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Original Article

Knockdown of E2F4 suppresses the growth of ovarian cancer cells through the cell cycle pathway

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Abstract: Ovarian cancer remains one of the major causes of death from gynecologic cancer in developed countries. The E2F family has been shown to have a central role in the control of cell proliferation, differentiation, and cell cycle progression in various types of cancer. Despite advances in cancer research, the carcinogenic role of E2F transcription factor 4 (E2F4) in ovarian cancer remains unclear. In this study, we investigated the underlying molecular mechanism of E2F4 in human ovarian cancer cells (OCC). E2F4 expression was demonstrated by quantitative real time polymerase chain reaction (qRT-PCR) in OCC. The alterations of expression values were determined using $2^{(-\Delta\Delta Ct)}$ method. The effects of suppressing E2F4 on cell proliferation, migration, and differentiation were evaluated by cell proliferation assay, colony formation assay and wound healing assay in vitro. Overexpression of E2F4 was found at both mRNA and protein levels in OCC. Small interfering RNA was used to suppress E2F4 expression. Depletion of E2F4 inhibited cell proliferation and suppressed the cell migration and colony formation ability compared to controls. The expression of cell cycle machinery including cyclin A, cyclin D and cyclin dependent kinase 2 (CDK2) was increased after E2F4 knockdown. E2F4 modulates ovarian cancer cell proliferation and migration through cell cycle components including cyclin A, cyclin D, and CDK2. Our findings indicate that E2F4 may serve as a valuable candidate and therapeutic target for ovarian cancer treatment in regard to cell cycle control.

Keywords: cell cycle, cell proliferation, E2F4 transcription factor, epithelial ovarian cancer

Introduction

Ovarian cancer presents the highest mortality rate among gynecological cancers, and is the fifth leading cause of cancer death among women in United States [1]. Although there have been significant improvements in diagnostics, surgery, and chemotherapy, the 5-year overall survival rate of ovarian cancer remains no more than 50%, dropping to 29% in advanced stage disease [2]. The underlying mechanisms of the development and progression of ovarian cancer are numerous and complex [3]. A proper knowledge of mechanism of occurrence and progression in ovarian cancer is crucialto improve the prognosis.

The E2F family is a group of transcription factors that play decisive roles in the regulation of cell proliferation and cell cycle progression through the transcriptional activation of target genes in higher eukaryotes [4]. The family is commonly divided by function into two classes: transcriptional activators and repressors. The former includes E2F1, E2F2, and E2F3a and the latter includes E2F3b, E2F4, E2F5, E2F6, E2F7, and E2F8. Moreover, E2F family genes regulate DNA repair, cell division, differentiation, and apoptosis [4].

Aberrant expression of several E2F family transcription factors was notably found in numerous human malignancies including ovarian, breast, bladder, and prostate cancer, as well as lung adenocarcinoma and colon cancer [5-10]. The E2F1 expression was increased in ovarian carcinoma, and it was believed that E2F1 stimulated the development of ovarian cancer [11].

Expression of E2F3a and E2F3b mRNA was also significantly higher in tumors than normal ovarian tissue [12]. Furthermore, E2F3 [12] and E2F5 [13] were evaluated as important targets to enhance the clinical outcome of ovarian cancer, including their roles in improving diagnostics. In contrary, little was known as to how E2F4 is regulated in ovarian cancer cells. Moreover, the exact underlying mechanism of E2F4 as oncogene needs further investigation.

The objective of this study was to investigate the molecular function of E2F4 and the mechanism modulating E2F4 through the cell cycle pathway in ovarian cancer cells.

Materials and methods

Cell lines and cell culture

The human ovarian cancer cell line A2780 was obtained from the European Collection of Cell Cultures (ECACC, Sigma-Aldrich, USA) and cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (pen/strep, Gibco, USA) in a 37°C incubator. The ovarian cancer cell line OVCA433 was obtained by the Korea Gynecologic Cancer Bank through the Bio & Medical Technology Development Program of the Minister of Science, Information and Communication Technology & Future Planning (MSIP) and cultured in Dulbecco's modified Eagle's medium (Gibco, USA).

Small interfering RNA (siRNA) transfection

E2F4 knockdown of the A2780 and OVCA433 cell line was performed using the E2F4 small interfering RNA (siRNA) duplex, purchased from Sigma-Aldrich (Cat. NM_001950). Transfection conditions were performed in 6-well, 12-well, or 96-well cell culture plates (Nunc, Denmark). Ovarian cancer cells were harvested and 1×10^6 (6-well), 1×10^3 (12-well) or 1×10^4 (96-well) cells were seeded to each well. These short RNA duplexes were transfected into cells with Lipofectamine 3000 transfection reagent (Invitrogen, USA) in Opti-MEM I Reduced Serum medium (Gibco, USA). A Lipofectamine 3000 reagent and siRNA were diluted in Opti-MEM medium at room temperature, then mixed with the diluted RNAi duplex with diluted Lipofectamine 3000. The siRNA-lipid complex was incubated for 10-15 minutes under the same conditions. siRNA-lipid complex was added to cells. The efficiency of siRNA and expression level of mRNA was observed 48-72 h post-transfection by qRT-PCR.

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cell lines using TRIzol® reagent (Invitrogen, USA). Complementary DNA was synthesized from 1 μg RNA and was reverse transcribed into first-strand cDNA by using RevertAid H Minus First Strand cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. The qRT-PCR was carried out in 96-well blocks with the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) using Power SYBR® Green Master Mix (Applied Biosystems, USA) in a final volume of 20 μl. All qRT-PCR was performed using SYBR Green, conducted at 95°C for 2 min followed by 40 cycles of 5 sec at 95°C, and 60°C for 15 sec. The expression of the genes was normalized to GAPDH. The primer sequences used for qRT-PCR were as follows: GAPDH: 5'-ACCCACTCCTCCACCTTTGA-3' (sense) and 5'-CTGTTGCTGTAGCCAAATTCGT-3' (antisense) E2F4: 5'-ATAGTCCTCAGCTCACTCCC-3' (sense) and 5'-GTCCTTGCTATCAGTCCCAG-3' (antisense) CDK2: 5'-CATTCCTCTTCCCCTCATCA-3' (sense) and 5'-CAGGGACTCCAAAAGCTCTG-3' (antisense) CDK6: 5'-GGATAAAGTTCCAGAGCCTGGAG-3' (sense) and 5'-CGATGCACTACTCGGTGTGAA-3' (antisense) Cyclin A1: 5'-ACCCCAAGAGTGGAGTTGTG-3' (sense) and 5'-GGAAGGCATTTTCTGATCCA-3' Cyclin D1: 5'-AACTACCTGGACCGCTTCCT-3' (sense) and 5'-CCACTTGAGCTTGTTCACCA-3' (antisense) Cyclin E1: 5'-CAGATTGCAGAGCTGTTGGA-3' (sense) and 5'-TCCCCGTCTCCCTTATAACC-3' (antisense). The relative changes in gene expression were determined using the $2^{(-\Delta\Delta Ct)}$ method and the mean fold changes of the three replicates were calculated.

Cell proliferation assay

Cell viability was established using the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan) following the manufacturer's protocol. A2780 and OVCA433 cells 48 hours post-transfection with negative control or E2F4-siRNA were seeded in a 96-well plate. After an additional 24, 48, 72, or 96 hours, the cells were added to 10 uL

of CCK-8 solution to a plate and then incubated at 37°C for 1 hour. The optical densities were measured using a microplate reader at 450 nm. The experiments for cell proliferation were quantified in triplicate.

Wound healing assay

Cells were seeded in 6-well culture plates (1 × 10^6 cells/well) with medium and allowed to grow to 90% confluency. Artificial wounds were made by scraping the monolayer with a 200 μL sterile pipette tip. The cells were washed with Phosphate-buffered saline (PBS) to remove the detached cells and the well was replenished with fresh medium. The wound healing was monitored at 0, 24, and 48 h under a microscope (Olympus, Japan). Each experiment was repeated three times. Data are presented as average ± SD of three independent experiments.

Colony formation assay

Cells were transfected with negative control or E2F4-siRNA for 2 days in 12-well plates at a low density (1 × 10^3 cells per well) at 37°C. The colonies were washed twice with PBS to remove cell debris, fixed with 4% (v/v) paraformaldehyde for 20 min, and stained with 0.1% crystal violet (Sigma-Aldrich, USA) for 10 min. The total number of colonies was counted visually by microscopic observation. Each experiment was performed three times.

Western blot assay

Cells were harvested for total protein using RIPA Lysis and Extraction buffer (Thermo Fisher Scientific, Inc., USA) and concentrations of protein were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., USA). Cell lysates containing equal amount of proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (SDS-PAGE) and then transferred onto polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 h and incubated with the primary antibody solution overnight at 4°C. Horseradish peroxidase-linked secondary antibody (1:5,000) was used and the membrane was incubated for 1 h at room temperature. The protein bands were detected by enhanced chemiluminescence solution (ECL), followed by detection with medical x-ray film (Fuji Film, Japan). All experiments were repeated three times.

Statistical analysis

Differences in measured data were assessed using Student's t-test using GraphPad Prism software (GraphPad, USA). Significant differences in multiple groups were analyzed by one-way ANOVA followed by Tukey's test or two-way ANOVA. A P value of <0.05 was considered significant.

Results

E2F4 mRNA and protein were overexpressed in ovarian cancer cell lines

In order to evaluate the biologic function of E2F4 in ovarian cancer cells (OCC), the transcript was analyzed in ovarian cancer cell lines. mRNA and protein expression level of E2F4 were investigated by qRT-PCR and western blot in four ovarian cancer cell lines and a human keratinocyte cell line. E2F4 gene expression was found to be higher in ovarian cancer cells. The mRNA showed more than eight-fold increase in transcription level in the A2780 and OVCA433 cell lines (**Figure 1A**). Western blot analysis revealed that the expression of E2F4 protein was higher in ovarian cancer cell lines than HaCaT cells. Both E2F4 mRNA and protein were increased in OCC (**Figure 1B**). These data showed that E2F4 was overexpressed in ovarian cancer cells. To further assess the function of E2F4 on cell proliferation in ovarian cancer, A2780 and OVCA433 were selected to reduce E2F4 expression by transfection with siRNA-E2F4. The expression of E2F4 in cells was validated by qRT-PCR. Compared with the controls, transfected cancer cells with siRNA markedly reduced the mRNA level (**Figure 1C**, **1D**).

Knockdown of E2F4 inhibited cell proliferation of OCC

The cell proliferation assay using Cell Counting Kit-8 (CCK-8) was conducted to investigate the effect of E2F4 on cell viability and growth of OCC. Negative control (siNC) or siE2F4 A2780 and OVCA433 cells were transfected with E2F4 siRNA or negative control. Here, OCC showed a lower proliferation rate than that in the control

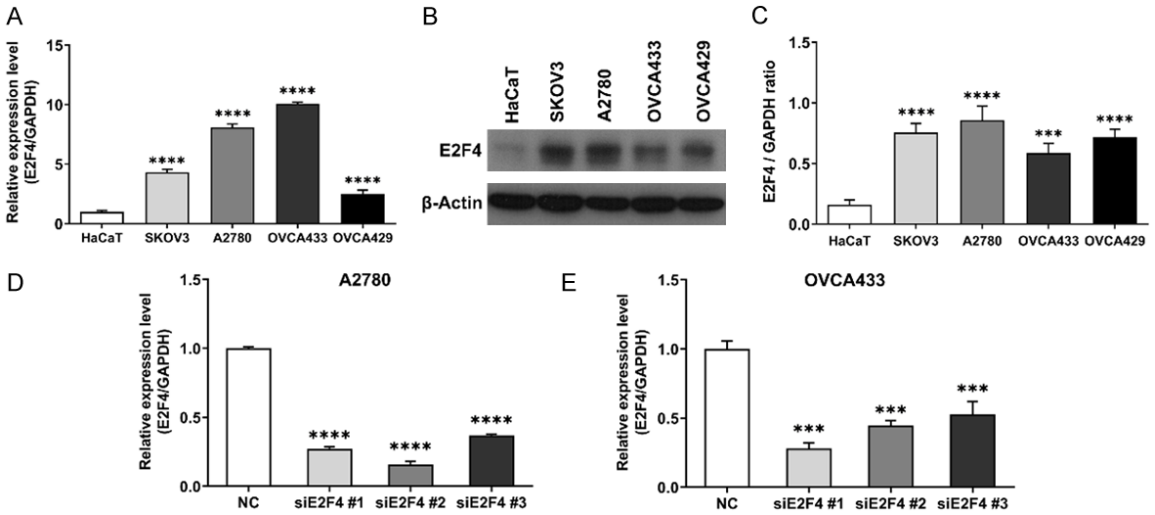

Figure 1. E2F4 is elevated in ovarian cancer cell lines. (A) E2F4 expression level in ovarian cancer cells was demonstrated by qRT-PCR. (B) E2F4 protein expression in a series of human OC cell lines was analyzed by western blot. Immunoblot analysis was performed on whole cell lysate for E2F4 expression and the relative protein level of E2F4 represented the protein level normalized to β-actin. (C) Quantitation of the relative gray scale of the expression of E2F4 in HaCaT, SKOV3, A2780, OVCA433, and OVCA429 cells. Data are presented as the mean ± SD (one-way ANOVA with Bonferroni post hoc multiple comparison test, ***P<0.001, ****P<0.0001; D), (E) The expression of E2F4 in A2780 and OVCA433 cells after transfection with E2F4-siRNA or negative control was analyzed by qRT-PCR. GAPDH was used for normalization.

group when E2F4 was knocked down (**Figure 2A**). The colony formation assay demonstrated that E2F4 knockdown diminished the formation of colonies in both A2780 and OVCA433 cell lines in vitro (**Figure 2B**, **2C**). Thus, the colony formation rate was significantly decreased in E2F4 knockdown cells compared with negative controls. The CCK-8 assay and colony formation assay indicated that the knockdown of E2F4 suppressed ovarian cancer cell proliferation.

E2F4 knockdown suppressed OC cell migration in vitro

To estimate the effects of E2F4 on migratory behavior of ovarian cancer, wound healing assay was performed to demonstrate the migration ability of A2780 and OVCA433 cells transfected by control-siRNA or E2F4-siRNA. Images showed that E2F4 knockdown decreased the rate of wound healing area at 48 h after the scratch compared to controls in OVCA433. Similar results were acquired in the A2780 cells as well (**Figure 3A**). The percentage change of wound width was higher in the depletion of E2F4 than controls (**Figure 3B**). The results suggested that E2F4 knockdown suppressed cell migration in ovarian cancer cells *in vitro*.

E2F4 regulated expression of cell cycle regulatory proteins

In order to examine whether E2F4 regulates cell proliferation by the cell cycle, the expression of cell cycle regulator was analyzed by qRT-PCR. Knockdown of E2F4 led to a marked upregulation of cyclin A1, D1, E1, CDK2 and CDK6, which are required for cell-cycle progression through S to M phases (**Figure 4A**). Whole-cell lysates were prepared following treatment with siE2F4 or control for 48 h, and the protein expression level of CDK2, CDK6, Cyclin A1, D1, and E1 was determined by western blot analysis (**Figure 4B**). Knockdown of E2F4 increased the expression of CDK2, CDK6, cyclin A1, D1, and E1. These results suggested that transcriptional upregulation in E2F4 may lead to cell cycle regulation and result in growth inhibition.

Discussion

In this study, the expression level of E2F4 was elevated in ovarian cancer cells compared to noncancerous cells. The depletion of E2F4 inhibited cell proliferation, migration, and colony formation in ovarian cancer cells. Moreover, E2F4 knockdown modulated ovarian cancer cell cycle components including cyclin D1, CDK2, and CDK6. Our study indicated that

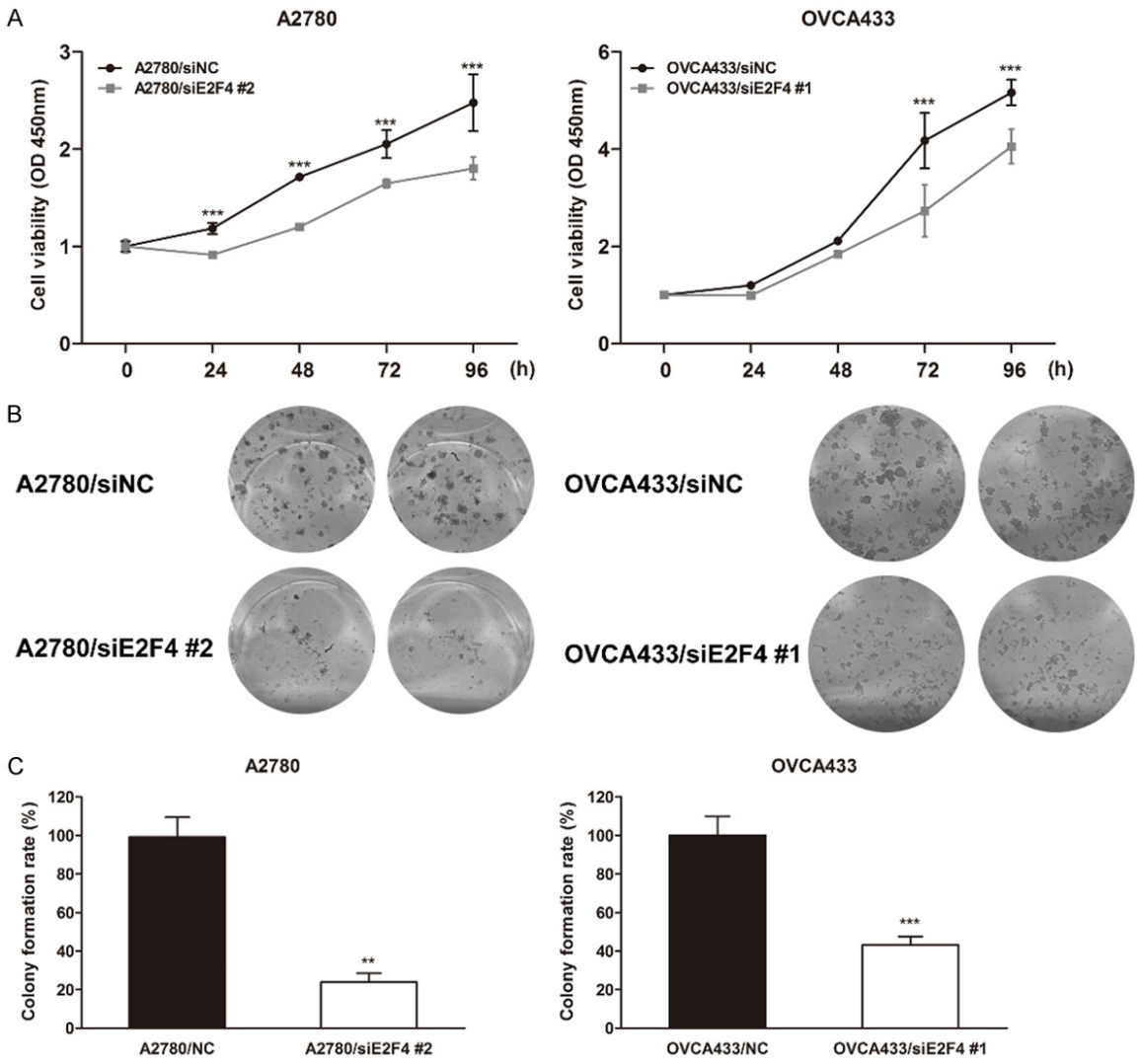

Figure 2. Knockdown of E2F4 in ovarian cancer cells inhibits cell proliferation. A. Determination of cell viability in ovarian cancer cell lines by CCK-8 kit. B. Colony formation assay showed that knockdown of E2F4 decreased cell proliferation in A2780 and OVCA433. Negative control or E2F4 knockdown cells were subjected to clonogenic assay. The colonies, observed by bright-field microscopy at day 12 of growth are also shown. C. Representative images for colony growth are shown. All values are expressed as mean ± SD. **P<0.01, ***P<0.001.

E2F4 has an oncogenic function by controlling cell proliferation, migration, and cell cycle regulation in ovarian cancer, which is compatible with previous studies in various cancers [14-16]. The knockdown of E2F4 showed overexpression of cell cycle regulatory cyclins and cyclin-dependent kinases. E2F4 facilitated the ovarian cancer cell cycle by modulating cell cycle components such as cyclin D1, CDK2, and CDK6.

E2F4, a member of the E2F family of transcription factors, is known to have upregulated gene expression in colorectal cancer, acute myeloid leukemia, breast cancer, prostate cancer, and gastric cancer [9, 17-20]. In addition, it exercises biologic functions in various processes such as the cell cycle, differentiation, proliferation, and apoptosis [21-24]. Previous studies have demonstrated that E2F4 may act as an oncogene and showed higher expression in breast and gastric cancer than the adjacent tissues [25, 26].

The E2 factor (E2F) family of transcription factors is composed of eight genes that are related to cell cycle progression and apoptosis regulating the transcription of DNA replication fac-

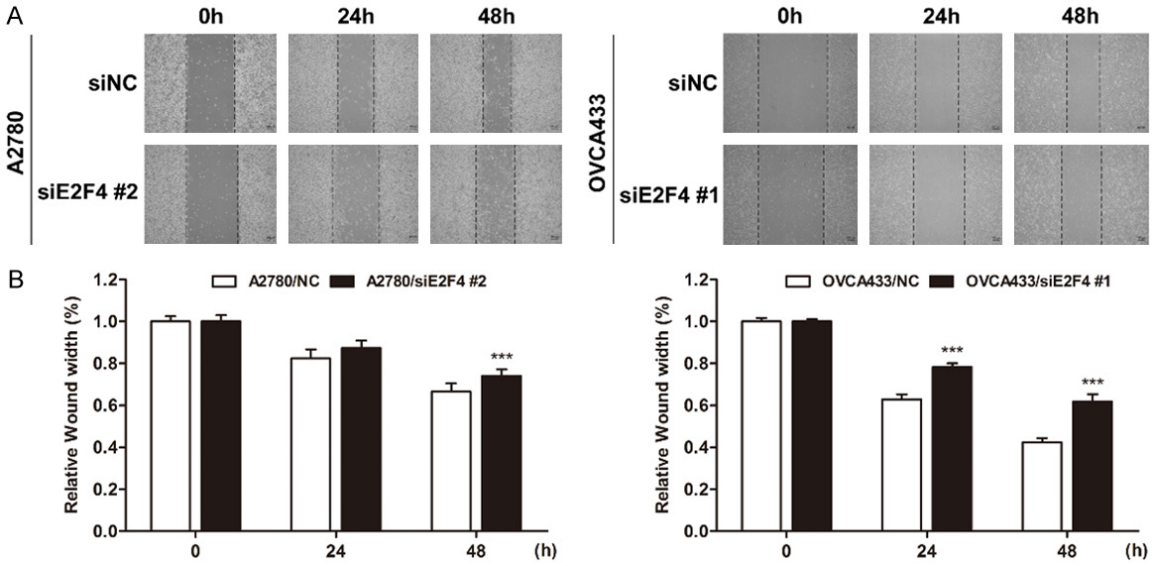

Figure 3. E2F4 knockdown suppresses cell migration ability of ovarian cancer cells. A. Wound-healing assay was performed in control siRNA or siE2F4 cells. Images were captured to display the process of gap closure at 0, 24 h, and 48 h. The area was measured by ImageJ software to evaluate the scratch by quantification of the areas in arbitrary units for three independent experiments performed in triplicate. Scale bar, 200 μm. B. Quantification of wound healing assay showed the cell migration ability of A2780 and OVCA433 cells. The results are from three independent experiments. Bars represent the mean ± SD (n=3). *P<0.05, **P<0.01.

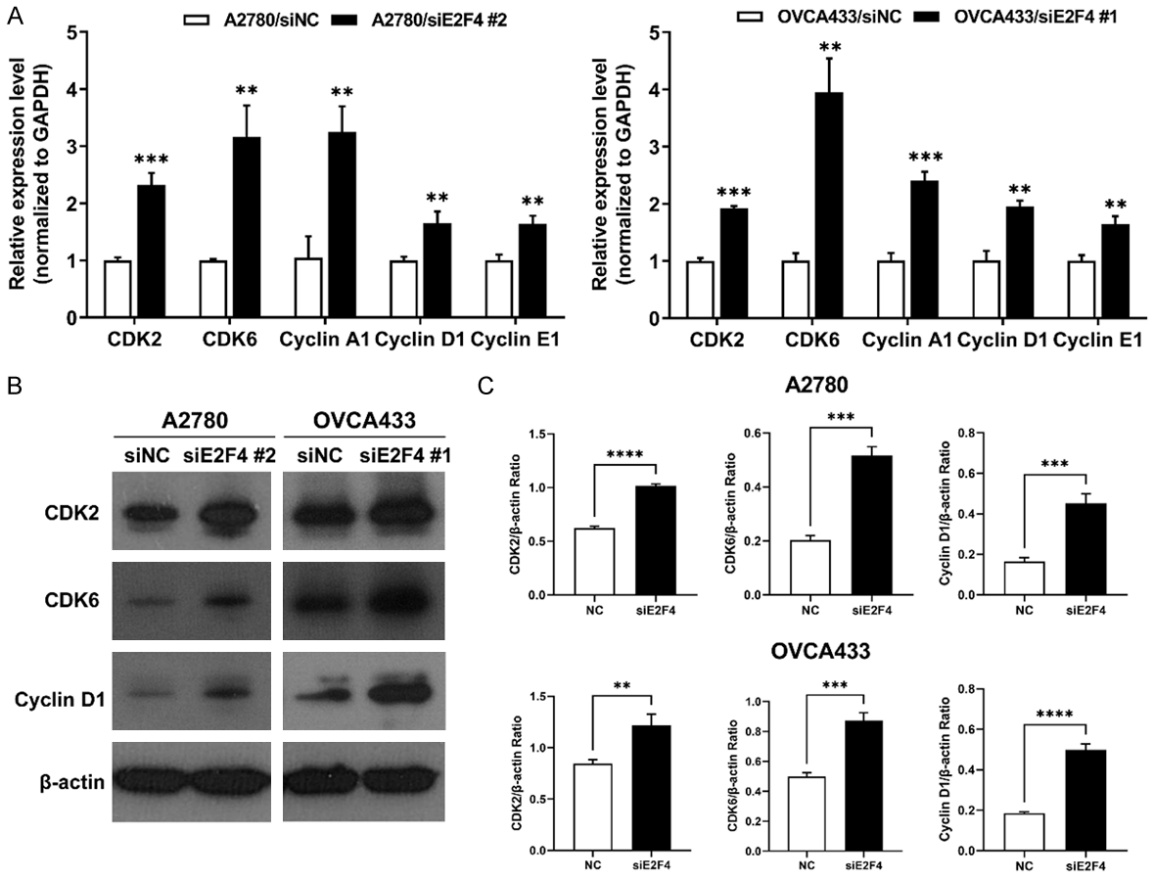

Figure 4. E2F4 knockdown induced expression of cell cycle markers in ovarian cancer cells. A. CDK2, CDK6, Cyclin A1, Cyclin D1, and Cyclin E1 mRNA expression pattern in A2780 and OVCA433 cells assessed by quantitative RT-

PCR. B. Western blot analysis of cell cycle regulators to evaluate the E2F4 knockdown effect of CDK2, CDK6, and Cyclin D1 in A2780 and OVCA433 cells treated with negative control or siRNA. β-actin levels are shown as loading controls. C. Gray-scale analysis of the level of CDK2, CDK6, and Cyclin D1. All data are presented as mean ± SD. **P<0.01, ***P<0.001, ****P<0.0001.

tors [27, 28]. E2F1-3, which are classified as activator E2Fs, are known to regulate the transcription of target proteins during the transition from G1 to S phase in the cell cycle [29, 30]. Previous research has shown increased expression of E2F1 in epithelial ovarian cancer patients and suggested its association with higher grade tumors and unfavorable overall survival [5]. In the case of E2F2, it was reported to regulate cell progression in areas such as cell proliferation, cell cycle, and tumorigenesis in ovarian cancer [31]. A recent study revealed that overexpression of E2F3 contributed to cell proliferation and invasive tumor growth in urinary bladder cancer [32]. Also, E2F3 gene expression was sufficient in anticipating cancer recurrences and overall survival of estrogen receptor-positive breast cancer patients [10].

As a member of the E2F group, E2F4 was found to generally act as a central regulator to promote E2F4 and Rb protein in the cell cycle pathway [33]. In breast cancer, the expression level of E2F4 was lower in primary and metastatic tissues compared to corresponding normal samples. Overexpression of E2F4 worsened survival outcomes of patients, indicating that E2F4 may serve as a prognostic biomarker and may be a therapeutic target for breast cancer. Moreover, study findings have shown that E2F4 is a modulator of human acute myeloid leukemia cell proliferation and differentiation by regulation of the MAPK signaling pathway, suggesting a crucial role in carcinogenesis [18].

To the best of our knowledge, this is the first study to explore the molecular mechanism of E2F4 in ovarian cancer cell lines, suggesting a role of E2F4 as a promoter during the occurrence of ovarian cancer. The knockdown of E2F4 induced the aberrant expression of ovarian cancer cell cycle regulators. Therefore, E2F4 may possess an oncogenic function in ovarian cancer cells. The limitation of this study is that the experiments were performed only *in vitro* using ovarian cancer cell lines. Hence, further research using ovarian cancer tissues is necessary to validate the findings of this study.

In conclusion, E2F4 modulates ovarian cancer cell proliferation and migration by controlling cell cycle components including cyclin A, cyclin D, and CDK2. Our findings suggest that E2F4 may represent a valuable therapeutic target in ovarian cancer therapy.

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Disclosure of conflict of interest

None.

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