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Suppressive Role of MicroRNA-148a in Cell Proliferation and Invasion in Ovarian Cancer Through Targeting Transforming Growth Factor-β-Induced 2

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Ovarian cancer (OC) is one of the most common gynecological malignancies. MicroRNAs (miRs) play a crucial role in the development and progression of OC, but the underlying mechanism remains largely unclear. Our study investigated the regulatory role of miR-148a in OC cell proliferation and invasion. We found that miR-148a was significantly downregulated in OC tissues compared to their matched adjacent nontumor tissues. In addition, its expression was also reduced in OC cell lines (SKOV3, ES-2, OVCAR, and A2780) compared to normal ovarian epithelial cells. Overexpression of miR-148a caused a significant decrease in OC cell proliferation and invasion, as well as reduced MMP9 protein levels. Transforming growth factor-β-induced 2 (TGFI2) was further identified as a target gene of miR-148a, and its protein expression was downregulated in OC cells after miR-148a overexpression. Restoration of TGFI2 attenuated the suppressive effects of miR-148a on OC cell proliferation and invasion. Moreover, we found that TGFI2 was remarkably upregulated in OC tissues when compared with their matched adjacent nontumor tissues, and observed a reverse correlation between miR-148a and TGFI2 expression in OC tissues. On the basis of these findings, we suggest that miR-148a inhibits OC cell proliferation and invasion partly through inhibition of TGFI2. Therefore, our study highlights the importance of the miR-148a/TGFI2 axis in the malignant progression of OC.

Key words: Ovarian cancer (OC); MicroRNAS (miRs); Transforming growth factor-β-induced 2 (TGFI2); Proliferation; Invasion

INTRODUCTION

Ovarian cancer (OC) is one of the most common gynecological malignancies and has become the fifth most common cause of death among female cancer patients (1,2). One reason for such mortality is that a majority of OC patients are diagnosed after tumors have widely spread within the peritoneal cavity (3). The patients are faced with poor therapeutic effect and a miserable prognosis in spite of the combination of surgery and chemotherapy (4,5). Fortunately, a promising treatment strategy may be introduced following the basic medical research of molecular mechanisms of OC.

MicroRNA (miR) is a kind of short, highly conserved, noncoding RNA molecule containing approximately 20–22 nucleotides that functions in the regulation of gene expression by interfering with the posttranscriptional process (6). miR can influence several kinds of biological processes, including cell proliferation, survival, differentiation, apoptosis, motility, and so forth (7,8). In addition, it can affect various pathophysiological courses such as tumorigenesis (9). miR-148a has been reported to be deregulated in many human malignancies and to play a role in cancer development and progression (10,11). Moreover, miR-148a was found to play a suppressive role in OC (12). Wen et al. reported that it could inhibit the migration and invasion of OC cells via targeting S1PR1 (12). However, the underlying mechanism of miR-148a in the regulation of proliferation and invasion of OC cells has not been adequately illuminated.

Transforming growth factor-β-induced 2 (TGIF2) lies on chromosome 20q11.2-12 and is a member of the TALE superfamily (13,14). It functions in various biological processes, including embryonic development, and cell proliferation and differentiation (13,14). Additionally, TGIF2 plays a major role in a variety of cancers such as gastric, breast, skin, and ovarian (15–18). Imoto et al. showed an increased expression of TGIF2 in OC cells, suggesting that TGIF2 may play an oncogenic role in the

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development and/or progression of OC (18). However, the relationship between TGIF2 and miR-148a in OC remains unknown, and evidence in the regulatory mechanism of TGIF2 expression is limited.

The present study aimed to reveal the exact role as well as the molecular mechanism of miR-148a in the regulation of OC cell proliferation and invasion.

MATERIALS AND METHODS

Tissue Collection

All protocols were approved by the Ethics Committee of The First Affiliated Hospital of Xiamen University, Xiamen, China. Informed consent was obtained from each patient within the guidelines of The First Affiliated Hospital of Xiamen University. A total of 29 OC tissue specimens and matched adjacent nontumor tissues were collected in our hospital. All patients were untreated, without medical history of other tumors. The tissues were immediately snap frozen in liquid nitrogen after surgical removal and stored at −80°C until use.

Cell Culture

Human OC cell lines (SKOV3, ES-2, OVCAR, and A2780) were purchased from the Chinese Academy of Sciences (Shanghai, China). Normal ovarian epithelial cells HUM-CELL-0088 were obtained from PriCells, Wuhan, China. The cells were cultured in DMEM (Life Technologies, USA) with 10% fetal bovine serum (FBS) (Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2.

RNA Extraction and Real-Time RT-PCR Analysis

Total RNA was extracted with TRIzol Reagent (Life Technologies). For miR expression detection, a miRNA Reverse Transcription Kit (Life Technologies) was used to convert RNA into cDNA. Real-time polymerase chain reaction (PCR) was then performed by using a miRNA Q-PCR Detection Kit (GeneCopoeia, Rockville, MD, USA) on ABI 7500 thermocycler. U6 gene was used as an internal reference. For mRNA detection, a Reverse Transcription Kit (Life Technologies) was used to convert RNA into cDNA. Real-time PCR was then performed by using the Q-PCR Detection Kit (Life Technologies). GAPDH was used as an internal reference. The relative expression was analyzed by the $2^{-\Delta\Delta Ct}$ method (19).

Transfection

Lipofectamine 2000 (Life Technologies) was used in the transfection processes under the manufacturer's instruction. SKOV3 and ES-2 cells were transfected with the scrambled miRNA mimics as a negative control (miR-NC), miR-148a mimics, or cotransfected with miR-148a and TGIF2 plasmid, respectively.

Cell Proliferation Assay

SKOV3 and ES-2 in each group were plated at a density of 10,000 cells per well in 96-well plates. After culture for 0, 24, 48, 72, and 96 h, the cells were incubated with MTT at a final concentration of 0.5 mg/ml for 4 h at 37°C. After the removal of the medium, 150 mM DMSO solutions were added to dissolve the formazan crystals. The absorbance was read at 570 nm using a BioTek ELX-800 Absorbance Microplate reader (BioTek, Winooski, VT, USA).

Cell Invasion Assay

This assay was performed in the 24-well Transwell chambers (Chemicon, Temecula, CA, USA) with 100 μg of Matrigel (Chemicon). Cell suspension of 5.0×10^5 cells/ml was prepared in the serum-free media, 300 μl of which was added into the upper chamber, with the bottom chamber supplemented with 0.5 ml of DMEM with 10% FBS. After incubation for 24 h at 37°C 5% CO, noninvading cells and the matrix gel on the interior of the inserts were removed, followed by staining on-the-bottom-chamber cells with trypan blue (Beyotime, Shanghai, China) for 15 min. Six fields were randomly selected, and the cell number was counted under the microscope (Nikon, Tokyo, Japan).

Western Blotting

Cells were solubilized in cold RIPA lysis buffer (Beyotime). Proteins were separated with 10% SDS-PAGE (Beyotime) and transferred onto a nitrocellulose membrane (Life Technologies), and then incubated with TBST (Beyotime) containing 5% nonfat milk (Yili, Beijing, China) at room temperature for 3 h. The membrane was then incubated with mouse anti-human TGIF2 antibody (Abcam, Cambridge, MA, USA), mouse anti-human MMP9 antibody (Abcam), and mouse anti-human GAPDH antibody (Abcam), respectively, at room temperature for 3 h. After washing three times with PBST, the membrane was incubated with the rabbit anti-mouse secondary antibody (Abcam) at room temperature for 1 h. Detection of immune complex was performed with an Pierce ECL Western Blotting KIT (Pierce, Thermo Fisher, USA), according to the manufacturer's instruction. Image-Pro plus software 6.0 was used to analyze the expression of the protein.

Dual Luciferase Reporter Assay

QuickChange Site-Directed Mutagenesis Kit (Stratagene, USA) was used to generate mutant-type 3′-UTR (3′-untranslated region) of TGIF2. The wild- or mutant-type 3′-UTR of TGIF2 was inserted into the psiCHECK-2 vector (Promega, USA), relatively. OC cells were transfected with psiCHECK-2-TGIF2-3′-UTR or psiCHECK-2-mutant TGIF2-3′-UTR vector, with or without miR-148a

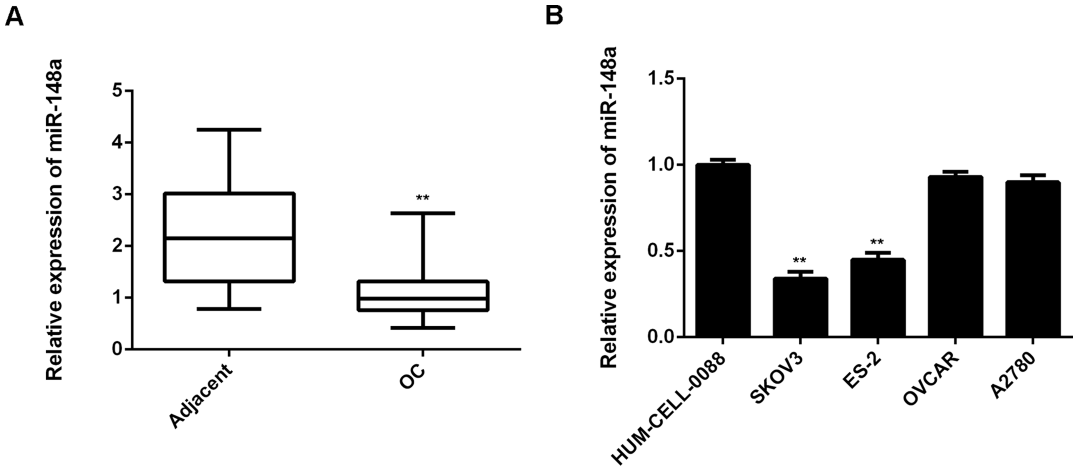

Figure 1. (A) Real-time PCR was used to examine the miR-148a expression in ovarian cancer (OC) tissues compared to matched adjacent nontumor tissues. $^{**}p<0.01$ versus Adjacent. (B) Real-time PCR was used to examine the miR-148a expression in OC cell lines compared to normal ovarian epithelial HUM-CELL-0088 cells. $^{**}p<0.01$ versus HUM-CELL-0088 cells.

mimics, separately. The luciferase activities were detected on an LD400 luminometer (Beckman Coulter, Fullerton, CA, USA) 48 h after cotransfection. Renilla luciferase activity was normalized to the firefly luciferase activity.

Statistical Methods

The data are expressed as mean ± SD. Statistical analysis of differences was performed by Student's *t*-test by using SPSS 21.0 software. A value of $p<0.05$ was regarded as statistically significant.

RESULTS

Downregulation of miR-148a Was Observed in OC Tissues and Cell Lines

First, the expression of miR-148a was examined in 29 cases of OC tissues and their matched adjacent nontumor

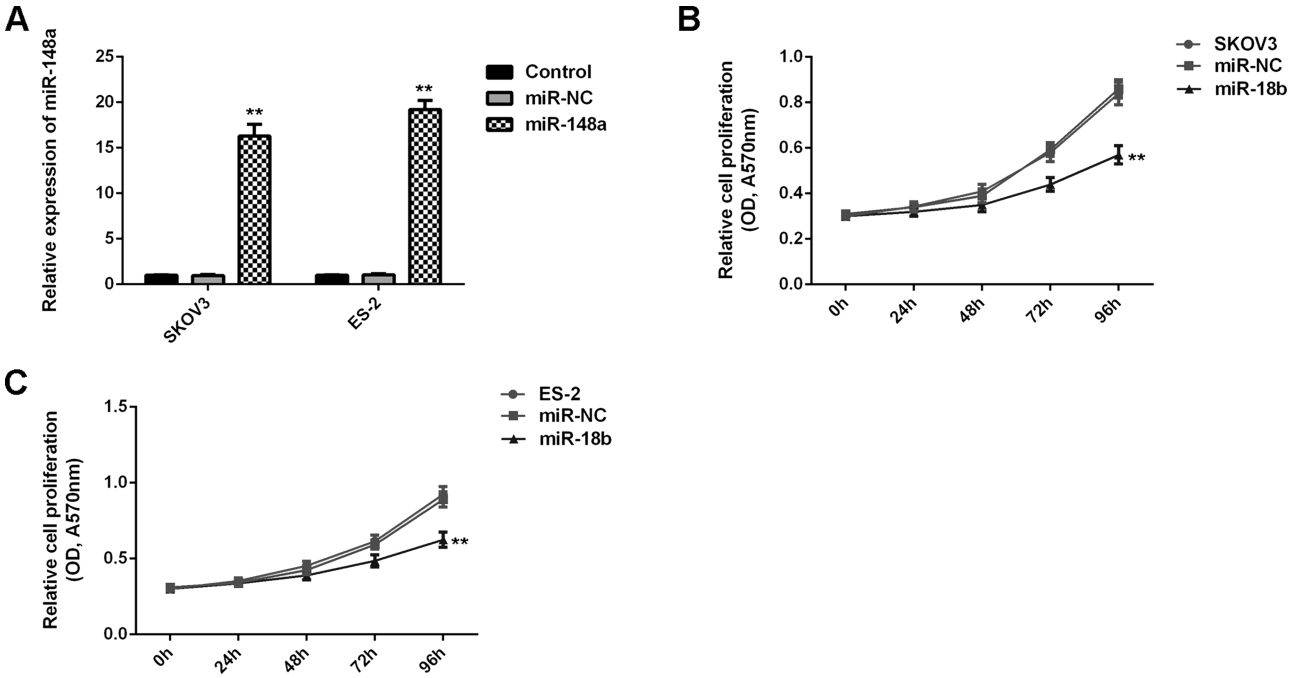

Figure 2. (A) Real-time PCR was used to examine the miR-148a expression in OC SKOV3 and ES-2 cells transfected with scramble miRNA (miR-NC) or miR-148a mimics. Nontransfected SKOV3 or ES-2 cells were used as Control, respectively. $^{**}p<0.01$ versus Control. (B, C) MTT assay was used to examine cell proliferation. $^{**}p<0.01$ versus SKOV3 or ES-2 cells, respectively.

tissues by real-time qRT-PCR. As shown in Figure 1A, the miR-148a levels were considerably decreased in the OC tissues when compared with those in their matched adjacent nontumor tissues. Sequentially, miR-148a expression was detected in OC cell lines, including SKOV3, ES-2, OVCAR, and A2780. Normal ovarian epithelial cell line HUM-CELL-0088 was used as a control. We found that SKOV3 and ES-2 cells also showed a significantly decreased expression of miR-148a compared with HUM-CELL-0088 cells (Fig. 1B). These results provide evidence in support of an opinion that miR-148a may play a suppressive role in OC.

Inhibitory Effect of miR-148a on Proliferation and Invasion of OC Cells

Since the expression of miR-148a was obviously reduced in OC, we further focused on its effects on the proliferation and invasion of OC cells, which are important for the malignant progression of this disease. SKOV3 and ES-2 cells were transfected with miR-148a mimics or scramble miRNA mimics, respectively. After transfection with miR-148a mimics, real-time RT-PCR data showed that the expression level of miR-148a was significantly increased (Fig. 2A). MTT assay further indicated that overexpression of miR-148a decreased

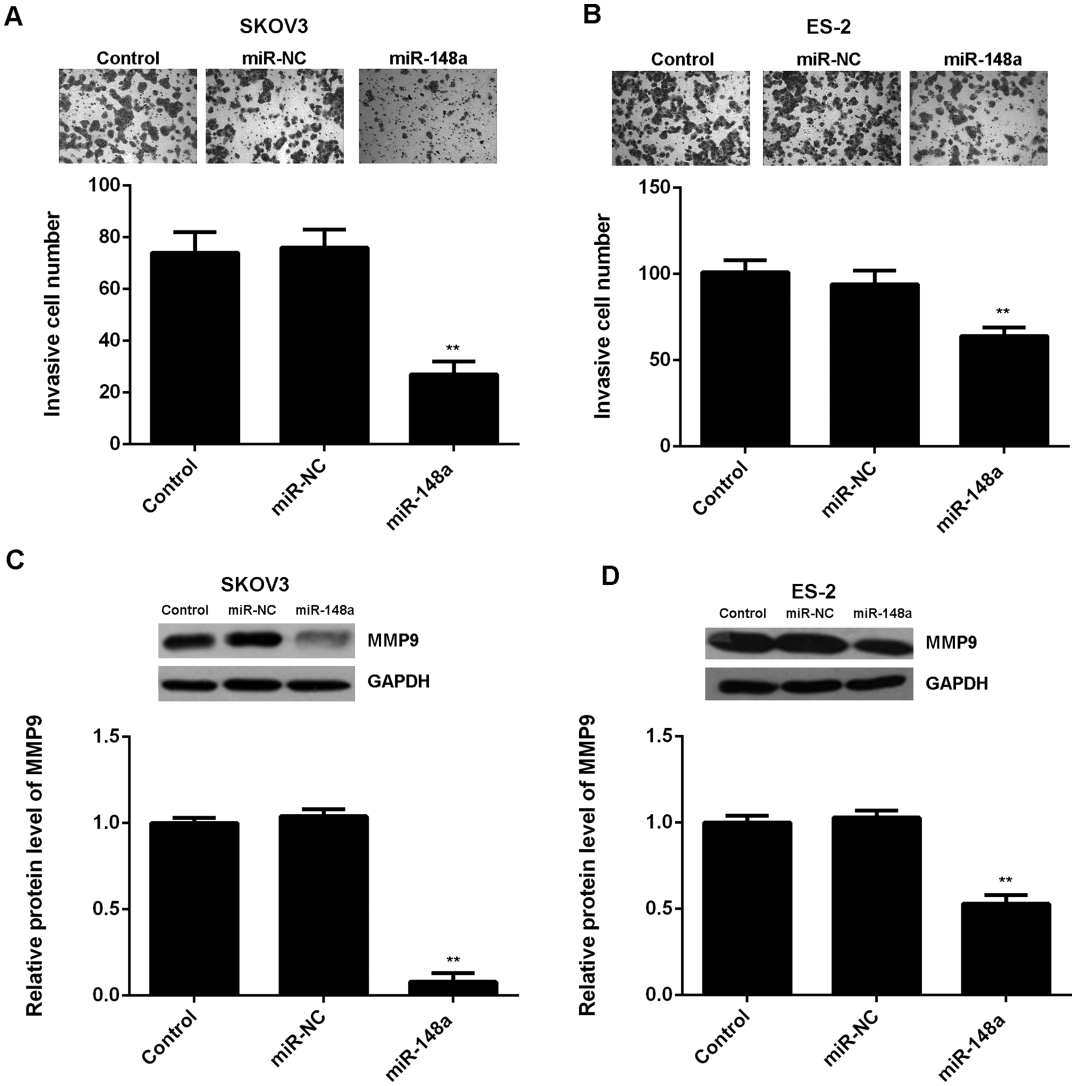

Figure 3. (A, B) Transwell assay was conducted to examine the cell invasion capacity of OC SKOV3 and ES-2 cells transfected with scramble miRNA (miR-NC) or miR-148a mimics. (C, D) Western blot was used to examine the protein expression of MMP9. Nontransfected SKOV3 or ES-2 cells were used as Control, respectively. $^{**}p < 0.01$ versus Control.

the proliferation of SKOV3 and ES-2 cells (Fig. 2B and C).

We then investigated the role of miR-148a in the regulation of OC cell invasion. As shown in Figure 3A and B, miR-148a overexpression significantly suppressed the invasion of SKOV3 and ES-2 cells compared with the control group. We further determined the protein expression of MMP9 in each group and found that their protein levels were significantly decreased after overexpression of miR-148a in OC cells (Fig. 3C and D). Taken together, these findings demonstrate that miR-148a has an inhibitory effect on OC cell proliferation and invasion.

TGIF2 Is a Direct Target Gene of miR-148a in SKOV3 and ES-2 Cells

TGIF2 was regarded as having an active role in the invasion, proliferation, and metastasis of cancer cells (15,20,21). Furthermore, it may play an important role in the development and/or progression of some ovarian tumors (18). Bioinformatics analysis predicted that TGIF2 was a direct target gene of miR-148 (Fig. 4A). To verify this prediction, we first detected the effects of miR-148a upregulation on the TGIF2 expression in SKOV3 and ES-2 cells. Our data showed that overexpression of miR-148a decreased the protein levels of TGIF2 in OC cells, while transfection with miR-NC did not affect the protein expression of TGIF2 (Fig. 4B). These results indicated that miR-148a negatively regulated the protein expression of TGIF2 in OC cells.

To further clarify whether TGIF2 was a direct target of miR-148a, the wild- or mutant-type 3′-UTR of TGIF2 was inserted into the psiCHECK-2 vector, respectively (Fig. 4C). OC cells were transfected with the psiCHECK-2-TGIF2-3′-UTR or psiCHECK-2-mutant TGIF2-3′-UTR vector, with or without miR-148a mimics, respectively. The luciferase reporter assay was then performed. The results showed that the luciferase activity was obviously reduced in OC cells cotransfected with the wild-type TGIF2-3′UTR vector and miR-148a mimics (Fig. 4D and E). However, the activity of luciferase had no difference in the other groups (Fig. 4D and E). These results indicated that TGIF2 is a direct target of miR-148a in OC cells.

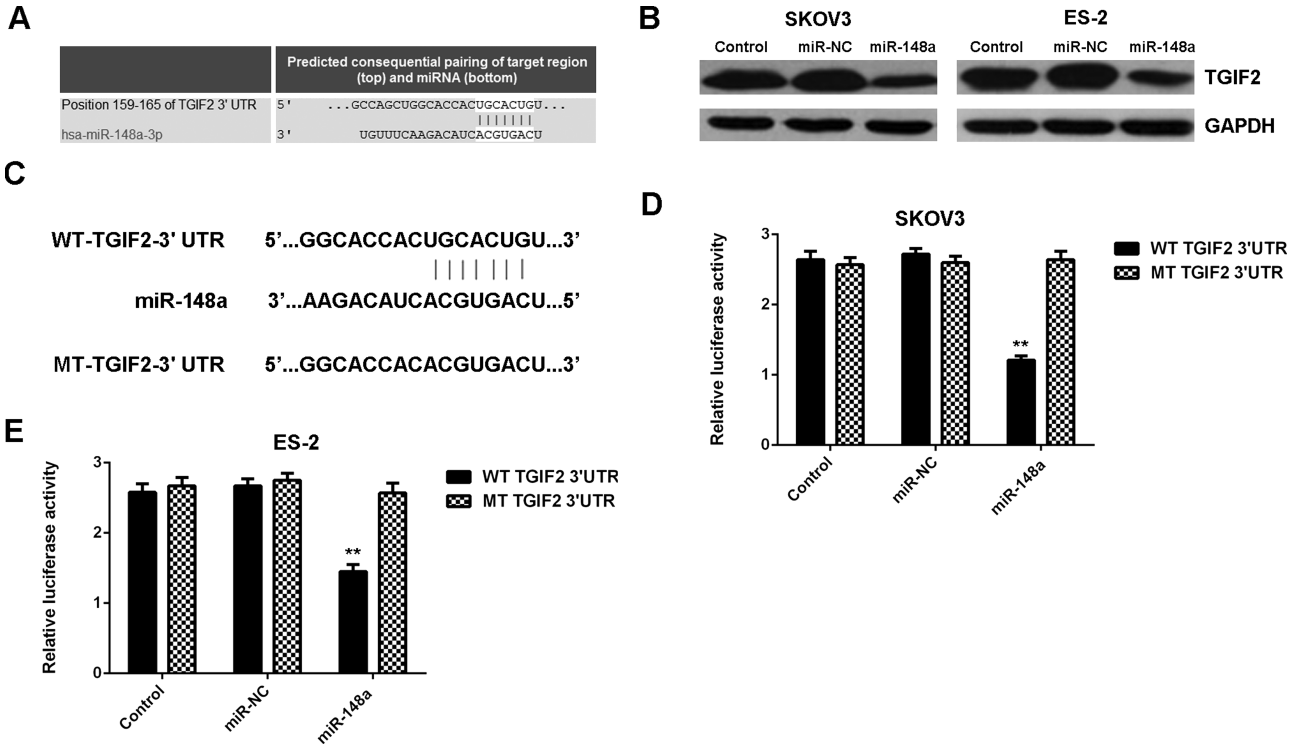

Figure 4. (A) Targetscan data indicated that TGIF2 was a target gene of miR-148a. (B) Western blot was used to examine the protein expression of TGIF2 in OC SKOV3 and ES-2 cells transfected with scramble miRNA (miR-NC) or miR-148a mimics. Nontransfected SKOV3 or ES-2 cells were used as Control, respectively. (C) The wild-type (WT) or mutant-type (MT) 3′-UTR of TGIF2 was inserted into the psiCHECK-2 vector, respectively. (D, E) The luciferase activity was obviously reduced in SKOV3 and ES-2 cells cotransfected with the WT TGIF2-3′-UTR vector and miR-148a mimics (C, D). However, the activity of luciferase had no difference in the other groups. $^{**}p < 0.01$ versus Control.

TGIF2 Is Involved in the miR-148a-Mediated Inhibition of OC Cell Proliferation and Invasion

The role of TGIF2 in the miR-148a-mediated inhibition of OC cell proliferation and invasion was further investigated. SKOV3 and ES-2 cells were transfected with miR-148a mimics or cotransfected with miR-148a mimics and TGIF2 plasmid, respectively. Subsequently, the protein level of TGIF2 was detected. As indicated in Figure 5A, the protein expression of TGIF2 was higher in the group of cells cotransfected with miR-148a mimics and TGIF2 plasmid, compared with that transfected only with miR-148a mimics. Furthermore, we found that TGIF2 overexpression led to a significant increase in OC cell proliferation and invasion, when compared to the control group that only transfected with miR-148a mimics (Fig. 5B–E). Therefore, our findings suggest that the suppressive effect of miR-148a on OC cell proliferation and invasion may be via inhibition of TGIF2.

Inverse Correlation Between the miR-148a and TDIF2 Expression in OC Tissues

The expression of TGIF2 was further studied in OC tissues. Our data indicated that the mRNA levels of TGIF2 were obviously upregulated in OC tissues compared to their matched normal adjacent tissues (Fig. 6A). Moreover, a significantly inverse correlation was observed between the miR-148a and TGIF2 expression in OC tissues (Fig. 6B), suggesting that the increased TGIF2 expression may be due to the downregulation of miR-148a in OC tissues.

DISCUSSION

The results of our study showed a downregulation of miR-148a in OC tissues and cell lines, when compared, respectively, with the adjacent nontumor tissues and normal ovarian epithelial cells. Overexpression of miR-148a suppressed OC cell proliferation and invasion, and decreased the protein levels of TGIF2 in OC cells. Furthermore, we identified TGIF2 as a direct target of miR-148a. Moreover, our data suggested that miR-148a inhibited OC cell proliferation and invasion partly via directly targeting TGIF2. Finally, we showed that TGIF2 displayed an increased expression in OC tissues when compared with that in adjacent tissues and a reverse correlation with miR-148a expression in OC tissues.

miRs, a group of conserved, small, noncoding RNAs, can modulate the target mRNA translation and stability (7). Factually, the effects of miRs have been demonstrated in various cancers (11,22,23). It has been reported that miR-504 inhibits cell proliferation and promotes apoptosis by targeting FOXP1 in human glioma (24). Zhang et al. reported that miR-506 suppressed the proliferation and epithelial–mesenchymal transition of cervical cancer cells by repressing forkhead box Q1 (25). Kang et al. provided evidence that miR-362-3p and miR-329 performed a tumor-suppressive function, and modulation of miR-362-3p/miR-329 might bring a novel therapeutic strategy against breast cancer (23). miR-148a plays an important regulatory role in the OC progression. Wen et al. found that the expression of miR-148a was diminished in OC tissues and cell lines, and found that upregulated miR-148a could inhibit OC cell invasion (12). Zhao et al. reported that overexpression of miRNA-148a inhibited the proliferation of OC cells (26). Consequently, miR-148a may play an important part in suppressing the OC progression.

Researchers are focusing on the effects of miR-148a on proliferation or invasion in cancer cells (27,28). The research of Jiang et al. indicated that miR-148a could suppress the invasion of breast cancer cells by targeting WNT-1 and inhibiting Wnt/β-catenin signaling pathway (27). Tsai et al. demonstrated that the overexpression of miRNA-148a inhibited proliferation and migration but not invasion in colon cancer cells (28). In our study, we found that overexpression of miR-148a repressed proliferation and invasion in two OC cell lines. The suppressive effect of miR-148a on cancer cell proliferation seems universal. The reason that Tsai et al. did not observe the inhibitory effect of miR-148a on the invasion in colon cancer cells is probably the difference between cancer types.

Our data indicated that the miR-148a-mediated suppression of proliferation and invasion in OC cells was mediated by TGIF2, a novel identified target gene of miR-148a in OC. TGIF2 plays an oncogenic role in the development and/or progression of OC (18,29). However, the relationship between TGIF2 and miR-148a has never been previously reported in OC. In skin cancer, TGIF2 was also found to be a target gene of miR-148a, and its expression was negatively regulated by miR-148a (17). In our study, we found that the expression level of TGIF2 was increased in OC cell lines and tissues, compared with normal ovarian cells and adjacent tissues, respectively, and overexpression of miR-148a decreased the expression of TGIF2 in OC cells. Subsequently, when miR-148a mimics and TGIF2 ORF plasmid were cotransfected into OC cells, we found that the ability of proliferation and invasion of OC cells was significantly increased, when compared with those of the cells transfected only with miR-148a mimics. These findings suggest that the suppressive effect of miR-148a on OC cell proliferation and invasion is probably via directly targeting TGIF2.

In summary, our study provided experimental evidence that miR-148a suppressed proliferation and invasion of OC cells via directly repressing the expression of TGIF2. Further investigation should focus on the effect of miR-148a/TGIF2 signaling in OC in vivo.

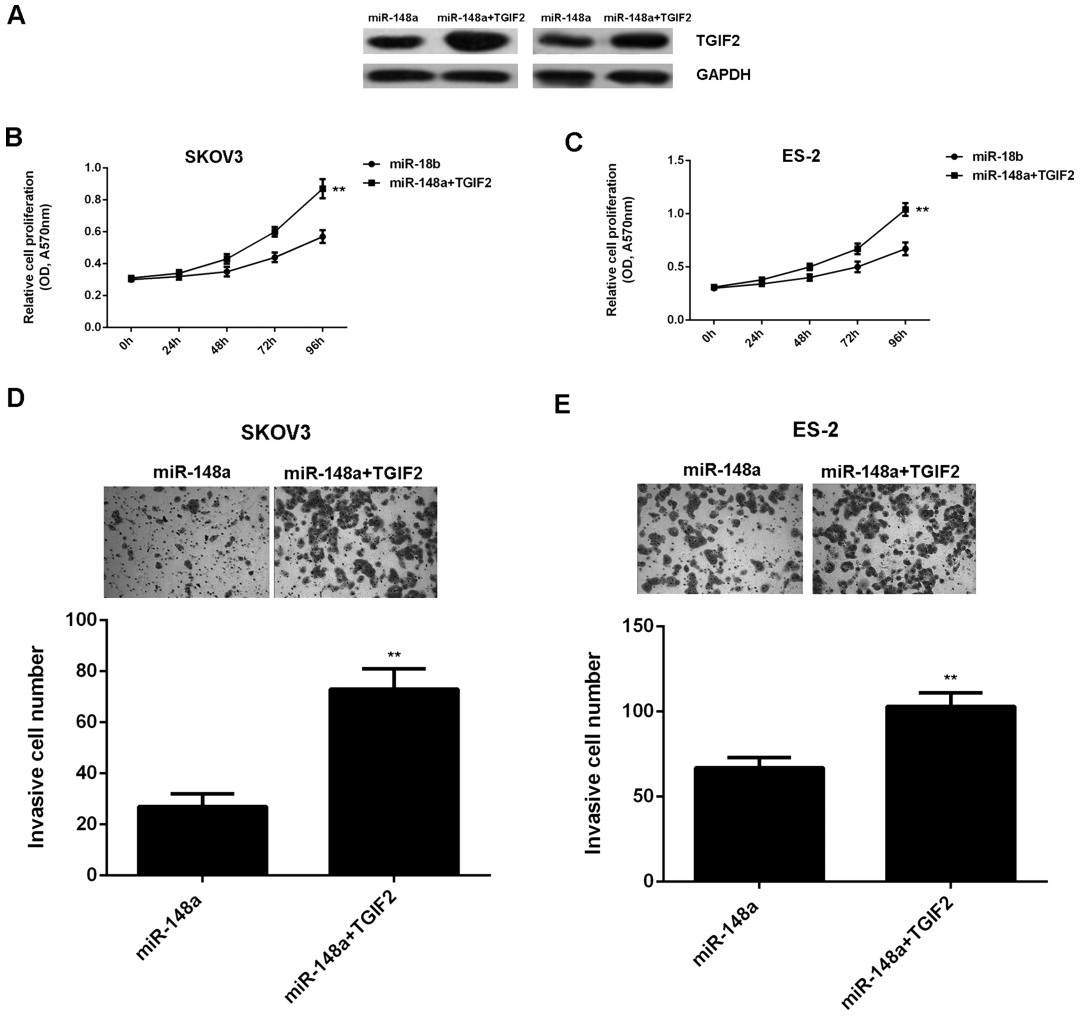

Figure 5. (A) Western blot was used to examine the protein expression of TGIF2 in OC SKOV3 and ES-2 cells transfected with miR-148a mimics or cotransfected with miR-148a mimics and TGIF2 plasmid. (B, C) MTT assay was used to determine cell proliferation. (D, E) Transwell assay was used to examine cell invasion capacity. $^{**}p<0.01$ versus miR-148a.

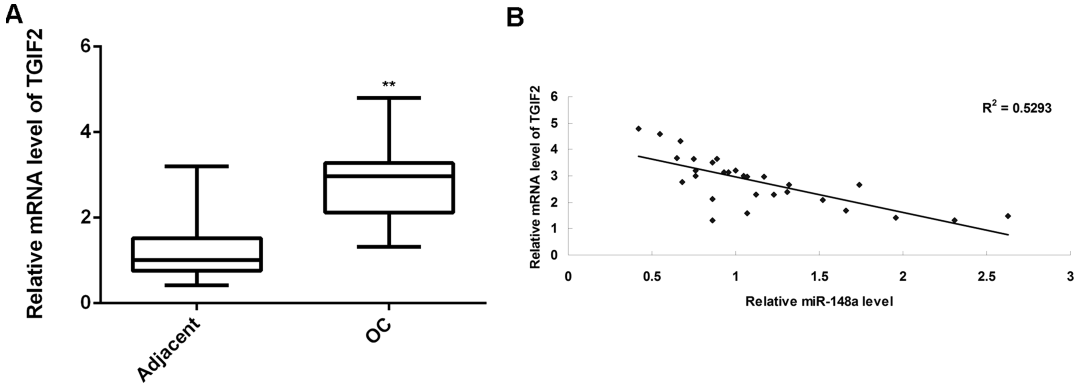

Figure 6. (A) Real-time PCR was used to examine the mRNA expression of TGIF2 in OC tissues compared to matched adjacent nontumor tissues. $^{**}p<0.01$ versus Adjacent. (B) Inverse correlation was observed between the miR-148a and TGIF2 expression in OC tissues.

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