

## Original Article

# miR-125b expression in ovarian cancer cell lines

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**Abstract:** Puerarin, a well-known isoflavone-C-glucoside isolated from *Pueraria radix*, has been demonstrated the anticancer activity in animal models as well as proliferation inhibition and apoptosis induction in a variety of cancer cell lines in vitro. However, the effect and mechanisms of puerarin on ovarian cancer (OC) have not been well understood. This study aimed to test whether puerarin could inhibit growth of ovarian cancer cells and reveal its underlying molecular mechanism. Cells viability was evaluated using cell counting Kit-8 in SKOV-3 and OVCAR-3 cells. Apoptosis of ovarian cancer cells was analyzed by flow cytometry. Bcl-2 levels were analyzed by Western blot and microRNAs levels were determined by real-time RT-PCR. Our study showed that puerarin inhibited SKOV-3 and OVCAR-3 cells growth in a concentration-dependent manner. Puerarin induced apoptosis by promoting expression of miR-125b, and then inhibiting expression of Bcl-2, a known target for miR-125b. Moreover, knockdown of miR-125b could reverse the reduction of cell viability induced by puerarin in SKOV-3 and OVCAR-3 cells. Taken together, our study unraveled the ability of puerarin to suppress ovarian cancer growth and elucidated the participation of miR-125b in the anti-cancer action of puerarin. Our findings suggest that puerarin can be considered to be a promising anticancer agent for ovarian cancer.

**Keywords:** Ovarian cancer, puerarin, miR-125b, proliferation, apoptosis

### Introduction

Ovarian cancer is the fifth leading cause of cancer-related death worldwide and is still the most lethal gynecologic malignancy among women [1]. Despite improved surgical technology and chemotherapy, the 5-year survival rate of patients with advanced ovarian cancer is only 25-35% [2]. One reason for this high mortality rate is lack of an effective early detection method for ovarian cancer. To improve therapeutics of ovarian cancer, it is important to explore the molecular mechanisms of ovarian cancer pathogenesis and to develop novel effective drugs for the treatment.

Currently, some natural bioactive phytochemicals are used to impede proliferation or metastasis of cancer cells [3]. Puerarin, a well-known isoflavone-C-glucoside, has been identified as a major constituent in *Pueraria radix* [4]. It has been shown to have beneficial effects on liver disease (cirrhosis) [5, 6], cardiovascular [7, 8],

neurological [9, 10], anti-platelet aggregation [11] and hyperglycemic disorders [12]. Recent studies have demonstrated that puerarin induces anticancer activity in animal models as well as proliferation inhibition and apoptosis induction in a variety of cancer cell lines in vitro [13]. Extensive studies have demonstrated that puerarin suppresses the growth of Human colon carcinoma cell line HT-29 [13]. However, limited studies have paid attention to the therapeutic effects and underlying mechanisms of puerarin on ovarian cancer.

MicroRNAs (miRNAs) are a kind of endogenous single-stranded RNAs of 19-25 nucleotides (~22nt) in length that regulate gene expression by multiple mechanisms [14]. Emerging evidence suggests that miRNAs play important roles in the development and progression of cancer, such as nasopharyngeal carcinoma, breast cancer, colorectal cancer and so on [15, 16]. A lot of studies over the comparison between normal ovary tissue and ovarian can-

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cer tissues in miRNA expression showed that there were significant changes of miRNA in ovarian cancer tissue [17, 18]. Lee et al. found that miR-125b inhibited ovarian cancer cells through post-transcriptional inactivation of EIF4EBP1 [19]. However, whether miR-125b participates in the anti-cancer effects of puerarin is unknown.

In this study, we evaluated whether puerarin could exert anti-tumor effects on ovarian cancer cells and further investigated the detailed mechanisms underlying this process. Considering the important roles of miR-125b in carcinogenesis, we investigated whether miR-125b contributes to the anti-cancer effect of puerarin.

### Materials and methods

#### *Patient samples*

All samples were handled and made anonymous according to the ethical and legal standards. Paired tissue specimens (tumor and adjacent normal tissues) from 20 patients with ovarian cancer were obtained and histologically confirmed by a pathologist at Department of Obstetrics and Gynecology, Anhui Provincial Hospital, China.

#### *Reagents and antibodies*

Puerarin was obtained from Nanjing Zelang Pharmaceutical Co., Ltd, China, and was dissolved in DMSO to prepare a 200  $\mu$ M stock solution which was stored at  $-20^{\circ}\text{C}$  prior to use. CCK-8 and DMSO were purchased from Sigma Chemical (St. Louis, MO, USA). Antibodies against Bcl-2 and  $\beta$ -actin were purchased from Cell Signaling technologies (Danvers, MA). Rabbit antibodies conjugated with horseradish peroxidase (HRP) and sheep anti-mouse-HRP were purchased from Zhongsanjinqiao (Beijing, China). All others chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

#### *Cell culture and treatments*

Ovarian cancer cell line SKOV-3, OVCAR-3, HO8901 and Caov-3 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), and cultured in DMEM/F12 (Invitrogen, Carlsbad, CA) containing 10% fetal

bovine serum (FBS) (Biowest, Nuaille, France), 100 U/ml penicillin, and 100 mg/ml streptomycin (Hyclone). All cell lines were maintained in a humidified atmosphere of 5%  $\text{CO}_2$ /air at  $37^{\circ}\text{C}$ . The cells treated with the indicated concentrations of puerarin were collected at 24 h, 36 h and 48 h for further measurements. Immortalized normal ovarian surface epithelial cell line IOSE80 was obtained as a generous gift from the laboratory of Dr Nelly Auersperg (The University of British Columbia, Vancouver, Canada). The cells were grown in a 1:1 combination of two media, Medium 199 (Invitrogen) and MCDB 105 (Cell Applications Inc., San Diego, CA) with 10% FBS in a humidified atmosphere containing 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

#### *Transfection*

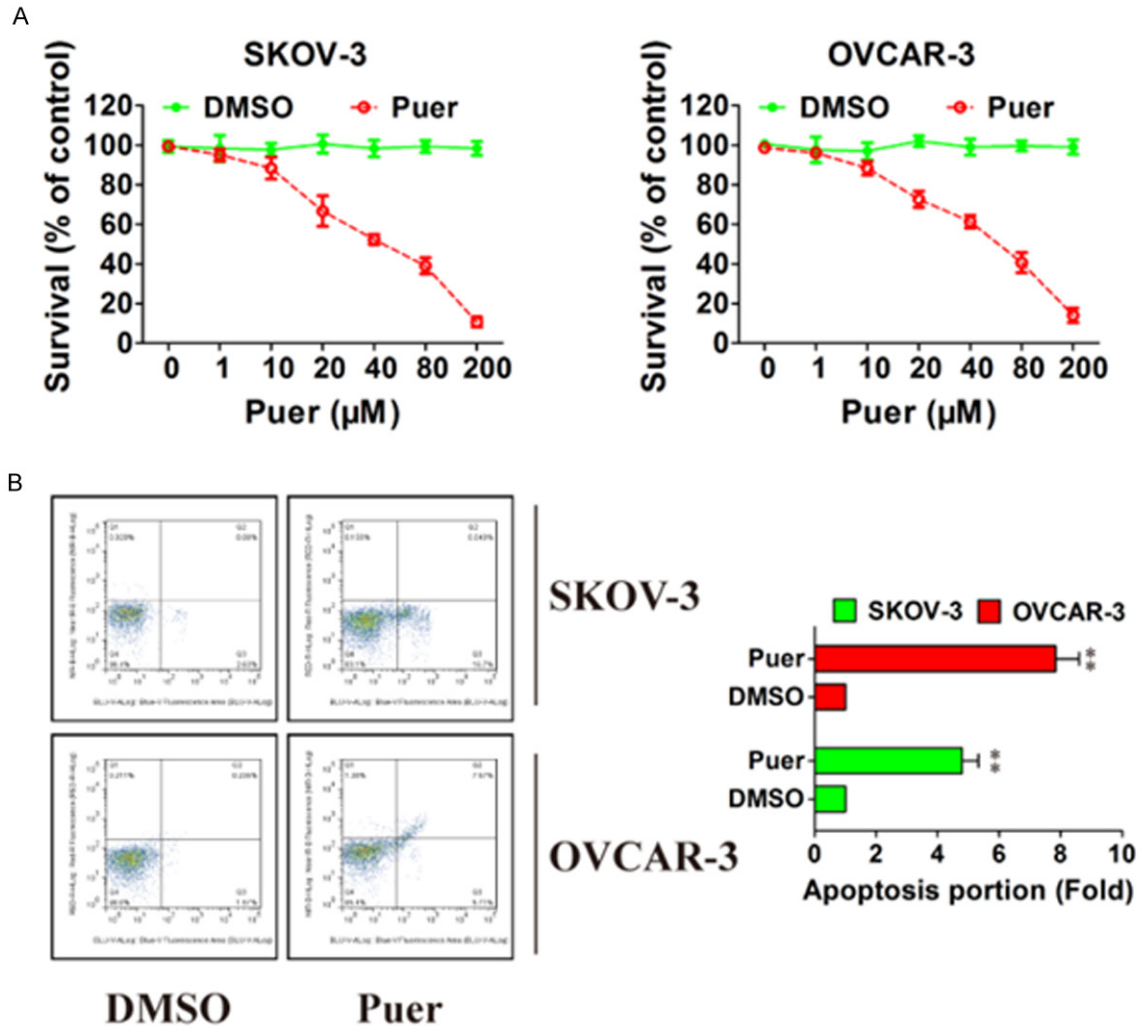
The DNA fragment encoding miR-125b mimics and miR-125b inhibitor were purchased from GenePharma (Shanghai, China). Cells were transfected with miR-125b inhibitor, miR-125b mimic or the nonspecific control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

#### *Cell proliferation assay*

Cells were seeded in 96-well plates at  $0.8-1 \times 10^3$  per well. Cell proliferation was evaluated using Cell Counting Kit-8 (Beyotime, China) according to the manufacturer's instructions. Briefly, 10  $\mu$ l of CCK-8 solution was added to culture medium, and incubated for 2 h. The absorbance at 450 nm wavelength was determined with a reference wavelength of 570 nm.

#### *Quantitative real-time RT-PCR analysis*

Total mRNA was extracted from ovarian cancer tissues and cultured cell lines using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The expression of mature miR-125b was quantified by real-time PCR using the miScript SYBR Green PCR Kit which contained 10 $\times$ miScript Universal Primer (Qiagen) and was performed according to the manufacturer's protocol. Quantization of U6 was used to normalize miRNA expression level. Real-time PCR was carried out in the Bio-Rad IQ5 amplification system (Bio-Rad, USA) and the results were calculated using  $\Delta\text{CT}$  method. Primer sequences were: U6 forward, 5'-gcttcggcagcacatataactaaaat-3'. U6 reverse, 5'-cgcttcacgaatttgcgtgtcat-3'. miR-125b RT pri-



**Figure 1.** The effect of Puerarin on ovarian cancer cell growth and apoptosis. A. Effects of different concentration of Puerarin on cell viability of SKOV-3 and OVCAR-3 cells, as assessed by CCK-8 assay. B. Puerarin increases apoptosis of SKOV-3 and OVCAR-3 cells, as assessed by flow cytometry. Data represent the mean  $\pm$  SD of three independent experiments, \* $P < 0.01$ , compared to the DMSO group.

mer: 5'-gtcgtatccagtgagggtccgaggtattcgactg-gatcagactcaca-3'. miR-125b forward, 5'-gc-cctccctgagacctcaa-3'. miR-125b reverse, 5'-gt-gcagggtccgaggt-3'.

*Cell apoptosis detection by flow cytometry*

Cell apoptosis was analyzed using an Annexin V-FITC/PI apoptosis detection kit (Beyotime Institute of Biotechnology China). The SKOV-3 and OVCAR-3 cells were seeded in 6-well plates overnight and then treated with puerarin at indicated concentration for 48 h. The following steps were conducted as the manufacturer's instruction. The cells were subjected to Annexin V-FITC/PI staining and analyzed using flow cytometer [20].

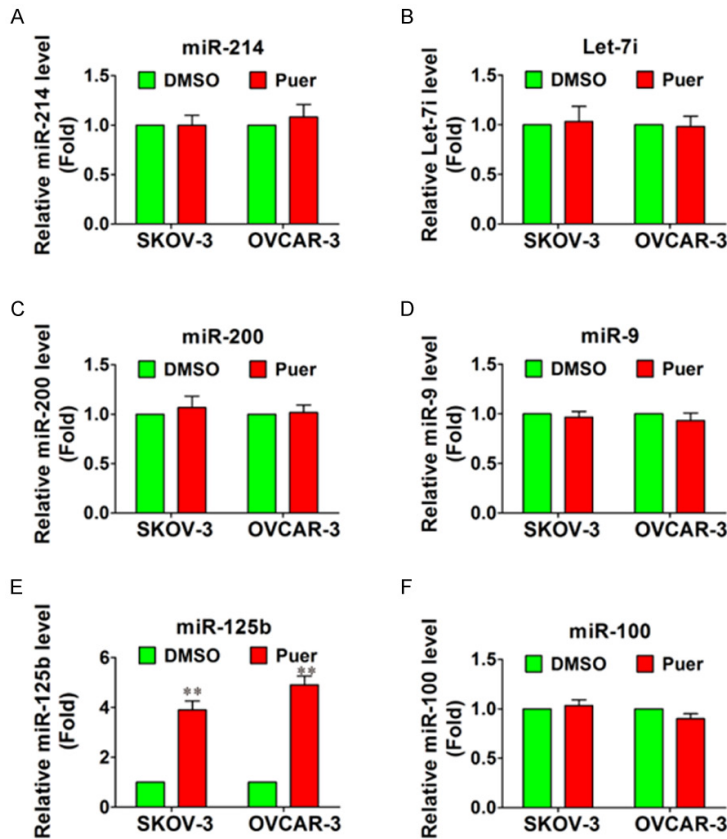
*Western blot analysis*

Protein extracts from SKOV-3 and OVCAR-3 cells were subjected to 10% SDS-PAGE and subsequently transferred to a PVDF membrane. This was followed by probing with mouse primary antibodies against Bcl-2. Anti- $\beta$ -actin antibody was used as an internal control. The immunoreactivity was detected using Odyssey Infrared Imaging System and analyzed using Odysseysoftware (Infrared Imaging System LI-COR Biosciences) [21].

*Statistical analysis*

Statistical analyses were performed with SPSS 13.0 software. The results were evaluated by  $\chi^2$

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**Figure 2.** Puerarin treatment increased miR-125b in OC cells. A-F. miR-214, let-7i, miR-200, miR-9, miR-125b, miR-100 mRNA levels were detected by qRT-PCR in puerarin treated SKOV-3 and OVCAR-3 cells respectively. GAPDH gene served as an internal control. The relative gene expression was calculated using  $2^{-\Delta\Delta Ct}$  method. Data represent the mean  $\pm$  SD of three independent experiments, \*\* $P < 0.01$ , compared to the DMSO group.

test and the other data were evaluated by Student's t-test and expressed as the mean  $\pm$  SD from three independent experiments. A  $P$ -value of less than 0.05 was considered statistically significant.

### Results

#### *Puerarin inhibited cell proliferation and induced cell apoptosis in OC cells*

To investigate the effect of puerarin on the growth of OC cells, we conducted CCK-8 assay to measure cell survival of SKOV-3 and OVCAR-3 cells after puerarin treatment for 72 h. Our results showed puerarin treatment caused cell proliferation inhibition in a dose-dependent manner in both SKOV-3 and OVCAR-3 cells (**Figure 1A**). The  $IC_{50}$  value of puerarin after 72 h of incubation was 40.2  $\mu$ M and 56.7  $\mu$ M for

SKOV-3 and OVCAR-3 cells, respectively. In the following studies, we chose puerarin with the concentration of 50  $\mu$ M for further investigation.

To determine whether apoptosis contributes to cell growth inhibition, flow cytometry assay was applied to detect apoptotic rate of SKOV-3 and OVCAR-3 cells treated with puerarin. As shown in **Figure 1B**, the percentage of apoptosis cells was increased by puerarin treatment in both SKOV-3 and OVCAR-3 cells. It suggests that the puerarin may induce cell apoptosis of ovarian cancer cells.

#### *Puerarin promotes expression of miR-125b*

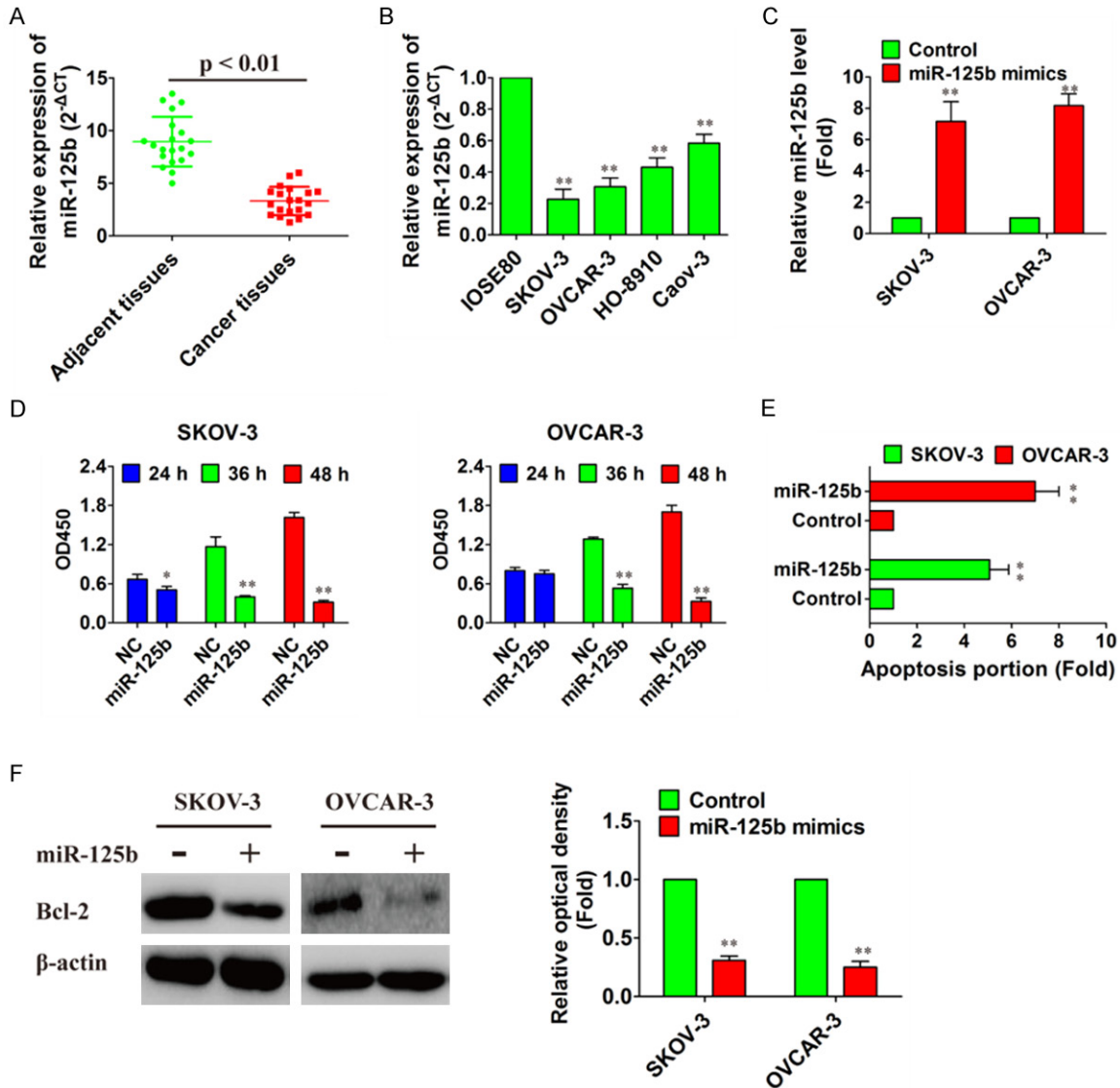
Studies have revealed that kinds of miRNA involved in tumorigenesis and the progression of ovarian cancer. To investigate whether puerarin treatment could regulate the expression of miRNA in OC cells, we selected six miRNAs and analyzed their expression using quantitative real-time polymerase chain reaction (qRT-PCR). As shown in **Figure 2E**, qRT-PCR analysis indicated that

puerarin treatment caused a significant elevation of miR-125b expression in both SKOV-3 and OVCAR-3 cells ( $P < 0.05$ ), whereas the expression of miR-124, let-7i, miR-200, miR-9 and miR-100 have no significant change (**Figure 2A-F**,  $P > 0.05$ ). In the following studies, we chose miR-125b for further investigation.

#### *Enforced expression of miR-125b inhibited cell proliferation in OC cells*

To assess the role of miR-125b in ovarian cancer progression, we first examined the miR-125b expression levels in ovarian cancer tissues and ovarian cancer cell using quantitative real-time PCR. We verified that miR-125b expression levels were significantly downregulated in ovarian cancer tissues and ovarian cancer cell lines (**Figure 3A**). Subsequently, we also detected the expression of miR-125b in 4

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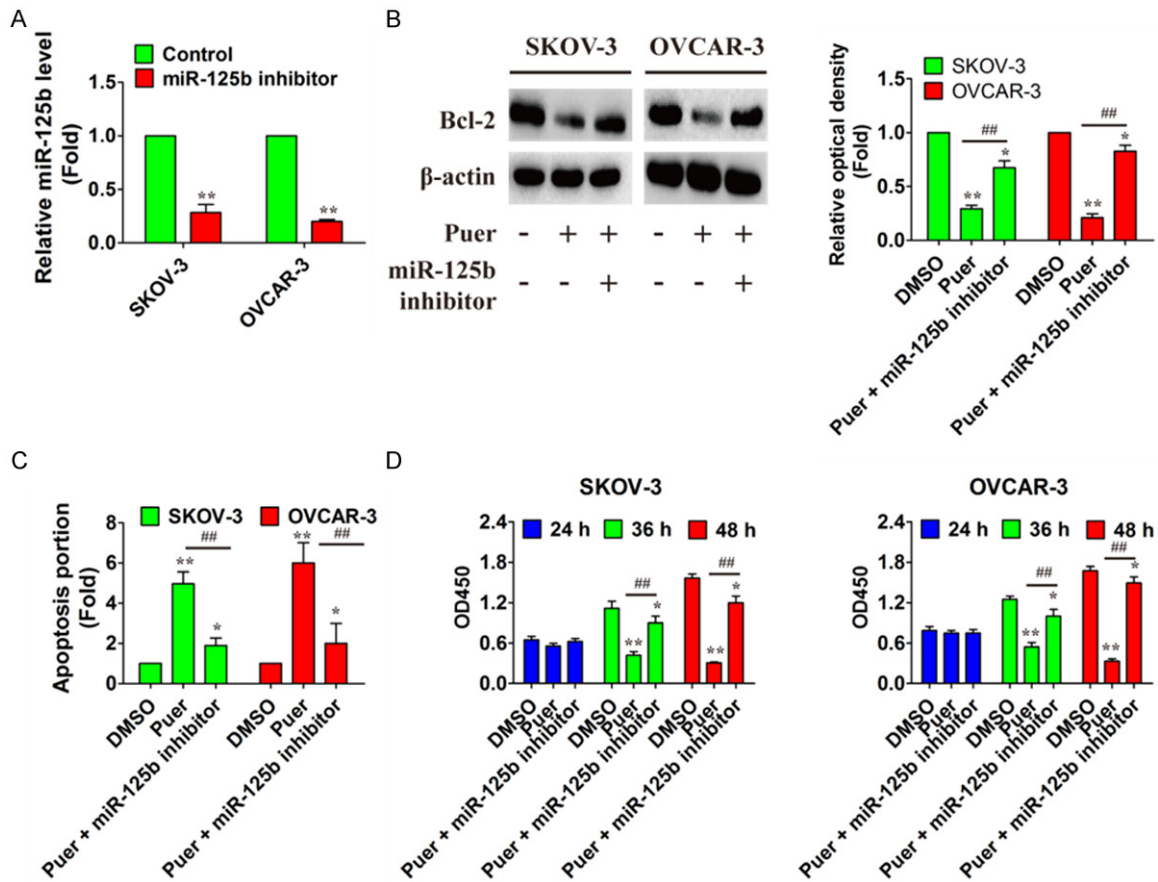


**Figure 3.** Overexpression of miR-125b inhibits cell proliferation in OC cells. A. miR-125b is down-regulated in ovarian cancer samples compared with adjacent samples, as measured by real-time PCR. N=20, \*\*P<0.01 vs. adjacent samples. B. miR-125b expression was measured by real-time PCR in four ovarian cancer cell lines (SKOV-3, OVCAR-3, HO8901 and Caov-3). The relative expression of miR-125b was normalized to the endogenous control U6. Each sample was analyzed in triplicate. (\*\*P<0.01). C. miR-125b expression in SKOV-3 and OVCAR-3 cells, following transfection with miR-125b mimic or control, was determined by qRT-PCR (\*\*P<0.01). D. Forced expression of miR-125b inhibits cell viability in SKOV-3 and OVCAR-3 cells, as assessed by CCK-8 assay. E. Overexpression of miR-125b increases apoptosis of SKOV-3 and OVCAR-3 cells, as assessed by flow cytometry. F. Effects of miR-125b on the expression of Bcl-2 protein in SKOV-3 and OVCAR-3 cells. Dates represent mean ± SD of three independent experiments (\*\*P<0.01).

human ovarian cancer cell lines including SKOV-3, OVCAR-3, HO8901 and Caov-3 using qRT-PCR. Immortalized normal ovarian surface epithelial cell line IOSE80 used as a negative control. Low expression of miR-125b in ovarian cancer cell lines were observed, especially in SKOV3 and OVCAR-3 cells (Figure 3B).

To examine the effect of miR-125b on cell proliferation, we transfected miR-125b mimics into SKOV3 and OVCAR-3 cells (Figure 3C). Indeed, we observed that miR-125b mimics decreased the cell viability and increased percentage of apoptotic cells in SKOV3 and OVCAR-3 cell cells (Figure 3D, 3E). And, forced expression of miR-

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**Figure 4.** Knock down of miR-125b alleviates the inhibition of cell proliferation induced by puerarin. A. miR-125b expression in SKOV-3 and OVCAR-3 cells, following transfection with miR-125b inhibitor or control, was determined by qRT-PCR (\*\* $P < 0.01$ ). B. Inhibition of miR-125b reverses dysregulation of Bcl-2 protein caused by puerarin in SKOV-3 and OVCAR-3 cells. C. Knock down of miR-125b alleviates the raise of cell apoptosis induced by puerarin in SKOV-3 and OVCAR-3 cell. D. Knock down of miR-125b mitigates the reduction of cell viability induced by puerarin in SKOV-3 and OVCAR-3 cell. Dates represent mean  $\pm$  SD of three independent experiments (\* $P < 0.05$ , \*\* $P < 0.01$  vs. Control; ## $P < 0.01$  vs. Pure).

125b by transfection markedly reduced the level of Bcl-2 protein, a known target for miR-125b (Figure 3F). These data suggest that upregulated expression of miR-125b is related to the progression of ovarian cancer.

### Knockdown of miR-125b alleviates inhibition of cell proliferation induced by puerarin

To examine whether the up-regulation of miR-125b contributes to the puerarin-induced cell inhibition in SKOV-3 and OVCAR-3 cells, we knocked down the expression of miR-125b by transfection of its specific inhibitor, miR-125b inhibitor, and then observed the alteration of puerarin-induced inhibition in OC cells (Figure 4A). The combination of miR-125b inhibitor transfection and puerarin treatment reversed

the dysregulation of Bcl-2 caused by puerarin alone in SKOV-3 and OVCAR-3 cells and alleviates the proapoptotic action of puerarin (Figure 4B and 4C). Moreover, the combination of miR-125b inhibitor transfection and puerarin treatment restored the reduction of cell viability induced by puerarin alone (Figure 4D). These results suggested that puerarin induced cell proliferation partly due to up-regulation of miR-125b in OC cells.

### Discussion

In the present study, we demonstrated that puerarin could inhibit the growth of ovarian cancer cell via up-regulating the expression of miR-125b. And, we investigated the altered expression pattern of miR-125b in ovarian can-

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cer cells and its indication of cancer cell apoptosis through suppression of Bcl-2 expression. Moreover, we found knock down of miR-125b could alleviate puerarin-induced proliferation. These findings should provide a new insight into the use of puerarin in the treatment of ovarian cancer.

Several reports revealed that puerarin exerted an intriguing role in inducing cell apoptosis and suppressing cell proliferation especially in tumor cells. More evidences have found that puerarin inhibited cell growth through inducing cell cycle arrest in breast cancer and glioblastoma cells [22, 23]. And, recent studies have found that puerarin can inhibit the adhesion, invasion and migration of HO-8910 cells, plays an antagonist effect against the stimulation of estrogen on the malignant behavior of tumor cells [24]. But there is little literature material about its antitumor property on ovarian cancer cells. Our data reinforce this anti-tumor action of puerarin by showing that the ability of puerarin to inhibit ovarian cancer cells growth, suggesting that puerarin could be used for the treatment of ovarian cancer.

Dysregulated expression of miRNAs has been observed in numerous human cancers including ovarian cancer. Fu X et al. found that miR-613 overexpression suppressed ovarian cancer cell proliferation, colony formation, and invasion by directly targeting KRAS [25]. A study from Sun KX et al. demonstrates that miR-186 may sensitize ovarian cancer cell to paclitaxel and cisplatin by targeting ABCB1 and modulating the expression of GST- $\pi$  [26]. Modulation of miRNA levels by a number of agents has been demonstrated to function as cancer chemopreventive agents, such as curcumin, isoflavone genistein, reversionerol and so on [27-29]. However, it is unknown whether miRNAs mediated the anti-cancer effect of puerarin.

MiR-125b is a tumour-suppressing miRNA downregulated in several cancers [30, 31]. For example, miR-125b was found to suppress the oncoproteins MUC1, ERBB2, and ERBB3, inhibiting the growth of breast cancer cells [31]. Recently, deregulation of miR-125b has been observed in invasive breast cancer, ovarian carcinoma, hepatocellular carcinoma, and thyroid carcinoma [32-35], and it is also associated with clinical outcome in liver cancer patients [34]. To date, Bak1, CYP24, and Bcl2 have been

identified as targets of miR-125b [36]. For further study, we chose to focus on Bcl-2, an important anti-apoptotic protein that is well characterized in many tumors. In this study, we found that reduced miR-125b expression is a frequent event in OVARIAN CANCER, which is consistent with other published studies [34, 37]. Moreover, our data showed that puerarin promoted the expression of miR-125b, which suppressed cancer growth by promoting cell apoptosis through the down-regulation of Bcl-2 expression. This is consistent with the current view that a single miRNA can regulate gene expression by targeting multiple mRNAs, which is collectively named a "targetome" [38].

In conclusion, our findings demonstrated that miR-125b was involved in the anti-cancer action of puerarin. However, emerging reports showed that other miRNAs participated in the progression of ovarian cancer. Maybe this is the reason why the dysregulation of Bcl2 was not fully recovered when we knocked down of miR-125b before treating with puerarin in OVARIAN CANCER cells. In addition, the evidences of puerarin negatively regulating the growth of ovarian cancer cells in xenograft models in the future will provide more convincing basis to support that puerarin may be a candidate to treat ovarian cancer.

### Disclosure of conflict of interest

None.

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