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LncRNA NR2F1-AS1 is involved in the progression of endometrial cancer by sponging miR-363 to target *SOX4*

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This study intended to investigate the role of lncRNA NR2F1-AS1 in endometrial cancer (EC). The expression level of NR2F1-AS1 in tumor tissues and EC cells was measured. After sh-NR2F1-AS1 transfection, the cell viability, apoptosis, migration and invasion of EC cells were analyzed. Luciferase reporter assay was conducted to investigate the target gene of miR-363. The expression levels of PI3K/AKT/GSK-3β pathway-associated factors were assayed using western blot. NR2F1-AS1 was significantly overexpressed in EC tissues and cells. NR2F1-AS1 inhibition decreased EC cell viability, migration and invasion, while promoted cell apoptosis. miR-363 was negatively regulated by NR2F1-AS1. *SOX4* was a target of miR-363. NR2F1-AS1 functioned on EC progression via PI3K/AKT/GSK-3β pathway. The results demonstrated that NR2F1-AS1 was highly expressed in EC, which involved in the proliferation and migration of EC cells through downregulation of miR-363 to target *SOX4* and regulating PI3K/AKT/GSK-3β pathway.

1. Introduction

Endometrial cancer (EC) one of the most frequent female reproductive system malignancy, and its incidence is increasing (Yeramian et al. 2013). EC is divided into two types: type I, estrogen-dependent endometriosis carcinoma, and type II, estrogen-independent non-endometriosis carcinoma (Hakim and Raboh 2015; Matias-Guiu et al. 2001). The type 1 EC has favorable prognosis, whose incidence is significantly higher than that of type 2 (Wang et al. 2016). Most of EC is diagnosed at early stage, with 5-year survival rates of over 95%. However, if there is regional spread or distant disease, the 5-year survival rates greatly decreased (Colombo et al. 2016). Presently, the molecular mechanisms of EC have been poorly understood, and finding the new biomarkers for EC progression is urgent.

Recently, extensive attention has been focused on long non-coding RNAs (lncRNAs) that could regulate many biological processes (Martin and Chang 2012). Aberrant expression of some lncRNAs may play an important functional role in cancer biology (Gutschner and Diederichs 2012). Additionally, lncRNAs act as new modulators in the tumorigenesis by involving in oncogenic and tumor suppressing pathways (Guerra et al. 2008; Wang and Chang 2011). Recently, Guo et al. reported that lncRNA GAS5 acted as an tumor suppressor lncRNA in endometrial cancer (Guo et al. 2015). Wang et al. (2016) have found that lncRNA BANCR is highly expressed in type 1 EC tissues, and involves in the progression of EC. More recently, lncRNA NR2F1-AS1 was found to regulate oxaliplatin resistance in hepatocellular carcinoma, while its role in EC has not been investigated.

Therefore, in this study, we aimed to explore the role of NR2F1-AS1 in EC progression. The expression level of NR2F1-AS1 in tumor tissues and EC cells was detected. Further *in vitro* studies were performed to identify the functional role of NR2F1-AS1 in cell proliferation, apoptosis and metastasis of EC cells, as well as to investigate the underlying molecular mechanism.

2. Investigations and results

2.1. NR2F1-AS1 was upregulated in human EC

To explore the role of NR2F1-AS1 in EC, we detected its expression level in tumor tissues and normal tissues using qRT-PCR. As shown in Fig. 1A, NR2F1-AS1 was upregulated in tumor tissues compared with non-tumor tissues ($P < 0.01$). Additionally, we also detected the expression level of NR2F1-AS1 in human EC cell lines (HHUA, KLE, Ishikawa, and ECC-1) and normal cycling endometrium (NE). The results show that the expression level of NR2F1-AS1 in EC cells was significantly downregulated compared with in NE ($P < 0.01$) (Fig. 1B). NR2F1-AS1 had the highest expression level in Ishikawa, so this cell line was selected for the following experiments.

2.2. Suppression of NR2F1-AS1 inhibited EC cell viability, migration, invasion and promoted cell apoptosis

To further explore the role of NR2F1-AS1 in EC progression, we transfected Ishikawa cells with shRNA-NR2F1-AS1 to investigate the effects of NR2F1-AS1 inhibition on cell viability, apoptosis, migration and invasion. The interference effect of NR2F1-AS1 transfection with shRNA was confirmed by qRT-PCR. As shown in Fig. 1C, the transfection efficiency of #1 was higher than the others, thus, #1 was used for the following experiments. NR2F1-AS1 inhibition significantly inhibited the viability of Ishikawa cells from 72 h after transfection ($P < 0.05$) (Fig. 1D), while significantly increased the number of apoptotic cells ($P < 0.001$) (Fig. 1E). The expression levels of apoptosis-associated proteins (Bcl-2, Bax, caspase-3 and caspase-9) were detected as well. As shown in Fig. 1E, NR2F1-AS1 inhibition promoted the expression of Bax, cleaved-caspase-3 and cleaved caspase-9 (pro-apoptotic proteins), but inhibited the expression of Bcl-2 (anti-apoptotic protein). Additionally, NR2F1-AS1 suppression significantly decreased cell migration and invasion ($P < 0.05$) (Fig.

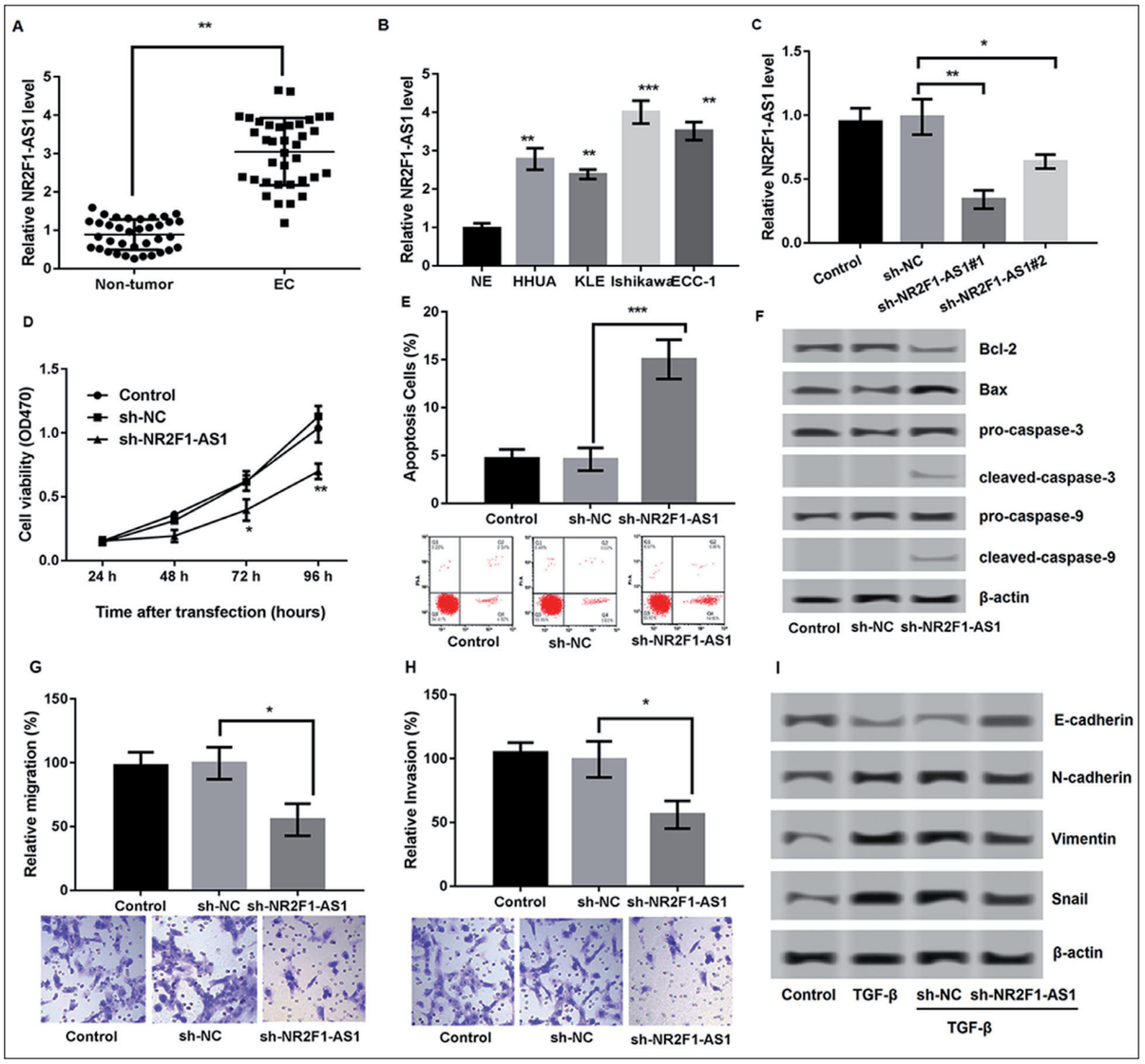

Fig. 1: Relative expression levels of NR2F1-AS1 in EC tumor tissues (A) and cell lines (B) detected by qRT-PCR and effects of NR2F1-AS1 inhibition on cell viability, apoptosis, migration and invasion of endometrial cancer (EC) cells. C: The interference effect of sh-RNA on the expression of NR2F1-AS1 in EC cells measured by qRT-PCR. D: NR2F1-AS1 inhibition significantly reduced cell viability in EC cells. E-F: NR2F1-AS1 inhibition significantly increased the number of apoptotic cells in EC cells; NR2F1-AS1 inhibition promoted the expression of pro-apoptotic proteins Bax, cleaved-caspase-3 and cleaved caspase-9, while inhibited the expression of anti-apoptotic protein Bcl-2 in EC cells. G and H: NR2F1-AS1 inhibition decreased cell migration and invasion in EC cells. I: NR2F1-AS1 inhibition promoted E-cadherin expression and inhibited N-cadherin, snail and vimentin expression in EC cells. For group A and B, *P < 0.05, **P < 0.01, ***P < 0.001 compared to control group. For group C-I, *P < 0.05, **P < 0.01, ***P < 0.001 compared to the corresponding control group.

1G and H). The expression of epithelial-mesenchymal transition (EMT) protein E-cadherin was promoted, and the expression of N-cadherin, snail and vimentin was inhibited (Fig. 1I).

2.3. NR2F1-AS1 regulated tumor growth and metastasis through miR-363

It was suggested that lncRNAs could act as competing endogenous RNAs to suppress the activities of miRNAs by binding to miRNAs (Karreth and Pandolfi 2013). A previous study has reported that NR2F1-AS1 functions through miR-363 (Huang et al. 2018). In this study, we investigated the regulatory relation between NR2F1-AS1 and miR-363, and found that there was a negative regulation between NR2F1-AS1 and miR-363 ($P < 0.01$) (Fig. 2A). To further confirm the relation, we detected the miR-363 expression in EC tissues and cells. As shown in Fig. 2B-C, miR-363 was significantly downregulated in EC tissues and cells ($P < 0.01$). To further reveal whether NR2F1-AS1 exerted functions by regulating miR-363, we co-transfected miR-363 inhibitor after NR2F1-AS1 suppression, and observed the changes of cell vitality, apoptosis, migration and invasion. As shown in Fig. 2E-I, miR-363 knockdown significantly reversed the effects of NR2F1-AS1 suppression on cell viability, apoptosis, migration and invasion in EC cells.

2.4. SOX4 was verified as a functional target of miR-363

SOX4 is an oncogene belonging to the SRY-related high mobility group box family, which has been found to be overexpressed in EC (Huang et al. 2009). A recent study revealed that miR-363-3p play roles in colorectal cancer by targeting *SOX4*. Therefore, we explored the relation between miR-363 and *SOX4* through targetscan (http://www.targetscan.org/cgi-bin/targetscan/vert_71/view_gene.cgi?rs=ENST00000244745.1&taxid=9606&members=miR-25-3p/32-5p/92-3p/363-3p/367-3p&showcnc=0&shownc=0&shownc_nc=&showncf1=&showncf2=&subset=1). *SOX4* was really one of target genes of miR-363 (Fig. 3A). Luciferase reporter assay showed that the group that co-transfected with miR-363 mimic and *SOX4*-WT significantly decreased the luciferase activity ($P < 0.05$) (Fig. 3B). miR-363 overexpression

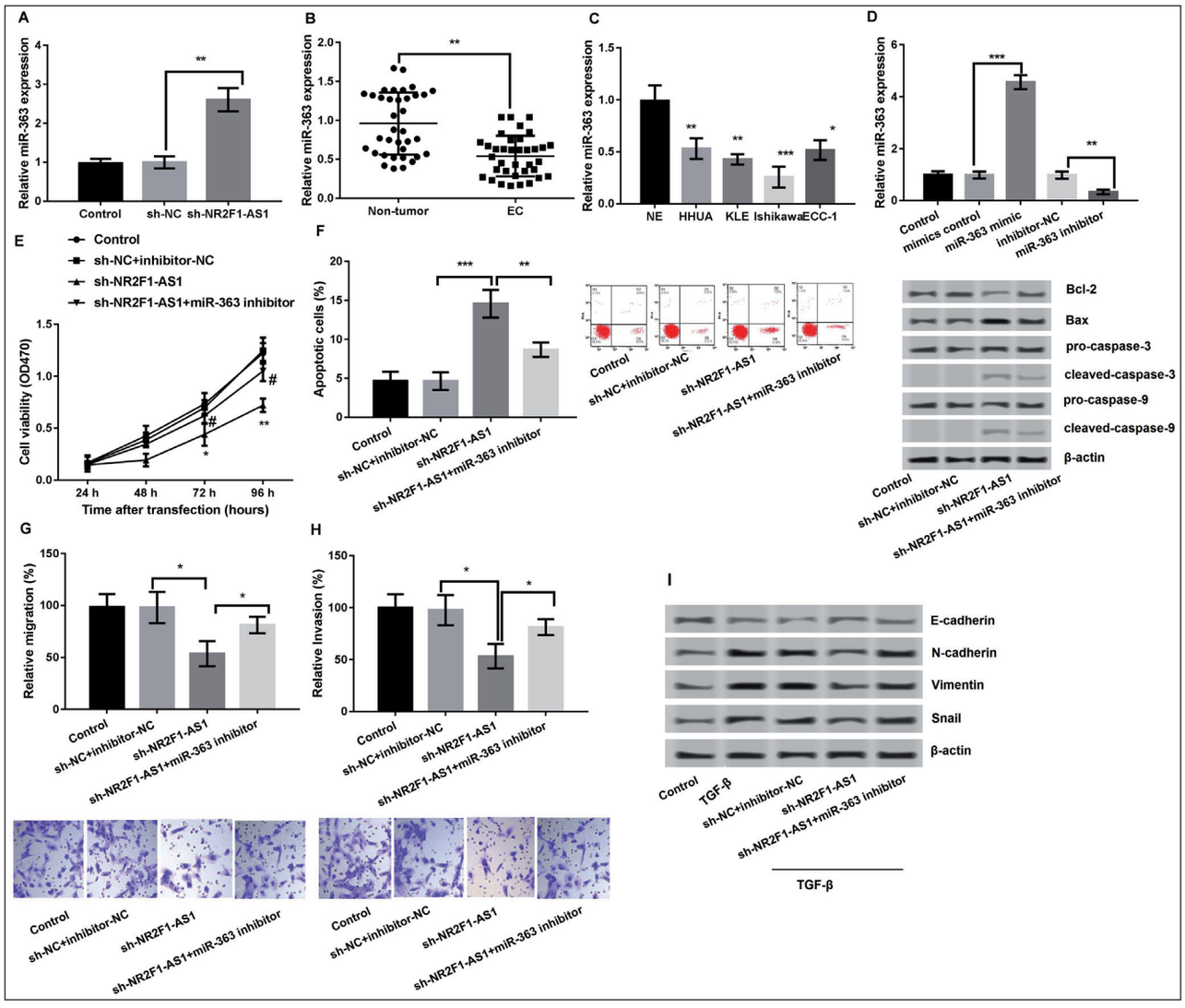

Fig. 2: NR2F1-AS1 regulated tumor growth and metastasis through miR-363 in endometrial cancer (EC) cells. A: There was a negative regulation between NR2F1-AS1 and miR-363 in EC cells. B and C: miR-363 was downregulated in EC tissues and cells. D: The transfection efficiency of miR-363 in EC cells measured by qRT-PCR. E-I: miR-363 knockdown significantly reversed the effects of NR2F1-AS1 inhibition on cell viability, apoptosis, migration and invasion in EC cells. *P < 0.05, **P < 0.01, ***P < 0.001 compared to the corresponding control group.

significantly inhibited the expression of *SOX4* (P < 0.01), while miR-363 inhibition significantly increased *SOX4* expression (P < 0.01) (Fig. 3C-D).

2.5. miR-363 regulatedstumor growth and metastasis through SOX4

To further verify whether miR-363 functions on EC through regulating *SOX4*, miR-363 and *SOX4* were silenced simultaneously. Then the changes of cell viability, apoptosis, migration and invasion were investigated. The silence efficiency of *SOX4* is shown in Fig. 4A (P < 0.001). As presented in Fig. 4B-F, miR-363 inhibition alone significantly increased cell viability, migration and invasion compared with control. However, simultaneously suppression of miR-363 and *SOX4* could reverse these effects of miR-363 inhibition alone remarkably (P < 0.05).

2.6. Effects of NR2F1-AS1 on EC cell viability, apoptosis, migration and invasion through PI3K/AKT/GSK-3β pathway

PI3K/AKT/GSK-3β pathway is an important signal transduction pathway in cells, which can regulate cell proliferation, apoptosis, differentiation, and migration (Jin et al. 2016). It plays an important role in the occurrence and development of various malignant tumors, including EC (Kang et al. 2012; Slomovitz and Coleman 2012). In this study, we investigated the effects of abnormal expression of NR2F1-AS1, miR-363 and *SOX4* on the expression of p-PI3K, p-AKT and p-GSK-3β. As shown in Fig. 4G, NR2F1-AS1 suppression could decrease the expression levels of p-PI3K, p-AKT and p-GSK-3β. Inhibition of NR2F1-AS1 and miR-363 simultaneously could increase the expression of p-PI3K, p-AKT and p-GSK-3β, whereas *SOX4* suppression reversed the effect of sh-NR2F1-AS1 and miR-363-inhibitor.

3. Discussion

Our results revealed that NR2F1-AS1 was overexpressed in EC tissues and cell lines, suppression of which inhibited viability, migration and invasion, but increased apoptosis of EC cells. Further study revealed that miR-363 was negatively regulated by NR2F1-AS1, and NR2F1-AS1 played roles in EC by regulating miR-363. Additionally, *SOX4* was a target gene of miR-363 and was negatively regulated by miR-363. Finally, we found that these effects may be associated with PI3K/Akt/GSK-3β pathway.

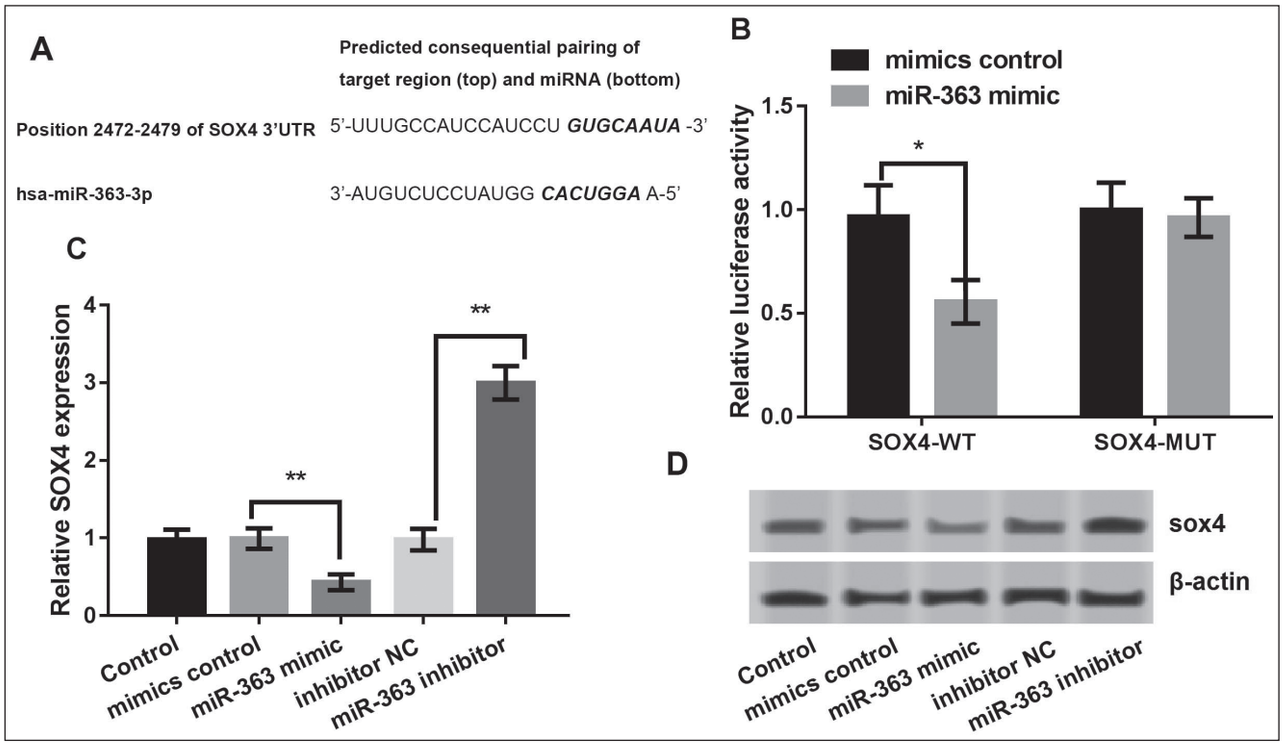

Fig. 3: *SOX4* was verified as a functional target gene of miR-363. A: Gene sequences of *SOX4* regulated by miR-363. B: The group that co-transfected with miR-363 mimic and *SOX4*-WT significantly decreased the luciferase activity. C and D: Overexpression of miR-363 inhibited *SOX4* expression, while suppression of miR-363 increased *SOX4* expression. *P < 0.05, **P < 0.01 compared to the corresponding control group.

Numerous lncRNAs have been identified in the human genome emerging and involved in some cell biology processes, dysregulation of which is implicated in many human cancers, including EC (Guo et al. 2015; Wang et al. 2016; Zhou et al. 2014). NR2F1-AS1 was recently found to be significantly upregulated in oxaliplatin-resistant hepatocellular carcinoma samples compared with oxaliplatin-sensitive samples (Huang et al. 2018). To our best knowledge, our study for the first time suggested its upregulation in EC, and demonstrated that this lncRNA was associated with the viability, migration and invasion, and apoptosis of EC cells.

Studies suggested that lncRNAs may be involved in the competing endogenous regulatory network and acts as endogenous miRNA sponges to bind to miRNAs and then regulate their function (Cesana et al. 2011; Sen et al. 2014; Wang et al. 2015). In this study we found that the expression levels of NR2F1-AS1 and miR-363 were inverse, indicating the antagonistic function. A previous study has reported that miR-363 is correlated with the outcome of gastric cancer (Chang et al. 2011). It was also found to function as a tumor suppressor in neuroblastoma (Qiao et al. 2013). Presently, we found that miR-363 was significantly upregulated by NR2F1-AS1 silencing, by which viability and metastasis of EC cells were significantly decreased while apoptosis of EC cells was significantly increased. Therefore, there may be a reciprocal repression between NR2F1-AS1 and miR-363 in EC cells.

miRNAs usually play roles by regulation of their target genes during tumor development and metastasis (Xin et al. 2014; Zhao et al. 2014). In this study, bioinformatics prediction tool TargetScan was used to discover the underlying molecular mechanism within miR-363 and its target genes. We found that miR-363 targeted the 3'-UTR of *SOX4* with complementary binding sites, which was in accordance with the study of Hu et al. (2016) who reported that miR-363-3p could target SOX4 to inhibit the epithelial-to-mesenchymal transition and suppresses metastasis in colorectal cancer. *SOX4*, an oncogene belonging to the SRY-related high mobility group box family, is located on chromosome 6p22 that is a region amplified frequently in bladder cancer, lung cancer, and EC (Heidenblad et al. 2008; Hurst et al. 2003; Levan et al. 2006; Medina et al. 2009). *SOX4* has been found to be overexpressed in EC compared with controls (Huang et al. 2009). Taken together, our data indicated that miR-363 may involve in tumor growth and metastasis in EC by regulating *SOX4*.

PI3K/AKT/GSK-3β pathway is an important signal transduction pathway in cells. It is associated with the regulation of cell proliferation, apoptosis, and migration (Jin et al. 2016). It has been implicated in the occurrence of many malignant tumors, including EC (Kang et al. 2012; Slomovitz and Coleman 2012). Presently, inhibitors of the PI3K/AKT/mTOR pathway are in mid- and late-stage trials in EC, as single agents and in combination with other

Table: Characteristics of endometrial cancer specimens

Total no.	36
Median age, years (range)	52.8 (38-77)
Pathological tumor stage	
I	18 (50%)
II	8 (22.2%)
III	5 (13.9%)
IV	5(13.9%)
Differentiation	
G1	13 (36.1%)
G2	12 (33.3%)
G3	11 (30.6%)
Lymphatic metastasis	
(+)	12 (33.3%)
(−)	22 (61.1%)
Unknown	2 (5.6%)

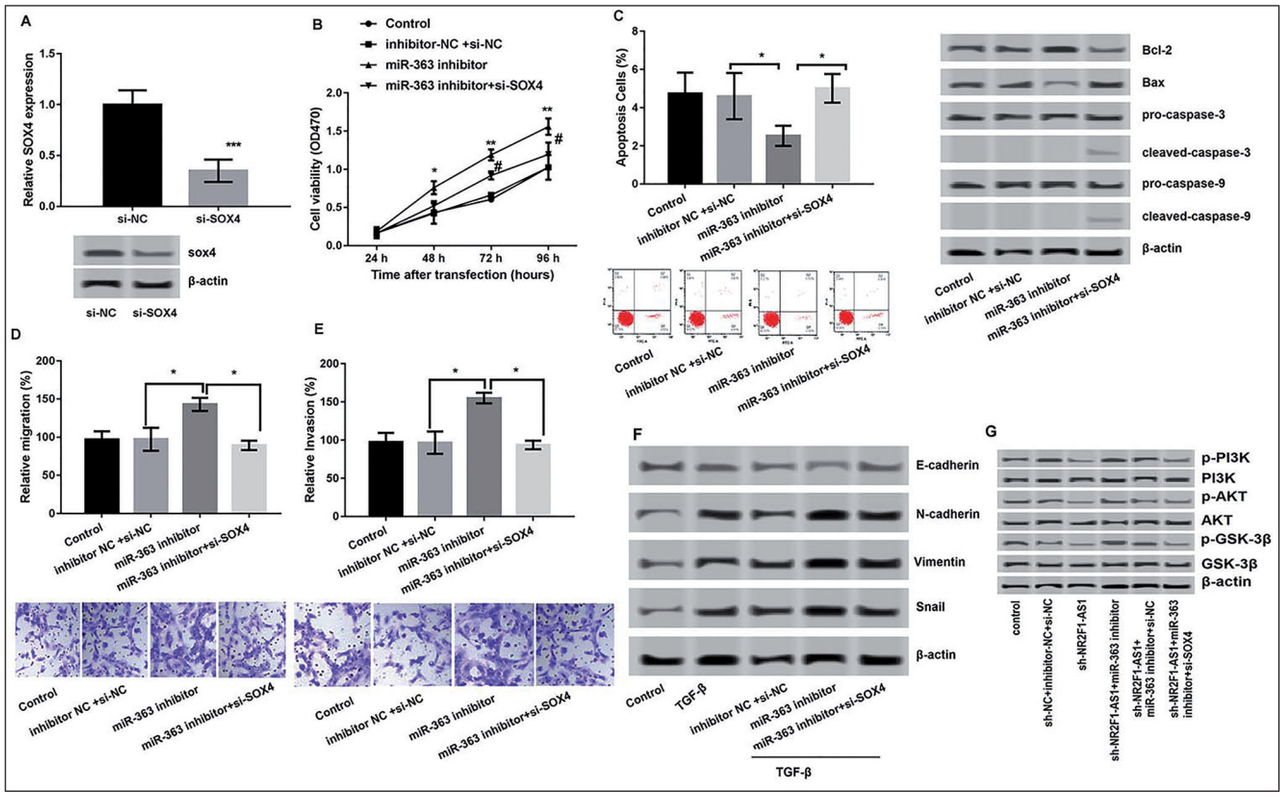

Fig. 4: miR-363 regulated endometrial cancer (EC) cell proliferation and metastasis through *SOX4*. A: The transfection efficiency of *SOX4*. B-F: miR-363 suppression significantly increased cell viability, migration and invasion compared with control. Simultaneously suppression of miR-363 and *SOX4* could reverse these effects remarkably. G: NR2F1-AS1 suppression decreased the expression levels of p-PI3K, p-AKT and p-GSK-3β, which was reversed by inhibition of NR2F1-AS1 and miR-363 simultaneously. *SOX4* suppression further reversed the effect of sh-NR2F1-AS1 and miR-363-inhibitor. *P < 0.05, **P < 0.01, ***P < 0.001 compared to the corresponding control group.

therapies (Slomovitz and Coleman 2012). GSK-3β is a serine/threonine protein kinase that operates in G1 to receive input from some signaling and developmental pathways (Doble and Woodgett 2003). PI3K/AKT signaling pathway can inactivate GSK-3β to promote cell-cycle entry by stabilizing proteins such as c-Myc and cyclin D1 (Diehl et al. 1998). Our study further found that that NR2F1-AS1 play its carcinogenesis role via the PI3K/AKT/GSK-3β pathway.

In conclusion, our study for the first time demonstrated that NR2F1-AS1 was overexpressed in EC, which involved in the proliferation and migration of EC cells by suppressing miR-363 to target *SOX4* and regulating PI3K/AKT/GSK-3β pathway. Our study may provide experimental basis for the targeted treatment of EC.

4. Experimental

4.1. Patients and samples

A total of 36 fresh-frozen samples were obtained from patients pathologically diagnosed with EC who underwent an initial hysterectomy at our hospital between April 2015 and July 2018. Additionally, 36 normal and age-matched endometrial tissues were obtained from patients who underwent hysterectomy due to benign diseases, such as myoma or adenomyosis. These tissue specimens were immediately frozen in liquid nitrogen after surgical excision, until total RNA extraction. The characteristics of EC specimens and normal specimens are summarized in the Table.

4.2. Cell lines and cell culture

The human EC cell lines HHUA, KLE, Ishikawa, and ECC-1, purchased from ATCC, were grown in Minimum Essential Medium Eagle (Sigma-Aldrich, UK) supplemented with 15% fetal bovine serum (Gibco, Darmstadt, Germany), 100 U/ml of penicillin and 100 µg/ml of streptomycin (Gibco). The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

4.3. Transient transfection and treatment

For the transfection of shRNA, miRNA mimics/inhibitor, siRNA or their corresponding controls, 2 × 10^5 Ishikawa cells were seeded in 6-well plates for 24 h. They were transfected with shRNA against NR2F1-AS1, 50 nM of miRNA mimic, 150 nM of miRNA inhibitor, or siRNA against SOX4 using Lipofectamine 3000 Reagent (Life Technologies, USA). In order to induce EMT, the medium was supplemented with 10 ng/mL of TGF-β1.

4.4. Cell viability assay

After 24 h of transfection, cells were seeded in 96-well plates at 10^4 cells/well. Cell viability was measured with a MTT assay. Briefly, cells were incubated with MTT (at a final concentration of 0.5 mg/ml) at 37 °C for another 4 h. The medium was then removed, and the precipitated formazan was dissolved in 100 µl dimethyl sulfoxide, followed with shaking for 15 min. Finally, the absorbance at 570 nm (A570) was detected using a µQuant universal microplate spectrophotometer (BioTekInstruments, Winooski, VT, USA).

4.5. Flow cytometry analysis of cell apoptosis

Apoptosis was detected by flow cytometric analysis. Briefly, cells were cultured in six-well plates for 48 h cells and then were collected and washed with PBS. After that, cells were stained with fluorescein isothiocyanate-labelled annexin V (Invitrogen, USA) and propidium iodide, which was followed by flow cytometry analysis.

4.6. Luciferase reporter assay

The full-length 3'-UTR segments of TCF21 mRNA containing the miR-363 binding site were amplified by PCR and inserted into the Xba1-site of pGL3 vector (Promega, WI, USA), which was named pGL3-TCF21. The pGL3-TCF21-mut reporter construct with point mutations in the seed sequence was synthesized with a site-directed mutagenesis kit (Stratagene, CA, USA). Total 1 × 10^6 cells were cotransfected with 50 pmol of miR-363 inhibitor (or control miRNA), 1 µg of pGL3-TCF21 (or pGL3-PTEN-mut) plasmid, and 1 µg of a Renilla luciferase expression construct pRL-TK (Promega, WI) using Lipofectamine 2000. Luciferase activity was measured after 36 h of transfection using the dual luciferase assay system (Promega, WI, USA) and normalized to Renilla luciferase activity.

4.7. qRT-PCR analysis

Total RNA was extracted using TRIzol Reagent (Life Technologies, USA), followed with quantity measurement with a SmartSpec Plus spectrophotometer (Bio-Rad, USA), and purity evaluation according to the A260/A280 ratio. qRT-PCRs of lncRNA and mRNA were performed using a GoTaq 2-Step RT-qPCR System (Promega, Madison, WI, USA) in a Mx3005P QPCR System

(Stratagene, La Jolla, CA, USA) with GAPDH as a control. qRT-PCR of the mature miRNAs was carried out using miScript SYBR Green PCR Kits (Qiagen, Hilden, Germany), miScript II RT Kits (Qiagen), and miScript Primer Assays in the Mx3005P QPCR System (Stratagene). The relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

4.8. Western blot analysis

Cells were collected and lysed with cell lysis buffer (Beyotime, Haimen, China) for 30 min at 48 h after transfection. The protein concentration of the lysates was measured using a Bradford protein assay kit (Bio-Rad, USA) and separated on 12 % SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Immunoblotting of the membranes was performed using the primary antibodies, followed with recommended secondary antibodies incubation. β-actin was used as control. Finally, the signals were detected using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA).

4.9. Statistical analysis

The data are presented as mean±SD from three independent experiments. The differences between groups were evaluated with two-tailed Student's t-tests using SPSS Statistics 20.0 software (IBM, Armonk, NY, USA). $P < 0.05$ was considered to be statistically significant.

Conflicts of interest: None declared.

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