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

Ziyao Ren[1], Yonghua Hu[1], Jian Chen[2], Lanying Jin[3]

[1]The First People's Hospital of Yongkang, China
[2]Guang Fu Hospital of Jinhua, China
[3]Jinhua Municipal Central Hospital, China

Corresponding author:
Lanying Jin
Jinhua Municipal Central Hospital, China
e-mail: jinlanyingmrs@163.com

Submitted: 2 February 2021; **Accepted:** 17 June 2021
Online publication: 2 July 2021

Arch Med Sci
DOI: https://doi.org/10.5114/aoms/139106

Abstract

Introduction: The microRNA-200 (miR-200) family and sex-determining region Y-box 4 (SOX4) could regulate epithelial-mesenchymal transition (EMT), which is important for the process of tumor pathology. This study explored the association of miR-200a with SOX4 in transforming growth factor (TGF)-β-induced EMT of ovarian carcinoma (OC) cells.

Material and methods: For the in vitro experiments, human CO cells subjected to TGF-β were used to induce EMT; the activity of miR-200a was selectively inhibited or overexpressed 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.

Results: 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 of interstitial markers (vimentin and n-cadherin), and enhancement of proliferation, migration and invasion. The OC cells were transduced 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.

Conclusions: 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.

Key words: 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, and is one of the most common tumors of the female reproductive organs, which 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 increases the migration rate of

tumor cells, which is the main factor responsible for the rapid progression of OC from an early to an advanced stage [2]. This enhanced migration ability is critical in tumor progression and affects 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 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 performed the following experiments to test the anti-EMT effects of miR-200a by targeting SOX4 in OC.

Material and methods

Tissue specimens

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 was 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. Ovarian cancer patients were assigned to the low expression group when miR-200a expression in the OC group was lower than the mean miR-200a level in the control group.

Cell culture

The 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% CO_2, and 95% humidity at 37°C. 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′-UAACACUGUCUGGUAACGAUGU-3′), mimic control (negative controls 1, NC1, 5′-GTGTAACACGTCTATACGCCCA-3′), inhibitor (5′-UAACCUCAUGGUGUACGAAUGU-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 the 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 use of the PrimeScript RT Reagent Kit (Takara, Dalian, China). Reverse transcription-quantitative PCR was carried out with the SYBR Premix Ex Taq II kit (Takara) based on cDNA with the 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: ab76011), 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

The sex-determining region Y-box 4 expression was detected by immunohistochemistry (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. Endogenous peroxidase was blocked 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 40× 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 score data, if the score < 6, it is regarded as low expression, and if the score ≥ 6, it is regarded as high expression [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 a 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 scraped across by the 200-µl plastic pipette tip. Subsequently, the scratched cells were washed with PBS and removed with serum-free medium, then the initial gap width (0 h) was photographed with an inverted microscope. At 48 h after scratching, the residual gap width was also recorded. The relative migration was calculated. Migration% = (gap at 0 h-gap at 48 h)/gap at 0 h.

Cell migration analysis was also performed by the transwell assay according to a previous study [19]. The number of cells that translocated to the lower compartment was calculated in five light microscopic fields selected randomly under the 40× 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 the χ^2 test, and other statistical significance between groups was determined by the unpaired Student's *t*-test or one-way ANOVA. All tests were performed in triplicate independently. A p value of less than 0.05 was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

Results

miR-200a expression is correlated with clinical characteristics of OC patients

To explore the relationship of the miR-200a and OC, we detected 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. We found that miR-200a expression was much lower in the OC group than in the healthy group (Figure 1 A, $p < 0.05$), indicating that miR-200a may be related to the initiation and pathogenesis of ovarian cancer. In the ovarian surface epithelium specimens of the OC group, there were

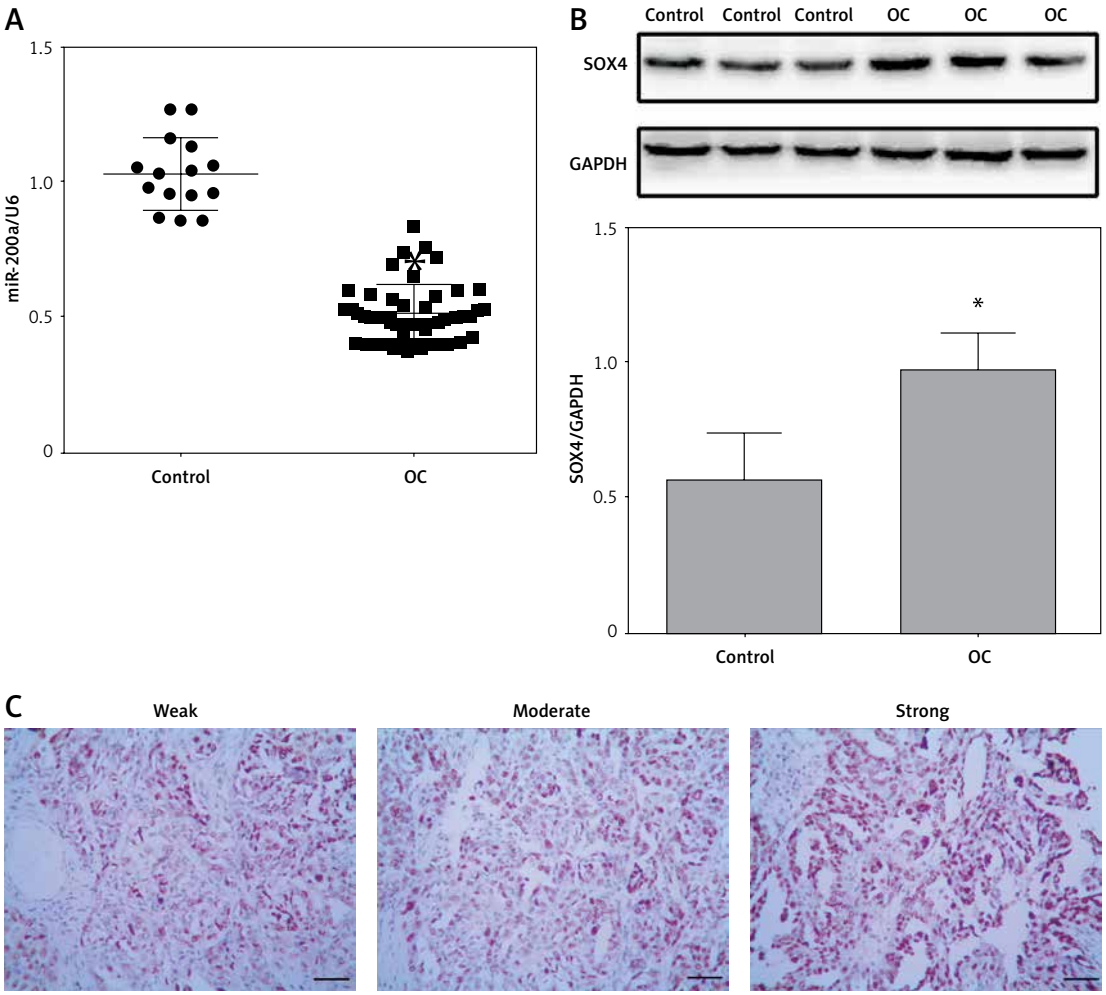


Figure 1. MiR-200a and SOX4 gene and protein expression levels in the ovarian surface epithelium specimens. **A** – miR-200a gene expression in the specimens of the control and OC groups. **B** – SOX4 protein expression in the specimens of the control and OC groups. **C** – Representative immunohistochemistry 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.*

Table I. Association of miR-200a and SOX4 expression with clinical characteristics of patients (n = 48) with ovarian carcinoma

Parameters	miR-200a expression			SOX4 expression		
	Low, *n*	High, *n*	*P*-value	Low, *n*	High, *n*	*P*-value
Total patients	30	18		11	37	
Age [years]	57.05 ±4.52	60.43 ±7.28	0.495	58.84 ±2.02	58.17 ±1.32	0.629
FIGO stage:			0.036			0.016
I–II	11	13		9	14	
III–IV	19	5		2	23	
CA125 [U/ml]	695.25 ±23.35	678.95 ±33.24	0.045	685.51 ±36.58	690.22 ±24.85	0.076
Lymph node metastasis:			0.008			0.040
negative	13	15		3	24	
positive	17	3		8	13	
Tumor size [cm]:			0.037			0.005
< 4	10	12		10	15	
≥ 4	20	6		1	22	

miR – microRNA, SOX4 – sex-determining region Y-box 4, FIGO – International Federation of Gynecology and Obstetrics.

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 I).

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

The sex-determining region Y-box 4 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 the SOX4 level in the OC group was higher than in the healthy group (Figure 1 B, $p < 0.05$). To further confirm the effect of SOX4 in OC, we detected the nuclear localization of the transcription factor SOX4. Immunofluorescence results showed that SOX4 was mainly located in the nucleus (Figure 1 C, $p < 0.05$). After score assignment, the ovarian surface epithelium specimens of the OC group were classified in the low SOX4 expression group (n = 13) and high (n = 35) SOX4 expression group in order to assess the relationship between SOX4 expression and OC patient characteristics. These data indicated that SOX4 expression was statistically correlated with FIGO staging, lymph node status and tumor size (Table I).

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 48-h treatment with TGF-β, we found that TGF-β treatment led to a marked decrease of E-cadherin expression compared with vehicle-treated cells; these results were further confirmed as evidenced by increased levels of the mesenchymal markers (vimentin and N-cadherin) (Figure 2 A, n = 8, $p < 0.05$), which suggested that TGF could induce EMT occurrence. In addition, increased proliferation (Figure 2 B, n = 8, $p < 0.05$) and migration (Figures 2 C, D, n = 8, $p < 0.05$) were detected after

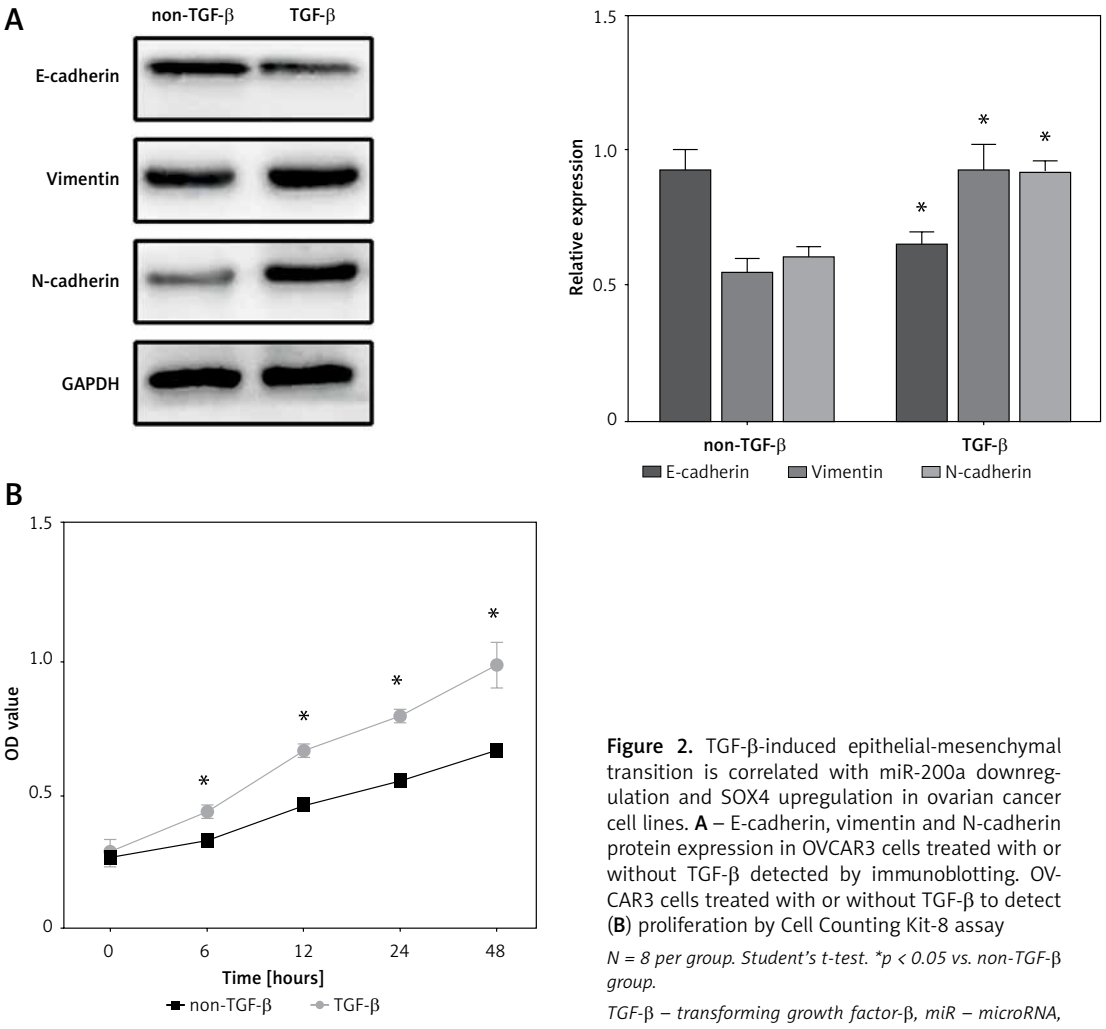


Figure 2. TGF-β-induced epithelial-mesenchymal transition is correlated with miR-200a downregulation and SOX4 upregulation in ovarian cancer cell lines. **A** – E-cadherin, vimentin and N-cadherin protein expression in OVCAR3 cells treated with or without TGF-β detected by immunoblotting. OVCAR3 cells treated with or without TGF-β to detect (**B**) proliferation by Cell Counting Kit-8 assay

*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.

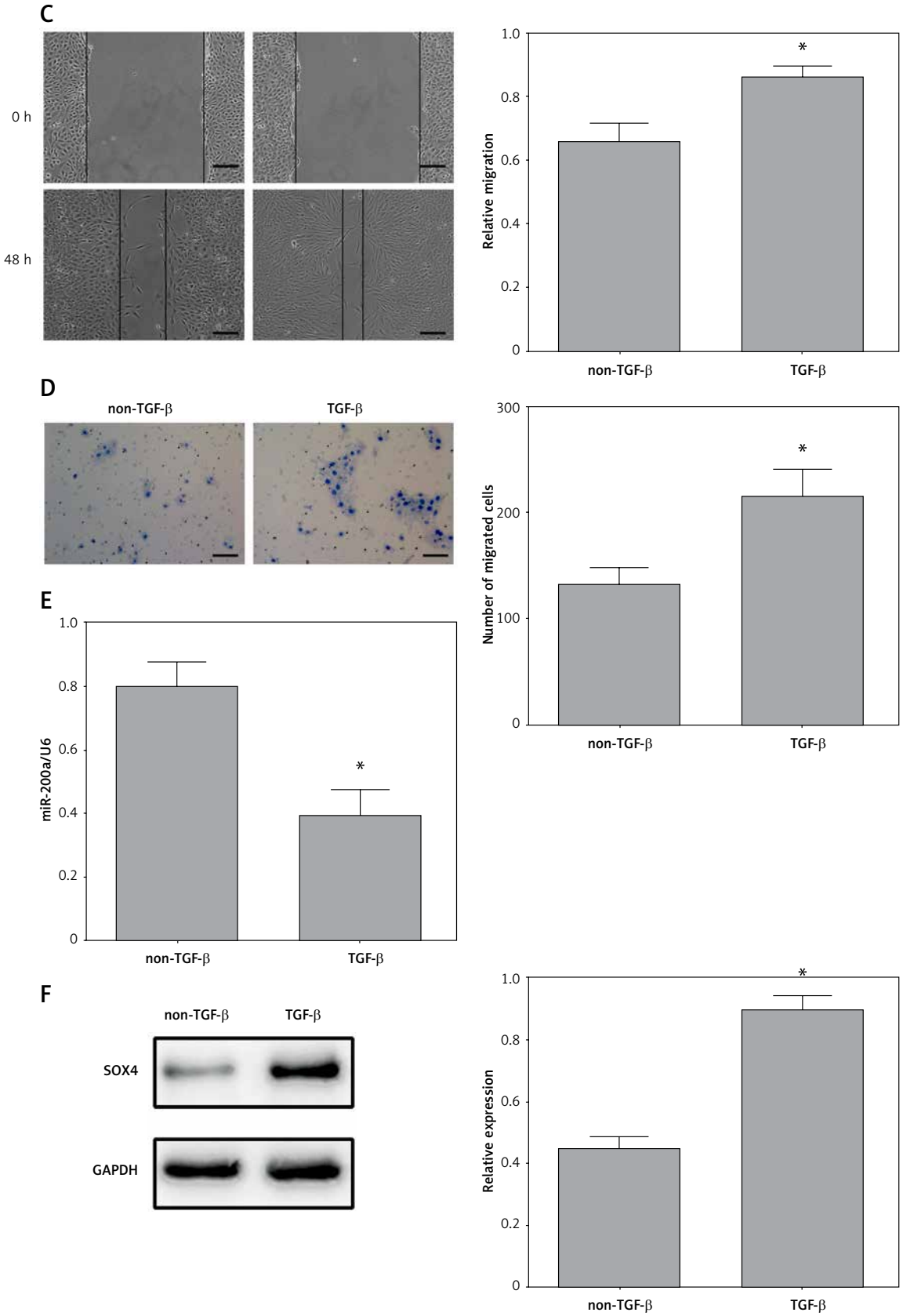


Figure 2. Cont. (**C**) migrated distance by wound-healing assay (scale bar = 100 µm) and (**D**) migrated cells by Transwell assay (scale bar = 100 µm). **E** – miR-200a gene expression in OVCAR3 cells treated with or without TGF-β detected by RT-qPCR. **F** – SOX4 protein expression in OVCAR3 cells 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.

treatment with TGF-β. Moreover, the TGF-β group had decreased miR-200a expression (Figure 2 E, $n = 8$, $p < 0.05$) and increased SOX4 expression (Figure 2 F, $n = 8$, $p < 0.05$) 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 the complementary area of the Mir-200a region on the 3-UTR of SOX4 (Figure 3 A). 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 (Figure 3 B, $p < 0.05$). Moreover, to determine whether miR-200a takes part in EMT in OC by regulating SOX4 expression, we transfected OVCAR3 cells with NC1, miR-200 mimics, NC2 and miR-200 inhibitor for 48 h after TGF-β treatment. As expected, the data demonstrated that miR-200a expression was enhanced in the mimic group, and markedly reversed in the inhibitor group compared to the respective control (Figure 3 C, $n = 8$, $p < 0.05$). In the mimic group, SOX4 level was suppressed compared with NC1 (Figure 3 D, $n = 8$, $p < 0.05$) and an opposite relationship was observed in the inhibition group.

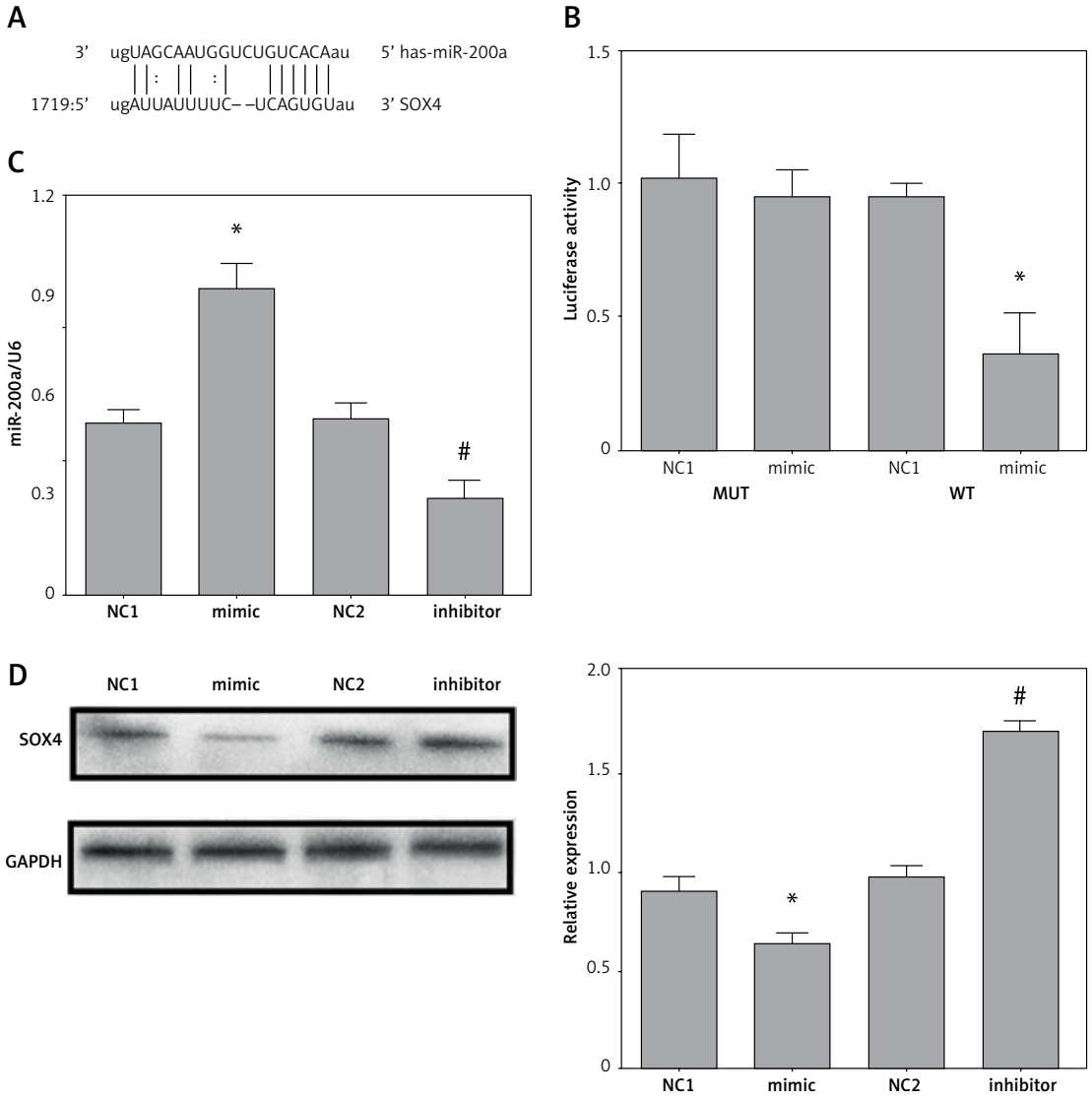


Figure 3. miR-200a is associated with EMT in ovarian cancer cell lines by regulating SOX4 expression. **A** – The 3'-untranslated region (UTR) of SOX4 with miR-200a-binding sites. **B** – The results of dual-luciferase reporter assay. **C** – miR-200a expression in TGF-β-treated OVCAR3 cells transfected 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

*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.*

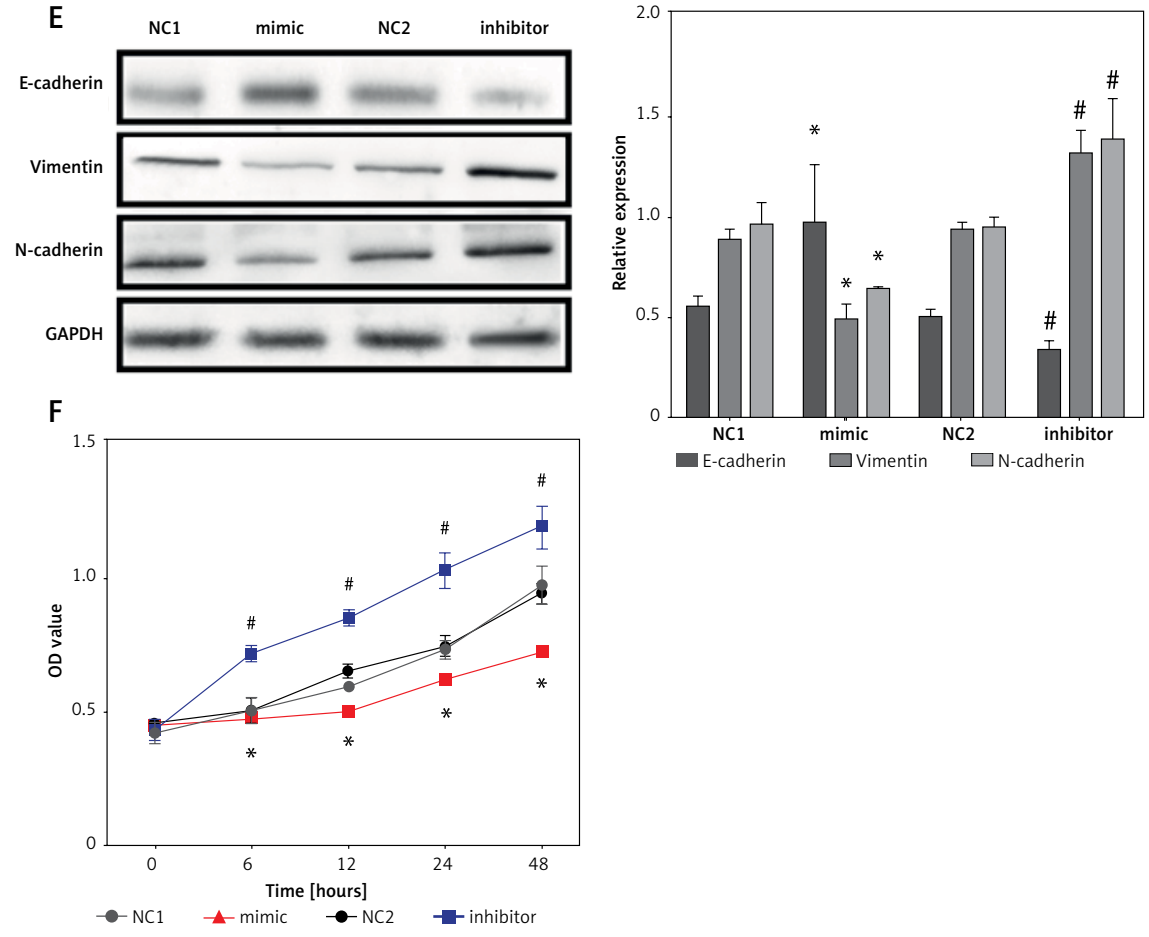


Figure 3. Cont. **E** – E-cadherin, vimentin and N-cadherin protein expression in TGF-β-treated OVCAR3 cells transfected with NC1, mimic, NC2 and inhibitor, detected by immunoblotting. TGF-β-treated OVCAR3 cells transfected with NC1, mimic, NC2 and inhibitor to detect (**F**) proliferation by cell counting kit-8 assay, (**G**) migrated distance by wound-healing assay (scale bar = 100 µm)

*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.

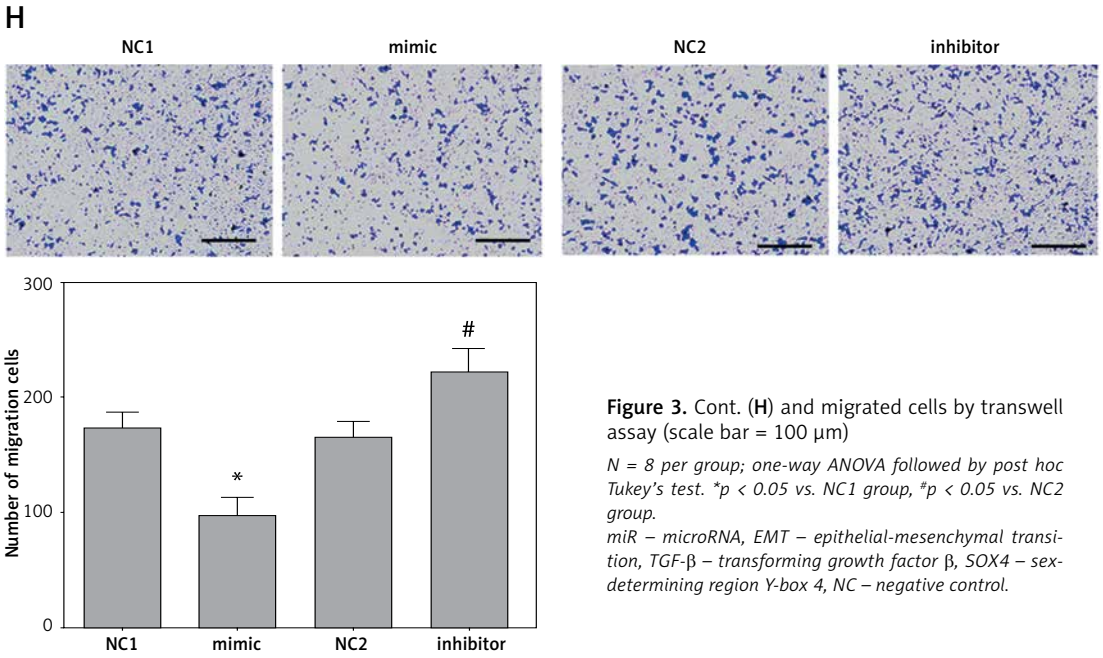


Figure 3. Cont. (**H**) and migrated cells by transwell assay (scale bar = 100 μm)

*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.

To assess the level of EMT, E-cadherin expression and decreased mesenchymal marker (vimentin and n-cadherin) were used. Increased E-cadherin expression and decreased vimentin and n-cadherin expression were detected when cells were subjected to miR-200a mimic (Figure 3 E, $n = 8$, $p < 0.05$). However, induction of E-cadherin expression and a reduction in vimentin and N-cadherin expression were detected after miR-200 was inhibited compared to the NC2 group (Figure 3 E, $n = 8$ group, $p < 0.05$). In addition, miR-200 overexpression diminished the ability of cell proliferation (Figure 3 F, $n = 8$, $p < 0.05$), and migration (Figures 3 G, H, $n = 8$, $p < 0.05$) when treated with the vehicle. However, promoted proliferation (Figure 3 F, $n = 8$, $p < 0.05$), as well as migration (Figures 3 G, H, $n = 8$, $p < 0.05$) were detected after cells were 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 (Figure 4 A, $n = 8$, $p < 0.05$). Subsequently, TGF-β-treated OVCAR3 cells were 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 compared to the inhibitor + si-SOX4 group (Figure 4 B, $n = 8$, $p < 0.05$). 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 (Figure 4 C, $n = 8$, $p < 0.05$). However, decreased E-cadherin expression and increased expression of mesenchymal markers (vimentin and N-cadherin) were observed in the inhibitor + NC3 group relative to the inhibitor + si-SOX4 group (Figure 4 C, $n = 8$, $p < 0.05$). In addition, NC2 + si-SOX4 administration showed significantly diminished proliferation (Figure 4 D, $n = 8$, $p < 0.05$) and migration (Figures 4 E, F, $n = 8$, $p < 0.05$) compared to the inhibitor + si-SOX4 group. However, increased proliferation (Figure 4 D, $n = 8$, $p < 0.05$) and migration (Figures 4 E, F, $n = 8$, $p < 0.05$) were observed in the inhibitor + NC3 group compared to the corresponding control group.

Discussion

The epithelial-mesenchymal transition is characterized by decreased characteristics of epithelial cells and development of 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 activation of SMAD2 and SMAD3 proteins [21]. These two proteins then combine with

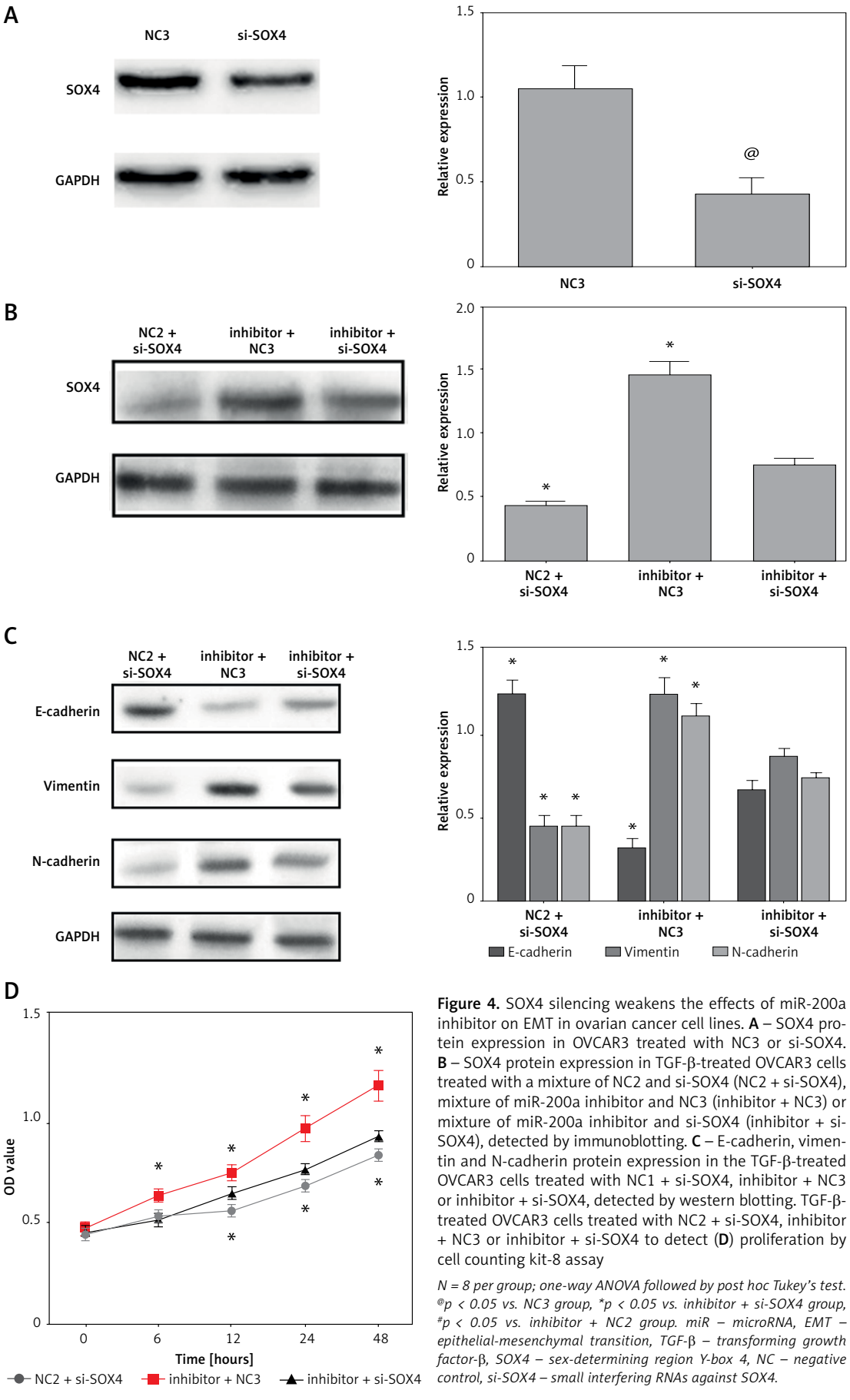
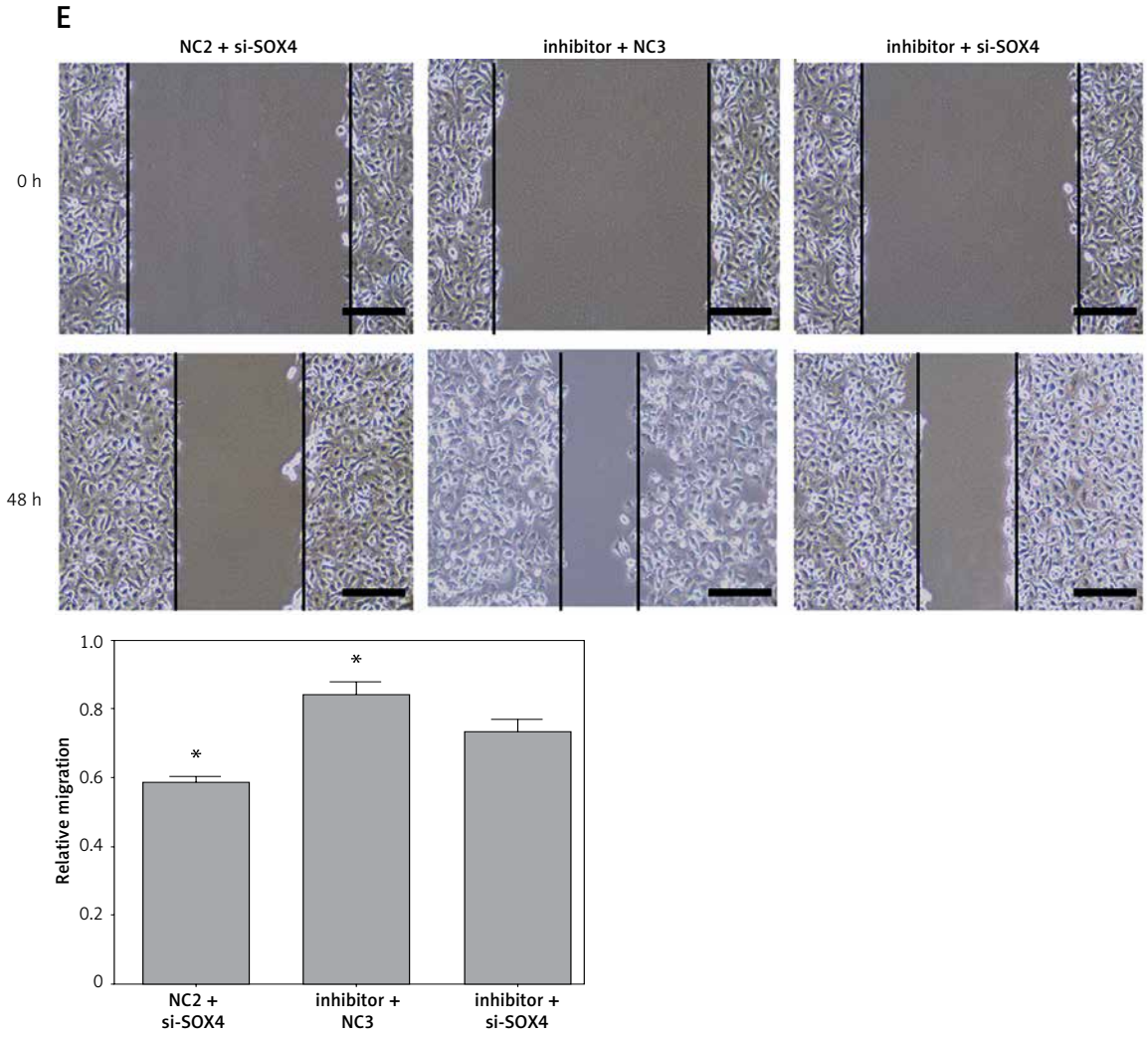


Figure 4. SOX4 silencing weakens the effects of miR-200a inhibitor on EMT in ovarian cancer cell lines. **A** – SOX4 protein expression in OVCAR3 treated with NC3 or si-SOX4. **B** – SOX4 protein expression in TGF-β-treated OVCAR3 cells treated with a mixture of NC2 and si-SOX4 (NC2 + si-SOX4), mixture 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

*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.*



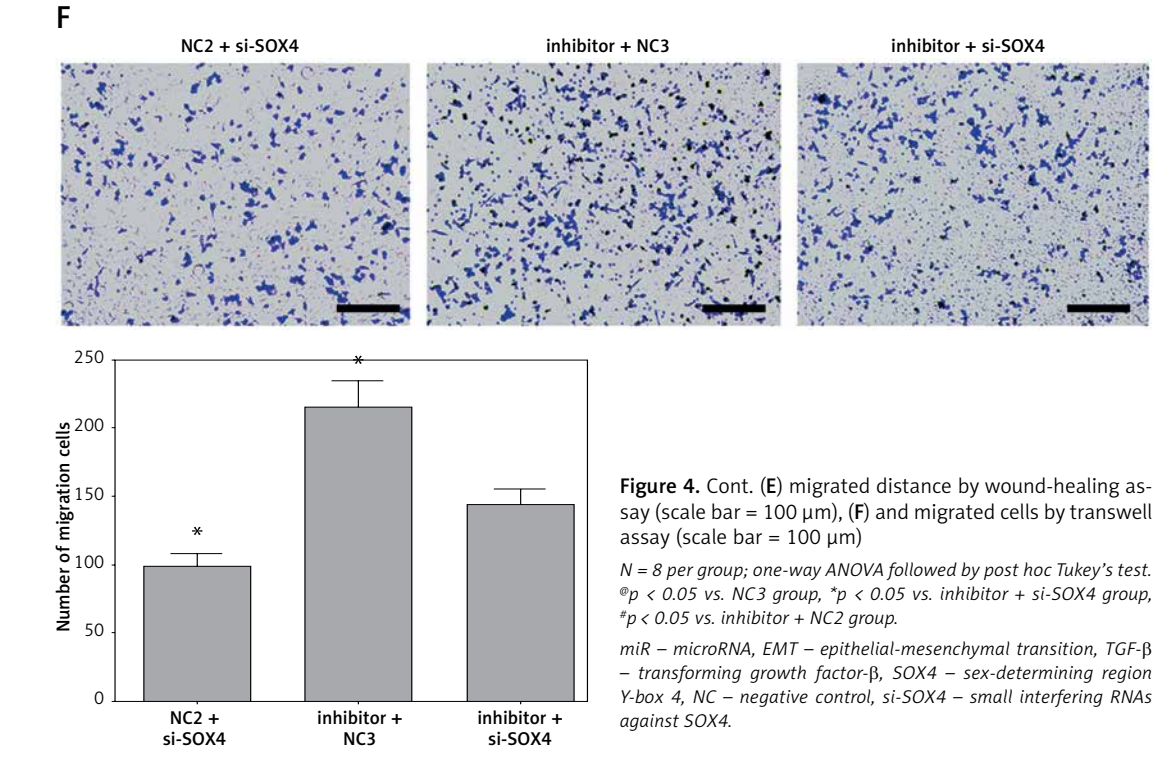


Figure 4. Cont. (**E**) migrated distance by wound-healing assay (scale bar = 100 µm), (**F**) and migrated cells by transwell assay (scale bar = 100 µm)

*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.

SMAD4 and regulate the levels of other target factors, including those that are commonly known as EMT inducers. Therefore, TGF-β is often used to induce EMT in different experiments. Thus, we used TGF-β to treat the human OC OVCAR3 cell line to generate an EMT model, resulting in EMT occurrence, increased proliferation, migration and invasion.

The sex-determining region Y-box 4 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 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 have revealed that miR-200 could increase epithelial cell integrity by regulating 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-administered 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 specimens of OC, which was closely associated with 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 the 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 to combat OC. However, these conclusions were only based on 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.

Conflict of interest

The authors declare no conflict of interest.

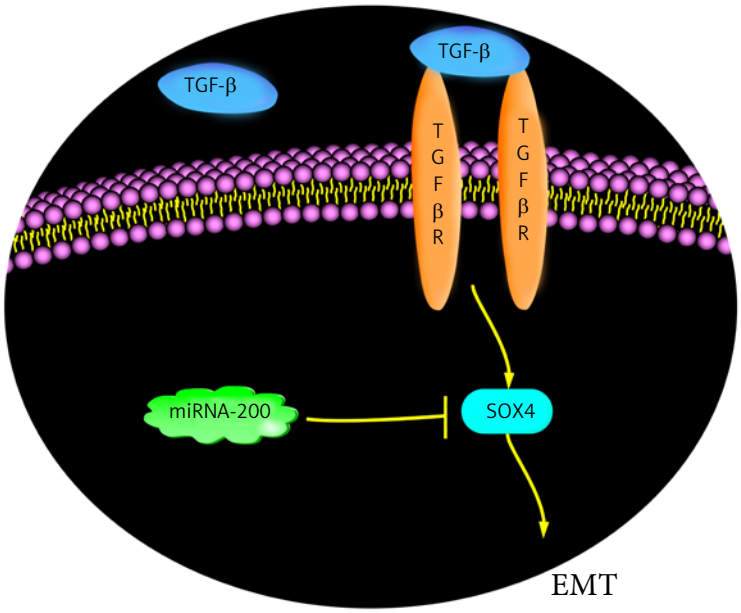


Figure 5. Effects of miR-200a on EMT in OC. TGF-β induces SOX4 up-regulation, which promotes EMT in OC. However, this promotion could be inhibited by miR-200a through targeting SOX4

EMT – epithelial-mesenchymal transition, TGF-β – transforming growth factor-β.

References

1. Mallen AR, Townsend MK, Tworoger SS. Risk factors for ovarian carcinoma. Hematol Oncol Clin North Am 2018; 32: 891-902.
2. Braga EA, Fridman MV, Kushlinskii NE. Molecular mechanisms of ovarian carcinoma metastasis: key genes and regulatory microRNAs. Biochemistry Mosc 2017; 82: 529-541.
3. Iwatsuki M, Mimori K, Yokobori T, et al. Epithelial-mesenchymal transition in cancer development and its clinical significance. Cancer Sci 2010; 101: 293-299.
4. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 2010; 11: 597-610.
5. Parikh A, Lee C, Joseph P, et al. MicroRNA-181a has a critical role in ovarian cancer progression through the regulation of the epithelial-mesenchymal transition. Nat Commun 2014; 5: 2977.
6. Ying X, Wei K, Lin Z, et al. MicroRNA-125b suppresses ovarian cancer progression via suppression of the epithelial-mesenchymal transition pathway by targeting the SET protein. Cell Physiol Biochem 2016; 39: 501-510.
7. Wu X, Ruan Y, Jiang H, Xu C. MicroRNA-424 inhibits cell migration, invasion, and epithelial mesenchymal transition by downregulating doublecortin-like kinase 1 in ovarian clear cell carcinoma. Int J Biochem Cell Biol 2017; 85: 66-74.
8. Koutsaki M, Spandidos DA, Zaravinos A. Epithelial-mesenchymal transition-associated miRNAs in ovarian carcinoma, with highlight on the miR-200 family: prognostic value and prospective role in ovarian cancer therapeutics. Cancer Lett 2014; 351: 173-181.
9. Gregory PA, Bert AG, Paterson EL, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol 2008; 10: 593-601.
10. Korpal M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J Biol Chem 2008; 283: 14910-14914.
11. Fu W, Tao T, Qi M, et al. MicroRNA-132/212 upregulation inhibits TGF-β-mediated epithelial-mesenchymal transition of prostate cancer cells by targeting SOX4. Prostate 2016; 76: 1560-1570.
12. Luan QX, Zhang BG, Li XJ, Guo MY. MiR-129-5p is downregulated in breast cancer cells partly due to promoter H3K27m3 modification and regulates epithelial-mesenchymal transition and multi-drug resistance. Eur Rev Med Pharmacol Sci 2016; 20: 4257-4265.
13. Zhang F, Luo Y, Shao Z, et al. MicroRNA-187, a downstream effector of TGFβ pathway, suppresses Smad-mediated epithelial-mesenchymal transition in colorectal cancer. Cancer Lett 2016; 373: 203-213.
14. Prat J; FIGO Committee on Gynecologic Oncology. Staging classification for cancer of the ovary, fallopian tube, and peritoneum. Int J Gynaecol Obstet 2014; 124: 1-5.
15. Yu SJ, Hu JY, Kuang XY, et al. MicroRNA-200a promotes anoikis resistance and metastasis by targeting YAP1 in human breast cancer. Clin Cancer Res 2013; 19: 1389-1399.
16. Wang Y, Zhang S, Dang S, Fang X, Liu M. Overexpression of microRNA-216a inhibits autophagy by targeting regulated MAP1S in colorectal cancer. Onco Targets Ther 2019; 12: 4621-4629.
17. Liu Y, Miao Y, Gao X, et al. MicroRNA-200a affects the proliferation of airway smooth muscle cells and airway remodeling by targeting FOXC1 via the PI3K/AKT signaling pathway in ovalbumin-induced asthmatic mice. Cell Physiol Biochem 2018; 50: 2365-2389.
18. Wang Y, Qiu C, Lu N, et al. FOXD1 is targeted by miR-30a-5p and miR-200a-5p and suppresses the proliferation of human ovarian carcinoma cells by promoting p21 expression in a p53-independent manner. Int J Oncol 2018; 52: 2130-2142.
19. Wang L, Zhang J, Yang X, et al. SOX4 is associated with poor prognosis in prostate cancer and promotes epithelial-mesenchymal transition in vitro. Prostate Cancer Prostatic Dis 2013; 16: 301-307.
20. Zaravinos A. The Regulatory role of microRNAs in EMT and cancer. J Oncol 2015; 2015: 865816.
21. Lourenço AR, Coffer PJ. SOX4: joining the master regulators of epithelial-to-mesenchymal transition. Trends Cancer 2017; 3: 571-582.
22. Vervoort SJ, van Boxtel R, Coffer PJ. The role of SRY-related HMG box transcription factor 4 (SOX4) in tumorigenesis and metastasis: friend or foe. Oncogene 2013; 32: 3397-3409.
23. Ruan H, Yang H, Wei H, et al. Overexpression of SOX4 promotes cell migration and invasion of renal cell carcinoma by inducing epithelial-mesenchymal transition. Int J Oncol 2017; 51: 336-346.
24. Zhang J, Liang Q, Lei Y, et al. SOX4 induces epithelial-mesenchymal transition and contributes to breast cancer progression. Cancer Res 2012; 72: 4597-4608.
25. Sasaki A, Abe H, Mochizuki S, Shimoda M, Okada Y. SOX4, an epithelial-mesenchymal transition inducer, transactivates ADAM28 gene expression and co-localizes with ADAM28 at the invasive front of human breast and lung carcinomas. Pathol Int 2018; doi: 10.1111/pin.12685.
26. Vervoort SJ, Lourenço AR, van Boxtel R, Coffer PJ. SOX4 mediates TGF-β-induced expression of mesenchymal markers during mammary cell epithelial to mesenchymal transition. PLoS ONE 2013; 8: e53238.
27. Peng X, Liu G, Peng H, Chen A, Zha L, Wang Z. SOX4 contributes to TGF-β-induced epithelial-mesenchymal transition and stem cell characteristics of gastric cancer cells. Genes Dis 2018; 5: 49-61.
28. Xi J, Feng J, Zeng S. Long noncoding RNA lncBRM facilitates the proliferation, migration and invasion of ovarian cancer cells via upregulation of SOX4. Am J Cancer Res 2017; 7: 2180-2189.
29. Yeh YM, Chuang CM, Chao KC, Wang LH. MicroRNA-138 suppresses ovarian cancer cell invasion and metastasis by targeting SOX4 and HIF-1α. Int J Cancer 2013; 133: 867-878.
30. Lin L, Wang Z, Jin H, Shi H, Lu Z, Qi Z. MiR-212/132 is epigenetically downregulated by SOX4/EZH2-H3K27me3 feedback loop in ovarian cancer cells. Tumour Biol 2016; doi: 10.1007/s13277-016-5339-9.
31. Choi PW, Ng SW. The functions of microRNA-200 family in ovarian cancer: beyond epithelial-mesenchymal transition. Int J Mol Sci 2017; 18: 1207.
32. Jabbari N, Reavis AN, McDonald JF. Sequence variation among members of the miR-200 microRNA family is correlated with variation in the ability to induce hallmarks of mesenchymal-epithelial transition in ovarian cancer cells. J Ovarian Res 2014; 7: 12.