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Original Article

Exosomes derived from human umbilical cord mesenchymal stem cells alleviate inflammatory bowel disease in mice through ubiquitination

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Abstract: Exosomes released from mesenchymal stem cells (MSCs) have an anti-inflammatory effect and can repair tissue injuries. Ubiquitination plays an important role in the regulation of various biological functions, including the regulation of inflammation. However, it remains unknown whether exosomes from human umbilical cord mesenchymal stem cells (hucMSC-ex) may cure the mice of DSS-induced inflammatory bowel disease (IBD) through the ubiquitin modification. In this study, we aimed to investigate the therapeutic effect and probe the mechanism relating to ubiquitination underlying the hucMSC-ex treatment in DSS-induced IBD of mice. HucMSC-ex significantly improved the symptoms of IBD in mice. In the IBD group, the gene expression levels of TNF-α, IL-1β, IL-6, NAe1, E2M and Uba3 dramatically increased while those of IL-10 and IP-10 decreased. The gene expression level in the group of hucMSC-ex treatment was adversed to that in the group of IBD. In the hucMSC-ex treated group, western blot results showed that the protein expressions of K48, K63 and FK2 have significantly decreased. Compared with that in the IBD mice, the mice treated by hucMSC-ex could recover the integrity of tissue structure. Our results indicated that exosomes from hucMSCs have profound effects on alleviating DSS-induced IBD and may exert their function by regulating the ubiquitin modification level.

Keywords: Mesenchymal stem cells, exosomes, ubiquitin, inflammatory bowel disease

Introduction

Inflammatory bowel disease (IBD), which primarily includes ulcerative colitis (UC) and Crohn's disease (CD), is a chronic idiopathic, systemic, heterogenetic and relapsing disorder of the gastrointestinal tract [1]. Although its etiology and pathogenesis remain poorly understood, the inflammatory response caused by abnormal reaction of intestinal mucosa immune system plays an important role in the pathogenesis of IBD [2]. It is believed that the development mechanisms result from many factors, including environmental, genetic, infectious and immune factors [3]. The current treatments for IBD mainly include anti-inflammatory drugs, immune-modifying agents, anti-tumor necrosis factor (TNF)-α, anti-interferon (IFN)-γ antibodies and anti-α-integrin drugs. However, IBD symptoms cannot be completely relieved [4]. Hence, an urgent need to find an alternative approach for IBD treatment.

Mesenchymal stem cells (MSCs) are cells with self-renewal ability and multi-directional differentiation potential of cell types [5]. Human umbilical cord MSCs (hucMSCs) have more advantages than other MSCs, including higher cell content and lower immunogenicity. Obtained at a low cost without a completed procedure, human umbilical cord MSCs (hucMSCs) play an important role in the formation of tissue regeneration from inflammation and tissue injuries [6]. Recent studies have shown that the most therapeutic benefits of MSCs are attributed to the release of paracrine soluble factors,

such as TGF-β, while microvesicles derived from MSCs could protect against tissue damage [7-9]. Similarly, it has been proved that MSC-derived exosomes (MSC-ex) can repair the tissue injuries including liver fibrosis [10], renal failure [11], acute tubular injury [12] and myocardial ischemia/reperfusion [13]. Therefore, it is suggested that MSC-ex should be used in the treatment of DSS-induced IBD.

Ubiquitination, the ubiquitin depending on enzymes to modify the target protein, plays an important role in the regulation of various biological functions such as injury repair and inflammatory immunity [14]. The complicated exosomes delivered components, including cytokine receptors, tumor-associated antigens, mRNA, and miRNA, are able to transfer these molecules to injury tissues [15]. Thus, we presume that some of the hucMSC-ex components may affect the DSS-induced IBD. However, it is not clear whether hucMSC-ex-mediated treatment involves ubiquitin modification. In this study, we demonstrated that hucMSC-ex could alleviate the symptoms of DSS-induced IBD in mice through ubiquitination.

Materials and methods

Cell culture and exosome isolation

Exosomes were isolated from human umbilical cord MSCs. Then, MSCs were isolated and cultured from human umbilical cords obtained from consenting women [16]. The cells were cultured for 48 h at 37°C in humidified atmosphere with 5% CO_2 in MEM alpha basic (low glucose, Gibco, USA) containing 10% FBS (fetal bovine serum) and 1% penicillin and streptomycin. The cell culture media was collected and centrifuged at 2,000 × g for 30 min to remove cell debris. Then, the cell culture media was centrifuged at 10,000 × g for 30 min. The supernatant was collected and concentrated using 100 KDa MWCO (Millipore, USA) at 1,500 × g for 30 min. Cell supernatant was concentrated for several times before the concentrated supernatant was loaded upon 5 ml of 30% sucrose/D_2O cushions and ultracentrifuged at 100,000 × g for 60 minutes. The exosomes-enriched fraction was harvested and diluted with PBS and then centrifuged thrice at 1,000 × g for 30 minutes using 100 KDa MWCO. Finally, the purified exosomes were collected, filtrated through 0.22 μm pore filter (Millipore, USA) and stored at -70°C for further use [17].

Characterization of exosomes derived from hucMSCs

The morphology of the exosomes was observed by transmission electron microscopy (FEI Tecnai 12, Philips, The Netherlands) [17]. 20 μl drops of purified exosomes were adsorbed onto copper grids, placed at room temperature for 1 min, adsorbed onto the superfluous exosomes, and stained with 30 g/L phosphotungstic acid (pH 6.8) for 5 minutes at room temperature, and finally the sample dried under a half-watt lamp. Samples were then imaged by transmission electron microscopy. The protein content of hucMSC-ex was tested by a BCA Protein Assay kit (CWbio), and the number of hucMSC-ex was quantified using nanoparticle tracking analysis, as described previously [11]. The presence of known exosome markers, including CD9 (Bioworld Technology, USA), CD-63 (Bioworld Technology, USA) and CD81 (Epitomics, USA), was analyzed by western blotting [18].

Animal models

A total of 18 Male BALB/c mice (Laboratory Animal Research Center of Jiangsu University, Jiangsu, China) were randomly assigned to three groups (n = 6/group): control group (Normal), IBD group (IBD), hucMSC-ex-treated IBD group (IBD + Ex). All experimental procedures were conducted in accordance with the Animal Use and Care Committee of Jiangsu University. To induce colitis, the mice received 3% DSS (MP, Cat NO: 160110, Canada) in their drinking water for 11 days. On Day 3, Day 6 and Day 9, 400 μg hucMSC-ex were injected into the mice of IBD + Ex group via the tail vein respectively [19]. The mice of IBD group received PBS per time through the tail vein. Weight measurements and evaluation of stool consistency were daily monitored. All animals were sacrificed on Day 11, and the colon and spleen tissues were collected for further studies.

Western blotting analysis

The colon mucosa tissues and spleen tissues were lysed in ice-cold RIPA lysis buffer containing phenylmethanesulfonyl fluoride (PMSF) (Vazyme biotech, Shanghai, China) by mechanical homogenization. The protein concentration of each sample was tested using the BCA assay kit. A total of 150 μg protein samples were separated on 10% SDS-PAGE (sodium dodecyl sul-

Table 1. Primer sequences for RT-PCR Primer sequences for the amplification of target genes and *β*-actin

Gene	Primer sequence (5'-3')	Annealing temp (°C)	Amplicon size (bp)
NAe1	FOR: GCAACGGCTACAGGAACTGA	60	146
	REV: GCTCGGTTCTTGCCAATACT		
E2M	FOR: AGACGACCTCCTCAACTTCA	60	257
	REV: TCGGCTCCAAGAAGAGATAC		
Uba3	FOR: GCTGGTGGCTTAGGATGTGA	60	199
	REV: GTACCACGTTGCAGTTAG		
TNF-α	FOR: AACTCCAGGCGGTGCCTATG	63	242
	REV: TCCAGCTGCTCCTCCACTTG		
IL-1β	FOR: AGCTTCAGGCAGGCAGTATC	61	215
	REV: TCATCTCGGAGCCTGTAGTG		
IL-10	FOR: CCTGGCTCAGCACTGCTATG	61	151
	REV: TCACCTGGCTGAAGGCAGTC		
IL-6	FOR: AAGTCCGGAGAGGAGACTTC	58	487
	REV: TGGATGGTCTTGGTCCTTAG		
IP-10	FOR:AAATCATCCCTGCGAGCCTATCC	64	413
	REV: ACAGCCATCCCAGCCACTTGAG		

fate-polyacrylamide gel electrophoresis), which the proteins transferred to PVDF (polyvinylidene difluoride) membrane (Millipore, IPVH-00010, USA). Membranes were blocked with 5% milk in Tris-buffered saline-Tween 20 (TBST) and incubated with corresponding primary antibodies at 4°C overnight. The primary antibody includes: anti-CD9 (Bioworld Technology, USA), anti-CD63 (Bioworld Technology, USA), anti-CD-81 (Abcam, USA), anti-β-actin (Santa Cruz Biotechnology), Ubiquitin (Bioworld Technology, USA) 1, K48 (Millipore, Canada), K63 (Millipore, Canada), and FK2 (Life Sensors, USA). The next day, the blots bound secondary antibodies for 1 h at room temperature. Western blotting detection was imaged using chemiluminescence (Millipore, USA).

H&E staining

To determine the injury of colon mucosa and spleen tissues, Hematoxylin and eosin (HE) staining was performed according to the standardized procedures. The tissues fixed in 4% paraformaldehyde (pH 7.4) were gradually dehydrated, embedded in paraffin, cut into 4-μM sections and stained with H&E stain.

Real-time PCR (RT-PCR)

The total RNA in the colon mucosa and splenic mononuclear cells was extracted with TRIzol Reagent (Life technologies, Carlsbad, CA, USA). The mRNA expression was analyzed using real-time PCR. The cDNA was performed using HiScript 1st Strand cDNA Synthesis Kit (Vazyme Biotech, Shanghai, China). The gene expression was analyzed in a Step One Plus Real-Time PCR System (Applied Biosystems, Life Technologies, USA) as previously described [20]. The sequences of primer pairs are listed in **Table 1**.

Statistical analysis

The experimental values were expressed as mean ± standard deviations (SD). To compare two groups, parametric and nonparametric analyses were performed using ANOVA for unpaired *t*-test followed by the Holm-Bonferroni. The significant difference was performed using Prism software (Graph Pad, SanDiego, USA), and $P < 0.05$ was considered statistically significant.

Results

Characterization of hucMSC-ex

Several methods can be used for hucMSC-ex isolation. Firstly, we used transmission electron microscopy to measure the size of the purified hucMSC-ex, with a mean diameter of 40-100 nm (**Figure 1A**). Next, we used Nanoparticle Tracking Analysis (NAT) to measure the diameter distribution of hucMSC-ex. As shown in **Figure 1B**, the diameter of isolated exosomes had a single peak at approximately 100 nm. Finally, the purified hucMSC-ex surface markers, including CD9, CD63 and CD81, were detected by western blotting. The results showed that the surface markers CD9, CD63 and CD81 were normally expressed in hucMSC-ex (**Figure 1C**). These results indicated that we have successfully isolated and identified exosomes from hucMSCs.

Exosomes from hucMSCs alleviated the severity of DSS-induced IBD in mice

To meet the strict diagnosis criteria and investigate the effects of exosomes from hucMSCs, we established a stable model with 3% DSS-induced inflammation bowel disease. Compar-

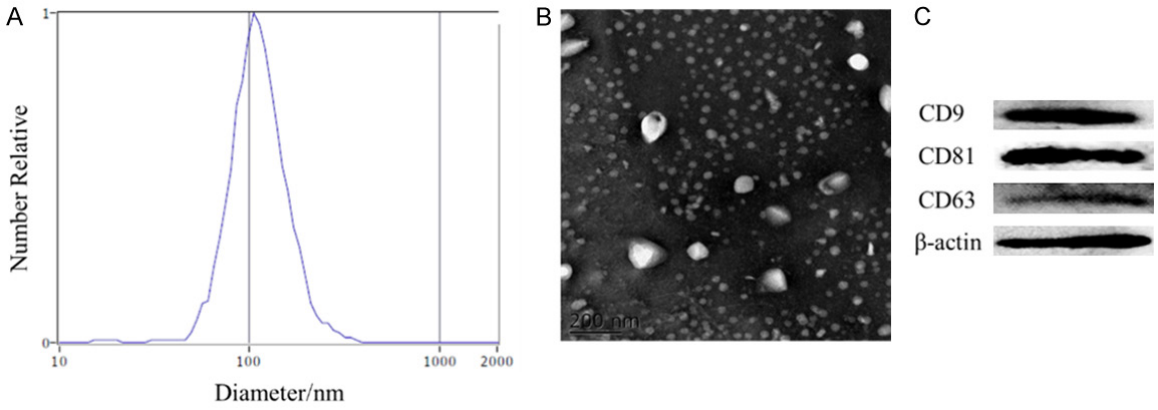

Figure 1. Characterization of exosomes derived from human umbilical cord mesenchymal stem cells. A. Transmission electron microscopy to identify the characteristics of hUCMSC-exosomes. Scale bar: 100 nm. B. Nanoparticle Tracking Analysis (NAT) of exosomes. C. CD9, CD63, and CD81 expressions of exosomes are detected using western blot assay.

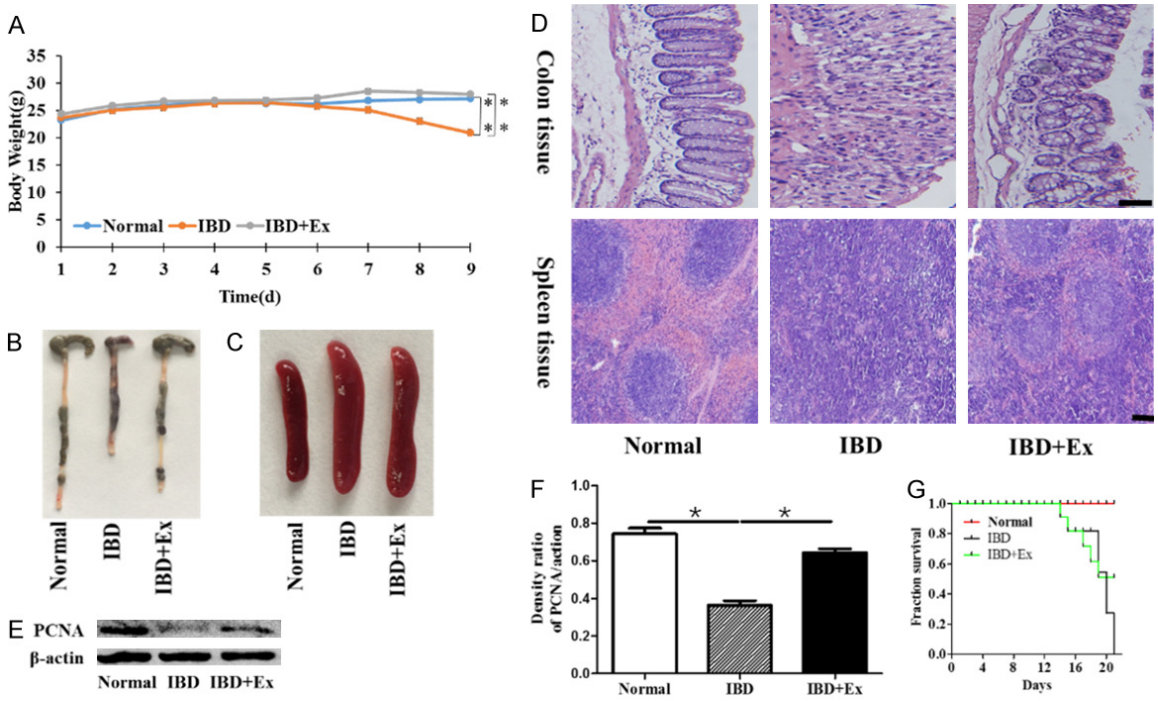

Figure 2. HucMSC-exosomes alleviate the DSS-induced IBD in mice. (A) The body weight of mice in normal, IBD and IBD + Ex groups. (B) The colon lengths of mice in normal, IBD and IBD + Ex groups. (C) The size of the spleens of mice in normal, IBD and IBD + Ex groups. (D) H&E staining of the colon and spleen tissues structure of mice in normal, IBD, IBD + Ex groups. (E) The western blot analysis of PCNA in the colon tissues of mice in normal, IBD and IBD + Ex groups. (F) Densitometric analyses of the protein bands in (E). (G) Survival curve of mice in normal, IBD and IBD + Ex groups (n = 6). Data shown are representative of three independent experiments. Bars represent the means ± SD. Scale bar = 100 μm. $^{*}P < 0.05$; $^{**}P < 0.01$.

ed with the mice in IBD group, the mice in IBD + Ex group had blood stool and began to lose weight on Day 5 after DSS exposure. Interestingly, hucMSC-ex administration dramatically inhibited the weight loss in IBD + Ex group (**Figure 2A**), suggesting that hucMSC-ex could alleviate the symptom of IBD. As shown in **Figure 2B** and **2C**, the size of spleen in hucMSC-ex treated group was significantly smaller than that in IBD group, and the length of colon in hucMSC-ex-treated group was longer than that in IBD group. Moreover, through examining

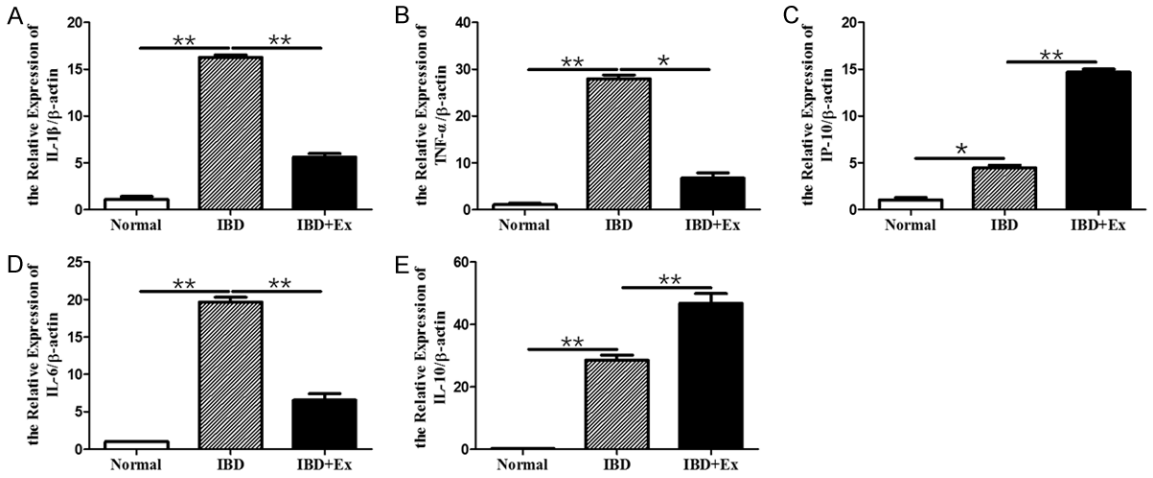

Figure 3. Quantitative analysis of inflammatory-associated gene expression level in the colon tissue of mice in normal, IBD and IBD + Ex groups. A. The level of IL-1β expression in normal, IBD and IBD + Ex groups. B. The level of TNF-α expression in normal, IBD and IBD + Ex groups. C. The level of IL-6 expression in normal, IBD and IBD + Ex groups. D. The level of IP-10 expression in normal, IBD and IBD + Ex groups. E. The level of IL-10 expression in normal, IBD and IBD + Ex groups. Bars represent the means ± SD. *P < 0.05; **P < 0.01.

the integral structure of splenic nodules and intestinal villus using H&E staining, we found that the integral structure was remarkably broken in the mice of IBD group compared with normal mice. However, treatment with hucMSC-ex successfully reduced this phenomenon (**Figure 2D**). Both of them suggested that DSS destroyed the integrity structure of colon and spleen tissues with increased inflammatory cell infiltration in the mice of IBD group. In contrast, hucMSC-ex-treated mice could recover the integrity of tissue structure and relieve the infiltration of inflammatory cells. Western blotting results of PCNA revealed that the percentage of proliferating cells decreased in the colon tissues of IBD mice but increased in hucMSC-ex-treated mice, suggesting that the proliferating ability of colon mucosa epithelial cells was recovered by exosomes treatment (**Figure 2E**, **2F**). Finally, we also compared the survival rate between IBD group and IBD + Ex group, indicating that the mice in IBD group died but hucMSC-ex-treated mice survived (**Figure 2G**). In general, hucMSC-ex administration significantly improved the symptoms of DSS-induced IBD in mice.

HucMSC-ex regulated the expression of cytokines in the colon tissues and spleen tissues of IBD mice

Then, we measured the expression of proinflammatory cytokines in the tissues of colon and spleen by qRT-PCR. By comparing the expression level of anti-inflammatory cytokines among the three groups, we found that the expression level of pro-inflammatory cytokines dramatically increased in IBD group compared with that in the IBD + Ex group in colon tissues, including TNF-α, IL-1β and IL-6 (**Figure 3A-C**). In contrast, anti-inflammatory cytokines, such as IL-10 and IP-10, increased obviously in IBD + Ex group than those in the IBD group (**Figure 3D**, **3E**). Similar results were found in the spleen tissues in the mice from the IBD group and the IBD + Ex group (**Figure 4**).

HucMSC-ex inhibited the expression of ubiquitin and its associated molecules in the colon tissues and spleens of IBD mice

Previous studies demonstrated that hucMSC-ex alleviates the severity of IBD [19]. It has been reported that ubiquitin plays an important role in the development of inflammation bowel disease [21]. Then we measured the expression of ubiquitin and some other molecules associated with ubiquitin. The results showed that the expression level of ubiquitin-associated genes, including NAe1, E2M and Uba3, decreased significantly in the IBD + Ex group compared with those in the IBD group (**Figure 5A-C**). Interestingly, the expression of ubiquitin protein significantly increased in the colon tissues of IBD mice but decreased in the colon tissues of IBD + ex mice. The protein expression level of P-NF-κB and P-mTOR also de-

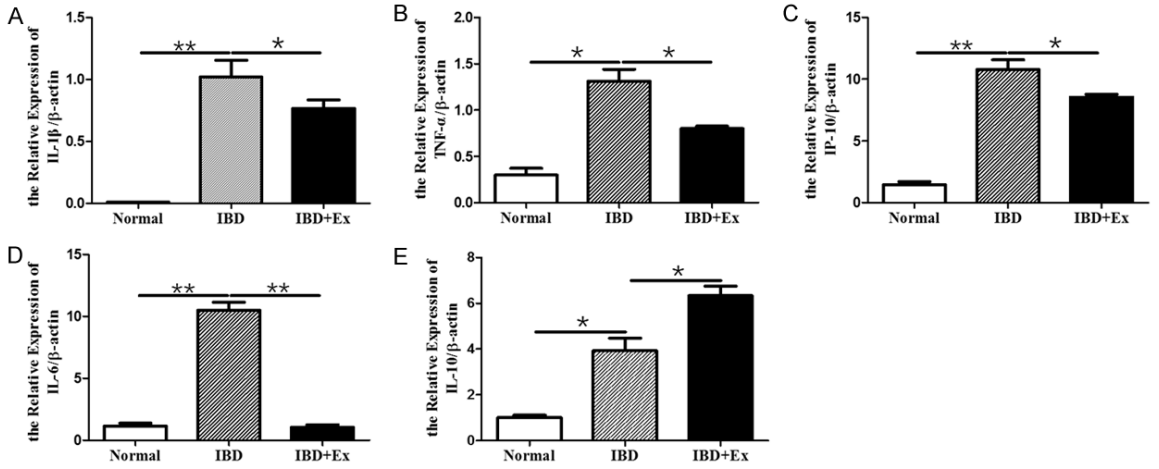

Figure 4. Quantitative analysis of inflammatory-associated gene expression level in the spleen tissue of mice in normal, IBD and IBD + Ex groups. A. The level of IL-1β expression in normal, IBD and IBD + Ex groups. B. The level of TNF-α expression in normal, IBD and IBD + Ex groups. C. The level of IL-6 expression in normal, IBD and IBD + Ex groups. D. The level of IP-10 expression in normal, IBD and IBD + Ex groups. E. The level of IL-10 expression in normal, IBD and IBD + Ex groups. Bars represent the means ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$.

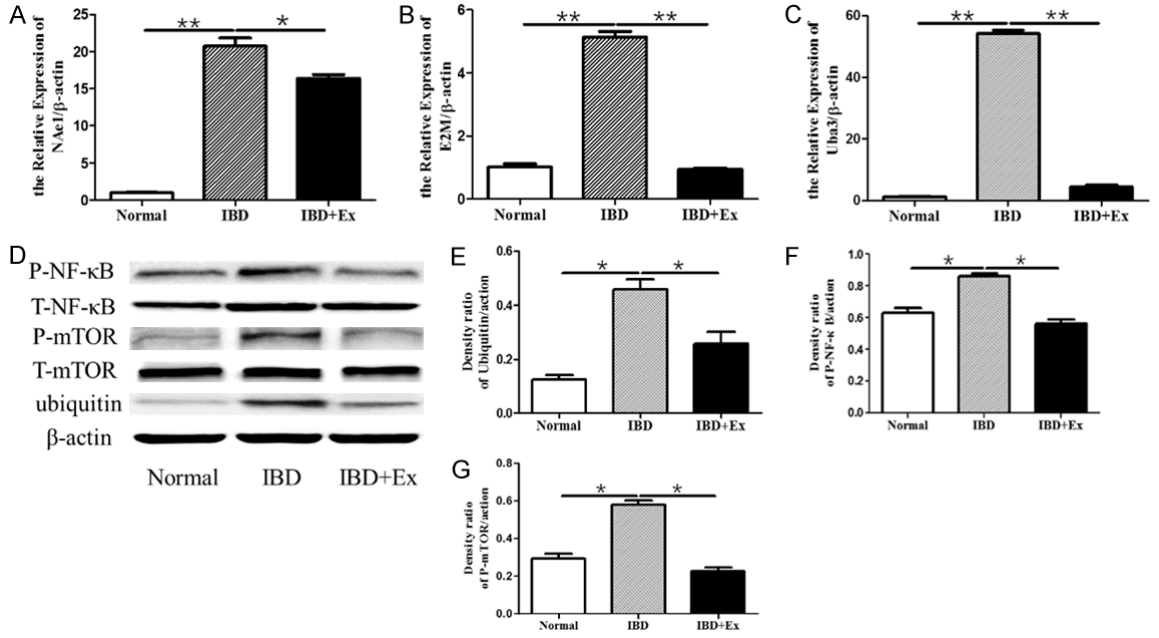

Figure 5. HucMSC-exosomes regulate the expression of ubiquitin-associated protein and gene. QRT-PCR analyses of the gene expression levels of ubiquitin-associated cytokines in the colon tissues of mice in normal, IBD and IBD + Ex groups. A. NAe1. B. E2M. C. Uba3. D. The western blot analysis of ubiquitin, NF-κB and mTOR in the colon tissues of mice in normal, IBD and IBD + Ex groups. E. Densitometric analyses of the protein bands in ubiquitin. F. Densitometric analyses of the protein bands in P-NF-κB. G. Densitometric analyses of the protein bands in P-mTOR. Data shown are representative of three independent experiments. Bars represent the means ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$.

creased in hucMSC-ex-treated IBD mice, suggesting that hucMSC-ex dramatically relieved the development of inflammation (**Figure 6D-G**). Similar results were found in the spleen tissues from the mice in IBD group and IBD + ex group (**Figure 6**).

Exosomes from hucMSCs alleviated inflammation through regulating the modification of ubiquitination

Different assembly modes of ubiquitin chain have different kinds of biological functions [22,

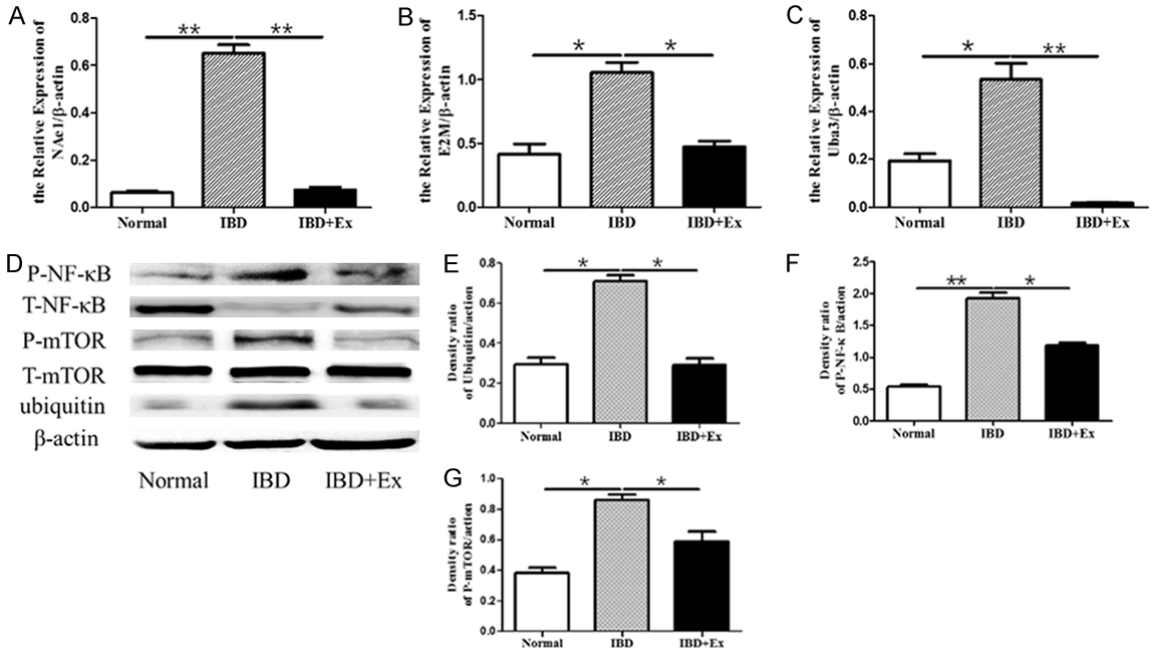

Figure 6. HucMSC-exosomes regulate the expression of ubiquitin-associated protein and gene. QRT-PCR analyses of the gene expression levels of ubiquitin-associated cytokines in the spleen tissues of mice in normal, IBD, and IBD + Ex groups. A. NAe1. B. E2M. C. Uba3. D. The western blot analysis of ubiquitin, NF-κB and mTOR in the colon tissues of mice in normal, IBD and IBD + Ex groups. E. Densitometric analyses of the protein bands in ubiquitin. F. Densitometric analyses of the protein bands in P-NF-κB. G. Densitometric analyses of the protein bands in P-mTOR. Data shown are representative of three independent experiments. Bars represent the means ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$.

23]. So, we detected the protein expression of ubiquitin-associated molecules such as K48, K63 and FK2 in different kinds of animal models. Compared with that in the mice of IBD group, western blotting results have shown that the protein expression of K48, K63 and FK2 have significant changes in the mice of IBD + ex group (**Figure 7**). In conclusion, hucMSC-ex has modulated the expression of ubiquitin-associated molecules.

Discussion

Recent studies have demonstrated that hucMSC can alleviate inflammation bowel diseases in mice [19, 24, 25], but the exact mechanism is unclear. HucMSCs significantly reduced systemic inflammation and attenuated DSS-induced inflammation bowel diseases in mice, and reduced inflammatory cytokine production. The majority of therapeutic benefits of MSCs on IBD rely on the release of paracrine soluble factors [26]. Exosomes, secreted by most cell types and formed by the fusion of multivesicular endosomes with the plasma membrane, play an important role in the regulation of cell-cell communication [27, 28]. Our group and the others demonstrated that hucMSC-derived exosomes have had a potential therapeutic effect on several tissue injury diseases such as liver fibrosis [29], cisplatin induced renal oxidative stress [30] and skin injury [31]. In this study, we showed that hucMSC-derived exosomes could ameliorate DSS-induced IBD in mice.

IBD is a chronic idiopathic, systemic, heterogenetic and relapsing disorder of the gastrointestinal tract, where the colon mucosal lesion is characterized by the infiltration of inflammatory cells [32]. We demonstrated that the expression level of inflammatory factor increased in inflammatory tissues of IBD mice and reduced in hucMSC-ex-treated IBD mice. Compared with that in the mice of IBD group, the hucMSC-derived exosomes decreased the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, but they increased the expression of anti-inflammatory cytokine such as IL-10 and IP-10. The results showed that hucMSC-derived exosomes could ameliorate colitis.

Ubiquitination is the process by which ubiquitin molecules modify the target protein by an enzy-

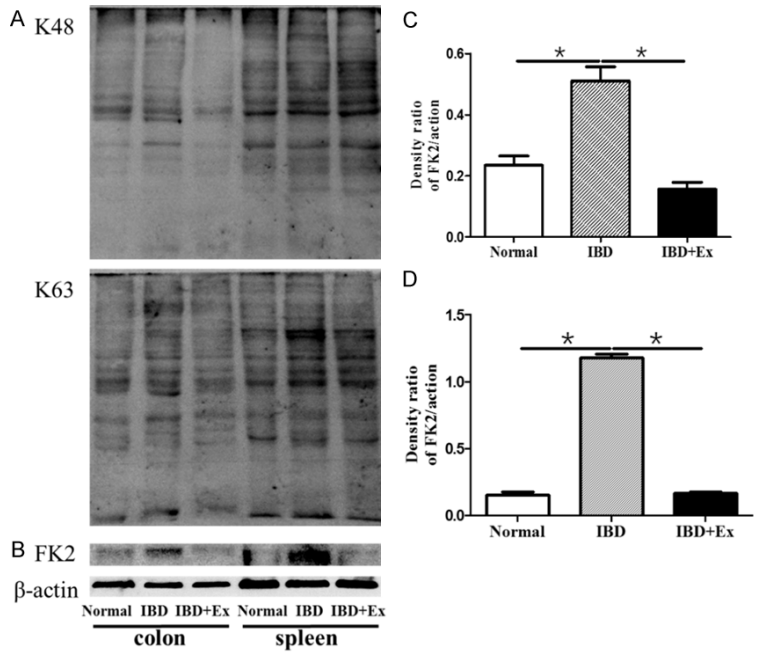

Figure 7. Exosomes from hucMSCs regulate the expression of ubiquitination linear modification protein. The western blot analyses of the different types of ubiquitination linear modification in normal, IBD, and IBD + Ex mice groups. A. K48 and K63. B. FK2. C. Densitometric analyses of the protein bands in FK2 in colon tissue. D. Densitometric analyses of the protein bands in FK2 in spleen tissue. Bars represent the means ± SD. $^{*}P < 0.05$.

matic reaction cascade [33, 34]. Ubiquitination regulates protein stability, activity and regulation of protein function, thereby controlling cellular function. Ubiquitin, as an essential protein associated with ubiquitination, plays a central role in inflammation response including IBD [21, 35, 36]. The downregulation of ubiquitin in mice with DSS-induced colitis could inhibit inflammation in the gastrointestinal tract. Studies showed that E3 Ubiquitin ligase promotes intestinal inflammation via the activation of the NF-κB pathway by increasing the ubiquitination and degradation of IκBα [37, 38]. Previous studies demonstrate that ubiquitin protein derived from inflammatory tissues is up-regulated in colitis mice [39]. And ubiquitin is associated with mTOR signaling pathway. For example, OTUB1 (dOTU domain-containing ubiquitin aldehyde-binding protein 1) as deubiquitinates, regulate mTOR activity-related inflammatory diseases through interaction with DEPTOR. The N-terminal domain and deubiquitinated DEPTOR stabilize DEPTOR in a Cys-91-independent through Asp-88-dependent manner, suggesting that OTUB1 targets DEPTOR for deubiquitination via a deubiquitinase activity. Therefore, Deubiquitin also regulates inflammation through mTOR signal [40]. In this study, we found that hucMSCs-derived exosomes significantly inhibited the expression of ubiquitin in the colon mucosa tissues and spleens of IBD mice. Different assembly modes of ubiquitin chain have various biological functions [41, 42]. K48-linked polyubiquitin chain (Lys 48) and K63-linked polyubiquitin chain (Lys 63) play an important role in regulating the activity process of NF-κB pathway [43, 44]. We further demonstrated that hucMSC-derived exosomes inhibited the expression of ubiquitin protein through regulating K48 and K63. Moreover, the attachment of UB to proteins is catalyzed by the action of Ubiquitin-activating enzyme E1, Ubiquitin-conjugating enzyme E2, and Ubiquitin ligase E3 [23]. The gene expression levels of NEDD8 activating enzyme E1 (NAe1), ubiquitin-conjugating enzyme E2M (UBE2M) and ubiquitin-like modifier activating enzyme 3 (Uba3) as the critical components of the ubiquitination, were much higher in the mice of IBD group than in the normal group but significantly lower in IBD + Ex group than in IBD group. Previous studies demonstrate that the FK1-FK2 domains adapt to the steric requirements in the FKBP52-rapamaycin-mTOR complex [45]. Our results suggested that the protein expression level of FK2 and P-mTOR increases in inflammatory tissues of IBD group but decreased significantly after the treatment with hucMSC-ex.

Conclusions

In summary, our finding suggested the importance of the mechanism by which hucMSC-derived exosomes decreased ubiquitin protein expression and subsequently reduced NF-κB and mTOR activation, an important mediator in regulating inflammatory factor expression. Moreover, hucMSC-ex also regulates the expression of polyubiquitination including K48,

K63 and monomeric ubiquitination including FK2. Therefore, the use of hucMSC-derived exosomes may provide a novel approach for the treatment of IBD.

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Disclosure of conflict of interest

None.

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