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Knockdown of E2F3 Inhibits Proliferation, Migration, and Invasion and Increases Apoptosis in Glioma Cells

Zhi-Gang Shen,^{*1} Xiao-Zhou Liu,^{†1} Chang-Xiu Chen[‡] and Jing-Min Lu[§]

^{*}Department of Neurosurgery, The Affiliated Hospital of Xuzhou Medical University, Xuzhou, Jiangsu Province, P.R. China

[†]Department of Neurology, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, P.R. China

[‡]Department of Pediatrics, 97th Hospital of PLA, Xuzhou, P.R. China

[§]Department of Neurology, Huai'an Second People's Hospital and The Affiliated Huai'an Hospital of Xuzhou Medical University, Huai'an, P.R. China

E2F3a, as a member of the E2F family, is essential for cell division associated with the progression of many cancers. However, the biological effect of E2F3a on glioma is not understood as well. To investigate the functional mechanism of E2F3a in glioma, we examined the expression of E2F3a in glioma tissue and cell lines. We found that E2F3a was upregulated in glioma tissue compared with adjacent tissue, and this was associated with a poor survival rate. E2F3a was highly expressed in glioma cell lines compared with normal HEB cell lines. Knockdown of E2F3a significantly inhibited cell proliferation, promoted G₀/G₁ phase arrest, elevated apoptosis rates, and suppressed cell migration and invasion. However, overexpression of E2F3a markedly promoted cell proliferation, migration, and invasion and inhibited apoptosis. Moreover, *in vivo* studies showed that knockdown of E2F3a expression dramatically inhibited U373 tumor growth in a nude mouse model. Results of real-time PCR and Western blot showed that the depletion of E2F3a upregulated the expression levels of cell apoptosis-related proteins and downregulated migration-related proteins. Conversely, E2F3a overexpression downregulated the expression levels of cell apoptosis-related proteins and upregulated migration-related proteins. In conclusion, our results highlight the importance of E2F3a in glioma and provide new insights into the diagnostics and therapeutics of gliomas.

Key words: Glioma; E2F3a; Apoptosis; Migration

INTRODUCTION

Malignant gliomas are the most common primary brain tumors, and they arise from the glial cells within the central nervous system, which are characterized by morphological and genetic complexities that contribute to uncontrolled proliferation, high resistance to apoptosis, and diffuse infiltration into normal brain parenchyma^{1,2}. Glioblastoma multiforme (GBM) is the most aggressive glioma with a survival time of only 12 months from the time of diagnosis, even with multimodal therapy such as irradiation and chemotherapy^{3,4}. Furthermore, conventional strategies are limited due to the glioma's ability to efficiently infiltrate surrounding normal brain tissue, making it difficult to fully resect the tumors during surgery. Recently, a wide range of other therapeutic strategies, including immunotherapy and gene therapy, have

been tried or are under evaluation. However, treatments for GBM remain essentially ineffective, and the recurrence of tumors is still poorly understood. Moreover, glioblastoma tumors are heavily infiltrated by microglia and macrophages, which contribute at least one third of the total tumor mass implicated in several roles in GBM progression including proliferation, survival, motility, and immunosuppression⁵⁻⁷, and secrete a soluble motogenic factor that acts on glioblastoma cells. Therefore, investigating the mechanism of glioblastoma cell invasion and discovering new therapeutic targets for glioma have received a great deal of interest.

The E2F family of transcription factors is essential for cell proliferation, differentiation, cell cycle, DNA replication, and apoptosis^{8,9}. To date, eight different E2F family members (E2F1–8) have been identified. Among them,

¹Co-first authors.

Address correspondence to Jing-Min Lu, Department of Neurology, Huai'an Second People's Hospital and The Affiliated Huai'an Hospital of Xuzhou Medical University, 62 Huaihai South Road, Qinghe District, Huai'an 223002, P.R. China. Tel: +86-0517-83943591; E-mail: lujmhy6@sina.com

E2F3 encodes two protein products, E2F3a and E2F3b, through the use of two distinct promoters¹⁰. E2F3a has its peak expression at the G₁/S transition, whereas E2F3b is constitutively expressed in quiescent cells that are functional in the G₀ phase and remain constant throughout the cell cycle¹¹. E2F3a transcriptionally activates E2F target genes such as thymidine kinase and cyclin E, unlike E2F3b, which represses the transcription of E2F target genes. Members of the E2F family have dual roles in tumorigenesis, as oncogenes and as tumor suppressor genes. E2F3a has been shown to be overexpressed in several types of human cancers, including liver, bladder, prostate, and ovarian cancers and acute lymphoblastic leukemia, and is correlated with cancer cell proliferation, apoptosis, invasion, and prognosis^{10,12–14}. E2F3a overexpression also induced DNA damage in primary human fibroblasts that were inhibited by blocking DNA replication¹⁵. Although E2F3a has been reported at a higher level in glioma tissue than in normal brain tissue, and proliferation rate and the percentage of S phase cells were reduced after E2F3a depletion¹⁶, no apoptosis or invasion functional studies of E2F3a, or the related molecular mechanism of glioma cells, have been performed.

In this study, we demonstrate the effects of silencing and overexpressing of E2F3a on cell proliferation, cell cycle, apoptosis, invasion, and migration, and the changes in the gene expression pattern in glioma cell lines. Our results suggest that E2F3a could regulate glioma carcinogenesis and may serve as a potential target for glioma therapies.

MATERIALS AND METHODS

Bioinformatics Analysis

TCGA RNA-Seq and the corresponding clinical data were downloaded from TCGA following the approval of this project by the consortium. RNA-Seq analysis used data from 68 GBM and 5 nontumor brain samples. Survival rate data were downloaded from the NCBI's Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series Accession No. GSE82009 following the approval of this project by the consortium.

Cell Lines and Culture Conditions

Human glioma cell lines T98G, U251, and U373 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured according to the guidelines recommended by the ATCC. SHG44 was from the Cell Bank of Shanghai Biological Institute, Chinese Academy of Science (Shanghai, P.R. China) and was cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone). All cells were maintained at 37°C under an atmosphere of 5% CO₂/95% air.

Protein Extraction and Western Blot

Total protein was extracted from cells using ice-cold radioimmunoprecipitation assay buffer (RIPA; Beyotime Institute of Biotechnology) containing 0.01% protease and phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO, USA). After being incubated on ice for 30 min, cell lysis was centrifuged 12,000×g at 4°C for 20 min, and proteins in the supernatant were collected for experiments. Lysate was separated by 10%–15% SDS-gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Boston, MA, USA). The protein was probed with anti-E2F3a (Cat. No. sc-879; 1:4,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Bim (Cat. No. ab7888; 1:1,000; Abcam, Cambridge, MA, USA), anti-Bax (Cat. No. ab69643; 1:1,000), anti-caspase 3 (Cat. No. ab2302; 1:500), anti-caspase 9 (Cat. No. ab2014; 1:1,000), MMP-2 (Cat. No. ab37150; 1:500), anti-MMP-9 (Cat. No. ab73734; 1:1,000), anti-MMP-14 (Cat. No. ab3644; 1:1,000), anti-MMP-19 (Cat. No. ab53146; 1:1,000), anti-PCNA (Cat. No. ab18197; 1:1,000), anti-Ki67 (Cat. No. ab15580; 1:1,000), and anti-GAPDH (Cat. No. 5174; 1:1,500; Cell Signaling Technology, Danvers, MA, USA).

Construction and Infection

Oligonucleotide encoding shRNA-targeted human E2F3a mRNA and a scramble shRNA were both obtained from Sangon Biotech Inc. (Beijing, P.R. China). These oligonucleotides were annealed and digested using *Age*I and *Eco*RI and were then ligated into pLKO.1-EGFP, giving rise to a pLKO.1-EGFP-E2F3a-shRNA construct. For E2F3a expression constructs, mRNA was isolated from adult human normal brain tissue and reversed transcribed, and the full-length cDNA was first amplified using the following primers: 5'-CGAGAGATGAGAAAGGGAATCCAG-3' (forward) and 5'-GAGAGTTCACACGAAGCATAATCAAC-3' (reverse), and then cloned into the pLVX-Puro vectors using *Xho*I and *Kpn*I (Shanghai Genomics Inc., Shanghai, P.R. China). The recombinant pLKO.1-EGFP-E2F3a-shRNA and pLVX-Puro-E2F3a were stably infected into U373 and U251 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The recombinant pLKO.1-EGFP-scramble shRNA (shNC) and black pLVX-Puro (NC) were used as the negative control.

CCK-8 Assay

Cell viability was determined using the cell counting kit-8 (CCK-8) in accordance with the manufacturer's protocol (Sigma-Aldrich). Cells infected with pLKO.1-EGFP-E2F3a-shRNA or pLVX-Puro-E2F3a were seeded into 96-well plates at 3×10³ per well for 48 h, and the CCK-8 assay was performed after 24, 48, and 72 h. The optical density (OD) was measured using a microplate

reader (Bio-Tek Company, Winooski, VT, USA) at 450 nm wavelength.

Cell Cycle and Cell Apoptosis Assay

Cells infected with pLKO.1-EGFP-E2F3a-shRNA or pLVX-Puro-E2F3a were seeded into 96-well plates at 3×10^3 per well for 48 h. For cell cycle analysis, cells were digested by trypsin, fixed with ice-cold 70% ethanol for 4 h, and stained with 50 $\mu\text{g/ml}$ propidium iodide (PI) in the presence of RNase A at 37°C for 30 min. For cell apoptosis analysis, cells were harvested and washed twice in phosphate-buffered saline (PBS), stained with 10 μl of annexin V-FITC and 5 μl of PI, and analyzed using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell Migration and Cell Invasion Assay

Cells (1×10^5) infected with pLKO.1-EGFP-E2F3a-shRNA or pLVX-Puro-E2F3a in 100 μl of MEM or DMEM without FBS were seeded into the Transwell upper chamber. In the lower chamber, 500 μl of MEM or DMEM with 10% FBS was added as a chemoattractant. After a 48-h incubation period, cells that did not migrate were scraped from the upper chamber, and the adherent cells were stained with crystal violet and photographed (200 \times). The procedure for the cell invasion assay was similar to the cell migration assay, except that the Transwell membranes were precoated with Matrigel (BD Biosciences, San Jose, CA, USA).

Real-Time PCR

Total RNA was isolated from tissue specimens or cells with the TRIzol reagent (Invitrogen) and reverse transcribed to cDNA according to the manufacturer's protocol (Fermentas, Hanover, MD, USA). Quantitative real-time PCR (qRT-PCR) was carried out using an ABI 7300 real-time PCR machine (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Kit (Thermo Fisher Scientific, Rockford, IL, USA). The relative expression of genes was calculated with the $2^{-\Delta\text{Ct}}$ method. The primers used are listed in Table 1.

In Vivo Tumor Growth

The in vivo tumor growth experiments were authorized by The Affiliated Hospital of Xuzhou Medical University. pLKO.1-EGFP-E2F3a-shRNA stable expressed U373 cells (1×10^6 cells/0.1 ml) were injected subcutaneously into the right armpit of 4-week-old male athymic nude mice ($n=6$; Shanghai Laboratory Animal Co., Ltd., Shanghai, P.R. China) every 3 days for 45 days. Afterward, the mice were sacrificed, and tumor tissues were excised and weighed. The shortest and longest diameters of the tumors were measured with calipers every 4 days, and tumor volume (mm^3) was calculated using the following

Table 1. Primer Sequences Used in This Study

Gene	Sequences
Bim forward	5'-CCACCAGCACCATAGAAG-3'
Bim reverse	5'-GAGCAGGCACAGAGAAAAG-3'
Bax forward	5'-AGCTGAGCGAGTGTCTCAAG-3'
Bax reverse	5'-TGTCCAGCCATGATGGTTC-3'
Caspase 9 forward	5'-CATGCTGGCTTCGTTTCTG-3'
Caspase 9 reverse	5'-AGGGGCAAACAACAGATGG-3'
Caspase 3 forward	5'-AACTGGACTGTGGCATTGAG-3'
Caspase 3 reverse	5'-ACAAAGCGACTGGATGAACC-3'
MMP-2 forward	5'-TTGACGGTAAGGACGGACTC-3'
MMP-2 reverse	5'-GGCGTTCCCATACTTCACAC-3'
MMP-9 forward	5'-AAGGGCGTCGTGGTTCCAACTC-3'
MMP-9 reverse	5'-AGCATTGCCGTCCTGGGTGTAG-3'
MMP-14 forward	5'-GAGCTCAGGGCAGTGGATAG-3'
MMP-14 reverse	5'-GGTAGCCCGTTTCTACCTTC-3'
MMP-19 forward	5'-CTTTCAAGGGGGACTATGTG-3'
MMP-19 reverse	5'-TATTCAGCTTCTTGGGGAAG-3'
GAPDH forward	5'-AATCCCATCACCATCTTC-3'
GAPDH reverse	5'-AGGCTGTGTCATACTTC-3'

standard formula: $(\text{the shortest diameter})^2 \times (\text{the longest diameter}) \times 0.5$.

Statistical Analyses

All data were obtained from at least three independent tests with triplicate samples and shown as mean \pm SD. GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis (one-way ANOVA for multiple groups and unpaired, two-tailed *t*-test for two groups). Overall survival in relation to WWP2 expression was evaluated by the Kaplan-Meier survival curve and log-rank nonparametric test. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

E2F3a Expression in Glioma Tissues and Cell Lines

E2F3a was significantly increased in glioma tissue when compared with the nontumor tissue of patients in the TCGA database (Fig. 1A). We next compared the survival time of patients from the GSE82009 database. The glioma patients were divided into two groups according to the median level of E2F3a. The cumulative survival rate was significantly higher in patients with lower E2F3a-expressing tumors than in those with higher E2F3a-expressing tumors (Fig. 1B). These results indicate that E2F3a expression could represent a new prognostic factor in glioma patients, and therefore we chose to focus our experimental research on E2F3a.

To investigate the expression pattern of E2F3a in glioma cell lines, we assessed the E2F3a protein levels in human embryonic brain (HEB) and four glioma cell lines. Four glioma cell lines showed a higher expression

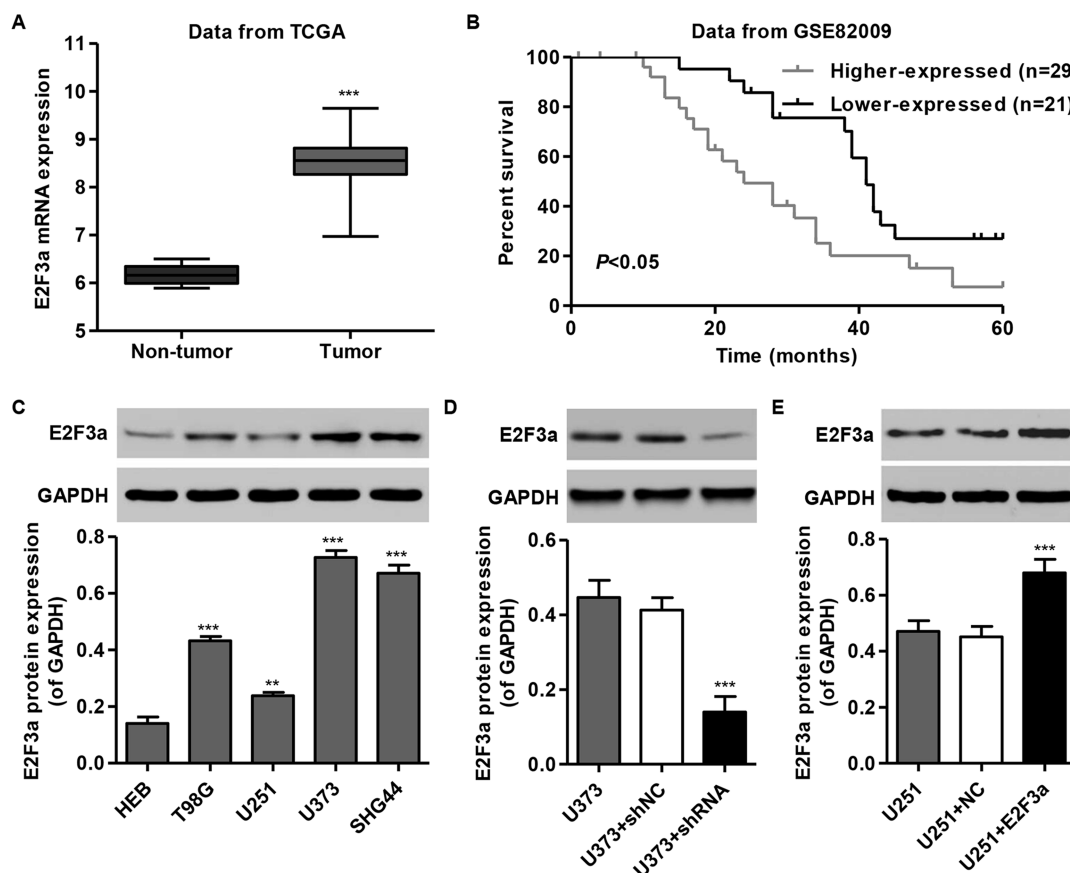


Figure 1. E2F3a expression in glioma tissues and cell lines. (A) Comparison of the expression level of E2F3a between glioma and nontumor tissues in TCGA. (B) Kaplan–Meyer survival curves for two groups of patients in GSE82009. Black, data for patients with low prognostic scores (group 1, higher survival time); gray, patients with high prognostic scores (group 2, poorer survival). (C) E2F3a expression level in four glioma cell lines and normal human embryonic brain (HEB) cells was analyzed by Western blot. (D, E). Expression of E2F3a in U373 and U251 cells was analyzed by Western blot. GAPDH was also detected as the internal control. Representative Western blots (top) and quantitative results are shown (bottom). ** $p < 0.01$ compared with HEB cells; *** $p < 0.001$ compared with HEB cells or corresponding control cells.

of E2F3a when compared with the normal HEB cell line, with the highest expression in U373 cells and the lowest expression in U251 cells (Fig. 1C). These two cell lines were therefore used in the following experiments. To study the function of E2F3a on glioma cell lines, construct and knockdown of E2F3a were generated and stably infected into U373 and U251 cells. E2F3a expression was significantly decreased in E2F3a-shRNA-infected U373 cells (Fig. 1D), whereas E2F3a expression was significantly increased in E2F3a construct-infected U251 cells (Fig. 1E).

Downregulation of E2F3a Inhibits Cell Proliferation of Glioma

To examine the effects of E2F3a knockdown and overexpression on the proliferation of glioma cell lines, CCK-8 assays were performed on both U373 and U251 cells following lentivirus infection for 48 h. E2F3a knockdown

can inhibit U373 cell proliferation, and E2F3a overexpression can promote U251 cell proliferation at both 48 and 72 h (Fig. 2A and B).

Considering that downregulation of E2F3a significantly slowed cell proliferation, we further performed cell cycle analysis to reveal the mechanism of the inhibitory effect of E2F3a shRNA lentivirus on cell proliferation. E2F3a shRNA lentivirus infection significantly increased the percentage of cells in the G_0/G_1 phase and decreased the percentage of cells in both the S and G_2/M phases, compared with the U373 control cells (Fig. 2C and D).

Downregulation of E2F3a Promotes Cell Apoptosis of Glioma

We then investigated the effect of E2F3a knockdown and overexpression on the apoptosis of glioma cell lines. E2F3a knockdown resulted in an approximately six-fold increase in the apoptotic ratio, compared with the

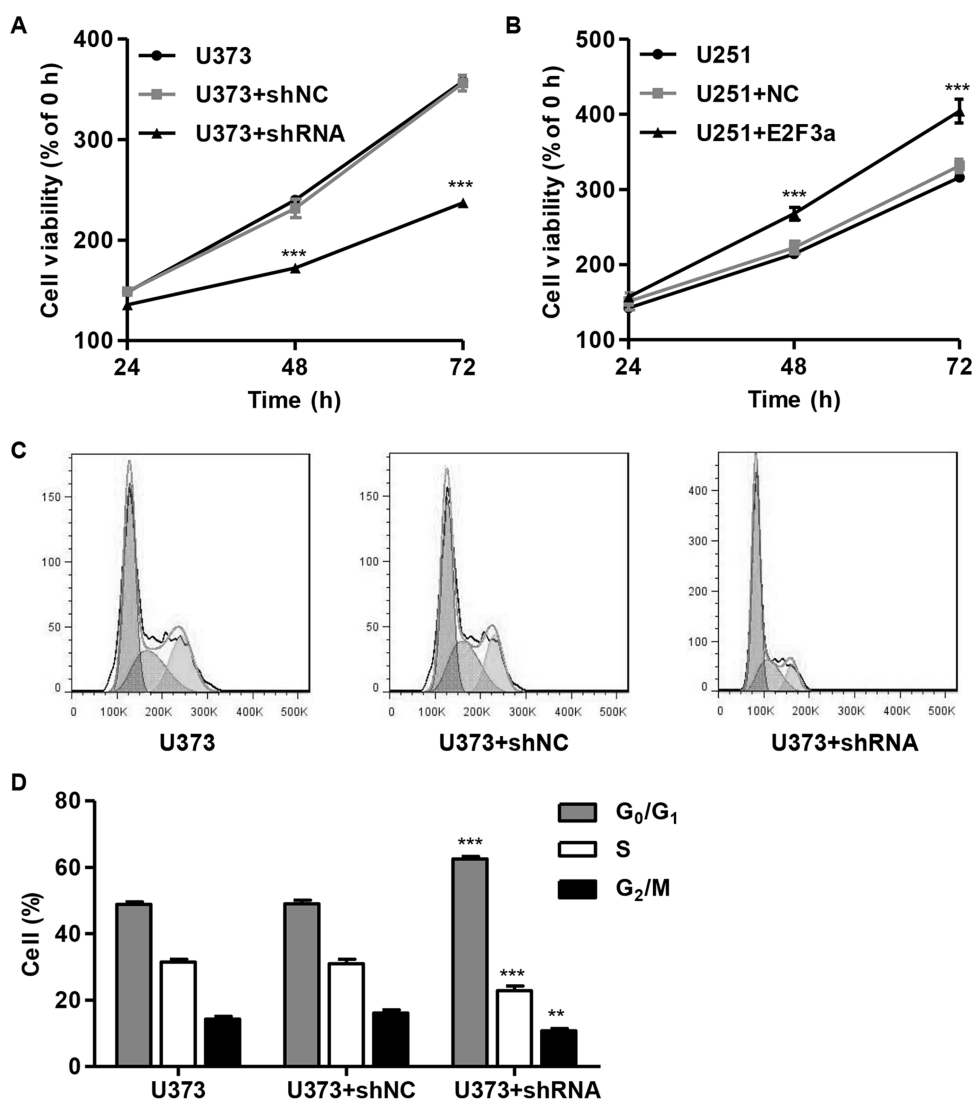


Figure 2. Suppressing E2F3a expression inhibited cell proliferation and induced the G₀/G₁ phase arrest. U373 cells were infected with E2F3a-shRNA and collected 48 h later, and U251 cells were infected with E2F3a constructs and collected 48 h later. (A, B) Cell proliferation was detected in U373 and U251 cells by cell counting kit-8 (CCK-8) assay, respectively. (C, D) Cell cycle profile of U373 cells was analyzed using flow cytometry. ** $p < 0.01$, *** $p < 0.001$ compared with corresponding control cells.

U373 control cells (Fig. 3A and C). E2F3a overexpression resulted in an approximately 54% decrease in the apoptotic ratio, compared with the U251 control cells (Fig. 3B and D).

Downregulation of E2F3a Reduces Cell Migration and Invasion of Glioma

To determine the function of E2F3a on glioma migration, Transwell assays were carried out to quantitatively determine the effect of E2F3a on cell migration and invasion. Similar numbers of control and shNC or vector (NC)-infected cells migrated to the lower face of the Transwell membrane, whereas the E2F3a knockdown cells exhibited a strongly inhibited motility, with only 43%

of cells migrating. E2F3a overexpression cells exhibited a strongly increased motility with approximately 1.6-fold cells migrating (Fig. 4A–D). Additionally, E2F3a knock-down cells exhibited a strongly inhibited invasion, with only 34% of cells invading, and the E2F3a overexpression cells exhibited a strongly increased invasion, with approximately 2.3-fold cells invading (Fig. 4E–H).

E2F3a Affected the Expression of Cell Apoptosis and Migration-Related Protein

The expression levels of apoptosis (Bim, Bax, cleaved caspase 3, and caspase 9) and migration-regulating proteins (MMP-2, MMP-3, MMP-9, and MMP-13) were then detected by RT-PCR and Western blotting,

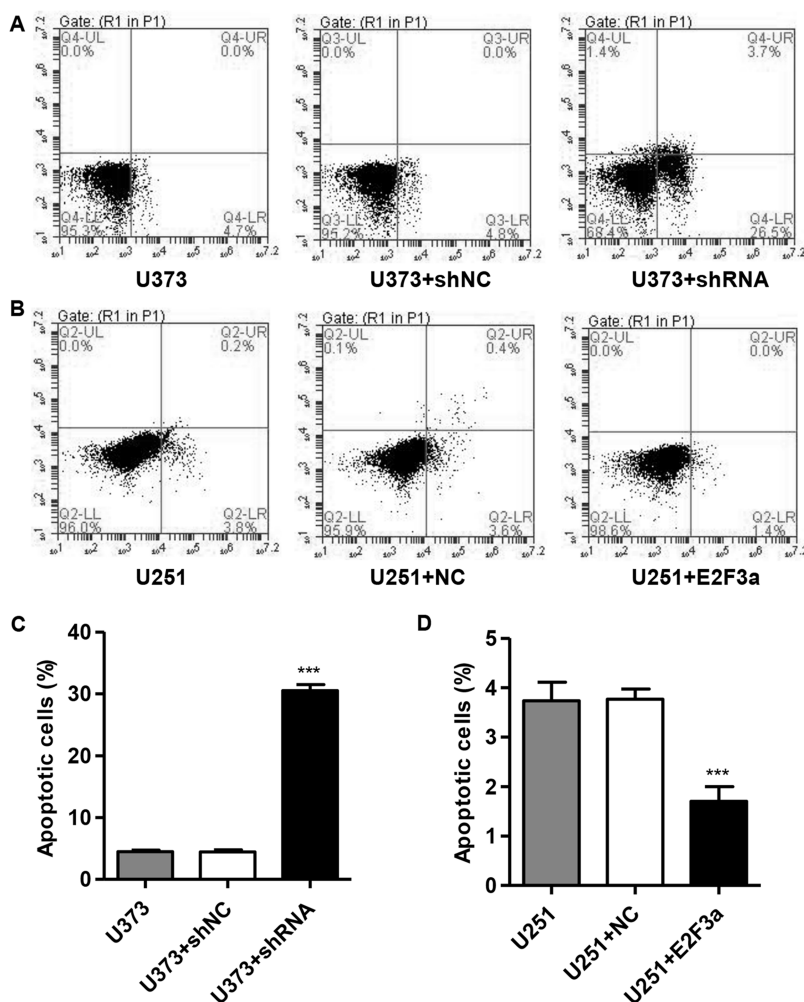


Figure 3. Suppressing E2F3a expression induced apoptosis in glioma cells. U373 cells were infected with E2F3a-shRNA and collected 48 h later, and U251 cells were infected with E2F3a constructs and collected 48 h later. Cell apoptosis of U373 (A, C) and U251 cells (B, D) was analyzed by annexin V/propidium iodide (PI) staining. *** $p < 0.001$ compared with corresponding control cells.

respectively. Interestingly, Bim, Bax, cleaved caspase 3, and caspase 9 mRNA and protein levels were increased by E2F3a knockdown in U373 cells (Fig. 5A, C, and D) but repressed by E2F3a overexpression in U251 cells (Fig. 5F, H, and I). While MMP-2, MMP-3, MMP-9, and MMP-13 mRNA and protein levels were repressed by E2F3a knockdown in U373 cells (Fig. 5B, C, and E), they were increased by E2F3a overexpression in U251 cells (Fig. 5G, H, and J). These data suggest antiapoptosis and migration-promoting roles for E2F3a in glioma cells.

Silencing of E2F3a Suppresses Tumorigenesis of Glioma Cells In Vivo

To confirm the growth inhibitory effect of E2F3a-shRNA in vivo, a xenograft tumor-bearing model was established by inoculating E2F3a-shRNA-infected U373 cells into nude mice. E2F3a-shRNA-treated tumors grew

much slower than the control shNC-treated tumors in mice (Fig. 6A), with average tumor weights of 0.42 ± 0.03 g and 0.16 ± 0.04 g in the shNC and E2F3a-shRNA mice, respectively (Fig. 6B). Furthermore, compared with shNC ones, a significant decrease in E2F3a, PCNA, and Ki-67 expression was observed in tumors formed from knock-down E2F3a cells by Western blot analysis (Fig. 6C–F) and immunohistochemical analysis (Fig. 6G–I).

DISCUSSION

It is known that members of the E2F family act individually or synergistically to regulate cell proliferation and apoptosis, as well as other cellular processes. Among these, depletion of E2F3 has emerged as profoundly suppressing ectopic proliferation and inappropriate apoptosis in the central nervous system (CNS), peripheral nervous system (PNS), placenta, and a number of other tissues

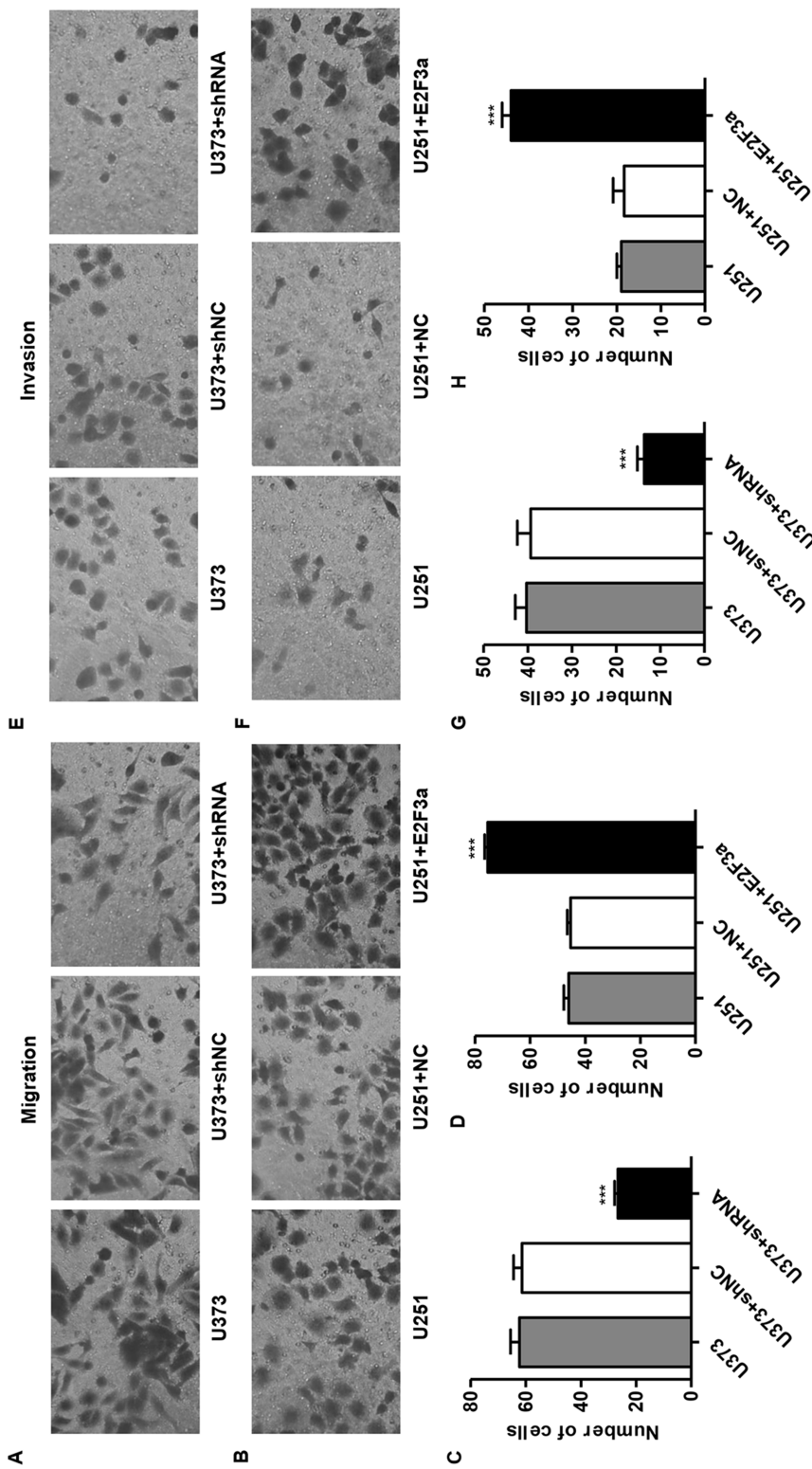


Figure 4. Silencing of E2F3a inhibited cell migration and invasion in glioma cells. U373 cells were infected with E2F3a-shRNA and collected 48 h later, and U251 cells were infected with E2F3a constructs and collected 48 h later. Migration (A–D) and invasion (E–H) of U373 and U251 cells were measured by Transwell assay. *** $p < 0.001$ compared with corresponding control cells.

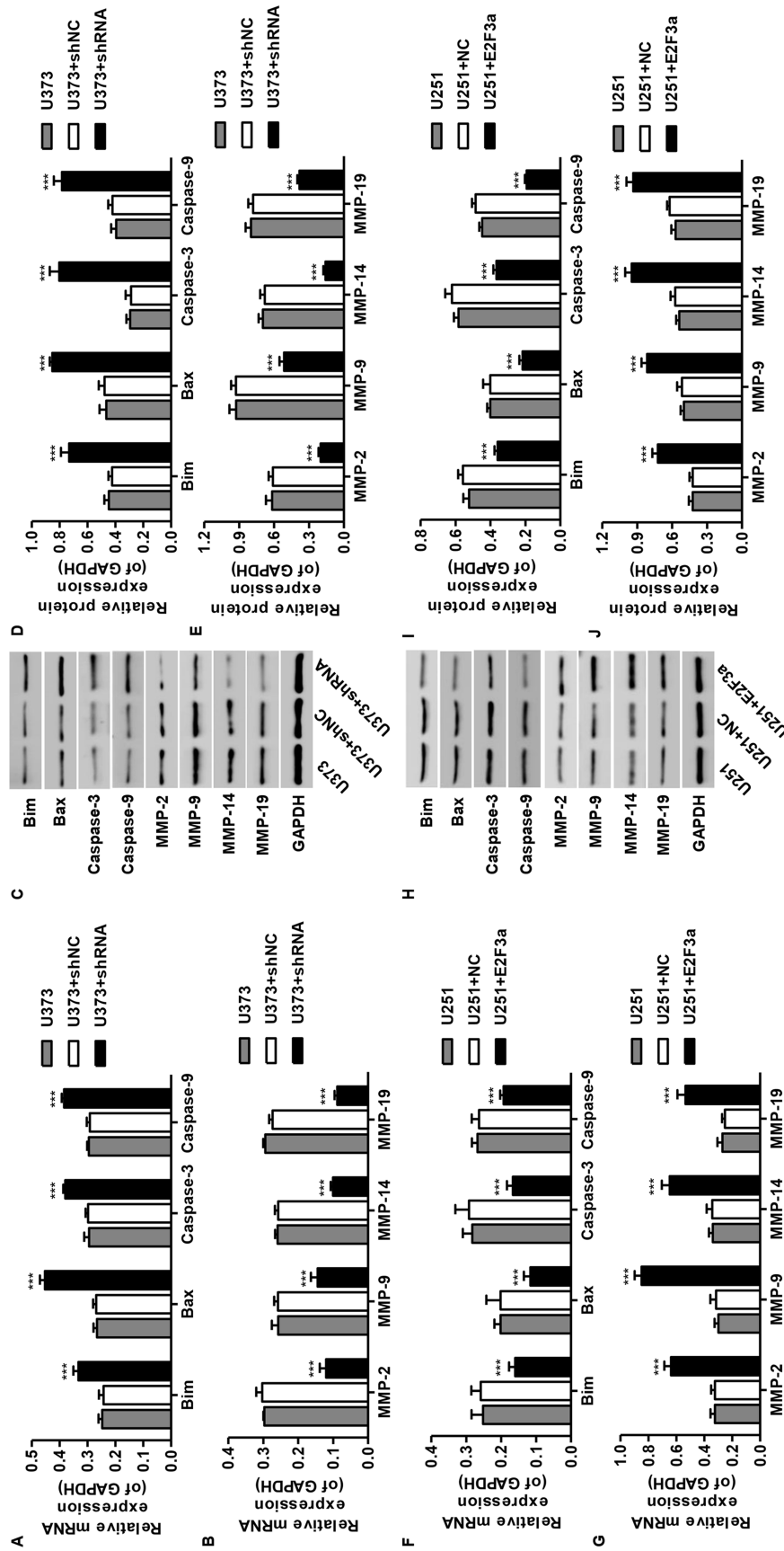


Figure 5. Mechanisms of E2F3a exert their functions in glioma cells. U373 cells were infected with E2F3a-shRNA and collected 48 h later, and U251 cells were infected with E2F3a constructs and collected 48 h later. The expression of apoptosis and migration-related protein in U373 (A–E) and U251 cells (F–J) was measured by real-time PCR and Western blot analysis. *** $p < 0.001$ compared with corresponding control cells.

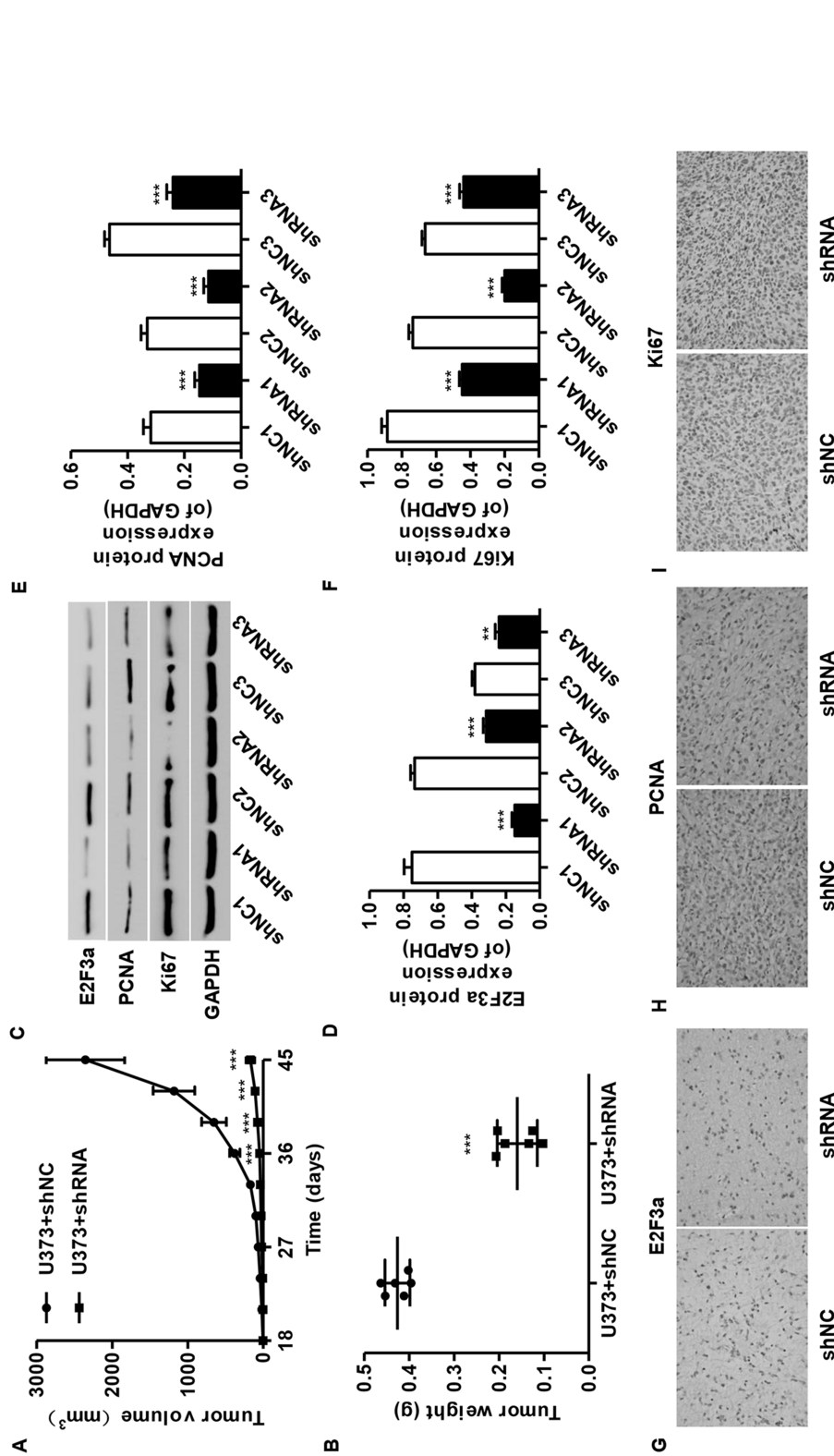


Figure 6. Knockdown of E2F3a in glioma cells reduced tumor growth in vivo. U373 cells infected with shRNA control (shNC) or E2F3a-shRNA were subcutaneously injected into athymic nude mice. Tumor growth was shown 45 days after injection. (A) Time course analysis of tumor growth after injection. (B) Tumor growth was significantly reduced in E2F3a knockdown tumors. (C–F) The expression of E2F3a, PCNA, and Ki-67 in xenografts from the nude mice was determined by Western blot and immunohistochemical analysis (G–I). ** $p < 0.01$, *** $p < 0.001$ compared with corresponding control cells.

including embryos^{17,18}. E2F3 can also function as tumor suppressors and oncogenes. For instance, lacking one or two copies of E2F3 (E2F3a and E2F3b) decreased the development of pituitary tumors, but the incidence and invasion abilities of medullary thyroid carcinomas are increased¹⁹. Low expression of E2F3a was associated with inferior prognosis in childhood acute lymphoblastic leukemia¹⁴ but was associated with improved progression-free survival and overall survival of patients with ovarian cancer¹³. Although E2F3a has been widely reported to be involved in tumorigenesis, there are few reports on its role in gliomagenesis. E2F3a protein expression is more upregulated in glioma tissues than in normal brain tissues and is greater in grade III and grade IV than in grade II, suggesting an important role of E2F3a in gliomagenesis¹⁶. In this study, the phenotypic and molecular effects of E2F3a knockdown and overexpression on glioma cells were characterized.

E2F3a plays an important role in regulating cell proliferation and cell cycle progression. E2F3a functions mainly at the G₁/S phase transition and is recognized as one of the key factors in cell cycle control. Transgenic mice expressing E2F3a have a hyperplastic and hyperproliferative epidermis and are predisposed to developing spontaneous tumors in the skin¹⁵. More recently, several miRNAs such as miR-34a, miR-20a, miR-125b, and miR-200c have been reported to inhibit cell proliferation by downregulating E2F3 in tumor cells^{20,21}. On the other hand, E2F3a overexpression in hepatocellular carcinoma did not lead to alterations in proliferation¹⁰. Thus, not all tissues respond to deregulated E2F3a expression in the same way. In this study, by taking advantage of the level of endogenous E2F3a expression in glioma cells, E2F3a was silenced in U373 cells and was overexpressed in U251 cells. We found that silencing E2F3a results in significantly decreased proliferation and percentage of S and G₂/M phase, and an increase in the percentage of the G₀/G₁ phase in U373 cells. In contrast, E2F3a overexpression results in increased proliferation in U251 cells. Moreover, our *in vivo* study showed that knockdown of E2F3a in U373 cell-injected mice showed decreased tumor growth *in vivo*, evidenced by decreased expression of PCNA and Ki-67. This suggests that E2F3a has a major effect on cell proliferation and cell cycle of the glioma cells both *in vitro* and *in vivo*.

Given that E2F3a plays an important role in the G₁/S transition and cell proliferation, low expression of this gene in glioma cells would result in an increase in apoptosis. This finding coincided with the results of many studies. For instance, E2F3a increases the apoptosis of the epidermis through the activation of caspase 3 in a transgenic mouse model independent of p53⁹. Very recently, E2F3a has been shown to mediate DNA damage-induced apoptosis²², which is significantly reduced by the absence

of ataxia telangiectasia mutated (ATM) kinase in transgenic mouse tissue and in primary human fibroblasts¹⁵. Consistent with these results, silencing E2F3a significantly increases the apoptotic events in U373 cells and decreases apoptotic events in U251 cells, suggesting an antiapoptotic role for the E2F3a proteins in glioma cells. However, overexpression of E2F3a proteins results in an increase in apoptotic events in HepG2 cells¹⁰, suggesting a contrary role for E2F3a in cell apoptosis between these two cancers.

To determine the oncogenic role of E2F3a in glioma cells, we detected the cell invasion and migration abilities of U373 and U251 cells with the knockdown or overexpression of E2F3a. We found that the invasive and migration activities were inhibited by the downregulation of E2F3a expression, but were promoted by the upregulation of E2F3a expression. In line with our findings, several miRNAs, such as miR-144, miR-497, miR-874, and miR-203, have been reported to inhibit cell migration and invasion of tumor cells by targeting E2F3^{23–26}, and E2F3 mediates neuronal migration in a manner beyond cell cycle regulation²⁷.

Many of the genes that are targets of E2F3a are involved in apoptosis, transcriptional control, and cell cycle regulation^{10,27}. XAF1 and TNFSF10, proapoptotic proteins, are the two most prominent genes upregulated by E2F3a. In this study, we found four genes associated with cell apoptosis progression, including Bim, Bax, caspase 3, and caspase 9, that were upregulated by E2F3a knockdown. Bim may act by directly binding and activating Bax and Bak²⁸. Also, previous data have shown that caspase 9 is a highly specific protease that only cleaves a few proteins, whereas caspase 3 and caspase 7 contribute to the majority of the cleavage that takes place during apoptosis²⁹. Thus, E2F3a may inhibit the apoptosis of glioma cells through the Bim, Bax, caspase 3, and caspase 9 pathways. Moreover, four genes associated with cell motility, including MMP-2, MMP-3, MMP-9, and MMP-13, were downregulated by E2F3a knockdown. It has been shown that the enhanced expression of MMPs might favor adhesion and migration not only of glioma-infiltrating microglia but also of glioma cells. Expression of the MMP-2 in human glioma tissues is upregulated and directly correlated to glioma invasiveness and the survival rate of tumor-bearing mice³⁰. A recent study showed that MMP-9 may play a special role in mediating glioma stem-like cells as its expression is induced by TGF- β secreted by glioma-infiltrating macrophages³¹. One of the main MMPs implicated in tumor invasion is the membrane-bound MMP, MMP-14, which is important for the glioma-infiltrating macrophage stimulation of GBM invasion³². MMP-19 is highly expressed in glioma tumors and enhances their invasive character *in vitro*³³.

In summary, we demonstrated that E2F3a functions as an inhibitor of apoptosis and motility in glioma cell lines. Western blot identified 10 target genes regulated by E2F3a, which are involved in cell apoptosis, migration, and invasion. These results provide significant insights into the functions of the E2F3a and the underlying molecular mechanisms. Future studies will focus on the elucidation of the specific E2F3a target genes and their exact roles in mediating apoptosis and motility in glioma.

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