Figure 5. Both wild type BM-MSCs or BM-MSCs transfected with HGF siRNA can inhibited IRI NRK cells expression of inflammatory factors. A. After 48 h of co-culture, the mRNA relative expression of HMGB1, IL-1β and TNF-α of the group B and group C were all significantly lower than that of the group A (P<0.05), this difference was also showed between the group B and group C (<0.05); B. After 48 h of co-culture, the protein relative expression of HMGB1, IL-1β and TNF-α of the group B and group C were all significantly lower than that of the group A (P<0.05), this difference was also showed between the group B and group C except for TNF-α (<0.05). *Indicated that when compared with the group A, P<0.05; # indicated that when compared with the group B, P<0.05.

142.44±3.18, respectively; Corresponding data of the group B and group C was 107.01±3.73, 123.75±3.95, 147.39±9.61, 172.36±2.17 and 112.18±3.27, 131.49±4.88, 164.87±2.85, 194.25±8.76, respectively. As we can see in the **Figure 4**, when compared with the group A, the proliferation rate of IRI NRK cells in the group B was significantly increased from 24 h of MSCs-NRK cells co-culture (P<0.05); While in the group C, this phenomenon was noticed from 4 h of co-culture; the proliferation rate of IRI NRK cells in the group C was significantly higher than that of the group B from 16 h of co-culture. This result demonstrated that BM-MSCs can promote IRI NRK cells proliferation, which was partly resulted from the effects of HGF secretion.

RT-PCR and Western blot results showed that, after 48 h of culture, mRNA and protein expression levels of HMGB1, IL-1β and TNF-α in the IRI NRK cells of the group B and group C were all significantly decreased when compared with the group A (P<0.05). Group B and group C showed similar change pattern. This means that, co-culture with MSCs can inhibit IRI NRK cells secretion of inflammatory factors such as HMGB1, IL-1β and TNF-α (**Figure 5**).

Discussion

HGF was firstly identified from the serum of hepatectomized rats in the early 1980s, which is a multifunctional cytokine that plays important roles in many kinds of physiological and pathological activities, such as liver growth and regeneration [21, 22], renal development and repair [23, 24], lung development and regeneration [25, 26]. Studies have showed that, except for triggering hepatocyte regeneration after liver injury, HGF also has positive roles in the regulation of the growth and migration of some cells such as epithelial cells and endothelial cells, which eventually resulted from the protection of organs [24, 27]. Recently, studies have demonstrated that MSCs can improve renal injury through paracrine mechanisms involving cytokines such as IL-6, HGF and VEGF [17, 28]. In this study, we found that co-culture with IRI NRK cells can promote BM-MSCs proliferation, which was similar with the results of the article of Luo et al [29], in that research their found in the circumstance of injured neurons, microglia tend to promote the MSCs proliferation. This phenomenon could be explained that, in the normal condition, somatic stem cells are mostly kept in a quiescent state; while

in the circumstance of pathological states (such as injury, degeneration and cancer); stem cells may be quickly mobilized to respond injury by accelerating proliferation. Besides, we also found that HGF secretion was increased after co-culture, which was in accordance with the current mainstream opinions that, in the condition of IRI, MSCs can improve cells injury through secretion of cytokines such as HGF [15-17, 28].

To further explore the roles of HGF secreted by MSCs on the proliferation of IRI NRK cells, we used HGF siRNA to knock down the HGF expression of MSCs; Transwell chamber were then used to build up co-culture systems of BM-MSCs and IRI NRK cells; After co-culture, we found that, both wild type BM-MSCs and BM-MSCs transfected with HGF siRNA can accelerate the proliferation of IRI NRK cells and this effect was more significant using wild type BM-MSCs, which means that the promotion role of BM-MSCs on the proliferation of IRI NRK cells was partially resulted from HGF secretion. Inflammatory factor plays critical roles in the process of IRI; among them, HMGB1, IL-β and TNF-α are three typical proinflammatory cytokines. Previous studies have demonstrated that MSCs could significantly inhibited the expression of HMGB1, IL-β and TNF-a in renal tubular epithelial cells after ischemia-reperfusion injury [30, 31], our study showed similar findings; Of those two types BM-MSCs, wild type BM-MSCs showed much more inhibition effects on the expression of HMGB1 and IL-β, but not TNF-a, which means that the anti-inflammatory effects of HGF secreted by BM-MSCs on IRI NRK cells was partially related to the inhibition of HMGB1 and IL-β.

In conclusion, this study found that, on one hand, co-culture with IRI NRK cells can significantly accelerate MSCs proliferation and HGF expression; one the other hand, MSCs can improve IRI NRK cells proliferation and inflammatory factors expression which was partially resulted from HGF secretion.

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Disclosure of conflict of interest

None.

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