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Original Article

Protection of mesenchymal stem cell against renal ischemia-reperfusion injury through activation of Notch intracellular domain-mediated Notch1 pathway

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Abstract: Renal ischemia-reperfusion injury (IRI) is the most common cause of acute kidney injury (AKI) with high mortality and morbidity. Due to currently limited treatment approaches, mesenchymal stem cells (MSC) are becoming a promising cell-based therapy for renal IRI. However, the role of Notch pathway in MSC repairing adjacent injured kidney cells remains poorly known. In this study, we isolated bone marrow-derived MSC (BMSC), then constructed NICD expression vector MigR1-NICD and BMSC transfection MigR1-NICD-BMSC which were transplanted into an established renal IRI model. The effects of MigR1-NICD-BMSC on renal IRI were evaluated by renal function and histopathology. The results showed that the isolated BMSC owned the expressions of typical markers, namely positive expressions of CD73, CD105, CD90 and negative expressions of CD44, CD34, CD11b and HLA-DR. The mRNA and protein expressions of NICD in MigR1-NICD-BMSC increased by 1.72- and 5.33-fold respectively compared with MigR1-BMSC. The proliferation of MigR1-NICD-BMSC was promoted significantly compared with MigR1-BMSC following 72 and 96 h of incubation. Both levels of Scr and BUN decreased remarkably after the treatments with MigR1-NICD-BMSC compared with the treatments of MigR1-BMSC post-injection of 72 h. The classical histological signs of tubulointerstitial injury in renal IRI were relieved by injection of MigR1-NICD-BMSC. The semi-quantitative scores showed IRI-rats treated with MigR1-NICD-BMSC exhibited significantly pathological improvements compared with those exposures to MigR1-BMSC. Therefore, activated Notch pathway by overexpression of NICD could promote BMSC proliferation and improve the renal function and histology. These observations suggested BMSC was protective against renal IRI via activation of NICD-mediated Notch pathway.

Keywords: BMSC, NICD, IRI, notch protein

Introduction

Ischemia and reperfusion is a common pathological condition often accompanying with an aggravation of tissue injury and a profound inflammatory response, leading to so called ischemia-reperfusion injury (IRI) [1]. Renal IRI usually appears in the clinical settings of kidney transplantation, hemorrhagic shock or cardiovascular surgery, constituting the most important cause of acute kidney injury (AKI) [2-4]. A series of pathophysiology are included in IRI-induced AKI, such as continued hypoperfusion, inflammation, oxidative stress, tubular epithelial apoptosis and microvascular thrombosis [3]. The current therapies for IRI-induced AKI are limited.

Cell-based treatment, especially the use of mesenchymal stem cell (MSC), is becoming a promising approach to protect systemically infused animals from IRI-induced AKI [5]. MSCs represent a heterogeneous population of multipotent stem cells which can secrete a spectrum of growth factors and mediators repressing local immunologic reactions, fibrosis and apoptosis, and differentiate into multiple cell types [4, 6, 7]. Increasing evidence from both *in vitro* and *in vivo* studies of AKI showed that MSC was able to improve greatly the tissue repair and renal function of injured kidney [4, 8-10]. It was formerly believed that the differentiation potential of MSC could be used to repair/replace the injured tissues [7]. More studies were inclined to consider the paracrine/endocrine action of

mediators/biologically active molecules as the main cause for beneficial effects of MSC [11-13]. However, the underlying mechanisms on how MSC mediate the repair of adjacent injured kidney cells are poorly known.

The Notch signaling pathway is evolutionarily conserved regulating a wide spectrum of cellular activities including cell proliferation, apoptosis, cell fate decisions, etc. Four Notch receptors and five ligands identified are implicated in the activation of Notch pathway. Canonical Notch pathway refers to a process that the γ-secretase cleaves receptors (Notch 1-4) before binding to the ligands (Delta-1, 3 and 4, Jagged-1 and 2) on adjacent cells which results in the release of Notch intracellular domain (NICD) that translocates to the nucleus and activates the Notch pathway [14-16]. Huang *et al*. reported previously that Notch2/Hes1 pathway contributes to inflammation and apoptosis induced by renal IRI [15]. Therefore, we presumed that the Notch pathway was associated with the protection of bone marrow-derived MSC (BMSC) against renal IRI through NICD.

In this study, NICD expression vector was constructed and transfected into BMSC. The NICD-transfected MSC were transplanted into an established renal IRI model. The protection of NICD-transfected MSC on renal IRI was then evaluated by renal function and histopathology.

Materials and methods

Animals and ethical statement

Two types of SD rats were purchased from Laboratory Animal Center of Medical College of Nanchang University (Jiangxi, China) including 1 week old weighing 20-30 g and 6-8 weeks old weighing 200-250 g. The rats were provided freely with a standard diet and water at a constant temperature (23±2°C) and humidity (50%) with a 12-12 h light-dark cycle. All animals were received humane care in compliance with the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Medical College of Nanchang University.

Isolation and culture of BMSC

The process was performed according to a previous report with minor modifications [17]. Briefly, the 1 week old SD rats were sacrificed by breaking neck. BM was collected from bilateral tibiaes and cultured in 5 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). After centrifugation and resuspension, the cells were incubated in a culture flask at 37°C with 5% CO_2 and 95% humidity in a temperature-constant incubator. The medium broth was changed every 3 days and the floating cells were discarded. The cells adhering to the flask bottom (BMSC) were cultured for another 12-14 days. When they grew up to 80% confluence, the cells were passaged for three times. The purified cells were harvested and used for further experiments.

Analysis of BMSC

The appearance of BMSC was confirmed by a light microscopy (Olympus CX23, Japan). The identification procedures by flow cytometry were described previously with minor modifications [18]. Briefly, the cell surface markers specific for BMSC were analyzed by flow cytometry. The BMSCs were fixed in 4% cold paraformaldehyde for 30 min and washed with PBS containing 2% FBS. BMSC concentration was adjusted to 1×10^6 cells/100 μL. Cells were incubated with phycoerythrin- or fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies against rat CD73, CD105, CD90, CD44, CD34, CD11b and HLA-DR (1:1000, Santa Cruz, CA, USA) and FITC-labeled goat anti-mouse secondary antibodies (1:100, Santa Cruz, CA, USA) for 60 min in the dark at 4°C at dilutions recommended by the manufacturers. Subsequently, the cells were washed with PBS and fixed in 2% paraformaldehyde. Immunoglobulin isotype incubation was performed as a negative control. Flow cytometry was performed with a FACSCalibur system (FC500; Beckman Coulter, Inc., Brea, CA, USA) and analyzed using FlowJo software version 7.6.5 (FlowJo, LLC, Ashland, OR, USA).

Construction, evaluation and transfection of MigR1-NICD

The crude RNA was extracted by phenol-chloroform method. Corresponding cDNA was reverse transcribed. Primers were devised by Primer premier 5.0 and synthesized by HuaGen (Shanghai, China) with sequences as follows: NICD forward 5'-CGCGGATCCATGCGCAAGCGCAGGCGGCAGC-3', reverse 5'-CCGTCTAGATT-

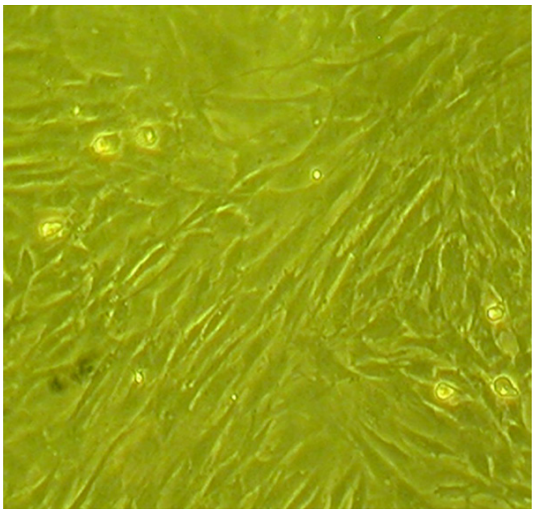

Figure 1. Representative image of BMSC observed by light microscopy.

ACTTAAATGCCTCTGGAATGGTGG-3'. The obtained NICD gene was introduced into MigR1 by Genechem (Shanghai, China). The introduced NICR gene was checked by quantitative RT-PCR using the 2-ΔΔCT method and Western blot. Total RNA was extracted using MagExtractor-RNA kit (ToyoBo, Tokyo, Japan). cDNA was obtained using ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (ToyoBo, Tokyo, Japan). The primer sequence of GAPDH was presented as forward: 5'-GTCTGCCACGATAACACC-3', reverse: 5'-CAATACAACAAGCCCACTC-3'. The experimental conditions of qRT-PCR were as follows: 95°C for 60 s, subsequently 95°C for 15 s, 55°C for 15 s, 72°C for 45 s for total of 40 cycles. GAPDH was set as reference gene.

The NICD protein was firstly separated by SDS-PAGE and then transferred to PVDF membrane (EMD Millipore, Billerica, MA, USA) incubating with monoclonal rabbit anti-NICD (1:500 dilution, Abcam) and secondary antibodies conjugated with horseradish peroxidase (1:2000, ZSGB-BIO, Beijing, China). The blots were stained with ECL assay (Amersham Pharmacia, NJ) and imaged by ChemiDocTM XRS Gel image system (Bio-Rad, Shanghai, China). The band intensities were measured using Quantity one v4.62 software.

The third generation BMSC growing close up to 70% fusion was incubated with MigR1-NICD and the same volume of Lipofectamine 2000 for 48 h according to the manufacturer's instructions. PCR and western blot were employed to evaluate the NICD level in BMSC.

Cell viability assay

The second generations of MigR1-BMSC and MigR1-NICD-BMSC were adjusted to 5×10^4 cells/mL. The inoculums were seeded into 96-well plates with 100 μL/well. Following incubation at 37°C with 5% CO_2 and 95% humidity, the medium was discarded. Cell Counting Kit 8 (10 μL, CCK8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) solution was then added to each well and incubated at 37°C for 2 h. Optical density (OD) values were measured spectrophotometrically with a MRX II absorbance reader (Dynex Technologies, Inc., Chantilly, VA, USA) at the wavelength of 450 nm. The viability assay was performed each day for consecutive 4 days.

Renal IRI model and transplantation

The 6-8 week old SD rats were used to establish renal IRI model. The rats were randomly divided into four groups: normal saline (NS)-treated sham group (sham, n=12), NS-treated renal IRI group (IRI+NS, n=12), MigR1-BMSC-treated group (IRI+MigR1-BMSC, n=12) and MigR1-NICD-BMSC treated group (IRI+MigR1-NICD-BMSC, n=12). Prior to operation, the rats were fasted for 8-12 h. The ischemia was caused by 45 min of atraumatic vascular clamp. Other procedures for establishing renal IRI models were described in a previous study [15]. After 45 min of ischemia, the adjusted cell suspensions of MigR1-BMSC and MigR1-NICD-BMSC (1 mL, 1.5×10^7 cell/mL) were injected through postcava.

Blood biochemical detection and renal histopathology evaluation

Following 24 h, 48 h, 72 h, 96 h of ischemia, the blood was collected via angular vein to measure the levels of Scr and BUN using a Biochemical Autoanalyzer (Olympus, Tokyo, Japan). After 48 h of ischemia, the rats were killed and the kidney samples were excised for histological examinations. The injured renal tubules were assessed by semiquantitative score from 0 to 5. The procedures of histological examination and the definition of semiquantitative score were described as previously [15].

Statistical analysis

All experiments were performed triplicate in three independent occasions. The data were

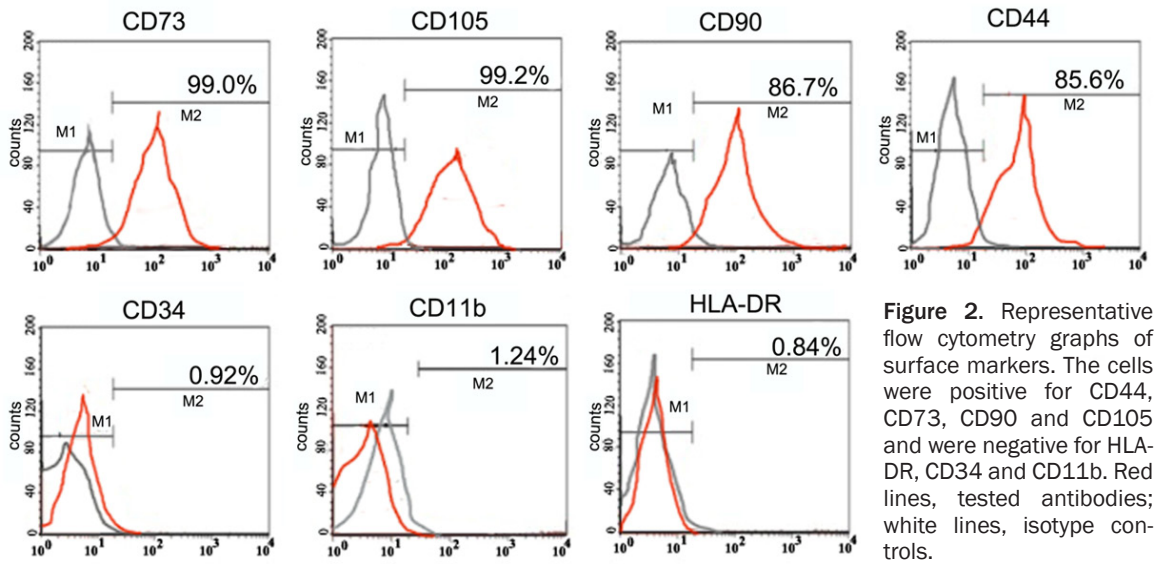

Figure 2. Representative flow cytometry graphs of surface markers. The cells were positive for CD44, CD73, CD90 and CD105 and were negative for HLA-DR, CD34 and CD11b. Red lines, tested antibodies; white lines, isotype controls.

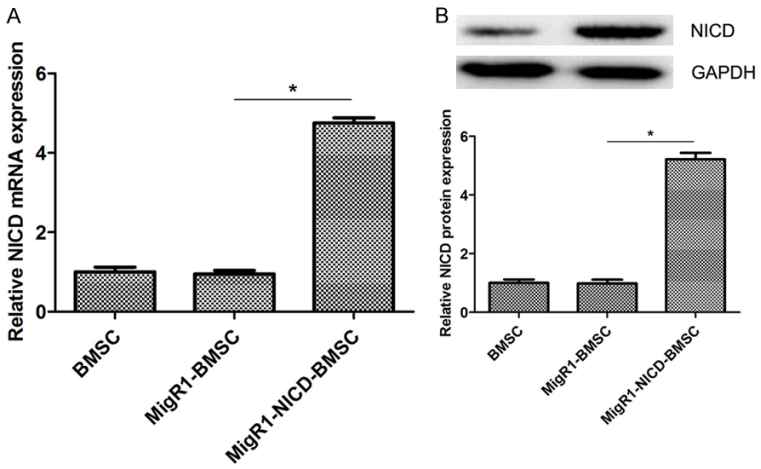

Figure 3. NICD levels after introduction into BMSC. Expressions of (A) NICD mRNA analyzed by qRT-PCR and (B) NICD protein measured by Western blot in BMSC, MigR1-BMSC, MigR1-NICD-BMSC. *, P<0.05, compared with MigR1-BMSC.

presented as mean ± standard deviation. The data between two groups were analyzed by the two-tailed Student's t-test, while the data among groups were analyzed by one-way analysis of variance (ANOVA). Statistical analyses were conducted by SPSS 19.0 (SPSS, Chicago, IL, USA). A value of $P<0.05$ was considered statistically significant.

Results

Characterization of BMSC

The typical spindle-shaped morphology of BMSC was confirmed by light microscopy (**Figure 1**). BMSCs were further identified via detecting their surface markers, showing positive expressions of CD73, CD105, CD44, and CD90 and negative expressions of CD34, CD11b and HLA-DR (**Figure 2**).

Overexpressed NICD promoted the proliferation of BMSC

The mRNA and protein expressions of NICD in MigR1-NICD-BMSC increased respectively by 5.01- and 5.33-fold (P< 0.05 and P<0.05, **Figure 3**) compared with MigR1-BMSC, inferring successful introduction of NICD in BMSC. The proliferation of BMSC was also assessed by CCK8 staining. We found that the growth of MigR1-NICD-BMSC was negligible till the cultivation continued for 48 h. Following 72 and 96 h of incubations, the proliferation of MigR1-NICD-BMSC was promoted significantly compared with MigR1-BMSC (P<0.05 and P<0.05, **Figure 4**).

BMSC containing overexpressed NICD improved renal function and histology in IRI model

As shown, both levels of Scr and BUN decreased notably after treatments of MigR1-NICD-BMSC compared with treatments of MigR1-BMSC

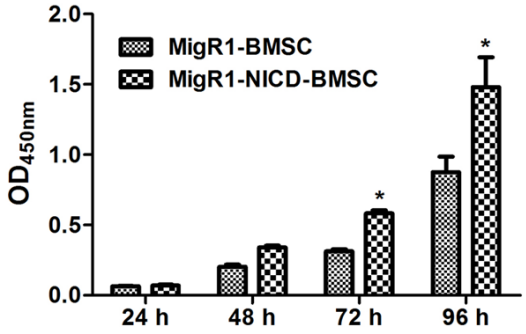

Figure 4. Proliferations of BMSC assayed by CCK8 (n=5). *, P<0.05, compared with MigR1-BMSC.

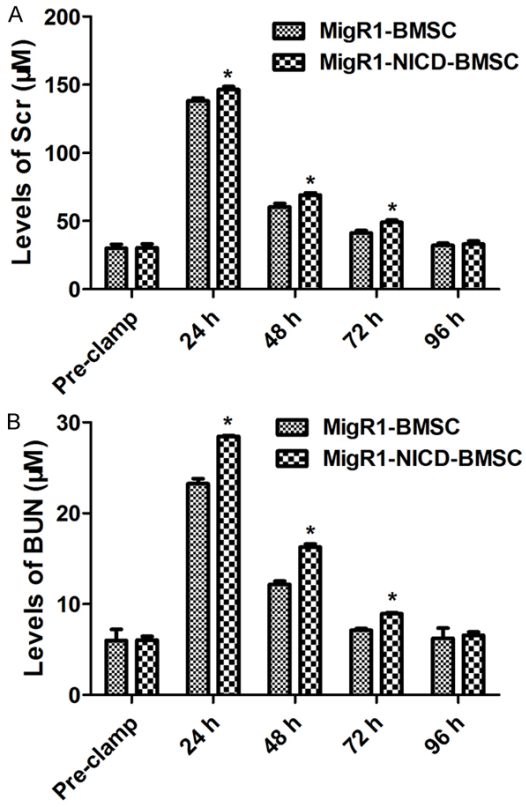

Figure 5. Levels of (A) serum creatinine (Scr) and (B) blood urea nitrogen (BUN) in renal IRI model (n=5). *, P<0.05, compared with MigR1-BMSC.

post-injection of 72 h (P<0.05). To be specific, the *p* values were 0.032, 0.041, 0.037 for Scr (**Figure 5A**) and 0.012, 0.027, 0.039 for BUN (**Figure 5B**). Following 96 h of exposure, both levels of Scr and BUN with treatments of MigR1-NICD-BMSC were insignificant compared with treatments of MigR1-BMSC and became almost equivalent to those prior to ischemia, suggesting that the renal function

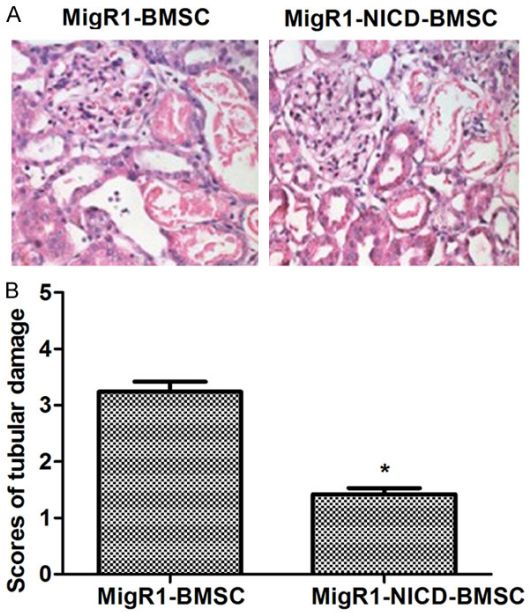

Figure 6. Effects of NICD on BMSC treatment in renal IRI. (A) Renal histological changes at 48 h in the renal IRI model (Magnification, ×200), and (B) semi-quantitative score of tubular damage in MigR1-BMSC, MigR1-NICD-BMSC. *, P<0.05, compared with MigR1-BMSC.

went back to normal gradually in IRI model (**Figure 5A** and **5B**). A series of typical histological sign of tubulointerstitial injury were apparent in renal IRI including focal areas of proximal tubular dilation and distal tubular casts, effacement and loss of proximal tubule brush border as well as interstitial inflammatory cell infiltration [19]. The injury signs were relieved by injecting MigR1-NICD-BMSC (**Figure 6A**). The semi-quantitative scores showed that IRI-rats treated with MigR1-NICD-BMSC exhibited strikingly pathological improvements compared with those exposure to MigR1-BMSC (1.42±0.11 vs. 3.24±0.18, P<0.05, **Figure 6B**).

Discussion

To verify the hypothesis proposed that NICD participated BMSC protection on kidney from IRI, NICD was cloned in MigR1 vector to screen MigR1-NICD which was confirmed by qRT-PCR and western blot. By flow cytometry, BMSC were then isolated and defined with positive expressions of CD105, CD73, CD90, CD44 and negative expressions of CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR [18]. Followed by transfected with MigR1-NICD vector, MigR1-NICD-BMSC was injected into animals with

renal IRI. It could be observed that introduced NICD promoted the proliferation of BMSC in four days which reduced the levels of Scr and BUN, improved the histopathology of renal IRI rat. The results indicated that BMSC revived animals from renal IRI through the activation of Notch1 pathway.

Cell delivery route and dosage are two crucial factors for the success of MSC therapy, impacting the distribution of donated MSC and the outcome of treatment [20]. The main obstacle for MSC-based cell treatment is the low efficiency of homing towards clamped kidneys. Currently, systemic injection of MSC, including intravenous and intra-arterial injections, is the most widely used approach for cell delivery in renal IRI treatment. Recently, Cai et al. provided an improved way of MSC injection through renal artery which was deemed to hold advantages of both intra-arterial and intraparenchymal injection [21]. Indeed, all of the current cell delivery routes have limitations so that they are not optimal for every disease condition or every donor cell type. Although the distribution of MSC was inhomogeneous in kidney and the lifetime of the remaining MSC was short, the manipulation of intravenous injection is simpler and accompanied injury is much less than other commonly used delivery routes. In this study, the dosage of BMSC was used up to 1.5×10^7/ml more than 1×10^6/ml (the amount commonly used for systemic delivery) through intravenous injection without observation of blood flow blocking and obvious tissue damages that could be encountered in renal artery injection [22]. The high dosage might offset at least partly the inhomogeneous distribution of BMSC.

As stated, MSC administration could reduce the danger of renal IRI and improve renal function in intro and in vivo. A great diversity of mammalian cells differentiates from common precursor cells during their developments. Based on different inductive cues in the environment and cell type-specific kinetics, some cell types differentiate only at early stages while others at later stages. This differentiation processes are associated with cell-to-cell interaction, namely “lateral inhibition”, controlled by Notch pathway, in which cells differentiated early inhibited adjacent cells by sending a signal from differentiating into the same cell types. That is to say, expressed Notch ligands on differentiating cells combine with activated Notch on neighboring cells to inhibit their differentiations. The Notch pathway can avoid generations of homogenously early-born cell types and maintain different cell types [23]. Several lines of evidence demonstrated that activated Notch pathway blocked the differentiation of BMSC [17, 24]. Although differentiation of human BMSC into cardiomyocytes was associated with overexpression of Notch1 signaling pathway [18], we assumed that the discrepancy was attributable to the effect of conditioned medium (CM) [12]. In this study, we observed that active Notch1 pathway could promote the proliferation of BMSC and improve renal IRI via overexpression of NICD, inferring that the protection of BMSC on renal IRI was unassociated with the differentiation of BMSC.

Hypoxia is predominant in clamped region of kidney. Several reports revealed that hypoxia could inhibit the differentiation of MSC via activation of Notch pathway [25, 26]. It was accepted that the mechanisms of BMSC based therapy were relevant with both differentiation and paracrine/endocrine. However, poor BMSC engraftment and survival at injury sites as well as poor differentiation possibility in a short time were suggested to be the reasons responsible for limited contribution of BMSC differentiation [27-29]. Therefore, it is reasonable to propose paracrine/endocrine as the principle mechanism accounting for the protection of BMSC on renal IRI in our experiments.

In conclusion, activated Notch pathway by overexpression of NICD could increase the growth of BMSC, decrease the levels of Scr and BUN and relieve renal IRI, suggesting BMSC was protective against renal IRI via activating NICD-mediated Notch pathway.

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Disclosure of conflict of interest

None.

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