Tumor Biol. (2016) 37:15719–15727
DOI 10.1007/s13277-016-5339-9

ORIGINAL ARTICLE

MiR-212/132 is epigenetically downregulated by SOX4/EZH2-H3K27me3 feedback loop in ovarian cancer cells

Lin Lin[1] · **Zhiwen Wang**[2] · **Haihong Jin**[2] · **Hongzhen Shi**[1] · **Zhihong Lu**[3] · **Zhenqin Qi**[4]

Received: 24 May 2016 / Accepted: 6 September 2016 / Published online: 3 November 2016

Abstract Both miR-212 and miR-132 are usually downregulated in ovarian cancer and act as tumor suppressors. However, the mechanism of their downregulation in ovarian cancer is not clear. In this study, we investigated the regulative effects of miR-212 and miR-132 on SOX4 expression in ovarian cancer cells and also studied whether there is a feedback regulation between miR-212/miR-132 and SOX4 via an epigenetic mechanism. The results showed that both EZH2 and SOX4 overexpressions significantly repressed miR-212 and miR-132 expressions in SKOV3 and OV2008 cells. Immunoprecipitation assay showed that there are interactions among SOX4, EZH2, and H3K27me3, and ChIP assay confirmed significant enrichment of EZH2 and H3K27me3 in the promoter region of miR-212/132. Both pri-miR-212 and pri-miR-132 expressions decreased after enforced EZH2 or SOX4 expression. Western blot and dual-luciferase assay confirmed that miR-212 and miR-132 can target the same sites in the 3′UTR of SOX4 mRNA and suppress its expression in ovarian cancer cells. MiR-132 or miR-212 overexpression or knockdown of endogenous SOX4 reduced epithelial-mesenchymal transition (EMT)-like properties. Therefore, we infer that the SOX4/EZH2 complex can silence miR-212 and miR-132 expressions via binding to the promoter region and promoting H3K27me3, while miR-212 and miR-132 can directly bind to the 3′UTR of SOX4 and suppress its expression. This forms a MiR-132/212-SOX4/EZH2-H3K27me3 feedback loop in ovarian cancer cells. Functionally, SOX4 is a downstream effector of miR-212/132 modulating EMT of ovarian cancer cells.

Keywords miR-212 · miR-132 · SOX4 · EZH2 · H3K27me3 · Ovarian cancer

✉ Zhiwen Wang
zhiwenwang66@outlook.com

[1] Department of Reproductive Medicine, The First Hospital of Qinhuangdao, Qinhuangdao, Hebei 066000, China

[2] Department of Gynaecology and Obstetrics, The First Hospital of Qinhuangdao, Qinhuangdao, Hebei 066000, China

[3] Department of Infectious Disease, The First Hospital of Qinhuangdao, Qinhuangdao, Hebei 066000, China

[4] B-ultrasound Room, The First Hospital of Qinhuangdao, Qinhuangdao, Hebei 066000, China

Introduction

Alterations of epigenetic modulation, such as methylation, histone modification, and non-coding RNAs, are widely seen in carcinogenesis [1, 2]. Ovarian cancer is a malignant and highly aggressive female neoplasm [3]. The molecular mechanisms underlying this malignancy have not been fully understood. MiR-212 and miR-132 are derived from the miR-212/132 cluster in chromosome 17 in human [4]. Previous studies reported that both miR-212 and miR-132 are usually downregulated in ovarian cancer and act as tumor suppressors by targeting E2F5 and HBEGF [5–7]. However, how these two microRNAs (miRNAs) are downregulated in ovarian cancer is not clear.

Enhancer of zeste homolog 2 (EZH2) is a subunit of the Polycomb repressor complex 2 (PRC2), which promotes DNA methylation via recruiting DNA methyl transferases (DNMTs) [8]. In addition, as a histone methyl transferase, EZH2 can specifically catalyze trimethylation (me3) of lysine 27 (K27) on histone 3 (H3) (H3K27me3), an epigenetic modification that silences gene transcription [9]. Recent studies found that this mechanism is involved in miRNA silencing in pathological development of multiple types of cancer, such

as miR-218 repression in malignant transformation of human bronchial epithelial cells [10], miR-31 downregulation in invasive esophageal cancer cells [11], and miR-200b/a loss in ovarian cancer [12]. SOX4 is a member of the highly conserved SoxC (SRY-related high-motility group box) transcription factor family [13]. Previous studies reported that aberrant SOX4 upregulation is associated with enhanced cancer cell invasion and metastasis [14, 15]. As a transcription factor, SOX4 can bind to the promoter region of EZH2 and enhance its expression [16]. In addtion, it also interacts with EZH2 and HDAC3, which forms a co-repressor complex to silence miR-31 expression via binding to the promoter region [11]. In addition, the regulative effects of miR-212 and miR-132 on SOX4 were also observed in breast cancer [17].

Based on these observations, we hypothesized that there might be mutual regulation between miR-212/miR-132 and SOX4. In this study, we firstly investigated the regulative effects of miR-212 and miR-132 on SOX4 expression in ovarian cancer cells and also studied whether there is a feedback regulation between miR-212/132 and SOX4 via an epigenetic mechanism. In addition, we further studied the role of SOX4 in the pathological development of ovarian cancer, typically in the epithelial-mesenchymal transition (EMT).

Materials and methods

Cell culture and treatment

The human ovarian cancer cell lines SKOV-3 and OV2008 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % fetal bovine serum at 37 °C in 5 % CO_2.

pWPXL-SOX4 and pCMVHA-hEZH2 expression vectors and the corresponding negative controls were obtained from Addgene (Cambridge, MA, USA). SOX4 small interfering RNA (siRNA) and the scramble negative controls, miR-212 mimics and miR-132 mimics, and the corresponding negative controls were purchased from RiboBio (Guangzhou, China). The lentiviral pWPXL-SOX4 was prepared according to manufacturer's instruction. SKOV3 and OV2008 cells were infected with pWPXL-Sox4 or transfected with pCMVHA hEZH2 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for overexpression. SKOV3 and OV2008 cells were transfected with 100-nM miR-212 mimics or miR-132 mimics using Lipofectamine 2000 (Invitrogen). SKOV3 cells were transfected with 100 nM SOX4 siRNA using Lipofectamine 2000 (Invitrogen) for knockdown. The morphological phenotype of the cells after transfection was captured using a CCD camera under a light microscopy.

Dual-luciferase assay

The binding sites between miR-212/132 and SOX4 3′UTR were predicted using TargetScan 7.0. Based on the results of prediction, a pair of oligonucleotides covering the wild-type or mutant miR-212/132 binding site of SOX4 3′UTR with flanking *SacI* and *XhoI* restriction enzyme digestion sites were chemically synthesized. Then, the sequences were inserted into the sites *SacI* and *XhoI* in pGL-3 promoter vector, respectively. The reconstructed dual-luciferase reporter plasmids were named as pGL3-SOX4-WT and pGL3-SOX4-MT, respectively. SKOV3 and OV2008 cells were co-transfected with 200-ng recombinant plasmids, 20-ng phRL-TK plasmid carrying the *Renilla* luciferase gene, and 100-nM miR-212 mimics or miR-132 mimics using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to that of *Renilla* luciferase.

The qRT-PCR analysis

Total RNA was extracted from the cell samples using TRIzol reagent (Invitrogen). Complementary DNA (cDNA) was reversely transcribed using the SuperScript™ First-Strand Synthesis System (Invitrogen). The primers for SOX4 and EZH2 were synthesized according to the design in one previous study [11]. The qRT-PCR was performed using SYBR® Premix Dimmer Eraser Kit (TaKaRa, Dalian, China) in an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). GAPDH was used as the endogenous control.

The levels of pri-miR-212 and pri-miR-132 were quantified using TaqMan Pri-miRNA Assays (Applied Biosystems, Foster City, CA, USA). The miRNA-specific cDNA was firstly synthesized using the stem-loop primers and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Then, mature miR-212 and miR-132 were quantified by qRT-PCT using the TaqMan MicroRNA Assays Kit (Applied Biosystems). The results were presented using $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Of the total protein extracted from cultured ovarian cancer cells, 20 μg was fractionated on 10 % SDS-polyacrylamide gel. Then, the proteins were transferred to nitrocellulose membrane and then subjected to primary antibody incubation. The primary antibodies used included anti-EZH2 (1:2000, ab3748, Abcam), anti-SOX4 (1:5000, ab134107, Abcam), anti-E-cadherin (1:1000, no. 3195, Cell Signaling, Danvers, MA, USA), anti-N-cadherin (1:1000, no. 13,116, Cell Signaling), anti-Vimentin (1:1000, no. 5741, Cell Signaling), and anti-β-actin (1:2000, ab8227, Abcam). The second HRP-conjugated

secondary antibodies were purchased from Abcam. The blot signals were visualized using the ECL Western Blotting Substrate (Promega, Madison, WI, USA). Then, signal intensity of the protein bands was quantified using densitometry (Quantity One software, Bio-Rad).

Immunoprecipitation

SOV3 and OV2008 cells were harvested and lysed using ice-cold immunoprecipitation (IP) lysis buffer (Pierce, Rockford, IL, USA). The lysate was incubated on ice for 5 min with periodic mixing. Then, the cell debris was removed by centrifugation at ~13,000×*g* for 10 min at 4 °C. Total protein lysate (500 μg) was immunoprecipitated with the agarose-immobilized antibody (6 μg of anti-SOX4, EZH2, or isotype control antibodies) and incubated overnight at 4 °C. Then, the immune complexes were eluted from the agarose beads and analyzed by SDS-PAGE followed by immunoblot analysis.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assays were performed by using the Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (Millipore) following the manufacturer's protocol. Briefly, SOV3 and OV2008 cells (1×10^7) were fixed using 1 % formaldehyde for 15 min. Then, the cell samples were lyzed and the protein-bound chromatin was fragmented by sonication into fragments ranging from 200 to 1000 bp. Equal volumes of chromatin were immunoprecipitated with magnetic Dynal bead (Invitrogen)-coupled antibody against EZH2 (Abcam), SOX4 (Abcam), H3K27me3 (Millipore), or isotype IgG at 4 °C overnight. The cross-links for the enriched and the input DNA were then reversed, and the DNA was cleaned by RNase A (0.2 mg/mL) and proteinase K (2 μg/mL). The DNA samples were further purified and then used for qRT-PCR analysis. A pair of primers that amplified a 152-bp sequence in the miR-212 upstream promoter region was designed (forward 5′-ACCTGATATTCAATAGACA-3′; reverse 5′-CAAATTAGCCCTAAAGAG-3′).

Fluorescence microscopy

SKOV3 cells with or without knockdown of SOX4 were grown on coverslips. Then, the cells were fixed in methanol, permeabilized in 0.1 % Triton X-100, and blocked in 1 % BSA. To investigate the expressions of E-cadherin and N-cadherin, the coverslips were probed with primary antibodies against E-cadherin (1:500, ab40772, Abcam) and N-cadherin (1:100, ab76011, Abcam), respectively, at 4 °C overnight. After the incubation, the coverslips were washed and further incubated with secondary Alexa Fluor®555-conjugated donkey anti-rabbit IgG H&L (1:500, ab150074, Abcam) and Alexa Fluor®488-conjugated donkey anti-rabbit polyclonal antibody (1:500, ab150073, Abcam), respectively, for 1 h at room temperature. Nuclei were stained using Prolong® Gold Antifade Reagent with DAPI (no. 8961, Cell Signaling). The images were captured using a fluorescence microscope (Olympus IX73, Olympus, Tokyo, Japan).

Statistical analysis

Data were presented in the form of means ± standard deviation (SD). Group difference was compared by using the unpaired *t* test. A two-sided *p* value of <0.05 was considered statistically significant.

Results

MiR-212 and miR-132 expressions are decreased due to EZH2 or SOX4 upregulation in ovarian cancer cells

Previous studies found that both miR-132 and miR-212 are two tumor suppressive miRNAs that are usually downregulated in ovarian cancer [5–7]. To investigate whether miR-212/132 expression is decreased due to EZH2-mediated H3K27me3 in ovarian cancer cells, SKOV3 and OV2008 cells were firstly transfected for EZH2 overexpression (Fig. 1a–c). Previous studies reported that SOX4 can bind to the promoter region of EZH2 and enhances its expression [16] and can also interact with EZH2 as a co-repressor complex to silence miRNA expression [11]. We overexpressed SOX4 in SKOV3 and OV2008 cells (Fig. 1d–f). SOX4 overexpression also led to enhanced EZH2 expression in the cells (Fig. 1e, f). Then, we performed qRT-PCR analysis to quantify miR-212/132 levels after EZH2 or SOX4 overexpression. Interestingly, we observed that both EZH2 and SOX4 overexpressions significantly repressed miR-212 and miR-132 expressions in SKOV3 and OV2008 cells (Fig. 1g–j).

MiR-212 and miR-132 are epigenetically silenced by via SOX4/EZH2-mediated H3K27me3

MiR-212 and miR-132 are two miRNAs clustered together. Previous studies found that hypermethylation in the promoter region of miR-212/132 is a mechanism of miR-212 and miR-132 downregulation in some types of cancers [18, 19]. EZH2-mediated H3K27 trimethylation is also a mechanism of miRNA suppression [10, 11]. Since we observed that EZH2 or SOX4 upregulation can result in decreased miR-212 and miR-132 in ovarian cancer cells, we hypothesized that EZH2-mediated H3K27 trimethylation might be a mechanism underlying the downregulation. By using anti-SOX4 and anti-EZH2 for coimmunoprecipitation (co-IP) in cell lysates from

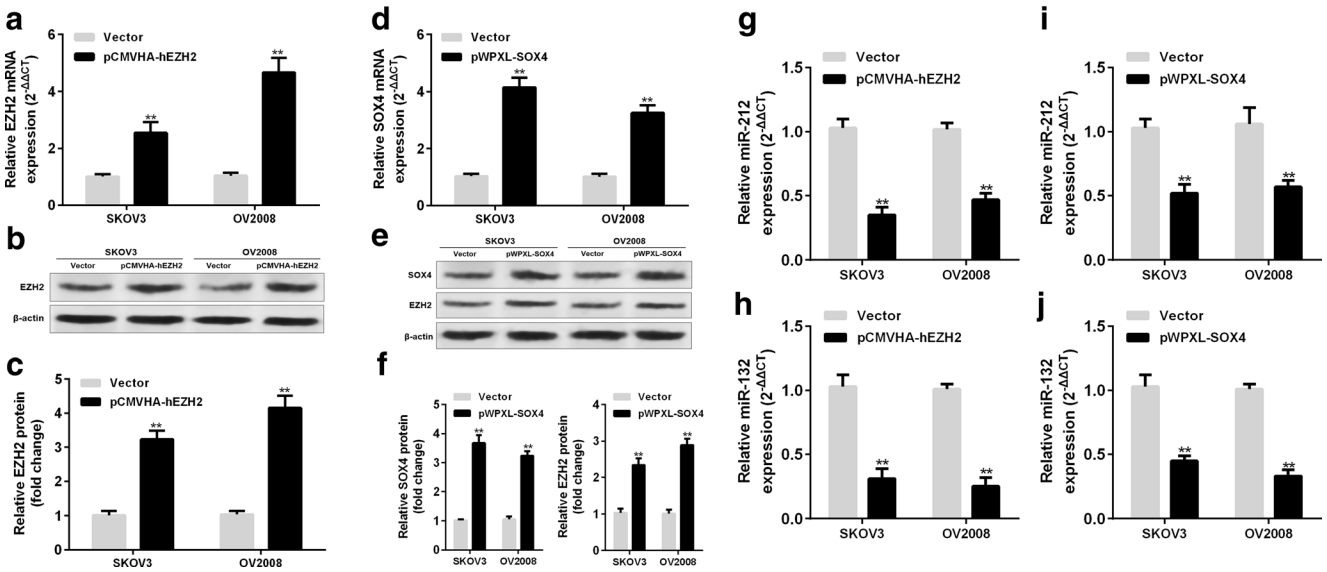

Fig. 1 MiR-212 and miR-132 expressions are decreased due to EZH2 or SOX4 upregulation in ovarian cancer cells. **a–c** The qRT-PCR analysis of EZH2 mRNA (**a**) and western blot images (**b**) and quantitation of band gray scale (**c**) of EZH2 protein expression in SKOV3 and OV2008 cells after transfection of EZH2 expression vectors (pCMVHA-hEZH2) or the negative control. **d–f** The qRT-PCR of SOX4 mRNA expression (**d**) and western blot images (**e**) and quantitation of band gray scale (**f**) of SOX4 and EZH2 (**f**) proteins in SKOV3 and OV2008 cells after infected with pWPXL-SOX4 or the negative control. **g–j** The qRT-PCR of miR-212 (**g**, **i**) and miR-132 (**h**, **j**) expressions in SKOV3 and OV2008 cells after transfection of pCMVHA-hEZH2 (**g**, **h**) or infected with pWPXL-SOX4 (**i**, **j**). $^{**}p < 0.01$

SKOV3 and OV2008 cells, we observed that there is an interaction among SOX4, EZH2, and H3K27me3 (Fig. 2a, b). Then, we detected whether SOX4 and EZH2 tethered to the promoter region of miR-212/132. By perform ChIP assay, we found significant enrichment of EZH2 and H3K27me3 in regions upstream of miR-212/132 (Fig. 2c). However, although SOX4 in the promoter region is slightly increased, the increase was not significant (Fig. 2c). To further verify the epigenetic silencing effect of SOX4/EZH2 complex, we further detected the levels of pri-miR-212 and pri-miR-132 in SKOV3 and OV2008 cells after EZH2 or SOX4 overexpression. The qRT-PCR results showed that both pri-miR-212 and pri-miR-132 expressions decreased after enforced EZH2 and SOX4 expressions (Fig. 2d–g). These results suggest that the SOX4/EZH2 complex can silence miR-212 and miR-132 expressions via binding to the promoter region and promoting H3K27me3.

SOX4 is a direct target of miR-212 and miR-132 in ovarian cancer cells

Since miR-212 and miR-132 share a part of same nucleotides, it is possible that they might synergistically suppress the same genes. In addition, the regulative effects of miR-212 and miR-132 on SOX4 were also observed in breast cancer [17]. By performing bioinformatic analysis, we found that the 3′UTR of SOX4 has a highly conserved putative target of miR-212 and miR-132 (Fig. 3a). Therefore, we further verify the regulative effects in ovarian cancer cells. SKOV3 and OV2008 cells were transfected with miR-212 (Fig. 3b) and miR-132 (Fig. 3c) for overexpression. Both enforced miR-212 and miR-132 expressions significantly decreased SOX4 level in these two cell lines (Fig. 3d). To further verify the direct binding between miR-212 or miR-132 and 3′UTR of SOX4, the luciferase reporters carrying either wild-type or mutant SOX4 3′UTR sequences (Fig. 3a) were constructed. Subsequent dual-luciferase assay showed that both miR-212 and miR-132 can suppress the luciferase activity of the reporter plasmids carrying wild-type sequences in SKOV3 and OV2008 cells (Fig. 3e, f). However, these two miRNAs had no suppressive effect on the reporters with mutant sequences (Fig. 3e, f). These results confirmed that SOX4 is a direct target of miR-212 and miR-132 in ovarian cancer cells. In combination with findings above, we infer that there is a reciprocal control between miR-212/miR-132 and SOX4 in ovarian cancer via the SOX4/EZH2-mediated H3K27me3 (Fig. 3g).

SOX4 is a functional downstream effector of miR-212 and miR-132 modulating EMT of ovarian cancer cells

Previous studies reported that SOX4 is an oncogene in ovarian cancer, and its upregulation is associated with enhanced cancer cell invasion and metastasis [14, 15]. In this study, we further studied the role of miR-212/miR-132/SOX4 axis in the pathological development of ovarian

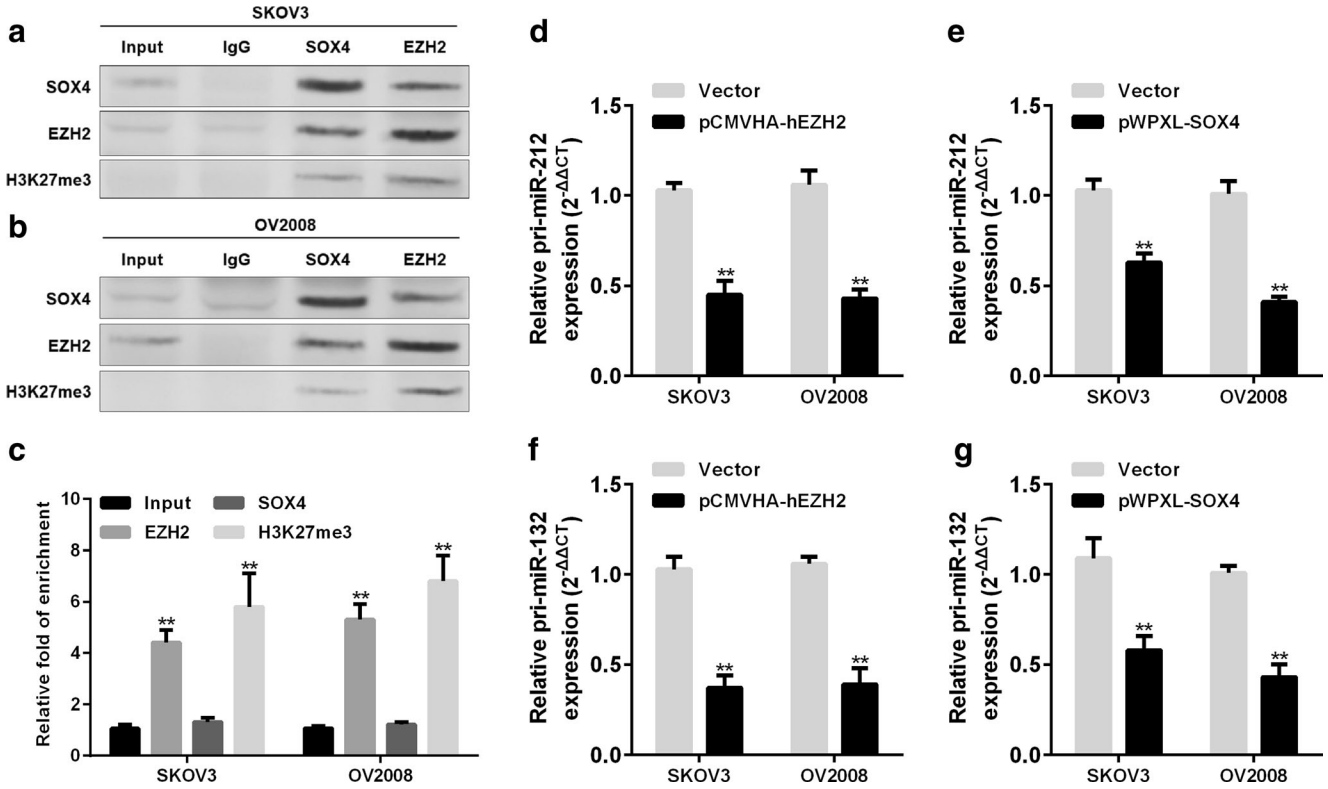

Fig. 2 MiR-212 and miR-132 are epigenetically silenced by via SOX4/EZH2-mediated H3K27me3. **a**, **b** SKOV3 (**a**) and OV2008 (**b**) cell lysates were immunoprecipitated with SOX4, EZH2, or IgG control antibodies, and immunoblots were probed for indicated antibodies including anti-SOX4, anti-EZH2, and anti-H3K27me3. **c** Quantitative ChIP assay showing H3K27me3 and EZH2 enrichment on the proximal promoter region of miR-212/132 in SKOV3 and OV2008 cells. **d**–**g** The qRT-PCR of pri-miR-212 (**d**, **e**) and pri-miR-132 (**f**, **g**) expressions in SKOV3 and OV2008 cells after transfection of EZH2 expression vectors (**d**, **f**) or infected with SOX4 lentiviral particles (**e**, **g**). $^{**}p < 0.01$

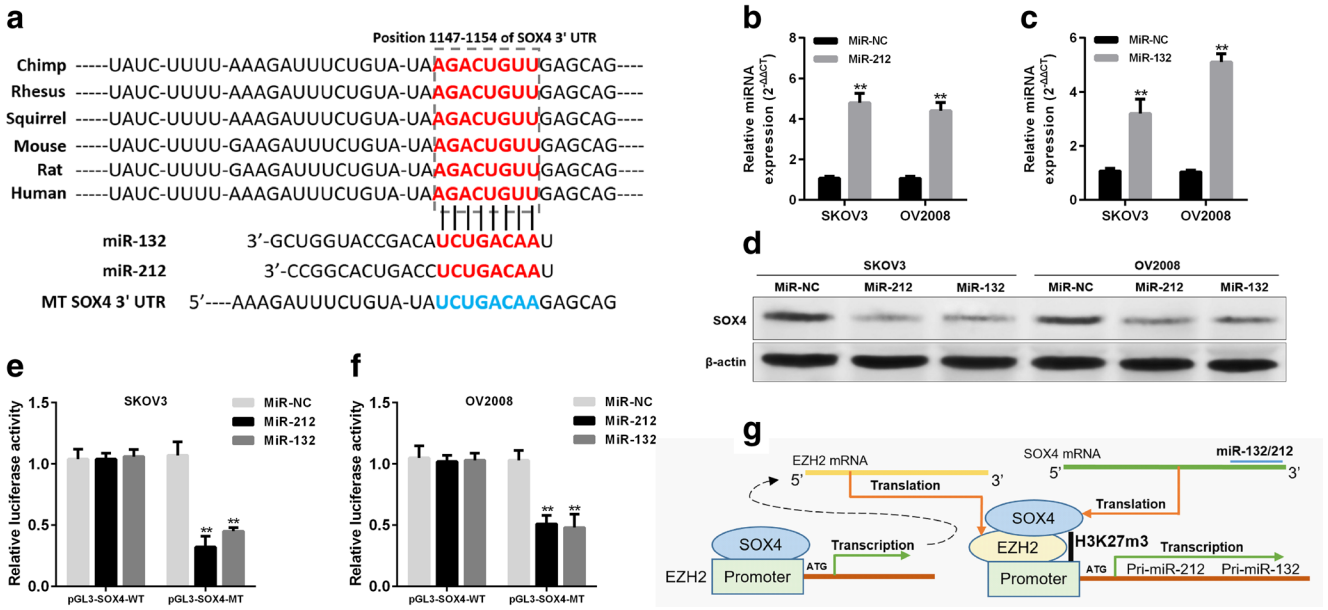

Fig. 3 SOX4 is a direct target of miR-212 and miR-132 in ovarian cancer cells. **a** The predicted binding sites between miR-212/132 and 3′UTR of SOX4. MT refers to the designed sequences with mutant binding sites. The 3′UTR of SOX4 in multiple species were compared. **b**, **c** The qRT-PCR of miR-212 (**b**) and miR-132 (**c**) expressions in SKOV3 and OV2008 cells after transfection of miR-212 (**b**) mimics or miR-132 (**c**) mimics. **d** Western blot analysis SOX4 protein levels in SKOV3 and OV2008 cells after transfection of miR-212 mimics or miR-132 mimics. **e**, **f** SKOV3 (**e**) and OV2008 (**f**) cells were co-transfected with 100 nM miR-212 mimics or miR-132 mimics and pGL3-SOX4-WT or pGL3-SOX4-MT. The relative luciferase activity was measured 24 h after transfection. **g** Schematic reciprocal control between miR-212/132 and SOX4 in ovarian cancer via the SOX4/EZH2-mediated H3K27me3. $^{**}p < 0.01$

cancer. EMT is an important mechanism of enahnced cancer cell invasion and metastasis [20]. SKOV3 cells transfected with miR-212 or miR-132 mimics showed elongated fibroblast-like morphologic alteration (Fig. 4a). In addition, enforced miR-212 or miR-132 expression significantly increased the expression of E-cadherin and suppressed the expressions of N-cadherin and Vimentin in SKOV3 cells (Fig. 4b, c). Then, we further investigated how SOX4 modulates EMT of ovarian cancer cells. Knockdown of endogenous SOX4 by transfection of SOX4 siRNA promoted elongated fibroblast-like morphologic alteration of SKOV3 cells (Fig. 4d). In contrast,

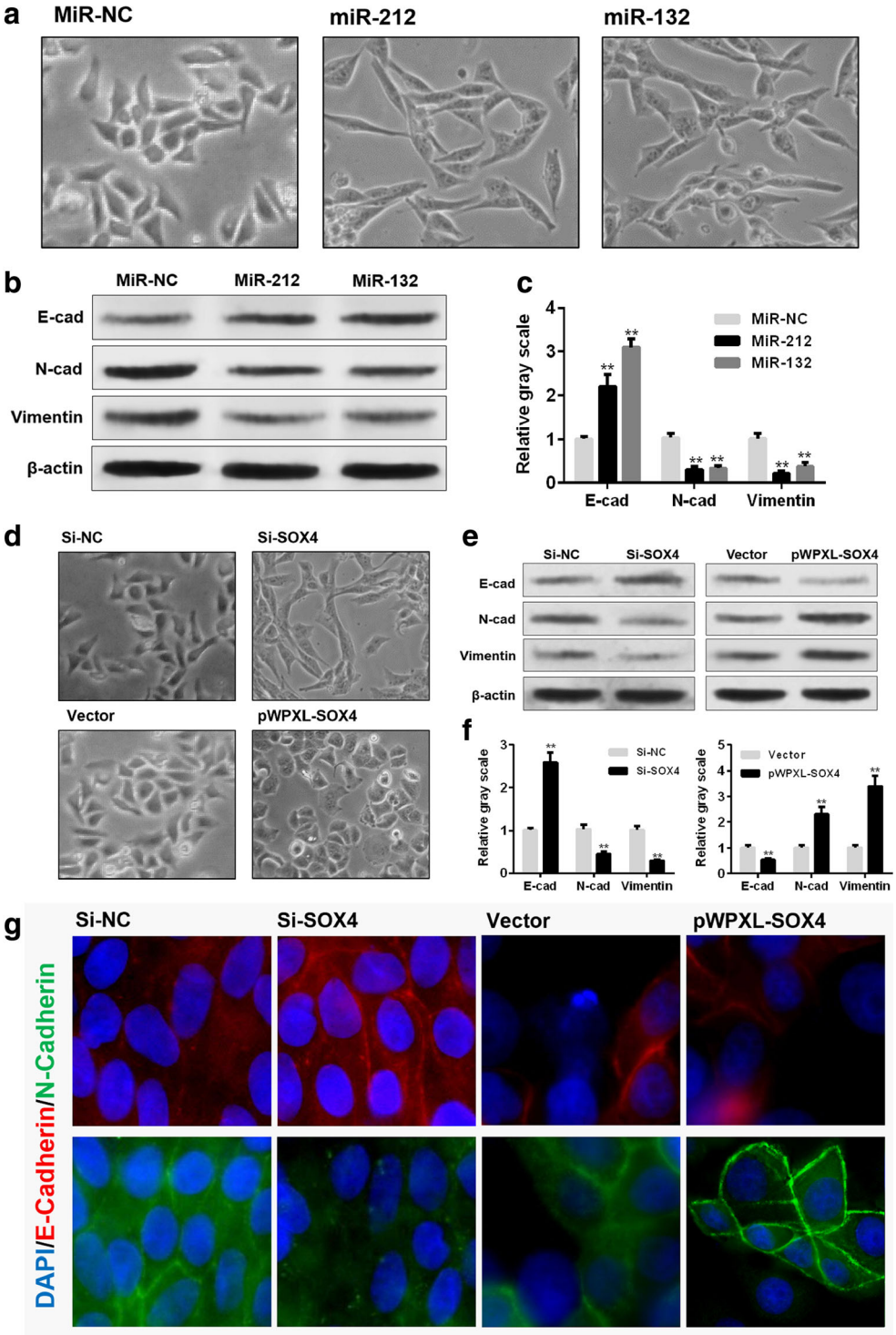

Fig. 4 SOX4 is a functional downstream effector of miR-212 and miR-132 modulating EMT of ovarian cancer cells. **a** Morphology of SKOV3 cells after transfection of miR-212 or miR-132 mimics. **b**, **c** Western blot analysis (**b**) and quantitation (**c**) of E-cadherin (E-cad), N-cadherin (N-cad), and Vimentin expressions in SKOV3 cells after transfection of miR-212 or miR-132 mimics. **d** Morphology of SKOV3 cells after transfection of SOX4 siRNA or infected with pWPXL-SOX4. **e**, **f** Western blot analysis (**e**) and quantitation (**f**) of E-cad, N-cad, and Vimentin expressions in SKOV3 cells after transfection of SOX4 siRNA or infected with pWPXL-SOX4. **g** Representative immunofluorescent images of E-cad and N-cad in SKOV3 cells after transfection of SOX4 siRNA or infected with pWPXL-SOX4. *Red* E-cad; *green* N-cad; *blue* DAPI. $^{**}p < 0.01$

SOX4 overexpression induced mesenchymal cell-like morphology (Fig. 4d). By using western blot analysis, we observed that knockdown of endogenous SOX4 increased the expression of E-cadherin and also significantly suppressed the expressions of N-cadherin and Vimentin (Fig. 4e, f). The following immunofluorescence staining also confirmed these changes (Fig. 4g).

Discussion

MiRNAs can be either tumor suppressors or oncogenes, depending on their downstream genes. MiR-212 and miR-132 have been previously identified as tumor suppressor in ovarian cancer and are both significantly downregulated in serum and tissues from epithelial ovarian cancer patients than the healthy controls [5, 7]. Enforced miR-212 expression can suppress SKOV3 cell proliferation, migration, and invasion by targeting the HBEGF transcript [7], while miR-132 also presented similar inhibitive effects on ovarian cancer cell proliferation, colony formation, migration, and invasion via targeting E2F5 [5]. However, how these two miRNAs are downregulated and whether other targets are involved in their regulation in ovarian cancer are not clear. MiR-212 and miR-132 are clustered together in the intro of chromosome 17 in human [4]. Previous studied found that epigenetic regulation is a possible mechanism of downregulated miR-212 and miR-132 in several types of cancer. One previous study based on non-small-cell lung carcinoma cells and normal human fibroblasts found that although the transcriptional start site of miR-212 is embedded in a CpG island, miR-212 suppression is not a result of DNA hypermethylation but associated with methylation status of histone tails in the promoter region [21]. MiR-132 is reduced due to promoter CpG island methylation in prostate cancer cells [19] and in HBV-related hepatocellular carcinoma [22]. In this study, we tried to further investigate the mechanism underlying miR-212 and miR-132 downregulation in ovarian cancer.

EZH2-mediated H3K27me3 is an epigenetic modification that leads to silence of multiple miRNA transcription in several disease models, such as miR-218 in non-small-cell lung cancer cells [10], miR-31 in invasive esophageal cancer cells [11], and miR-101 in embryonal rhabdomyosarcoma [23]. EZH2 upregulation is also observed in ovarian cancer tissue and cells [24]. Therefore, we hypothesized that the EZH2-mediated H3K27me3 might also be a mechanism of miR-212/132 downregulation in ovarian cancer cells. The results showed that EZH2 overexpression significantly repressed miR-212 and miR-132 expressions in SKOV3 and OV2008 cells. Interestingly, by performing ChIP assay, we also verified the interaction between EZH2 and SOX4. In fact, SOX4 can enhance the expression of EZH2 via binding to the promoter region [16] and can also interact with EZH2 and HDAC3 to form a co-repressor complex to silence miRNA expression [11]. In this study, co-IP assay confirmed the interaction among SOX4, EZH2, and H3K27me3 in both SKOV3 and OV2008 cells. Following ChIP assay also detected significant enrichment of EZH2 and H3K27me3 in regions upstream of miR-212/132. One previous study studied the regulative effects of the SOX4, EZH2, and HDAC3 complexes on miR-31 expression in esophageal cancer cells [11]. Their IP assay study observed that there is a direct interaction between EZH2 and H3K27me3 marks and between HDAC3 and H3K27me3 marks but not between SOX4 and H3K27me3. These findings suggest that SOX4 is a functional part of the complex but without direct interaction with the DNA. This might be the reason why the quantitative ChIP assay did not observed significant increase of SOX4 in the promoter region of miR-212/132. In addition, our qRT-PCR results further demonstrated that both pri-miR-212 and pri-miR-132 expressions were decreased after enforced EZH2 and SOX4 expressions. Therefore, we infer that the SOX4/EZH2 complex can silence miR-212/132 expression via binding to the promoter region and promoting H3K27me3.

Two previous studies reported that both miR-212 and miR-132 can directly bind to the 3′UTR of SOX4 in breast cancer [17], osteosarcoma [25], and lung cancer [26] and in B cell development [27]. This triggered our interest to further investigate whether there is a reciprocal regulation between miR-212/132 and SOX4. By performing western blot and dual-luciferase assay, we confirmed that miR-212 and miR-132 can target the same sites in the 3′UTR of SOX4 messenger RNA (mRNA) and suppress its expression in ovarian cancer cells. This finding validated our hypothesis that there is a reciprocal control between miR-212/miR-132 and SOX4 in ovarian cancer via the SOX4/EZH2-mediated H3K27me3.

SOX4, as a transcription factor, upregulation in ovarian cancer is associated with enhanced cancer cell invasion and metastasis [14, 15]. However, its pathological role in ovarian cancer has not been fully revealed. One recent study reported that SOX4 is a master regulator that is indispensable for EMT in cancer cells [16]. Several miRNAs with suppressive effect on SOX4 expression also showed inhibitive effect on EMT [25, 28, 29]. We also investigated the effect of the miR-212/miR-132/SOX4 axis on EMT of ovarian cancer cells. The results showed that miR-212 or miR-132 overexpression or knockdown of endogenous SOX4 increased the expression of E-cadherin and also significantly suppressed the expressions of N-cadherin and Vimentin in SKOV3 cells. Therefore, we infer that SOX4 is a

functional downstream effector of miR-212 and miR-132 modulating EMT of ovarian cancer cells.

Conclusion

SOX4/EZH2 complex can silence miR-212/132 expression via binding to the promoter region and promoting H3K27me3, while miR-212 and miR-132 can directly bind to the 3′UTR of SOX4 and suppress its expression. This forms a MiR-132/212-SOX4/EZH2-H3K27me3 feedback loop in ovarian cancer cells. Functionally, SOX4 is a downstream effector of miR-212 and miR-132 modulating EMT of ovarian cancer cells.

Compliance with ethical standards

Conflicts of interest None

References

1. Shao C, Lacey M, Dubeau L, Ehrlich M. Hemimethylation footprints of DNA demethylation in cancer. Epigenetics. 2009;4:165–75.
2. Chmelarova M, Krepinska E, Spacek J, Laco J, Beranek M, Palicka V. Methylation in the p53 promoter in epithelial ovarian cancer. Clinical & translational oncology: official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico. 2013;15:160–3.
3. Zaman MS, Maher DM, Khan S, Jaggi M, Chauhan SC. Current status and implications of microRNAs in ovarian cancer diagnosis and therapy. Journal of ovarian research. 2012;5:44.
4. Nakahama T, Hanieh H, Nguyen NT, Chinen I, Ripley B, Millrine D, Lee S, Nyati KK, Dubey PK, Chowdhury K, Kawahara Y, Kishimoto T. Aryl hydrocarbon receptor-mediated induction of the microrna-132/212 cluster promotes interleukin-17-producing T-helper cell differentiation. Proc Natl Acad Sci U S A. 2013;110:11964–9.
5. Tian H, Hou L, Xiong YM, Huang JX, Zhang WH, Pan YY, Song XR. Mir-132 targeting e2f5 suppresses cell proliferation, invasion, migration in ovarian cancer cells. Am J Transl Res. 2016;8:1492–501.
6. Chung YW, Bae HS, Song JY, Lee JK, Lee NW, Kim T, Lee KW. Detection of microrna as novel biomarkers of epithelial ovarian cancer from the serum of ovarian cancer patients. International journal of gynecological cancer: official journal of the International Gynecological Cancer Society. 2013;23:673–9.
7. Wei LQ, Liang HT, Qin DC, Jin HF, Zhao Y, She MC. Mir-212 exerts suppressive effect on skov3 ovarian cancer cells through targeting HBEGF. Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine. 2014;35:12427–34.
8. Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, Morey L, Van Eynde A, Bernard D, Vanderwinden JM, Bollen M, Esteller M, Di Croce L, de Launoit Y, Fuks F. The polycomb group protein ezh2 directly controls DNA methylation. Nature. 2006;439:871–4.
9. Cao R, Zhang Y. The functions of e(z)/ezh2-mediated methylation of lysine 27 in histone h3. Current opinion in genetics & development. 2004;14:155–64.
10. Wang B, Liu Y, Luo F, Xu Y, Qin Y, Lu X, Xu W, Shi L, Liu Q, Xiang Q. Epigenetic silencing of microrna-218 via ezh2-mediated h3 k27 trimethylation is involved in malignant transformation of hbe cells induced by cigarette smoke extract. Arch Toxicol. 2016;90:449–61.
11. Koumangoye RB, Andl T, Taubenslag KJ, Zilberman ST, Taylor CJ, Loomans HA, Andl CD. Sox4 interacts with ezh2 and hdac3 to suppress microRNA-31 in invasive esophageal cancer cells. Mol Cancer. 2015;14:24.
12. Chung VY, Tan TZ, Tan M, Wong MK, Kuay KT, Yang Z, Ye J, Muller J, Koh CM, Guccione E, Thiery JP, Huang RY. Grhl2-mir-200-zeb1 maintains the epithelial status of ovarian cancer through transcriptional regulation and histone modification. Scientific reports. 2016;6:19943.
13. Bowles J, Schepers G, Koopman P. Phylogeny of the sox family of developmental transcription factors based on sequence and structural indicators. Dev Biol. 2000;227:239–55.
14. Yeh YM, Chuang CM, Chao KC, Wang LH. MicroRNA-138 suppresses ovarian cancer cell invasion and metastasis by targeting sox4 and hif-1alpha. Int J Cancer. 2013;133:867–78.
15. Jin Y, Zhao M, Xie Q, Zhang H, Wang Q, Ma Q. MicroRNA-338-3p functions as tumor suppressor in breast cancer by targeting sox4. Int J Oncol. 2015;47:1594–602.
16. Tiwari N, Tiwari VK, Waldmeier L, Balwierz PJ, Arnold P, Pachkov M, Meyer-Schaller N, Schubeler D, van Nimwegen E, Christofori G. Sox4 is a master regulator of epithelial-mesenchymal transition by controlling ezh2 expression and epigenetic reprogramming. Cancer Cell. 2013;23:768–83.
17. Hanieh H. Aryl hydrocarbon receptor-microRNA-212/132 axis in human breast cancer suppresses metastasis by targeting sox4. Mol Cancer. 2015;14:172.
18. Xu L, Wang F, XF X, Mo WH, Xia YJ, Wan R, Wang XP, Guo CY. Down-regulation of mir-212 expression by DNA hypermethylation in human gastric cancer cells. Med Oncol. 2011;28(Suppl 1):S189–96.
19. Formosa A, Lena AM, Markert EK, Cortelli S, Miano R, Mauriello A, Croce N, Vandesompele J, Mestdagh P, Finazzi-Agro E, Levine AJ, Melino G, Bernardini S, Candi E. DNA methylation silences mir-132 in prostate cancer. Oncogene. 2013;32:127–34.
20. Liu X, Dong R, Jiang Z, Wei Y, Li Y, Wei L, Sun H, Li Y, Yang N, Yang Q, Liu Z, Kong B. Mdc1 promotes ovarian cancer metastasis by inducing epithelial-mesenchymal transition. Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine. 2015;36:4261–9.
21. Incoronato M, Urso L, Portela A, Laukkanen MO, Soini Y, Quintavalle C, Keller S, Esteller M, Condorelli G. Epigenetic regulation of mir-212 expression in lung cancer. PLoS One. 2011;6:e27722.
22. Wei X, Tan C, Tang C, Ren G, Xiang T, Qiu Z, Liu R, Wu Z. Epigenetic repression of mir-132 expression by the hepatitis B virus × protein in hepatitis B virus-related hepatocellular carcinoma. Cell Signal. 2013;25:1037–43.
23. Vella S, Pomella S, Leoncini PP, Colletti M, Conti B, Marquez VE, Strillacci A, Roma J, Gallego S, Milano GM, Capogrossi MC, Bertaina A, Ciarapica R, Rota R. MicroRNA-101 is repressed by ezh2 and its restoration inhibits tumorigenic features in embryonal rhabdomyosarcoma. Clinical epigenetics. 2015;7:82.
24. Liu L, Guo J, Yu L, Cai J, Gui T, Tang H, Song L, Wang J, Han F, Yang C, Chen C, Marks A, Wang Z. Mir-101 regulates expression of ezh2 and contributes to progression of and cisplatin resistance in epithelial ovarian cancer. Tumour biology: the journal of the

International Society for Oncodevelopmental Biology and Medicine. 2014;35:12619–26.
25. Liu Y, Li Y, Liu J, Wu Y, Zhu Q. MicroRNA-132 inhibits cell growth and metastasis in osteosarcoma cell lines possibly by targeting sox4. Int J Oncol. 2015;47:1672–84.
26. Li Y, Zu L, Wang Y, Wang M, Chen P, Zhou Q. Mir-132 inhibits lung cancer cell migration and invasion by targeting sox4. Journal of thoracic disease. 2015;7:1563–9.
27. Mehta A, Mann M, Zhao JL, Marinov GK, Majumdar D, Garcia-Flores Y, Du X, Erikci E, Chowdhury K, Baltimore D. The microRNA-212/132 cluster regulates b cell development by targeting sox4. J Exp Med. 2015;212:1679–92.
28. Hu F, Min J, Cao X, Liu L, Ge Z, Hu J, Li X. Mir-363-3p inhibits the epithelial-to-mesenchymal transition and suppresses metastasis in colorectal cancer by targeting sox4. Biochem Biophys Res Commun. 2016;474:35–42.
29. Li S, Qin X, Li Y, Zhang X, Niu R, Zhang H, Cui A, An W, Wang X. Mir-133a suppresses the migration and invasion of esophageal cancer cells by targeting the emt regulator sox4. Am J Transl Res. 2015;7:1390–403.