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Bone marrow-derived mesenchymal stem cells ameliorate nephrosis through repair of impaired podocytes

Abstract

Purpose: The purpose of this study was to investigate the effects of bone marrow-derived mesenchymal stem cells (BMSC) on podocytes of puromycin amino nuclear glucoside (PAN) -induced nephrosis in mice.

Methods: Mice were randomly divided into Control, PAN and BMSC groups. Mice were injected with PAN (0.5 mg/g weight) via the tail vein. The 24-h urinary protein was obtained after modelling, and urinary protein excretion was determined. The blood and kidney specimens were isolated after the tenth day of modelling. Blood samples were collected for measuring serum creatinine (SCr) and blood urea nitrogen (BUN). A sample of kidney was taken for observing pathological changes through hematoxylin-eosin staining and electron microscopy, and the rest of the kidney was used for detecting the protein and mRNA expression of nephrin, CD2AP, synaptopodin, TRPC6 by real-time quantitative PCR, Western-blot and immunohistochemistry.

Results: After PAN injection, podocyte foot process fusion was detected by electron microscopy, and the 24 h urinary protein excretion increased compared with control mice on days 3, 7 and 10 post-PAN injection ($P<0.05$). Serum albumin decreased compared with control mice after day 10 ($P<0.05$). The phenomenon of foot process fusion was ameliorated after administration of BMSC, and 24 h urinary protein decreased ($P<0.05$), while serum albumin increased ($P<0.05$). Nephrin, CD2AP and synaptopodin in the glomerular slit diaphragm were up-regulated compared to PAN nephropathy model mice ($P<0.05$) while TRPC6 was down-regulated ($P<0.05$).

Conclusions: Administration of BMSC reduced foot process fusion and urine protein excretion, and protected against podocyte damage caused by PAN.

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Podocytes are epithelial cells of the renal corpuscle, which covers the outer aspect of the glomerular basement membrane (GBM). Together with the GBM and the capillary endothelium, they form the blood filtration barriers of the glomerulus. Recent studies have consistently shown that damage or loss of podocytes lead to proteinuria [1]. From past studies, we know that podocytes are highly differentiated cells. Importantly, defects during mitosis that impede cell division prevent the reproduction of mature podocytes.

Stem cells are un-differentiated cells that have the ability to self-renew, give rise to multiple cell lineages and proliferate. Under specific conditions, they can differentiate into many different cell types with specific functions. Within the stem cell family, bone marrow-derived mesenchymal stem cells (BMSC) are an important group and originate from the mesoderm and ectoderm during early development. BMSCs are the only cell group, other than hematopoietic stem cells, found in the bone marrow that give rise to blood cells. Studies on the cell cycle of BMSC show that approximately 20% of the cell population is in a resting state, G₀, suggesting that BMSCs have a large potential to proliferate. Under specific *in vitro* or *in vivo* conditions, BMSCs can be induced to differentiate into various cell types including fat, bone, muscle, nerve, heart and endothelial cells [2]. This ability to differentiate is retained even after continuous passage and multiple freeze-thaw cycles. BMSCs are easy to obtain, isolate, culture, amplify and purify. These benefits, together with the ability for BMSCs to differentiate in the absence of immune rejection after autologous transplant, mean that BMSCs could have wide clinical application in treating kidney diseases [3].

Few studies have looked at whether BMSCs, with multi-lineage differentiation potential, can repair damaged podocytes. Using a model of sheep kidney ischemia, Behr *et al.* [4] showed that implanted BMSCs exhibited epithelial cell markers observed in the renal tubules and podocyte phenotypes. Furthermore, studies by Bruno *et al.* [5] showed that renal resident mesenchymal stem cells (MSCs) in human glomeruli can differentiate into capillary endothelial cells in the kidneys and also express cell surface proteins characteristic of podocytes; such as nephrin, podocin, and synaptopodin. In this study, we investigated the potential of cultured BMSCs to repair podocytes in mice with nephrosis induced by puromycin amino nuclear glucoside (PAN).

Materials and Methods

Animals

BALB/C mice (18-20g), provided by the Wu Experimental Animal Center of Fujian were used in this study. To reduce the experiment bias and ensure that the causes of some symptoms in the process of experiment are objective, rather than the inherent factors of mice, considering that the health index of the male mice is better than female mice, we use only male mice. The sex of these animals was assigned following external examination of body characteristics such as external genitalia. Animals were maintained in an animal facility at the First Affiliated Hospital of Fujian Medical University. All mice were housed under controlled temperature (22-25°C) and light conditions with 12-h day/night cycle, and given *ad libitum* access to pellets and water. The bedding of the home cages were kept dry at all times.

Chemicals

Puromycin amino nuclear glucoside (PAN) was purchased from Sigma-Aldrich (St. Louis, MO, USA), L-DMEM culture medium from Hyclone, fetal bovine serum (FBS) and 0.25% trypsin from Gibco, First Strand cDNA Synthesis kit and PCR kit from TaKaRa, polyclonal rabbit anti-mouse nephrin, CD2AP, synaptopodin, and TRPC6 from Abcam, and horseradish peroxidase conjugated goat anti-rabbit secondary antibody from Zhongshan Golden Bridge.

Isolation, culture, and identification of mouse BMSC

Six to eight week old male BALB/C mice were sacrificed by cervical dislocation to obtain the femur and tibia. Bone marrow cells were collected by flushing the marrow cavity from one end using 2 mL L-DMEM, and subsequently filtered through a 150 copper mesh to obtain single cell suspension. The supernatant was discarded, and the cells were re-suspended in L-DMEM (+10% FBS) before being cultured in 25 mL culture flasks using additional L-DMEM +10% FBS. Culture media was changed every 3 days. The cells were passaged at 80-90% confluence and collected after three generations. The cells were collected using 2.5 g/mL trypsin, and L-DMEM was added to inactivate the trypsin. Cells were re-suspended by pipetting up and down, and the resulting suspension was centrifuged at 2000 rpm for 5 min. The supernatant was then discarded and the pellets were washed twice with 2 mL PBS. The final suspension (density of 2x10⁹/L in PBS) was filtered through a 300 µm nylon mesh, and aliquoted into ten 1.5 mL Eppendorf tubes. The following monoclonal antibodies and

their respective isotype controls (10 ml) were added to the tubes: fluorescently-tagged rat anti-mouse CD90-PE; rat anti-mouse CD34-FITC; rat anti-mouse CD44-PE; rat anti-mouse CD11b/c-FITC; and, rat-anti mouse CD29-FITC. The mixture was incubated in the dark at 37°C for 15 min. One milliliter PBS was then added to each tube, the solution mixed and then centrifuged at 2000 rpm for 5 min and the supernatant was discarded. The wash was repeated to remove unbound antibody. After re-suspending the cells with 1 ml PBS, flow cytometry was performed to detect cell surface antigens: CD11b/c; CD34; CD44; CD90; and, CD29.

Creating the PAN mouse model and study groups

Thirty male BALB/C mice were randomly divided into Control, PAN and BMSC groups (n=10/group). Mice in the Control group were given a single injection of 0.5 mL saline via the tail vein. Mice in the PAN group were given a single tail vein injection of PAN (0.5 mg/g), followed by 0.3 mL saline on the same day. Mice in the BMSC group were given a single injection of PAN (0.5 mg/g), followed by 0.3 mL BMSC suspension (2×10^6 /L) on the same day. Urine samples were collected daily from all animals and stored at -80°C with preservatives (37% concentrated hydrochloric acid) until analysis at the end of the modelling period. During this time, the animals were monitored for activity levels, fur quality, body weight and survival.

Analysis of urine protein and kidney function

Urine samples were collected for 24 h from mice 1 day prior to PAN injection, and day 1, 3, 7, and 10 of the modelling period and analyzed for urine protein using an automatic analyzer. On day 10 of the modelling period, 0.5 mL blood was collected retro-orbitally from each mouse. Blood samples were centrifuged at 3000 rpm for 10 min to extract plasma, which was then stored at -80°C, thawed, and analyzed for serum creatinine (SCr) and blood urine nitrogen (BUN) content using an automated analyzer.

Kidney tissue collection

Following retro-orbital blood collection, the renal capsules were vertically incised and both kidneys were rapidly removed. The left kidneys were numbered, stored in 10% formaldehyde, paraffin-embedded, sectioned at 3 μ m thickness using an ultramicrotome, and stained with hematoxylin and eosin (H&E) and immunohistochemistry. Using a sharp blade, 1 mm³ renal cortex tissue was extracted from the right kidneys

and immediately placed into fixative for electron microscopy analysis. The remaining tissue from the right kidneys were stored at -80°C for mRNA and protein expression analysis using PCR and Western blot, respectively.

Electron microscopy analysis of podocytes

Renal cortex tissues (1 mm³) were fixed at 4°C for 2 h in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) for electron microscopy. The tissues were then washed three times in 0.1 mol/L sodium cacodylate buffer (pH 7.4) and fixed at 4°C for another 2 h in 1% osmium tetroxide in the same buffer. Next, the tissues were dehydrated in increasing alcohol concentrations then soaked in pure embedding medium for 2-4 h. The embedded tissues were polymerized at 60°C for 24 h, cut into ultrathin slices, double stained with lead and uranium and analyzed using transmission electron microscopy.

Immunohistochemistry

Immunohistochemistry was performed to assess nephrin, CD2AP, synaptopodin, and TRPC6 antigen expression in kidney tissues. Paraffin slices 3 μ m thick were dewaxed with xylene and dehydrated in a series of graded ethanol. To block endogenous peroxidase, tissue was exposed to 3% H₂O₂ and the slices were incubated in deionized water for 10 min. The slices were then washed in PBS before incubation in 50 μ l diluted primary antibody for 1 h at 37°C. Following this incubation period, tissues were washed with PBS and incubated in secondary antibody for 30 min at 37°C. After washing with PBS, DAB was used for visualizing the immunolabelling. Staining was observed under the microscope and the reaction was stopped using distilled water followed by a wash with tap water. H&E staining was performed followed by graded ethanol dehydration, xylene clearing and neutral gum cementing. PBS replaced primary antibody as a negative control. The presence of dark brown colour represented a positive labelling reaction and light blue indicated the nucleus. Under 200X magnification, five non-overlapping fields were selected in each slice for the imaging software to allow data extraction from pathology images. The data were input into the image analysis system Motic Images Advances for optical density (OD) calculations. The average OD values were used for statistical analysis.

Real-time quantitative PCR

Roughly 50 mg kidney tissue stored in liquid nitrogen was added to 1 mL triol lysate. RNA extraction and reverse

transcription were performed according to the reagent manufacturer's instructions. The primer sequences used and the length of the amplifications are shown in Table 1 (designed and synthesized with assistance from TaKaRa). Quantitative amplification was performed on an ABI PRISM 7500 Fast Fluorescent PCR instrument using SYBR Green. PCR proceeded according to the following steps: initiation at 95°C for 30 s, denaturation at 95°C for 5 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, for 30 cycles. At the end of the reactions, PCR amplification and melting curves were analyzed. The cycle threshold (Ct) values were compared, and the results were represented as the ratio of RQ values between two groups [6]. Each cDNA template was tested three times and the results were averaged. Each reaction had three genes, and each gene had a negative control.

Western blot

From each sample, 50 mg of tissue was placed into protein lysates, and analyzed for protein content following instructions in the Bradford Protein Assay kit. The protein extracts were separated by SDS-PAGE on 10% separating/5% stacking gels and blotted on a nitrocellulose (NC) membrane. NC membranes were blocked for non-specific antibody binding, and incubated in primary monoclonal antibodies diluted with blocking solution: rabbit anti-mouse nephrin (1:400), rabbit anti-mouse CD2AP (1:1000), rabbit anti-mouse synaptopodin (1:2000), and rabbit anti-mouse TRPC6 (1:1000). TIPC-labeled goat anti-rabbit IgG was used as the secondary antibody (1:2000). Blots were developed using enhanced chemiluminescence (ECL), scanned, and analyzed with a White/Ultraviolet Transilluminator software system. Protein expression was represented as integrated optical density (IOD). β -actin was used as an internal control for semi-quantitative analysis; the ratio of proteins to β -actin indicated their relative content.

Statistical analysis

All data are presented as mean $\pm$ SD. Following test for homogeneity of variance, t-test was used to compare the means of two groups; one-way analysis of variance (ANOVA) was used to compare the values at various time points within groups, followed by least significant difference (LSD) *post hoc* test for pair-wise comparison. Data were analyzed using SPSS15.0. Statistical significance was set at $p < 0.05$ ($\alpha = 0.05$).

FIGURE 1. The flow cytometry characterization of surface antigens in BMSCs, among the third passage (P3) BMSCs $13.2 \pm 0.6\%$ were CD11b/c positive, $1.2 \pm 0.5\%$ were CD34 positive, $97.8 \pm 0.9\%$ were CD44 positive, $96.8 \pm 1.4\%$ were CD90 positive, and $97.6 \pm 2.4\%$ were CD29 positive.

Results

Isolation, culture, and identification of mouse BMSC

The cellular morphological characteristics of bone marrow-derived MSCs changed over the course of 7 days: the cells of the original generation were flat in shape, the third generation of cell subculture after 3 days were spindling in shape, the third generation of cell subculture after 7 days were arranging spirally or radially. Using flow cytometry, the third

TABLE 1. PCR primers and product sizes

Target mRNA	Primer	Sequence	PCR product (bp)
Nephrin	F	5'-CGAGGCACTCCGTGAAAC-3'	175
	R	5'-GCCTCGGAAGGTCCAGTT-3'	
CD2AP	F	5'-AGGAATTCAGCCACATCCACA-3'	113
	R	5'-CGATCAATTCCAGTTCGTCCTC-3'	
Synaptopodin	F	5'-GCTCGAATTCGGATGCAAATAAAC-3'	168
	R	5'-CAGGCCACAGTGAGATGTGAAGA-3'	
TRPC6	F	5'-GCATCCAAAGCTCAGAGCATCA-3'	155
	R	5'-GCGATTGCATAAAGACCTTCAGAGA-3'	
GAPDH	F	5'-TGTGTCCGTCGTGGATCTGA-3'	150
	R	5'-TTGCTGTTGAAGTCGCAGGAG-3'	

PCR, polymerase chain reaction; CD2AP, CD2-associated protein; TRPC6, transient receptor potential channel 6; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

FIGURE 3. Comparison of serum albumin in three mice groups. Control group, mice were administrated with 0.5 ml 0.9% sodium chloride via tail vein; PAN group, mice were administrated with puromycin amino nuclear glucoside according to 0.5 mg/g weight via tail vein; BMSC group, mice were administrated with puromycin amino nuclear glucoside according to 0.5 mg/g weight and 0.3 ml 2×10^6 /L cell suspension of bone marrow-derived mesenchymal stem cells via tail vein. Data are mean $\pm$ SD (n=10). *P < 0.05 versus control mice. #P < 0.05 versus PAN mice. PAN, puromycin amino nuclear glucoside; BMSC, bone marrow-derived mesenchymal stem cells

FIGURE 2. Urinary protein excretion at different time points after PAN administration and BMSC transplantation. 24 hours urine samples were collected before 1 day of PAN administration and after 1, 3, 7, 10 days of PAN administration and BMSC transplantation. Data are mean $\pm$ SD (n=10). #P < 0.05 versus control mice. *P < 0.05 versus PAN mice. PAN, puromycin amino nuclear glucoside; BMSC, bone marrow-derived mesenchymal stem cells

FIGURE 4. Glomerular ultrastructure of kidneys at the 10th day after PAN administration and BMSC transplantation. (A) Glomerular ultrastructure from a control mice. No foot process effacement or flattening was seen. (B) Glomerular ultrastructure from PAN mice. Foot process effacement was detected. (C) Glomerulus from a BMSC mice. Podocyte foot process fusion gradually restored, the original fusion of foot processes produce protuberant. PAN, puromycin amino nuclear glucoside; BMSC, bone marrow-derived mesenchymal stem cell.

FIGURE 5. Relative expression of mRNA by real-time quantitative PCR. Real-time quantitative PCR was performed as described in Materials and Methods using RNA prepared from isolated kidney tissue. Control group, mice were administrated with 0.5 ml 0.9% sodium chloride via tail vein; PAN group, mice were administrated with puromycin amino nuclear glucoside according to 0.5 mg/g weight via tail vein; BMSC group, mice were administrated with puromycin amino nuclear glucoside according to 0.5 mg/g weight and 0.3ml 2×10^6 /L cell suspension of bone marrow-derived mesenchymal stem cells via tail vein. Data are mean $\pm$ SD (n=10). *P < 0.05 versus control mice. #P < 0.05 versus PAN mice.

passage (P3) BMSCs $13.2 \pm 0.6\%$ were found to be CD11b/c positive, $1.2 \pm 0.5\%$ were CD34 positive, $97.8 \pm 0.9\%$ were CD44 positive, $96.8 \pm 1.4\%$ were CD90 positive and $97.6 \pm 2.4\%$ were CD29 positive (Figure 1).

Effect of BMSC transplant on urine protein and kidney function in PAN mice

Compared with the Control mice, the mice in the PAN group had significantly higher urine protein levels starting from day 3 of the modelling period ($p < 0.05$). Additionally, compared to Control mice, PAN mice had significantly lower plasma protein from day 10 after modelling ($p < 0.05$), (Table 2, Figures 2 & 3). Mice in the BMSC group had significantly lower urine protein levels and higher levels of plasma protein than PAN mice at any particular time point ($p < 0.05$) (Table 2, Figures 2 and 3). The data for SCr and BUN, with $p > 0.05$ (Table 3), indicate that the differences are insignificant.

Effect of BMSC on kidney tissue podocytes in PAN mice

Ten days into the modelling period, kidney tissue biopsies were analyzed from the three mice groups. H&E staining indicated that there was no significant difference in glomerular morphology between the three groups.

Ten days following PAN injection, ultrastructure examination of the kidney tissues from the three groups of mice using electron microscopy revealed various characteristics. Podocytes in the Control group were shaped like crossed fingers or fences with obvious slits on the foot processes. In the same group, the basement membrane exhibited normal thickness and was tightly connected to the foot processes. Glomerular visceral epithelial cells in the PAN group were swollen. In addition, the majority of podocytes lost their original morphology and fused into a sheet-like structure lacking filtration slits. In the BMSC group, the podocyte foot processes were restored. Processes that were previously fused began reforming finger-like extensions. (Figure 4).

Effect of BMSC transplantation on podocyte-specific proteins in PAN mice

Data from fluorescence quantitative PCR showed that mice in the PAN group had significantly lower mRNA expression of nephrin, CD2AP, and synaptopodin compared with mice in the Control group ($p < 0.05$). These levels were restored in the BMSC group ($p < 0.05$). TRPC6 mRNA in the PAN group was significantly greater than that in the Control group ($p < 0.05$). This increase was significantly attenuated in the BMSC-treated mice ($p < 0.05$) (Figure 5).

FIGURE 6. Expression of nephrin protein by western blot. Western blot was performed as described in Materials and Methods using protein prepared from isolated kidney tissue. Control group, mice were administrated with 0.5 ml 0.9% sodium chloride via tail vein; PAN group, mice were administrated with puromycin amino nuclear glucoside according to 0.5 mg/g weight via tail vein; BMSC group, mice were administrated with puromycin amino nuclear glucoside according to 0.5 mg/g weight and 0.3ml 2×10^6 /L cell suspension of bone marrow-derived mesenchymal stem cells via tail vein. Data are mean $\pm$ SD ($n=10$). * $P < 0.05$ versus control mice. # $P < 0.05$ versus PAN mice.

TABLE 2. Effect of BMSC transplant on urine protein(g/24 h) ($\bar{x} \pm s$, $n=10$)

Groups	day -1	day 1	day 3	day 7	day 10
Control	3.14 $\pm$ 0.12	3.19 $\pm$ 0.17	4.32 $\pm$ 0.59	4.70 $\pm$ 0.53	5.09 $\pm$ 0.65
PAN	3.05 $\pm$ 0.20	5.00 $\pm$ 1.15	35.98 $\pm$ 2.15*	73.20 $\pm$ 3.64*	113.46 $\pm$ 7.78*
BMSC	3.15 $\pm$ 0.23	4.23 $\pm$ 0.91	19.32 $\pm$ 2.13#	24.00 $\pm$ 1.83 #	32.48 $\pm$ 2.17 #

* $P < 0.05$ vs Control; # $P < 0.05$ vs PAN

FIGURE 7. Expression of nephrin, CD2AP, synaptopodin and TRPC6 protein by immunohistochemical staining. Control group: mice were administrated 0.5 ml 0.9% sodium chloride via tail vein; PAN group: mice were administrated puromycin amino nuclear glucoside according to 0.5 mg/g weight via tail vein; BMSC group: mice were administrated puromycin amino nuclear glucoside according to 0.5 mg/g weight and 0.3ml 2×10^6 /L cell suspension of bone marrow-derived mesenchymal stem cells via tail vein.

TABLE 3. Comparison of renal function in three mice groups

Groups	SCr ($\mu\text{mol/L}$)	BUN (mmol/L)
Control	8.75 ± 0.56	36.04 ± 1.51
PAN	$8.69 \pm 0.38^*$	$35.89 \pm 0.92^*$
BMSC	$8.50 \pm 0.40^\#$	$36.28 \pm 0.96^\#$

* $P > 0.05$ vs Control; # $P > 0.05$ vs PAN; Control group, mice were administrated 0.5 ml 0.9% sodium chloride via tail vein; PAN group, mice were administrated puromycin amino nuclear glucoside (0.5 mg/g weight) via tail vein; BMSC group, mice were administrated puromycin amino nuclear glucoside (0.5 mg/g weight) and cell suspension of bone marrow-derived mesenchymal stem cells (0.3 ml 2×10^6 /L) via tail vein. Data are mean $\pm$ SD (n=10). * $P > 0.05$ versus control mice. # $P > 0.05$ versus PAN mice.

Western blot data showed that PAN-injected mice had significantly lower protein expression of nephrin, CD2AP, and synaptopodin compared Control mice ($p < 0.05$). These levels were partially restored following BMSC transplantation ($p < 0.05$ vs. PAN). TRPC6 protein level in the PAN group was significantly elevated compared with the Control group ($p < 0.05$). This increase was significantly reduced in the BMSC group ($p < 0.05$) (Figure 6).

Nephlin expression in kidney tissues, as shown by the IHC data in Figure 8 was concentrated in podocytes and the cytoplasm of tubule epithelial cells. The occurrence of nephlin-positive vacuoles was significantly less in the PAN group than both the Control and BMSC groups. The expression of CD2AP in kidney tissues was concentrated in podocytes, the nuclei of epithelial cells and areas surrounding the nuclei. CD2AP-positive vacuoles were significantly reduced in the PAN group compared to both the Control and BMSC groups. Synaptopodin expression in kidney tissues occurred primarily in podocytes and tubule epithelial cell cytoplasm. Synaptopodin-positive vacuoles were significantly decreased in PAN mice compared to the Control and BMSC groups. TRPC6 expression in kidney tissues was localized to podocytes and the cytoplasm of epithelial cells in the tubules. The occurrence of TRPC6-positive vacuoles in the PAN group was greater than in the Control and BMSC groups (Figure 7).

Discussion

The major finding of the present work was that the phenomenon of foot process fusion was ameliorated after administration of BMSC, and 24 h urinary protein decreased, while serum albumin increased. Nephlin, CD2AP, synaptopodin in the glomerular SD were up-regulated while TRPC6 was down-regulated. Administration of BMSC reduced foot process fusion and urine protein excretion, and protected against podocyte damage caused by PAN.

Podocyte damage has been demonstrated in minimal change disease (MCD), focal segmental glomerulosclerosis, membranous nephropathy and other glomerular diseases [7-8]. Studies have also shown that damage or loss of podocytes critically influenced occurrence and development of proteinuria [8-9]. PAN-induced kidney disease in rats is a classic model of MCD in animals [10]. Its clinical manifestation and pathological changes, such as foot process fusion and disappearance of SD in podocytes, are reminiscent of MCD in humans. As the pathological changes associated with the model are simple, this is ideal for investigating primary podocyte damage. Jones et al. [11] showed that a single intraperitoneal injection of PAN (0.1 mg/g) in adult

Sprague-Dawley rats can directly damage podocytes, leading to a release of large quantities of proteins in the urine that diminished gradually within 20 days after injection. A second injection of PAN irreversibly damaged podocytes and induced glomerulosclerosis. Due to species differences, proteinuria is not observed in adult mice given the same dose of PAN (0.1 mg/g) [12]. In addition, intraperitoneal injection could lead to tolerance, which prevents the reproduction of MCD model in mice [13]. Wang *et al.* [14] showed that a single epidermal injection of PAN (0.5 mg/g) in mice can generate excess urine protein, thus reproducing the classic rat disease model in mice. Following our earlier experiments, we gave mice single injections of PAN (0.5 mg/g), and from a 24 h urine collection 1 day prior to, and 1, 3, 7 and 10 days after injection, as well as blood samples collected 10 days following injection, we saw a steady increase in urine protein. Compared with the Control group, PAN treated animals had lower plasma protein, while no significant changes in SCr or BUN were observed. The microscopic appearance of glomeruli were generally healthy; however, under electron microscopy, foot processes of podocytes were fused, indicating that damage to the kidneys was minimal and did not significantly alter kidney function. This further suggests that adverse effects of PAN were limited to podocytes only, creating a minimally changing mouse disease model.

BMSC are the only cells other than hematopoietic stem cells found in the bone marrow capable of self-renewal and generating multiple cell lineages. Recent studies have investigated the efficacy of BMSC therapy in restoring function in various kidney diseases [15-16]. Zoja *et al.* [17] used doxorubicin to create a rat model of focal segmental glomerulosclerosis. In this study, death and detachment of podocytes were reduced, glomerulosclerosis was ameliorated, and expression of CD2-associated protein (CD2AP) and nephrin increased after injecting rats with BMSCs. Similarly, *in vitro* co-culture of BMSC and podocytes treated with doxorubicin showed elevated endothelial growth factor and reduced injury in podocytes.

Nephrin was the first globulin transmembrane protein found specifically on the SD of podocytes and is important in maintaining slit integrity [18-19]; therefore, nephrin serves as an early marker of podocyte damage [20]. In nephrin knockout mice, podocyte foot processes disappear, SD are deformed and excess proteinuria occurs. These findings support the importance of nephrin in maintaining the structural integrity and function of SD [21]. Studies have also shown that, together, nephrin and podocin associate with CD2AP to influence the cytoskeleton and tight

junction-associated proteins and maintain the integrity and function podocytes. Chuang *et al.* [22] showed that, in the presence of CD2AP, nephrin could activate phosphatidylinositol-3-kinase (PI3K)/AKT signalling, which suppresses cytoskeletal rearrangement associated with podocyte effacement. This also highlights the importance of nephrin in maintaining podocyte integrity and function. In a MCD model, nephrin expression decreases with increasing proteinuria severity [23-24]. In the present study, we showed that proteinuria in PAN mice was accompanied by a decrease in both nephrin protein and mRNA expression. BMSC transplantation reversed this and increased the expression of nephrin in PAN mice. These results suggest that BMSC transplantation may repair damaged podocytes.

CD2AP belongs to the immunoglobulin superfamily. As CD2AP is rich in Src homology 3 (SH3) domains, it functions as an adaptor protein found abundantly throughout the body. In the kidneys, CD2AP is distributed in SD of podocytes and tubular cells and functions to connect neighboring foot processes. It crosslinks with transmembrane adhesion proteins, such as podocin, nephrin, and NEPH1-2, to maintain the integrity of the podocyte SD, and is involved in the formation and maintenance of glomerular filtration barriers [25-26]. Studies by Shih *et al.* [27] showed that, in mouse pups lacking CD2AP (CD2AP^{-/-}), proteinuria develops immediately after birth and rapidly evolves into nephrotic syndrome within 3 weeks, and the pups die by 6 weeks from kidney failure. Studies by Zhang *et al.* [28] showed that, following down-regulation of CD2AP, podocyte proliferation was severely suppressed and podocyte cytoskeleton collapsed, leading to proteinuria. Tossidou *et al.* [29] showed that in CD2AP^{-/-} mice, some signaling pathways were terminated, interrupting podocyte function. Damaged podocytes can up-regulate CIN85 protein expression to restore signaling, implicating the importance of CD2AP in intracellular signal transduction. Moreover, CD2AP plays an important role in cytoskeleton formation by interacting directly with F-actin to maintain normal cytoskeletal architecture in podocytes [30]. In rat PAN models, the CD2AP expression is inversely correlated with the proteinuria severity [31, 32]. In the present study, both the mRNA and protein expression of CD2AP decreased in the mouse PAN model; however, with BMSC treatment, CD2AP expression increased, indicating that BMSCs may repair damaged podocytes.

Synaptopodin is a 73.7 kDa, linear, proline-rich protein that associates with actin filaments to stabilize cytoskeletal architecture. During fetal development, immature podocytes lack synaptopodin, which is expressed only when foot

processes start forming; thus, synaptopodin is an important marker of podocyte maturation [33]. When studying synaptopodin knockout podocytes, Kim *et al.* [34] discovered that synaptopodin can associate with SD proteins, such as nephrin, Neph1, MAGI-1, and TRPC6, through BKCa channel proteins to sustain SD function. Furthermore, synaptopodin can combine with α -actinin-4, CD2AP, and MAGI-1 to maintain normal cytoskeletal architecture and strengthen the glomerular filtration barrier. Studies have shown that synaptopodin expression decreases in renal MCD, focal segmental glomerulosclerosis, membranous nephropathy and other glomerular diseases. Zuo *et al.* [35] demonstrated a significant reduction in synaptopodin expression in rat PAN models. Wagrowska-Danilewicz *et al.* [36] reported similar findings. As a result, decreased synaptopodin has been implicated in the progression of podocyte damage. In the present study, when we injected PAN, the mRNA and protein expression levels of synaptopodin decreased in kidney tissues; however, with BMSC transplantation, both levels increased, further suggesting that BMSC transplant may repair damaged podocytes.

TRPC6 is a protein recently discovered in the podocyte filtration membrane. It belongs to the superfamily of transient receptor potential cation channel proteins and is non-selective for cations. Winn *et al.* [37] first identified TRPC6 and assigned the gene to chromosome 11q21-22. In patients with segmental glomerulosclerosis, MCD, and membranous nephropathy, Moller *et al.* [38] discovered that the TRPC6 mRNA and protein expression increased and exhibited a linear relationship with the severity of podocyte pathology. The same group also showed that plasmid-mediated overexpression of TRPC6 in healthy mice induced proteinuria, which indicates that overexpression of TRPC6 damages podocytes and causes subsequent proteinuria. Through the identification of a wild-type and two mutant TRPC6 (P111Q and E896K) over-expressing mice, Krall *et al.* [39] showed that overexpression of TRPC6 induced fusion of foot processes in podocytes, causing proteinuria. Therefore, overexpression of TRPC6, wild-type or mutant, is sufficient to cause proteinuria.

Current studies show that TRPC6 can interact with G-protein coupled receptors to activate tyrosine kinases, which then promotes influx and intracellular accumulation of calcium ions by regulating cation-dependent phospholipase C channels; thus, implicating TRPC6 in pathophysiology [40-41]. Through plasmid-mediated overexpression of TRPC6 in podocytes, Wiggins *et al.* [42] proved that TRPC6-mediated calcium influx is involved in restructuring the podocyte cytoskeleton, leading to F-actin derangement and

decreased expression of SD proteins nephrin and synaptopodin. Wilson *et al.* [43] discovered that TRPC6 coding gene mutations lead to the disorder of glomerular filtration barrier and actin cytoskeleton rearrangement, and the occurrence of kidney diseases. Research by Moller *et al.* [39] further established that over-expression of TRPC6 affects podocyte foot process integrity through its interaction with nephrin. Jiang *et al.* [44] utilized RhoA inhibitors to suppress the effect of TRPC6 over-expression, implicating RhoA as an important component in TRPC6-induced proteinuria, derangement of podocyte cytoskeleton, decreased expression of nephrin and foot process effacement. Zhu *et al.* [45] also showed that TRPC6 overexpression increases podocyte intracellular calcium to activate RhoA signaling pathways and subsequent kidney damage.

In our mouse PAN model, proteinuria, overexpression of TRPC6, and downregulation of nephrin, CD2AP, and synaptopodin were all observed. These findings strongly suggest that TRPC6 overexpression induces calcium overload-induced apoptosis. More importantly, they show that TRPC6 overexpression decreases filtration membrane proteins, which disrupts integrity of SD, foot process and glomerular filtration barrier, and causes proteinuria. Following BMSC transplantation, TRPC6 mRNA and protein expression decreased significantly, indicating that BMSCs may repair damaged podocytes.

Some studies have suggested that MSCs represent a useful strategy to preserve podocyte viability and reduce glomerular inflammation and sclerosis. Potential mechanisms include the following: a) MSCs may have the tropism to arrive at damaged kidney; and, b) MSCs have the ability to provide a local pro-survival environment. Similarly, *in vitro* co-culture of BMSC and podocytes treated with doxorubicin showed elevated endothelial growth factor and reduced injury in podocytes [17]. Another study indicates that BMSC undergo a degree of podocytic differentiation *in vitro* [46]. In another study [47] using a model of autosomally-recessive Alport syndrome mice, Prodromidi *et al.* showed that a) an apparent podocyte phenotype was showed in mice transplanted with bone marrow, b) the podocyte defect was rectified and c) glomerular scarring and interstitial fibrosis were also significantly decreased. These improvements in mice resulted from bone marrow transplantation from wild-type donors, and the mechanism by which these above-mentioned phenomena occurred may in part involve generation of podocytes without the gene defect [47].

Our study was limited by the lack of identifying BMSC markers that could allow us to determine whether BMSCs

colonized kidney tissue after transplantation, and to assess whether BMSCs underwent phenotypic changes to produce molecules that promote podocyte repair. Ongoing and future studies will help shed light on these remaining questions, and further our understanding of BMSC therapy on treating kidney disease. Another limitation is that only male animals were used in this study. To translate our results to the clinic, it will be necessary to add female animals and to carefully compare the data with those from male animals.

In summary, proteinuria, overexpression of TRPC6 and downregulation of nephrin, CD2AP, and synaptopodin were all observed in our mouse PAN model. Following BMSC transplantation, TRPC6 mRNA and protein expression decreased significantly, expressions of nephrin, CD2AP, and synaptopodin increased. These results show that administration of BMSC has certain protection effect on podocyte SD related protein and cytoskeleton proteins, to maintain the structure stability and sufficient cell normal function of SD, and has repairing effect on the damaged podocyte.

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