Original Article
Mechanisms of miR-145 regulating invasion and metastasis of ovarian carcinoma

Ling Wang, Xiaotong Wu, Bowei Wang, Qiang Wang, Liying Han

Department of Obstetrics and Gynecology, The Second Hospital of Jilin University, Changchun, China

Abstract: MicroRNA-145 (miR-145) can regulate tumor cell invasion, metastasis, apoptosis, proliferation and stem cell differentiation. However, the molecular mechanisms of miR-145 used to regulate ovarian invasion and metastasis remain to be determined. In this study, Transwell cell migration and wound healing assays were used to detect the effects of miR-145 upregulation on ovarian carcinoma cell invasion and metastasis, respectively. The MUC1 expression vector, together with quantitative real-time PCR and Western blotting, was used to investigate the effects of miR-145 on E-cadherin (E-cad)-induced cell invasion and the related molecular mechanisms. The results showed that miR-145 mimics could inhibit SKOV3 cell invasion and metastasis. MiR-145 inhibited mucin 1 (MUC1) post-transcriptional expression by binding to its 3'-untranslated region. The epithelial mesenchymal transition marker E-cad, which is a downstream molecule of MUC1, was promoted by miR-145 overexpression. Furthermore, the E-cad protein level was inversely correlated with MUC1 expression in SKOV3 cells. These observations indicated that promotion of E-cad signaling induced by miR-145 was restrained by MUC1 inhibition. Thus, miR-145 may serve as a tumor suppressor which can downregulate E-cad expression by targeting MUC1, leading to the inhibition of tumor invasion and metastasis. Using miR-145 mimics may be a rational approach for therapeutic applications in ovarian carcinoma in the future.

Keywords: Ovarian carcinoma, miR-145, MUC1, E-cadherin, epithelial mesenchymal transition

Introduction

In China, 52,100 new cases of ovarian cancer were diagnosed, and an estimated 22,500 of them resulted in death in 2015 [1]. The majority of patients were diagnosed at advanced stages (III or IV) with peritoneal seeding. Despite accepting conventional therapy such as surgery and chemotherapy, the 5-year overall survival (stage III: 40%-60%; stage IV: 17%) for patients has been unchanged over the past 30 years due to chemoresistance and lack of screening tests [2].

MicroRNAs (miRNA) form a class of small non-coding RNAs that have been identified as a new type of gene expression regulator through targeting mRNAs for translational repression or degradation. Each individual miRNA has the potential to modulate multiple genes that harbor the target sequence located in their 3' untranslated region (UTR) and is complementary to the seed region of the miRNA [3]. MiR-145 is transcribed from a putative cluster on chromosome 5 in humans (5q33) and chromosome 18 in mice (18qE1) and is conserved across species [4]. Several target genes of miR-145 have been verified, including DNA Fragmentation Factor-45 (DFF45), catenin δ-1, histone deacetylase 2 (HDAC2), v-ets avian erythroblastosis virus e26 oncogene homolog (ERG), N-cadherin (CDH2) and POU class 5 homeobox 1 (OCT4) [5-10]. Based on these observations, miR-145 has been acknowledged as a tumor suppressor. Recently, miR-145 was validated to play pivotal roles in regulating various cellular functions such as cell apoptosis, cell proliferation, invasion and metastasis in different malignant tumors, including breast cancer, glioma, colon carcinoma and cervix cancer [3]. However, the patho-biological significance of aberrant miR-145 expression in serous epithelial ovarian carcinoma (SEOC) is not clearly understood, and the role of miR-145 in SEOC remains to be demonstrated.

In the current study, we investigated the aberrant expression and functional role of miR-145 in SEOC. Overexpression of miR-145 in SEOC was demonstrated to significantly suppress migration and invasion *in vitro*. Furthermore, mucin 1 (MUC1), an oncogene that is highly expressed in SEOC, was identified as a direct target of miR-145.

Materials and methods

Cell culture

All cell lines were purchased from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (NTCC). Ovarian cancer cell lines SKOV3, LvX-MOCK-CMV-RFP SKOV3 and LvX-miR-145-CMV-RFP SKOV3 were grown in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA). HEK-293T cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS. All media contained 2 mM glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Cells were incubated at 37°C and supplemented with 5% CO_2 in a humidified chamber.

Transfection

SKOV3 cells were transfected with miR-145 mimics using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR)

MiRNAs were reverse transcribed in a 20-μl reaction using the One-Step Primescript miRNA cDNA Synthesis Kit (Takara, Tokyo, Japan) and the forward primer sequence has-miR-145: GTCCAGTTTTCCCAGGAATC. The qRT-PCR was performed on a Bio-Rad Real-Time PCR System (Bio-Rad Inc. California, USA) using Power-SYBR Green PCR Master Mix (Takara) in a 20-μl reaction and U6 as an endogenous control, as well as miRlet-7 as a positive control. The results were determined using the $2^{-\Delta\Delta CT}$ method. The qRT-PCR experiments were performed in triplicate within each experiment run, and relative expression values were normalized to standard deviations from the mean.

Western blot

Total protein was extracted from frozen tissues. Protein contents were measured with a DC Protein Assay Kit. Ten micrograms of lysate proteins for Western blotting of MUC1 were separated by SDS-PAGE using polyacrylamide gels and electroblotted onto a PVDF membrane. The antibodies used were anti-human MUC1 mouse monoclonal antibody properly diluted with PBS-T containing 2% bovine serum albumin and 0.01% sodium azide. Loading control was conducted by re-incubating the same membrane with anti-human tubulin antibody. Expression levels of MUC1 were calculated by densitometry and expressed as the MUC1/tubulin ratio.

Wound healing assay

The wound healing migration assay was used to evaluate the effect of miR-145 transfection on the migratory ability of cancer cells. Cells were transfected with miR-145 mimics or negative control sequence. Transfected cells were plated, and the cell monolayer was scraped using a micropipette tip. The initial (0 h) and residual gap lengths at 12 to 48 h after wounding were calculated from photomicrographs.

Invasion assay

Matrigel chambers (BD Biosciences, San Jose, CA) were used to determine the effect of miR-145 or MUC1 on invasiveness per the manufacturer's protocol. In brief, infected cells were harvested, resuspended in serum-free medium and then transferred to the hydrated matrigel chambers (25,000 cells/well). The chambers were then incubated for 24 h in culture medium with 10% FBS in the bottom chambers before examination. The cells on the upper surface were scraped and washed away while the invaded cells on the lower surface were fixed and stained with 0.05% crystal violet for 2 h. Finally, invaded cells were counted under a microscope and the relative number was calculated.

Plasmids

To ectopically express MUC1, we cloned the MUC1 coding region in pCMV-Myc. The PCR product for MUC1 without an UTR was obtained by primers MUC1-R1-5.1 (5'GAATTCTGACACCG-GGCACCCAGTCTC) and MUC1-Not1-3.1 (5'GC-GGCCG CCTACAAGTTGGCAGAAGTGGC). For MUC1 with an UTR, we used primers MUC1-R1-5.1 and MUC1-UTR-Not1-3.1. The PCR product was first cloned into a PCR cloning
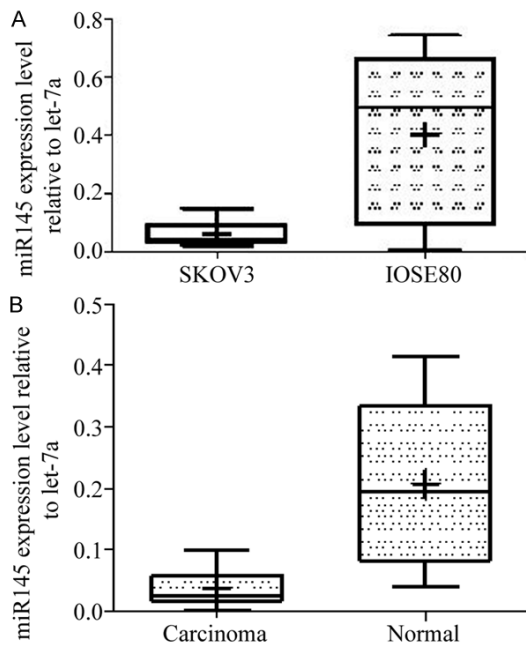
Figure 1. Downregulation of miR-145 in ovarian cancer tissue and cell lines. A. Relative miR-145 expression levels of ovarian cancer cell line, SKOV3 cells and normal ovarian epithelial cell line IOSE80 were examined by qRT-PCR. B. qRT-PCR validation of miR-145 expression in normal ovarian tissue and SEOC tissue.

vector (pCR8) and then subcloned into pCMV-Myc at *Eco*RI and *Not*I sites. The luciferase-UTR reporter constructs were generated by introducing the MUC1 3'-UTR carrying a putative miR-145 binding site into the pGL3 control vector (Promega, Madison, WI, USA). We first amplified the MUC1 3'-UTR sequence by PCR using primers MUC1-UTR-5.1 (5'TCTGCCAACTTGTA-GGGGCAC) and MUC1-UTR-Not1-3.1 (5'GCG-GCCGCTTTTTTGGCGCAGTGGGAGAC), and MC-F10A cDNA as a template. The PCR product was also first cloned into a PCR cloning vector (pCR8) and then subcloned into a modified pGL3 control vector where *Eco*RI and *Not*I sites were introduced into the original *Xba*I site. To delete the putative miR-145 binding site in the MUC1 3'-UTR, we amplified the UTR by using primers MUC1-UTR-5.1 and MUC1-UTR-Not-13.2 (5'GCGGCCGCCAGGATCCCC GCTATCT-CAGG), which was then cloned into the modified pGL3 control vector at the *Eco*RI and *Not*I sites. Site-directed mutagenesis of the miR-145 binding site in the MUC1 3'-UTR was carried out by the two-step PCR approach. All PCR products were verified by DNA sequencing.

Luciferase reporter assay

The 3'UTR of MUC1 containing two putative miR-145 binding sites (580 bp) was amplified and cloned into pmirGLO vector (Promega) using the SacI and XbaI sites to generate the wild-type (WT) construct. For the mutant-type (MT) plasmid, overlapping extension PCR assay was used. Cells were cultured in 96-well plates and transfected with 100 ng of WT or MT MTDH 3'UTR constructs by using Lipofectamine 2000. Twenty-four hours after transfection, luciferase activity was measured using the Dual-Glo Luciferase Assay System. Renilla luciferase activity was normalized to corresponding firefly luciferase activity and plotted as a percentage.

Statistical analysis

Data were presented as mean ± standard deviation (SD; $\chi \pm s$), and analyses were carried out using the statistical software SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Comparison between two groups was conducted by independent sample *t*-test, while comparisons among three or more groups were analyzed by one way analysis of variance (ANOVA). The enumeration data among groups were compared by χ^2 test. The risk ratio and its 95% confidence interval were recorded for each marker. *P*-values < 0.05 were considered statistically significant in all of the analyses.

Results

MiR-145 is decreased in clinical samples and ovarian carcinoma cell line

MiR-145 was reported to be downregulated in freshly frozen ovarian carcinoma samples based on microarray analysis. To further elucidate the ectopic expression of miR-145 in ovarian carcinoma, we detected the expression level of miR-145 in the human SEOC cell line SKOV3 and the normal human ovarian epithelial cell line IOSE80. Moreover, we detected the expression level of miR-145 in 50 freshly frozen human SEOC samples and 30 normal human ovarian epithelial biopsies. MiR-145 was demonstrated to be significantly reduced in the SKOV3 cell line (**Figure 1A**, *P* < 0.05). The qRT-PCR results also showed that in 50 freshly frozen SEOC biopsies, expression of miR-145 was downregulated with a relative fold change of
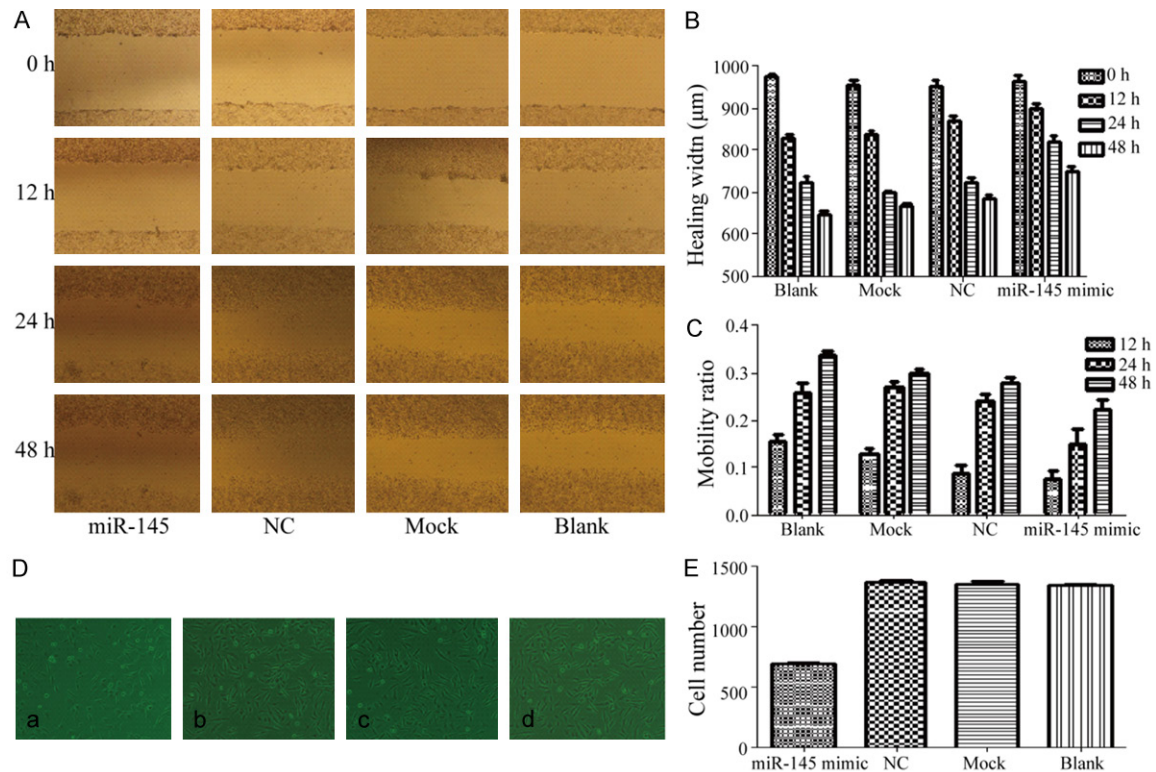
Figure 2. MiR-145 regulates MUC1 to suppress migration of SKOV3 cells. A. Wound healing assay. Gap length of the initial (0 h) and the residual gap length of 12 h, 24 h, 48 h after wounding were analyzed from photomicrographs. B, C. Influence of miR-145 to SKOV3 cells migration. D. Effects of ectopic expression of miR-145 on the migration of SKOV3 cells. a. miR-145 mimic (×40); b. NC (×40); c. Mock (×40); d. Blank (×40). E. Influence of miR-145 to SKOV3 cells metastasis.

0.15 (**Figure 1B**, $P < 0.05$), indicating that miR-145 expression was clearly decreased in SEOC tissues compared with normal ovarian tissue samples.

MiR-145 suppresses SKOV3 cell migration

To investigate whether miR-145 affects the migratory ability of SEOC cells, we transiently transfected SKOV3 cells with human miR-145 mimics or a negative control and then performed wound healing and Transwell migration assays. In the wound healing assay, the ratio of SKOV3 cells transfected with miR-145 mimics that had migrated (0.074, 0.148, 0.220) was much lower than those of cells transfected with the negative control (0.087, 0.242, 0.276), mock group (0.125, 0.269, 0.301) and blank group (0.152, 0.260, 0.339) at 12, 24 and 48 h after incubation (**Figure 2A-C**, $P < 0.05$). The Transwell migration assay further validated that transfection of miR-145 mimics (693.75 ± 29.75) significantly reduced the migratory ability of SKOV3 cells transfected with the negative

control (1360 ± 70.4), mock group (1340 ± 44.16) and blank group (1347 ± 57.01) (**Figure 2D**, **2E**, $P < 0.05$). These results indicated that miR-145 could significantly inhibit the migration ability of SEOC cells *in vitro*.

MiR-145 suppresses SKOV3 cell invasion

To further test whether miR-145 affects the invasive ability of SEOC cells, we transiently transfected SKOV3 cells with human miR-145 mimics or negative control and then conducted the Transwell invasion assay. The Matrigel Transwell invasion assay showed that the invasive ability of SKOV3 cells transfected with miR-145 mimics (33.47 ± 3.292) was significantly reduced compared with those of cells transfected with negative control (63.87 ± 4.42), mock group (67.87 ± 3.502) and blank group (65.53 ± 3.796) (**Figure 3**, $P < 0.05$). These findings suggested that miR-145 could significantly suppress the invasive ability of SEOC cells *in vitro*.
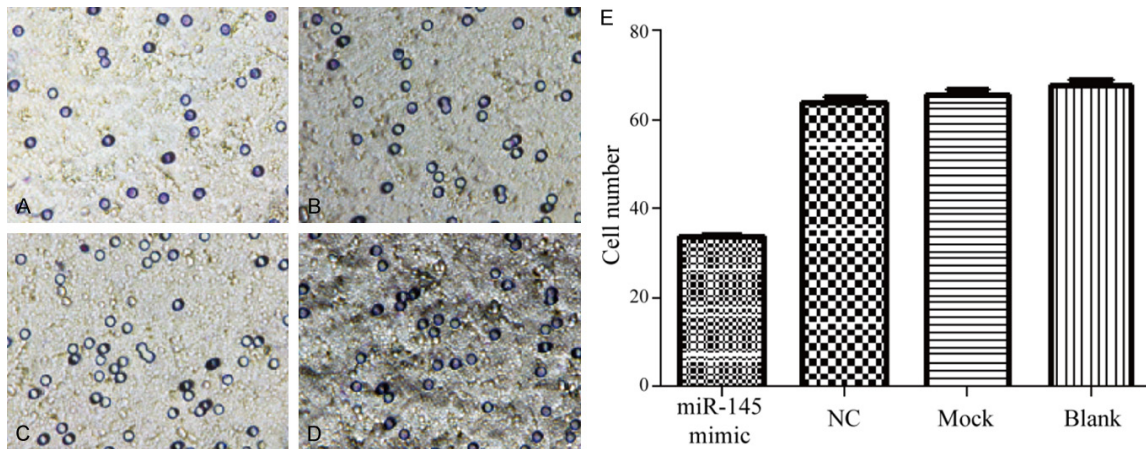
Figure 3. MiR-145 regulates MUC1 to suppress invasion of SKOV3 cells. A. miR-145 mimic (×40); B. NC (×40); C. Mock (×40); D. Blank (×40). E. Influence of miR-145 on SKOV3 cell invasion.
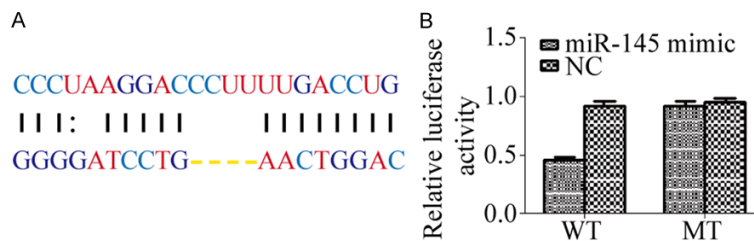

Figure 4. MiR-145 targets MUC1. A. Predicted complementary sequence of miR-145 to predicted target gene MUC1. B. Luciferase reporter assay revealed miR-145 suppressed MUC1 3'UTR luciferase activity. Histogram illustrates the relative luciferase activity in SKOV3 cells with and without miR-145 overexpression when transfected with WT or MT luciferase plasmids.

MUC1 is a direct target of miR-145

To further explore the mechanism by which miR-145 inhibits invasion and metastasis, we used three public databases (TargetScan, MicroCosm and miRanda) to identify potential targets of miR-145 and selected MUC1 for further analysis. As shown in **Figure 4A**, we first cloned the WT and MT miR-145 target sequences of the MUC1 3'UTR into luciferase reporter vectors and performed the luciferase reporter gene assay. Co-transfection with human miR-145 mimics (0.452 ± 0.066) clearly reduced the luciferase activity of the WT reporter gene compared with the negative control (0.912 ± 0.087). However, when co-transfected with the MT reporter gene, the luciferase activity of the miR-145 mimic group (0.918 ± 0.100) showed no difference compared with the negative control (0.942 ± 0.086) (**Figure 4B**, $P < 0.05$). Taken together, these findings demonstrated that miR-145 could negatively regulate the expression of MUC1 by directly targeting the MUC1 3'UTR.

Expression of E-cadherin and MUC1

To test whether miR-145 regulates the expression of MUC1 and E-cad at the protein level in ovarian carcinoma, SKOV3 cells were transfected with miR-145 mimics and negative control sequence and then analyzed by qRT-PCR and Western blot. The results showed that overexpression of miR-145 remarkably reduced the protein but not mRNA expression of MUC1 (**Figure 5A-C**, $P < 0.05$). We found that the E-cad protein expression was significantly increased by miR-145 compared to the negative control in SKOV3 cells. However, overexpression of MUC1 by transfection of MUC1 plasmids into SKOV3 cells downregulated protein expression of E-cad compared to negative control plasmids. Meanwhile, the protein expression of E-cad in SKOV3 cells transfected with both miR-145 mimics and MUC1 plasmid was lower than that in cells transfected with miR-145 mimics only (**Figure 5D, 5E**). The Western blotting analysis revealed that E-cad expression levels were decreased in SKOV3 cells with increased MUC1 expression. Taken together, these findings showed that the promotion of E-cad signaling induced by miR-145 was restrained by MUC1 inhibition.
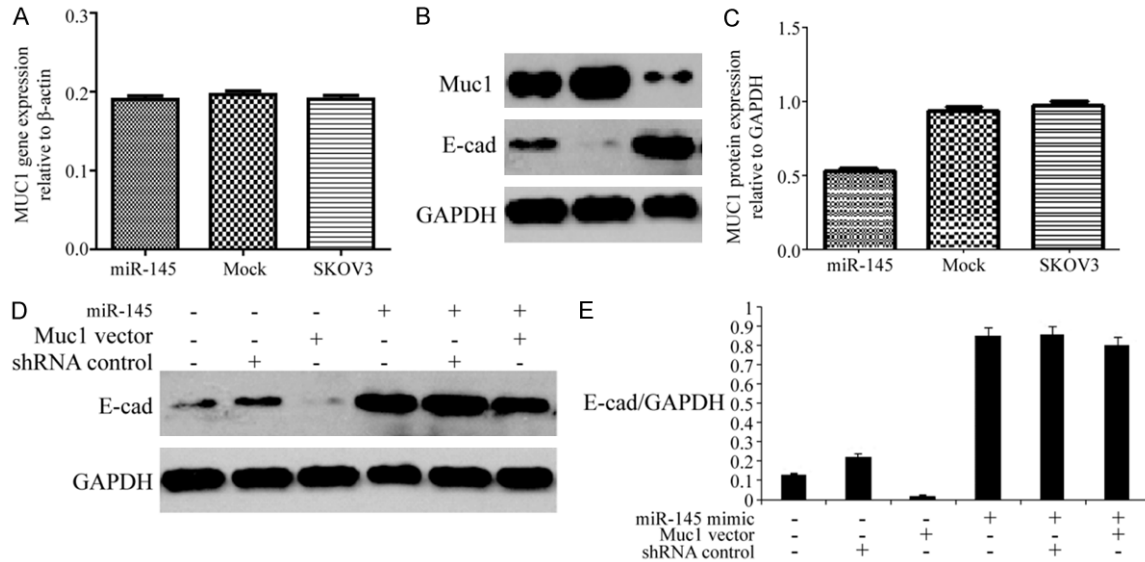
Figure 5. Comparisons of protein expression of MUC1 and E-cad of SKOV3 cells with miR-145 overexpression. A. Expression of MUC1 gene in SKOV3 cells with and without miR-145 overexpression. B, C. Protein expression of MUC1 and EMT marker E-cad in SKOV3 cells with or without miR-145 overexpression. D, E. Different expression of EMT marker E-cad with miR-145 or MUC1 overexpression in SKOV3 cells.

Discussion

MiRNAs are known for their multifunctional roles in cell differentiation, apoptosis, proliferation and metabolism [11, 12]. MiR-145, a member of the 143/145 cluster, is downregulated in many cancers, including glioma [13], breast cancer [14], renal cell carcinoma [15] and prostate cancer [16]. In our study, expression of miR-145 was found to be dramatically low in SKOV3 cells and SEOC tissue and was correlated with poor prognosis of SEOC patients, similar to findings from previous studies [17].

Transcriptional regulation of miR-145 appears to be complex. Since TP53 mutations have been found to occur in nearly all SEOC (96%), downregulation of miR-145 in SEOC may be related to p53 mutations. Sachdeva et al. found that p53 could increase miR-145 expression by directly binding to the p53 response elements-2 (p53RE-2) in the miR-145 promoter, which is possibly the mechanism of p53-mediated repression of c-Myc [18]. Moreover, the positive feedback regulation between miR-145 and p53 partially deals with impairing the murine double minute 2 (MDM2)-p53 feedback loop [19, 20]. In addition to p53, other transcription factors participate in the regulation of miR-145, including CCAAT/enhancer-binding

protein-beta (C/EBP-β), β-catenin/T cell factor 4 (TCF4) and forkhead transcription factors of the O class 1 and 3 (FoxO1 and FoxO3) [21-23]. Moreover, DNA methylation in the upstream sequence of miR-145 contributing to the downregulation of miR-145 in prostate cancer [24]. Post-transcriptional regulation is also critical for miR-145 expression. Breast cancer 1 (BRCA1) recognizes the root of the stem-loop structure of miR-145, directly associates with Drosha and DDX5 of the Drosha complex, and interacts with Smad3, p53 and DHX9 RNA helicase, promoting miR-145 processing [25]. Conversely, BCDIN3D is a methyltransferase that modifies the 50-monophosphate end of miRNAs, including pre-miR-145, which affects their recognition by Dicer. BCDIN3D depletion was found to reduce the level of pre-miR-145 and increased the level of mature miR-145 in breast cancer cells [26].

To determine the potential roles of miR-145 in SEOC, SKOV3 cells were separately transfected with miR-145 mimics and negative control sequence and were challenged in Transwell matrigel chambers and wound healing assay. Assessments of the invasion and migration reveal that upregulated miR-145 significantly inhibited the migration and invasion of SKOV3 cells.

Many studies have demonstrated that miR-145 may influence tumor development by mediating different downstream targeting mRNAs, including c-Myc in breast cancer [27] and human lung cancer [28], FSCN1 in prostate cancer [29], ANGPT2 and NEDD9 in renal cell carcinoma [30], and Sox2 in human choriocarcinoma cells [31]. Our results validated that miR-145 mimics could inhibit the protein expression level of MUC1 in SKOV3 cells as the dual luciferase reporter gene assay demonstrated that miR-145 was able to directly bind with the 3'UTR of *MUC1* mRNA. Furthermore, E-cadherin, an EMT marker, could be upregulated by overexpression of miR-145, while this upregulation could be repressed by MUC1. Thus, our study provides a new link between miR-145, MUC1 and E-cadherin in the regulation of tumor invasion and metastasis in SEOC.

MUC1 is a heterodimeric type I transmembrane glycoprotein expressed on the apical surface of most epithelia, including mammary gland, lung, pancreas, kidney, female reproductive tract and stomach [32]. MUC1 was found to be over-expressed in breast, colon, pancreas and bladder tumors and was often associated with EMT of different cancer cells [33, 34]. By promoting focal adhesion assembly, MUC1 also promotes tumor cell growth and facilitates metastasis [35]. A critical step preceding metastasis is adhesion of circulating tumor cells to the vascular endothelium. Tumor-specific forms of MUC1 promote tumor cell adhesion to the endothelium through interactions with E-selectin and the intercellular adhesion molecule-1 (ICAM-1), two receptors expressed on the surface of endothelial and peritumoral stromal cells. The interaction between MUC1 and ICAM-1 facilitates cell-cell adhesion and trans-endothelial migration [36, 37]. *MUC1* is considered as an important metastasis gene.

Previous studies demonstrated that over-expression of miR-145 can repress EMT in both prostate cancer [38, 39] and breast cancer [14]. EMT not only triggers the invasion and metastasis of tumors, but it also endows cancer cells with immortalized proliferation [40]. Importantly, many studies have validated that miRNAs play a key role in tumor metastasis by regulating EMT. Although miR-145-based therapy for ovarian cancer is at an early stage, further research on miR-145 may lead to novel therapeutic strategies.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Liying Han, Department of Obstetrics and Gynecology, The Second Hospital of Jilin University, Changchun 130000, China. Tel: 86 13321547030; E-mail: zanghu@163.com

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