MicroRNA-326 inhibits endometrial fibrosis by regulating TGF-β1/Smad3 pathway in intrauterine adhesions

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Received August 31, 2017; Accepted November 11, 2017

DOI: 10.3892/mmr.2018.9187

Abstract. Intrauterine adhesion (IUA), characterized by endometrial fibrosis, may lead to infertility and recurrent pregnancy loss. At present, there is no ideal therapy for IUA. Recent findings have revealed that microRNAs (miRNAs) have a decisive role in the regulation of fibrosis. The aim of the present study was to investigate the molecular mechanism of miRNAs in endometrial fibrosis. The present study compared the expression profiles of miRNAs between endometrial tissues from patients with IUA and normal endometrial tissues using microarray analysis. Validation of miR-326 level in endometrial tissues was performed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Subsequently, the effects of miR-326 on fibrotic markers including α-smooth muscle actin (α-SMA), collagen type I α 1 chain (COL1A1), transforming growth factor-β1 (TGF-β1) and fibronectin (FN), were evaluated in endometrial tissues and endometrial stromal cells (ESCs) from patients with IUA. Additional bioinformatics analysis, luciferase reporter assays, RT-qPCR and western blotting were performed to identify target genes. Additionally, the expression levels of TGF-β1, p-Smad3 and Smad3 were quantified to determine whether the anti-fibrotic role of miR-326 was associated with the activity of the TGF-β1/Smad3 signaling pathway. The present study determined that miR-326 was downregulated in endometrial tissues from patients with IUA and miR-326 levels were inversely correlated with the expression of TGF-β1, α-SMA, COL1A1 and FN. Additional findings revealed that overexpression of miR-326 inhibited endometrial fibrosis by downregulating these pro-fibrotic genes. TGF-β1, an important pro-fibrogenic mediator, was identified as a direct target of miR-326. Additionally, overexpression of miR-326 blocked the activation of the TGF-β1/SMAD family member 3 (Smad3) signaling pathway by suppressing the expression of TGF-β1 in ESCs from patients with IUA. The findings of the present study indicated that miR-326 inhibited endometrial fibrosis by suppressing the TGF-β1/Smad3 signaling pathway, suggesting that miR-326 may be a prognostic biomarker and therapeutic target for IUA.

Introduction

Intrauterine adhesions (IUA) frequently occur as a result of trauma to the basal layer of the endometrium that may lead to a range of symptoms, such as amenorrhea, hypomenorrhea, infertility or recurrent pregnancy loss (1,2). Additionally, it may lead to a variety of subsequent sequelae and severely affect women’s health and fertility (3). Despite the wide use of diagnostic and operative hysteroscopy that have improved the functional outcome of patients with IUA, advances in the management of IUA remain limited. Endometrial fibrosis is the primary pathological feature of IUA characterized by excessive deposition and reorganization of extracellular matrix (ECM) replacing the normal endometrium. The prevention of endometrial fibrosis may potentially lead to endometrial repair and promote the formation of fibrous scar adhesions (4). However, the specific mechanisms of underlying endometrial fibrosis remain to be elucidated.

Transforming growth factor (TGF)-β1 is a secreted protein expressed in most cell types, which has been implicated in endometrial fibrosis, by inducing fibroblast synthesis and by induction of epithelial mesenchymal transition (EMT) (5). It has been previously established that TGF-β1 exert its biological activity by binding to its receptor through two critical downstream mediators, SMAD family member 2 (Smad2) and Smad3, receptor-regulated Smads (6,7). Previous studies have reported that TGF-β1 has an important role in the development of fibrosis diseases (8-10). Increasing TGF-β1 expression in the mouse lung leads to severe interstitial and pleural fibrosis (11). Previous studies demonstrated that TGF-β1/Smad3 pathway mediated fibrosis by directly binding to various ECM promoters, including collagen I, II, and III (12-15). It has also been demonstrated the expression of TGF-β in the endometrial tissue of IUA was significantly increased compared with normal endometrium and uterine septum (5). However,
whether the TGF-β1/Smad3 pathway is involved in endometrial fibrosis remains to be determined.

MicroRNAs (miRNAs) are a class of endogenous small (19-24 nucleotide) non-coding RNAs that suppress translation of target messenger RNAs (mRNAs) or induce degradation of target miRNAs by binding to the 3‘-untranslated region (UTR) of target mRNAs (16). Previous studies revealed that aberrant expression or dysfunction of miRNAs has an important role in various types of tissue fibrosis, such as in the heart (17,18), kidney (19,20), liver (21) and lung (22). However, to the best of our knowledge, at present, there have only been preliminary studies on the biological roles of miRNAs in IUA. MiR-29b was determined to be downregulated in TGF-β1-treated endometrial stromal cells (ESCs) and inhibited TGF-β1-induced fibrosis via regulation of the TGF-β1/Smad pathway in ESCs (23). Therefore, more extensive investigation on the identification and the functions of miRNAs involved in the development of IUA are required.

The present study focused on the expression level of miR-326 in IUA and investigated its role in endometrial fibrosis. The present findings revealed that overexpression of miR-326 may inhibit endometrial fibrosis by inactivating the TGF-β1/Smad3 pathway. These findings suggest that miR-326 may be a novel therapeutic target for IUA.

Materials and methods

Clinical subjects. All the subjects were recruited from Hainan Branch of PLA General Hospital (Sanya, China) between January 2015 and July 2016. Written informed consent was obtained prior to inclusion and the present study was approved by the Ethics Committee of Hainan branch of PLA General Hospital. A total of 30 endometrial tissues were collected from IUA patients diagnosed by hysteroscopy, with mean age 28.06 years (range 19-34). Normal endometrial tissues from individuals without IUA (n=15) in the same period were used as controls. All samples were flash-frozen in liquid nitrogen, and stored at -80°C until subsequent molecular analysis.

MicroRNA expression profiling. Total RNA was extracted from endometrial tissues using the miRcute miRNA Isolation kit (Tiangen Biotech Co., Ltd., Beijing, China). RNA quality was determined using an Agilent Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Samples were labeled using a miRCURY Hy3/Hy5 Power labeling kit and hybridized to the miRCURY LNA® Array version 18.0 (Agilent Technologies, Inc.). The chips were scanned with the Axon GenePix 4000B Microarray Scanner (Axon Instruments, Foster City, CA, USA). The procedure and image processing method was performed as previously described (24). The miRNA expressions of all differentially expressed samples were clearly displayed by a hierarchical clustering heat map.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from endometrial tissues using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. For the detection of miR-326, RT-qPCR assays were performed using the miRcute miRNA qPCR Detection kit (Tiangen Biotech Co., Ltd.) following the manufacturer's protocol. For detection of the TGF-β1, α-smooth muscle actin (α-SMA), collagen type I α 1 chain (COL1A1) and fibronectin (FN) mRNA levels, 1 µg of total RNA was reverse transcribed at 37°C for 60 min using the miRcute miRNA First-Strand cDNA Synthesis kit (Tiangen Biotech Co., Ltd.), then amplified on an Applied Biosystems 7900HT cycler using SuperReal PreMix Plus (Tiangen Biotech Co., Ltd.). The PCR conditions consisted of 5 min at 94°C for one cycle followed by 40 cycles of 94°C for 20 sec and 60°C for 20 sec. U6 and GAPDH were used as normalization controls for miRNA qPCR and qPCR, respectively. The relative expression was calculated using the 2^-ΔCq method (25). Each reaction was conducted in triplicate. The primers for RT-qPCR analysis were as follows: miR-326 forward primer: 5'-GGCGCCCAAGUAAUGGC-3'; miR-326 reverse primer: 5'-CGTGGAGGTCGGAGTC-3'; U6 forward primer: 5'-TGCAGGTGCAGCTTCGAGAC-3'; U6 reverse primer: 5'-CCAGTGCGAGGTCGGAGT-G-3'; TGF-β1 forward primer: 5'-TGGAGCAGCAACACACAC-3'; TGF-β1 reverse primer: 5'-CAGATCGATGCAGTCGCAC-3'; α-SMA forward primer: 5'-GGCTCTGGGCTCTGTAAG-3'; reverse primer: 5'-CTCTGCTGCTGCTGCTAC-3'; fibronectin forward primer: 5'-GAGATCGTGGAGGATC-3'; reverse primer: 5'-GGGTGCCACCTGAGTGA-3'; GAPDH reverse primer: 5'-CGGAGTCAAGGATGTTTGCTAT-3'; reverse primer: 5'-AGCCTTTCCATGGGTGGAAGAC-3'.

Primary ESC isolation and culture. ESCs were isolated from the endometrial tissues as previously described (26). ESCs were maintained in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2. The cells reached confluence in 2-3 days, and the passage 3-6 were used for subsequent experiments.

Cell transfection. MiR-326 mimics, miR-326 inhibitor and controls were purchased from Shanghai GenePharma Co., Ltd., (Shanghai, China). ESCs were seeded at 2x10^5 cells/well in 6-well plates and cultured in antibiotic-free DMEM at 37°C and 5% CO2. Next, the cells were incubated overnight to a confluence of 30-50%. cell transfection was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were collected for further experiments 48 h after the transfection.

Luciferase reporter assay. A cDNA fragment of the TGF-β1 3'-UTR mRNA containing the seed sequence of the miR-326-binding site or a mutated binding site was cloned into the pmirGLO dual-luciferase vector (Promega Corporation, Madison, WI, USA). The constructed dual-luciferase vector was co-transfected with miR-326 mimics, miR-326 inhibitor or NC into ESCs isolated from patients with IUA using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. The relative firefly luciferase activity normalized with Renilla luciferase was measured 48 h after transfection by using the Dual-Luciferase
Reporter system (Promega Corporation) following the manufacturer's protocol. All of the dual-luciferase reporter assays were performed in triplicate for each experiment and three independent experiments were conducted.

**Western blotting.** Cells were lysed using radioimmunopreципitation assay protein extraction reagent (Beyotime Institute of Biotechnology, Haimen, China). The concentration of proteins was determined using a bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL, USA). Protein extracts (50 µg per lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred to nitrocellulose membranes (Sigma-Aldrich, Merck Millipore; Darmstadt, Germany) and incubated with the following primary antibodies: TGF-β1 monoclonal antibody (cat. no. sc-52892; 1:300; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-phosphorylated (p)-Smad3 monoclonal antibody (cat. no. sc-517575; 1:1,000; Santa Cruz Biotechnology, Inc.) and Smad3 monoclonal antibody (cat. no. sc-101154; 1:1,000; Santa Cruz Biotechnology, Inc.). Subsequently, the membranes were incubated with peroxidase conjugated goat anti-rabbit IgG (cat. no. TA130023; 1:5,000; OriGene Technologies, Inc., Beijing, China) conjugated to horseradish peroxidase for 1 h at 37°C. ECL chromogenic substrate (Thermo Fisher Scientific, Inc.) was used to visualize the bands and the intensity of the bands was quantified by densitometry using ImageJ software (version 2.1.4.7; National Institutes of Health, Bethesda, MD, USA). Control antibody used was anti-β-actin (cat. no. sc-8432; 1:1,000; Santa Cruz Biotechnology, Inc.).

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Differences were analyzed with Student's t-test between two groups or with one-way analysis of variance among four groups. Correlation analyses were performed using Spearman's correlation coefficient test. P < 0.05 was considered to indicate a statistically significant difference.

**Results**

*MiR-326 is downregulated in endometrial tissues from patients with IUA.* To determine the expression of miRNAs associated with IUA, the present study performed a miRNA microarray on endometrial tissues from pairs of patients with or without IUA. The current findings revealed that the expression of 56 miRNAs was significantly different in the IUA group compared with the normal group. Among these miRNAs, 28 miRNAs were downregulated, whereas 28 miRNAs were upregulated (Fig. 1A). From these aberrantly expressed miRNAs, miR-326 was one which was significantly downregulated. In addition, a previous study reported that miR-326 had a key role in alleviating lung fibrosis by regulating TGF-β1 expression and other pro-fibrotic genes (27). Therefore, the present study selected miR-326 for further investigation.

To validate the expression trend identified for miR-326 in endometrial tissues obtained from miRNA microarray assay RT-qPCR was performed to detect miR-326 expression levels in 30 IUA endometrial tissues and 15 normal endometrial tissues. As presented in Fig. 1B, miR-326 was significantly downregulated in the IUA group compared with the normal group. These findings indicated that miR-326 may be involved in IUA progression.

**Correlation between miR-326 and the level of fibrotic markers in endometrial tissues.** The present study examined whether abnormal expression of miR-326 was associated with the endometrial fibrosis of patients with IUA following confirmation of the reduced expression of miR-326 in endometrial tissues. Previous studies revealed that TGF-β1 may promote the endometrial fibrosis, and α-SMA, COL1A1 and FN are the primary fibrotic marker genes (9,20,28). Thus, the miR-326 expression level and the fibrotic markers (TGF-β1 α-SMA, COL1A1, and FN) mRNA expression level in 30 IUA endometrial tissues were determined. Using Spearman's correlation coefficient, a negative correlation was identified between TGF-β1, α-SMA, COL1A1, and FN mRNA expression levels and the miR-326 expression level (Fig. 2). These findings indicate that miR-326 may potentially serve as an effective biomarker for the prognosis of patients with IUA. Additionally, miR-326 may be relevant to fibrotic processes in IUA pathology.

*miR-326 inhibits the fibrosis of endometrial stromal cells (ESCs).* Previous studies have reported that deregulated proliferation and differentiation of ESCs are important factors contributing to the development of endometrial fibrosis (29,30). Therefore, the present study used the primary ESCs isolated from patients with IUA to investigate function of miR-326 in endometrial fibrosis. ESCs were transfected with miR-326 mimics to overexpress miR-326. The miR-326 mimic group had a significantly increased the expression level of miR-326 (Fig. 3A). The alteration of the expression of fibrotic-associated markers in ESCs were investigated following miR-326 overexpression. As presented in Fig. 3B-D, the mRNA expression levels of α-SMA, COL1A1 and FN in ESCs isolated from patients with IUA was increased when compared with normal subjects, whereas overexpression of miR-326 was able to suppress the mRNA expression levels of α-SMA, COL1A1 and FN. These findings suggest that miR-326 may inhibit the fibrosis of endometrial stromal cells.

*TGF-β1 is a direct target of miR-326 in ESCs.* TGF-β1, an important pro-fibrogenic mediator (31), was previously reported to be involved in the development of fibrosis and organ dysfunction, by upregulating the extracellular matrix (ECM) protein expression and α-SMA (8). As miR-326 regulates the TGF-β1 level and attenuates bleomycin-induced lung fibrosis mice (27), it was hypothesized that TGF-β1 may also be involved in the anti-fibrotic role of miR-326 in IUA. In order to investigate the association between miR-326 and TGF-β1, a luciferase reporter assay was performed in ESCs isolated from patients with IUA. The findings revealed that overexpression of miR-326 significantly reduced the luciferase activity of TGF-β1 with wild-type (wt) 3'-UTR, whereas miR-326 inhibition increased the luciferase activity of TGF-β1 with wt 3'-UTR (Fig. 4A). Cells co-transfected with miR-326 mimics, miR-326 inhibitor, and TGF-β1-mut-3'UTR showed no obvious change in their luciferase activity (Fig. 4A). These findings indicate that miR-326 directly targeted the 3'-UTR of TGF-β1. The present study further
examined whether miR-326 may modulate the expression of TGF-β1 in ESCs by RT-qPCR and western blotting. The findings revealed that the mRNA and protein expression levels of TGF-β1 in ESCs were significantly downregulated following transfection with miR-326 mimics, compared with the negative control, whereas transfection with the miR-326 inhibitor increased TGF-β1 expression (Fig. 4B and C). Collectively, these findings suggest that TGF-β1 is a direct downstream target of miR-326 in ESCs.

**miR-326 protects ESCs from fibrosis by regulating the TGF-β1/Smad3 pathway.** Due to the important role of the TGF-β1/Smad3 pathway in organ fibrosis, including endometrial fibrosis (23,32,33), subsequent experiments were designed to investigate the effects of miR-326 on the activity of the TGF-β1/Smad3 pathway in ESCs. Western blotting was performed to determine the protein expression levels of TGF-β1, p-Smad3 and Smad3 in ESCs. The present study confirmed that TGF-β1 and p-Smad3 protein expression levels were increased in ESCs from patients with IUA (Fig. 5A and B). However, overexpression of miR-326 effectively downregulated TGF-β1 and p-Smad3 protein expression levels (Fig. 5A and B). In addition, no change was observed in the expression levels of Smad3. These findings suggest that miR-326 may inhibit endometrial fibrosis via inactivating the TGF-β1/Smad3 pathway.

**Discussion**

The present study identified that miR-326 was downregulated in endometrial tissues and ESCs isolated from patients with IUA. Additionally, miR-326 expression levels were inversely correlated with the expression levels of fibrotic markers including TGF-β1, α-SMA, COL1A1 and FN. The present findings also demonstrated that miR-326 exerted an anti-fibrotic effect by inhibiting TGF-β1, thus negatively regulating the TGF-β1/Smad3 signaling pathway in IUA. Therefore, the miR-326/TGF-β1/Smad3 axis may be a potential target for the prevention and treatment of IUA.

Previous studies suggest that miRNAs have important roles in fibrosis diseases. For example, Pandit et al described a novel role for miR-21 in the context of pulmonary fibrosis.
fibrosis by regulating EMT TGF-β1 signaling activity (34). Additionally, Tao et al have determined the involvement of miR-433 in the regulation of cardiac fibrosis through TGF-β1 and the mitogen-activated protein kinase/extracellular-signal regulated kinase/P38 pathways (35). However, the role of miRNAs in endometrial fibrosis remains to be determined. In order to identify miRNAs which could potentially regulate endometrial fibrosis in the present study, a miRNA array in endometrial tissues from patients with IUA was performed and determined that miR-326 was significantly downregulated. Additionally, it was observed that an inverse relationship between miR-326 expression and the...
expression levels of fibrotic markers including TGF-β1, α-SMA, COL1A1 and FN in endometrial tissues and ESCs. These findings indicated that miR-326 have an important role in endometrial fibrosis.

Previous studies have reported the involvement of the TGF-β1/Smad pathway in fibrosis diseases. For example, Zhou et al revealed that casticin attenuated liver fibrosis by blocking the TGF-β1/Smad signaling pathway (36). A study by Choi et al reported that capsaicin was able to ameliorate hepatic fibrosis by inhibiting the TGF-β1/Smad pathway via peroxisome proliferator-activated receptor-γ activation (37). Additionally, Gao et al determined that anoctamin-1 inhibited cardiac fibrosis after myocardial infarction via the TGF-β1/Smad3 pathway (32). Previous studies have made significant progress in identifying the core signaling pathways in endometrial fibrosis, including the wnt/β-catenin, nuclear factor-κB and TGF-β1/Smad pathways (5,23,28,38). These pathways are frequently constitutively activated in subsets of endometrial tissues and cell lines, particularly in the TGF-β1/Smad pathway. Therefore, understanding the molecular mechanisms involved in the TGF-β1/Smad pathway in endometrial fibrosis is necessary. Das et al reported that miR-326 physiologically reduced TGF-β1 expression and attenuated the fibrotic process in a bleomycin-induced lung fibrosis model (27). However, it remains to be determined whether miR-326 is also involved in endometrial fibrosis via TGF-β1/Smad pathway. To the best of our knowledge, the present study is the first to report that miR-326 regulates TGF-β1 expression post-transcriptionally and overexpression of miR-326 may inhibit TGF-β1 and p-Smad3 protein expression in ESCs. These findings indicated that miR-326 protects ESCs from fibrosis by inactivating the TGF-β1/Smad3 pathway.

In conclusion, the findings of the current study suggest that miR-326 inhibits endometrial fibrosis by negatively regulating the TGF-β1/Smad3 pathway via direct targeting of TGF-β1, confirming that miR-326 may be an anti-fibrotic element in endometrial fibrosis. Therefore, the present study proposed that the miR-326/TGF-β1/Smad3 axis may represent a promising therapeutic target for the future treatment of IUA.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Planning and performance of experiments, contribution of reagents or other essential materials and writing the paper: JN. Data analysis and manuscript review: JN, HZ and HY.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Hainan branch of PLA General Hospital.

Consent for publication

Written informed consent was obtained prior to inclusion to the present study.

Competing interests

The authors declare that they have no competing interests.

References

fibrosis in endometriosis


