

Micro-vesicles from mesenchymal stem cells over-expressing miR-34a inhibit transforming growth factor- β 1-induced epithelial-mesenchymal transition in renal tubular epithelial cells *in vitro*

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Abstract

Background: The use of microRNAs in the therapy of kidney disease is hampered by the difficulties in their effective delivery. Micro-vesicles (MVs) are known as natural carriers of small RNAs. Our prior research has demonstrated that MVs isolated from mesenchymal stem cells (MSCs) are capable of attenuating kidney injuries induced by unilateral ureteral obstruction and 5/6 subtotal nephrectomy in mice. The present study aimed to evaluate the effects of miR-34a-5p (miR-34a)-modified MSC-MVs on transforming growth factor (TGF)- β 1-induced fibrosis and apoptosis *in vitro*.

Methods: Bone marrow MSCs were modified by lentiviruses over-expressing miR-34a, from which MVs were collected for the treatment of human Kidney-2 (HK-2) renal tubular cells exposed to TGF- β 1 (6 ng/mL). The survival of HK-2 cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and Annexin V-Light 650/propidium iodide (PI) assays. The expression levels of epithelial markers (tight junction protein 1 [TJP1] and E-cadherin) and mesenchymal markers (smooth muscle actin alpha (α -SMA) and fibronectin) in HK-2 cells were measured using Western blot analysis and an immunofluorescence assay. In addition, changes in Notch-1/Jagged-1 signaling were analyzed using Western blotting. Data were analyzed using a Student's *t* test or one-way analysis of variance.

Results: MiR-34a expression increased three-fold in MVs generated by miR-34a-modified MSCs compared with that expressed in control MVs ($P < 0.01$, $t = 16.55$). In HK-2 cells, TJP1 and E-cadherin levels decreased to 31% and 37% after treatment with TGF- β 1, respectively, and were restored to 62% and 70% by miR-34a-enriched MSC-MVs, respectively. The expression of α -SMA and fibronectin increased by 3.9- and 5.0-fold following TGF- β 1 treatment, and decreased to 2.0- and 1.7-fold after treatment of HK-2 cells with miR-34a-enriched MSC-MVs. The effects of miR-34a-enriched MSC-MVs on epithelial-mesenchymal transition (EMT) markers were stronger than control MSC-MVs. The effects of miR-34a-enriched MSC-MVs on these EMT markers were stronger than control MSC-MVs. Notch-1 receptor and Jagged-1 ligand, two major molecules of Notch signaling pathway, are predicted targets of miR-34a. It was further observed that elevation of Notch-1 and Jagged-1 induced by TGF- β 1 was inhibited by miR-34a-enriched MSC-MVs. In addition, TGF- β 1 exposure also induced apoptosis in HK-2 cells. Although miR-34a-modified MSC-MVs were able to inhibit TGF- β 1-triggered apoptosis in HK-2 cells, the effects were less significant than control MSC-MVs (control:TGF- β 1:miR-nc-MV:miR-34a-MV = 1.3:0.6:1.1:0.9 for MTT assay, 1.8%:23.3%:9.4%:17.4% for apoptosis assay). This phenomenon may be the result of the pro-apoptotic effects of miR-34a.

Conclusions: The present study demonstrated that miR-34a-over-expressing MSC-MVs inhibit EMT induced by pro-fibrotic TGF- β 1 in renal tubular epithelial cells, possibly through inhibition of the Jagged-1/Notch-1 pathway. Genetic modification of MSC-MVs with an anti-fibrotic molecule may represent a novel strategy for the treatment of renal injuries.

Keywords: Micro-vesicles; Mesenchymal stem cells; miR-34a; Epithelial-mesenchymal transition

Introduction

Kidney disease is among the greatest challenges within healthcare systems. Despite advances in care, mortality rates of patients with acute kidney injury^[1] or chronic kidney disease (CKD)^[2] have not reduced significantly in recent decades. Mesenchymal stem cells (MSCs) belong to a unique cell population that can self-renew, proliferate,

and differentiate into multiple cell lineages.^[3] Transplantation of MSCs is considered a potential strategy for slowing or preventing the progression of kidney disease.^[4]

It has been suggested that MSCs exert their therapeutic effects by releasing factors beneficial for organ repair. Pre-clinical studies have established that micro-vesicles (MVs) secreted by MSCs can promote functional improvement in injured kidneys.^[5] Our previous study also demonstrated

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that MVs derived from MSCs attenuate experimental renal injury in mice.^[6,7] Several previously published studies indicate that MVs isolated from genetically engineered MSCs exhibit better therapeutic efficiency in attenuating myocardial and cerebral injuries.^[8,9] Here, we sought to improve the therapeutic efficiency of MSC-MVs via a genetic modification-based strategy.

Fibrosis resulted from dysfunctional tissue repair processes is a life-threatening pathological condition,^[10] commonly occurring in CKD.^[11] During embryogenesis, renal epithelial cells arise through a process known as mesenchymal to epithelial transition (MET). In injured renal tissue, these cells switch back to a mesenchymal phenotype, a process called epithelial-mesenchymal transition (EMT) that contributes to renal fibrosis.^[12,13] MicroRNAs (miRNAs) are small non-coding single-stranded RNAs that regulate gene expression.^[14] MVs collected from MSCs modified using an anti-fibrotic miRNA exhibit therapeutic properties superior to unmodified MVs in CKD. In a study of Wang *et al*, extracellular vesicles were extracted from miR-let-7c-over-expressing MSCs and used to treat renal epithelial cells exposed to pro-fibrotic transforming growth factor beta 1 (TGF- β 1).^[15] TGF- β 1-induced EMT was substantially reduced by vesicles from miR-let-7c-modified MSCs.^[15] A previous report suggests that MV-based miRNA delivery may provide a clinically relevant gene-therapy strategy for the treatment of renal fibrosis.

Besides miR-let-7c, many other types of miRNA have also been identified as anti-fibrotic miRNAs, such as miR-221/222 and miR-217.^[16,17] The research of our group has focused on the properties of miR-34a-5p (hereafter miR-34a). This miRNA has been shown to be down-regulated in the renal tissues of patients with CKD compared with those of living donors, and to exhibit direct anti-fibrotic effects on hypoxia-induced fibrosis in human Kidney-2 (HK-2) renal tubular cells.^[18] On the basis of this previous study, we hypothesize that MVs isolated from miR-34a-modified MSCs contain additional miR-34a and so have greater anti-fibrotic activity.

Therefore, over-expression of miR-34a was induced in MSCs derived from bone marrow via a lentivirus expression system. MVs were then collected from the culture media of MSCs to treat HK-2 cells exposed to TGF- β 1. We found that miR-34a-enriched MSC-MVs promoted MET in HK-2 cells, the efficiency of which was stronger than that of control MVs.

Methods

Isolation and culture of bone marrow MSCs

MSCs were isolated and cultured in accordance with a protocol published in *Nature*.^[19] Briefly, to isolate bone marrow, male C57BL/6J mice (6 weeks old) were sacrificed by cervical dislocation to harvest their femurs and tibias. The bones were placed in 75% ethanol for 5 min, after which muscle and connective tissue were removed, and then the bones were washed in 1× phosphate-buffered saline (PBS; Double-Helix Biotech Co., Ltd, Shanghai, China) several times. The ends of the tibias and femurs were

cut to expose the marrow cavity, into which a needle attached to a 2-mL syringe containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) culture media (Gibco, Grand Island, NY, USA) was placed. The marrow plug was flushed vigorously with DMEM/F12 culture media several times, and the bone marrow cells collected by centrifugation (300 × g, 10 min). The bone marrow cells were re-suspended in Ammonium-Chloride-Potassium (ACK) lysis buffer (Beyotime Biotech Co., Ltd, Shanghai, China) for 3 min, and then the cells were pelleted by centrifugation (300 × g, 3 min). The cells (2 × 10⁵ in 2-mL DMEM/F12 supplemented with 10% fetal bovine serum [FBS]) were seeded into six-well plates and incubated in a humidified chamber at 37°C and 5% CO₂. Non-adherent cells were removed by replacing the culture media. Thereafter, the culture medium was replaced with fresh culture medium every 8 h for the first 3 days then every 3 days for an additional 10 days (Passage 0, P0). The cells (P0) were passaged by incubating in 0.25% trypsin for 90 s which was then neutralized using complete media. Cells were sub-cultured to P7. Animal experiments were conducted in accordance with the *Guidelines for the Care and Use of Laboratory Animals*.

Flow cytometry

All antibodies used for flow cytometry were purchased from eBioscience™ (San Diego, CA, USA). MSCs (P3) were incubated with anti-CD34 polyclonal antibody and anti-CD105 monoclonal antibody, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated immunoglobulin G (IgG) (H+L). Cells were also incubated with anti-CD45-FITC and anti-CD11b-FITC monoclonal antibodies. The cells were then analyzed using a flow cytometer. Non-specific IgG-treated cells were used to set the threshold fluorescence.

Determination of multi-potential differentiation of MSCs

MSCs (P3) were incubated in osteogenic and adipogenic induction culture media for 3 weeks as described in our previous study.^[6] The cells were then fixed in 10% formalin for 20 min at room temperature, and their mineralization and lipid generation determined using Alizarin Red S and Oil Red O staining.

Lentiviral infection of MSCs and collection of MVs

Lentiviral particles (10⁸ U/mL; green fluorescent protein (GFP) over-expressing miR-34a (5'-UGGCAGUGU-CUUAGCUGGUUGU-3') or miR-nc (negative control) were purchased from WanleiBio (Shenyang, China), which were termed lenti-miR-34a and lenti-miR-nc, respectively. MVs were collected from MSCs (P3) 48 h post-viral infection. The culture media were collected from P3-P7 MSCs every 48 h to isolate the MVs, in accordance with methods described in a previous study.^[6] Briefly, the culture medium was centrifuged at 3000 × g for 10 min to collect cell and debris-free supernatants. After centrifugation at 100,000 × g at 4°C for 1 h (Beckman Coulter, Indianapolis, IN, USA, Optima L-90 K ultra-centrifuge), the supernatants were carefully discarded. The pellets were re-suspended in serum-free M199 medium (Invitrogen, Carlsbad, CA, USA) containing 25 mmol/L N-2-hydrox-

yethylpiperazine-*N*-2-ethanesulfonic acid (Sigma, Burlington, MA, USA), and then ultra-centrifuged for a second time, with the supernatants again discarded. Suspensions of MVs were then re-suspended in M199 medium, protein content quantified using a bicinchoninic acid assay, then stored at -80°C . Micrographs of MVs were acquired by transmission electron microscopy.

Reverse transcription and real-time polymerase chain reaction

MSCs were harvested, and their total RNA isolated with Trizol (Invitrogen), in accordance with the manufacturer's protocol. The RNA of the MVs was isolated using an exosome DNA-RNA Out kit (Catalog number: 180808; Tiandz, Beijing, China) in accordance with the supplier's protocol. To determine the expression levels of miR-34a, a specific primer for miR-34a (5'-GTTGGCTCTGGTGCAGGTCGAGGTATTCGCACCCAGAGCCAACACAACC-3') was used in the reverse transcription polymerase chain reaction (PCR) step. The expression levels of miR-34a were determined using semi-quantitative real-time PCR ($2^{-\Delta\Delta\text{CT}}$ method; forward primer: 5'-TGGCAGTGTCTTAGCTGGTTGT-3'; reverse primer: 5'-GTGCAGGGTCCGAGGTATTC-3'). U6, a house keeping gene, was used as a control reference.

Culture and experimental conditions of HK-2 cells

Proximal tubular epithelial HK-2 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in M199 medium (Invitrogen) supplemented with 10% Fetal bovine serum (FBS, Gibco, Burlington, Canada) and 2% penicillin/streptomycin (Hyclone Labs, Logan, UT, USA). For a number of experiments, HK-2 cells were stimulated with 6 ng/mL recombinant human TGF- β 1 (R&D, Minneapolis, MN, USA) for 48 h in the presence or absence of MSC-MVs (30 $\mu\text{g}/\text{mL}$ protein concentration).

A Jagged-1 (NM_000214) encoding fragment was inserted into eukaryotic over-expression plasmids (Jagged-1 vector), with an empty vector as a control. To mediate the over-expression of Jagged-1, a Jagged-1 vector was transfected into HK-2 cells using Lipofectamine 2000 (Invitrogen). After 24 h, HK-2 cells were treated with 6 ng/mL recombinant TGF- β 1 for 48 h with or without miR-34a-modified MSC-MVs.

Assessment of viability and apoptosis of HK-2 cells

Cell viability and apoptosis were measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and Annexin V-Light 650/PI kit, in accordance with standard protocols (WanleiBio).

Western blot analysis

Proteins (25 μg) isolated from HK-2 cells were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were treated with 10% fat-free milk in Tris-buffered saline prior to incubating with primary antibodies, including anti-E-cadherin (1:800; Bio-world, Visalia, CA, USA), anti-

α -SMA (1:2000; ProteinTech, Rosemont, IL, USA), anti-tight junction protein 1 (TJP1; 1:1000; CST, Danvers, MA, USA), anti-fibronectin (1:500; WanleiBio), anti-Notch1 antibody (1:500; WanleiBio), and anti-Jagged-1 antibody (1:500; WanleiBio). The membranes were then incubated with horseradish-peroxidase-conjugated secondary antibody (1:5000; Santa, Dallas, TX, USA). Protein signals were then visualized using an Enhanced chemiluminescence (ECL) kit, then normalized to β -actin.

Immunofluorescence assay

HK-2 cells were seeded onto glass slides, and then treated with MSC-MVs with or without recombinant TGF- β 1 for 48 h. The cells were then fixed in 4% paraformaldehyde for 15 min, washed with PBS three times, and then permeabilized with 0.1% Triton X-100. The slides were soaked in goat serum for 15 min and then incubated with anti-TJP1 (1:200; ProteinTech), anti-E-cadherin (1:200; ProteinTech), anti- α -SMA (1:200, ProteinTech), or anti-fibronectin (1:200; ProteinTech) at 4°C overnight. Excess primary antibody was removed by washing with PBS several times, after which each membrane was incubated with a corresponding Cy3-labeled goat anti-rabbit secondary antibody (1:200; Beyotime Biotech Co.). Cell nuclei were stained with 2-(4-Amidinophenyl)-6-indolecarbamide dihydrochloride (DAPI) (Beyotime Biotech Co.).

Statistical analysis

All data are presented as mean and standard deviations, and were analyzed using GraphPad Prism version 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data fitted a normal distribution and had equal variances. A Student's *t* test (two-tailed) was used to compare differences between two groups, while a one-way analysis of variance followed by a Tukey's multiple comparison test was used to compare values from three groups. The statistical significance level was defined as $P < 0.05$.

Results

MV extraction from miR-34a-modified MSCs

Isolated MSCs were positive for CD105 and negative for the hematopoietic surface markers CD11, CD45, and CD34 [Figure 1A]. Images of oil red and alizarin red staining confirmed the adipogenic and osteogenic potential of the MSCs *in vitro* [Figure 1B and 1C].

The over-expression of miR-34a in MSCs was mediated using a lentiviral vector. After 48 h, the miR-34a levels in non-infected MSCs and those infected with lenti-miR-34a or lenti-miR-nc were determined using a real-time PCR assay. The data indicated a three-fold elevation of miR-34a in lenti-miR-34a-infected MSCs (lenti-miR-nc *vs.* lenti-miR-34a: 0.9 ± 0.06 *vs.* 3.2 ± 0.25 , $P < 0.01$) [Figure 2A]. The diameters of isolated MVs were found by electron microscopy to be < 200 nm [Figure 2B]. Furthermore, as shown in Figure 2C, a greater quantity of miR-34a was packaged in the MVs from MSCs infected with lenti-miR-34a (lenti-miR-nc-MV *vs.* lenti-miR-34a-MV: 1.0 ± 0.03 *vs.* 3.1 ± 0.21 , $P < 0.01$).

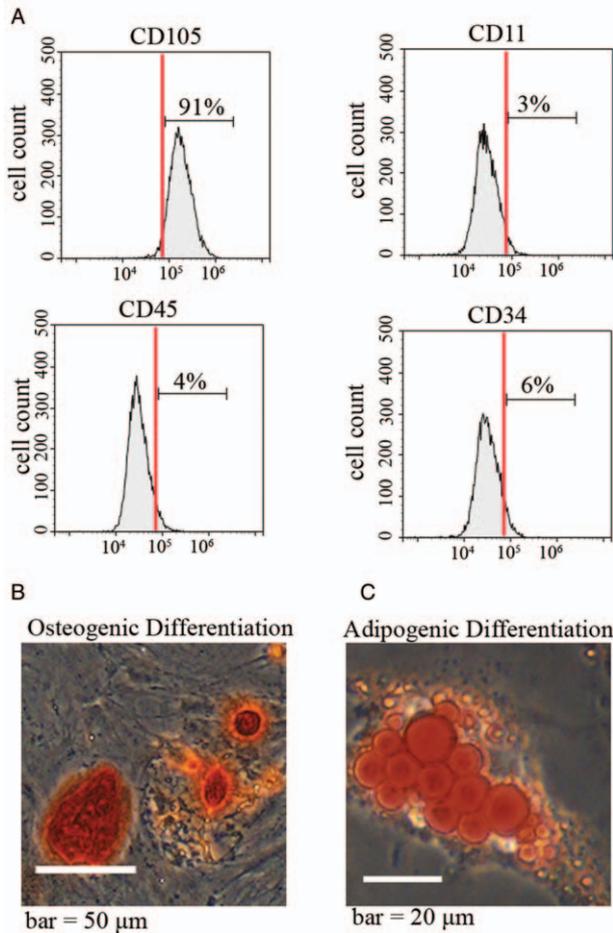


Figure 1: Identification of mesenchymal stem cells. The surface markers, CD105, CD34, CD45, and CD11b, were determined with flow cytometry assay (A). Representative images of alizarin red (B) and oil red (C) staining.

MiR-34a-modified MSC-MVs attenuated EMT in TGF-β1-stimulated HK-2 cells

HK-2 cells were treated with TGF-β1 to simulate an *in vivo* microenvironment. The expression levels of a group of EMT-associated molecules were measured by Western blot analysis and/or immunofluorescence (IF) assay [Figure 3B]. In HK-2 cells, levels of TJP1 and E-cadherin decreased to 31% and 37% of baseline levels after administration of TGF-β1, and were restored to 62% and 70% with miR-34a-enriched MSC-MVs. The expression of α-SMA and fibronectin increased 3.9- and 5.0-fold following TGF-β1 treatment, and decreased to 2.0- and 1.7-fold when HK-2 cells were treated with miR-34a-enriched MSC-MVs [Figure 3A]. TGF-β1 also decreased miR-34a expression to 29% in HK-2 cells [Supplementary Figure 1, <http://links.lww.com/CM9/A190>]. MiR-34a-modified MSC-MVs counteracted the effects of TGF-β1 significantly.

Furthermore, alteration in Notch signaling of HK-2 cells was determined by Western blot analysis. As shown in Figure 3C and 3D, up-regulation of Notch-1 (3.4-fold, $P < 0.01$ vs. control cells) and Jagged-1 (5.0-fold, $P < 0.01$ vs. control cells) was observed in response to TGF-β1 treatment, whereas they were down-regulated in cells co-treated with MSC-MVs. A reduction in these two factors

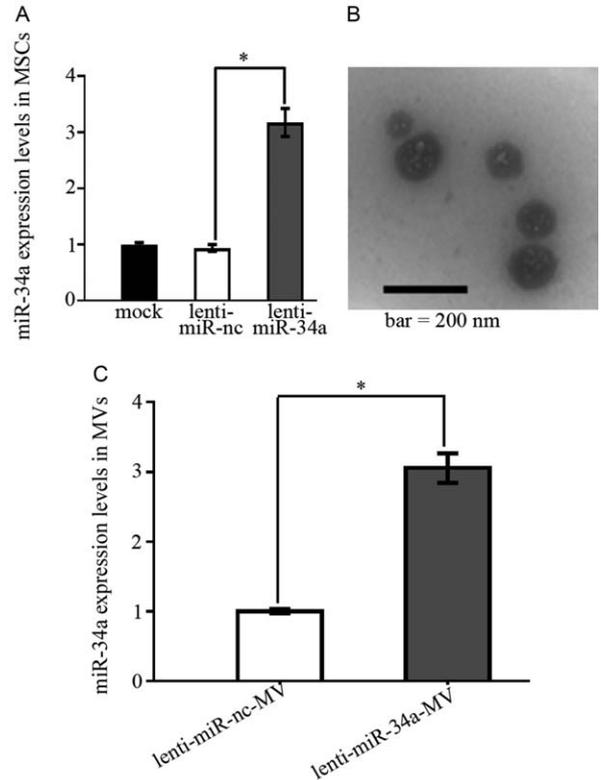


Figure 2: MV extraction from MSCs. MSCs were infected with lenti-miR-34a or lenti-nc, and the MVs in cell supernatant were collected. The miR-34a expression was first determined with real-time PCR, and shown as a relative value to the reference U6 according to $2^{-\Delta\Delta CT}$ methods (A). Representative transmission electron microscopy micrographs of MVs (B). MSC-MVs were collected, and the miR-34a expression levels were also determined with real-time PCR (C; $t = 16.55$; two-tailed). * indicated $P < 0.01$. Differences were considered significant when P -value was less than 0.05. MV: Micro-vesicles; MSC: Mesenchymal stem cells; PCR: Polymerase chain reaction.

was more significant in cells treated with miR-34a-modified MSC-MVs (1.3- and 1.2-fold; both $P < 0.05$ vs. control MV-treated cells). To restore the expression of Jagged-1, a Jagged-1 over-expression vector was transfected into HK-2 cells. Jagged-1 over-expression weakened the anti-fibrotic effects of miR-34a-modified MSC-MVs in HK-2 cells [Figure 4].

Effects of miR-34a-modified MSC-MVs on TGF-β1-induced apoptosis in HK-2 cells

HK-2 cells were treated with recombinant TGF-β1 for 48 h with or without MSC-MVs. As demonstrated by MTT analysis, both modified and unmodified MSC-MVs were able to enhance cell survival, but the protective effects of miR-34a-modified MSC-MVs were less significant than those that were unmodified (control:TGF-β1:miR-nc-MV:miR-34a-MV = 1.3:0.6:1.1:0.9) [Figure 5A]. Data from additional apoptosis analysis supported the MTT results (control:TGF-β1:miR-nc-MV:miR-34a-MV = 1.8%:23.3%:9.4%:17.4%) [Figure 5B].

Discussion

MVs can be secreted by nearly all cells in an organism, which regulate multiple physiological and pathophysio-

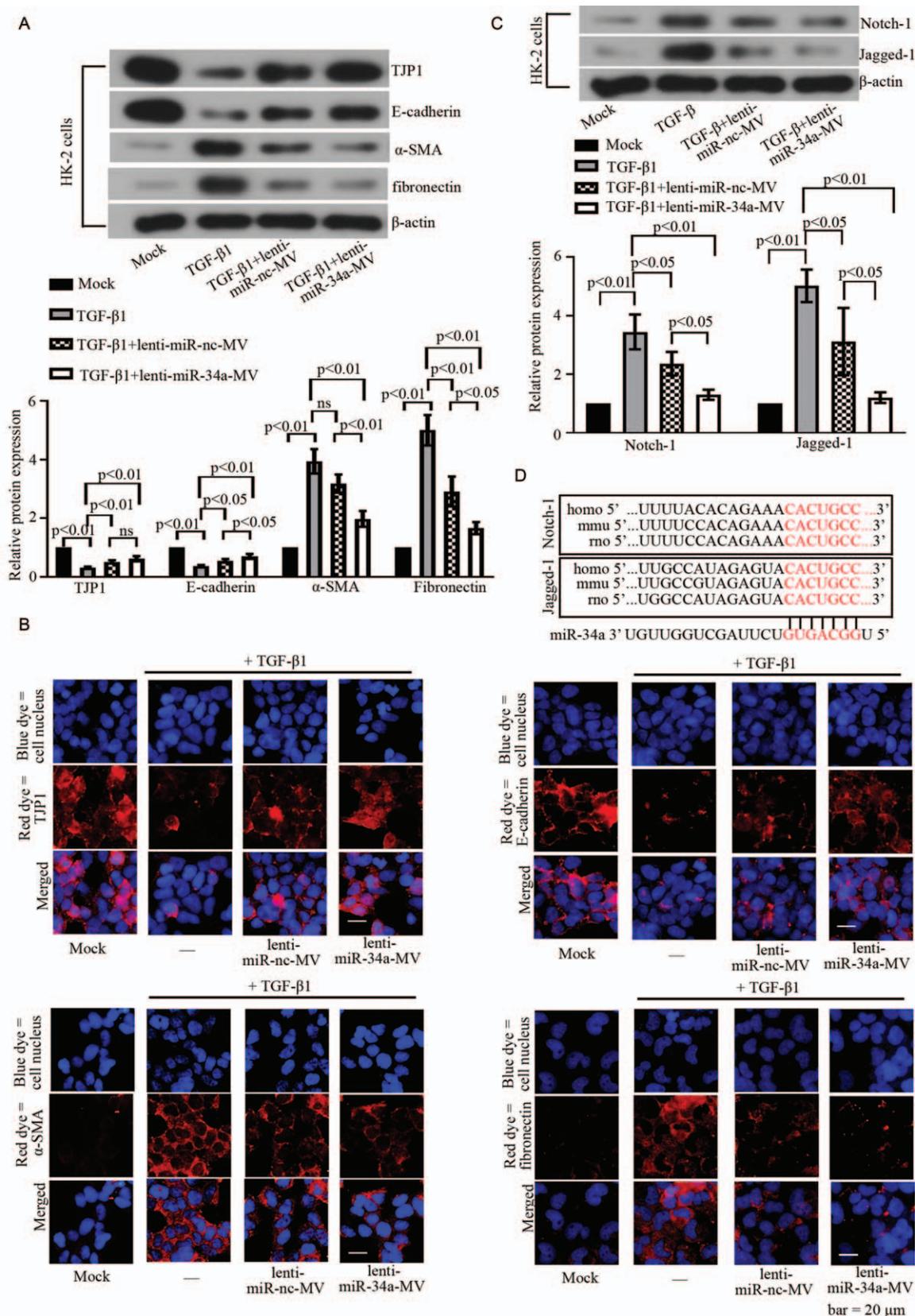


Figure 3: MiR-34a-modified MSC-MVs initiated MET in TGF-β1-stimulated HK-2 cells. HK-2 cells were treated with recombinant 6 ng/mL TGF-β1 for 48 h with or without MSC-MVs. Then, total protein fractions were isolated from these cells, and analyzed via western blot (A, C). The expression levels of TJP1, E-cadherin, α-SMA, and fibronectin were additionally determined with immunofluorescence assay (B). The predicted results of miR-34a-Notch-1/Jagged-1 were shown (D). Differences were considered significant when P-value was less than 0.05. HK-2: human Kidney-2; MSC: Mesenchymal stem cells; MV: Micro-vesicles; MET: Mesenchymal to epithelial transition; TGF: Transforming growth factor.

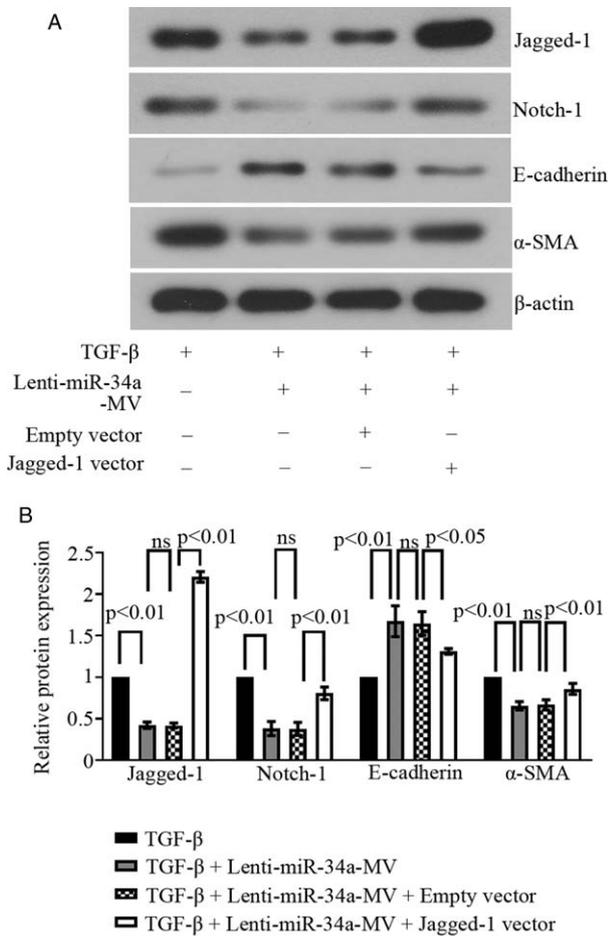


Figure 4: Jagged-1 over-expression weakened the anti-fibrotic effects of miR-34a-modified MSC-MVs in HK-2 cells. Empty control or Jagged-1 over-expression vector was transfected into HK-2 cells via Lipofectamine 2000. After 24 h, HK-2 cells were treated with recombinant 6 ng/mL TGF-β1 for 48 h with or without miR-34a-modified MSC-MVs. The expression levels of E-cadherin, α-SMA, Jagged-1, and Notch-1 were determined with western blot assay (A), and quantified (B). Differences were considered significant when *P*-value was less than 0.05. HK-2: Human Kidney-2; MSC: Mesenchymal stem cells; MV: Micro-vesicles; TGF: Transforming growth factor.

logic processes. The discovery of nano-sized MVs as natural carriers of small proteins and RNAs has raised a great deal of interest in the field of drug delivery.^[20] Our group has demonstrated that MSC-MVs can alleviate unilateral ureteral obstruction (UUO)- and 5/6 sub-total nephrectomy (Nx)-induced renal injury in mice, improving renal function.^[6,7] In the present study, we isolated MVs from control MSCs and those over-expressing anti-fibrotic miR-34a then compared the effects of the two types on TGF-β1-induced EMT and apoptosis in renal tubular epithelial cells *in vitro*. We noted that miR-34a over-expression slightly reduced the pro-survival effects of MSC-MVs, and significantly enhanced their inhibitory properties towards EMT in HK-2 cells.

Treatment of injured renal cells with MSC-MVs *in vitro* lays the foundation for establishing a cell-free clinical MSC therapy in kidney disease. The diameters of the isolated MVs were approximately 50 to 200 nm. By gradient separation, Collino *et al* established that the diameters of the extracellular vesicles peaked at 90 to 110 and 170 to 190 nm, consistent with our data. Such nano-sized MVs

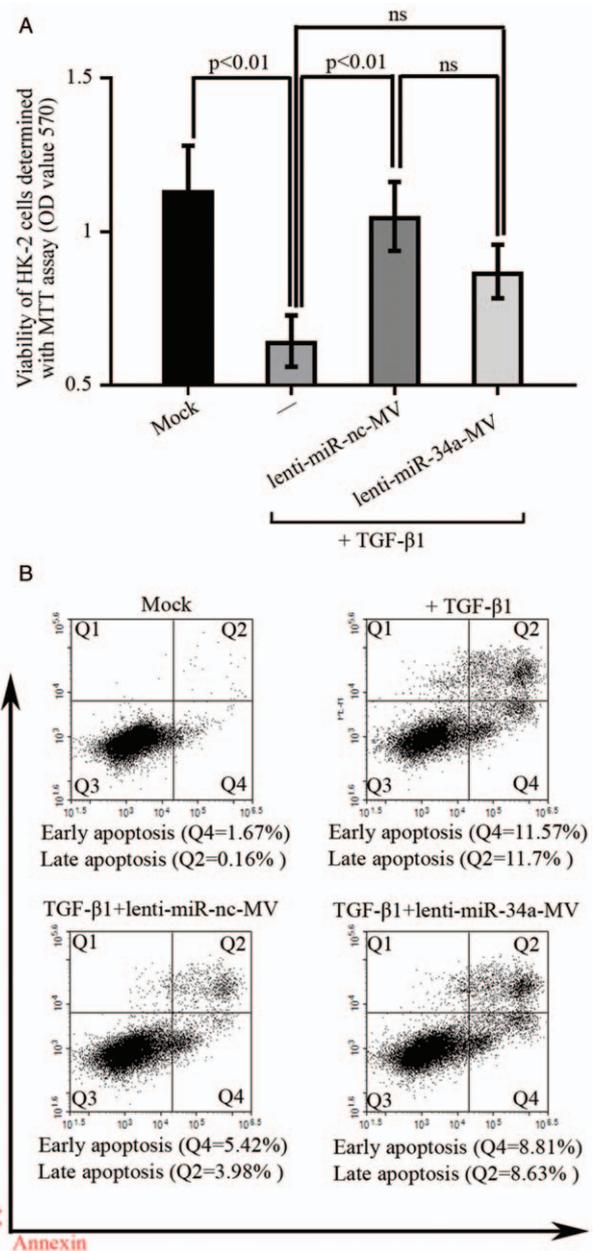


Figure 5: Effects of miR-34a-modified MSC-MVs on TGF-β1-induced apoptosis in HK-2 cells. HK-2 cells were treated with recombinant 6 ng/mL TGF-β1 for 48 h with or without MSC-MVs. Then, cells were incubated with MTT, and their viabilities were determined (A). Cell apoptosis was determined with an Annexin V/PI kit (B). Cells in quadrants (Q) 4 and 2 underwent early and late apoptosis, respectively. Differences were considered significant when *P*-value was less than 0.05. HK-2: Human Kidney-2; MSC: Mesenchymal stem cells; MV: Micro-vesicles; TGF: Transforming growth factor; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

can easily be internalized by renal cells.^[21] A greater quantity of miR-34a was packaged into MVs from MSCs over-expressing miR-34a.

TGF-β1 drives fibrosis in the kidney.^[22] In an injured kidney, TGF-β1 interacts with the TGF-β receptor 1 (TGFB1, also known as ALK5) which activates the canonical down-stream effector, SMAD2/3, and drives a program of pathologic renal extracellular matrix synthesis leading to advancing fibrosis.^[23,24] Abnormal EMT in

tubular epithelial cells is a key contributor to tubulointerstitial fibrosis, which can be induced by TGF- β 1.^[25] Herein, HK-2 cells were stimulated with TGF- β 1, and as expected, EMT was successfully triggered. Both our earlier^[7] and present data confirmed that MSC-MVs can suppress TGF- β 1-induced EMT.

MiR-34a-5p and miR-34c-5p (hereafter miR-34c) share the same seed sequence (5'-GGCAGUG-3') and are broadly conserved (online database: <http://mirbase.org/>). Given the function of miRNAs as gene suppressors by binding to the 3'-UTR of a gene containing the complementary sequence to the seed sequences, we assume that miR-34a and miR-34c exert biologic functions in a similar fashion. Unexpectedly, we found opposite effects of miR-34a and miR-34c toward EMT in renal tubular epithelial cells. In HK-2 cells, Liu *et al* found that miR-34a over-expression promoted EMT.^[26] Zhao *et al* showed that miR-30c over-expression suppressed high glucose-induced EMT^[27] and Du *et al* demonstrated that miR-34a mimics effectively inhibited hypoxia-induced EMT.^[18] Our present data demonstrated that miR-34a-enriched MVs inhibited TGF- β 1-induced EMT in HK-2 cells. Our observations, together with the findings from the latter two studies, demonstrate an anti-fibrotic role for miR-30 in renal tubular epithelial cells. Additionally, we also found that the basal expression of miR-34a in HK-2 cells was suppressed by TGF- β 1 stimulation. These findings suggest that miR-34a down-regulation occurs concurrently with TGF- β 1-initiated EMT.

In mammals, Notch ligands (Jagged-1/2 and Delta-1/-3/-4) bind to their receptors (Notch-1/-2/-3/-4) to initiate subsequent endocytosis of the Notch-ligand complex.^[28] Following endocytosis, Notch is cleaved by a gamma-secretase complex that releases the intra-cellular fragment.^[28] In the nucleus, the Notch intra-cellular fragment co-activates the Su(H)/RBPjk/CBf1 transcription factor that promotes the activation of Snail/Slug, the transcriptional repressors of E-cadherin, resulting in EMT.^[29,30] In addition to canonical SMAD2/3 signaling, TGF- β 1 also activates non-canonical pathways that drive the fibrotic genomic program, one of which Jagged/Notch are participants.^[31] The expression of Jagged-1 was up-regulated in the renal tissues of mice with UUO-induced renal fibrosis and in renal cortical epithelial cells treated with recombinant TGF- β 1.^[31] The results of the present study also showed that TGF- β 1 activated Jagged-1/Notch-1 signaling in HK-2 cells. Interestingly, both *homo*, *mmu*, and *rno* Jagged-1 and Notch-1 are predicted to be direct targets of miR-34a (<http://www.targetscan.org>), suggesting that miR-34a is widely conserved among different species. Similar to the direct inhibitory effect of miR-34a,^[18,32] we found that miR-34a-modified MSC-MVs also inhibit TGF- β 1-induced increase in the expression of Jagged-1 and Notch-1. Furthermore, Jagged-1 over-expression weakened the anti-fibrotic effects of miR-34a-modified MSC-MVs in HK-2 cells. These findings suggest that miR-34a can be successfully delivered into HK-2 cells, where it functions as a suppressor of the Jagged-1/Notch-1 signaling pathway.

Tubular degeneration during CKD is considered to occur as a result of EMT and apoptosis of tubular epithelial cells.^[33] In addition to EMT, the pro-fibrotic TGF- β 1 also induces a mild degree of apoptosis in cultured renal tubular

epithelial cells.^[33,34] Therefore, to evaluate the therapeutic effects of miR-34a-enriched MSC-MVs on tubular cells, we additionally analyzed the viability of HK-2 cell. Two previous studies demonstrated that miR-34a targets the pro-survival molecule Bcl-2 directly, and exacerbates renal cell apoptosis.^[35,36] We noted that the pro-survival effects of miR-34a-enriched MSC-MVs were less effective than those of control MSC-MVs. Such observations may result from the direct pro-apoptotic role of miR-34a. Of note, the gene expression patterns of MSCs that over-express miR-34a changed, which may have led to an altered secretome profile. MVs from miR-34a-overexpressing MSCs not only delivered miR-34a, but also messenger RNA, long non-coding RNAs, and small proteins to the recipient cells.^[5] This may explain why miR-34a is a pro-apoptotic molecule, but miR-34a modified MSC-MVs did not reduce TGF- β 1-induced apoptosis in HK-2 cells.

We are aware that miR-34a over-expression can promote the senescence of MSCs, probably by targeting Sirtuin 1.^[37,38] In addition to the pro-fibrotic Jagged-1 and Notch-1, miR-34a also targeted pro-survival and anti-senescence molecules, such as Bcl-2 and Sirtuin 1, as described earlier. Insights into the potential therapeutic effects of genetically modified MSC-MVs can be ascertained by further establishing the secretome profile of MSC-MVs that over-express miR-34a. In addition, validation of the anti-fibrotic effects of miR-34a-modified MSC-MVs in animals with experimental renal fibrosis is also urgently needed.

In conclusion, the present study demonstrates that MSC-MVs with miR-34a over-expression inhibit EMT induced by TGF- β 1 in cultured renal tubular epithelial cells. Normally elevated levels of Jagged-1 and Notch-1 were reduced after administration of miR-34a-modified MSC-MVs. These results suggest that genetic modification of MSC-MVs with anti-fibrotic molecules may represent a novel strategy for the treatment of renal fibrosis.

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Conflicts of interest

None.

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