Induced Pluripotent Stem Cells (iPSC)-derived Mesenchymal Stem Cells (MSCs) Showed Comparable Effects in Repair of Acute Kidney Injury as Compared to Adult MSCs

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Purpose: The study aimed to compare the therapeutic effects of iPSC-derived MSCs (iPSC-MSCs) and adult MSCs for acute kidney injury (AKI) therapy.

Materials and Methods: Model rats with ischemia/reperfusion (I/R)-induced AKI were randomly divided into three groups (n=15 for each group) to receive transplantation of iPSC-MSCs, adult MSCs, or the saline control. After transplantation, engraftment and differentiation of both iPSC-MSCs and adult MSCs were detected in the transplanted sites. Serum creatinine and blood urea nitrogen (BUN) for renal function evaluation were measured, and histological assays were performed as well.

Results: Compared with the saline control, both iPSC-MSCs and adult MSCs significantly ($p < 0.05$ or 0.01) improved the renal function. Furthermore, iPSC-MSCs showed comparable effects in ameliorating tissue damage, reducing cell apoptosis and promoting vascularization with adult MSCs.

Conclusion: This study compared the therapeutic effects of iPSC-MSCs and adult MSCs for AKI treatment. Both iPSC-MSCs and adult MSCs were observed with comparable effects in repair of AKI. The results indicated that iPSC-MSCs may serve as an alternative source of MSCs for stem cell-based therapy for AKI therapy.

Keywords: acute kidney injury; induced pluripotent stem cells; mesenchymal stem cells; paracrine effects; stem cell-based therapy

INTRODUCTION

Acute kidney injury (AKI) affects up to 7% of hospitalized patients [(1)], and leads to mortality rates from 15 to 60% [(2)]. Stem cell-based therapy has been regarded as one promising strategy for AKI treatment, with mesenchymal stem cells (MSCs) as one of the most studied cell types. It was reported that transplantation of MSCs prevented and ameliorated renal damage during AKI induced by cisplatin[(3-6)] or ischemia-reperfusion (IR) [(7-10)].

MSCs have self-renewal and multipotency potential, and can differentiate into multiple cell types, such as osteoblasts, chondrocytes and adipocytes. Normally, transplanted MSCs might differentiate to the functional cells to replace injured cells. Emerging studies suggested that paracrine secretion was an alternative mechanism for MSC-based tissue repair [(8,11)]. The beneficial functions of cytokines secreted by MSCs included promoting angiogenesis, inhibiting apoptosis, suppressing inflammation and scavenging reactive oxygen species (ROS)[(12,13)]. However, even though MSCs can be conveniently isolated from various tissue sources, including bone marrow, adipose tissue and umbilical cord (14), the differentiation potential of MSCs was reduced during in vitro expansion, and the generation of quantity and quality required MSCs from adult faces a great deal of challenges. As a result, the therapeutic efficacy of adult MSCs were highly limited[(15-17)]. Furthermore, aging and aging-related disorders could also adversely impact the differentiation potential and therapeutic efficacy of MSCs[(18-20)].

Induced pluripotent stem cells (iPSCs) generated from adult somatic cells provide an alternative source of patient-specific MSCs [(21-23)]. It was reported that MSCs derived from human iPSCs shared similar performance with adult MSCs and were proved to be differentiated into osteoblasts, chondrocytes and adipocytes[(24)]. For example, transplantation of iPSCs-derived MSCs (iPSC-MSCs) significantly ameliorated limb ischemia and promoted vascular and muscle regeneration in mice [(24)]. However, the therapeutic effects of iPSC-MSCs for AKI remain to be clarified, especially for comparing with adult MSCs.

To address this issue, in this study human iPSC- MSCs and adult MSCs were generated and investigated for their biological function for AKI treatment. The differentiation potential and paracrine secretion were compared between iPSC-MSCs and adult MSCs in vitro. Two sources of MSCs were then injected into the kidney of rats with I/R-induced AKI. Renal function, tissue damage, cell apoptosis, and vascularization of ischemic kidneys were evaluated thereafter.

MATERIALS AND METHODS

Cultivation and Characterization of iPSC-MSCs and adult MSCs

Human iPSC (hiPSC) cell line was purchased from Shanghai Cell Bank. (Shanghai, China). The hiPSC

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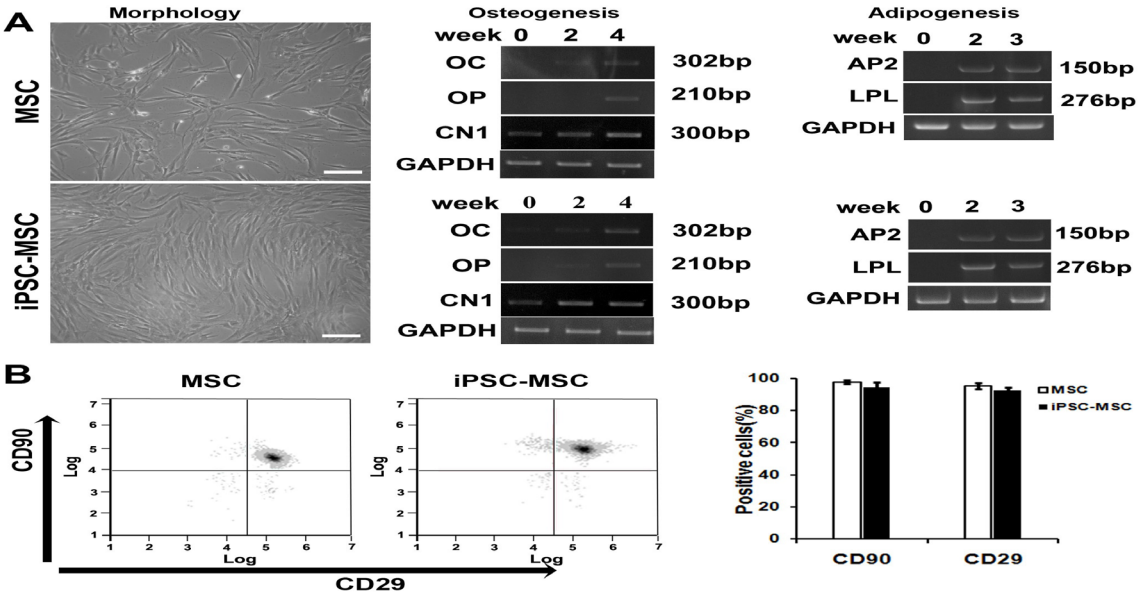

Figure 1. Characterization of iPSC-MSCs and adult MSCs in vitro. **(A)** Morphology and multi-potency of MSCs. Both iPSC-MSCs and adult human MSCs showed fibrous morphology (Bar = 200 μm). They could differentiate into adipogenic and osteogenic lineages when cultured in differentiation media. **(B)** The immunophenotype of iPSC-MSCs and adult human MSCs was analyzed by flow cytometry and most cells expressed CD29 and CD90.

cells were cultivated in suspension culture to form embryoid bodies, and then seeded into 0.1% gelatin-coated dishes in α-MEM (Gibco) containing 8 ng/mL of fibroblast growth factor 2 (FGF2, Invitrogen). Cells were passaged using 0.25% trypsin when they became confluent. Cell passage was continuously performed until uniform fibrous cell layer was formed. Adult human MSCs were isolated from raw human lipo-aspirates (healthy female, 22 years old). Briefly, adipose tissues were washed with sterile phosphate buffer solution (PBS), cut into pieces with scissors, and then digested with 0.1% collagenase I (Sigma) in PBS. The solution was filtered with 80 μm meshes and centrifuged at 800×g for 8 min. The pellet was re-suspended in fresh medium (α-MEM/10% FBS) and seeded in tissue culture plates. The plates were placed into an incubator

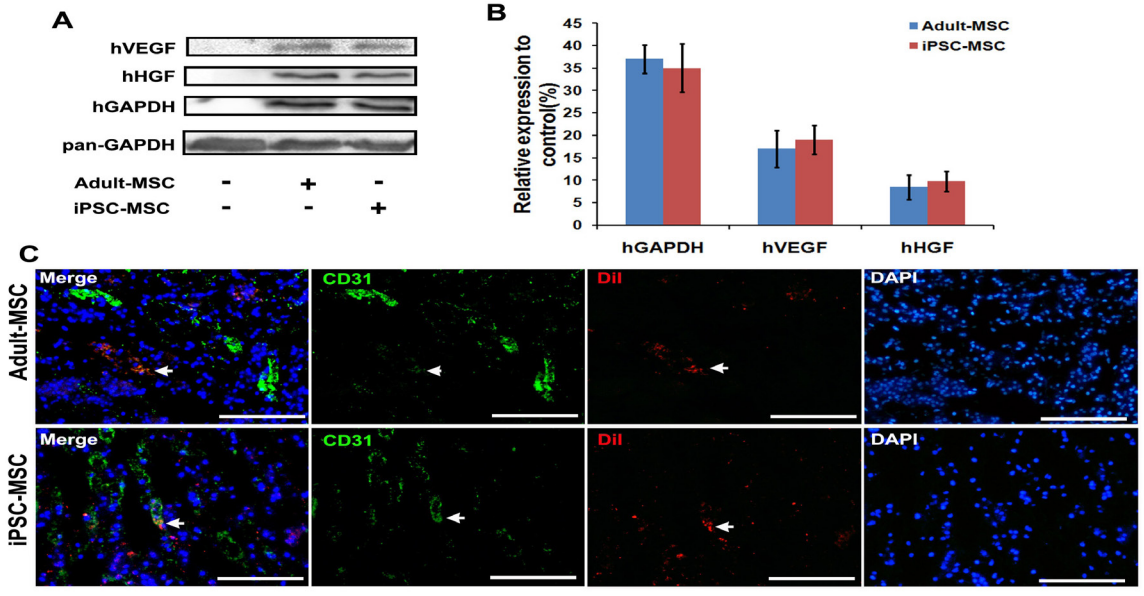

Figure 2. Engraftment and differentiation of iPSC-MSCs and adult MSCs in vivo. **(A)** Western blotting analysis of human GAPDH, VEGF and HGF proteins in host renal tissues one week after MSC transplantation, **(B)** and its quantification analysis of Western blotting. Band intensities of human GAPDH, VEGF and HGF were quantified and normalized to the internal control, pan-GAPDH (n=5). **(C)** Differentiation of iPSC-MSCs and adult MSCs within host renal tissues (Bar = 100 μm). Two weeks after MSC transplantation, paraffin-embedded sections of kidney were prepared, and immunostaining against the vascular marker CD31 was performed. Co-localization of DiI and CD31 was observed under fluorescence microscopy.

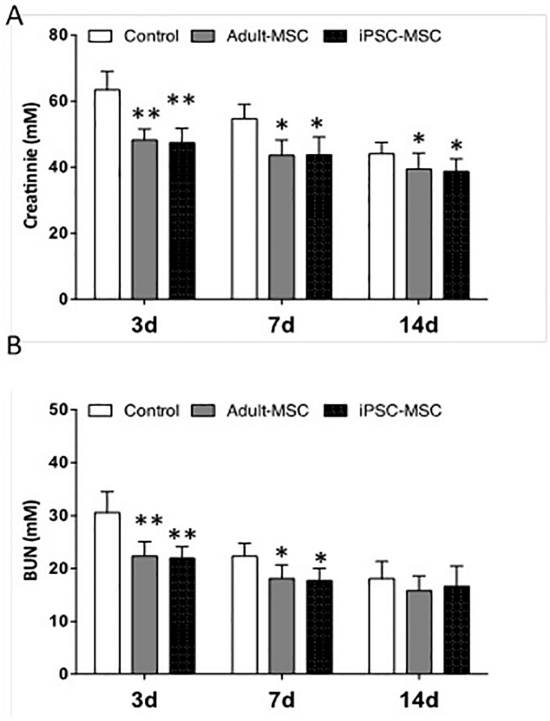

Figure 3. Renal function after MSC transplantation.
The rats with I/R-induced AKI were injected with saline (control), iPSC-MSCs or adult MSCs. Blood was taken 3 days, 7 days and 14 days after the surgery. **(A)** Serum creatinine and **(B)** BUN were measured. The results were expressed as mean ± SD (n = 5). *P < 0.05 compared with control; **P < 0.01 compared with control.

at 37°C with 5% CO_2. Cells were passaged when they reached 90% confluence, using 0.25% trypsin at a 1:3 split ratio.
For osteogenic differentiation assays, iPSC-MSCs and adult MSCs were seeded onto tissue culture plates and cultured in α-MEM media containing 10% FBS, 1% antibiotics, 50 μM ascorbic acid, 10 mM β-glycerol phosphate and 0.01 μM dexamethasone. For adipogenic differentiation assays, iPSC-MSCs and adult MSCs were seeded into tissue culture plates and cultured in α-MEM media containing 10% FBS, 1% antibiotics, 0.5 mM isobutylmethyl xanthine, 1 μM dexamethasone, 200 μM indometacin and 10 μM insulin. The immunophenotype of iPSC-MSCs and adult MSCs was analyzed by flow cytometry. CD29 and CD90 were analyzed using anti-CD29-FITC and anti-CD90-PE antibodies.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted by using an RNAprep Pure Cell/Bacteria Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. Reverse transcription was performed using the QuantScript RT Kit (TIANGEN) for cDNA synthesis. Quantitative real-time PCR was conducted on a Bio-Rad CFX manager system (Bio-Rad, USA) using TB Green® Fast qPCR Mix (Takara, China). The cyclin g amplification was performed with a hot start at 95.0 °C for 30 s, followed by 40 cycles at 95.0°C for 5 s and 60.0 °C for 30 s. Three independent experiments were performed for each sample. The PCR primers were selected according to the previous report[25].

AKI induction, cell injection and renal function evaluation

Adult male SPF Sprague-Dawley rats (9 weeks of age) were purchased from the Experimental Animal Center, Academy of Military Medical Science (Beijing, China). All the experiments in the study were approved by Animal Care and Use Committee of Chinese PLA General Hospital.
Rats were anesthetized with sodium pentobarbital (30 mg/kg), and the acute renal ischemia/reperfusion (I/R) injury was performed. Briefly, after laparotomy and exposure of the kidney, atraumatic vascular clamps were used to clamp the bilateral renal pedicles. The kidney was subjected to 40 min of ischemia, followed by reperfusion. The rats with I/R-induced AKI were randomly divided into three groups (n = 15 for each group). At the time of reperfusion, 100 μL of saline or 2×10^6 of iPSC-MSCs or 2×10^6 of adult MSCs were injected into the kidney cortex using a 1 ml syringes. For in vivo tracking, cells were labeled with DiI dyes according to the manufacturer's guideline (Invitrogen). After surgery, the abdomen was closed and rats were allowed to recover with cautious care. Blood was taken 3 days, 1 week and 2 weeks after the surgery (n = 5 for each group at each time points). Serum creatinine and blood urea nitrogen (BUN) were measured with standard protocols in our hospital.

Western blotting

One week after surgery, renal tissues were sampled (n = 5 per group) and lysed in Laemmli Sample Buffer (Bio-Rad). Total proteins were extracted and quantified using the BCATM Protein Assay Kit (Thermo Scientific). Human GAPDH, HGF and VEGF were detected using human-specific antibodies (R&D Systems) as our previous report[26]. pan-GAPDH was used as internal control (Abcam). Band intensities were quantified and normalized to the internal control.

Histological, TUNEL and immunohistochemical analysis

Three days after surgery, the kidneys were obtained and fixed in 4% paraformaldehyde (n = 5 for each group). 4 μm paraffin-embedded sections were prepared for histological analysis and TUNEL assay. To determine tissue damage, hematoxylin and eosin (H&E) staining was performed on paraffin-embedded sections. Tubular injury was evaluated by an experienced technician as previously reported. The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed using the TUNEL Staining Kit (Invitrogen) according to manufacturer's instructions. Positive-stained cells were counted under microscopy. For each animal, 5 discontinuous sections were observed and 5 random fields were selected on each section.

Statistical analysis

All data are expressed as mean ± SD. The SPSS 17.0 software was used for statistical analysis. One-way analysis of variance (ANOVA) followed by a post hoc Student-Newman-Keuls test was used for the comparison of multiple groups. Unpaired student t-test was used for compare the mean of two groups. A value of $p < 0.05$ was considered statistically significant and $p < 0.01$ was considered statistically highly significant.

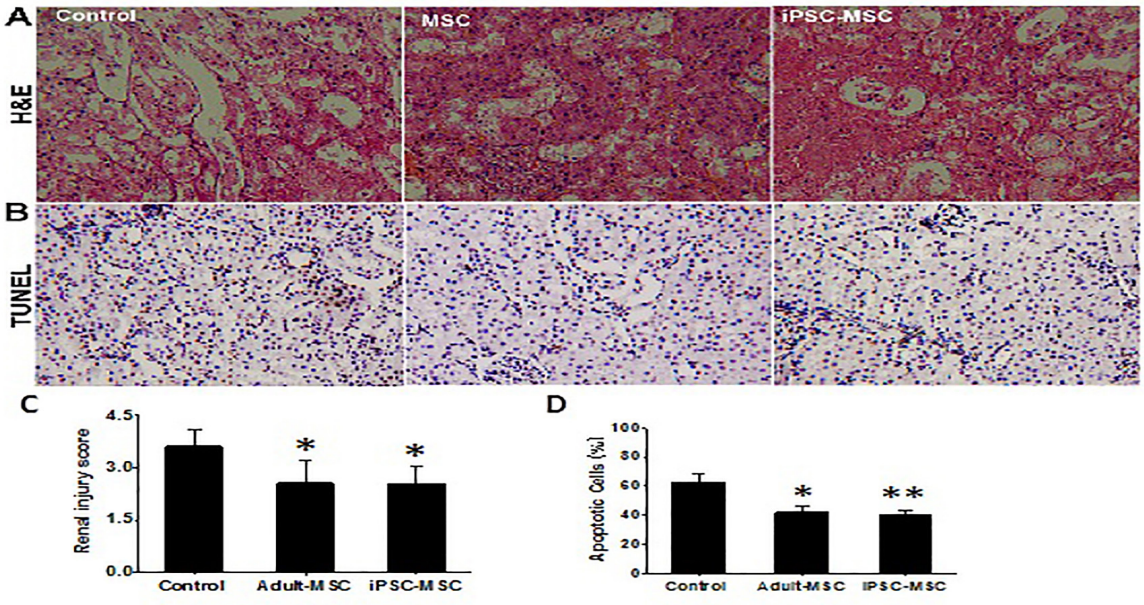

Figure 4. Histological and TUNEL assay of injured renal tissues. **(A)** Representative images of H&E staining and **(C)** statistics of renal damage score accordingly (n = 5); **(B)** Representative images of TUNEL assays, **(D)** and statistics of cell apoptosis analysis accordingly (n = 5). *$P < 0.05$, and **$P < 0.01$, as compared with control.

RESULTS

In vitro comparison of the properties of iPSC-MSCs and adult MSCs

As shown in Figure 1A, both human iPSC-MSCs and adult MSCs showed fibrous morphology. When cultured in appropriate differentiation media, the two types of MSCs exhibited comparable differentiation capability towards osteogenic and adipogenic lineages (**Figure 1A**), which indicated their similar multi-potency. Flow cytometry analysis further demonstrated that iPSC-MSCs also possessed similar immune-phenotype with adult MSCs (**Figure 1B**).

In vivo comparison of the engraftment and differentiation of iPSC-MSCs and adult MSCs

The new arrived SPF rats were observed with similar normal weight of 220-250 g and good health conditions (including of moving well, breathing well, sitting normally, with bright eyes shiny coat etc.) prior to I/R treatment. After I/R-induced AKI treatment, the model rats were randomly divided into three groups to receive injection of DiI-labeled iPSC-MSCs, DiI-labeled adult MSCs, or the saline control. One week after transplantation, the engraftment and cytokine secretion of injected MSCs were evaluated by Western blotting. Human GAPDH, VEGF and HGF proteins were detected in the rat kidney for both iPSC-MSCs and adult MSCs transplantation groups (**Figure 2A**). Quantification of human GAPDH, VEGF and HGF protein bands demonstrated no significant differences between these two groups (**Figure 2B**), which suggested that iPSC-MSCs and adult MSCs engrafted in the host tissues and produced paracrine effects comparably.

Two weeks after transplantation, paraffin-embedded sections of kidney were prepared, and immunostaining against the vascular marker CD31 was performed. Under fluorescence microscopy, co-localization of DiI and CD31 was observed in both iPSC-MSCs and adult MSCs transplantation groups. The results indicated that both iPSC-MSCs and adult MSCs differentiated towards the vascular lineage within the host renal tissues (**Figure 2C**).

Renal function

To compare the effects of iPSC-MSCs and adult MSCs in ameliorating renal function, the levels of serum creatinine and blood urea nitrogen (BUN) in each group were measured to evaluate the renal function at 3 days, 7 days and 14 days after MSC transplantation. For the saline control, both creatinine and BUN levels tended to decrease from day 3 to day 14, suggesting the renal function recovered to a certain extent by itself after the AKI induction **(Figure 3A and 3B)**. Compared with the saline control, the creatinine levels were significantly decreased ($p < 0.05$ or $p < 0.01$) in both iPSC-MSCs and adult MSCs injected groups at each time point (**Figure 3**A). Meanwhile, transplantation of iPSC-MSCs and adult MSCs also reduced the BUN levels significantly ($p < 0.05$ or $p < 0.01$) at day 3 and day 7 (**Figure 3B**). It was noteworthy that iPSC-MSCs showed comparable effects in ameliorating renal function with adult MSCs (**Figure 3A and 3B**).

Histological and immune-histochemical analysis

The therapeutic effects of iPSC-MSCs and adult MSCs for AKI were further investigated in several perspectives, including tissue damage, tissue apoptosis, and vascularization. Three days after MSC transplantation, H&E staining and TUNEL assay were performed to evaluate tissue damage and cell apoptosis, respectively. Compared with the saline control, the I/R-induced renal injury was significantly attenuated ($p < 0.05$) in iPSC-MSCs and adult MSCs injection groups, as indicated by less tubular cell necrosis, less loss of brush border, lower grade of tubular dilatation, and lower

quantitative histological scores (**Figure 4A and 4C**). Transplantation of iPSC-MSCs and adult MSCs also reduced number of apoptotic cells significantly (**Figure 4B and 4D**). The results indicated that iPSC-MSCs and adult MSCs exhibited similar effects in protecting the I/R kidney against apoptosis, reducing tissue damage and promoting vascularization.

DISCUSSION

AKI is associated with high mortality rates, with no effective treatment methods so far[2]. The potential application of MSC-based therapy during AKI treatment has been widely investigated[3-10]. Normally, MSCs can be isolated from different adult human tissues[14]. However, the therapeutic efficacy of MSCs is limited, because the differentiation potential of MSCs is reduced during in vitro expansion,[15-17] and is also adversely impacted by aging and aging-related disorders[18-20]. Therefore, it is of significance to improve the therapeutic efficacy of MSCs or to find alternative sources of MSCs.

In this study, iPSCs derived MSCs was used to treat I/R-induced AKI in rats and compared with adult human MSCs as well. The differentiation potential and paracrine secretions of iPSCs-MSCs and adult human MSCs were similar in vitro, which was consistently with previously study[27]. What's more important, after being transplanted into the I/R rat kidney, iPSCs-MSCs were observed with comparable therapeutic effects in improving renal function, ameliorating tissue damage, reducing cell apoptosis, and promoting vascularization as compared with adult MSCs. Our results suggested that iPSCs-MSCs can be an alternative source of patient-specific MSCs other than adult human tissues.

Large number of studies proved that MSCs exert several beneficial effects, such as via differentiating into targeted cells to repair the injure tissue and paracrine actions[8,11]. As compared with adult, it was reported that iPSCs-MSCs could survive for a longer time after transplantation into ischemia limb, and their therapeutic effects might be direct de novo vascular and muscle differentiation or paracrine mechanisms[24]. Our results indicated that the expression levels of cytokines in iPSC-MSCs were comparable to adult MSCs, which was consistent with their similar therapeutic efficacy. Besides, the results also indicated that the transplanted MSCs might differentiate into the vascular lineage in the injured kidney tissue. The mechanism through which iPSCs-MSCs ameliorate tissue ischemia and damage remains to be further investigated.

It is important to address the safety issues before iPSCs and their derivatives can be applied clinically. iPSCs can be generated from human somatic cells by introducing the induced genes using viral integration, nonintegrating episomal vectors and direct reprogramming protein delivery[21,22]. As a result, the application of iPSCs may cause mutation of the host genome and increase the risk of tumorigenicity. Although further differentiation of iPSC into functional cell types, like iPSCs derived MSCs in the present study, could decrease the tumorigenic with highly proliferative[28,29], the long term of iPSCs derived MSC for I/R-induced AKI needs to be further evaluation.

In conclusion, our studies showed that iPSCs-MSCs exhibited similar differentiation potential, immunophenotype and paracrine effects as compared with adult MSCs. Importantly, the two sources of MSCs also showed comparable therapeutic effects for AKI. Therefore, considering the unlimited capacity for proliferation of iPSCs, iPSC-derived MSCs are promising to overcome the shortcomings of adult MSCs and may serve as an alternative source of MSCs for stem cell-based therapy.

CONFLICT OF INTEREST

The authors declare that we have no conflict of interest.

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