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MiR-200a promotes epithelial-mesenchymal transition of endometrial cancer cells by negatively regulating FOXA2 expression

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Endometrial cancer is the most common gynecological cancer. Epithelial–mesenchymal transition (EMT) plays a critical role in tumor invasion and metastasis, which limits the success of treatment. Here, we investigated the roles of forkhead box A2 (FOXA2) and microRNA-200a (miR-200a) in regulating the EMT of endometrial cancer cells RL95-2. Empty vector or FOXA2 was stably transfected into RL95-2 cells. MTT assay measured cell proliferation, apoptosis assay measured apoptosis, Transwell invasion assay measured cell invasion, and Western blot measured the protein expression of FOXA2, E-cadherin, and vimentin. ChIP assay determined the binding of FOXA2 to E-cadherin promoter. For miR-200a analysis, the cells with stable FOXA2 expression were transfected with miR-negative control or miR-200a. Forced expression of FOXA2 decreased the proliferation and invasion, and increased the apoptosis of RL95-2 cells. FOXA2 also affected the EMT-associated proteins: FOXA2 increased the protein expression of E-cadherin and decreased the expression of vimentin. Moreover, FOXA2 positively regulated the promoter of E-cadherin in RL95-2 cells. Luciferase reporter assay identified FOXA2 as a target of miR-200a, which negatively regulated FOXA2. Western blot results showed that overexpression of miR-200a decreased the expression of E-cadherin but increased the expression of vimentin in the endometrial cancer cells by downregulating FOXA2 expression. FOXA2 may act as a tumor suppressor and inhibit EMT of endometrial cancer cells. FOXA2 expression is controlled by miR-200a, which promotes EMT of the endometrial cancer cells.

1. Introduction

Endometrial cancer arises from the lining of the uterus. It is the most common gynecological cancer, with over 60,000 new cases and 10,000 deaths each year (Torre et al. 2015). Common symptoms include pelvic pain, gastrointestinal symptoms, palpable mass, abdominal distension, vaginal bleeding, and newly developed or exacerbated dysmenorrhea and dyspareunia (Lim et al. 2010). Endometrial cancer is classified into two types: endometrioid adenocarcinoma (type I) and serous carcinoma (type II) (Jurcevic et al. 2014). Among these two types, endometrioid adenocarcinoma is the most common endometrial cancer, with approximately 75% to 80% of cases (Srikantia et al. 2009). Endometrioid adenocarcinoma develops from endometrial hyperplasia and can occur in both pre- and post-menopausal women. Endometrioid adenocarcinoma is estrogen dependent and has good prognosis. Serous carcinoma which is estrogen dependent, develops from atrophic endometrium, and occurs mainly in post-menopausal women, and has poor prognosis (Cavanagh et al. 1999; Emons et al. 2000). Five-year survival rate in patients with endometrioid adenocarcinoma is 80% among early diagnosed cases (Amant et al. 2005). In patients with advanced-stage or recurrent endometrioid adenocarcinoma, prognosis is very poor (Jurcevic et al. 2014). Therefore, development of novel biomarkers for early detection, prognosis, and treatment of endometrial cancer could reduce the mortality.

The forkhead box A (FOXA) family of transcription factors regulates chromatin structure and gene expression during embryonic development. FOXA proteins are involved in cell cycle progression, proliferation, migration, metabolism, senescence, and apoptosis (Friedman and Kaestner 2006; Ionescu et al. 2012; Nakshatri and Badve 2007). FOXA2 is a member of the FOXA family. FOXA2 acts as a tumor suppressor in various types of solid tumors including breast cancer, liver cancer, lung cancer, and pancreatic cancer (Jang et al. 2015; Kondratyeva et al. 2016; Perez-Balaguer et al. 2015; Wang et al. 2014). Smith et al. (2016) found that FOXA2 is frequently mutated in endometrioid endometrial cancer; and the patterns of FOXA2 mutation and expression in different tumors suggest complex regulation and a haplo-insufficient or dominant-negative tumor suppressor function.

MicroRNAs (miRNAs) are small, non-coding RNA molecules that regulate gene expression at a post-transcriptional level (Lee et al. 2002). MiRNAs play important roles in various biological processes, including cellular differentiation, proliferation, and apoptosis, indicating that aberrant expression of miRNAs can contribute to the development of human cancer (Sassen et al. 2008). Aberrant expressions of miRNAs have been reported in several types of human cancer, including endometrial cancer. Reports suggest that miR-200a is upregulated in endometrial cancer (Dong et al. 2015; Yoneyama et al. 2015).

Epithelial–mesenchymal transition (EMT) is a crucial process during development by which epithelial cells acquire mesenchymal properties and show reduced intercellular adhesion and increased motility. Growing evidence suggests that EMT plays an important role during tumor progression and malignant transformation, thereby providing invasive and metastatic properties to the embryonic cancer cells. E-cadherin is a cell adhesion molecule present in the plasma membrane of normal epithelial cells. E-cadherin is downregulated by the EMT. In cancer, E-cadherin plays a tumor suppressor role, inhibiting invasion and metastasis. E-cadherin is frequently repressed or degraded during cell transformation (Larue and Bellacosa 2005).

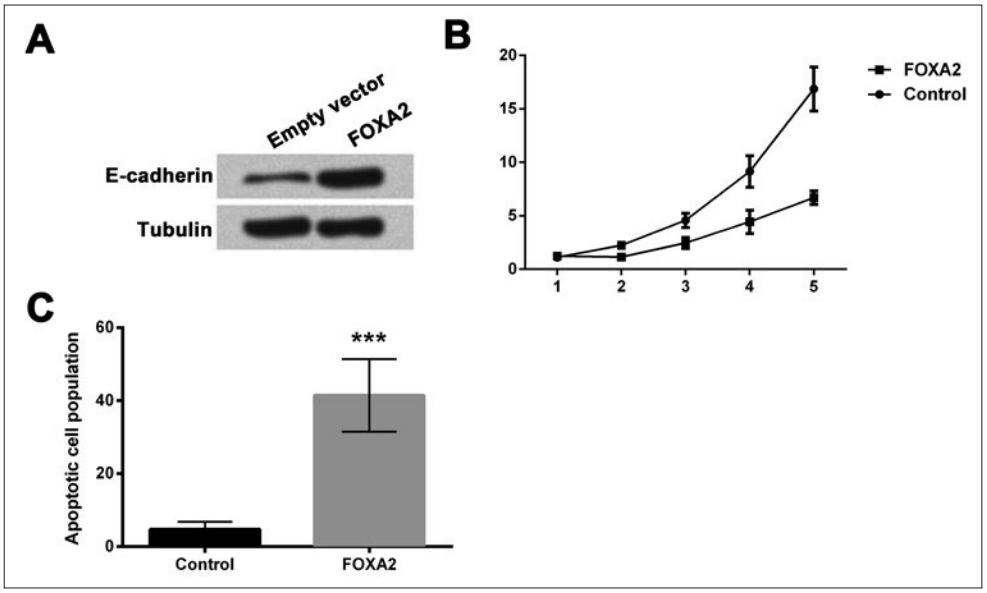

Fig. 1: FOXA2 acts as a tumor suppressor in human endometrial cancer RL95-2 cells. An empty vector (control) or FOXA2 was stably transfected into RL95-2 cells. (A) Weatern blot analysis; (B) MTT assay was conducted to determine cell proliferation and (C) apoptosis assay to determine cell apoptosis. FOXA2: Forkhead box A2. **: P<0.01 and ***: P<0.001.

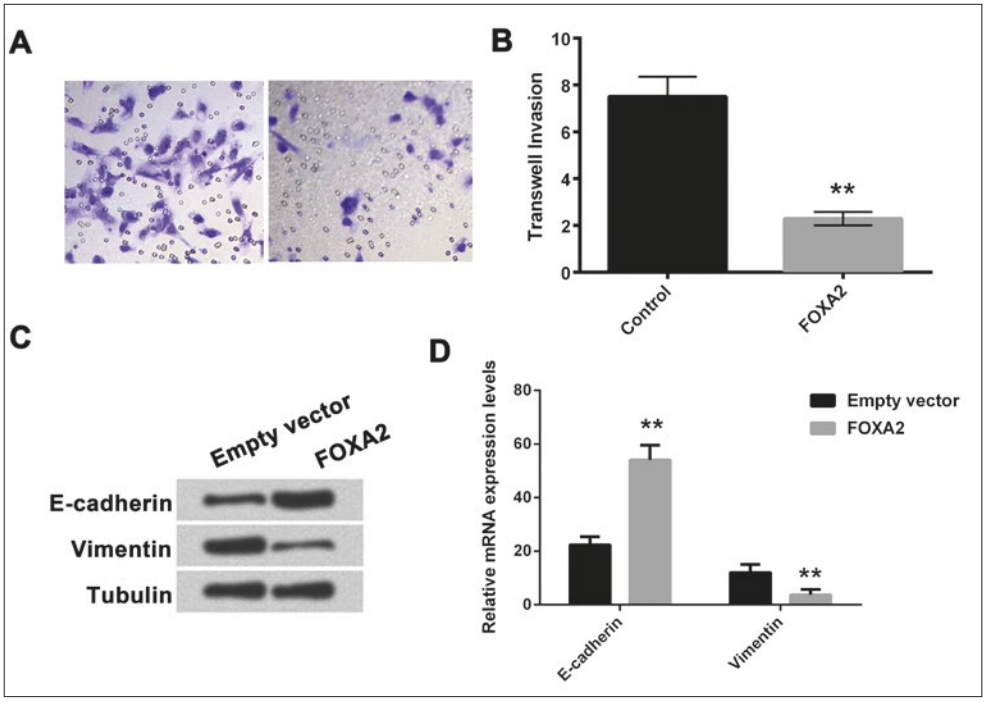

Fig. 2: FOXA2 inhibits EMT of RL95-2 cells. (A and B) An empty vector (control) or FOXA2 was stably transfected into RL95-2 cells and Transwell invasion assay was performed to measure cell invasion. (C) Western blot was performed to determine the protein expression and (D) qRT-PCR was performed to measure the mRNA expression of E-cadherin and vimentin. FOXA2: Forkhead box A2. **: P<0.01.

The mechanism by which FOXA2 inhibits the development and progression of endometrial cancer is still unknown. In the present study, we investigated the roles of FOXA2 and miR-200a in regulating the EMT of endometrial cancer cells.

2. Investigations and results

2.1. FOXA2 acts as a tumor suppressor in human endometrial cancer RL95-2 cells

An empty vector (control) or FOXA2 was stably transfected into RL95-2 cells. A MTT assay was conducted to determine cell proliferation and apoptosis assay to determine cell apoptosis. FOXA2 decreased the cell proliferation (P<0.01, Fig. 1A and 1B) and increased the apoptosis (P<0.001, Fig. 1C) in RL95-2 cells as compared to the control group of cells. These results indicate that FOXA2 suppresses tumor cell growth of endometrial cancer cells.

2.2. FOXA2 inhibits EMT of RL95-2 cells

An empty vector (control) or FOXA2 was stably transfected into RL95-2 cells and a Transwell invasion assay was performed to measure cell invasion. As shown in Fig. 2A and 2B, FOXA2 decreased the invasion of RL95-2 cells as compared to the control group of cells (P<0.01). Western blot was performed to determine the protein expression of EMT-associated proteins, E-cadherin and vimentin. As shown in Fig. 2C and 2D (P<0.01), FOXA2 increased the protein expression of E-cadherin but decreased the expression of vimentin, as compared to the control. These results indicate that FOXA2 inhibits EMT (through alteration of the expressions of EMT-associated proteins) and thus suppresses invasion of endometrial cancer cells.

2.3. FOXA2 regulates the promoter of E-cadherin in RL95-2 cells

RL95-2 cells were harvested and ChIP assay was performed to determine the binding of FOXA2 to the E-cadherin promoter. As shown in Fig. 3A, the density of FOXA2 was increased at the E-cadherin promoter site. Then, FOXA2 and E-cadherin promoter plasmids were co-transfected into RL95-2 cells and luciferase assay was performed to measure the luciferase activity of E-cadherin promoter (P<0.001). As shown in Fig. 3B, FOXA2 increased

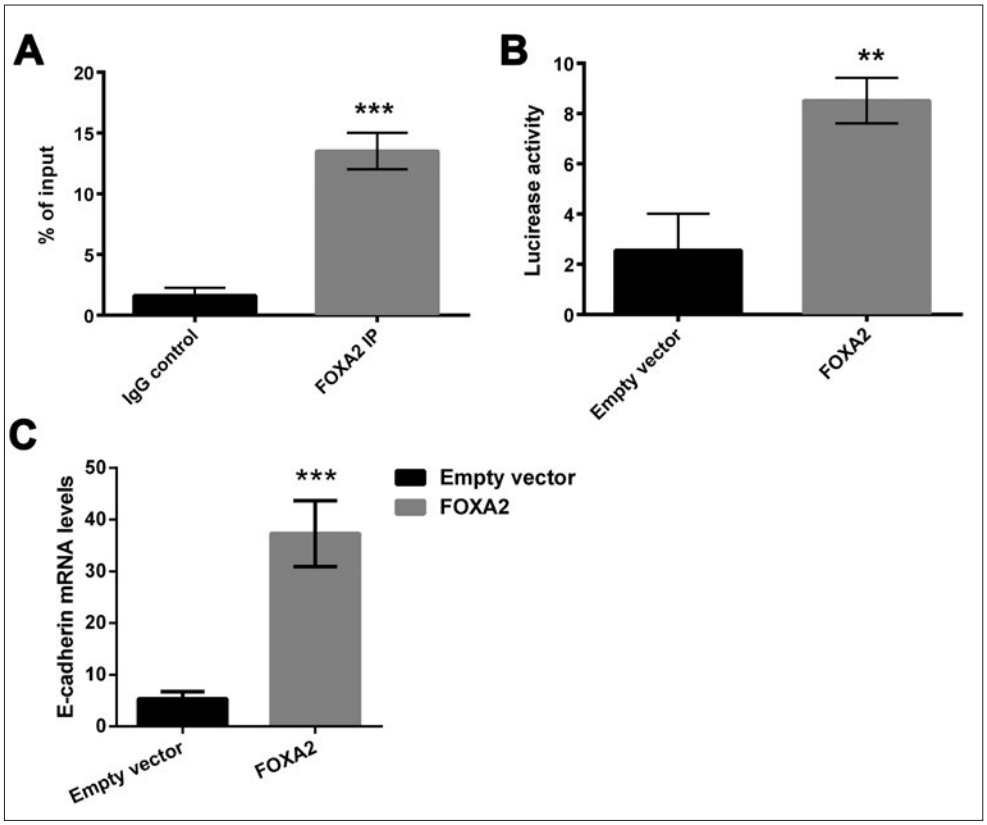

Fig. 3: FOXA2 regulates the promoter of E-cadherin in RL95-2 cells. (A) RL95-2 cells were harvested and ChIP assay was performed to determine the binding of FOXA2 to the E-cadherin promoter. (B) FOXA2 and E-cadherin promoter plasmids were co-transfected into RL95-2 cells and luciferase assay was performed to measure the luciferase activity of E-cadherin promoter. (C) Quantitative RT-PCR was conducted to measure the mRNA expression of E-cadherin in the cells transfected with FOXA2 or control. FOXA2: Forkhead box A2; RT-PCR: reverse transcription polymerase chain reaction. **: P<0.01 and ***: P<0.001.

the luciferase activity of E-cadherin promoter as compared to the control. Meanwhile, mRNA was purified and RT-PCR was conducted to measure the mRNA expression of E-cadherin in the cells transfected with FOXA2 or control (P<0.001). As expected, FOXA2 increased the mRNA expression of E-cadherin in the cells as compared to the control group (P<0.01, Fig. 3C). These results indicate that FOXA2 positively regulates the expression of promoter of E-cadherin in RL95-2 cells.

2.4. FOXA2 is a direct target of miR-200a

In bioinformatics analysis, we found that miR-200a forms base-pair with the 3' UTR of FOXA2 (P<0.01, Fig. 4A). Then, miR-200a and 3' UTR of FOXA2 were co-transfected into RL95-2 cells and luciferase activities were measured. As shown in Fig. 4B, miR-200a + wt 3' UTR decreased the luciferase activity of FOXA2. Western blot analysis was performed to determine the expression of FOXA2 and tubulin in RL95-2 cells. For this, miR-200a mimics were transfected into the cells (P<0.001). The results showed that miR-200a mimic decreased the protein expression of FOXA2 compared to the negative control. These results indicate that FOXA2 is negatively regulated by miR-200a (**Figures 4C and 4D**).

2.5. MiR-200a promotes EMT of RL95-2 cells by targeting FOXA2 expression

RL95-2 cells with stable FOXA2 expression were transfected with miR-NC or miR-200a. The cells were then harvested to measure the protein expression of E-cadherin and vimentin using Western blot. The results (P<0.01, Fig. 5A and 5B) showed that miR-200a mimic decreased the expression of E-cadherin but increased the expression of vimentin. In contrast, overexpression of both miR-200a mimic and FOXA2 increased the expression of E-cadherin and decreased the expression of vimentin. These results indicate that forced expression of miR-200a promotes EMT of RL95-2 cells by down-regulating FOXA2 expression.

3. Discussion

To our knowledge, our data provide the first evidence that FOXA2 and miR-200a together play a critical role in endometrial cancer. First, we determined the effect of FOXA2 on the proliferation, apoptosis, and viability of human endometrial cancer RL95-2 cells as well as on the expression of EMT-associated proteins – E-cadherin and vimentin. Then, we studied the effects of miR-200a on the expression of FOXA2, E-cadherin, and vimentin.

FOXA2 has been shown to inhibit tumor progression and development. Kondratyeva et al. (2016) reported that FOXA2 was downregulated in pancreatic cancer cells. Basseres et al. (2012) showed that FOXA2 was downregulated in lung cancer cells. In our study, FOXA2 decreased the cell proliferation and increased the apoptosis in RL95-2 cells, indicating that FOXA2 suppresses tumor cell growth in endometrial cancer. Similar findings have been reported by other researchers. Villacorte et al. (2013) showed that knockdown of FOXA2 leads to defects in cell cycle regulation of endometrial adenocarcinoma cells, suggesting a role of FOXA2 in the control of cell proliferation. Smith et al. (2016) reported that FOXA2 is mutated in endometrioid endometrial cancer cells, suggesting the tumor suppressor role of FOXA2.

Invasion and metastasis are two major reasons for treatment failure in endometrial cancer (Felix et al. 2010). EMT plays a critical role in tumor invasion and metastasis. During EMT process, epithelial cells downregulate the expression of cell adhesion molecules (such as E-cadherin) and acquire migratory and invasive properties (Puisieux et al. 2014; Yilmaz and Christofori 2009). Tang et al. (2011) reported that overexpression of FOXA2 inhibits EMT and decreases invasion of lung cancer cells. Zhang et al. (2015) showed that overexpression of FOXA2 decreased EMT in breast cancer cells. Similarly, in our study, forced expression of FOXA2 suppressed the invasion of endometrial cancer cells and altered the expression levels of EMT-associated proteins (E-cadherin and vimentin). These data suggest that FOXA2 suppresses endometrial cancer cell invasion via blocking the EMT phenotype.

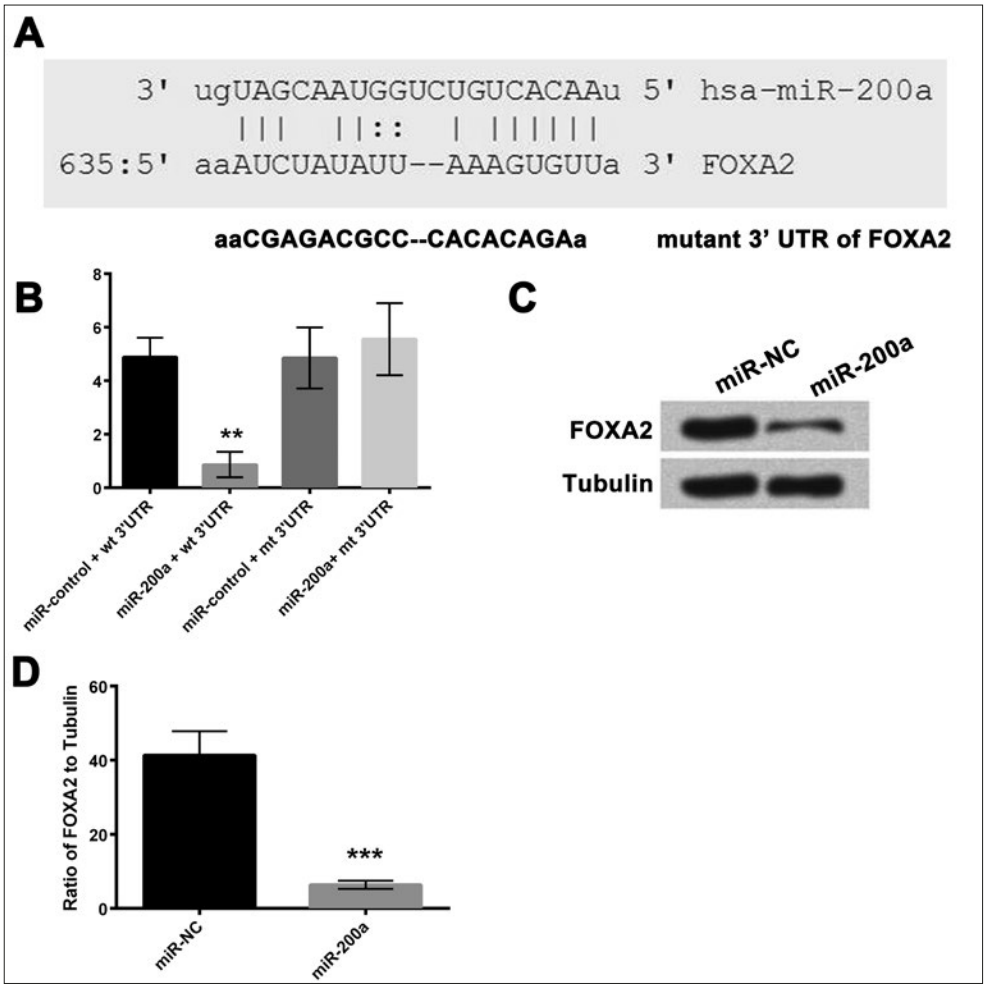

Fig. 4: FOXA2 is a direct target of miR-200a in RL95-2 cells. (A) Bioinformatics analysis to identify the binding site of miR-200a in FOXA2. (B) MiR-200a and 3' UTR of FOXA2 were co-transfected into RL95-2 cells and luciferase activities were measured. (C and D) Western blot analysis was performed to determine the expression of FOXA2 and tubulin in RL95-2 cells. For this, miR-200a mimics were transfected into the cells. FOXA2: Forkhead box A2; NC: negative control. **: P<0.01 and ***: P<0.001.

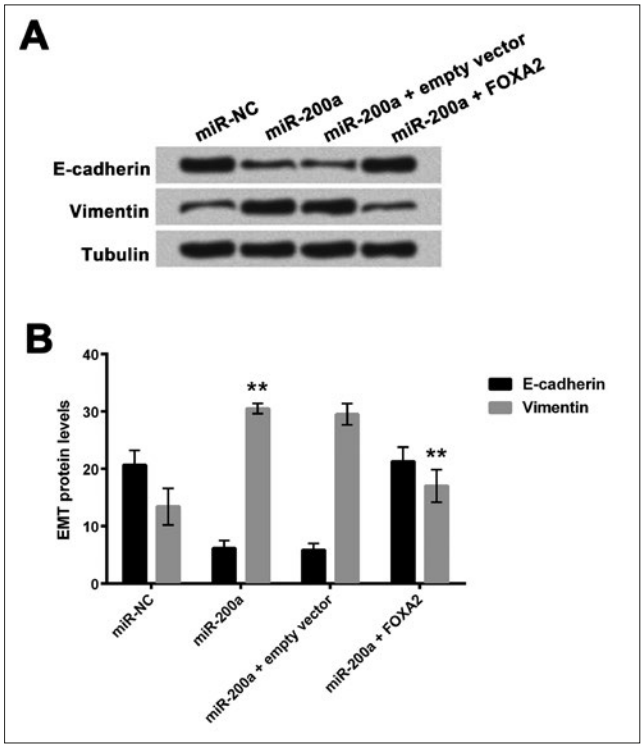

Fig. 5: MiR-200a promotes EMT of RL95-2 cells by targeting FOXA2 expression. RL95-2 cells with stable FOXA2 expression were transfected with miR-NC or miR-200a. The cells were then harvested to measure the protein expression of E-cadherin and vimentin using western blot. **: P<0.01.

In most tumors, the expressions of miR-200 family members are downregulated; however, miR-200 is upregulated in a few tumors, including endometrial cancer, melanoma, ovarian cancer, and colorectal cancer (Snowdon et al. 2011). In our study, we performed bioinformatics analysis, luciferase reporter assay, and Western blotting to identify that miR-200a binds to FOXA2 3'UTR site and overexpression of miR-200a in RL95-2 cells decreases the protein expression of FOXA2. Similar findings have been reported by Chen et al. (2017) who found that FOXA2 is negatively regulated by miR-200a. Moreover, the miR-200 family is also involved in the EMT process (Gregory et al. 2008; Korpal et al. 2008; Park et al. 2008). Park et al. (2008) reported that ectopic expression of miR-200 upregulated the expression of E-cadherin in cancer cells and reduced their motility. In contrast, in our study, overexpression of miR-200a decreased the expression of E-cadherin in the endometrial cancer cells, by decreasing the expression of FOXA2. Our results indicate that miR-200a promotes EMT of endometrial cancer cells by targeting FOXA2.

In conclusion, our data revealed that FOXA2 may function as a tumor suppressor by inhibiting EMT of human endometrial cancer RL95-2 cells. Additionally, FOXA2 regulates EMT by directly binding to the promoter of E-cadherin in endometrial cancer cells. Furthermore, FOXA2 expression is controlled by miR-200a in endometrial cancer cells. MiR-200a negatively regulates the FOXA2 expression, and promotes EMT of the cells. Thus, FOXA2 and miR-200a might be novel therapeutic targets for the diagnosis, prognosis, and treatment of endometrial cancer.

4. Experimental

4.1. Cell culture

Human endometrial cancer cells – RL95-2 cells – were cultured in DMEM/Hams F12, supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator.

4.2. Cell transfection

MiR-200a miRNA precursors were purchased from ThermoFisher. Synthetic miR-200a mimics and scrambled negative control RNA were purchased from Genepharma (Shanghai, China). Cells were seeded in 6-well plates and were transfected with Lipofectamine 2000 (Invitrogen) on the next day when the cells were approximately 70% confluent. In each well, equal amount (100 pmol) of miR-200a mimic and scrambled negative control RNAs was used.

4.3. MTT assay

Cells transfected with siRNA-FOXA2 at logarithmic stage were cultured in DMEM medium mixed with 10% FBS. Cells were adjusted to 5×10^3 cells for injection into the 96-well plates. After being cultured for 24 h, cells were centrifuged at 12,000 rpm, and then supernatant was removed. Followed with addition into 20 μL MTT and then cultured for 4 h. Finally, 150 μL dimethylsulfoxide (DMSO) was used to mix with the cells for 10 min. Absorbance of cells in each well was observed at 570 nm under an absorption spectrophotometer (Olympus, Japan). All experiments were conducted independently for 3 times.

4.4. Cell apoptosis assay

Briefly, the cells were transfected with vectors for 36 h, followed by the replacement of cell culture medium with serum-free RPMI 1640 medium. Total cells were harvested and then washed using PBS buffer (PH 7.4) for 3 times, and then resuspended in the staining buffer. After that, 5 μL of annexin-V-FITC and 5 μL of propidium iodide (PI) were mixed with the cells. After being cultivated at room temperature for 10 min, mixtures were analyzed using the FACScan flow cytometry. Annexin V-positive and propidium iodide-negative cells were considered to be apoptotic cells.

4.5. Cell migration assessment

Shortly, cells in each group were cultured in 6-well plates with the density of 5×10^5 per well. After being confluented, a wound was made across the well with a 200 μL pipette tip. The wound was photographed immediately and then observed the document cellular migration cells across the gap wound using an inverted microscope (× 10 magnification). The width of the gap was detected in 5 different visuals and the average width was calculated. All experiments were conducted independently for 3 times.

4.6. Stable expression of FOXA2

Full-length FOXA2 was cloned into the retroviral pBABE vector backbone. Retroviruses were generated from the 293T cells after transfection with pBABE.FOXA2 using Lipofectamine 2000, according to the manufacturer's protocol. The T24 cells were infected with virus containing RKIP. At 48 hours after the infection, the cells were selected using puromycin (2 μg/ml) for 10 days, and then were used for the experiments.

4.7. Western blot analysis

The cells were washed 2 times with phosphate buffered saline and then lysed with 1× SDS-loading buffer (50 mM Tris-Cl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol and 0.1% bromophenol blue) as the whole-cell sample. The protein samples were processed using SDS-PAGE gel electrophoresis. Immunoblotting assays were carried out with primary antibodies (anti-SRCIN1, anti-beta-Tubulin, Santa Cruz). The proteins were detected by enhanced chemiluminescence (ECL-plus, Amersham Pharmacia Biotech).

4.8. Luciferase activity assay

Human E-cadherin promoters were amplified from human genomic DNA by PCR with the following primers: Forward 5′-CCG CTC GAG TGG GCA AGA CAG AGC GAG AC-3′ and Reverse 5′-CCC AAG CTT CTT CCG CAA GCT CAC AGG-3'. The PCR products were digested and cloned into XhoI and HindIII sites of pGL3 basic Luciferase vector (Promega, USA).

The 3'UTR segment of the RKIP gene, containing the miR-200a binding site, was amplified through PCR and inserted into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI). RL95-2 cells were co-transfected with S100A16 3'UTR and Pre-miR-455 or miR-NC using Lipofectamine 2000 (Invitrogen). Luciferase activity was analyzed at 48 hours post transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). For each transfection, the luciferase activity was averaged from three replicates.

4.9. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted with Trizol reagent (Invitrogen, Grand Island, NY). RNA (500 ng) was polyadenylated and reverse transcribed to cDNA using an NCode miRNA First-Strand cDNA synthesis kit (Invitrogen). cDNA was used as the template for real-time PCR FastStart Universal SYBR green Master (Roche) with the universal reverse primer provided in the kit. Real-time PCR was performed using Applied Biosystems real-time detection system (Applied Biosystems); the thermocycling parameters were 95 °C for 3 min and 40 cycles of 95 °C for 15 s followed by 60 °C for 30 s. Each sample was run in triplicate and was normalized to U6 snRNA levels (U6 primers 5′-CTTCGGCAGCACATATACT-3′ [forward] and 5′-AAAATATGGAACGCTTCACG-3′ [reverse]). Melting curve analysis was performed to confirm the specificity of the PCR products. The replicates were then averaged, and fold induction was determined by a threshold cycle ($\Delta\Delta CT$)-based fold change calculation.

4.10. Chromatin-immunoprecipitation (ChIP)

ChIP assays were performed using the ChIP Assay Kit following the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY). The mouse monoclonal antibody to human FOXA2 was used to precipitate the protein-DNA complexes. DNA was purified and subjected to qPCR with primers E-cadherin promoter forward: 5′-AAA AAT ACA AAC AAA ACA AAC A-3′ and reverse: 5′-TCC TGG GCT GAA GCG ATC-3′. The abundance of FOXA2 binding was calculated by 2^-ΔCt, where ΔCt was determined by subtraction of the Ct of DNA of FOXA2 antibody precipitation from that of DNA from control IgG incubation. The ChIP assay was repeated two times.

4.11. Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). Independent sample t-test was used to calculate the difference between two groups using the graph prism 5.0 software (GraphPad Prism, San Diego, CA). Post-hoc Tukey-test was used to calculate the difference among groups. The $P<0.05$ was considered as statistically significant.

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