MiR-10b Directly Targets ZEB1 and PIK3CA to Curb Adenomyotic Epithelial Cell Invasiveness via Upregulation of E-Cadherin and Inhibition of Akt Phosphorylation

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Key Words
MiR-10b • Adenomyosis • E-cadherin • Akt

Abstract
Background/Aims: Adenomyosis is a disease in which ectopic endometrial glands and stromal cells appear in the uterine myometrium. Despite its prevalence, the molecular mechanisms involved in the development of adenomyosis are largely unknown. The aim of this study was to investigate the role of miR-10b and its target genes ZEB1 and PIK3CA in adenomyosis.

Methods: 1387 miRNAs in human normal endometrium and ectopic endometrial lesions of adenomyosis using a microarray screen assay. The significant differential expression of 10 miRNAs was confirmed by qRT-PCR. The expression of miR-10b in endometrial epithelial cells isolated from normal endometrium and paired eutopic and ectopic endometrium of adenomyosis was measured by qRT-PCR. Subsequently, the targets of miR-10b were predicted by bioinformatics and confirmed using a luciferase assay, and the mRNA and protein expression of ZEB1 and PIK3CA were assessed in the endometrium or endometrial epithelial cells by qRT-PCR and western blotting or immunohistochemical analysis. Cell migration and cell invasion of endometrial epithelial cells with different treatments by Transwell assays. The expression of p-AKT, Akt and E-cadherin proteins was determined by Western blot analysis.

Results: MiR-10b expression was significantly downregulated in both adenomyotic lesions and adenomyotic epithelial cells. MiR-10b overexpression in adenomyotic epithelial cells inhibited cell migration and invasion. We then demonstrated that miR-10b directly targets the 3′-UTRs of Y. Guo and X. Lang contribute equally to this work.
of ZEB1 and PIK3CA, and downregulates ZEB1 and PIK3CA in adenomyotic epithelial cells, leading to increased E-cadherin expression and decreased Akt phosphorylation. **Conclusions:** miR-10b directly targets ZEB1 and PIK3CA to curb adenomyotic epithelial cell invasiveness via upregulation of E-Cadherin and inhibition of Akt phosphorylation.

**Introduction**

Adenomyosis is a common, benign, gynecological disorder characterized by the invasion of endometrial epithelial cells into the myometrium of uterus. Adenomyosis can cause menorrhagia, dysmenorrhea, chronic pelvic pain, and preterm labor in women of reproductive age, and is associated with an increased risk of anemia [1, 2]. Substantial evidence indicates that adenomyosis is prompted and driven by the effects of estrogen [3]. Oestrogen-enhanced migratory and invasive properties of endometrial epithelial cells underlie the ability of the endometrium to infiltrate the myometrical junctional zone and the growth of ectopic tissue [3, 4]. However, the specific signaling pathways involved in the regulation of the motility and invasiveness of endometrial epithelial cells are not well understood.

MicroRNAs (miRNAs) are single-stranded noncoding RNAs of about 22 nucleotides that suppress the expression of target genes to regulate diverse cellular processes. Many miRNAs are implicated in tumor growth and metastasis [5]. Of these miRNAs, miR-10b is upregulated in a number of human cancers, promoting cancer cell migration and invasion [6-10]. However, miR-10b is found to be downregulated in endometrial cancers such as endometrial serous adenocarcinoma (ESA) and endometrioid endometrial carcinoma [11, 12]. In particular, the lower expression of miR-10b is significantly correlated with poor overall survival in ESA [11]. These data suggest that, opposite of its oncogenic activity in other cancer types, miR-10b acts as a tumor suppressor in cancers of endometrium origin. MiR-10b is also found to be downregulated in endometriotic tissues of women with endometriosis [13]. **In vitro** studies reveal that miR-10b directly targets syndecan-1 to curb invasiveness of human endometriotic cells [14], suggesting that miR-10b plays an inhibitory role in the development of endometriosis. However, the expression and function of miR-10b in adenomyosis, a condition closely related to endometriosis, have not been reported.

Epithelial–mesenchymal transition (EMT) is characterized by loss of cell-to-cell adhesion and increased cell motility, and is closely associated with the invasion and metastasis of several cancers [15]. One of the hallmarks of EMT is the down-regulation of E-cadherin, which is repressed by several transcription factors including ZEB1, ZEB2, SNAI1, SNAI2, Twist1, Twist2 and E12/E47 [16]. Interestingly, several recent studies have shown that E-cadherin expression is downregulated in epithelial component of human adenomyotic lesions, suggesting that EMT plays a crucial role in the development of adenomyosis [4, 17]. The PI3K/Akt survival pathway, which is activated by estrogen, is also implicated in the pathogenesis of adenomyosis [18, 19].

In the present work, we first compared the expression of miR-10b in human adenomyosis versus healthy controls. We subsequently investigated the function of miR-10b in the development of adenomyosis in the context of the migration and invasion of adenomyotic epithelial cells. Finally, we examined the regulatory relationship between miR-10b and putative target genes ZEB1 and PIK3CA and explored the involvement of E-cadherin and Akt, which act downstream of ZEB1 and PIK3CA in miR-10b-induced effects.

**Materials and Methods**

**Patients and tissue collection**

The study protocol was approved by the Ethics Review Board of JiangWan Hospital, Shanghai, China. The study was conducted in accordance with the Declaration of Helsinki and applicable regulations in China. All study participants gave written informed consent.
Paired eutopic and ectopic endometrial tissues were collected from premenopausal women with adenomyosis (n = 22, 30 - 39 years old) who underwent hysterectomy at JiangWan Hospital of Shanghai. These tissues were from different phases of the menstrual cycle. Normal endometrial tissues were collected from 13 premenopausal women without evidence of adenomyosis (n = 13, 29 – 41 years old) who underwent hysterectomy for subserousal leiomyoma at the same hospital. Samples were immediately frozen and stored in liquid nitrogen until analysis.

Microarray and analysis
Fresh-frozen normal endometrial tissues and ectopic endometrial tissues of adenomyosis in TRIzol reagent were shipped to KangChen Bio-tech (Shanghai, China). MiRNAs were extracted, labeled, and hybridized to the Phalanx human miRNA OneArray (v5.1) containing probes for 1387 human miRNAs. Data were normalized using median normalization method. A heat map was created using Cluster 3.0 and JAVA Treeview software. All experiments and data analysis were carried out at KangChen Bio-tech.

Quantitative real time PCR
Total RNA was extracted from tissues and cells using Trizol reagent (Invitrogen, USA) according to the manufacturer’s instructions. MiRNA cDNA was synthesized using a Mir-X miRNA First-Strand synthesis kit (Clontech, USA). Real-time quantitative PCR (qRT-PCR) was performed using a SYBR green qPCR kit (TaKaRa, Japan) on an Applied Biosystems 7300 QPCR System (Foster City, CA, USA). qRT-PCR was performed for 40 cycles (miRNAs: 95°C for 15 s and 60°C for 1 min after an initial 15 min incubation at 95°C; mRNAs: 95°C for 10 s and 72°C for 30 s after an initial 3 min incubation at 95°C). The data were analyzed according to the $2^{-\Delta\Delta Ct}$ method [20], and the fold change in the expression of each miRNA or mRNA was normalized to RNU6B or GAPDH. Sequences of primers used in PCR are shown in Table 1.

Isolation and culture of human endometrial epithelial cells
Freshly collected normal endometrium and paired eutopic and ectopic endometrial tissues were processed and separated into stromal and epithelial cell fractions following previously described procedures [21]. The identity of the epithelial cell fraction was confirmed by morphological analysis and the expression of the glandular epithelial marker CD9 by immunofluorescence staining. Cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) at 37°C, 5% CO$_2$ in a humidified incubator.

RNA oligoribonucleotides and plasmids
The shRNAs targeting human ZEB1 and PIK3CA transcripts were designated as shRNA-ZEB1 and shRNA-PIK3CA, respectively. The anti-miR-10b that had a sequence complementary to mature miR-10b was a 2′-O-methyl-modified oligoribonucleotide. The shRNAs and negative control shRNA-NC, anti-miR-10b and negative control anti-miR-NC, and miR-10b mimics (miR-10b) and negative control miR-NC mimics (miR-NC) were synthesized at Genepharma (Shanghai, P. R. China). All negative control RNAs had sequences that were non-homologous to any human genome sequences.

**Table 1.** Sequences of primers used in qRT-PCR

|        | Forward               | Reverse               |
|--------|-----------------------|-----------------------|
| miR-10b| 5′-CCAGAAGGTTGTAAGCTTGTCT-3′ | 5′-TGTCAGTCGCCATATATTCCTCC-3′ |
| miR-371b-5p | 5′-GGTAAACCTCAAAAGATGGCAGG-3′ | 5′-GCCACTAAGCCTGTTGAGG-3′ |
| miR-92b-5p | 5′-CTTTAAAGGCTGGGGAGGGG-3′ | 5′-TCCAGTCATATAGCTTGGTCCAGG-3′ |
| miR-30c | 5′-ACCACCGTGGTATGTTGAA-3′ | 5′-AGTAAACACCGTCTTCCAGG-3′ |
| miR-100 | 5′-AAACAAAGGGGAGAGCTGG-3′ | 5′-GCATTAAAGGAGAGGACCCAGG-3′ |
| miRNA-143 | 5′-CAATGTGCTGATCTGGTACA-3′ | 5′-TGGCAACAGGAATCTCCCGTTCTC-3′ |
| miR-513-3p | 5′-ACTTGCTTTTTCTGCTGATCC-3′ | 5′-CTCCGGGTGCCCAGGGGTATA-3′ |
| miR-513a | 5′-CCACATTCCAGGCCATCAGC-3′ | 5′-TGAGCACTGACAGCTCATT-3′ |
| miR-466 | 5′-ATACATAAAGGTCACAGCAT-3′ | 5′-GGAAACCGATGATGGTCC-3′ |
| miR-451a | 5′-GAACCCGTATTCTGTATAC-3′ | 5′-TCTTGTCTCAGGACAGCTC-3′ |
| RNU6B | 5′-CTGGCTTCGCGGCCAGCAG-3′ | 5′-AAAGCTTCAGATGTGTTCCG-3′ |
| ZEB1 | 5′-GAGCGGAGTGGGACCTTGAGA-3′ | 5′-GGAGATGGAGGAGGACTGATG-3′ |
| mRNM | 5′-CCAGATCATGCTTGGCGAGGT-3′ | 5′-CTTCAAAAAGGAGCCCAAACC-3′ |
| GAPDH | 5′-GTCAAAGGCTGAGAACGGGA-3′ | 5′-ATGAGAAGAAGTTGAGGG-3′ |
The wild type 3′-UTRs of human ZEB1 and PIK3CA were amplified from human genomic DNA by PCR using the following primers: pGL3-ZEB1 forward, 5′-GGCGAGATGGACGGAAGT-3′, reverse, 5′-ACCTGTATGCGATGCAAA-3′; pGL3-PIK3CA forward, 5′-TCATGGCTGGACAAACAA-3′, reverse, 5′-ACCTAAAGCATAACATGAATTGCG-3′. Mutant 3′-UTRs of ZEB1 and PIK3CA containing mutations at the miR-10b binding site were created using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA) following manufacturer’s instructions. The wild type and mutant 3′-UTRs were introduced into the pGL3 luciferase reporter vector (Promega, USA) downstream of the luciferase gene.

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**Cell transfections**

Endometrial epithelial cells isolated from ectopic endometrial tissues of adenomyosis patients were seeded in 12-well plates, cultured for 24 h, and then transfected with RNA oligoribonucleotides or plasmids. All transfections were performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions. Cells were collected 48 h after transfection and used in subsequent studies.

**Cell migration and invasion assays**

Cell migration and invasion assays were performed as previously described using uncoated and matrigel-coated Transwell systems, respectively (8-μm pore size, polycarbonate membrane; BD Biosciences, USA) [22]. Briefly, 5 × 10⁴ cells resuspended in 200 μl serum-free DMEM/F12 medium were added to the upper inserts whilst the lower chamber was loaded with 500 μl medium supplemented with 10% FBS, serving as a chemoattractant. After 24 h incubation at 37°C, cells that had moved through the membrane and adhered to the lower surface of the membrane were counted under a microscope.

**Luciferase reporter assay**

Dual-Luciferase reporter assays (Promega, USA) were performed following manufacturer’s instructions as previously described [23]. Briefly, HEK293T cells were seeded in 12-well plates and cultured in DMEM/F12 at 37°C for 24 h. Cells were subsequently co-transfected with 50 nM of miR-10b or miR-NC and 100 ng of firefly luciferase reporter plasmid comprising the wild-type or mutant 3′-UTR using Lipofectamine 2000 (Life Technologies, USA). Luciferase activity was detected 48 h after transfection and normalized to Renilla activity. Each experiment was conducted as duplicate and was repeated three times.

**Western blot analysis**

Whole cell lysates were obtained using the M-Per Mammalian Protein Extraction Reagent (Pierce Biotechnology, USA). Total proteins (20 μg) were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies to ZEB1 (1:2000; Abcam, Cambridge, MA), PIK3CA (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), Akt (1:10000; Abcam), phospho-Akt (Ser 473; 1:5000; Abcam), E-cadherin (1:500; Abcam), and β-actin (1:2000; Abcam), respectively. After extensive washing, membranes were incubated for 1 h at room temperature with goat polyclonal anti-rabbit IgG–H&L-pre-adsorbed (HRP) at 1:2000 dilution (Abcam). Bands were detected using the enhanced chemiluminescence (ECL) blotting analysis system (Amersham Pharmacia Biotech, UK). The densitometric values were determined using a gel image analysis system (Bio-Rad, Hercules, CA) normalized to β-actin.

**Immunohistochemical analysis**

Formalin-fixed, paraffin-embedded blocks of normal endometrium and paired eutopic and ectopic endometrial tissues of adenomyosis were subjected to immunohistochemical analysis for the expression of ZEB1 and PIK3CA. The streptavidin–biotin peroxidase complex technique was used for staining as previously described [24]. The formalin-fixed, paraffin-embedded sections were stained with rabbit monoclonal antibody to ZEB1 (1:100; Abcam) or PIK3CA (1:200; Santa Cruz Biotechnology). The degree of the sections was evaluated independently by two observers using light microscopy (Olympus BX-50; Olympus Optical, Tokyo, Japan) (10 fields per section, 200×). Sections were scored according to the percentage of stained cells: <1%, negative (-); 1–5%, borderline (+); 5–25%, intermediate (+); 25–50%, moderate (++); 50–75%, strong (+++); 75–100%, very strong (++++). Cells were then classified as either negatively stained (-/±) or positively stained (+/++/+++/++++).
Fig. 1. Identification of deregulated miRNAs in human adenomyosis. (A) Heat map showing the unsupervised filtered clustering of 156 differentially expressed microRNAs between human normal endometrium (Normal) and ectopic endometrial lesions of adenomyosis (Lesions). (B) Heat map showing the clustering of 46 significantly deregulated miRNAs. (C) The normalized absolute and relative signal intensity of 10 most significantly deregulated miRNAs. The relative signal intensity is presented as the ratio and log-2 transformed ratio of Lesions to Normal. (D) Expression of the 10 most significantly deregulated miRNAs in Normal and Lesions by qRT-PCR. Data were normalized to RNU6B. *p<0.05. (E) MiR-10b expression in normal endometrium (n = 13) and paired eutopic and ectopic endometrial tissues of adenomyosis (n = 22, respectively) by qRT-PCR. Data were normalized to RNU6B. *p<0.05.

Statistical analysis

Data are interpreted using the GraphPad Prism 5 software (GraphPad Software, Inc, San Diego, CA). All results are presented as the mean ± SD (standard deviation). To determine statistical differences between two groups Student’s t-test was used. ANOVA (one-way analysis of variance or repeated measures) was used with the post hoc Bonferroni’s test for the comparison of more than two groups. Differences with a p value less than 0.05 were considered statistically significant.

Results

MiR-10b expression is downregulated in human adenomyosis

To identify miRNAs deregulated in human adenomyosis, we analyzed the expression of 1387 miRNAs in human normal endometrium and ectopic endometrial lesions of adenomyosis using a microarray screen assay. Of the 156 miRNAs that showed differential expression (Fig. 1A), 46 miRNAs were found to be significantly deregulated in ectopic endometrial lesions of adenomyosis versus normal endometrium (p<0.05) (Fig. 1B).
In particular, miR-10b, miR-371b-5p, miR-92b-5p, miR-30c, and miR-100 were most significantly downregulated whilst miR-143, miR-532-3p, miR-513a, miR-466, and miR-451a were most significantly upregulated (Fig. 1C). The significant differential expression of...
MiR-10b inhibits adenomyotic epithelial cell migration and invasion

Previous studies have reported that miR-10b is downregulated in human endometriosis [13]. Additionally, miR-10b functions to curb the invasiveness of human endometriotic epithelial cells [14]. Having found that miR-10b is also downregulated in human adenomyotic lesions, we proceeded to test the effects of miR-10b on the migration and invasion of adenomyotic epithelial cells. Our qRT-PCR results showed that miR-10b was expressed in endometrial epithelial cells isolated from normal endometrium with a progressive decline in expression in cells isolated from paired eutopic and ectopic endometrium of adenomyosis (Fig. 2A), and the rate of cell migration (Fig. 2B) and invasion (Fig. 2C) was inversely correlated with the level of miR-10b expression. Furthermore, in endometrial epithelial cells isolated from ectopic endometrium of adenomyosis, miR-10b overexpression significantly diminished the rate of cell migration and invasion (Fig. 2D-2F). Taken together, these data suggest that...
miR-10b represses the invasiveness of adenomyotic epithelial cells, potentially playing an inhibitory role in the development of adenomyosis.

**MiR-10b downregulates ZEB1 and PIK3CA expression by directly targeting their 3'-UTRs**

To explore the mechanisms underlying miR-10b-induced inhibition of the migration and invasion of adenomyotic epithelial cells, we used computational algorithms to identify potential miR-10b target genes. Our *in silico* analyses using three target prediction softwares (TargetScan, DIANA-microT and microRNA.org) identified putative binding sites for miR-10b in the 3'-UTRs of ZEB1 and PIK3CA. To test whether ZEB1 and PIK3CA are direct targets of miR-10b, we prepared luciferase reporter systems using the 3'-UTRs of human ZEB1 and PIK3CA, respectively, containing the putative miR-10b binding site (Fig. 3A). We also created luciferase expression systems using mutant 3'-UTRs in which several nucleotides at the putative binding site were mutated to disrupt miR-10b binding (Fig. 3A). Co-transfection of the luciferase reporter system containing the wild type 3'-UTR of ZEB1 or PIK3CA with miR-10b in HEK293T cells resulted in significant loss of luciferase reporter expression compared with miR-NC (p<0.05, Fig. 3B). However, luciferase expression was not affected by co-transfection with miR-10b when the wild type 3'-UTR was replaced with mutant 3'-UTR.

**Fig. 4.** ZEB1 and PIK3CA expression is upregulated in human adenomyosis. (A) Images of normal endometrium and paired eutopic and ectopic endometrial tissues of adenomyosis subjected to immunohistochemical analysis. (B) Semi-quantitative assessment of ZEB1 and PIK3CA protein expression using immunostaining scores. (C) ZEB1 and PIK3CA mRNA expression determined by qRT-PCR. Data were normalized to GAPDH. *p<0.05, **p<0.01 versus normal.
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ZEB1 and PIK3CA expression is upregulated in human adenomyosis

To explore the potential role of ZEB1 and PIK3CA in human adenomyosis, we analyzed their protein expression in human normal endometrium and paired eutopic and ectopic endometrial tissues of adenomyosis by immunohistochemical analysis. Visual examination of stained tissues revealed progressively increased protein expression of both ZEB1 and PIK3CA in paired human eutopic and ectopic endometrium of adenomyosis compared with that in normal endometrium (Fig. 4A). These results were confirmed by semi-quantitative assessment of protein expression using immunostaining scores (Fig. 4B). qRT-PCR analysis revealed similar progressive increases in mRNA expression of ZEB1 and PIK3CA in these tissues (Fig. 4C). Thus, ZEB1 and PIK3CA expression was upregulated in human adenomyosis and was inversely correlated with miR-10b expression, suggesting that they potentially play a role in the development of human adenomyosis.
**MiR-10b upregulates E-cadherin expression and inhibits Akt phosphorylation in adenomyotic epithelial cells**

The cell-cell adhesion molecule E-cadherin and the PI3K/Akt pathway are implicated in the development of adenomyosis [4, 17-19]. E-cadherin is negatively regulated by transcription factor ZEB1 whilst the p110α catalytic subunit of PI3K is encoded by PIK3CA. Our findings that ZEB1 and PIK3CA are direct targets of miR-10b suggest that E-cadherin and Akt might be involved in the mechanisms underlying miR-10b-induced effects on adenomyotic epithelial cells. Indeed, in endometrial epithelial cells isolated from ectopic endometrial tissues of adenomyosis, miR-10b overexpression-induced inhibition of cell migration and invasion was accompanied by increased E-cadherin expression and decreased Akt phosphorylation. ZEB1 and PIK3CA knockdown in these cells produced similar effects on E-cadherin expression and Akt phosphorylation, respectively, and diminished cell migration and invasion. Collectively, these data provided evidence that the ZEB1/E-cadherin and PI3K/Akt signaling pathways are involved in miR-10b-induced inhibition of adenomyotic epithelial cell migration and invasion.

**Discussion**

MiRNAs have been implicated in endometriotic lesion development [25], regulating endometriosis-associated processes including inflammation, tissue repair, cell growth and apoptosis, and angiogenesis [26]. In particular, miR-10b is downregulated in human endometriosis [13] and directly targets syndecan-1 to inhibit endometriotic epithelial cell invasiveness via downregulation of metalloproteinase activity and interleukin-6 [14]. However, the role of miR-10b in adenomyosis, a condition closely related to endometriosis, is unknown. In the present study, we found that miR-10b expression was significantly downregulated in human adenomyotic lesions and adenomyotic epithelial cells. Compared with normal endometrial tissues, eutopic and ectopic endometrial tissues of adenomyosis showed progressively declined miR-10b expression and progressively enhanced cell migration and invasion. Additionally, miR-10b overexpression reduced the migratory and invasive capacity of adenomyotic epithelial cells. To explore the mechanisms underlying these effects, we searched for potential targets of miR-10b and demonstrated that miR-10b directly targets the 3'-UTRs of ZEB1 and PIK3CA using a luciferase reporter assay. We also showed that in adenomyotic epithelial cells, miR-10b overexpression downregulated ZEB1 and PIK3CA expression, leading to increased E-cadherin expression and reduced Akt phosphorylation. Collectively, these results suggest that miR-10b directly targets ZEB1 and PI3K to curb adenomyotic epithelial cell invasiveness via upregulation of E-cadherin and inhibition of Akt phosphorylation.

Adenomyosis is enigmatic in terms of etiology. Oestrogen-enhanced migratory and invasive properties of endometrial epithelial cells play a crucial role in the development of adenomyosis [3, 4]. Our results suggest that miR-10b inhibits the development of adenomyosis by curtailing the invasiveness of adenomyotic epithelial cells. To our knowledge, this is the first report on the role of miRNAs in human adenomyosis. The major limitation of our study is the sample size. It only comprised 22 adenomyosis patients and 13 control women. And the study should be followed by larger population-based and multiethnic studies to confirm our findings. Moreover, the function of miR-10b and other miRNAs in adenomyosis needs to be further investigated.

Current treatment for adenomyosis includes anti-inflammatory medications or hormone therapy to control the pain associated with the disease. The only definitive cure for adenomyosis is a hysterectomy. Uterus-sparing operative treatment is feasible but shows only limited efficacy [27]. Our findings suggest that pharmaceutical interventions targeting miR-10b may provide novel therapy for adenomyosis treatment.
Acknowledgements

This study was supported by grants from the Science and Technology Commission of Shanghai Municipality (No. 12401902901) and the National Natural Science Foundation of China (No. 81441097).

Disclosure Statement

All authors are in agreement with the content of this manuscript. The authors declare no conflict of interest.

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