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MiR-145 is downregulated in human ovarian cancer and modulates cell growth and invasion by targeting p70S6K1 and MUC1

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ABSTRACT

MicroRNAs (miRNAs) are a family of small non-coding RNA molecules that regulate gene expression at post-transcriptional levels. Previous studies have shown that miR-145 is downregulated in human ovarian cancer; however, the roles of miR-145 in ovarian cancer growth and invasion have not been fully demonstrated. In the present study, Northern blot and qRT-PCR analysis indicate that miR-145 is downregulated in ovarian cancer tissues and cell lines, as well as in serum samples of ovarian cancer, compared to healthy ovarian tissues, cell lines and serum samples. Functional studies suggest that miR-145 overexpression leads to the inhibition of colony formation, cell proliferation, cell growth viability and invasion, and the induction of cell apoptosis. In accordance with the effect of miR-145 on cell growth, miR-145 suppresses tumor growth in vivo. MiR-145 is found to negatively regulate P70S6K1 and MUC1 protein levels by directly targeting their 3′UTRs. Importantly, the overexpression of p70S6K1 and MUC1 can restore the cell colony formation and invasion abilities that are reduced by miR-145, respectively. MiR-145 expression is increased after 5-aza-CdR treatment, and 5-aza-CdR treatment results in the same phenotype as the effect of miR-145 overexpression. Our study suggests that miR-145 modulates ovarian cancer growth and invasion by suppressing p70S6K1 and MUC1, functioning as a tumor suppressor. Moreover, our data imply that miR-145 has potential as a miRNA-based therapeutic target for ovarian cancer.

1. Introduction

Human ovarian cancer is one of the most common gynecologic malignancies in women [1]. The morphological origin of ovarian cancer is complicated, including cancers of epithelial, germ cell, undifferentiated and differentiated mesenchymal origin. Epithelial ovarian cancer (EOC), including serous adenocarcinoma, clear cell carcinoma and endometrial adenocarcinoma, is the most common type of ovarian cancer and ranks as the 7th leading cause of cancer-related death in women [2,3]. Because the early symptoms of EOC are not obvious, it is often diagnosed at a later stage. It often metastasizes to distant organs, leading to a high mortality and low 5-year survival rate and seriously threatens human health. Therefore, it is crucial to identify a novel molecule which might serve as a diagnostic biomarker and therapeutic target that can be used to elucidate the mechanism of EOC growth and metastasis.

MicroRNAs (miRNAs) are a family of endogenous, small non-coding RNAs that post-transcriptionally regulate gene expression through base pairs complementary to the binding sites on the 3′UTR of the target mRNA, leading to target mRNA cleavage or translational repression [4–6]. They have been identified to be involved in biological processes, including cell proliferation, apoptosis as well as cell differentiation [7,8]. Accumulating evidence shows that the abnormal expression of miRNAs occurs frequently in cancer, and they can serve either as tumor suppressors or oncogenes depending on the functions of their target genes [9,10]. Originally, tumor suppressive miRNAs are validated to be downregulated in cancers; in contrast, oncogenic miRNAs have relatively high levels. For example, miR-122 is downregulated in human liver cancer and serves as a tumor suppressor by inhibiting tumorigenesis and metastasis [11]. MiR-223 has been found to be upregulated in gastric cancer and promotes tumor invasion and metastasis, functioning as an oncogene [12]. So far, approximately 60% of protein-coding genes have been shown to be under the control of miRNAs [13]. Previous data demonstrate that several miRNAs are dysregulated in ovarian cancer [14–18],

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including miR-145. Despite the fact that several studies have validated the downregulation of miR-145 in ovarian cancer, the potential roles of miR-145 in ovarian cancer cell growth and metastasis are less known.

In this study, we found that miR-145 is downregulated in ovarian cancer tissues and cell lines, as well as in serum specimens of ovarian cancer patients. There existed an inverse relationship between miR-145 expression and CA125, a novel marker for ovarian cancer. The overexpression of miR-145 led to a reduction of cell growth and invasion abilities, and overexpression was also found to induce cell apoptosis. Using bioinformatics, we identified two direct target genes of miR-145.

2. Materials and methods

2.1. Tissue specimens and cell culture

Three normal ovarian tissues, four serous ovarian cancer tissue specimens, four clear cell ovarian cancer tissues specimens, four endometrioid ovarian cancer tissues, as well as seven normal serum samples, and twelve serum samples from serous and clear cell ovarian cancer patients were acquired from Tianjin Medical University Cancer Institute and Hospital. All of the tissue specimens were obtained after consent was given by patients, and they were then analyzed by immunohistochemistry.

Normal human ovarian cells, OECs (ovarian epithelial cell), were cultured in RPMI 1640 medium. Human ovarian cancer cells, IGROV1 (from endometrioid carcinoma) and OVCAR3 (from serous carcinoma) cells, were cultured in Dulbecco's Modified Eagle Medium (DMEM), whereas ES-2 (from clear cell carcinoma) and SKOV3 cells (from serous carcinoma) were cultured in McCoy's 5a Medium. All medium was supplemented with 10% fetal bovine serum (FBS). The cells were maintained in a humidified incubator at 37 °C with 5% CO2.

2.2. RNA isolation and Northern blot analysis

Total RNA (including miRNA) was extracted from cells, tissues and serum samples using TRIzol (Invitrogen) according to the manufacturer's protocol. The Northern blot analysis of miRNAs was performed in accordance with previously published methods [19].

2.3. Reverse transcription and real-time PCR analysis

For the reverse transcription (RT) of miRNA, a special RT primer for miR-145 was used. The real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, USA) according to the manufacturer's instructions. The reaction conditions were as follows: 95 °C for 5 min followed by 40 cycles of amplification at 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s. RNU6B (U6 small nuclear B non-coding RNA) was used as an internal control for the normalization of miR-145 expression.

2.4. Plasmid construct and transfection

MiR-145 mimics and the mimic controls were purchased from GenePharma Company (Shanghai, China). p70S6K1 or the MUC1 gene containing only the coding region was cloned and inserted into the pCMV6 vector. Cell transfection was performed using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's protocols.

2.5. Western blot analysis

The transfected cells were harvested at 48 h after transfection and lysed using RIPA buffer (50 mM Tris–HCl, pH 8.8, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Protein concentration was measured using the BCA method. A total of 20 μg of protein was loaded into the SDS–PAGE gel for analysis. The primary antibodies used were rabbit monoclonal anti-candidate gene (Abcam, USA) or anti-GAPDH antibody (Abcam, USA, 1:1000 dilutions). The secondary antibody was goat anti-rabbit IgG conjugated with HRP (horseradish peroxidase) at a dilution of 1:1000. The bound antibodies were detected with an ECL Plus western blotting Detection system (GE Healthcare), and the chemiluminiscent signals were detected with the use of high-performance chemiluminescence film (GE Healthcare). GAPDH was used as an internal control for the normalization of candidate genes.

2.6. Colony formation assay

The transfected cells were plated into 12-well plates at a density of 200 cells/well. The medium was refreshed every three days until most of the cell colonies contained at least 50 cells. The colonies were stained with 5% crystal violet and counted.

2.7. Cell cycle analysis using FACS

At 48 h after transfection, the cells were added with EdU (5 μM). After incubation for 2 h, the cells were harvested, fixed and used for cell cycle analysis using EdU DNA Proliferation in vitro Detection kit according to the manufacturer's instructions.

2.8. Cell growth curve

The transfected cells were plated into 24-well plates at a density of 4000 cells/well, and each group consisted six replicates to perform cell counts at different time points after transfection. Day 1 was defined as the time of cell plating.

2.9. Ovarian cancer xenograft model in nude mice

The mice used in this study were fed with normal diet under specific pathogen-free conditions. Animal studies were conducted after approval by the Animal Care Committee and were performed in accordance with NIH animal use guidelines. Briefly, 10^6 SKOV3 cells that ectopically expressed miR-145 were suspended with 100 μl serum-free medium and subcutaneously injected into nude mice. About three weeks after injection, the animals were sacrificed and tumor volumes were calculated by measuring the tumor length and width with calipers.

2.10. Annexin-V FITC apoptosis assay

At 24 h after transfection, the cells were incubated with staurosporine (Sigma, USA) for approximately 24 h. Then, the cells were harvested and assayed using an Annexin-V FITC kit on a BD FACS Calibur Flow Cytometry System (Becton Dickson, USA) according to the manufacturer's protocols.

2.11. Cell invasion assay

The cell invasion assay was performed using a transwell chamber coated with Matrigel (Millipore, USA). The transfected cells were plated into the upper chamber at a density of 2.5×10^4 - cells/well with serum-free medium, while the lower chamber con-

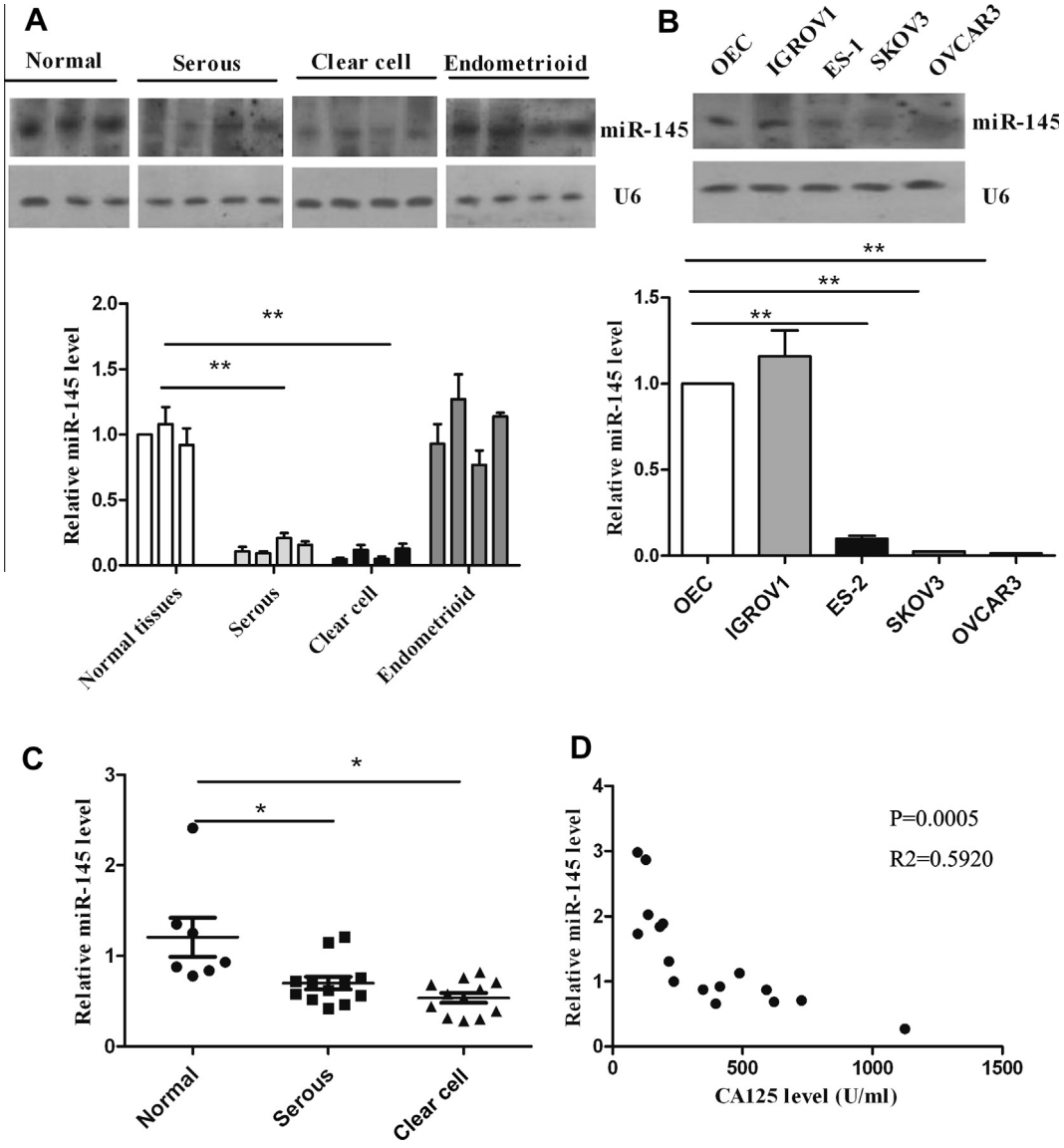

Fig. 1. MiR-145 is downregulated in human ovarian cancer. (A) The expression of miR-145 was determined by Northern blot in normal ovarian tissues and tissues from different types of ovarian cancer. (B) The expression of miR-145 was determined by Northern blot in normal ovarian cells (OECs) and ovarian cancer cells. (C) The expression of miR-145 was determined by qRT-PCR in normal serum and serum samples from Serous and Clear cell ovarian cancer. (D) The relationship between miR-145 and CA125 levels was determined in serum samples from ovarian cancer patients. U6 was used as an internal control. $^{*}P < 0.05$, $^{**}P < 0.01$.

tained medium with 10% FBS. When the cells were allowed to invade the Matrigel for approximately 20 h, the invasive cells were fixed and stained using crystal violet, while the noninvasive cells were scraped with cotton tips. Finally, the invasive cells were taken imaged and counted.

2.12. Luciferase reporter assay

The 3′UTR of the candidate gene was cloned and inserted into the downstream of luciferse gene in pGL3/luciferase vector. The mutant 3′UTR of the candidate gene (several nucleotides within binding sites were mutated) was cloned using the wild type 3′UTR as a template and inserted into pGL3/luciferase as described for the wild type 3′UTR. The cells were co-transfected with miRNA mimics and the wild type or mutant 3′UTR of the candidate gene. At 48 h after transfection, the cells were harvested and lysed using RIPA buffer. The luciferase intensity was measured using a Dual Luciferase® Reporter Assay System (Promega) following the manufacturer's instructions.

2.13. 5-aza-CdR treatment

The cells were plated into 24-well plates at a density of 5×10^4 cells/well, and 5-aza-CdR (5-aza-2′-deoxycytidine, 5 μM) was added to the cells after they had adhered to the plate. The cells were incubated with 5-aza-CdR for approximately 96 h and then harvested to assess miR-145 expression using real-time PCR.

2.14. Statistical analysis

Each group was formed in triplicate and all experiments were repeated at least twice. The difference was measured using Student's *t* test, and $P < 0.05$ was considered significantly statistical.

3. Results

3.1. MiR-145 is downregulated in human ovarian cancer

Our comparison of miR-145 expression by Northern blot between normal ovarian tissues and ovarian tissue specimens

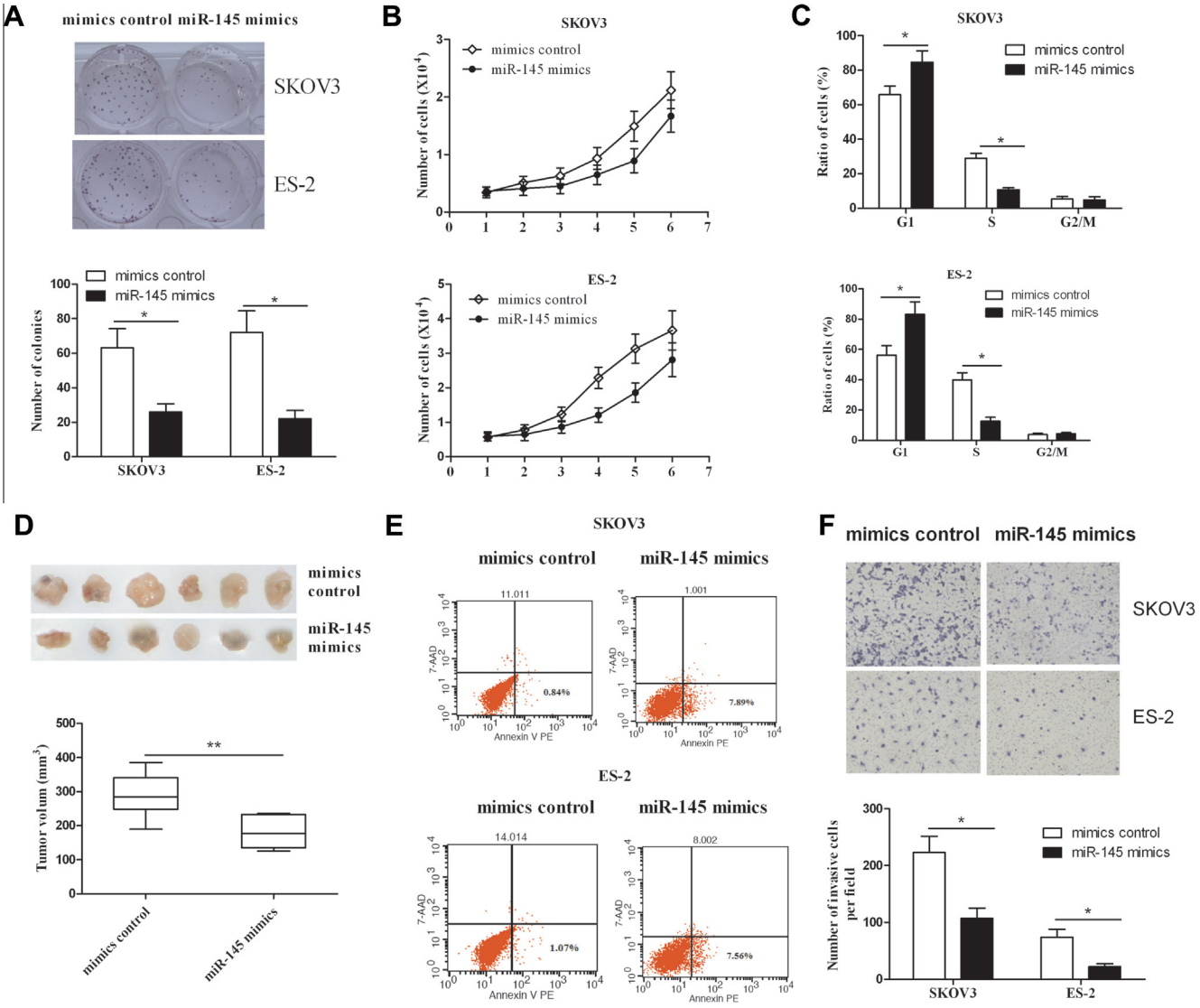

Fig. 2. MiR-145 inhibits ovarian cancer cell proliferation, tumor growth and invasion, but promotes apoptosis. SKOV3 and ES-2 cells were transfected with miR-145 mimics, and the cell proliferation was determined by colony formation assay (A), cell growth curve (B) and cell cycle analysis (C). (D) The sizes of xenografts were measured after the injection of SKOV3 cells with miR-145 mimics or mimic controls. (E) Cell apoptosis was determined by flow cytometry using Annexin V-PE staining. The percentage of apoptotic SKOV3 cells that had been transfected with the miR-145 mimics increased from 0.84% to 7.89%; the percentage of apoptotic cells transfected with the miR-145 mimics increased from 1.079% to 7.56%. (F) The cell invasion ability was determined with transwell chambers coated with Matrigel. The pictures represent the invasive SKOV3 and ES-2 cells stained with crystal violet. The right graph shows the relative number of invasive cells per field. $^{*}P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(including serous ovarian cancer, clear cell ovarian cancer and endometrioid ovarian cancer) indicated that miR-145 is downregulated in serous and clear cell ovarian cancer tissues, except endometrioid ovarian cancer, compared to normal tissues (Fig. 1A). In accordance with the results obtained in cancer tissues, miR-145 was validated to be downregulated in ovarian cancer cells, ES-2, SKOV3 and OVCAR3, but not IGROV1 cells, compared to normal ovarian epithelial cells (OECs) (Fig. 1B).

In addition, serum expression levels of miR-145 were also determined by real-time PCR among normal serum specimens and samples from serous and clear cell ovarian cancer patients. As shown in Fig. 1C, miR-145 was expressed at a lower level in ovarian cancer serum samples than in normal serum. CA125, a main indicator of ovarian cancer, was present at a relatively high level. We found that there existed a converse relationship between miR-145 and CA125 levels (Fig. 1D), indicating that the downregulation of miR-145 might play an important role in ovarian cancer development.

3.2. MiR-145 inhibits ovarian cancer cell proliferation and tumor growth

To investigate the functions of miR-145 in ovarian cells, cell proliferation was tested in miR-145 mimic-transfected ES-2 and SKOV3 cells. Colony formation showed that miR-145 mimic-transfected SKOV3 cells had a lower colony number than control mimic-transfected cells; accordingly, miR-145 transfection resulted in a similar effect on the colony formation ability of ES-2 cells (Fig. 2A). Upon the construction of cell growth curves, we found that miR-145 inhibited the number of SKOV3 cells compared to control cells. MiR-145 also inhibited the number of ES-2 cells (Fig. 2B). Based on the cell cycle analysis conducted using an EdU DNA proliferation detection kit, we discovered that miR-145 increased the number of cells in the G1 phase but inhibited the number of S phase cells in ES-2 and SKOV3 cells, respectively, suggesting that miR-145 could cause G1/S arrest. Overall, these data show that miR-145 inhibits ovarian cancer cell proliferation.

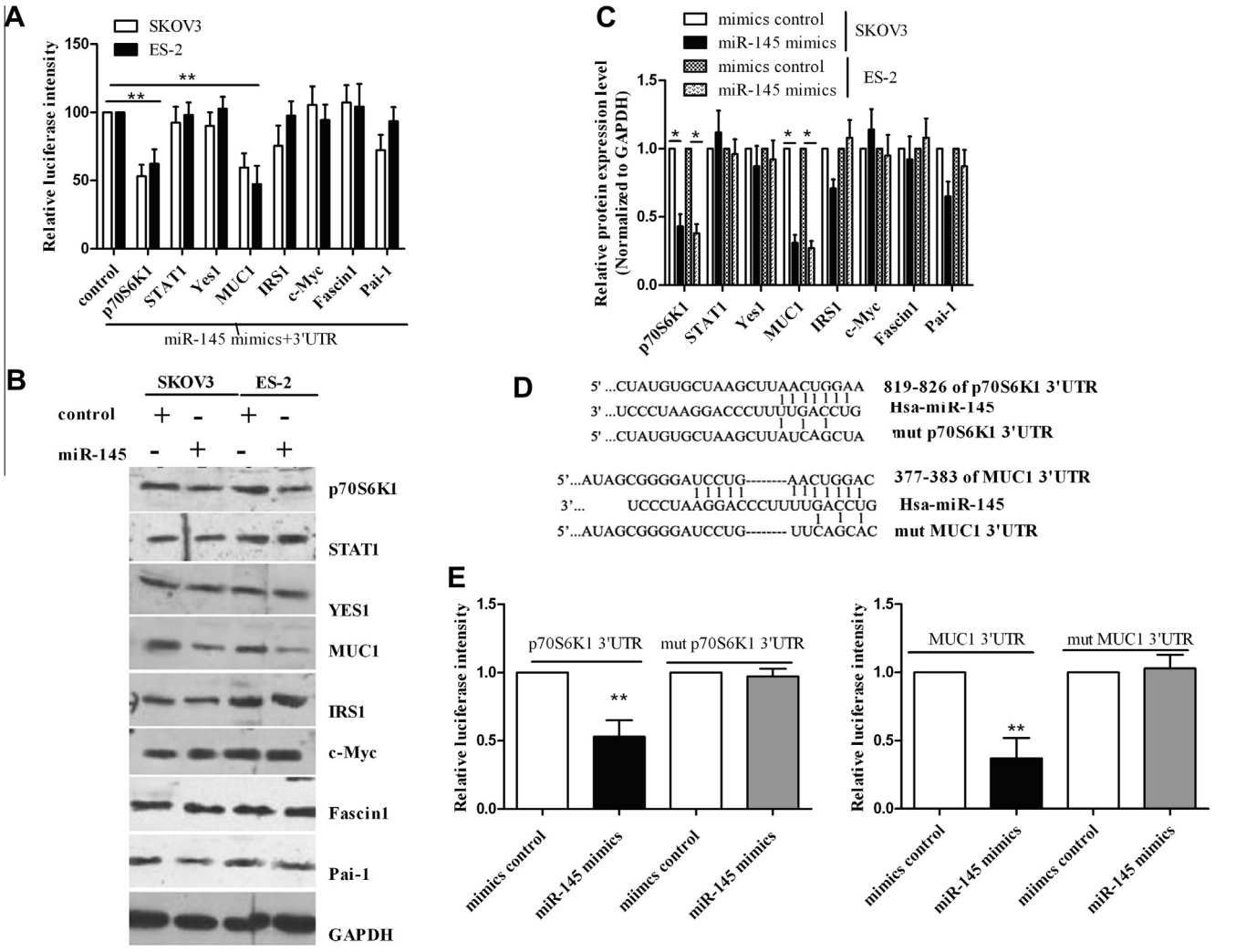

Fig. 3. p70S6K1 and MUC1 are direct target genes of miR-145. (A) Both SKOV3 and ES-2 cells were co-transfected with miR-145 mimics and the 3′UTRs of candidate genes, and the luciferase intensity was measured after transfection for 48 h. The luciferase intensities of cells transfected with the mimic control and target 3′UTR were normalized to 1. (B and C) The effect of miR-145 on the protein levels of candidate genes was determined by Western blot in both SKOV3 and ES-2 cells. GAPDH was used as an internal control. (D) MiR-145 was found to have a binding site in the 3′UTR of p70S6K1 and MUC1, and several point mutations were generated within the binding site. (E) The effect of miR-145 on luciferase intensity controlled by the wild type or mutant 3′UTR of p70S6K1 and MUC1 was determined by luciferase assay. $^{**}P < 0.01$.

Furthermore, we determined the effect of miR-145 on tumor growth. As shown in Fig. 2D, the xenografts from mice injected with the miR-145 mimic were smaller than those from the animals injected with the control mimic. These results indicate that the overexpression of miR-145 in ovarian cancer cells inhibits tumor growth.

3.3. MiR-145 promotes ovarian cancer cell apoptosis

We performed Annexin-V FITC/PE double staining to detect the effect of miR-145 on cell apoptosis. As shown in Fig. 2E, we observed that miR-145 significantly promoted SKOV3 cell apoptosis compared to cells transfected with the control mimic. The apoptosis results were confirmed in ES-2 cells.

3.4. MiR-145 inhibits ovarian cancer cell invasion

Cell invasion is an essential characteristic for tumor metastasis; therefore, we attempted to observe the effect of miR-145 on cell invasion ability. Fig. 2F showed that SKOV3 cells transfected with the miR-145 mimics were less invasive than the cells with transfected with the mimic control. Consistently, miR-145 had similar effect on ES-2 cell invasion (Fig. 2F). These data suggest that miR-145 may be an important mediator for tumor metastasis.

3.5. p70S6K1 and MUC1 are direct targets of miR-145 in ovarian cancer cells

We used bioinformatics to predict candidate target genes of miR-145 to investigate the mechanism for miR-145's effect on cell growth and invasion. Through target prediction and the potential roles of candidate genes, several genes shown in Fig. 3A were selected as candidate genes. We then constructed luciferase reporter genes with the 3′UTRs of the targets in the downstream of the reporter. Cells were co-transfected with miR-145 mimics and the 3′UTRs of candidate genes for the luciferase reporter assay. As shown in Fig. 3A, miR-145 inhibited the luciferase intensity controlled by the p70S6K1 and MUC1 3′UTRs. Western blot analysis showed that miR-145 also inhibited the expression of p70S6K1 and MUC1 protein levels (Fig. 3B and C).

To identify the direct interaction between miR-145 and p70S6k1 and MUC1, a binding site mutation (Fig. 3D) was

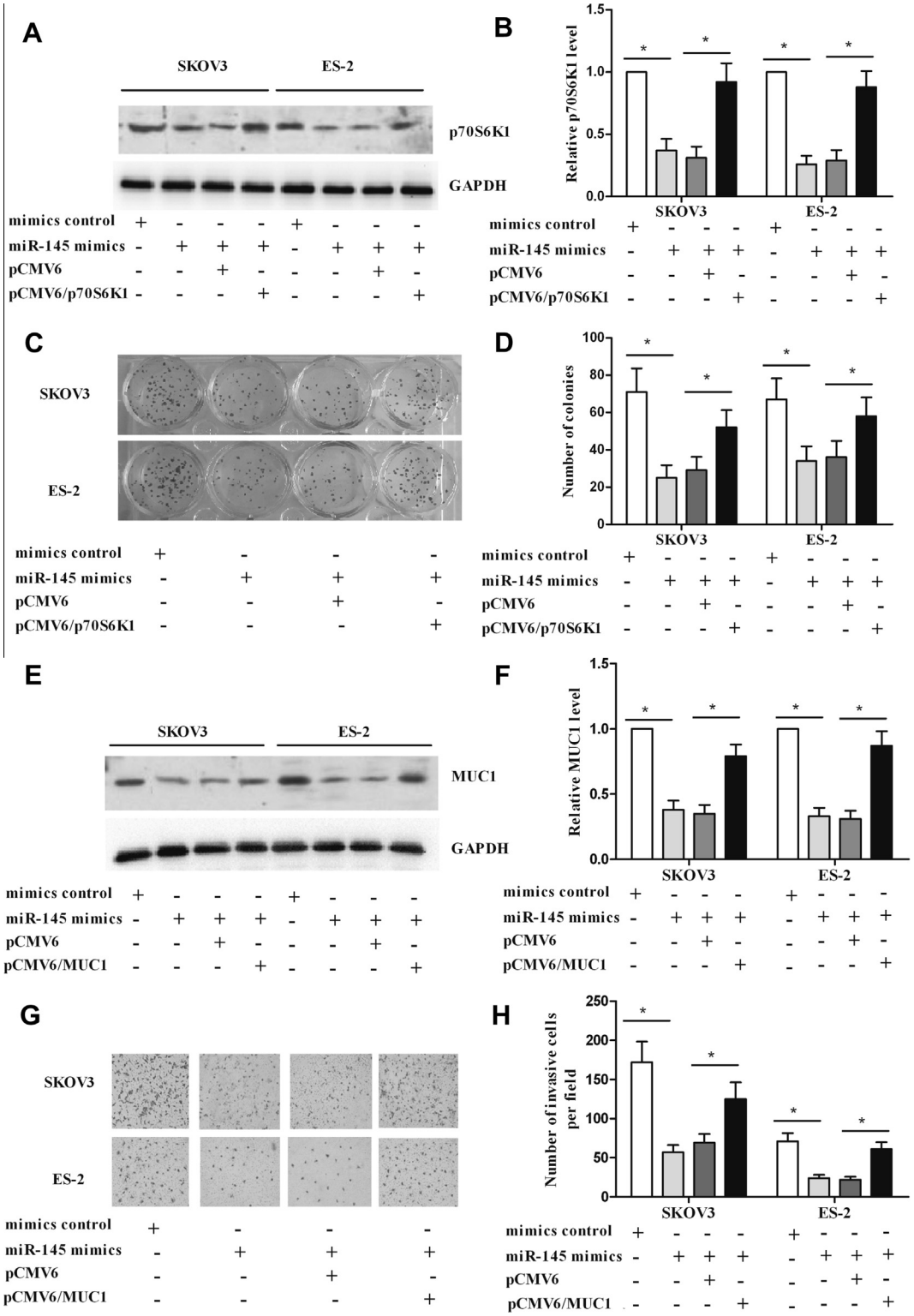

Fig. 4. The overexpression of p70S6K1 and MUC1 alleviates the inhibitory effects induced by miR-145. The cells were either transfected with miR-145 and the control, or were co-transfected with miR-145 and pCMV6/p70S6K1or pCMV6/MUC1. (A and B) p70S6K1 expression levels were determined by western blot. GAPDH was used as an internal control. (C and D) Cell proliferation was determined by colony formation assay. Each group was formed in triplicate and all experiments were repeated at least twice. (E and F) MUC1 expression levels were determined by western blot. GAPDH was used as an internal control. (G and H) Cell invasion was determined by using a transwell chamber coated with Matrigel. $^{*}P < 0.05$.

generated. The luciferase reporter assay indicated that miR-145 led to the inhibition of wild type 3′UTR, but it had no effect on the luciferase intensity controlled by the mutant 3′UTR (Fig. 3E). Taken together, these results indicate that miR-145 negatively regulates p70S6k1 and MUC1 by directly targeting their 3′UTRs.

3.6. Overexpression of p70S6K1 and MUC1 can rescue the inhibitory effect induced by miR-145

We next attempted to validate whether p70S6k1 and MUC1 could mediate the effect of miR-145 on cells. pCMV6/p70S6K1

and pCMV6/MUC1 were constructed to overexpress the genes. Fig. 4A and B confirmed that p70S6K1 was overexpressed and also confirmed that it can rescue the inhibition of p70S6K1 protein levels caused by miR-145. In addition, the colony formation assay indicated that p70S6K1 reduced the inhibitory effect of miR-145 on colony formation (Fig. 4C and D), suggesting that p70S6K1 mediates the cell proliferation induced by miR-145. Then, overexpression of MUC1 was confirmed by western blot (Fig. 4E and F), and MUC1 was found to alleviate the miR-145-mediated inhibition of MUC1 expression and cell invasion (Fig. 4G and H), suggesting that MUC1 mediates the cell invasion induced by miR-145.

4. Discussion

A large amount of evidence has demonstrated that miRNAs are key regulators of protein coding genes in various cancers, including human ovarian cancer. In the present study, our data indicated that miR-145 was downregulated in ovarian cancer tissues, cell lines and serum samples. MiR-145 overexpression was found to reduce cell proliferation, cell invasion, tumor growth, and induce cell apoptosis, functioning as a tumor suppressor in ovarian cancer. All of these results were supported by the data from other reports. For example, miR-145 is found to suppress cell proliferation, migration and invasion of prostate cancer [20]. MiR-145 is also shown to inhibit glioma cell migration and invasion by targeting ADAM17, serving as a tumor suppressor [21].

As for the mechanism of miRNA regulation in cancer, it is crucial to validate target genes. Using the luciferase report assay, we observed that miR-145 reduced the luciferase intensity controlled by the wild type 3′UTRs of p70S6K1 and MUC1, while there was no effect on the mutant 3′UTR with several binding sites mutated, indicating that p70S6K1 and MUC1 were direct target genes for miR-145. Then, western blotting demonstrated that miR-145 also reduced the protein levels of both genes and negatively affected gene expression. Through functional studies about p70S6K1 and MUC1, we discovered that the overexpression of p70S6K1 and MUC1 can restore the inhibitory effects of miR-145 on their protein levels, cell colony formation and invasion abilities, further validating the conclusion that miR-145 regulates cell growth and invasion by directly targeting p70S6K1 and MUC1.

Previous report suggests that P70S6K1 inhibits cell proliferation, cell cycle progression and tumor growth through the suppression of HIF-1 expression, which plays an important role in tumor growth [22]. In human ovarian cancer, the inhibition of PI3K suppresses the phosphorylation of AKT and p70S6K1, leading to the suppression of the G1 phase and cell cycle progression, indicating that p70S6K1 plays a crucial role in cell growth [23]. MUC1 has been validated to promote cell invasion through upregulating the metastasis-related gene, matrix metalloproteinase 13 [24]. Consistent with these data, our study also showed that p70S6K1 promoted ovarian cancer cell proliferation and MUC1 promoted cell invasion. All of these data suggest that miR-145 regulates cell processes through the modulation of different target genes.

Studies have demonstrated that hypermethylation is one of the main contributors to the downregulation of miRNAs in cancers. The hypermethylation of CpG islands leads to the silencing of miR-124 and miR-203 in human hepatocellular carcinoma, and 5-aza-CdR increases miR-124 and miR-203 expression levels [25]. MiR-335 is downregulated in breast cancer due to the regulation of methylation [26]. In accordance with the data reported in previous studies, we discovered that 5-aza-CdR treatment significantly increased miR-145 expression. The cells incubated with 5-aza-CdR had relatively lower levels of p70S6K1 and MUC1 expression, and fewer colonies and invasive cells (shown in Supplementary Fig. 1). These results are similar to those observed for miR-145 overexpression. However, the 5-aza-CdR results alone are not enough to validate the methylation status of miR-145, and further studies should focus more on this topic.

In conclusion, the present study indicates that 5-aza-CdR increases miR-145 expression, and miR-145 inhibits cell proliferation, invasion and tumor growth through the suppression of p70S6K1 and MUC1, functioning as a tumor suppressor (shown in Supplementary Fig. 2). Our data imply that the overexpression of miR-145 may serve as a novel biomarker for ovarian cancer and may also be a potential target for a miRNA-based molecular therapeutic strategy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.053.

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