

## miR-940 Upregulation Suppresses Cell Proliferation and Induces Apoptosis by Targeting PKC- $\delta$ in Ovarian Cancer OVCAR3 Cells

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Ovarian cancer remains as one of the most threatening malignancies for females in the world. This study investigated the pivotal role of miR-940 in the progression of ovarian cancer and to reveal the possible molecular mechanism of its action. Ovarian cancer OVCAR3 cells were transfected with the miR-940 vector, miR-940 inhibitor, and/or small interfering RNA (siRNA) targeting PKC- $\delta$  (si-PKC- $\delta$ ) respectively. After transfection, cell viability and cell apoptosis were analyzed, as well as cell proliferation and apoptosis related protein expression. Compared to the control, miR-940 upregulation suppressed cell viability but induced cell apoptosis. miR-940 upregulation increased the expression of p27, Hes1, survivin, and caspase 3, but decreased the expression of PKC- $\delta$ . In addition, elevated cell viability induced by the miR-940 inhibitor was significantly decreased by knockdown of PKC- $\delta$ , and reduced cell apoptosis induced by the miR-940 inhibitor was increased by knockdown of PKC- $\delta$ . Taken together, the results of our study suggest that upregulation of miR-940 may function as a suppressor in the progression of ovarian cancer by inhibiting cell proliferation and inducing apoptosis by targeting PKC- $\delta$ . This study may provide a basis for the possible application of miR-940 in illustrating the molecular pathogenic mechanism of ovarian cancer.

**Key words:** Ovarian cancer; miR-940; Cell proliferation; Cell apoptosis; Protein kinase C- $\delta$  (PKC- $\delta$ )

### INTRODUCTION

Ovarian cancer remains as one of the most threatening malignancies affecting females worldwide, and the 5-year survival rate is high (1). The biology and the mechanism for development and progression of ovarian cancer are complicated, such as DNA hypomethylation, epigenetics, and genetic polymorphism (2,3). Only about 19% of patients are diagnosed at the early stage because of the difficulty in detecting ovarian cancer, including the obscure self-conscious symptoms, less effective diagnostic methods, and drug resistance (4). Therefore, to explore the deep pathogen mechanism for ovarian cancer is of great significance for the diagnosis and treatment of ovarian cancer.

MicroRNAs (miRNAs) are small noncoding RNAs that potentially play a pivotal role in the biology and biological processes at the transcriptional or posttranscriptional level by targeting the 3'-UTR of genes (5). Mounting evidence indicates that many miRNAs are playing pivotal roles in the tumorigenesis and biology of cancers, including ovarian cancer (6,7). The molecular diagnosis in cancers provides the highest specificity and sensitivity in classification and early detection (8). For

example, Chitkara et al. reported that several miRNAs are involved in the progression and biology of pancreatic ductal adenocarcinoma (9). Corney et al. reported the important role of downregulation of the miR-34 family in ovarian cancer (10). Recently, studies reported that the specific miRNA miR-940 is downregulated in numerous human cancers, including hepatocellular carcinoma and pancreatic ductal adenocarcinoma (11,12). miR-940 functions as a biomarker in the biology of the cancer by complicated mechanisms (13). Nonetheless, the expression of miR-940, as well as the role of miR-940 in ovarian cancer, has not been reported before.

In this study, we analyzed the expression of miR-940 in the ovarian cancer OVCAR3 cells and investigated the effects of miR-940 expression on the OVCAR3 cell biological processes, including cell proliferation and apoptosis, using comprehensive experimental methods. We further investigated the expression of miR-940 on OVCAR3 cell apoptosis and proliferation-related protein expression. The goals of this study were to better clarify the role of miR-940 in tumor growth of ovarian cancer cells and to reveal its potential mechanism. This study

may provide a basis for elucidating the important role of miR-940 in the pathogenesis of ovarian cancer.

## MATERIALS AND METHODS

### Cell Culture and Transfection

Human ovarian cancer OVCAR3 cells obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured in DMEM supplemented with 20% fetal bovine serum (FBS; Invitrogen, USA) in an atmosphere of 5% CO<sub>2</sub> at 37°C.

For cell transfection, the vectors were transfected into the OVCAR3 cells using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). The miR-940 vector, miR-940 inhibitor, and small interfering RNA (siRNA) targeting protein kinase C- $\delta$  (si-PKC- $\delta$ ) were purchased from Sangon Biotech (Shanghai, P.R. China). Cells without any vector transfection were considered as the control.

### MTT Assay

The effect of target gene expression on cell proliferation was assessed using MTT assay as previously described (14). Briefly, OVCAR3 cells cultured in DMEM containing 20% FBS at logarithmic stage ( $5 \times 10^3$ ) were transfected into 96-well plates. After 24 h of cultivation, the supernatant was abandoned and followed with the addition of 20  $\mu$ l of MTT every 24 h and incubation for 4 h. Afterward, 150  $\mu$ l of dimethyl sulfoxide (DMSO) was used to mix with the cells for 10 min. Absorbance of cells in each well was observed at 450 nm under an absorption spectrophotometer (Olympus, Japan).

### BrdU Assay

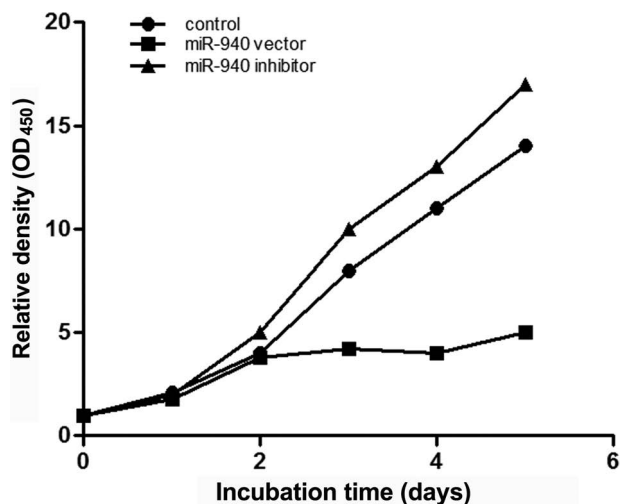
The influence of miR-940 expression on cell proliferation in each group was analyzed using the BrdU assay as previously described (15). Briefly, cells were seeded in the 24-well plates at a density of  $1 \times 10^4$  cells/well. After 72 h of incubation, BrdU (Sigma-Aldrich) was added to the medium at a final concentration of 10  $\mu$ M and incubated at 37°C for 5 h. Cells were then fixed in cold 70% ethanol for 5 min, and DNA was denaturated by adding 1.5 M HCl to the cells for 30 min at room temperature. Immunofluorescence to visualize incorporated BrdU was performed as described above using a mouse anti-BrdU antibody (BD Biosciences, San Jose, CA, USA), under the conditions suggested by the manufacturer. Primary antibody was followed by 45-min incubation with secondary green fluorescent dye-conjugated antibody (Invitrogen). VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) was used to stain the nuclei. Images were visualized using a Leica inverted fully automated microscope (DMI6000B) with digital camera DFC 420 RGB (Leica Microsystems, Wetzlar, Germany).

### Cell Apoptosis

Cell apoptosis was quantified with flow cytometry using an Annexin-V-FITC Cell Apoptosis Kit (Invitrogen) according to the manufacturer's protocol (16). After collection, cells were fixed with 100% ice-cold methanol at -20°C overnight. Cells were then incubated for 30 min in 50  $\mu$ g/ml propidium iodide (PI) with 1 mg/ml RNase. Apoptotic cell analyses were performed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Data were analyzed with the CellQuest software.

### Western Blotting

OVCAR3 cells cultured at 48 h in each group were lapped with RIPA assay (radioimmunoprecipitation; Sangon Biotech) lysate containing PMSF (phenylmethanesulfonyl fluoride; Sigma-Aldrich, USA) and then were centrifuged at 12,000 rpm for 10 min at 4°C. Supernatant was collected for the measurement of protein concentrations using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). For Western blotting (17), 50  $\mu$ g of protein per cell lysate was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). The PVDF membranes were then blocked in Tris-buffered saline Tween (TBST) containing 5% nonfat milk for 1 h at room temperature. Consequently, the membrane was incubated with rabbit anti-human antibodies (p27, p21, caspase 3, survivin, Hes1, and PKC- $\delta$ ; 1:100 dilution; Invitrogen) overnight at 4°C. Then the membrane was incubated with horseradish peroxidase-labeled goat anti-rat secondary antibody (1:1,000 dilution) at room temperature for 1 h. Finally, the PVDF membranes were washed three times

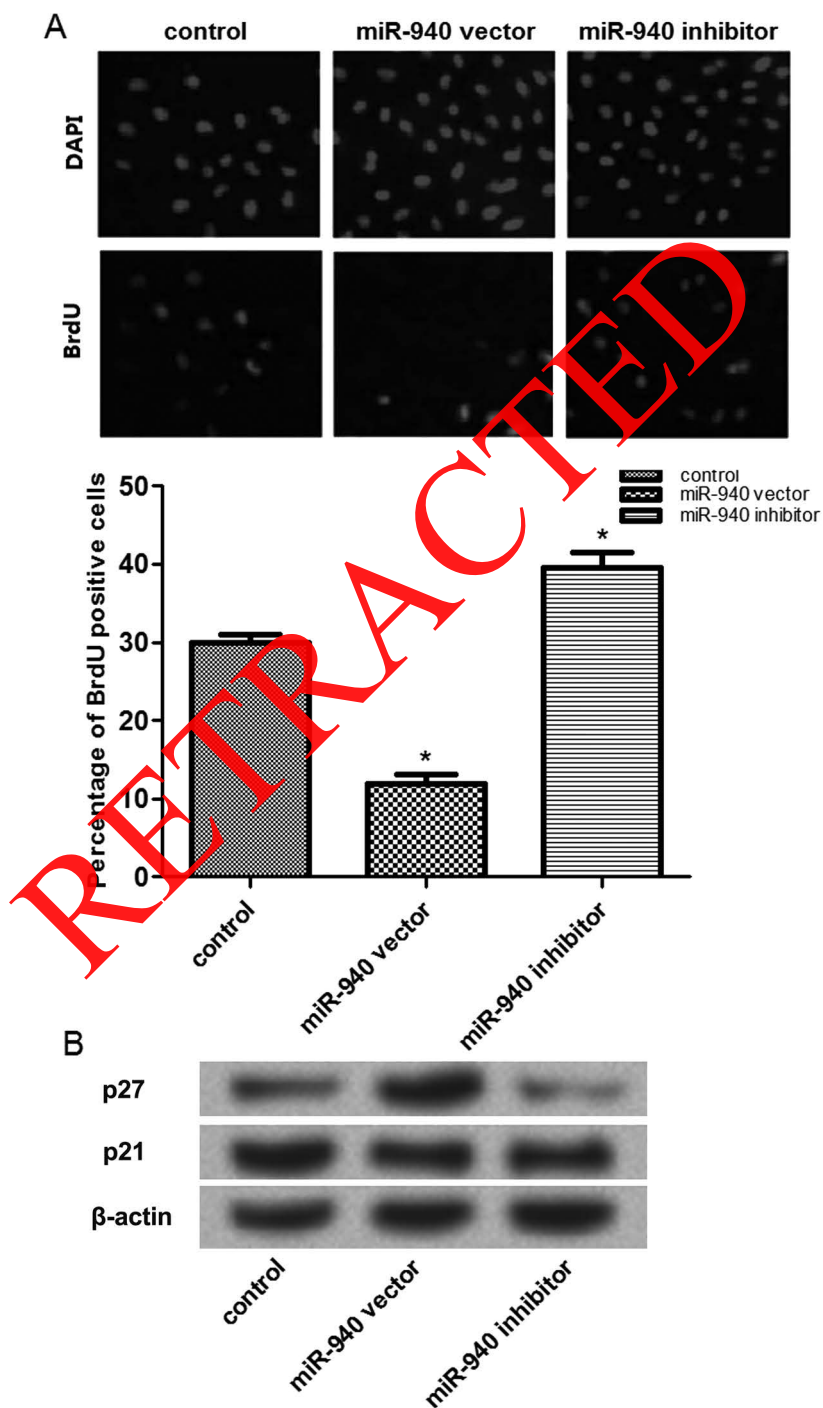


**Figure 1.** Effects of miR-940 expression on OVCAR3 cell viability. miR-940 upregulation suppressed cell viability, but miR-940 suppression promoted cell viability with increasing time.

with 1× TBST buffer for 10 min each. The signals were detected after incubation with a chromogenic substrate using the enhanced chemiluminescence (ECL) method. Additionally, GAPDH served as the internal control, and  $\beta$ -actin (Sigma-Aldrich) served as the internal control.

#### Statistical Analysis

All experiments were conducted independently three times in this study. Data were expressed as mean  $\pm$  standard error of the mean (SEM). Independent sample *t*-test was used to calculate the difference between two groups



**Figure 2.** Effects of miR-940 expression on OVCAR3 cell cycle and proliferation. (A) BrdU assay revealed that the number of OVCAR3 cells in the miR-940 upregulation group was less than that in the control. (B) miR-940 upregulation increased the protein level of p27. \* $p < 0.05$  compared to the control.

using the GraphPad Prism 5.0 software (GraphPad Prism, San Diego, CA, USA). A value of  $p < 0.05$  was defined as statistically significant.

## RESULTS

### *miR-940 Overexpression Reduced OVCAR3 Cell Proliferation*

The MTT assay was used to detect the effects of miR-940 expression on OVCAR3 cell proliferation (Fig. 1). Cell proliferation ability was increased by the miR-940 downregulation but was decreased by the overexpression of miR-940 with increasing time.

### *miR-940 Overexpression Induced Cell Growth Arrest by Upregulating p27 in OVCAR3 Cells*

Flow cytometry was used to assess the influence of miR-940 expression on OVCAR3 cells (Fig. 2). DAPI assay revealed that the total amount of cells in each group was approximately the same, namely, there was no significant difference for the number of active cells. Subsequently, we analyzed the cell proliferation using the BrdU assay, and the results showed that the percentage of positive BrdU cells was significantly decreased by the miR-940 overexpression, while it was significantly increased by the miR-940 suppression compared to the control ( $p < 0.05$ ) (Fig. 2A). Moreover, we detected the expression of p27 signal pathway-related proteins to analyze the possible mechanism of miR-940 expression on cell viability. p27 was highly expressed in the miR-940 overexpression group, while it was slightly expressed in the miR-940 suppression group (Fig. 2B). However, another major protein of the CDKN1 family member, p21, was unaffected by the expression of miR-940.

### *miR-940 Expression Was Positively Correlated to Cell Apoptosis in OVCAR3 Cells*

The effects of miR-940 expression on cell apoptosis were further analyzed using flow cytometry (Fig. 3). The apoptotic cells in the overexpressed miR-940 group was more (about 24.3%) than that in the control (about 5.3%), whereas the apoptotic cells in the miR-940 suppression group (about 4.2%) were less than that in the control group, suggesting that the overexpression of miR-940 may induce the apoptosis of OVCAR3 cells.

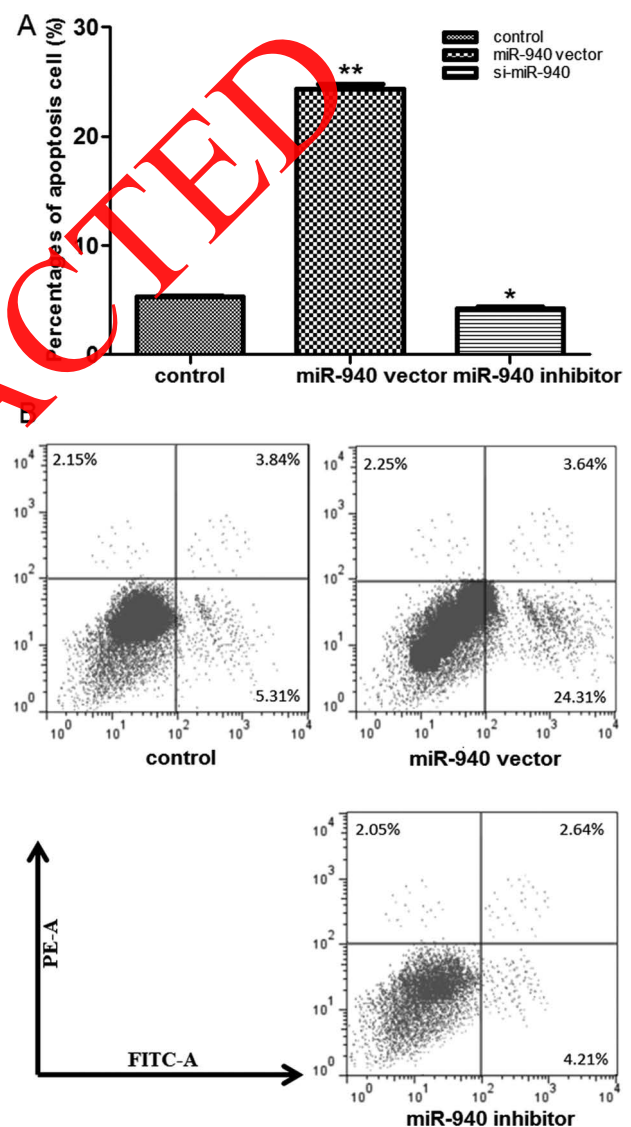
### *Influences of miR-940 Expression on PKC- $\delta$ Expression in OVCAR3 Cells*

To understand the possible mechanism of miR-940 expression on cell apoptosis, the expression of cell apoptosis-related protein in OVCAR3 cells was further detected (Fig. 4). Proteins including survivin, Hes1, procaspase 3, and active caspase 3 were highly expressed by the overexpressed miR-940, whereas their expression was significantly decreased by the miR-940 suppression.

Otherwise, expression of PKC- $\delta$  was significantly decreased by overexpression of miR-940 but was significantly increased by the suppression of miR-940.

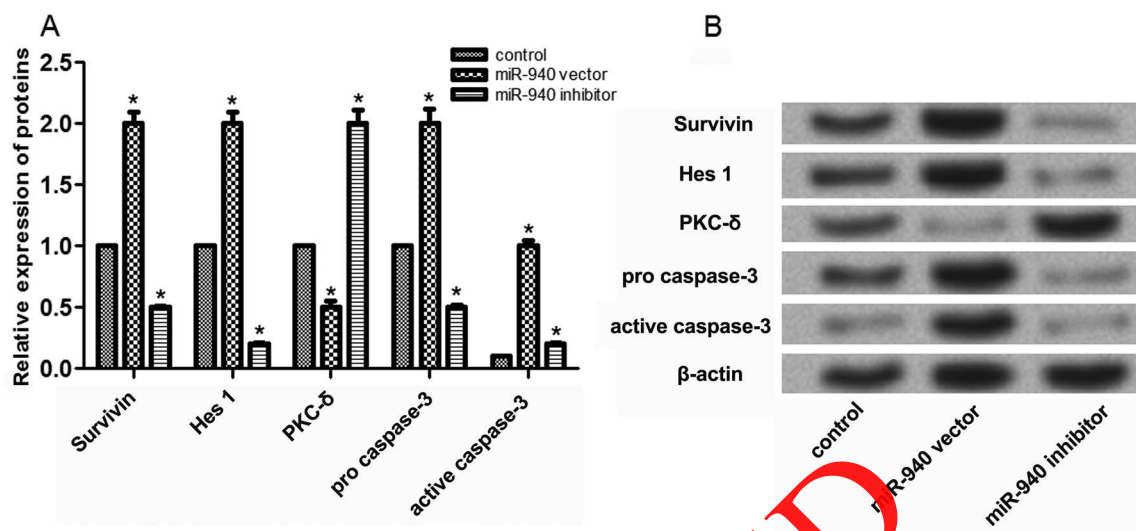
### *Knockdown of PKC- $\delta$ Reversed the Effects of miR-940 Inhibitor on Cell Viability and Cell Apoptosis*

To further confirm whether the effects of miR-940 expression on cell viability and cell apoptosis might be by targeting PKC- $\delta$ , the expression of PKC- $\delta$  was knocked down by siRNA technology, and then the effects of miR-940 inhibitor on cell viability and cell apoptosis were



**Figure 3.** miR-940 upregulation induced OVCAR3 apoptosis. (A) The percentage of apoptotic cells was increased by the overexpressed miR-940. (B) Flow cytometry analysis revealed that the apoptotic cells were increased by the overexpressed miR-940. \* $p < 0.05$  compared to the control; \*\* $p < 0.01$  compared to the control.





**Figure 4.** Influence of miR-940 expression on the expression of cell apoptosis-related protein in OVCAR3 cells. (A, B) The protein levels of Hes1, survivin, and caspase 3 were increased by the upregulation of miR-940, while the PKC- $\delta$  expression was suppressed by the overexpressed miR-940. \* $p < 0.05$  compared to the control.

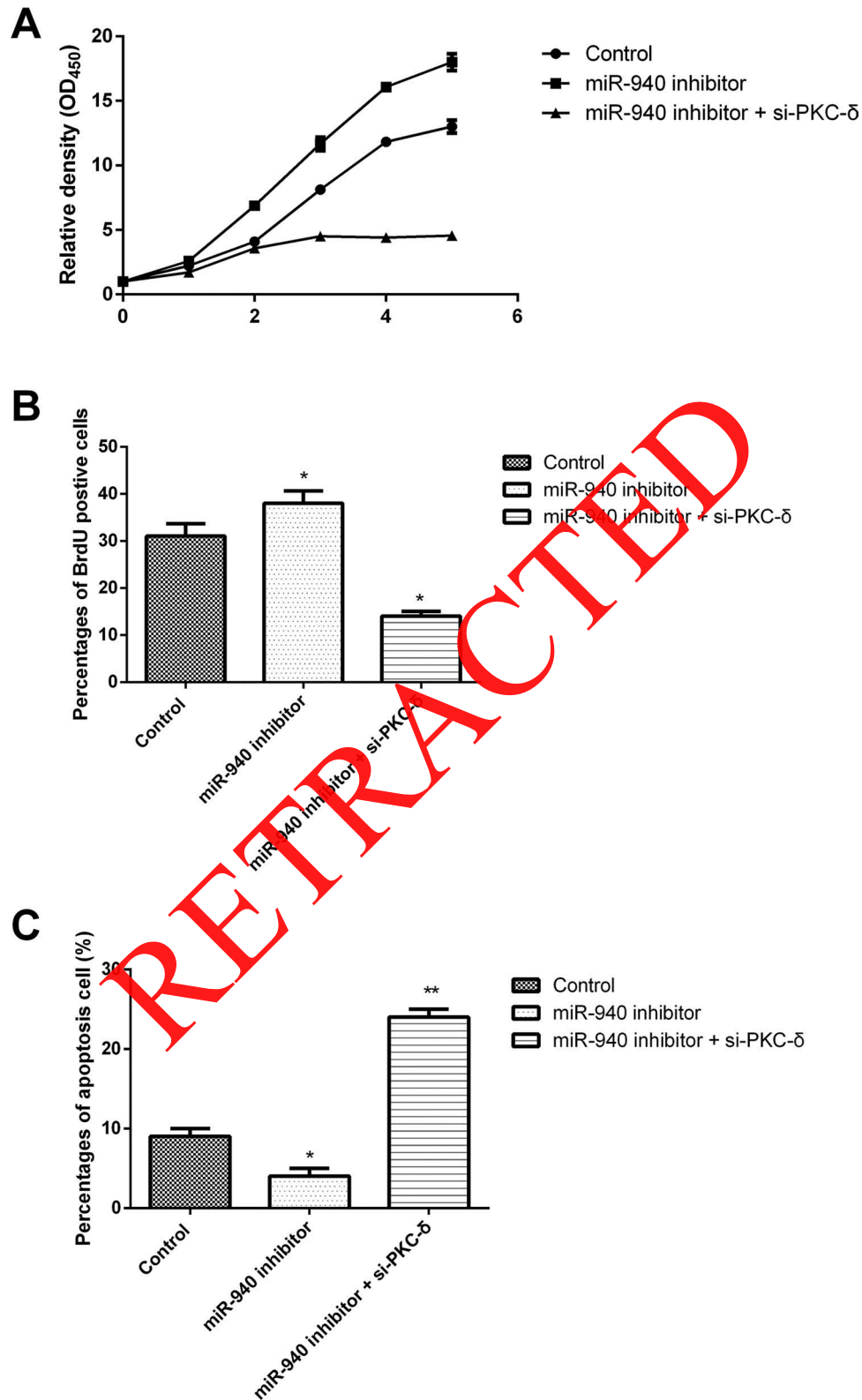
analyzed again. In Figure 5A and B, the results showed that the elevated cell viability induced by the miR-940 inhibitor was significantly decreased by knockdown of PKC- $\delta$  ( $p < 0.05$ ). Similarly, we found that the reduced percentages of apoptotic cells induced by the miR-940 inhibitor were statistically increased by knockdown of PKC- $\delta$  ( $p < 0.01$ ) (Fig. 5C). The results suggested that the effects of miR-940 expression on cell viability and cell apoptosis might be by targeting PKC- $\delta$ .

## DISCUSSION

Increasing studies have demonstrated the pivotal role of miRNAs in the molecular biology of tumorigenesis (18,19). It has been reported that miR-940 is downregulated in many kinds of cancer, such as thyroid follicular tumor and pancreatic cancer (12,20). This study investigated the potential roles of miR-940 expression on cell viability and apoptosis of the OVCAR3 cells and illustrated the possible molecular mechanism of miR-940 in the progression of ovarian cancer. Accordingly, we analyzed the effects of miR-940 abnormal expression on OVCAR3 cell viability. In agreement with previous data (11,12), our study showed that cell viability was suppressed by the overexpressed miR-940 (Fig. 1), indicating that miR-940 abnormal expression was correlated to the decrease in cell viability. In order to further verify the influence of miR-940 overexpression on inhibiting OVCAR3 cell viability, we further analyzed the number of proliferated cells by BrdU assay (Fig. 2A), and the results showed that the number of positive BrdU cells was significantly decreased by the overexpressed miR-940 compared to the control. Moreover, we analyzed the influences of miR-940 abnormal expression

on apoptosis. Hepatocellular cell apoptosis was induced by the overexpressed miR-940 (11), as well as the pancreatic ductal adenocarcinoma apoptosis (12). In this study, our results also revealed that the number of apoptotic cells was increased by the overexpressed miR-940 (Fig. 3), indicating that miR-940 upregulation induced cell apoptosis.

We further analyzed the expression of cell proliferation and apoptosis-related protein in cells from different groups. p27 (cyclin-dependent kinase inhibitor 1B) is a cell cycle protein regulator that is involved in G<sub>1</sub> arrest (21). Previous evidence demonstrated that the loss of p27 produced cell proliferation acceleration in ovarian cancer (22,23). In this study, our results showed that the protein level of p27 was significantly increased by the overexpressed miR-940 (Fig. 2B); hence, we speculated that miR-940 upregulation may suppress cell proliferation by increasing the expression of p27. Hes1 is a member of the Hes family BHLH transcription factor and is involved in cell proliferation and apoptosis (24), while survivin is another cell apoptosis-related protein (25). Hes1 was highly expressed in ovarian cancer and was correlated to the poor prognosis of ovarian cancer (26). Caldas et al. proved that survivin induced apoptosis in ovarian cancer (27). In addition, the cell apoptosis executor of caspase 3 was significantly increased by the overexpressed miR-940, which is in accordance with previous evidence (28), indicating the contributory role of miR-940 in ovarian cancer cell apoptosis. PKC- $\delta$  is a member of the PKC family of proteins, which serves as the major receptor for phorbol esters and is involved in regulating cell growth, apoptosis, and differentiation (29,30). Our study showed that the expression of Hes1,



**Figure 5.** Knockdown of PKC- $\delta$  reversed the effects of the miR-940 inhibitor on cell viability and cell apoptosis. (A, B) The elevated cell viability induced by the miR-940 inhibitor was significantly decreased by knockdown of PKC- $\delta$ . (C) The reduced percentages of apoptotic cells induced by the miR-940 inhibitor were statistically increased by knockdown of PKC- $\delta$ . \* $p < 0.05$  compared to the control; \*\* $p < 0.01$  compared to the control.

survivin, and caspase 3 was increased, whereas PKC- $\delta$  was decreased by miR-940 upregulation, implying that miR-940 may induce cell apoptosis by increasing the cell apoptosis-related proteins including Hes1, survivin, and caspase 3 and decreasing the PKC- $\delta$  signal pathway. To further confirm whether the effects of miR-940 expression on cell viability and cell apoptosis might be by targeting PKC- $\delta$ , we suppressed the expression of PKC- $\delta$  and then determined cell viability and apoptosis again. As expected, the results showed that the effects of the miR-940 inhibitor on cell viability and apoptosis were reversed by knockdown of PKC- $\delta$ .

To sum up, the data presented in this study revealed that miR-940 upregulation may play a crucial role in suppressing the progression of ovarian cancer via the involvement in cell proliferation and apoptosis by increasing p27, Hes1, survivin, and caspase 3 expression but suppressing the PKC- $\delta$  signal pathway. This study may provide a theoretical basis for the possible application of miR-940 in illustrating the molecular biology of ovarian cancer. Further studies are needed to explore the deep molecular mechanism at the transcriptional level.

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