

Effects of dexamethasone on C6 cell proliferation, migration and invasion through the upregulation of AQP1

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Abstract. Dexamethasone (Dex) is commonly used to treat glioma; however, the mechanism underlying the action of Dex remains unclear. In the present study, the hypothesis that aquaporin-1 (AQP1) may participate in tumor cell proliferation, apoptosis, migration and invasion was tested using small interfering RNA (siRNA). The results of the current study indicated that Dex could inhibit the proliferation, in addition to promoting the migration, of C6 cells. Dex was indicated to promote the expression of AQP1. Downregulation of AQP1, achieved using siRNAs, demonstrated the inhibition of cell proliferation, promotion of cell migration and suppression of invasion; therefore, Dex was indicated to serve a role in these effects in the C6 cells, via the upregulation of AQP1. This demonstrated that AQP1 could be utilized as a novel therapeutic target, with the aim of inhibiting the proliferation and metastasis of gliomas.

Introduction

Malignant glioma is the most common type of intracranial primary tumor, with a high mortality rate and poor treatment outcomes (1,2). The mean survival time of patients following surgery, combined with chemotherapy and/or radiotherapy,

is still <1 year (2,3). Glucocorticoids are a type of steroid hormone with extensive physiological and pharmacological effects (4). One function is an anti-proliferative effect that may be observed in a variety of tissues and cells, including lymphoid tissue, fibroblast tissue and epithelial cells, through inducing cell cycle arrest in the G₁ phase, or through apoptosis (5-7). Furthermore, previous studies have demonstrated that glucocorticoids have an inhibitory effect on the growth of various tumor cell types, and have been clinically used in the treatment of a number of malignant tumors, including chronic lymphocytic leukemia and prostate cancer (8-10). Dexamethasone (Dex) is frequently used as a clinical synthetic glucocorticoid, as it has a stronger efficacy than natural glucocorticoids (11,12). It is now a commonly used agent for clinical hormone therapy; however, the mechanism underlying Dex is still unclear (13,14).

Aquaporin-1 (AQP1) is a highly conserved membrane-bound protein with a molecular size of 28 kDa. This protein serves an important role in the specific transmembrane transport of water molecules (15). The gene is located on the human chromosome 7p14 (15). In the central nervous system (CNS), AQP1 is mainly expressed in choroid plexus epithelial cells, fulfilling a dual role as a water channel and in the regulation of c-GMP ion channels, which are involved in the formation of cerebrospinal fluid (16). Malignant gliomas originate in the neural epithelium and are the most common primary CNS tumors. According to the histological origin, glioma can be divided into astrocytoma, oligodendroglioma, oligodendrocytoma and ependymoma (17). The majority of low-grade astrocytomas can develop into malignant gliomas (17). The malignant glioma phenotype is characterized by rapid tumor growth, high glucose consumption, tumor necrosis, hypoxia, an increase in microvessel density, and the destruction of the blood-brain barrier (17). Prior studies have demonstrated that AQP1 is expressed in astrocytoma cells and vascular endothelial cells, and is increased in parallel with the histological grade of gliomas (18,19). Further studies revealed that AQP1 is closely associated with tumor angiogenesis, tumor-associated brain edema and tumor metastasis (20,21). AQP1 expression in the gliosarcoma cell line is induced by Dex, platelet-derived growth factors, sodium chloride, hypoxia, D-glucose and fructose (22). Based on the expression of AQP1 in gliomas, and on the existing studies regarding its function, the hypothesis

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Abbreviations: AQP1, aquaporin-1; CNS, central nervous system; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA

Key words: dexamethasone, AQP1, C6 cells, metastasis, siRNA, glioma

investigated in the present study was that AQP1 may participate in tumor cell proliferation, apoptosis, migration and invasion.

The present study aimed to investigate the function of AQP1 and its mechanism using C6 cells. C6 cells are rat brain glioma cells obtained following N-nitrosomethylurea-induced glioma in Wistar rats (23,24). These cells were subcultured *in vitro* and *in vivo*, are stable in growth and express glioma specific markers, including glial fibrillary acidic protein and the S-100 protein; therefore, C6 cells are widely used in experimental studies for the treatment of glioma (25-27). The roles of AQP1 and Dex in cell proliferation, apoptosis, migration and invasion in C6 cells were investigated, to determine whether AQP1 can be used as a novel therapeutic target to inhibit the proliferation and metastasis of gliomas.

Materials and methods

Cell culture. Rat glioma C6 cells (iCell Bioscience Inc., Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 100 ml/l fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 kU/l penicillin and 100 mg/l chloramphenicol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in a humidified 5/95% CO₂/air incubator at 37°C. The cell growth state was observed under an inverted light microscope (x100 magnification; Olympus Corporation, Tokyo, Japan). To detect the role of AQP1, small interfering RNA (siRNA) was transfected into the C6 cells causing the knockdown of AQP1; the transfected cells were used for subsequent studies with 1 μM Dex treatment.

MTT test. Cell proliferation was detected using an MTT assay (Beyotime Institute of Biotechnology, Haimen, China). A total of 100 μl DMEM with 10% FBS containing a concentration of 1x10⁵ cells/ml C6 cells were added to 96-well plates and were cultured for 24 h; following this, the cells were treated with Dex (0, 0.01, 0.1 and 1 μM; Sigma-Aldrich; Merck KGaA) for 24 or 48 h in a humidified 5/95% CO₂/air incubator at 37°C. A total of 20 μl MTT solution (0.5 mg/ml) was added into each well and incubated for 4 h at 37°C, followed by a 150-μl dimethylsulfoxide culture on an oscillator for 15 min, to dissolve the formazan crystals. Finally, absorbance of the solution was measured at 570 nm using an ST-360 microplate reader (Shanghai Kehua Bio-Engineering Co., Ltd., China) and the rates of cell proliferation inhibition were calculated. The formula used was as follows: The inhibition rate of cell proliferation (%)=(1-absorbance value of the experimental group/that of the control group) x100%.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), homogenized using a pipette, and the RNA concentrations were measured with a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Following this, the extracted RNA was transcribed into cDNA using a PrimeScript™ RT-PCR kit (RR014A; Takara Bio, Inc., Otsu, Japan) and the RT-qPCR reactions were performed on a T100™ qPCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using TransStart® Top Green qPCR SuperMix AQ131 (TransGen Biotech, Inc.,

Beijing, China). Pre-denaturation consisted of 1 cycle at 95°C and 5 sec; and RT-qPCR reactions consisted of 35 cycles: 95°C for 30 sec, followed by 55°C for 30 sec, 72°C for 1 sec, 72°C for 5 sec, and 16°C for 30 sec. The primers used were as follows: Forward 5'-CTGTGGTGGCTGAGTTCCTG-3' and reverse 5'-ACCTCGGCCAAGTGAGTTCTC-3' for AQP1; and forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3' for GAPDH. Gene expression was normalized to the level of GAPDH within each sample using the relative 2^{-ΔΔC_q} method (28). Gene expression is presented as relative to the control, and the data are representative of three independent experiments.

Transwell assay for migration and invasion. After being pretreated with Dex (0, 0.01, 0.1 and 1 μM) for 48 h, the C6 cells were digested with 0.25% EDTA trypsin, then collected and C6 cells were resuspended in DMEM with 10% FBS at a density of 5x10⁵ cells/ml. Following this, C6 cells (5x10⁵) were seeded into the upper chambers of a Transwell plate (200 μl/well). Subsequently, 600 μl DMEM containing 20% FBS was added into each lower chamber. After being cultured for 24 h in a humidified 5/95% CO₂/air incubator at 37°C, the cells were fixed in 4% paraformaldehyde for 10 min, washed twice with PBS and then stained with 0.05% crystal violet for 10 min at 37°C. After removing the cells that had not migrated, the cells on the lower surfaces were quantified and images were obtained. To study invasion, 50 μl of 1 g/l Matrigel was used to coat the upper chambers of the Transwell plate at 37°C for 1 h.

Apoptosis rate. The rate of C6 cell apoptosis was detected using the terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) method with In Situ Cell Death Detection Kit (cat no. 11684795910, Roche Applied Science, Penzberg, Germany) according to the manufacturer's instruction. C6 cells at a concentration of 1x10⁵/ml were inoculated into 24-well plates prior to culturing with Dex (0, 0.01, 0.1 and 1 μM) for 24 h in a 5% CO₂ saturated humidity incubator at 37°C. Following 2 washes with PBS, cells were fixed using 4% paraformaldehyde in PBS (pH 7.4) for 1 h at 22-25°C. Permeabilisation solution with 0.1% Triton X-100 in 0.1% sodium citrate were used for incubation on ice for 2 min. Cell samples were resuspended in 50 μl/well TUNEL reaction mixture containing enzyme solution with terminal deoxynucleotidyl transferase from calf thymus in storage buffer and label solution with nucleotide mixture in reaction buffer (cat no. 11684795910, Roche Applied Science, Penzberg, Germany) mixed at a ratio of 1:9. Samples were incubated for 60 min at 37°C in the dark and washed twice using PBS. 50 μl of DAPI Staining Solution WH1150 (Biotechwell, Shanghai, China) were added into every well, incubation for 10 min at room temperature. After 3 washes with PBS, 50 μl of fluorescein (F6182, Sigma) were added to preserve fluorescence of cell smears. The green fluorescence at 520±20 nm and the blue DAPI staining at 460 nm were observed via fluorescence microscopy using a fluorescein isothiocyanate (FITC)-labeled TUNEL Apoptosis Detection kit (Roche Applied Science, Penzberg, Germany). Three different fields of view were randomly photographed per sample. DAPI can stain apoptotic and non-apoptotic cells blue, and the green fluorescence of

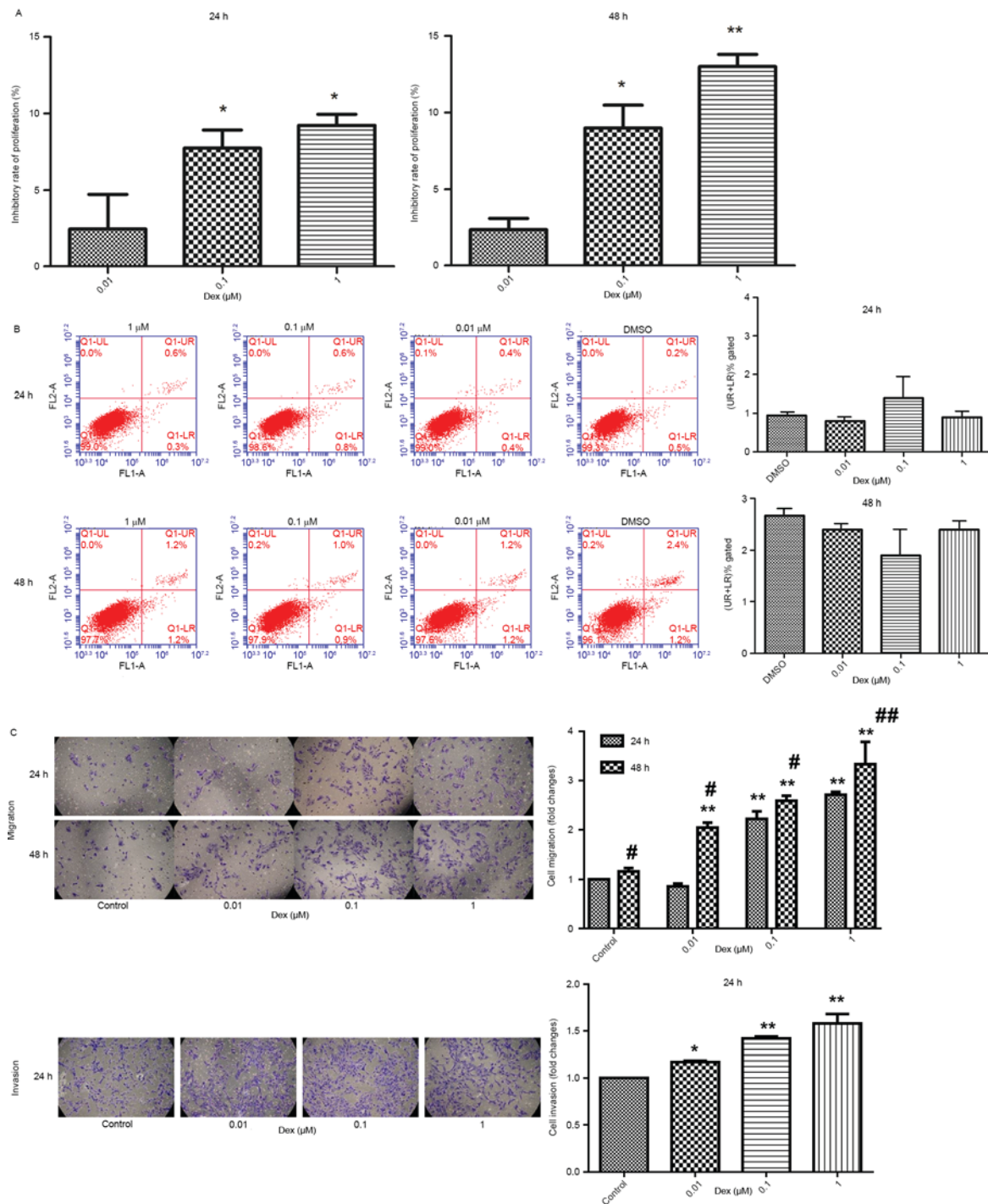


Figure 1. Effects of Dex on cell proliferation, cell apoptosis, cell migration and invasion. (A) Inhibitory effect of Dex on C6 cell proliferation. *P<0.05, **P<0.01 vs. 0.01 μM. (B) The apoptosis rate of C6 cells induced by Dex. (C) Cell migration and invasion induced by Dex. The quantitative data regarding the apoptosis rate [(UR+LR)%Gated], cell migration and invasion were also depicted. *P<0.05, **P<0.01 vs. 0.01 control; #P<0.05, ##P<0.01 vs. 24 h. Dex, dexamethasone; DMSO, dimethyl sulfoxide.

FITC-12-dUTP is incorporated and localized in the apoptotic nuclei. Cell apoptosis was confirmed by Hoechst staining.

Western blot analysis. After pretreatment with benzo[a]pyrene (0.5,10,50,100 and 200 μM) for various lengths of time, C6 cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology) and centrifuged at 12,000 x g for 10 min at 4°C. Protein concentration was measured using a BCA assay (Pierce; Thermo Fisher Scientific, Inc.), according to the

manufacturer's instructions. A total of 30 μg protein from each sample was resolved via 10% SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane at 100 V for 120 min. After blocking with 5% non-fat dry milk for 1 h at room temperature, the membranes were incubated with anti-AQP1 (cat no. ab122821; 1:100; Abcam, Cambridge, MA, USA) and anti-GAPDH (cat no. ab8245; 1:1,000; Abcam) antibodies overnight at 4°C. After 3 washes with PBS, the membranes were incubated with goat anti-rabbit (cat no. 32460;

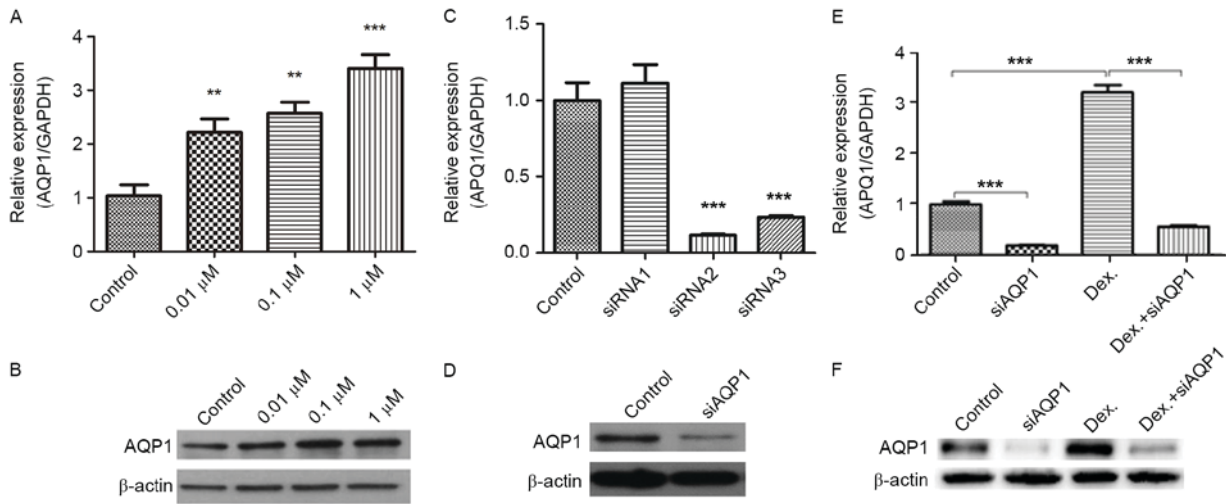


Figure 2. (A) Effect of Dex on the expression of AQP1 in C6 cells, and silence of AQP1. Effect of Dex on the expression of AQP1 mRNA in C6 cells. (B) Effect of Dex on the expression of AQP1 protein in C6 cells. ** $P < 0.01$, *** $P < 0.001$ vs. control. (C) Identification of the interference efficiency of AQP1 siRNA on mRNA level. (D) Identification of the interference efficiency of AQP1 siRNA on protein level *** $P < 0.001$ vs. control. (E) Effect of AQP1 siRNA on $1 \mu\text{M}$ Dex-induced expression of AQP1 mRNA. (F) Effect of AQP1 siRNA on $1 \mu\text{M}$ Dex-induced expression of AQP1 mRNA *** $P < 0.001$. Dex, dexamethasone; AQP1, aquaporin-1.

1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.) or goat anti-mouse IgG (cat no. A0216; 1:1,000; Beyotime Institute of Biotechnology) secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. Detection was performed using the ChemiDoc™ XRS+ enhanced chemiluminescent system (Bio-Rad Laboratories, Inc.).

Statistical analysis. Data are presented as the mean \pm standard deviation. A value of $P < 0.05$ was considered to indicate statistical significance, using one-way analysis of variance with least significant difference post-hoc test in SPSS version 12.0 package (SPSS, Inc. Chicago, IL, USA). Images were prepared using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Effects of Dex on C6 cell proliferation, apoptosis, migration and invasion. The inhibitory effect of Dex on C6 cell proliferation was studied (Fig. 1A). The results indicated that Dex had a significant dose-dependent inhibitory effect on the proliferation of C6 cells at 24 and 48 h ($P < 0.05$).

There were no significant changes in cell apoptosis with various concentrations of Dex (0, 0.01, 0.1 and $1 \mu\text{M}$) for 24 and 48 h ($P < 0.05$; Fig. 1B). Cell migration and invasion were also studied (Fig. 1C). At 24 and 48 h, the migration of C6 cells was significantly increased ($P < 0.05$), in a dose-dependent manner. In addition, cell migration at 48 h was greater than cell migration at 24 h, demonstrating that the promotion of cell migration by Dex was dose- and time-dependent; similarly, Dex promoted C6 cell invasion.

Dex upregulates the expression of AQP1. The expression level of AQP1 induced by Dex was studied (Fig. 2A and B). The results indicated that the transcription and translation of AQP1 gene could be enhanced by treatment with Dex for 24 h. Regarding mRNA and protein levels, the expression levels of AQP1 significantly increased along with the Dex

dose, demonstrating that the changes in AQP1 expression were dose-dependent. Due to $1 \mu\text{M}$ Dex having the greatest significance in inhibiting cell proliferation ($P < 0.05$), enhancing the expression of AQP1 and promoting cell migration and invasion, $1 \mu\text{M}$ Dex was used to detect the roles of AQP1 in the proliferation, migration and invasion of C6 cells.

To further study the role of AQP1, three siRNAs used to silence AQP1 in C6 cells were designed (Fig. 2C and D). The results identified that siRNA-2 and -3 could significantly reduce the transcription level of AQP1 in C6 cells (Fig. 2C) ($P < 0.05$). Additionally, siRNA-2 was demonstrated to significantly reduce the levels of AQP1 protein expression ($P < 0.05$); therefore, siRNA-2 was used in subsequent studies.

In the C6 cells transfected with AQP1 siRNA (siAQP1), $1 \mu\text{M}$ Dex did not increase the expression of AQP1 (Fig. 2E and F); thus, AQP1 siRNA antagonized the Dex-induced upregulation of AQP1.

Effects of siAQP1 on Dex-induced C6 cell proliferation, migration and invasion. Dex inhibited the proliferation of cells, whereas siAQP1 promoted proliferation (Fig. 3A), indicating that Dex-inhibited cell proliferation is mediated by AQP1. The proliferation of cells caused by Dex was confirmed by a clonogenicity assay (Fig. 3B). siAQP1 increased clonal formation, which was inhibited by Dex, and Dex-inhibited cell proliferation.

The results for flow cytometry (Fig. 3C) and Hoechst staining (Fig. 3D) demonstrated that cell apoptosis was promoted by siAQP1, but treatment with Dex did not further enhance cell apoptosis; this confirmed the effect on cell apoptosis was not Dex dose-dependent.

The results revealed that the migration of C6 cells increased after treatment with Dex (Fig. 3E). siAQP1, having the ability to inhibit Dex-induced migration, demonstrated that Dex facilitates cell migration through the upregulation of AQP1 expression levels. Similar to cell migration, siAQP1 inhibited Dex-induced invasion, which indicates that Dex also facilitates cell invasion through the upregulation of AQP1 expression levels.

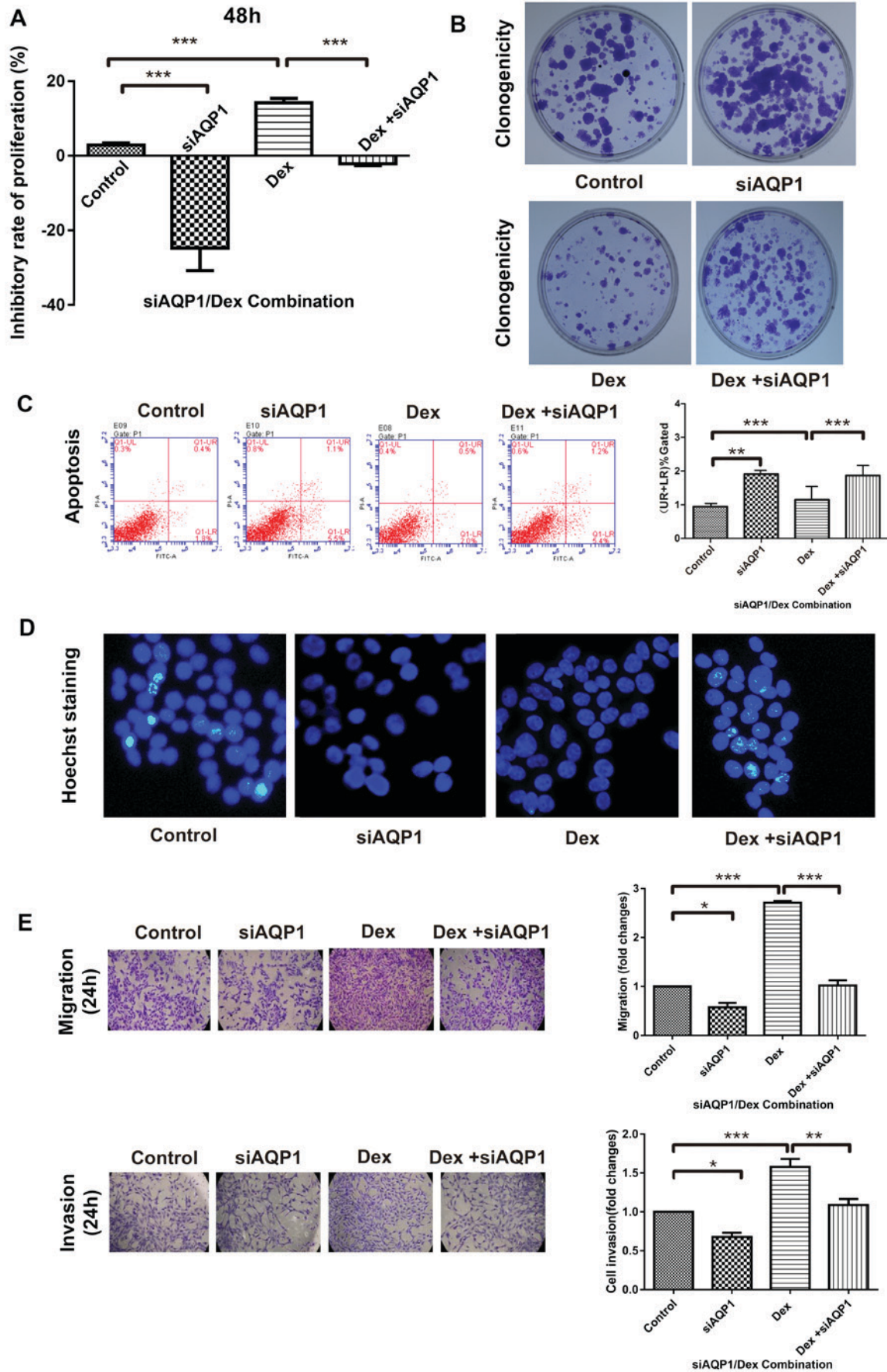


Figure 3. AQP1 siRNA antagonized the roles of Dex in C6 cell proliferation, migration and invasion. (A) Effects of Dex and AQP1 siRNA on the proliferation of C6 cells, as well as the inhibitory effect, were evaluated. (B) Clonogenicity. The apoptosis of C6 cells in the combination of AQP1 siRNA and Dex was detected by flow cytometry (C) and Hoechst staining (D). Apoptosis rate was present as (UR+LR)%Gated. (E) Effects of Dex and AQP1 siRNA on the migration and invasion of C6 cells. *P<0.05, **P<0.01, ***P<0.001 vs. control. Dex, dexamethasone; siRNA, small interfering RNA; AQP1, aquaporin-1.

Discussion

Dex inhibited the proliferation of C6 cells, and promoted the migration and invasion of C6 cells. In addition, Dex promoted the expression of AQP1. With the downregulation of AQP1 by using siRNA, the inhibition of cell proliferation and promotion of cell migration and invasion were reversed; therefore, Dex inhibited the proliferation of C6 cells and promoted the migration and invasion of C6 cells via AQP1.

Studies have indicated that Dex can inhibit the proliferation of a variety of cancer cell lines (29,30). Dex also inhibits the proliferation of Chinese hamster ovary cells, HeLa S3 cervical cancer cells, and the human ovarian cancer cell line 3AO (8). The results of the present study demonstrated that Dex had a significant inhibitory effect on the proliferation of C6 cells at 24 and 48 h. The inhibitory rate was dose-dependent. Cell apoptosis and cell cycle progression are closely associated (31). When the DNA is damaged or replication is incomplete, the cell cycle will remain in a certain phase, until DNA repair or replication is complete, prior to progressing onto the next phase (31,32). If the DNA cannot be repaired or cannot be completely replicated, apoptosis is initiated (33,34). Recent studies on fibroblasts, lung cancer, bladder cancer and hepatocarcinoma cells revealed that glucocorticoids can induce an increase in p21/WAF1 levels, causing the cell cycle to become arrested in the G₁ phase (11,35). It was demonstrated that high-dose Dex also caused C6 cell apoptosis (36,37). In the present study, it was demonstrated that 0-1 μ M Dex did not induce apoptosis of C6 cells. Clinically, Dex is only used as an adjuvant drug for the treatment of brain glioma, to reduce the toxicity of anti-tumor drugs and to reduce the inflammatory reaction. Migration and invasion of cells are important markers for metastasis (38,39). Dex also inhibits the sodium nitroprusside-induced invasion of U87MG cells (40). The addition of carmustine and Dex inhibited invasion in the C6, U251, U373 and A172 glioma cell lines (41). It was also reported that Dex administered orally to patients with glioma resulted in positive outcomes (42); however, whether Dex inhibited glioma cell invasion without the involvement of other factors was previously unclear (22). The sensitivity of different glioma cells to Dex may differ; it was demonstrated that 1 μ M Dex significantly inhibited the invasion and migration of U373MG cells, and that 10 μ M Dex inhibited cell proliferation (43). The present findings indicated that Dex could be used to inhibit the proliferation and promote the metastasis of the C6 glioma cell line. The difference between the results for U373MG and C6 cells may be due to the cell type. The results of the present study demonstrated that Dex functions as an enhancer for metastasis in C6 cells; therefore, the use of Dex needs further consideration due to it inhibiting cell proliferation, but also inducing cell migration and invasion.

AQP1 expression was detected in astrocytomas, ependymomas and oligodendrogliomas (20,44). AQP1 expression increases with tumor grade. For low-grade astrocytomas, AQP1 is only located in the tumor cell membrane, whereas for high-grade astrocytoma, AQP1 is also distributed in the cytoplasm (18,19). Compared with cells in the necrotic area of the tumor center, the expression of AQP1 in cells near the tumor periphery increased (21). By contrast, in the low-grade glioma, AQP1 expression in the non-tumor adjacent tissue was

greater compared with that in the high-grade astrocytoma (20). AQP1 is expressed in reactive astrocytes in the paracancerous tissues of astrocytomas, indicating a distribution of microvascular colonies (20,21). In AQP1-knockout mice, tumor angiogenesis and endothelial cell migration was significantly reduced; therefore, aquaporin-dependent cell migration may be a common phenomenon in tumors. Regarding the migration process, through rapid formation and contraction, cells constantly adjust their own volume to adapt to the external small space and promote the forward movement of cells (45). AQP1 binds to Lin7/ β -catenin, causing actin recombination to form plasma membrane processes and mediating rapid transport of water molecules into pseudopodia, by promoting the rapid renewal of plasma membrane processes and increasing water permeability, which eventually leads to promotion of tumor cell migration (46,47). Through the knockdown of AQP1 in glioma cells, cell migration and invasion decreased significantly, indicating that AQP1 mediated the migration and invasion of glioma cells in the brain, which is consistent with previous studies (22,48). Hayashi *et al* (36) tested the effect of Dex (10⁻³-10 μ M) on the expression of AQP1 in rat 9L cells and determined that the expression of AQP1 induced by 10 μ M Dex was lower than that recorded for 0.01-1 μ M Dex. The presence of AQP1 in glioma cell lines is closely associated with enhanced cell migration and invasion (21,48). Consistently, the present results further indicated that Dex inhibits proliferation, and promotes the migration and invasion of C6 cells via the induction of AQP1. Therefore, Dex can inhibit proliferation and induce apoptosis in cultured C6 glioma cells via upregulating the expression of AQP1, thus laying a foundation for the treatment of glioma. In the present study, only C6 cells were used; as a result, more and varied cell lines should be used for further investigations.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JC and HL designed the experiments, YG carried out all the experiments, analyzed the data and wrote the paper.

YZ interpreted the results and contributed to writing of the paper. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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