MiR-195 inhibits migration, invasion and epithelial-mesenchymal transition (EMT) of endometrial carcinoma cells by targeting SOX4

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MicroRNAs (miRNAs) have been identified as potential biomarkers for endometrial carcinoma (EC) diagnosis, prognosis and therapy. The purpose of the present study was to investigate the detailed role and molecular mechanism of miR-195 in EC metastasis. qRT-PCR assay was performed to assess the expression of miR-195 and SRY-related high-mobility group box 4 (SOX4) mRNA in EC tissues and cells. The levels of N-cadherin, Vimentin, E-cadherin and SOX4 protein were determined by western blot. SOX4 protein expression in EC tissues was also determined by Immunohistochemistry (IHC) experiment. Transwell assay was used to analyze cell migration and invasion abilities. Dual-luciferase reporter assay and RNA Immunoprecipitation (RIP) assay were performed to confirm the targeted interaction between miR-195 and SOX4. Our data supported that miR-195 was downregulated and SOX4 was upregulated in EC tissues and cell lines. Upregulation of miR-195 inhibited migration, invasion and epithelial-mesenchymal transition (EMT) of EC cells. Moreover, SOX4 was a direct target of miR-195. MiR-195 overexpression-mediated anti-migration, anti-invasion and anti-EMT effects were antagonized by SOX4 restoration in EC cells. In conclusion, our study suggested that miR-195 inhibited the migration, invasion and epithelial mesenchymal transition (EMT) of EC cells at least partly by targeting SOX4. Our study provided a novel underlying mechanism for EC metastasis and a promising therapeutic target for EC management.

Keywords. Endometrial carcinoma (EC); epithelial-mesenchymal transition (EMT); miR-195; SRY-related high-mobility group box 4 (SOX4)

Abbreviations: EC, endometrial carcinoma; UTR, untranslated region; Ago, Argonaute; EMT, epithelial mesenchymal transition; HRP, horseradish peroxidase; IHC, Immunohistochemistry; BSA, bovine serum albumin; RIP, RNA Immunoprecipitation

1. Introduction

Endometrial carcinoma (EC), arising from the lining of the uterus, is one of the most common malignancies among women worldwide, with a 2–3% lifetime risk of developing the disease (Amant *et al.* 2005, Torre *et al.* 2015). In recent years, with the changes in living environment, the incidence of EC has been upward trend in the number of annual diagnoses in China (Chen *et al.* 2016b). Although the developments of diagnosis and therapeutic methods have improved the prognosis of EC patients, the 5-year survival rate of EC patients is still unsatisfactory mainly owing to the metastasized property (Amant *et al.* 2005). Therefore, it is essential to identify novel effective biomarkers for improving EC treatment.

MicroRNAs (miRNAs), a large family of small non-coding RNAs with ~ 22 nucleotides in length, play crucial roles in a wide array of biological processes (Hammond 2015). The mature miRNA forms a ribonucleoprotein complex, called RNA-induced silencing complex (RISC), which also harbored Argonaute2 (Ago2) protein (Finnegan and Pasquinelli 2013). MiRNAs negatively regulate genes

expression by binding to the 3′-untranslated region (3′-UTR) of target mRNAs, leading to translational repression and/or target mRNA degradation (Hammond 2015). Increasing number of evidence has shown that miRNAs function as potential biomarkers for human cancer diagnosis, prognosis and treatment, including EC (Calin and Croce 2006, Kaur *et al.* 2015). MiR-195 has been identified as a tumor-suppressive miRNA in a series of human cancers, such as non-small cell lung cancer (Liu *et al.* 2015), prostate cancer (Cai *et al.* 2015) and cervical cancer (Shen *et al.* 2017). Previous studies manifested that miR-195 was downregulated in EC tissues compared with normal endometrial tissues, indicating its role as a potential early diagnostic target for EC (Jayaraman *et al.* 2017, Kong *et al.* 2018, Tsukamoto *et al.* 2014). The purpose of the present study was to investigate the detailed role and molecular mechanism of miR-195 in EC metastasis. SRY-related high-mobility group box 4 (SOX4) has been identified as an oncogene in many human cancers, including breast cancer (Mehta *et al.* 2017), esophageal cancer (Li *et al.* 2015b) and gastric cancer (Peng *et al.* 2018). SOX4 expression was found to be upregulated in EC tissues, and it involved in the regulation of miR-129-2 on the proliferation of EC cells (Huang *et al.* 2009). However, the effect of interplay between miR-195 and SOX4 in EC metastasis remains undefined.

In the present study, our data supported that miR-195 was downregulated and SOX4 was upregulated in EC tissues and cell lines. SOX4 was a direct target of miR-195. Furthermore, our data suggested that miR-195 inhibited the migration, invasion and epithelial mesenchymal transition (EMT) of EC cells at least partly by targeting SOX4.

2.   Materials and methods

2.1   *Tissue specimens*

30 cases of EC tissues and 26 cases of normal proliferation endometrium tissues from hysterectomy specimens were obtained from the First Hospital of Hebei Medical University. All participants did not receive any therapy before surgery. The clinical characteristics of all participants were shown in table 1. All specimens were stored at -80°C until use. Written informed consent was obtained from all participants prior to their surgical procedure, and this study was approved by the Human Studies Ethical Committee at the First Hospital of Hebei Medical University.

2.2   *Cell culture*

Primary human endometrial epithelial cell line (hEEC) was isolated as described previously (Marwood *et al.* 2009). Briefly, endometrium tissues were minced and digested with collagenase I (Roche, Mannheim, Germany), and the suspension was filtered through nylon mesh, followed by

Table 1.   Relationship of miR-195 level with pathologic parameters of tumor patients

Clinicopathologic features	N (%)	Relative miR-195 level		P value
		Low (%)	High (%)	
Total cases	30 (100.0)	18 (60.0)	12 (40.0)	
Age (years)				P = 0.6926
≥55	20 (66.7)	12 (60.0)	8 (40.0)	
<55	10 (33.3)	6 (60.0)	4 (40.0)	
Clinical stage				P = 0.0422
I+III	17 (56.7)	7 (41.2)	10 (58.8)	
III-IV	13 (43.3)	11 (84.6)	2 (15.4)	
Lymphatic Metastasis				P = 0.0249
Yes	11 (36.7)	10 (90.9)	1 (9.1)	
No	19 (63.3)	8 (42.1)	11 (57.9)	
Differentiation				P = 0.9404
High	16 (53.3)	9 (56.3)	7 (43.7)	
Low+Middle	14 (46.7)	9 (64.3)	5 (35.7)	

purification using selective adherence to collect hEEC. Four EC cell lines HEC-1A (human endometrial epithelium-derived cell line), KLE (human endometrial adenocarcinoma cell line), RL95-2 (human endometrial cancer cell line) and Ishikawa (human endometrial adenocarcinoma cell line) and 293T cells were purchased from American Tissues Culture Collection (ATCC, Manassas, VA, USA).All cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, UK) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 1% pencillin/streptomycin (Hyclone) at 37°C in a humidified incubator with 5% CO_2.

2.3   *Cell transfection*

For miR-195 investigation in EC, miR-195 mimics (Genepharma, Shanghai, China), miR-195 inhibitors (anti-miR-195, Genepharma) or respective negative control (miR-NC mimics or anti-miR-NC, Genepharma) were transfected into EC cells using Lipofectamine™ 3000 Transfection Reagent (Invitrogen, Karlsruhe, Germany) in accordance with the instructions of manufacturers. For SOX4 expression alteration, SOX4 overexpression plasmid (pcDNA-SOX4) was synthesized by Genepharma and transfected into EC cells, and pcDNA (Genepharma) was used as negative control.

2.4   *RNA extraction, reverse transcription PCR and quantitative real-time PCR (qRT-PCR)*

Total RNA was extracted from tissues and cells using Trizol Reagent (Life Technologies, Frederick, MD, USA) according to the protocol of manufacturers. For miR-195 detection, RNA was reverse-transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystem, Madrid,

Spain), and qRT-PCR was performed with TaqMan universal master mix (Applied Biosystem) with U6 as a housekeeping gene for normalization on a RealPlex4 real-time PCR detection system (Eppendorf, Hamburg, Germany). For SOX4 mRNA detection, the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) and TaqMan universal master mix (Thermo Fisher Scientific) were used, and GAPDH was used as an endogenous control. The relative expressions of miR-195 and SOX4 mRNA were defined from the threshold cycle (Ct) and were calculated by using the $2^{-\Delta\Delta Ct}$ method.

2.5 *Transwell assay of cell migration and invasion*

Cell migration and invasion abilities were detected using transwell assay with a 24-Transwell insert containing an 8 μm pore size of polycarbonate filter (Corning, NY, USA). Briefly, cells were transfected with miR-NC mimics, miR-195 mimics, miR-195 mimics+pcDNA and miR-195 mimics+pcDNA-SOX4. For migration ability, cells (2×10^5) suspended in 200 μl serum-free medium were added into the upper chamber with non-coated membrane in 24-Transwell plate. For invasion ability, 200 μl of serum-free medium containing 2×10^5 cells was seeded into the upper chamber with Matrigel-coated membrane (Corning) in 24-Transwell plate. In both assays, 600 μl of DMEM medium containing 10% FBS was added to the lower chamber. After 24 h, the migrated or invaded cells were fixed in 95% ethanol and stained with 0.1% crystal violet. The images were captured by FSX100 (Olympus, Tokyo, Japan) and the numbers were counted in 10 random fields.

2.6 *Western blot*

Total cellular proteins were prepared using RIPA lysis buffer (50 mM Tris-HCl, pH=7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF) supplemented with 1% protease and phosphatase inhibitor cocktail (Roche). Protein extracts were resolved on a 10% SDS polyacrylamide gel and blotted onto a Hybond P PVDF membrane (Millipore, Darmstadt, Germany). The membranes were incubated with anti-E-cadherin (Cell Signaling Technology, Danvers, MA, USA; dilution 1:1000), anti-N-cadherin (Cell Signaling Technology; dilution 1:1000), anti-Vimentin (Cell Signaling Technology; dilution 1:1000), anti-SOX4 (Abcam, Cambridge, UK; dilution 1:500) and anti-β-actin (Abcam, dilution 1:5000), and then probed with horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology; dilution 1:1000). The protein bands were visualized using an enhanced chemiluminescence reagent kit (GE Healthcare, Munich, Germany) and the images were analyzed with an ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.7 *Immunohistochemistry (IHC)*

SOX4 protein expression in EC tissues was determined using immunohistochemical examination. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin blocks, and then were dewaxed and rehydrated. Heating the slides was used for antigen retrieval. After blocking with bovine serum albumin (BSA, Thermo Fisher Scientific), the slides were incubated with anti-SOX4 overnight at 4°C, followed by the incubation with HRP-conjugated secondary antibody. The DAB substrate (Thermo Fisher Scientific) was used for color development. SOX4 protein expression was observed in 10 randomly different microscopic fields respectively.

2.8 *Bioinformatics*

Analysis of miR-195 targets was performed using the Starbase 3.0 software available at *http://starbase.sysu.edu.cn/*.

2.9 *Dual-luciferase reporter assay*

To verify whether SOX4 was a target of miR-195, dual-luciferase reporter plasmids containing the fragment of SOX4 3′-UTR with the predicted wild-type miR-195 binding sites (SOX4-WT) and its mutant-type in seeded region (SOX4-MUT) were designed and synthesized by Genepharma, and then transfected into 293T cells, respectively, together with miR-195 mimics or miR-NC mimics using Lipofectamine™ 3000 Transfection Reagent. The luciferase activity was analyzed 24 h after transfection using the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

2.10 *RNA Immunoprecipitation (RIP) assay*

RIP assay was employed to explore the endogenous interaction between miR-195 and SOX4 using an Imprint RNA immunoprecipitation kit (Thermo Fisher Scientific). Briefly, cells were transfected with miR-195 mimics or miR-NC mimics and then were lysed with ice-cold RIPA lysis buffer. Cell lysates were incubated with RIP buffer containing magnetic beads conjugated with anti-Ago2 (Cell Signaling Technology) or negative control anti-IgG (Cell Signaling Technology).The beads were harvested by centrifugation and washed three times with ice-cold PBS. Total RNA was extracted from the beads, and the enrichment of SOX4 mRNA was determined by qRT-PCR.

2.11 *Statistical analysis*

All statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) and Graphpad

Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). All data were shown as mean ± standard deviation (SD) with at least three times repetition. Differences between two groups were analyzed by a Student's *t*-test and P values < 0.05 was considered statistically significant.

3. Results

3.1 *Downregulation of miR-195 in EC tissues and cell lines*

Firstly, we detected the expression of miR-195 in EC tissues and normal proliferation endometrium tissues by qRT-PCR. As shown in figure 1A, these data revealed a significant decrease of miR-195 level in EC tissues compared with normal control. Then, miR-195 expression was assessed in EC cell lines. As demonstrated by qRT-PCR, miR-195 level was significantly downregulated in EC cell lines compared with negative control (figure 1B). Moreover, miR-195 level was significantly associated with the clinical stage and lymphatic metastasis of EC patients (table 1).

3.2 *Upregulation of miR-195 inhibited migration, invasion and EMT of EC cell lines*

To further observe the role of miR-195 on EC metastasis, gain-of-function experiments were carried out. HEC-1A and Ishikawa cells were transfected with miR-NC mimics or miR-195 mimics. As presented by qRT-PCR, transfection of miR-195 mimics, but not a scrambled control sequence, resulted in a about 3.6-fold increase of miR-195 expression in HEC-1A cells and 4.6-fold increase in Ishikawa cells. Subsequent transwell assays demonstrated that compared to negative control, miR-195 overexpression significantly repressed the migration and invasion abilities of HEC-1A and Ishikawa cells (figure 2B and C). Also, western blot results presented that miR-195 overexpression led to a significant decrease of N-cadherin and Vimentin levels and an increase of E-cadherin level, indicating that miR-195 overexpression significantly suppressed the EMT of HEC-1A and Ishikawa cells (figure 2D–F).

3.3 *Upregulation of SOX4 in EC tissues and cell lines*

To determine whether SOX4 was aberrantly expressed in EC tissues, qRT-PCR and IHC assays were performed. These results revealed that the expression levels of SOX4 mRNA and protein were significantly upregulated in EC tissues compared with negative control (figure 3A and B). Consistently, qRT-PCR assays showed that SOX4 mRNA expression was significantly increased in EC cell lines compared with hEEC (figure 3C).

3.4 *SOX4 was a direct target of miR-195*

To further understand the role of miR-195, we carried out a detailed analysis for its molecular targets. The prediction by Starbase 3.0 software revealed that the 3′-UTR of SOX4 mRNA harbored a putative target sequence for miR-195 (figure 4A). To confirm this, SOX4 3′-UTR luciferase reporter plasmid containing the putative target sequence of miR-195 (SOX4-WT) or site-directed mutation in seeded region (SOX4-MUT) was cotransfected into 293T cells, together with miR-195 mimics or miR-NC mimics. Transfection of SOX4-WT in the presence of miR-195 mimics induced almost a 62% decrease of relative luciferase activity, while the effect was completely abolished by SOX4-MUT (figure 4B). Then, RIP assay was employed to verify the endogenous correlation between SOX4 and miR-195 in RISC using anti-Ago2 or IgG antibody. These results showed that transfection of miR-195 mimics, but not a miR-NC mimics control, significantly enriched SOX4 mRNA using anti-Ago2 antibody in both HEC-1A and Ishikawa cells (figure 4C and D).

Further, we observed whether miR-195 regulated SOX4 expression in EC cells. HEC-1A and Ishikawa cells were transfected with miR-195 mimics, anti-miR-195 or respective
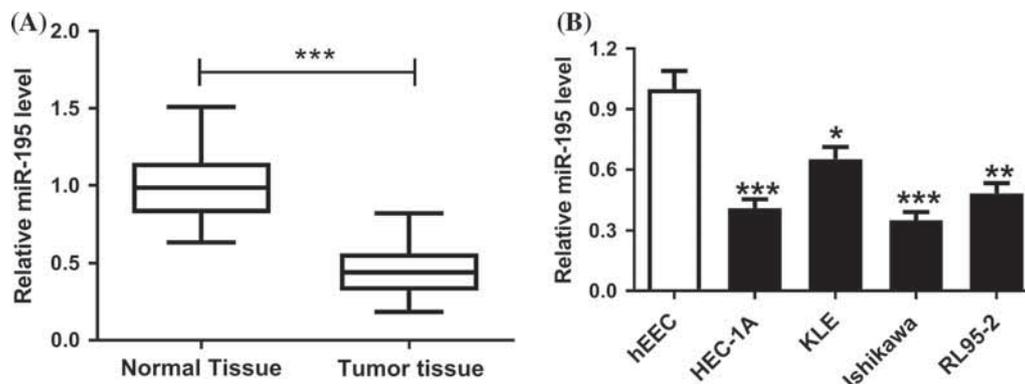

Figure 1. MiR-195 was downregulated in EC tissues and cell lines. The expression of miR-195 was detected by qRT-PCR assay in 30 cases of EC tissues and 26 cases of normal proliferation endometrium tissues (**A**), EC cell lines (HEC-1A, KLE, RL95-2 and Ishikawa) and human endometrial epithelial cell line (hEEC) (**B**). *P < 0.05 or **P < 0.01 or ***P < 0.001 vs. normal tissue or hEEC.
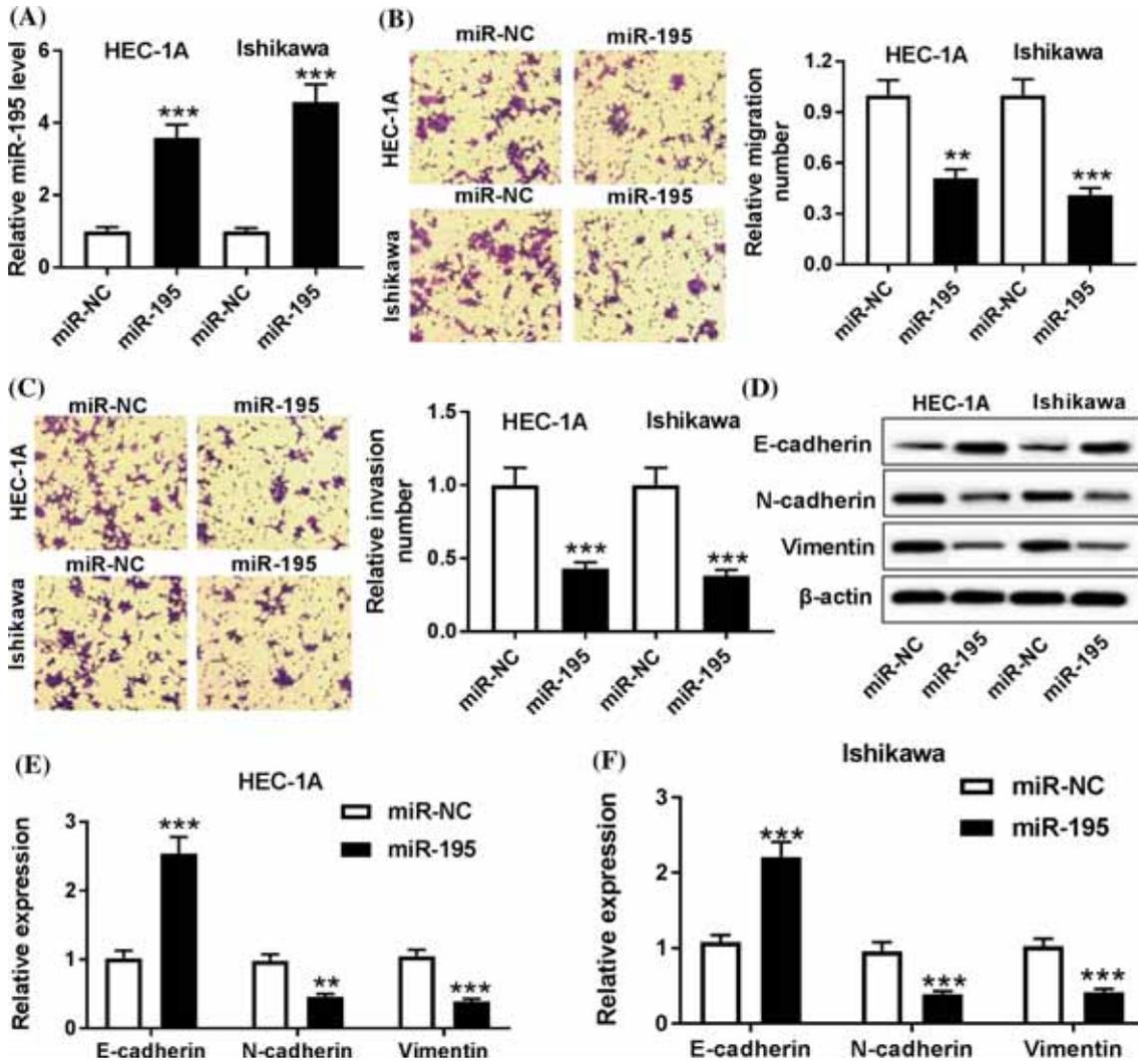
Figure 2. The role of miR-195 overexpression was determined on migration, invasion and EMT of HEC-1A and Ishikawa cells. HEC-1A and Ishikawa cells were transfected with miR-NC mimics or miR-195 mimics, followed by the measurement of miR-195 level by qRT-PCR assay (**A**), cell migration (**B**) and invasion (**C**) abilities by transwell assay, the expression levels of N-cadherin, Vimentin and E-cadherin by western blot (D-F). **$P < 0.01$ or ***$P < 0.001$ vs. miR-NC mimics.
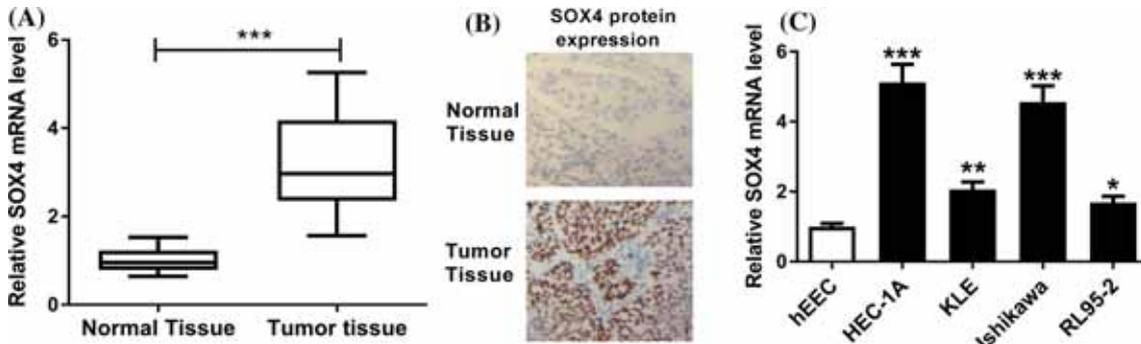

Figure 3. The expression of SOX4 in EC tissues and cell lines. SOX4 mRNA level was determined by qRT-PCR assay (**A**) and SOX4 protein level was assessed by IHC assay (**B**) in EC tissues and normal proliferation endometrium tissues. (**C**) qRT-PCR assay for SOX4 mRNA level in EC cell lines (HEC-1A, KLE, RL95-2 and Ishikawa) and human endometrial epithelial cell line (hEEC). *$P < 0.05$ or **$P < 0.01$ or ***$P < 0.001$ vs. normal tissues or hEEC.

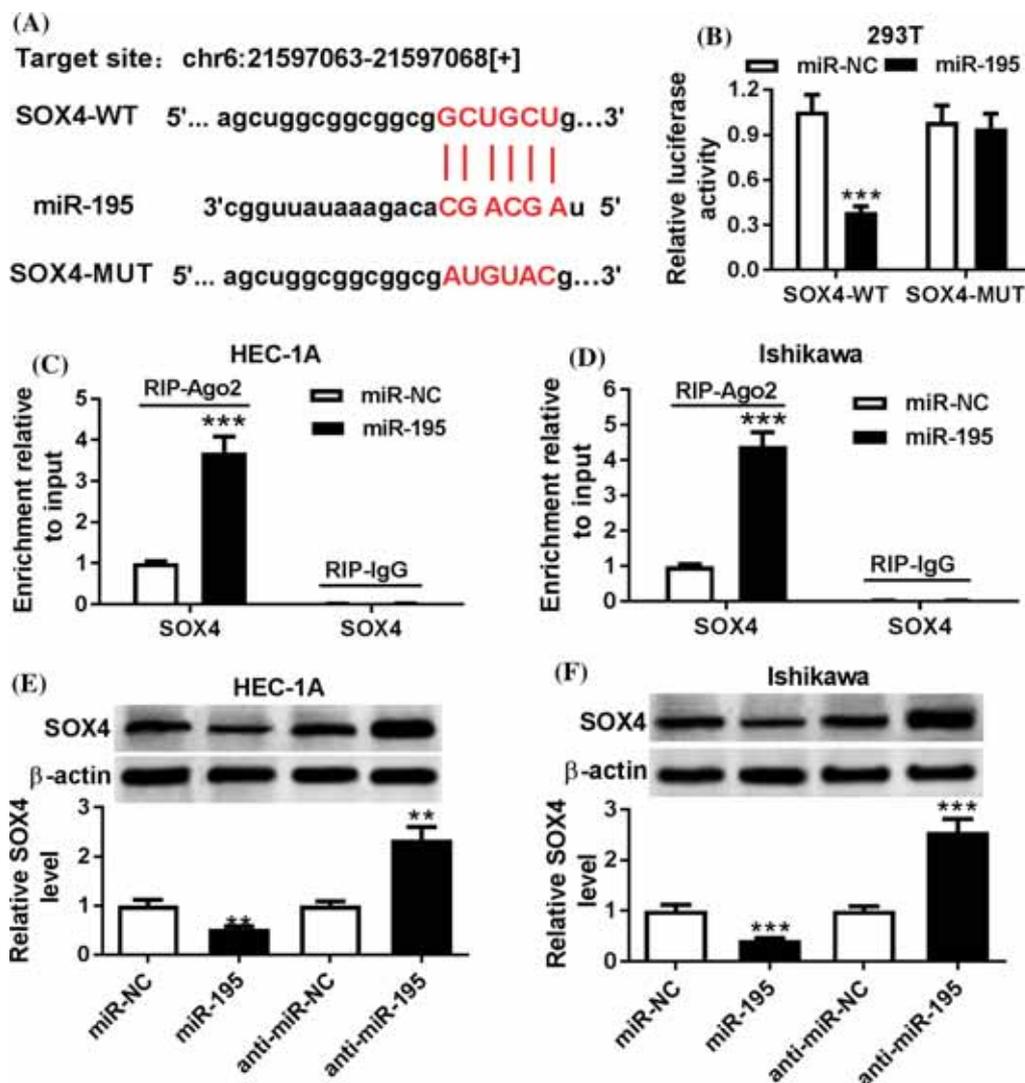**Figure 4.** SOX4 was a direct target of miR-195. (**A**) Predicted miR-195 target sequence in the SOX4 3′-UTR and site-directed mutant in seeded region. (**B**) 293T cells were cotransfected with wild-type or mutant-type luciferase reporter construct (SOX4-WT or SOX4-MUT) with miR-NC mimics or miR-195 mimics, and then the relative luciferase activity was measured. HEC-1A (**C**) and Ishikawa (**D**) cells were transfected with miR-NC mimics or miR-195 mimics, followed by the determination of SOX4 mRNA level in RISC with anti-Ago2 or anti-IgG antibody. HEC-1A (**E**) and Ishikawa (**F**) cells were transfected with miR-NC mimics, miR-195 mimics, anti-miR-NC or anti-miR-195, followed by the measurement of SOX4 level by western blot. **P < 0.01 or ***P < 0.001 vs. respective control.

negative control. Western blot data showed that in comparison to their counterparts, SOX4 expression was significantly reduced by transfection of miR-195 mimics, while it was significantly elevated following miR-195 silencing (figure 4E and F). These data indicated that miR-195 negatively regulated SOX4 expression in both HEC-1A and Ishikawa cells.

3.5 *MiR-195 mimics-mediated anti-migration, anti-invasion and anti-EMT effects were abated by SOX4 in EC cell lines*

To provide further mechanistic insight into the link between miR-195 and SOX4 on EC metastasis, HEC-1A and

Ishikawa cells were cotransfected with miR-195 mimics and pcDNA-SOX4. Subsequent transwell assays and western blot experiments revealed that transfection of pcDNA-SOX4 significantly antagonized the miR-195 overexpression-mediated suppression effects on cell migration, invasion and EMT in HEC-1A and Ishikawa cells compared with negative control (figure 5A–H).

4. Discussion

Until now, miRNAs have been widely accepted to be frequently dysregulated and play important roles in tumorigenesis and development of human cancers, including EC.
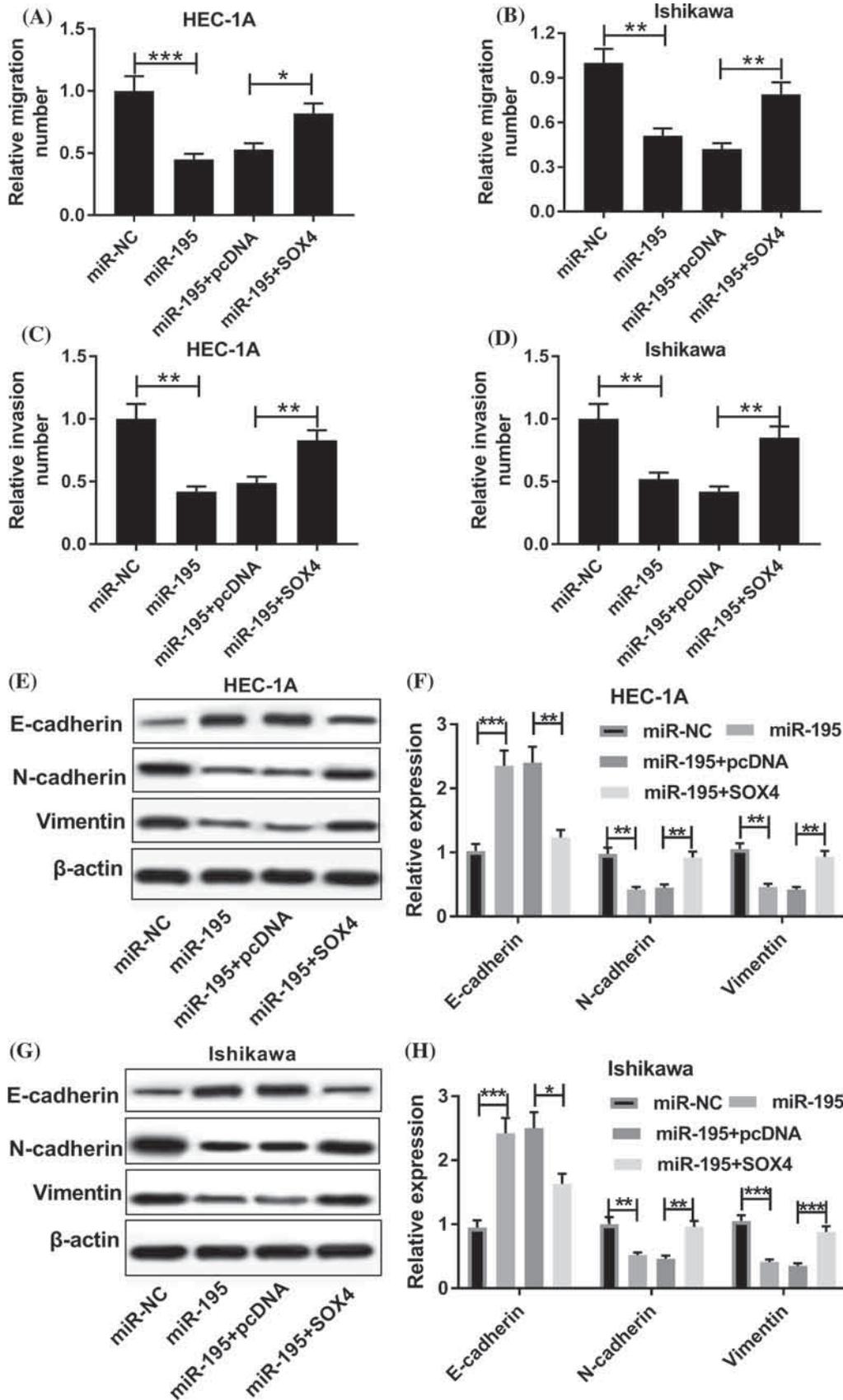
◀ **Figure 5.** MiR-195 exerted its effects on cell migration, invasion and EMT by SOX4. HEC-1A and Ishikawa cells were transfected with miR-195 mimics, miR-NC mimics, miR-195 mimics+pcDNA or miR-195 mimics+pcDNA-SOX4, followed by the detection of cell migration (**A** and **B**) and invasion (**C** and **D**) abilities by transwell assay, the expression levels of N-cadherin, Vimentin and E-cadherin by western blot (E-H). *P < 0.05 or **P < 0.01 or ***P < 0.001 vs. miR-NC mimics or miR-195 mimics+pcDNA.

Some miRNAs were identified as potent tumor-suppressors involved in EC, such as miR-424 (Li *et al.* 2015a), miR-29b (Chen *et al.* 2017) and miR-340 (Xie *et al.* 2016). Moreover, miR-320d repressed EMT of EC cells by targeting PBX3 (Wang *et al.* 2017). Some high expression miRNAs were highlighted as potential oncogenes in EC progression, including miR-200a, miR-200b and miR-429 (Yoneyama *et al.* 2015), and miR-125b (Jiang *et al.* 2011). Additionally, miR-93 overexpression promoted EC cell migration, invasion and EMT through inhibiting FOXA1 expression (Chen *et al.* 2016a).

MiR-195 has been identified as a tumor-suppressor in a large number of human cancers (Cai *et al.* 2015, Liu *et al.* 2015). Accumulating evidence has also suggested that downregulated miR-195 play an important role in EC tumorigenesis (Hiroki *et al.* 2010, Jayaraman *et al.* 2017, Tsukamoto *et al.* 2014). Moreover, a previous document reported that knockdown of plasmacytoma variant translocation 1 combined with miR-195 overexpression suppressed EC progression (Kong *et al.* 2018). In the present study, our data supported that miR-195 was significant downregulated in EC tissues and cells. Moreover, we verified that miR-195 inhibited the migration, invasion and EMT of EC cells, highlighting its role as a potential tumor-suppressor in EC metastasis.

SOX4, a member of the SOXC group, functions as an important regulator in diverse developmental processes by facilitating protein-protein or protein-DNA interactions (Kamachi and Kondoh 2013). SOX4 has been established as a master regulator of EMT through governing the expression of the epigenetic modifier Ezh2 (Tiwari *et al.* 2013). SOX4 overexpression has been reported in a variety of human cancers, such as melanomas (Jafarnejad *et al.* 2010) and leukemias (Zhang *et al.* 2013), suggesting a fundamental role in the development of these malignancies. A previous document demonstrated that SOX4 acted as an activator of β-catenin signaling by inducing TCF4 expression during transdifferentiation toward the morular phenotype of EC (Saegusa *et al.* 2012). In addition, SOX4 was found to be upregulated in EC tissues compared with uninvolved controls (Huang *et al.* 2009). Moreover, SOX4 overexpression was concomitant with miR-129-2 depletion in majority of EC tissues, and miR-129-2 restoration inhibited SOX4 expression and reduced EC cells proliferation (Huang *et al.* 2009). In this study, we validated that SOX4 was upregulated in EC tissues and cell lines, consistent with previous work (Huang *et al.* 2009).

It is widely accepted that miRNAs play crucial roles in a wide array of biological processes by negatively regulating their target genes (Finnegan and Pasquinelli 2013). Hence, Starbase 3.0 software was used to predict the targets of miR-195. Intriguingly, the predicted data revealed that the 3′-UTR of SOX4 mRNA harbored a putative target sequence for miR-195. Subsequently, we confirmed that SOX4 was a direct target of miR-195. Further, our data firstly revealed that miR-195 mimics-mediated anti-migration, anti-invasion and anti-EMT effects were abated by SOX4 expression restoration in EC cells. Similar with our findings, Huang and colleague (Huang *et al.* 2009) manifested that miR-129-2 inhibited EC cell proliferation though targeting SOX4. Li *et al* (Yeh *et al.* 2013) found that miR-138 repressed ovarian cancer cell migration, invasion and metastasis via targeting EMT regulator SOX4.

In conclusion, our data supported that miR-195 was downregulated and SOX4 was upregulated in EC tissues and cell lines. Furthermore, we firstly demonstrated that miR-195 repressed the migration, invasion and EMT of EC cells at least in part by targeting SOX4. Our study provided a novel underlying mechanism for EC metastasis and a promising therapeutic target for EC management.

Acknowledgements

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