

Original Article

Transplantation of human umbilical cord mesenchymal stem cell relieves the symptoms of lupus nephritis by inhibiting osteopontin expression

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Abstract: Systemic lupus erythematosus (SLE) is a systemic autoimmune disease which may develop into lupus nephritis (LN) and subsequently lead to injury of renal microstructure. It has been indicated that mesenchymal stem cell (MSC) showed therapeutic effect in LN. Here, we present evidence of mechanisms of human umbilical cord derived MSC (Huc-MSC) in ameliorating symptoms of LN. Our results demonstrated that proteinuria concentration is significantly decreased after Huc-MSC transplantation. Moreover, we observed a significant reduction of osteopontin (OPN) expression in renal tissues from Huc-MSC-transplant mice. The expression of matrix metalloproteinases 2 (MMP-2) and MMP-9 were inhibited in renal tissues of Huc-MSC transplantation by the similar fashion. Our results also indicated that the expression of MMP-2 and MMP-9 correlated with basal OPN expression. Finally, our results showed that Huc-MSC treatment can inhibit the expressions of transcription factors AP-1 and SP-1 as well as NF- κ B, which were also associated with the low levels of OPN. Taken together, we report an alternative mechanism of Huc-MSC treatment in LN possibly through inhibiting OPN expression.

Keywords: Osteopontin, mesenchymal stem cell, lupus nephritis, matrix metalloproteinase

Introduction

Mesenchymal stem cell (MSC) was implicated in functions that related to tissue repair, regeneration and regulation of the immune system. Because of its capacities of self-renewal, differentiation and immune-regulation, MSC has been used in cell-based therapies to treat varieties of inflammatory and autoimmune diseases, such as colitis [1], rheumatoid arthritis [2] and systemic lupus erythematosus (SLE) [3]. In addition, due to the lack of cell-surface human leukocyte antigen class DR (HLA-DR) and co-stimulatory molecules including CD80 and CD86, MSC is suitable for allogeneic cell-based therapy [4].

SLE, characterized by multiple organs injury and dysfunction of B cell and T cell, is a multi-systemic autoimmune disorder [5]. LN is a leading complication of SLE with high morbidity and mortality. Renal biopsy demonstrated that LN belongs to glomerulonephritis triggered by

circulating immune complexes [6]. Recent studies have suggested that the autoreactive high-affinity B and T cell clones in SLE patients resulted in autoantibody production and immune complex formation [7, 8]. These results strongly suggests that the loss of self-immunotolerance and the formation of autoantibodies deposited in the kidney play an important role in LN pathogenesis. At present, many drugs, such as cellcept, cyclophosphamide, have been used in SLE. In addition, MSC transplantation has been reported that can greatly ameliorate symptoms of LN through its immunosuppressive effect [9]. However, the exact mechanisms of MSC in SLE therapy are not well characterized.

Osteopontin (OPN), known as secreted phosphoprotein 1 (SPP1), is a soluble, highly modified, secreted extracellular matrix glycoprotein [10]. It has been reported that OPN is widely distributed among different cell types and tissues, including bone cell, immune cell,

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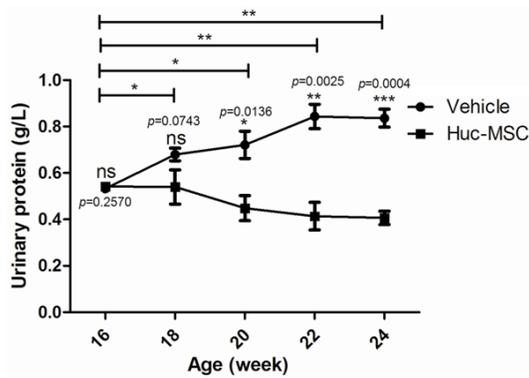


Figure 1. Huc-MSC transplantation promoted the decrease of urinary protein concentration in MRL/lpr mice. Treated MRL/lpr mice with Huc-MSC, and then observed urinary protein in different times. (Student t-test, one-way ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

fibroblast, endothelial cell brain, intestine, lung and kidney [11]. Research has shown that OPN, as a cytokine, is strongly associated with various inflammatory and autoimmune diseases [12]. Moreover, OPN plays a major role in cardiovascular disease [13], diabetic nephropathy [14], and SLE [15]. Research suggested that OPN is highly expressed in SLE patients and MRL/lpr mice, which develop into a lupus-like disease [10, 16]. Xu and colleagues demonstrated that the polymorphism of *OPN* gene appears to be closely associated with the susceptibility to SLE [17]. It is known that the expression of OPN can be stimulated by multiple factors. OPN phosphorylation can be regulated by Ca^{2+} signaling, which is considered the major contributing factor [18]. Research has shown that OPN participates in intracellular signaling pathways, such as MER/ERK pathway, MAPK pathway, PI3K/Akt pathway and JNK pathway [19]. In addition, activation of OPN promoter can be directly regulated by transcription factors, particularly AP-1 and SP-1, which play a critical role in acute or chronic nephritis [12, 19]. Importantly, OPN silencing suppresses the NF- κ B activity, which further leads to decreasing expression of downstream factors such as matrix metalloproteinase (MMP) [20].

Here, our present study indicated that human umbilical cord derived MSC (Huc-MSC) effectively relieved the symptoms of LN, revealing a novel mechanism of Huc-MSC in LN treatment

through inhibiting OPN expression in renal tissues.

Materials and methods

Isolation and culture of Huc-MSC

Human umbilical cord derived MSC (Huc-MSC) was purchased from Beike Biology Technology Company in Shenzhen. The Huc-MSCs were cultured in DMEM/F12 medium with 10% fetal bovine serum, 100 U/mL penicillin and 100 μ g/mL streptomycin. Cells were incubated at 37°C with 5% CO_2 and saturating humidity. When reaching 60% confluence, the cells were trypsinized with 0.25% trypsin-EDTA (Invitrogen Life Science) and plated into a new culture flask. The surface immunophenotype and multilineage differentiation potential of Huc-MSC were confirmed prior to the commencement of the transplantation experiments.

Animal model of LN

Two-month-old female MRL/lpr mice were obtained from Model Animal Research Center of Nanjing University in a pathogen-free facility. All mice used in the experiments were housed in the same room and fed with an identical diet. At 18 weeks of age, the MRL/lpr mice were randomized and divided into the following groups: (i) Huc-MSC-transplant mice ($n=8$); (ii) wild-type control mice ($n=8$). The experimental protocols conformed to the animal care guidelines of the China Physiologic Society and were approved by our Institutional Animal Research Committee.

Transplantation of Huc-MSC

In group (i), 18-week-old MRL/lpr mice were received transplantation of Huc-MSC (5×10^5 cells) via intravenous injection. In group (ii), 18-week-old MRL/lpr mice were received 0.5ml saline via intravenous injection. All mice were sacrificed at 24-week-old to evaluate the therapeutic effectiveness of Huc-MSC transplantation.

Renal function assessments

To analyze the renal function, urine of mice was collected and urinary protein was measured every two weeks. Proteinuria concentration

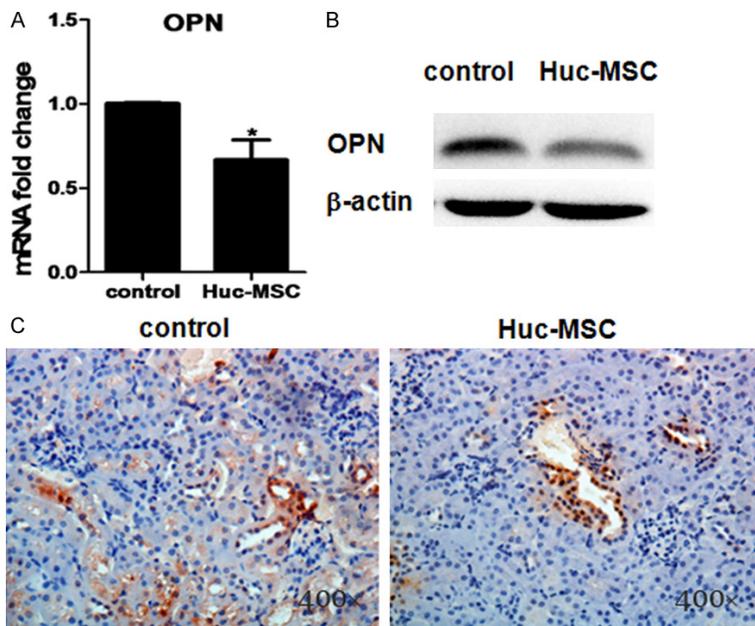


Figure 2. Transplantation of Huc-MSc reduced OPN level in MRL/lpr mice. A. Real-time PCR confirmed that the OPN mRNA level reduced after transplanting Huc-MSc into MRL/lpr mice. Error bars indicate SE. *, $P < 0.05$ (Student t-test). B. The protein level of OPN diminished significantly in Huc-MSc transplantation mice. Renal tissue lysates were used for Western blotting with anti-OPN antibody, β -actin was used as a control. C. Huc-MSc transplantation decreased the OPN intensity (brown) significantly. MRL/lpr mice were transplanted with Huc-MSc and harvested for immunohistochemical staining assay with anti-OPN antibody. Bar: $400\times$.

was tested using Coomassie Brilliant Blue. All of the results were based on three independent trials.

Quantitative reverse transcription polymerase chain reaction analysis

Total RNA of kidney tissues were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Integrity of RNA was tested by formaldehyde denaturalization agarose gel electrophoresis. Concentration of RNA was measured by the Smart Spec™ Plus spectrophotometry (Bio-Rad). For Quantitative Reverse Transcription Polymerase Chain Reaction (Q-PCR) analysis of OPN, AP-1, SP-1, p65, MMP-2 and MMP-9, 1 μ g of total RNA was reverse-transcribed to cDNA. Q-PCR was performed on an Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) using SYBR green dye (Bio-Rad). For quantification, the relative mRNA levels of specific gene expression were obtained using the $2^{-\Delta\Delta C_t}$ method to obtain relative variables, further make the results more scie-

ntific. All of the results were based on three independent trials.

Immunohistochemical analysis

Serial sections (4- μ m thickness) were cut on a paraffin block. Endogenous peroxidase activity was quenched by incubation with 3% H_2O_2 and non-specific antibody binding was blocked with 5% normal goat serum for 30 min. The sections were incubated with primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C and were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (dilution 1:500) (Santa Cruz Biotechnology, Santa Cruz, CA) for two hours at room temperature. For the samples incubated with horseradish peroxidase (HRP)-conjugated secondary antibody, the reactions were visualized by devel-

opment in DAB reagent (Boster Biological Technology, Ltd, Wuhan, China), and the nuclei were counterstained with hematoxylin. The sections were examined under a light microscope (Olympus, Tokyo, Japan); the brown indicated positive expression of the antibody, and the blue staining represented the nuclei. All of the results were based on three independent trials.

Statistical analysis

Quantitative data were expressed as mean \pm SEM. All graphing and statistical analyses were performed using GraphPad prism 5. The Student t-test was used to evaluate the significant differences between the experiment values of the two samples, and comparisons among groups were performed using one-way ANOVA followed by the Student-Newman-Keuls multiple comparison tests. Pearson's correlation coefficient was used to assess the strength of associations between variables. Significance was considered with P value < 0.05 .

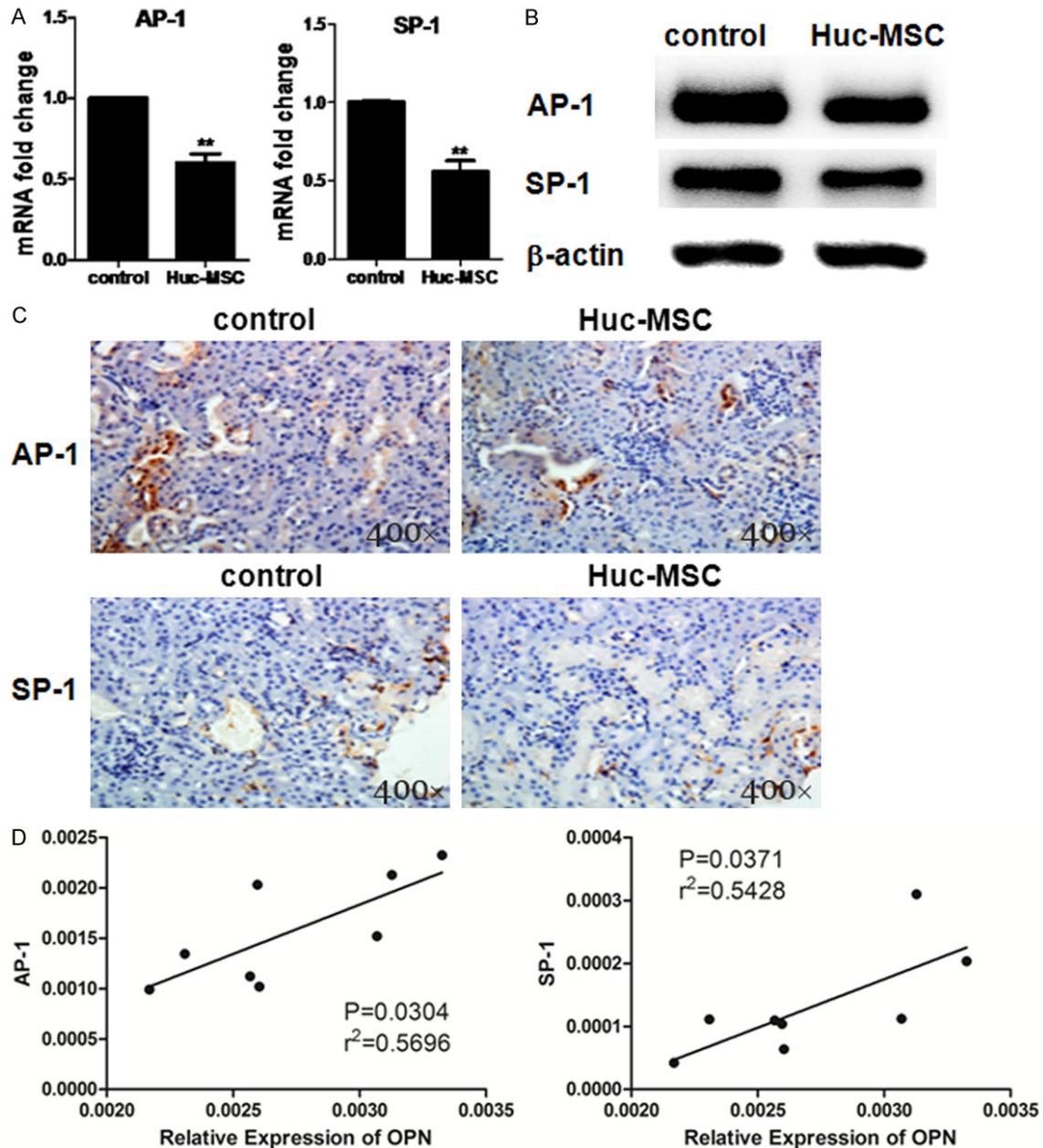


Figure 3. Huc-MSC inhibited AP-1 and SP-1 in mRNA and protein levels. A. Loss of AP-1 and SP-1 mRNA in MRL/lpr mice treated with Huc-MSC by Real-time PCR. Error bars indicate SE. **, P<0.01 (Student t-test). B. AP-1 and SP-1 were detected by Western blotting in control renal tissues and in Huc-MSC-transplantation renal tissues. C. Immunohistochemical staining analysis of AP-1 and SP-1 expression in control renal tissues and in Huc-MSC-transplantation renal tissues. Bar: 400 \times . D. Correlation analysis (Pearson's correlation scatter plot). Examined the expression of AP-1, SP-1 and OPN in each group of each mice, to analyze the correlation AP-1, SP-1 to OPN. There was positive correlation between AP-1 and OPN ($r^2=0.5696$, $P=0.0304$), there was positive correlation between SP-1 and OPN ($r^2=0.5428$, $P=0.0371$). Huc-MSC-transplant mice number (n=8).

Results

Huc-MSC transplantation alleviated proteinuria concentration in MRL/lpr mice

In MRL/lpr mice, proteinuria concentrations were significantly elevated, which resulted from

renal damage. We found that proteinuria in the control group was progressive enhanced at 16th week. Meanwhile, the increase of proteinuria reached a peak at 24th week. Moreover, mild proteinuria was also detected at 16th week in response to Huc-MSC transplantation. Our results showed a lower proteinuria concen-

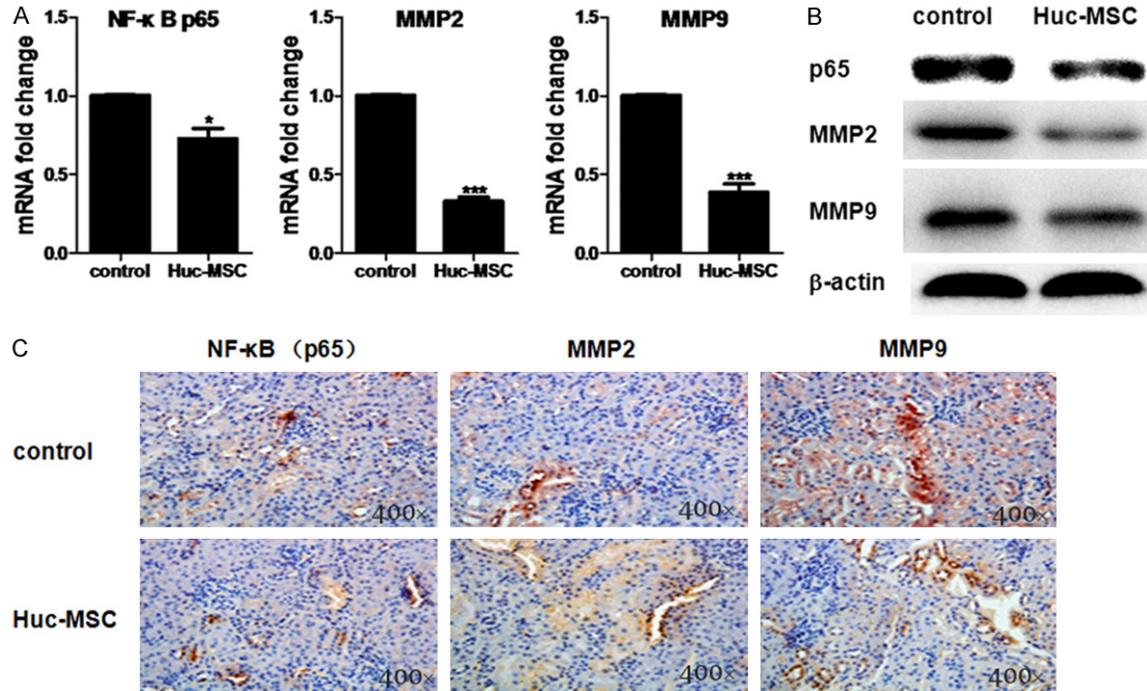


Figure 4. Transplantation of Huc-MSC inhibited MMP-2 and MMP-9 through NF-κB pathway. A. Real-time PCR confirmed that the p65, MMP-2 and MMP-9 mRNA levels reduced via transplanting Huc-MSC into MRL/lpr mice. Error bars indicate SE. *, P<0.05, ***, P<0.001 (Student t-test). B. The protein levels of p65, MMP-2 and MMP-9 were diminished significantly after Huc-MSC transplantation. Renal tissue lysates were used for Western blotting, β-actin was used as a control. C. Huc-MSC transplantation decreased the p65, MMP-2 and MMP-9 intensities (brown) significantly. MRL/lpr mice were transplanted with Huc-MSC and harvested for immunohistochemical staining assay. Bar: 400×.

tration in Huc-MSC transplantation group than in the control group without Huc-MSC at 24th week (**Figure 1**). Given these data, the results indicated that transplantation of Huc-MSC attenuated proteinuria concentrations in MRL/lpr mice, relieving the symptoms of LN.

Huc-MSC transplantation reduced OPN levels in MRL/lpr mice

Recently studies have revealed OPN was significantly increased in SLE mouse, which may be served as a potential marker for the susceptibility, severity of SLE [10], we wanted to know whether OPN was related to the efficacy of Huc-MSC transplantation. Real-time PCR from three independent trials confirmed that the levels of OPN mRNA were markedly decreased in renal tissues from Huc-MSC-transplant mice compared to control mock (**Figure 2A**). Similarly, the protein levels of OPN were significantly diminished in Huc-MSC transplantation mice (**Figure 2B**). To further identify the above results, we performed immunohisto-

chemistry which also showed significantly decreased of OPN intensity in Huc-MSC-transplant mice; control experiments, on the other hand, revealed no significant OPN change (**Figure 2C**). These results demonstrated that the expression of OPN was decreased after transplanting Huc-MSC into MRL/lpr mice.

Transplantation of Huc-MSC inhibited transcription factors AP-1 and SP-1 in MRL/lpr mice renal tissues

It has been suggested that the promoter activation and the expression of OPN can be directly regulated by transcription factors AP-1 and SP-1, representative members of the major contributing factors in renal pathogenicity [12]. Previous research showed that AP-1 and SP-1 expression were progressively enhanced in MRL/lpr mice. We also tested Lupus Nephritis treatment with Huc-MSC inhibited the expression of transcription factors AP-1 and SP-1 [12]. Our results indicated that both of mRNA and protein expression levels of AP-1 and SP-1

were obviously decreased after Huc-MSCTransplantation compared to that in the control group (**Figure 3A** and **3B**). To further confirm the interaction between Huc-MSCTransplantation and AP-1, SP-1, we carried out immunohistochemical staining. The positive staining of transcription factor AP-1 and SP-1 were much weaker in renal tissues from Huc-MSCTransplant mice than that in the control renal tissues (**Figure 3C**). In brief, these results implied that AP-1 and SP-1 expressions were decreased in renal tissues treated with Huc-MSCTransplantation. As we have confirmed that Huc-MSCTransplantation promoted the dramatic reduction of OPN, we wondered if AP-1 and SP-1 were related to OPN. Pearson's correlation analysis showed that both of AP-1 and SP-1 in renal tissues from Huc-MSCTransplant mice were positively correlated with OPN expression levels, indicating OPN expression may be regulated by AP-1 and SP-1 (**Figure 3D**, [Supplementary Figure 1](#)).

Transplantation of Huc-MSCTransplantation inhibited expression of MMP-2 and MMP-9 through NF- κ B pathway

OPN has been proposed to promote the expressions of matrix metalloproteinase 2 (MMP-2) and MMP-9 through activating NF- κ B and AP-1 pathways [20, 21]. Moreover, the increase of MMP-2 and MMP-9 has been demonstrated to be greatly associated with the development of LN [22-24]. We found that the mRNA expression levels of MMP-2 and MMP-9 were significantly lower in renal tissues from Huc-MSCTransplant mice than that from the control group (**Figure 4A**). Consistently, both of MMP-2 and MMP-9 protein expression in renal tissues were also suppressed by transplanting Huc-MSCTransplantation (**Figure 4B**). Furthermore, we treated MRL/lpr mice with Huc-MSCTransplantation, and MMP-2 and MMP-9 were weaker compared to the control group via immunohistochemical staining (**Figure 4C**). As we know, MMP-2 and MMP-9 are considered the major contributing factors of NF- κ B pathway [20]. We assessed the expression of NF- κ B and found that it was affected by transplantation of Huc-MSCTransplantation. The NF- κ B family is comprised of five different members p65, Rel B, C2Rel, p50 and p52, in which p65 is an important member. Therefore, we detected the expression of p65. The loss of p65 in mRNA and protein levels in response to Huc-MSCTransplantation was observed (**Figure 4A** and

4B). Importantly, immunohistochemical staining showed in contrast to the control, the level of p65 in response to Huc-MSCTransplantation in MRL/lpr mice was also decreased (**Figure 4C**). In conclusion, these results indicated that Huc-MSCTransplantation inhibited expression of MMP-2 and MMP-9, further suppressed OPN expression, through NF- κ B pathway.

Discussion

In the present study, we have demonstrated that Huc-MSCTransplantation significantly alleviated the development of urinary protein resulted from renal damage of MRL/lpr mice. For the first time, we have confirmed that OPN expression is markedly decreased in renal tissues from Huc-MSCTransplant mice compared to the control renal tissues. Secondly, the expression of AP-1 and SP-1 were lower in Huc-MSCTransplant mice than the control renal tissues. Meanwhile, our current study shows that the expression of AP-1 and SP-1 were strongly positive correlated with OPN expression level in Huc-MSCTransplant mice. Lastly, our results indicated that the both of MMP-2 and MMP-9, are significantly down-regulated by transplantation of Huc-MSCTransplantation, in which may be due to the inhibition of NF- κ B pathways.

Recently, more and more evidence have shown that secreted OPN has an important effect on regulating the differentiation of helper T1 lymphocyte (Th1) and Th17 cells [25]. Previous work have suggested that OPN gene polymorphism and OPN protein play crucial roles in pathogenesis and clinical manifestation of SLE. Uede T and colleagues have found that OPN highly expressed in serum in MRL/lpr mice as well as in SLE patients. Moreover, OPN have been found to induce the proliferation of B1 cell and the increase the expression of IgM and IgG₃ [26]. Research have showed that the 3'UTR of OPN (rs9138C) is associated with higher serum OPN and interferon α (IFN- α) in SLE patients [27]. Moreover, there are reports suggesting that OPN protein is expressed highly in MRL/lpr mice [10, 28, 29]. We found that OPN expression decreased in MRL/lpr mice renal tissues treated with Huc-MSCTransplantation, providing a new insight of how Huc-MSCTransplantation to alleviates the symptoms of LN. Research has shown that the activation of transcription factors is involved in the pathogenesis of SLE,

particularly NF- κ B which increased highly in the lupus lymphocytes from SLE patients [30]. Activated NF- κ B is up-regulated in renal tubular cells and interstitial cells in parallel with over-activation of AP-1 in LN. In addition, the downstreams of NF- κ B and AP-1 targeted to pro-inflammatory factors were markedly enhanced in LN compared with the normal control [31]. We found that Huc-MSc treatment significantly decreased the expression of AP-1 and NF- κ B. It has been reported that OPN expression could be directly regulated by AP-1 and SP-1 [12, 19]. We observed that the expression levels of AP-1 and SP-1 were positive correlated with OPN expression level in renal tissues from Huc-MSc-transplantation mice. These results suggested that Huc-MSc may be inhibit the expression of OPN through down-regulated the expression of AP-1 and SP-1.

MMPs is a zinc-dependent enzymes family involved in various pathological processes such as inflammation and autoimmunity [23]. MMP-2 and MMP-9 have been reported to play crucial role in the pathogenesis of SLE [23, 32, 33]. Moreover, Liu J and colleagues have suggested that OPN promotes the expression of MMP-2 and MMP-9 through NF- κ B pathway [20]. Our results indicated that Huc-MSc transplantation leads to decrease the expression of MMP-2 and MMP-9 in renal tissues of MRL/lpr mice. Importantly, the down-regulation of MMP-2 and MMP-9 is significantly correlated with the reduction of OPN in treatment group, suggesting that the effective treatment with Huc-MSc on LN may be partly mediated by inhibiting OPN expression.

In summary, Huc-MSc may be an effective treatment for LN and we have provided a new perspective on the study of the participation of Huc-MSc in relieving LN symptoms. Huc-MSc is able to decrease the urine protein due to reducing OPN expression. Meanwhile the loss of OPN inhibits NF- κ B expression and activation, further depresses MMP-2 and MMP-9 in MRL/lpr mice in response to Huc-MSc. These findings will aid our understanding of the treatment of SLE with MSC.

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Disclosure of conflict of interest

None.

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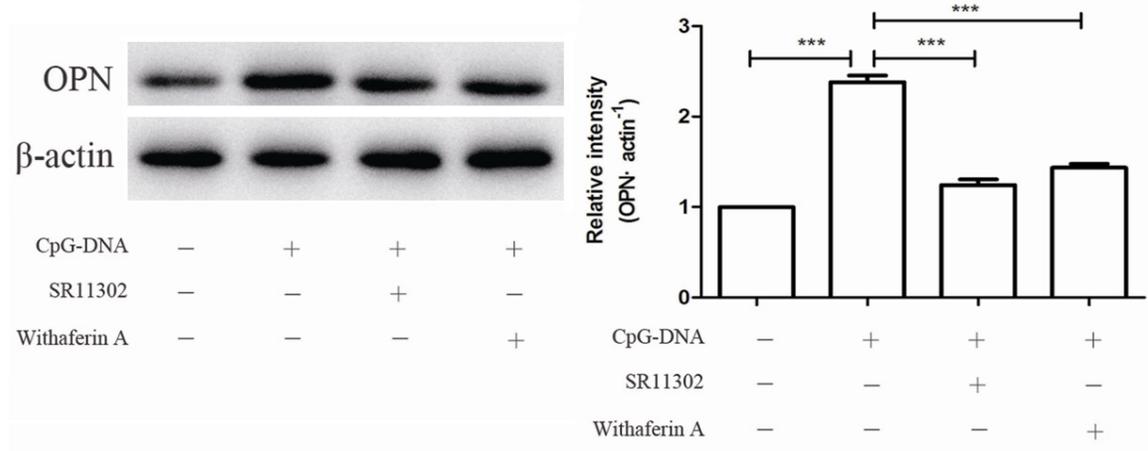
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Supplementary Figure 1. OPN were regulated by the AP-1/SP-1. Used the CpG-DNA to treat the mononuclear cells of mice as the SLE model of inducing the expression of OPN. And added the inhibitor of AP-1 (SR 11302) and Sp-1 (Withaferin A) to pretreat the cells. The proteins were analyzed by Western blot.