

Original Article

HucMSC exosomes-delivered 14-3-3ζ enhanced autophagy via modulation of ATG16L in preventing cisplatin-induced acute kidney injury

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Abstract: The clinical application of cisplatin is restricted by its side effects of nephrotoxicity. Human umbilical cord mesenchymal stem cell-derived exosomes (hucMSC-ex) have an important effect in tissue injury repair. Our previous work discovered that pretreatment with human umbilical cord mesenchymal stem cell-derived exosomes (hucMSC-ex) alleviated cisplatin-induced acute kidney injury (AKI) by activating autophagy both *in vitro* and *in vivo*. In this study, we further explored the mechanisms of hucMSC-ex in autophagy for preventing cisplatin-induced nephrotoxicity. We discovered that 14-3-3ζ was contained in hucMSC-ex, and knockdown and overexpression 14-3-3ζ reduced and enhanced the autophagic activity respectively. Furthermore, Knockdown of 14-3-3ζ alleviated the preventive effect of hucMSC-ex. In contrast, overexpression of 14-3-3ζ enhanced the effect. Further results confirmed that hucMSC-ex increased ATG16L expression and that 14-3-3ζ interacted with ATG16L, promoting the localization of ATG16L at autophagosome precursors. In this study, we revealed that hucMSC-ex-delivered 14-3-3ζ interacted with ATG16L to activate autophagy. Our findings suggest that 14-3-3ζ is a novel mechanism for MSC-exosomes-activated autophagy and provides a new strategy for the prevention of cisplatin-induced nephrotoxicity.

Keywords: Exosomes, mesenchymal stem cells, autophagy, 14-3-3ζ, nephrotoxicity

Introduction

Kidney injury induced by ischemia, drugs, or sepsis leads to cell death, tissue damage, and loss of renal function or renal failure [1]. Acute kidney injury (AKI) remains a common clinical problem with high morbidity and mortality, and few options for prevention and treatment [2]. Cisplatin is a chemotherapeutic reagent that is most widely used for the treatment of neoplastic diseases. However, its application is restricted by its side effects of nephrotoxicity for which there is still a lack of effective preventive measures.

Mesenchymal stem cells (MSCs) are a class of tissue stem cells that have potential for self-renewal and multiple differentiation. Human umbilical cord MSCs (hucMSCs) which are harvested at low cost and noninvasively can ame-

liorate liver fibrosis and AKI [3, 4]. In our previous study, we discovered that hucMSCs ameliorated ischemia and reperfusion-induced acute renal failure in rats through paracrine actions [5]. Furthermore, exosomes derived from MSCs play a crucial role in cell-to-cell communication, which can transport proteins and RNAs to target cells. We had already demonstrated that hucMSC derived exosomes (hucMSC-ex) alleviated liver fibrosis [6], cutaneous wound healing [7], and acute myocardial ischemic injury [8]. These findings suggested that hucMSC-ex play an important role in tissue damage and repair. We also indicated that hucMSC-ex protected against cisplatin-induced renal oxidative stress and apoptosis *in vivo* and *in vitro* [9]. Moreover, in recent work we discovered that pre-incubation with hucMSC-ex prevented cisplatin-induced kidney injury by activating autophagy [10]. This demonstrated that hucMSC-ex might

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play a critical role in cisplatin induced nephrotoxicity. However, the exact mechanisms have not been thoroughly explored.

Exosomes contains different types of cargos. Our team has found that 14-3-3 ζ was delivered by hucMSC-ex and regulated cutaneous regeneration [11]. The 14-3-3 proteins comprise a family of highly conserved proteins that widely exist in different eukaryotic cells. 14-3-3 proteins have several isoforms such as β , ϵ , η , γ , τ , σ , ζ and their expression levels vary in different tissues [12, 13]. The 14-3-3 family participates in the regulation of diverse processes, such as protein trafficking, signal transduction, the cell cycle, and apoptosis [14, 15]. The protein 14-3-3 ζ (gene symbol YWHAZ) is known to protect cells from different types of stress, including hypoxia, chemotherapy-induced death and growth factor deprivation [16-18]. Several studies have demonstrated that 14-3-3 proteins play a critical role in regulating autophagy [14, 19, 20]. However, whether hucMSC-ex delivered 14-3-3 ζ can induce the activation of autophagy for preventing cisplatin-induced kidney injury is unclear.

In this study, we optimized the experimental conditions and discovered that pre-incubation with hucMSC-ex which transferred 14-3-3 ζ protein to renal cells, promoted cells proliferation and inhibited cells apoptosis by activating autophagy. Further results confirmed that 14-3-3 ζ interacted with autophagy-related protein 16L (ATG16L) and promoted the formation of autophagosomes and autolysosomes. These findings provide a new strategy for the prevention of nephrotoxicity induced by cisplatin.

Materials and methods

Ethics

The study was approved by the ethical committee of Jiangsu University (2012258).

Isolation and characterization of exosomes derived from hucMSCs

After obtaining parental and ethics committee consent, the fresh umbilical cords were collected and processed within 6 h and detected as previously described [9]. HucMSCs were cultured in serum medium which were exosomes-free for 48 h, cell culture media were collected and centrifuged to remove cell debris and then

passed through a 0.22-mm filter. Final exosomes were diluted by PBS and stored at -80°C. According to the directions, the exosomes was isolated by ExoQuick kit (SBI, USA). The protein concentration of exosomes was quantified using a BCA protein assay kit (CWBio, Beijing, China). The characterization of exosomes was detected as previously described [9, 10].

Knockdown or overexpress of 14-3-3 ζ in hucMSCs

A lentiviral expression vector containing the 14-3-3 ζ shRNA sequence (Sigma, USA) was selected for targeting 14-3-3 ζ genes silence, Lenti-GFP-shRNA as negative control vector. The Lenti-14-3-3 ζ shRNA vectors were generated by ligating the vector Tet-pLKO-puro with 14-3-3 ζ shRNA oligonucleotides. The sequences of 14-3-3 ζ shRNA oligonucleotides was as follows: forward, 5'-CCGGGCAGAGAGCAAAGTCTTCTATCTCGAGATAGAAGACTTTGCTCTCTGCTTTTG-3' and reverse, 5'-AATTCAAAAAGCAGAGAGCAAAGTCTTCTATCTCGAGATAGAAGACTTTGCTCTCTGC-3'. The shRNA lentivirus were produced using lentivirus packaging mix (ViraPower, Invitrogen). HucMSCs were transduced with the prepared lentivirus (Lenti-14-3-3 ζ shRNA or Lenti-GFP shRNA) and selected with 1 μ g/mL of puromycin (Invitrogen) for 15 days. The expression of shRNA was induced by addition 80 μ g/mL doxycycline for 2 days. The efficiency of 14-3-3 ζ knockdown was evaluated by Western blot.

Adenovirus expression vectors containing 14-3-3 ζ (Ad-14-3-3 ζ) expression sequence and adenovirus empty vector (Ad-GFP) used as a comparison were transfected into hucMSCs according to manufacturer's guidelines. After 24 h, the medium was changed, and after 48 h of transfection, cells were used for further analysis. Adenovirus vectors were purchased from Geneway (Shanghai, China). According to the previous method to separate exosomes and named as shGFP-ex, sh14-3-3 ζ -ex, Ad-GFP-ex and Ad-14-3-3 ζ -ex.

Preventive models in vitro and in vivo

Rat renal proximal tubular (NRK-52E) cells were purchased from Stem Cell Bank of Chinese Academy of Sciences and maintained in high glucose Dulbecco's modified Eagle's medium (H-DMEM; Gibco, USA) containing 10% fetal

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bovine serum (FBS, ExCell Biology, China) and 1% penicillin and streptomycin at 37°C with 5% CO₂. For *in vitro* treatments, NRK-52E cells were seeded in six-well plates at 5×10⁴ cells per well. After 24 h, cells were divided into seven groups, control group: normally cultured; PBS group: add the equal volume of PBS, and then treated with 20 μ M cisplatin for 16 h; hucMSC-ex group: pretreated with 200 μ g/ml exosomes for 24 h and then treated with 20 μ M cisplatin for 16 h; Ad-GFP-ex group: 24 h before cisplatin administration, cells were incubated with 200 μ g/ml exosomes from Ad-GFP-hucMSCs; Ad-14-3-3 ζ -ex group: 24 h before cisplatin treatment, cells were incubated with 200 μ g/ml exosomes from Ad-14-3-3 ζ -hucMSCs; shGFP-ex group: 200 μ g/ml shGFP-ex incubated for 24 h and then treated with 20 μ M cisplatin for 16 h; sh14-3-3 ζ -ex group: 200 μ g/ml sh14-3-3 ζ -ex incubated for 24 h and then treated with 20 μ M cisplatin for 16 h.

Female Sprague-Dawley rats (weighing 220±20 g) were purchased from the Animal Centre of Chinese Academy of Sciences (Shanghai, China). The animals were kept under standard laboratory conditions (12 h light, 12 h dark and 21±2°C). All protocols and surgical procedures were approved by the Institutional Animal Care Committee of Jiangsu University. The rats were randomly divided into five groups (n = 6): control group: normal rats; PBS group: both kidneys in one rat received a renal capsule injection of PBS. After 24 h, intraperitoneal injection of a single dose of 5 mg/kg cisplatin; hucMSC-ex group: 24 h before cisplatin administration, both kidneys in one rat received a renal capsule injection of 200 μ g hucMSC-ex; Ad-GFP-ex group: 24 h before cisplatin administration, both kidneys in one rat received a renal capsule injection of 200 μ g exosomes from Ad-GFP-hucMSCs; Ad-14-3-3 ζ -ex group: 24 h before cisplatin administration, both kidneys in one rat received a renal capsule injection of 200 μ g exosomes from Ad-14-3-3 ζ -hucMSCs.

Blood samples were collected from the rats via the tail vein every day and the serum was separated and detected the blood urea nitrogen (BUN) and creatinine (Cr) levels a Biochemistry Autoanalyzer (Beckman, America). Rats were sacrificed at 3 days after administration of cisplatin. The kidneys were fixed in 4% paraformaldehyde (pH 7.4) gradually dehydrated, embedded in paraffin, cut into 4 μ m sections and stained with H&E stain for light microscopy.

Sections were analyzed and at least 20 random fields were scored by a nephropathologist in a blinded manner. Tubular cell necrosis, tubular dilation, and tubular protein casts (200× magnification) in sections were observed and analyzed. Abnormalities were graded by a semi-quantitative score from 0 to 4: 0, no abnormalities; 1+, changes affecting less than 25% of the sample; 2+, changes affecting 25 to 50%; 3+, changes affecting 50 to 75%; 4+, changes affecting more than 75%.

Apoptosis assay

Cisplatin-induced apoptosis of NRK-52E cells pretreated with exosomes was determined from using an Annexin V/PI Apoptosis Detection Kit (Molecular Probes, USA). According to the manufacturer's instructions, cells were suspended in the buffer analyzed with flow cytometer (BD, USA). According to the manufacturer's instructions, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was used to measure renal tubular cell apoptosis using cell apoptosis detection kit (Boster, Wuhan, China). The number of apoptotic cells was counted in 20 randomly selected visual fields of blinded samples, using 200× magnification.

Western blot

The cells and tissues were lysed in a RIPA buffer with proteinase inhibitors. An equal amount of protein samples was electrophoresed on 12% SDS-polyacrylamide gels, which were transferred to polyvinylidene fluoride membranes, subsequently blocked with 5% (w/v) nonfat milk and then blotted against primary antibodies at 4°C overnight. The primary antibodies were anti-caspase 3 (Bioworld, USA), anti-14-3-3 ζ (Bioworld, USA), anti-LC3B (CST, USA), anti-ATG16L (AbSci, USA) and anti- β -actin (Bioworld, USA). After washing three times with Tris-buffered saline/Tween (TBST), the membranes were incubated with goat anti-rabbit (CW BIO, China) for 1 h at 37°C. The proteins were detected by enhanced chemiluminescence. Band intensities were quantified using ImageJ software. Each experiment was performed 3 separate times.

Immunoprecipitation (IP)

Cell pellets were resuspended in co-IP buffer, then lysates were centrifuged and incubated with 25 μ l of protein A/G gel at 4°C for 1.5 h.

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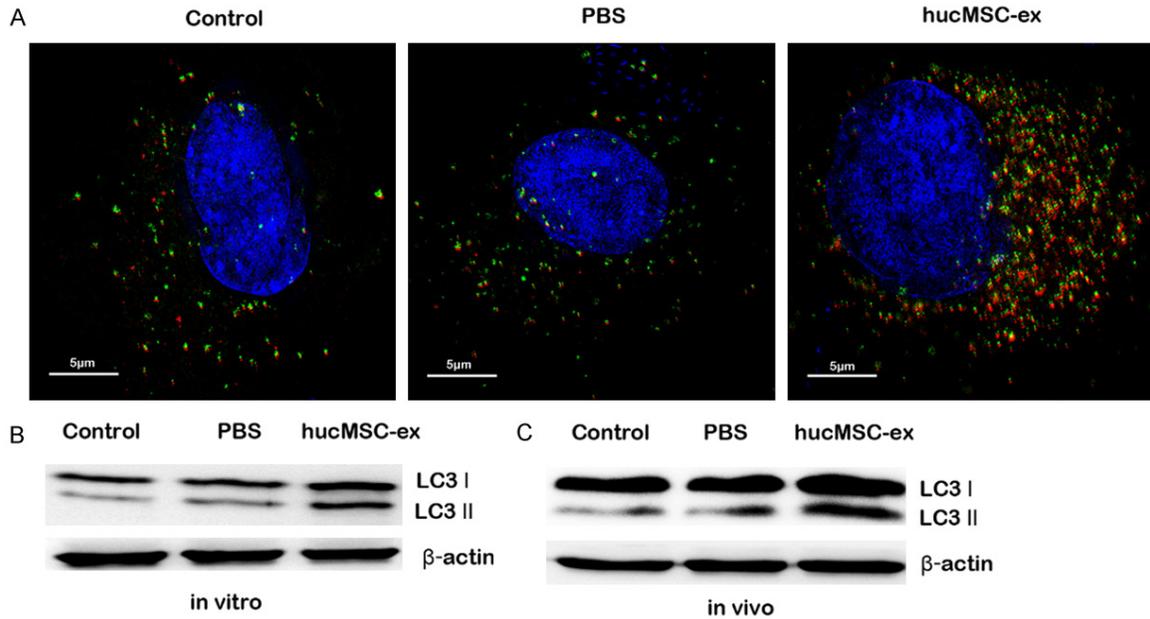


Figure 1. HucMSC-ex activated autophagy in NRK-52E cells and in rats in response to cisplatin. NRK-52E cells and rats were treated with PBS and hucMSC-ex before treating with cisplatin. A. Representative images of fluorescent LC3 puncta by SIM examination in NRK-52E cells. Yellow dots represent autophagosome, red dots represent the autolysosome (bar = 5 μm). B. Western blotting analyses of the expression of autophagic markers LC3 in NRK-52E cells. C. Western blotting assay for LC3 expression in kidney.

The supernatant was subjected to IP using the indicated first antibodies at 4°C overnight. Subsequently, the proteins were incubated with 30 μl of Protein A/G gel at 4°C for 2 h. The collected protein complexes were washed for 4 times with co-IP buffer and analyzed by Western blot.

Cell transfection and structured illumination microscopy assay

NRK-52E cells seeded at 24 well plates and cultured for 24 h, then transfected mRFP-GFP-LC3 adenovirus according to the manufacturer's protocol (Han Heng Biology, China). After treatment, the cells were washed with PBS and fixed with 4% paraformaldehyde. Finally, the cells were then stained with Hoechst33342 for nuclear staining. The images were acquired with a structured illumination microscopy (Nikon, SIM). The yellow puncta were autophagosomes and the red puncta were autolysosomes.

Immunofluorescence and Immunohistochemistry analysis

NRK-52E cells washed with PBS and were fixed in 4% paraformaldehyde for 30 minutes. The PCNA (CST, USA), ATG16L (AbSci, USA), ATG16L (Santa Cruz, USA) and 14-3-3 ζ antibody was

incubated at 4°C overnight. After washing with PBS, cells were incubated with Donkey anti-Mouse IgG, Alexa Fluor 488 (Invitrogen, USA) or Donkey anti-rabbit IgG, Alexa Fluor 555 (Invitrogen, USA) at 37°C for 1 h. Nuclei were counterstained with Hoechst33342 (Sigma, USA). Images were captured with a fluorescent microscope (Nikon, Tokyo, Japan) or a structured illumination microscopy (Nikon, SIM).

The kidney tissue slices were incubated with the primary antibodies against PCNA (1:100, CST), 14-3-3 ζ (1:100, Bioworld) followed by biotinylated sheep anti-rabbit or mouse IgG (Bostar, Wuhan, China). The antibody binding sites in the tissue slices were visualized with DAB, and the nuclei were slightly counterstained with hematoxylin. The number of PCNA and 14-3-3 ζ positive cells were counted in 20 randomly selected visual fields of blinded samples, using 200× magnification.

Statistical analysis

All Data are expressed as mean ± standard deviation. The statistically significant differences between groups were assessed using analysis of variance or t test by using Prism software

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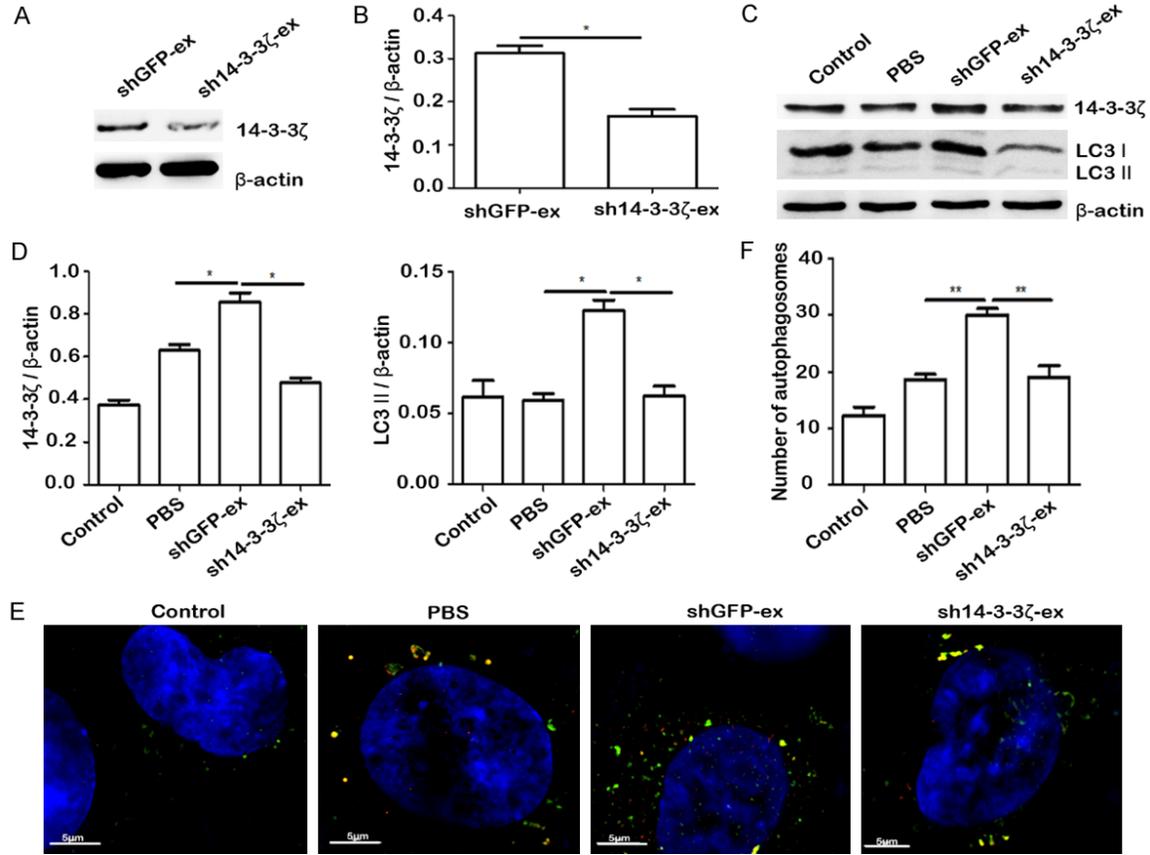


Figure 2. Knockdown of 14-3-3 ζ alleviated the autophagic activity in NRK-52E cells. NRK-52E cells were treated with PBS, shGFP-ex and sh14-3-3 ζ -ex for 24 h, and then treated with cisplatin for 16 h. A, B. Western blotting assay for 14-3-3 ζ expression in shGFP-ex and sh14-3-3 ζ -ex. C, D. Western blotting analyses of the expression of 14-3-3 ζ and LC3 in NRK-52E cells. E, F. Representative images of fluorescent LC3 puncta by SIM examination in NRK-52E cells (bar = 5 μ m). * P <0.05; ** P <0.01.

(GraphPad, San Diego, USA). P <0.05 was considered statistically significant.

Results

HucMSC-ex induced autophagic activity in vitro and in vivo

Autophagy is crucial in maintaining cell homeostasis and may contribute to anti-apoptotic effects. We therefore examined the autophagosomes and autolysosomes in NRK-52E cells transiently transfected with mRFP-GFP-LC3 by SIM. SIM images showed that there were numerous of yellow and red dots in hucMSC-ex group. The results revealed that the numbers of autophagosomes and autolysosomes puncta were higher in hucMSC-ex group (**Figure 1A**). Western blotting results suggested that the autophagy marker LC3 II was increased in the hucMSC-ex group compared with the PBS

group (**Figure 1B**). To verify the *in vitro* results, we measured the levels of LC3 II through western blotting in kidney tissues. HucMSC-ex increased the expression of LC3 II contrast to the PBS group in renal tissues (**Figure 1C**).

Knock down or overexpression the expression of 14-3-3 ζ in hucMSC-ex alleviated or enhanced autophagic activity in vitro

14-3-3 ζ was determined to be crucial in autophagy [14, 19, 20] and 14-3-3 ζ was contained in hucMSC-ex [11]. To further indicated the role of 14-3-3 ζ delivered by hucMSC-ex in autophagy, we knocked down 14-3-3 ζ in hucMSCs through lentivirus transduction (sh14-3-3 ζ) and confirmed the expression of 14-3-3 ζ protein in hucMSC-ex by western blotting, the expression of 14-3-3 ζ was decreased in sh14-3-3 ζ -ex compared with shGFP-ex (**Figure 2A, 2B**). The expression of 14-3-3 ζ and LC3 II protein was

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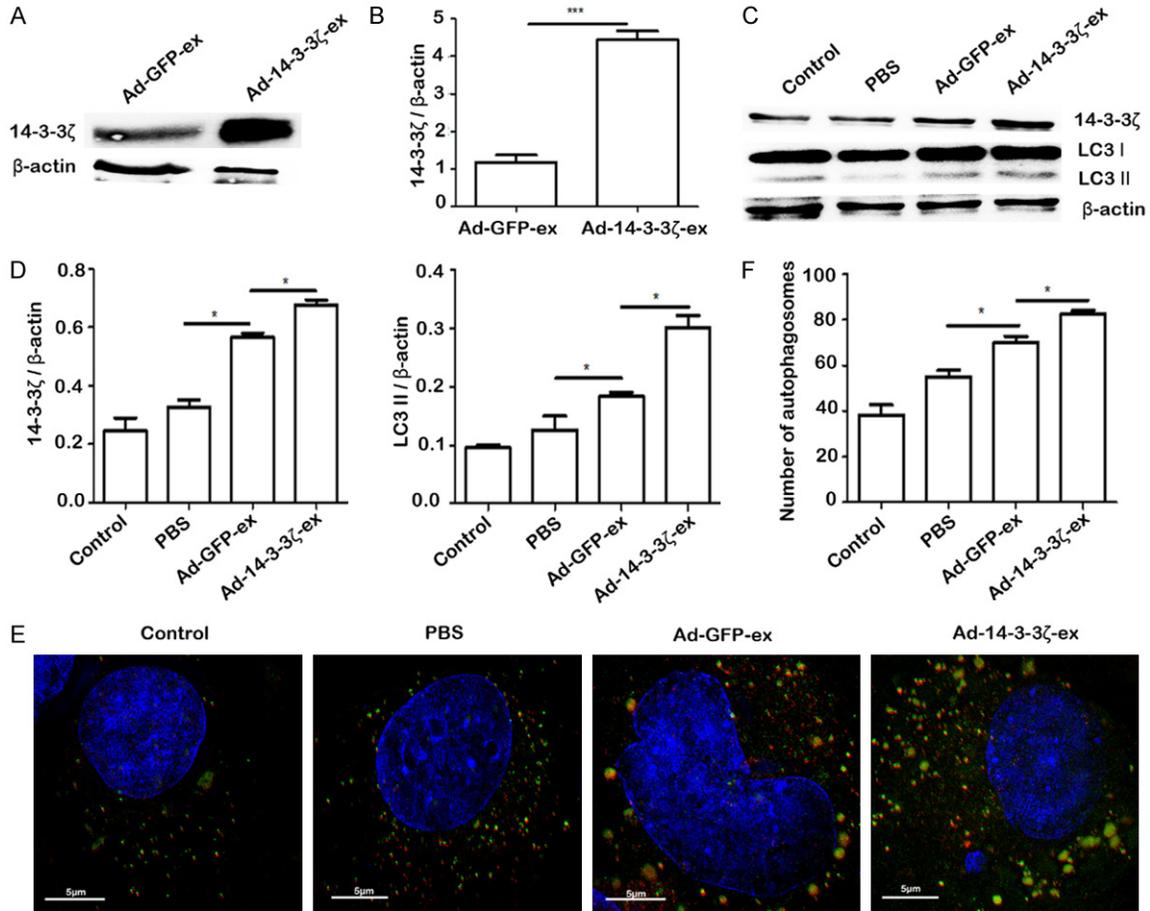


Figure 3. Overexpression of 14-3-3 ζ increased the autophagic activity in NRK-52E cells. NRK-52E cells were treated with PBS, Ad-GFP-ex and Ad-14-3-3 ζ -ex for 24 h, and then treated with cisplatin for 16 h. A, B. Western blotting analyses of the expression of 14-3-3 ζ in Ad-GFP-ex and Ad-14-3-3 ζ -ex. C, D. Western blotting assay for 14-3-3 ζ and LC3 expression in NRK-52E cells. E, F. Representative images of fluorescent LC3 puncta by SIM examination in NRK-52E cells (bar = 5 μ m). The results are shown as the mean values \pm SD of 3 independent experiments, * P <0.05; *** P <0.001.

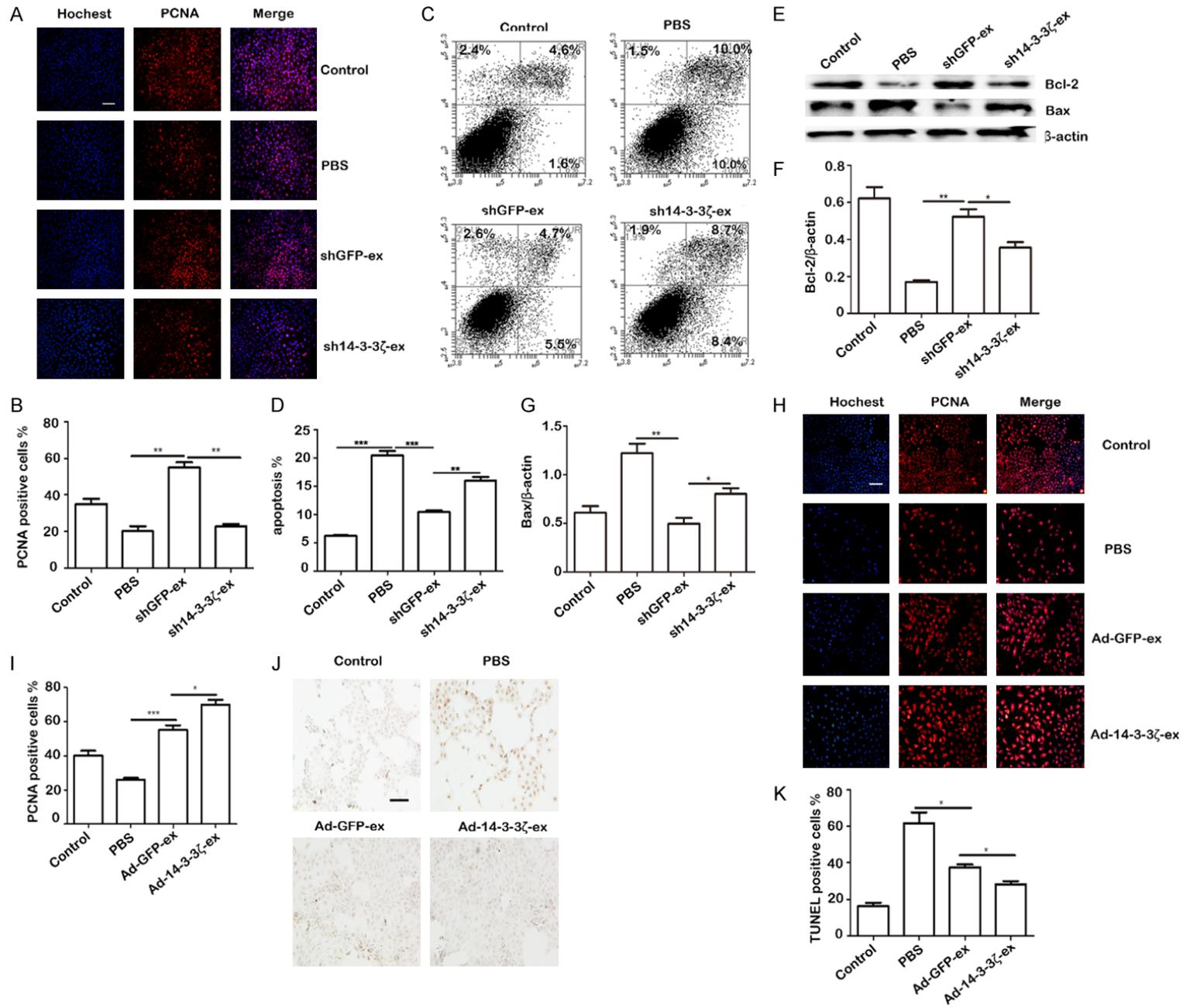
observed by Western blotting in NRK-52E cells. Expression of these two proteins was lower in the sh14-3-3 ζ -ex and the PBS groups than in the shGFP-ex group (Figure 2C, 2D). In addition, the number of autophagosome was decreased in both the sh14-3-3 ζ -ex and PBS groups compared with the shGFP-ex group (Figure 2E, 2F).

To determine the effect of 14-3-3 ζ on autophagy, we overexpressed 14-3-3 ζ in hucMSCs through adenoviral transduction (Ad-14-3-3 ζ) and confirmed that transduced 14-3-3 ζ could be parceled into exosomes by Western blotting (Figure 3A, 3B). Western blotting also showed that the protein of 14-3-3 ζ increased in the Ad-14-3-3 ζ -ex group compared with the Ad-GFP-ex group, but its expression was lower in the PBS group (Figure 3C, 3D). LC3 II was increased

in the Ad-14-3-3 ζ -ex group compared with the PBS and the Ad-GFP-ex groups (Figure 3C, 3D). SIM images showed that there were numerous of yellow and red dots in the Ad-GFP-ex treated NRK-52E cells compared with the PBS group. Moreover, the number of autophagosomes was higher in the Ad-14-3-3 ζ -ex group compared with the Ad-GFP-ex group (Figure 3E, 3F). This indicated that overexpression of 14-3-3 ζ increased the autophagic activity.

In addition, we investigated the effect of 14-3-3 ζ in cell proliferation and apoptosis. The expression of PCNA was decreased in the sh14-3-3 ζ -ex group compared with the shGFP-ex group (Figure 4A, 4B). Thereafter, we investigated changes in cell viability following 14-3-3 ζ silencing by using Annexin V/PI staining. Sh14-3-3 ζ -

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Figure 4. 14-3-3 ζ enhanced the preventive effect of hucMSC-ex in NRK-52E cells. A. The expression of PCNA was detected by immunofluorescence staining in NRK-52E cells after treating with shGFP-ex or sh14-3-3 ζ -ex. B. Percentage of PCNA positive cells in A. C. Flow cytometric assay for apoptotic cells in NRK-52E cells after treating with shGFP-ex or sh14-3-3 ζ -ex. D. The percentage of apoptosis cells in C. E-G. Western blotting assay for Bcl-2 and Bax expression in NRK-52E cells. H. Immunofluorescence staining assay for the expression of PCNA in NRK-52E cells after treating with Ad-GFP-ex or Ad-14-3-3 ζ -ex. I. Percentage of PCNA positive cells in E. J. TUNEL assay for the apoptosis of cells in each group. K. The percentage of TUNEL positive cells. Original magnification, 200 \times , Scale bars = 50 μ m. The results are shown as the mean values \pm SD of 3 independent experiments, * P <0.05; ** P <0.01; *** P <0.001.

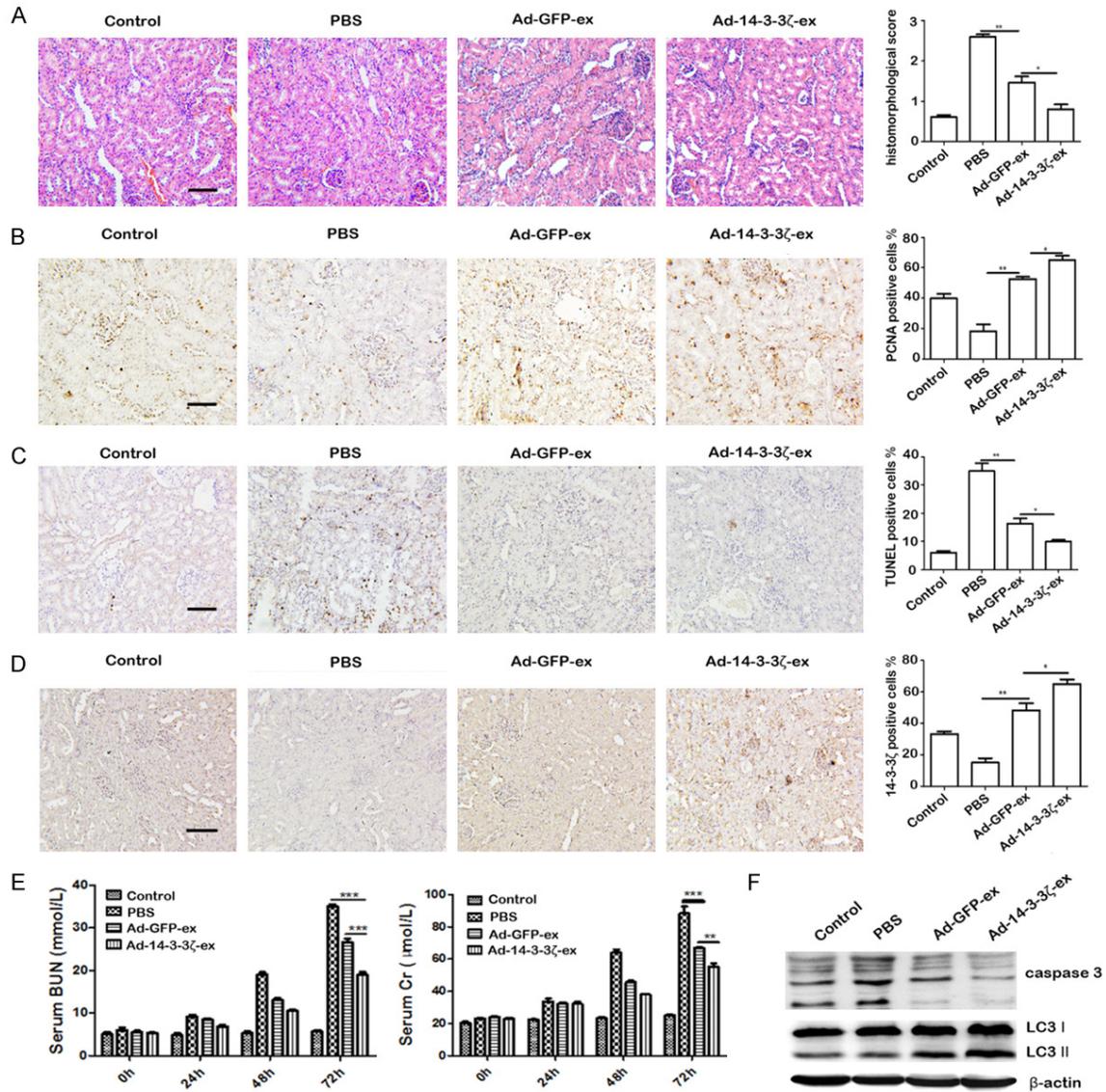


Figure 5. Overexpression of 14-3-3 ζ promoted the preventive effect of hucMSC-ex in rats. Rats were treated with PBS, Ad-GFP-ex and Ad-14-3-3 ζ -ex for 24 h, and then treated with cisplatin for 72 h. A. Representative images of renal histology and the histomorphological score. B. Immunohistochemical analysis of PCNA expression in kidney tissues and the percentage of PCNA positive cells. C. The apoptotic cells in rat renal tissues were detected by TUNEL assay and the percentage of apoptotic cells. D. Immunohistochemical analysis of 14-3-3 ζ expression in kidney tissues and the percentage of 14-3-3 ζ positive cells. E. Serum blood urea nitrogen and creatinine levels were measured at different times after cisplatin injection. Original magnification, 200 \times , Scale bars = 50 μ m. The results are shown as the mean values \pm SD of 6 independent experiments, * P <0.05; ** P <0.01; *** P <0.001. F. The protein of LC3 II and caspase 3 were detected by Western blotting.

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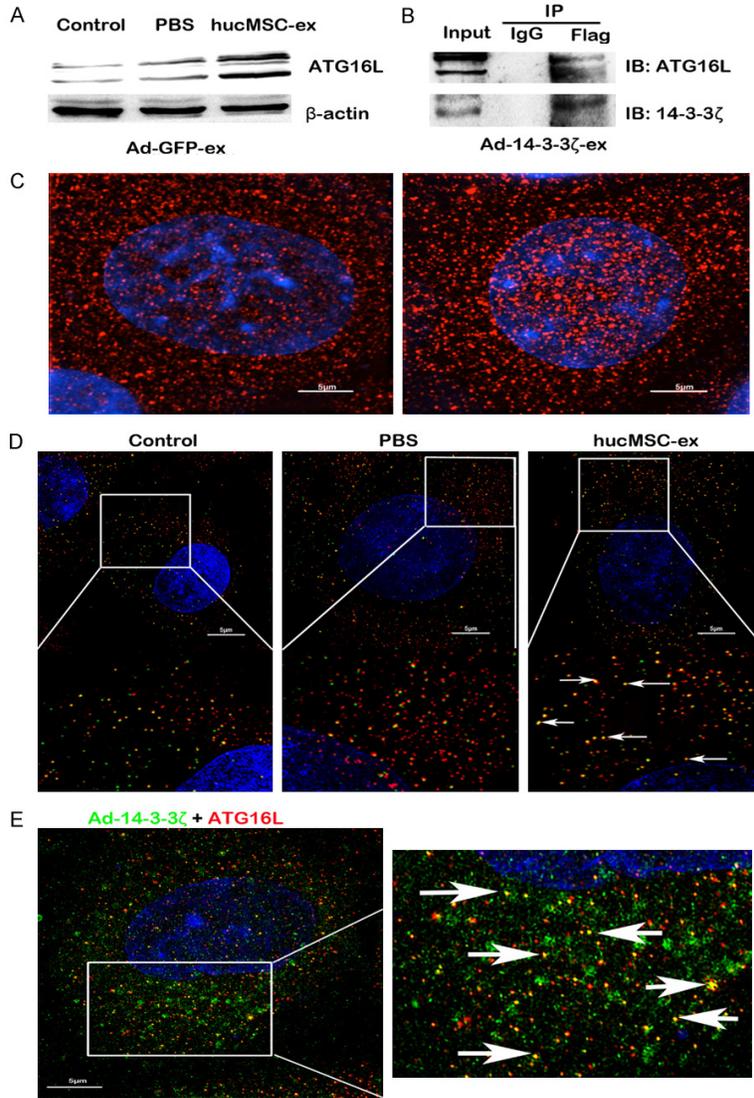


Figure 6. 14-3-3 ζ enhanced the autophagy by interacting with ATG16L. A. Western blotting assay for ATG16L in NRK-52E cells. B. The interaction between 14-3-3 ζ and ATG16L by Western blotting in 293T cells. C. Representative images of ATG16L by SIM examination in Ad-GFP-ex group and Ad-14-3-3 ζ -ex group in NRK-52E cells. D. Representative images of the location between endogenous 14-3-3 ζ (red) and endogenous ATG16L (green) by SIM in NRK-52E cells in different groups. E. Representative images of the location between exogenous 14-3-3 ζ (green, which derive from Ad-14-3-3 ζ -ex) and ATG16L (red) by SIM in Ad-14-3-3 ζ -ex group in NRK-52E cells. Original magnification, 63 \times , bar = 5 μ m.

ex increased cell death compared with the shGFP-ex group (Figure 4C, 4D). The expression of Bcl-2 and Bax proteins were observed through western blotting in NRK-52E cells. The level of Bcl-2 was evidently higher and the level of Bax was lower in the shGFP-ex group compared with the PBS group and sh14-3-3 ζ -ex group (Figure 4E-G). However, the expression of PCNA protein was significantly enhanced in the

Ad-14-3-3 ζ -ex group (Figure 4H, 4I). As illustrated in Figure 4J and 4K the number of TUNEL-positive cells in the Ad-14-3-3 ζ -ex group was lower than that in PBS group. This suggests that 14-3-3 ζ may play a renoprotective role in cisplatin induced nephrotoxicity by activating autophagy.

Overexpression of 14-3-3 ζ in hucMSC-ex enhanced autophagic-activity and alleviated cell death in vivo

H&E staining was employed to observe lesions of kidney tissue slices taken from rats 3 days after cisplatin injection. Numerous necrotic areas of the proximal epithelium and abundant tubular protein casts had appeared in the PBS group. By contrast, there were fewer tubular lesions in the Ad-GFP-ex group and considerable fewer in the Ad-14-3-3 ζ -ex group (Figure 5A). In addition, the PCNA positive cells were significantly increased in Ad-GFP-ex group and Ad-14-3-3 ζ -ex group compared with PBS group (Figure 5B). The number of TUNEL-positive cells had significantly increased after cisplatin treatment compared with the Control group, whereas pretreatment with Ad-14-3-3 ζ -ex resulted in fewer TUNEL-positive cells (Figure 5C). Immunohistochemically detection revealed that the Ad-14-3-3 ζ -ex group had higher expression 14-3-3 ζ than the PBS group (Figure 5D).

Three days after cisplatin injection, we observed a significant rise in blood BUN and Cr levels in the PBS group. However pretreatment with Ad-GFP-ex and Ad-14-3-3 ζ -ex resulted in lower BUN and Cr levels (Figure 5E). Additionally, the expression of caspase 3 protein was observed through western blotting in kidney tissues. The level of caspase 3 was evidently higher in the PBS group compared with the Control group,

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but pretreatment with Ad-14-3-3 ζ -ex reduced this expression (**Figure 5F**). This suggests that overexpression of 14-3-3 ζ -ex promoted the preventive effect of exosomes during cisplatin treatment *in vivo*. Moreover, Ad-14-3-3 ζ -ex more markedly increased the expression of LC3 protein compare with the PBS group, Ad-GFP-ex also activated autophagy (**Figure 5F**).

14-3-3 ζ increased ATG16L expression and promoted the location of ATG16L to autophagosome precursors

To further investigate the regulatory mechanism of 14-3-3 ζ on autophagy, we found that pretreatment with hucMSC-ex increased the expression of ATG16L compared with the PBS group in NRK-52E cells during cisplatin treatment by Western blotting (**Figure 6A**). Furthermore, 14-3-3 ζ interacted with ATG16L in the co-immunoprecipitation assay (**Figure 6B**). To better illustrate the change of ATG16L, we used SIM, which allowed us to acquire super-resolution 2D images. The expression and the puncta of ATG16L (labeled with red) were increased in the Ad-14-3-3 ζ -ex group compared with the Ad-GFP-ex group (**Figure 6C**). In addition, the SIM images showed that endogenous 14-3-3 ζ (labeled with red) interacted with endogenous ATG16L (labeled with green) in NRK-52E cells, the co-localization puncta were yellow. There was less co-localization in control and PBS groups than that in hucMSC-ex group (**Figure 6D**). But, the SIM images showed that exogenous 14-3-3 ζ (labeled with GFP, derived from Ad-14-3-3 ζ -ex) bound with endogenous ATG16L (labeled with red) in the Ad-14-3-3 ζ -ex group in NRK-52E cells, with the yellow puncta representing this colocalization (**Figure 6E**).

Discussion

Exosomes are small vesicles that can be released to the extracellular environment by all cell types [21]. Exosomes carry specific repertoires of proteins, mRNAs, long non-coding RNAs and small non-coding RNAs to neighboring cells [22]. Exosomes have been found to play a role in tissue repair [23], immune responses [24], organism development [25] and neuronal communication [26]. Our study showed that MSC-ex promote AKI repair via inhibition of endothelial cell apoptosis [9]. However, few studies have focused on the function of protein molecules transported by MSC-ex in preventive medicine.

Autophagy is an evolutionarily conserved process, many misfolded proteins and damaged organelles are degraded through lysosomes for the maintenance of cellular homeostasis [27]. In the kidneys, autophagy is essential to the homeostasis and physiological function of podocytes [28]. Notably, autophagy induction has been demonstrated in renal tubular cells in experimental models of AKI induced by nephrotoxicity, ischemia-reperfusion and sepsis [29-32].

In our previous research, we discovered that the preventive effects of hucMSC-ex on cisplatin-induced apoptosis were dependent on autophagy. Both *in vitro* and *in vivo*, 3MA inhibited the hucMSC-ex-mediated activation of the autophagy. However, treatment with the autophagic inducer rapamycin mimicked the effect of hucMSC-ex, with an increase of autophagy [10]. The exact mechanism of how pretreatment with hucMSC-ex inhibited kidney injury by activating autophagy is unclear.

In this study we demonstrated that hucMSC-ex-derived 14-3-3 ζ significantly alleviated cisplatin-induced nephrotoxicity though activating autophagy. 14-3-3 proteins are known to be involved in a growing number of cell biology processes, suggesting the multifunctionality of this ubiquitous eukaryotic adaptor protein family [33]. In our previous study, we discovered that hucMSC-ex contained 14-3-3 proteins using LC-MS/MS analysis and that the expression of 14-3-3 ζ was very high. The 14-3-3 ζ protein derived from hucMSC-ex could self-control Wnt response though modulation of YAP in cutaneous regeneration models [11]. Additionally, our research found that the 14-3-3 ζ proteins were upregulated in the hucMSC-ex group, which indicated that hucMSC-ex may transport 14-3-3 ζ proteins to renal tubular cells. In addition, overexpression of 14-3-3 ζ enhanced the preventive effect of hucMSC-ex in cisplatin-induced kidney injury both *in vitro* and *in vivo*. By contrast, knockdown of 14-3-3 ζ aggravated the renal injury *in vitro*. These results suggested that 14-3-3 ζ derived from hucMSC-ex played a beneficial role in the prevention of nephrotoxicity.

Beclin1 also plays an essential role in autophagy. Wang et al. found that 14-3-3t upregulated beclin1 though E2F1 [34]. In contrast, 14-3-3 ϵ siRNA enhanced the formation of autophago-

14-3-3 ζ enhanced autophagy to preventing AKI by interacting with ATG16L

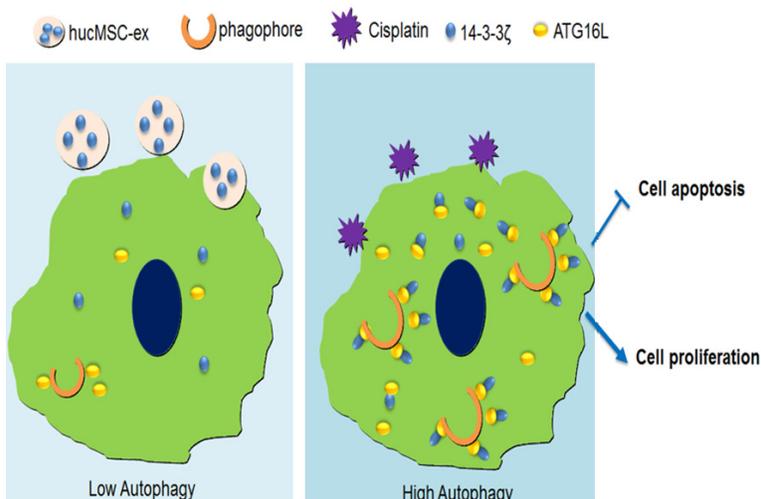


Figure 7. A proposed model for hucMSC-ex-specific 14-3-3 ζ mediation of autophagy. Pre-treating with hucMSC-ex, less 14-3-3 ζ binding with ATG16L, but when treating with cisplatin, 14-3-3 ζ binding with ATG16L induced autophagy to inhibit cell apoptosis.

some in neurodegenerative disease [35]. Thus, different subtypes of 14-3-3 proteins had different regulator function in autophagy. The role of 14-3-3 ζ is dynamic in promoting the adaptation of cells to stress. Weerasekara et al. showed that 14-3-3 ζ promoted autophagy in hypoxia via interaction with phosphorylated Atg9 [19]. Another study found that 14-3-3 interacted with Raptor, a component of the mTOR complex, and activated autophagy, leading to Raptor sequestration and mTOR inhibition in amino acid deficiency [36]. Conversely, 14-3-3 ζ was reported to inhibit autophagy by interacting with the class III PI3K human vacuolar sorting protein 34 (hVps34) during nutrient deprivation [37]. hVps34 was involved in the vesicle nucleation process during autophagy. 14-3-3 ζ interacted with hVps34 in a phosphorylation-dependent manner under normal growth condition. In contrast during nutrient starvation hVps34 was dissociation from 14-3-3 ζ to promote autophagy [38]. Thus, the role of 14-3-3 ζ is dynamically and promotes a pro-survival cellular program for adapting to a variety of pathological conditions. In this study, we demonstrated that overexpression of 14-3-3 ζ enhanced the activity of autophagy. In addition, we found that 14-3-3 ζ knockdown inhibited the autophagic activity. This indicated that 14-3-3 ζ was crucial for regulating autophagy in hucMSC-ex preventing cisplatin-induced kidney injury in the rat model. The ATG12-ATG5-ATG16 complex is a ubiquitin-like conjugation system that is

involved in the elongation and expansion steps of autophagosome formation. In our study, we revealed that 14-3-3 ζ may activate autophagy by binding with ATG16L and promoting ATG16L localization to the outer surface of the phagophore. Although exactly how 14-3-3 ζ interacts with ATG16L is unclear, this binding may be a phosphorylation-dependent manner. The incomplete combination indicates that other proteins are involved in the process which requires further research.

In summary, 14-3-3 ζ derived from hucMSC-ex was transported to renal tubular cells.

There were few interaction between 14-3-3 ζ and ATG16L and the autophagy level was low. However, there was greater colocalization following treating with cisplatin. Furthermore the combination inhibited cell apoptosis and promoted cells proliferation by activating autophagy (**Figure 7**). These experiments led to the discovery of a novel mechanism of autophagy regulation through ATG16L. These findings may provide a new strategy for kidney injury prevention.

Conclusion

This study reveals that overexpression of 14-3-3 ζ in hucMSC-ex enhanced the preventive role of hucMSC-ex *in vitro* and *in vivo*. In contrast, knockdown of 14-3-3 ζ in hucMSC-ex was discovered to aggravate the cell injury *in vitro*. Furthermore the study suggested that 14-3-3 ζ derived from hucMSC-ex activates autophagy by interacting with ATG16L. These findings provide a new approach for exosomes in the treatment of renal diseases and injuries.

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Disclosure of conflict of interest

None.

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