**MicroRNA-200a inhibits TGF-β-induced epithelial-mesenchymal transition in human ovarian carcinoma cells by downregulating SOX4 expression**

**Type**
Research paper

**Keywords**
Ovarian cancer, Epithelial-mesenchymal transition, MicroRNA-200a, Transforming growth factor-β, SOX4

**Abstract**

**Introduction**
The microRNA-200 (miR-200) family and sex-determining region Y-box 4 (SOX4) could regulate EMT phenotypes, which is important to the process of tumor pathological. This study explored the association of miR-200a with SOX4 in transforming growth factor (TGF)-β-induced EMT of OC cells.

**Material and methods**
For the in vitro experiments, human CO cells subjected to TGF-β was used to induce EMT; the activity of miR-200a was selectively inhibited or overexpressioned by miR-200a inhibitor and mimics, respectively. Small interfering RNAs against SOX4 (si-SOX4) were used to inhibit SOX4 expression in human OC cell lines.

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Decreased miR-200a and increased SOX4 levels were detected in patients with OC and these changes were closely related to the International Federation of Gynaecology and Obstetrics stage, ovarian tumor biomarker CA125 level, lymph node status and tumor size. The TGF-β-treated cells increased the miR-200a level, decreased the SOX4 level and prompted EMT properties, including a reduction in epithelial marker (e-cadherin), induction in interstitial markers (vimentin and n-cadherin), and enhancement of proliferation, migration and invasion. The OC cells were transducted with miR-200a mimic and the overexpression cells were subsequently treated with TGF-β, decreased SOX4 expression and EMT properties were detected. Also, in miR-200a inhibited cells, TGF-β increased SOX4 expression and EMT properties. Moreover, SOX4 silencing weakened the effect of the miR-200a inhibitor.

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Overall, these results provide a link between miR-200a and SOX4 in OC pathogenesis and indicate that miRNA-200a inhibits EMT by downregulating SOX4 expression in human OC cells.
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Running title: MiR-200a downregulates SOX4

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**Keywords:** Ovarian cancer; MicroRNA-200a; Transforming growth factor-β; Epithelial-mesenchymal transition; SOX4

**Introduction**

Ovarian cancer is a malignant tumor that occurs in the epithelium of the ovary, is one of the most common tumors of the female reproductive organs that affects ~200,000 women worldwide every year; ~60% of women with ovarian cancer (OC) die due to diagnosis at an advanced stage [1]. Tumor metastasis increased migration rate of tumor cells, which is the main factor of the rapid progression of OC from an early to an advanced stage. [2]. This enhanced migration ability is critical in tumor progression and effect the epithelial-mesenchymal transition (EMT) process [3]. Although many researchers have pointed out the that EMT is mediated by complex molecular networks [3], additional studies are needed to understand its underlying mechanism in OC.

MicroRNAs (miRNAs) lead to the inhibition or degradation of translation by regulating
mRNA [4]. A large number of miRNAs, including miR-181a [5], miR-125b [6] and miR-424 [7], are involved in OC metastasis through an EMT-regulated mechanism. In addition, abnormal miR-200 expression in OC and its suppressive roles in tumor metastasis have been demonstrated [8]. Studies have revealed that miR-200 is significantly decreased after transforming growth factor (TGF)-β treatment, whereas the level of the cadherin transcriptional repressors ZEB1 and ZEB2, was inhibited by the miR-200 overexpression, which in turn inhibits EMT [9, 10]. Moreover, the sex-determining region Y-box 4 (SOX4) is a recognized upstream factor of EMT, and this process is mediated by miRNAs, including miRNA132 [11], miR-129-5p [12] and miR-187 [13]. However, whether miR-200a is involved in triggering EMT through SOX4 regulation in OC remains unknown. Thus, to illuminate this issue, we have performed the following experiments to test the anti-ETM effects of miR-200a by targeting SOX4 in OC.

**Materials and methods**

**Tissue specimen.**

Specimens of ovarian surface epithelium were collected from 48 patients (age range, 39-71 years; mean age ± SD, 48.63 ± 5.02 years) with OC and 15 patients (age range, 32-55 years; mean age ± SD, 40.95 ± 4.38 years) with benign gynecological diseases (control group), who underwent oophorectomy in our hospital between January 2013 and January 2018. These people provided signed informed consent and no other treatment performed before oophorectomy. Ovarian surface epithelium was collected and then stored in liquid nitrogen, then analyzed by
some clinical features: age, International Federation of Gynaecology and Obstetrics (FIGO) stage [14], CA125 level, lymph node status and tumor size. OC patients were classified as low expression group when miR-200a expression in OC group was lower than mean miR-200a level in control group.

**Cell culture.**

OVCAR3 cell line was obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were placed into 6-well plates and routinely cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher scientific, Inc.), penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The cells were incubated in 5% CO₂, and 95% humidity at 37 ℃. Additionally, for TGF-β treatment, TGF-β (10 ng/ml; BD Biosciences) was administered to the cells for 2 days, which were then harvested for further experiments.

**Cell transfection and luciferase assays.**

MiR-200a mimic (5′-UAACACUGUCUGGAACGUGU-3′), mimic control (negative controls 1, NC1, 5′-GTGTAACACGTCTATACGGCCCA-3′), inhibitor (5′-UAACCUCAUGUGUGUACGUAUGU-3′) and inhibitor control (negative controls 2, NC2, 5′-UUGUACUACACAAAAGUACUG-3′) were purchased from Guangzhou RiboBio Co. Ltd. Small interfering RNAs for SOX4 (si-SOX4,
5-GCAAACGCTGGAAGCTGCTCAAAGA-3′) and plasmid vector containing non-targeting sequences (negative controls 3, NC3, 5′-TGGGTCGACTCAGAACGACGAAACA-3′) were purchased from Shanghai GenePharma Co., Ltd [15]. Cell transfection was performed using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions.

The fragment of the 3′-untranslated region (UTR) of SOX4 containing miR-200a-binding sites was amplified by PCR and inserted downstream of the firefly luciferase gene in the pGL3-promoter vector (Promega Corporation). miRanda (www.microRNA.org) was used to explore the potential targets of miR-200a, and a luciferase reporter assay was conducted according to a previous study [16].

Reverse transcription-quantitative PCR (RT-qPCR).

The detailed procedure and the primers used to detect miR-200a and U6, used as reference, were both used according to a previous study [17]. Briefly, RNAs were acquired by Trizol reagent (Invitrogen, New York, CA, USA), and then cDNA was obtained by PrimeScript™ RT Reagent Kit (Takara, Dalian, China). And qRT-PCR was carried out by SYBR® Premix Ex Taq™ II kit (Takara) base on cDNA with ABI 7500 Fast Real-Time PCR system (ABI, USA) according to the manufacturer’s instructions. U6 was used as a relative control because it is stably expressed in the cells.

Western blotting.

Total supernatants of protein were prepared using RIPA lysis buffer (Boster Biological
Technology) and the concentration was measured by Bradford assay (Boster Biological Technology). After total proteins (25 μg protein of each sample per lane) were loaded and segregated using a 10% gel in SDS-PAGE, then PVDF membranes was used for transfer (Boster Biological Technology). Membranes were blocked with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 for 1 h, the PVDF was incubated with rabbit anti-SOX4 (1:1,000; Abcam; Catalogue number: ab86809), rabbit anti-E-cadherin (1:1,000; Abcam; Catalogue number: ab15148), rabbit anti-N-cadherin (1:1,000; Abcam; Catalogue number: ab7601), rabbit anti-vimentin (1:1,000; Abcam; Catalogue number: ab92547) and rabbit anti-GAPDH (1:3,000; Abcam; Catalogue number: ab181602) primary antibodies overnight at 4˚C. After washing in TBST, the PVDF membrane was placed on goat anti-rabbit HRP secondary antibody (1:3,000; Boster Biological Technology; Catalogue number: BA1055). Signals were detected using Ultra-sensitive ECL chemiluminescence Reagent (Boster Biological Technology; Catalogue number: AR1190) and proteins were normalized to GAPDH. The optical density of bands was analyzed by Fusion FX5 software (Vilber Lourmat Deutschland GmbH, Germany).

**Immunohistochemistry (IHC).**

SOX4 expression was detected by IHC in the human ovarian surface epithelium from the patients with OC. The collected specimens were embedded in paraffin and then sliced into 5-μm sections. The slices were deparaffinized with xylol and rehydrated with different concentration of
ethanol. Block endogenous peroxidase with 3% hydrogen peroxide for 10 min, then the sections were soaked in antigen retrieval with sodium citrate buffer (pH 6.0) for 5 min, 96°C. Then sections were permeabilized in PBS with 0.2% Triton X-100 for 15 min at room temperature and blocked with PBS containing 0.5% bovine serum albumin. anti-rabbit IHC kit (Boster Biological Technology; Catalogue number: SV0002) and rabbit anti-SOX4 (1:50; Abcam; Catalogue number: ab86809) was used. The intensity of the positively cells was visualized with 40x magnification of the light microscope (Olympus Corporation), which was divided into 4 levels: negative; weak; moderate; and strong. The ratio of positively stained cells was counted and then divided into 4 levels: 1-0-25%; 2-26-50%; 3-51-75%; and 4-76-100%. SOX4 staining scores = the scores of the staining intensity (0-3) × the scores of the percentage of positively stained cells (1-4). According to the scores data, If the score <6, it is regarded as a low expression group, and if the score ≥6, it is regarded as a high expression group [18].

Proliferation and migration.

Cell proliferation was detected by Cell Counting kit-8 (CCK-8, Boster Biological Technology). OVCAR3 Cells were cultured in 2.0*10^3 per wells of 96-well plates and were treated with mimic, NC1, inhibitor, NC2, si-SOX4) and NC3. After 24 h, CCK-8 solution (10 μL) was added, and after another 24 h, the absorbance was measured under the absorbance of 450 nm.

Cell migration analysis was detected by wound-healing experiment. After cells achieved to 90-100% confluence, cells were treated with mimic, NC1, inhibitor, NC2, si-SOX4) and NC3,
and the confluent cell layer was scrape across by the 200-µl plastic pipette tip. Subsequently, the scratched-cells were washed by PBS and removed with serum-free medium, then the initial gap width (0 h) was photographed with an inverted microscope. After 48 h scratching, and residual gap width (48 h) was also recorded. The relative migration was accounted. Migration\% = (gap at 0 h-gap at 48 h)/gap at 0 h.

Moreover, cell migration analysis was also measured by the transwell assay according to a previous study [19]. The number of the cells that translocated to the lower compartment was calculated in five light microscopic fields selected randomly under 40X microscope.

Statistical analysis.

Analyses were carried out through GraphPad Prism 5 software. All results were expressed as mean ± standard error of the mean (S.E.M.). The relationship between SOX4 expression and clinical characteristics was assessed using Fisher’s exact test or \( \chi^2 \) test, and other statistical significance between groups was determined by unpaired student t-test or one-way ANOVA. All tests were performed in triplicates independently. \( P \) value of less than 0.05 was considered to a statistically significant difference. All experiments were repeated at least three times.

Results

miR-200a expression is correlated with the clinical characteristics of OC patients.
To explore the relationship of the miR-200a and OC, we detect the expression of miR-200a in the specimens of ovarian surface epithelium from the control (n=15) and OC groups (n=48) by RT-qPCR. And found that miR-200a expression was much lower in OC group than in healthy group (Fig. 1A; P<0.05), indicating that miR-200a may be related to the initiation and the pathogenesis of ovarian cancer. In the ovarian surface epithelium specimens of the OC group, there were 30 cases in the low-expression group and 18 cases in the high-expression group. In addition, we found that the downregulation of miR-200a in the patients was statistically correlated with the stage of FIGO, CA125 level, lymph node status and tumor size (Table 1).

Increased SOX4 expression is correlated with the clinical characteristics of patients with OC.

SOX4 expression in the ovarian surface epithelium specimens of the control group (n=15) and the OC group (n=48) was detected. Immunoblotting results showed that SOX4 level in OC group was higher when compared with healthy group (Fig. 1B; P<0.05). To further confirm the effect of SOX4 in OC, we detected the nuclear localization of transcription factor SOX4. Immunofluorescence results showed that SOX4 was mainly located in the nucleus (Fig. 1C; P<0.05). After score assignment, the ovarian surface epithelium specimens of the OC group were classified to low SOX4 expression groups (n=13) and high (n=35) SOX4 expression groups in order to assess the relationship between SOX4 expression and OC patient characteristics. These data indicated that SOX4 expression was obviously related to FIGO staging, CA125 level, lymph node status and tumor size (Table 1).
TGF-β-induced EMT is accompanied by miR-200a downregulation and SOX4 upregulation in OC cell lines.

To detect whether tumor EMT was accompanied by altered expression of miR-200a and SOX4, EMT was induced in OVCAR3 cells by TGF-β. After a 48-h treatment with TGF-β, we found that TGF-β treatment led to a markedly decrease of e-cadherin expression compared with vehicle-treated cell, these results were further confirmed as evidenced by increased levels of the mesenchymal markers (vimentin and N-cadherin) (Fig. 2A; n=8; P<0.05), which suggested that TGF could induced EMT occurrence. In addition, increased proliferation (Fig. 2B; n=8; P<0.05) and migration (Fig. 2C, 2D; n=8; P<0.05) were detected after treatment with TGF-β. Moreover, the TGF-β group had decreased miR-200a expression (Fig. 2E; n=8; P<0.05), increased SOX4 expression (Fig. 2F; n=8; P<0.05) compared to the non-TGF-β group.

miR-200a is associated with EMT in OC cell lines by regulating SOX4 expression.

To explore the targets of miR-200a, miRanda assay was performed. We found that the complementary area of Mir-200a region on the 3-UTR of SOX4 (Fig. 3A). To further confirm their relationship, we generated a dual-luciferase reporter gene containing the SOX4 3’-UTR. In the OVCAR3 cells, miR-200a mimic showed a lower luciferase activity, which suggested that miR-200a overexpression reduced SOX4 activity (Fig. 3B; P<0.05). Moreover, to determine whether miR-200a takes part in EMT in OC by regulating SOX4 expression, we transfecting
OVCAR3 cells with NC1, miR-200 mimics, NC2 and miR-200 inhibitor for 48 h after TGF-β treatment. As expect, the data demonstrated that miR-200a expression was enhanced in the mimic group, and markedly reversed in the inhibitor group than the relative control, respectively (Fig. 3C; n=8; P<0.05). In the mimic group, SOX4 level was suppressed compared with the NC1 (Fig. 3D; n=8; P<0.05) and an opposite trend was observed in the inhibition group. To assess the level of EMT, E-cadherin expression and decreased mesenchymal marker (vimentin and n-cadherin) was used. Increased E-cadherin expression and decreased vimentin and n-cadherin expression were detected when cells subjected to miR-200a mimic (Fig. 3E; n=8; P<0.05). However, an induction in E-cadherin expression and a reduction in vimentin and N-cadherin expression were detected after miR-200 was inhibited than NC2 group (Fig. 3E; n=8 group; P<0.05). In addition, miR-200 overexpression diminished the ability of cells proliferation (Fig. 3F; n=8; P<0.05), and migration (Fig. 3G, 3H; n=8; P<0.05) when treated with the vehicle. However, promoted proliferation (Fig. 3F; n=8; P<0.05), as well as migration (Fig. 3G, 3H; n=8; P<0.05) were detected after cells subjected to the inhibitor. These results revealed that miR-200a overexpression repressed EMT in OC by regulating SOX4 expression.

*Inhibition of SOX4 weakens the effect of miR-200a inhibitor on EMT in OC cell lines.*

To further detect whether miR-200a regulated EMT in OC by targeting SOX4, si-SOX4 was used to inhibit SOX4 expression in the OVCAR3 cells. SOX4 inhibition significantly decreased SOX4 expression (Fig. 4A; n=8; P<0.05). Subsequently, TGF-β-treated OVCAR3 cells
transduced with a mixture of NC2 and si-SOX4 (NC2+si-SOX4), a mixture of miR-200a inhibitor and si-SOX4 control scramble(inhibitor+NC3), as well as a mixture of miR-200a inhibitor and si-SOX4 (inhibitor+si-SOX4). We found that the expression of SOX4 was significantly decreased in the NC2+si-SOX4 group and increased in the inhibitor+NC3 group than the inhibitor+si-SOX4 group (Fig. 4B; n=8; P<0.05). E-cadherin expression was upregulated and mesenchymal markers (vimentin and N-cadherin) expression was downregulated in the NC2+si-SOX4 group compared with the inhibitor+si-SOX4 group (Fig. 4C; n=8; P<0.05). However, decreased E-cadherin expression and increased expression of mesenchymal markers (vimentin and N-cadherin) were observed in the inhibitor+NC3 group relative to inhibitor+si-SOX4 group (Fig. 4C; n=8; P<0.05). In addition, NC2+si-SOX4 administration showed obviously diminish in proliferation (Fig. 4D; n=8; P<0.05) and migration (Fig. 4E, 4F; n=8; P<0.05) than inhibitor+si-SOX4 group. However, increased proliferation (Fig. 4D; n=8; P<0.05), migration (Fig. 4E, 4F; n=8; P<0.05) were showed in the inhibitor+NC3 group than the corresponding control group.

Discussion

EMT is characterized by decreased the characters of epithelial cells and to gain the phenotype of mesenchymal cells, leading to enhanced cellular motility and invasion, which occurs during embryogenesis, tissue repair and tumorigenesis [20]. Although the molecular mechanism of EMT during tumorigenesis is extremely complex and remains unknown, it is
widely accepted that TGF-β is an effective inducer of EMT by binding to its receptor, resulting in the activation of SMAD2 and SMAD3 proteins [21]. These two proteins then combine with SMAD4 and regulate the levels of other target factors, including the ones that are commonly known as EMT inducers. Therefore, TGF-β is often used to induce EMT in different experiments. Thus, we used TGF-β-treat human OC OVCAR3 cell line to generated EMT model, resulting in EMT occurrence, increased proliferation, migration and invasion.

SOX4 is closely associated with tumor progression [22]. In many tumor types, such as renal [23], prostate [11], breast [24] and lung [25] cancer, a high expression level of SOX4 results in the loss in epithelial cell properties and increased in interstitial properties. Additionally, SOX4 is a key factor for the process of TGF-β-induced EMT [21, 26]. Previous studies reported that TGF-β upregulates SOX4, which promotes EMT in gastric cancer cells [27]. Also, a growing body of literature shows that the SOX level was high in the TGF-β-treated OVCAR3 cells. Moreover, SOX4 expression was increased in patients with OC and its expression was closely related to FIGO stage, CA125 levels, lymph node status as well as tumor size, which was similar to previous studies [28, 29]. These data indicated that SOX4 may participate in OC development through EMT regulation. Therefore, inhibition of SOX4 may be expected to suppress the progression of OC.

Several studies reveal that miRNAs also regulate EMT in OC through targeting SOX4. Lin et al [30] indicated that miR-212 and miR-132 target SOX4, suppress its activation and decrease the EMT-like properties. Although many studies revealed that the miR-200 could increase epithelial cell integrity by regulated the level of target genes thereby suppressing EMT in OC by
[31, 32], the involvement of miR-200a in triggering EMT in OC by SOX4 regulation is still unknown. In the present study, TGF-β-treated OVCAR3 cells reduced miR-200a expression and induced SOX4 expression. When miR-200a was overexpressed in the TGF-β-administrated OVCAR3 cells, SOX4 expression was decreased and OVCAR3 cells showed EMT properties. However, miR-200a downregulation increased SOX4 expression, promoted EMT, and enhanced proliferation, migration and invasion. siRNA was also used to downregulate SOX4, in order to assess the relationship between SOX4 and miR-200a; the data indicated that SOX4 silencing impaired the influence of the miR-200a inhibitor. Additionally, the expression of miR-200a was decreased in specimen of OC, which was closely involved in the FIGO stage, CA125 levels, lymph node status and tumor size.

Our study demonstrated that miRNA-200a could inhibit EMT in TGF-β-induced human OC cells by downregulating SOX4 level (Figure 5). We provide a potential functional linkage between the miRNA-200a and OC and miRNA-200a might be a new therapeutic factor for combat OC. However, these conclusions were only verified by in vitro experiments. It is still unknown whether miRNA-200a plays an anti-EMT role in OC in vivo, which might be a potential future research direction.

Acknowledgements

Not applicable.
References


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**Figure legends**

**Figure 1.** miR-200a and SOX4 gene and protein expression levels in the ovarian surface epithelium specimens. (A) miR-200a gene expression in the specimens of the control and OC
groups. (B) SOX4 protein expression in the specimens of the control and OC groups. (C) Representative immunohistochemistry images of weak, moderate and strong SOX4 staining (scale bar = 50 μm). *P<0.05 vs. control group. miR, microRNA; SOX4, sex-determining region Y-box 4; OC, ovarian cancer.

Figure 2. TGF-β-induced epithelial-mesenchymal transition is correlated with miR-200a downregulation and SOX4 upregulation in ovarian cancer cell lines. (A) E-cadherin, vimentin and N-cadherin protein expression in OVCAR3 cells treated with or without TGF-β detected by immunoblotting. OVCAR3 cells treated with or without TGF-β to detect (B) proliferation by Cell Counting Kit-8 assay, (C) migrated distance by wound-healing assay (scale bar = 100 μm) and (D) migrated cells by Transwell assay (scale bar = 100 μm). (E) miR-200a gene expression in OVCAR3 cells treated with or without TGF-β detected by RT-qPCR. (F) SOX4 protein expression in OVCAR3 cells treated with or without TGF-β detected by immunoblotting. n=8 per group. Student’s t-test; *P<0.05 vs. non-TGF-β group. TGF-β, transforming growth factor-β; miR, microRNA; SOX4, sex-determining region Y-box 4.

Figure 3. miR-200a is associated with EMT in ovarian cancer cell lines by regulating SOX4 expression. (A) The 3’-untranslated region (UTR) of SOX4 with miR-200a-binding sites. (B) The results of dual-luciferase reporter assay. (C) miR-200a expression in TGF-β-treated OVCAR3 cells transfected with NC1, mimic, NC2 and inhibitor, detected by RT-qPCR. (D) SOX4 protein expression in TGF-β-treated OVCAR3 cells transfected with NC1, mimic, NC2
and inhibitor, detected by western blotting. (E) E-cadherin, vimentin and N-cadherin protein expression in TGF-β-treated OVCAR3 cells transfected with NC1, mimic, NC2 and inhibitor, detected by immunoblotting. TGF-β-treated OVCAR3 cells transfected with NC1, mimic, NC2 and inhibitor to detect (F) proliferation by Cell Counting Kit-8 assay, (G) migrated distance by wound-healing assay (scale bar = 100 μm), (H) and migrated cells by Transwell assay (scale bar = 100 μm). n=8 per group; one-way ANOVA followed by post hoc Tukey’s test. *P<0.05 vs. NC1 group. #P<0.05 vs. NC2 group. miR, microRNA; EMT, epithelial-mesenchymal transition; TGF-β, transforming growth factor-β; SOX4, sex-determining region Y-box 4; NC, negative control.

**Figure 4. SOX4 silencing weakens the effects of miR-200a inhibitor on EMT in ovarian cancer cell lines.** (A) SOX4 protein expression in OVCAR3 treated with NC3 or si-SOX4. (B) SOX4 protein expression in TGF-β-treated OVCAR3 cells treated with a mixture of NC2 and si-SOX4 (NC2+si-SOX4), mixture of miR-200a inhibitor and NC3 (inhibitor+NC3) or mixture of miR-200a inhibitor and si-SOX4 (inhibitor+si-SOX4), detected by immunoblotting. (C) E-cadherin, vimentin and N-cadherin protein expression in the TGF-β-treated OVCAR3 cells treated with NC1+si-SOX4, inhibitor+NC3 or inhibitor+si-SOX4, detected by western blotting. TGF-β-treated OVCAR3 cells treated with NC2+si-SOX4, inhibitor+NC3 or inhibitor+si-SOX4 to detect (D) proliferation by Cell Counting Kit-8 assay, (E) migrated distance by wound-healing assay (scale bar = 100 μm), (F) and migrated cells by Transwell assay (scale bar = 100 μm). n=8 per group; one-way ANOVA followed by post hoc Tukey’s test. @P<0.05 vs. NC3 group.
*P<0.05 vs. inhibitor+si-SOX4 group. #P<0.05 vs. inhibitor+NC2 group. miR, microRNA; EMT, epithelial-mesenchymal transition; TGF-β, transforming growth factor-β; SOX4, sex-determining region Y-box 4; NC, negative control; si-SOX4, small interfering RNAs against SOX4.

**Figure 5. Effects of miR-200a on EMT in OC.** TGF-β induces SOX4 up-regulation, which promotes EMT in OC. However, this promotion could be inhibited by miR-200a through targeting SOX4.
Table 1. Expression of miR-200a and SOX4 is associated with clinical characteristics of patients (n=48) with ovarian carcinoma.

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Figure 2. TGF-β-induced epithelial-mesenchymal transition is correlated with miR-200a downregulation and SOX4 upregulation in ovarian cancer cell lines. (A) E-cadherin, vimentin and N-cadherin protein expression in OVCAR3 cells treated with or without TGF-β detected by immunoblotting. OVCAR3 cells treated with or without TGF-β to detect (B) proliferation by Cell Counting Kit-8 assay, (C) migrated distance by wound-healing assay (scale bar = 100 μm) and (D) migrated cells by Transwell assay (scale bar = 100 μm). (E) miR-200a gene expression in OVCAR3 cells treated with or without TGF-β detected by RT-qPCR. (F) SOX4 protein expression in OVCAR3 cells treated with or without TGF-β detected by immunoblotting. n=8 per group. Student’s t-test; *P<0.05 vs. non-TGF-β group.
TGF-β, transforming growth factor-β; miR, microRNA; SOX4, sex-determining region Y-box 4.
Figure 3. miR-200a is associated with EMT in ovarian cancer cell lines by regulating SOX4 expression. (A) The 3’-untranslated region (UTR) of SOX4 with miR-200a-binding sites. (B) The results of dual-luciferase reporter assay. (C) miR-200a expression in TGF-β-treated OVCAR3 cells transfected with NC1, mimic, NC2 and inhibitor, detected by RT-qPCR. (D) SOX4 protein expression in TGF-β-treated OVCAR3 cells transfected with NC1, mimic, NC2 and inhibitor, detected by western blotting. (E) E-cadherin, vimentin and N-cadherin protein expression in TGF-β-treated OVCAR3 cells transfected with NC1, mimic, NC2 and inhibitor to detect (F) proliferation by Cell Counting Kit-8 assay, (G) migrated distance by wound-healing assay (scale bar = 100 μm), (H) and migrated cells by Transwell assay (scale bar = 100 μm). n=8 per group; one-way ANOVA followed by post hoc Tukey’s test. *P<0.05 vs. NC1 group. #P<0.05 vs. NC2 group. miR, microRNA; EMT, epithelial-mesenchymal transition; TGF-β, transforming growth factor-β; SOX4, sex-determining region Y-box 4; NC, negative control.
Figure 4. SOX4 silencing weakens the effects of miR-200a inhibitor on EMT in ovarian cancer cell lines. (A) SOX4 protein expression in OVCAR3 treated with NC3 or si-SOX4. (B) SOX4 protein expression in TGF-β-treated OVCAR3 cells treated with a mixture of NC2 and si-SOX4 (NC2+si-SOX4), mixture of miR-200a inhibitor and NC3 (inhibitor+NC3) or mixture of miR-200a inhibitor and si-SOX4 (inhibitor+si-SOX4), detected by immunoblotting. (C) E-cadherin, vimentin and N-cadherin protein expression in the TGF-β-treated OVCAR3 cells treated with NC1+si-SOX4, inhibitor+NC3 or inhibitor+si-SOX4, detected by western blotting. TGF-β-treated OVCAR3 cells treated with NC2+si-SOX4, inhibitor+NC3 or inhibitor+si-SOX4 to detect (D) proliferation by Cell Counting Kit-8 assay, (E) migrated...
distance by wound-healing assay (scale bar = 100 μm), (F) and migrated cells by Transwell assay (scale bar = 100 μm). n=8 per group; one-way ANOVA followed by post hoc Tukey’s test. @P<0.05 vs. NC3 group. *P<0.05 vs. inhibitor+si-SOX4 group. #P<0.05 vs. inhibitor+NC2 group. miR, microRNA; EMT, epithelial-mesenchymal transition; TGF-β, transforming growth factor-β; SOX4, sex-determining region Y-box 4; NC, negative control; si-SOX4, small interfering RNAs against SOX4.
Figure 5. Effects of miR-200a on EMT in OC. TGF-β induces SOX4 up-regulation, which promotes EMT in OC. However, this promotion could be inhibited by miR-200a through targeting SOX4.