Knockdown of eIF4E suppresses cell proliferation, invasion and enhances cisplatin cytotoxicity in human ovarian cancer cells

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Abstract. Eukaryotic initiation factor 4E (eIF4E) plays an important role in cap-dependent translation. The overexpression of *eIF4E* gene has been found in a variety of human malignancies. In this study, we attempted to identify the potential effects of eIF4E and explore the possibility of eIF4E as a therapeutic target for the treatment of human ovarian cancer. First the activation of eIF4E protein was detected with m^7-GTP cap binding assays in ovarian cancer and control cells. Next, the *eIF4E*-shRNA expression plasmids were used to specifically inhibit eIF4E activity in ovarian cancer cells line A2780 and C200. The effects of knockdown *eIF4E* gene on cell proliferation, migration and invasion were investigated *in vitro*. Moreover, the changes of cell cycle and apoptosis of ovarian cancer cells were detected by flow cytometry. Finally, we investigated the effect of knockdown of eIF4E on the chemosensitivity of ovarian cancer cells to cisplatin *in vitro*. Our results show there is elevated activation of eIF4E in ovarian cancer cells compared with normal human ovarian epithelial cell line. The results of BrdU incorporation and FCM assay indicate that knockdown of eIF4E efficiently suppressed cell growth and induce cell cycle arrest in G_1 phase and subsequent apoptosis in ovarian cancer cells. From Transwell assay analysis, knockdown eIF4E significantly decrease cellular migration and invasion of ovarian cancer cells. We also confirmed that knockdown eIF4E could synergistically enhance the cytotoxicity effects of cisplatin to cancer cells and sensitized cisplatin-resistant C200 cells *in vitro*. This study demonstrates that the activation of *eIF4E* gene is an essential component of the malignant phenotype in ovarian cancer, and aberration of eIF4E expression is associated with proliferation, migration, invasion and chemosensitivity to cisplatin in ovarian cancer cells. Knockdown *eIF4E* gene can be used as a potential therapeutic target for the treatment of human ovarian cancer.

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Introduction

Epithelial ovarian cancer is the most lethal gynecological cancer and has the highest mortality rate in the world (1,2). Due to the asymptomatic nature of early stages of this disease and lack of a reliable method for early diagnosis, majority of patients with ovarian cancer are not effectively treated in the early stage (3,4). Although epithelial ovarian cancer is a chemosensitive tumor, with initial overall response rate to systemic therapy exceeds 80%, the development of acquired platinum-resistance has become a major obstacle for the clinical management of ovarian cancer (5,6). Platinum-resistance has resulted in a decrease in the overall survival rate, with a significant decrease to 30% for patients in advanced stages (5,7).

To improve its diagnosis and survival, it is necessary to better understand the mechanism of ovarian cancer development and to find novel ways for more effectively diagnose and treatm ovarian cancer. Based on the fact that cap-dependent translation of protein synthesis is a key regulatory step in the flow of genetic information from cellular genome to proteome, it is generally accepted that cap-dependent translation plays a central role in tumorigenesis or other tumor phenomenon (8,9). On the initiation of cap-dependent translation, the eukaryotic translation initiation factor 4E (eIF4E) protein recognizes the 7-methyl-GTP (m^7-GTP) cap structure at the 5'-end of mRNA molecules and associates with the eukaryotic translation initiation factor 4G (eIF4G) protein to form the translation initiation complex. Thus, eIF4E protein plays a central role in the process of protein synthesis.

The most frequently aberrant change in the translational apparatus is the upregulated levels of eIF4E expression, which selectively affects transport of specific transcripts, increases cap-dependent translation, suppresses apoptosis and induces malignant transformation (10). Although the mechanism whereby eIF4E acts in tumorigenesis is still not understood, it has been established to be rate-limiting for cell growth and transformation in many types of human tumors, such as breast cancer, lung cancer, colon carcinoma, bladder carcinoma, and cervical carcinoma (11-13). Several research groups have reported eIF4E overexpression was correlated with malignant progression and poor prognosis in various cancers (14-16). In tissue culture and xenograft mouse models, eIF4E overexpression led to oncogenic transformation, and to increased tumor

size, invasion, and metastases (17,18). Inhibition of eIF4E effectively suppresses tumor growth and invasiveness in many types of cancers (19,20). The elevated eIF4E greatly increases translation of some mRNA encoding proteins contributing to angiogenesis and proliferation such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-2) and cyclin D1, which are key stimulators of migration and angiogenesis in tumors (21,22). These results suggest that *eIF4E* may play an important role in malignant progression and even drug resistance of ovarian cancer cells. However, the precise function and mechanisms of *eIF4E* in tumorigenesis of ovarian cancer has not been characterized well so far.

To directly examine the biological effects of eIF4E in epithelial ovarian cancer cells, specific shRNA targeting *eIF4E* gene was used in our study to knock down eIF4E expression and disturb its binding to eIF4G, blocking the cap-dependent translation initiation. To investigate the functional relevance of eIF4E to resistance of ovarian cancer cells, our comparison studies between platinum-sensitive ovarian cancer cells A2780 and the platinum-resistant cell line C200 were explored with shRNA/cisplatin combinative treatment in this study. Finally, we wished to confirm that the deregulation of initiation of cap-dependent translation was required to develop ovarian tumorigenesis and maintain the resistance to platinum in ovarian cancer cells, and to demonstrate that the inhibitors of cap-dependent translation may be a therapeutic target against epithelial ovarian cancer.

Materials and methods

Cell culture and reagents. Paired isogenic cisplatin-sensitive human ovarian cancer cell line A2780 and its cisplatin-resistant clone C200 were used in this study (23). As the control cells an immortalized normal human ovarian epithelial cell line (IOSE) was used. All cells were generously provided by Dr S. Ramakrishnan (Minnesota University). The cancer cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Gibco), insulin and 1% penicillin/streptomycin at 37˚C in a humidified 5% CO_2 atmosphere. IOSE was maintained in 50% M199, 50% MCDB105 medium supplemented with 5% FBS and 0.1% $NaHCO_2$. Primary antibodies against eIF4E (C46H6, rabbit mAb, #9742), eIF4G (C65H5, rabbit mAb, #9742), 4E-BP_1 (53H11, Rabbit mAb, #9644), PARP (46D11, rabbit mAb, #9532) and goat peroxidase (HRP)-conjugated secondary antibodies (#7074) and antibody against β-actin (D6A8, rabbit mAb, #8457) were all purchased from Cell Signaling Technology.

eIF4E/eIF4G interaction inhibitor 4EGI-1 was purchased from Selleck Chemicals (Selleck China, S7369). 4egi-1 was dissolved in DMSO at concentration of 10 mM, and stored at -80˚C. Cisplatin was purchased from Sigma-Aldrich (P4394), and was dissolved in 0.9% NaCl solution to make a 1 mM stock solution. Chemiluminescence substrate was obtained from Pierce. The DC Bio-Rad protein quantization reagents were from Bio-Rad.

m^7-GTPcap binding assays. A total of $5x10^6$ cells were lysed in 300 μl lysis buffer. The supernatants were incubated with 20 μl Sepharose beads (Amersham Pharmacia Biotech) at 4˚C for 1 h, and washed twice in 1 ml PBS buffer. Also, 10 μl of m^7-GTP-Sepharose beads were washed with 500 μl of PBS buffer three times. Then m^7-GTP-Sepharose beads were added into the cell extracts pre-cleared with Sepharose beads, and rotated overnight at 4˚C. The beads were washed three times with PBS buffer. Then beads were denatured, and the supernatants were resuspended in Laemmli sample buffer with 2% β-ME and were resolved on SDS-PAGE for western blot analysis.

Plasmid and transfection. The pGIPZ shRNA plasmid and recombinant pGIPZ shRNA plasmid targeting eIF4E and non-silencing negative control shRNA plasmid were kindly provided by Dr S. Ramakrishnan (Minnesota University). DNA template oligonucleotides corresponding to *eIF4E* gene (GenBank NM_001968.3) was designed as follows: *eIF4E*-shRNA1 (sense, 5'-AAGCAAACCUGCGGCUGAUCU-3'), *eIF4E*-shRNA2 (sense, 5'-ACAGCAGAGACGAAGUGAC-3'), and a non-specific shRNA (sense, 5'-GGACGUGGUCGCCA CCCUGCCC-3'). These recombinant plasmids were named pGIP-4e1, pGIP-4e2 and pGIP-NS. For temporal transfection, $2x10^5$ skov-3 cells were seeded in 60-mm plates. The cells (60-70% confluent) were transfected with 2 μg of plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Selecting a population of cells that stably express siRNA. Cells ($2x10^5$) were plated into 6-well plates. Total 2 μg of pGIP-4e and vector plasmid of eIF4E was, respectively, transfected into C200 and A2780 cells using Lipofectamine 2000 (Invitrogen) and cultured for 24 h without antibiotic selection. Then the cells were cultured in medium containing 5 μg/ml puromycin (Sigma) until all the non-transfected cells were killed. The antibiotic-resistant cells were pooled and passaged in medium containing 5 μg/ml puromycin. Stable transfectants were termed as C200-4e1 or A2780-4e1 (transfected with pGIP-4e1 plasmid), C200-NS or A2780-NS (transfected with pGIP-NS plasmid) respectively.

Western blot analysis. Approximately $5x10^5$ cells were harvested and boiled in 200 μl lysis buffer containing protease inhibitors. Samples were subjected to SDS-PAGE and electrophoretically transferred to a PVDF (polyvinylidene difluoride) membrane. Membranes were incubated with the primary antibodies (1:1,000) at 4˚C overnight, washed with TBST buffer, and incubated again with an appropriate HRP-conjugated secondary antibody (1:5,000) at room temperature for 1 h. The membranes were washed and examined by chemiluminescence detection.

Cell proliferation assay. The cell proliferation was analyzed with 5-bromo-2'-deoxy-uridine (BrdU) incorporated into the newly synthesized DNA of replicating cells. In brief, cells were cultured in a 96-well plate for 24 h and pulse-labeled with BrdU for 4 h according to the manufacturer's instructions. The BrdU label in the DNA was detected using a peroxidase and FITC-conjugated anti-BrdU second antibody subjected to immunodetection and immunofluorescence assays (24). For peroxidase-conjugated anti-BrdU immunodetection, the values of relative proliferation was quantified with a peroxidase substrate and measured at 370 nm with a wavelength

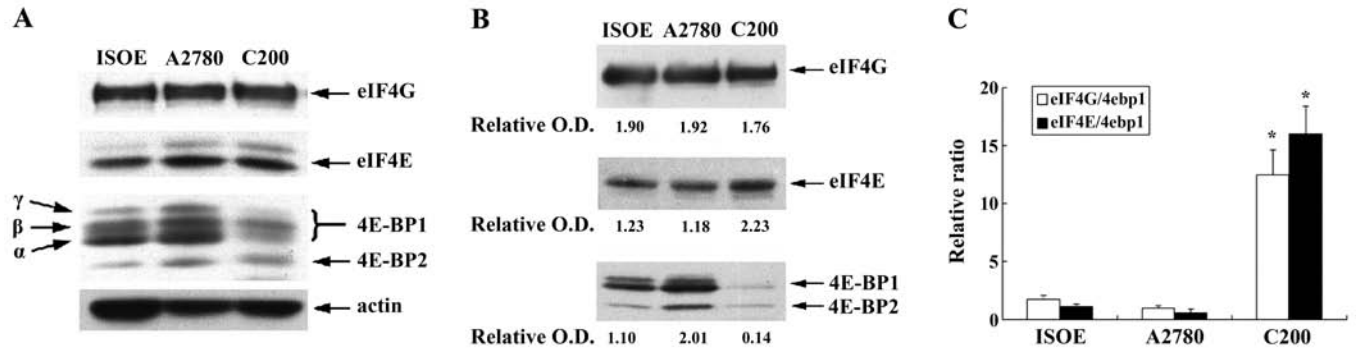

Figure 1. Analysis of the eIF4F translation complex in non-transformed and malignant ovarian epithelial cells. (A) Immunoblotting results showing the expression levels of total and phosphorylated eIF4E, eIF4G and 4E-$BP_{1/2}$ in non-transformed ovarian epithelial and cancer cells. The results showed much lower amounts of 4E-$BP_{1/2}$ proteins in C200 cells. A2780 and C200 cells all possessed much higher levels of eIF4E than ISOE cells. (B and C) Examination of eIF4F integrity. Cell lysates were incubated with m^7-GTP-Sepharose resin to capture eIF4E and its binding partners, and then probed for eIF4E, eIF4G and 4E-$BP_{1/2}$ in western blotting. The results show that C200 cells were translationally activated at a significantly higher level.

of 540 nm. For BrdU-FITC immunofluorescence assay, cells were cultured on cover slips until reaching 60% confluence, and then BrdU incorporated into cellular DNA was detected after shRNA plasmid transfection for 24 h with immunofluorescence assay kit (Roche Applied Science, USA). The cells were analyzed using a Nikon confocal microscope, at the wavelength of excitation and emission of 488 and 525 nm, respectively. The positive BrdU cells were counted from five randomly selected fields by direct counting cells in each sample in a blinded manner. The percentage of positive cells was calculated as the number of positive cells divided by the number of total cells. Each assay was repeated at least 3 times.

Adherent colony formation assay. For cell colony formation assay on plastic surface, cells were trypsinized and plated in triplicate in 6-well culture plates at 500 cells per dish. Cells were allowed to grow at 37˚C, 5% CO_2 with media changes every 3-4 days until colonies were visible by eye. After 14-day incubation, cells were fixed with 4% paraformaldehyde at 4˚C, then stained with 0.1% crystal violet for 20 min. Cells were washed and plates were scanned, and colonies consisting of ≥30 cells were counted using ImageJ software.

Flow cytometric analysis of cell cycle and apoptosis. Cells were collected and fixed with 70% ice-cold ethanol overnight at -20˚C. Cells were centrifuged, resuspended in 1 ml of PBS mixed with propidium iodide (PI) and RNaseA (10 μg of propidium iodide, 10 μg of RNase A, and 0.5% Tween-20), and incubated at 37˚C for 40 min. Cell cycle distribution was determined by analyzing 10,000-20,000 cells using a FACScan flow cytometer and CellQuest software (Becton-Dickinson, San Jose, CA, USA). The percentage of apoptotic cells was determined by the subG_1 peak in the DNA histogram.

Growth inhibition assay. For the cisplatin-siRNA combination experiments, a 3-(4,5-dimethythazolz-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to analyzed the effects of *eIF4E*-shRNA on cell viability *in vitro*. All cells were pre-treated with *eIF4E*-shRNA plasmid for 24 h before cisplatin treatment. Drug-treated cells were incubated for 48 h and then MTT was added to the cultures for an additional 2 h. The results were assessed in a 96-well microreader (Bio-Rad Co.) by measuring the absobance at a wavelength of 570 nm. The inhibitory rate of cell growth was calculated as: inhibitory rate = (1-$A_{treated\ group}/A_{untreated\ group}$) x 100%. The IC_{50} was determined based on the dose response curves by Graphpad Prism software. Combination index (CI) for drug interaction was calculated using CompuSyn software (CompuSyn, Inc.). The experiments were performed three times independently.

Cell migration and invasion assays. *In vitro* cell migration and invasion assays were performed using Transwell chambers. For the migration assays, $2x10^4$ cells were added to the upper chamber of 8-μm pore size Transwells (BD Biosciences, Franklin Lakes, NJ, USA). For the invasion assays, $1x10^5$ cells were added to the upper chamber of 8-μm pore size Tranwells precoated with Matrigel (BD Biosciences). In these assays, cells were suspended in RPMI-1640 medium containing 10%, FBS was added to the upper chambers, and RPMI-1640 medium containing 10% FBS was placed in the lower well, and then incubated at 37˚C in 5% CO_2. After 24 h of incubation, non-migrated or non-invaded cells were removed carefully. The filters were then fixed in 4% paraformaldehyde and stained with crystal violet. Five random fields were counted per chamber using an inverted microscope (Olympus Corp., Tokyo, Japan) at x200 magnification for each membrane. All of the analysis was performed in triplicate.

Statistical analysis. All statistical analyses were performed using SPSS (version 16, SPSS Inc., Chicago, IL, USA). The values were expressed as the means ± SE and statistical significance was analyzed using two-tailed Student's t-test. A p-value of <0.05 was considered as significant and indicated by asterisks in the figures.

Results

Cisplatin-resistant ovarian cancer cells display a hyperactivated eIF4E and elevated cap-dependent translation. We first detected eIF4E and 4E-$BP_{1/2}$ protein expression in A2780 and C200 cells in comparison with an immortalized normal human ovarian epithelial cell line (IOSE) with western blot analysis. As presented in Fig. 1A, we found there were significant differences between IOSE cells and carcinoma cell lines,

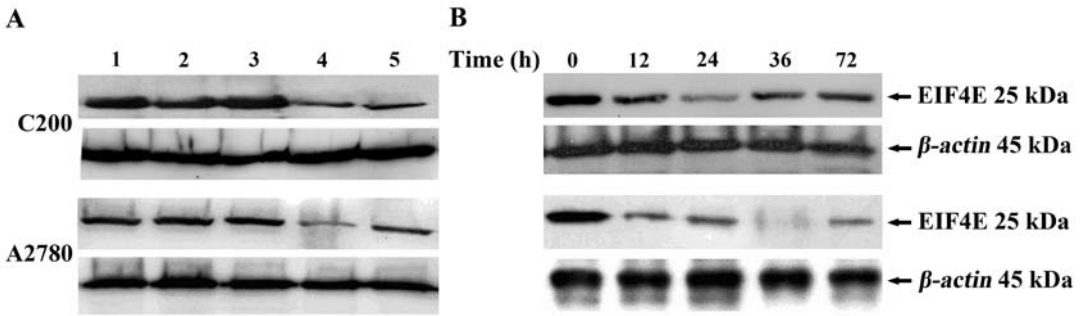

Figure 2. The expression of eIF4E protein was inhibited by knockdown of eIF4E in A2780 and C200 cells. (A) The protein expression of eIF4E was obviously reduced by shRNAs. Lane 1, Lipofectamine 2000 control; lane 2, pGIPZ vector control; lane 3, pGIP-NS; lane 4, pGIP-4e1; lane 5, pGIP-4e2. (B) C200 cells were transfected with pGIP-4e1 for the indicated times. The protein expression of eIF4E was reduced to almost zero after 36-h transfection in A2780 cells.

the levels of eIF4E were substantially increased in A2780 and C200 cell lines. Specially, we detected much lower amounts of 4E-$BP_{1/2}$ protein in C200 cells. It indicates that C200 cells possessed much higher levels of eIF4E than A2780 cells, and an increasing activity of eIF4F in C200 cells. Interestingly, we found that all cells expressed similar levels of eIF4G in addition to eIF4E (Fig. 1A).

To further compare the pattern and integrity of eIF4F in IOSE and the other two carcinoma cell lines, we assessed formation of the m^7GTP-eIF4F complex in an m^7-GTP cap binding assay. The levels of cap-bound eIF4E, eIF4G and 4E-$BP_{1/2}$ were analyzed by immunoblotting. The relative amounts of cap captured eIF4E and eIF4G in cells could be regarded as indicator of the integrity and functional potency of eIF4F. The amounts of cap-associated 4E-BP_1 showed negative impact on eIF4E assembly and function. The cap-bound fraction from IOSE and A2780 cells contained significant amounts of 4E-BP_1 (Fig. 1B), indicating that in these cells, cap-dependent translation was under strong 4E-BP_1-mediated negative control. In contrast, the cap-bound complexes from C200 cells were at a significantly lower level of 4E-$BP_{1/2}$ indicating that cisplatin-resistant cells existed in a translationally activated state. These results clearly indicated that eIF4E expression was upregulated in platinum-resistant ovarian cancer cells. Moreover, eIF4E and 4E-BPs displayed a deregulated activity in platinum-resistant ovarian cancer cells.

Knockdown of eIF4E inhibits the proliferation of A2780 and C200 cells in vitro. If elevated eIF4E was critical for the protein synthesis of ovarian cancer cells, we hypothesized that downregulation of eIF4E would result in inhibition of the growth of ovarian cancer cells. To verify this, we employed two different sequences of *eIF4E* shRNA plasmid pGIP-4e1 and pGIP-4e2 to knock down eIF4E expression and determined its impact on the growth in our study. The efficiency of silencing and the expression level of eIF4E protein was measured with expression of green fluorescent proteins. In order to analyze the downregulation of eIF4E expression in cells, western blot assay was performed. As shown in Fig. 2A, the levels of eIF4E protein expression in A2780 and C200 cells were both significantly reduced by pGIP-4e1 and pGIP-4e2 in comparison with its negative control cells, and the inhibitory rates were ~65% and 55% ($p<0.05$) in C200 cells, and 75 and 55% ($p<0.05$) in A2780 cells, respectively. Moreover, pGIP-4e1 showed stronger inhibition effect in both cell lines. Thus, we focused our further analyses on the pGIP-4e1 plasmid at the next biological assay. By the time-course analysis, we observed that downregulation of eIF4E protein expression level occurred at 12 h after transfection. The eIF4E reduction peak was at 24 h in C200 cells and 36 h in A2780 cells, respectively. The eIF4E reduction in both A2780 and C200 cells was sustained up to 72 h (Fig. 2B). The results indicated successful knockdown of eIF4E by pGIP-4e1 in ovarian cancer cells. In addition, no effects of shRNA plasmid were observed on the expression of β-action used as an internal control gene in this study.

We examined the anti-proliferate response of pGIP-4e1 in ovarian cancer cell lines C200 and A2780 with BrdU proliferation assay. The results showed that the growth of both A2780 and C200 cells were inhibited by pGIP-4e1 plasmid after transfection for 24 h (Fig. 3A). BrdU-FITC immunofluorescence assay indicated BrdU incorporation was significantly decreased in cells that were transfected with pGIP-4e1 and displayed less density than that of control cells (Fig. 3B).

Knockdown of eIF4E inhibits the colony formation of C200 and A2780 cells. To investigate the potential of knockdown of *eIF4E* in reducing colony formation of tumor cells *in vitro*, we examined the stably transfected C200 and A2780 cells. First, we generated stable cell lines expressing shRNA specific to *eIF4E*, C200-4e1 and A2780-4e1, C200-NS and A2780-NS (negative plasmid control). The expression of eIF4E protein was analyzed by western blot analysis to test the inhibitive effect of pGIP-4e1 in these stable cell lines. Results of western blot analysis showed that eIF4E protein level was significantly decreased by pGIP-4e1shRNA in ovarian cancer cells (Fig. 4A). Colony formation assay was analyzed two weeks after plating. All cells were fixed in paraformaldehyde and stained with crystal violet (Fig. 4B). The mean values from plates are shown in Fig. 4B. In our results, pGIP-4e1 reduced the colony formation, the number of colonies of both cancer cells was much lower than those of control cell lines, respectively ($p<0.05$). It further indicated that inhibition of eIF4E expression suppresses the growth of cancer cells.

Knockdown of eIF4E inhibits the migration and invasion of C200 and A2780 cells. Some research has shown that eIF4E might be involved in regulation of cancer metastasis. Therefore, we evaluated whether the role of eIF4E knockdown affected

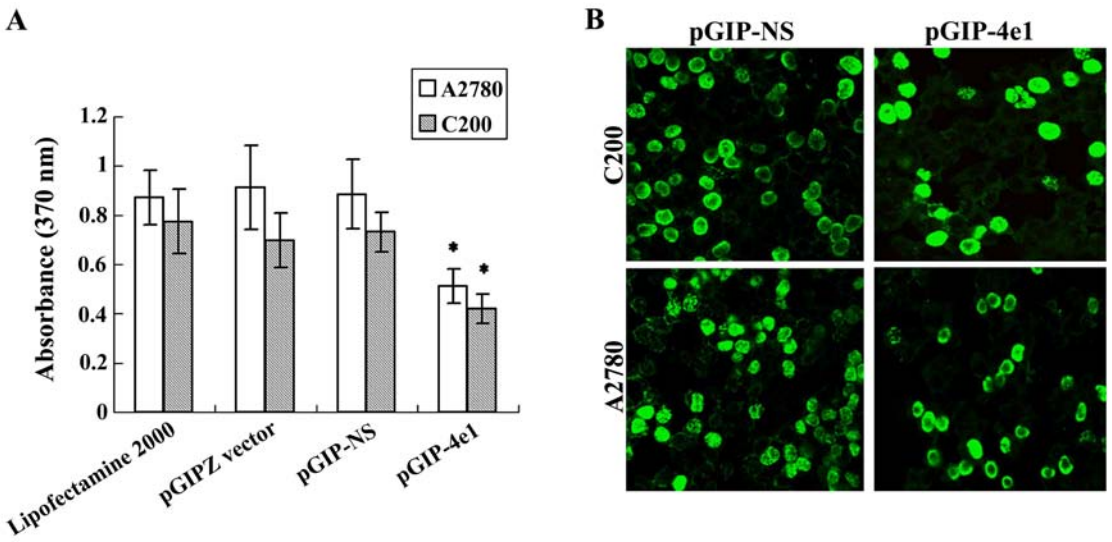

Figure 3. Knockdown of eIF4E inhibits the proliferation of A2780 and C200 cells. (A) The absorbance was measured when all cells were transfected with pGIP-4e1 and control plasmids, respectively. The asterisk indicates a significant different between pGIP-4e1 transfected groups and all control groups ($p<0.05$). (B) After 24-h transfection, the immunofluorescence images of positive BrdU incorporated cells *in vitro*. The pGIP-NS control cells display stronger fluorescence density than that of pGIP-4e1 transfected cells ($p<0.05$).

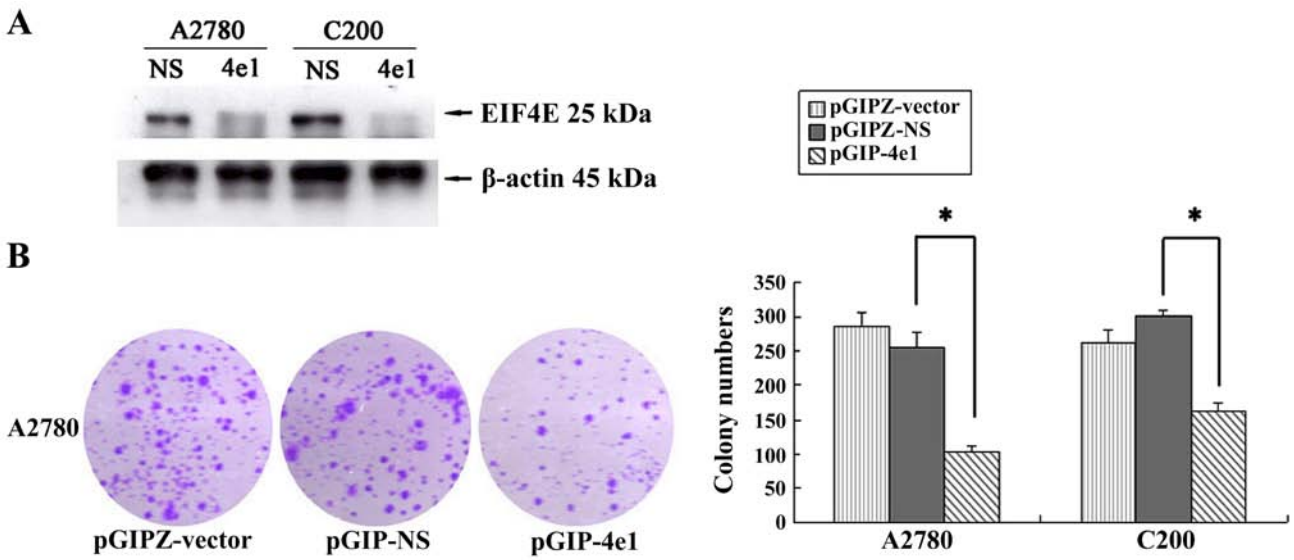

Figure 4. Specific shRNA against eIF4E inhibits colony formaion of C200 and A2780 cells. (A) The cells stably transfected with pGIP-4e1 and pGIP-NS plasmid were cultured in RPMI-1640 medium with 10% FBS for 24 h. Total 50 μg of cellular protein was analyzed by immunoblotting. EIF4E protein level was significantly decreased in C200 and A2780 cells ($p<0.05$). (B) C200 and A2780 cells were seeded into 6-well plates at a concentration of 200 cells/well and cultured for 2 weeks. The means ± SD of colony number was counted in three wells and average number was plotted. The colony number of C200 and A2780 was significantly reduced compared with other control groups (the asterisk indicates $p<0.05$). Representative image of A2780 cell colonies is shown.

the migration and invasion of ovarian cancer cells. The C200 and A2780 cells stably transfected pGIP-4e1 shRNA were analyzed by Transwell assays. Western blot analysis showed that eIF4E protein level was significantly decreased by pGIP-4e1 shRNA in C200 and A2780 cells (Fig. 4A). The results of transwell assays showed that knockdown of eIF4E significantly reduced the number of migration and invasion in both cell lines compared with the control cells (Fig. 5). It suggested that elevated eIF4E expression was associated with positive regulation of ovarian cancer cell migration and invasion.

Knockdown of eIF4E arrests ovarian cancer cells in G_1 phase and induces apoptosis. To elucidate the mechanisms underlying the siRNA mediated growth inhibition, further analysis was performed to test the effects of eIF4E-shRNA on the cell cycle progression of C200 and A2780 cells and each assay was performed three times. The mean values of triplicate experiments are shown in Fig. 6. Flow cytometric cell cycle analysis of logarithmically growing C200 cells revealed a distribution of continuously proliferating cells in G_1 phase, S phase and the mitotic G_2/M phase as evaluated in Fig. 6A. Compared with that of transfected C200-NS cells,

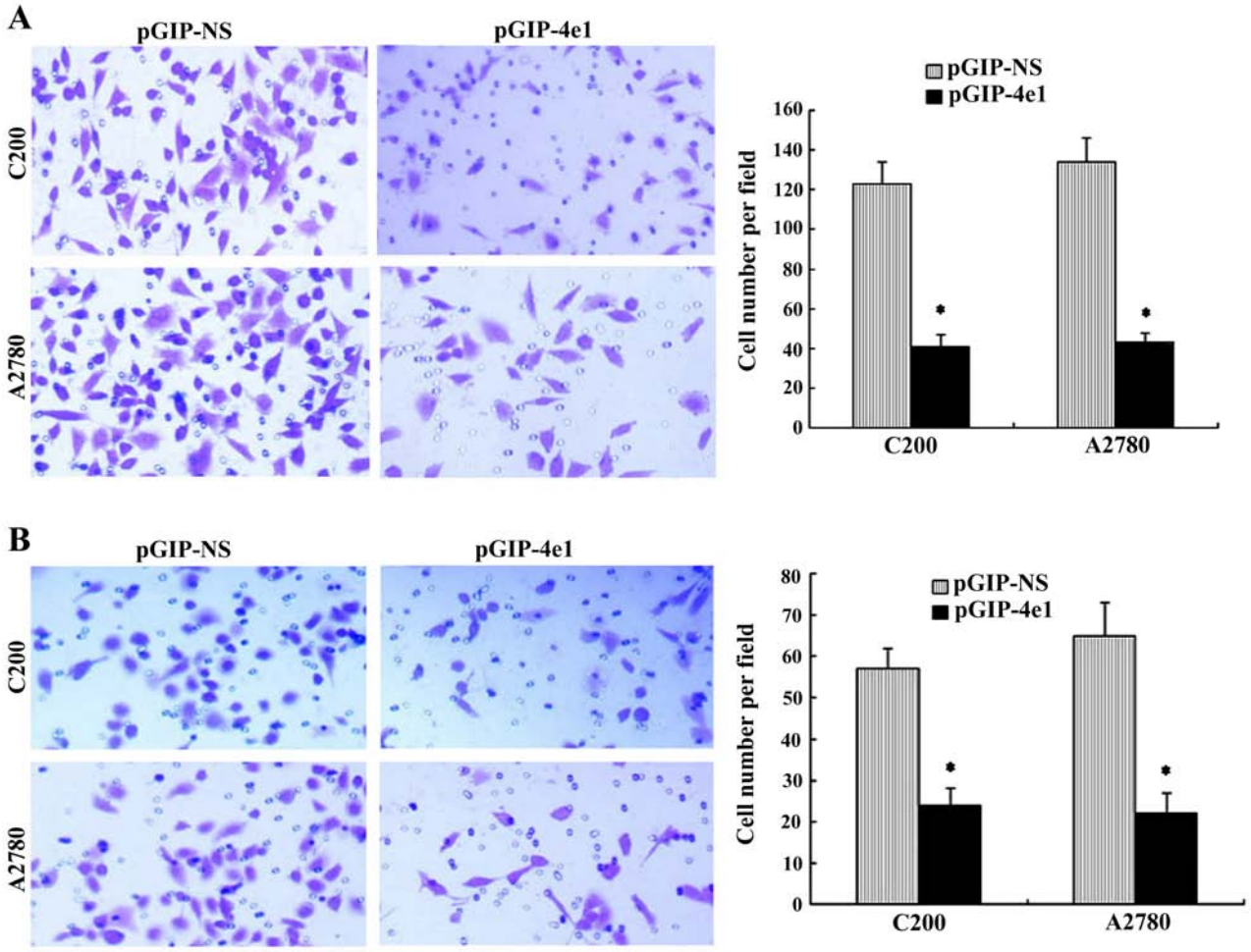

Figure 5. Specific shRNA against eIF4E inhibits the migration and invasion of ovarian cancer cells. The migration and invasion assays were carried out. The migrated cells were fixed in 4% paraformaldehyde and stained with crystal violet. The representative fields were photographed and counted at x200 magnification. The cells were counted in five different fields per assay under the microscope. The mean values and standard error were obtained from three independent experiments. (A) The migrated cells of C200-4e1 and A2780-4e1 cells were significant less than those of control cells. (B) The invasion cells of C200-4e1 and A2780-4e1 cells were significant less than those of control cells. The asterisk indicates $p<0.05$.

the percentage of C200-4e cells in the G_1 phase increased by 17.2±2.1%, while the percentage of C200-4e cells in S phase significantly reduced by 18.5±1.2% ($p<0.05$). As shown in Fig. 6A, cell cycle analysis showed a significantly greater distribution in subG_1 phase indicating more apoptotic cells in C200-4e cells than in C200-NS cells ($p<0.05$). However, a significant difference was not found in G_2/M phase between C200-4e and C200-NS cells, suggesting that knockdown of eIF4E induces G_1 cell arrest and apoptosis but does not increase G_2/M phase in which mitotic arrest occurs. Using cleaved PARP as readout of apoptosis, we further determined whether knockdown of eIF4E induces apoptosis in the tested cell lines. As presented in Fig. 6B, we detected cleaved form of PARP in C200-4e or A2780-4e cells. Interestingly, we found the stronger expression of cleaved-PARP in C200-4e cells. As a positive control, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induced strong cleavage of PARP in the both cell lines.

Knockdown of eIF4E enhances cisplatin cytotoxicity of ovarian cancer cells. The lower level of 4E-$BP_{1/2}$ and elevated eIF4E proteins indicated that cisplatin-resistant C200 cells showed a translational activated state, and it was involved in development of acquired resistance to cisplatin. We speculated that inhibition of eIF4E would overcome cisplatin resistance and enhance the sensitivity of ovarian cancer cells to cisplatin. To test this hypothesis, an MTT assay was used to examine the impact on cell response to cisplatin by knockdown of eIF4E in C200 and A2780 cells. Generally, the approximate cisplatin IC_{50} value of A2780 cell line was 10-20 μM, and the equivalent IC_{50} value for C200 was ~200 μM. The C200 cell lines were ~20-fold resistant to cisplatin compared with the A2780 (23). As presented in Fig. 7A, we tested the cisplatin IC_{50} of stable transfected C200-4e1 cells compared with C200-NS cells, the cisplatin IC_{50} value of C200-4e1 cells was significantly decreased by 28.2% ($p<0.05$). Accordingly, the cisplatin IC_{50} also significantly decreased by 18.2% in A2780 cells (Fig. 7B). Thus, it was clear that knockdown of eIF4E expression was able to enhance the effect of cisplatin in resistant ovarian cancer cells.

As a small molecule inhibitor of eIF4E/eIF4G interaction, 4egi-1 behaves as 4ebp1 mimetic to inhibit cap-dependent translation initiation. Thus, we further analyzed whether 4egi-1 could enhance the cytotoxic effects of cisplatin on C200

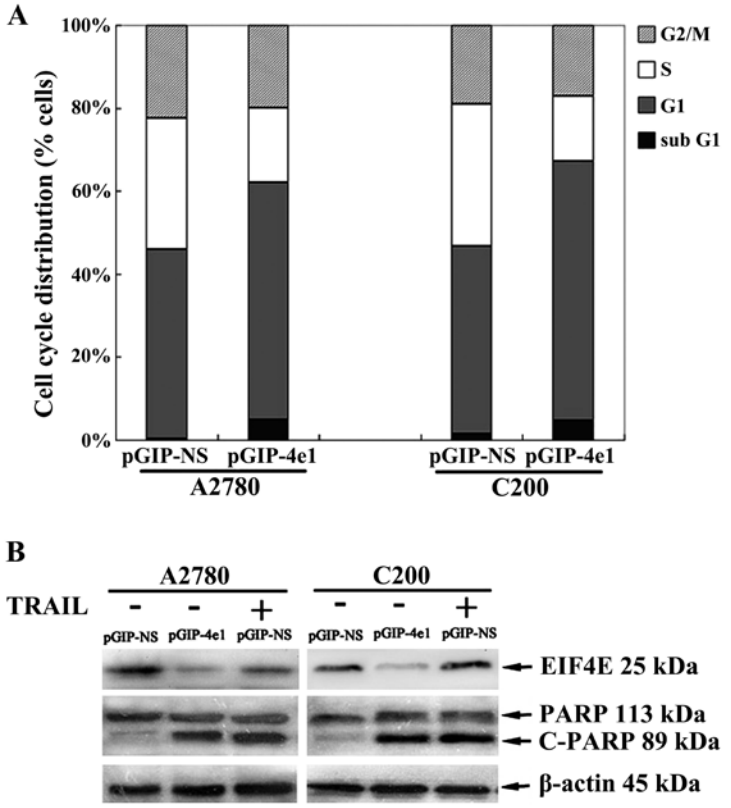

Figure 6. Effect of eIF4E knockdown on cell cycle and apoptosis in ovarian cancer cells. A2780 and C200 cells were transfected with pGIP-4e1 and control pGIP-NS, respectively. Twenty-four hours after transfection, the attached cells were harvested, and FACS analysis was performed. (A) Knockdown of eIF4E induced increased percentage of cells in G_1 phase ($p<0.05$). In both cell lines, the percentage of apoptotic cells in the subG_1 phase were significantly increased by knockdown of eIF4E ($p<0.05$). Each column represents the mean of the results of three separate experiments. (B) Knockdown of eIF4E induced apoptosis of A2780 and C200 cells. The cleaved form of PARP in cancer cells was detected, and control cells (transfected with pGIP-NS) were exposed to 50 ng/ml tumor necrosis factor related apoptosis inducing ligand (TRAIL) for 12 h before harvesting the cells.

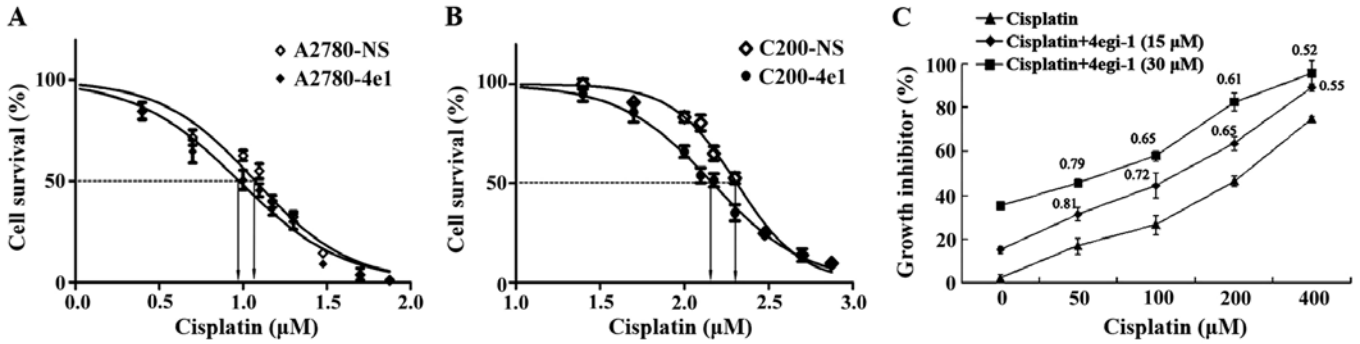

Figure 7. Knockdown of eIF4E enhances cisplatin cytotoxicity in cisplatin-resistant C200 cells. The IC_{50} of cisplatin was determined based on the dose response curves by Graphpad Prism software. Arrows indicated IC_{50} concentrations. The cell survival and growth inhibition were analyzed by the MTT assay. (A) The IC_{50} of cisplatin for A2780-NS cells was decreased from 11.86 to 9.48 μM ($p<0.05$). (B) The IC_{50} of cisplatin for C200-NS cells was 204.6 μM, and IC_{50} concentration for stably transfected C200-4e1 cells significantly decreased to 147 μM, ($n=3$, $p<0.05$). (C) C200 cells were treated with indicated concentrations of cisplatin in the absence and presence of 4egi-1 for 48 h. The numbers above the lines are combination indexes for the combination of cisplatin and 4egi-1.

cells. Our results showed that the combination of 4egi-1 and cisplatin was more potent than either agent alone in inhibiting the growth of C200 cells. The combination indexes were <1 for combination treatments (Fig. 7C), indicating synergy between 4egi-1 and cisplatin in cytotoxicity. This result further suggested that inhibition of cap-dependent translation indeed overcame cisplatin resistance and enhanced the sensitivity of ovarian cancer cells to cisplatin.

Discussion

Activation of translation initiation is critical for cancer cell growth, survival, and tumor progression. There is increasing evidence in the literature linking altered activation of the cap-dependent translation machinery to cell transformation and human cancer (25,26). Here, our results showed that the activation of cap-dependent translational initiation was

significantly different in platinum-sensitive and resistant ovarian cancer cell lines. The exciting finding in this study was the significantly higher activated eIF4E and lower level of 4E-$BP_{1/2}$ in cisplatin-resistant C200 cells (Fig. 1A). It is well known that the expression inhibition or hyper-phosphorylation of 4E-BP_1 can cause a decreasing affinity to eIF4E sequence to release the eIF4E to promote assembly of the eIF4F, and initiate translation (27). It suggested that platinum-resistant cells possessed a hyper-activated eIF4E and eIF4F complex. To the best of our knowledge, this is the first study that links eIF4E and cap-dependent translation to the acquired cisplatin-resistant ovarian cancer cells.

As a rate-limiting factor in cap-dependent translation and a focal downstream point of multiple signal pathways in cells, eIF4E might be a rational target for novel cancer therapeutics (28). It is well known that the altered expression of eIF4E contributes to cancer progression by enabling the translation of a limited pool of mRNAs encoding key proteins involved in cellular malignancy. Overexpression of eIF4E resulted in enhanced translation of mRNAs containing extensive secondary structure in their 5'-UTR. These mRNAs encode growth-promoting gene products such as cyclin D1, EGF-R/erb-b, c-Myc, c-myc, and VEGF (21,29-31). Thus, we hypothesized that eIF4E could plausibly serve as an integrator and amplifier of a broad range of diverse neoplastic phenomena in naturally occurring ovarian cancer. In this study, our results showed that knockdown of eIF4E significantly suppressed cell proliferation, colony formation, migration and invasion of both A2780 and C200 cells *in vitro*. To explore the mechanism of growth inhibition, we detected the changes of cell cycle in A2780 and C200 cells stably expressing eIF4E shRNA with FCM assay. Results indicated that the knockdown of eIF4E significantly induced accumulation of the cells in the G_1 phase, and induced increased number of apoptotic cells in the subG_1 phase. Coincidentally, we found that the expression of cleaved PARP was significantly upregulated by eIF4E shRNA inducing downregulation of eIF4E expression and apoptosis in ovarian cancer cells. Our study suggested that eIF4E played a critical role in inhibiting the growth of cancer cells through growth arrest or/and apoptosis in both tested ovarian cancer cells lines. The exact mechanism of the eIF4E shRNA-induced proliferation and apoptosis needs to be further clarified.

Platinum-based chemotherapy plays a pivotal role as first line chemotherapy option and is usually combined with taxanes to treat human ovarian cancer (5,32,33). However, the clinical therapeutic results of ovarian cancer are not satisfactory due to development of resistance to chemotherapeutic treatments, resulting in a dramatic decrease in the overall survival rate (34,35). It has been reported that the chemoresistance maybe a manifestation of inherent properties, but also the ability of responding to an apoptotic stimulus that rendered chemotherapy ineffective. Generally, the anticancer effect of cisplatin was mediated by the formation of functionally lethal intrastrand DNA cross-links, which resulted in many cellular biological effects including DNA synthesis inhibition, RNA transcription and proteins translation suppression, cell cycle arrest, and apoptosis (5,36,37). Despite the substantial data accumulated over the past years, the mechanisms responsible for platinum-resistance remains unclear. As a focal downstream point of multiple signal pathways, we hypothesized eIF4E was an ideal breakthrough point to offer valuable clues of platinum resistance or targets for chemotherapeutics of epithelial ovarian cancer. Here, we investigated the efficacy of eIF4E knockdown to cisplatin cytotoxicity in different ovarian cancer cell subtypes including cisplatin-sensitive and cisplatin-resistant ovarian cancer cells. An important finding in our study was the anticancer activity of eIF4E knockdown in cisplatin-resistant ovarian cancer cells. Our results showed that the in stable transfected C200-4e1 cells compared with C200-NS cells, the cisplatin IC_{50} value of C200-4e1 cells was decreased by 28.2%. Others have found that the overexpression of eIF4E induces the upregulated levels of cyclin D1 expression which confers many tumors cisplatin resistance (38,39). It is likely that the aberration of eIF4E caused the alteration of cap-dependent translation, and then resulted in the different sensitivity to platinum in ovarian cancer cells due to the alteration of platinum accumulation, detoxification and metabolism, even DNA damage repair.

On the other hand, 4EGI-1, a small molecule inhibitor that specially prevents eIF4E from binding to eIF4G, was used in our study to block the cap-dependent translation initiation and eIF4F assembly (40,41). Thus, we further determined whether addition of 4EGI-1 would enhance the growth inhibitory effects of cisplatin on C200 cells. The combination of cisplatin and 4EGI-1 was more potent than either agent alone in inhibiting the growth of C200 cells. The combination indexes were <1 for all combination treatments (Fig. 7C), indicating synergy between cisplatin and 4EGI-1 in inhibiting the growth of C200 cells. These data showed that cisplatin chemotherapy could be more effective in combination with inhibition of translation initiation or eIF4F assembly. As reported on other human malignancies by other researchers, the data reported here provide direct evidence that inhibition of translation initiation and eIF4F assembly with eIF4E shRNA or 4EGI-1 could abrogate ovarian cancer cell growth *in vitro* and synergistically led to enhancement of chemosensitivity to cisplatin in ovarian cancer cells.

Taken together, our study demonstrated that eIF4E expression was elevated in human ovarian cancer cells. An increasing activity of eIF4F was detected in cisplatin-resistant C200 cells. The elevated eIF4E expression was associated with positive regulation of cell proliferation, migration and invasion of ovarian cancer cells. Our results suggested that the sustained activation of eIF4E in cancer cells might be essential for expression of a transformed phenotype. Our study further suggested that knockdown eIF4E could effectively lead to growth suppression, apoptosis and enhancement of chemosensitivity to cisplatin of ovarian cancer cells. Eukaryotic initiation factor eIF4E should be an ideal breakthrough point to offer valuable clues of ovarian tumorigenesis, and downregulated eIF4E expression might a useful approach to improve the therapeutic responsiveness of ovarian cancer.

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