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# Kinesin Family Member C1 (KIFC1) Regulated by Centrosome Protein E (CENPE) Promotes Proliferation, Migration, and Epithelial-Mesenchymal Transition of Ovarian Cancer

Authors' Contribution:

Study Design A

Data Collection B

Statistical Analysis C

Data Interpretation D

Manuscript Preparation E

Literature Search F

Funds Collection G

**ABE** Jiangning Li  
**AE** Haidan Diao  
**BF** Xin Guan  
**CD** Xiaofang Tian

Department of Gynecology, The Third People's Hospital of Dalian, Dalian, Liaoning, P.R. China

**Corresponding Author:** Haidan Diao, e-mail: [haidanhh@126.com](mailto:haidanhh@126.com)  
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**Background:** Centrosome amplification is recognized as a hallmark of cancer. Kinesin family member C1 (KIFC1), a centrosome-clustering molecule, is essential for the viability of extra centrosome-bearing cancer cells and may be the basis for the progression of ovarian cancer. However, its biological function and mechanism in ovarian cancer have not yet been studied.

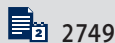
**Material/Methods:** Quantitative reverse-transcription polymerase chain reaction was performed to detect the levels of KIFC1 and centrosome protein E (CENPE). Further, cell viability was analyzed with CCK-8 assay, and immunofluorescence was used to measure the expression of Ki67 and PCNA. Cell migration was analyzed with wound healing and transwell assays. Western blot analysis was performed to measure the expression of proteins in ovarian cancer cells. The relationship between KIFC1 and CENPE was investigated by performing co-immunoprecipitation.

**Results:** KIFC1 was upregulated in ovarian cancer cells, especially in SKOV3 cells. Additionally, we found that KIFC1 silencing in SKOV3 cells inhibited cell proliferation and downregulated the expression of Ki67 and PCNA. Further, the knockdown of KIFC1 suppressed cell migration and epithelial-mesenchymal transition (EMT) and regulated the expression of matrix metalloproteinase (MMP)2, MMP9, E-cadherin, N-cadherin, Snail, and ZEB1. Next, we found that KIFC1 bound to and positively regulated CENPE, a tumor promoter in certain human cancers. All the suppressive effects triggered by KIFC1 inhibition were reversed by CENPE overexpression.

**Conclusions:** KIFC1 contributed to cell proliferation, migration, and EMT via interacting with CENPE in ovarian cancer. KIFC1 might be a potential biomarker and therapeutic target in ovarian cancer patients.

**MeSH Keywords:** **Cell Proliferation • Epithelial-Mesenchymal Transition • Ovarian Neoplasms**

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## Background

Ovarian cancer is the sixth most common cancer in women worldwide and the fifth leading cause of death due to gynecological malignancies, with a total cure rate of less than 40% [1]. Although significant progress has been made in surgery and treatment methods, no fundamental changes in the overall mortality rate of ovarian cancer have been observed in the past few decades [2]. Credible biomarkers for early detection, prognosis, and prediction of disease aggressiveness are still lacking despite years of extensive research [1]. Also, the mechanisms underlying ovarian cancer onset and progression are not fully understood; thus, in-depth clarification of its mechanisms may contribute to the development of novel therapeutic strategies for improving the diagnosis, treatment, and prognosis of ovarian cancer patients.

Centrosome amplification is considered a hallmark of cancer [3,4]. Recent evidence has suggested that amplified centrosomes may drive malignant transformation and fuel metastatic dissemination [5]. Kinesin family member C1 (KIFC1) belongs to the kinesin-14 family of motor proteins and is involved in centrosome clusters, microtubule transport, and spindle formation during mitosis [6]. It has been shown that KIFC1 is overexpressed in diverse carcinomas, including breast cancer, gastric cancer, and ovarian adenocarcinoma, and promotes tumor cell proliferation and/or drug resistance [7–9]. Data from the Gene Expression Omnibus database showed that the expression level of KIFC1 was significantly upregulated in the tissues of patients with ovarian cancer (GSE12470). In clinical epithelial ovarian cancer samples, the expression level of KIFC1 was significantly higher than that of normal tissues, and the expression of KIFC1 also increased with tumor grade. In patients with serous ovarian adenocarcinoma, poor overall survival was correlated with a higher expression of KIFC1, indicating that the aggressive course of ovarian adenocarcinoma in patients may be due to high KIFC1 levels [9,10]. The high expression level of KIFC1 was also related to poor prognosis in patients with hepatocellular carcinoma. Other results showed that KIFC1 promotes cell proliferation of hepatocellular carcinoma *in vitro*, while the knockdown of KIFC1 inhibits tumor formation and growth in mice [8]. Therefore, these results identified KIFC1 as a potential therapeutic target for the treatment of hepatocellular carcinoma. In addition, the mRNA and protein levels of KIFC1 were significantly upregulated in bladder cancer specimens. The overexpression of KIFC1 phosphorylated GSK3 $\beta$  and promoted proliferation and epithelial-mesenchymal transition (EMT) by activating AKT and enhancing Snail expression, thereby greatly promoting the migration and transfer of bladder cancer [11]. However, the role of KIFC1 in the progression of ovarian cancer and its related mechanisms remain unknown.

Centrosome protein E (CENPE, also known as KIF10, kinesin family member 10) is a type of kinesin-like motor protein that accumulates in the G2 phase of the cell cycle [12]. Unlike other centrosome-related proteins, it first appears in the centromeric region of the chromosome in the prometaphase, but not in the mesophase (9). In different types of solid tumors, cancerous cells with a rapid proliferation ability exhibit higher levels of CENPE expression; for example, CENPE is upregulated in lung cancer cells [13,14]. One recent study reported that the levels of CENPE and CENPF mRNA in the entire ovarian subtype are significantly reduced compared with those in native cells [15]. In addition, in breast cancer, overexpressed KIF members, including CENPE and KIFC1, are significantly associated with poor overall survival, recurrence-free survival, and distant migration-free survival [16]. However, the relationship between KIFC1 and CENPE has not yet been elucidated. The present study showed that KIFC1 promoted the proliferation, migration, and EMT of ovarian cancer, which may be attributed to the activation of CENPE.

## Material and Methods

### Cell culture

The human ovarian epithelial cell line (IOSE386) and 5 ovarian cancer cell lines, ES-2, SKOV3, OVCAR3, HEY, and A2780, were procured from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA, USA) and maintained at 37°C in a 5% CO<sub>2</sub> atmosphere.

### Cell transfection

The KIFC1 short hairpin RNAs (shRNAs; shKIFC1-1: 5'-CCGGTCCCTCAACTCTCTACGCTTTCTCGAGAAAGCGTAGAGAGTTGAGGGATTTTG-3' and shKIFC1-2: 5'-CCGGCTGTCCACCAATGCTCGATATGCTCGAGCATATCGAGCATTGGTGACAGTTTTTG-3') and CENPE overexpression (pcDNA-CENPE) plasmid together with their negative controls (shRNA and pcDNA) were purchased from GenePharma (Shanghai, China). The sequence of CENPE was amplified and inserted into the pcDNA3.1(+) vector. For transfection, SKOV3 cells were grown in a 6-well plate until 50% to 70% confluence, and then Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to transfect the specified vectors, according to the manufacturer's instructions. Briefly, 50 pmol shRNAs and 1.5  $\mu$ L Lipofectamine 3000 were diluted in 25  $\mu$ L FBS-free DMEM, separately. After complete mixing, the 50  $\mu$ L of transfection solution was added to the culture medium. At 48 h after transfection, the cells were ready for the experiments.

### Cell proliferation assay

The cell proliferation of SKOV3 cells was tested by CCK-8 assay (Sigma-Aldrich, St Louis, MO, USA). Cells were seeded in 96-well plates ( $1 \times 10^4$  cells/well) and cultured in appropriate medium containing 10% FBS for 24 h. CCK-8 reagent (10  $\mu$ l/well) was added, and was followed by the detection of the absorbance (optical density) at 450 nm on an enzyme immunoassay analyzer (Bio-Rad, USA).

### Wound healing assay

For the wound healing assay, SKOV3 cells were seeded into 6-well plates ( $5 \times 10^5$  cells/well). A pipette tip was used to create a wound after 48 h of transfection. The cells were then cultured in serum-free medium. Cell migration was determined by detecting the average distance of cells migrating into the wound surface under an inverted microscopy at 0 h and 72 h.

### Cell migration assay

Cell migration assays were performed using 24-well culture plates with 8-mm pore inserts (Transwell; Falcon, BD Biosciences). The lower chamber was filled with 600  $\mu$ l DMEM containing 10% FBS. SKOV3 cells ( $1 \times 10^5$  cell/well) were seeded into the upper chamber. After 24 h of incubation, the migrated cells were fixed with 5% glutaraldehyde and then stained with crystal violet, and the number of cells in the bottom well were counted by a counting chamber.

### Real-time reverse-transcription polymerase chain reaction

Following the manufacturer's instructions, Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total the RNA from the cells. Then, the total RNA was reverse transcribed into cDNA using a PrimeScript RT kit (Invitrogen, USA), and real-time reverse-transcription polymerase chain reaction (RT-qPCR) was conducted using SYBR Premix Ex Taq (TaKaRa, China), according to the manufacturer's instructions. GAPDH was used as an internal control. The primer sequences were as follows: KIFC1 forward, 5'-GGTGC AACGACCAAAATTACC-3' and reverse, 5'-GGGTCTGTCTTCTTGGAAAC-3'; CENPE forward, 5'-GATTCTGCCATACAAGGCTACAA-3' and reverse, 5'-TGCCCTGGGTATAACTCCCAA-3'. The RT-qPCR was carried out using the ABI PRISM 7500 PCR System (Applied Biosystems, Foster City, CA, USA) and the  $2^{-\Delta\Delta Ct}$  method was utilized in each sample for quantification.

### Western blot analysis

Ovarian cancer cells were lysed using RIPA lysis buffer (Beyotime, China) with the addition of a protease inhibitor cocktail (Thermo Fisher Scientific), and total protein concentration

was detected using a BCA Protein Assay kit (Pierce, Rockland, IL, USA). Extracted proteins were separated by 12% SDS-PAGE gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (Roche, Basel, Switzerland). After blocking with dried skimmed milk powder dissolved in TBST solution, the membranes were incubated with specific primary antibodies against KIFC1, CENPE, E-cadherin, N-cadherin, Snail, ZEB1, matrix metalloproteinase (MMP)2, and MMP9 (Cell Signaling Technology) at 4°C overnight. Subsequently, membranes were cultured with peroxidase-conjugated secondary antibodies (Cell Signaling Technology) at room temperature for 2 h. Chemiluminescence (Millipore Corporation) and densitometry analysis by ImageJ software were applied to measure the protein expression.

### Immunofluorescence

The SKOV3 cells were fixed with 4% paraformaldehyde, blocked with 3% bovine serum albumin (Sigma-Aldrich), and incubated with polyclonal rabbit anti-Ki67 and anti-PCNA primary antibodies (1: 200) (Abcam, Cambridge, UK) overnight at 4°C. Secondary antibody Alexa Fluor 488 donkey anti-rabbit (1: 200) was applied for 1 h at 37°C. Nuclei were visualized by staining with DAPI (Sigma-Aldrich). InCell 2200 (GE Healthcare Life Sciences, UK) was used to acquire images, and analyses (cell counting and islet area calculations) were performed using the GE InCell Developer Toolbox.

### Co-immunoprecipitation

The co-immunoprecipitation of proteins was performed using a Pierce Co-Immunoprecipitation Kit (Thermo Fisher Scientific). The rabbit KIFC1 and CENPE antibodies (Sigma-Aldrich) and negative control rabbit IgG antibody were used in ASVEC. The complexes after immunoprecipitation were subjected to western blotting for detecting mutual binding between KIFC1 and CENPE.

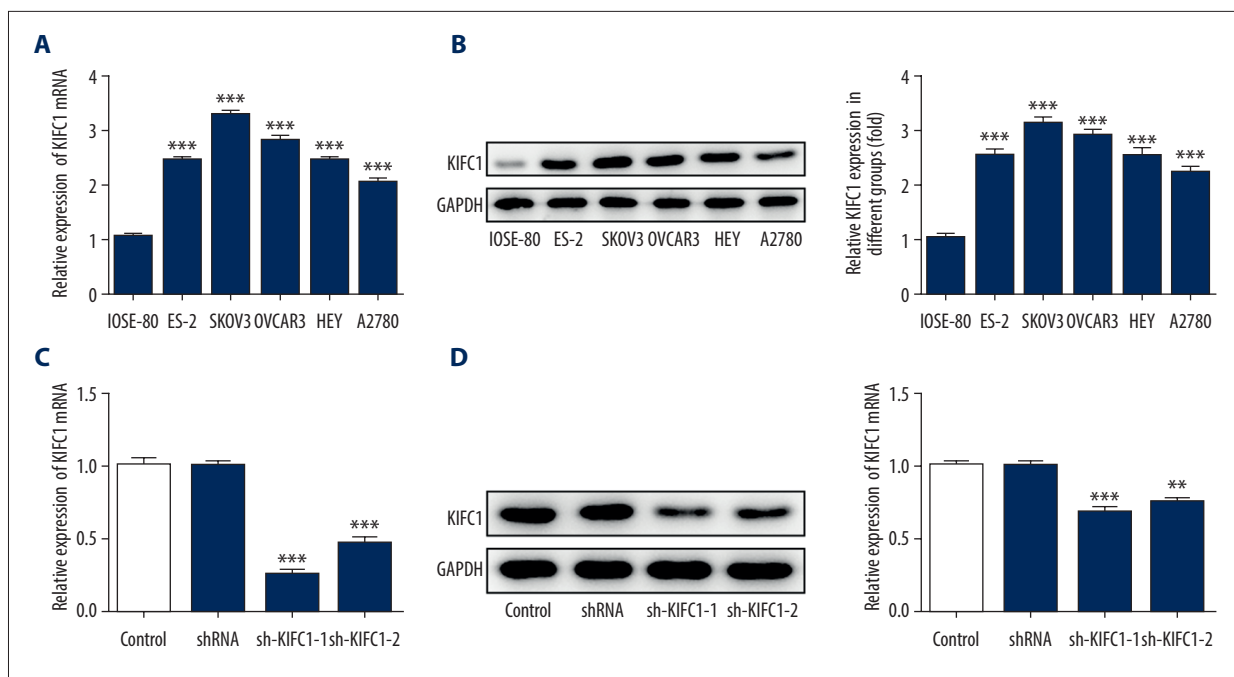
### Statistical analysis

Statistical analysis was conducted using SPSS 22.0 (IBM, Armonk, NY, USA). All data were presented as mean  $\pm$  standard deviation (SD). The differences between 2 groups were analyzed by *t* test, and the differences among more than 2 groups were analyzed by one-way ANOVA following Student Newman-Keuls analysis.  $P < 0.05$  indicated statistical significance.

## Results

### KIFC1 was highly expressed in ovarian cancer cells

We first estimated the expression pattern of KIFC1 in human ovarian cancer. The mRNA and protein expression of KIFC1 in



**Figure 1.** Kinesin family member C1 (KIFC1) was highly expressed in ovarian cancer cells. (A) mRNA and (B) protein expression of KIFC1 in human ovarian epithelial cell line (IOSE386) and 5 ovarian cancer cell lines (ES-2, SKOV3, OVCAR3, HEY, and A2780). \*\*\*  $P < 0.001$  vs. IOSE386. The (C) mRNA and (D) protein expression of KIFC1 in SKOV3 cells with knockdown of KIFC1 or no knockdown. \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$  vs. control.

ovarian cancer cell lines ES-2, SKOV3, OVCAR3, HEY, and A2780 were measured by RT-qPCR and western blotting. As shown in Figure 1A and 1B, the expression of KIFC1 in the 5 ovarian cancer cell lines was significantly increased compared with that in IOSE386 cells. Among all cancerous cells, SKOV3 presented the highest expression of KIFC1. Therefore, we chose to perform further analysis using SKOV3.

#### KIFC1 knockdown inhibited the proliferation of ovarian cancer cells

Next, we generated KIFC1 shRNA plasmids to perform knockdown experiments in SKOV3 cells. The RT-qPCR and western blotting results showed that KIFC1 expression in the sh-KIFC1-1 and sh-KIFC1-2 groups was markedly downregulated, compared to the KIFC1 expression in cells with an empty vector (shRNA group) and the control group. Next, the sh-KIFC1-1 group, which exhibited the lowest expression of KIFC1, was chosen for subsequent analyses (Figure 1C, 1D).

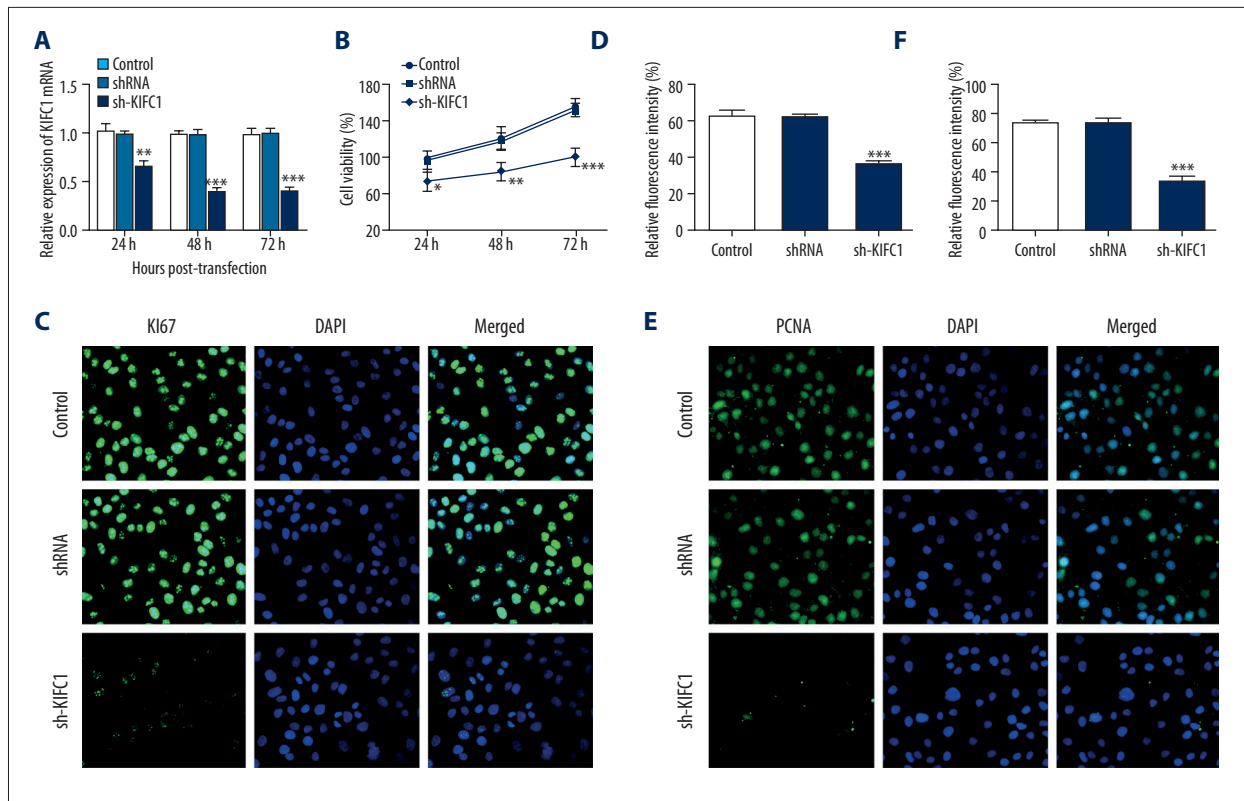
To determine the effects of KIFC1 on ovarian cancer cell proliferation, the SKOV3 cells were transfected with sh-KIFC1-1, and the KIFC1 mRNA expression and cell viability was measured after 24, 48, and 72 h. Figure 2A shows that the silencing effect reach a maximum and then remained stable at 48 h after transfection. The CCK-8 results revealed that the knockdown of KIFC1 significantly decreased the proliferation of

SKOV3 cells from 24 h to 72 h after transfection (Figure 2B). In addition, the results of immunofluorescence showed that proliferation markers Ki67 and PCNA had lower expression in the sh-KIFC1-1 group than in the shRNA and control groups (Figure 2C–2F). Thus, these results showed that KIFC1 promotes ovarian cancer cell proliferation.

#### KIFC1 silencing repressed the migration and EMT of ovarian cancer cells

Wound healing and transwell assays were applied to detect cell migration in SKOV3 cells. No significant difference between the control group and shRNA group was found. However, the knockdown of KIFC1 dramatically inhibited the cell migration abilities of SKOV3 cells compared with those of the negative controls (Figure 3A–3D). Next, we investigated the expression of MMPs associated with the degradation of the extracellular matrix and tumor migration. The knockdown of KIFC1 significantly decreased MMP2 and MMP9 protein expression, as determined by the western blot assay (Figure 3E). Thus, the results suggested that KIFC1 promotes migration in ovarian cancer.

Further, the effect of KIFC1 depletion on EMT was determined. We examined several biomarkers for the process of EMT. As shown in Figure 3F, the protein expression of N-cadherin, Snail, and ZEB1 were decreased by sh-KIFC1-1 transfection, while E-cadherin protein expression was increased by sh-KIFC1-1



**Figure 2.** Kinesin family member C1 (KIFC1) silencing inhibited the proliferation of ovarian cancer cells. (A) The relative expression of KIFC1 mRNA in SKOV3 cells with and without knockdown of KIFC1 at different timepoints after transfection. (B) The cell viability of SKOV3 cells in the absence or presence of KIFC1 knockdown at different timepoints. (C–F) Representative images of immunofluorescence staining ( $\times 200$ ) for (C–D) Ki67 and (E, F) PCNA, together with quantitative analyses. \*  $P < 0.05$ , \*\*  $P < 0.01$ , and \*\*\*  $P < 0.001$  vs. control.

transfection. Therefore, these results demonstrated that KIFC1 might enhance EMT in ovarian cancer cells.

### KIFC1 knockdown suppressed the expression of CENPE in ovarian cancer cells

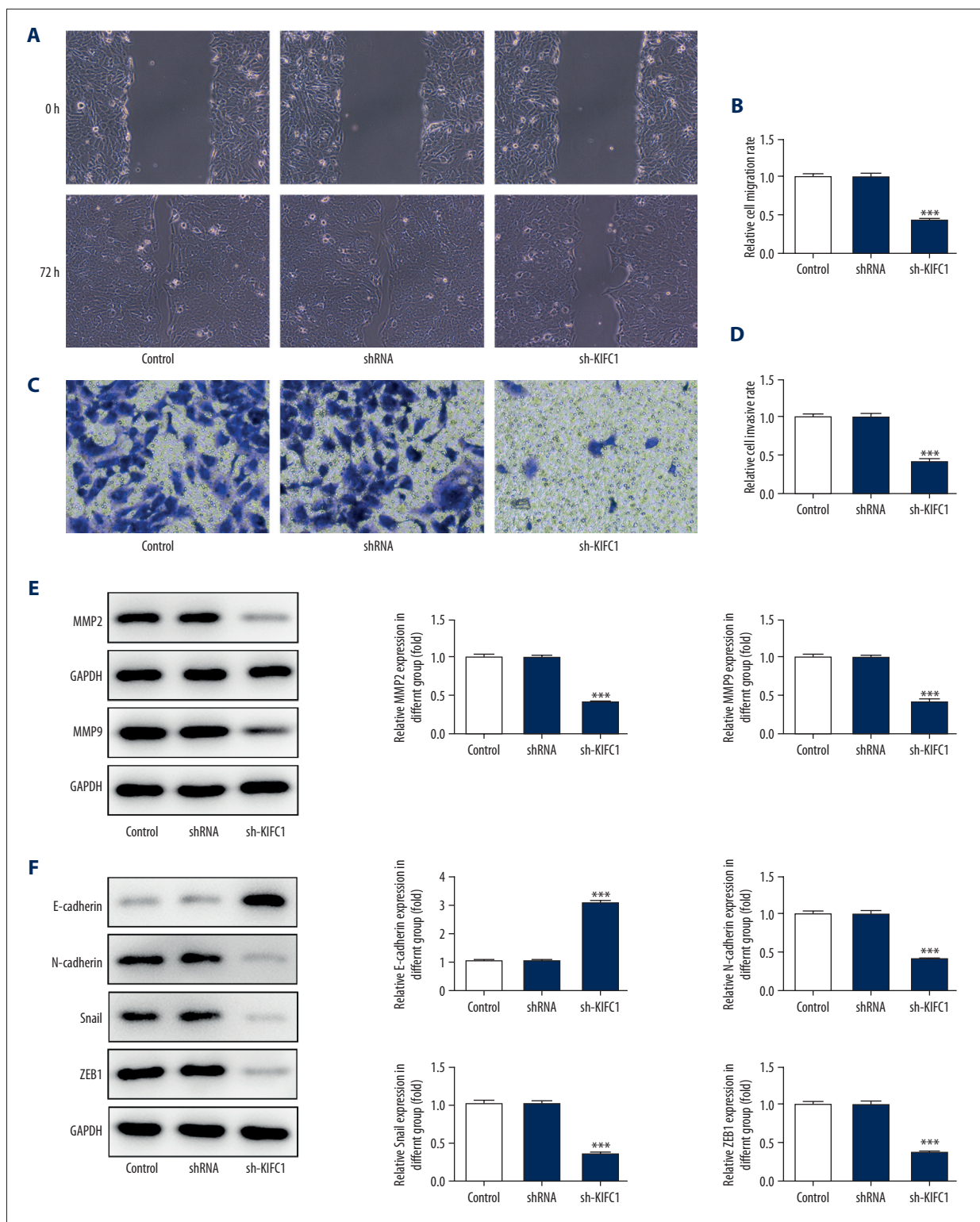
The protein-protein interaction (PPI) network of differentially expressed genes constructed by the STRING database predicted that KIFC1 might target CENPE. First, the expression of CENPE in the IOSE386 cell line and various ovarian cancer cells was estimated. As shown in Figure 4A and 4B, the expression of CENPE in ovarian cancer cell lines was significantly increased compared with that in IOSE386 cells. SKOV3 and OVCAR3 cells showed the highest expression of CENPE among all ovarian cancer cells. Co-immunoprecipitation was used to detect the relationship between KIFC1 and CENPE and we found that KIFC1 could directly bind to CENPE in SKOV3 cells (Figure 4C, 4D). To determine whether KIFC1 mediated the expression of CENPE, we further detected the expression of CENPE after KIFC1 regulation by western blotting and RT-qPCR. The knockdown of KIFC1 significantly decreased CENPE mRNA and protein expression (Figure 4E, 4F). Therefore,

knockdown of KIFC1 might directly inhibit the expression of CENPE in ovarian cancer cells.

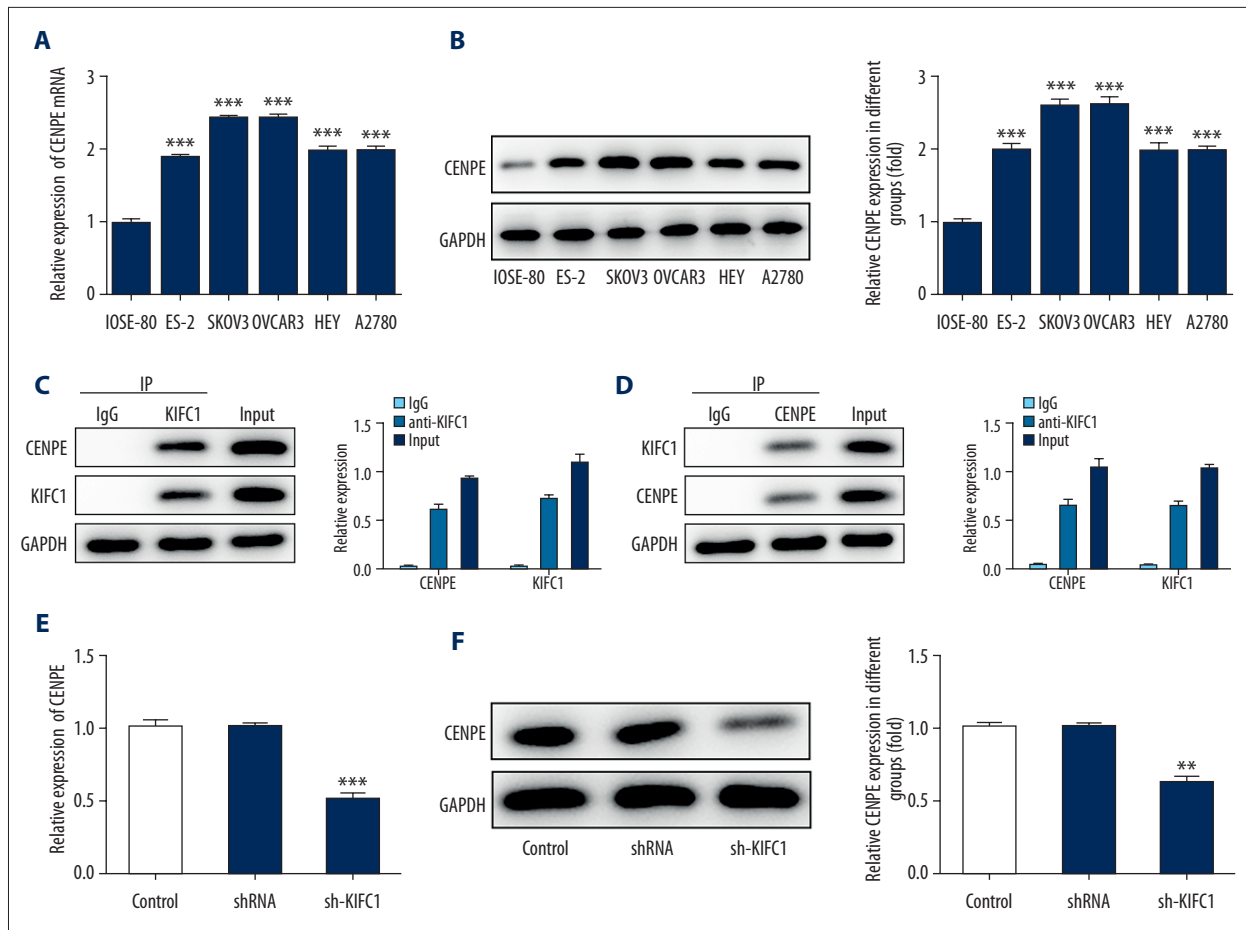
### KIFC1 knockdown hampers the proliferation, migration, and EMT by downregulating CENPE expression in ovarian cancer cells

Next, we investigated whether CENPE was critical for the functioning of KIFC1 in ovarian cancer cells. Figure 5A and 5B demonstrate the overexpression of CENPE in SKOV3 cells. Findings of the CCK-8 assay showed that KIFC1 knockdown significantly reduced the viability of SKOV3 cells, and this inhibitory effect was reversed by CENPE overexpression (Figure 5C). The expressions of Ki67 and PCNA were lower in the sh-KIFC1-1 group than in the shRNA and control groups, which could also be reversed by CENPE (Figure 5D–5G). Transwell and wound healing assays also demonstrated that co-transfection of sh-KIFC1-1 and pcDNA-CENPE reversed the KIFC1 knockdown-inhibited migration in SKOV3 cells (Figure 6A–6D). And the knockdown of KIFC1 significantly decreased MMP2 and MMP9 expression, which could be restored by overexpressing CENPE (Figure 6E). Moreover, the protein expression of N-cadherin,





**Figure 3.** Knockdown of Kinesin family member C1 (KIFC1) inhibited the migration and epithelial-mesenchymal transition of ovarian cancer cells. **(A, B)** The migration rate of SKOV3 cells with and without knockdown of KIFC1 was detected by wound healing ( $\times 100$ ). **(C, D)** The representative images for transwell assay ( $\times 200$ ) of SKOV3 cells with and without KIFC1 silencing. **(E, F)** The protein expression of MMP2, MMP9, E-cadherin, N-cadherin, Snail, and ZEB1 was measured by western blot assay. \*\*\*  $P < 0.001$  vs. control.



**Figure 4.** The relationship between Kinesin family member C1 (KIFC1) and centrosome protein E (CENPE) in ovarian cancer cells. **(A)** mRNA and **(B)** protein expression of CENPE in human ovarian epithelial cell line (IOSE386) and 5 ovarian cancer cell lines (ES-2, SKOV3, OVCAR3, HEY, and A2780). \*\*\*  $P < 0.001$  vs. IOSE386. **(C, D)** The interaction between KIFC1 and CENPE was tested by co-immunoprecipitation assay. The **(E)** mRNA and **(F)** protein expression of CENPE in SKOV3 cells in the with and without knockdown of KIFC1. \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$  vs. control.

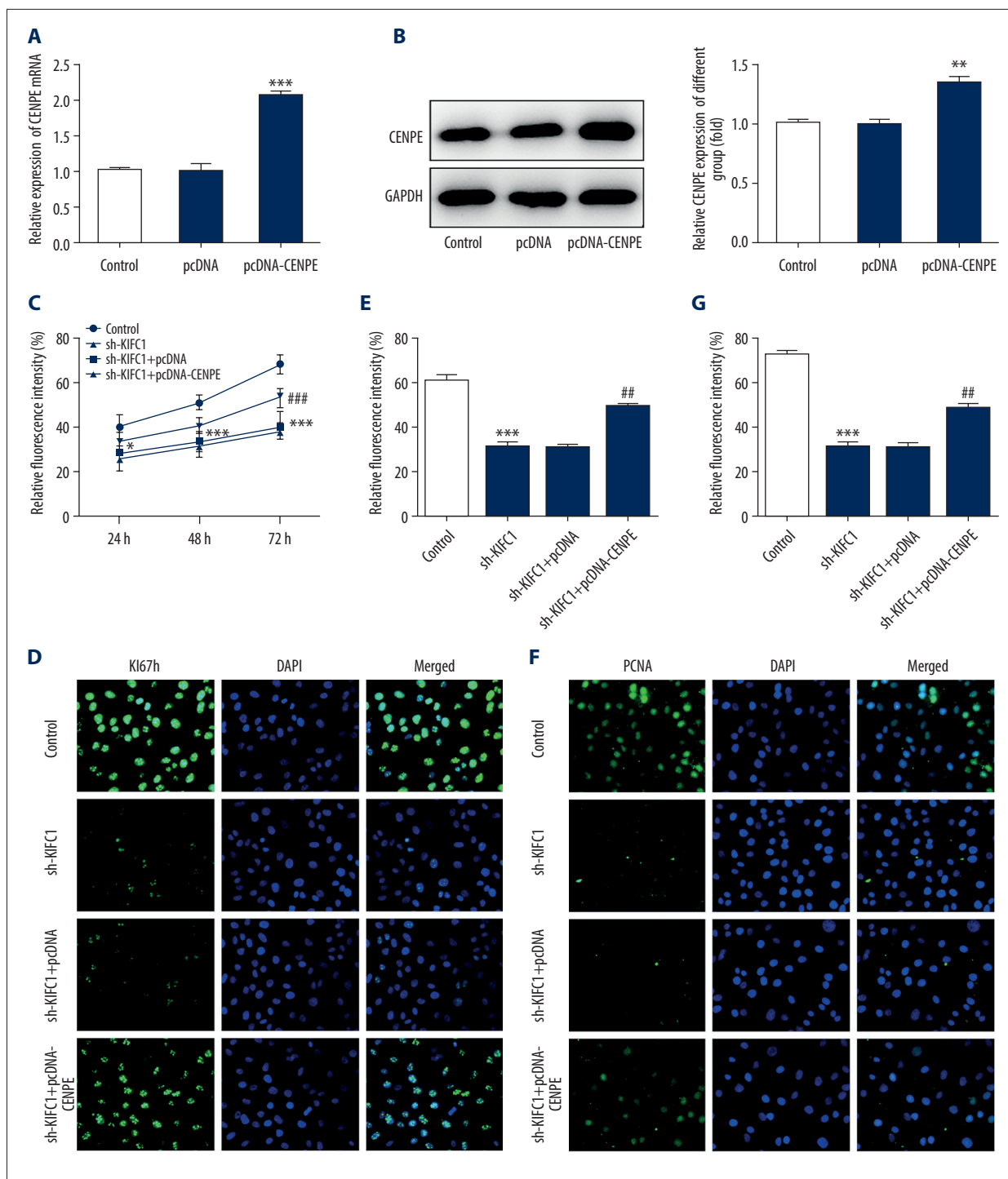
Snail, and ZEB1 were decreased, whereas that of E-cadherin was increased after the silencing of KIFC1, which could be recovered when CENPE was overexpressed (Figure 6F). These results demonstrated that KIFC1 regulated the proliferation, migration, and EMT of ovarian cancer cells via CENPE.

## Discussion

Although significant achievements have been made in identifying biomarkers for the diagnosis and prognosis of ovarian cancer, comprehension of the molecular mechanisms potentiating ovarian cancer development is limited. In this study, we revealed that KIFC1 was upregulated in human ovarian cancer cell lines and promoted the proliferation, migration, and EMT of SKOV3 cells. Next, we investigated the downstream molecular mechanisms of KIFC1 in ovarian cancer. A regulatory relationship between KIFC1 and CENPE was found. In addition,

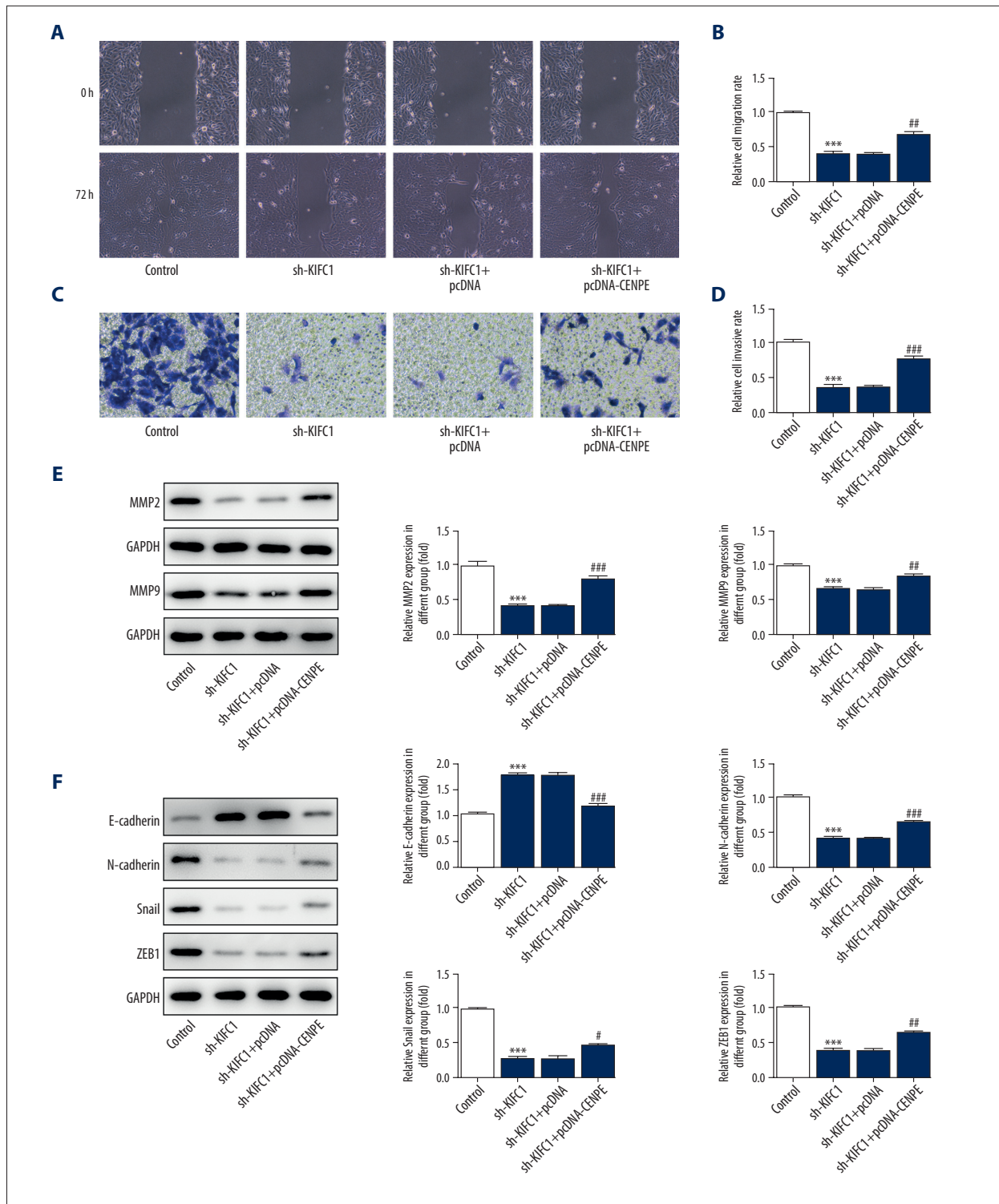
the knockdown of KIFC1 exerted a potential inhibition on the proliferation, migration, and EMT of ovarian cancer cells, and these effects could be reversed by upregulating CENPE. Taken together, these findings suggest that KIFC1 developed an oncogenic function by positively regulating CENPE during ovarian cancer progression.

Chromosomal instability is the major trigger for complex genomic changes in tumorigenesis [17]. The role of centrosome amplification-driven karyotype diversity in pancreatic cancer, ductal cancer, breast cancer, and colon cancer has been fully studied owing to its effect on chromosomal instability [18]. Many studies have emphasized the existence of redundant centrosomes in ovarian cancer, indicating that centrosome amplification is a sign of ovarian cancer [4,19]. Excess centrosomes in cancer cells tend to gather to control the centrosome load, thereby escaping the possibility of mitotic catastrophe. The role of KIFC1 in clustering hypercentrosomes has been well studied [7,20].



**Figure 5.** Overexpression of centrosome protein E (CENPE) reversed the inhibitory effect of Kinesin family member C1 (KIF1C1) silencing on ovarian cancer cell proliferation. **(A)** mRNA and **(B)** protein expression of CENPE in SKOV3 cells with and without overexpression of CENPE. **(C)** The cell viability of SKOV3 cells in different groups. **(D–G)** Representative images of immunofluorescence staining ( $\times 200$ ) for Ki67 and PCNA, with respective quantitative analyses. \*  $P < 0.05$ , \*\*  $P < 0.01$ , and \*\*\*  $P < 0.001$  vs. control. ##  $P < 0.01$  and ###  $P < 0.001$  vs. sh-KIF1C1+pcDNA.





**Figure 6.** Overexpression of centrosome protein E (CENPE) reduced the inhibitory effects of Kinesin family member C1 (KIFC1) silencing on ovarian cancer migration and epithelial-mesenchymal transition. **(A, B)** The migration rate of control cells or SKOV3 cells silencing KIFC1 in the presence or absence of CENPE overexpression was detected by wound healing ( $\times 100$ ). **(C, D)** The representative images for transwell assay ( $\times 200$ ) of control cells or SKOV3 cells silencing KIFC1 in the presence or absence of CENPE overexpression. **(E, F)** The protein expression of MMP2, MMP9, E-cadherin, N-cadherin, Snail, and ZEB1 was measured by western blot assay. \*\*\*  $P < 0.001$  vs. control. #  $P < 0.05$ , ##  $P < 0.01$  and ###  $P < 0.001$  vs. sh-KIFC1+pcDNA.

Recently, KIFC1 was discovered to be a biomarker for predicting the aggressive course of ovarian adenocarcinoma [10]. In the present study, we confirmed that KIFC1 was significantly highly expressed in ovarian cancer cell lines, especially in SKOV3 cells. Moreover, the knockdown of KIFC1 restricted the proliferation, migration, and EMT of ovarian cancer cells. These findings provided important evidence that KIFC1 was frequently upregulated in ovarian cancer, acting as a tumor promoter in the progression of ovarian cancer. Hence, KIFC1 may be a novel independent biomarker of ovarian cancer.

CENPE (KIF10) is an essential and end-directed microtubule motor and acts by aligning chromosomes on the metaphase plate [21,22]. CENPE is highly expressed in a variety of tumors, such as glioma, breast cancer, and gastric cancer [23,24]. CENPE overexpression in esophageal adenocarcinoma is significantly associated with tumor grade and prognosis [25]. Interestingly, both KIFC1 and CENPE are required for chromosome congression and alignment [26]. Through the PPI network

constructed by STRING, we discovered that KIFC1 might directly target CENPE. Thus, we supposed that KIFC1 might directly interact with CENPE to exert its function in ovarian cancer. In our study, we verified that KIFC1 could interact with CENPE in SKOV3 cells through co-immunoprecipitation. More importantly, we performed rescue experiments, which revealed that CENPE could reverse the suppressive effects of cell proliferation, migration, and EMT triggered by KIFC1 silencing. Taken together, our findings showed that KIFC1 may exert its tumor-promotive functions in ovarian cancer cells through targeting CENPE.

## Conclusions

We demonstrated that KIFC1 was upregulated in ovarian cancer cells. KIFC1 might promote cell proliferation, migration, and EMT via binding to CENPE. Our results revealed a novel strategy for targeting KIFC1 as a potential biomarker and a therapeutic target in ovarian cancer patients.

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