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Adult kidney stem/progenitor cells contribute to regeneration through the secretion of trophic factors

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ABSTRACT

Adult kidney stem cells are known to have important roles in renal regeneration after acute kidney injury. Although trophic factors from tissue stem cells have been reported to promote the regeneration of other organs, there is limited number of evidence of this phenomenon in the kidneys. Here, we explored the effects of secreted factors from kidney stem cells. We intraperitoneally administered culture supernatant obtained from adult rat kidney stem/progenitor cells into rat kidney ischemia/reperfusion injury models, and the treatment significantly ameliorated renal tubulointerstitial injury, suppressed tubular cell apoptosis, diminished inflammation and promoted the proliferation of both residual renal cells and immature cells. *In vitro,* treatment with culture supernatant from kidney stem cells significantly promoted cell proliferation and suppressed cisplatin-induced cell apoptosis in both normal rat kidney cells and kidney stem cells. In addition, treatment with culture supernatant increased the expression of nestin in normal rat kidney cells, suggesting the dedifferentiation of tubular cells into stem-like cells. Analysis of the culture supernatant revealed that it contained a variety of growth factors. Taken together, the results suggest that these factors together lead to renal regeneration. In conclusion, adult kidney stem cells contribute to renal regeneration indirectly through the secretion of regenerative factors.

1. Introduction

Acute kidney injury (AKI) is a major healthcare problem associated with prolonged hospitalization and high mortality rates (DuBose et al., 1997; Thadhani et al., 1996). Ischemic renal injury is one of the common causes of AKI (Aydin et al., 2007; Kazmers et al., 1997; Mangano et al., 1998). Despite advances in medical technology, there are no better options for treating AKI than supportive treatments, such as renal replacement therapy (DuBose et al., 1997). On the other hand, the recovery of renal function after AKI is sometimes observed. Improvements in renal function require the recovery of injured renal tubules and the regeneration of these tubules. Although it is controversial which cell type can reconstruct damaged tubules and promote tubular regeneration, three candidate cell types, residual tubular cells, bone marrow-derived stem cells and kidney stem cells, have been reported. For example, previous reports have revealed that surviving tubular cells around injured tubules dedifferentiate and replace lost tubular cells (Humphreys et al., 2008; Kusaba et al., 2014). Others have demonstrated that bone marrow-derived cells promote regeneration via the replacement of lost tubular cells (Iwasaki et al., 2005; Kale et al., 2003). More recently, Lazzeri et al. demonstrated progenitor-driven renal regeneration after AKI that can be pharmacologically enhanced (Lazzeri et al., 2018). Taken together, the repair system involved in renal regeneration after kidney injury might be complex and several cell types, including residual tubular cells, bone marrow-derived stem cells and kidney stem cells, might contribute to the regeneration together.

As a regenerative treatment, mesenchyme stem cell (MSC) therapy has been reported to be effective in different models of kidney diseases (Asanuma et al., 2010; Bi et al., 2007; Herrera et al., 2004; Kunter et al., 2007; Morigi et al., 2008; Togel et al., 2009, 2005) through the direct differentiation of stem/progenitor cells into renal mature cells. However, recent evidence suggests that both the number of stem cells that undergo direct differentiation into mature tubular cells and the rate of this process are limited (Humphreys et al., 2008; Lange et al., 2005; Togel et al., 2005; Vogetseder et al., 2008). Instead, other reports have revealed that MSC therapy protects residual cells via trophic factors secreted from MSCs (Bi et al., 2007; Caplan and Dennis, 2006; Gnecchi et al., 2005; Rabb, 2005; Tang et al., 2005; Togel et al., 2005). These

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findings indicate a strong potential of stem cells for mediating tissue regeneration indirectly via trophic factors.

Regarding kidney stem cells, several research groups revealed the presence of adult renal stem/progenitor cells using different kinds of technologies. (Maeshima et al., 2014) (Bussolati and Camussi, 2015; Tsuji and Kitamura, 2015). For example, Inowa et al. identified kidney side population (SP) cells using Hoechst 33,342 staining and indicated that the SP cell population in human kidney is 1.3% (Inowa et al., 2008). Dekel et al. isolated adult kidney stem/progenitor cells as stem cell antigen-1 (sca-1)-positive cells (Dekel et al., 2006). Gupta et al. isolated multipotent renal progenitor cells (MRPC) using specific cell culture conditions (Gupta et al., 2006) and reported that injection of MRPC differentiated into renal tubules. Maeshima et al. also identified adult kidney stem/progenitor cells using DNA labeling with BrdU and named the cells slow cycling label-retaining cells (LRCs) (Maeshima et al., 2003). In addition, several groups identified CD133(+)CD24(+) adult kidney stem cells in Bowman's capsule, inner medullary papilla, and proximal tubular cells (Bussolati et al., 2005; Lindgren et al., 2011; Sagrinati et al., 2006; Sallustio et al., 2013, 2010). Although these reports suggest the regenerative potential of adult kidney stem/progenitor cells under renal injury, the contribution of these stem cells to regeneration is still controversial and unclear.

Our group previously established an adult rat renal stem/progenitor-like cell line (KS cells) from the S3 segment of the renal proximal tubules of adult rat kidneys (Kitamura et al., 2005). KS cells have stem cell-like characteristics, including self-renewal ability, prolonged cell proliferation and regenerative ability. When we injected LacZ-labeled KS cells into cisplatin-induced AKI rats at the subcapsule of the left kidney, the treatment with KS cells implantation improved renal function, induced cell proliferation, and limited tubulointerstitial injury and tubular cell apoptosis (Kinomura et al., 2008; Kitamura et al., 2005). In addition, Bluo-gal(+) KS cells with AQP1 expression were observed in the corticomedullary junction. Although there is a controversy of the capacity of differentiation of the stem cells into mature cells, the result suggested that implanted KS cells migrate to injured tubules and then differentiate into mature renal cells (Kinomura et al., 2008; Kitamura et al., 2005). Interestingly, adjacent tubular epithelial cells of Bluo-gal(+) KS cells expressed AQP1 more intensely than KS cells, and the amelioration of tubular cell apoptosis and tubular injury was also observed in the right kidney without KS cell implantation. Taken together with the fact that the number of Bluo-gal(+) KS cells in the corticomedullary junction are limited, we hypothesized that there might be an indirect regenerative pathway that involves the secretion of trophic factors from kidney stem/progenitor cells during renal regeneration. Here, we examined the effect of the culture supernatant of KS cells in rat models of kidney ischemia/reperfusion (I/R) and *in vitro*.

2. Material and methods

2.1. Animal experimental design

The experimental protocol was approved by the Animal Ethics Review Committee of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (OKU-2012106 and OKU-2018579). Studies were performed on 5-week-old male Sprague-Dawley rats obtained from CLEA (CLEA Japan, Tokyo, JAPAN). The animals had free access to tap water and standard rat chow.

2.2. Cell culture

KS cells were isolated from adult rat kidneys as previously reported (Kitamura et al., 2005). KS cells were cultured in a 1:1 mixture of culture supernatant of mouse MCSs [Dulbecco's modified Eagle medium (DMEM; Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS; Gibco, Grand Island, NY)] and modified K1 medium [1:1 mixture of DMEM and Ham's F12 medium (Gibco), supplemented with 10% FCS, 5 μg/ml insulin, 2.75 μg/ml transferrin, 3.35 ng/ml sodium selenium acid (ITS-X, Gibco), 50 nM hydrocortisone (Sigma), 25 ng/ml hepatocyte growth factor (HGF, Sigma), 2.5 mM nicotinamide (Sigma)], penicillin G (100 U/ml) and streptomycin (100 μg/ml) on type IV collagen-coated disks or 75 cm^2 flasks (BD Bioscience, San Jose, CA). NRK-52E cells were cultured in DMEM and Ham's F12 culture medium containing 10% FCS and penicillin G (100 U/ml) and streptomycin (100 μg/ml).

2.3. Collection of cell culture supernatant

Concentrated supernatant of KS cells (CS-KS) and concentrated supernatant of NRK-52E cells (CS-NRK) were generated using the following protocol for *in vivo* and *in vitro* experiments. KS cells on type IV collagen coated 75 cm^2 flasks and NRK-52E cells on 75 cm^2 flasks were cultured until they were subconfluent and were washed with phosphate-buffered saline (PBS; Gibco). Then, the cells were incubated with 20 ml of mixed medium (DMEM/F12, Gibco) without serum for 24 h. The supernatant was harvested, and the cellular debris was removed using MediaKap-2 filters (Spectrum Laboratories, Inc.). The supernatant was concentrated (40×) using Amicon Ultra-15 Centrifugal Filter Units with Ultracel-3 membranes (Millipore) according to the manufacturer's instructions. For control vehicle, 20 ml of medium (DMEM/F12, Gibco) was concentrated (40×) using Amicon Ultra-15 Centrifugal Filter Units with Ultracel-3 membranes.

2.4. Induction of acute kidney injury by ischemia-reperfusion and treatment with cell supernatant

Kidney ischemia/reperfusion (I/R) injury was induced in rats by clamping the left renal artery for 45 min and then removing the right kidney. Because some rats did not show the reperfusion even after the clamp removal most likely due to renal artery thrombus, we only chose rats with good perfusion for the experiment and separated these rats into four groups: the sham-operated group (sham group, n = 6), vehicle treatment group (I/R plus intraperitoneal administration of 500 μl of vehicle 3 h after I/R; I/R group, n = 7), KS cell supernatant treatment group (I/R plus intraperitoneal administration of 500 μl of CS-KS 3 h after I/R; I/R-CS-KS group, n = 6), and CS-NRK treatment group (I/R plus intraperitoneal administration of 500 μl of CS-NRK 3 h after I/R; I/R-CS-NRK group, n = 5). At the end of each experimental period, 24-hour urine samples were collected. The rats were sacrificed 2 days after I/R, and the kidney tissues were obtained for paraffin embedding or frozen sectioning. Blood samples were drawn from the vena cava inferior while the rats were under anesthesia. The levels of serum creatinine (S-Cr), blood urea nitrogen (BUN), urinary creatinine (U-Cr) and urinary N-acetyl-β-D-glucosaminidase (U-NAG) were measured by Oriental Yeast CO., LTD. (Tokyo, Japan). We also conducted an experiment in which rats from 3 groups, namely, the sham group (n = 6), I/R group (n = 5), and I/R-CS-KS group (n = 6), were sacrificed 4 days after I/R.

2.5. Histological examination

Collected kidney tissues were fixed in 10% buffered formalin and were embedded in paraffin. Sections (4 μm thick) were stained with hematoxylin and eosin (H&E) for light microscopy observation. Tubular damage was scored by calculation of the percentage of tubules in the corticomedullary area that displayed cell necrosis, loss of brush border, tubular dilation, and cast formation: 0, none; 1, < 10%; 2, 10%–25%; 3, 26%–45%; 4, 46%–75%; 5, > 76%. 10 high-power fields (200×) per section for each samples were examined (n = 3–6).

2.6. Immunohistochemical staining

Frozen kidney sections were subjected to cryosectioning (4 μm) and

immunofluorescence staining with primary antibody [rabbit polyclonal anti-histone H3 p-Ser10 (Millipore, Darmstadt, Germany), mouse monoclonal anti-nestin (R&D Systems, MN, USA), guinea pig polyclonal anti-nephrin (PROGEN Biotechnil GmbH, Heidelberg, Germany), rat monoclonal anti-F4/80 (Abcam) antibody, hepatocyte growth factor (HGF) (Institute of Immunology, Tokyo, Japan), epidermal growth factor (EGF) (Institute of Immunology), transforming growth factor-β (TGF-β) (Bio Vision, Milpitas, CA, USA), and erythropoietin (Epo) (Santa Cruz, CA, USA)] overnight at 4 °C after incubation with 1% bovine serum albumin (BSA) in PBS. After three washes with PBS, the sections were incubated with secondary antibody conjugated to FITC (sheep IgG; Chemicon, Temecula, CA, USA) or rhodamine (goat IgG; Chemicon) for 1 h at room temperature. The sections were washed and counterstained with 4′,6′-diamidino-2-phenylindole (DAPI; Roche Diagnostics GmbH, Mannheim, Germany). The number of histone H3 p-Ser10-positive and HGF-positive cells in 10 randomly selected fields in each kidney section was counted at 200× magnification. Nestin-positive area was evaluated in 10 randomly selected fields at 200× magnification in each kidney section using the ImageJ software. The F4/80 positive area in 5 randomly selected fields from each section was evaluated at 200× magnification using ImageJ software.

KS cells and NRK-52E cells were plated on chamber slides (16 well, ThermoFisher Scientific), allowed to grow to 90% confluence. Then, 0.05 μg/ml cisplatin with or without 10 μl of CS-KS or CS-NRK was added to the NRK-52E cells. After 24 h, the cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min, and then permeabilized with 0.01% Triton X-100 in PBS for 4 min. After incubation with 1% BSA in PBS, the cells were incubated with primary antibody (HGF, EGF, and Epo antibody) overnight at 4 °C. The cells were washed with PBS three times and then incubated with a secondary antibody conjugated to FITC or rhodamine for 1 h at room temperature. The sections were washed and counterstained with DAPI. Images were captured using an FSX100 microscope (Olympus Corporation, Tokyo, Japan).

2.7. TUNEL staining

Degrees of tubular apoptosis were assessed using *in situ* DNA nick end labeling (TUNEL) technique. Paraffin-embedded sections (4 μm thick) were processed for immunohistochemistry using the DeadEnd fluorometric TUNEL system (Promega) according to the manufacturer's instructions and were counterstained with DAPI (Roche Diagnostics GmbH). The number of TUNEL-positive cells in the outer stripe of the outer medulla in 10 randomly selected fields was counted at 200× magnification in each kidney section.

2.8. In vitro assay using electric cell-substrate impedance sensing

For the investigation of real-time cell behavior, we applied the electric cell-substrate impedance sensing (ECIS) method (Applied Biophysics, Troy, NY, USA). 1.0×10^4 KS cells were placed in each well of an 8W10E + ECIS array (Applied Biophysics) and cultured with 400 μl of medium with or without 10 μl of KS supernatant (n = 3, each) containing cisplatin (0.05 μg/ml) on type IV collagen (BD Bioscience)-coated dishes. We collected resistance, impedance, and capacitance data every 90 s. The measurement of cell adhesion was based on the changes in the ratio of resistance/capacitance to the current flow applied to the electrode arrays at different frequencies (Heijink et al., 2010). A frequency scan was performed to determine the frequency at which the greatest difference in R_{ep} was obtained between the cell-covered and cell-free electrodes. The baseline was established using culture medium (400 μl / well) alone and was compared with values obtained using electrodes covered with a monolayer of cells in 400 μl of medium.

2.9. Immunoblot analysis

SDS-PAGE and immunoblot analysis were performed as described previously (Rabb, 2005). Protein concentration was calculated using a modified Lowry Protein Assay Kit (Thermo Scientific) and we loaded 20 μg protein of CS-KS and KS cells lysate for each lane. Primary rabbit antibodies against HGF (1000× dilution), bone morphogenetic protein-7 (BMP-7, 1000× dilution) (AVIVA systems biology, San Diego, CA, USA), EGF (1000× dilution), TGF-β (1000× dilution), Epo (1000× dilution), and vascular endothelial growth factor (VEGF, 1000× dilution) (Abcam, Cambridge, MA) were used. Secondary anti-rabbit IgG antibodies (10,000× dilution) conjugated to horseradish peroxidase (BIO-RAD) were used. The images were developed using the ECL plus western blotting detection system (GE Healthcare). The bands were detected using ImageQuant LAS 4000 mini (GE Healthcare) and were evaluated using the ImageJ software.

2.10. Statistical analysis

Statistical analysis was performed by JMP (SAS Institute Inc. version 11.0.0 for Windows software). Statistical significance was defined by one-way analysis of variance (ANOVA) with the *post-hoc Turkey's* test. Data are shown as the mean ± SE. Significance was defined as $p < 0.05$.

3. Results

3.1. Culture supernatant from adult rat kidney stem cells ameliorates tubulointerstitial injury

SD rats were subjected to I/R injury and we examined the regenerative effect of culture supernatant from KS cells. Histological analysis by hematoxylin and eosin (H&E) staining showed renal tubular injury, including renal tubular necrosis, cast formation, loss of brush border and dilatation in the cortico-medullary junction area, in the rats that underwent I/R injury compared to the sham group (Fig. 1A). The damage was extensive in the I/R group and I/R-CS-NRK groups compared to the I/R-CS-KS group on day 2. Analysis by tissue injury scores revealed that the damage was significantly lower in the I/R-CS-KS group than in the I/R and I/R-CS-NRK groups on day 2 (Fig. 1B), indicating that CS-KS treatment ameliorates tubulointerstitial injury and that CS-NRK does not have this effect. Tissue damage was also lower in the I/R-CS-KS group compared to the I/R group on day 4 (Fig. 1B). Next, we examined the renal function. BUN and S-Cr levels were significantly elevated in the rats that underwent I/R injury compared to the sham group on day 2 (Fig. 1, C and D). Importantly, S-Cr and BUN levels in the I/R-CS-KS group were significantly lower than those in the I/R group on day 2 (Fig. 1C and D). In addition, U-NAG levels were significantly lower in the I/R-CS-KS group compared to the I/R group 2 days after I/R (Fig. 1E), suggesting that CS-KS treatment attenuates renal tubulointerstitial injury.

3.2. Culture supernatant from adult rat kidney stem cells inhibits apoptosis and induces proliferation of tubular epithelial cells

Tubular apoptosis was evaluated by counting the number of TUNEL-positive cells in the I/R and I/R-CS-KS groups. On day 2, the number of TUNEL-positive renal tubular cells was significantly lower in the I/R-CS-KS group compared to the I/R group (Fig. 2A), indicating that CS-KS treatment protects against tubular cell apoptosis.

Cell proliferation is indispensable for the regeneration and repair of injured renal tubules. Cell proliferation within the tubules was evaluated by immunohistochemical staining for a mitosis marker, histone H3 p-Ser10. The number of histone H3 p-Ser10-positive cells was significantly higher in the I/R-CS-KS group compared to the I/R group on day 2 and 4 (Fig. 2B), indicating that CS-KS treatment promotes tubular cell proliferation after I/R injury.

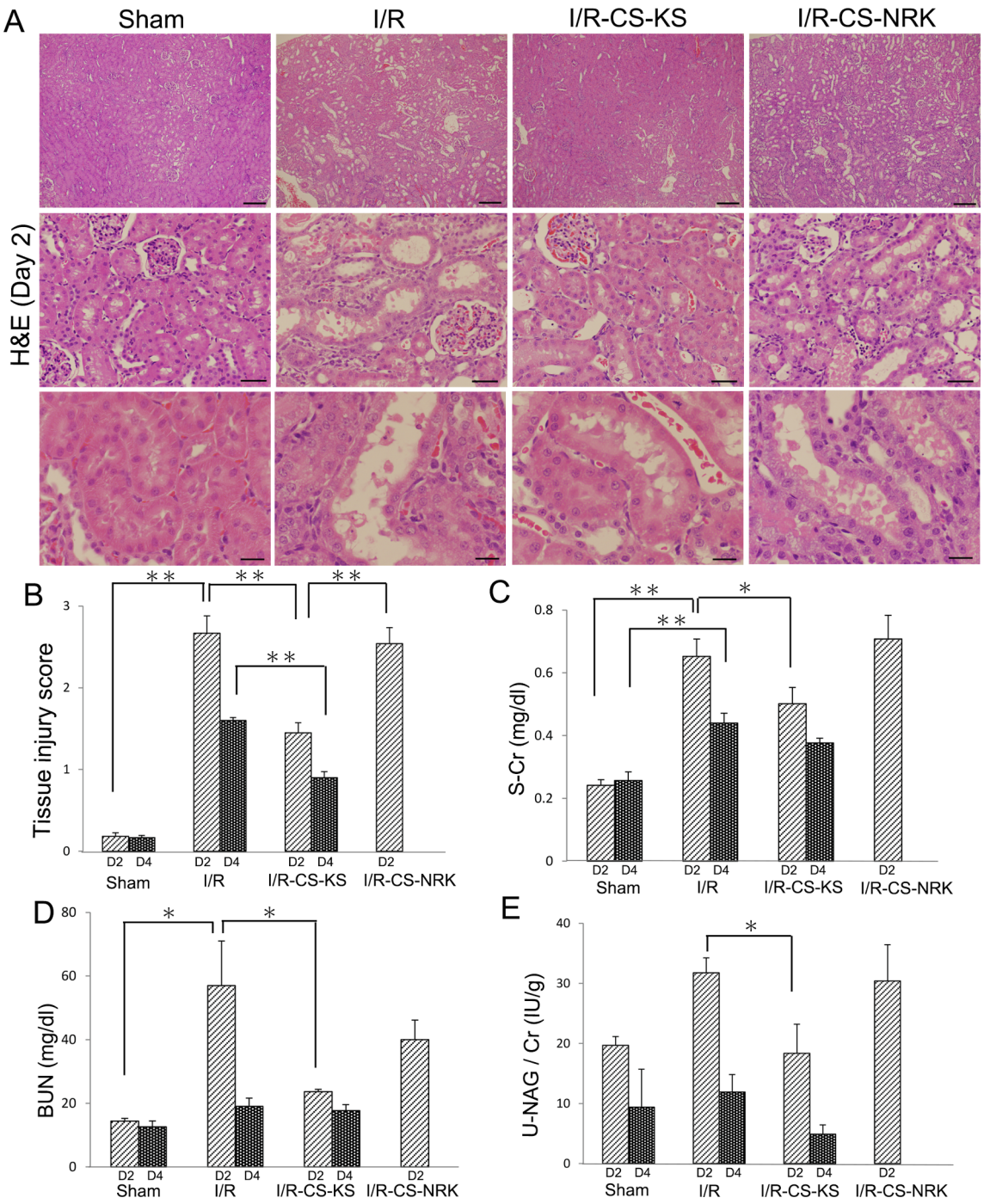

Fig. 1. Culture supernatant of adult kidney stem/progenitor cells attenuates renal function and tubular injury in an I/R model. (A) Tubulointerstitial alterations were examined by hematoxylin and eosin (H&E) staining in the sham-operated group (sham group), the group that underwent rat kidney ischemia-reperfusion (I/R) plus intraperitoneal administration of vehicle (I/R group), the group that underwent I/R plus intraperitoneal administration of CS-KS (I/R-CS-KS group), and the group that underwent I/R plus intraperitoneal administration of CS-NRK (I/R-CS-NRK group). Representative images on day 2 after kidney I/R are shown. Scale bars, 200 μm in upper panels; 50 μm in middle panels; 20 μm in lower panels. (B–E) Tissue injury scores (B), serum creatinine (S-Cr) (C), blood urea nitrogen (BUN) (D), and urinary NAG (U-NAG) (E) levels in the sham group, I/R group, I/R-CS-KS group and I/R-CS-NRK group were measured on day 2 and 4. D2; day2, D4; day4. **: $P < 0.01$; *: $P < 0.05$.

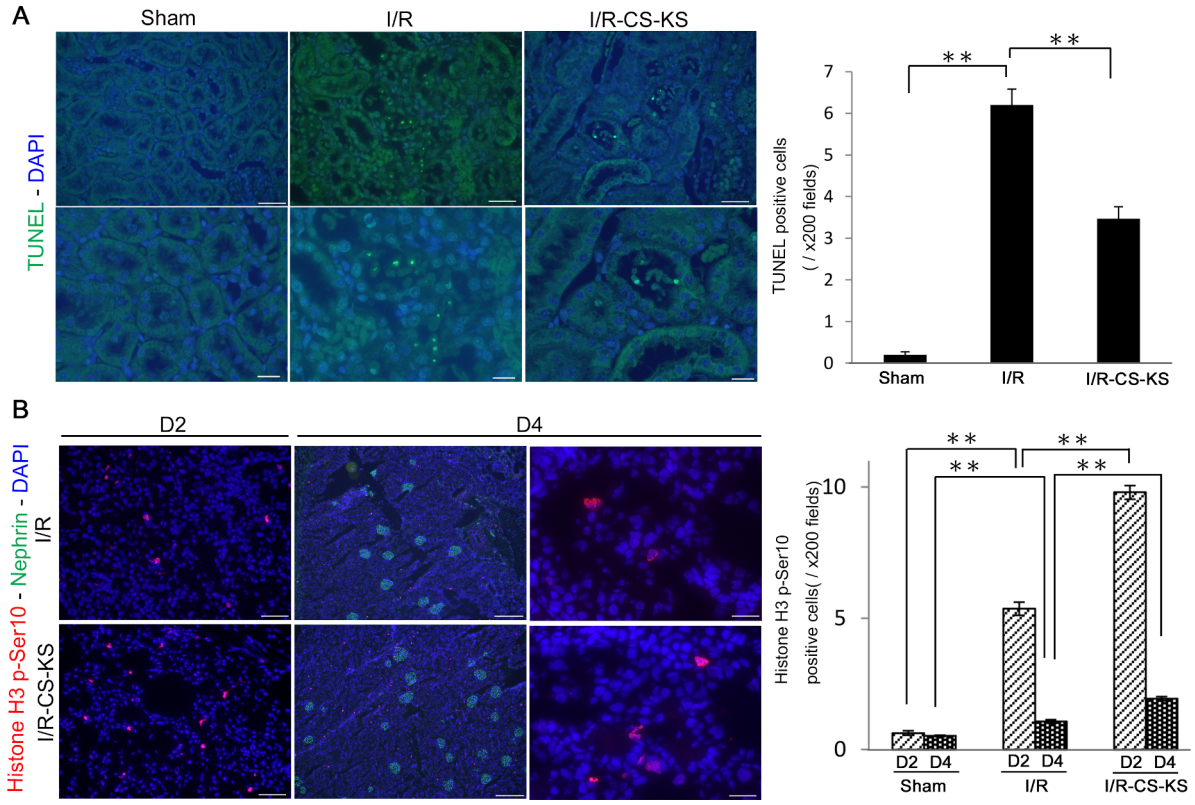

Fig. 2. Culture supernatant of adult kidney stem/progenitor cells attenuates renal cell apoptosis and activates renal cell proliferation in an I/R model. (A) Renal cell apoptosis in the group that underwent rat kidney ischemia-reperfusion (I/R) plus intraperitoneal administration on vehicle (I/R group) and the group that underwent I/R plus intraperitoneal administration of CS-KS (I/R-CS-KS group) was evaluated by Terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining (green) with DAPI staining (blue) on day 2. Scale bars, 50 μm in upper panels; 25 μm in lower panels. The graph shows the number of tubular TUNEL-positive cells/200 × field measured on day 2. (B) Anti-histone H3 p-Ser10 staining (red) with nephrin (green) and DAPI staining (blue) was performed on day 2 and 4 in the sham, I/R and I/R-CS-KS groups. Scale bars, 25 μm in left panels; 250 μm in middle panels; 50 μm in right panels. The graph shows the number of tubular histone H3 p-Ser10-positive cells/200 × -field measured on day 2 and 4. **: $P < 0.01$.

3.3. Culture supernatant from adult rat kidney stem cells attenuates renal tubulointerstitial inflammation and promotes immature renal cell proliferation

We evaluated renal tubulointerstitial inflammation. The area positive for F4/80, a marker of macrophage infiltration, was smaller in the I/R-CS-KS group than in the I/R group on day 4 (Fig. 3A and B), suggesting that CS-KS has the potential to attenuate renal tubulointerstitial inflammation in I/R injury.

A previous report revealed an increase in stem/progenitor cells, which promote renal regeneration, in rodent AKI models. Nestin has been reported to be a stem cell marker (Chen et al., 2006). Therefore, we evaluated the number of nestin-positive immature cells in the I/R model. A significant increase in the nestin-positive area was observed in the I/R-CS-KS group compared to the I/R group on day 4 (Fig. 3A and C), indicating that CS-KS has the potential to activate immature cell proliferation and/or dedifferentiation of residual tubular cells in an I/R model.

3.4. Adult rat kidney stem cells secrete growth factors

These results strongly suggest that CS-KS contains trophic factors that protect against kidney injury. Some kinds of growth factors have been reported to ameliorate kidney injuries. Therefore, we examined the expression of several growth factors, such as HGF, BMP-7, EGF, TGF-β, EPO and VEGF, which have been reported to be regenerative factors (Hammerman and Miller, 1994; Kang et al., 2001; Luo et al., 2005; Miller et al., 1994; Mohamed et al., 2013; Zeisberg et al., 2003). Immunoblot analysis revealed that CS-KS contained HGF, EGF, TGF-β and Epo (Fig. 4A). The expression of HGF, EGF, Epo in KS cells were also confirmed by immunostaining (Fig. 4B). These factors might play important roles in the regeneration process during renal injury.

We also analyzed growth factor expression in the sham, I/R, and I/R-CS-KS groups by immunofluorescence staining. While no different expression pattern was observed in EGF staining among these groups, increased Epo and TGFβ1 expressions were observed in the I/R and I/R-CS-KS groups compared to the sham group on day 2 (Supplementary Fig. 1), most likely due to renal ischemia caused by I/R injury. In addition, increased HGF expression was observed in the rats that underwent I/R injury compared to the sham group (Fig. 4C). Interestingly, the number of HGF-positive cells was higher in the I/R-CS-KS group compared to the I/R group (Fig. 4C). These results suggested that CS-KS might affect the secretomes of HGF from tubular cells or kidney stem cells.

3.5. Culture supernatant from adult rat kidney stem cells protects against cisplatin-induced cell injury and promotes the dedifferentiation of renal cells in vitro

To further confirm the trophic effect of CS-KS, immunofluorescence analyses were carried out to further evaluate the effect of CS-KS. Treating NRK-52E cells with CS-KS significantly increased the number of histone H3 p-Ser10-positive cells (Fig. 5A–C and G), indicating that CS-KS promotes mature cell proliferation. Interestingly, treating NRK-52E cells with CS-KS significantly increased the number of Nestin-positive cells (Fig. 5D–F and H), suggesting that CS-KS might lead to the transition of mature cells to immature cells, or dedifferentiation, which has been reported to play important roles during renal regeneration

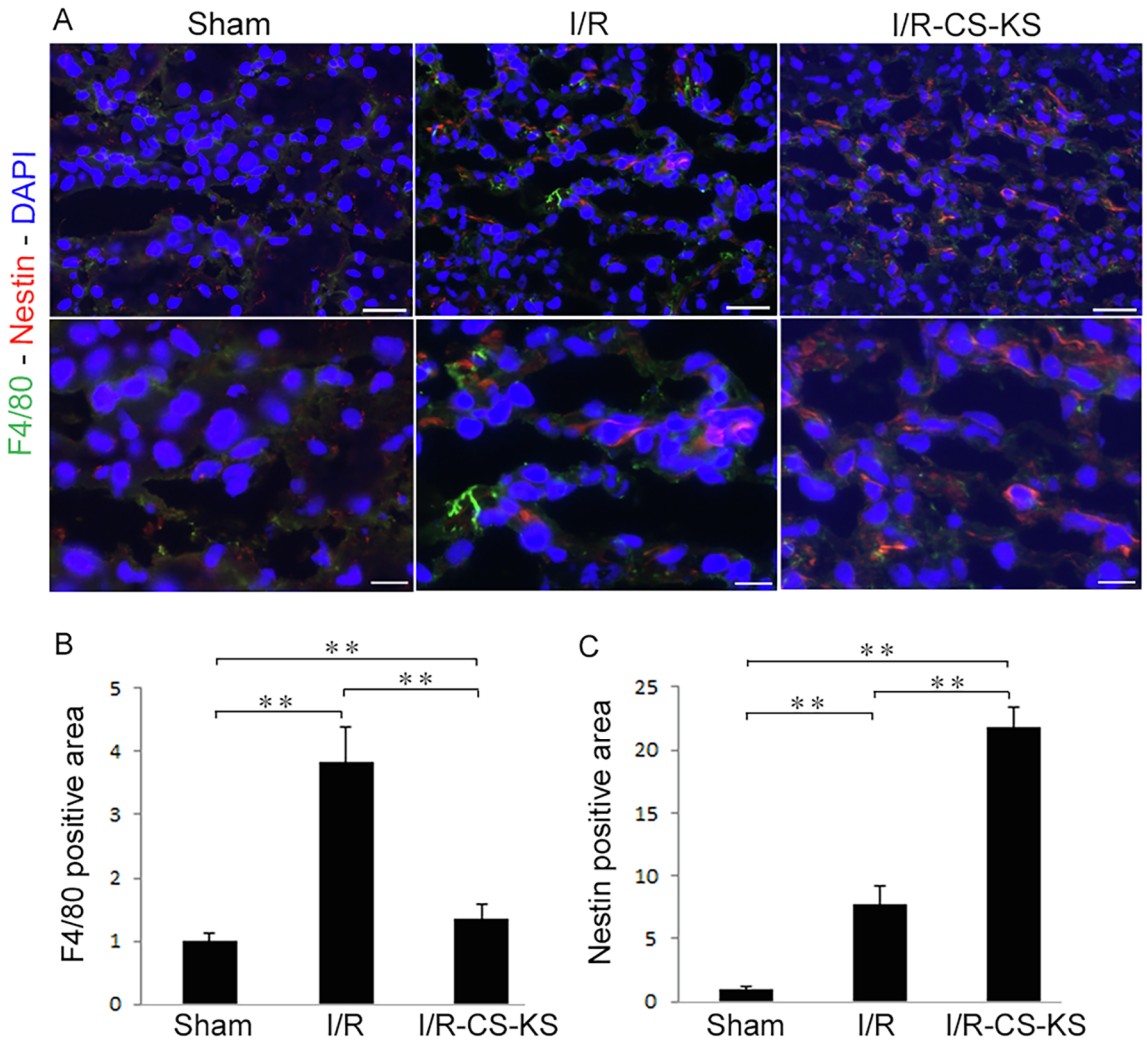

Fig. 3. Culture supernatant of adult kidney stem/progenitor cells attenuates renal inflammation and activates immature cell activity. (A) F4/80 (red) and nestin (green) staining with DAPI staining (blue) was performed in the sham-operated group (sham group), the group that underwent rat kidney ischemia-reperfusion (I/R) plus intraperitoneal administration on vehicle (I/R group) and the group that underwent I/R plus intraperitoneal administration of CS-KS (I/R-CS-KS group) on day 4. Scale bars, 25 μm in upper panels; 10 μm in lower panels. (B) The F4/80-positive area/200 × field was determined on day 4. (C) The nestin-positive area/200 × field was determined on day 4. **: $P < 0.01$.

(Kusaba et al., 2014).

To examine the autocrine effect of KS cells, we examined the effect of CS-KS *in vitro* by monitoring cell behavior after cisplatin-induced cell injury in real time using the ECIS method. In this system, high resistance indicates increased strength of intracellular junctions and connection strength between cells and their scaffolds, and low capacitance indicates active cell proliferation. KS cells were cultured in the presence of 0.05 μg/ml cisplatin with or without 10 μl of CS-KS. CS-KS treatment elevated the resistance and decreased the capacitance more rapidly than the control (no treatment) or CS-NRK treatment (Fig. 6A and B), suggesting that CS-KS might promote KS cell proliferation and attenuate cisplatin-induced KS cell apoptosis. Taken together, the results suggested that adult kidney stem cells might work in both an paracrine and autocrine manner to promote renal regeneration.

4. Discussion

Treatment with stem cells has been reported to be beneficial in various kidney disorders. However, the protective mechanisms of these stem cells are still controversial. Several reports have suggested the involvement of different mechanisms, including the direct differentiation of stem/progenitor cells and the protection of residual cells by regenerative growth factors secreted from stem cells (Tsuji and Kitamura, 2015). We previously reported that implanted adult kidney stem/progenitor cells, which directly contribute to renal regeneration. Tögel et al. reported that the paracrine effect is primarily responsible for the organ-protective actions of administered mesenchymal stem cells (Togel et al., 2005). Bi et al. reported that conditioned medium from bone marrow mesenchymal stem cells (BMSCs) decreases tubular cell apoptosis, increases the survival time of animals and limits renal injury (Bi et al., 2007). Along this line, MSCs have been reported to secrete a variety of factors, such as HGF, insulin-like growth factor-1 (IGF-1), EGF, VEGF, prostaglandin E2 (PGE2), granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M−CSF) (Aggarwal and Pittenger, 2005; Caplan and Dennis, 2006; Groh et al., 2005; Togel et al., 2005). Moreover, the injection of HGF and IGF-1 has been reported to reduce renal tubular injury (Miller et al., 1992; Miller et al., 1994). Taken together, the evidence suggests that trophic factors from stem cells promote renal regeneration after injury. In the present study, we focused on factors secreted by adult kidney stem/progenitor cells and revealed that these cells produce a variety of trophic factors, that lead to renal regeneration by promoting cell proliferation and inhibiting renal cell apoptosis both *in vivo* and *in vitro*. Interestingly, the trophic mechanisms of renal repair were similar to

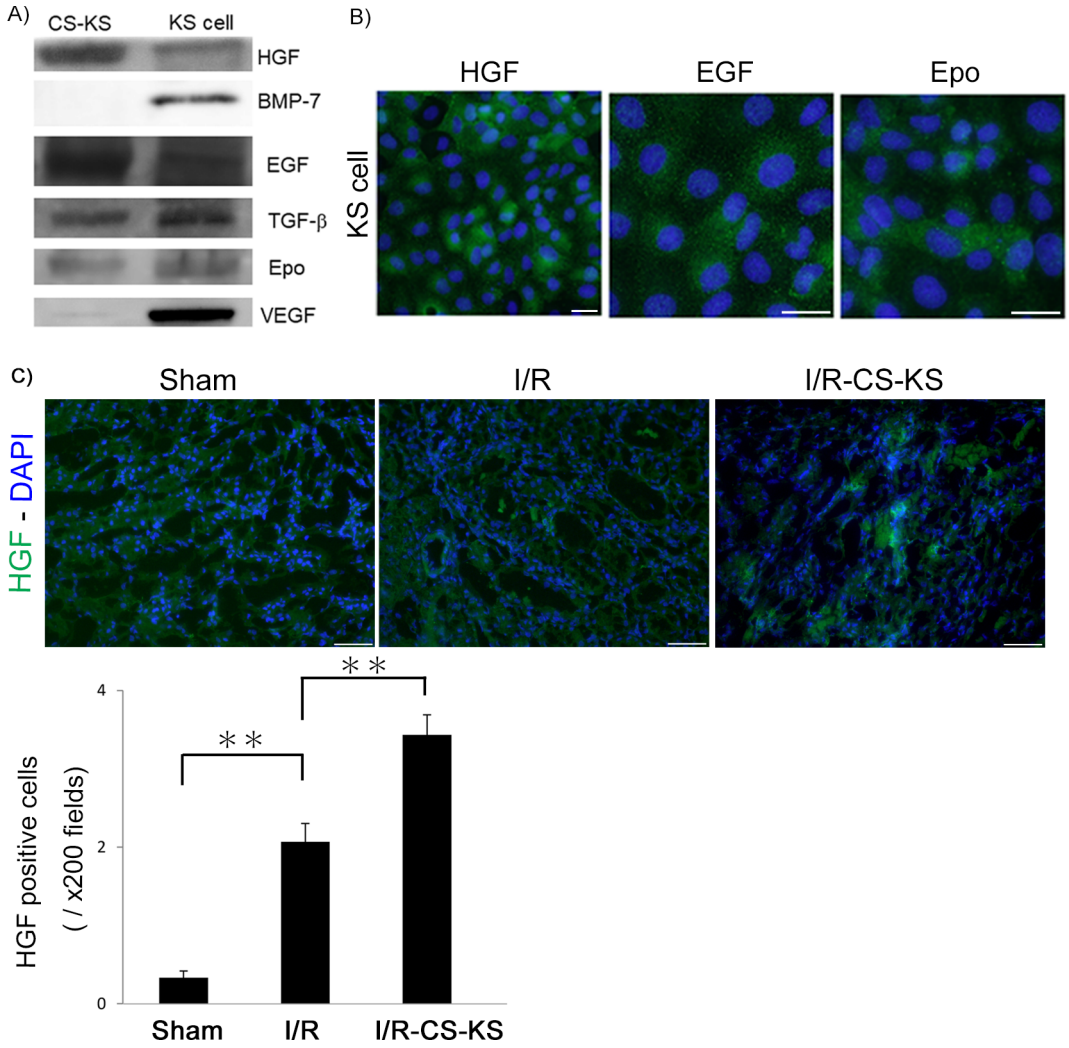

Fig. 4. Trophic factors in the culture supernatant of adult kidney stem/progenitor cells. (A) HGF, BMP-7, EGF, TGF-β, Epo, and VEGF expression in CS-KS and KS cells was analyzed by immunoblot analysis. 20 μg of the protein lysate was loaded in each lane. (B) Immunofluorescence staining for HGF, EGF and Epo in KS cells. Scale bars, 25 μm. (C) HGF (green) staining with DAPI staining (blue) was performed in the sham-operated group (sham group), the group that underwent rat kidney ischemia-reperfusion (I/R) plus intraperitoneal administration on vehicle (I/R group) and the group that underwent I/R plus intraperitoneal administration of CS-KS (I/R-CS-KS group) on day 2. Scale bars, 50 μm. The graph shows the HGF-positive cells/200 × field determined on day 2. **: $P < 0.01$.

those previously reported to underlie the effects of the injection of KS cells themselves. Based on these results together with the previous report that the number of implanted KS cells that directly differentiated into mature cells was limited, we conclude that the indirect therapeutic mechanism involved in renal regeneration induced by trophic factors from KS cells might be the major contributor to renal repair under KS cell therapy. Importantly, we also suggest that the dedifferentiation of residual tubular cells might occur via trophic factors. Kusaba et al. previously reported that tubular epithelial cells contribute to renal repair through dedifferentiation and replacement with stem cell markers (Kusaba et al., 2014). Taken together, these findings suggest that renal stem/progenitor cells promote this process through the secretion of trophic factors.

Sallustio et al. previously reported that CD133(+) human renal stem/progenitor cells repair tubular injury through TLR2-driven inhibin-A and microvesicle-shuttled decorin (Sallustio et al., 2013). Aggarwal et al. reported that human CD133(+) renal progenitor cells limit renal fibrosis after tubular injury through the secretion of erythropoietin (Aggarwal et al., 2016). More recently, Ranghino et al. reported that CD133(+) cells, but not extracellular vesicles from CD133(+) cells, contribute to renal regeneration after ischemia/reperfusion renal injury (Ranghino et al., 2017). These CD133(+) progenitor cells lack six2 expression (Brossa et al., 2018), while KS cells have high six2 expression, indicating a difference in the characteristics of CD133(+) progenitor cells and KS cells. Indeed, CS-KS treatment might activate tubular cell dedifferentiation, which was not reported with CD133(+) cell treatment.

We injected the 40× concentrated culture supernatant of KS cells *in vivo* based on the fact that the injected supernatant would be diluted by absorption in the intraperitoneal cavity before ultimately reaching the injured kidney. Our study revealed indirect regenerative effects of trophic factors from adult kidney stem/progenitor cells, which were evaluated based on improvements in tubular injury markers and renal function. We assumed that adult kidney stem/progenitor cells in the S3 segment proliferate and secrete trophic factors in both a paracrine and autocrine manner and that these processes promote renal regeneration and attenuate renal injury during or after kidney injury, including I/R injury. Therefore, it is important to further explore how adult kidney stem/progenitor cells in the S3 segment are activated during kidney injury and how we can promote stem cell-mediated renal regeneration.

Adult kidney stem/progenitor cells secrete a variety of trophic factors, which make it difficult to identify the main factor that contributes to the promotion of regeneration. Based on these results showing some of the therapeutic mechanisms of CS-KS, including anti-inflammation,

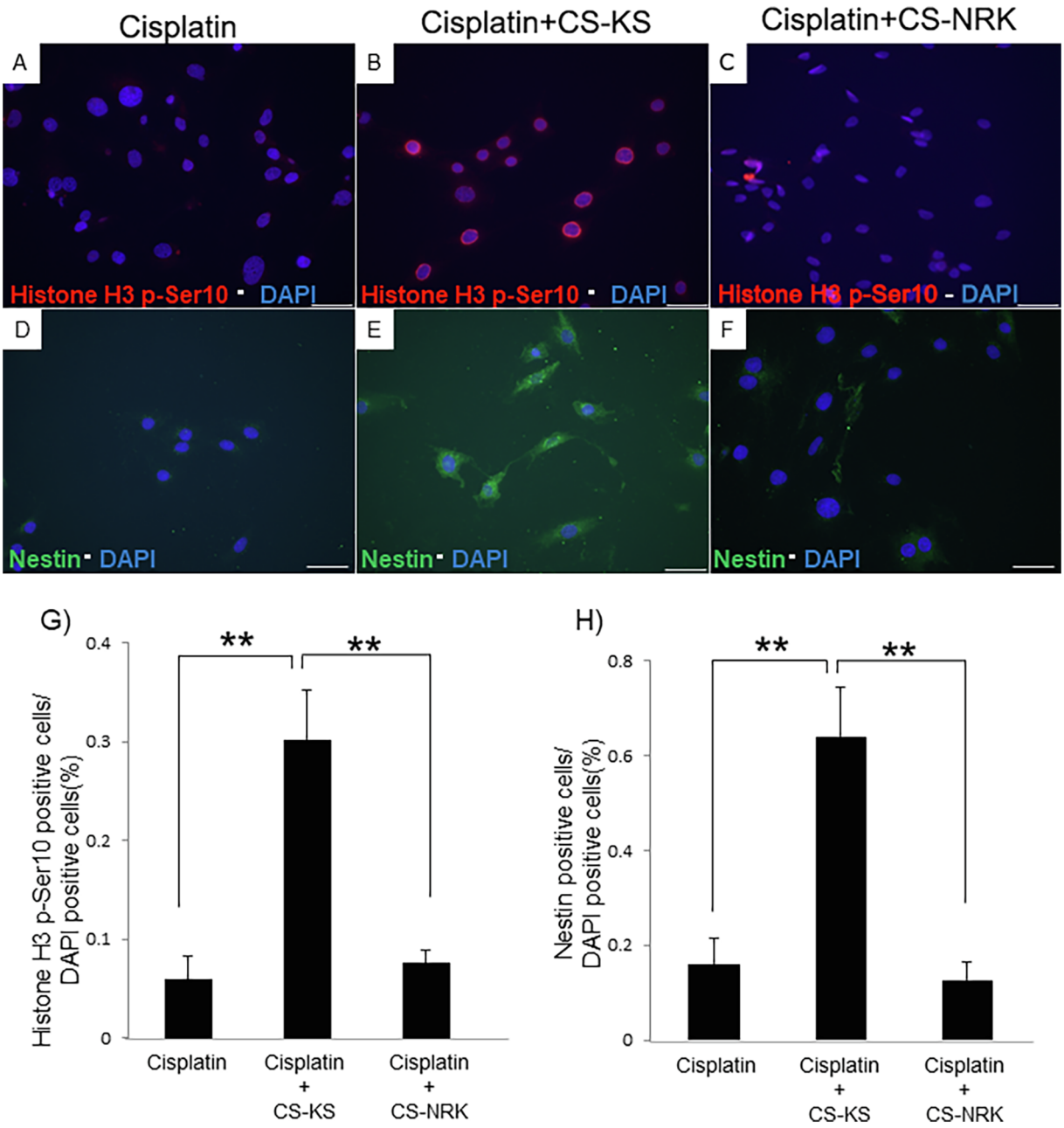

Fig. 5. Effects of culture supernatant of adult kidney stem/progenitor cells on NRK-52E cells. Anti-histone H3 p-Ser10 staining (red) or anti-Nestin staining (green) with DAPI staining (blue) was performed on NRK-52E cells treated with 0.05 μg/ml cisplatin (cisplatin, A; red, D; green), NRK-52E cells treated with 0.05 μg/ml cisplatin containing 10 μl of KS supernatant (cisplatin + CS-KS; red, B; green, E), and NRK-52E cells treated with 0.05 μg/ml cisplatin containing 10 μl of CS-NRK (cisplatin + CS-NRK; red, C; green, F). Scale bars, 50 μm in each panels. The ratios of histone H3 p-Ser10-positive cells (G) and nestin-positive cells/DAPI-positive area (H) were analyzed. CS-KS treatment resulted in a significant increase in the number of histone H3 p-Ser10-positive cells and nestin-positive area. The number of histone H3 p-Ser10-positive cells was determined in 5 randomly selected fields from each section at 200× magnification. The area of nestin-positive area was evaluated in 5 randomly selected fields at 200× magnification in each section. **: $P < 0.01$.

anti-apoptosis, and proliferation, several secreted factors from adult kidney stem/progenitor cells likely affect the regeneration of residual renal cells and immature cells together (Fig. 7). Importantly, the factors secreted from NRK-52E cells do not have regenerative effects, indicating that adult kidney stem/progenitor cells specifically indirectly contribute to regeneration. Interestingly, the number of HGF-positive cells was higher in the I/R-CS-KS group compared to the I/R group, suggesting that trophic factors from kidney stem cells might affect the secretomes from cells in the kidney. It was difficult to distinguish which cells produce HGF after CS-KS treatment, but it might be the adult stem/progenitor cells stimulated by CS-KS or dedifferentiated tubular cells by CS-KS.

Recent reports have revealed that stem cells secrete extracellular vesicles containing mRNA, protein and micro RNAs, which have important roles in cell–cell communication, in addition to trophic proteins, including growth factors. These factors have been reported to have essential roles during tissue injury and regeneration (Tsuji and Kitamura, 2015). For example, a recent report revealed that adult kidney stem/progenitor cells have the potential to secrete microvesicles that contain protective mRNAs (Orlic et al., 2003). CS-KS might contain exosomes, which might be regenerative factors. Recent studies have revealed that exosomes from MSCs attenuate renal injury (Bruno et al.,

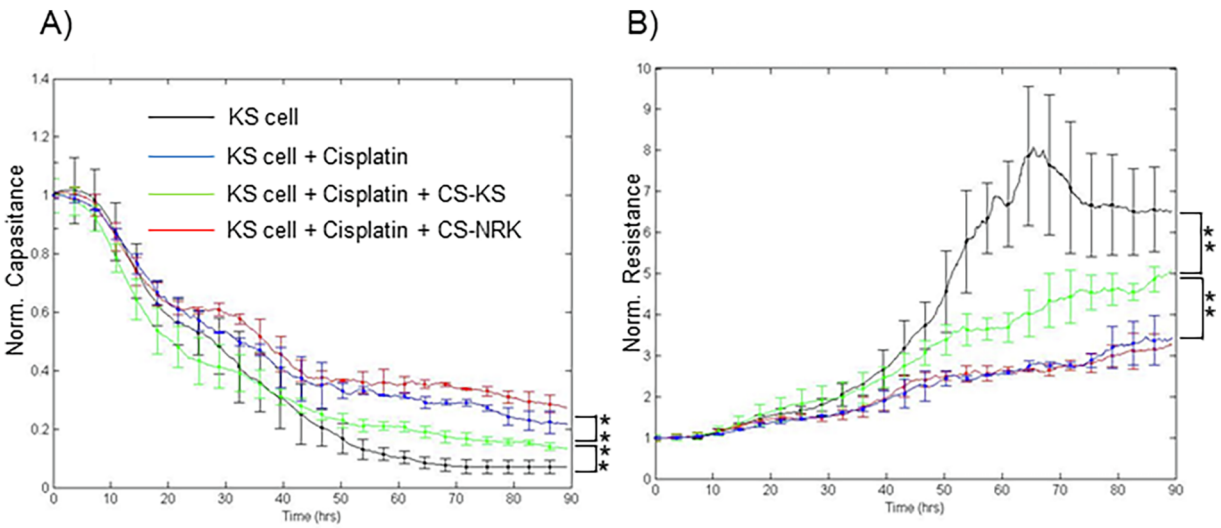

Fig. 6. Culture supernatant of adult kidney stem/progenitor cells attenuates cisplatin-induced cell apoptosis *in vitro*. Real-time monitoring of cell behavior after cisplatin-induced cell injury was conducted using the electric cell-substrate impedance sensing (ECIS) method. Capacitance (64 kHz; A) and resistance (4000 Hz; B) data were collected every 90 s for control cells (KS cells), cells treated with 0.05 μg/ml cisplatin (KS cells + cisplatin), cells treated with 0.05 μg/ml cisplatin containing 10 μl of KS supernatant (KS cells + cisplatin + CS-KS), and cells treated with 0.05 μg/ml cisplatin containing 10 μl of CS-NRK (KS cells + cisplatin + CS-NRK). **: $P < 0.01$.

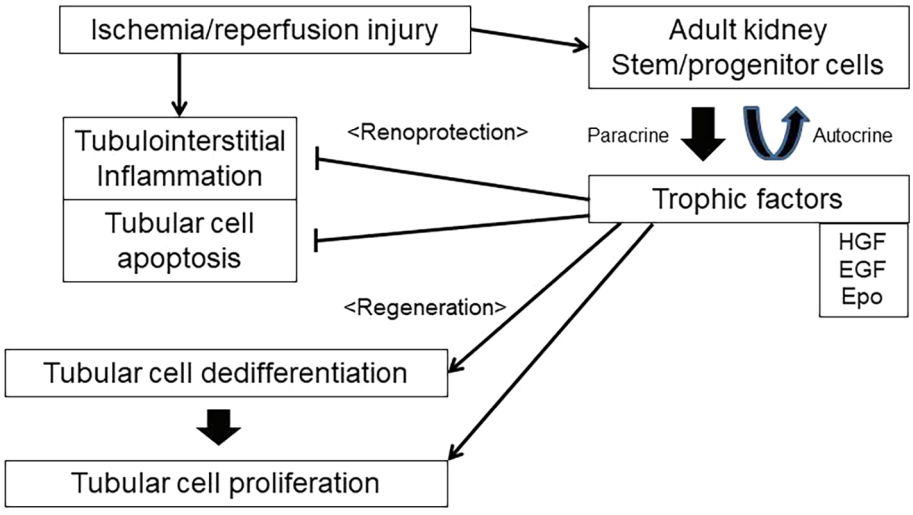

Fig. 7. Therapeutic mechanism of the effects of culture supernatant from adult kidney stem/progenitor cells against I/R injury. Rat kidney ischemia-reperfusion injury (I/R) causes tubulointerstitial inflammation and tubular cell apoptosis. Trophic factors from adult kidney stem/progenitor cells promote renal regeneration in both paracrine and autocrine manner. These mechanisms include the attenuation of tubular cell apoptosis and renal inflammation as well as the activation of immature cell proliferation and residual tubular cell proliferation partly via tubular cell dedifferentiation.

2012, 2009; Gatti et al., 2011; He et al., 2012; Zhou et al., 2013). The exploration of exosomes in CS-KS might have the potential to open the new therapies for renal regeneration.

There are several limitations to be discussed in the study. First, CS-KS was injected only once (3 h after I/R injury) *in vivo*. Because the supply of trophic factors from stem/progenitor cells might be continuous during the regenerative period under physiological conditions, we need to keep in mind that there might be differences in both the concentration of these factors and the timing of trophic factor supply between intraperitoneal treatment and physiological conditions during renal regeneration. Second, we only focused on the acute phase of renal regeneration in this experiments, since we evaluated the renal tissue in 2 time points of day 2 and day 4. Although we elucidated that CS-KS treatment promoted regeneration through a variety of different mechanisms, including promotion of proliferation and de-differentiation, and inhibition of cell apoptosis and renal interstitial inflammation, further analysis is required for elucidating how long these trophic factors can maintain regenerative effects and whether CS-KS can protect from renal injury in the chronic stage.

5. Conclusions

Adult kidney stem/progenitor cells contribute to renal regeneration through the secretion of protective factors.

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Availability of data and materials

All data generated and analyzed in this study are included in this publication.

Authors' contributions

KT: study design, conduct experiment, data analysis, writing and revising the manuscript. YS: conduct experiment. KF: conduct experiment. SK: study design, data evaluation, revising the manuscript. JW: revising manuscript. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Kenji Tsuji: Investigation, Writing - original draft, Funding acquisition. **Shinji Kitamura:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision, Project administration. **Yizhen Sang:** Investigation, Writing - review & editing. **Kazuhiko Fukushima:** Investigation, Writing - review & editing. **Jun Wada:** Conceptualization, Methodology, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2020.101865.

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