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miR-326 regulates EMT and metastasis of endometrial cancer through targeting TWIST1

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Abstract. – OBJECTIVE: Endometrial carcinoma is the most common malignancy of the female genital tract. Therefore, there is an urgent need to understand the molecular mechanism of its metastasis. This study is aimed to explore the function and underlying mechanism of miR-326 in endometrial cancer (EC).

PATIENTS AND METHODS: RT-PCR was used to evaluate the miR-326 expression in EC tissues and cell lines. The CKK-8 was used to detect the EC cells proliferation. Transwell assay was performed to evaluate the metastasis of EC cells. Targeted genes were predicted by a bioinformatics algorithm. Dual-luciferase reporter assays were performed to examine the regulation of putative miR-326 targets. The expression of TWIST1 and EMT-related proteins was assayed using Western blot.

RESULTS: Our results proved that miR-326 expression was downregulated in EC cell lines and tissue samples. *In vitro* assays, our results indicated that over-expression of miR-326 inhibited cell proliferation, migration, invasion, and EMT. Moreover, Bioinformatics analysis revealed Twist homolog 1 (TWIST1), a putative tumor promoter, to be a potential target of miR-326. Results from a dual-luciferase reporter system supported TWIST1 as a direct target gene of miR-326. In addition, Western blot showed that over-expression of miR-326 resulted in decreased TWIST1 expression in EC cells. Final *in vitro* assays revealed that knockdown of TWIST1 inhibited EC cell, migration, invasion and EMT, suggesting that miR-326 exerted its tumor-suppressive role by targeting TWIST1.

CONCLUSIONS: We demonstrate that miR-326 served as a tumor suppressor by targeting TWIST1, and may serve as a biomarker or therapeutic target for patients with EC.

Key Words
MiR-326, Endometrial cancer, EMT, Metastasis, Proliferation.

Introduction

Endometrial cancer (EC) is one of the most frequent gynecologic malignancy, and its incidence is increasing[1]. In Europe, there are approximately 9000 women dying from EC each year[2]. EC includes a broad range of histologic subtypes, the most common being endometrioid[3]. Because few diagnostic or therapeutic biomarkers were identified or applied in clinics, poor prognosis is observed in a large portion of EC patients, especially for those patients diagnosed with EC at an advanced[4-6]. Therefore, it is critical to better understand the molecular mechanisms of endometrial cancer and explore new therapeutic target.

miRNAs are a class of single stranded small noncoding RNAs, 18-25 nucleotides (nt) in length, which exist widely in the eukaryotic organisms[7]. It has been confirmed that regulate gene expression at the transcriptional or post-transcriptional level by binding to the complementary 3'-UTR of mRNAs[8]. Growing findings suggest that miRNA deregulation participates in various diseases, including human tumors[9]. Additionally, miRNAs have identified as important regulators of proliferation, migration, invasion, and apoptosis in tumor cells[10,11]. Previous studies[12,13] indicated that miRNAs served as either oncogene or tumor suppressor according to their targeting genes. Thus, miRNAs have great potential for early diagnosis, prognosis, and treatment of EC.

TWIST1, a transcription factor, plays an important role in cancer development and progression[14]. Up-regulation of TWIST1 induces EMT and E-cadherin repression, indicating that TWIST1 promotes metastasis by inducing EMT[15]. Previous investigations[16,17] have suggested that some miRNAs can regulate the expression levels of TWIST1

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by bind to TWIST1 mRNA. Previous reports demonstrated that dysregulation of miR-326 is associated with various tumor progression, including non-small cell lung cancer[18], osteosarcoma[19], and glioblastoma[20]. However, the expression pattern, possible functions and underlying mechanisms of miR-326 in EC have not been reported. In the present work, we identified a previously unknown miR-326/TWIST1/EMT molecular network may be involved in EC development.

Patients and Methods

Patients and Tissue Samples

Tumor samples and the adjacent normal tissues were obtained from 56 EC patients who underwent a hysterectomy at Shandong Provincial Qianfoshan Hospital. None of these patients had received chemotherapy before surgery. All specimens were immediately frozen in liquid nitrogen and stored at -70°C until use. All specimens had been histologically and clinically diagnosed by two independent experienced pathologists. Written consent was always obtained in a formal style after approval by the local Ethics Committee.

Cell Culture and Transfection

Human EC cell lines (HEC-1B, RL95-2, and AN3CA) and normal human endometrial epithelial cells (NEEC) were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). These cell lines were cultured in RPMI 1640 or DMEM (Gibco Brl, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, GE Healthcare Life Sciences, HyClone Laboratories, South Logan, UT, USA). All cells were grown under sterile conditions at 37°C in a humidified atmosphere of 5% CO^2 and 95% air. The miR-326 mimic and a negative control were all purchased from Genecopoeia (Guangzhou, China). Transfection of cells with oligonucleotides was performed using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. TWIST1 siRNAs were purchased from GenePharma Company (Shanghai, China). Transfection was performed in the same way.

RNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from tumor tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified with an RNeasy Maxi kit (Qiagen, Valencia, CA, USA). cDNAs were synthesized using 1 μg total RNAs and PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, Liaoning, China). The levels of miR-326 were detected by Real-time polymerase chain reaction (RT-PCR), which was performed using an ABI 7900 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). GAPDH was used as the reference gene for normalization of expression. The relative amount of miR-326 to GAPDH was calculated using the equation $2^{-\Delta Ct}$, where $\Delta CT = (CT^{miR\text{-}326} - CT^{GDAPH})$. The PCR primers for mature miR-326 and GDAPH were purchased from Invitrogen (Carlsbad, CA, USA). All samples were performed in triplicate and independently repeated three times.

Cell Counting Kit-8 (CCK-8)

Cell proliferation was quantified using CCK-8. Briefly, cells were plated per well in 96-well plates, cultured for 24 h. After transfection and incubation for 48 h, 10 μL of CCK-8 reagent was added to each well and incubated at 37 °C for 2 h. The absorbance was determined by scanning at 450 nm with a microplate reader. The results represent the average of three replicates under the same conditions.

Transwell Assay

Migration and invasion assays were performed as described previously using transwell chambers[21].

Western Blot Analysis

Western blot was performed using Mini-PROTEAN® Tetra Cell Systems (Bio-Rad, Hercules, CA, USA) on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The cells were transfected with either miR-NC or miR-326. 72 hours post-transfection, the cells were collected and lysed. The proteins were resolved by 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. Skim milk (5%) was added to block nonspecific binding. After 1 hour incubation in the dark at room temperature, 1:200 diluted rabbit anti-human TWIST1 polyclonal antibody was added. After washing with PBS 3 times, the membranes were incubated with their corresponding secondary antibodies at room temperature for 1 h. For the EMT-related proteins, the first antibody was used according to the specific proteins. Finally, The bands were then visualized by chemiluminescence (Millipore, Billerica, MA, USA) after washing in TBS.

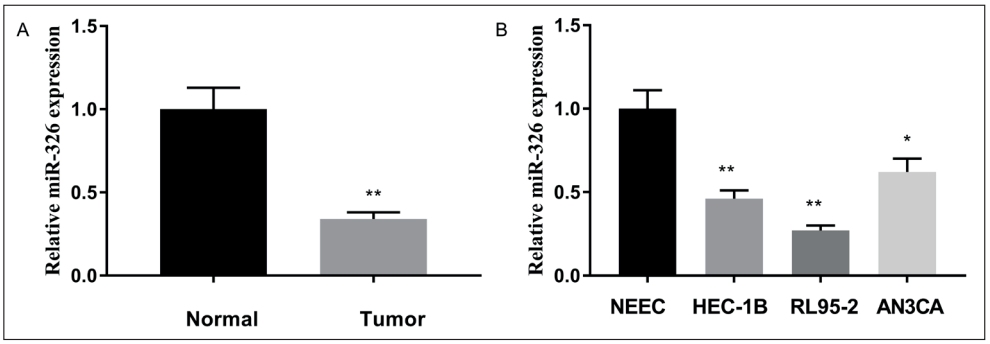

Figure 1. miR-326 was down-regulated in EC tissues and cell lines. **A**, Relative levels of miR-326 in 36 paired samples of EC tissues and adjacent non-tumorous tissues were evaluated by qRT-PCR. **B**, Expression of miR-326 in EC cell lines, as detected by quantitative real-time PCR. $**p < 0.01$, $*p < 0.05$.

Target Prediction Analysis

PicTar, TargetScan, and MicroRNA.org were used for miR-326 target prediction, and to verify whether TWIST1 is a direct target gene of miR-326.

Luciferase Reporter Assay

For dual-luciferase reporter assay, 3×10^4 cells were seeded in 24-well plates and incubated for about 12 h. The miR-326 mimics or NC were transiently transfected into cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Co-transfections of TWIST1 3'-UTR or mut-TWIST1 3'-UTR plasmid with miR-326 lentivirus vector into the cells were accomplished in the same way. Firefly luciferase activity was standardized to the renilla activity as the control.

Statistical Analysis

All statistical analyses were carried out using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Counting data were reported as means ± standard deviation (SD). Student's *t*-test was used to analyze the difference between two groups. $p < 0.05$ was considered statistically significant.

Results

miR-326 was Down-Regulated in Human EC Tissues and Cell Lines

We first detected the expression level of miR-326 in EC tissues and human EC cells (HEC-1B, RL95-2, and AN3CA), as well as matched normal endometrial tissues and NEEC. As shown in Figure 1A, the results showed that expression of miR-326 was downregulated in EC tissues compared with the adjacent normal bone tissues. Similarly, we also observed that miR-326 expression was lower in human EC cell lines compared with normal cells (Figure 1B).

miR-326 Suppressed the Proliferation of EC Cells

Next, we investigated the biological function of miR-326 in EC. The HEC-1B and RL95-2 cells were transfected with miR-326 mimics or the respective controls. RT-PCR was used to determine the transfection effect, and miR-338-3p expression was restored in EC cell lines (Figure 2A). Then, we investigated the effect of miR-326

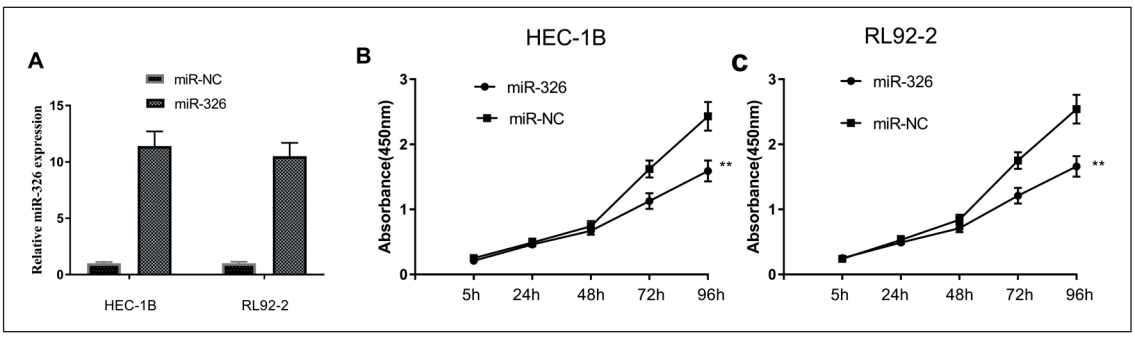

Figure 2. Over-expression of miR-326 inhibited proliferation of HEC-1B and RL92-2 cells. **A**, Transfection efficiency was evaluated by qRT-PCR in HEC-1B and RL92-2 cells transfected with miR-326 mimics. **B-C**, The CCK-8 assay was performed to analyze the effect of miR-326 on the proliferation of HEC-1B (**B**) and RL92-2 (**C**) cells. $**p < 0.01$, $*p < 0.05$.

on the growth of HEC-1B and RL95-2 cells using CKK-8. As shown in Figure 2B and 2C, forced of miR-326 significantly suppressed proliferation of HEC-1B and RL95-2 cells compared with the control group ($p < 0.01$, respectively).

miR-326 inhibited EC cell migration and invasion by suppressing EMT

To further characterize the functional importance of miR-326 in EC progression, we examined its effect on invasion and migration of HEC-1B and RL95-2 cells by transwell assays. It was found that overexpression of miR-326 significantly inhibited the migration (Figure 3A) and invasion(Figure 3B) capacities in both HEC-1B and RL95-2 cells. Then, we further investigated whether miR-485-suppressed EC metastasis was mediated by the EMT process. As shown in Figure 3C and 3D, The results of western blot showed that upregulated miR-326 expression resulted in evaluated E-cadherin expression and decreased N-cadherin, fibronectin and vimentin expression. This finding indicated that miR-326 exerted its tumor-suppressive role by modulating EMT process.

TWIST1 was a Direct Target of miR-326

We performed bioinformatics analysis to identify potential targets of miR-326 using the website microRNA.org. As shown in Figure 4A, we found that miR-326 has a putative binding site with the 3'-UTR of TWIST1. Then, we performed luciferase reporter assay to further validate whether TWIST1 is a direct target of miR-326. As shown

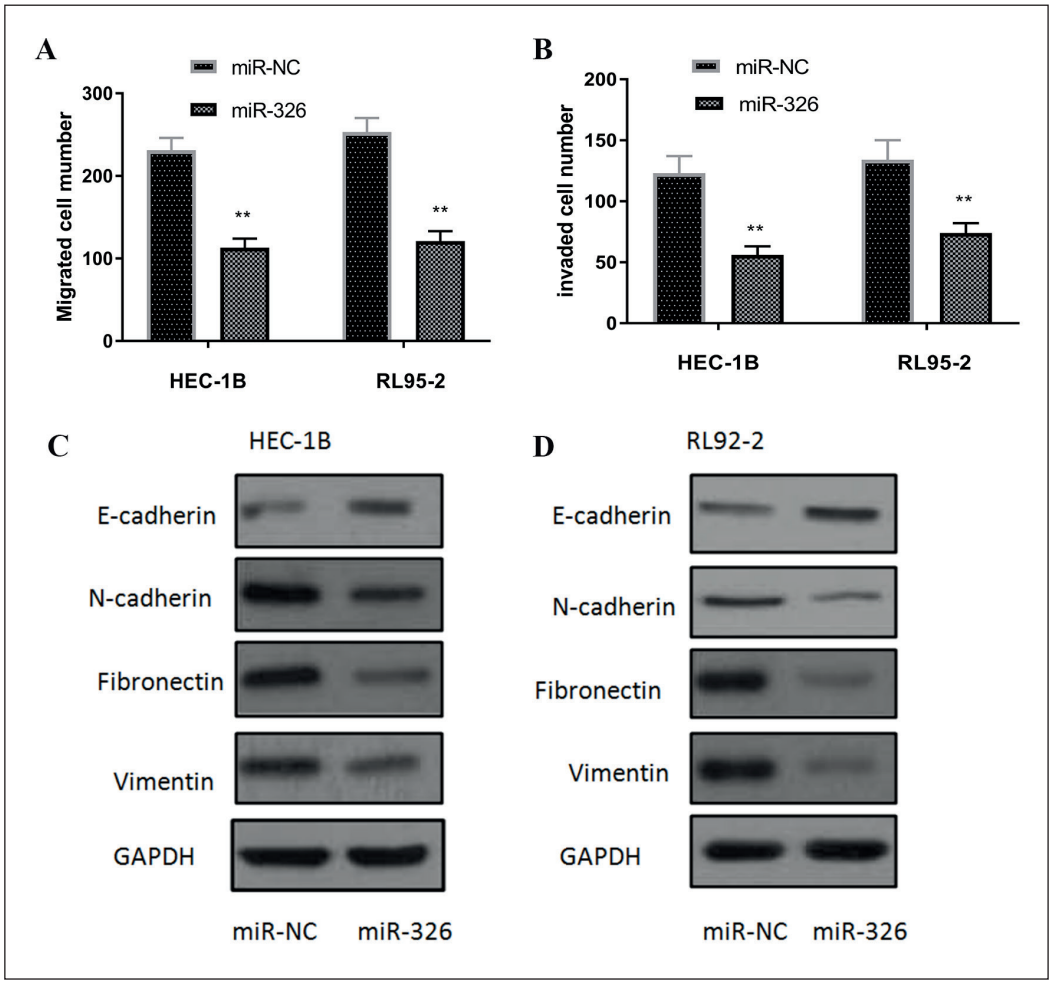

Figure 3. miR-326 inhibited invasion and migration of EC cells via down-regulation of EMT. **A-B**, Transwell assays were performed to investigate the migratory and invasive ability in HEC-1B and RL92-2 cells. **C-D**, Western blot analysis of the phenotypic markers, including E-cadherin, N-cadherin, fibronectin and Vimentin in HEC-1B and RL92-2 cells transfected with miR-326 mimics. $^{**}p < 0.01$, $^{*}p < 0.05$.

in Figure 4B, our results showed that overexpression of miR-326 in EC cells suppressed the activity of reporter gene ($p < 0.05$), whereas mutants plasmids showed no changes in the reporter gene activity in EC cells. Next, we performed western blot to confirm whether mR-326 could influence the expression level of TWIST1. As predicted, we observed that overexpression of miR-326 significantly downregulated the expression of TWIST1 at protein levels in EC cell lines(Figure 4C). Then, we used si-TWIST1 to knock down the expression of TWIST1 and explore the biological effect of TWIST1. The effects of TWIST1 knockdown on cell migration and invasion were evaluated by a transwell assay. As shown in Figure 4D and 4E, it was observed that knockdown of TWIST1 significantly decreased the EC cell migration and invasion ($p < 0.05$, respectively). Finally, we explored whether TWIST1 affected EMT process. Similar to the effect of miR-326 overexpression,

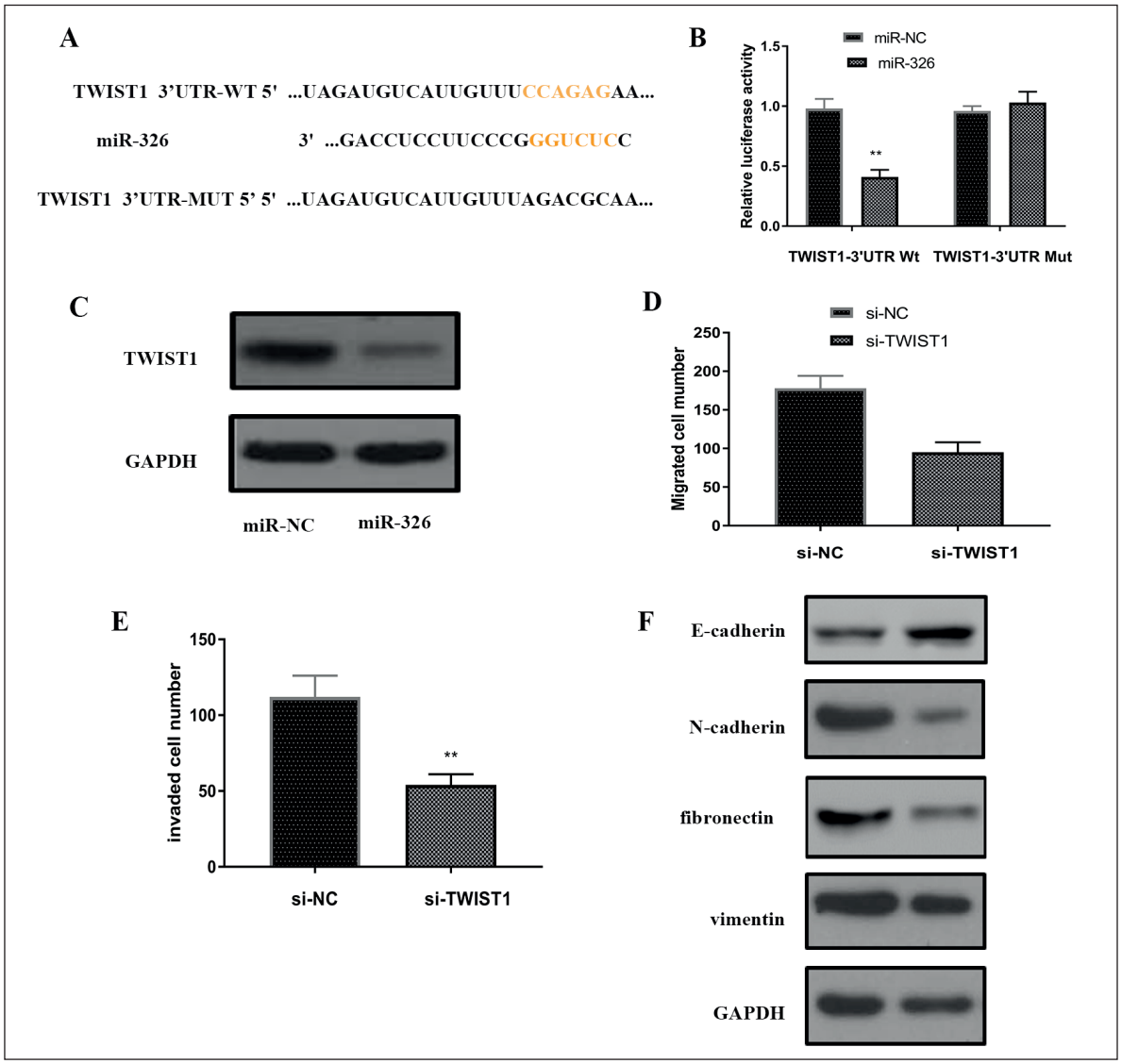

Figure 4. The 3'-UTR of TWIST1 mRNA is a target for miR-326. **A**, Bioinformatic analysis revealed that the targeting association between miR-326 and TWIST1 is evolutionarily conserved. **B**, Relative luciferase activity in HEC-1B cells co-transfected with miR-326 and a luciferase reporter containing the Slug 3'UTR (WT) or a mutant (Mut). **C**, miR-326-mimics inhibited the expression of TWIST1 at the protein level in the transfected HEC-1B cells. **D-E**, The transwell invasion assay showed that in the si-TWIST1 group, the number of invaded and migrated cells decreased significantly compared to cells transfected with si-NC. **F**, Western blot analysis of the phenotypic markers, including E-cadherin, N-cadherin, fibronectin, and vimentin in HEC-1B cells transfected with si-TWIST1. $^{**}p < 0.01$, $^{*}p < 0.05$.

knockdown of TWIST1 also exerted the same effect of suppressing EMT process(Figure 4E). Taken together, the above findings indicated that TWIST1 was an authentic target of tumor suppressor miR-326.

Discussion

A better understanding of the biological mechanisms of miRNAs may provide a novel way for early diagnosis and therapy of various malignance. Increasing researches suggested miR-326 as a tumor suppressor in various tumors. For instance, Zhou et al[22] reported that in glioma miR-326 was downregulated and it suppressed the proliferation of glioma cells by blocking NOB1. Li et al[23] found that the expression of miR-326 in gastric cancer tissues was significantly down-regulated, and its down-expression was associated with a poorer overall survival. Moreover, *in vitro* experiment showed that forced expression of miR-326 suppressed gastric cancer proliferation and metastasis by targeting FSCN1. Another study by Wu et al[24] confirmed miR-326 as a tumor suppressor in colorectal cancer by targeting the nin one binding protein. All of these findings provide strong evidence that miR-326 played a critical negative role in the development and progression of tumors. In the present investigation, to our best knowledge, we firstly showed down-expression of miR-326 in EC tissues and cell lines, suggesting that miR-326 may serve as a tumor suppressor in EC progression. *In vitro* assay confirmed our hypothesis.

The EMT is a complex process in which epithelial cells lose their identity and undergo a transition to mesenchymal[25]. TWIST1 is a critical oncogene that is overexpressed and plays critical roles in EMT process in various tumors, including EC[26,27]. Recent studies showed that some miRNAs may exert its role in regulating tumor proliferation and metastasis by targeting TWIST1. For instance, Sun et al[28] reported that up-regulation of miR-548c significantly suppressed whereas knockdown of miR-548c dramatically induces ovarian cancer cell proliferation, migration and invasion downregulation of Twist. More importantly, recent findings by Dong et al[29] showed that miR-106b inhibited endometrial cancer proliferation, invasion, and EMT by targeting TWIST1. In the present work, we checked TargetScan, and MicroRNA.org and found that TWIST1 may be a target of miR-326. Thus, our attention focused on the association between miR-326 and TWIST1, as well as EMT.

To confirm the above hypothesis, we performed luciferase reporter assay, and the results showed suggest TWIST1 as the potential target gene of miR-326. Forced expression of miR-326 significantly decreased the levels of TWIST1. To further explore the role of TWIST1 in EC cells, we used specific siRNA targeting TWIST1 to inhibit TWIST1 protein expression. Further *in vitro* assay showed that knockdown of TWIST1 suppressed EC cells migration, invasion, and EMT. These results imply that miR-326 might be a good metastasis suppressor in EC.

Conclusions

We first demonstrated that miR-326 was low expressed in EC tissues and cell lines, and forced expression of miR-326 significantly suppressed EC proliferation and metastasis. We further provide the first evidence that the tumorigenesis of EC may be partly due to the down-expression of miR-326 to promote downstream TWIST1/EMT signaling, suggesting the role of miR-326 as a theoretical basis of a novel targeted therapy for EC.

Conflict of Interest:

The Authors declare that there are no conflicts of interest.

References

1) Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012; 62: 10-29.
2) Lette M, Bemelmans WJ, Breda J, Slobbe LC, Dias J, Boshuizen HC. Health care costs attributable to overweight calculated in a standardized way for three European countries. Eur J Health Econ 2016; 17: 61-69.
3) Ray M, Fleming G. Management of advanced-stage and recurrent endometrial cancer. Semin Oncol 2009; 36: 145-154.
4) Yoon A, Park JY, Park JY, Lee YY, Kim TJ, Choi CH, Bae DS, Kim BG, Lee JW, Nam JH. Prognostic factors and outcomes in endometrial stromal sarcoma with the 2009 FIGO staging system: a multicenter review of 114 cases. Gynecol Oncol 2014; 132: 70-75.
5) Chen Y, Ren YL, Li N, Yi XF, Wang HY. Serum human epididymis protein 4 vs. carbohydrate antigen 125 and their combination for endometrial cancer diagnosis: a meta-analysis. Eur Rev Med Pharmacol Sci 2016; 20: 1974-1985.

6) Banno K, Nogami Y, Kisu I, Yanokura M, Umene K, Masuda K, Kobayashi Y, Yamagami W, Susumu N, Aoki D. Candidate biomarkers for genetic and clinicopathological diagnosis of endometrial cancer. Int J Mol Sci 2013; 14: 12123-12137.
7) Plaisance-Bonstaff K, Renne R. Viral miRNAs. Methods Mol Biol 2011; 721: 43-66.
8) Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215-233.
9) Kwak PB, Iwasaki S, Tomari Y. The microRNA pathway and cancer. Cancer Sci 2010; 101: 2309-2315.
10) Almeida MI, Reis RM, Calin GA. MicroRNA history: discovery, recent applications, and next frontiers. Mutat Res 2011; 717: 1-8.
11) Xia M, Li H, Wang JJ, Zeng HJ, Wang SH. MiR-99a suppress proliferation, migration and invasion through regulating insulin-like growth factor 1 receptor in breast cancer. Eur Rev Med Pharmacol Sci 2016; 20: 1755-1763.
12) Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 2006; 6: 259-269.
13) Guo YJ, Liu JX, Guan YW. Hypoxia induced upregulation of miR-301a/b contributes to increased cell autophagy and viability of prostate cancer cells by targeting NDRG2. Eur Rev Med Pharmacol Sci 2016; 20: 101-108.
14) Zhao Z, Rahman MA, Chen ZG, Shin DM. Multiple biological functions of Twist1 in various cancers. Oncotarget 2017; 8: 20380-20393.
15) Yuan W, Li T, Mo X, Wang X, Liu B, Wang W, Su Y, Xu L, Han W. Knockdown of CMTM3 promotes metastasis of gastric cancer via the STAT3/Twist1/EMT signaling pathway. Oncotarget 2016; 7: 29507-29519.
16) Guo XF, Wang AY, Liu J. HIFs-MiR-33a-Twsit1 axis can regulate invasiveness of hepatocellular cancer cells. Eur Rev Med Pharmacol Sci 2016; 20: 3011-3016.
17) Drasin DJ, Guarnieri AL, Neelakantan D, Kim J, Cabrera JH, Wang CA, Zaberezhnyy V, Gasparini P, Cascione L, Huebner K, Tan AC, Ford HL. TWIST1-induced miR-424 reversibly drives mesenchymal programming while inhibiting tumor initiation. Cancer Res 2015; 75: 1908-1921.
18) Sun C, Huang C, Li S, Yang C, Xi Y, Wang L, Zhang F, Fu Y, Li D. Hsa-miR-326 targets CCND1 and inhibits non-small cell lung cancer development. Oncotarget 2016; 7: 8341-8359.
19) Cao L, Wang J, Wang PQ. MiR-326 is a diagnostic biomarker and regulates cell survival and apoptosis by targeting Bcl-2 in osteosarcoma. Biomed Pharmacother 2016; 84: 828-835.
20) Nawaz Z, Patil V, Paul Y, Hegde AS, Arivazhagan A, Santosh V, Somasundaram K. PI3 kinase pathway regulated miRNome in glioblastoma: identification of miR-326 as a tumour suppressor miRNA. Mol Cancer 2016; 15: 74.
21) Li Y, Zhang Z, Liu X, Huang T, He W, Shen Y, Liu X, Hong K, Cao Q. miR-124 functions as a tumor suppressor in the endometrial carcinoma cell line HEC-1B partly by suppressing STAT3. Mol Cell Biochem 2014; 388: 219-231.
22) Zhou J, Xu T, Yan Y, Qin R, Wang H, Zhang X, Huang Y, Wang Y, Lu Y, Fu D, Chen J. MicroRNA-326 functions as a tumor suppressor in glioma by targeting the Nin one binding protein (NOB1). PLoS One 2013; 8: e68469.
23) Li Y, Gao Y, Xu Y, Ma H, Yang M. Down-regulation of miR-326 is associated with poor prognosis and promotes growth and metastasis by targeting FSCN1 in gastric cancer. Growth Factors 2015; 33: 267-274.
24) Wu L, Hui H, Wang LJ, Wang H, Liu QF, Han SX. MicroRNA-326 functions as a tumor suppressor in colorectal cancer by targeting the nin one binding protein. Oncol Rep 2015; 33: 2309-2318.
25) Smith BN, Bhowmick NA. Role of EMT in metastasis and therapy resistance. J Clin Med 2016; 5: E17.
26) Tran DD, Corsa CA, Biswas H, Aft RL, Longmore GD. Temporal and spatial cooperation of Snail1 and Twist1 during epithelial-mesenchymal transition predicts for human breast cancer recurrence. Mol Cancer Res 2011; 9: 1644-1657.
27) Dong P, Konno Y, Watari H, Hosaka M, Noguchi M, Sakuragi N. The impact of microRNA-mediated PI3K/AKT signaling on epithelial-mesenchymal transition and cancer stemness in endometrial cancer. J Transl Med 2014; 12: 231.
28) Sun X, Cui M, Zhang A, Tong L, Wang K, Li K, Wang X, Sun Z, Zhang H. MiR-548c impairs migration and invasion of endometrial and ovarian cancer cells via downregulation of Twist. J Exp Clin Cancer Res 2016; 35: 10.
29) Dong P, Kaneuchi M, Watari H, Sudo S, Sakuragi N. MicroRNA-106b modulates epithelial-mesenchymal transition by targeting TWIST1 in invasive endometrial cancer cell lines. Mol Carcinog 2014; 53: 349-359.