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From Cell Biology to Tissue Engineering

Transplantation of mesenchymal stem cells preserves podocyte homeostasis through modulation of parietal epithelial cell activation in adriamycin-induced mouse kidney injury model

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Summary. To determine the role of the transplantation of bone marrow-derived mesenchymal stem cells (MSCs) in podocyte renewal, we studied BALB/C mice with or without adriamycin-induced acute kidney injury. MSCs were transplanted ectopically under the capsule of the left kidney or into the peritoneal cavity after the onset of kidney injury to test their local or systemic paracrine effects, respectively. Adriamycin produced increases in urine protein: creatinine ratios, blood urea nitrogen, and blood pressure, which improved after both renal subcapsular and intraperitoneal MSCs transplants. The histological changes of adriamycin kidney changes regressed in both kidneys and in only the ipsilateral kidney after intraperitoneal or renal subcapsular transplants indicating that the benefits of transplanted MSCs were related to the extent of paracrine factor distribution. Analysis of kidney tissues for p57-positive podocytes showed that MSC transplants restored adriamycin-induced decreases in the abundance of these cells to normal levels, although after renal subcapsular transplants these changes did not extend to contralateral kidneys. Moreover, adriamycin caused inflammatory activation of PECs with coexpression of CD44 and phospho-ERK, which was normalized in both or only ipsilateral kidneys depending on whether MSCs were transplanted in the peritoneal cavity or subcapsular space, respectively.

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Introduction

Mesenchymal stem cells (MSCs) are non-hematopoietic bone marrow cells (BMC) with the ability to differentiate into multiple lineages, e.g., adipocytes, osteoblasts, chondrocytes, etc. (Tropel et al., 2004; Herrera et al., 2007; Bussolati et al., 2009; Singaravelu and Padanilam, 2009; Roobrouck et al., 2011). These MSCs have been of interest for their therapeutic potential, including in kidney injury (Bian et al., 2014; Erpicum et al., 2014; Rowart et al., 2015; Anan et al., 2016; Gregorini et al., 2016). We and others previously reported the beneficial effects of transplanting MSCs in animal models of acute kidney injury (Faubel et al., 2007; Herrera et al., 2007; Morigi et al., 2008; Eliopoulos et al., 2010; Cheng et al., 2013a). As due to their size-structure relationships, targeting of MSCs to glomerular capillaries via intravascular administration was generally unsuccessful, in our studies, we transplanted MSCs by the intraperitoneal route, which was helpful for defining their paracrine benefits in cisplatin-induced kidney injury (Cheng et al., 2013b). When MSCs were transplanted subcapsularly into one kidney, their benefits were restricted to only that kidney, and the contralateral kidney served as a control for evaluating differences in injury (Cheng et al., 2013b). This offered suitable models for testing local or systemic

benefits of transplanted MSCs in the case of specific cell types, such as the role of parietal epithelial cells (PECs) in renal homeostasis.

Recent reports have suggested that kidneys harbor progenitor cells locally with the potential to replace lost podocytes (Smeets et al., 2014; Eng et al., 2015; Kaverina et al., 2016). For instance, PECs adjacent to injured podocytes have been considered to play that role (Eng et al., 2015; Kaverina et al., 2016). During podocyte losses, PECs may acquire profibrotic properties and also assume the phenotype of progenitor cells. Profibrotic cells may contribute matrix proteins and other molecules to promote sclerosis, whereas activation of cells with regenerative capacity may promote podocyte replenishment. Since MSCs may delay the progression of kidney injury by regulating abundances of profibrotic PECs or by restoring podocyte populations, we hypothesized that if paracrine factors from MSCs contributed to these processes, we should be able to define these events in suitable experimental models.

In the present study, we evaluated the benefits of MSCs in mice with adriamycin toxicity with or without cell transplantation into renal subcapsular or intraperitoneal locations. We evaluated changes in renal histology urinary protein excretion rates, blood urea nitrogen, blood pressure, and podocyte markers to identify profibrotic or regenerative forms of PECs.

Materials and methods

The Animal Care and Use Committees of the Albert Einstein College of Medicine and the Feinstein Institute of Medical Research approved study protocols. BALB/C mice were purchased from Jackson Labs (Bar Harbor, ME). Mice aged 6-8 weeks were used and were housed under standard lighting conditions with unlimited access to pelleted food and water.

Isolation and characterization of mouse MSCs

Primary MSCs were isolated and expanded from long bones of C57BL/6J mice according to the method described previously (Cheng et al., 2009). Briefly, compact bones were excised into fragments and suspended in Mesencult basal medium (StemCell Technologies Inc; Vancouver, CA) containing 10% FBS and 1 mg/ml of collagenase II (Sigma-Aldrich, USA), followed by digestion for 1-2 hours at 37°C. The released cells were discharged, and bone fragments were incubated in Mesencult basal medium with stimulatory supplements (StemCell Technologies Inc., CA) in a humidified 37°C incubator with 4% O_2 and 5% CO_2 for three days. The non-adherent cells and tissue debris were removed at three days by changing medium and replacing with fresh medium. The cells were used for studies after three to five passages in culture. To analyze cellular phenotype, MSCs were stained with PE-conjugated monoclonal antibodies specific for CD11b, CD14, CD34, CD86, CD90, and CD105 (BD Pharmingen) and analyzed by flow cytometry (Becton Dickinson) as described previously (Cheng et al., 2013a,b).

Mouse models

BALB/C mice of 8-10 weeks of age were distributed randomly to establish groups as follows. Healthy control mice received normal saline via tail vein (n=6). Another group of mice (n=18) was given 10 mg/Kg adriamycin (Sigma-Aldrich), by tail vein with additional manipulations after three weeks: injection of normal saline via tail vein (group A, n=6), transplantation of 5×10^6 MSC mixed with Cytodex 3 microcarriers, as described previously (Cheng et al., 2009) into either subcapsular space of left kidney (group B, n=6) or intraperitoneally (i.p.) (group C, n=6). After one week, blood, urine, and kidneys were collected for assays.

Histological evaluation of nephrotoxicity

Kidneys were removed and fixed in 10% buffered formalin for 24 hours and then transferred to 100% ethanol. Tissues were embedded in paraffin to cut 5 μm thick sections and stained with hematoxylin and eosin and Periodic Acid Schiff (PAS). The extent of injury in cortex and outer medulla was graded by identifying fractions of tubules with loss of brush borders, necrosis, cast formation, or dilatation, as follows: 0, none; 1, <10%; 2, 10-25%; 3, 26-45%; 4, 46-75%; and 5, >75%. At least 20 microscopic fields were reviewed per tissue section under high power by blinded observers.

Protein extraction and western blotting

Renal cortical tissues were treated with lysis buffer (1×PBS pH 7.4, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, and 0 µl of protease inhibitor cocktail; 100×; Calbiochem and 100 µg/ml PMSF), homogenized with a Dounce homogenizer, and then lysed for 30 min on ice. The samples were centrifuged at 15,000 g for 20 min at 4°C. The lysate was assayed for protein content by Bradford assay (Bio-Rad). The proteins, 20-40 µg/lane, were separated in 10% SDS-PAGE and transferred onto nitrocellulose membrane using a Bio-Rad Western blotting apparatus. After transfer, blots were stained with Ponceau S (Sigma, St. Louis, MO) for protein transfer and loading. The blots were blocked with 5% BSA and 0.1% Tween 20 in 1×PBS for 60 min at room temperature and then incubated with anti-podocin antibody (goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-WT1 antibody (rabbit polyclonal, Santa Cruz) overnight at 4°C. A horseradish peroxidase-conjugated appropriate secondary antibody was applied for one h at room temperature. The blots were developed using a chemiluminescence detection kit (ECL; Amersham, Arlington Heights, IL) and exposed

to Kodak X-OMAT AR film. Densitometric scans were performed on the identified band using a computer-based measurement system

Immunohistochemical studies of kidney tissues

Renal cortical sections from control and experimental mice were de-paraffinized, and antigen retrieval was performed in a double boiler system. The endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature. Sections were washed in phosphate-buffered saline (PBS) three times and incubated in avidin and biotin blocking solution and then in blocking serum solution for 60 minutes at room temperature, followed by incubation with primary antibodies against either p57 (goat polyclonal; sc-1037; Santa Cruz Biotechnology, Santa Cruz, CA) or podocin (goat polyclonal, sc-22296) overnight at 4°C in a moist chamber. Each of the sections was washed three times with PBS and incubated with an appropriate secondary antibody at 1:250 dilutions at room temperature for 1 hour. After washing with PBS three times, sections were incubated in ABC reagent (Vector Laboratories, Burlingame, CA) for half an hour. Sections were washed three times in PBS and then placed in Nova Red/hydrogen peroxide solution, counterstained with methyl green, dehydrated, and mounted with a xylene-based mounting media (Permount; Fisher Scientific Corporation, Fair Lawn, NJ). Appropriate positive and negative controls were used.

Immunofluorescence of kidney tissue

Tissue slides of renal cortical sections from control and experimental mice were immersed in 100% xylene for 5 minutes four times, then placed in 100% ethanol for 5 minutes two times, and then into 70% ethanol 5 minutes two times, followed by 50% ethanol for 5 minutes twice. Slides were washed with double-distilled water for 1 minute. Then slides were subjected to 1×retrieve-All antigen unmasking system buffer (catalog no. SIG-31910-50; Covance, Dedham, MA) at 100°C for 90 minutes. Slides were kept at room temperature for 20 to 30 minutes for cooling and washed with 1×PBS. Slides were immersed in 0.3% Triton X-100 for 20 minutes at room temperature. Slides were blocked with 2% bovine serum albumin for 2 hours. After blocking, primary antibody CD44 (mouse monoclonal, sc-7297; Santa Cruz Biotechnology) and phos-ERK (rabbit polyclonal, ab-65142, Abcam) were added overnight at 4°C. Next day, slides were washed with 0.1% Triton X-100 for 5 minutes three times on shaker at room temperature. Secondary antibody was added with fluorescence conjugated at 1:500 dilution for 1 hour at room temperature. Slides were washed with 0.1% Triton X-100 for 5 minutes three times on a shaker at room temperature and mounted for fluorescence microscopy.

BUN assay

Serum BUN was measured as previously described (Cheng et al., 2009). Briefly, 0.15 mL Urease Buffer Reagent (U-3383, Sigma) was added to 50 μL serum for 20 minutes at room temperature followed by 0.3 mL Phenol Nitroprusside Solution (P-6994, Sigma), 0.3 mL alkaline hypochlorite solution (A-1727, Sigma), and 1.5 mL deionized water, in that order. The mixture was incubated for 30 minutes at room temperature, and absorbance at 540 nm was measured. Standard stock solution was prepared at the concentration of 10 mg/mL urea (U-5128, Sigma).

Statistical analysis

Data were expressed as means ± standard deviation. Significance was determined by Student's t-test or analysis of variance with SigmaStat 3.1 (Systat Software Inc., Point Richmond, CA); $P<0.05$ was considered significant. Mann-Whitney test was used to analyze statistical significance for analyzing data in Figs. 4, 5.

Results

Effect of MSCs transplantation on adriamycin-induced renal injury

Adriamycin induced renal injury (renal histology) with or without MSC transplantation has been displayed in representative microphotographs and in the bar graphs (Fig. 1). Mice receiving MSCs, i.p. + adriamycin displayed less severe histological renal lesions (both FSGS and GSG, Fig. 1A,B; tubular dilation, Fig. 1A,C) in comparison with adriamycin-treated controls (n=6, in each group). Moreover, in these mice with i.p. transplantation of MSCs, urine protein/creatinine ratios (Fig. 1D), as well as BUN levels (Fig. 1E) and blood pressure changes, both systolic and diastolic, were lower (Fig. 1F), which was in agreement with decreases in adriamycin-induced kidney injury. Similarly, after subcapsular transplants of MSCs, renal histology improved with less severe lesions in the left kidney, which received MSCs, compared with contralateral right kidney without MSC transplants (FSGS, GSG, Fig. 2A,B; tubular dilation, Fig. 2A,C). The urine/creatinine ratio (Fig. 2D), BUN (Fig. 2E), and blood pressure (Fig. 2F) were all decreased in mice receiving MSCs compared with adriamycin alone.

Effect of transplanted MSCs on Podocin and WT1 expression in adriamycin-treated mice

These studies were restricted to recipients of MSCs (n=6) via renal subcapsular space with an opportunity to compare outcomes in contralateral kidneys distal to transplanted MSCs. Representative renal sections labeled for podocin in control and experimental mice are shown in Fig. 3A. Podocin expression was preserved in

glomeruli of the left kidney, which received MSCs when compared with that of the contralateral right kidney. Western blot displayed relative preservation of podocin in the left kidney in adriamycin-treated animals (Fig. 3B). Cumulative densitometric analysis (n=3) is shown in bar graphs in Fig. 3C. Renal tissues from the right kidney of adriamycin-treated mice revealed downregulation of podocin expression; however, this effect of adriamycin was partially reversed by MSC transplantation in the left kidney. Similarly, we observed decreases in renal WT1 expression after adriamycin, which was partially reversed by MSC transplantation in the left kidney (Fig. 3D,E).

Transplantation of MSC preserved p57 +ve cells in glomerular tufts

The abundance of podocytes with p57 marker is

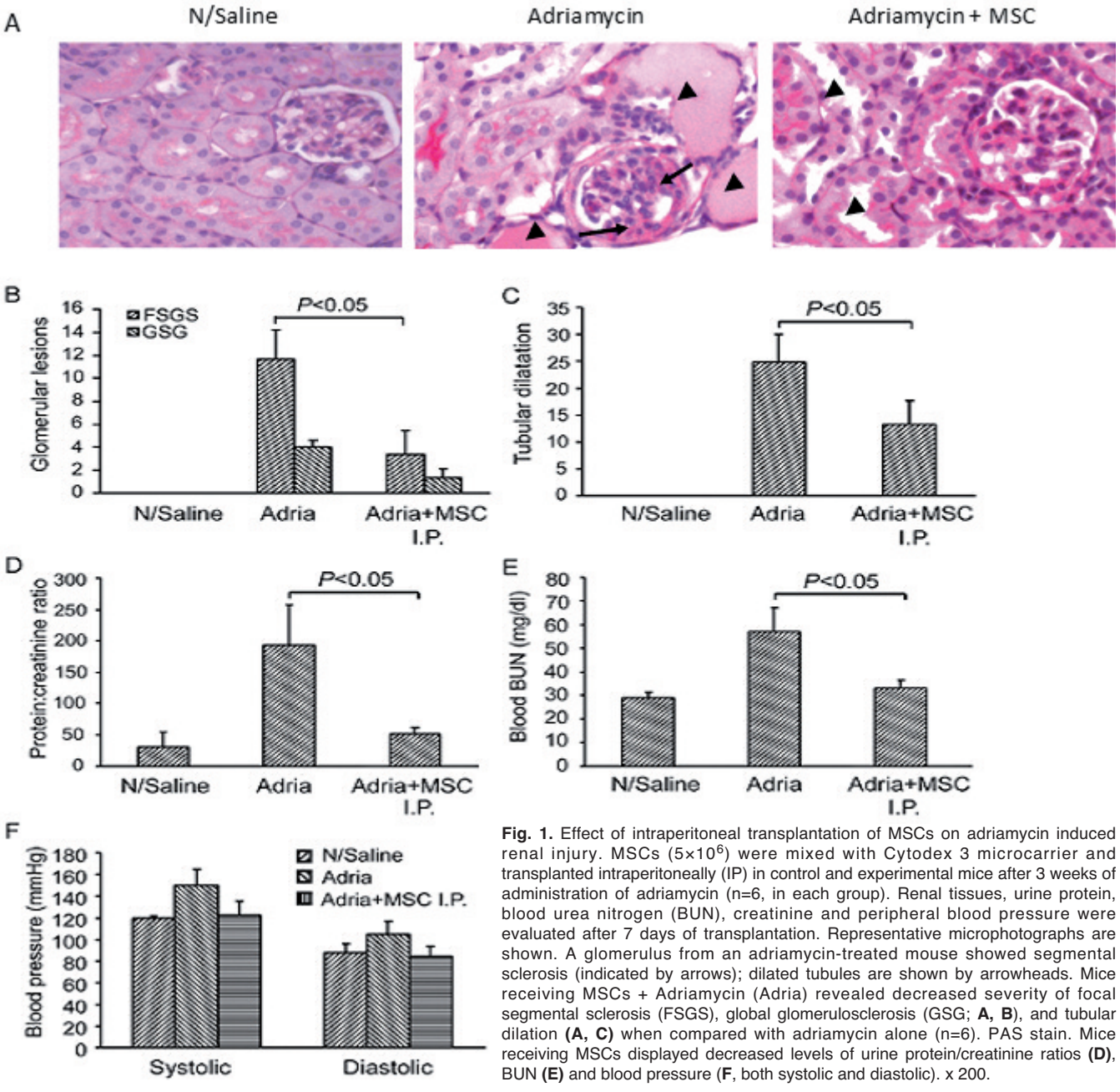

Fig. 1. Effect of intraperitoneal transplantation of MSCs on adriamycin induced renal injury. MSCs (5×10^6) were mixed with Cytodex 3 microcarrier and transplanted intraperitoneally (IP) in control and experimental mice after 3 weeks of administration of adriamycin (n=6, in each group). Renal tissues, urine protein, blood urea nitrogen (BUN), creatinine and peripheral blood pressure were evaluated after 7 days of transplantation. Representative microphotographs are shown. A glomerulus from an adriamycin-treated mouse showed segmental sclerosis (indicated by arrows); dilated tubules are shown by arrowheads. Mice receiving MSCs + Adriamycin (Adria) revealed decreased severity of focal segmental sclerosis (FSGS), global glomerulosclerosis (GSG; **A, B**), and tubular dilation **(A, C)** when compared with adriamycin alone (n=6). PAS stain. Mice receiving MSCs displayed decreased levels of urine protein/creatinine ratios **(D)**, BUN **(E)** and blood pressure (**F**, both systolic and diastolic). x 200.

shown in Fig. 4A. Cumulative data (n=30, number of glomeruli) are in Figs. 4B and 4C. In control mice, 12±4 p57 +ve cells/glomerulus were observed (Fig. 4B). In adriamycin-treated mice, the number of podocytes decreased, 3.9±0.4 p57 +ve cells/glomerulus, P<0.01. By contrast, after intraperitoneal transplantation of MSCs in adriamycin-treated mice (group C), the number of podocytes was greater, although remaining below that in healthy controls, 6.7±0.5 p57 +ve cells/glomerulus, P<0.05. In animals with subcapsular transplants of MSCs, (Fig. 4C, group B) the number of podocytes was greater in the ipsilateral kidney with MSC transplants although again remaining below that in healthy controls, 5.4±0.5 p57+ve cells/per glomerulus, versus the contralateral right kidney, 3.4±0.8 p57 +ve cells/glomerulus, P<0.01.

MSC transplantation decreased activation of profibrotic PECs

To determine the role of profibrotic PECs in adriamycin-induced podocyte injury, renal tissues were

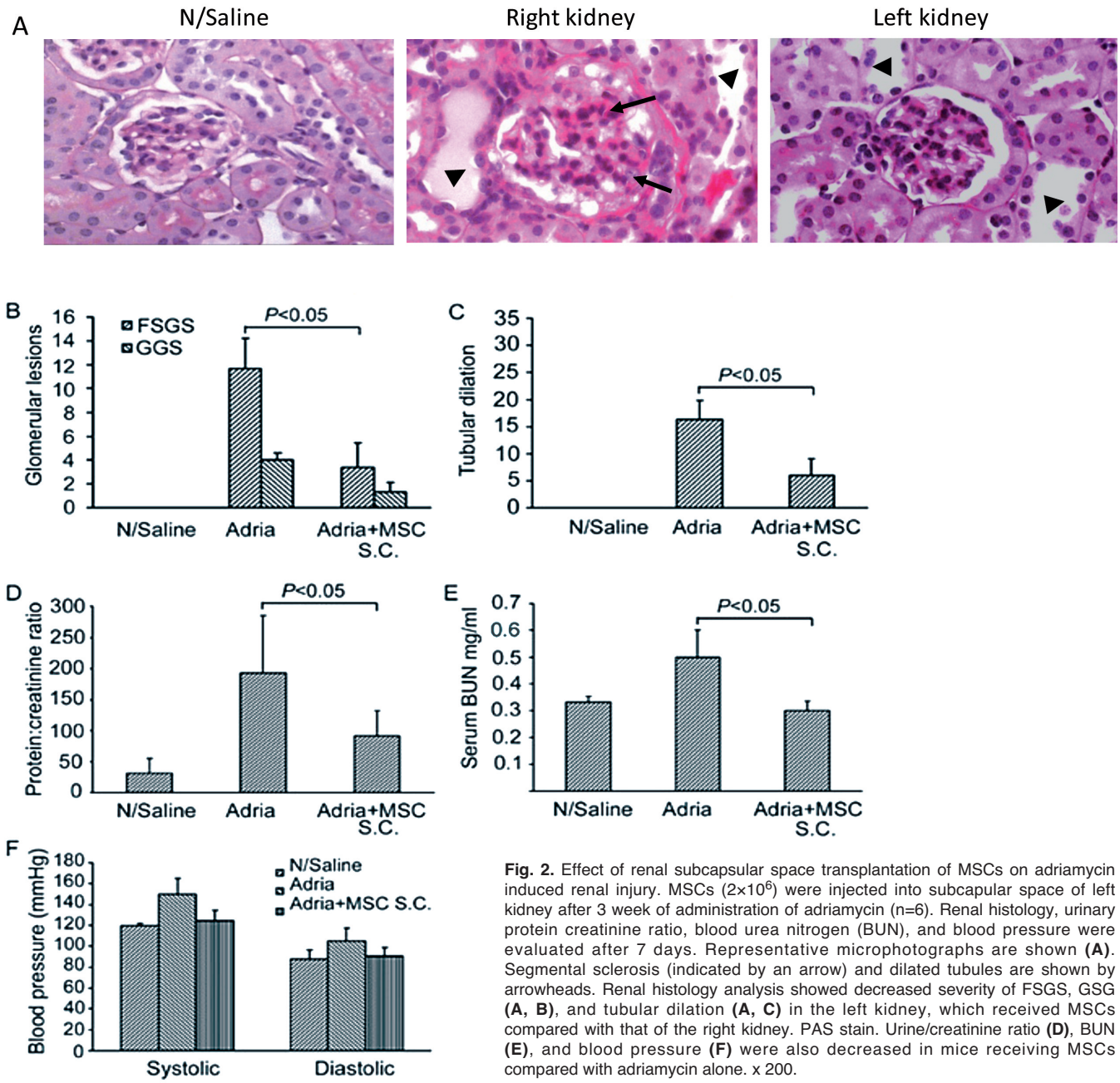

Fig. 2. Effect of renal subcapsular space transplantation of MSCs on adriamycin induced renal injury. MSCs (2×10^6) were injected into subcapular space of left kidney after 3 week of administration of adriamycin (n=6). Renal histology, urinary protein creatinine ratio, blood urea nitrogen (BUN), and blood pressure were evaluated after 7 days. Representative microphotographs are shown **(A)**. Segmental sclerosis (indicated by an arrow) and dilated tubules are shown by arrowheads. Renal histology analysis showed decreased severity of FSGS, GSG **(A, B)**, and tubular dilation **(A, C)** in the left kidney, which received MSCs compared with that of the right kidney. PAS stain. Urine/creatinine ratio **(D)**, BUN **(E)**, and blood pressure **(F)** were also decreased in mice receiving MSCs compared with adriamycin alone. x 200.

co-stained for CD44 and phos-ERK (PERK). Representative microphotographs of control and experimental mice are shown in Fig. 5A. Cumulative data (n=16, number of glomeruli) are shown in bar graphs (Fig. 5B). Control mice did not show activated profibrotic PECs expressing both CD44 and PERK. On the other hand, adriamycin-treated mice in both groups A and group B (right kidney) displayed 5 to 7 CD44 and PERK double-labeled cells in Bowman's capsule (Fig. 5B). However, MSCs transplantation significantly decreased the number of these CD44 and PERK double-labeled cells in group B (left kidney transplanted with MSC) as well as in group C (MSCs transplanted i.p.). This indicated that MSC transplantation decreased the abundance of profibrotic PECs after adriamycin injury.

Discussion

In the present study, we demonstrated that the transplantation of MSCs both in the peritoneal cavity as well as renal capsular space decreased proteinuria and also partially preserved the podocyte phenotype after adriamycin injury. Interestingly, the transplantation of MSCs either intraperitoneally or subcapsularly, reduced the number of adriamycin-induced profibrotic PECs compared to mice without MSC transplantation. These PECs did not display podocyte markers, i.e., p57 and podocin. Since transplanted MSCs did not have direct

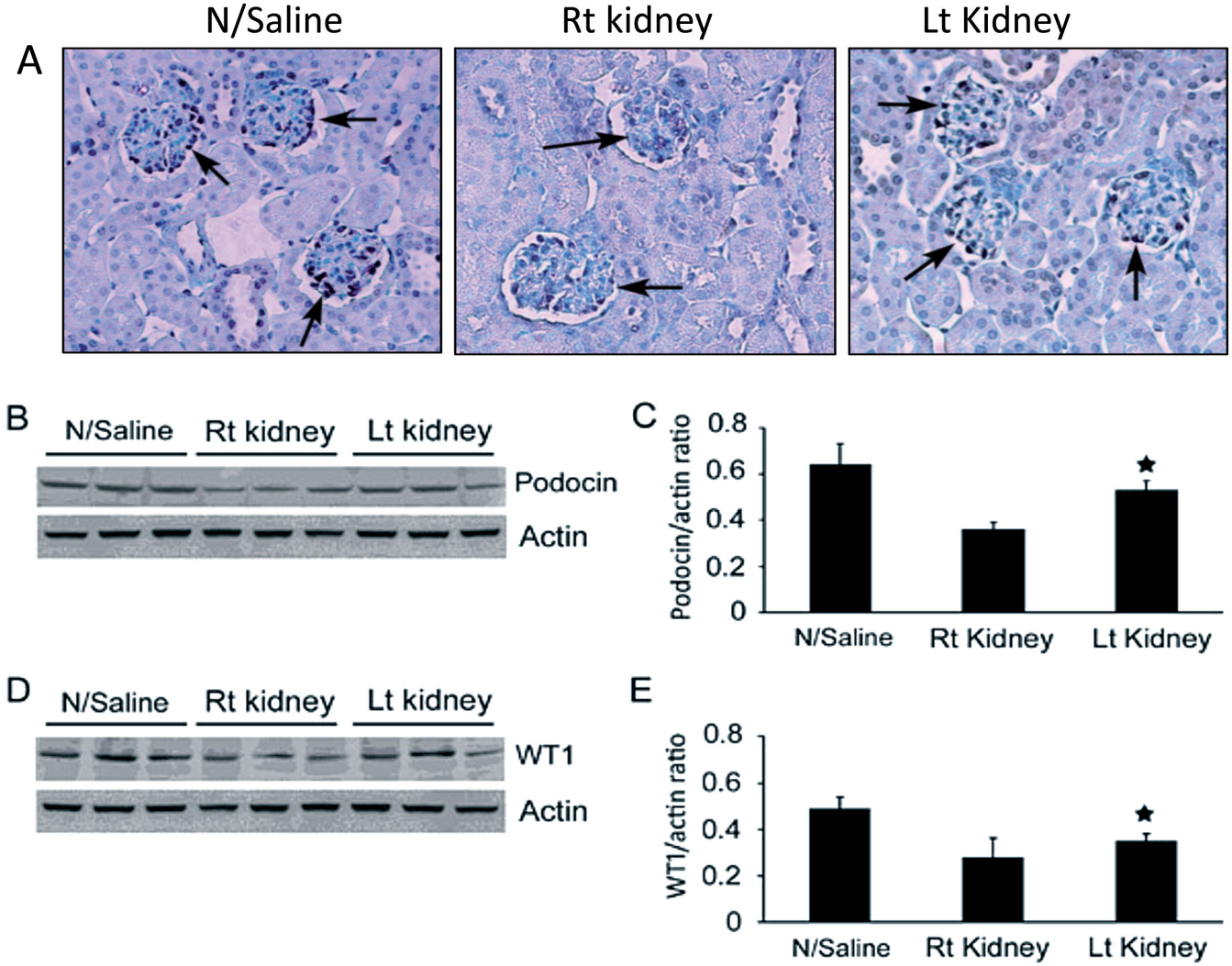

Fig. 3. Effect of single kidney renal subcapsular space (SCS) transplantation of MSCs on Podocin and WT1 expression in Adriamycin-treated mice. **A.** Representative renal cortical sections were immune-labeled for podocin. Podocin expression (indicated by arrows) was relatively preserved in the left kidney, which received MScs when compared with that of the right kidney. **B, D.** Western blot displayed relative preservation of Podocin and WT1 in the left kidney. Cumulative densitometric analysis (n=3) is shown in bar graphs, Podocin **(C)**, and WT1 **(E)**. *P<0.05 compared to the right kidney. × 200.

contact with podocytes in the kidneys, it is likely that these effects of MSCs on PECs and podocytes were mediated by cytokines and growth factors released by transplanted MSCs.

Normally, PECs and tubular cells express CD44, although greater expression of CD44 in PECs has been suggested to be injurious to podocytes (Okamoto et al., 2013). Other investigators suggested that CD44+ve cells with phosphorylation of ERK are profibrotic cells that participate in glomerular sclerosis (Eng et al., 2015; Roeder et al., 2017). In the present study, the number of CD44+ve cells with phosphorylated ERK expression was greater in kidneys with adriamycin injury, whereas the number of such cells decreased in kidneys after MSC transplants. Therefore, transplanted MSCs likely preserved the phenotype of podocytes by decreasing the profibrotic activation of PECs. Previously, the participation of PECs in fibrogenesis was evaluated in four models of podocyte injury (Ohse et al., 2010). While normal glomeruli did not display any overlapping of PEC and podocyte markers in Bowman's capsule or glomerular tufts, overlapping markers were observed in PECs and podocytes after renal injury in TGF-beta1 transgenic, anti-GBM, and membranous nephropathy mouse models. As an exception, Bowman's capsule cells did not show overlapping markers of PECs and podocytes in an FSGS mouse model (Ohse et al., 2010). These findings are consistent with our observations on PECs and podocytes in adriamycin-induced injury.

In the past, several investigators evaluated the role of MSCs in prevention of podocyte injury both in rat and mouse models (Chang et al., 2012; Zoja et al., 2012; Li et al., 2013; Wang et al., 2013; Migliorini et al, 2013; Okamoto et al., 2013; Hamatani et al., 2014; Hakroush

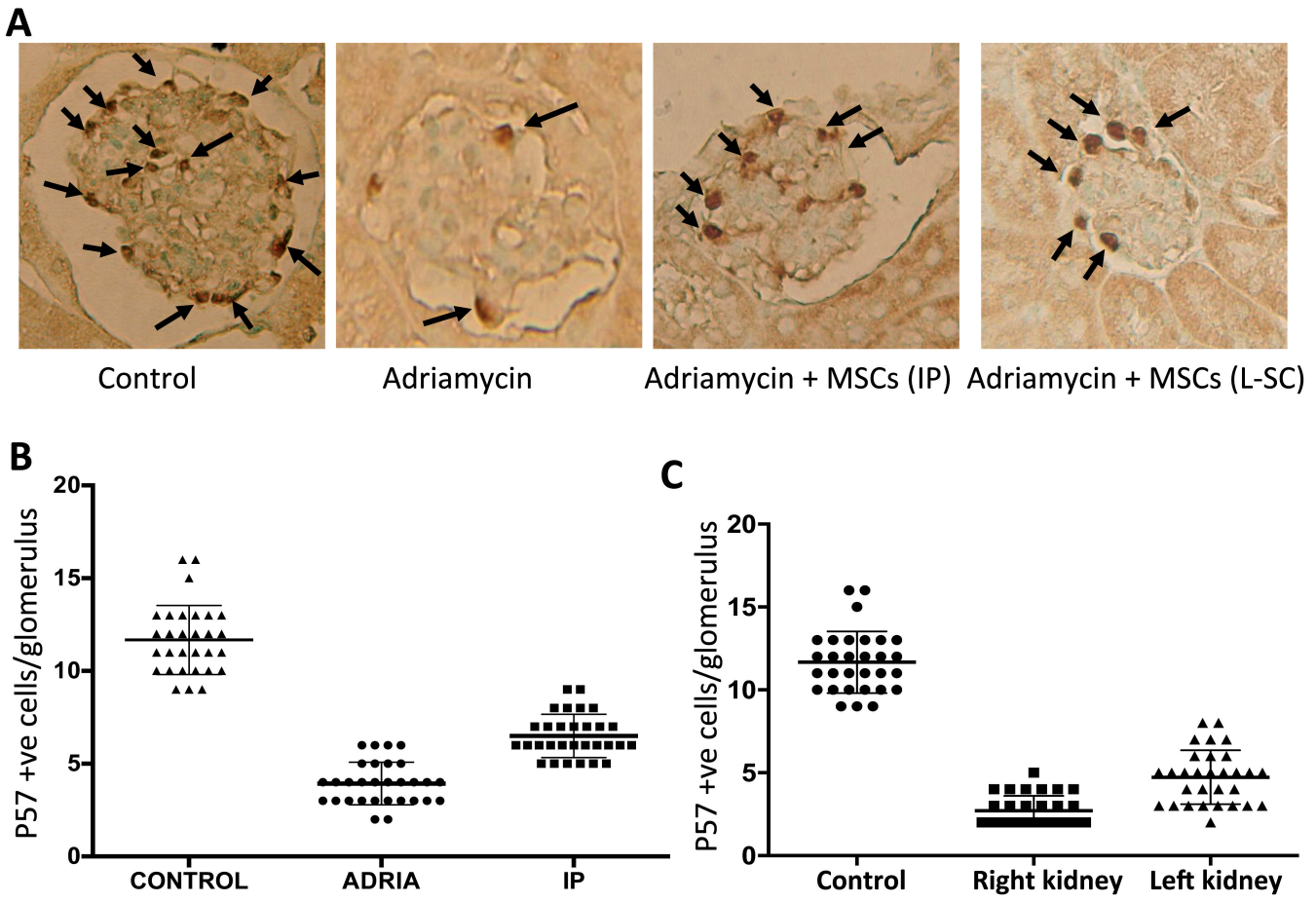

Fig. 4. MSCs preserve p57 +ve cells in glomerular tufts. Renal cortical sections of kidneys from control and experimental (adriamycin, ADRIA; adriamycin + MSCs administered, intraperitoneal, IP; MSCs transplanted in left subscapular space, L-SC) mice were immunolabeled for p57 (n=30, number of glomeruli). **A.** Representative microphotographs are displayed. Dark brown staining indicated p57 +ve cells (indicated by arrows). **B.** A number of p57 +ve cells per glomerulus are shown in scattergrams in control, adriamycin treated, and adriamycin + MSCs administered intraperitoneal. p57 +ve cells (nuclei) are stained dark brown. *P<0.01 vs control; **P<0.05 vs adriamycin (ADRIA). **C.** A number of p57 +ve cells/glomerulus in control left and right kidney kidneys of group C (MSCs were transplanted in the subcapsular region of the left kidneys. Cumulative data are shown as scattergrams.*P<0.001 vs. control; **P<0.01 vs. R-SC. Mann-Whitney test was used to analyze statistical significance. x 400.

et al., 2014; Yamazaki et., 2016). Repeated administration of MSCs in a rat model of adriamycin toxicity helped preserve podocytes (Zoja et al., 2012); similarly, studies in vitro showed MSCs provided protection against apoptosis in podocytes with adriamycin toxicity, and this effect of MSCs was attributed to the release of vascular endothelial growth factor (VEGF) by MSCs (Zoja et., 2012). In our earlier work (Zoja et al., 2012), we found MSCs secreted >40 cytokines, chemokines and growth factors, including VEGF, which added to previous knowledge (Chen et al., 2008; Eliopoulos et al., 2010; Yoon et al., 2010). Certainly, VEGF could have contributed to the preservation of podocyte integrity. Similarly, increasing VEGF secretion enhanced the protective effect of MSCs in kidney cell injury (Yuan et al., 2011). On the other hand, after knockout of insulin-like growth factor-I (IGF-I) or VEGF, MSCs were unable to prevent kidney cell injury (Tögel et al., 2005; Imberti et al., 2007). Moreover, hepatocyte growth factor (HGF) and epidermal growth factor (EGF) have been demonstrated to participate in tubular cell repair and regeneration (Deuse et al., 2009). Our studies differed from other investigators in terms of the administration of MSCs with extracellular matrix support. We previously reported that the use of microcarrier scaffolds markedly improved the survival of transplanted cells over the long-term (Cheng et al., 2009). Transplanted cells immobilized with microcarrier scaffolds did not migrate into kidneys or other locations, which was similar to the fate of other cell types, e.g., intraperitoneally transplanted hepatocytes did not migrate into the liver, but supported organ regeneration by paracrine mechanisms (Bandi et al., 2011).

In the current study, MSCs were transplanted 3 weeks after adriamycin administration; therefore, prevention of podocyte injury was not expected. To evaluate the role of MSCs in the prevention of podocyte injury, one needs to transplant MSCs before the onset of the injury. In real-life situations, patients typically come to medical attention after disease onset. Thus, our study suggests that this strategy could be used to slow down the progression of kidney disease.

In the present study, transplanted MSCs preserved the integrity of podocytes while simultaneously decreasing the profibrotic activation of PECs. However, there was no increase in the number of regenerative PECs. Moreover, it is not clear whether a decrease in the

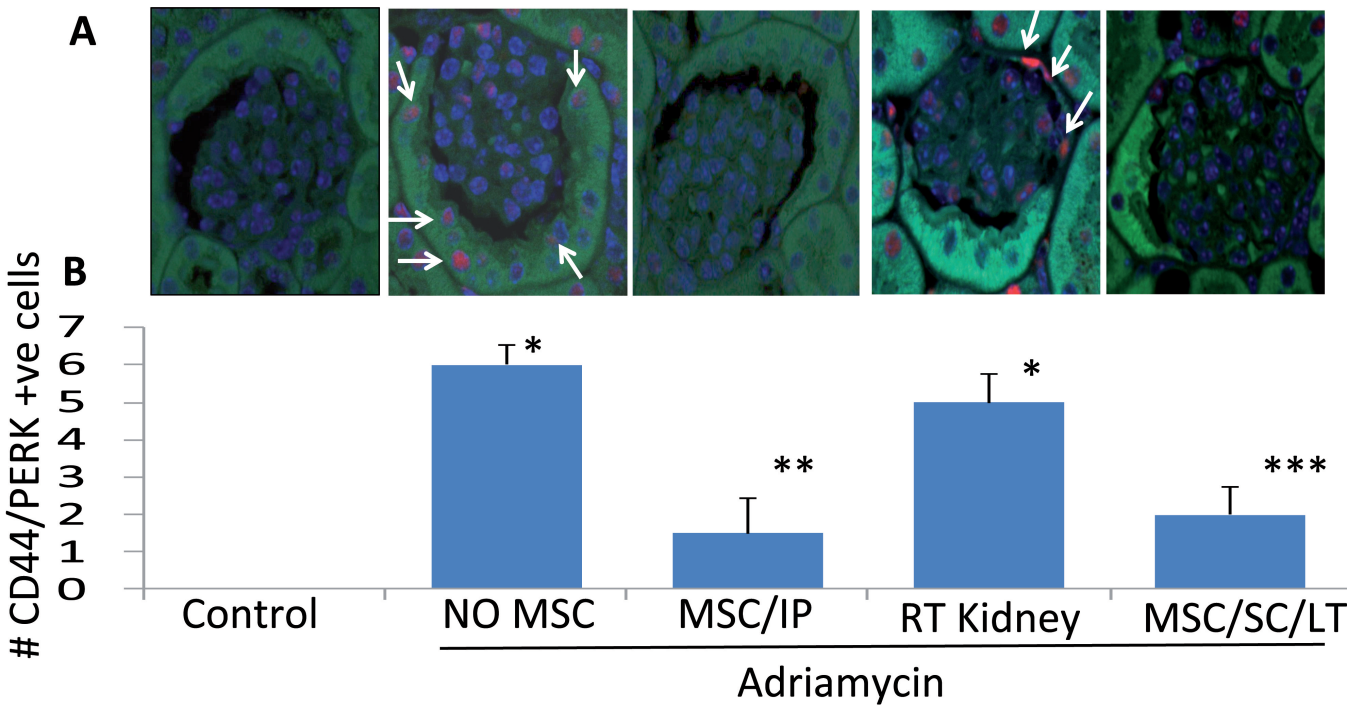

Fig. 5. MSCs transplantation down-regulates number of profibrotic PECs in adriamycin-administered mice. Renal cortical sections of control and experimental mice were co-immunolabeled for CD44 and phos-ERK and examined under a confocal microscope. Co-labeled (CD44 and PERK +ve) cells in Bowman's capsule were counted in each glomerulus. Nuclei were stained by DAPI (blue). **A.** Representative microphotographs from control, Adriamycin-receiving, Adriamycin + MSCs (IP), Adriamycin-receiving right (RT) kidney, and Adriamycin-receiving left (LT) kidney + MSCs transplanted subcapsular (SC). Cells labeled for both CD44 (green fluorescence) and phos-ERK (red fluorescence) are indicated by white arrows. **B.** Cumulative data of mean number (± SD) of double-labeled cells in Bowman's capsule/glomerulus of control and experimental mice (n=16, number of glomeruli). *P<0.001 compared with control; **P<0.005 vs. NO MSCs; ***P<0.05 vs RT Kidney. Mann-Whitney test was used to analyze statistical significance. x 400.

number of profibrotic PECs was a consequence of a lesser requirement of progenitor cells because of the preservation of a higher number of podocytes in animals with transplanted MSCs. Therefore, we consider it likely that decreases in profibrotic cells slowed the progression of sclerosis, and that, in turn, preserved the integrity of podocytes after transplantation of MSCs.

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Conflict of interest. None of the authors has any conflict of interest.

Authors' Contribution. Rukhsana Aslam and Ali Hussain carried out staining, imaging, and analysis of data, Kang Cheng and Vinod Kumar harvested stem cells and participated in animal experimentation; Ashwani Malhotra, Sanjeev Gupta, and Pravin C. Singhal designed the experiments and prepared the manuscript.

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