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miR-15a-5p suppresses endometrial cancer cell growth via Wnt/β-catenin signaling pathway by inhibiting WNT3A

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Abstract. – OBJECTIVE: Endometrial cancer is one of the three most common types of gynecologic cancer. The global incidence has increased in recent years. microRNAs (miRNAs) regulate numerous biological processes by binding to the 3'UTR of target mRNA to down-regulate protein synthesis.

PATIENTS AND METHODS: Endometrial cancer patients received surgeries in our hospital were enrolled. MiR-15a-5p mimic or miR-15a-5p inhibitor was transfected into HEC-1-A cells by lentivirus. Colony formation assay was applied for detecting cell proliferation. Real-time PCR was performed to test miRNA and mRNA expression. Western blot was used to detect protein level. ChIP was adopted to test transcription activation. TOP/FOP was tested to determine Wnt signaling pathway activity. A dual-luciferase reporter assay was used to confirm miRNA target.

RESULTS: miR-15a-5p was decreased in endometrial cancer cells and tissues. miR-15a-5p overexpression restrained HEC-1-A cell proliferation and stemness. miR-15a-5p mimic transfection reduced mRNA and protein levels of the proteins which are related to cell proliferation and Wnt signaling pathway. MiR-15a-5p targeted a putative binding site in the 3'-UTR of Wnt3a gene, thus regulating Wnt signaling pathway. miR-15a-5p overexpression decreased Wnt3a protein expression. Wnt3a presented significant negative correlation with the miR-15a-5p level in endometrial cancer patients.

CONCLUSIONS: miR-15a-5p is a regulator of endometrial cancer cell proliferation by directly targeting Wnt3a to block Wnt signaling pathway.

Key Words:
Endometrial cancer, Wnt signaling pathway, Proliferation, miR-15a-5p, Wnt3a.

Introduction

Endometrial cancer is one of the three most common types of gynecologic cancer. The global incidence has increased in recent years[1]. It is known that endometrial cancer is divided into two types, estrogen-dependent (type I) and non-estrogen dependent (type II), respectively[2]. The pathogenesis of type I endometrial cancer is that the body is under the long-term effects of estrogen, but without the antagonistic effects of progesterone. Thus the endometrium progressively increases in thickness and therefore becomes more susceptible to be cancerous[3]. FIGO stage I (2009) endometrial cancer is considered to have a good prognosis with a 5-year survival rate of up to 96%[4]. Unfortunately, as multiple factors affect recurrence, including surgical stage, differentiation and lymph node metastasis, effective therapies for patients with advanced-stage endometrial cancer or disease recurrence still lack[5], the 5-year OS rate of the FIGO stages II-IV was 76.0%[6]. The identification and further elucidation of the molecular mechanisms responsible for endometrial cancer tumorigenesis and progression may have a major impact on the health of females.

microRNAs (miRNAs) are small non-coding RNA molecules at 21-25 nucleotides. miRNAs either suppress translation or degrade mRNAs by recognizing the specific and complementary sequences of the 3'UTR on target mRNAs[7,8]. miRNAs target approximately 20-30% of genes. A single miRNA targets at least 200 genes, and a single gene can be regulated by many RNAs[9]. Several researches[10-13] have demonstrated that dysregulation of microRNAs promotes tu-

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morigenesis and metastasis. Recent papers have demonstrated that miRNAs act as either potent oncogenes or tumor suppressor genes[14].

Numerous studies have proved that miR-15a-5p inhibits cell proliferation division in various cancers[15,16]. Also, miR-15a-5p has been identified to function as an important regulator of endometriosis[17,18]. Especially, miRNA-15a-5p regulates VEGFA in endometrial mesenchymal stem cells[17]. Moreover, miRNA microarray data from Uyghur Population in China indicate an association between miR-15a-5p and human cervical cancer infected by HPV[19]. Another worky recently provided evidence that miR-15a-5p could regulate viability and matrix degradation of human osteoarthritic chondrocytes via targeting VEGFA[20]. However, little is known about the biological function and target genes of miR-15a-5p in endometrial cancer.

In this research, we found that miR-15a-5p was down-regulated in endometrial cancer cells and tissues. Then miR-15a-5p mimics or inhibitor were transfected into HEC-1-A cells using a lentivirus vector. The transfected endometrial cancer cells were further assessed for the effect of miR-15a-5p on cell proliferation. The results indicated that miR-15a-5p regulated endometrial cancer cell proliferation through Wnt signaling pathway. In the present work, we aimed to provide proof-of-concept and experimental methods of combining miRNA technique in treating endometrial cancer.

Patients and Methods

Clinical Information

Endometrial cancer tissues were obtained from patients who underwent a hysterectomy in the Gynecology Department of The First Affiliated Hospital of Xinjiang Medical University from 2011 to 2016. The endometrial cancer was diagnosed by pathology. The tissue samples comprised of 8 endometrial cancer tissues and 3 normal control tissues. Informed consent was obtained from the patients for the use of their tissues. The tissue samples were used for RNA studies. The Institutional Ethics Board (IRB) at The First Affiliated Hospital of Xinjiang Medical University approved the investigation.

Cell Culture

The HEC-251, AN3CA, RL95-2, HEC-1-A, ISK, Ishikawa, and JEC cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37˚C.

Lentivirus Vector Construction

The 293T cells were transfected with psPAX, Pmd2.G lentivirus package system. In brief, the recombinant lentivirus containing the entire coding sequence of miR-15a-5p or miR-15a-5p inhibitor (miR-15a-5p-in) were digested, conjugated, and transformed. Then, the 293T cells were cultured in a 60-mm plate and transfected with the miR-15a-5p plasmid at a confluency of 40%. The transfection reaction was carried out using the lipofectamine® 2000 reagent as described by the manufacturer. The viruses were harvested from the supernatant after 24 h using a 0.2 μm filter.

Spheroid Formation Assay

HEC-1-A cells containing GFP were seeded into 6-well Ultra Low Cluster plate (Corning, NY, USA) at 500 cells/well and were maintained in serum-free DMEM medium (BioWhittaker, Walkersville, MD, USA) for 10-12 days. After 10-12 days, the number of HEC-1-A cell spheres characterized as tight, spherical, and non-adherent masses > 50 μm in diameter was counted. The images of the spheres were scored under an inverse microscope. Sphere formation efficiency = colonies/input cells × 100%.

Immunofluorescence

The expression levels of β-catenin were detected by immunofluorescence. In brief, the HEC-1-A cells were seeded in 12 well chamber (ibidi, Martinsried, Germany) and then transfected with miR-15a-5p. The cells were fixed with 4% paraformaldehyde in PBS, were permeabilized with 0.1% Triton X-100 and were blocked with 10% serum of goat. Then, the cells were incubated with primary antibody β-catenin (Abcam, Cambridge, MA, USA) overnight and, then, incubated with secondary antibody Goat Anti-Rabbit IgG (Alexa Fluor 488) (Abcam, Cambridge, MA, USA).

Real-time PCR

Total RNA was extracted from the cells with TRizol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized following the manufacturer's protocol (Fermentas, Glen Burnie, MD, USA). Real-time PCR (qRT-PCR) analyses were conducted with SYBR Green Realtime PCR

Master Mix (Toyobo, Ohtsu, Japan) according to the manufacturer's instructions. qRT-PCR reactions were performed in the PTC-220 Real-Time PCR Machine (Bio-Rad Laboratories, Hercules, CA, USA). Results were normalized to the expression of U6 or GAPDH and were calculated with the $2^{-\Delta\Delta Ct}$ method[21].

Western Blot

Proteins (30 μg) were separated by electrophoresis on 12% polyacrylamide gels containing 0.1% SDS and transferred to nitrocellulose membrane. The membranes were incubated overnight at 4 °C in blocking buffer (5% nonfat dry milk) and probed with antibodies against the following proteins: Wnt3a, Cyclin D1, p21, p-pRb, pRb, β-catenin, and GAPDH. The blots were developed with a peroxidase-conjugated secondary antibody, and reacted proteins were visualized using a Millipore ECL Western Blotting Detection System (Millipore, Billerica, MA, USA).

TOP/FOP Detection

TOPglow/FOPglow TCF reporter kit (Millipore, Billerica, MA, USA) was applied for detection of Wnt signaling pathway activity. HEC-1-A cells were seeded in 6-well plate and transfected with TOP-glow and FOP-glow according to the manual. All transfections were performed in triplicates and repeated at least 3 times

ChIP

ChIP assays were performed as described previously[22]. Briefly, HEC-1-A cells were cross-linked for 10 minutes with 1% formaldehyde and quenched with 125 mM glycine. After nuclei were isolated by centrifugation, the pellet was re-suspended in lysis buffer containing 0.1% SDS and sonicated to achieve fragment sizes of 200-600 bp. The assay was conducted with ChIP-grade protein G magnetic beads using an antibody against β-catenin. IgG protein was used as the negative control. To validate the enrichment, qPCR was performed with tiled primers.

Dual-luciferase Reporter Gene Assay

For luciferase activity analysis, HEC-1-A cells (2×10^5 cells/well) were co-transfected with luciferase reporter constructs, phRL-TK (Promega, Madison, WI, USA) Renilla luciferase plasmid and Wnt3a-3'UTR with Lipofectamine 2000 according to the manufacturer's instructions (Promega). After incubation for 24 h, the luciferase assay was carried out using dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Measurements of luminescence were performed on the luminometer and calculated through Dual-Glo. Three independent experiments were performed in triplicate.

Statistical Analysis

Data were depicted as the mean ± SD and analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Statistical comparisons were made between two groups with *t*-test and among multiple groups with one-way ANOVA. Tukey post-hoc test was used for one-way ANOVA. A value of $p < 0.05$ was considered as statistical significance.

Results

miR-15a-5p was Downregulated in Human Endometrial Cancer Cells and Tissues

To identify miR-15a-5p expression in human endometrial cancer, we searched TCGA database. miR-15a-5p levels in human endometrial cancer tissues and normal endometrium tissues were compared. It was showed that miR-15a-5p level was significantly reduced in endometrial cancer tissues compared with healthy control ($p < 0.05$, Figure 1A). Next, 6 endometrial cancer cell lines were selected, and we found that miR-15a-5p level was decreased compared with normal endometrium cell line HEC-251 ($p < 0.05$, Figure 1B). Moreover, we tested 8 endometrial cancer tissues and 3 healthy controls from our hospital using qRT-PCR. It was demonstrated that miR-15a-5p level in endometrial cancer tissues was lower than that of healthy control, which further confirmed its expression in endometrial cancer ($p < 0.05$, Figure 1C).

miR-15a-5p Suppressed Endometrial Cancer Cells Proliferation and Stemness

As miR-15a-5p was down-regulated in endometrial cancer tissues and cells, we hypothesized that miR-15a-5p may be involved in endometrial cancer. We adopted lentivirus for transfection to increase or reduce miR-15a-5p expression in HEC-1-A cells. Real-time PCR confirmed that we successfully changed miR-15a-5p level in HEC-1-A cells (Figure 2A). Furthermore, we tested the influence of miR-15a-5p on cell proliferation. Immunofluorescence assay revealed that miR-15a-5p suppressed cell proliferation of HEC-1-A,

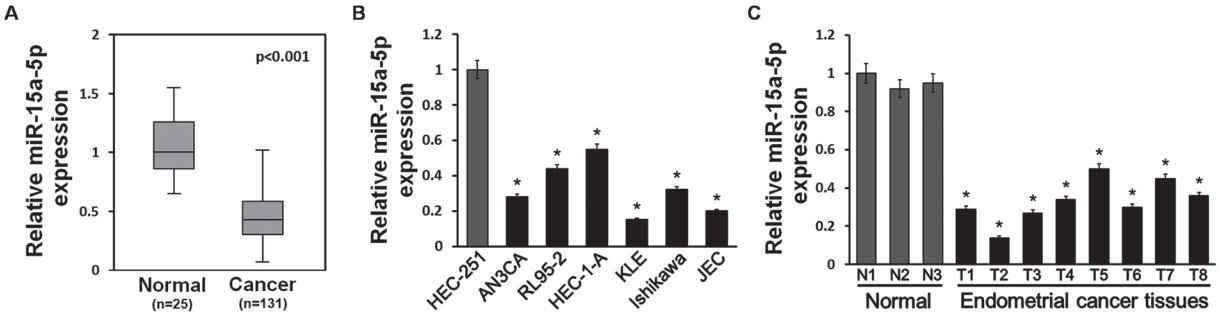

Figure 1. miR-15a-5p downregulated in human endometrial cancer cells and tissues. ***A,*** miR-15a-5p expression in endometrial cancer tissues and normal control from TCGA. ***B,*** miR-15a-5p expression in endometrial cancer and normal endometrium epithelial cell lines. ***C,*** miR-15a-5p expression in endometrial cancer and normal tissues from our hospital. *$p < 0.05$, compared with control.

while miR-15a-5p-in markedly enhanced the cell proliferation (Figure 2B). Colony formation assay revealed that miR-15a-5p apparently suppressed cell colony formation, whereas miR-15a-5p-in significantly facilitated cell colony formation (Figure 2C). Also, miR-15a-5p and miR-15a-5p-in also restrained or accelerated HEC-1-A cell proliferation, respectively (Figure 2D). Moreover, the expression of several genes which are related to cell proliferation and stemness, such as Twist,

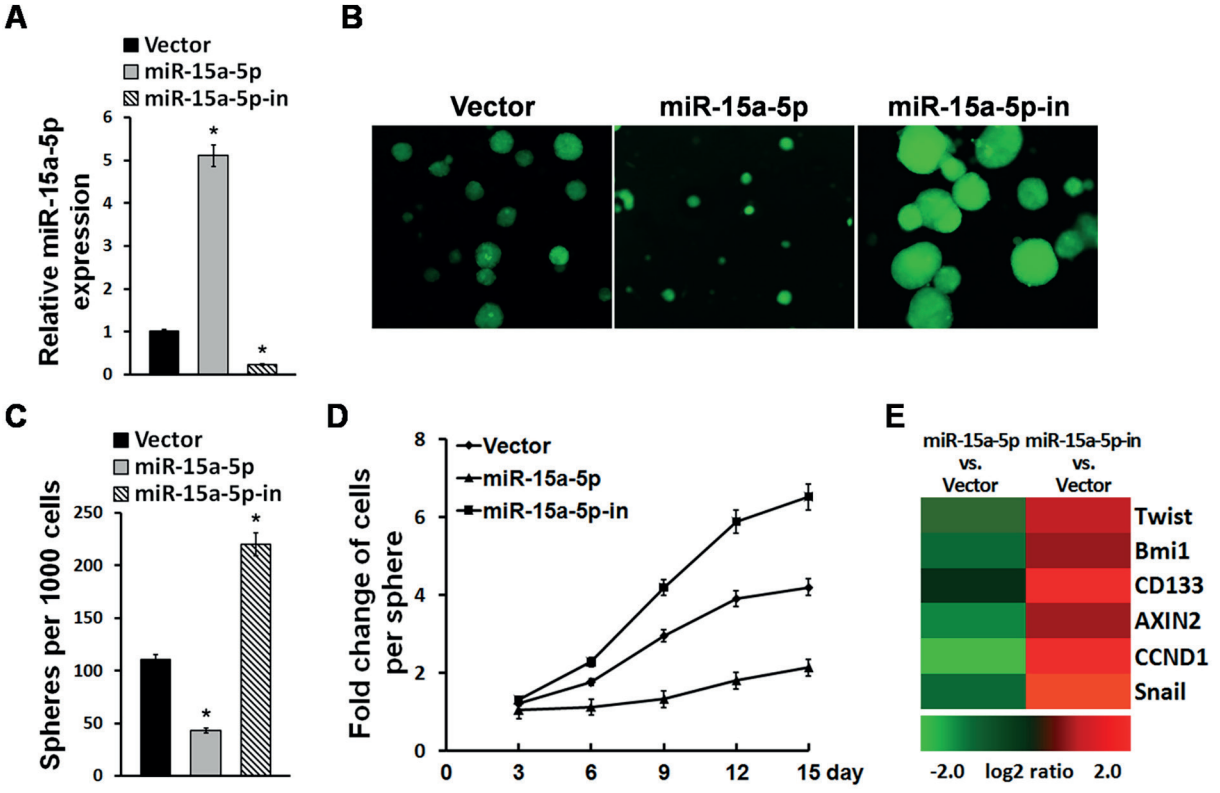

Figure 2. miR-15a-5p suppressed endometrial cancer cells proliferation and stemness. ***A,*** miR-15a-5p expression in HEC-1-A cells after miR-15a-5p or miR-15a-5p-in transfection. ***B,*** HEC-1-A cell colony formation after miR-15a-5p or miR-15a-5p-in transfection. ***C,*** HEC-1-A cell colony formation analysis after miR-15a-5p or miR-15a-5p-in transfection. ***D,*** HEC-1-A cell count in sphere after miR-15a-5p or miR-15a-5p-in transfection. ***E,*** Real-time PCR analysis revealed that miR-15a-5p regulates the expression levels of numerous stem cell regulators. The pseudocolors represent the intensity scale of expression in miR-15a-5p *vs.* Vector-transfected cells or miR-15a-5p-in *vs.* Vector-transfected cells generated by log2 transformation. *$p < 0.05$, compared with control.

BMI1, CD133, AXIN2, CCND1, and Snail, were markedly enhanced in HEC-1-A cells after miR-15a-5p-in transfection, suggesting that miR-15a-5p plays a role in regulating endometrial cancer cell proliferation (Figure 2E).

miR-15a-5p Blocked Wnt Signaling Pathway

Wnt signaling pathway plays an important role in endometrial cancer pathogenesis. Thus, we intended to explore whether miR-15a-5p may regulate Wnt signaling pathway activity. TOP/FOP ratio was significantly decreased in HEC-1-A after miR-15a-5p overexpression, while it was up-regulated after miR-15a-5p-in transfection (Figure 3A). Western blot demonstrated that Cyclin D1 and p21 were overexpressed, while pRb phosphorylation was enhanced in HEC-1-A cells which were transfected with miR-15a-5p-in. In addition, miR-15a-5p showed the opposite impact (Figure 3B). Next, we examined the influence of miR-15a-5p on β-catenin nuclear translocation. Immunofluorescence assay indicated that more β-catenin protein entered nucleus after miR-15a-5p-in transfection, whereas miR-15a-5p exhibited the inhibitory effect on β-catenin nuclear translocation (Figure 3C). After extracted from cytoplasm and nucleus, the protein was detected by Western blot. It was showed that nuclear β-catenin content was markedly increased in in miR-15a-5p-in group, revealing that miR-15a-5p suppression facilitated β-catenin entering nucleus in endometrial cancer cells (Figure 3D). Furthermore, we applied ChIP assay to evaluate activation of Wnt signaling pathway. It was found that miR-15a-5p overexpression suppressed nuclear β-catenin binding with the promoter of OCT-4, SOX-2, and Nanog (Figure 3E). Meanwhile, TOP/FOP ratio was declined by transfection of β-catenin or TCF-4 siRNA in HEC-1-A cells transfected by miR-15a-5p, suggesting that miR-15a-5p played a role in endometrial cancer tumorigenesis through regulating Wnt signaling pathway.

miR-15a-5p Targeted Wnt3a to Regulate Wnt Signaling Pathway

Our previous results showed that miR-15a-5p may affect Wnt signaling pathway activity. Therefore, we proposed that miR-15a-5p may influence Wnt3a. Western blot revealed

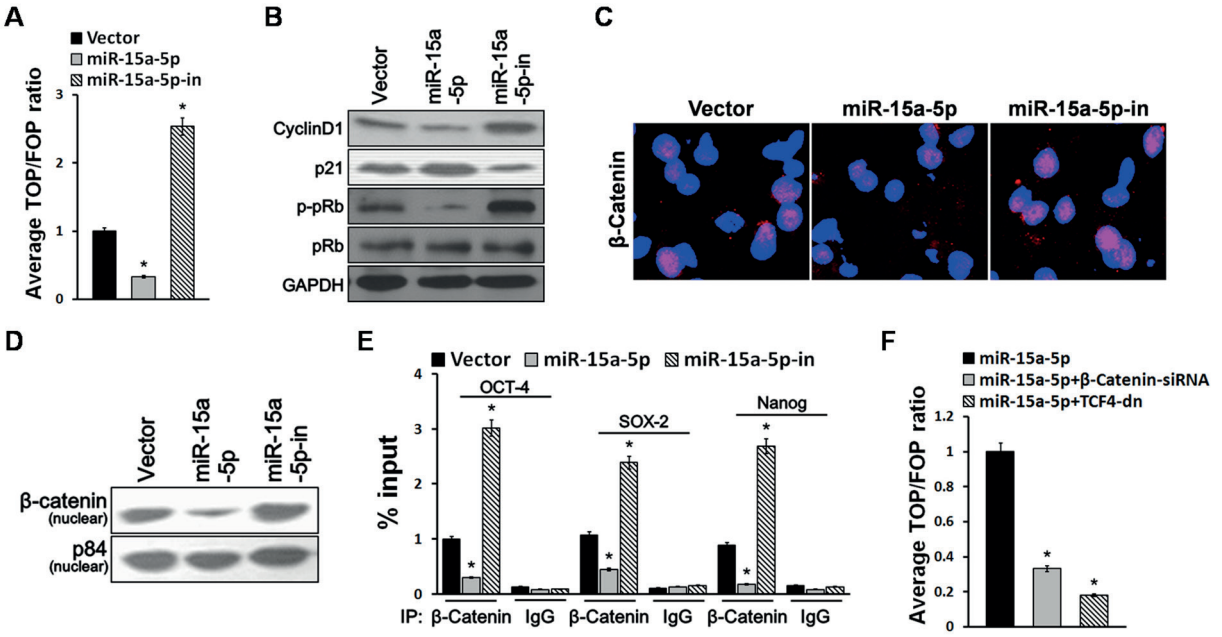

Figure 3. miR-15a-5p blocked Wnt signaling pathway. ***A,*** TOP/FOP ratio determination of Wnt signaling pathway activity in miR-15a-5p or miR-15a-5p-in transfected HEC-1-A cells. ***B,*** Western blot detection of target proteins of Wnt signaling pathway in miR-15a-5p or miR-15a-5p-in transfected HEC-1-A cells. ***C,*** Immunofluorescence detection of β-catenin nuclear translocation. ***D,*** Western blot detection of nuclear β-catenin protein expression. ***E,*** ChIP detection of OCT-4, SOX-2, and Nanog in miR-15a-5p or miR-15a-5p-in transfected HEC-1-A cells. ***F,*** TOP/FOP ratio determination of Wnt signaling pathway activity in HEC-1-A cells transfected by miR-15a-5p together with β-catenin or TCF4 siRNA. $*p < 0.05$, compared with control.

that miR-15a-5p inhibited Wnt3a expression in HEC-1-A cells, while miR-15a-5p-in up-regulated Wnt3a expression (Figure 4A). Moreover, we searched the miRNA database and found that miR-15a-5p was correctively pairing with the 3'UTR of Wnt3a mRNA (Figure 4B). Luciferase assay showed that the level of Wnt3a 3'UTR was reduced in miR-15a-5p transfected HEC-1-A cells (Figure 4C). Whereas the luciferase activity in HEC-1-A cells transfected with Wnt3a-3'UTR-mut was not influenced (Figure 4D), suggesting that miR-15a-5p regulated Wnt3a expression by directly binding with its 3'UTR. Meanwhile, TOP/FOP ratio was also decreased in HEC-1-A cells which were co-transfected with Wnt3a siRNA and miR-15a-5p-in (Figure 4E). Correlation analysis demonstrated that Wnt3a expression was negatively correlated with miR-15a-5p expression in endometrial cancer tissue (Figure 4F), suggesting the relationship between miR-15a-5p and Wnt3a in endometrial cancer.

Discussion

As endogenous inhibitory molecules of gene expression, miRNAs play critical roles in cancer cell proliferation and invasion[23,24]. In this study, we proved that miR-15a-5p suppressed endometrial cancer cell proliferation by directly targeting Wnt3a at the post-transcriptional level, thus regulating Wnt signaling pathway. It was also showed that miR-15a-5p markedly restrained cell proliferation rate in HEC-1-A cells. These results indicated that miR-15a-5p affected endometrial cancer progression through regulating cell proliferation. In conclusion, our results showed that miR-15a-5p acted as a negative regulator of endometrial cancer by blocking cell proliferation.

Our Real-time PCR data demonstrated that miR-15a-5p was declined in endometrial cancer tissues both from TCGA and our hospital. It was further revealed that miR-15a-5p was markedly down-regulated in endometrial cancer cells compared with normal endometrium

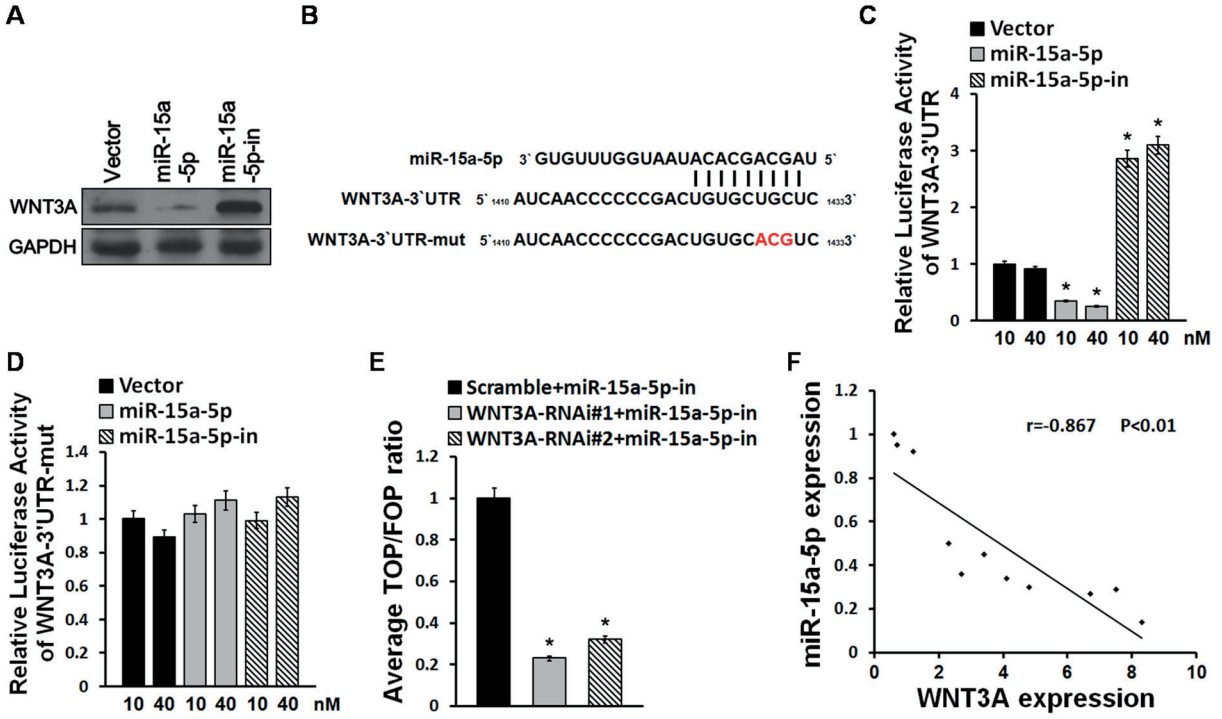

Figure 4. miR-15a-5p targeted Wnt3a to regulate Wnt signaling pathway. ***A,*** Western blot detection of Wnt3a expression. ***B,*** The predicted miR-15a-5p target sequence in the 3' UTR of Wnt3a. ***C,*** Dual-luciferase reporter assay of the HEC-1-A cells transfected with the Wnt3a-3' UTR reporter and miR-15a-5p or miR-15a-5p-in. ***D,*** Dual-luciferase reporter assay of the HEC-1-A cells transfected with the Wnt3a-3' UTR mutation reporter and miR-15a-5p or miR-15a-5p-in. ***E,*** TOP/FOP ratio determination of Wnt signaling pathway activity in HEC-1-A cells transfected by miR-15a-5p-in together with Wnt3a siRNA. ***F,*** Correlation analysis of miR-15a-5p and Wnt3a expression in endometrial cancer tissue. *$p < 0.05$, compared with control.

epithelial cells. Therefore, miR-15a-5p exhibited as a candidate with significant potential to participate in the regulation of endometrial cancer. Although miR-15a-5p has been known to be involved in the carcinogenesis of various types of cancer and endometriosis[15,17], we have identified another function for the treatment of endometrial cancer.

Mounting evidence has shown that aberrant activation of the Wnt/β-catenin signaling has associated with human cancers including endometrial cancer[25]. Therefore, targeting Wnt/β-catenin signaling cascade is a potential effective therapeutic approach to human cancers. β-catenin is the primary mediator of the oncogenic effect in this signaling pathway. Numerous studies[26,27] demonstrated that targeting the upstream effectors can inhibit Wnt/β-catenin signaling activity by reducing the level of β-catenin. However, genetic mutation of β-catenin was found in some human cancers such as endometrial cancer. It hinders the therapeutic approach of using inhibitors against upstream effectors in Wnt/β-catenin signaling cascade[28]. On the other hand, targeting the β-catenin/TCF protein complex is a better choice for suppression of Wnt/β-catenin signaling activity. Indeed, several studies showed that Wnt/β-catenin signaling activity is successfully inhibited by small molecules[29]. Here we report miR-15a-5p is a negative regulator of Wnt/β-catenin signaling and may be a potential target for inhibiting the activity of Wnt/β-catenin pathway. The present work provided several lines of evidence which suggest that miR-15a-5p suppressed the transcriptional activity of β-catenin. According to our immunofluorescent microscopy, the nuclear co-localization indicated that there was a functional interaction among miR-15a-5p and β-catenin. Based on this evidence, we hypothesized that miR-15a-5p might disrupt the transcriptional activity of β-catenin.

Our results indicated that overexpression of miR-15a-5p caused suppression of endometrial cancer cell proliferation, as reflected by a decreased mRNA expression of cell proliferation and stemness markers including Twist, Bmi1, CD133, AXIN2, CCND1, and Snail. In contrast, inhibition of miR-15a-5p enhanced endometrial cancer cell proliferation as evidenced by the remarkably up-regulated mRNA expression of Twist, Bmi1, CD133, AXIN2, CCND1, and Snail. Furthermore, miR-15a-5p has an evident effect on restraining β-catenin nuclear translocation and binding with the promoter of OCT-4, SOX-2, and Nanog. The results suggested that miR-15a-5p had a specific influence on genes which are associated with endometrial cancer cell growth and stemness. Taken together, miR-15a-5p represented as a regulator for suppressing endometrial cancer cell proliferation by targeting Wnt signaling pathway.

The luciferase reporter analysis proved that exogenous miR-15a-5p and miR-15a-5p-in regulated the activity of luciferase when the miR-15a-5p miRNAs regulatory element (MRE) of Wnt3a 3'UTR was fused to luciferase. These results indicated that miR-15a-5p potentially regulated Wnt3a expression by binding the MREs of Wnt3a 3'UTR. The results prompted us to explore whether miR-15a-5p affects endometrial cancer cell proliferation through targeting Wnt3a. Consistent with the prominent role of Wnt signaling pathway in the regulation of endometrial cancer, our results showed that miR-15a-5p overexpression resulted in inhibition of endometrial cancer cell proliferation, whereas miR-15a-5p inhibitor accelerated cell proliferation.

microRNAs inhibit targeted gene expression through two distinct pathways, which are dependent on whether the miRNAs and the target mRNAs are completely complementary. When the miRNA and the target mRNAs are imperfect complementary, miRNAs inhibit mRNA translation. When the miRNA and the target mRNAs are perfectly complementary, miRNAs promote mRNA degradation[30,31]]. Computational algorithms predicted that miR-15a-5p binds to 3'-UTR of Wnt3a with perfect complementation, indicating that it potentially promote the degradation of Wnt3a mRNA. Consistent with the mechanism of miRNAs regulation, we found Wnt3a differentially expressed at both the protein and mRNA levels. Correlation analysis indicated that Wnt3a mRNA presented significantly negative correlation with miR-15a-5p. This study revealed that miR-15a-5p repressed Wnt3a expression through binding with complementation to the 3'-UTR of Wnt3a mRNA.

Conclusions

Our research demonstrated that miR-15a-5p level was decreased in endometrial cancer. Inhibition of miR-15a-5p expression negatively regulated its direct target gene Wnt3a, and resulted in facilitating Wnt signaling pathway

in endometrial cancer cell proliferation. Our findings indicated that miR-15a-5p played an important role in endometrial cancer. The results may provide a novel mechanism in miRNA mediated regulation of endometrial cancer cells. However, an *in vivo* study is still need to be clarified.

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Conflict of Interest
The Authors declare that they have no conflict of interests.

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