Eukaryotic initiation factor 3C silencing inhibits cell proliferation and promotes apoptosis in human glioma

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Abstract. Eukaryotic initiation factor 3, subunit c (eIF3c), an oncogene overexpressed in human cancers, plays an important role in cell tumorigenesis and proliferation. However, studies assessing its function in gliomas are scarce. The present study evaluated for the first time, the role of eIF3c in gliomas. Immunohistochemistry was carried out to assess eIF3c expression in 95 human glioma samples and normal brain tissues. Then, the eIF3c mRNA levels were detected in tumor and normal brain specimens by quantitative RT-PCR. In addition, eIF3c mRNA levels were assessed in four glioma cell lines (U87, U251, A172 and U373) by semi-quantitative RT-PCR. The RNA interference (RNAi) technology was employed to knock down the eIF3c gene in the U251 cells. Western blot analysis, BrdU assay and flow cytometry were used to measure eIF3c protein levels, cell proliferation, cell apoptosis and cell cycle, respectively. The eIF3c protein was overexpressed in the human glioma specimens. In agreement, the eIF3c mRNA expression levels were significantly higher in the human glioma tissues compared with the normal brain samples ($P<0.0001$). In addition, eIF3c mRNA was detected in all the glioma cell lines. Silencing the eIF3c gene in the U251 cells by RNAi significantly suppressed cell proliferation ($P<0.01$) and increased apoptosis ($P<0.01$). Finally, a stark decrease was observed in the G1 phase cell number ($P<0.01$), while the S and G2 phase cells were significantly increased ($P<0.01$) after eIF3c knockdown. These findings suggest that eIF3c is overexpressed in human gliomas and essential for their proliferation and survival. Therefore, inhibiting eIF3c expression may constitute an effective therapy for human glioma.

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Introduction

Human glioma remains a refractory and life-threatening cerebral disease with poor prognosis, despite the improvement in current available therapies, including surgery, radiotherapy and chemotherapy. It is a histologically and molecularly heterogeneous central nervous system (CNS) malignancy (1). Malignant glioma accounts for 32-45% of all primary brain tumors and 70-80% of malignant cerebral tumors (2-5); it is the second major cause of cancer-related deaths in both children and young adults. The overall survival time of most patients with glioma is less than two years after diagnosis (6), with a the median life expectancy of only 12-14 months (7) and a 5-year survival rate of less than 10% (8). This poor prognosis illustrates the urgent need to unveil the novel molecular mechanisms involved in glioma, with the hope of finding novel molecular targets for the treatment of this disease.

Close associations have been found between several cancers and eukaryotic initiation factors (eIFs) (9), which are the key factors of translation initiation, particularly in the first steps of translation; indeed, eIFs regulate protein synthesis, and control cell growth, size and proliferation. Specifically, the mRNA is activated for pre-initiation complex (PIC) binding by eIFs that recognize the mRNA m7G cap structure at the 5'-end or the poly(A) tail at the 3'-end (10). Among eIFs, eIF2 brings the Met-tRNA to the 40S ribosomal subunit; the elF4 complex stabilizes the mRNA by binding to the cap [7-methylguanosine (m7GpppN)] (11,12); eIF4G establishes a bridge between eIF3 and eIF4E (13,14); the eIF3 complex which comprises 13 subunits (eIF3a to m) (9) serves as a scaffold to mediate translation initiation and recognizes the first AUG initiation codon closest to the 5'-end of the mRNA (9). It has been suggested that interactions between eIF3 and other translation initiation factors control the binding of the ternary complex (eIF2-GTP-methionine) to the small (40S) ribosomal subunit, position the mRNA on the 40S subunit and modulate the stringency of the start codon selection (15,16). Importantly, misregulation of eIF3 expression has been implicated in oncogenesis and the maintenance of the cancerous state (17-19), indicating the importance of the eIF3 complex.

eIF3c (913 amino acids, located at 16p11.2), a housekeeping gene localized in the cytoplasm, is essential for the assembly of the eIF3 complex, as well as the general initiation complex (20-22). In the past few years, the eIF3c gene has been demonstrated to be essential for cell proliferation in numerous human tumors, including testicular seminomas (23), meningiomas (24) and colon cancer cells (25). However, little is known concerning the relationship between eIF3c and human glioma.

In the present study, we first assessed the eIF3c expression in human glioma tissues and determined its correlation with pathologic grades. Then, the eIF3c gene was knocked down in the human glioma U251 cells using the RNA interference (RNAi) technology in order to explore its functions in cell tumorigenesis, proliferation, apoptosis and cycle. The findings presented here provide new insights into the biological role of the eIF3c gene in human gliomas and identify eIF3c as a potential diagnostic or therapeutic target for human gliomas.

Materials and methods

Patients and glioma specimens. The present study included 95 Chinese patients with cerebral glioma treated surgically at the Department of Neurosurgery, the Second Affiliated Hospital of Hebei Medical University between January 2008 and December 2013. There were 50 males and 45 females, aged 49.65±15.15 (ranging from 16 to 73 years). Inclusion criteria; all the specimens were obtained at the initial surgery and the patients had not received preoperative radiotherapy, chemotherapy or immunotherapy. The pathologic grades of the samples were confirmed independently by two pathologists according to the revised World Health Organization criteria of tumors for the central nervous system (1): respectively 11, 23, 26 and 35 patients were found with grade I-IV tumors. Normal cerebral tissues used as controls were obtained from 31 patients suffering from severe cranio-cerebral injury who underwent internal decompression operation. For each resected specimen, a portion was immediately snap-frozen in liquid nitrogen, and the remaining part was fixed with formalin and embedded in paraffin for histological studies. Written informed consent forms were obtained from all the patients involved in the present study. All the experiments using human samples were approved by the Ethics Committee of the Second Hospital of Hebei Medical University and complied with the current laws of China.

Immunohistochemistry staining. Immunohistochemistry (IHC) staining of pathological sections was performed following the standard procedures. Paraffin blocks were cut in 4-μm sections and mounted on glass slides. After being dewaxed in xylene, the sections were rehydrated in ethanol gradients and immerged in water. Antigen retrieval was performed by microwave exposure in 0.01 mmol/l sodium citrate buffer solution (pH 6.0). Afterwards, the sections were blocked with 10% bovine serum albumin for 30 min and incubated overnight with primary rabbit polyclonal anti-eIF3c antibodies (1:50), followed by biotinylated secondary anti-goat antibodies (1:200) for 60 min. Then, the sections were treated with diaminobenzidine (DAB) activated with H_2O_2, used according to the manufacturer's instructions (Vector Laboratories). The slides were finally counterstained with hematoxylin and examined using a Leica DM1000 microscope (Leica, Germany).

Assessment of eIF3c gene expression in 95 glioma tissue samples and 31 normal cerebral specimens by real-time quantitative PCR. Total RNA was extracted from the clinical specimens (glioma and normal cerebral tissues) using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed with M-MLV reverse transcriptase (Promega, USA) and oligo(dT) primers (Sangon, Shanghai). cDNA (1 μl) was used for real-time PCR, which was performed to detect eIF3c using SYBR Master Mixture (Takara, Japan) according to the manufacturer's protocol. Sequences of eIF3c and GAPDH primers were as follows: GAPDH (internal control forward, 5'-TGAC TTCAACAGCGACACCCA-3' and reverse, 5'-CACCCTGTT GCTGTAGCCAAA-3'; eIF3c forward, 5'-AGATGAGGAT GAGGATGAGGAC-3' and reverse, 5'-GGAATCGGAAGAT GTGGAACC-3'.

Real-time PCR was carried out with initial denaturation at 95˚C for 15 sec, followed by 45 cycles of 95˚C for 5 sec and 60˚C for 30 sec. Data were analyzed using GraphPad Prism 4.0 software. The $2^{-\Delta\Delta Ct}$ method was used to quantitate the relative gene expression.

Cell culture. Human glioma cells (U87, U251, A172 and U373) were purchased from the National Platform of Experimental Cell Resources for Sci-Tech (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (all from Gibco, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gen-View, USA). All the cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2.

Assessment of eIF3c gene expression in four human glioma cell lines (U87, U251, A172 and U373) by semi-quantitative RT-PCR. Total RNA from the human glioma cell lines U87, U251, A172 and U373 was extracted under RNase-free conditions using TRIzol reagent according to the manufacturer's instructions. For semi-quantitative RT-PCR analysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Total RNA (2 μ) from each sample was reverse transcribed to single-stranded cDNA. cDNA (1 μg) was used as template for PCR with the following primers: eIF3c forward, 5'-AGATGAGGATGAGGATGAGGAC-3' and reverse, 5'-GGAATCGGAAGATGTGGAACC-3', product size of 175 bp; GAPDH forward, 5'-TGACTTCAACAGCGAC ACCCA-3' and reverse, 5'-CACCCTGTTGCTGTAGCCA AA-3', product size of 121 bp. The semi-quantitative RT-PCR was carried out with initial denaturation at 95˚C for 15 sec followed by 22 cycles of 95˚C for 5 sec, and 60˚C for 30 sec. PCR products were separated on a 2% agarose gel and analyzed by imaging.

Construction and transfection of the eIF3c-siRNA lentivirus. The sequences used for the eIF3c-siRNA and scrambled siRNA were: 5'-GAC CAT CCG TAA TGC CAT GAA-3' and 5'-TTC TCC GAA CGT GTC ACG T-3', respectively. These nucleotide sequences were inserted into the plasmid using the siRNA expressing vector pGCSIL-GFP and lentivirus packing eIF3c-siRNA Lentivector Expression Systems (both from GeneChem, Shanghai, China). The identities of the generated lentiviral based siRNA expressing vectors were confirmed by

DNA sequencing. Human renal epithelial 293T cells were infected with eIF3c-siRNA lentivirus and scramble siRNA lentivirus (negative control) and the interference efficiency was determined by western blot analysis.

Human glioma U251 cells were infected with eIF3c-siRNA lentivirus and scrambled siRNA lentivirus (negative control) at a multiplicity of infection (MOI) of 100. Non-transfected cells were also included as controls. After three days of infection, GFP expression was observed by fluorescence microscopy. After five days of infection, the cells were harvested to determine knockdown efficiency by real-time quantitative PCR.

Western blot analysis. Western blot analysis was performed to evaluate the eIF3c expression levels in the eIF3c-siRNA lentivirus-infected 293T cells compared to the scrambled siRNA lentivirus (negative control)-infected cells. For protein isolation, the cells were washed with cold phosphate-buffered saline (PBS) and lysed with radio-immunoprecipitation assay (RIPA) buffer [100 mmol/l Tris-HCl (pH 6.8), 2% nonidet P-40, 4% sodium dodecyl sulfate (SDS)]. Proteins were separated by SDS-PAGE, transferred onto PVDF membranes (Amersham, USA), probed with anti-eIF3c antibody (1:200; Abcam, USA), and detected using the electrochemiluminescence (ECL) kit (Amersham). Bands were obtained after exposure to X-ray film. GAPDH was used as a control and was detected by anti-GAPDH antibody (Santa Cruz Biotechnology, USA). The bands on the X-ray film were quantified with an ImageQuant densitometric scanner (Molecular Dynamics, USA).

BrdU cell proliferation assay. The U251 cells infected with lentivirus expressing eIF3c-siRNA or scrambled siRNA (negative control) were cultured for 48 h. Cell proliferation was assessed with the BrdU Cell Proliferation ELISA kit (Roche Applied Science, Switzerland) according to the manufacturer's instructions. The cells were seeded at appropriate density into 96-well plates and cultured for 1-4 days. During the final 2-24 h, BrdU reagents were diluted at 1:100 and added to the cells (10 μl/well). Then, the cells were fixed with FixDenat (200 μl/well) for 30 min and blocked with 5-10% BSA for 30 min at room temperature. Anti-BrdU-POD antibody was added (100 μl/well) to the cells for 90 min at room temperature. After three washes with washing buffer (200-300 μl/well), the substrate solution (100 μl/well) was added and incubated for 5-30 min in the dark. Color was developed with 10% H_2SO_4 (50 μl/well) for 30 min and BrdU amounts were determined at 450 nm on an ELx800 Absorbance Mircoplate Reader (BioTek, USA).

Colony formation assay. The U251 cells that were transfected with eIF3c-siRNA lentivirus or scrambled siRNA lentivirus (negative control) for five days were harvested and seeded in 6-well plates at a density of 500 cells/well. The medium was changed every three days. After two weeks of culture, the cells were fixed with 4% paraformaldehyde and stained by adding freshly prepared diluted Giemsa stain for 20 min. Finally, the cells were rinsed with distilled water and colonies with >50 cells were counted by fluorescence microscopy (Olympus IX71, Japan).

Cell cycle analysis. The evaluation of cell cycle distribution was carried out using flow cytometry. The U251 glioma cells infected with lentivirus expressing eIF3c-siRNA or scrambled siRNA (negative control) were incubated for 96 h with conditioned medium. Then, the cells were resuspended, seeded in 6 cm dishes, and grown until ~80% confluency. Afterwards, the cells were harvested and fixed with 70% ice-cold alcohol for at least 1 h. After being washed with PBS, the cells were treated with the staining solution containing propidium iodide (PI), RNase and PBS. Finally, the cells were filtered through a 50-μm nylon mesh and cell cycle profiles were analyzed by flow cytometry (FACSCalibur; Becton-Dickinson, USA). At least $1x10^6$ cells/sample were prepared for cell cycle analysis and triplicate experiments were performed.

Evaluation of cell apoptosis. Apoptosis in the cells was assessed using the Annexin V-APC apoptosis detection kit (BioVision, USA). The U251 cells infected with lentivirus expressing eIF3c-siRNA or scrambled siRNA (negative control) were incubated for five days, harvested and washed with PBS. Then, the cells at a final density of $1x10^6$-$1x10^7$/ml were resuspended in staining buffer. For the apoptosis assessment, 100 μl of cell suspension were mixed with 5 μl of Annexin V solution at room temperature in the dark for 10-15 min. Finally, cell apoptosis was analyzed by flow cytometry (FACSCalibur). All the assays were performed in triplicates.

Statistical analysis. Data were analyzed using the statistical package SAS version 8 software (SAS, USA). All the data were expressed as mean ± standard deviation (SD). Significant differences between the groups were analyzed by a Chi-square test and the one-way ANOVA followed by the Student-Newman-Keuls (SNK) test. P<0.05 was considered to indicate a statistically significant result.

Results

eIF3c is overexpressed in glioma and associated with pathologic grade. As shown in Fig. 1, the eIF3c protein was overexpressed in the human glioma samples, yet hardly detected in the normal brain specimens. In immunopositive glioma cells, the labeling was primarily cytoplasmic as observed by light microscopy. The positive expression rate of the eIF3c protein was significantly different (χ^2=55.0385, P<0.0001) between the human glioma (83.16%, 79/95) and the normal brain (3.23%, 1/31) tissues. In addition, a significant difference (χ^2=9.0958, P=0.0026) was found between the low grade (grade I and II, 23/34, 67.65%) and the high grade (grade III and IV, 56/61, 91.80%) gliomas. Notably, no significant correlation between eIF3c protein level and gender, age, tumor size and site was found (all P>0.05). Representative images of the eIF3c immunostaining are shown in Fig. 1A-K, and the related results are summarized in Tables I and II.

Glioma tissues and cells display higher eIF3c mRNA expression. Real-time quantitative PCR data showed that eIF3c mRNA levels were significantly higher in human gliomas compared with the normal cerebral tissues (P<0.01, Fig. 1L).

In addition, semi-quantitative RT-PCR revealed the presence of the eIF3c gene in all the human glioma cell lines tested, including U87, U251, A172 and U373 (Fig. 2).

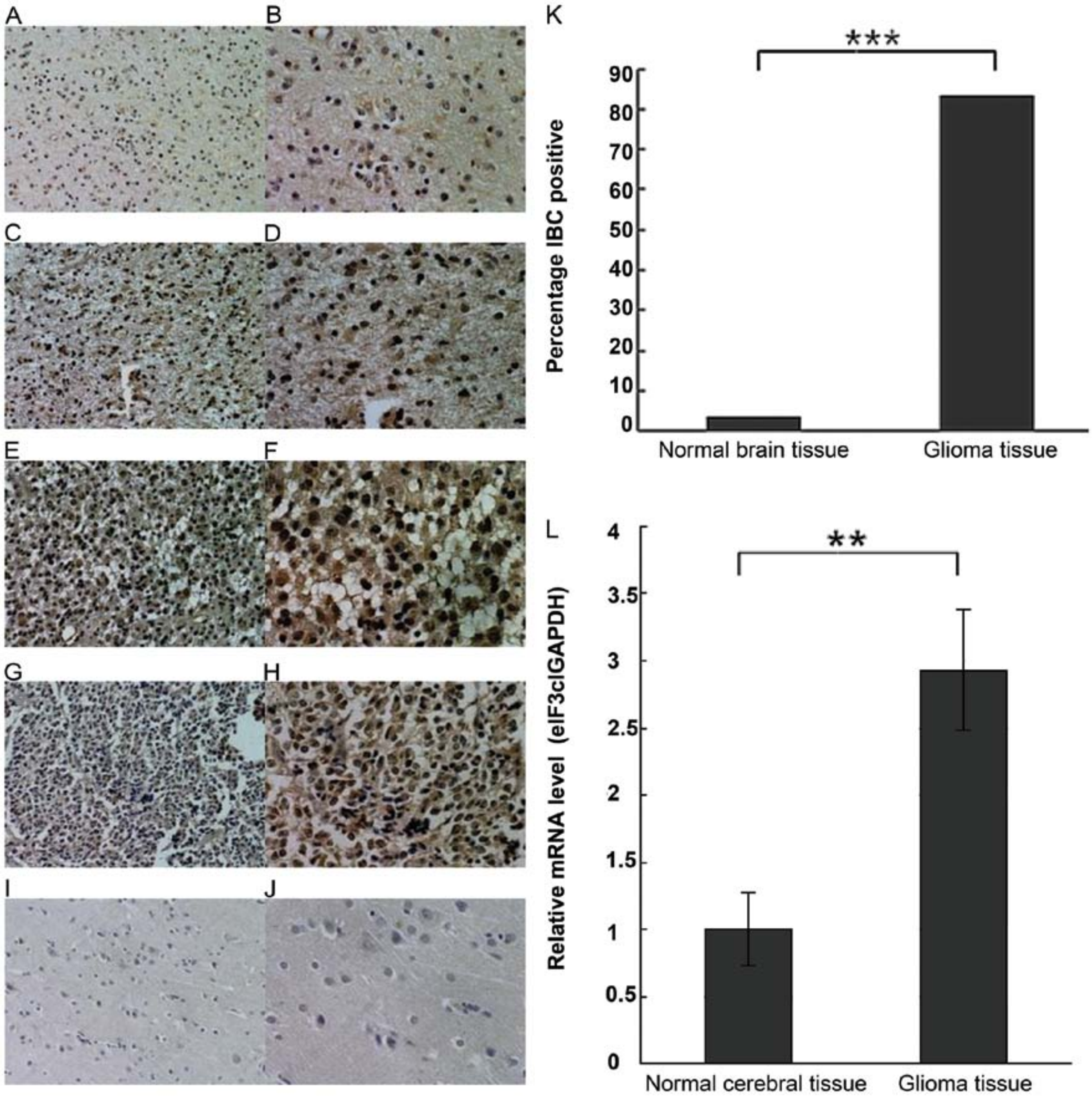

Figure 1. eIF3c protein and mRNA expression in glioma and normal brain tissues. (A-J) Representative sections of eIF3c immunoreactivity. Immunohistochemical expression of eIF3c in (A-H) glioma specimens and (I-J) normal cerebral tissues. The eIF3c-positive cells contain homogeneous brown-yellow areas in the cytoplasm. WHO I grade (A) x200; (B) x400; WHO II grade (C) x200; (D) x400; WHO III grade (E) x200; (F) x400; WHO IV (G) x200; (H) x400, normal cerebral tissue; (I) x200; and (J) x400. (K) The eIF3c protein levels were detected by immunohistochemistry in glioma and normal brain tissues. (L) The eIF3c mRNA levels in human glioma samples and normal cerebral tissues were detected by real-time quantitative PCR (glioma tissue vs. normal brain tissue, $^{**}P<0.01$, $^{***}P<0.001$). eIF3c, eukaryotic initiation factor 3, subunit c.

Table I. Expression of eIF3c in human gliomas and normal brain samples by IHC staining.

		Expression status of eIF3c				
Group	Case no.	Negative	Positive	Percentage (%)	χ^2	P-value
Glioma tissues	95	16	79	83.16	55.0385	<0.0001
Normal brain tissues	31	30	1	3.23		

eIF3c, eukaryotic initiation factor 3, subunit c; IHC, immunohistochemistry.

eIF3c-siRNA delivery results in decreased eIF3c expression in 293T and U251 cells. Human renal epithelial 293T cells were infected with eIF3c-siRNA lentivirus or scrambled siRNA lentivirus (negative control). As shown in Fig. 3, the eIF3c protein levels detected by western blot analysis were markedly decreased in eIF3c-siRNA infected cells compared with those infected with scrambled siRNA. These data indicated that the eIF3c gene was effectively silenced.

Table II. Relationship between eIF3c expression and clinicopathological parameters (cases,%) by IHC staining.

Clinicopathological parameters	Case no.	eIF3c expression pattern		Positive percentage (%)	χ^2	P-value
		Negative	Positive			
Age (years)						
<50	39	7	32	82.05	0.0578	0.8099
≥50	56	9	47	83.93		
Gender						
Male	50	8	42	84.00	0.0534	0.8172
Female	45	8	37	82.22		
Tumor diameter (cm)						
<3	43	6	37	86.05	0.4680	0.4939
≥3	52	10	42	80.77		
Glioma grades						
I+II	34	11	23	67.65	9.0958	0.0026
III+IV	61	5	56	91.80		

eIF3c, eukaryotic initiation factor 3, subunit c; IHC, immunohistochemistry.

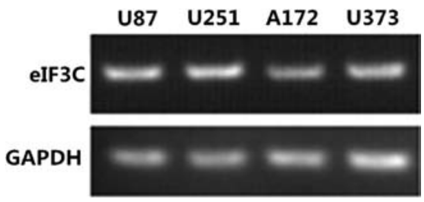

Figure 2. eIF3c gene expression in selected human glioma cell lines. This was determined by semi-quantitative RT-PCR. GAPDH was used as an internal control. eIF3c, eukaryotic initiation factor 3, subunit c.

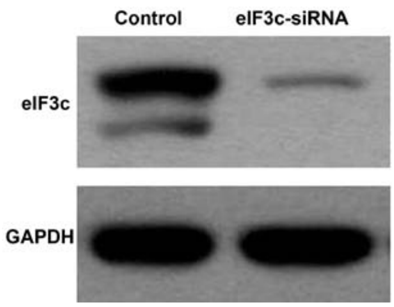

Figure 3. eIF3c protein levels in 293T cells after silencing. Protein levels were determined by western blot analysis. Compared with negative control (NC), eIF3c protein levels in 293T cells were markedly reduced after eIF3c silencing by RNAi. GAPDH was used as an internal control. eIF3c, eukaryotic initiation factor 3, subunit c.

The successful transfection of eIF3c-siRNA or scrambled siRNA into the U251 cells was confirmed by microscopic green fluorescence detection and real-time PCR. As shown in Fig. 4A-E, transfection efficiency was >80% by 3 days after infection for both eIF3c-siRNA and scrambled siRNA sequences. As shown in Fig. 4F, eIF3c-siRNA treatment resulted in markedly downregulated gene expression of eIF3c, i.e. by 80%, compared with the scrambled siRNA group. Therefore, the high expression of eIF3c in U251 cells was suppressed by siRNA infection.

eIF3c silencing results in decreased proliferation of the U251 cells (BrdU assay). In order to determine the eIF3c function on the cell growth, the U251 cells expressing either eIF3c-siRNA or scrambled siRNA sequences were analyzed by BrdU incorporation. The amounts of DNA synthesized decreased significantly on days 1 and 4 after infection with eIF3c-siRNA lentivirus ($P<0.01$, Fig. 4G) compared with the scrambled siRNA lentivirus (negative control) group. These results indicated that cell proliferation and DNA synthesis were significantly suppressed by silencing eIF3c in the human glioma U251 cells.

eIF3c silencing decreases U251 cell proliferation as assessed by colony formation assay. As shown in Fig. 5, no typical clone formation was achieved, indicating that the U251 cells have poor ability to form colonies. However, U251 cell numbers decreased significantly in eIF3c-siRNA infected-cells when compared with the scrambled siRNA (negative control) group. These results further indicated that eIF3c gene knockdown resulted in decreased U251 cell proliferation.

eIF3c gene silencing results in cell cycle arrest in the U251 cells. We assessed the role of eIF3c in cell cycle progression of the human glioma by PI-FACS in U251 cells (Fig. 6A). In the scrambled siRNA (negative control) group, 64.05±2.20, 23.54±2.31 and 11.08±0.69% cells were found in the G0/G1, S and G2/M phases, respectively; in the eIF3c-siRNA group, 50.83±0.55, 35.26±0.82 and 15.64±1.38% cells were in the G0/G1, S and G2/M, respectively. As shown in Fig. 6B, eIF3c-siRNA lentivirus cultures displayed a significant increase in the percentage of S ($P<0.01$) and G2 ($P<0.01$ phase cells, concomitantly with a significant decrease in G1 phase

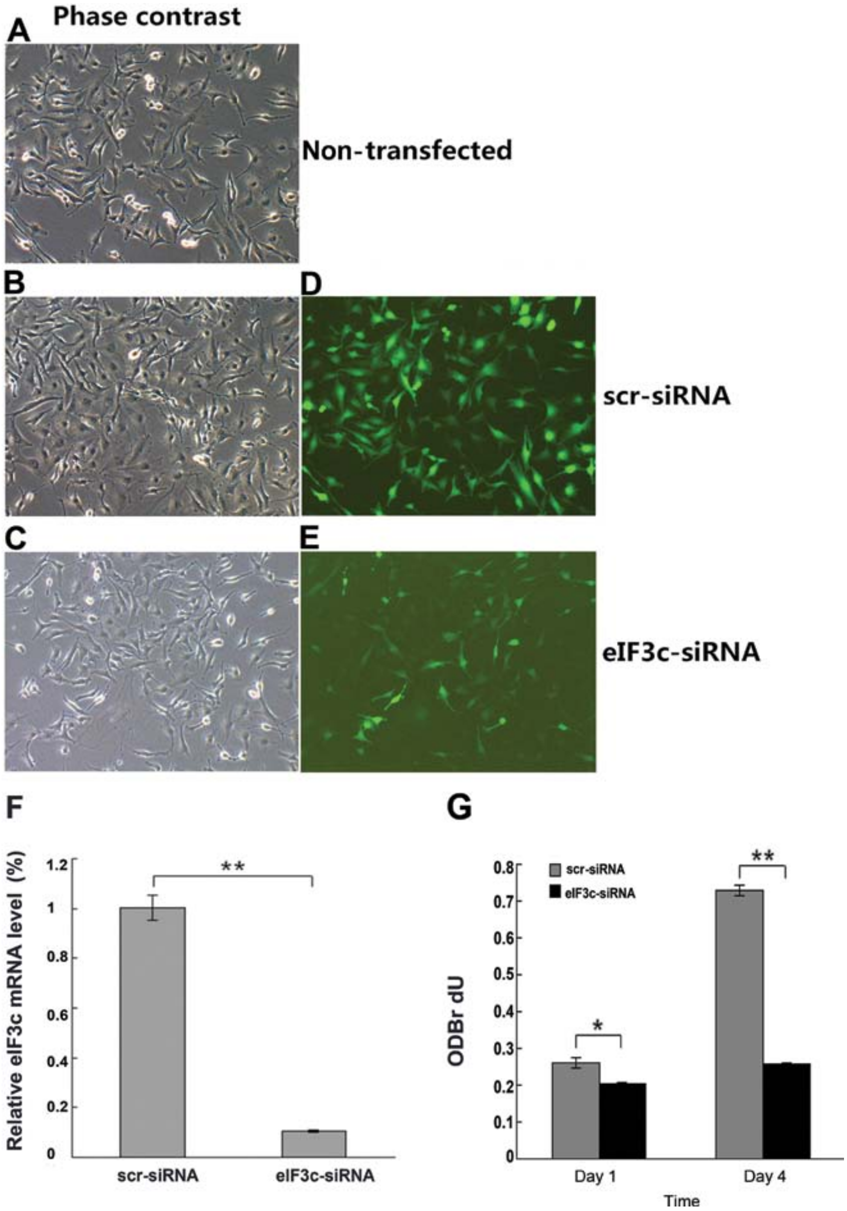

Figure 4. Lentivirus-mediated knockdown of the eIF3c gene in U251 glioma cells results in reduced cell proliferation. (A-E) Knockdown efficiency of Lenti-eIF3c-shRNA. The U251 cells were transfected with Lenti-Scr-siRNA or Lenti-eIF3c-siRNA as indicated in Materials and methods. More than 80% U251 cells expressed GFP. Light (left) and fluorescence (right) micrographs of human glioma U251 cells. (A) x100; (B) NC x100; (C) KD x100; (D) NC x100; (E) KD x100. (NC, negative control; KD, knockdown). (F) The total cell mRNA levels were analyzed by RT-PCR. (G) Lenti-eIF3c-shRNA inhibited U251 cell proliferation by BrdU incorporation assay. The U251 cells in the logarithmic phase were trypsinized and seeded onto the 96-well plates. The cells were incubated at 37˚C for 4 days and OD 450 nm was measured. *P<0.05, **P<0.01, compared with the negative control group (Scr, siRNA). eIF3c, eukaryotic initiation factor 3, subunit c.

cells (P<0.01). Therefore, the eIF3c gene is closely related to U251 cell cycle distribution.

The eIF3c gene knockdown induces apoptosis in human glioma U251 cells. Cell apoptosis was assessed by Annexin V staining and flow cytometry (Fig. 7A). As shown in Fig. 7B, cell apoptosis was significantly increased in the eIF3c-siRNA group when compared with the cells that were transfected with scrambled siRNA (4.34±0.27 vs. 2.62±0.11, P<0.01). Therefore, knockdown of the eIF3c gene induced apoptosis in human glioma U251 cells. These data suggested a major role for the eIF3c gene in U251 cell survival; in its absence, decreased survival rates resulted from apoptosis induction.

Discussion

Malignant glioma, one of the most common and fatal types of primary brain tumors in humans (26), arises in a multistep process which relates to sequential and cumulative genetic alterations resulting from intrinsic and environmental carcinogenic factors. Gene expression is regulated at multiple levels, including the translation of mRNAs into the proteins. Protein translation is known to be the critical step of gene expression and translational regulation for normal cell homeostasis and physiology (10). Remarkable progress has been made in understanding the role of mRNA translation and protein synthesis in human types of cancers. Studies have described that the

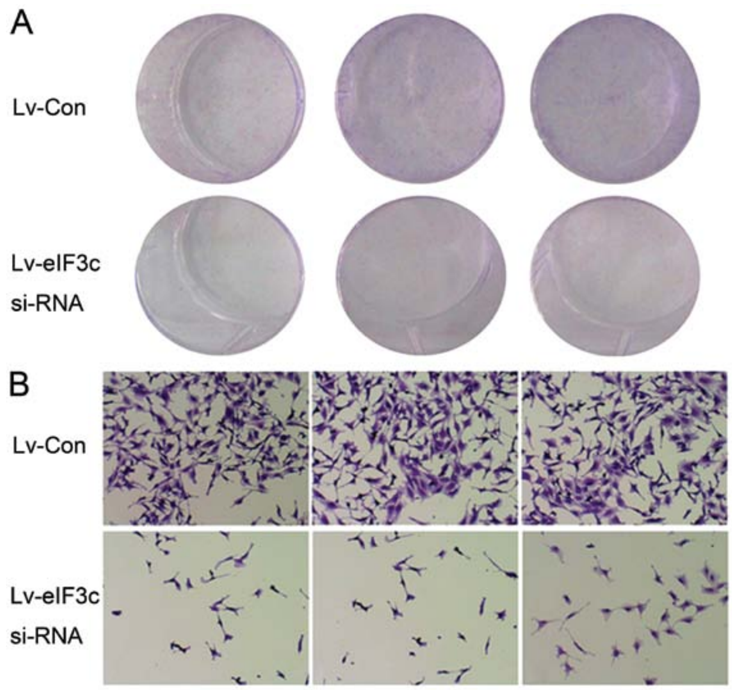

Figure 5. Effect of eIF3c knockdown on colony formation in U251 cells. (A) Giemsa staining of glioma cell colonies observed by light microscopy; (B) micrographs show a reduced number of U251 cells following the Giemsa staining in the eIF3c knockdown group (U251 cells were unable to form colonies in the present study). Lv-Con, scrambled siRNA. eIF3c, eukaryotic initiation factor 3, subunit c.

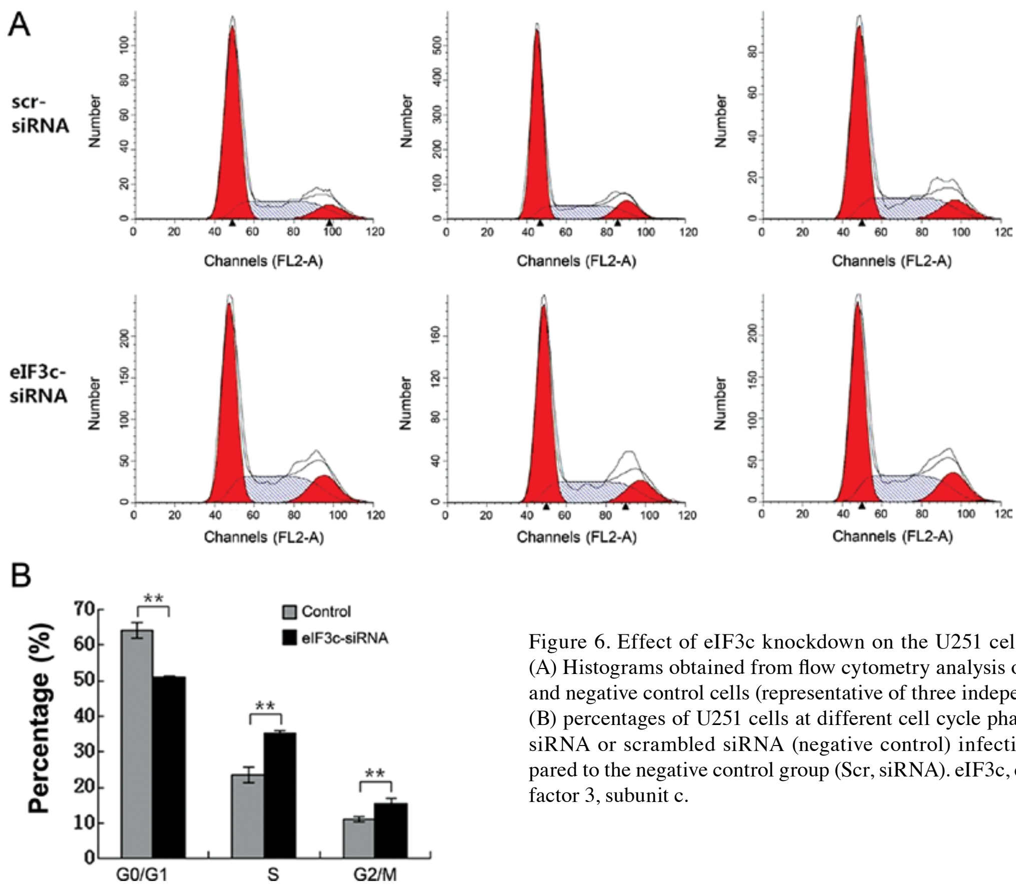

Figure 6. Effect of eIF3c knockdown on the U251 cell cycle distribution. (A) Histograms obtained from flow cytometry analysis of eIF3c knockdown and negative control cells (representative of three independent experiments); (B) percentages of U251 cells at different cell cycle phases following eIF3c siRNA or scrambled siRNA (negative control) infection. $^{**}P<0.001$ compared to the negative control group (Scr, siRNA). eIF3c, eukaryotic initiation factor 3, subunit c.

abnormal mRNA transcription and protein synthesis play a key role in the tumorigenesis pathways (10). In the present study, we showed that eIF3c was expressed in initial surgery specimens from 95 patients with glioma and four glioma cell lines. Knockdown of eIF3c by siRNA subsequently resulted in decreased proliferation, increased cycle arrest and induced

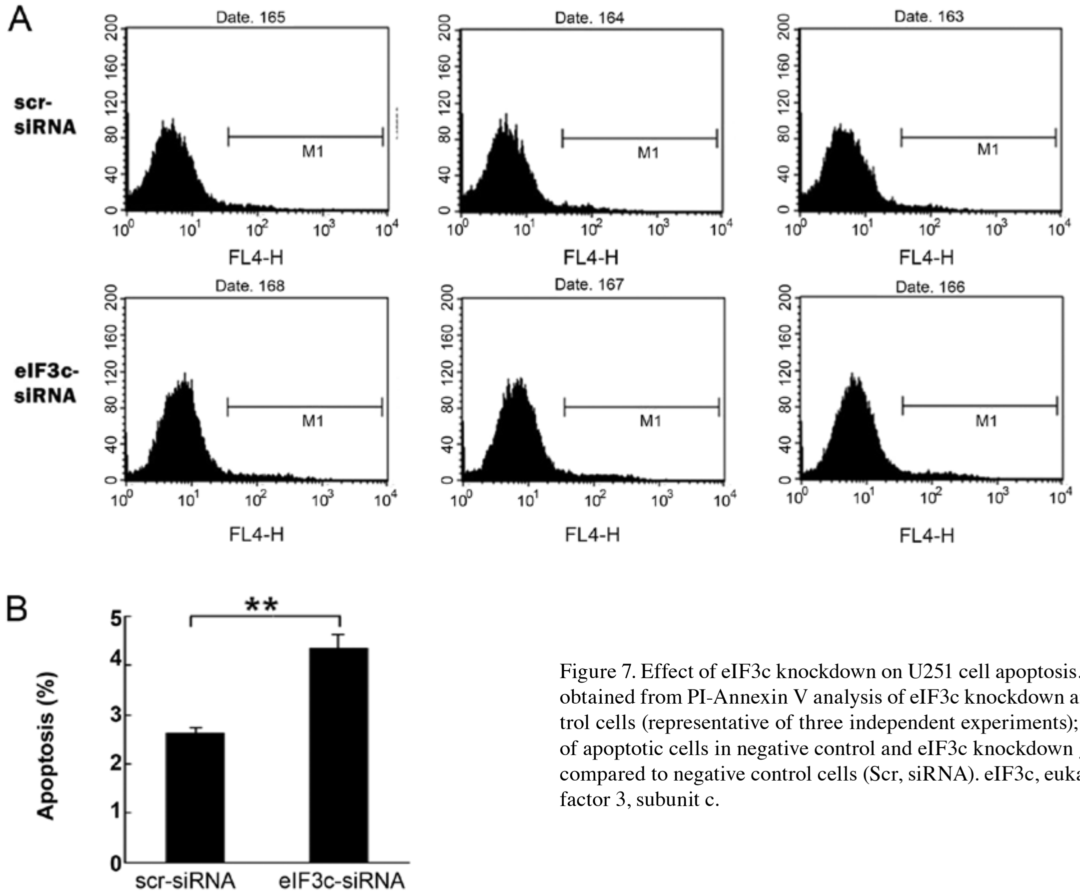

Figure 7. Effect of eIF3c knockdown on U251 cell apoptosis. (A) Histograms obtained from PI-Annexin V analysis of eIF3c knockdown and negative control cells (representative of three independent experiments); (B) percentages of apoptotic cells in negative control and eIF3c knockdown groups. **P<0.01 compared to negative control cells (Scr, siRNA). eIF3c, eukaryotic initiation factor 3, subunit c.

apoptosis in the U251 cells. These results picture eIF3c as a tumor-related gene in glioma cells and suggest this gene to be a potential target for anti-glioma therapy.

To date, only few studies have investigated the relationship between eIF3c and tumorigenesis, showing that eIF3c gene expression is upregulated in several tumor types including colon cancer (25), testicular seminomas (23), meningiomas bearing mutation in the tumor suppressor protein neurofibromatosis 2 (NF2) (25) and immortal fibroblast cells (27). After exploring the therapeutic potential of the eIF3c gene in five different cancer cell lines [NCI-ADR/RES (NAR), HeLa, MCF7, HCT116 and B16F10] and analyzing the polysome profile following downregulation of eIF3c gene expression in NAR cells, Emmanuel *et al* found that eIF3c regulated cell cycle progression; indeed, eIE3c knockdown caused polysome run-off and resulted in cell death, justifying the conclusion that the translation machinery was inhibited at the initiation stage and presenting eIF3c as an anticancer target in different malignancies (20). However, in human glioma, the expression, function and molecular mechanisms of the eIF3c gene have not been reported and remain largely unknown.

In the present study, we found significantly higher eIF3c expression in human glioma tissues. In addition, the positive expression rate of eIF3c in high glioma grades (WHO III and IV) was significantly higher than in low grades (WHO I and II) (P<0.001). It is therefore possible that eIF3c plays a critical role in the survival of the glioma cells as described above with other types of cancer, likely by promoting translation initiation.

In agreement, eIF3c mRNA were detected in all four human glioma cell lines studied, including U87, U251, U373 and A172, again suggesting a critical role for this gene in glioma. To further characterize the eIF3c gene function in the glioma, we used RNAi to knockdown this gene in human glioma U251 cells. Compared to the control group, eIF3c-siRNA cells displayed reduced proliferation, decreased S phase cell numbers, increased G1 phase rates and enhanced apoptosis. These findings suggest that eIF3c may be related to cell cycle checkpoints in the U251 cells. Indeed, the S phase represents a critical period for cells to commit to proliferation or undergo growth arrest (18). Therefore, these findings demonstrate that silencing the eIF3c subunit causes a dramatic reduction of translation initiation in glioma cells. Thus, the eIF3c gene plays an important role in promoting U251 cell growth and is significantly associated with U251 cell cycle distribution. Clearly, the important oncogene eIF3c overexpressed in human gliomas plays a critical role in proliferation and apoptosis of the glioma cells through translational control in the protein translation initiation phase.

Notably, a few studies have assessed miRNA profiling in gliomas and found that miRNA regulates various cancer-associated genes and oncogenic functions in gliomas (28). It is reasonably deduced that eIF3c by dysregulating translational initiation with other cancer-associated genes correlates significantly with tumorigenesis, proliferation and apoptosis in human gliomas. Therefore, eIF3c may be a key regulator of human gliomas and is a novel and attractive therapeutic target to be used for designing anticancer therapies. Inhibition of eIF3c may help substantially to improve the clinical outcome and prognosis of patients with gliomas.

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