Microvesicles from Mesenchymal Stem Cells Overexpressing MiR-34a Ameliorate Renal Fibrosis *In Vivo*

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Introduction. We recently discovered that microvesicles (MVs) derived from mesenchymal stem cells (MSCs) overexpressing miRNA-34a can alleviate experimental kidney injury in mice. In this study, we further explored the effects of miR34a-MV on renal fibrosis in the unilateral ureteral obstruction (UUO) models.
Methods. Bone marrow MSCs were modified by lentiviruses overexpressing miR-34a, and MVs were collected from the supernatants of MSCs. C57BL6/J mice were divided into control, unilateral ureteral obstruction (UUO), UUO + MV, UUO + miR-34a-MV and UUO + miR-34a-inhibitor-MV groups. MVs were injected to mice after surgery. The mice were then euthanized on day 7 and 14 of modeling, and renal tissues were collected for further analyses by Hematoxylin and eosin (H&E), Masson's trichrome, and Immunohistochemical (IHC) staining.
Results. The UUO + MV group exhibited a significantly reduced degree of renal interstitial fibrosis with inflammatory cell infiltration, tubular epithelial cell atrophy, and vacuole degeneration compared with the UUO group. Surprisingly, overexpressing miR-34a enhanced these effects of MSC-MV on the UUO mice.
Conclusion. Our study demonstrates that miR34a further enhances the effects of MSC-MV on renal fibrosis in mice through the regulation of epithelial-to-mesenchymal transition (EMT) and Notch pathway. miR-34a may be a candidate molecular therapeutic target for the treatment of renal fibrosis.

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INTRODUCTION

Fibrosis resulting from dysfunctional tissue repair processes is the most common pathophysiological manifestation of chronic kidney disease (CKD). In injured renal tissue, renal epithelial cells undergo an epithelial-to-mesenchymal phenotype transition, also known as EMT, contributing to renal fibrosis.[1] Unfortunately, data regarding treatment strategies for renal fibrosis are currently relatively limited. Recently, a molecular-targeted therapy has been proposed as a potential new strategy for treating renal fibrosis;[2] however, there is still no clinically validated molecularly targeted therapy. Thus, identifying new and effective molecular markers for the diagnosis and prognosis of renal fibrosis is required, which may contribute to a better understanding of the pathogenesis of CKD.

MicroRNAs (miRNAs), small non-coding single-stranded RNAs, are important regulators of gene expression that affect many biological processes such as growth, development and immunity.[3] Abnormally expressed miRNAs can be used as a diagnostic biomarker of various diseases.[4] It has been previously demonstrated that miRNAs

serve substantial effects on the progression of renal fibrosis and are potential candidates for antifibrotic therapies in CKD.[5] For example, studies have demonstrated that miR-34a, which acts as a tumor-suppressive miRNA that contributes to p53-mediated apoptosis,[6] is abundant in mammalian cardiac tissues and exacerbates the progression of cardiac fibrosis.[7] In addition, it has been demonstrated that miR-34a can regulate EMT-related transcriptional factors.[8] However, the exact role of miR-34a in the progression of renal fibrosis is still unclear and requires further investigation.

Mesenchymal stem cells (MSCs) are multipotent adult cells that differentiate into multiple mesodermal germ layer cell types[9] and participate in the regeneration of injured tissue as well as immune regulation.[10] Microvesicles (MVs) have been described as a one-of-a-kind paracrine factor derived from MSCs (MSC-MVs), which may act as paracrine mediators between MSCs and target cells.[11] MSC-MVs were demonstrated to be effective against various diseases,[12] including kidney disease. For example, Imafuku *et al*. showed that MVs isolated from genetically engineered MSCs suppress renal fibrosis via microvascular protection.[13] Moreover, our previous studies have demonstrated that MSC-MVs could attenuate experimental renal injury in mice, and MVs with the function of transferring cytokines were superior to MSCs alone.[14,15,18] In this study, we further investigated whether miR-34a improves the therapeutic efficiency of MSC-MVs for treating renal fibrosis using the unilateral ureteral obstruction (UUO) model. Our results suggested that miR-34a is a promising target for the development of therapeutics for renal fibrosis. These data provide a basis for the biotherapy and molecular mechanism of renal fibrosis.

MATERIALS AND METHODS

Animal Treatments and Surgical Methods

Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006), and were approved by the animal ethics committee of Northwestern University. A healthy specific pathogen-free (SPF) C57BL6/J mice (n = 30, 6-week-old, body weight of 15 to 23 g) were purchased from Huagengkang Biotechnology Co., Ltd. The environment in which all the animals were kept had a temperature of 20 ± 1 ℃, a relative humidity of 50 ± 1%, and a 12-hour light-dark cycle. All experimental animals were conducted in accordance with the guidelines of the Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council of China) and comply with the Guide for the Care and Use of Laboratory Animals.

The animals were randomly divided into five groups (n = 6 per group): control, UUO, UUO + MV, UUO + miR-34a-MV, and UUO + miR-34a-inhibitor-MV (miR-34a inhibitor, WanleiBio, China). After a week of adaptive feeding, all the mice showed a quick response, good activity, smooth fur, normal water intake and food intake; also, no weight loss and diarrhea were observed. The UUO model was conducted under tiletamine/zolazepam anesthesia (VIRBAC Laboratories, Carros, France) administered via the tail vein at 37 °C following procedures outlined in a prior study.[14] Briefly, the left ureter was ligated with silk (4/0) at two points and treated with antibiotics to prevent urinary tract infection. The contralateral ureter underwent identical surgical procedures without ureteral ligation. After modeling, there was no wound infection in all groups. After the procedure, mice were given amoxicillin orally three times a day. MVs-treated groups were injected intraperitoneally with 30 mg MV/mouse. On day 7 and 14 after the first injection, the mice were exposed to light ether anesthesia for about 20 seconds and euthanized after 4 minutes. Following the removal of the kidneys, one section was preserved in 4% paraformaldehyde and the other section was snap-frozen in liquid nitrogen before being stored at –80 °C for subsequent use.

Isolation and Culture of Bone Marrow MSCs and MVs

The primary bone marrow mesenchymal stem cells (MSCs) from mice were collected, separated, and fragmented as described before. The collected cells were subsequently examined by flow cytometry to evaluate their osteogenesis and adipogenesis. Microvesicles (MVs) were collected from mesenchymal stem cell (MSC) supernatants using an established method.[14] The MSCs were cultured in DMEM/F12 (Gibco, USA) for 2 to 3 generations. The culture media were collected, centrifuged at 2000 g for 20 min, and stored at

-80 °C. The supernatants containing free-cell debris were centrifuged at 100,000 g using a Beckman Coulter Optima L-90 K ultracentrifuge at 4 °C for 1 hour. They were then washed with serum-free medium 199 (M199) supplemented with 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) from Sigma, USA, and subjected to a second ultracentrifugation under the same conditions before being washed again with M199. MV suspensions were kept at -80 °C and quantified by using the bicinchoninic acid (BCA) method. The structure of MVs was evaluated as outlined before. [15]

Lentiviral Infection of MSCs and Collection of Genetically Modified MVs

Lentiviral particles (108 TU/mL; GFP) overexpressing miR-34a (5′-UGGCAGUGUCUUAGCUGGUUGU-3′) or miR-NC (negative control) were purchased from WanleiBio (Shenyang, China), and labeled lenti-miR-34a and lenti-miR-NC, respectively. MVs were obtained from MSCs (P3) 48 h post-viral infection. The culture media were collected from P3-P7 MSCs every 48 h to extract the MVs as previously described.[15]

MV Extraction from MiR-34a-modified MSCs

The extraction, identification, and quantification of MSC and MV have been described in detail in previous references. Also, MV extraction from miR-34a-modified MSCs was also described in another study.[15]

Biochemical Experiments

Quantitative real-time PCR (qRT-PCR), hematoxylin and eosin (H&E), Masson's trichrome staining, and immunohistochemical (IHC) staining were described in Supplementary Methods. Histological changes were assessed by Histological Score of Kidney (HSK) scoring: the non-overlapping field of view (up to 10 fields per mouse) of the entire section was analyzed at high magnification using a × 20 objective (HPF). Renal histological score (HSK) was used to define: 0, no damage; 1, unicellular, patchy solitary necrosis; 2, < 25% tubular necrosis; 3, renal tubular necrosis of 25 to 50%; 4, > 50% of renal tubule necrosis and infarct tissue present were photographed using a VKC150 color camera (Hitachi, Tokyo, Japan) connected to an AX70 microscope (Olympus, Center Valley, PA, USA). The sections were analyzed by a pathologist in a single-blind manner.

RESULTS

Animal Condition After Modeling and Treatment

In order to explore the effect of miR-34a overexpression in microvesicles on renal fibrosis *in vivo*, the UUO mouse model was established. The mice of the UUO group presented with a sluggish response, reduced activity, weakness, and lassitude and showed reduced food and water intake compared with that in the control group, which suggested that the UUO model was successfully created. As compared to the UUO group, these symptoms were all improved in the UUO + MV group. Also, mice in the UUO + miR-34a-MV group showed increased activity and significantly improved water and food intake. The reaction was slightly more sensitive than the UUO group and UUO + MV group, and the state was similar to the control group. Interestingly, UUO + miR-34a-inhibitor-MV mice were less responsive, less active, less fed, emaciated and tired, which was similar to the UUO mice.

Overexpression of MiR-34a Alleviates Renal Fibrosis in UUO Mice

To observe the effects of miR-34a-MVs on the degree of interstitial fibrosis, H&E and Masson's staining were performed on the kidney sections at day 7 or 14 after UUO. As shown in Figure 1A, glomeruli showed no obvious pathological changes among 5 groups at day 7, while swelling of renal tubular epithelial cells, brush border sloughing, and renal tubular lumen dilatation were observed after UUO. Moreover, the pathological changes gradually became aggravated. On day 14, kidney sections of mice model showed slight glomerular shrinkage, slight enlargement of renal cyst cavity, obvious swelling of most renal tubular epithelial cells with brush borders detachment, renal tubular dilatation, and a mild lymphocytes and monocytes infiltration of renal interstitium. Surprisingly, treatment with MV significantly attenuated the kidney injury, especially in the UUO + miR-34a-MV group. Additionally, few red blood cells, monocytes and lymphocytes were dispersed in the interstitial tissue space in the UUO + miR-34a-MVgroup, and the original normal structure of glomeruli

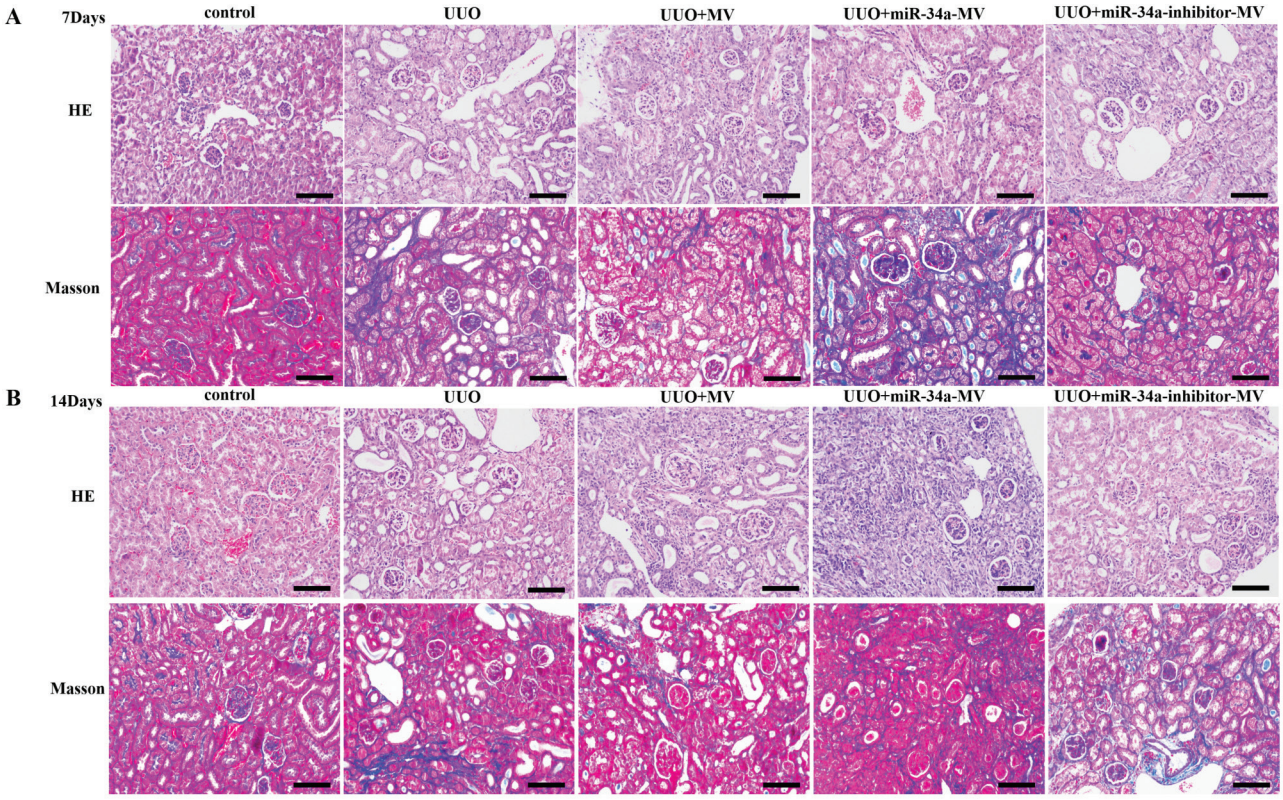

Figure 1. Overexpression of miR-34a alleviated renal fibrosis *in vivo* [A) Representative images of HE staining of kidney injury in five groups (control, UUO, UUO + MV, UUO + miR-34a-MV, and UUO + miR-34a-inhibitor-MV) on day 7 or 14 post-modeling (magnification, ×400; scale bar, 50 μm); B) Representative images of Masson's trichrome staining of kidney injury in five groups on day 7 or 14 post-modeling (magnification, ×200; scale bar, 50 μm)]

and most renal tubules could be seen, with only a few atrophied renal tubules and collecting ducts. However, pathological changes in the UUO + miR-34a-inhibitor-MV group were similar to those in the UUO + miR-34a-MV group, suggesting the effect of miR-34a was not reversed by the administration of miR-34a inhibitor. Overall, these results suggested that overexpression of miR-34a could reduce the progression of interstitial area in UUO mice.

The assessment of the histological changes was graded by HSK. UUO scored 2.95 ± 1.00 (day 7) and 3.98 ± 0.50 (day 14); UUO + MV scored 1.38 ± 0.55 (day 7) and 1.98 ± 0.67 (day 14); UUO + miR-34a group scored 1.29 ± 0.30 (day 7) and 2.26 ± 1.15 (day 14), and the UUO + miR-34a-inhibitor group scored 2.29 ± 0.36 (day 7) and 2.16 ± 1.15. Scores in the UUO + MV, UUO + miR-34a group, and UUO+miR-34a-inhibitor group were significantly different compared with the UUO group (all $P < .05$). However, pathological changes in the UUO + miR-34a-inhibitor-MV group were similar to those in the UUO + miR-34a-MV group, suggesting the effect of miR-34a was not reversed by the administration of miR-34a inhibitor, which is consistent with the results of our previous *in vitro* studys.

Masson's trichrome staining showed normal tissue in the control group (Figure 1B). On day 7, the UUO group showed obvious bluish fibroid material in renal tubules and interstitial regions. Moreover, the blue-stained filaments around the renal capsule and the interstitial area gradually increased with time, and the degree of fibrosis was more severe (Figure 1B). On day 7, compared with UUO group, blue filaments staining substances were found in renal tubules and renal interstitial area in UUO + MV group, and the degree of fibrosis was lighter than that in UUO group. Also, in this group, the tissue structure atrophied obviously, and the blue-stained area decreased on day 14 (Figure 1B). In the UUO + miR-34a-MV group, blue-stained fibers distributed in glomeruli, tubules and interstitium simultaneously, and the degree of fibrosis decreased compared to the UUO + MV mice (Figure 1B). It is noteworthy that the degree of

renal fibrosis in the UUO + miR-34a-inhibitor-MV group was similar to the UUO + MV group and increased compared to the UUO + miR-34a-MV group. These results showed that renal fibrosis was improved by overexpression of miR-34a in MSCs.

Overexpression of MiR-34a Modulates Renal Injury Through EMT

To examine whether genetically engineered microvesicles modulate renal injury through EMT, the expression levels of EMT-related proteins α-SMA, E-cadherin, and fibronectin were detected by IHC staining. α-SMA was expressed in the cytoplasm and cytoskeleton. As shown in Figure 2A and 2B, the expression levels of α-SMA and fibronectin increased on day 7 and 14 after UUO compared with a control group, while the expression level of E-cadherin decreased. Treatment with MV reversed this process; it significantly decreased the positive areas of α-SMA and fibronectin in UUO kidneys and upregulated fibronectin on day 7 and 14 (Figure 2A, B). Remarkably, α-SMA and fibronectin levels were significantly reduced at day 7 in the UUO + miR-34a-MV group, while E-cadherin expression increased (Figure 2B). Also, the UUO + miR-34a-MV group exhibited a time-dependent decrease in the expression of α-SMA as well as a time-dependent increase in the expression of fibronectin and E-cadherin (Figure 2A, B); however, the fibronectin expression of UUO + miR-34a-MV mice tissue at day 14 was higher compared to day 7 (Figure 2A, B) and UUO + MV

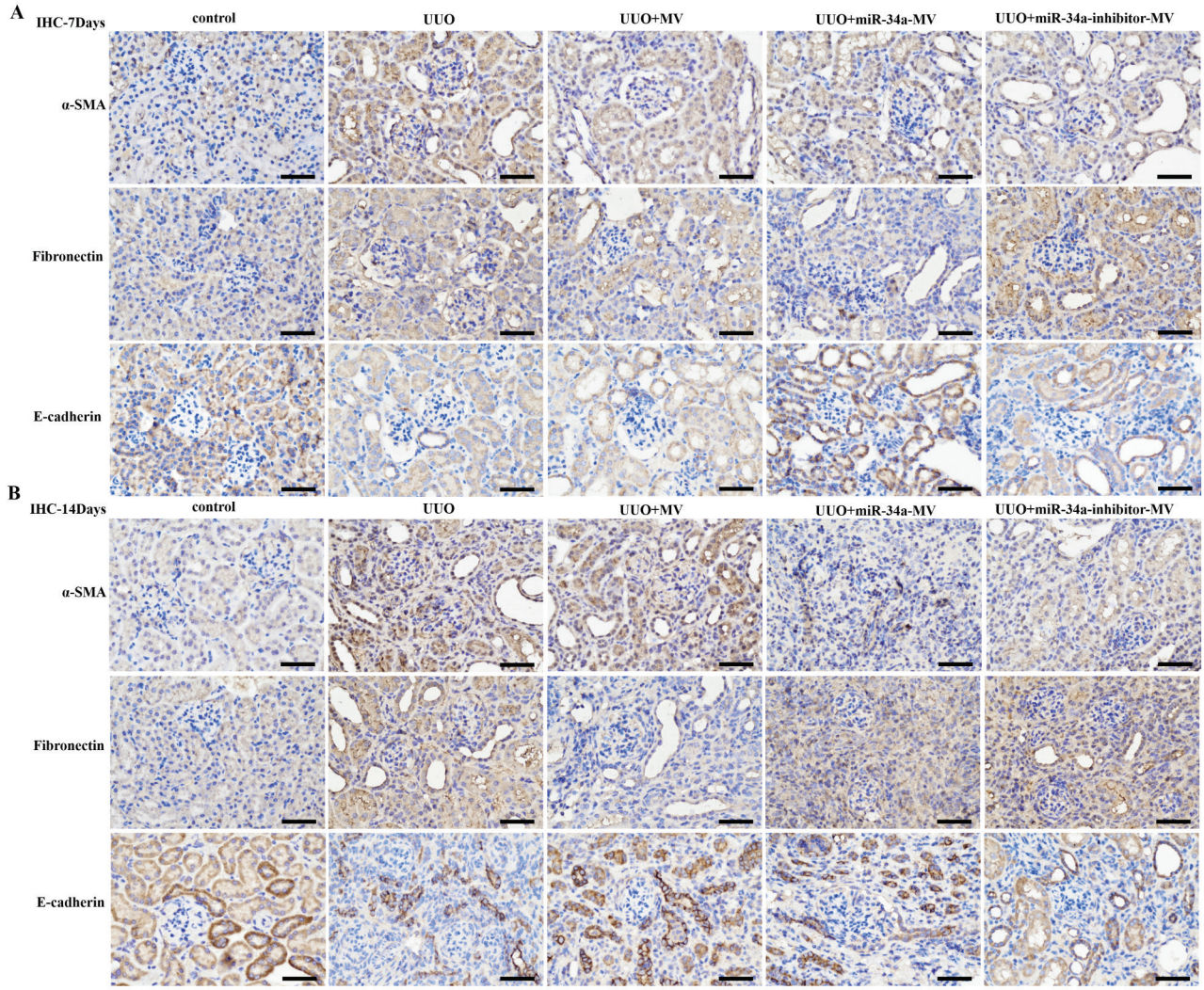

Figure 2. Effects of EMT on renal fibrosis *in vivo* [The expression levels of EMT-related proteins α-SMA, E-cadherin, and fibronectin in five groups (control, UUO, UUO + MV, UUO + miR-34a-MV and UUO + miR-34a-inhibitor-MV) were examined by IHC staining at 7 days after UUO (A) or 14 days after UUO (B) (magnification, ×400; scale bar, 50 μm)]

mice (Figure 2B). On the other hand, the UUO + miR-34a-inhibitor-MV group induced higher expression levels of α-SMA and fibronectin and a lower expression level of E-cadherin than those of the UUO + miR-34a-MV group. Broadly speaking, these results suggest that overexpression of miR-34a suppressed EMT during fibrosis, suggesting miR-34a-MV could suppress the progression of renal fibrosis presumably through regulation of EMT.

MiR-34a-MV Modulates Renal Injury Through Notch Pathway

Our previous studies indicated that Notch-1 and Jagged-1, as major molecules of the Notch signaling pathway, are targets of miR-34a.[15] In order to further the role of the Notch pathway in the modulation of miR-34a on renal injury, the expression of Notch1, Jagged1 and cytoplasmic adhesion protein Zo-1 were detected by IHC staining. As shown in Figure 3A, the expression of Notch1 in the UUO group on day 7 was basically the same as in the control group, while it was increased on day 14 (Figure 3B). Notably, the expression level of Notch1 in the UUO + miR-34a-inhibitor-MV group was similar to the UUO+MV group and UUO + miR-34a-MV group at day 7, while the expression levels were lower than the first two groups at day 14 (Figure 3A, B). Additionally, the expression range of Jagged1 was widespread in the UUO group compared with the

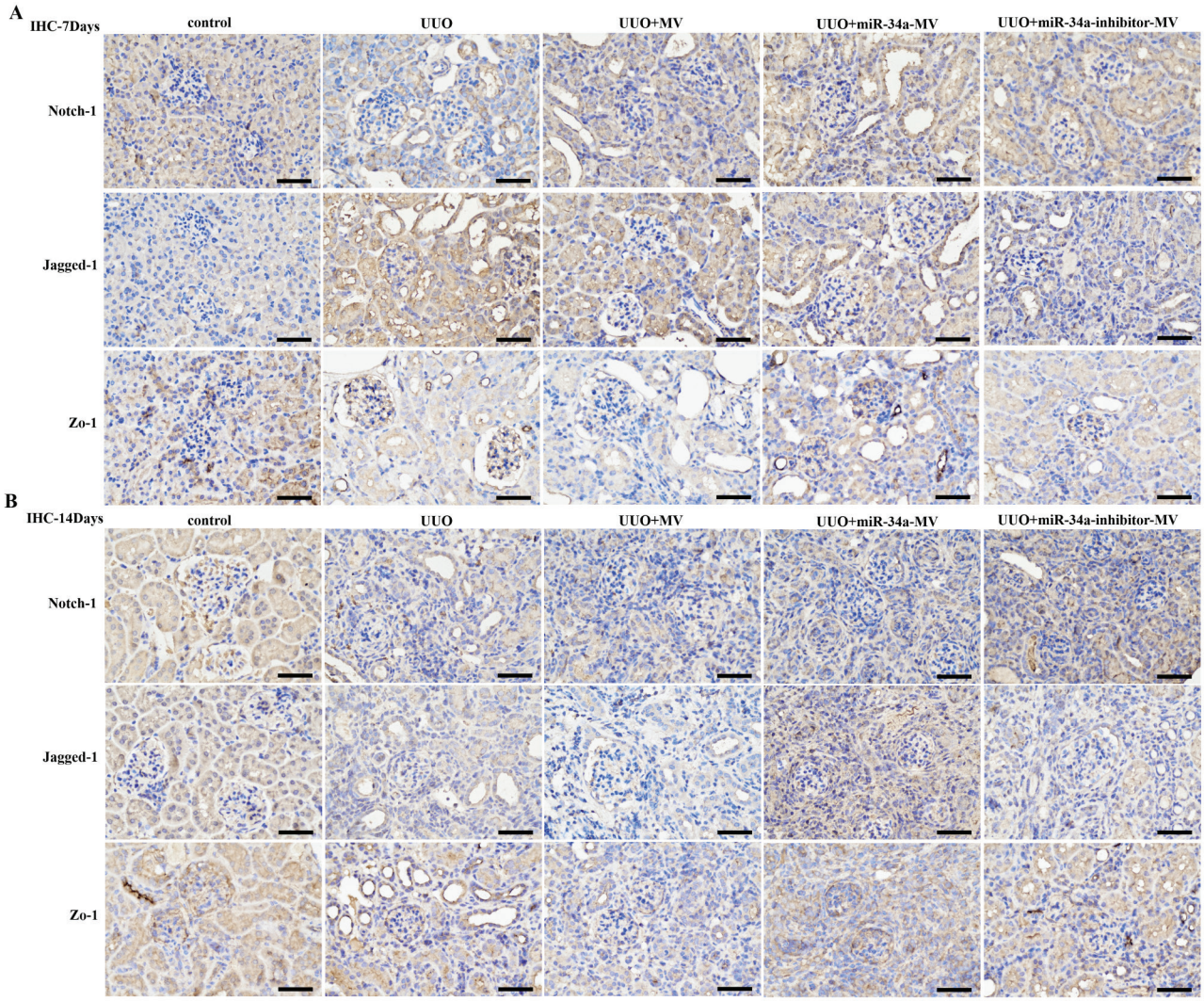

Figure 3. Effects of Notch pathway on the renal fibrosis *in vivo* [The expression levels of Notch1, Jagged1 and Zo-1 in five groups (control, UUO, UUO + MV, UUO + miR-34a-MV and UUO + miR-34a-inhibitor-MV) were examined by IHC staining on day 7 (A) and day 14 after UUO (B) (magnification, ×400; scale bar, 50 μm)]

control group (Figure 3A, B). The expression level of Jagged1 in the UUO + miR-34a-MV group was higher than that of the UUO+MV group while lower than that of the UUO group (Figure 3A, B). Moreover, the expression of Jagged1 in the UUO + miR-34a-inhibitor-MV group was significantly decreased compared with UUO+MV and UUO + miR-34a-MV groups and increased compared with the control group. Furthermore, the expression of Zo-1 in the control group was mainly expressed in renal tubular epithelial cells and glomerulus (Figure 3A, B). Compared with the control group, Zo-1 expression of the UUO mice was significantly decreased on day 7 and 14 after UUO (Figure 3A, B). Notably, the Zo-1 expression level of the glomerulus in the UUO + MV group showed no significant difference between the 7-day group and the 14-day group (Figure 3 A, B), while lower than that of the UUO group at day 7 (Figure 3A). Compared with the UUO + MV group, the expression of Zo-1 in the UUO + miR-34a-MV group was increased at 7 and 14 days after UUO (Figure 3C). In addition, the expression of Zo-1 in the UUO + miR-34a-inhibitor-MV group was similar to the UUO + miR-34a-MV group at 7 and 14 days after UUO (Figure 3C). Taken together, these results suggested that miR-34a-MV could suppress the progression of renal fibrosis, presumably through regulation of the Notch pathway.

DISCUSSION

In the current study, we obtained MVs from control MSCs or MSCs overexpressing miR-34a and compared the effects of two types on the UUO models. We found that a single administration of genetically modified MVs derived from MSCs could protect mice against kidney failure. Our results suggested that miR-34a exhibited anti-fibrotic effects on the UUO models of renal fibrosis *in vivo*. Furthermore, miR-34a-EVs reduced renal fibrosis through EMT and Notch signaling pathways.

MVs isolated from MSCs are natural carriers of small RNAs that transfer molecules between originator and recipient cells and could modify the phenotype and function of recipient cells.[16] Preclinical studies showed that MSC-EVs exhibit regenerative effects and can inhibit the progression of kidney injury.[17] Kholia *et al.* found that MSC-EVs significantly reduce tubular necrosis and interstitial fibrosis and participate in renal regeneration in a mouse model of aristolochic acid nephropathy.[17] Besides, our previous study indicated that MSC-MVs could alleviate mice renal injury induced by UUO and 5/6 subtotal nephrectomy. [18] In this study, we found that MSC-EVs contribute to the amelioration of renal fibrosis in the UUO mice. Moreover, MV is rich in miRNAs with biological effects. For example, highly elevated expression levels of miR34a have been found during mouse mesothelial peritoneal cell (MMC) fibrosis caused by high glucose, while suppression of miR34a expression partially inhibited MMC fibrosis.[19] Notably, Liu *et al.* indicated that aberrant upregulation of miR-34a has an important role in the progression of renal fibrosis.[20] This study further supports that miR-34a overexpression significantly enhances the anti-fibrotic effect of MSC-EV on renal fibrosis *in vivo*, while miR34a inhibitors have no significant effect on the repair of renal parenchyma.

EMT is considered a hallmark of tubulointerstitial renal fibrosis that contributes to the kidney population of myofibroblasts in mice.[21] A recent study disclosed that miR-30c overexpression suppresses high glucose-induced EMT.[22] Additionally, another study showed that miR-145 is involved in fibrosis by promoting the EMT process.[23] Takano *et al.* found that miR-34a possibly participates in lung fibrosis and drug-induced EMT in alveolar epithelial cells.[24] MiR-34a is also shown to strongly regulate EMT by directly targeting Notch1 and Jagged1.[8,25] In this study, we found that overexpression of miR-34a inhibited renal fibrosis in UUO mice via regulation of EMT-related proteins α-SMA, E-cadherin, and fibronectin, suggesting miR-34a overexpression could attenuate renal fibrosis by inhibiting EMT *in vivo*.

The notch signaling pathway participates in fibrosis in several tissues and organs,[26] including the kidney.[27] *Morizane et al.* suggested that miR-34c suppresses EMT and kidney fibrosis by suppressing the Notch/Jag1 pathway.[28] In addition, miR-34a may regulate the differentiation process and RCC cell proliferation by influencing the Notch signaling pathway.[29] Notably, we found significantly different expression of Notch1, Jagged1, and cytoplasmic adhesion protein Zo-1 between the UUO + MV group and UUO + miR-34a-MV group, suggesting miR-34a may regulate the Notch signaling pathway. All the above findings reveal that miR-34a may inhibit renal fibrosis through EMT and Notch

signaling pathways. Nevertheless, more studies are required to explore the effects and mechanisms of miR-34a in kidney diseases. Additionally, it has been reported that a single miRNA could simultaneously target multiple genes.[30] Therefore, further study is needed to identify whether overexpression of miR-34a inhibited the progression of renal fibrosis via targeting other genes. The expression of Jagged1 was upregulated in UUO-induced renal fibrosis mice, and TGF- β 1 treated renal cortical epithelial cells. Previous research results of our group also showed that TGF- β 1 activated the Jagged-1/Notch-1 signaling pathway in HK-2 cells.[14] Also, homo, mmu, and rno were predicted for the 34-a direct target of miR34 - (http://www.targetscan.org), which suggests that miR-34 is a widely conservative in different species. Similar to the direct inhibition of miR-34a,[31,32] we found that mir-34a-modified MSC-MV inhibited TGF- β 1-induced increases in jaggeed1 and Notch1 expression. In addition, Jagged1 overexpression weakened the antifibrotic effect of mir-34a-modified MSC-MV in HK-2 cells. These findings suggest that miR-34a can be successfully delivered to HK-2 cells, where it functions as a suppressor of the Jagged-1/Notch-1 signaling pathway, which is consistent with the results of our previous in vitro experiments.

CONCLUSION

miR-34a overexpression-MV in mice with UUO could partially suppress the progression of renal fibrosis, presumably through regulation of EMT and Notch pathway. These findings suggest that miR-34a may be a candidate molecular therapeutic target for treating renal fibrosis and may provide new insight into developing therapeutic strategies against renal fibrosis.

STUDY LIMITATIONS

Although our findings supported that miR-34a might further improve the repar effects of MSC, future experiments are needed to prove the functional roles of miR-34a in renal fibrosis. In addition, the pro-fibrotic Jagged1 and Notch1, miR-34a, also target pro-survival and anti-senescence molecules, such as klotho, which may inhibit the anti-fibrotic effect of MV to some extent. However, this inhibitory effect may be offset due to MV being rich in micro-RNA. MSC secretes MV in the process of growth. However, MV cannot exert its biological function *in vitro*. Therefore, we will try to improve the ability of MSC to secrete MV in the future, thus making the repair effect of cell-free and cell-derived more stable and efficient.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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