Upregulation of miR-33b promotes endometriosis via inhibition of Wnt/β-catenin signaling and ZEB1 expression

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Abstract. The present study aimed to investigate the role and mechanisms of microRNA (miR)-33b in endometriosis (Ems). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR), MTT assays, flow cytometry, caspase-3/9 activity assays and western blotting were performed in the present study. Initially, miR-33b expression in an Ems rat model was investigated by RT-qPCR and was demonstrated to be upregulated in Ems tissue samples of rats compared with the control group. In addition, miR-33b upregulation inhibited cell growth and enhanced apoptosis in an Ems model (primary cell cultures) compared with the control group. In addition, miR-33b up-regulation reduced Wnt/β-catenin signaling pathway and suppressed zinc finger E-box-binding homeobox 1 (ZEB1) protein expression in the in vitro Ems model (primary cell cultures) compared with the control group. Furthermore, small interfering-ZEB1 ameliorated the effects of miR-33b downregulation on Ems cell growth in the in vitro Ems model. Additionally, a Wnt agonist reduced the effects of miR-33b upregulation on Ems cell growth in the in vitro Ems model. In conclusion, the present study demonstrated that upregulation of miR-33b may promote Ems through Wnt/β-catenin by ZEB1 expression.

Introduction

Endometriosis (Ems) is a common benign gynecological disease in women of child-bearing age. It indicates that endometrial tissues (glands and mesenchyme) with growth function are coated with endometrium in the uterine cavity (1). Additionally, endometrial tissues may grow in other sites apart from the myometrium. Ems morbidity has notably increased in recent years, with the major clinical symptoms of infertility, chronic pelvic pain and dysmenorrhea (1). Ems frequently occurs in the ovary, rectovaginal pouch and vesicouterine pouch (1). It is a benign disease, but certain of its biological behaviors resemble those of malignancies (2). It is extremely invasive, and may lead to extensive and severe adhesion. Additionally, Ems is regarded as a benign tumor, which severely affects patient quality of life (2).

miRNA s (miRNAs/miRs) are a class of endogenous RNAs with regulatory functions in eukaryotes (3). They are ~22-23 nucleotides in length (4). miRNAs are extensively distributed in plants, animals and viruses, and negatively regulate gene expression at the post-transcriptional level through complementary pairing of mRNA (3). Eventually, they may lead to mRNA degradation or translational inhibition (5). As important regulatory molecules, miRNAs are involved in a series of vital life processes, such as virus defense, hematopoiesis, organogenesis, cell proliferation, apoptosis, fat metabolism and tumorigenesis (5).

The Wnt/β-catenin signaling pathway is a key pathway regulating cell growth and proliferation (6). A previous study demonstrated that such a pathway serves a vital role in the genesis, metastasis and invasion of multiple tumors (6). In addition, it may be involved in the adhesion, invasion and angiogenesis of the ectopic endometrium (7). Wnt/β-catenin signaling pathway serves a decisive role in endometrial gland formation and mesenchymal development (7).

Transcription factor zinc-finger E-box binding homeobox (ZEB)1 located in the short arm of human chromosome 10, is also referred to as TCF8 or δEF1 (8). Recently, it was indicated that ZEB1 serves a crucial role in tumorigenesis (8) in different cancer types, including breast cancer, prostate cancer, lung cancer and endometrial cancer. Transcription factor ZEB-1 is one of the factors inducing Ems (9). A previous study on the role of ZEB1 in promoting tumor cell metastasis focused on its inhibitory effect on E-cadherin (10). Cadherins are a class of Ca2+-dependent transmembrane glycoproteins (10). E-cadherin marks epithelial cells and its deletion may be observed in Ems (10). Mesenchymal cell markers, including N-cadherin, are upregulated in Ems (11). Wang et al (12) demonstrated that miRNA-33b is able to mediate the cellular apoptosis of endometrial cells. The present study aimed to investigate the role and mechanism of miRNA-33b in Ems.
Materials and methods

Animals and rat model. Female Sprague-Dawley rats (6-7 weeks old; 180-200 g; n=12) were housed at 22-24°C, 55-60% humidity with a 12-h/12-h light/dark cycle, and were given a regular chow diet and water ad libitum. The present study was approved by the Animal Care and Use Committee of Affiliated Qilu Hospital of Shandong University (Jinan, China). All rats were randomly assigned to control (n=6) and Ems (n=6) groups. In the control group, rats were anesthetized with 35 mg/kg pentobarbital sodium without intervention. Rats were anesthetized with 35 mg/kg pentobarbital sodium, and a vertical incision in the abdomen was made. The uterus was removed and immediately washed with PBS, the endometrium was cut into 0.5x0.5-cm sections and uterine segments were sutured onto the peritoneum close to blood vessels. 0.5 µg/kg/day of Estradiol benzoate (MedChem Express, Shanghai, China) was subcutaneously injected for 3 days following the surgery. Following treatment with Estradiol benzoate for 3 days, rats were sacrificed using decollation under 35 mg/kg pentobarbital sodium.

Cell culture and cell transfection. Endometrial stromal cells were separated from the isolated endometrial tissues, and tissue was finely minced. Cells were dispersed and incubated in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (Gibco; Thermo Fisher Scientific, Inc.), penicillin-streptomycin solution, and 2 mg/ml collagenase II for 1 h at 37°C. Subsequently, endometrial stromal cells were separated using a 100 µm filter. Stromal cells were pelleted by centrifugation at 200 x g for 10 min at 4°C and incubated with DMEM/F-12 containing fetal bovine serum (10%, v/v; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37°C in a humidified atmosphere containing 5% CO2. Then, transfection with 100 ng of mir-33b (5'-UGUGCAUUGCUGAUUGCAUUGC-3' and 5'-AAUGCAACAGCAUUGCU-3'), anti-mir-33b (forward, 5'-CCA AGATCTCCAGGCTCGA-3' and reverse, 5'-TTCCGAG CCTGGAGACTCCTTG-3'), small interfering (si) -ZEB1 (sc-38643; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and negative mimics (forward, 5'-TTCCCGAGGCTCGA-3' and reverse, 5'-TTCGAG CGGTTGAGACTTCTG-3'), small interfering (si)-ZEB1 (sc-4920; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and negative mimics (forward, 5'-TTCCGAGGCTCGA-3' and reverse, 5'-TTCGAGCGGTTGAGACTTCTG-3') was performed for 4 h. For Wnt activation, 10 µM of SKL2001 (Biolandia, Darmstadt, Germany) at 37˚C in a humidified atmosphere. Following transfection for 4 h, old medium was removed and new DMEM/F-12 was added into cell. Next, transfection of miR-33b or negative mimics was performed for 4 h. For Wnt activation, 10 µM of SKL2001 (MedChemExpress, Shanghai, China) was added to the cells for 72 h.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from frozen tissues or cells using TRIzol® reagent (Life Technologies; Thermo Fisher Scientific, Inc.). Total RNA (1 µg) was transfected to first-strand cDNA using an RT kit (Toyobo Life Science, Osaka, Japan). The RT-qPCR protocol was performed using SYBR-Green PCR master mix (Bio-Rad Laboratories, Hercules, CA, USA) and an Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific, Inc.). The primer sequences were: miR-33b forward, 5'-ATT CTTTCAACTGTCTTG-3' and reverse, 5'-TCACCCCTCG GCTGTCCTGACA-3'; U6 forward, 5'-AGTACAGTCG TTGCTTG-3' and reverse, 5'-TAATAGCCCGATGTCT GGT-3'. The qPCR cycle was set to an initial 95°C for 10 min, followed by 40 cycles of 95°C for 25 sec, 60°C for 30 sec, and 72°C for 30 sec. Gene expression levels were measured using the 2-ΔΔCq method (13).

Gene microarray hybridization. Isolated RNA was reverse transcribed into cDNA and hybridized to Affymetrix HG-U133 Plus 2.0 GeneChip arrays 1 (Affymetrix, Santa Clara, CA, USA). Data were analyzed through TargetScan version 7.1 (http://www.targetscan.org) and QIAGEN’s Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, USA) (14).

Hematoxylin and eosin (H&E) staining. Endometriosis tissue was collected and fixed with 4% paraformaldehyde for 24 h. The colonic sections of 4 µm were cut from formalin-fixed, paraffin-embedded tissue blocks. Tissue samples were stained with HE assay for 15 min and evaluated under the light microscope (Olympus BX51).

Cell viability and apoptosis. MTT (5 mg/ml; Sigma Aldrich; Merck KGaA) was added to the cells for incubation at 37°C for 4 h. Subsequently, the old medium was removed and dimethyl sulfoxide was added to the cells for 20 min at 37°C. The absorbance was measured by spectrophotometry with a microplate reader (model 680; Bio-Rad Laboratories, Inc.) at 490 nm. Endometrial stromal cells were washed with PBS and resuspended in 100 µl 1X binding buffer (BestBio, Shanghai, China) and incubated with Annexin V-fluorescein isothiocyanate (FITC; BestBio) and propidium iodide (PI; BestBio) for 15 min at room temperature in the dark. The apoptosis rate was analyzed using a flow cytometer (C6; Beckman Coulter Inc., Brea, CA, USA) analyzed using Image Lab version 3.0 (Bio-Rad Laboratories, Inc.).

Lactate dehydrogenase (LDH) activity. LDH activity levels were measured using LDH activity kits (C0016; Beyotime Institute of Biotechnology, Haimen, China). The absorbance was measured by spectrophotometry with a microplate reader (model 680; Bio-Rad Laboratories, Inc.) at 450 nm.

Caspase 3/9 activity assay. Endometrial stromal cells were lysed with lysis buffer (radioimmunoprecipitation assay buffer) containing a protease inhibitor cocktail (phenylmethanesulfonyl fluoride) and EDTA at 4°C for 30 min. Cells were centrifuged at 12,000 x g for 10 min at 4°C. Subsequently, the concentration of total protein was determined using a bicinchoninic acid (BCA) assay, and 10 µg/lane total protein was used to analyze caspase 3/9 activity levels using caspase 3/9 activity kits (C1115 and C1158, Beyotime Institute of Biotechnology). The absorbance was measured by spectrophotometry with a microplate reader (model 680; Bio-Rad Laboratories, Inc.) at 405 nm.

Western blot analysis. Endometrial stromal cells were lysed with lysis buffer (radioimmunoprecipitation assay buffer) containing protease inhibitor cocktail (phenylmethanesulfonyl fluoride) and EDTA at 4°C for 30 min. Cells were centrifuged

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at 12,000 x g for 10 min at 4˚C. Subsequently, the concentration of total protein was determined using the BCA assay, and 40 µg/lane total protein was subjected to 10‑12% SDS‑PAGE and subsequently transferred onto a polyvinylidene difluoride membrane (Bio‑Rad Laboratories, Inc.). The membrane was blocked with 5% non‑fat milk in TBS containing 0.1% Tween‑20 for 1 h at 37˚C, and subsequently incubated with antibodies against Wnt (sc‑376029; 1:1,000), β‑catenin (sc‑65480; 1:1,000), ZEB1 (sc‑515797; 1:1,000), apoptosis regulator BAX (Bax; sc‑20067; 1:1;000) and GAPDH (sc‑51631; 1:5,000; all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4˚C. Following this, membranes were incubated with anti‑rabbit immunoglobulin G secondary antibody (sc‑2004; 1:5,000; Santa Cruz Biotechnology, Inc.) at 37˚C for 1 h and were developed using an enhanced chemiluminescence kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Protein levels were quantified using Bio‑Rad Laboratories, Inc. Quantity One software (version 3.0).

Statistical analysis. All data are expressed as the mean ± standard error using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Statistical differences were measured using one way analysis of variance with Bonferroni's correction for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

miR‑33b expression in Ems. Firstly, H&E staining suggested that Ems was successfully induced in the Ems group compared with the control group (Fig. 1A). As demonstrated in Fig. 1B and C, miR‑33b expression was upregulated by 4.29±0.05 fold in the Ems rat model compared with the control group.

Effect of miR‑33b on cell growth in Ems. Notably, miR‑33b expression was investigated using miR‑33b mimics or anti miR‑33b mimics in vitro. Fig. 2A and B indicate that miR‑33b expression was upregulated by 5.26±0.04 fold or downregulated by 0.26±0.03 fold in the in vitro Ems model compared with the control group. Overexpression of miR‑33b suppressed cell viability by 0.82±0.06 fold (48 h) or 0.51±0.06 fold (72 h), and enhanced the lactate dehydrogenase (LDH) activity of Ems by 4.70±0.29 fold; additionally, downregulation of miR‑33b promoted cell viability by 1.23±0.06 fold (48 h) or 1.28±0.03 fold (72 h), and decreased the LDH activity of Ems by 0.37±0.03 fold (Fig. 2C‑F).

Effect of miR‑33b on apoptosis in Ems. Subsequently, it was demonstrated that overexpression of miR‑33b increased the apoptosis rate by 2.05±0.15-fold and promoted caspase-3 and 9 activity by 3.97±0.17 and 3.70±0.24-fold, respectively, in the Ems model compared with the control group (Fig. 3A‑D). However, downregulation of miR‑33b decreased the apoptosis rate by 0.54±0.04-fold (Fig. 3E and F) and the caspase-3 and 9 activity by 0.39 ± 0.03 and 0.31 ± 0.05-fold, respectively, in the Ems model (Fig. 3G and H).

Effect of miR‑33b on Wnt/β‑catenin in Ems by ZEB1 expression. To analyze the mechanism of miR‑33b in Ems, the Wnt/β‑catenin signaling pathway was investigated. It was demonstrated that miR‑33b was able to target the 3'UTR of ZEB1 (Fig. 4A). Subsequently, overexpression of miR‑33b suppressed ZEB1 protein expression by 0.34±0.04 fold, reduced Wnt and β‑catenin protein expression by 0.44±0.06 and 0.32±0.12 fold, respectively, and induced Bax protein expression by 2.56±0.13 fold in the in vitro Ems model (Fig. 4B‑F). Furthermore, downregulation of miR‑33b induced ZEB1 protein expression by 1.94±0.17 fold, reduced Wnt/β‑catenin protein expression by 2.57±0.07 and 2.72±0.14 fold, respectively, and suppressed Bax protein expression by 0.43±0.02 fold in the in vitro Ems model compared with the control group (Fig. 4G‑K).

ZEB1/Wnt/β‑catenin influences the effect of miR‑33b in Ems. To examine the role of ZEB1/Wnt/β‑catenin in the effect of miR‑33b on Ems, si-ZEB1 or Wnt agonist were co‑transfected with
Figure 2. Effect of miR-33b on cell growth in endometriosis. Quantification of miR-33b expression following treatment with its (A) mimic and (B) anti-mimic as measured by RT-qPCR. Effect of (C) miR-33b mimic and (D) anti-mimic on cell viability. Effect of (E) miR-33b mimic and (F) anti-mimic on LDH activity levels. "P<0.05 vs. respective control. miR, microRNA. LDH, lactate dehydrogenase.

Figure 3. Effect of miR-33b on apoptosis in Ems. Effects of overexpression of miR-33b on (A and B) apoptotic rate, (C and D) caspase-3 and caspase-9 activity respectively; and of downregulation of miR-33b on (E and F) apoptotic rate, (G and H) caspase-3 and caspase-9 activity, respectively. "P<0.05 vs. control. Ems, endometriosis; miR, microRNA.
miR-33b mimics in Ems. As illustrated in Fig. 5A-E, compared with the miR-33b downregulation group, si-ZEB1 suppressed ZEB1 by 0.52±0.08 fold, decreased Wnt and β-catenin protein expression by 0.57±0.04 and 0.71±0.05 fold, respectively, and induced Bax protein expression by 2.36±0.07 fold in the Ems model. Furthermore, compared with the miR-33b overexpression group, the Wnt agonist induced Wnt and β-catenin protein expression by 2.01±0.04 and 0.71±0.05 fold, respectively, and suppressed Bax protein expression by 0.61±0.07-fold in the in vitro Ems model compared with miR-33b (Fig. 5F-I).

ZEB1 inhibition reduces the effect of anti-miR-33b on cell viability in Ems. To gain insight into the function of miR-33b on Ems, the effect of the ZEB1 inhibitor on the function of miR-33b in Ems was analyzed. Compared with the miR-33b downregulation group, ZEB1 inhibition reduced cell viability
by 0.81±0.05 (48 h) or 0.71±0.08-fold (72 h), increased LDH activity by 2.12±0.04 fold, increased caspase-3 and 9 activity by 2.72±0.14 and 2.16±0.12-fold, respectively, and apoptosis rate by 2.26±0.07 fold in the in vitro Ems model (Fig. 6).
Wnt activation inhibits the effect of miR-33b on cell viability in Ems. Furthermore, miR-33b mimics were co-transfected with Wnt agonist in the in vitro Ems model. Compared with the miR-33b overexpression group, Wnt activation inhibited cell growth by 1.46±0.06 (48 h) or 1.86±0.05 fold (72 h), LDH activity by 0.36±0.13 fold, caspase-3 and 9 activity by 0.39±0.03 and 0.48±0.12 fold, respectively, and apoptosis rate by 0.61±0.07 fold in the in vitro Ems model following miR-33b (Fig. 7).
Discussion

Ems is a common gynecological disease, which has similar features to malignancies, including cell growth, invasion, distant metastasis and recurrence, affecting the quality of life of patients (15). Ems is a benign disease with malignant behavior. Its morbidity has exhibited an increasing trend (15). However, its pathogenesis remains unclear (15). miRNAs are endogenous RNAs with regulatory functions discovered in eukaryotes and are able to suppress gene expression at the transcriptional level (5). As important regulatory molecules, miRNAs are involved in a series of vital life processes, including antiviral defense, hematopoiesis, organogenesis, cell proliferation, apoptosis, fat metabolism and tumorigenesis (5).

In the present study, it was demonstrated that miR-33b expression was upregulated in an Ems rat model. Wang and Ren (12) demonstrated that miRNA-33b is able to mediate the apoptosis of endometrial stromal cells. In the present study, miR-33b expression was examined in a rat model, and miR-33b expression in vivo may be analyzed in further studies.

A previous study has indicated that the Wnt/β-catenin signaling pathway may induce epithelial-mesenchymal transition (EMT) in epithelial cells (6). Research on the interaction between β-catenin and estrogen is available. The Wnt/β-catenin signaling pathway is highly conserved (16). Additionally, a previous study has verified that the Wnt/β-catenin signaling pathway serves a crucial role in ectopic endometrial cell adhesion, invasion and angiogenesis (17). The intracellular accumulation of β-catenin accounts for its principal mechanism of action (17). It activates its downstream target genes, including vascular epithelial growth factor and matrix metalloproteinases (MMPs), leading to abnormal cell proliferation, differentiation and maturation (18). Of these genes, MMP-9 is able to degrade extracellular matrix components, including type IV and type V collagen and gelatin (19). In addition, the Wnt/β-catenin signaling pathway may promote vascular endothelial cell growth, thus leading to angiogenesis (6). Furthermore, the Wnt/β-catenin signaling pathway is able to strengthen intercellular adhesion through mutual activation with integrin and, as a result, it serves a key role in ectopic adhesion, planting and the growth of endometrial cells (6). The present study demonstrated that overexpression of miR-33b may suppress ZEB1 protein expression and may reduce Wnt/β-catenin protein expression in Ems in vitro. Wang et al (20) demonstrated that miRNA-33b inhibited lung adenocarcinoma cell growth and invasion by suppressing the Wnt/β-catenin/ZEB1 signaling pathway. These results are consistent with the present ones, demonstrating that miRNA-33b may regulate the ZEB1/Wnt/β-catenin signaling pathway to induce cell death in Ems.

High ZEB1 expression has been demonstrated in numerous malignancies, including lung, colorectal, prostate and ovarian cancer (21). ZEB1 is able to promote the malignant phenotype of Ems mainly by regulating EMT (11). Similarly, high ZEB1 expression may be detected in breast cancer, and may regulate uterine cell adhesion and polarity alterations (21). Furthermore, it may promote the abnormal proliferation of uterine stem cells (22). A previous study demonstrated that ZEB1 may regulate estrogen receptor-α silencing (22). A previous study indicated that ZEB1 may also be involved in epigenetic regulation during Ems (21). In the present study, it was demonstrated that ZEB1 inhibition decreased the effect of anti-miR-33b on cell growth in Ems. Wang et al (20) demonstrated that miRNA-33b inhibited lung adenocarcinoma cell growth and invasion by suppressing Wnt/β-catenin/ZEB1 signaling. Wang et al (12) reported that the miR-33b/HMG12/Twist1/ZEB1 axis serves a critical role in regulating melanoma dissemination. The results of the present study suggested that miRNA-33b/ZEB1/Wnt/β-catenin inhibited cell growth in an Ems rat model. However, the down-stream molecular pathway of miRNA-33b/ZEB1/Wnt/β-catenin requires further study.

In conclusion, the present results demonstrated that upregulation of miRNA-33b may promote Ems via Wnt/β-catenin by ZEB1 expression. Thus, restoration of miRNA-33b expression may be used as a novel strategy for the treatment of Ems, although this requires further investigation.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors’ contributions

SZ designed the experiment; HZ, GL and XS performed the experiment; SZ and HZ analyzed the data; SZ wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Care and Use Committee of Affiliated Qilu Hospital of Shandong University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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