MicroRNA-29b inhibits TGF-β1-induced fibrosis via regulation of the TGF-β1/Smad pathway in primary human endometrial stromal cells

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Abstract. Transforming growth factor (TGF)-β1 has a key role in the regulation of fibrosis and organ dysfunction. During the pathogenesis and progression of vital organ fibrosis, the microRNA (miR)-29 family is irregularly downregulated and exogenous supplementation of miR-29b has a strong anti-fibrotic capacity. However, whether TGF-β1 is able to provoke endometrial fibrosis, and the role of miR-29 in endometrial fibrosis remain unclear. In the present study, RT-qPCR, immunocytochemistry, western blot analysis, scanning electron microscopy, immunofluorescence staining, cell proliferation assay and flow cytometric analysis were employed. The results demonstrated that the expression levels of collagen, type 1, alpha 1 (COL1A1), α-smooth muscle actin (α-SMA) and phosphorylated (p)-Smad2/3 were increased, whereas miR-29b and maternally expressed gene 3 (MEG3) were decreased in primary endometrial stromal cells (ESCs) in response to TGF-β1 stimulation, in a time and dose-dependent manner. Furthermore, overexpression of miR-29b markedly reduced the expression levels of COL1A1 and α-SMA, and decreased the expression and nuclear accumulation of p-Smad2/3. In addition, ectopic overexpression of miR-29b increased the expression levels of MEG3, inhibited myofibroblast-like cell proliferation and induced apoptosis. These findings indicated that miR-29b may have a significant anti-fibrotic role, and may attenuate TGF-β1-induced fibrosis in ESCs. Therefore, exogenous miR-29b may serve as a potential therapeutic agent for the treatment of endometrial fibrosis.

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Introduction

Trauma to the basal layer can lead to the development of intrauterine scars, resulting in Asherman syndrome (1), which is associated with secondary infertility and miscarriage. The risk of Asherman syndrome has increased in recent years due to intrauterine operations becoming increasingly common. Endometrial fibrosis is the main pathological characteristic of Asherman syndrome, which consists of excessive deposition and reorganization of the extracellular matrix (ECM), in place of normal endometrium (2). At present, surgical removal of scar tissue and ancillary treatments, including physical barriers and hormone therapy, are recommended to treat endometrial fibrosis. However, the recurrence of the disease remains high, particularly in severe cases. Therefore, novel therapies that target the cellular mechanisms underlying the development of these pathologies are required. Epithelial and stromal cells are two main cell populations present in the endometrium. During homeostasis, a subset of stromal cells differentiates and incorporates stably into the epithelial compartment (3). Furthermore, stromal cells are critical regulators of endometrial function via the paracrine pathway, which regulates epithelial and leukocyte functions (4). Therefore, post-injury stromal cell dysfunction may have a key role in the development of endometrial fibrosis, which is considered a failure of tissue regeneration.

Transforming growth factor (TGF)-β1 is known to regulate various cellular responses, including ECM production, cell proliferation, apoptosis and differentiation (5). In the TGF-β1 pathway, Smad2 and Smad3 are receptor-regulated effector proteins (R-Smads), which are phosphorylated by the activated TGF-β type I receptor at a C-terminal SSXS motif, resulting in R-Smad nuclear accumulation (6). Notably, TGF-β1 is a critical fibrotic cytokine that has a significant role in organ fibrosis and dysfunction. In the context of fibrosis, TGF-β1 upregulates numerous fibrogenic genes, including collagenous ECM proteins and α-smooth muscle actin (α-SMA), via the Smad2/3 signaling pathway (7). It has also been demonstrated that the concentration of TGF-β1 is significantly higher in the intrauterine fluid of patients with endothelial fibrosis, as compared with in healthy individuals (8). However, whether the TGF-β1/Smad pathway is involved in endometrial fibrosis remains unclear.

The microRNA (miR)-29 family shares the same seed binding sequence, and is well-characterized by its ability to regulate ECM proteins, including collagens, elastin and fibrillins (9). The miR-29 family has been shown to be reduced in various types of tissue fibrosis, and their anti-fibrotic role has been demonstrated in the heart (10), kidney (11), liver (12), lung (13) and peritoneum (14). Long non-coding RNAs (lncRNA) have recently been reported to regulate gene expression. Among them, maternally expressed gene 3 (MEG3), which may be regulated by miR-29 (15), has been shown to induce caspase-3-dependent apoptosis in TGF-β1-treated LX-2 cells, thus inhibiting stellate cell activation and liver fibrosis progression (16). In our previous study, the expression levels of TGF-β1 were increased, whereas miR-29 was decreased in the clinical samples of patients with Asherman syndrome (17). However, the mechanisms underlying the interaction between miR-29 and TGF-β1, and the functional importance of miR-29 in endometrial fibrosis remain unexplored. Knowledge of these mechanisms is indispensable for the development of novel strategies for the prevention and treatment of endometrial fibrosis.

The present study aimed to investigate the TGF-β1-induced development of endometrial fibrosis using primary human endometrial stromal cells (ESCs). Furthermore, the preventive and therapeutic potential of miR-29b mimics, and the associated underlying mechanisms, were determined in the established cell model of endometrial fibrosis.

Materials and methods

Clinical subjects and ethics statement. Human endometrial tissue samples were obtained by curettage from six patients who had undergone a hysterectomy. The patients were between 30 and 45 years old, had a normal menstrual cycle, and underwent hysterectomy for conditions other than endometrial disease. Patients with subserous or intramural leiomyoma, or cervical intraepithelial neoplasia, who were estimated to be in the mid or late proliferative phase of the menstrual cycle were selected for the present study. The present study was approved by the Ethics Committee of Zhujiang Hospital affiliated to Southern Medical University (Guangzhou, China).

Primary ESC isolation, purification and culture. The protocol for stromal cell isolation was similar to that described in a previous study (18). Full-thickness endometrial tissue samples were collected under aseptic conditions and placed immediately in phosphate-buffered saline (PBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing streptomycin (100 mg/ml; Gibco; Thermo Fisher Scientific, Inc.). The samples were transported to the laboratory within 30 min. After several washes with PBS, the tissue samples were minced into 0.5-1 mm^3 pieces, and were incubated in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 0.2% collagenase I (Sigma-Aldrich China, Inc., Shanghai, China) for 60 min at 37˚C with agitation. Subsequently, the resulting suspension was filtered through sterile 100- and 40-μm nylon strainers (BD Biosciences, Shanghai, China) in turn, in order to remove undigested material and epithelial cells. Following centrifugation at 150 x g for 5 min at room temperature, the supernatant was discarded and the ESCs were resuspended in DMEM/F12 supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), plated into 25 cm^2 flasks, and incubated at 37˚C in an atmosphere containing 5% CO_2. After 24 h, the medium was replaced and the unattached cells were removed. The remaining attached stromal cells were supplemented with fresh culture medium every three days until they reached confluence. The 3rd-6th passages were used for subsequent experiments.

Treatment with TGF-β1. ESCs were seeded in 6-well plates (or on sterile cover glass) at a density of $2x10^5$ cells/well in order to investigate the function of TGF-β1 in primary ESCs. The cells were initially incubated with TGF-β1 (1.5 or 10 ng/ml) for 48 h and the vehicle (10 mM citric acid; Peprotech, Inc., Rocky Hill, NJ, USA) was used as a negative control. In addition, the ESCs were incubated with 10 ng/ml TGF-β1 for 12, 24, 48 or 72 h. Cells in the 6-well plates were harvested for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting, and the cover glass of cells were used for scanning electron microscopy.

Transfection. The cultured ESCs were seeded into 96-well plates ($3x10^3$ cells/well) for cell proliferation analysis, into 6-well plates ($2x10^5$ cells/well) for cell cycle distribution and apoptosis analyses, RT-qPCR and western blotting, or into 30 mm dishes ($2x10^5$ cells/dish) for immunofluorescence staining. For the preventive treatment, the cells were incubated overnight to a density of 30-50%, and were then incubated for a further 24 h in serum-free culture media, in order to synchronize the cell cycle. Subsequently, miR-29b mimics and a scrambled miRNA control designed by Guangzhou RiboBio Co., Ltd. (Guangzhou, China) were transfected into the cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. A total of 6 h post-transfection, the media were refreshed and ESCs were stimulated with TGF-β1 (10 ng/ml; Peprotech, Inc.) for 48 h for RT-qPCR analysis, and for 72 h for western blot analysis. For the therapeutic treatment, ESCs were treated with TGF-β1 (10 ng/ml) for 24 h ahead of miR-29b transfection as described. The media were refreshed and ESCs were continuously stimulated with TGF-β1 (10 ng/ml) for 24 h for RT-qPCR, and for 48 h for western blot analysis. For the therapeutic treatment, ESCs were initially treated with TGF-β1 (10 ng/ml) for 24 h, then transfected with miR-29 as described. Thereafter, the medium was changed with TGF-β1 (10 ng/ml) for another 24 h.

Immunocytochemistry. Immunocytochemical staining was performed, in order to determine the purity of the stromal cells following isolation. The cells were cultured on cover slides in 30 mm culture dishes (Corning China, Shanghai, China), and fixed with 4% paraformaldehyde. Cells were permeabilized with 0.5% Triton X-100 (Shanghai Solarbio Bioscience & Technology Co., Ltd., Shanghai, China) for 10 min, rinsed with PBS three times, incubated with 3% H_2O_2 for 15 min to remove endogenous peroxidase and incubated for 30 min with 5% BSA for antigen blocking (Beyotime Institute of Biotechnology). Primary antibodies targeting mouse monoclonal cytokeratin 18 (cat. no. sc-32329; 1:400

Table I. Quantitative polymerase chain reaction primer sequences.

Name	Sequence (5'-3')
COL1A1	F: 5'-GAGGGCCAAGACGAAGACATC-3'
	R: 5'-CAGATCACGTCATCGCACAAC-3'
α-SMA	F: 5'-GGCTCTGGGCTCTGTAAGG-3'
	R: 5'-CTCTTGCTCTGGGCTTCATC-3'
MEG3	F: 5'-GCTCTACTCCGTGGAAGCAC-3'
	R: 5'-CAAACCAGGAAGGAGACGAG-3'
GAPDH	F: 5'-CGGAGTCAACGGATTTGGTCGTAT-3'
	R: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'

F, forward; R, reverse; COL1A1, collagen, type 1, alpha 1; α-SMA, α-smooth muscle actin; MEG3, maternally expressed gene 3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse monoclonal vimentin (cat. no. sc-373717; 1:400 dilution; Santa Cruz Biotechnology, Inc.) were incubated with cells for 2 h at room temperature followed by incubation with peroxidase-conjugated goat anti-mouse IgG secondary antibody (cat. no. ZDR-5307; ZSGB-BIO, Beijing, China) for 40 min at room temperature, as previously described (19). Images were captured using a Leica DM3000 microscope (Leica Microsystems GmbH, Wetzlar, Germany).

RNA extraction and RT-qPCR. Total RNA was extracted from the cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. All of the mRNA, lncRNA and miRNA from each sample were reverse transcribed into cDNA using the PrimeScript RT Reagent kit (Takara Bio Inc., Otsu, Japan). The RNA was initially treated with gDNA Eraser according to the manufacturer's instructions. Thereafter, the RT reaction was conducted at 37˚C for 15 min, 85˚C for 5 sec and 4˚C prior to qPCR analysis. qPCR was performed using the SYBR *Premix Ex Taq* (Takara Bio, Inc.). The mRNA PCR primers (Invitrogen; Thermo Fisher Scientific, Inc.) used in the present study are summarized in Table I. For analysis of miR-29b expression, miRNA-specific stem-loop RT primers and qPCR primers provided in the miRNA quantification kit (Bulge-loop™ miRNA qRT-PCR Primer Sets, one RT primer and a pair of qPCR primers for each set) and specific for miR-29b were used and designed by Guangzhou RiboBio Co., Ltd. For mRNA and lncRNA, qPCR was conducted at 95˚C for 15 sec followed by 40 cycles at 95˚C for 5 sec and 60˚C for 60 sec, and a final extension step at 72˚C for 10 sec in a Roche LightCycler480 Real-Time PCR system (Roche Diagnostics, Basel, Switzerland). For miR-29 and U6, qPCR was conducted at 95˚C for 15 sec followed by 40 cycles at 95˚C for 5 sec, 57˚C for 20 sec and 72˚C for 10 sec. The relative levels of the RNAs of interest were normalized with internal controls [U6 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH)], and gene expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (20).

Western blot analysis. Cells were scraped off the plates, centrifuged at 200 x g for 5 min at 4˚C and total protein was extracted using 50 μl radioimmunoprecipitation assay buffer (Wuhan Boster Biological Technology, Ltd., Wuhan, China) supplemented with 0.5 μl protease inhibitors (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, China) for 30 min. The lysates were collected by centrifugation at 13,523 x g for 20 min at 4˚C. Total protein concentrations were quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). Total protein from each sample (40 μg) was separated by 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline 1 h prior to incubation with the following primary antibodies: Rabbit polyclonal anti-collagen, type 1, alpha 1 (COL1A1; 1:1,000; cat. no. ab34710; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-α-SMA (1:1,000; cat. no. ab5694; Abcam), rabbit polyclonal anti-phosphorylated (p)-Smad2/3 (1:1,000; cat. no. sc-11769-R; Santa Cruz Biotechnology, Inc.) and rabbit polyclonal GAPDH (1:1,000 dilution; Santa Cruz Biotechnology, Inc.; cat. no. sc-25778) for 12 h at 4˚C. Subsequently, the blots were incubated with peroxidase-conjugated secondary antibody (1:5,000 dilution; cat. no. ZDR-5307; ZSGB-BIO, Beijing, China) for 1 h at room temperature, and the proteins were visualized using Chemiluminescent Horseradish Peroxidase Substrate (EMD Millipore), according to the manufacturer's protocol. Chemiluminescence measurements and semi-quantitative values were obtained using the ChemiDoc™ XRS+ Imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and Image Gauge V3.12 (Fujifilm, Tokyo, Japan). The densitometric analyses were repeated three times, and protein expression levels were quantified relative to the internal control GAPDH.

Scanning electron microscopy (SEM) of cell morphology. The cells were fixed with cold 2.5% glutaraldehyde in 0.1 M PBS (4˚C, 24 h), and were post-fixed in 1% osmium tetroxide with 0.1% potassium ferricyanide. The samples were dehydrated through a graded series of ethanol concentrations, and embedded in hexamethyl-disilazane, followed by air-drying. The samples were gold-coated prior to examination under a KYKY-EM3200 scanning electron microscope (Kyky Technology Development Co., Ltd., Beijing, China) at an accelerating voltage of 10 keV.

Immunofluorescence staining. ESCs were fixed and permeabilized in 0.5% Triton X-100/PBS. Following treatment with blocking buffer (BIOSS, Beijing, China) supplemented with 5% goat serum for 30 min, the cells were incubated with the specific primary antibodies against p-Smad2/3 (1:100; cat. no. sc-11769-R), α-SMA (1:100; cat. no. ab5694) and COL1A1 (1:100; cat. no. ab34710) for 1 h at room temperature, followed by incubation with a secondary antibody (Cy3 AffiniPure Goat Anti-Rabbit Immunoglobulin G; Earthox Life Sciences, Milbrae, CA, USA; cat. no. E031640-01) for 30 min in the dark. Finally, the cells were incubated with 4',6-diamidino-2-phenylindole (BestBio, Shanghai, China) for 15 min. Images were captured under an inverted fluorescence microscope (IX71; Olympus Corporation, Tokyo, Japan).

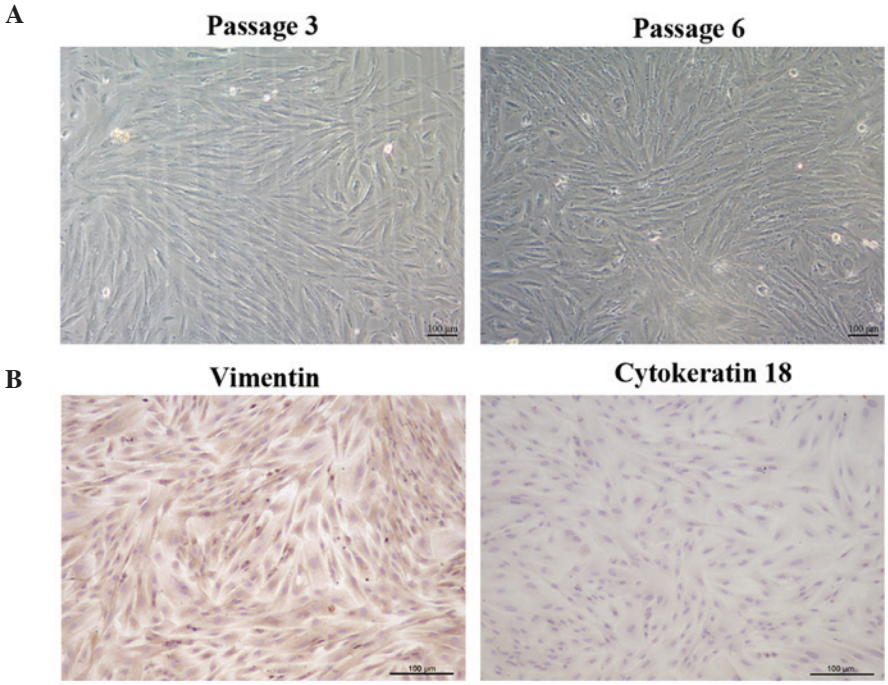

Figure 1. Observation and identification of endometrial stromal cells (ESCs) (magnification, x200). (A) 3rd and 6th generation ESCs were observed under an inverted phase contrast microscope. The cells exhibited a fiber-like morphology, and the majority of ESCs formed tightly parallel arrays and were able to grow to confluence. (B) Immunocytochemical staining demonstrated that the ESCs were positively stained for the stromal marker vimentin (brown), and negative for the epithelial cell marker cytokeratin 18. The purity of the stromal cells was >98%. Scale bar, 100 μm.

Cell proliferation assay. Cell counting kit-8 (CCK8) assay was performed to analyze cell proliferation. CCK-8 solution (10 μl; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well, and the cells were incubated for 2 h. Optical densities (OD) were recorded at 450 nm using a microplate reader (model 680; Bio-Rad Laboratories, Inc.), according to manufacturer's protocol. Cell proliferation was plotted as OD450 compared to untreated control cells.

Flow cytometric analysis. For cell cycle analysis, the cells were fixed with 70% ethanol overnight, treated with RNaseA (50 μg/ml; Sigma-Aldrich) in PBS for 20 min and incubated with propidium iodide (PI, 50 μg/ml; Sigma-Aldrich) for 30 min in the dark. Finally, the stained cells were analyzed by fluorescence-activated cell sorting using a flow cytometer (BD FACSCanto™ II; BD Biosciences, Franklin Lakes, NJ, USA), and the percentage of cells at G_0/G_1, S and G_2/M phases were quantified using ModFit software (ModFit LT version 3.2; BD Biosciences). For apoptosis analysis, the cells were stained with Annexin V and propidium iodide, using the Annexin V Apoptosis Detection kit (BestBio), according to the manufacturer's protocol. The rate of apoptosis was analyzed using a flow cytometer.

Statistical analysis. All the experiments were performed at least three times using independent cell cultures of samples from a different individual. Statistical analyses were performed using SPSS 19.0 software (SPSS IBM, Armonk, NY, USA). Data are presented as the mean ± standard error of the mean. One-way analysis of variance was used to determine the relationship between different groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Morphological observation and identification of ESCs. To identify and characterize fiber-like morphology of the ESCs, immunocytochemical and microscopic observations were performed. The ESCs were able to attach and grow 24 h after seeding. Subsequently, ESC cultures of the 3rd and 6th generation were observed under a microscope. As shown in Fig. 1A, the cells exhibited a fiber-like morphology, with the majority of the ESCs forming tightly parallel arrays. In addition, the cells were able to grow to confluence. Immunocytochemical staining demonstrated that the ESCs were positively stained for the stromal marker vimentin (brown), and were negative for the epithelial cell marker cytokeratin 18 (Fig. 1B). In addition, the purity of the stromal cells was detected to be >98%.

TGF-β1 increases the expression levels of COL1A1, α-SMA and p-Smad2/3 in the ESCs and promotes transdifferentiation of the cells. The well-known powerful pro-fibrotic effects of TGF-β1 make it a promising candidate for the induction of a cell model of endometrial fibrosis. In order to study the function of TGF-β1 in primary ESCs, the cells were initially incubated with TGF-β1 (1, 5 or 10 ng/ml) for 48 h, with vehicle (10 mM citric acid; Peprotech, Inc.) used as a negative control. In addition, the ESCs were incubated with TGF-β1 at 10 ng/ml for 12, 24, 48 or 72 h. As shown in Fig. 2A and B, COL1A1 and α-SMA expression were increased in response to TGF-β1, in a time- and dose-dependent manner. Furthermore, the addition of 1, 5 and 10 ng/ml TGF-β1 significantly increased the protein expression levels of p-Smad2/3 in ESCs after 24, 48 and 72 h of treatment. Notably, consistent with the phenotypic changes mentioned previously, the primary ESCs

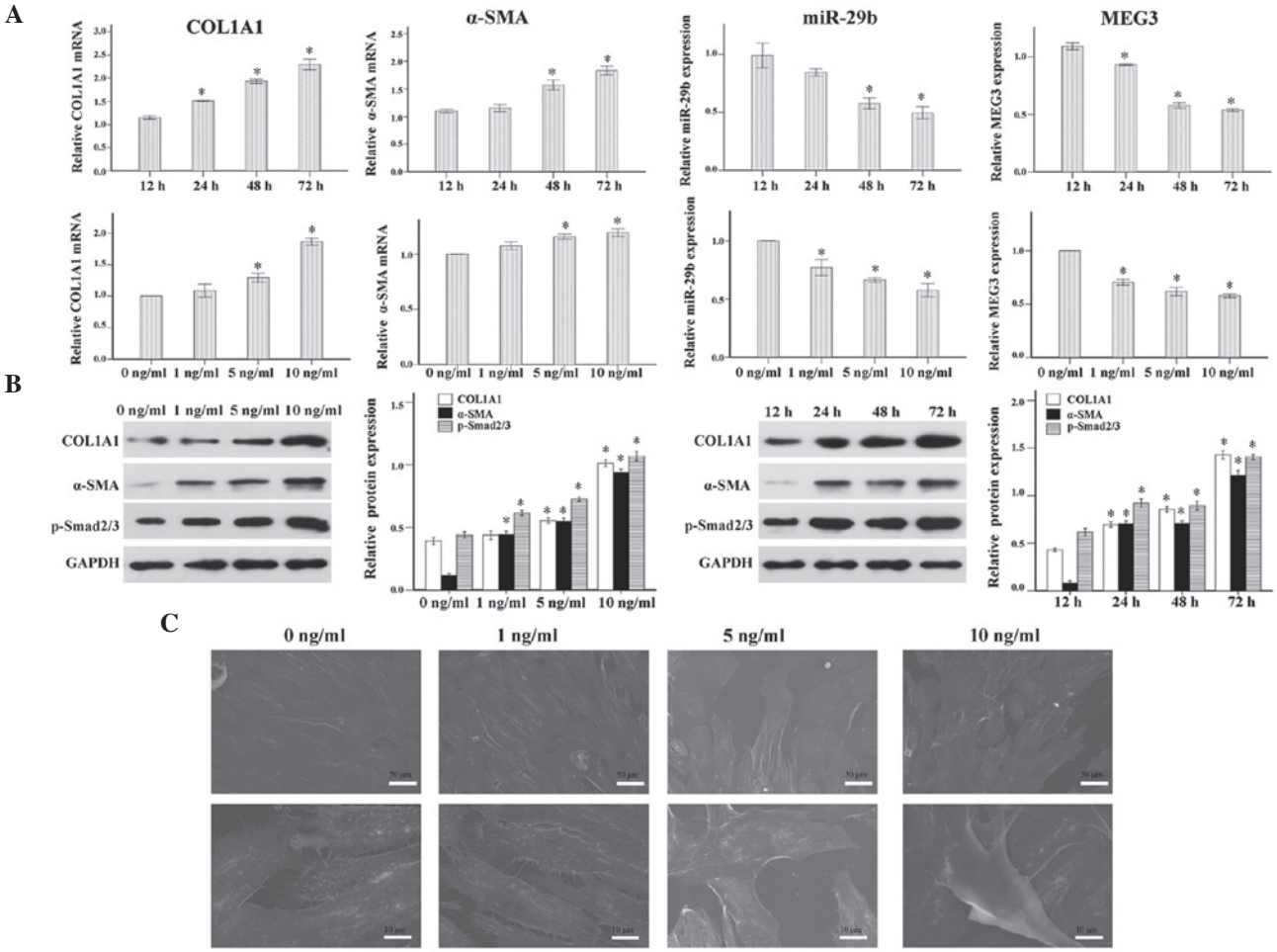

Figure 2. Pro-fibrotic effects of transforming growth factor (TGF)-β1 in endometrial stromal cells (ESCs). Primary ESCs were treated with TGF-β1 (0, 1, 5 or 10 ng/ml) for 48 h, or TGF-β1 (10 ng/ml) for the indicated time (12, 24, 48 or 72 h) in Dulbecco's modified Eagle's medium containing 1% fetal bovine serum. (A) Time and dose-dependent effects of TGF-β1 on the mRNA expression levels of collagen, type 1, alpha 1 (COL1A1), α-smooth muscle actin (α-SMA), microRNA (miR)-29b and maternally expressed gene 3 (MEG3), as analyzed by reverse transcription-quantitative polymerase chain reaction. (B) Time and dose-dependent effects of TGF-β1 on the protein expression levels of COL1A1, α-SMA and p-Smad2/3 as analyzed by western blotting. (C) Scanning electron microscopy images demonstrated the morphological changes of primary ESCs into spindle-shaped myofibroblast-like cells in response to continuous TGF-β1 stimulation (10 ng/ml) for 4 days. The results are expressed as relative expression against control expression without treatment. Data are presented as the mean ± standard error of the mean. *P<0.05 vs. the control group (12 h or 0 ng/ml). Scale bar, 10 or 50 μm.

underwent morphological changes to become spindle-shaped myofibroblast-like cells in response to continuous TGF-β1 stimulation for 4 days, as observed under an electronic microscope (Fig. 2C). These results indicate that TGF-β1 may promote myofibroblast transdifferentiation of ESCs, activate the TGF-β1/Smad signaling pathway and increase COL1A1 synthesis.

miR-29b and MEG3 are downregulated in TGF-β1-treated ESCs. To determine whether the TGF-β1/Smad pathway interacts with miR-29b in order to mediate endometrial fibrosis, the expression levels of miR-29b were detected following treatment with various doses of TGF-β1 for various durations. Treatment with TGF-β1 significantly reduced the expression levels of miR-29b in a time- and dose-dependent manner (Fig. 2A). Notably, MEG3 was downregulated in a similar manner following treatment of the cells with TGF-β1 (Fig. 2A).

Overexpression of miR-29b exerts anti-fibrotic effects in ESCs. Significantly decreased miR-29b expression was detected in TGF-β1-treated ESCs; therefore, the possible functional role of miR-29b in ESCs activation was determined. miR-29b mimics were transfected into ESCs before or after TGF-β1 treatment (10 ng/ml). In the ESCs that underwent preventive treatment, overexpression of miR-29b significantly suppressed transcription and protein synthesis of COL1A1 (Fig. 3A and B). Notably, α-SMA, an important factor of myofibroblast transdifferentiation, was decreased post-transfection of ESCs with miR-29b (Fig. 3A and B), although it is not predicted to be a direct target of miR-29b by several computational algorithms (data not shown). In the ESCs that underwent therapeutic treatment, overexpression of miR-29b exerted a significant anti-fibrotic effect on ESCs, via suppression of COL1A1 expression; however, its inhibitory effects were less marked, as compared with those induced by the preventive treatment (Fig. 3A and C). The decreased expression levels of COL1A1 and α-SMA were further confirmed by immunofluorescence staining (Fig. 4A).

miR-29b protects ESCs from fibrosis by suppressing TGF-β1/Smad signaling. To explore the possible

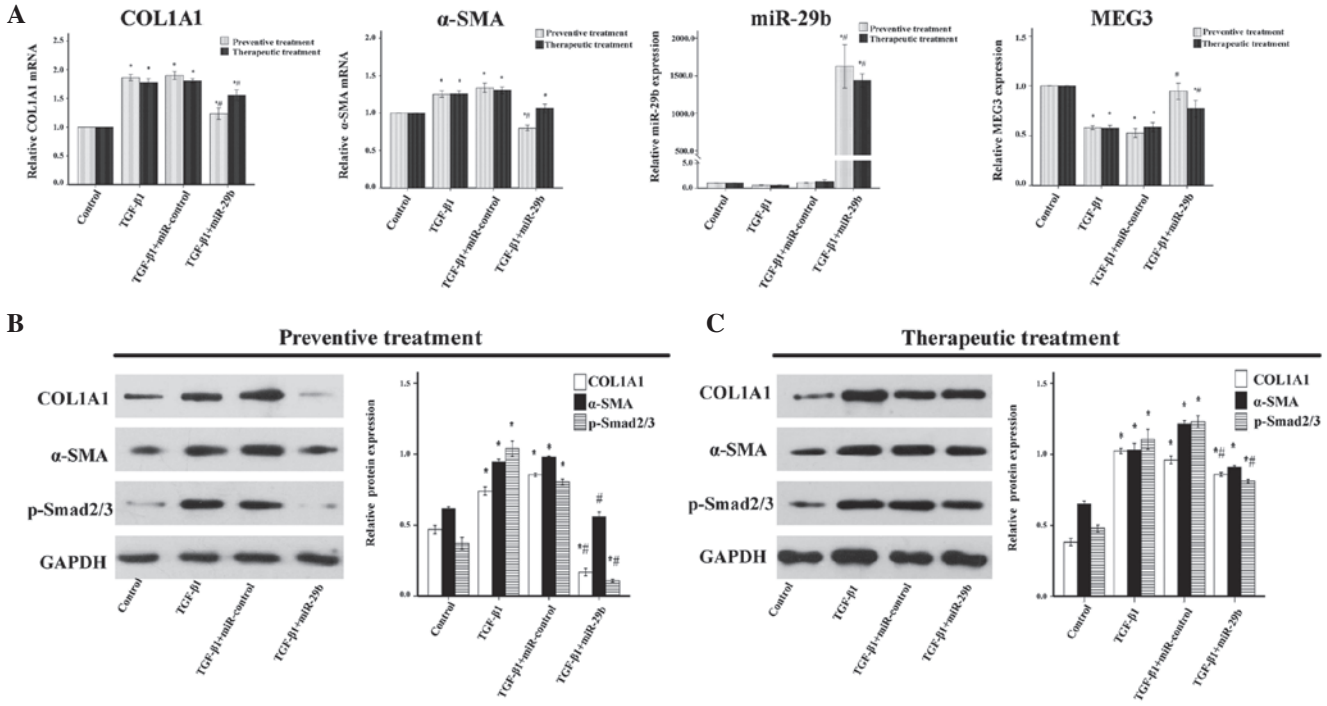

Figure 3. Anti-fibrotic effects of microRNA (miR)-29b on transforming growth factor (TGF)-β1-treated endometrial stromal cells (ESCs). miR-29b mimics (50 nM) were transfected before or after TGF-β1 (10 ng/ml) treatment of ESCs. (A) In the cells that underwent preventive treatment, exogenous miR-29b significantly decreased the mRNA expression levels of collagen, type 1, α 1 (COL1A1) and α-smooth muscle actin (α-SMA), and increased the expression levels of maternally expressed 3 (MEG3), as compared with the TGF-β1-treated group, as determined by reverse transcription-quantitative polymerase chain reaction analysis. Similar results were observed for the therapeutic treatment. (B) In the cells that underwent preventive treatment, western blot analysis detected a marked downregulation of COL1A1, α-SMA and phosphorylated (p)-Smad2/3 protein expression. (C) In the cells that underwent therapeutic treatment, COL1A1, α-SMA and p-Smad2/3 protein expression levels were decreased, as assessed by western blotting in ESCs transfected with miR-29b mimics following pretreatment with TGF-β1 for 24 h, as compared with the TGF-β1 group. Data are presented as the mean ± standard error of the mean. *P<0.05, compared with the control group; $^{\#}$P<0. 05 compared with the TGF-β1 group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

mechanism by which miR-29b protects ESCs from fibrosis, apart from its direct effect on ECM mRNAs via binding the 3'-untranslated region (UTR) (21), alterations to the TGF-β1/Smad signaling pathway were detected post-transfection. Exogenous overexpression of miR-29b suppressed the TGF-β1-induced pro-fibrotic effects by inhibiting the TGF-β1/Smad signaling pathway, as determined by a marked reduction in p-Smad2/3 protein expression (Fig. 3B and C). In addition, the decreased p-Smad2/3 nuclear translocation and accumulation detected by immunofluorescence staining supported this theory (Fig. 4B). These results suggest that inhibition of the TGF-β1/Smad signaling pathway may be considered a key mechanism by which miR-29b inhibits endometrial fibrosis in response to TGF-β1 treatment *in vitro*.

Overexpression of miR-29b upregulates MEG3 in TGF-β1-stimulated ESCs and mediates suppression of cell proliferation and promotion of cell apoptosis. To identify the alterations in MEG3 expression post-transfection, RT-qPCR analysis was performed. Overexpression of miR-29b increased the expression levels of MEG3 in both the preventive and therapeutic treatment groups (Fig. 3A). Furthermore, to investigate whether miR-29b ectopic expression had an effect on cell proliferation and apoptosis following treatment with TGF-β1, a cell proliferation assay, and cell cycle and apoptosis analyses were conducted. The proportion of apoptotic cells in the miR-29b intervention group was greater than that in the miR-control group (Fig. 5A), thus suggesting that miR-29b may induce apoptosis in activated ESCs. In addition, the overexpression of miR-29b significantly increased the percentage of activated ESCs in G_0/G_1 and G_2/M phases, and decreased the percentage of cells in S phase (Fig. 5B). Furthermore, cell proliferation was significantly suppressed in TGF-β1-treated ESCs transfected with miR-29b mimics (Fig. 5C).

Discussion

TGF-β1 is recognized as an important pro-fibrogenic mediator, which regulates ECM production and triggers myofibroblast transition (22). The results of the present study strongly suggested that TGF-β1 was able to exert a significant pro-fibrotic effect on ESCs, induce myofibroblast transdifferentiation of ESCs, activate the TGF-β1/Smad signaling pathway and increase COL1A1 synthesis. Concordant with the findings of previous studies, which demonstrated that TGF-β1 is a key profibrotic factor in various cell types ranging from human hepatic stellate cells (23) to human conditionally immortalized podocytes (11), the results of the present study demonstrated that TGF-β1 was able to promote fibrogenesis in ESCs. In the present study, ESCs transdifferentiation into myofibroblast-like cells was activated by TGF-β1, as further verified by increased α-SMA expression, which is an important factor contributing to the development of endometrial fibrosis. This phenotypic transformation results in hypertrophy accompanied by the increased secretion of ECM components and inflammatory cytokines, which results in a vicious circle that promotes

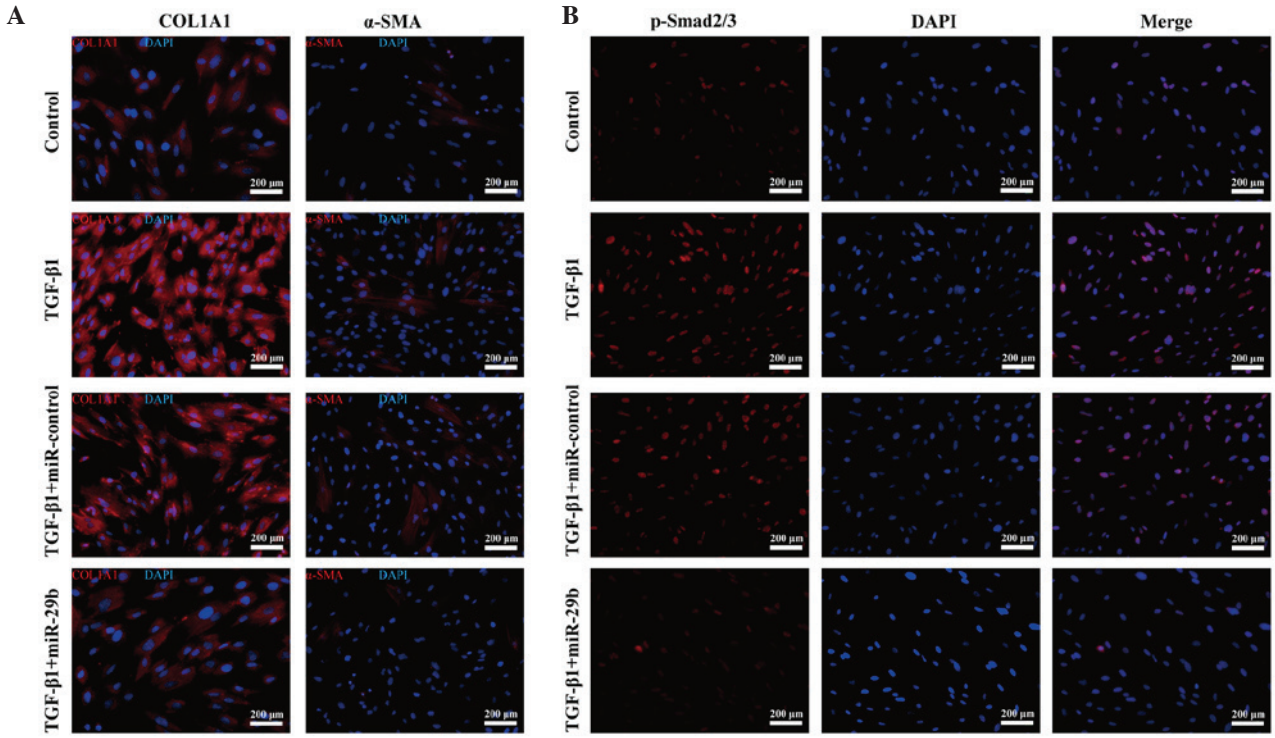

Figure 4. Collagen, type 1, alpha 1 (COL1A1), α-smooth muscle actin (α-SMA) and phosphorylated (p)-Smad2/3 protein expression was detected by immunofluorescence staining. (A) Transforming growth factor (TGF)-β1-treated group exhibited high expression of COL1A1 and α-SMA. Transfection with microRNA (miR)-29b mimics following TGF-β1 stimulation resulted in downregulation of the expression of COL1A1 and α-SMA. (B) TGF-β1-treated group exhibited high expression of intranuclear p-Smad2/3; however, overexpression of miR-29b decreased p-Smad2/3 nuclear translocation and accumulation. Scale bar, 200 μm. DAPI, 4',6-diamidino-2-phenylindole.

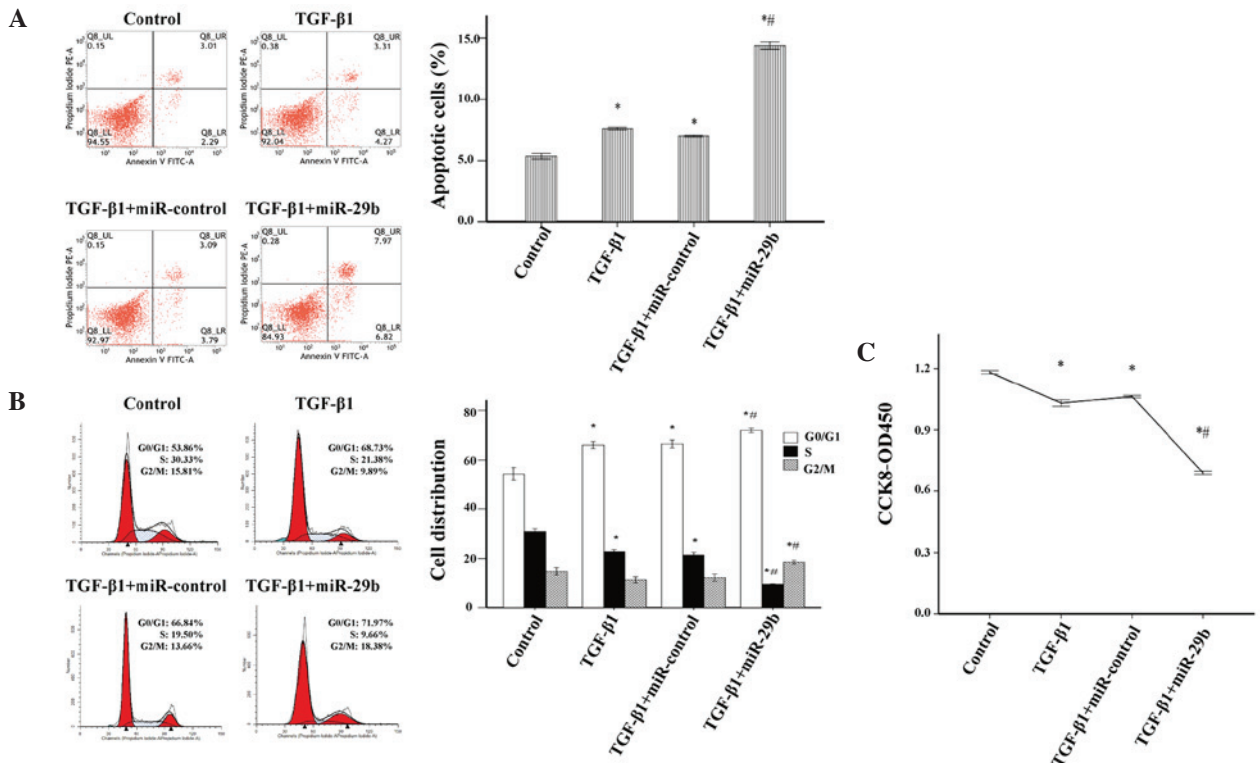

Figure 5. Effects of microRNA (miR)-29b on cell proliferation, cell cycle distribution and apoptosis of transforming growth factor (TGF)-β1-treated endometrial stromal cells (ESCs). (A) Effects of miR-29b on apoptosis in TGF-β1-treated ESCs. miR-29b exhibited significant pro-apoptotic effects on activated ESCs, as compared with the control and TGF-β1 groups. (B) Effects of miR-29b on cell cycle distribution in TGF-β1-treated ESCs. Transfection with miR-29b increased the percentage of activated ESCs in G_0/G_1 and G_2/M phases, and decreased the population of cells in S phase, as compared with the control and TGF-β1 groups. (C) Role of miR-29b in regulating TGF-β1-treated ESCs proliferation was determined by Cell Counting kit (CCK)-8 assay. Overexpression of miR-29b significantly inhibited the proliferation of fibrotic ESCs. Data are presented as the mean ± standard error of the mean. *P<0.05, compared with the control group; #P<0. 05, compared with the TGF-β1 group. OD, optical density.

transdifferentiation. A similar situation has been detected in the fibrotic liver, where hepatic stellate cells constitute the main matrix-producing cell type of the fibrotic liver following transdifferentiation into myofibroblasts (24). In addition to the transdifferentiation effect, TGF-β1 also induces the synthesis of ECM components, including COL1A1 in ESCs, via the TGF-β1/Smad signaling pathway. Similar results have been reported in various other disease models, including post-myocardial infarction (10), obstructive nephropathy (25) and pulmonary fibrosis (13). Notably, downregulation of miR-29b and MEG3 were inversely correlated with the increased expression levels of COL1A1 and α-SMA when the cells were treated with TGF-β1, thus indicating the complexity of the TGF-β1-induced fibrotic process in ESCs with the participation of mRNA, miRNA and lncRNA.

The miR-29 family has garnered attention regarding research into fibrotic disease in various organs, since no other miRNA has been predicted to possess the unique characteristic of targeting >11 of the 20 collagen genes by binding to their 3'-UTR (21). Although the anti-fibrotic effects of miR-29 agree with the phenomenon that TGF-β1 promotes collagen gene expression by downregulating miR-29, it does not rule out the possibility that miR-29 modulates fibrosis via other biological pathways. Among the miR-29 family members, miR-29b is intriguing due to its tendency for nuclear localization (26). Cytosolic and nuclear miR-29b may interact with diverse mRNAs, or DNA and lncRNAs, to elicit various biological effects. In line with previous studies (22,24), the present study demonstrated that loss of miR-29b in ESCs following TGF-β1 exposure led to the increased expression of COL1A1 and α-SMA, via activation of the TGF-β1/Smad signaling pathway. Conversely, overexpression of miR-29b in ESCs was capable of preventing TGF-β1-induced endometrial fibrosis *in vitro* via suppression of the TGF-β1/Smad signaling pathway. In addition, the present study detected anti-proliferative and pro-apoptotic roles for miR-29b in activated ESCs.

In addition to the well-accepted mechanism of miR-29b on suppression of collagen matrix expression by directly targeting the 3'-UTR of collagen genes (10), targeting bone morphogenetic protein 1, a known activator of TGF-β1, in order to inhibit TGF-β1 transcription, may be another mechanism by which miR-29b inhibits the pro-fibrotic effects of TGF-β1 (27). A similar recently reported mechanism suggests that miR-29b targets the TGF-β1 coding sequence region exon 3, thereby inhibiting the TGF-β1/Smad signaling pathway (28). Furthermore, overexpression of miR-29 is able to modulate DNA methyltransferase 1 and 3, and thus increase the expression of MEG3 (15). The upregulation of MEG3 can further induce the accumulation of p53 protein, leading to the inhibition of cell growth (29). In the present study, it was suggested that upregulation of MEG3 induced by the overexpression of miR-29b may be associated with anti-fibrotic effects in ESCs. He *et al* (16) reported that MEG3 inhibited cell proliferation, increased cell apoptosis, and decreased α-SMA and COL1A1 expression in TGF-β1-treated hepatic stellate cells. In this respect, miR-29b may contribute to the suppression of proliferation and promote apoptosis of activated ESCs by upregulating MEG3.

In conclusion, the present study suggested that loss of miR-29b expression in ESCs following treatment with TGF-β1 may lead to the transdifferentiation of ESCs into myofibroblast-like cells, and the increased expression of COL1A1 and α-SMA, via activation of the TGF-β1/Smad signaling pathway. Conversely, overexpression of miR-29b efficiently overcomes the pro-fibrogenic influence of TGF-β1 on ESCs. Overexpressing miR-29 may be considered a promising therapeutic strategy for the treatment of endometrial fibrosis.

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