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ORIGINAL RESEARCH PAPER

Pretreatments with injured microenvironmental signals altered the characteristics of human umbilical cord mesenchymal stem cells

Huiling Cao · Qian Hui · Yongmin Yan · Chunbing Zhang · Xuewen Yang · Liang Ge · Wenrong Xu

Abstract

Objective Human umbilical cord mesenchymal stem cells (hUCMSCs) have renoprotective effects but the influence of the microenvironment on characteristics of hUCMSCs has not been well studied. Here, we investigate the effects of injury conditions on properties of hUCMSCs.

Results hUCMSCs were treated in vitro under conditions mimicking the injury microenvironment of acute kidney injury. Cells stimulated with factor-treated medium proliferated slowly at first but quickly afterwards their morphology subsequently changed from spindle to stellate shape. Increased number of cells with strong expression of thymine-1 (Thy-1) or α-smooth muscle actin (α-SMA) was detected at 1 or 2 weeks after stimulation. Hepatocyte growth factor (HGF) level markedly increased after culture for 6 h under hypoxia condition. The expressions of HGF and insulin growth factor-1 (IGF-1) were significantly up-regulated from 0.22 ± 0.03 to 0.9 ± 0.02 and 0.07 ± 0.03 to 0.19 ± 0.01 in H/R-treated hUCMSCs respectively. Co-culture with injured renal tubular epithelial cells significantly promoted the expression of HGF (1.19 ± 0.21) and IGF-1 (0.24 ± 0.03) in hUCMSCs.

Conclusion The characteristics of hUCMSCs change in response to inured conditions, which may enhance the efficacy of stem cell therapy and provide novel strategies in maximizing biological and functional properties of hUCMSCs

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Introduction

Mesenchymal stem cells are widely used in the repair of tissue injuries. Bone-marrow derived MSCs (BMMSCs) are the most studied candidates because of already existing clinical experience and their successful use in both autologous and allogeneic settings (Sampson et al. 2013). However, MSCs isolated from abandoned umbilical cord (human umbilical cord MSCs, hUCMSCs) have been of high

interest for regenerative therapies because they are easy to harvest, can be readily expanded in culture and differentiate into a number of cell types in vitro (Wu et al. 2014a). Furthermore, their harvests do no harm to the donor and are free of ethical problems. In recent years, a large number of publications have demonstrated the therapeutic capacity of hUCMSCs (Sabapathy et al. 2014).

Acute kidney injury (AKI) is a critical clinical condition associated with a high degree of morbidity and ischemia/reperfusion (I/R) injury is the most common cause for AKI. MSC transplantation for AKI is an emerging technology in cell biological engineering, in which MSCs are transplanted into the damaged kidney and differentiate into local cell types or improve the partial microenvironment via their paracrine function, both of which consequently improve kidney function (Liu et al. 2012; Tsuda et al. 2014). We have shown that hUCMSCs have renoprotective effects and enhance regeneration after AKI in an ischemia/reperfusion model in rats (Cao et al. 2010a). However, the influence of the highly complex environment in injured tissue on the biological characteristics of hUCMSCs has not been evaluated in vitro. Accordingly, the current study was designed to investigate the effects of different microenvironmental signals on the biological properties of hUCMSCs.

Materials and methods

Cell culture

All experiment protocols were approved by the ethical committee of Jiangsu University (2012258). Fresh umbilical cords were obtained from consenting mothers and processed within 5 h. The cord was rinsed by phosphate-buffered saline (PBS) containing penicillin and streptomycin (1000 U/ml) until the blood was removed. Then, the stromas of the cord rather than blood vessels were selected and cut into 1–2 mm^3-sized pieces. The pieces were cultured in low glucose Dulbecco's modified Eagle's medium (L-DMEM) containing 10 % (v/v) fetal bovine serum (FBS) at 37 °C in humidified air with 5 % CO_2. After 3 days, the medium were changed and non-adherent cells were removed. Subsequently, the medium was changed every 3 days. When the cord pieces adhered

to the plastic flask, MSCs would grow from the tissues and developed clones about 10 days after initial plating (Cao et al. 2010b). Cultured cells were examined using a transmission electron microscope as described previously (Cao et al. 2009). The harvested cells (1.5×10^6 cells) were trypsinized, washed twice with PBS and immunostained for 30 min on ice with monoclonal antibodies against CD13, CD29, CD38, CD44, CD45, CD105, NGFR (CD271) (PE-conjugated), CD31, CD34, and HLA-DR (FITC-conjugated) (Becton–Dickinson, San Jose, CA). Labeled cells were analyzed using a flow cytometer. Background signals were established using control cells incubated with isotype-specific IgGs. The cells at passage 3 were used for subsequent experiments. Rat renal tubular epithelial cells (NRK) were purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China).

Cytokine induction

10^5 hUCMSCs were seeded onto coverslips in 6-well plate and allowed to attach overnight in L-DMEM with 10 % FBS. Media were then replaced with factor-treated medium containing platelet-drevied growth factor-BB (PDGF-BB) (PeproTech, USA). Two different groups due to the concentration of factor (PDGF-BB 50 or 200 ng/ml) were used. HUCMSCs were collected for protein expression after induced for certain times (1 and 2 week). Then, proliferation of hUCMSCs was measured using the Cell Counting Kit-8 (Dojindo, Japan). The protein expressions of thymine-1 (Thy-1) and alpha-smooth muscle actin (α-SMA) (eBioscience, USA) were detected by immunohistochemical staining.

Hypoxia/reoxygenation procedure

HUCMSCs were kept at 37 °C in a 5 % CO_2 atmosphere. For hypoxia exposure, cells at 80 % confluence were maintained under hypoxic conditions in an airtight chamber (Billups–Rothenberg, Del Mar, CA, USA) gassed with a mixture of 5 % CO_2/1 % O_2/95 % N_2 for the indicated times (2, 4, 6 h). For reoxygenation, plates were removed from hypoxic chamber and were incubated under standard conditions (95 % air/5 % CO_2). At intervals (3, 18, 24 h), the expression of hepatocyte growth factor (HGF) and insulin growth factor-1 (IGF-1) in hUCMSCs were

detected. Gene expression levels were calculated and normalized relative to the levels of β-actin gene expression.

Cell co-culture

Rat renal tubular epithelial cells (NRK) were seeded in L-DMEM including H_2O_2 (500 ng/ml) over 24 h and the injured cells were observed by Hoechst 33342/PI (Sigma). Then, the damaged cells were co-cultured with hUCMSCs for 72 h in 6-well culture plates (at 1:1 ratio) to investigate the certain gene expressions of hUCMSCs. The normal NRK were co-cultured with MSCs as a control. Gene expression levels were calculated and normalized relative to the levels of GAPDH gene expression.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software. Data are expressed as mean ± SD. Student's *t* test was used to compare two groups. ANOVA followed by a multiple comparison test was used to analyze variance among multiple groups. Each experiment was repeated at least twice, and each data point represents three independent samples. The *p* value of <0.01 and 0.05 were considered statistical significant.

Results

Morphology and surface antigens of hUCMSCs.

After 10 days of primary culture, the MSC-like cells migrated from the tissues and adhered to the plastic surface, resulting in a small population of single, spindle-shaped cells (Fig. 1A-a). After replating, the fibroblast-like cells appeared polygonal or spindly with a long process, and formed an orderly pattern at confluence (Fig. 1A-b). Transmission electron micrographs showed that hUCMSCs appeared to be in a quiescent stage, with a large cell nucleus and a few cellular organelles (Fig. 1A-c). HUCMSCs were positive for CD13, CD29, CD44 and CD105, but were negative for CD31, CD34, CD38, CD45, NGFR and HLA-DR (Fig. 1B). The hUCMSCs phenotype and purity were further confirmed by their ability to differentiate into osteocytes and adipocytes using respective induction media (Supplementary Fig. 1).

Morphologic change and growth curves of hUCMSCs after inducing by PDGF-BB.

When cultured in the growth factor-treated medium, hUCMSCs displayed slower proliferation at first and subsequently morphological changes differing from spindle to stellate (Fig. 2A). Growth curves are shown in Fig. 2B. At culture days 0-3, the number of hUCMSCs decreased in the experiment groups even in the 50 ng PDGF-BB/ml group. However, the cell counts increased rapidly from days 5 and presented a higher cumulative population level in 200 ng/ml group.

Overexpression of Thy-1 and α-SMA in hUCMSCs after exposure to growth factor-treated medium.

Since stellate cells in the growth factor-treated medium were reminiscent of mesangial cells, the expressions of Thy-1 and α-SMA were examined by using immunohistochemical staining. In the control groups, Thy-1 and α-SMA-negative (Thy-1⁻ and α-SMA⁻) or Thy-1 and α-SMA-weakly positive (Thy-1⁺ and α-SMA⁺) populations were expressed (Fig. 3A, B). Treatment with growth factor produced more cells with strong expression of Thy-1 (Thy-1^{++}) and α-SMA (α-SMA^{++}) at 1 or 2 week after induction (Fig. 3A, B, C-b, c). The effect of 50 ng of PDGF-BB/ml was indistinguishable from that of 200 ng PDGF-BB/ml (1-week: Thy-1^{++} 73.3 ± 4 vs 70.4 ± 6.1 %, α-SMA^{++} 68.3 ± 6.5 vs 71.4 ± 17.3 %; 2-week: Thy-1^{++} 86.3 ± 7 vs 90.5 ± 8.4 %; α-SMA^{++} 76.3 ± 6.3 vs 86.5 ± 14.6 %). However, the number of stellate cells was greater with the 200 ng/ml group (55.5 ± 6.5 % at 1 week, 79.2 ± 17.5 % at 2 week) than in 50 ng/ml group (25.6 ± 3.5, 56.2 ± 13.4 %) ($p < 0.01$ at 1 week, $p < 0.05$ at 2 week, Fig. 3C-a).

While 50 ng PDGF-BB/ml was sufficient to produce Thy-1^{++} or α-SMA^{++} cells but not to produce more stellate cells, the effect of 500 ng/ml PDGF-BB was indistinguishable from that of 200 ng PDGF-BB/ml (data not shown). Therefore, there might be a critical concentration between 50 and 200 ng/ml.

Increased expressions of HGF and IGF-1 in hUCMSCs under hypoxia/reoxygenation exposure.

As shown in Fig. 4A, the level of HGF protein markedly increased after culture for 6 h under hypoxia conditions ($p < 0.01$). As shown in Fig. 4B, the level of HGF gene expression was significantly up-regulated from 0.22 ± 0.03 to 0.9 ± 0.02 in hUCMSCs under H/R exposure after 18 h. However, the level of IGF-1 gene was increased at 18 h (from 0.07 ± 0.03
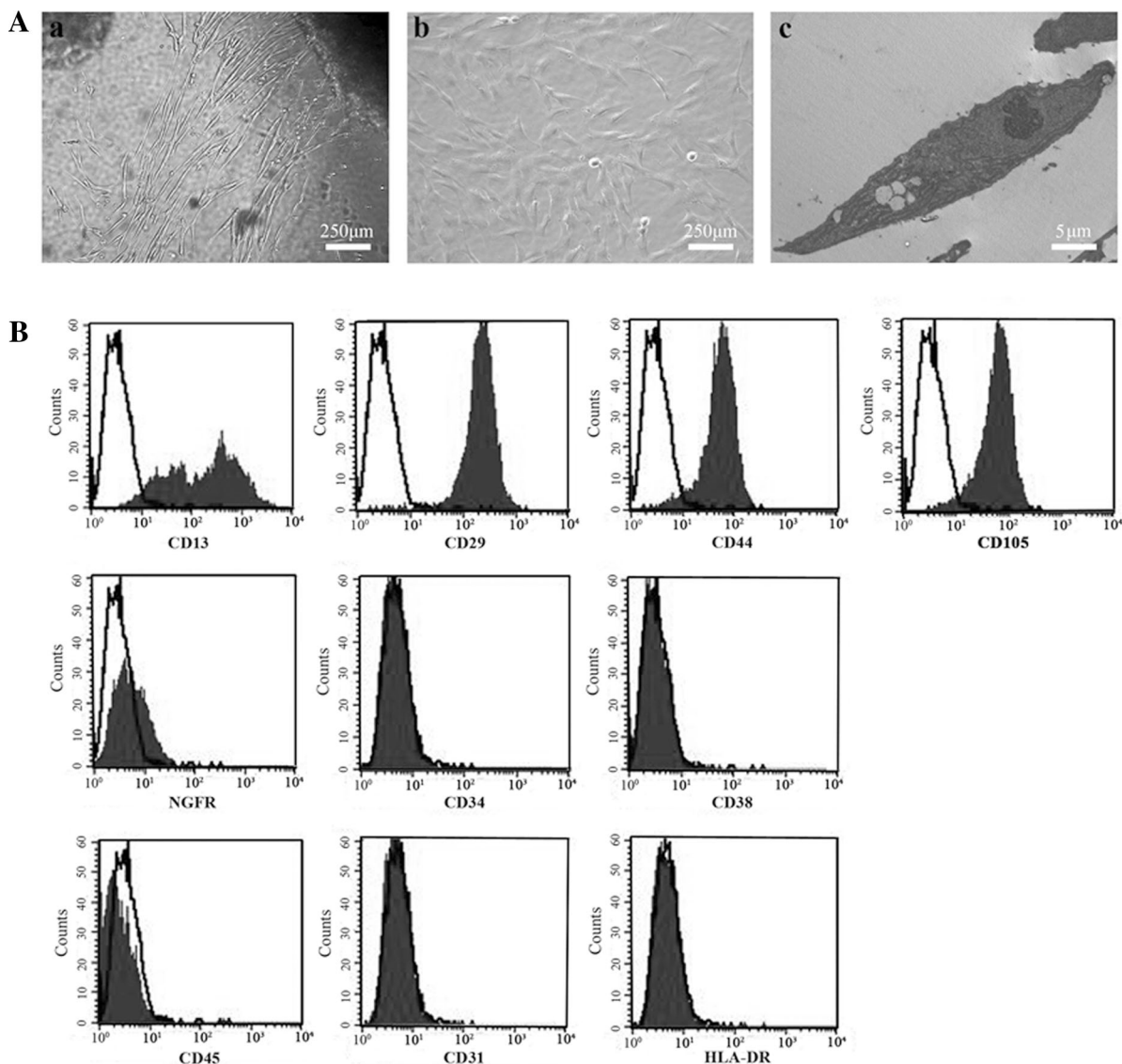
Fig. 1 Morphology, ultrastructure and surface antigens of hUCMSCs. **A** The morphology and ultrastructure of hUCMSCs after 10 days of primary culture. *a* hUCMSCs were fibroblastic in appearance after 10 days of primary culture. *b* The appearance of hUCMSCs at passage 2. *c* Transmission electron micrographs. **B** The surface antigens of hUCMSCs. HUCMSCs were positive for CD13, CD29, CD44 and CD105, and negative for CD31, CD34, CD38, CD45, NGFR and HLA-DR

to 0.19 ± 0.01 ($p < 0.01$) and decreased at 24 h (0.09 ± 0.02) under H/R exposure.

Up-regulation of HGF and IGF-1 in hUCMSCs after co-cultured with injured NRK cells.

To identify the interaction between hUCMSCs and NRK cells, hUCMSCs were co-cultured with normal (Fig. 5D) or H_2O_2-damaged NRK cells (Fig. 5C). The injured NRK cells were stained with Hoechst 33342/PI dye. The results of fluorescence microscopy revealed that H_2O_2 induced significant apoptosis in NRK cells (Fig. 5A) compared to the normal NRK cells (Fig. 5B). Co-culture with injured NRK cells resulted in a significant up-regulation of HGF and IGF-1 gene expressions in hUCMSCs (HGF: 1.19 ± 0.21 vs 0.27 ± 0.05; IGF-1: 0.24 ± 0.03 vs 0.01 ± 0.001) ($p < 0.01$, Fig. 5E).

A

B
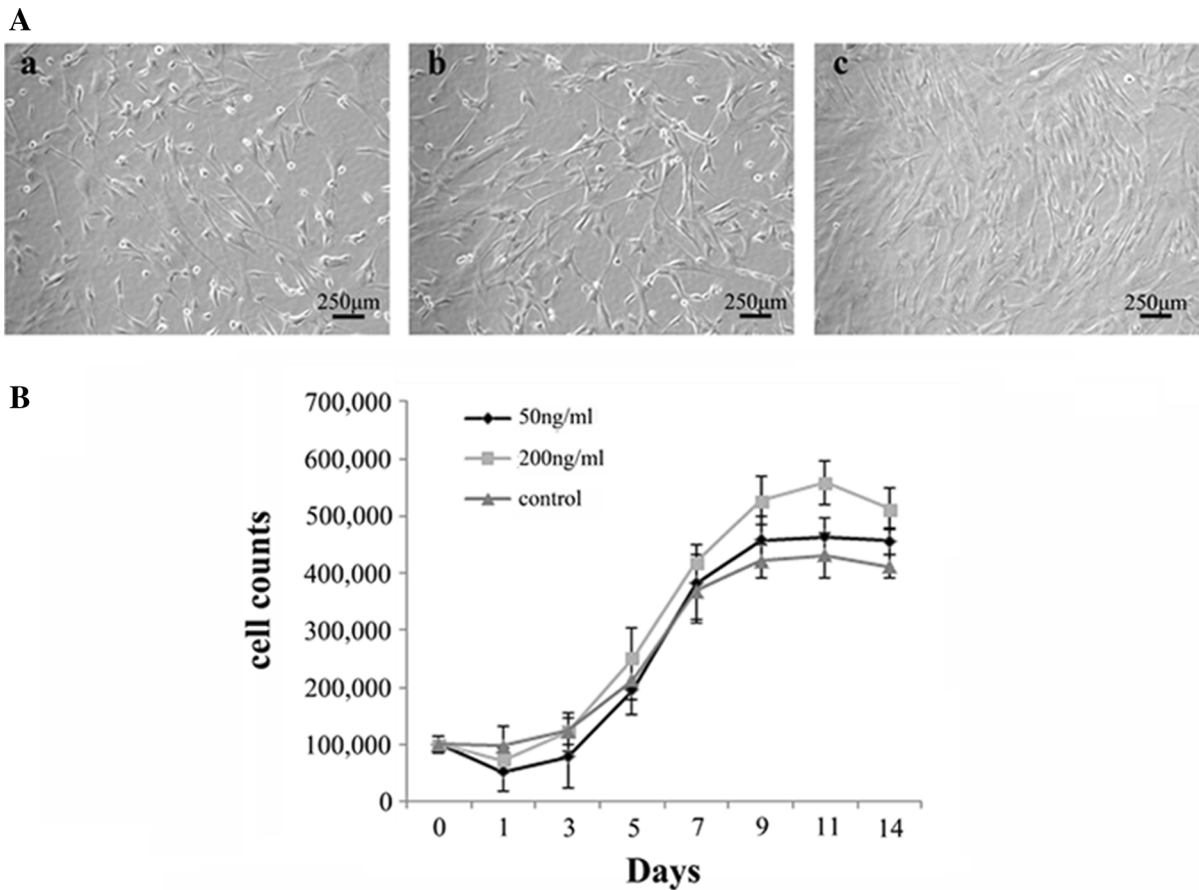
Fig. 2 The morphology and growth curves of hUCMSCs treated by PDGF-BB. **A** The morphology of hUCMSCs in factor-treated groups and control groups (*a* 50 ng PDGF-BB/ml; *b* 200 ng PDGF-BB/ml; *c* non-induced); **B** The growth curves of hUCMSCs among groups

Discussion

Mesenchymal stem cell-based therapies are promising new treatment approaches for a large number of diseases (Silva et al. 2014). However, controversy remains as to the mechanisms whereby MSCs mediate the obtained therapeutic actions. MSCs home to injured tissues and exert their beneficial effects by differentiating into target cells and then replacing injured tissue (Abd-Allah et al. 2014; Liu et al. 2014). On the other hand, the transplanted stem cells do not replace injured resident cells but mitigate injury and hasten repair by a paracrine mechanism (Galie and Stegemann 2014).

MSCs have a potential to differentiate into different lineages in response to different environments.

Restricted microenvironments, including distinct types of growth factors and cell–cell interactions, influence the fate of MSCs and give them the competence to contribute to more specific lineages (Silva et al. 2014; Wu et al. 2014b). So far, however, little is known about the events regulating the acquisition of the competence that is required for hUCMSCs to differentiate into other lineages. Here, we established the similar microenvironment as the injured tissues after AKI in vitro.

Injury of glomerular is one presentation of AKI. Several lines of evidence show that overactivity of PDGF is involved in the pathogenesis of several glomerular diseases that are characterized by mesangial cell proliferation (Eisel et al. 2013). Furthermore, mesenchymal stem cells were predominantly located
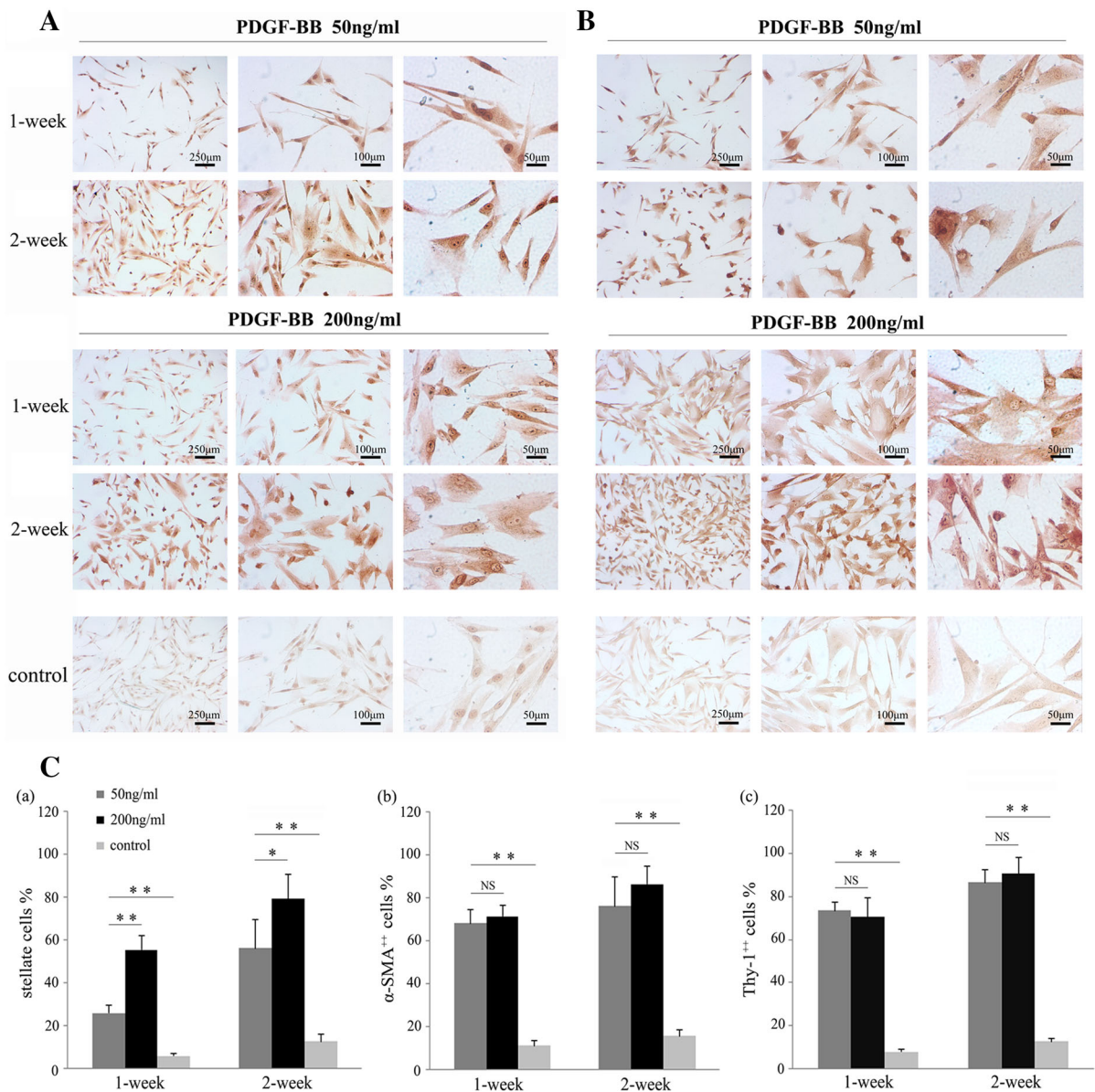
Fig. 3 The expressions of Thy-1 and α-SMA in hUCMSCs after induction with cytokines. **A** The morphology and expression of Thy-1 protein in hUCMSCs at different times after induction; **B** the expression of α-SMA protein at different times. **C** The percentage of stellate cells (*a*), α-SMA^{++} (*b*) and Thy-1^{++} (*c*) cells in hUCMSCs among groups induced with PDGF-BB at 50, 200 and non-induced groups. *$p < 0.05$, **$p < 0.01$, *NS* not significant

in glomerular capillaries, indicating likely mesangial cell transdifferentiation. PDGF-BB is important to the microenvironment of renal generation. It is produced physiologically or pathologically by several types of renal cells in vivo, which include mesangial, glomerular and tubular epithelial cells. PDGF-BB mediates

mitogenic and chemotactic effects on many mesenchymal cells, including vascular smooth muscle cells, fibroblasts and mesangial cells in vitro (Eisel et al. 2013; Cui et al. 2014). In the present study, we have demonstrated that hUCMSCs may differentiate into mesangial-like cells in response to PDGF-BB.
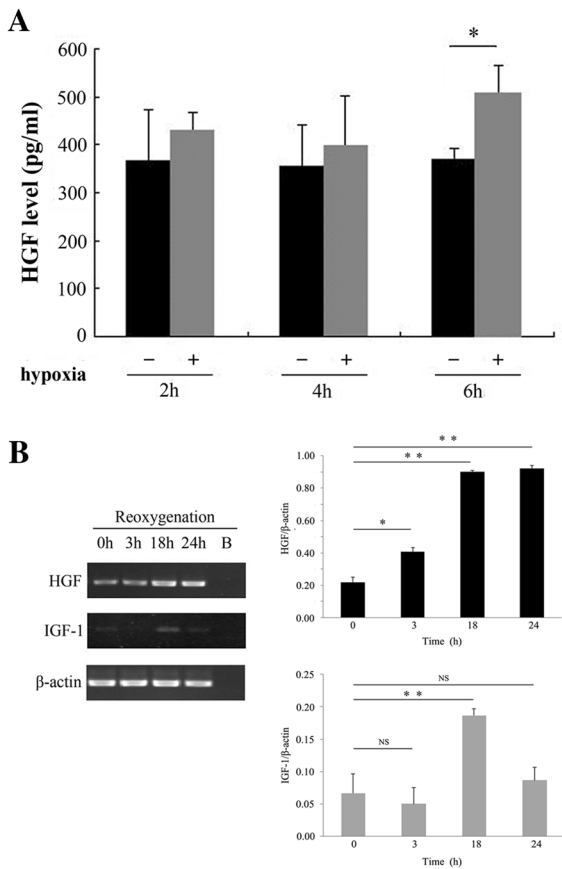
Fig. 4 The effect of hypoxia/reoxygenation on cytokine secretion and gene expression in hUCMSCs. **A** The level of HGF in supernatant of hUCMSCs at different time points after hypoxia; **B** the expression levels of HGF and IGF-1 genes in hUCMSCs at different time points after hypoxia/reoxygenation. $*p < 0.05$, $**p < 0.01$, *NS* not significant

Here, these cells were identified mainly by the expression of Thy-1 and α-SMA, and by the stellate appearance. Thy-1 is a well-established marker for mesangial cells in vitro (Wu et al. 2014b). Expression of α-SMA is positive in glomerular endothelium (Batchelder et al. 2010). Thus, hUCMSCs derived Thy-1^{++} or α-SMA^{++} cells were more mesangial-like also because most of them were stellate. The results showed that the medium containing both 50 and 200 ng PDGF-BB/ml could produce more Thy-1^{++} or α-SMA^{++} cells. Furthermore, more stellate cells could be produced after treating by 200 ng PDGF-BB/ml which suggested that PDGF-BB is important for the differentiation. In addition, PDGF-BB is a serum component widely described as a potent

mitogen and also shown to prevent apoptosis (Eisel et al. 2013), so the factor-treated medium facilitates the proliferation of hUCMSCs.

Histologically, dysfunction and loss of tubular epithelial cells play central roles in the process underlying the failure of the kidney after ischemic or toxic challenge. Growth factors, such as IGF-1, HGF have been used consistently to potentiate tubular regeneration in ARI (Srisawat et al. 2014). Thus, the therapeutic effects of MSCs transplantation in ischemic disease may be enhanced by more production of paracrine bioactive factors. Other than in vitro, once localized to the ischemic tissues, MSCs encounter severe hypoxic conditions, ranging from 0.5 to 3 % O_2. Otherwise, the culture in hypoxic conditions (<5 % O_2) may also be beneficial for MSCs, as the O_2 tension is similar to the physiologic niche for MSCs in the bone marrow. In our work, we tested whether hypoxia/reoxygenation culture affect the characteristics of hUCMSCs: HGF gene expression in hUCMSCs was increased in response to the hypoxic exposure (6 h). Furthermore, HGF and IGF-1 gene expressions of hypoxic MSCs were markedly enhanced after reoxygenation, which suggest that hUCMSCs might be influenced by the microenvironment in renal I/R injured tissues. Over-expression of HGF and IGF-1 genesm in turnm might be protective for injured renal tissues.

In our previous study, we found hUCMSCs could reside in local renal tubular injury sites and had renoprotective effects in an ischemia/reperfusion model in rats (Cao et al. 2010a). Thus, the interactions between injured rat renal tubular epithelial cells (NRK) and hUCMSCs were observed. Compared to the control group, expression levels of HGF and IGF-1 in hUCMSCs were enhanced by interacting with injured NRK cells. We conclude that a paracrine mechanism may be mainly responsible for the enhanced proliferation and protection of renal cells by hUCMSCs. This may explain how hUCMSCs administration promotes significant renoprotection despite the low level of differentiation (Cao et al. 2010a).

In conclusion, hUCMSCs changed to renoprotective characteristics after priming with conditions mimicking the microenvironment of injured renal tissues, which may enhance the efficacy of stem cell therapy and provide novel strategies in maximizing biological and functional properties of hUCMSCs.

Fig. 5 The expression of HGF and IGF-1 genes in hUCMSCs after co-culture with NRK cells. **A**, **B** Hochest33342/PI staining; **A** NRK oxidative damaged by H$_2$O$_2$ (*white arrow* apoptotic cells, *gray arrow* necrotic cells); **B** NRK in normal culture; **C** hUCMSCs co-cultured with damaged NRK cells; **D** hUCMSCs co-cultured with normal NRK cells; **E** The expression levels of HGF and IGF-1 genes in hUCMSCs after co-culture with NRK cells. **p < 0.01
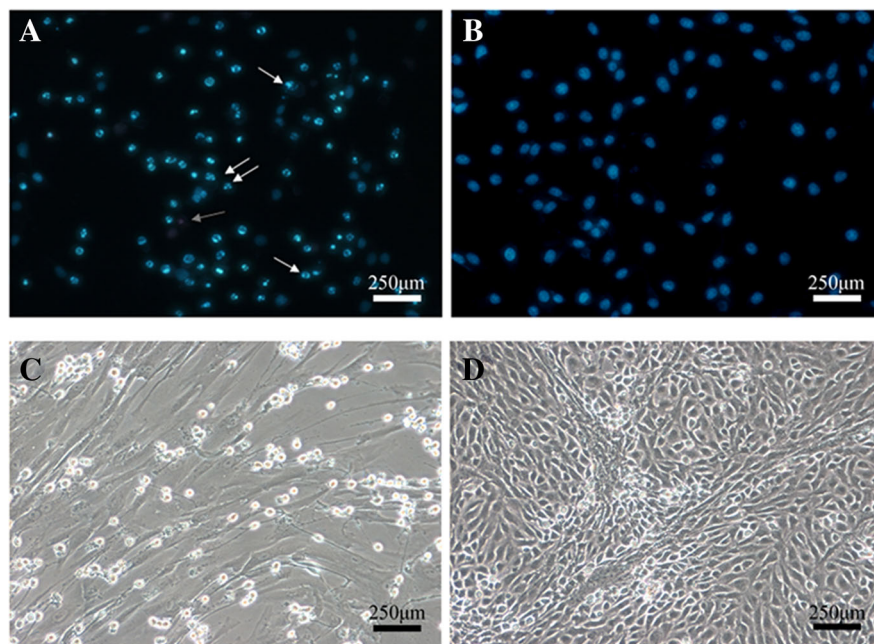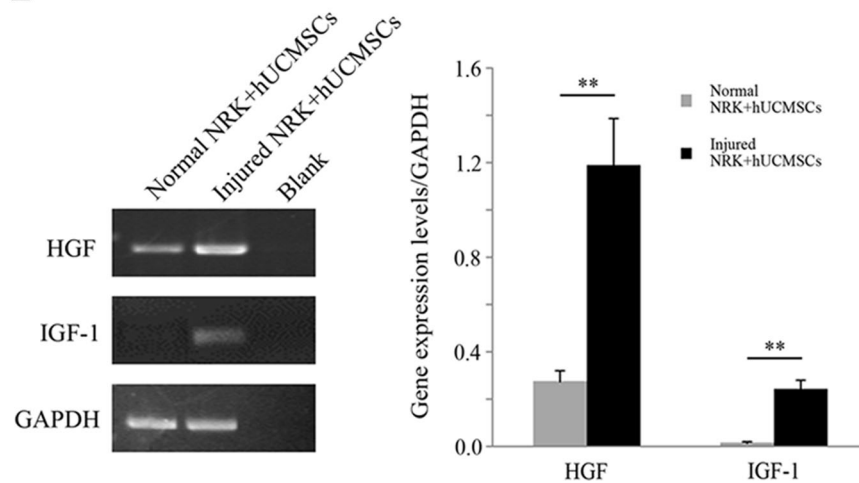
Supporting information Supplementary Figure 1—Most hUCMSCs were positive for alkaline phosphatase after being induced to differentiate into osteoblasts, and were positive for Oil-Red-O staining after being induced to differentiate into adipocytes for 14 days.

Supplementary Table 1—Primer sequences of target genes.

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