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Downregulation of Ezh2 expression by RNA interference induces cell cycle arrest in the G0/G1 phase and apoptosis in U87 human glioma cells

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Abstract. The Ezh2 gene is an important member of the polycomb-group (PcG) family. As a newly identified oncogene, the expression of Ezh2 has been shown to be significantly increased in prostate cancer, breast cancer, renal cell carcinoma and hepatic cancer; however, a role for Ezh2 in the occurrence of glioma has not yet been reported. In this study, we found that the Ezh2 gene is highly expressed in U87 human glioma cells. Using RNA interference, we demonstrated that the downregulation of Ezh2 expression in U87 human glioma cells resulted in apoptosis and a cell cycle arrest in the G0/G1 phase. In addition, we found that silencing of the Ezh2 gene altered the mitochondrial membrane potential and promoted the release of cytochrome *c* from the mitochondria. Furthermore, the reduced expression of Ezh2 altered the Bax and Bcl-2 protein levels and led to the activation of caspase 9 and 3. These results indicate that the apoptosis induced in U87 human glioma cells by the silencing of the Ezh2 gene is related to the mitochondrial pathway.

Introduction

Glioma is the most common type of primary tumour found in the central nervous system, where it accounts for 45-55% of all primary tumours and is a leading cause of mortality in patients with intracranial tumours (1,2). Currently, conventional treatments for glioma include surgery, radiotherapy, and chemotherapy (3,4); however, the efficacy of these treatments remains poor. In addition, the molecular mechanisms that result in the occurrence and development of glioma are not yet well understood. Therefore, the discovery of molecules with roles in the occurrence and development of glioma is required to understand the malignant biological behaviour of glioma. These novel molecules may provide valuable, reliable molecular targets for future targeted therapies.

The Ezh2 gene is an important member of the polycomb-group (PcG) family. PcG genes regulate the transcription process and thereby play an important role in the regulation of cell proliferation and the cell cycle. The Ezh2 gene is often expressed at low levels in normal cells but at high levels in a variety of stem cells (5-7). In addition, expression of the Ezh2 gene has been found in many tumour cells, including hepatic cancer (8), breast cancer (9), renal cell carcinoma (10), prostate cancer (11), and lymphoma cells (12). In neuroblastoma, increased expression of the Ezh2 gene has been shown to enhance the proliferation, migration, and angiogenesis capacity of the tumour cells, and these phenotypes could be reduced by the downregulation of Ezh2 gene expression (13). These results indicate that the Ezh2 gene plays a key role in maintaining the growth and invasion of neuroblastoma. Studies have also shown that the apoptosis of glioma cells is closely related to the mitochondrial pathway (14,15). In this study, we showed that the silencing of the Ezh2 gene leads to changes in the levels of Bax and Bcl-2. The translocations of Bax and Bcl-2 have been shown to alter the mitochondrial membrane potential, cause the release of cytochrome *c*, activate the caspase family, and eventually lead to apoptosis (16).

We investigated the effects of the downregulation of the Ezh2 gene on the proliferation, apoptosis, and cell cycle of human glioma cells. We also explored the signal transduction pathway induced by the silencing of the Ezh2 gene that results in apoptosis.

Materials and methods

Cells, antibodies, and reagents. The U87 and U251 human glioma cells were purchased from the American Type Culture Collection (ATCC; USA). Lipofectamine™ 2000 was purchased from Invitrogen (USA), the siRNAs were synthesised by GenePharma (China), the RT-PCR kit [Takara

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RNA PCR kit (AMV) Ver. 3.0] was purchased from Takara Biotechnology (Japan), and propidium iodide was purchased from Sigma (USA). The antibodies against Ezh2, Bax, Bcl-2, caspase 3 and 9, CDK4, CDK6, and cyclin D1 were purchased from Cell Signaling Technology, Inc., USA.

Cell culture. The U87 and U251 human glioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C with 5% CO_2. All of the experiments were performed with logarithmically growing cells.

Detection of cellular viability. To measure the cellular viability using the MTT assay, the cells were seeded in 96-well culture plates at a density of $1x10^5$ cells/ml, and Ezh2 siRNA was added when the cells were determined to be logarithmically growing. There were 5 groups of 6 wells of cells for each time point (24, 48, 72, 96 and 120 h) within each experiment. After 20 μl of a 5-mg/ml solution of 5-diphenyltetrazolium bromide (MTT) was added to each well, the cells were cultured for an additional 4 h. Then, the culture medium was removed, and 150 μl of dimethyl sulphoxide (DMSO) was added to each well. The cells were subsequently vortexed at room temperature for 10 min, and the OD value (570 nm) of each well was detected with a microplate reader.

siRNA transfection. For the siRNA transfections, the cells were seeded in 6-well plates at a density of $5x10^5$ cells/ml and cultured in DMEM without antibiotics. The transfection was performed when the confluence of the cells was ≥60%, and 2 individual groups of cells were either transfected with an Ezh2 siRNA or a non-targeting oligonucleotide control using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The media were changed prior to the transfection with serum-free DMEM without antibiotics and replaced 4-6 h after the transfection with serum-containing DMEM medium. The sequence of the Ezh2 siRNA was 5'-AAG ACT CTG AAT GCA GTT GCT-3', and a non-targeting siRNA served as a negative control. The efficiency of the siRNA was determined using reverse transcription polymerase chain reaction (RT-PCR) and western blotting.

RNA extraction and RT-PCR. The extraction of the total-RNA was performed using an RNAiso™ Plus kit (Takara) according to the manufacturer's instructions. After calculating the RNA concentration, the RT-PCR was performed using an RT-PCR kit (Takara) according to the product manual. The primers for the Ezh2 and β-actin RT-PCR reaction were synthesised by Invitrogen and were as follows: Ezh2, forward primer 5'-GCC AGA CTG GGA AGA AAT CTG-3' and reverse primer 5'-TGT GCT GGA AAA TCC AAG TCA-3'; β-actin, forward primer 5'-CTG GGA CGA CAT GGA GAA AA-3' and reverse primer 5'-AAG GAA GGC TGG AAG AGT GC-3'. The PCR reaction was then performed in a 50-μl volume with the following reaction conditions: an initial denaturing step at 94˚C for 2 min and 30 cycles of denaturing at 94˚C for 30 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 30 sec. The PCR products were separated on a 1.0% agarose gel by electrophoresis and analysed with a gel imaging scanning system.

Detection of apoptosis by flow cytometry. The cells were collected by trypsinisation and washed twice in ice-cold PBS. Next, the cells were resuspended in PBS, and a single-cell suspension was prepared by pipetting the medium up and down. An Annexin-V and PI staining solution was then added according to the manufacturer's instructions (Annexin-V-FITC kit, Biosea Biotechnology, China). The cells were stained for 15 min in the dark at room temperature, and the apoptotic cells were detected using a flow cytometer (Becton-Dickinson, USA).

Detection of mitochondrial membrane potential. JC-1 staining and flow cytometry were used to detect changes in the mitochondrial membrane potential according to a previously published protocol (17). The fluorescence signals of the JC-1 monomer and polymer were detected by the FL1 and FL2 detectors, respectively. FL1-H and FL2-H represent the green and red fluorescence intensities, respectively. CellQuest software was used for the quantification of the results.

Cell cycle analysis by flow cytometry. For the cell cycle analysis, the cells were trypsinised with 0.25% trypsin, collected, washed twice in PBS, and fixed with a 70% ethanol solution at 4˚C overnight. The following day, the ethanol was discarded. Subsequently, the cells were washed twice with PBS, stained with 1 ml of PI dye that contained 10 μg of RNaseA and 5 μl of Triton X-100 for 30 min at 4˚C in the dark, and analysed by flow cytometry.

Western blotting. For the western blotting, the transfected U87 cells from each group were collected and washed twice in PBS. Then, 2 ml of lysis buffer (50 mM Tris-HCl pH 7.4, 137 mM NaCl, 10% glycerol, 100 mM sodium vanadate, 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1% NP-40, and 5 mM cocktail) was added to the cells. The protein concentration was determined using the BCA method, and the proteins were stained with bromophenol blue. Equal amounts of protein were loaded and separated on a 10% polyacrylamide gel by SDS-PAGE electrophoresis, and the proteins were transferred onto a PVDF membrane using the semi-dry method. The membrane was then blocked with 5% non-fat dry milk overnight. The following day, the membrane was washed with TBST, incubated for 2 h with the primary antibodies, washed with TBST, and incubated for an additional 2 h with the secondary antibody. After adding the chemiluminescence reagent, X-ray autoradiography was performed, the bands were scanned, and the gray scale images were analysed.

Statistical analysis. SPSS 16.0 statistical software was used for the statistical analysis. The values are shown as the mean ± SD. Statistical analysis was performed using the Student's t-test, and the differences between the groups were considered to be statistically significant at $p<0.05$.

Results

The Ezh2 gene is highly expressed in U87 human glioma cells. The Ezh2 mRNA and protein expression levels in U87 and U251 human glioma cells were examined using RT-PCR and western blotting, respectively. We found that Ezh2 mRNA

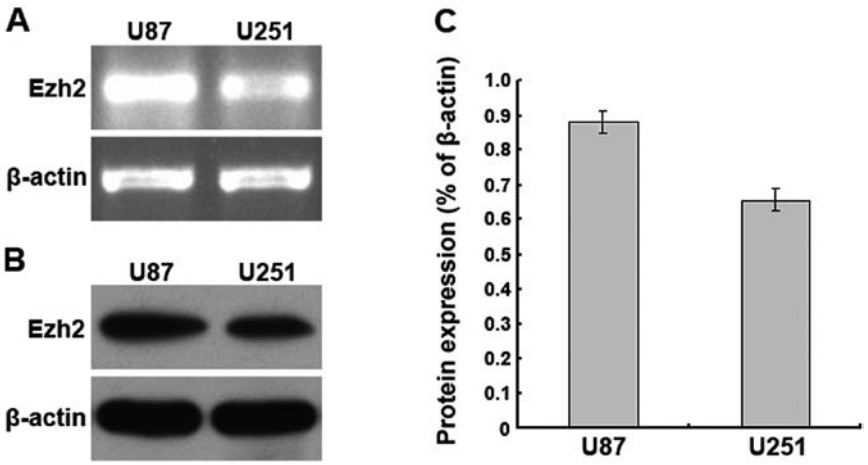

Figure 1. Ezh2 mRNA and protein expression levels in U87 and U251 cells. (A) The Ezh2 mRNA expression level as detected by RT-PCR. (B) Ezh2 protein expression level as detected by western blotting. (C) An analysis of the western blotting results with Gel-Pro Analyzer 4.0 software. The results shown are representative of 3 independent experiments.

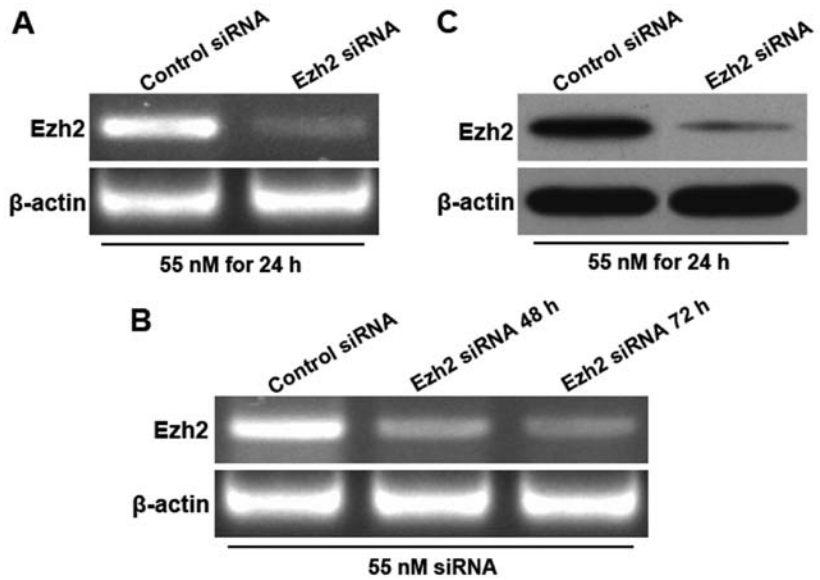

Figure 2. Ezh2 mRNA and protein expression levels in U87 cells after siRNA treatment. (A and B) After the U87 cells were transfected with siRNA targeting Ezh2, RT-PCR was used to determine the efficacy of the siRNA treatment. With a 55-nM siRNA transfection, the expression level of the Ezh2 mRNA was significantly reduced, and this reduction in mRNA expression lasted for at least 72 h. (C) The Ezh2 siRNA efficacy was further analysed by western blotting. Twenty-four hours after the transfection with 55 nM Ezh2 siRNA, the Ezh2 protein expression level was significantly reduced.

was highly expressed in the U87 and U251 cells, and a higher level was present in the U87 cells. Additionally, western blot analysis demonstrated that the expression levels of the Ezh2 protein in the U87 and U251 cells correlated with the mRNA expression levels. These results suggest that Ezh2 is highly expressed in the U87 and U251 human glioma cells (Fig. 1).

Downregulation of Ezh2 expression in U87 human glioma cells by RNA interference. An siRNA targeting Ezh2 and a non-targeting oligonucleotide were individually transfected into U87 cells, and the changes in the Ezh2 mRNA and protein expression levels after transfection were analysed by RT-PCR and western blotting, respectively. The results demonstrated that at 24 h after the transfection of the Ezh2 siRNA (55 nM), the Ezh2 mRNA expression level was significantly reduced. The silencing of Ezh2 lasted for at least 72 h after the siRNA transfection (Fig. 2). These results suggest that after the transfection of the siRNA targeting Ezh2 at 55 nM for 24 h, the Ezh2 mRNA and protein expression levels are effectively downregulated.

Downregulation of Ezh2 expression inhibits the proliferation of U87 human glioma cells. MTT assay was used to determine the effects of the downregulation of Ezh2 expression on the proliferation of U87 cells. We found that compared to the mock-transfected cells or the cells transfected with the non-targeting siRNA, the proliferation of the U87 cells transfected with the Ezh2 siRNA was significantly reduced at 24, 48, 72, 96, or 120 h after the transfection. These results suggest that the silencing of the Ezh2 gene inhibits the proliferation of U87 cells (Fig. 3).

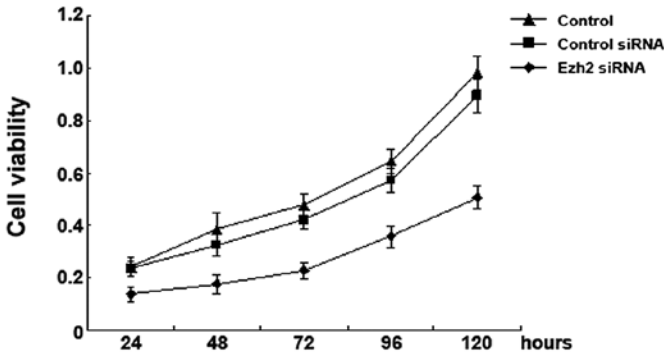

Figure 3. Downregulation of Ezh2 expression inhibits the proliferation of U87 cells. MTT assay was used to measure the proliferation of the U87 cells that had been transfected with either the Ezh2 siRNA or a non-targeting siRNA control. The proliferation level in the cells with downregulated Ezh2 expression was significantly reduced compared to the proliferation level in the mock-transfected cells or the cells that had been transfected with a non-targeting siRNA as a negative control. The results shown are representative of 3 independent experiments.

Downregulation of Ezh2 expression induces apoptosis in U87 human glioma cells. To investigate whether the level of Ezh2 expression was related to the level of apoptosis found in the U87 cells, the expression of the Ezh2 gene was downregulated by RNA interference, and the indicators of apoptosis were analysed. We found that after the silencing of the Ezh2 gene, a significantly higher rate of apoptosis was present in the U87 cells as detected by flow cytometry (Fig. 4A and B). In addition, western blot analysis indicated that the protein expression levels of procaspase 9 and 3 were significantly reduced after the downregulation of Ezh2 expression (Fig. 4C and D). These results suggest that the silencing of the Ezh2 gene leads to apoptosis in U87 cells.

Downregulation of Ezh2 expression induces apoptosis in the U87 human glioma cells through the mitochondrial pathway. To verify whether the apoptosis induced by the silencing of the Ezh2 gene in the U87 cells was related to the mitochondrial pathway, the changes in the mitochondrial membrane potential were detected using JC-1 staining. The results demonstrated that the downregulation of Ezh2 expression significantly reduced the mitochondrial membrane potential (Fig. 5A). In addition, the levels of Bax, Bcl-2, and cytochrome *c* were analysed by western blotting. The silencing of Ezh2 expression decreased the level of Bax and increased the levels of Bcl-2 and cytochrome *c* in the cytoplasm. However, the changes in the levels of Bax, Bcl-2, and cytochrome *c* in the mitochondria were the opposite of the changes found in the cytoplasm (Fig. 5B-E). Therefore, our data indicate that the downregulation of Ezh2 expression promotes the translocation of Bax and Bcl-2, causes the release of mitochondrial cytochrome *c*, reduces the mitochondrial membrane potential, and results in the apoptosis of human glioma cells.

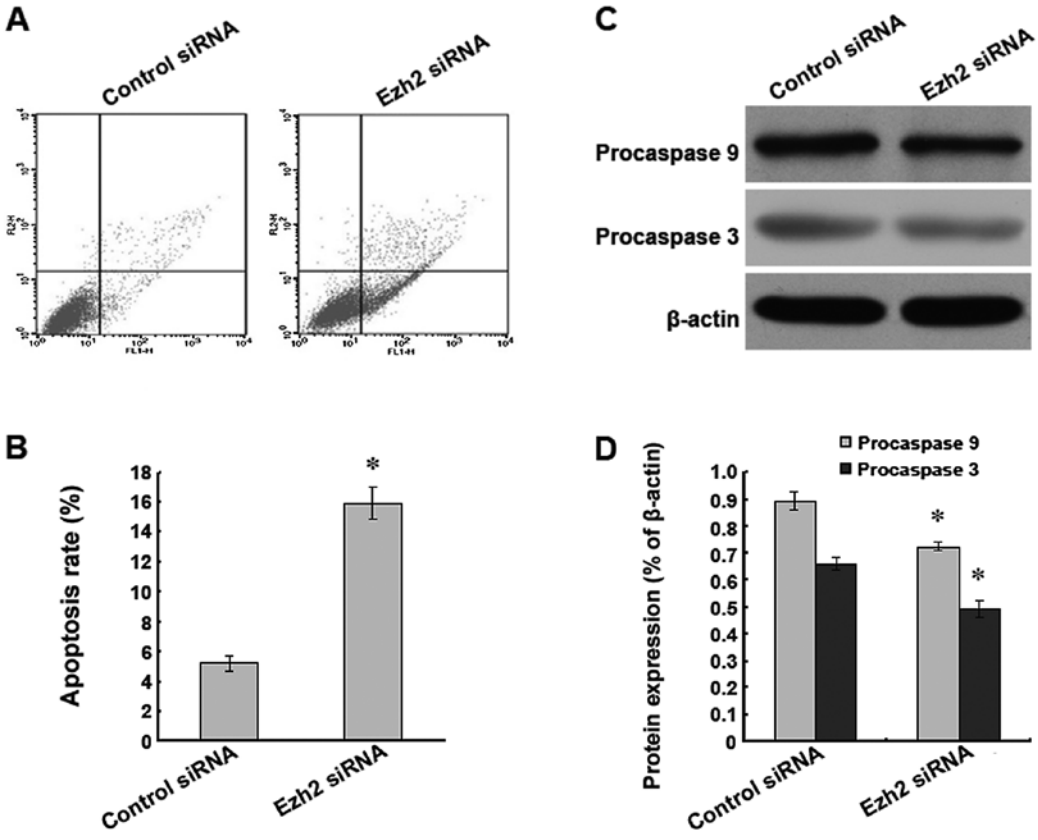

Figure 4. Downregulation of Ezh2 expression leads to apoptosis in U87 cells. (A) The apoptosis rate in U87 cells was analysed by flow cytometry. (B) A histogram representing the apoptosis rate in the U87 cells (%). (C) The changes in the protein expression levels of procaspase 9 and 3 after Ezh2 siRNA treatment were detected by western blotting. (D) The western blotting results were analysed using Gel-Pro Analyzer 4.0 software. $^{*}p<0.05$. The results shown are representative of 3 independent experiments.

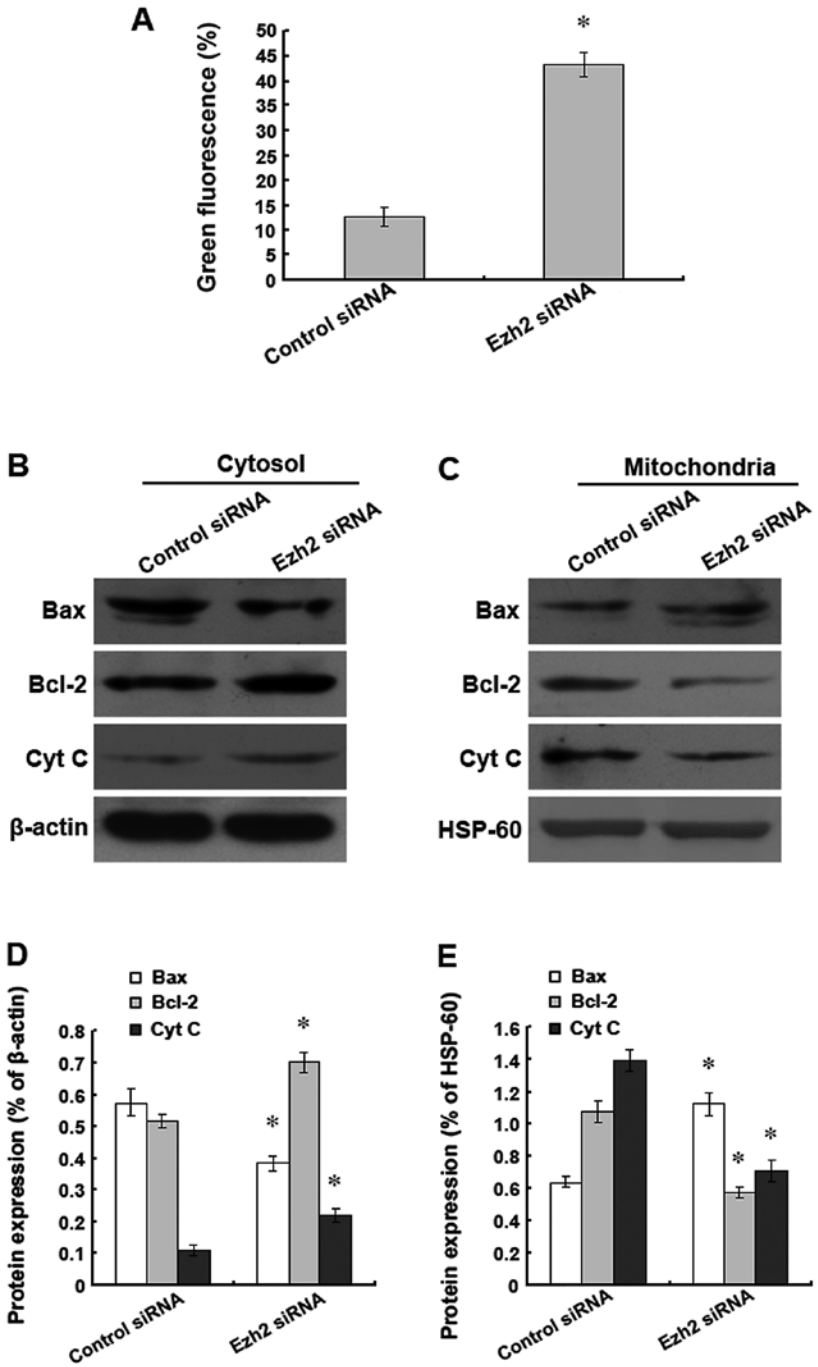

Figure 5. Downregulation of Ezh2 expression induces apoptosis in U87 cells through the mitochondrial pathway (A) Changes in the mitochondrial membrane potential were analysed by JC-1 staining and subsequent flow cytometry. (B and C) The expression levels of Bax, Bcl-2, and cytochrome *c* in the cytoplasm and mitochondria were analysed by western blotting. (D and E) The western blotting results were analysed with Gel-Pro Analyzer 4.0 software. *p<0.05. The results shown are representative of 3 independent experiments.

Downregulation of Ezh2 expression causes a cell cycle arrest in the G0/G1 phase in U87 human glioma cells. To further investigate the inhibition of proliferation caused by Ezh2 siRNA treatment, U87 cells were individually transfected with 55 nM Ezh2 siRNA or a non-targeting siRNA as a negative control, and the cell cycle was analysed by flow cytometry. We found that upon Ezh2 siRNA treatment, the percentage of cells in the G0/G1 phase was significantly higher than the percentage found in the mock-transfected cells or the cells treated with the non-targeting siRNA (Fig. 6A). Additionally, we found that the downregulation of Ezh2 expression altered the levels of several cell cycle proteins, including cyclin D1, CDK4, and CDK6, all of which have roles in the G0/G1 phase. Western blot analysis revealed that compared with the mock-transfected cells or the cells transfected with the non-targeting siRNA, the protein expression levels of cyclin D1, CDK4, and CDK6 were significantly reduced in the cells transfected with the Ezh2 siRNA (Fig. 6B and C). These results suggest that the inhibition of proliferation caused by the downregulation of Ezh2 expression in the U87 cells may be mediated by the reduced expression levels of cyclin D1, CDK4, and CDK6, which result in an arrest of the cell cycle in the G0/G1 phase.

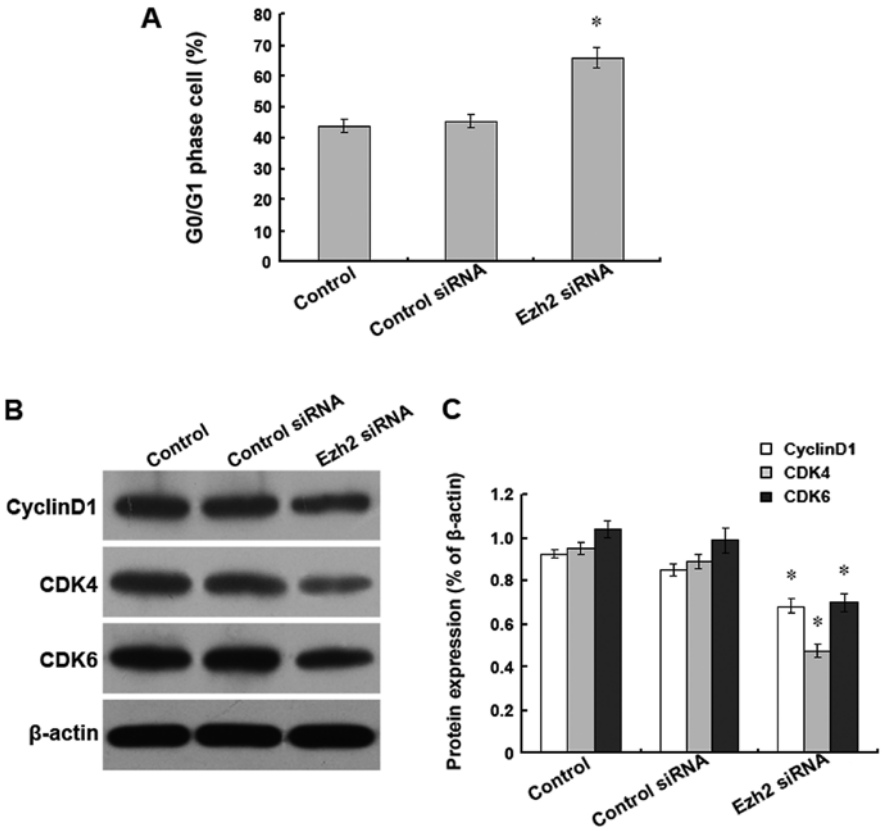

Figure 6. Downregulation of Ezh2 expression causes a cell cycle arrest in U87 cells in the G0/G1 phase. (A) The cell cycle distribution of U87 cells that had been treated with Ezh2 siRNA was analysed by flow cytometry. (B) The protein expression levels of cyclin D1, CDK4, and CDK6 were analysed by western blotting. (C) The western blotting results were examined using Gel-Pro Analyzer 4.0 software. *$p<0.05$. The results shown are representative of 3 independent experiments.

Discussion

In the present study, we explored the role of the Ezh2 gene for the treatment of human glioma. We demonstrated that Ezh2 was highly expressed both at the mRNA and protein level in human glioma cells, which suggests that the Ezh2 gene is closely related to the occurrence and development of glioma. Additionally, we hypothesised that the Ezh2 gene may serve as a potential biomarker and therapeutic target for the treatment of glioma. To further elucidate the detailed mechanism of the function of Ezh2 in human glioma, we used RNA interference to downregulate the expression of Ezh2 and observe the resulting changes in human glioma cells. We found that the Ezh2 mRNA and protein expression levels were significantly reduced with the siRNA treatment, and the downregulation of Ezh2 expression led to a reduction in the proliferation of the glioma cells. These data suggest that the Ezh2 gene plays a role in promoting the proliferation of glioma cells. Consistent with our findings, previous studies have also shown that the Ezh2 gene promotes the proliferation of lung (18), breast (9), thyroid (19), colon (20), ovarian (21), and pancreatic cancer cells (22). Therefore, we concluded that the Ezh2 gene plays an important role in the regulation of the proliferation of glioma cells.

To clarify how the downregulation of Ezh2 expression inhibited the proliferation of glioma cells, we used flow cytometry to analyse the cell cycle and determine the level of apoptosis. Our results demonstrated that the silencing of Ezh2 expression led to an induction of apoptosis in the glioma cells. To date, research regarding the role of Ezh2 in the induction of apoptosis is very limited. One study demonstrated that the silencing of the Ezh2 gene led to increased levels of apoptosis in renal cancer cells (23). In addition, the suppression of Ezh2 expression has been reported to cause apoptosis by downregulating the expression levels of Bax and caspase 3 (24). Consistent with these studies, we also found that the reduced proliferation caused by Ezh2 siRNA treatment correlated with an increased level of apoptosis in the glioma cells. We also found that the downregulation of Ezh2 expression caused an arrest of the cell cycle in the G0/G1 phase and a reduction in the protein levels of the cell cycle regulatory proteins, cyclin D1, CDK4, and CDK6. These results indicate that the Ezh2 gene is essential for cellular proliferation, and the downregulation of Ezh2 expression leads to a reduced number of dividing glioma cells, which is consistent with previous reports (25,26).

Tumour cells can undergo apoptosis through the mitochondrial pathway or the death receptor pathway (27). We found that in glioma cells, the silencing of Ezh2 expression led to the translocation of Bax and Bcl-2, which was followed by a decrease in the mitochondrial membrane potential and the release of cytochrome *c*. These results suggest that the apoptosis of glioma cells induced by the downregulation of Ezh2 expression is dependent on the mitochondrial pathway. Bcl-2 and Bax play key regulatory roles in the mitochondrial apoptosis pathway (28). When Bax translocates from the cytoplasm to the mitochondrial membrane, it changes the permeability of the mitochondrial membrane and promotes the release of cyto-

chrome *c* from the mitochondria into the cytoplasm (29) to initiate the events that lead to cellular apoptosis. The activation of the caspase family is necessary to induce apoptosis. Herein, we analysed the protein levels of procaspase 9 and procaspase 3 after Ezh2 siRNA treatment and found that the levels of these proteins were significantly reduced. Cytochrome *c* released into the cytoplasm activates caspase 9 and caspase 3, which play key roles in the apoptosis pathway (30). Therefore, these results suggest that the downregulation of Ezh2 expression induces apoptosis through the mitochondrial pathway in glioma cells.

Collectively, we demonstrated that the Ezh2 gene is highly expressed in human glioma cells, and the downregulation of Ezh2 expression induces apoptosis through the mitochondrial pathway by regulating the Bcl-2/Bax family in human glioma cells. Furthermore, this study provides a new approach for the clinical treatment of glioma.

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