

Full Length Research Paper

Impact of pEGFP mediated ING4 gene on growth of glioma U251 cells and its potential molecular mechanism

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To investigate the impact of the inhibitor of growth family 4 (ING4) on growth of glioma cells (U251 cells) and its potential molecular mechanism, total RNA was extracted from the embryonic tissues and ING4 was amplified by RT-PCR and cloned into pEGFP-C2 vector. U251 cells were transfected with eukaryotic expression vector pEGFP-ING4 mediated by cationic polymers polyethylenimine (PEI). Flow cytometry and G418 were used to screen the cells successfully transfected with pEGFP-ING4. pEGFP-ING4 group, pEGFP group and blank control group (without transfection) were included in this study. Morphological examination, MTT assay and Hoechst staining were employed to detect the proliferation and apoptosis of U251 cells. RT-PCR, immunohistochemistry and western blot were recruited to determine the mRNA and protein expression of ING4, respectively. ELISA was performed to measure the VEGF level in the supernatant of U251 cells. Sequencing of pEGFP-ING4 showed sequence correctness and the transfection efficiency was 84% for pEGFP-ING4 and 82% for pEGFP. Results from RT-PCR, immunohistochemistry and western blot revealed significantly increased ING4 expression. When compared with the pEGFP group and blank group, the growth inhibition rate and apoptotic rate 72 h after transfection in the pEGFP-ING4 group were significantly higher ($P < 0.05$), but the VEGF content was not significantly changed ($P > 0.05$). ING4 can be highly expressed in the pEGFP-ING4 group and remarkably suppress the growth of U251 cells through inducing apoptosis but not suppressing VEGF expression.

Key words: Inhibitor of growth family member 4, glioma, angiogenesis, vascular endothelial growth factor.

INTRODUCTION

Glioma, the most common intracranial malignant tumor, is often associated with poor prognosis. At present, it has been evidenced that the occurrence of glioma is a polygenic and multi-step process. Not only the activation of proto-oncogenes but the inactivation of tumor suppressor genes is involved in the occurrence and development of glioma. Recent studies have shown that the inhibitor of growth family member 4 (ING4) plays an important role in the suppression of tumor growth, promotion of tumor cell apoptosis, presence of contact inhibition between cells, cell cycle procession, inhibition of angiogenesis and

enhanced sensitivity of cancer cells to cytotoxic agents (Kim et al., 2004; Zhang et al., 2004; Li et al., 2008, 2009). We speculate that ING4 may play an important role in the pathogenesis of glioma. In the present study, the effect of ING4 on glioma cells (U251 cells) and its potential mechanism were investigated, which may provide experimental and theoretical evidence for gene therapy of glioma.

MATERIALS AND METHODS

Plasmid, bacterial strain and cell line

pEGFP-C2 (Invitrogen, USA), pEGFP-ING4, DH5 α *Escherichia coli* and U251 cell line (from the Department Of Neurosurgery, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Zhongshan

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510120, Guangzhou, China) were used in the present study.

Reagents

Trizol reagent (Invitrogen, USA), RT-PCR kit (Nanjing Keygentec, China), primers for ING4 (Invitrogen, USA), GoTaq DNA polymerase (Promega, USA), pGEM-T vector systems (Promega, USA), T4 DNA ligase (TaKaRa, Japan), ampicillin, kanamycin (Sigma, USA), G418 (Merck, USA), plasmid DNA extraction mini kit (Qiagen, Germany), EcoRI and Sall restriction endonucleases (TaKaRa, Japan), DNA gel extraction kit (Qiagen, Germany), DMEM high glucose (GIBICO, USA), trypsin (Invitrogen, USA), poly-ethylen-imine (PEI; China), rabbit anti-human ING4 antibody (PTG, USA), goat anti-rabbit IgG (Boster, China) and SABC immunohistochemistry kit (Boster, China) were used in this study.

Instruments

PCR instrument (Bio-Rad, USA), gel imager (UVIDOC, UK), gel imaging system (Cole Parmer, USA), Universal 32 R refrigerated centrifuge (Hettich, Germany), UV-1206 ultraviolet spectrophotometer (Shimadzu, Japan), clean bench (Airtech, Japan), inverted microscope (Leica, Germany), constant temperature and humidity incubator (Forma scientific, USA) and converted fluorescence microscope (Nikon, Japan) were used in this study.

Cell culture and immunohistochemistry

When cell confluence reached nearly 100%, U251 cells were fixed in 4% paraformaldehyde for 90 min and then, the endogenous peroxidase in these cells was inactivated by treatment with hydrogen peroxide in methanol solution at room temperature. These cells were blocked with 5% BSA and then, treated with rabbit anti-human GFAP antibody (1:200) at room temperature for 2 h followed by washing three times (2 min per time). Subsequently, the cells were incubated with biotin conjugated secondary antibody for 20 min at room temperature followed by washing three times (2 min per time). Then, the cells were treated with the streptavidin-biotin-peroxidase complex for 20 min at room temperature followed by washing four times (5 min per time). Color development was performed with DAB at room temperature for 10 min. After washing with ddH₂O, cells were counterstained with hematoxylin.

Construction and confirmation of pEGFP-ING4

Total RNA was extracted from the human placenta and reversely transcribed into cDNA which then served as templates for amplification by PCR. The target fragment was connected to the T vectors by TA cloning followed by transformation, amplification and plasmid retrieval. Positive colonies were identified after restriction enzyme digestion. After digestion with both restriction enzymes, the positive plasmid and vector pEGFP were retrieved by gel excision after electrophoresis. Then, the target fragment was connected to the vector pEGFP followed by transformation, amplification and plasmid retrieval. Finally, enzyme digestion and sequencing were conducted to confirm the positive colonies.

Cell transfection and screening of positive colonies

U251 cells were transfected with pEGFP-ING4 (positive control) or pEGFP (negative control) and those without transfection served as blank controls. Forty eight hours after transfection, the expression of green fluorescent protein (GFP) was detected under

fluorescence microscope. After G418 (800 µg/ml) screening for 14 days, flow cytometry was performed to measure the GFP expression aiming to detect the transfection efficiency. Then, RT-PCR, western-blot and immunohistochemistry were employed to determine the mRNA and protein expression of ING4, respectively.

Detection of cell proliferation by MTT assay

After digestion with trypsin, U251 cells were harvested and single suspension was prepared with the medium containing 10% newborn calf serum. These cells were seeded into a 96-well plate at a density of 1×10⁴ (200 µl/well) and the experiment was conducted in triplicates. After 1, 3 or 5 days of culture, 20 µl of MTT solution (final concentration: 5 mg/ml) were added to each well followed by incubation for 4 h. Then, the supernatant was removed and 150 µl of DMSO were added to each well followed by shaking until the crystals resolved. The absorbance (A) of each well was determined at 492 nm with a microplate reader. The A was recorded as means ± standard deviation (SD).

Detection of apoptosis by Hoechst staining

After digestion with trypsin, cells were harvested, resuspended and seeded at a density of 1×10⁵ followed by incubation for 3 days. Then, the medium was removed and cells were fixed in 0.5 ml of fixative solution for 10 min. The fixative solution was removed and cells were rinsed with PBS two times (3 min per time). Subsequently, these cells were stained with 0.5 ml of Hoechst solution for 5 min followed by rinsed with PBS two times (3 min per time). These cells were observed under a fluorescence microscope and positive cells had blue nuclei. Viable cells have large nuclei and the fluorescence is evenly diffuse. Apoptotic cells have shrunk nuclei or karyorrhexis or pyknosis. Five fields at high magnification were selected randomly from each group and the number of cells (viable or apoptotic) and that of cells having karyorrhexis or pyknosis were detected. The apoptosis rate was calculated as follow and expressed as means ± SD: apoptosis rate = number of apoptotic cells/total number of cells.

Detection of VEGF content by ELISA

After screening with G418 (800 µg/ml) for 14 days, the supernatant was collected and the VEGF content was determined by ELISA. The difference in the VEGF content was compared among different groups.

Statistical analysis

Statistical analysis was performed with SPSS version 13.0 statistical software and comparisons were done with one way analysis of variance. A value of P < 0.05 was considered statistically significant.

RESULTS

Identification of U251 cells

The morphological features of U251 cells were consistent with those of glioma cells (Figure 1a,b). GFAP staining showed brown granules in the cytoplasm of U251 cells, suggesting glioma cells (Figure 1c,d).

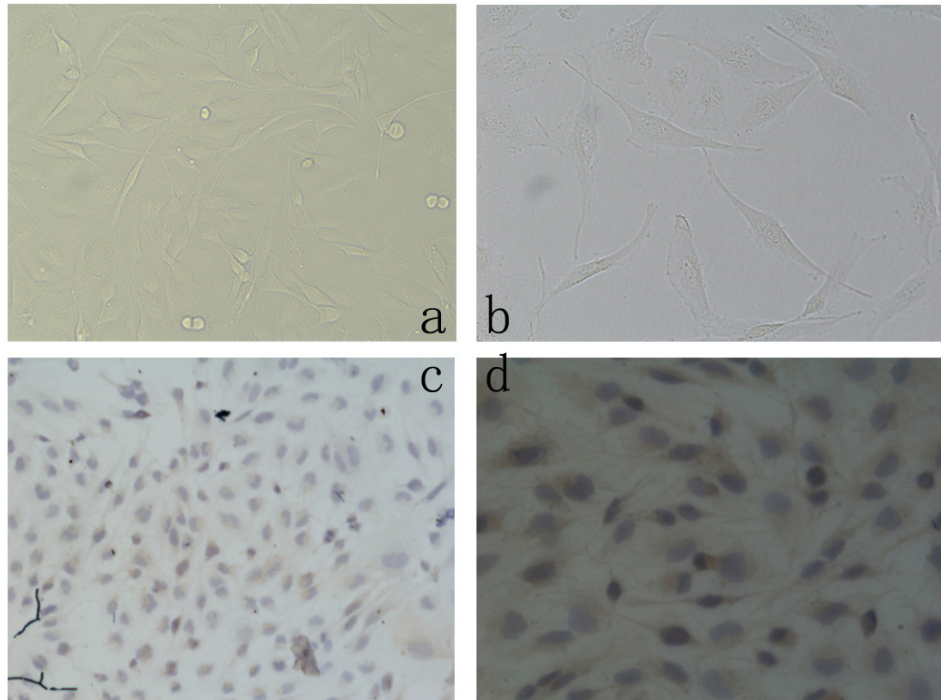


Figure 1. Morphological features of U251 cells: A, viable U251 cells ($\times 100$); B, viable U251 cells ($\times 200$); C, GFAP immunohistochemistry: brown granules in the cytoplasm ($\times 100$); D, GFAP immunohistochemistry: Brown granules in the cytoplasm ($\times 200$).

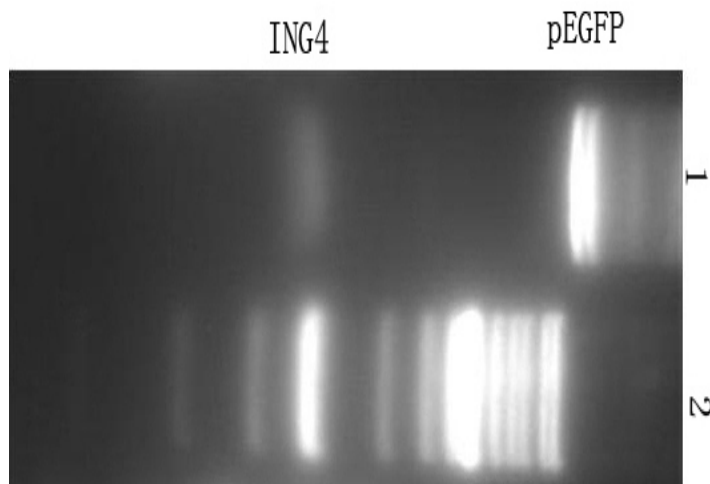


Figure 2. Electrophoresis after digestion with both restriction enzymes. Lane 2: Marker (1 kb): from top to bottom 10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 750, 500 and 250 bp. The brightest bands in the lane 2 were at 3000 and 1000 bp, respectively. Lane 1: products after digestion with both restriction enzymes. A band at about 10000 bp represented pEGFP plasmid with open loop and another band at about 1000 bp was ING4.

Construction and identification of pEGFP-ING4 eukaryotic expression vector

The reverse transcribed cDNA was used for PCR for

amplification. The target fragments were then connected to T vectors with a TA cloning technique, followed by transduction, amplification and plasmid extraction. The positive colonies were screened by enzyme digestion. The recombinant plasmid pEGFP-ING4 was digested with two enzymes and then subjected to agarose gel electrophoresis. Results showed two bands; one band was about 10000 bp which was similar to the length of pEGFP plasmid and the other band (about 1000 bp) was similar to the ORF of ING4 in the length (Figure 2).

Screening of positive colonies by G418 and flow cytometry

Plasmids pEGFP and pEGFP-ING4 were used to transfect U251 cells which were then, screened by G418 (800 $\mu\text{g/ml}$). GFP positive cells could be identified. Subsequently, flow cytometry was performed to acquire GFP positive colonies. Results showed that the proportion of GFP positive U251 cells was greater than 90% (Figure 3).

ING4 expression in U251 cells

Immunohistochemistry was performed for ING4. Positive staining was observed in cells after pEGFP-ING4. The nucleus was stained dark and apoptotic body was evident. However, there was no positive staining in the pEGFP group and blank control group (Figure 4).

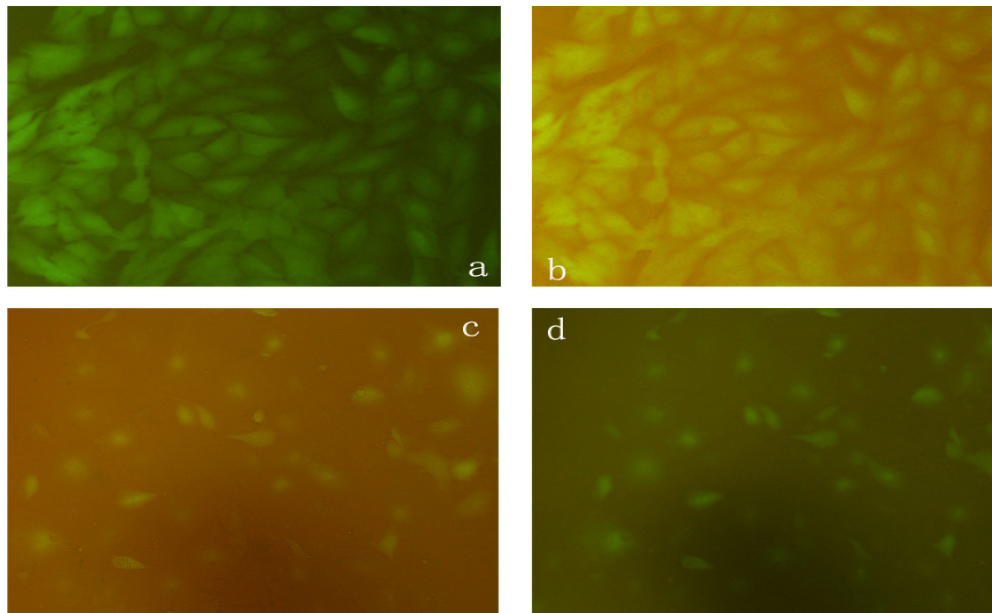


Figure 3. Screening of positive colonies by G418 and flow cytometry. A, Cells in the pEGFP group under a fluorescence microscope after screening; B, merged photograph of cells in the pEGFP group. The proportion of GFP positive cells was greater than 90% and the cell confluence reached nearly 100% ($\times 200$). C, cells in the pEGFP-ING4 group under fluorescence microscope after screening; D, merged photograph of cells in the pEGFP-ING4 group. The proportion of GFP positive cells was greater than 90% and the cell confluence was obviously lower than that in the pEGFP group ($\times 200$).

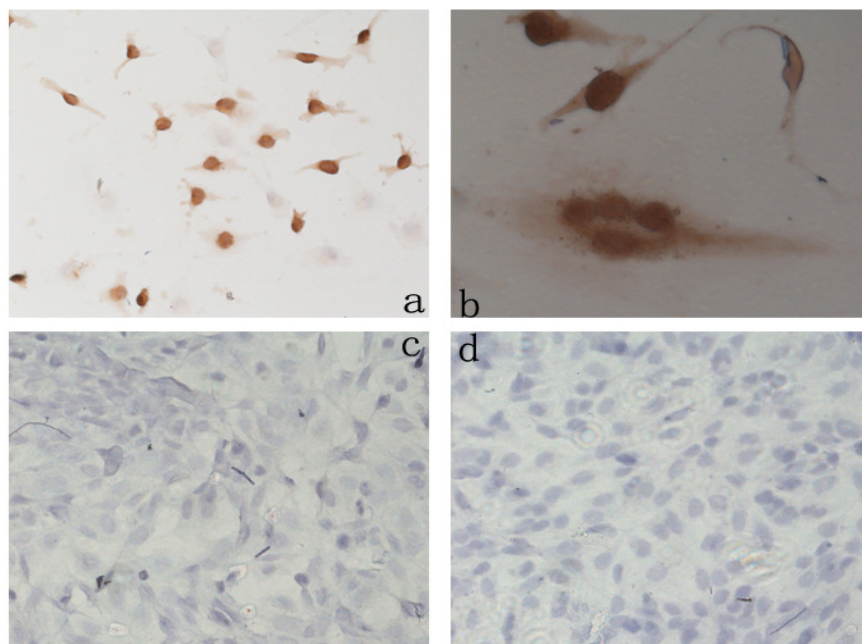


Figure 4. Detection of ING4 expression in the U251 cells by immunohistochemistry. A, Immunohistochemistry for ING4 in the pEGFP-ING4 group. Brown granules were observed in the cytoplasm, the nuclei were dark and the amount of cells was small ($\times 100$); B, Immunohistochemistry for ING4 in the pEGFP-ING4 group. Brown granules were observed in the cytoplasm, and apoptotic body was present in the cells ($\times 400$); C, Immunohistochemistry for ING4 in the blank controls. No brown granules were noted in the cytoplasm ($\times 100$); D, Immunohistochemistry for ING4 in the pEGFP group. There were not brown granules in the cytoplasm ($\times 100$).

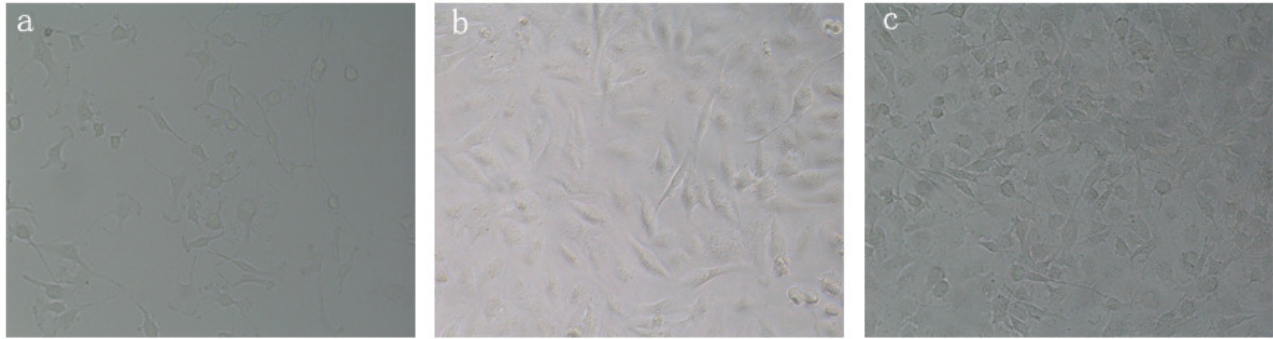


Figure 5. U251 cells after transfection. A, One day after transfection, the amount of cells was still small ($\times 200$); B, three days after transfection, the cell confluence reached nearly 100%; C, seven days after transfection, formation of overlapping cell layers were observed suggesting the absence of contact inhibition ($\times 200$).

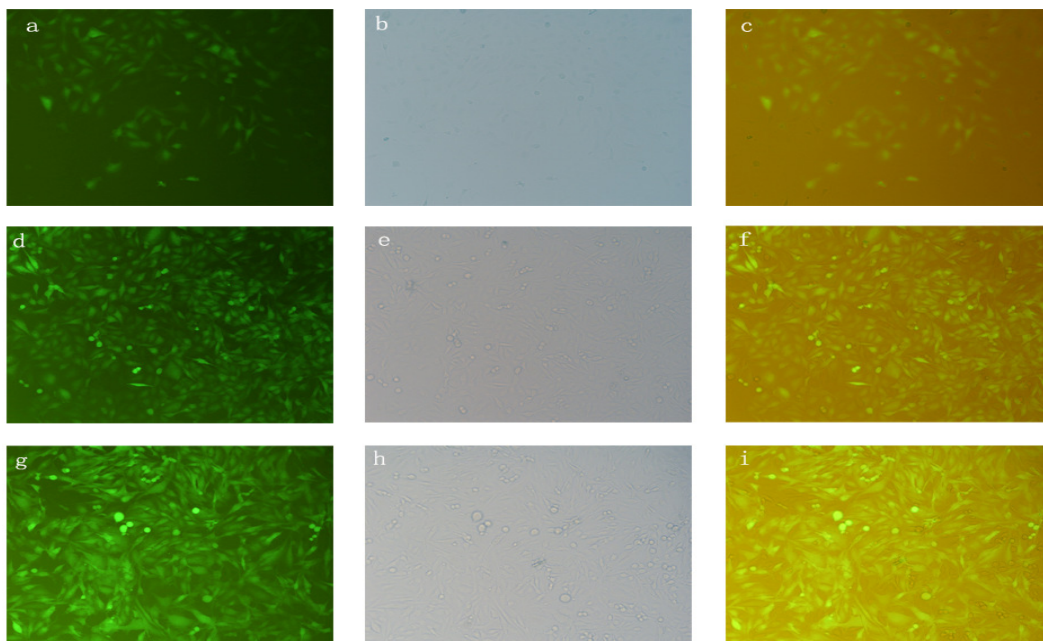


Figure 6. U251 cells in the pEGFP group under fluorescence microscope. A, U251 cells under a fluorescence microscope one day after transfection; B, U251 cells in the same field under a light microscope one day after transfection ($\times 100$); C, merged photograph of U251 cells one day after transfection ($\times 100$) and the amount of cells was small; D, U251 cells under a fluorescence microscope three days after transfection; E, U251 cells in the same field under a light microscope three days after transfection ($\times 100$); F, merged photograph of U251 cells three days after transfection ($\times 100$) and the cell confluence reached nearly 100%; G, U251 cells under a fluorescence microscope seven days after transfection; H, U251 cells in the same field under a light microscope seven days after transfection ($\times 100$); I, merged photograph of U251 cells seven days after transfection ($\times 100$) and overlapping cell layers were observed suggesting the absence of contact inhibition.

Morphological characteristics of U251 cells after transfection with pEGFP-ING4

It was reported that ING4 could change the cell morphology and disable the contact inhibition of cells. Fluorescence microscopy was conducted to observed cells in the blank control group (Figure 5), pEGFP group (Figure 6) and pEGFP-ING4 group (Figure 7) at days 1, 3

and 7. Results showed cell confluence did not reach 100% at day 1 and GFP expressing cells were observed in pEGFP-ING4 group and pEGFP group. Three days after transfection, the cell confluence reached nearly 100% in blank control group and pEGFP group, but not in the pEGFP-ING4 group. Fluorescence microscopy showed GFP expressing cells in pEGFP-ING4 group and pEGFP group. Seven days after transfection, the cell

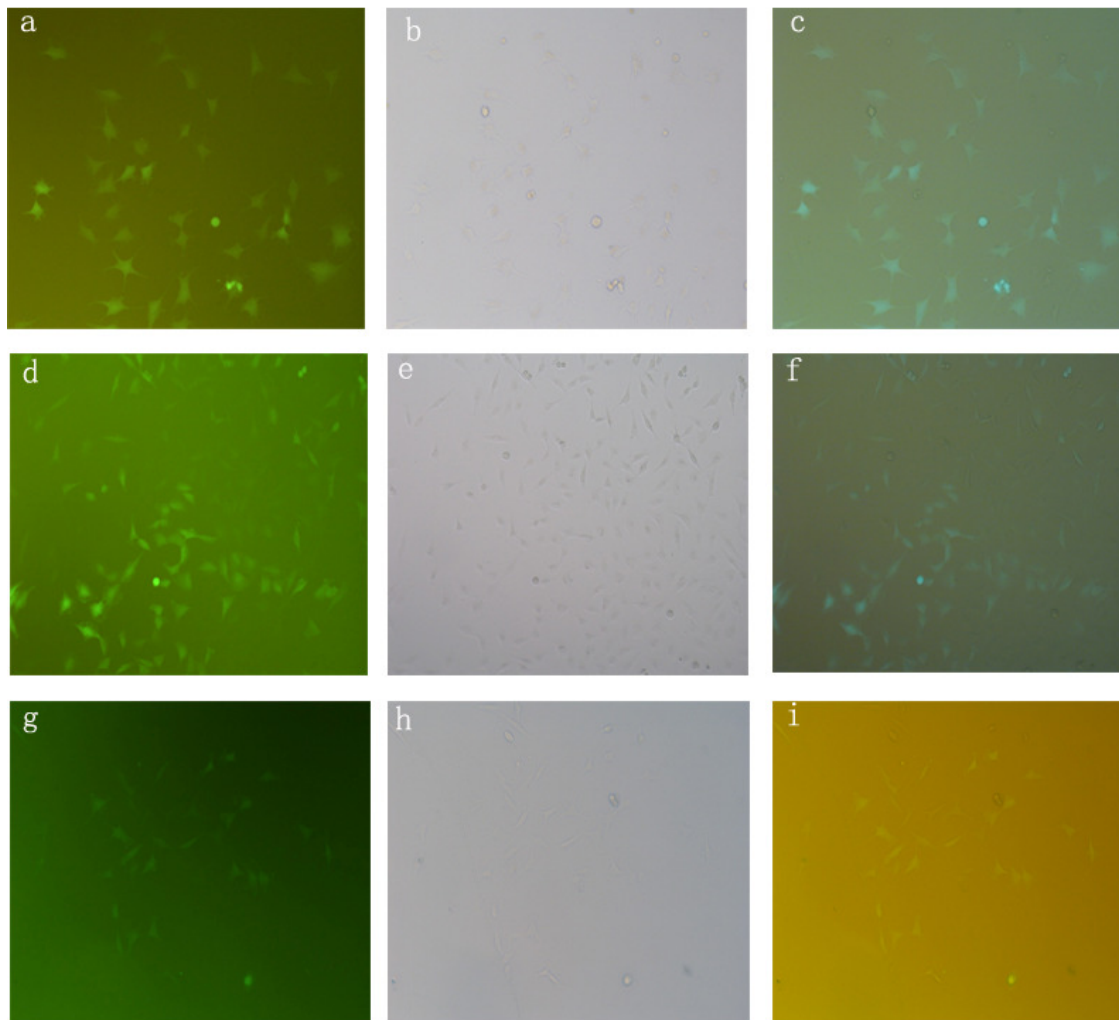


Figure 7. U251 cells in the pEGFP-ING4 group under a fluorescence microscope. A, U251 cells under a fluorescence microscope one day after transfection; B, U251 cells in the same field under a light microscope one day after transfection ($\times 100$); C, merged photograph of U251 cells one day after transfection ($\times 100$) and the amount of cells was little; D, U251 cells under a fluorescence microscope three days after transfection; E, U251 cells in the same field under a light microscope three days after transfection ($\times 100$); F, merged photograph of U251 cells three days after transfection ($\times 100$) and the amount of cells was still small; G, U251 cells under a fluorescence microscope seven days after transfection; H, U251 cells in the same field under a light microscope seven days after transfection ($\times 100$); I, merged photograph of U251 cells seven days after transfection ($\times 100$) and the amount of cells was still small suggesting inhibition of cell proliferation.

confluence reached 100% and overlapping growth was also noted in blank control group and pEGFP group, which were not observed in pEGFP-ING4. GFP expressing cells in pEGFP-ING4 group and pEGFP group were still found. These findings further demonstrated the absence of contact inhibition after pEGFP-ING4 transfection.

Effect of pEGFP-ING4 transfection on the growth of U251 cells

One, three and five days after transfection, the A in the pEGFP-ING4 group had a decreased tendency (Figure 8).

The A in the pEGFP-ING4 group was $0.2607 \pm 0.0464\%$ at day 3 and $0.1920 \pm 0.0105\%$ at day 5, which were significantly higher than those in the pEGFP group and blank control group ($P < 0.05$). There was no marked difference between pEGFP group and blank control group ($P > 0.05$).

Effect of pEGFP-ING4 transfection on the apoptosis of U251 cells

Hochest staining revealed apoptotic characteristics including pyknosis and karyorrhexis in 3 groups 3 days after transfection, which however, were more obvious in

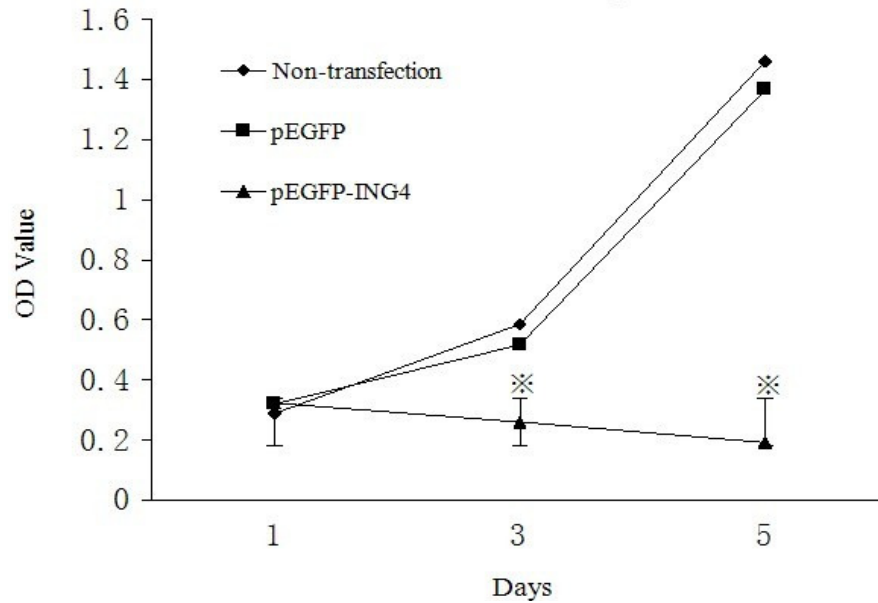


Figure 8. Growth curve of U251 cells. One, three and five days after transfection, the cell proliferation decreased in the pEGFP-ING4 group but had an increased tendency in the pEGFP group and the blank control group. The A in the pEGFP-ING4 group 3 and 5 days after transfection were significantly higher than those in the pEGFP group and blank control group ($P < 0.05$). There was no marked difference between pEGFP group and blank control group ($P > 0.05$). * $P < 0.05$.

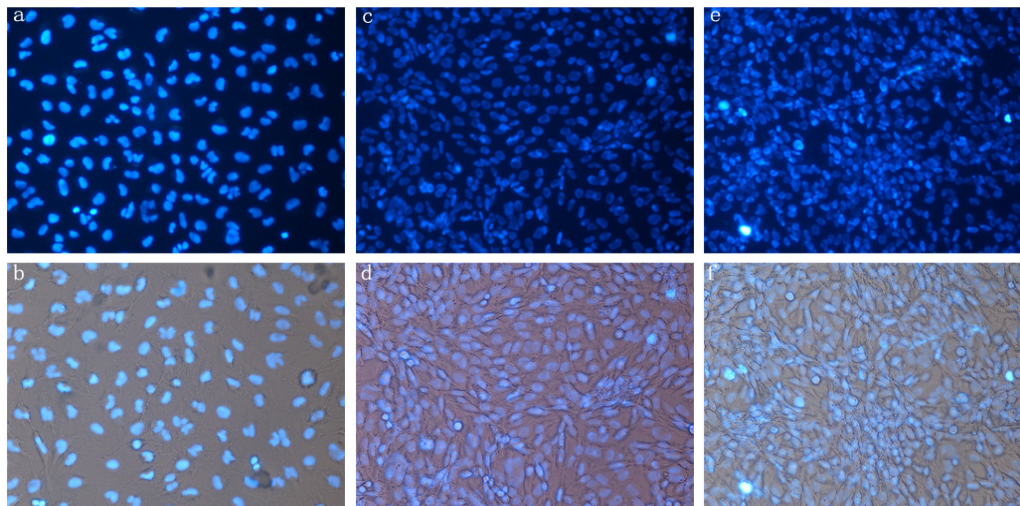


Figure 9. Hoechst staining. A, Three days after transfection, cells in the pEGFP-ING4 group were observed under a fluorescence microscope and a lot of apoptotic cells were observed ($\times 100$); B, Merged photograph from the same field in the pEGFP-ING4 group ($\times 100$); C, Three days after transfection, cells in the pEGFP group were observed under a fluorescence microscope and a small amount of apoptotic cells were observed ($\times 100$); D, Merged photograph from the same field in the pEGFP group ($\times 100$); E, Three days after transfection, cells in the blank control group were observed under a fluorescence microscope and a small amount of apoptotic cells were observed ($\times 100$); F, Merged photograph from the same field in the blank control group ($\times 100$).

pEGFP-ING4 than in blank control group and pEGFP group (Figure 9). The apoptosis rate was $29.0 \pm 1.2\%$ in pEGFP-ING4 group and markedly higher than the

remaining two groups ($P < 0.05$). There was no significant difference between blank control group and pEGFP group in the apoptosis rate ($P > 0.05$) (Figure 10).

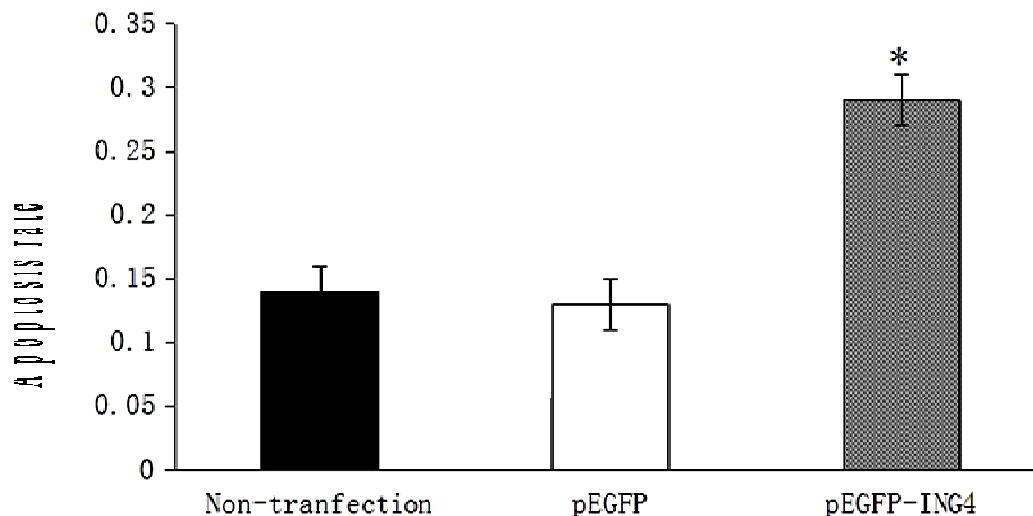


Figure 10. Apoptosis rate of U251 cells. The apoptosis rate in the pEGFP-ING4 group was significantly higher than that in the blank control group and the pEGFP group ($P < 0.05$). There was no marked difference in the apoptosis rate between the blank control group and the pEGFP group ($P > 0.05$). * $P < 0.05$.

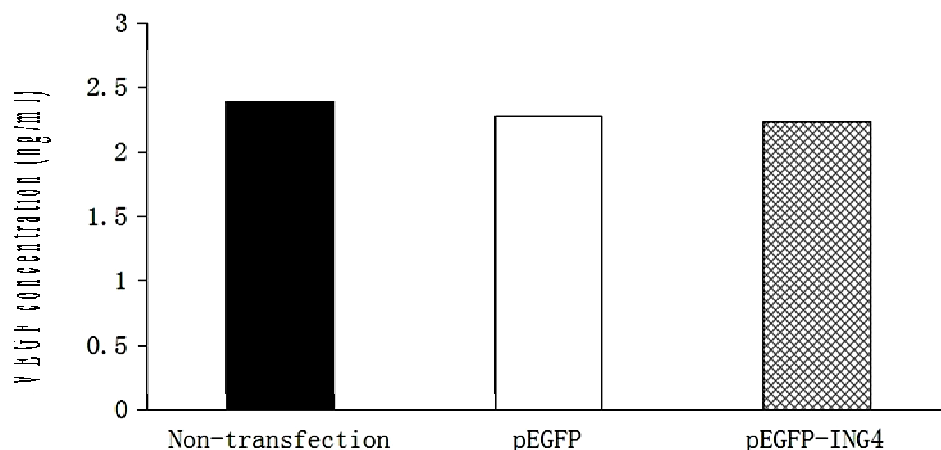


Figure 11. The mean VEGF content was 2.239 ng/ml in the pEGFP-ING4 group, 2.279 ng/ml in the pEGFP and 2.390 ng/ml in the blank control group.

Effect of pEGFP-ING4 transfection on the VEGF expression

After screening with G418 (800 $\mu\text{g/ml}$) for 14 days, ELISA was performed to detect the VEGF content in the supernatant. Statistical analysis showed there was no significant difference in the VEGF content between three groups ($P > 0.05$). This result suggested that pEGFP-ING4 transfection did not suppress the VEGF expression (Figure 11).

DISCUSSION

In 1996, Garkavtsev et al. identified the inhibitor of growth

family member 1 (ING1) in the normal mammary epithelial cells and breast cancer cells through subtractive hybridization (Garkavtsev et al., 2004). Later, the other members of the inhibitor of growth family were found and they have been shown to play an important role in the suppression of cancer growth. Therefore, the inhibitor of growth family has been a hot topic in the recent research. ING4 is a recently found tumor suppressor gene and evidence revealed ING4 that it is involved in the pathogenesis of cancers, acclimatization to hypoxia and other processes (Garkavtsev et al., 2004; Zhang et al., 2010; Xie et al., 2009; Zhao et al., 2010; Ozer and Bruick, 2005). Studies have demonstrated that ING4 could suppress the cell growth and apoptosis in liver cancer cells (HepG2), human lung cancer cells (A594) and rectal cancer cells

(RKO) (Xie et al., 2009; Zhang et al., 2004; Shen et al., 2007).

Garkavtsev et al. investigated the relationship between ING4 and glioma. Garkavtsev et al. (2004) demonstrated that the ING4 expression was negatively associated with the severity of glioma by PCR and ING4 could markedly inhibit the growth of U87 cells (Chen and Deng, 2008). In China, Zhao et al. (2007) also indicated that adenovirus mediated ING4 gene transfection could suppress the proliferation of glioma cells (C6 cells) and induce apoptosis. In the present study, after ING4 transfection, the morphology of U251 cells was observed and the proliferation and apoptosis were detected through MTT assay and Hoechst staining, respectively. These results also revealed that the growth of U251 cells was dramatically suppressed accompanied by evident apoptosis induction.

There was evidence that ING4 could suppress the angiogenesis. Suppressed angiogenesis was observed in the human glioma cells (U87 cells) after ING4 treatment which may be related to the suppressed VEGF activity or other pathways (Chen and Deng, 2008; Xie et al., 2008). In the present study, over expression of ING4 did not influence the VEGF expression in the U251 cells and the exact mechanism underlying the effect of ING4 on the angiogenesis should be further study (Xie et al., 2009).

In the present study, the eukaryotic expression vector carrying ING4 gene was successfully constructed and transfected into glioma cells (U251 cells). The impacts of ING4 on the growth and apoptosis of U251 cells and on the VEGF expression were investigated, which may provide basis for the further studies on ING functions as well as the potential mechanism and also present evidence for the therapy of glioma.

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