

# Mesenchymal stem cell injection ameliorates chronic renal failure in a rat model

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## A B S T R A C T

CKD (chronic kidney disease) has become a public health problem. The therapeutic approaches have been able to reduce proteinuria, but have not been successful in limiting disease progression. In this setting, cell therapies associated with regenerative effects are attracting increasing interest. We evaluated the effect of MSC (mesenchymal stem cells) on the progression of CKD and the expression of molecular biomarkers associated with regenerative effects. Adult male Sprague–Dawley rats subjected to 5/6 NPX (nephrectomy) received a single intravenous infusion of  $0.5 \times 10^6$  MSC or culture medium. A sham group subjected to the same injection was used as the control. Rats were killed 5 weeks after MSC infusion. Dye tracking of MSC was followed by immunofluorescence analysis. Kidney function was evaluated using plasma creatinine. Structural damage was evaluated by H&E (haematoxylin and eosin) staining, ED-1 abundance (macrophages) and interstitial  $\alpha$ -SMA ( $\alpha$ -smooth muscle actin). Repairing processes were evaluated by functional and structural analyses and angiogenic/epitheliogenic protein expression. MSC could be detected in kidney tissues from NPX animals treated with intravenous cell infusion. This group presented a marked reduction in plasma creatinine levels and damage markers ED-1 and  $\alpha$ -SMA ( $P < 0.05$ ). In addition, treated rats exhibited a significant induction in epitheliogenic [Pax-2, bFGF (basic fibroblast growth factor) and BMP-7 (bone morphogenetic protein-7)] and angiogenic [VEGF (vascular endothelial growth factor) and Tie-2] proteins. The expression of these biomarkers of regeneration was significantly related to the increase in renal function. Many aspects of the cell therapy in CKD remain to be investigated in more detail: for example, its safety, low cost and the possible need for repeated cell injections over time. Beyond the undeniable importance of these issues, what still needs to be clarified is whether MSC administration has a real effect on the treatment of this pathology. It is precisely to this point that the present study aims to contribute.

**Key words:** angiogene, chronic kidney disease, epitheliogene, mesenchymal stem cell, renal functional recovery.

**Abbreviations:** Ab, antibody; ARF, acute renal failure; AU, arbitrary units; BCIP, 5-bromo-4-chloroindol-3-yl phosphate; bFGF, basic fibroblast growth factor; BMP-7, bone morphogenetic protein-7; CKD, chronic kidney disease; CMFDA, 5-chloromethylfluorescein diaconate; DAPI, 4',6-diamidino-2-phenylindole; EMT, epithelial mesenchymal transition; ESRD, end-stage renal disease; GFP, green fluorescence protein; H&E, haematoxylin and eosin; IHC, immunohistochemistry; MSC, mesenchymal stem cell(s); NBT, Nitro Blue Tetrazolium; NPX, nephrectomy; Oct-4, octamer-binding protein-4; PAP, peroxidase-anti-peroxidase; SC, stem cell(s); SD, Sprague–Dawley;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; VEGF, vascular endothelial growth factor; WB, Western blotting.

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## INTRODUCTION

CKD (chronic kidney disease) affects millions of people in the world and represents an important public health problem [1,2], owing to its increasing incidence and prevalence and the high costs of treatment. The latest estimates of the World Health Organization suggest a CKD prevalence of over 10%, including ESRD (end-stage renal disease) [3].

Many therapies have been developed in an attempt to reduce damage progression in CKD. The major impact on reducing the risk of developing ESRD has been achieved by renin-angiotensin-aldosterone system blockers [4,5]. Although administration of these drugs reduces proteinuria, they have not shown significant improvement in the GFR (glomerular filtration rate) or tubular function; moreover, the chronic administration of these drugs increases the risk of hyperkalaemia [5]. These facts have fuelled the search for new therapeutic strategies that could help achieve better results.

Recent studies have reported on resident kidney SC (stem cells), supporting the idea of a possible physiological mechanism of kidney repair, based on the proliferation and differentiation of SC [6–10]. MSC (mesenchymal stem cells) are involved in the regeneration of many tissues subjected to different types of injury [11–15].

On the other hand, previous investigations have reported the ability of the kidney to repair itself after ischaemic ARF (acute renal failure) [7,16,17]. We have demonstrated in an experimental model of ARF the re-expression of epitheliogenic and angiogenic proteins involved in kidney development in a similar pattern to that observed during embryogenesis [17], suggesting a pathway by which the repair might be achieved.

The aim of the present study was to evaluate the effect of MSC on the progression of damage in CKD. We hypothesized that the MSC injection may have positive effects delaying or even abolishing tissue injury in this disease. We propose that MSC may stimulate kidney repair by inducing the expression of repairing proteins that in turn would activate genes involved in the regeneration processes. To evaluate the potential action of MSC, functional, morphological and molecular studies were conducted.

## MATERIALS AND METHODS

### Animals

Adult male SD (Sprague-Dawley) rats (220–250 g) were maintained under a 12 h light/12 h dark cycle, with food and water *ad libitum* at the University animal care facilities. All procedures were in accordance with institutional and international standards for the human care and use of laboratory animals [Animal Welfare

Assurance Publication A5427-01, Office for Protection from Research Risks, Division of Animal Welfare, NIH (National Institutes of Health), Bethesda, MD, U.S.A.], as described previously [18].

### MSC isolation and *in vitro* expansion

Bone marrow cells from adult SD rats were collected by flushing femurs and tibias with sterile PBS passed through a 70  $\mu\text{m}$  Falcon cell strainer (BD Biosciences) and centrifuged at 350 g for 10 min. After centrifugation, cells were resuspended in  $\alpha$ -MEM ( $\alpha$ -modified Eagle's medium; Gibco), supplemented with 10% heat-inactivated MSC-qualified FBS (fetal bovine serum; Gibco) with 100 units/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin (Gibco), and then cultured at a density of  $10^6$  nucleated cells/ $\text{cm}^2$  at 37°C in a 5%  $\text{CO}_2$  atmosphere. Non-adherent cells were removed after 48 h and culture medium was changed every 3 days. At 80% confluency, cells were subcultured by treatment with triple™ selects (Gibco), washed and cultured at  $10^4$  cells/ $\text{cm}^2$ . After three passages, adherent cells were detached, washed and resuspended in X-Vivo medium.

### MSC characterization

MSC are characterized by their adherence, fibroblast-like morphology and capacity to differentiate into three specific cell lineages: adipocytes, chondrocytes and osteoblasts. To induce adipogenic differentiation, confluent cells were cultured in a medium supplemented with  $10^{-6}$  M dexamethasone, 0.02 mg/ml indomethacin and 10  $\mu\text{g}/\text{ml}$  insulin (Sigma-Aldrich). After 12 days, cell differentiation into lipid-laden adipocytes was confirmed by Oil Red O staining (Sigma-Aldrich). For chondrogenic differentiation, cells were incubated at  $5 \times 10^3$  cells/ $\mu\text{l}$  in 10  $\mu\text{l}$  of culture medium for 1 h to achieve the conditions for micromass formation. Cells were cultured in a medium supplemented with  $10^{-7}$  M dexamethasone, 50  $\mu\text{g}/\text{ml}$  ascorbic acid and 10 ng/ml of TGF- $\beta$ 3 (transforming growth factor- $\beta$ 3; Sigma-Aldrich) for 7 days, assessing chondrogenic differentiation with Safranin O staining (Merck). To induce osteogenic differentiation, adherent cells were grown at  $3 \times 10^4$  cells/ $\text{cm}^2$  in culture medium with  $10^{-7}$  M dexamethasone, 50 g/ml ascorbic acid and 10 mM 2-glycerophosphate (Sigma-Aldrich). After 21 days of culture, calcium deposits were detected by Alizarin Red staining (Sigma-Aldrich).

### *In vivo* MSC tracking

To assess the migration of MSC to the kidney, we performed cell tracking experiments with dye-labelled cells [19]. The long-term tracker Green CMFDA (5-chloromethylfluorescein diaconate; Molecular Probes) was used since it is retained through several generations in living cells [20,21].

Briefly, once detached, the suspension of MSC in X-Vivo medium was incubated with 5  $\mu\text{M}$  of Green CMFDA or with DMSO (vehicle) for 30 min at 37 °C, according to the manufacturer's recommendations. Then, the MSC suspension was centrifuged at 350 *g* for 5 min and resuspended in PBS. After repeating this washing twice, the MSC suspension was centrifuged again and the pellet resuspended in X-Vivo medium and was equilibrated at 37 °C for 30 min. Finally, rats were injected with labelled cells or MSC vehicle as described above. After 24 h, rats were killed and kidneys were quickly dissected, embedded in Tissue-Tek, frozen with liquid nitrogen and stored at -80 °C. Sagittal cryostat sections (5  $\mu\text{m}$  thick) were cut and fixed with ethanol (70%) for 20 min at -20 °C. After washing in PBS, slides were washed with distilled water and counterstained with H&E (haematoxylin and eosin) as described previously [22]. Slides were observed in an Olympus BX 51W11 upright microscope with water immersion lenses. The NIH Image program ImageJ (<http://rsbweb.nih.gov/ij/>) was used to perform the imaging analyses.

### Immunofluorescence analysis

To corroborate that infused MSC home to the kidney, we performed immunofluorescence analysis in kidney sections fixed with ethanol as described above. Briefly, tissue sections were incubated overnight at 4 °C with the primary anti-CD73 Ab (antibody) (R&D Systems) which has been described as an MSC marker [23]. After washing with PBS, the secondary Ab was incubated at room temperature (25 °C). We used an F(ab')<sub>2</sub> goat anti-mouse secondary Ab from Jackson ImmunoResearch to avoid non-specific Fc binding. Nucleic acid staining was done, after washing secondary Ab, by incubating kidney sections with DAPI (4',6-diamidino-2-phenylindole; 5  $\mu\text{M}$ ) from Molecular Probes. Slides were observed in an Olympus BX 51W11 upright microscope with water immersion lenses.

Immunofluorescence analyses of MSC growth on coverslips previous to injection were performed and CD73 reactivity was observed (results not shown).

### CKD induced by NPX (nephrectomy) and MSC injection

A model that mimics the structural and functional damage of CKD was used [24]. Rats were anaesthetized with ketamine/xylazine (10:1 mg/kg of body weight, intraperitoneal); then a retroabdominal incision in the left flank was performed and the kidney mass was reduced by clamping two renal artery subdivisions; after 1 week, rats were subjected to a contralateral NPX. This moment was considered as the initiation of kidney damage, which was prolonged for 5 weeks. Animals were randomized in four groups: (i) NPX rats injected in the tail vein with 450  $\mu\text{l}$  (0.5  $\times 10^6$  cells) of MSC in X-Vivo medium

[25], (ii) NPX rats injected with fresh X-Vivo medium (without MSC); (iii) sham rats injected in a tail vein with 450  $\mu\text{l}$  of MSC (0.5  $\times 10^6$ ) in X-Vivo medium; and (iv) sham rats injected with X-Vivo medium (without MSC) ( $n = 7$  for all groups). All rats were injected with the corresponding solution immediately after contralateral NPX. Rats were killed 35 days after NPX; the kidney was processed for IHC (immunohistochemistry) and WB (Western blotting).

### Functional and histological damage assessment

Functional damage was assessed through plasma creatinine levels [26]. Tissue damage was evaluated by morphological analysis using H&E staining and IHC of ED-1 and  $\alpha$ -SMA ( $\alpha$ -smooth muscle actin).

### Tissue processing and IHC analysis

IHC studies in Paraplast-embedded sections were conducted as previously described [27]. Immunolocalization studies were conducted using an indirect immunoperoxidase technique as described in [27]. Briefly, tissue sections were incubated with the primary Ab, followed by incubation with the corresponding secondary Ab and with the PAP (peroxidase-anti-peroxidase) complex, revealed using DAB (3,3'-diaminobenzidine). For some Abs, immunoreactivity was revealed using a secondary Ab conjugated with alkaline phosphatase in the presence of NBT (Nitro Blue Tetrazolium)/BCIP (5-bromo-4-chloroindol-3-yl phosphate) (9:7  $\mu\text{l}/\text{ml}$ ) in Tris buffer (100 mM; pH 9.5). Controls for the immunostaining procedure were prepared by excluding the first Ab by replacing it with normal or preimmune serum of the same species [28].

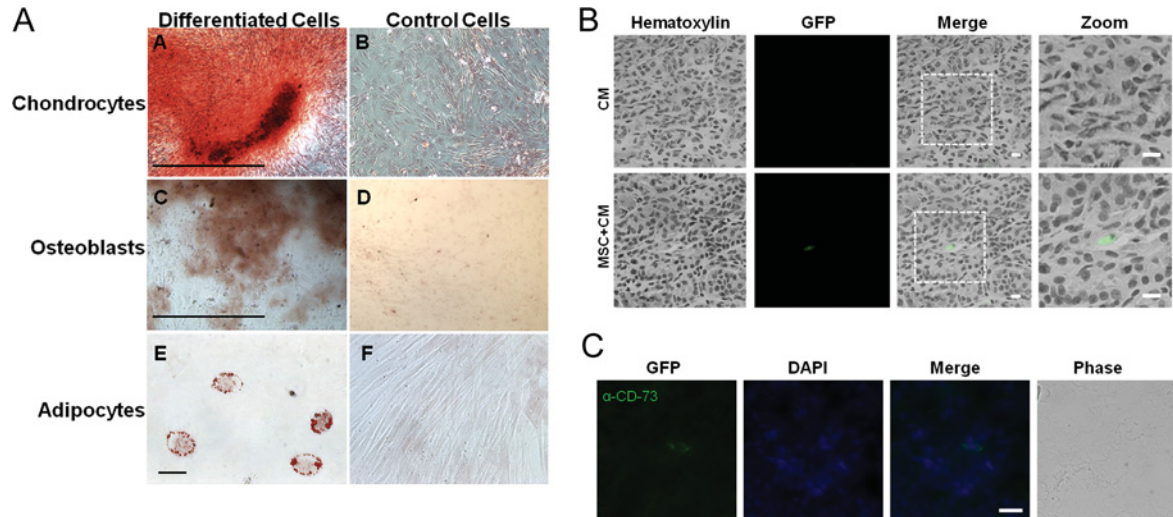
### Antibodies

The primary Abs used were the same as we have used previously [17,18,26,27]: Pax-2, BMP-7 (bone morphogenetic protein-7), bFGF (basic fibroblast growth factor), Tie-2 and VEGF (vascular endothelial growth factor) (all from Santa Cruz Biotechnology). The anti-ED-1 Ab was from Biosource and the anti- $\alpha$ -SMA Ab was from Sigma-Aldrich. The presence of SC was determined using an anti-Oct-4 (octamer-binding protein-4) Ab (Santa Cruz Biotechnology).

The secondary Ab and the corresponding PAP complexes were purchased from MP Biomedicals. Other chemicals were purchased from Sigma-Aldrich.

### Immunoblotting

Kidney sections were homogenized and protein concentration was determined as previously described [27]. WB was performed as described by Harlow and Lane [29]. For SDS/PAGE, proteins were mixed with sample buffer [100 mM Tris/HCl, pH 6.8, 200 mM



**Figure 1** MSC characterization (A), MSC dye tracking *in vivo* (B), and CD73 + MSC in the kidney (C)

(A) MSC were characterized by their capacity to differentiate into three specific cell lineages: chondrocytes, osteoblasts and adipocytes. The chondrogenic differentiation was confirmed after 7 culture days by Safranin O staining (AA). The osteogenic differentiation was confirmed after 21 culture days by Alizarin Red staining (AC). The adipogenic differentiation was confirmed after 12 culture days by Oil Red O staining (AE). (AB, AD and AF) The corresponding controls. Scale bar, 200  $\mu\text{m}$  (AA–AD) and 50  $\mu\text{m}$  (AE and AF). (B) Microscopic images show kidney sections 24 h after MSC injection incubated with vehicle (upper panel) or with the cell tracker Green CMFDA (lower panel). The nuclei were counterstained with H&E (dark). The right panel (zoom) corresponds to the area enclosed by the segmented square in the merge panel. CM corresponds to kidney injected with culture medium and CM + MSC corresponds to kidney injected with MSC. Scale bar, 10  $\mu\text{m}$ . (C) CD73, an MSC marker, was observed in kidney sections 24 h after MSC injection (GFP panel). DAPI nucleic acid staining is shown in blue. The right panels correspond to the Merge and Phase microscope techniques. Scale bar, 20  $\mu\text{m}$ .

dithiothreitol, 4% (w/v) SDS, 0.2% Bromophenol Blue and 20% (v/v) glycerol], transferred to nitrocellulose membranes and blocked as previously described [27]. After blocking, membranes were probed with the Ab, washed with TBS-T (Tris-buffered saline containing 1% Tween 20) and incubated with HRP (horseradish peroxidase)-conjugated secondary Ab for 2 h at room temperature. Immunoreactivity was detected using enhanced chemiluminescence obtained from Pierce. Basal controls were obtained from sham kidneys.

Densitometry analyses were performed using the NIH Image program v1.61 (NIH, <http://rsb.info.nih.gov/nih-image>).  $\alpha$ -Tubulin total protein levels were used to correct variation in sample loading.

### Statistical analysis

The Mann–Whitney *U* test was used with a significance level:  $P < 0.05$ . Densitometry values are presented as means  $\pm$  S.D. Values are presented in AU (arbitrary units).

## RESULTS

### Functional characterization of MSC

Bone marrow-derived MSC from SD rats with a stable fibroblast-like phenotype were isolated by adherence separation. The MSC were able to differentiate into adipocytes, chondrocytes and osteoblasts (Figure 1).

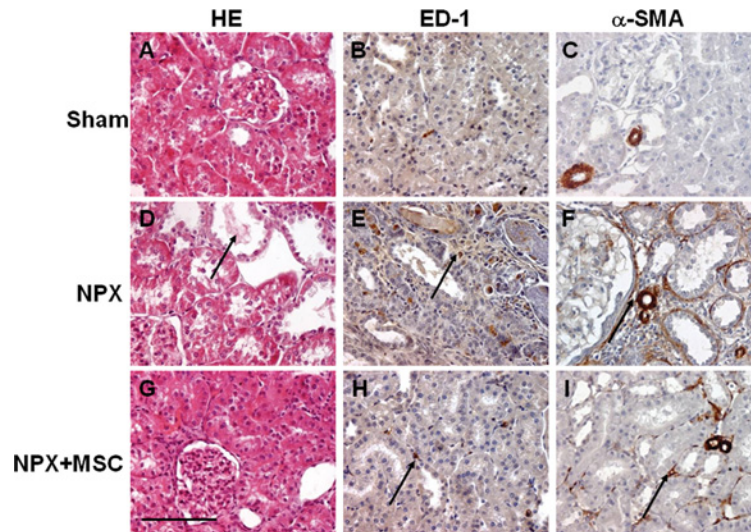
### MSC dye tracking

To assess MSC localization in kidney tissues, we used the CMFDA cell tracker in dye-tracking experiments [19]. At 24 h after MSC injection, kidney sections counterstained with H&E showed that the presence of GFP (green fluorescent protein)-positive cells was only observed in animals receiving Green CMFDA-treated cells (approx. 1%), but not in those treated with vehicle-incubated cells, indicating the migration of injected MSC to the kidney (Figure 1B).

CD73 is a membrane protein involved in the proteolytic activity of extracellular nucleotides and has been described as a positive antigen present in MSC [23]. Immunofluorescence analysis was performed to corroborate the migration of unlabelled MSC to the kidneys at 24 h after the injection. In agreement with the previous results, CD73-positive cells were observed in kidney sections at 24 h after the MSC injection (Figure 1C). DAPI nucleic acid stain allows the cellular identification and evaluation of tissue morphology. The CD73 reactivity of cells in kidney showed a pattern similar to what is seen *in vitro* in cultured MSC (results not shown).

### Functional renal damage

Renal function was assessed by plasma creatinine levels in sham animals (Sham) infused with fresh X-Vivo medium, Sham infused with MSC (Sham + MSC), NPX



**Figure 2** Histological NPX-induced damage in CKD kidneys

The IHC in kidney samples at day 35 post-NPX, injected with fresh X-Vivo medium (NPX) or MSC (NPX + MSC). Positive staining for macrophages (ED-1) and miofibroblasts ( $\alpha$ -SMA) markers was observed in the interstitium of internal and external medulla in NPX kidneys injected with X-Vivo medium (E and F). A decreased staining of these markers was observed in NPX + MSC kidneys (H and I), comparable with kidneys of Sham rats (B and C). The tissue damage was also assessed by H&E staining: NPX rats showed the disappearance of the brush border, epithelium, tubular and glomerular alterations (D). NPX + MSC showed morphological characteristics (G) similar to that observed in Sham rats (A). ED-1 and  $\alpha$ -SMA markers were detected using peroxidase and developed with DAB (brown reaction). Scale bar, 100  $\mu$ m. Arrows show the marker localization.

rats infused with fresh X-Vivo medium and NPX injected with MSC (NPX + MSC). Sham rats injected with MSC or X-Vivo had normal plasma creatinine levels (0.3 mg/dl). In NPX animals, creatinine levels increased to  $1.0 \pm 0.3$  mg/dl, which was significantly higher than in both Sham groups ( $P < 0.05$ ). In rats subjected to NPX + MSC, the creatinine level was reduced to  $0.4 \pm 0.1$  mg/dl ( $P < 0.05$  compared with NPX group). Furthermore, creatinine values of the NPX + MSC were not significantly different from those in Sham animals.

### Histological studies

H&E staining of NPX renal sections showed alterations consistent with chronic damage: epithelia flattening, dilated tubules and increased fibrotic areas (Figure 2D). NPX treated with a single dose of MSC had normal morphology (Figure 2G), comparable with that observed in Sham kidneys (Figure 2A and Table 1). We did not find morphological, functional or histological differences between Sham and Sham + MSC kidneys (results not shown); for this reason, this latter group was excluded in the following IHC analyses.

Damage markers ED-1 (macrophages) and  $\alpha$ -SMA (myofibroblasts) were widely distributed in renal parenchyma of NPX animals (Figures 2E and 2F). However, both markers had a minor expression in NPX + MSC animals (Figures 2H and 2I). Figures 2(B) and 2(C) are Sham rats, showing normal IHC distribution of these markers.

### Renal SC detection

The presence of the renal SC marker Oct-4 was evaluated 5 and 35 days after injection. The IHC staining observed in Sham animals was minimal (Figure 3AA and 3AD). NPX animals showed a positive stain 5 days after NPX (Figure 3AB) that was completely inhibited 35 days after surgery (Figure 3AE). NPX animals injected with MSC presented a marked stain at 5 days after injection (Figure 3AC) that was maintained 35 days after injection (Figure 3AF). Oct-4 expression evaluated by WB 35 days after injection is shown in Figure 3(AG): Sham and NPX animals had reduced Oct-4 expression ( $15 \pm 8$  and  $24 \pm 8$  AU respectively), which was increased with MSC injection ( $74 \pm 8$  and  $185 \pm 16$  AU in Sham + MSC and NPX + MSC respectively). These differences were statistically significant ( $P < 0.05$ ).

Additionally, we analysed the co-localization of CD-73 and Oct-4 35 days after injection (Figure 3B). The co-expression of CD73/Oct-4 was observed in a reduced cell number (0.2%).

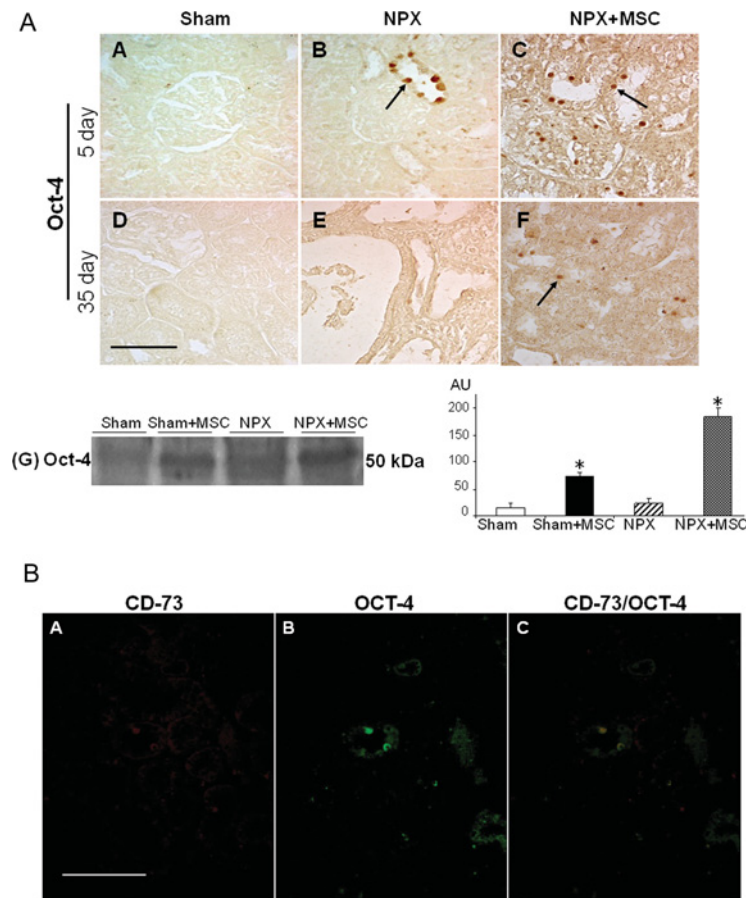
### Localization of the angiogenic markers: VEGF and Tie-2

At 5 weeks after NPX, the presence of transcriptional factors involved in angiogenesis, VEGF and Tie-2, was analysed. The IHC of VEGF and Tie-2 in Sham kidneys revealed that the levels of both factors were observed as a weak signal (Figures 4A and 4D); however, in NPX animals, VEGF and Tie-2 were completely abolished

**Table 1 Renal damage indices**

A comparison of the renal morphology in the NPX model treated with MSC (NPX + MSC) or vehicle (NPX) and sham animals, 35 days after damage, was determined. One-way ANOVA and a Tukey test were used. \* $P < 0.05$  compared with Sham; † $P < 0.05$  compared with NPX. ED-1 and  $\alpha$ -SMA are markers of macrophage abundance and myofibroblasts respectively.

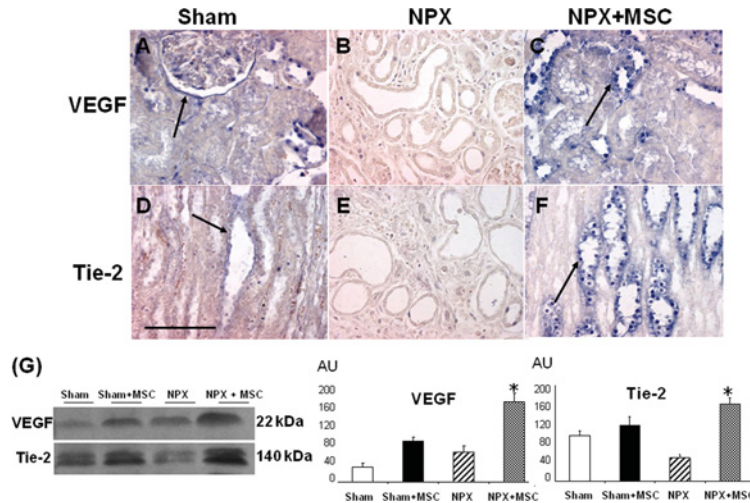
Group	Indices of damage (score 0–5)			
	ED-1	$\alpha$ -SMA	Dilated tubules	Protein cast
Sham ( $n = 7$ )	$0.7 \pm 0.5$	$1.0 \pm 0.0$	$1.1 \pm 0.7$	$0.3 \pm 0.5$
NPX ( $n = 7$ )	$4.6 \pm 0.5^*$	$4.7 \pm 0.5^*$	$4.1 \pm 0.9^*$	$3.6 \pm 0.8^*$
NPX + MSC ( $n = 7$ )	$2.4 \pm 0.5^{\dagger}$	$2.6 \pm 0.8^{\dagger}$	$2.1 \pm 0.7^{\dagger}$	$1.7 \pm 0.5^{\dagger}$

**Figure 3 Expression and localization of renal SC (A), and CD73/Oct-4 + MSC in kidney (B)**

(A) The presence of renal MSC was evaluated by using the Oct-4 marker. Oct-4 localization was evaluated by IHC in Sham (Aa and Ad), NPX (Ab and Ae) and NPX + MSC (Ac and Af) kidney samples at 5 days (Aa–Ac) and 35 days (Ad–Af) after injection. An induction of Oct-4 staining was observed at 5 days in NPX animals (Ab) that was not maintained at 35 days after injection (Ae). The staining was higher in NPX + MSC kidneys 5 days after injection (Ac) and was maintained at 35 days after injection (Af). The stain was not observed in Sham animals (Aa and Ad). Scale bar, 100  $\mu$ m. Arrows show the marker localization. The immunoblot (Ag) shows increased expression of Oct-4 in animals injected with MSC (Sham and NPX) compared with the corresponding controls. \* $P < 0.05$ . (B) CD73 (Ba, red) and Oct-4 (Bb, green) were observed in kidney sections 35 days after MSC injection. The right-hand panel (Bc) corresponds to Merge microscope techniques.

(Figures 4B and 4E). The addition of MSC in NPX rats increased the levels of both proteins (Figures 4C and 4F). As shown in Figures 4(A)–4(F), VEGF and Tie-2 were localized in proximal tubule cells in the inner and outer medulla.

The expression of angiogenic markers is shown in Figure 4(G). VEGF and Tie-2 levels were minimal in Sham ( $31 \pm 7$  and  $84 \pm 9$  AU respectively) and NPX animals ( $61 \pm 12$  and  $43 \pm 6$  AU respectively) measured 35 days after damage. However, Sham + MSC



**Figure 4 Immunolocalization and expression of endothelial cell markers during MSC-induced recovery in CKD**

Sham, NPX and NPX + MSC samples were stained for the angiogenic factors VEGF (A–C) and Tie-2 (D–F). Maximum expression was observed in kidneys treated with MSC (C and F). The expressions of these markers were observed in the thick ascending limb of Henle and proximal tubule cells localized primarily in the internal and external medulla. The staining was performed using alkaline phosphatase and developed with NBT/BCIP (blue reaction). Scale bar, 100  $\mu$ m. Arrows show the marker localization. Immunoblot studies were conducted to evaluate the expression of VEGF and Tie-2 (G): in animals treated with MSC (Sham + MSC and NPX + MSC), increased expression of these markers was observed, in comparison with corresponding controls. \* $P < 0.05$ .

had augmented angiogenic proteins ( $83 \pm 8$  and  $102 \pm 16$  AU for VEGF and Tie-2 respectively). All markers were elevated in NPX + MSC animals ( $165 \pm 16$  and  $142 \pm 11$  AU for VEGF and Tie-2 respectively). The differences between NPX and NPX + MSC were statistically significant ( $P < 0.05$ ). The  $\alpha$ -tubulin total protein level was used to correct for variation in sample loading.

### Expression of epithelial markers: Pax-2, bFGF and BMP-7

A significant difference was found in epithelial markers among controls and MSC kidneys treated. As indicated in Figure 5, rats treated with MSC showed a characteristic pattern of distribution of epithelial markers Pax-2, bFGF and BMP-7. The IHC of these proteins showed a minimal staining in Sham rats (Figures 5A, 5D and 5G) and even a weaker staining in NPX kidneys (Figures 5B, 5E and 5H). The injection with MSC in NPX animals (NPX + MSC) induced an increase in these markers (Figures 5C, 5F and 5I). As reported previously [30], in adult kidneys of Sham animals, bFGF, Pax-2 and BMP-7 are detected in the nucleus of collecting duct cells, but not in proximal tubular cells. A similar pattern was observed in the present study for Sham rats (Figures 5A, 5D and 5G), whereas the expression pattern was changed to proximal tubular cells in NPX + MSC (Figures 5C, 5F and 5I).

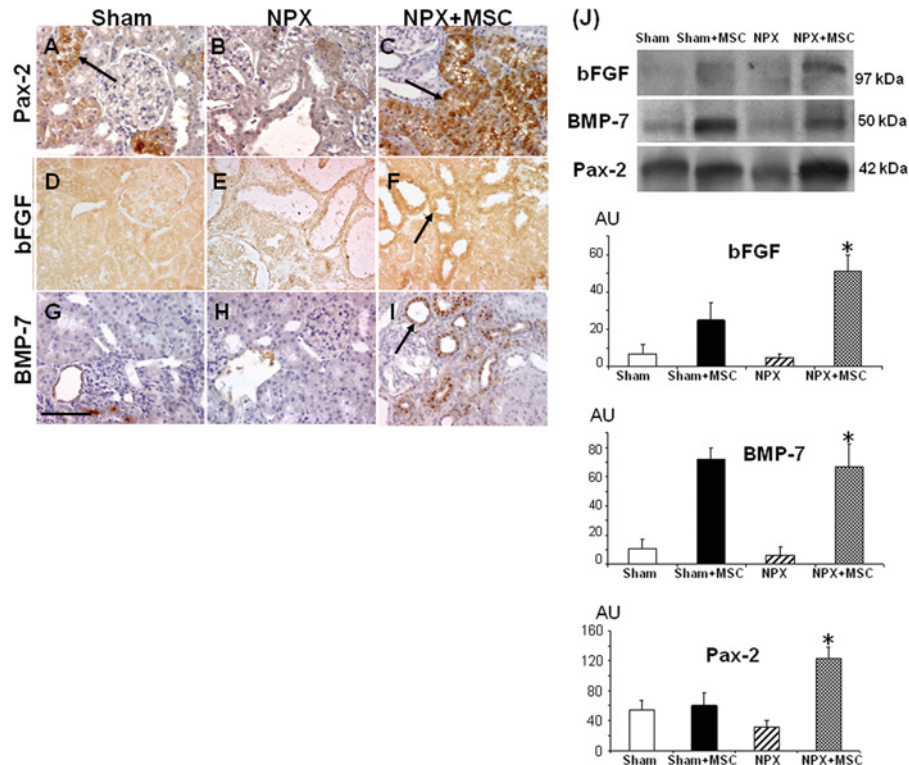
The expression of bFGF, BMP-7 and Pax-2 in Sham kidneys was scarce, whereas in NPX animals the expression level was even lower ( $8 \pm 4$ ,  $9 \pm 3$  and  $32 \pm 8$  AU respectively; Figure 5J). However, in Sham + MSC,

an increased expression of epitheliogenic markers was observed ( $25 \pm 3$ ,  $68 \pm 13$  and  $51 \pm 9$  AU for bFGF, BMP-7 and Pax-2 respectively) that was higher in NPX + MSC rats ( $52 \pm 7$ ,  $67 \pm 15$  and  $123 \pm 15$  AU for bFGF, BMP-7 and Pax-2 respectively; Figure 5J). These differences were statistically significant ( $P < 0.05$ ). The expression of  $\alpha$ -tubulin was used to correct for variation in sample loading.

### DISCUSSION

During kidney development, proliferation of undifferentiated cells and subsequently differentiation into specific cell types occurs, achieved by the sequential expression of a large number of renal genes [17]. Previously, we have shown that in the repairing phases of acute kidney damage, a recapitulation of the genetic programme expressed in the organogenesis is activated [17]. In the present paper, we have studied the renal effect of MSC injection in rats subjected to NPX by the measurement of functional, histological and molecular parameters. As shown by the results, a single intravenous infusion of MSC was able to enhance renal reparative processes and markedly improve renal function with this approach.

MSC have been a subject of much interest over the last few years because of their potential role in regeneration and tissue repair [31]. Their experimental and/or clinical use in acute myocardial infarction, skin and skeletal muscle regeneration and ARF has shown encouraging results [11–15]. Moreover, the positive effects of MSC



**Figure 5 Immunolocalization and expression of epithelial markers in MSC-mediated recovery of CKD**

The samples were stained for Pax-2 (A–C), bFGF (D–F) and BMP-7 (G–I) in Sham, NPX and NPX + MSC kidneys. Pax-2 and bFGF were localized in proximal tubular cells and BMP-7 in the collector tubular cells of internal and external medulla. The staining for these markers was increased in NPX + MSC (C, F and I) when compared with Sham (A, D and G) and NPX (B, E and H) kidneys. The staining was done using peroxidase and developed with DAB (brown reaction). Scale bar, 100  $\mu$ m. Arrows show the marker localization. Immunoblot studies were conducted to evaluate the expression of bFGF, BMP-7 and Pax-2 (J). In Sham and NPX rats, the expression of these markers were minimal; however, the injection with MSC in Sham and NPX kidneys induces an increased expression of all of the markers assessed. \* $P < 0.05$ .

in different CKD animal models were reported recently, as evaluated by the reduction in plasma creatinine levels [31], improvements of proteinuria [32,33], renal fibrosis [34], glomerulosclerosis, macrophage infiltration [35], improvements of renal filtration [36] and the reduction of pro-inflammatory cytokines [37], but the mechanisms involved in these reparative processes are not yet well understood.

As shown in the present study, CKD rats injected with MSC have an increased expression of VEGF and Tie-2; these results are in agreement with *in vitro* studies [38], suggesting that there is a pathway related to vascular protection induced by MSC. VEGF is an essential factor in endothelial and vascular system development, and in glomerulogenesis. Its expression in adult kidneys is restricted to glomerular and some tubular cells, where it has been implicated in the maintenance of the permeability and integrity of the capillaries that constitute the glomerulus. Previous results from our laboratory and others have shown a protective role of VEGF against glomerular injury [17,39] and its expression has been inversely related to the generation of glomerulosclerosis in progressive

kidney diseases [40]. The angiopoietin receptor Tie-2 is also associated with glomerulogenesis [41] and the maintenance of the glomerular filtration membrane integrity. Furthermore, in the NPX + MSC group, we observed an increased expression of BMP-7, Pax-2 and bFGF that is related to tubular development. We have also shown increased expression of these factors in the repairing processes after acute kidney damage [17,42,43]. Therefore, if these proteins are overexpressed, the EMT (epithelial mesenchymal transition) processes might be activated, supporting a reparation and regeneration hypothesis underlying the effects of MSC therapy [42]. *In vitro* studies have shown that MSC can induce the expression of important trophic factors such as IGF-1 (insulin-like growth factor-1), EGF (epidermal growth factor), VEGF and bFGF [38]. It is known that BMP-7 and bFGF participate in kidney development, protecting against cellular apoptosis [44] and promoting the epithelialization processes [17,27,45]. The injection of recombinant bFGF induces an earlier expression of the other nephrogenic proteins, favouring kidney recovery and repair after injury [27]. The considerable increase in bFGF and BMP-7 in the NPX + MSC kidneys can be



interpreted as an indicator of a cellular repair response to kidney damage. One of the biggest challenges with respect to cell therapy, and specifically cell therapy in the kidney, is the knowledge of the mechanisms involved in the therapeutic effect described, which may include a pharmacological effect given by the secretion of cytokines, chemokines and several mediators that would activate genes involved in the regeneration processes. However, it seems technically difficult to decide which of these possible factors plays a dominant role. Further studies should be directed to previously modified MSC, either genetically or by pre-incubation with specific Abs directed against factors such as VEGF or BMP-7. Additionally, comparing the effect of the MSC with the direct administration of one or a combination of these factors could provide information on the importance of the cellular effect, i.e. that mediated by homing, replication and differentiation of SC in kidney cells.

Different studies have described that HSC (haematopoietic stem cells), EPC (endothelial progenitor cells)/haemangioblasts and multipotent mesenchymal stromal cells are SC completely devoid of nephrogenic potential, but may enhance the intrinsic reparative capabilities of the kidney [46–50]. However, other authors have described that MSC can express tubular epithelial cell markers and podocyte phenotype [51,52]. In addition, it has been reported that bone marrow-derived cells contribute to podocyte regeneration and amelioration of renal disease in a mouse model of the Alport syndrome [53].

A mild but significant increase in the renal SC marker, Oct-4 [8], is observed after the injection of MSC in uraemic rats, suggesting the induction of tubular and epithelial cells to render EMT processes, to proliferate and differentiate with a consequent renal reparative phenomenon, or may indicate the induction of resident kidney SC to proliferate and mediate kidney repair. In this regard, we must emphasize that these two pathways are not mutually exclusive and that probably both play a role in tissue regeneration.

It has been proposed that the proliferation and differentiation of MSC and resident kidney SC into mature and functional epithelial cells capable of promoting repair are given by the secretion of the anti-inflammatory protein TSG-6 due to the embolization of the injected MSC in the lung [54]. In the present study, we have shown the migration of MSC to kidney, although we cannot rule out the possible embolization of injected MSC in the lungs and the subsequent secretion of paracrine factors. In fact, after 35 days we found a minimal presence of MSC in the kidney, which favours previous data on a paracrine mechanism of action [55,56].

The present study opens a new perspective in stem cell therapy and regenerative medicine in CKD, in which MSC could play a therapeutic beneficial role.

## AUTHOR CONTRIBUTION

Sandra Villanueva, Flavio Carrión and Carlos Vio conceived the experiments and co-wrote the paper; Ernesto Ewertz, Andrés Tapia, César Vergara, Carlos Céspedes, Pablo Sáez, Patricia Luz and Carlos Irrarázabal carried out the study; and Juan Carreño and Fernando Figueroa analysed the data and co-wrote the paper.

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