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Upregulation of MicroRNA-34a Sensitizes Ovarian Cancer Cells to Resveratrol by Targeting Bcl-2

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Purpose: Resveratrol (REV), a natural compound found in red wine, exhibits antitumor activity in various cancers, including ovarian cancer (OC). However, its potential anti-tumor mechanisms in OC are not well characterized. Here, we tried to elucidate the underlying mechanisms of REV in OC cells.

Materials and Methods: The anti-proliferative effects of REV against OC cells were measured using CCK-8 assay. Apoptosis was measured using an Annexin V-FITC/PI apoptosis detection kit. The anti-metastasis effects of REV were evaluated by invasion assay and wound healing assay. The miRNA profiles in REV-treated cells were determined by microarray assay.

Results: Our results showed that REV treatment suppresses the proliferation, induces the apoptosis, and inhibits the invasion and migration of OV-90 and SKOV-3 cells. miR-34a was selected for further study due to its tumor suppressive roles in various human cancers. We found miR-34a overexpression enhanced the inhibitory effects of REV on OC cells, whereas miR-34a inhibition had the opposite effect in OC cells. In addition, we verified that *BCL2*, an anti-apoptotic gene, was found directly targeted by miR-34a. We also found that REV reduced the expression of Bcl-2 in OC cells. Further investigations revealed that overexpression of Bcl-2 significantly abolished the anti-tumor effects of REV on OC cells.

Conclusion: Overall, these results demonstrated that REV exerts anti-cancer effects on OC cells through an miR-34a/Bcl-2 axis, highlighting the therapeutic potential of REV for treatment of OC.

Key Words: Resveratrol, ovarian cancer, microRNA-34a, Bcl-2

INTRODUCTION

Ovarian cancer (OC) is one of the most common gynecological malignancies in women worldwide, with the highest mortality rate of all gynecologic neoplasms.[1] Although remarkable advances have been made in the treatment of this tumor type, 5-year survival rates are less than 30–50% due to recurrence and severe metastasis.[2] Accordingly, there is an urgent need to search for more effective therapeutic drugs for treating OC.

A large number of natural products have gained attention due to their potent anticancer activities.[3,4] Resveratrol (REV) (trans-3,4,5-trihydroxystilbene) is a natural phytoalexin product found in many plants, including peanuts, grapes, and red wine.[5,6] Research has indicated that REV can inhibit initiation and progression in a wide range of malignancies, including OC.[7] Clinical trials have assessed the use of REV as a cancer preventive and therapeutic agent: Zhu, et al.[8] indicated the beneficial roles of REV in breast cancer through altering mammary promoter hypermethylation. Currently, several phase I and phase II clinical trials dealing with REV have been conducted for colon cancer and lymphoma patients (according to ClinicalTrials.gov). In addition, previous studies have reported the anti-tumor efficacy of REV on OC.[9] However, underlying mechanisms need further study.

MicroRNAs (miRNAs) are single-stranded non-coding RNAs that regulate gene expression by directly binding to target mRNAs and by interfering with the translation process.[10] Re-

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cent studies have discovered that REV exerts its anti-cancer properties in various cancers through their regulation of microRNAs (miRNAs).[11] Wu, et al.[12] demonstrated that miR-326 participates in apoptosis induced by REV treatment in breast cancer and cervical cancer cells. MiR-520h-mediated forkhead box C2 (FOXC2) regulation was found to be involved in the suppression of lung cancer progression by REV.[13] REV was also found to suppress colon cancer growth and invasion by promoting miR-663 expression.[14] These observations clearly suggest that miRNAs play important roles in the anti-tumor activity of REV. However, whether miRNAs contribute to the anti-tumor activities of REV in OC remains unknown.

In this study, we investigated the anti-tumor effects of REV on OC cells and explored the potential contribution of miR-34a to the anti-tumor activities of REV in OC. Our findings provide a theoretical basis for the therapeutic effects of REV in OC treatment.

MATERIALS AND METHODS

Cell culture and treatments

OC cell lines SKOV-3 and OV-90 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Biowest, Nuaillé, France) and 1% antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in 5% incubator. Cells were treated with different concentrations of REV (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Anhui Medical University (IRB no. 2018-03-016).

Cell viability

The anti-proliferative effects of REV against SKOV-3 and OV-90 cells were measured using a Cell Counting Kit 8 (CCK-8) assay. At the end of transfection, 10 μL of CCK-8 solution (Beyotime, Jiangsu, China) was added to each well (1×10^5/well), and SKOV-3 and OV-90 cells were cultured for another 2 h. Then, optical density (OD) absorbance at 450 nm was detected using an iMark microplate reader (Bio-Rad, Hercules, CA, USA).

Cell apoptosis

Apoptosis was measured using an Annexin V-FITC/PI apoptosis detection kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. Finally, cell apoptosis was measured on a FACScan flow cytometer (FCM; Bechman Coulter, Fullerton, CA, USA), and the data were analyzed by FlowJo 8.7.1 software (TreeStar Inc., Ashland, OR, USA).

Wound healing assay

SKOV-3 and OV-90 cells (2×10^6/well) were seeded in 6-well plates overnight to allow cells to attach. The cell monolayer was scratched with a 10-μL pipette tip, and then, the wound area was measured at 0 h and 24 h under a fluorescence microscope (Olympus Corp., Tokyo, Japan). Finally, the migration distances were calculated using Image J analysis software version 1.46 (National Institute of Health).

Invasion assay

We used transwell chambers (8-μm pore; BD Biosciences, San Jose, CA, USA) coated with Matrigel (BD Biosciences) for invasion assay. Briefly, a total of 8×10^4 OC cells was added in the top chamber, while the lower chamber was treated with DMEM/F12 containing 20% FBS. After incubation for 24 h by REV treatment, the cells were stained and photographed with a CKX41 inverted microscope (Olympus Corp.).

miRNA microarray

Total RNA was isolated from SKOV-3 cells treated with or without REV using an miRNeasy kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. The samples were assessed using miRCURY LNA™ Array v. 18.0 (Agilent, Santa Clara, CA, USA). The procedure and imaging processes were described previously.[15]

qRT-PCR

Total RNA was extracted from cells with an miRNeasy isolation kit (Qiagen) according to the manufacturer's protocol. For the synthesis of cDNA, 1 μg of extracted RNAs was transcribed using the PrimeScript reverse transcription reagent kit (Takara). Real-time PCR was performed using an miScript SYBR® Green PCR Kit (Exiqon; Qiagen) on an ABI 7300 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers for qRT-PCR analysis were as follows: miR-34a forward: 5'-CCCAGAACATAGACACGCTGGA-3'; miR-34a reverse: 5'-ATCAGCTGGGCACCTAGGACA-3'; U6 forward: 5'-TGCGGGTGCTCGCTTCGCAGC-3'; U6 reverse: 5'-CCAGTGCAGGGTCCGAGGT-3'. The thermo cycle was conducted at 95°C (1 min) and 40 cycles of 95°C (30 s), 58°C (30 s), and 68°C (3 min/kb), followed by 68°C (10 min). The relative expression levels were calculated based on the $2^{-\Delta\Delta}$Ct method.[16] U6 was set as an internal control.

Cell transfection

The miR-34a mimics/inhibitor and corresponding negative control were designed from GenePharma Co., Ltd. (Shanghai, China). SKOV-3 and OV-90 cells that had reached 80% confluence in 6-well plates were transfected with 20-nmol miR-34a mimics/inhibitor and 2 μg of pcDNA-Bcl-2 using Lipofectamine® 2000 (Invitrogen). Subsequently, the cells were treated with REV for 24 h and utilized for further analysis.

Dual-luciferase reporter assays

TargetScan Release 7.0 (http://targetscan.org/) and Miranda (http://miranda.org.uk) were used to search for putative targets

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of miR-34a. The dual-luciferase reporter assay was performed as described previously.[17] HEK-293T cells were co-transfected with 20-nM miR-34a mimics/inhibitor and 400 ng of either pGL-Bcl-2-3'-UTR or pGL-Bcl-2-mut-3'-UTR using Lipofectamine 2000 (Invitrogen). At 48 h post-transfection, luciferase activities were measured with a dual luciferase reporter kit (Beyotime Institute of Biotechnology). Renilla activity was used to normalize Firefly luciferase activity.

Immunofluorescence assay

After treatment, cells were fixed with absolute alcohol for 30 min, followed by incubation in a solution containing primary antibodies specific for Bcl-2 (cat no.#15071; Cell Signaling Technology, Danvers, MA, USA, 1:2000 dilution) at room temperature. Subsequently, the cells were incubated with an Alexa fluorescein-labeled secondary antibody for 1 h at 37°C. The cells on slides were mounted with buffer containing DAPI (5.0 μg/mL). Immunofluorescence was detected using a fluorescence microscope (Bx51, Olympus) at 200× magnification.

Western blot analysis

Western blot was performed as previously described.[18] Briefly, 40 μg of protein sample was separated by 12% SDS-PAGE gel and then transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA). Subsequently, these membranes were blocked with 5% skim milk for 2 h at room temperature and then incubated with primary antibodies against Bcl-2 (cat no.#15071; Cell Signaling Technology, 1:2000 dilution) and β-actin (cat no.#3700; Cell Signaling Technology, 1:2000 dilution) at 4°C overnight. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (cat no.#8887; Cell Signaling Technology, 1:2000) were used as secondary antibodies. All antibodies were obtained from Cell Signaling Technology. The bands were detected using an enhanced chemiluminescence kit (GE Healthcare, Freiburg, DE, Germany). The intensities of bands of interest were analyzed using Image J analysis software version 1.46 (National Institute of Health).

Statistical analysis

Statistical analysis was performed using Prism 7 (GraphPad Software, Inc., San Diego, CA, USA). All data are presented as a mean±standard deviation. Student's t-test was used for intergroup comparisons. Continuous data from multiple groups were calculated by one-way analysis of variance, followed by Tukey's post-hoc test. $p<0.05$ was considered statistically significant.

RESULTS

REV exhibits an anti-tumor effect in both SKOV-3 and OV-90 cells

To explore the anti-tumor effects of REV on OC cells, we first investigated the effect of REV on cell viability using CCK8 assay. As shown in Fig. 1A, REV suppressed the cell viability of SKOV-3 and OV-90 cells in a dose dependent manner. The half inhibitory concentration (IC50) values of REV against SKOV-3 and OV-90 cells were 163.31±11.09 μM and 132.26±15.34 μM, respectively. Based on these results, we found that low-dose REV pretreatment partially inhibited cell proliferation, and therefore, the optimal treatment dose of REV (100 μM) was used in subsequent experiments. Next, we determined whether the decreased cell viability caused by REV was due to induction of apoptosis. The results showed that compared with controls, REV markedly promoted the apoptosis of SKOV-3 and OV-90 cells (Fig. 1B and C). These data suggest that REV may inhibit OC cell proliferation by inducing apoptosis.

Next, the influences of REV on cell migration and invasion were also examined using wound healing assay and transwell assay, respectively. Wound healing assay indicated that REV significantly inhibited the migration of SKOV-3 and OV-90 cells, compared with controls (Fig. 1D). Transwell assay showed that the number of cells that invaded into transwell chambers was significantly decreased by REV in both SKOV-3 and OV-90 cells, compared with controls (Fig. 1E). All data suggest that REV could affect the migration and invasion of OC cells.

REV increases the expression of miR-34a in OC cells

Previous studies have indicated that REV exhibits an anti-tumor role in several types of cancers through modulation of miRNAs.[12] Thus, we performed a microarray analysis to determine miRNA levels in OC cells after REV treatment. The data revealed that compared with controls, 30 miRNAs were significantly up-regulated and that 26 miRNAs were markedly down-regulated (Fig. 2A). Among them, miR-34a, a well-known tumor suppressor, was the most significantly up-regulated after REV treatment. Previous studies have revealed that REV inhibits human colorectal cancer cell growth and induces apoptosis by up-regulating miR-34a.[19] Meanwhile, it has been reported that miR-34a exerts suppressive effects on OC cells. Therefore, miR-34a was selected for further study. To further verify the results of miRNA microarray analysis, the expression levels of miR-34a was measured by qRT-PCR in SKOV-3 and OV-90 cells treated with different concentrations of REV. As expected, REV elicited dose-dependent increases in miR-34a expression levels in OC cells (Fig. 2B and C). All data suggested that miR-34a may be involved in the anti-tumor role of REV in OC cells.

miR-34a overexpression enhances the anti-tumor effects of REV in OC cells

To further examine whether REV exerts its anti-tumor effects by regulating miR-34a, miR-34a mimics was transfected to SKOV-3 and OV-90 cells 24 h prior to REV treatment. As shown in Fig. 3A, miR-34a mimics notably increased miR-34a levels in both SKOV-3 and OV-90 cells. Using CCK-8 assay, we found that miR-34a overexpression significantly reduced the cell viability of

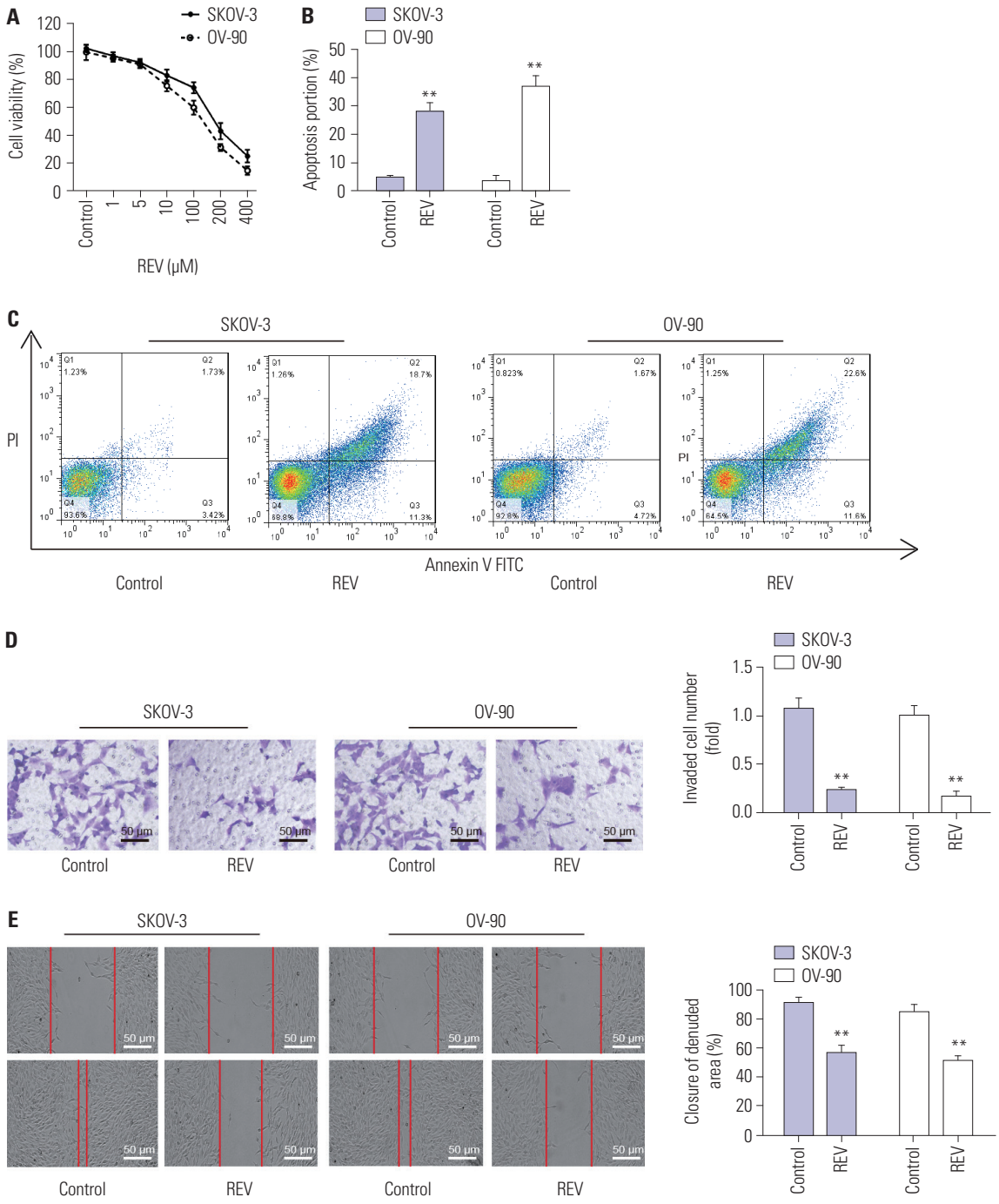

Fig. 1. REV inhibits cell viability, migration, and invasion and induces cell apoptosis in ovarian cancer cells. (A) SKOV-3 and OV-90 cells were treated with REV (0, 5, 10, 100, 200, and 400 μM) for 24 h, after which cell viability was measured by CCK-8 assay. (B) SKOV-3 and OV-90 cells were treated with 100 μM REV, and flow cytometry analysis was performed to determine apoptotic cells. (C and D) Cell invasion was detected by transwell assay in SKOV-3 and OV-90 cells after 100 μM REV treatment. (E) Cell migration was assessed by wound healing assay in SKOV-3 and OV-90 cells after 100 μM REV treatment. Data are represented as the mean±SD of three individual experiments. $^{**}p<0.01$ vs. controls. REV, resveratrol.

SKOV-3 and OV-90 cells, compared with REV group (Fig. 3B). Additionally, the induction of cell apoptosis caused by REV was significantly enhanced by miR-34a overexpression in SKOV-3 and OV-90 cells (Fig. 3C). Further investigation showed that miR-34a overexpression also markedly decreased the invasion and migration in SKOV-3 and OV-90 cells, compared with REV group (Fig. 3D and E). These findings suggested that overexpression of miR-34a enhances the anti-tumor effects of REV in OC cells.

miR-34a inhibition alleviates the anti-tumor effects of REV in OC cells

Next, we added miR-34a inhibitor into SKOV-3 and OV-90 cells to assess the effects of miR-34a knockdown on the anti-tumor

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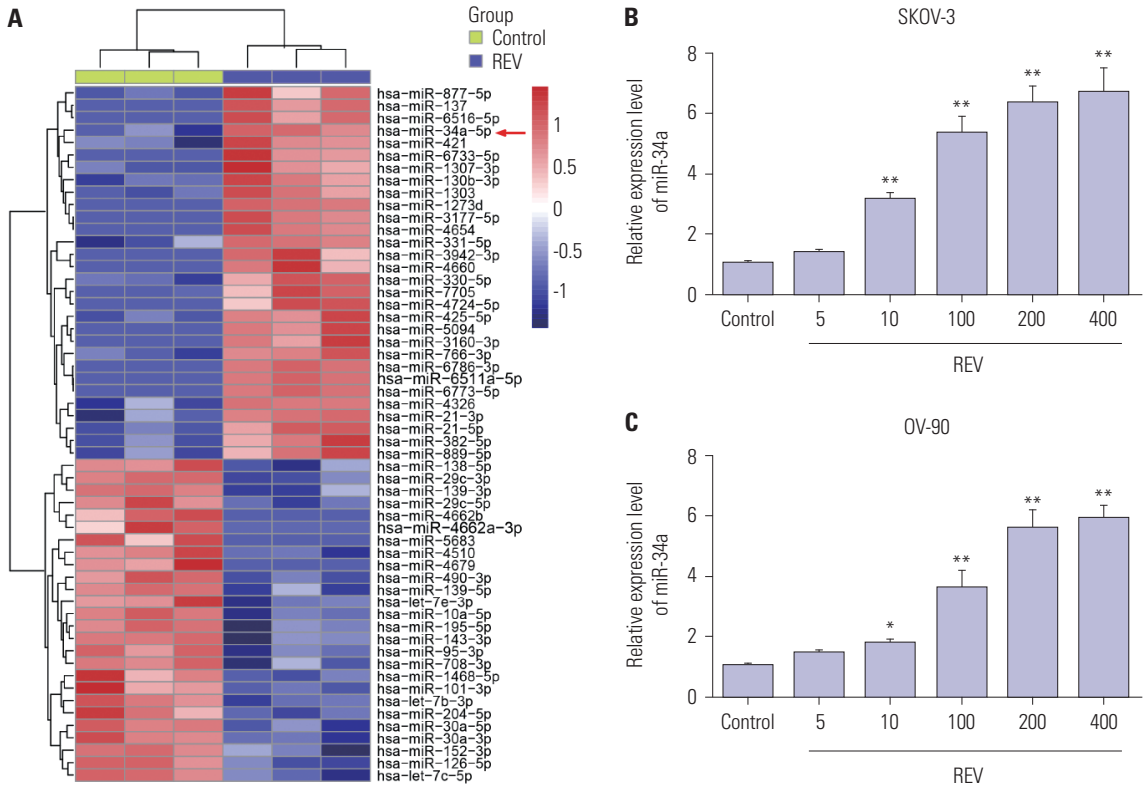

Fig. 2. REV increases the expression of miR-34a in ovarian cancer cells. (A) SKOV-3 cells were treated with REV (100 μM) for 24 h, and microarray analysis was used to determine miRNA levels. A heat map shows significant expressional changes in miRNAs in SKOV-3 cell treated with REV. The color code is linear within the heat map: blue represents the lowest level of expression and red the highest. The miRNAs that were upregulated are shown from blue to red, whereas the miRNAs that were downregulated are shown from red to green. (B and C) The SKOV-3 and OV-90 cells were treated with 5-400 μM of REV for 24 h, and qRT-PCR was conducted to determine miR-34a expression. Data are presented as the mean±SD of three individual experiments. *p<0.05, **p<0.01 vs. control. REV, resveratrol.

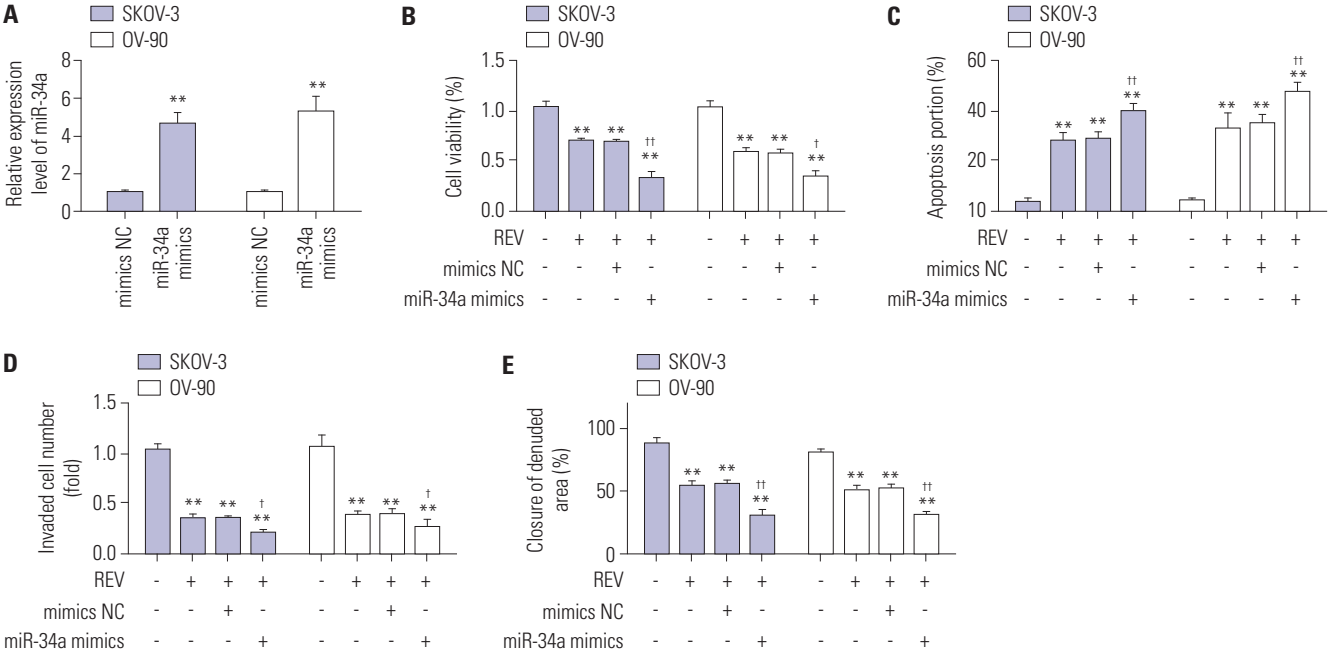

Fig. 3. miR-34a overexpression enhances the anti-tumor effects of REV in ovarian cancer cells. The SKOV-3 and OV-90 cells were transfected with miR-34a mimics or mimic NC for 24 h, followed by treatment with 100 μM REV for 24 h. (A) The expression of miR-34a was measured by qRT-PCR. (B) Cell viability was detected by CCK-8 assay. (C) Flow cytometric analysis was performed to determine apoptotic cells among SKOV-3 and OV-90 cells. (D) Cell invasion was detected by transwell assay in SKOV-3 and OV-90 cells. (E) Cell migration was assessed by wound healing assay in SKOV-3 and OV-90 cells. Data are presented as the mean±SD of three independent experiments. **p<0.01 vs. control, $^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01 vs. REV group. REV, resveratrol.

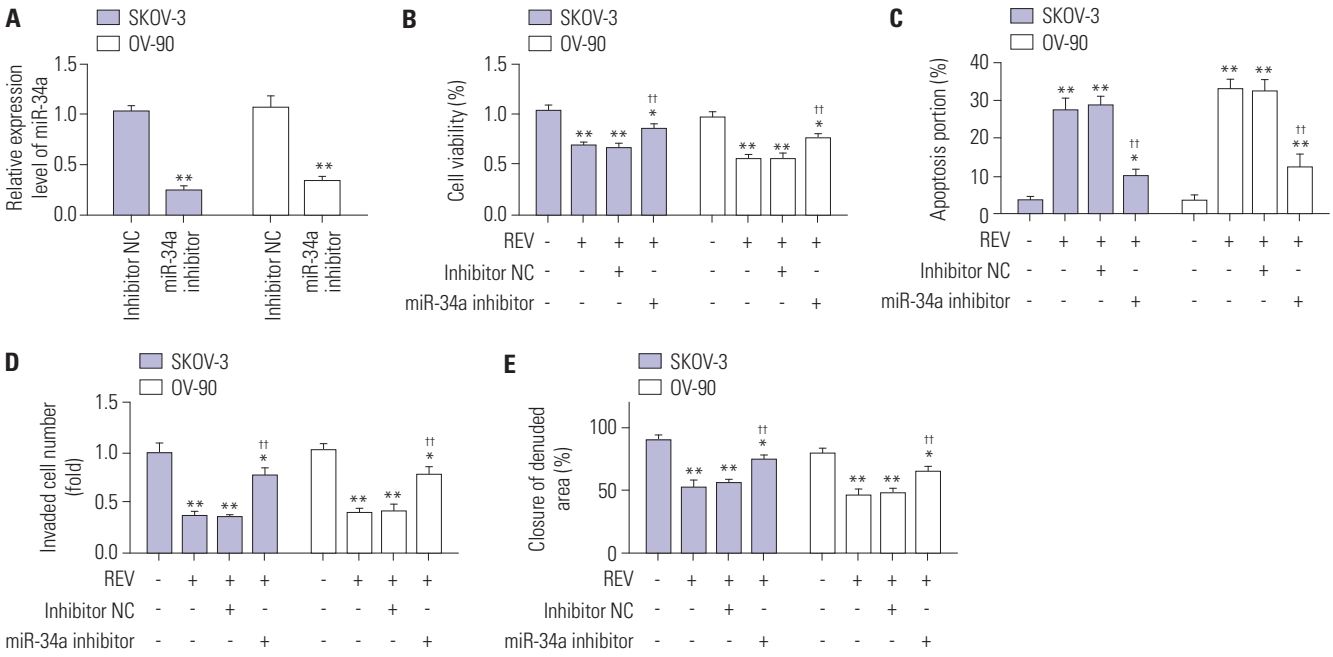

Fig. 4. miR-34a inhibition attenuated the anti-tumor effects of REV in ovarian cancer cells. The SKOV-3 and OV-90 cells were transfected with miR-34a inhibitor or inhibitor NC for 24 h, followed treatment with 100 μM REV for 24 h. (A) The expression of miR-34a was measured by qRT-PCR. (B) Cell viability was detected by CCK-8 assay. (C) The flow cytometric analysis was performed to determine apoptotic cells among SKOV-3 and OV-90 cells. (D) Cell invasion was detected by transwell assay in SKOV-3 and OV-90 cells. (E) Cell migration was assessed by wound healing assay in SKOV-3 and OV-90 cells. Data are presented as the mean±SD of three independent experiments. *p<0.05, **p<0.01 vs. control, $^{\dagger\dagger}p$<0.01 vs. REV group. REV, resveratrol.

effects of REV in OC cells. As shown in Fig. 4A, miR-34a expression was notably decreased in both SKOV-3 and OV-90 cells after transfection with miR-34a inhibitor. The CCK-8 and flow cytometry assays showed that miR-34a inhibition attenuated the anti-proliferative and pro-apoptotic effects of REV in SKOV-3 and OV-90 cells (Fig. 4B and C). The transwell and wound healing assays revealed that miR-34a inhibition alleviated the anti-invasive and anti-migratory effects of REV in SKOV-3 and OV-90 cells (Fig. 4D and E). All these results suggested that REV exerts its anti-tumor activity by promoting miR-34a expression in OC cells.

Bcl-2 targeted by miR-34a in OC cells

In order to elucidate the mechanisms by which miR-34a inhibits tumorigenesis, TargetScan Release 7.0 (http://targetscan.org/) and Miranda (http://miranda.org.uk) online software were employed to identify the target mRNAs of miR-34a.[10] We found a putative target site of miR-34a in the 3'-UTR of Bcl-2 mRNA (Fig. 5A). To verify whether miR-34a directly binds to Bcl-2, we performed a dual luciferase reporter assay. As shown in Fig. 5B, miR-34a mimics markedly inhibited the luciferase activity of the Bcl-2-3'UTR Wt reporter, whereas co-transfection with the miR-34a inhibitor and Wt reporter resulted in increased luciferase activity; however, no evident changes were observed after co-transfection of Bcl-2 3'-UTR-Mut with miR-34a mimics or inhibitor. To further confirm whether Bcl-2 is regulated by miR-34a, the protein expression of Bcl-2 was measured by Western blot. Upon doing so, we discovered that the protein levels of Bcl-2 were significantly decreased by miR-34a mimics, but increased by miR-34a inhibitor in SKOV-3 cells (Fig. 5C). To further investigate whether REV affects Bcl-2 expression in OC cells, we measured the protein levels of Bcl-2 in SKOV-3 cells after treatment of REV at different concentrations using Western blot and indirect immunofluorescence assay, respectively. The results showed that REV suppresses the expression of Bcl-2 protein in a dose-dependent manner (Fig. 5D and E). All these results suggested that REV may inhibit Bcl-2 expression by upregulating miR-34a.

Overexpression of Bcl-2 reverses the anti-tumor effects of REV in OC cells

As mentioned above, Bcl-2 was regulated by REV in OC cells; therefore, we further investigated whether REV exerts its anti-tumor effects by downregulating Bcl-2. The Bcl-2 expression vector pcDNA-Bcl-2 was transfected into SKOV-3 and OV-90 cells for 24 h, which notably increased the expression of Bcl-2 (Fig. 6A). Functionally, the reduction of cell viability caused by REV was partly abrogated by overexpression of Bcl-2 in SKOV-3 and OV-90 cells (Fig. 6B). Subsequently, the induction of apoptosis by REV was reversed by overexpression of Bcl-2 in SKOV-3 and OV-90 cells (Fig. 6C). We also found that the reductions in invasion and migration induced by REV were markedly attenuated by Bcl-2 overexpression in SKOV-3 and OV-90 cells (Fig. 6D and E). These data suggested that REV exerts anti-tumor effects by downregulating Bcl-2. In conclusion, our results revealed that REV inhibits OC cell proliferation and invasion and

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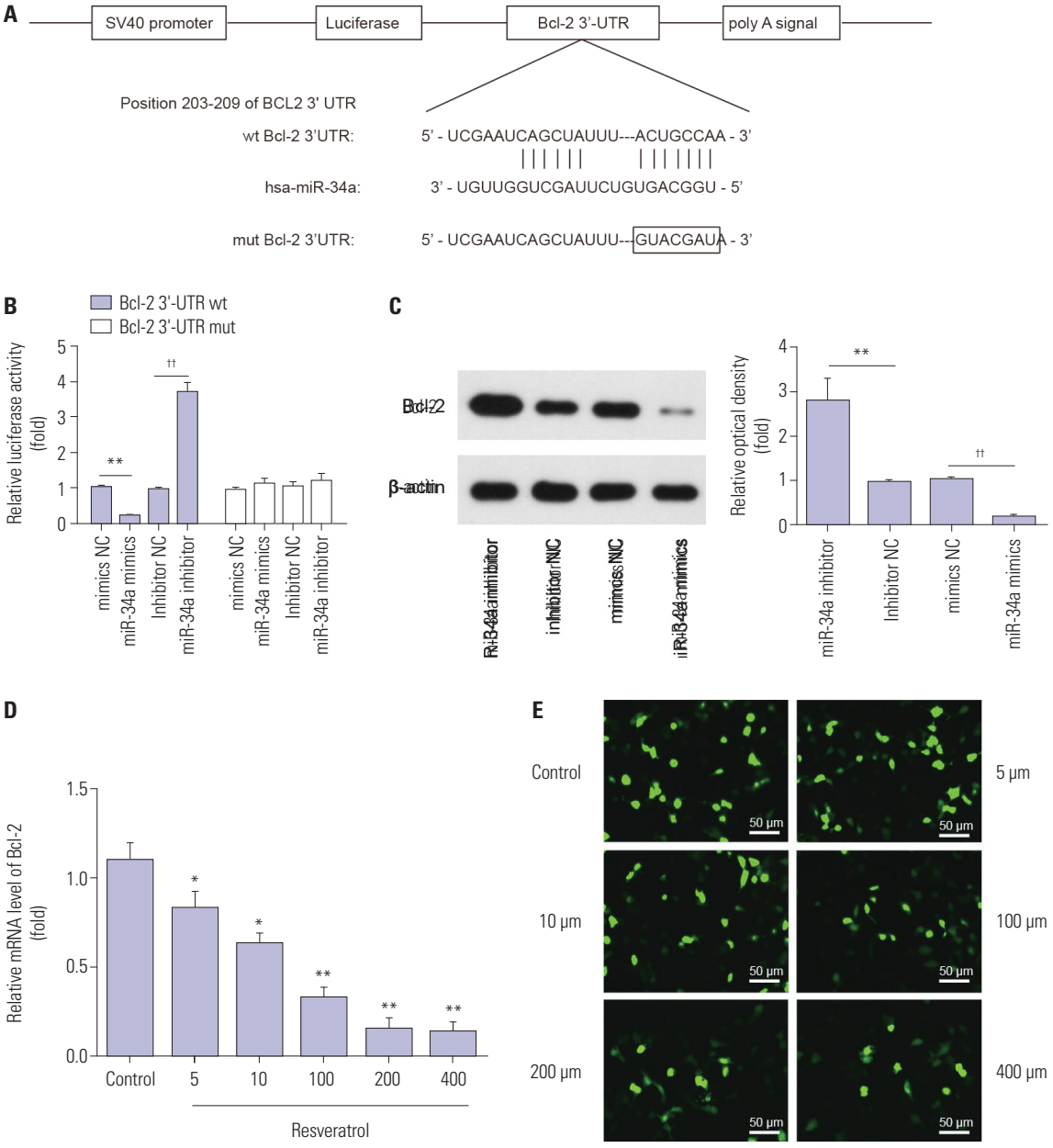

Fig. 5. Bcl-2 is a direct target of miR-34a in ovarian cancer cells. (A) The predicted miR-34a binding sites on Bcl-2. (B) Luciferase reporter assay was conducted to detect luciferase activity of SKOV-3 cells co-transfected with miR-34a mimics, miR-34a inhibitor or miR-NC, and WT-Bcl2-3'-UTR or MUT-Bcl2-3'-UTR. (C) Western blot analysis was performed to determine the protein levels of Bcl-2 in SKOV-3 cells transfected with miR-34a mimics, miR-34a inhibitor, or miR-NC. $^{**}p<0.01$ vs. mimics NC, $^{\dagger\dagger}p<0.01$ vs. inhibitor NC. (D and E) The SKOV-3 cells were treated with 5-400 μM of REV for 24 h, after which Western blot and indirect immunofluorescence assays were conducted to determine Bcl-2 expression. Data represent the mean±SD of three individual experiments. $^{*}p<0.05$, $^{**}p<0.01$ vs. control.

promotes cell apoptosis through an miR-34a/Bcl-2 axis (Fig. 7)

REV suppresses Bcl-2 signaling by upregulating miR-34a expression in OC cells

Since miR-34a is involved in the anti-cancer effect of REV in OC and since Bcl-2 is a direct target of miR-34a, we further investigated whether miR-34a sensitizes OC cells to REV by inducing Bcl-2 signaling. As expected, REV treatment significantly decreased the expression levels of Bcl-2 and markedly increased the accumulation of Bax and cleaved caspase 3 in SKOV-3 and OV-90 cells. However, treatment with REV and miR-34a inhibitor promoted the expression of Bcl-2 and reduced the expression of Bax and cleaved caspase 3 in SKOV-3 and OV-90 cells (Fig. 7). All these data suggested that REV could activate apoptotic pathway by suppressing Bcl-2-dependent signaling via the upregulation of miR-34a expression in OC cells (Fig. 8).

DISCUSSION

In the present study, we revealed the REV inhibits cell proliferation, induces cell apoptosis, and suppressed the migration and invasion of OC cells, demonstrating that it is a highly potent anti-tumor agent. Moreover, we discovered that an miR-34a/

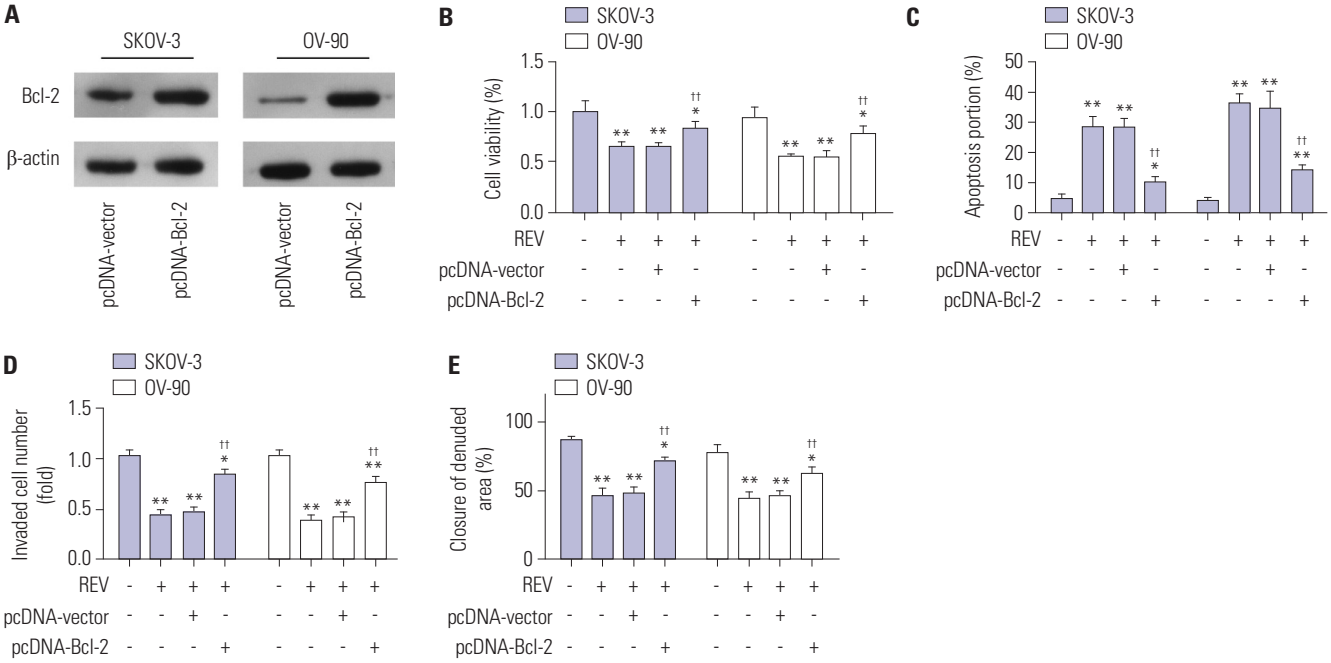

Fig. 6. Bcl-2 overexpression reverses the anti-tumor effects of REV in ovarian cancer cells. The SKOV-3 and OV-90 cells were transfected with pcDNA-Bcl-2 or pcDNA-vector for 24 h, followed by treatment with 100 μM REV for 24 h. (A) The expression of Bcl-2 was measured by Western blot. (B) Cell viability was detected by CCK-8 assay. (C) Flow cytometric analysis was performed to determine apoptotic cells among SKOV-3 and OV-90 cells. (D) Cell invasion was detected by transwell assay in SKOV-3 and OV-90 cells. (E) Cell migration was assessed by wound healing assay in SKOV-3 and OV-90 cells. Data represent the mean±SD of three independent experiments. *p<0.05, **p<0.01 vs. control, ††p<0.01 vs. REV group. REV, resveratrol.

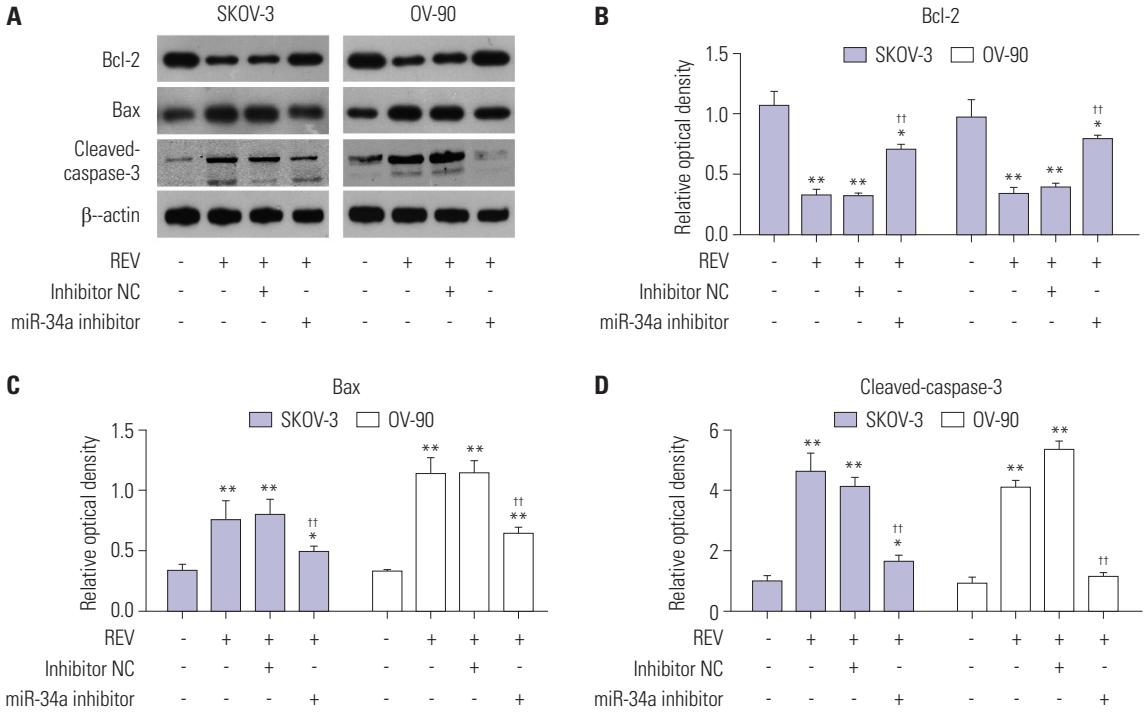

Fig. 7. REV suppresses Bcl-2 signaling by upregulating miR-34a expression in ovarian cancer cells. SKOV-3 and OV-90 cells were transfected with miR-34a inhibitor or inhibitor NC for 24 h, followed by treatment with 100 μM REV for 24 h. (A-D) The expression levels of Bcl-2, Bax, and cleaved caspase 3 were measured by Western blot. Data represent the mean±SD of three independent experiments. *p<0.05, **p<0.01 vs. control, ††p<0.01 vs. REV group. REV, resveratrol.

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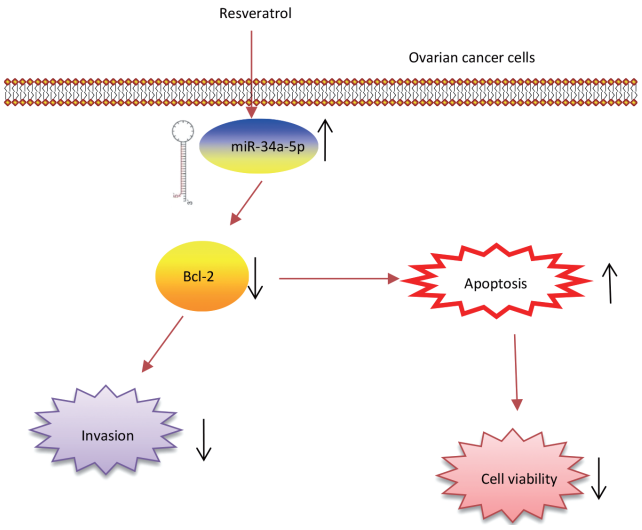

Fig. 8. Schematic diagram of the molecular mechanism by which REV exerts its anti-tumor effects in OC cells. REV inhibits OC cell proliferation, invasion and migration and promotes cell apoptosis through an miR-34a/Bcl-2 axis. REV, resveratrol; OC, ovarian cancer.

Bcl-2 axis mediates the anti-tumor effects of REV in OC cells. These findings may highlight a novel molecular mechanism underlying the anti-tumor effects of REV.

REV is a nonflavonoid polyphenol that exerts antibacterial, anti-inflammatory, and immunoregulatory effects.[20] It was well-widely recognized to have antitumor activity in connection with the lung, gastric, prostate, and breast cancer.[21,22] Furthermore, the use of REV has been reported in phase I trials in colon cancer and phase II trials in lymphoma patients.[23] Another study from Patel, et al.[24] measured concentrations of REV in colorectal cancer patients who ingested REV daily for 8 days at 0.5 g or 1.0 g and found that highest mean concentrations of parent REV in plasma was 22.3 nmol/mL. In our study, we found that the IC50 values of REV were 163.31±11.09 μM and 132.26± 15.34 μM in SKOV-3 and OV-90 cells, respectively. Although our effective concentration of REV was higher than that found in the serum of clinical colorectal cancer patients, REV is still a potential antitumor agent. More importantly, the anticancer doses (100 μM to 200 μM) of REV have little harmful effect on glial cells and neurons in the central nervous system and transitional epithelial cells of the urinary bladder.[25,26] These data indicate that REV may be a potential therapeutic agent in the treatment of OC.

The anticancer efficacy of REV on OC cells has been documented previously. Liu, et al.[27] showed that REV induces apoptosis and inhibits cell migration and invasion in OC cells via impaired glycolysis. However, limited information is available regarding the molecular mechanism of REV against OC. Increasing evidence has reported that the antitumor effect of REV is well regulated by miRNAs. For example, REV ameliorates the invasive and migratory abilities of pancreatic cancer cells by suppressing miR-21 expression.[28] Venkatadri, et al.[29] showed that several miRNAs have key roles in REV-mediated effects on cell apoptosis in breast cancer cells. Recent studies have uncovered that several miRNAs play important roles in the development of OC, such as miR-182, miR-338, and miR-590.[30-32] In accordance with our microarray analysis, miR-34a was significantly increased in REV-treated OC cells. The miR-34 family is composed of three members, miR-34b, -34c, and -34a, and has been reported to regulate both tumor cell apoptosis and proliferation in multiple studies.[33-35] A polymorphism in miR-34b/c has been found to be closely associated with an increased risk of hepatocellular carcinoma.[36] Notably, miR-34a/b/c has recently been found to be downregulated in OC tissues and has been linked to worse overall survival and progression free survival in patients with OC.[37] Interestingly, within the miR-34 family, miR-34a has been well studied in several cancers, with marked effects on cell proliferation, apoptosis and invasion.[38-40] Also, miR-34a was documented to function as a tumor suppressor in OC.[41] Yet, whether REV exhibits its suppressive role in OC through regulation of miR-34a remains unclear. Therefore, we selected miR-34a for further studies. In our study, we observed that REV increased the expression of miR-34a in OC cells, suggesting that high miR-34a expression might be associated with anti-tumor effects of REV in OC cells. Our study further demonstrated that miR-34a upregulation enhances the anti-tumor activity of REV, while the knockdown of miR-34a attenuated the anti-tumor activity of REV in OC cells. Collectively, these data suggest that miR-34a is a viable target of REV for mediating its antitumor actions against OC.

BCL2 is a well-known anti-apoptotic gene, and the miR-34a/Bcl-2 axis has been shown to play important roles in a wide variety of human tumors. For example, miR-34a overexpression inhibited the proliferative and invasive of HCT116 cells by targeting Bcl-2.[42] Similarly, in meningioma, miR-34a-3p modulated cell proliferation and apoptosis through regulation of Bcl-2.[43] Of note, in OC, miR-34a suppressed cell proliferation and induced apoptosis by regulating Bcl-2.[44] Thus, we sought to determine whether miR-34a/Bcl-2 axis mediates the antitumor activity of REV in OC. Firstly, Bcl-2 was identified as a direct target of miR-34a, and its translation was suppressed by miR-34a in OC cells. We also found that REV treatment dose-dependently increased the expression levels of Bcl-2 protein in OC cells, indicating REV may regulate Bcl-2 expression through miR-34a. Finally, we found that Bcl-2 overexpression reversed the anticancer effects of REV, which is similar with the role of miR-34a inhibition in OC cells. Taken together, our results suggest that REV exerts its antitumor activity through regulation of the miR-34a/Bcl-2 pathway in OC cells.

However, there are some limitations to the present study. First, epithelial OC (EOC) constitutes at least five different histological subtypes, including serous cystadenocarcinoma, mucinous, endometrioid, clear cell, and mixed phenotype tumors.[45] In addition to their distinct morphological appearance and clinical differences, there is molecular evidence for heterogene-

ity between different EOC subtypes.[46] Moreover, previous study has indicated that the anticancer efficiency of drugs and biomarker profiles are different for histologic subtypes of EOC.[47] In this study, we only focused on two OC cell lines: SKOV-3 represents clear-cell adenocarcinoma type and OV-90 represents high-grade serous type. In the future, we will further investigate the inhibitory effects of REV on OC cells of each histologic type.

In conclusion, our results revealed that REV inhibits OC cell proliferation and invasion and promotes cell apoptosis through the miR-34a/Bcl-2 axis. To our knowledge, this is the first report on the effects of REV as a miRNA activator in OC cells. These data also indicated that miR-34a is a potential candidate for combination therapy of miRNA and REV in cancers. Moreover, our data suggest that miRNA may increase the sensitivity of tumor cells to drugs, suggesting a new direction to solve the problem of clinical drug resistance. Our findings suggest that REV could be a potent agent for treating OC.

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AUTHOR CONTRIBUTIONS

Conceptualization: Shangli Yao and Bing Wei. Data curation: Shangli Yao, Ming Gao, and Zujun Wang. Formal analysis: Shangli Yao and Wenyan Wang. Funding acquisition: Bing Wei. Investigation: Shangli Yao and Bing Wei. Methodology: Shangli Yao and Ming Gao. Project administration: Lei Zhan and Bing Wei. Resources: Wenyan Wang, Lei Zhan, and Bing Wei. Software: Zujun Wang, Wenyan Wang, and Lei Zhan. Supervision: Lei Zhan and Bing Wei. Validation: Zujun Wang and Bing Wei. Visualization: Shangli Yao and Ming Gao. Writing—original draft: Shangli Yao. Writing—review & editing: Shangli Yao, Ming Gao, and Bing Wei. Approval of final manuscript: all authors.

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