Human urine-derived stem cells contribute to the repair of ischemic acute kidney injury in rats

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Abstract. Acute kidney injury (AKI) is a clinical syndrome associated with high rates of morbidity and mortality. It has previously been reported that stem cells may be considered a potential therapeutic strategy for the treatment of AKI. The present study aimed to determine whether administration of urine-derived stem cells (USCs) to rats with ischemia/reperfusion (I/R)-induced AKI could improve renal function. USCs were isolated and cultured from 8 healthy men. Subsequently, USCs transduced with green fluorescent protein were mixed with hydrogel and were injected into rats with renal I/R injury. Renal tubular injury, proliferation and apoptosis were detected in the I/R model. Hematoxylin and eosin staining was used to detect the morphological of kidney injury. Immunohistochemistry and TUNEL kits used to evaluate the proliferation and apoptosis of the I/R model. The results demonstrated that USCs could be detected in the tubular epithelial lining of the rats and administration of USCs was able to improve renal function in the I/R model. The USCs-treated group exhibited significantly reduced serum creatinine and blood urea nitrogen levels, decreased tubular injury score, an increased number of proliferating cells and a decreased number of apoptotic cells. Compared with the control group, the mRNA expression levels of the anti-inflammatory factors interleukin (IL)-10 and transforming growth factor-β1 were significantly upregulated, whereas the expression levels of the proinflammatory factors interferon-γ and IL-1β were significantly reduced in the USCs-treated group. These findings suggested that USCs may promote kidney repair and improve function following ischemic AKI, which may be useful in treating human kidney disease.

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Introduction

Acute kidney injury (AKI) is a multifactorial syndrome, which is classically described as a rapid and progressive loss of renal function. Despite improvements in intensive care and dialysis, the prevalence of cardiovascular disease and sepsis has increased, and the rates of mortality and morbidity associated with AKI remain elevated, which may be attributed to an ageing population (1). Between 1988 and 2002, the incidence of AKI in America rose from 61 to 288 per 100,000 individuals (2) and in England it was 577 per 100,000 in 2015 (3). Patients with AKI who are treated with renal replacement therapy still have a mortality rate between 50 and 60% (4). Therefore, the generation of more effective therapeutic strategies for the treatment of AKI is required.

Renal tubular impairment is thought to be the main pathological alteration associated with AKI. The regeneration of necrotic tubular epithelial cells directly affects the prognosis of patients with AKI. In recent years, novel strategies have been explored that promote the repair of renal tubular epithelial cells. Stem cells or progenitor cells, such as embryonic stem cells (ESCs), bone marrow-derived mesenchymal stem cells (BMSCs), adipocyte-derived mesenchymal stem cells (AMSCs) and induced pluripotent stem cells (iPSCs), are considered a promising strategy in kidney regeneration (5-8). ESCs are an inexhaustible source for cell replacement therapy; however, the use of human ESCs is controversial due to the opposition surrounding use of embryos. In addition, isolation of BMSCs and AMSCs is painful and also raises ethical issues. iPSCs are easily generated from human cells, without fear of immunological rejection; however, their safety is questionable due to the risk of teratoma formation. Therefore, a more reliable method for generating *in vitro* stem cells is required.

Urine may be a convenient source of stem cells, which could be obtained noninvasively. Urine-derived stem

cells (USCs) belong to a stem cell population that exhibits self-renewal and multilineage differentiation abilities. USCs are isolated from voided urine, which may be considered for tissue engineering applications in urology. Due to the differentiation potential of USCs, they could be considered a potential therapy for the treatment of kidney disease (9-11). The present study aimed to test the hypothesis that treatment with USCs could improve renal function and attenuate tubular injury in rats with ischemia/reperfusion (I/R)-induced AKI. If found effective, USCs may be considered useful for the development of bioartificial organs or cell-based therapies in kidney disease treatment.

Materials and methods

Ethics statement. The individuals recruited to the present study provided written informed consent prior to donating urine for stem cell generation. All experiments involving human subjects and animals were approved by the Ethics Committee and Medical Laboratory Animal Center of Shanghai Jiao Tong University Affiliated Sixth People's Hospital (Shanghai, China).

Isolation and proliferation of USCs. Urine samples were donated from 8 healthy men with a mean age of 25 years (age, 22-28 years). USCs were collected, cultured and identified as previously described (12). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; 11960-051; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 2% (v/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 10 ng/ml human epidermal growth factor (Peprotech, Inc., Rocky Hill, NJ, USA), 2 ng/ml platelet-derived growth factor (EMD Millipore, Billerica, MA, USA), 1 ng/ml transforming growth factor (TGF)-β (Peprotech, Inc.), 2 ng/ml basic fibroblast growth factor (bFGF; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 0.5 μM cortisol (Sigma-Aldrich; Merck KGaA), 25 μg/ml insulin (Humulin; Eli Lilly and Company, Indianapolis, IN, USA), 20 μg/ml transferrin, 549 ng/ml adrenaline, 50 ng/ml triiodothyronine, 4 mM L-glutamine (Thermo Fisher Scientific, Inc.) and 20 units/ml PEN/STREP (Thermo Fisher Scientific, Inc.). Cells from passage 4 were used for subsequent experiments.

Flow cytometry. Cell surface marker proteins of USCs were detected using flow cytometry, according to a previously reported method (12). The following monoclonal antibodies were used: Cluster of differentiation (CD) 29-phycoerythrin (PE; 561795; BD Biosciences, Franklin Lakes, NJ, USA), CD34-allophycocyanin (cat. no. 560940; BD Biosciences), CD45-PE (560975; BD Biosciences), CD73-PE (cat. no. 561014; BD Biosciences), CD90-FITC (cat. no. 561969; BD Biosciences) and human leukocyte antigen (HLA)-DR-PE (cat. no. 560943; BD Biosciences).

USCs transduction. USCs at logarithmic growth phase, with a density of 30-50% in a 25-cm^2 flask were transduced with green fluorescent protein (GFP) using a GFP lentivirus (Shanghai GeneChem Co., Ltd., Shanghai, China) according to the manufacturer's protocol. Briefly, the GFP lentivirus (1x10^7 TU/ml) was suspended in 100 ml DMEM, and the cells were cultured at 37˚C in an incubator containing 5% CO_2 for 4 h. Subsequently, the supernatant was discarded and the cells were further cultured for 24-48 h.

Rat model of renal I/R injury. A total of 36 healthy Male Sprague-Dawley rats (weight, 180-200 g; aged 8-10 weeks) were provided by Shanghai Jiao Tong University Medical Laboratory Animal Center (Shanghai, China). During the experiment, the rats were housed at 20-25˚C and 40-70% humidity, with a 12-h light/dark cycle. All rats had free access to standard rat chow and water. To generate an AKI rat model, following a mid-abdominal laparatomy, the kidneys were exposed and the right kidney was removed. The left renal pedicle was dissected to expose the left renal artery, which was clamped with an atraumatic vascular clamp for 45 min under a surgical microscope. Then the artery clamp was loosened and the hydrogel injected. The wound was sutured immediately after confirming that there was no active bleeding in the abdominal cavity, ~2 min.

30 rats were randomly divided into the treated and control groups (n=15 rats/group), the other 6 rats were considered as sham-operated group. In the treated group 1x10^5 USCs mixed with 50 μl sucrose solution (10%, w/v), 50 μl hydrogel and 100 μl PBS were immediately injected into the upper, middle, and lower cortex of the left kidney. Then 5 rats were sacrificed at 1, 7 and 14 days following the injection and blood and kidney tissues were collected. The blood serum creatinine (Scr) and blood urea nitrogen (Bun) levels were detected by fully automatic biochemical analyzer (AU2700; Olympus Corporation, Tokyo, Japan). In the control group, rats were treated identically; however, the rats were treated with mixture of 50 μl sucrose solution (10% w/v), 50 μl hydrogel and 100 μl PBS. Sham-operated rats underwent similar operative procedures but without clamping of the left renal artery and right kidney nephrectomy.

Tubular injury morphological evaluation. Kidney samples were fixed by 4% paraformaldehyde for 24 h at room temperature immediately after separation off the organism, dehydrated by a graded series of ethanol, embedded in paraffin and then sectioned at 5 μm. The sections were stained with hematoxylin and eosin (H&E) and the degree of tubular injury was scored according to a previously reported method (13). The observation and image collection were performed using an inverted microscope (DM4000B; Leica Microsystems GmbH, Wetzlar, Germany) For each kidney, 100 cortical tubules from ≥10 different areas were scored; the maximum score per tubule was 10 and higher scores indicated more severe damage. Points were given for the presence and extent of tubular epithelial cell flattening (1 point), brush border loss (1 point), cell membrane bleb formation (1 or 2 points), interstitial edema (1 point), cytoplasmic vacuolization (1 point), cell necrosis (1 or 2 points) and tubular lumen obstruction (1 or 2 points).

Immunohistochemistry. The fresh kidney tissues were frozen to -25˚C using a freezing microtome (CM1520; Leica Microsystems GmbH), embedded in OTC tissue freezing medium (0201 08926; Leica Microsystems GmbH). When the OCT embedded sections turned into a white solid, the embedded tissue blocks

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

Gene name	Primer sequence (5'-3')	GenBank accession no.
IL-10	F: TAACTGCACCCACTTCCCAGT R: TGGCAACCCAAGTAACCCTTA	NM_012854.2
TGF-β1	F: GCCCTGGATACCAACTACTGCT R: GTTGGTTGTAGAGGGCAAGGAC	NM_021578.2
bFGF	F: CGACCCACACGTCAAACTAC R: CCAGGCGTTCAAAGAAGAAA	NM_019305.2
IFN-γ	F: TCTGGAGGAACTGGCAAAAG R: GTGCTGGATCTGTGGGTTG	NM_138880.2
IL-1β	F: GGACCCAAGCACCTTCTTTT R: AGACAGCACGAGGCATTTTT	NM_031512.2
TNF-α	F: CTCGAGTGACAAGCCCGTG R: CCTTGAAGAGAACCTGGGAGTAG	NM_012675.3
Bcl-2	F: GTGTGTGGGGAGCGTCAACA R: GGCATCCCAGCCTCCGTTAT	NM_016993.1
PDGF	F: ATCGAGCCAAGACACCTCAAAC R: ATGGGGGCAGTACAGCAAATAC	NM_031524.1
GAPDH	F: AGTGCCAGCCTCGTCTCATAG R: CGTTGAACTTGCCGTGGGTAG	NM_017008.4

F, forward; R, reverse; IL, interleukin; TGF-β1, transforming growth factor-β1; bFGF, basic fibroblast growth factor; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; Bcl-2, B-cell lymphoma 2; PDGF, platelet-derived growth factor.

were sectioned at 5-7 μm. Then the sections were fixed in 4% paraformaldehyde for 15 min at room temperature. The paraffin-embedded kidney sections were deparaffinized with xylene and rehydrated in a graded series of alcohol and water. Successive incubations in 3% H_2O_2-methyl alcohol solution (IHC WORLD, LLC, Woodstock, MD, USA) for 10 mifn and in 5% BSA-PBS solution (IHC WORLD, LLC) for 30 min at room temperature were used to blocking the endogenous peroxidase and nonspecific proteins, respectively. The kidney sections were incubated at 4˚C overnight with the following antibodies: Anti-rat proliferating cell nuclear antigen (PCNA; ab29; 1:200; Abcam, Cambridge, UK) and anti-rat GFP (ab290; 1:75; Abcam) were used as the primary antibodies, and goat anti-mouse IgG (H + L) (ab96879; 1:500; Abcam) with was used as the secondary antibody. Following washing in PBS, the kidney sections were incubated with secondary antibodies for 1 h at room temperature. The sections were visualized with 3,3-diaminobenzidine (DAB; ZLI-9019; OriGene Technologies, Inc., Beijing, China). PCNA-positive cell scoring was performed by counting the number of positive nuclei in 10 random sections in the cortex and outer medulla under magnification, x40. The observation and image collection were performed using a fluorescence microscope (DMI6000B; Leica Microsystems GmbH).

Tubular epithelial cell apoptosis was quantitated using a TUNEL assay (G3250; Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. Briefly, kidney sections were deparaffinized, rehydrated, digested with protein K and labeled with TUNEL reaction mixture for 60 min at 37˚C. Then the sections were stained by DAB and the reaction was terminated when a brown background was observed under the microscope (DMI6000B; Leica Microsystems GmbH). Subsequently, TUNEL-positive cells were counted in 20 random sections in the cortex and outer medulla under magnification, x40. The number of apoptotic cells, as defined by nuclear fragmentation, was counted.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the kidney tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, 1 μg RNA was reverse transcribed using the Fermentas RevertAid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) according to the manufacturer's protocols. RT-qPCR was performed on a 7900 Real-Time PCR system using TaqMan Universal PCR Master Mix (Roche Diagnostics, Indianapolis, IN, USA). The thermocycling conditions were 95˚C for 60 sec, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 15 sec and 72˚C for 45 sec. Primer sequences used for RT-qPCR are presented in Table I. GAPDH expression was used as an endogenous control and the 2-ΔΔCq method was used to quantified to the results (14).

Statistical analysis. Data of 3 times repeated experiments are presented as the mean ± standard error of the mean. Statistical analyses were performed using SPSS version 11.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The Mann-Whitney U test was used to statistically analyze the results. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Culture and expansion of USCs. Cell colonies were observed in the USCs culture plates 3-6 days after initial plating.

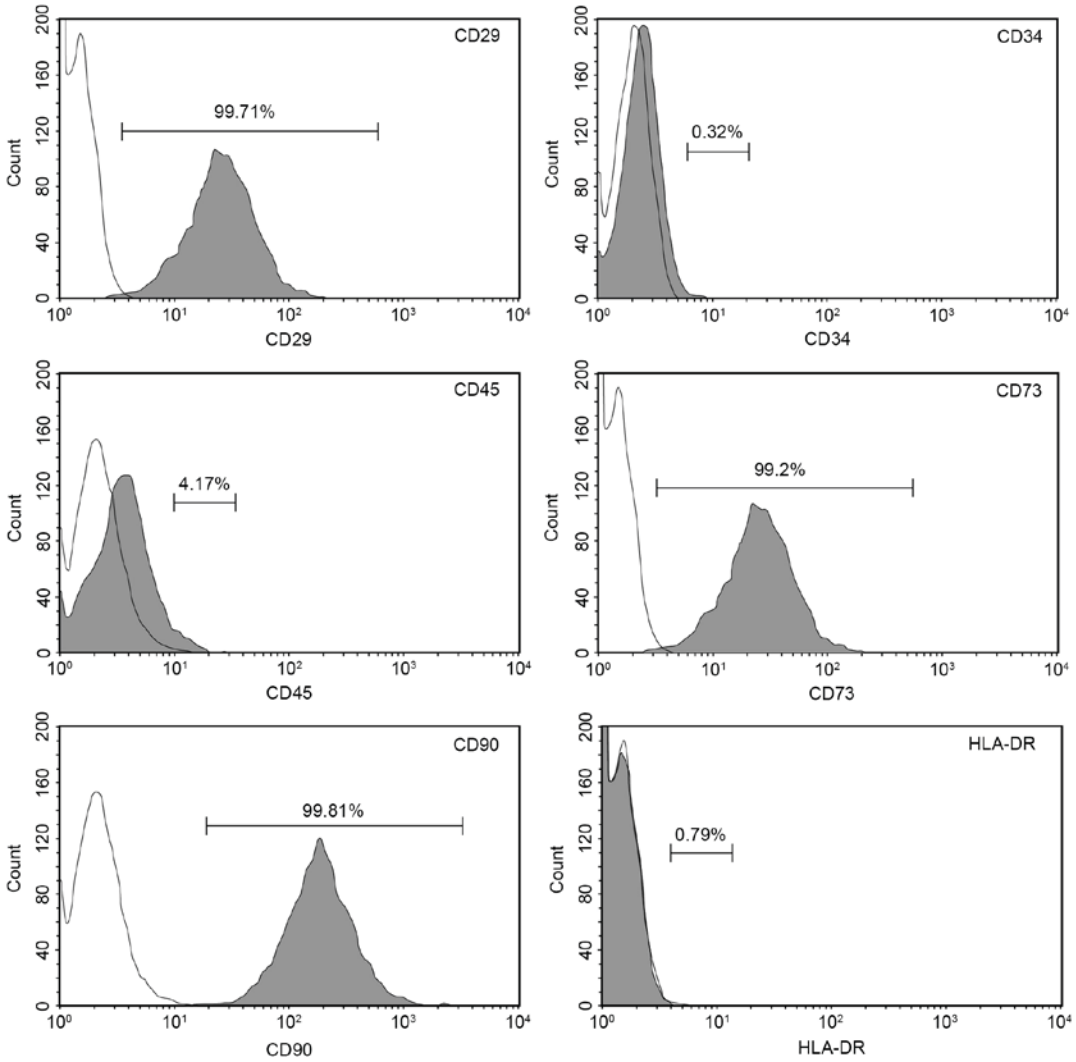

Figure 1. Urine-derived stem cells were analyzed for surface markers using flow cytometry. CD, cluster of differentiation; HLA, human leukocyte antigen.

USCs exhibited typical fibroblast-like morphology, exhibited proliferative capabilities and reached 80-90% confluence after 10 days. USCs retained their elongated morphology after numerous passages.

Cell surface marker expression in USCs. Among the predefined CD markers, USCs exhibited positive expression of CD29, CD73 and CD90 antigens, and negative expression of CD34, CD45 and HLA-DR (Fig. 1). The present study demonstrated that USCs have similar surface antigen markers to ASCs.

Treatment with USCs improves kidney function following I/R injury. Administration of USCs improved renal function in rats at day 1 and 7 (Fig. 2). Serum creatinine (Scr) and blood urea nitrogen (Bun) levels were significantly decreased in the treated group at day 1 (Fig. 2B and C). As determined by H&E staining, tubular necrosis with lumen expansion, flattened tubular epithelial cells and brush border loss were observed in the treated and control groups, compared with in the sham-operated rats (Fig. 2A). However, tubular injury scores in the treated group were significantly lower than in the control group (Fig. 2D).

Apoptosis and proliferation of tubular epithelial cells. The apoptosis and proliferation of tubular epithelial cells from the kidneys of control and USCs-treated rats were subsequently determined. Tubular epithelial cells from the kidneys of USCs-treated rats exhibited a lower apoptotic index compared with the control rats, as determined by TUNEL assay (Fig. 3A and B). In addition, administration of USCs simultaneously increased the number of PCNA-positive cells compared with in the control group (Fig. 3C and D). However, no apoptosis and proliferation were found in the tubular epithelial cells of the sham group.

mRNA expression levels of inflammatory factors. To further investigate the potential underlying mechanisms of USCs, kidneys underwent RT-qPCR to determine alterations in the expression levels of growth factor-associated and inflammatory-associated genes. The genes assessed included anti-inflammatory cytokines [interleukin (IL)-10, bFGF, TGF-β1] and proinflammatory cytokines (TNF-α, IFN-γ, IL-1β).

Kidneys of the rats treated with USCs exhibited a significant reduction in the mRNA expression levels of the proinflammatory cytokines IFN-γ and IL-1β, whereas the

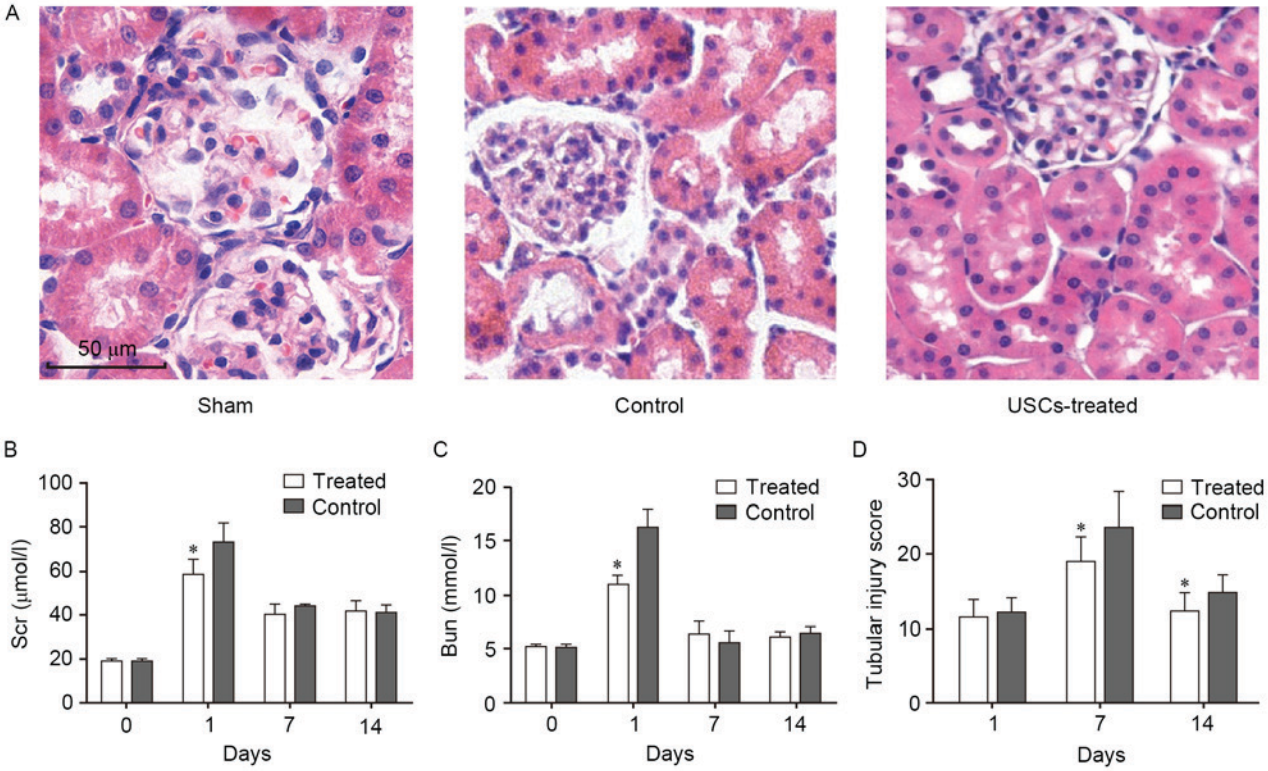

Figure 2. USCs improve renal function following I/R injury. (A) Pathological alterations in the kidney 7 days after surgery (H&E staining; scale bar, 50 μm). (B) Scr and (C) Bun levels were detected at various time points following I/R injury. Administration of USCs significantly improved renal function 1 day after I/R injury. (D) Administration of USCs reduced tubular injury scores. *P<0.05 vs. Control group. Bun, blood urea nitrogen; I/R, ischemia/reperfusion; Scr, serum creatinine; USCs, urine-derived stem cells.

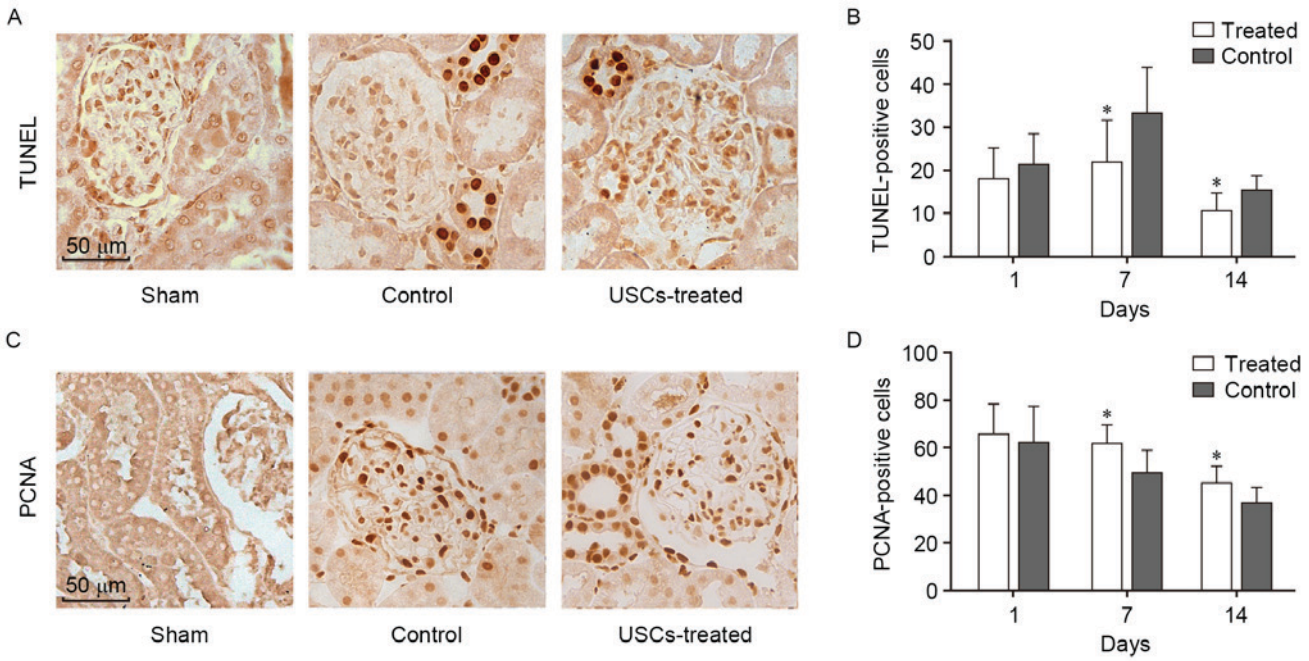

Figure 3. Apoptosis is decreased and cell proliferation is increased in response to USCs administration. (A) Renal tubular epithelial cell apoptosis was decreased in the USCs-treated group 7 days after surgery, as determined by TUNEL assay. Scale bar, 50 μm. (B) Quantitative analysis of the number of TUNEL-positive cells at the various time points after surgery. (C) Renal tubular epithelial cell proliferation was increased in the USCs-treated group 7 days after surgery. Scale bar, 50 μm. (D) Quantitative analysis of the number of PCNA-positive cells at the various time points after surgery. *P<0.05 vs. Control group. PCNA, proliferating cell nuclear antigen; USCs, urine-derived stem cells.

expression of anti-inflammatory cytokines IL-10 and TGF-β1 were increased in USCs-treated rats (Fig. 4).

Detection of USCs in the kidney by in vivo microscopy. Injected GFP-labeled USCs were examined by immunostaining. A

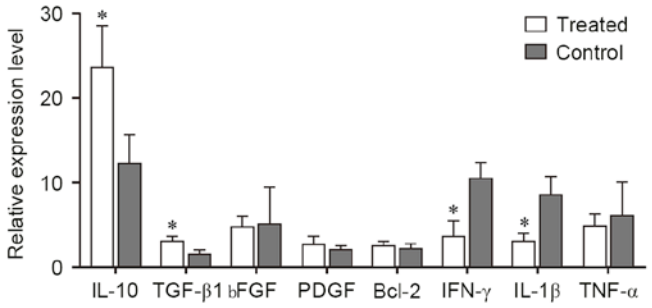

Figure 4. Alterations in the expression of growth factor-associated and inflammatory-associated genes by reverse transcription-quantitative polymerase chain reaction. mRNA expression levels of IL-10 and TGF-β1 were upregulated, whereas INF-γ and IL-1β were significantly downregulated in the urine-derived stem cells-treated group. *P<0.05 vs. Control group. Bcl-2, B-cell lymphoma 2; bFGF, basic fibroblast growth factor; IFN-γ, interferon-γ; IL, interleukin; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor-α; PDGF, platelet-derived growth factor.

total of 7 days after USCs administration, GFP was detected (Fig. 5A). Immunohistochemistry of kidney specimens demonstrated that GFP-positive staining was present in tubular epithelial cells, not in glomerular cells (Fig. 5B).

Discussion

The present study demonstrated that administration of USCs was able to improve renal function in an I/R model. Following injection of USCs into the kidney of a rat model, USCs were detected in the tubular epithelial lining. The USCs-treated group exhibited significantly reduced Scr and Bun levels, a decreased Paller's tubular injury score, an increased number of proliferating cells and a reduced number of apoptotic cells. Compared with the control group, in the USCs-treated group, the mRNA expression levels of the anti-inflammatory factors IL-10 and TGF-β1 were significantly upregulated, whereas the expression levels of the proinflammatory factors interferon (INF)-γ and IL-1β were significantly reduced.

I/R injury largely contributes to AKI in clinical practice, and the main pathological alterations are acute tubular necrosis, apoptosis and inflammation. At present, renal replacement therapy remains the standard treatment for renal I/R injury (15). Recovery from renal I/R injury depends predominantly on the replacement of necrotic tubular epithelial cells with functional cells. However, the kidney itself has a limited regenerative capacity for increasing the number of tubular cells after AKI (16,17). Although numerous drugs and biological factors have been revealed to be effective in the amelioration of AKI in animal models, there remains no significantly effective novel therapy that has been applied in clinical practice (18). Notably, the present study demonstrated that GFP-labeled USCs were localized to the tubular epithelial lining, indicating that some USCs may differentiate into tubular epithelial cells and participate in reconstruction of the injured kidney, thereby restoring renal function.

In the present study, USCs markedly promoted kidney tubular cell proliferation to repair I/R injury, as suggested by the increased number of PCNA-positive cells detected in the USCs-treated rats compared with the control group rats that were administered hydrogel with sucrose solution.

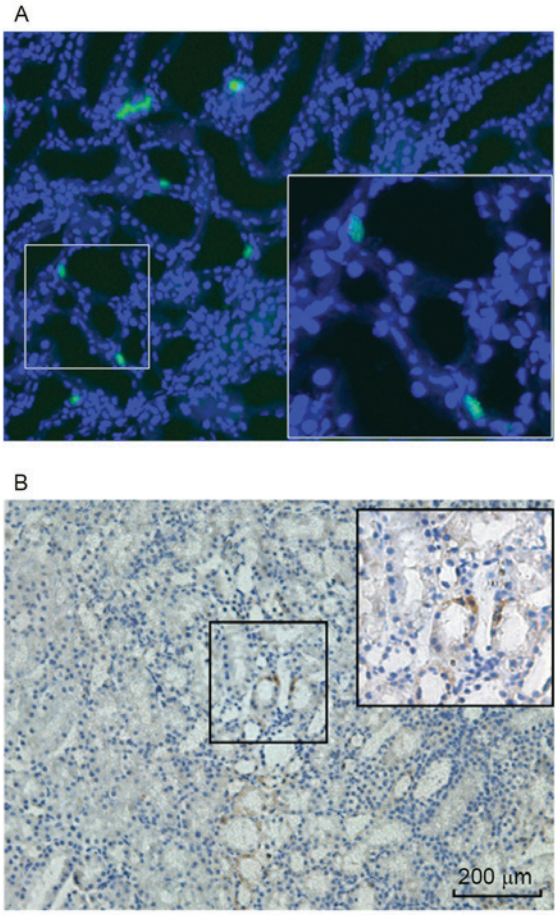

Figure 5. Detection of USCs in the kidney *in vivo*. (A) GFP-labeled USCs were detected in renal frozen sections by immunostaining. (B) Renal paraffin-embedded sections underwent immunohistochemistry with anti-GFP antibodies and DAB. Immunohistochemistry confirmed that GFP-positive cells were detected in tubular epithelial linings. Scale bar, 200 μm.

Furthermore, reductions in Paller's tubular injury score and the number of TUNEL-positive apoptotic cells indicated that USCs could alleviate renal tubular epithelial cell injury and apoptosis, thereby ameliorating I/R injury.

The specific mechanisms underlying renal I/R injury have yet to be fully elucidated (19,20). Previous studies regarding the pathogenesis of I/R injury have focused on the following aspects: Mechanisms of action of inflammatory cytokines, and the function of neutrophils, oxygen-free radicals and other factors (21-25). Among these aspects, the production of inflammatory cytokines is considered an important factor in the development of I/R injury. Numerous studies have reported that BMSCs are effective in the treatment of AKI (6,26,27). The effects of MSCs are primarily mediated via complex paracrine actions, including the secretion of beneficial factors and the activation of signal proteins (28). In addition, a previous report indicated that adipose derived stem cells (ASCs) therapy may improve functional parameters and reduce the progression of renal fibrosis during the early and late stages following injury, decrease the expression levels of IL-6 and TNF, and increase the expression levels of IL-4, IL-10 and heme oxygenase-1 (29).

Our previous study demonstrated that USCs have similar surface antigen markers to ASCs (12). However, to the best

of our knowledge, the beneficial effects of USCs on kidney disease have yet to be thoroughly investigated. USCs have numerous advantages to MSCs and ASCs, as they have a rich source, and are non-invasive and low cost. Therefore, the present study focused on the mechanism of action of USCs on AKI.

The present study observed significant differences in the expression of inflammatory cytokines, including IL-10, TGF-β1, IFN-γ and IL-1β, in kidneys from USCs-treated rats compared with control rats. It is well known that IL-10 and TGF-β1 exert protective effects on I/R injury. It has been reported that deficiency in TGF-β1 expression may increase the severity of renal injury, as indicated by more severe renal tubular damage, and increased levels of Scr and Bun, and TGF-β1 deficiency may result in an increased risk of renal I/R injury (30). In addition, it has been demonstrated that increased IL-10 production may protect animals from renal I/R injury (31). IL-10 may attenuate inflammation by suppressing the activation of nuclear factor-κB, which is involved in renal I/R injury (32). IFN-γ is an important signal in I/R injury, which contributes to reperfusion injury, as well as IL-1β. Day *et al* reported that $CD4^+$ T lymphocytes were important in the early stage of reperfusion-induced inflammation, and IFN-γ was an essential mediator of $CD4^+$ T cell-induced reperfusion injury (33). Previous studies have demonstrated that AKI was associated with increases in the levels of IL-1β in the kidney, and IL-1β has been revealed to act as a proximal mediator of inflammatory events associated with infection, sepsis and ischemia (34).

The present study demonstrated that administration of USCs is associated with increased expression of the anti-inflammatory factors IL-10 and TGF-β1, and decreased expression of the proinflammatory cytokines IL-1β and IFN-γ, in the kidney. These findings suggested that the mechanism by which USCs protect the kidney against I/R injury may be associated with the promotion of IL-10 and TGF-β1 expression, and the inhibition of IL-1β and IFN-γ expression.

In conclusion, the present study demonstrated that USCs could differentiate into renal cells and alleviate renal I/R injury in rats. The effects of USCs were mediated by their anti-apoptotic and mitogenic actions, and their effects on cytokine production. The successful treatment of I/R injury with USCs strongly indicates that USCs may be considered a well-suited type of stem cell for the treatment of kidney disease.

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