Umbilical Cord-Derived Mesenchymal Stem Cell-Derived Exosomal MicroRNAs Suppress Myofibroblast Differentiation by Inhibiting the Transforming Growth Factor-β/SMAD2 Pathway During Wound Healing

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ABSTRACT

Excessive scar formation caused by myofibroblast aggregations is of great clinical importance during skin wound healing. Studies have shown that mesenchymal stem cells (MSCs) can promote skin regeneration, but whether MSCs contribute to scar formation remains undefined. We found that umbilical cord-derived MSCs (uMSCs) reduced scar formation and myofibroblast accumulation in a skin-defect mouse model. We found that these functions were mainly dependent on uMSC-derived exosomes (uMSC-Exos) and especially exosomal microRNAs. Through high-throughput RNA sequencing and functional analysis, we demonstrated that a group of uMSC-Exos enriched in specific microRNAs (miR-21, miR-23a, miR-125b, and miR-145) found to play key roles in suppressing myofibroblast formation by inhibiting the transforming growth factor-β2/SMAD2 pathway. Finally, using the strategy we established to block miRNAs inside the exosomes, we showed that these specific exosomal miRNAs were essential for the myofibroblast-suppressing and anti-scarring functions of uMSCs both in vitro and in vivo. Our study revealed a novel role of exosomal miRNAs in uMSC-mediated therapy, suggesting that the clinical application of uMSC-derived exosomes might represent a strategy to prevent scar formation during wound healing.

SIGNIFICANCE

Exosomes have been identified as a new type of major paracrine factor released by umbilical cord-derived mesenchymal stem cells (uMSCs). They have been reported to be an important mediator of cell-to-cell communication. However, it is still unclear precisely which molecule or group of molecules carried within MSC-derived exosomes can mediate myofibroblast functions, especially in the process of wound repair. The present study explored the functional roles of uMSC-exosomal microRNAs in the process of myofibroblast formation, which can cause excessive scarring. This is an unreported function of uMSC exosomes. Also, for the first time, the uMSC-exosomal microRNAs were examined by high-throughput sequencing, with a group of specific microRNAs (miR-21, miR-23a, miR-125b, and miR-145) found to play key roles in suppressing myofibroblast formation by inhibiting the transforming growth factor-β2/SMAD2 signaling pathway.

INTRODUCTION

Myofibroblasts appear during the contraction stage of wound healing. For optimal healing of a cutaneous wound, the processes occurring during the contraction stage can reduce the surface area and facilitate re-epithelialization. However, aberrations in the wound healing program or other pathological states can lead to the recruitment and maintenance of active myofibroblasts and result in fibrotic diseases [1, 2]. The transforming growth factor-β (TGF-β) family has been recognized as a pivotal regulator of cellular proliferation, differentiation, and metabolism in wound healing and tissue repair [3]. Inappropriately high levels of TGF-β activity at wound sites have been associated with excessive scarring and fibrosis. TGF-β binds and activates a membrane receptor serine/threonine kinase complex that phosphorylates various SMAD family proteins [4]. Phosphorylated...
SMAD2 (p-SMAD2) levels have been proposed as a positive prognostic marker in myofibroblast differentiation [2, 5]. In this regard, interfering with the activity of the TGF-β/SMAD2 signaling pathway might suppress myofibroblast differentiation and overaggregation to reduce excessive fibrosis or scar formation.

Mesenchymal stem cells (MSCs) have been reported to be suitable for treating tissue defects and excessive fibrosis because of their ability to migrate to the site of injury, their potential to differentiate into cells needed for tissue repair, and their relative ease of expansion in vitro. Nevertheless, with the recognition that only a small number of MSCs are retained in the injury site after MSC treatment, many investigators [6], including our group, have suggested that a strong paracrine capacity of MSCs might be the principal mechanism responsible for the clinical benefits of stem cell-based therapies [7].

Recently, exosomes have been identified as a new kind of major paracrine factor released by the outward budding of various types of cells, including MSCs, and important for various cellular functions. Exosomes are a type of membrane vesicle with diameters of 40–150 nm that are surrounded by a phospholipid bilayer [8]. They have been reported to be an important mediator of cell-to-cell communication. They protect the bioactive substances they carry from high temperatures, a variety of pH environments, repeated freezing and thawing, and other adverse conditions. Exosomes have been found to play key roles in normal physiology and in diseases such as myocardial fibrosis, renal fibrosis, and hepatic fibrosis [9–12]. However, it is still unclear precisely whether MSC-derived exosomes can mediate myofibroblast functions, especially in the process of wound repair.

Previous studies have implicated uMSC-derived exosomes (uMSC-Exos), which contain proteins, mRNAs, and microRNAs (miRNAs), to have functions in diverse biological processes [13–15]. We identified that uMSC-Exos-derived miRNAs can function mainly through suppressing the differentiation of fibroblasts to myofibroblasts. In the present study, through high-throughput sequencing, we identified a group of specific miRNAs carried by uMSC-Exos as key components contributing to the fibroblast/myofibroblast transition by inhibiting excess α-smooth muscle actin (α-SMA) and collagen deposition associated with activity of the TGF-β/SMAD2 signaling pathway. Thus, our findings suggest that applying the uMSC-derived exosomes could be a potential strategy to prevent scar formation or even tissue fibrosis during wound healing in patients.

**Materials and Methods**

**Mouse Model**

All procedures using animal subjects were performed under an institutionally approved protocol deemed in accordance with the guidelines of the Institute of Laboratory Animal Resources, the Second Military Medical University. Mice were obtained from the Shanghai Laboratory Animal Research Center (SIPPR-BK Laboratory Animal Corp., Shanghai, China, http://www.sippr.org.cn) and then housed in a specific pathogen-free environment with 12-hour photoperiods and ad libitum access to standard chow and water. Adult male ICR mice (Swiss-Hauschka mice) and nude mice (BALB/c-nu) were used for the present study. In brief, the mice were anesthetized using 10% chloral hydrate (0.3 ml/100 g). After hair was removed from the dorsal surface, 1.5 cm of skin, uniform in diameter, was removed from the back of mice to create full-thickness skin defects. The wounds were washed with Dulbecco’s modified Eagle’s medium (DMEM; HyClone Laboratories; Thermo Fisher Scientific Life Sciences, Waltham, MA, http://www.thermo.com) to remove excess blood. After another wash with 70% ethanol, the tissues were minced into small pieces (2–4 mm) and incubated with standard culture medium in dishes at 37°C. When the fibroblasts and uMSCs reached 80% confluence, they were trypsinized and prepared for subculture. Thereafter, the medium was changed every 3 days. Only uMSCs and fibroblasts in passages 2–5 were used. HEK293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, http://www.atcc.org) and maintained in DMEM (Thermo Fisher Scientific Life Sciences) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific Life Sciences) at 37°C with 5% CO2. Fibroblast culture medium consisted of high-glucose DMEM supplemented with 10% FBS and 100 µg/ml streptomycin and penicillin (Thermo Fisher Scientific Life Sciences). uMSC culture medium consisted of CMRL (Connaught Medical Research Laboratories developed medium, Thermo Fisher Scientific Life Sciences) with 10% FBS, 2% antibiotic-antimycotic solution, and 1% L-glutamine.

**Exosome Isolation**

Before isolation, the FBS used was depleted of host exosomes by ultracentrifugation at 120,000g for 3 hours at 4°C. Cell suspension medium was collected every 2 days. Collected culture suspension was transferred to conical tubes for centrifugation at 300g for 10 minutes at 4°C to pellet the cells. The supernatant was then filtered through a 0.22 µm filter and the flow was transferred to new tubes and then ultracentrifuged again at 120,000g for 70 minutes at 4°C in a SW32Ti rotor (Beckman Coulter, Inc., Pasadena, CA, http://www.beckman.com) to pellet the exosomes. The supernatant was immediately aspirated on completion of the first ultracentrifugation and then ultracentrifuged again as described previously. For maximal exosome retrieval, the exosome-enriched pellet was resuspended in a small volume (approximately 100 µl) of an appropriate buffer. This buffer depends on the downstream experiments planned after exosome isolation. The exosomes were measured for their protein content using the BCA protein assay kit.
The presence of the exosomes was subsequently confirmed by us-ing a NanoSight NS300 (Malvern Instruments, Ltd., Malvern, U.K., http://www.malvern.com) and detection of exosomal surface markers CD81 using Western blot.

**Fluorescence-Activated Cell Sorting and Cell Cycle Analysis**

Flow cytometry analysis were performed as follows. For cell cycle analysis, approximately $1 \times 10^5$ cells were fixed in 75% alcohol, rehydrated, and incubated with 1 ml of PI (Cell Signaling Technology, Danvers, MA, http://www.cellsignal.com). Approximately $1.5 \times 10^6$ cells were counted for each test. For quantification of SMA and p-SMAD2 using cytometry, $5 \times 10^4$ isolated cells from each sample were collected and fixed in 4% paraformaldehyde. The cells were washed, permeabilized, and blocked with goat serum before specific antibody incubation. Unconjugated anti-phosphate SMAD2 (at 1:25 dilutions; Abcam, Cambridge, UK, http://www.abcam.com) and anti-SMA (at 1:25 dilutions; Abcam) were incubated with the cells. After washing, Alexa Fluor...
Figure 2. uMSC-Exosomes suppress TGF-β-induced myofibroblast formation in vitro. (A): α-SMA expression in different TGF-β dosage-stimulated fibroblasts. Immunohistochemistry images of stimulated fibroblasts (left) and RNA level of SMA and collagen I (right). Scale bar = 20 μm. (B): Exosomes were added to fibroblasts labeled with PKH67. Nuclei were counterstained with Hoechst 33342. The cells were subject to fluorescence microscopy after 12 hours. Scale bar = 20 μm. (C): Fluorescent microscopy images illustrate the expression of SMA (green) followed by indicated treatment. Scale bar = 20 μm. (D): Flow cytometry comparing the percentage of SMA-negative cells of differently treated fibroblasts. Percentage of SMA-negative cells shown in upper left corner as standardized using the isotype control antibody-incubated cells (NC). (E): Expression levels of α-SMA and collagen I in different treatments using quantitative reverse transcription-polymerase chain (Figure legend continues on next page.)
488-conjugated anti-rabbit secondary antibody (at 1:2,000 dilutions; Abcam) were stained before detection. A rabbit isotype control antibody was used as the control (at 1:25 dilutions; Abcam). For each experiment, the isotype control was performed first to determine the negative region (shown in each histogram of the cytometry results), then the samples were run, and only the percentage of negative cells was labeled in the related figures.

**Immunofluorescence and Fluorescent In Situ Hybridization**

These assays were performed according to a previous report [16]. For the detection of protein, anti-phosphate SMAD2 (at 1:1,000 dilutions; Abcam) was used. For the detection of microRNAs, the probes were transcribed and labeled with digoxigenin-uridine triphosphate (UTP) (Roche, Basel, Switzerland, http://www.roche.com) using the mMESSAGE T7 Ultra In Vitro Transcription Kit (Ambion; Thermo Fisher Scientific Life Sciences) in accordance with the manufacturer’s directions.

**Data and Material Availability**

Small RNA sequencing data were deposited in the GEO database as GSE69909. For reviewer access, the following link can be used to view the raw data: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=svwvcuffpvev&acc=GSE69909. The processed total count data can also be found in supplemental data file 3. The microRNA expression data of uMSC and HEK293T cells were obtained from GEO DataSets GSE46989 [17] and GSE56862. The processed data files were downloaded to generate the results, which can be found at the same site. More detailed material and methods can be found in the supplemental data file. The primers used in the article are listed in supplemental data file 1.

**Statistical Analysis**

The data are expressed as the mean ± SD. Differences among groups were determined using analysis of variance two-factor for repeated measurements. Results were considered significant at \( p < .05 \).

**RESULTS**

**uMSC-Exos Suppress Myofibroblast Aggregation and Scar Formation in a Full-Thickness Skin Defect Mouse Model**

In order to clarify the functions of uMSCs in the regulation of scar formation during wound healing, we established a full-thickness skin defect nude mouse model and compared the effects of hydrogel-coated uMSCs with those of HEK-293T cells or PBS as controls to study the effects of uMSCs on wound healing. At the 14th day after treatment, we found that, on average, the edges of the cuts were smoother in the uMSC group than were those in the control groups. At the 25th day after treatment, the skin defects of the uMSC group were closed and exhibited smaller scars than those of the other groups (Fig. 1A, 1B). We evaluated the expression of \( \alpha \)-SMA by immunohistochemical (IHC) staining and found a strong reduction of \( \alpha \)-SMA expression in the uMSC-treated group compared with the HEK293T- and PBS-treated groups (Fig. 1C).

With the recognition that transplanted MSCs are not retained in organs for longer periods [18–20] (supplemental online Fig. 1A), we then suggested that their paracrine ability might play a key role in exerting their functions in promoting wound repair. Considering the important role of exosomes as a secreted factor, we therefore studied the functions of uMSC-Exos in wound repair. We collected and purified the exosomes from the culture supernatant of uMSCs and HEK293 cells and validated their existence using NanoSight, Laser Vertriebsgesellschaft (ALV-Laser Vertriebsgesellschaft mbH, Langen, Germany, http://www.alvgmbh.de), and Western blot analysis (Fig. 1D–1F; supplemental online Fig. 2A). Next, we tried to elucidate the functions of uMSC-Exos in vivo. We injected equal quantities of hydrogel-coated uMSC-Exos, HEK-293T cell-derived exosomes (HEK293-Exos), PBS, or UDF (the concentrated medium left after exosome removal) around the wounds. The results showed that at the 14th day after treatment, the uMSC-Exo group had the smallest mean wound area and much smoother edges of the cuts among all the groups. After 25 days, the defect of the uMSC-Exo group was closed and exhibited highly reduced scar formation compared with that of the control groups. IHC staining suggested that the expression of \( \alpha \)-SMA was also strongly reduced in the uMSC-Exo-treated group and that the healed tissue was more neatly arranged (Fig. 1G). These findings indicated that uMSC-Exos can promote wound healing and also reduce scarring and in situ myofibroblast formation.

**uMSC-Exos Suppress TGF-\( \beta \)-Induced Myofibroblast Formation In Vitro**

In order to validate the in vivo findings and unveil the underlying mechanism, we established a myofibroblast differentiation model by treating fibroblasts with recombinant TGF-\( \beta \) protein. The cell model was validated using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and IHC analysis, showing increased levels of \( \alpha \)-SMA and collagen I expression with increased dosage of TGF-\( \beta \) used, which indicated that the cell model is reliable (Fig. 2A). Next, we tried to validate the paracrine function of uMSCs using a Transwell-based myofibroblast differentiation assay. The results showed that an uMSC-conditioned cell culture environment did relieve the TGF-\( \beta \)-induced elevation of \( \alpha \)-SMA (supplemental online Fig. 3A–3C).

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reaction. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control, \(*\) \( p < .01 \). (F): Representative photographs of collagen gel contraction assay in the indicated treatment groups (left). The contracted gel diameter was measured 24 hours after treatment and is presented as the fold change of diameter compared with contraction inhibitor (1 M BDM) (right). (G): Cell cycle assay of differently treated fibroblasts showing representative images (left) and percentage of G2 population (right). \( **\) \( p < .01 \). (H): Scratch wound assay of differently treated fibroblasts showing representative images 48 hours after treatment (left) and the internal distance (right). Data are presented as mean ± SD; \( n = 3 \). \( **\) \( p < .01 \) compared with negative controls. Scale bar = 200 \( \mu \)m. Abbreviations: BDM, 2,3-butanedione monoxime; h, hours; NC, negative control; SMA, smooth muscle actin; TGF-\( \beta \), transforming growth factor-\( \beta \); UDF, umbilical cord-derived mesenchymal stem cell exosome-free supernatant; uMSC-Exos, umbilical cord-derived mesenchymal stem cell-derived exosomes.
Figure 3. The myofibroblast-suppressing ability of uMSC-Exos mainly depends on its RNA components. **(A):** Gel electrophoresis (left) showing the RNase digested exosomes were depleted of RNAs compared with proteinase and control treatment. Silver staining (right) showing that after proteinase treatment, exosomes were degraded thoroughly. **(B):** NanoSight analysis showing that proteinase treatment did not compromise exosome integrity (additional data shown in supplemental online Fig. 3). **(C):** Quantitative reverse transcription-polymerase chain reaction showing α-SMA and collagen I expression in the indicated enzyme digestion groups. Data are presented as mean ± SD; n = 3; **, p < .01. **(D):** Western blot analysis showing that RNase-treated exosomes cannot downregulate the protein level of SMA induced by TGF-β. **(E):** Collagen gel contraction assay assessing the contraction inhibitory effect of different enzyme-digested exosomes showing representative images (left) and measurement of gel diameter (right). Data are presented as mean ± SD; n = 3; **, p < .01. **(F):** Cell cycle analysis of different enzyme-treated exosomes showing representative images (left) and percentage of G2

(Figure legend continues on next page.)
Aiming to determine the effects of uMSC-Exos on myofibroblast formation, we first verified the integration ability of our purified exosomes by PHK67 assay, using a membrane labeling dye (PHK67) that integrates specifically into the membrane bilayer structure on fusion. After staining, washed and ultracentrifuged uMSC-Exos were added to the fibroblasts. Fluorescence microscopy analysis revealed that cells treated with stained uMSC-Exos showed prominent PHK67 fluorescence located in the cytoplasm (Fig. 2B), but the UFS group showed no obvious fluorescence, indicating that purified exosomes do have cellular transmission activity. Next, we added equal quantities of uMSC-Exos, HEK293T-Exos, UFS, and PBS to TGF-β-treated cells. Immunofluorescence analysis showed that uMSC-Exo treatment greatly inhibited the TGF-β-induced elevation of α-SMA, although no significant effects were found in the other groups (Fig. 2C). Flow cytometry analysis showed that by comparing the percentage of α-SMA-negative cells in each group, uMSC-Exo treatment resulted in significantly increased amount of α-SMA-negative cells under TGF-β stimulation (Fig. 2D). For qRT-PCR analysis, we also found that the mRNA expression of both α-SMA and collagen I was greatly decreased after uMSC-Exo treatment (Fig. 2E). Therefore, our findings suggest that uMSC-Exos could suppress TGF-β-induced fibroblast differentiation and possibly even reverse myofibroblast formation.

To further verify the influence of uMSC-Exos on the contraction ability of TGF-β-treated fibroblasts, we performed a three-dimensional collagen contraction assay. The cells were treated with uMSC-Exos, HEK293T-Exos, UFS, or a contraction inhibitor in the presence of TGF-β for 48 hours. The results showed that the uMSC-Exo group exhibited significantly reduced gel contraction compared with that of the HEK293T and TGF-β only groups, and the contraction inhibitor-treated group (2,3-butanedione monoxime) showed no contraction (Fig. 2F). Collectively, these data indicate that uMSC-Exos could suppress TGF-β-induced fibroblast differentiation and possibly even reverse myofibroblast formation.

The Myofibroblast-Suppressing Ability of uMSC-Exos Mainly Depends on the RNA Components

It was previously confirmed that exosomes contain numerous proteins and RNA components [6]. We next explored which molecules carried by the uMSC-Exos were key for suppressing myofibroblast formation. We administered equal amounts of purified uMSC-Exos, together with either proteinase K or RNase A supplemented with 0.05% Triton X-100, for different periods of time and tested the efficacy of the enzyme treatments by silver staining and gel electrophoresis, respectively. The results showed that the protein and RNA components in the uMSC-Exos were degraded thoroughly by the indicated enzyme treatments (Fig. 3A). In addition, the gel electrophoresis results showed that the RNA components carried by the uMSC-Exos were mainly small RNAs (<100 base pairs [bp]; Fig. 3A), consistent with other reports [14]. To determine the structural integrity of enzyme-treated exosomes, we also performed NanoSight analysis and showed that intact exosomes were observed after enzyme treatments (Fig. 3B; supplemental online Fig. 4A, 4B).

Next, we added untreated, proteinase-treated, or RNase-treated uMSC-Exos into the TGF-β-induced cell models and tested their effects on fibroblast differentiation and proliferation. The qRT-PCR analysis showed that the RNase-treated uMSC-Exos lost the ability to suppress myofibroblast formation, indicated by the significantly elevated expression of α-SMA in that group (Fig. 3C). Western blot analysis also confirmed this change (Fig. 3D). The results of the gel contraction assay also supported this conclusion, because the RNase-treated uMSC-Exos failed to inhibit gel contraction, but the untreated uMSC-Exo group did inhibit this process (Fig. 3E).

For cell cycle analysis using flow cytometry, the protease-treated uMSC-Exos, but not the RNase-treated uMSC-Exos, showed a much weakened ability to promote cell cycle progression (Fig. 3F), indicating that the proliferation-promoting ability was mainly dependent on the protein components in the uMSC-Exos. Taken together, these results indicate that the RNA components of uMSC-Exos play a key role in suppressing myofibroblast formation.

Identification of a Group of uMSC-Exo-Specific MicroRNAs by High-Throughput Sequencing

It has previously been shown that the main type of functional RNA component in exosome is microRNA, which can be efficiently transmitted to other cells and functions diversely through exosome integration [22]. Our gel electrophoresis analysis correspondingly showed that RNA molecules carried by uMSC-Exos were mostly small fragment RNAs of <100 bp. We therefore analyzed the global expression of microRNAs in uMSC-Exos via high-throughput sequencing approaches using HEK293T-Exos as a control. We also analyzed the microRNA expression patterns in uMSCs and HEK-293T cells using existing GEO DataSets (GSE46989 [17] and GSE56862, all from http://www.ncbi.nih.gov/geo/). We found that uMSC-Exos had a specific miRNA abundance signature that was quite different from that of HEK293T-Exos and even uMSCs (Fig. 4A, 4B). Among the most abundant 10 miRNAs in the uMSC-Exos, only miR-21 was also highly expressed in uMSCs (Fig. 4C, 4D). These findings indicated that most of the exosomal microRNAs might be actively secreted into exosomes by uMSCs, which also supports our hypothesis that these microRNAs have biological functions.

According to these results, using qRT-PCR, we evaluated miRNA and pre-miRNA expression levels in the fibroblasts after treating them with uMSC-Exos or HEK293T-Exos for 48 hours.
The results showed that the expression of miR-21, miR-23a, miR-125b, and miR-145 in the uMSC-Exo group was significantly increased compared with that in the HEK293T-Exos group; pre-miRNAs were not affected (Fig. 4E), confirming the ability of exosomes to transport its contained mature miRNAs into target cells.

To further reveal the possible roles of these miRNAs, we predicted their target genes and their functions using TargetScan (available at http://www.targetscan.org/) and Gene Ontology (GO) analysis. The analysis showed that the TGF-β/SMAD2 pathway was highly enriched in the GO analysis (Fig. 4F), and the several most abundant microRNAs, such as miR-21, miR-23a, miR-125b, and miR-145, were all found to be directly targeted to genes involved in the TGF-β/SMAD2 pathway, such as TGF-β2, TGF-βR2, and SMAD2 (Fig. 4G; supplemental data file 2). Because this pathway is a well-known regulator of myofibroblast formation [5, 23], we believe that these specific miRNAs could inhibit fibroblastic differentiation to myofibroblasts by suppressing TGF-β/SMAD2 pathway activities.

**uMSC-Exo-Specific MicroRNAs Target the TGF-β/SMAD2 Pathway to Suppress Myofibroblast Formation**

To validate the functions of these exosomal microRNAs, we synthesized agomirs to achieve stable overexpression. To investigate the functions of specific microRNAs in uMSC-Exos, we first
overexpressed candidate agomirs to test whether these candidate miRNAs could affect the expression of α-SMA. The qRT-PCR analysis showed that 4 of 7 candidate miRNAs, namely miR-21, miR-23a, miR-125b, and miR-145, significantly suppressed the expression of α-SMA (Fig. 5A). We therefore suggest that these four abundantly expressed exosomal miRNAs might contribute to the function of uMSC-Exos, and we tested them further.

According to the TargetScan prediction, these candidate miRNAs target TGF-β2, TGF-βR2, and SMAD2 differently (Fig. 5B). Some findings validating these functions have been previously reported [24, 25]. However, to address the relationship of the functions of these miRNAs to myofibroblast differentiation, we constructed firefly luciferase reporter vectors carrying the respective microRNA-binding sites of SMAD2, TGF-β2, and TGF-βR2 3′-untranslated regions (3′-UTRs). These vectors were transfected into fibroblasts, together with the indicated agomirs and a renilla luciferase vector. The renilla luciferase vector was used as an endogenous reference control to monitor the transfection efficiency. The results showed that the relative firefly luciferase activity was drastically reduced in the 3′-UTR overexpressing group compared with that in the control group, in which a scrambled agomir was overexpressed but was predicted not to bind any targets (Fig. 5C).

To evaluate the functions of the candidate miRNAs during myofibroblast differentiation, we first used Western blot analysis to assess α-SMA, SMAD2, and p-SMAD2 protein levels during TGF-β stimulation (Fig. 5D). The finding was further investigated by a SMAD2 reporter assay. The results showed that the candidate miRNAs could reduce the activity of the SMAD2 luciferase reporter during differentiation compared with the effect of TGF-β treatment alone (Fig. 5E). In addition, we investigated whether the individual overexpression of miR-21, miR-23a, miR-125b, or miR-145 could modulate the contraction ability during TGF-β-mediated myofibroblast differentiation. Gel contraction experiments showed that after treatment with specific microRNAs, the contracted gel was increased approximately 2- to 2.5-fold in area compared with that in the scrambled negative control group, as calculated using the mean diameter measured (Fig. 5F).

Together, these findings demonstrate that overexpressing these uMSC-Exo-specific miRNAs (miR-21, miR-23a, miR-125b, and miR-145) in uMSC-Exos could suppress the activation of TGF-β/SMAD2 pathway and thereby inhibit the differentiation of fibroblasts to myofibroblasts by targeting TGF-β2, TGF-βR2, and/or SMAD2.

Inhibition of uMSC-Exo-Specific miRNAs Abolished the Ability of uMSC-Exos to Suppress TGF-β/SMAD2 Activation In Vitro

To validate the critical roles of these exosomal miRNAs in the functions of uMSC-Exos in vitro, we developed a strategy to stably inhibit these specific miRNAs inside the uMSC-Exos. The uMSC-Exos were transfected with a mixture of antagomir RNAs (Antago-uMSC-Exos) that blocked miR-21, miR-23a, miR-125b, and miR-145 (Fig. 6A). In the control group, uMSC-Exos were transfected with a scrambled agomir as a negative control (NC-uMSC-Exos). All the transfected exosomes were ultracentrifuged again to exclude
Figure 5. uMSC-Exo-specific microRNAs target the TGF-β/SMAD2 pathway to suppress myofibroblast formation. (A): The effect of uMSC-Exo-specific miRNAs on TGF-β-stimulated SMA expression. The miRNAs were overexpressed using agomirs. Data are presented as mean ± SD; n = 3; **, p < .01. (B): A list of predicted binding sites of uMSC-Exo-specific miRNAs and their targets. (C): Luciferase reporter assay showing exosomal miR-21, miR-23a, miR-125b, and miR-145 regulates the target gene reporters’ luciferase activities. Data are presented as mean ± SD; n = 4; **, p < .01; ***, p < .001. (D): Western blot analysis showing the effect of exosomal miRs.
residual antagonirs outside the exosomes. After adding these modified exosomes into fibroblasts, qRT-PCR analysis showed that the detected levels of all four miRNAs were greatly decreased in Antago-uMSC-Exos compared with those in the negative control group (Fig. 6B), indicating the successful inhibition of these miRNAs with Antago-uMSC-Exos treatment.

After testing the efficacy of miRNA inhibition in the modified uMSC-Exos, we administered them to the cell model to test their effects on the differentiation of fibroblasts into myofibroblasts. Compared with the NC-uMSC-Exo group, Antago-uMSC-Exo treatment failed to suppress the expression of α-SMA and collagen I at both the RNA and the protein levels (Fig. 6C) during TGF-β stimulation. Flow cytometry analysis also showed that in the Antago-uMSC-Exo group, the percentage of p-SMAD2-negative cells was significantly decreased compared with those in the NC-uMSC-Exo group. Also the percentage of α-SMA-negative cells was significantly decreased in the Antago-uMSC-Exo group compared with the percentage in the NC-uMSC-Exo group (Fig. 6D).

To initially investigate whether the expression and activation levels of SMAD2, TGF-β2, and TGF-βR2 were directly regulated by the exosomal miRNAs during differentiation, we also transfected luciferase reporter vectors carrying the specific miRNA binding sites into fibroblasts and then treated the cells with wild-type uMSC-Exos, NC-uMSC-Exos, or Antago-uMSC-Exos. The dual-luciferase analysis showed that wild-type uMSC-Exos and NC-uMSC-Exos significantly suppressed the relative luciferase activities of the reporters and the Antago-uMSC-Exos failed to do so (Fig. 6E). We also performed a SMAD2 reporter analysis and found that Antago-uMSC-Exos failed to do so (Fig. 6F). We also performed a SMAD2 reporter analysis showing the luciferase level of SMAD2-binding sequence-contained luciferase reporters under the indicated treatment.

Taken together, these data revealed that the depletion of exosomal miR-21, miR-23a, miR-125b, and miR-145 greatly abolished the ability of uMSC-Exos to inhibit the TGF-β/SMAD2 pathway and indicated that these microRNAs might play key roles in the inhibition of myofibroblast formation in vitro.

uMSC-Exo-Specific miRNAs Play Essential Roles in Their Myofibroblast-Suppressing Functions In Vivo

Finally, we examined whether the uMSC-Exo-specific miRNAs contributed to TGF-β/SMAD2 suppression and myofibroblast formation in vivo. We studied the expression of p-SMAD2 (red signals) and miRNAs (green signals) in the normal and wounded skin of mice. Fluorescence in situ hybridization analysis showed little expression of miRNAs and p-SMAD2 in normal tissue. In contrast, the wounded skin exhibited only widespread p-SMAD2 expression. However, in the wounded skin treated with hydrogel-coated uMSC-Exos, p-SMAD2 was hardly detected but the miR-145, miR-125b, miR-21, and miR-23 expression levels had increased significantly (Fig. 7A). These results provided direct evidence that uMSC-Exo-specific miRNAs correlate inversely with the level of SMAD2 phosphorylation in the wound.

To further confirm the critical roles of these exosomal microRNAs in vivo, we also injected the formerly established Antago-uMSC-Exos coated with hydrogel. At 25 days after the initial injection, we found that the antagomir-modified uMSC-Exos had failed to produce any decrease in α-SMA expression or suppress SMAD2 phosphorylation. In contrast, the uMSC-Exo-treated group showed the opposite effects (Fig. 7B). These in vivo findings addressed the critical roles of miR-21, miR-23a, miR-125b, and miR-145 derived from uMSC-Exos in suppressing TGF-β/SMAD2 activation and myofibroblast differentiation during scar formation.

**DISCUSSION**

The repair of wounds usually results in scar formation. During scar development, myofibroblasts accumulate quickly and became the dominant cell phenotype. After scar development, the myofibroblasts either undergo apoptosis or revert into fibroblasts over time [26]. However, healing of severe tissue loss or conditions of inflammation can induce abnormal myofibroblast formation and can result in excessive scarring or even organ or tissue contraction. Approaches to control myofibroblast formation have been investigated to prevent excessive scarring. MSC-based therapies have been shown to induce a complex process of interactions among numerous types of cells, components of the extracellular matrix, and signaling molecules after injury. Such therapies have been reported to promote wound healing, maintain cutaneous homeostasis, and reduce scar formation [27, 28]. Most previous studies were aimed at deciphering the mechanism of the healing promoting effects of MSC-based therapies. In contrast, few attempts have been made to study the effects of MSCs on scar formation. The present report, for the first time, has substantiated that uMSCs suppress myofibroblast formation during wound healing, which can be, in part, via uMSC-Exos. We also sought to clearly distinguish the ability of exosomal miRNAs from exosomal protein. To a certain extent, our study provides new insights into the potential prevention of scar formation using umbilical cord-derived MSCs.

Among the different available sources of MSCs, the umbilical cord represents a cost-effective, productive, feasible, accepted, and universal source to isolate MSCs. Also, uMSCs are considered to be advantageous compared with bone marrow-derived MSCs and adipose-derived MSCs owing to their better potential to differentiate into other tissues and their higher capacity in proliferation [29–31]. However, the difference in function among these cells and especially the difference in exosomes produced is still unclear, which could be a goal of our future studies.
Figure 6. Inhibition of uMSC-Exo-specific miRNAs abolished the ability of uMSC-Exos to suppress TGF-β/SMAD2 activation in vitro. (A): Schematic showing the procedure of preparing Antago-uMSC-Exos. (B): Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) showing the miRNA level of fibroblasts treated with modified uMSC-Exos. The inhibitory efficiency of Antago-uMSC-Exos is shown compared with NC-uMSC-Exos. Equal amounts of phosphate-buffered saline (PBS) were used as exosome control (Mock). Data are presented as mean ± SD; n = 3; **, p < .01. (C): The effect of modified exosomes on SMA expression level assessed by both Western blot (upper) and qRT-PCR (lower). Equal amounts of PBS served as negative control. Data are presented as mean ± SD; n = 3; **, p < .01. (D): Flow cytometry analysis showed the
In the case of wound healing, uMSCs attenuate tissue damage, inhibit fibrotic remodeling and apoptosis, promote angiogenesis, stimulate endogenous stem cell recruitment and proliferation, and reduce immune responses. However, the prevalent hypothesis has shown that, when used in cell-based therapy, uMSCs mainly provide benefits for wound healing via paracrine mechanisms [18, 32]. Despite the early accumulation of systemically administered uMSCs at the site of injury, few uMSCs become permanently engrafted within the tissue. In our previous study, we used a murine model to implant green

(Figure legend continued from previous page.) percentage of p-SMAD2-negative and SMA-negative cells for the indicated groups; the bar region was standardized using the isotype antibody incubated cells as controls. (E): Reporter assay showing the effect of modified uMSC-Exo on TGFβ2, TGFβR2, and SMAD2 3’UTR reporters’ luciferase activities. Data are presented as mean ± SD; n = 4; **, p < .01. (F): SMAD2 reporter analysis showing the luciferase level of SMAD2-binding sequence-contained luciferase reporter under the modified uMSC-Exo treatment. Data are presented as mean ± SD; n = 4; **, p < .01. Abbreviations: Antago-uMSC-Exo, antagonir contained uMSC-Exo; Blank, no treatment; miR, microRNA; Mock, treatment using equal amounts of phosphate-buffered saline as exosome control; N, normal region; NC-uMSC-Exo, scramble antagonim contained uMSC-Exo; p-SMAD2, phosphorylated SMAD2; SMA, α-smooth muscle actin; uMSC-Exo, umbilical cord-derived mesenchymal stem cell-derived exosome; W, wound region.

(Figure 7). uMSC-Exo-specific miRNAs play essential roles in the myofibroblast-suppressing function of uMSC-Exos in vivo. (A): Fluorescence in situ hybridization assay showing the existence and abundance of miR-145, miR-125b, miR-21, miR-23a (green) and p-SMAD2 (red) in mouse skin wound model treated with uMSC-Exos or phosphate-buffered saline (PBS) control. The nucleus was counterstained with 4’,6-diamidino-2-phenylindole. Scale bars = 200 µm. (B): Representative images of immunohistochemistry showing SMA and p-SMAD2 expression in normal and wound skin tissue. Wounded mice were treated with either NC-uMSC-Exo or Antago-uMSC-Exo. Equal amounts of PBS served as negative control. Scale bars = 500 µm. Abbreviations: Antago-uMSC-Exo, antagonim contained uMSC-Exo; Blank, no treatment; miR, microRNA; Mock, treatment using equal amounts of phosphate-buffered saline as exosome control; N, normal region; NC-uMSC-Exo, scramble antagonim contained uMSC-Exo; p-SMAD2, phosphorylated SMAD2; SMA, α-smooth muscle actin; uMSC-Exo, umbilical cord-derived mesenchymal stem cell-derived exosome; W, wound region.

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miR-23a and miR-125b act as inhibitors of the TGF-
lar regulations. In addition, we also found that the functions of
RNAs through uMSC-derived exosomes might improve our
be diverse among different cells from different organs and tis-
therefore also suggest that the functions of microRNAs might
in the previous studies were mostly abnormal types of cells, such
and its functions might be alterable according to the state of cells
known that a miRNA could target different mRNAs at the
naling [41], consistent with our present findings. As it is well
exosome-mediated intercellular transfer, miR-21, miR-
23a, miR-125b, and miR-145 from uMSC-Exos inhibited TGF-
R2, and SMAD2 and thereby suppressed expression of
TGF-
23a, miR-125b, and miR-145 from uMSC-Exos inhibited TGF-
miR-21 and miR-145 have been previously reported to promote
wound healing and prevent scar formation. As an alternative to cell therapy, administering modified uMSC-Exos
transfected miRNAs to wounds might have a clinically benefi-
cial anti-scarring effect.

CONCLUSION

The present report sheds light on the specific microRNAs of
uMSC-Exos and clarified a new approach for using stem cell ther-
apy to promote wound healing and prevent scar formation.

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AUTHOR CONTRIBUTIONS

S.F.: conception and design, collection of data, data analysis
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study material or patients, data analysis and interpretation,
collection of data; Y. Zhang: provision of study material or pa-
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tients; M.W.: performance of uMSC-related experiments,
collection and/or assembly of data; K.J. and Y. Zhao: proofread-
ing and manuscript writing; Y.W.: collection and/or assembly
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H.L.: conception and design, data analysis and interpretation, financial
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X.X.: conception and design, administrative support, final ap-
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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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