Silencing of IKKε using siRNA inhibits proliferation and invasion of glioma cells *in vitro* and *in vivo*

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Abstract. Recent studies implicated IKKε in the pathogenesis of many human cancers by promoting cell proliferation, increasing tumor angiogenesis and metastasis, and generating resistance to cell apoptosis. However, whether IKKε can influence the invasive ability and proliferation of glioma cells remains largely unknown. In this study, we showed that overexpression of IKKε is positively correlated to glioma pathological grade, suggesting that IKKε plays a role in tumor progression, rather than tumor initiation. Targeted knockdown of IKKε in human glioma cells using siRNA, was associated with inhibition of cell growth, cell cycle arrest and decreased cell invasion; however, notable apoptosis was not observed. Furthermore, we demonstrated that transposition of NF-κB p65 resulted in the alteration of these phenotypes. Tumor growth was attenuated in established subcutaneous gliomas in nude mice treated with IKKε siRNA *in vivo*. Collectively, our results suggest that deregulation of IKKε plays a pivotal role in the uncontrolled proliferation and malignant invasion of glioma cells *in vitro* and *in vivo* by targeting NF-κB. Silencing of IKKε using synthetic siRNAs may offer a novel therapeutic strategy for the treatment of glioma.

Introduction

Gliomagenesis is a complex and multifactorial process with multiple genetic alterations, and is characterized by rapid cell proliferation, high invasion, genetic alteration and increased angiogenesis (1,2). Despite technical advances in neurosurgery, radiation therapy and clinical trials, the prognosis of patients with glioma is poor, with a mean survival time of 9-12 months (3,4). IKKε is involved in regulating the activation of the type I IFN and NF-κB signaling pathways, and was initially identified in a subtractive hybridization screen as a lipopolysaccharide (LPS)-inducible gene and as a PMA-inducible protein. The kinase domain of IKKε is 27% identical to IKKα and IKKβ (5,6). It has been suggested that IKKε is involved in TNFα and LPS-induced matrix metalloproteinase-3 and matrix metalloproteinase-13 gene expression via phosphorylation and activation of the c-JUN pathway. This pathway may be responsible for synovial inflammation and extracellular matrix destruction in rheumatoid arthritis, as well as being involved in tumor invasion and metastasis (7). Eddy *et al* demonstrated that overexpression of a kinase-inactive form of IKKε in breast cancer cells reduced the expression of NF-κB target genes, cyclin D1 and RelB, as well as anchorage-independent growth and invasion in Matrigel (8). Subsequently, IKKε was shown to activate basal levels of NF-κB via phosphorylation of serine 536, and inhibition of this activity significantly suppressed cancer cell proliferation (9). IKKε was identified as a breast oncogene using complementary genomic approaches in over 30% of breast cancer cell lines and carcinomas. Abnormal upregulation of NF-κB activity by IKKε was presumed to be an essential step for cell transformation (10). Guo *et al* showed that IKKε is overexpressed in a significant proportion of ovarian carcinomas (63/95), and elevated IKKε levels served as a marker for poor prognosis (11). IKKε is also reported to promote prostate cancer progression, by inducing secretion of IL-6, which may act as a positive growth factor in prostate cancer (12). Taken together, these data strongly support the role of IKKε in tumorigenesis, and indicate that blocking IKKε expression may represent a rational therapeutic strategy.

In the present study, we demonstrated that IKKε is frequently overexpressed in human glioma. Targeted knockdown of IKKε using synthetic siRNAs dramatically inhibits the proliferative and invasive ability of tumor cells and the expression of proteins associated with these phenotypes *in vitro* and *in vivo*. Our data indicated that IKKε may represent a promising therapeutic target for the treatment of glioma.

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Materials and methods

Tissue samples. Seven normal brain tissue and 51 neurospongioma specimens were obtained during surgical resection from patients at Huanhu Hospital during 2010. The pathological diagnosis and grading of glioma specimens was assessed according to WHO guidelines (2000). Glioma specimens consisted of 5 cases of pilocytic astrocytoma (Grade I), 11 cases of astrocytoma (Grade II), 8 cases of oligodendroglioma (Grade II), 9 cases of anaplastic astrocytoma (Grade III), 6 cases of anaplastic oligodendroglioma (Grade III) and 12 cases of glioblastoma (Grade IV). All tissue samples were collected in accordance with institutional review board-approved protocols. A sample of all tissues was snap-frozen and stored at -80˚C, and the remaining portion was fixed with 10% formalin for histopathological and immunohistochemical examination.

Cell culture and transfection. The human U251 and LN229 glioblastoma cell lines were purchased from the Institute of Biochemistry and Cell Biology. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS), and maintained at 37˚C in 5% CO_2. Prior to transfection, cells were plated in six-well plates and incubated for 24 h. Cells were transfected in serum-free medium with 10 ng/5 μl siRNA using Lipofectamine (Invitrogen, Carlsbad, CA, USA). After 4 h, medium was replaced with DMEM supplemented with 10% FBS and cells were incubated at 37˚C in 5% CO_2. The siRNA sequences targeting IKKε were: siR-1: 5'-GGUCUUCAACACUACCAGCTT-3'; siR-2: 5'-GAGCTATCTCACCAGCTCC-3'; siR-3': 5'-GCUGAACCACCAGAACAUCTT-3'; siR-4: 5'-GAAGCATCCAGCAGATTCA-3'; siR-5: 5'-TGGCAGGAGCTAATGTTTCG-3'.

Real-time PCR analysis. Total RNA was extracted with TRIzol (Gibco, Carlsbad, CA, USA) 48 h after transfection, and reverse transcription was performed using AMV reverse transcriptase (Takara, Shiga, Japan) according to the manufacturer's protocol. Quantitative real-time PCR was performed with 100 ng of cDNA using SYBR Green-1 dye universal Master mix on a real-time PCR cycler (7500 ABI, USA). Primer sequences used for quantitative PCR are shown in Table I. The reaction conditions were as follows: 95˚C 10 min, 95˚C 15 sec, 60˚C 1 min, 60˚C 1 min, 40 cycles. β-actin was used as control. The results of real-time PCR were analyzed using the ΔΔCT method: $\Delta CT=CT_{selected\ gene}-CT_{\beta\text{-}actin}$, $\Delta\Delta CT=\Delta CT_{therapy\ group}-\Delta CT_{control\ group}$, RQ (relative quantitation) therapy group $=2^{-\Delta\Delta CT}$, RQ control group = 1. Real-time results are presented as the ratio between the selected genes and β-actin transcripts.

Western blot analysis. Total cell lysates were prepared using RIPA lysis buffer containing freshly added protease and phosphatase inhibitors (GENMED, Shanghai, China). Cell lysates were clarified by centrifugation at 4˚C for 10 min at 12,000 rpm. Cytoplasmic and nuclear NF-κB p65 protein sub-fractions were prepared using a commercial extraction kit (Active Motif, Tokyo, Japan) according to the manufacturer's instructions. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins (20 μg) were resolved by SDS-PAGE and electrotransferred to PVDF membranes (Millipore, Billerica, MA, USA). Antibodies used were IKKε (Sigma, St. Louis, MO, USA), CCND1, VEGF, MMP2/9 MYC, BCL2, IκBα, NF-κB (Zhongshan, Beijing, China) and GAPDH (Santa Cruz). All antibodies were used at a dilution of 1:1000. Proteins were detected using the SuperSignal protein detection kit (Pierce).

Focus formation assays. Cells/well ($5x10^3$) were seeded in six-well plates 48 h after transfection. Growth medium was changed every 2 days. After 9 days, cells were washed twice with ice-cold growth medium, fixed in ice-cold methanol and stained with 0.5% crystal violet. Colonies (≥50 cells) were scored using a microscope (Olympus, Japan). Plate clone formation efficiency was calculated as follows: [(number of colonies/number of cells inoculated) x100]. Each experiment was performed in triplicate.

Cell viability assays. Cell viability was assessed over a 6-day period. Cells were seeded ($2x10^3$/well) into 96-well plates 48 h after transfection. 20 μl of MTT reagent (Sigma) was added into each well and incubated for 4 h at 37˚C in a CO_2 incubator. DMSO (200 μl) was added to each well and optical density was measured at the wavelength of 570 nm. The data are presented as the mean ± SD, derived from triplicate samples of three independent experiments.

Cell cycle analysis. Cell cycle analysis was performed 48 h after transfection. Cells were trypsinized, washed with PBS and fixed with 75% ethanol overnight at 4˚C. Cells were washed twice with PBS and incubated with 200 μl RNase (1 mg/ml) at 37˚C for 30 min. Cells were stained with 800 μl propidium iodide staining solution for 30 min at 4˚C. A total of 10,000 nuclei were analyzed by use of a FacsCalibur flow cytometer (Bio-Rad, Hercules, CA, USA) and the DNA histograms were generated by Modifit software (Becton-Dickinson, USA).

Apoptosis assays. Apoptotic cells were identified 48 h after transfection. In brief, after siRNA treatments, both floating and attached cells were collected and subject to Annexin V/PI staining using the Annexin V-FITC Apoptosis Detection kit I (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. Flow cytometric analysis was performed on a FACS flow cytometer (Becton-Dickinson). Data were analyzed by CellQuest software.

Wound healing assays. U251 and LN229 cells were seeded in 6-well plates ($2x10^5$ cells/well) and transfected with 10 ng/5 μl siRNA. 48 h after transfection (~90% confluency), a wound area was carefully created by scraping the cell monolayer with a sterile 100 μl pipette tip. The cells were then washed with PBS to remove detached cells and fresh growth media was added. Cells were then incubated at 37˚C and the width of the wound area was monitored with an inverted microscope at various time points. The normalized wound area was calculated using TScratch software (13).

Cell invasion assays. Transwell filters (Costar, USA) were prepared by coating with Matrigel on the upper surface of the polycarbonic membrane. The Matrigel was set for 30 min at 37˚C, allowing it to be used as an extracellular matrix for tumor cell invasion analysis, and 48 h after transfection, $1x10^5$ cells

Table I. Gene specific primers.

Gene	Forward primer	Reverse primer
IKBKE	5'-TGCGTGCAGAAGTATCAAGC-3'	5'-TACAGCAGCCACAGAACAG-3'
Cyclin D1	5'-AACTACCTGGACCGCTTCCT-3'	5'-CCACTTGAGCTTGTTCACCA-3'
MMP9	5'-TTGGTCCACCTGGTTCAACT-3'	5'-ACGACGTCTTCCAGTACCGA-3'
VEGF	5'-CCCACTGAGGAGTCCAACAT-3'	5'-TTTCTTGCGCTTTCGTTTTT-3'
Bcl-2	5'-GAGGATTGTGGCCTTCTTTG-3'	5'-ACAGTTCCACAAAGGCATCC-3'
MYC	5'-TTCGGGTAGTGGAAAACCAG-3'	5'-CAGCAGCTCGAATTTCTTCC-3'
β-actin	5'-CTCCATCCTGGCCTCGCTGT-3'	5'-GCTGTCACCTTCACCGTT-3'

were resuspended in 100 μl of serum-free DMEM and added to the upper chamber of transwells. Conditioned medium from U251 and LN229 cells (200 μl) was used as chemoattractant and placed in the bottom chamber. Cells were allowed to migrate for 48 h at 37˚C in a CO_2 incubator. Non-invading cells were removed from the upper side of the chamber with a cotton swab and inserts containing migrated cells were then fixed with 100% methanol, stained with hematoxylin, mounted and dried at 80˚C for 30 min. The number of cells invading the Matrigel were counted from ten randomly selected visual fields, each from the central and peripheral portion of the filter, using an inverted microscope at x200 magnification. Experiments were performed in triplicate.

Immunofluorescence and immunohistochemical staining. For immunofluorescence staining, cells were seeded onto sterile cover slips 48 h after transfection and incubated for 2 h at 37˚C. Cells were washed twice with ice-cold PBS and fixed with 2% formaldehyde for 30 min. Cells were then permeabilized with 0.1% Triton X-100 and blocked in 2% BSA for 30 min at room temperature. Cells were incubated with primary anti-NF-κB p65 antibody (Sigma, 1:100 dilution) at 4˚C overnight. Samples were washed with PBS and incubated with species-specific secondary antibodies (1:100 dilution) in PBS for 30 min. Immunofluorescence was examined using a confocal microscope (Olympus FV1000S, Japan). For immunohistochemistry, sections were dewaxed, treated with 3% H_2O_2 for 10 min and incubated with appropriate antibodies (1:100 dilution) at 4˚C overnight. Sections were incubated with biotinylated secondary antibody (1:100 dilution) for 1 h at room temperature, followed by incubation with ABC-peroxidase for 1 h. After washing with Tris-buffer, sections were incubated with DAB (3,3'-diaminobenzidine, 30 mg dissolved in 100 ml Tris-buffer containing 0.03% H_2O_2) for 5 min, washed in water and counterstained with hematoxylin.

Xenograft tumor assay. Six-week old female immune-deficient nude mice (BALB/C-nu) were purchased from the animal center of the Cancer Institute, Chinese Academy of Sciences. Animals were housed in individual micro-isolator cages at the facility of laboratory animals, Tianjin Medical University. All experiments were carried out according to the regulations and internal biosafety and bioethics guidelines of Tianjin Medical University and Tianjin Municipal Science and Technology Commission. Mice (n=3) were injected subcutaneously with $6x10^6$ U251 cells in a 200 μl volume. Mice were monitored daily for tumor formation. Tumors of approximately 5 mm in diameter were surgically removed, and 1-2 mm^3 sections were implanted into individual mice (n=12). When tumors reached approximately 5 mm in diameter, mice were divided into two groups, IKKε siRNA (n=6) and siRNA-NC (non-sense siRNA). Twenty five microliters of a mixture containing 150 μl siRNA (IKKε siRNA or siRNA-NC, 20 pmol/μl) and 60 μl oligofectamine was injected into subcutaneous tumors using a multi-site-injection method. Tumor volume was assessed for a period of three weeks by measurement with a caliper every 2 days using the formula: volume = length x $width^2/2$. Mice were sacrificed after three weeks and tumors were excised and prepared as paraffin-embedded sections for detection of the expression of IKKε, MMP2/9, cyclin D1, PCNA, VEGF and c-myc by immunohistochemical staining.

Table II. IKKε expression and clinical pathological characteristics.

Variable	IKKε Low/no	IKKε High/moderate	P-value
Sex			
Male	9	19	
Female	8	15	0.538
Age			
≤43	8	11	
>43	10	22	0.313
Grade (WHO)			
I/II	21	3	
III/IV	4	23	0.000
Histology			
Astrocytic	7	30	
Oligodendroglioma	5	9	0.185

Statistical analysis. All data represent the average of at least three biological replicates or as indicated ± standard deviation (SD). Statistical analyses were carried out using the SPSS 16.0. $P<0.05$ is considered to be statistically significant by ANOVA and χ^2 test.

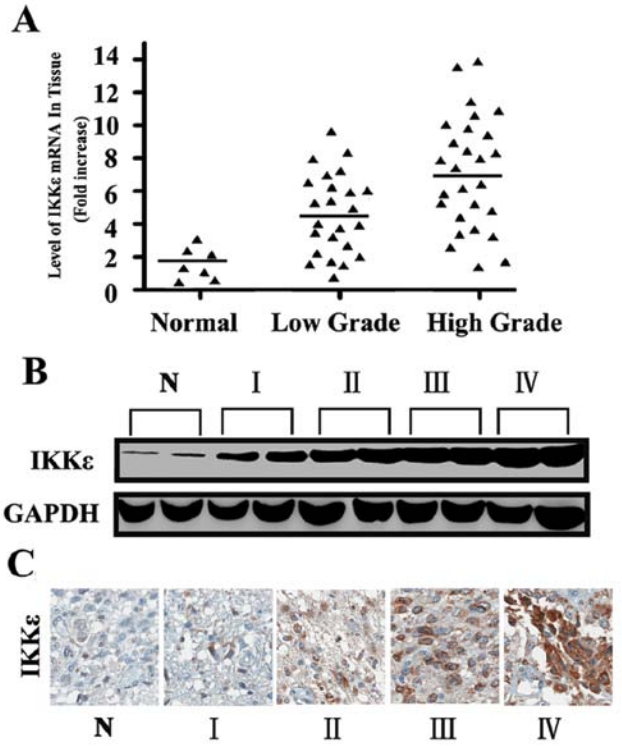

Figure 1. IKKε is highly expressed in human gliomas specimens. (A) Expression of IKKε mRNA in different grades of human gliomas specimens examined by quantitative real-time PCR. (B) Western blot analysis show that human gliomas specimens grade I-IV express higher levels of IKKε compared with normal brain tissues. (C) IKKε expression in different grades of human glioma specimens detected by immunohistochemistry (x200).

Results

IKKε is frequently overexpressed in glioma and correlates with tumor grade. IKKε was recently identified as a breast cancer oncogene, with amplification detected in 16% breast cancers and overexpression of IKKε protein observed in nearly half of primary breast tumors (10). In our study, overexpression of IKKε mRNA and protein was detected in 45 of 51 frozen glioma specimens (88.2%), whereas IKKε expression was either absent or expressed at low levels in normal brain tissue (1/7) (Fig. 1A and B). Immunohistochemical staining of the same panel of glioma specimens with anti-IKKε antibody also showed that IKKε expression was significantly increased in glioma compared with normal brain, with expression restricted to the cytoplasm (Fig. 1C). While Guan *et al* previously demonstrated that IKKε is upregulated in glioma cell lines and primary human glioma specimens, no significant difference in IKKε expression among different grades of glioma was observed (14). In contrast, our analyses show that IKKε expression positively correlated with tumor grade (Fig. 1A-C). IKKε overexpression was relatively low in low grade glioma and increased in high grade tumors (P=0.000; Table II), suggesting that alterations of IKKε may be involved in glioma progression rather than initiation. We next examined the relationships between IKKε overexpression and clinicopathological features of glioma. We observed that IKKε is frequently overexpressed in glioblastoma (11/12, 91.6%) compared with other histological types of glioma. However, we did not observe any differences in IKKε expression related to age, gender and histology.

Silencing of IKKε expression in glioma cells by siRNA knockdown. We tested the ability of five siRNAs designed to target different regions of the IKKε mRNA, to reduce IKKε expression in U251 and LN229 cells. Analysis of IKKε expression 48 h after transfection by quantitative real-time PCR, revealed that all five siRNAs were capable of silencing IKKε expression to varying degrees when compared with control scrambled siRNA (Figs. 2A and B). The most efficient knockdown was observed with siRNA-3 ($p<0.05$), which was selected for use

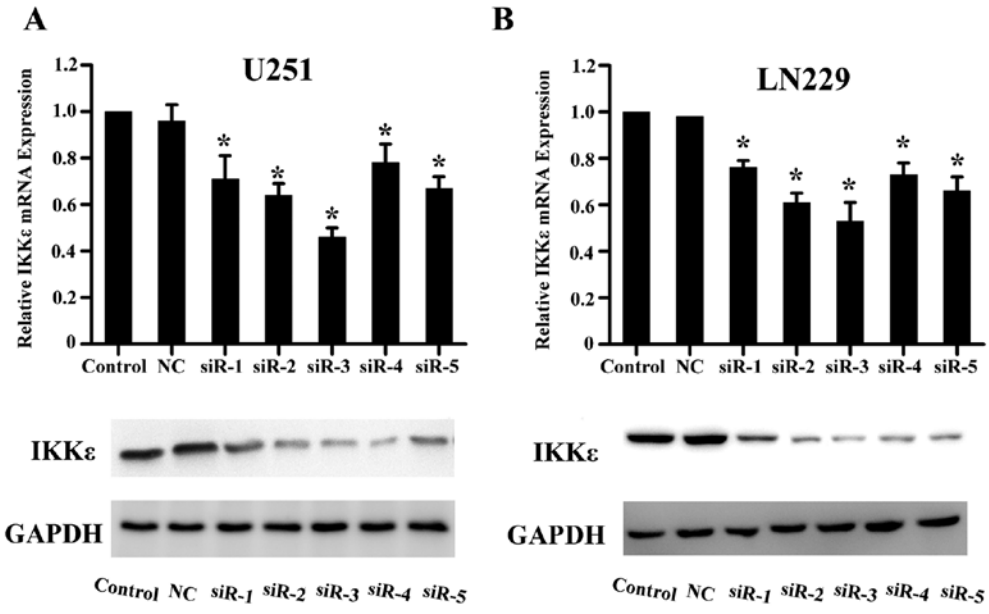

Figure 2. IKKε expression is effectively inhibited by targeted knockdown using siRNA. (A and C) IKKε expression in U251 treated with siRNA-NC and siRNA1-5 was determined by quantitative real-time PCR and western blot analysis, all five siRNAs showed a significant silencing effect (P<0.05) and in comparison with scrambled siRNA. (B and D) IKKε expression in LN229 treated with siRNA-NC and siRNA1-5 was determined by quantitative real-time PCR and western blot analysis. Among them, siR-3 showed the greatest suppression of IKKε and therefore siR-3 was selected for subsequent biological studies.

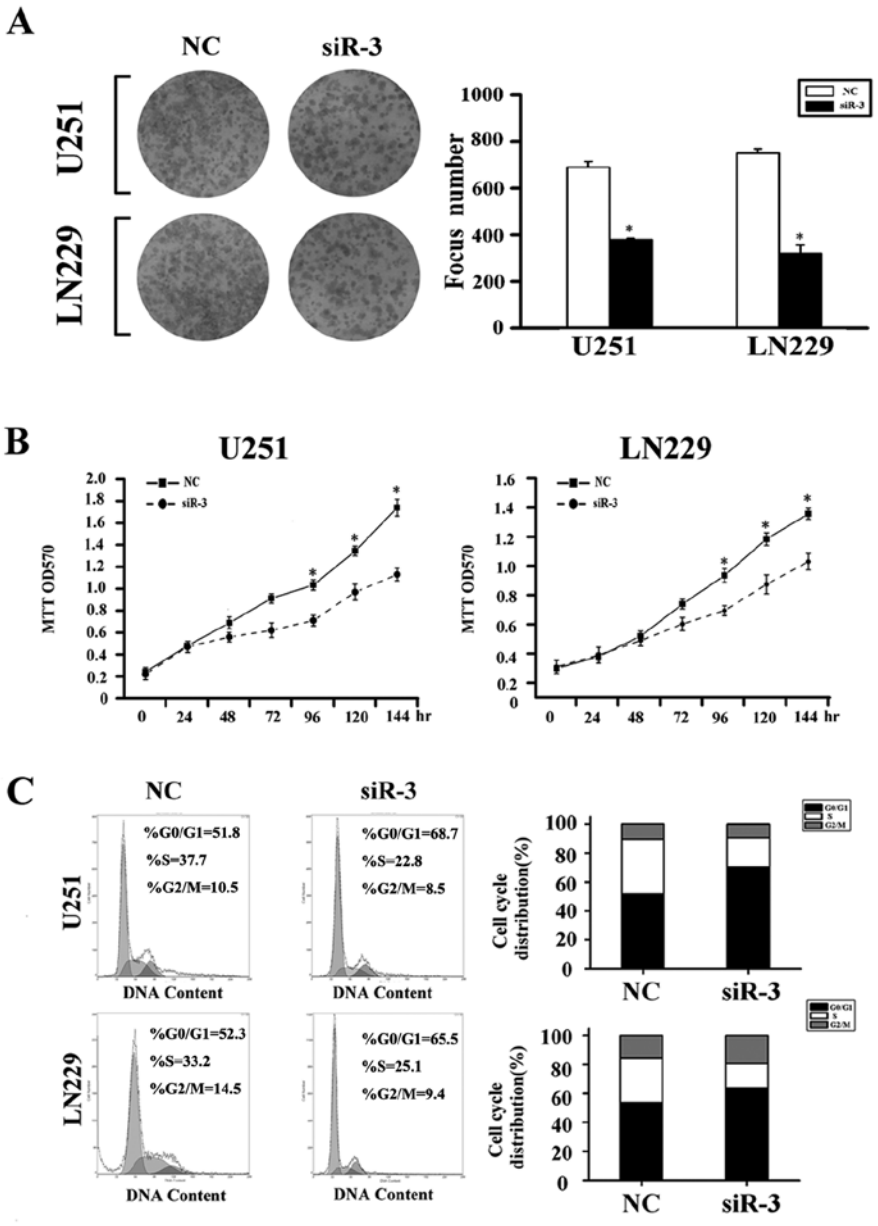

Figure 3. Silencing IKKε expression suppresses proliferation *in vitro*. (A) Focus formation assays in U251 and LN229 cells transfected with siR-3 or scrambled control (siRNA-NC). (B) Proliferation rate of U251 and LN229 cells transfected with IKKε siRNA determined by MTT assay, knockdown of IKKε significantly inhibited cell proliferation compared to the cells treated with scramble oligonucleotides. (C) Cell cycle analysis examined in U251 and LN229 cells transfected with siRNA-3 or siRNA-NC ($P<0.05$). Flow cytometry data represented low expression of IKKε induced G0/G1 cell cycle arrest.

in future loss of function experiments. Western blot analysis confirmed knockdown of IKKε at the protein level (Figs. 2C and D).

Downregulation of IKKε inhibits glioma cell proliferation and inhibits foci formation. To test whether downregulation of IKKε in glioma cells affects the anchorage-independent growth capability, we performed focus formation assays in cells transfected with IKKε siRNA. As shown in Fig. 3A, U251 cells treated with IKKε siRNA exhibited decreased foci formation and smaller foci diameter compared with control siRNA cells. Similar results were also observed in LN229 cells. These data indicate that inhibition of IKKε markedly decreases anchorage-independent growth potential. We next assessed the effect of loss of IKKε on U251 and LN229 cell proliferation by MTT assay. Cell proliferation was significantly impaired in cells transfected with IKKε siRNA in a time-dependent manner, compared with control cells (Fig. 3B). These results demonstrate that IKKε plays a pivotal role in the proliferation and survival of glioma cells, and indicate that suppression of IKKε leads to inhibition of cell proliferation.

Suppression of IKKε induces cell cycle arrest. To identify the mechanism for this effect on cell proliferation, we performed cell cycle analysis. We observed a significant decrease in the proportion of cells in G0/G1 in cells transfected with IKKε siRNA-3 compared with scrambled siRNA controls (U251; 68.7% vs 51.8%, $p<0.05$, LN229; 65.5% vs 52.3%, $p<0.05$) (Fig. 3C). This was also accompanied by a reduction in the percentage of S-phase cells. These results suggest that knock-

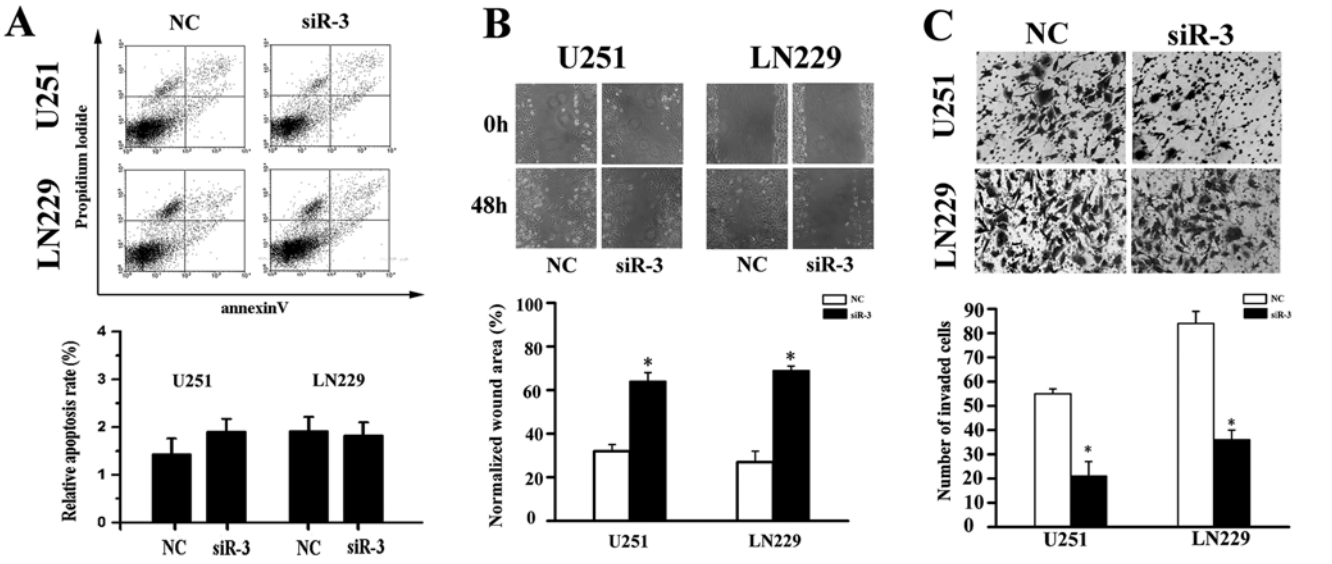

Figure 4. Effect of IKKε knockdown on invasion and apoptosis of glioma cells. (A) Apoptosis assay in U251 and LN229 cells after siRNA-NC or IKKε siRNA treatments. (B) IKKε knockdown inhibits migration of U251 and LN229 cells. Compared with cells transfected with scrambled siRNA, the cells treated with siR-3 showed a wider wound area 48 h after wound generation. The normalized wound area was calculated by the software Tscratch. (C) Cell invasion assays using transwell coated with Matrigel. Significant reduction of invasion was observed after silencing IKKε expression in U251 and LN229 cells.

down of IKKε expression can induce cell arrest in the G0/G1 phase, delay the progression of cell cycle, and inhibit cell proliferation.

Knockdown of IKKε induces negligible apoptosis. To determine whether IKKε knockdown affects apoptosis, we performed Annexin-V staining of glioma cells 48 h after transfection with IKKε siRNA-3. We observed no significant difference in the percentage of Annexin-V-positive apoptotic cells in IKKε siRNA-treated cells compared with controls (U251; 1.9% vs 1.4%, p=0.43, LN229; 1.9% vs 1.8%, p=0.78) (Fig. 4A). No significant distinction was observed in this study, suggesting that knockdown of IKKε alone may not be involved in glioma cell apoptosis.

Silencing of IKKε inhibits cell migration and invasion. To investigate the role of IKKε in glioma cell migration, we adopted a classic wound healing assay in which a monolayer of adherent cells is scratched and the migration of cells to the wound area was analyzed at different time points. Knockdown of IKKε inhibited wound healing compared with cells transfected with scrambled siRNA control after 48 h, indicating impaired migration (Fig. 4B). Because both cell migration and invasion are critical properties for metastases, we further investigated the invasive capacities of glioma cells using cell invasion assays. Knockdown of IKKε significantly impaired the invasion of both U251 and LN229 cells across a trans-well chamber compared with control siRNA by 62% and 57%, respectively (Fig. 4C). These data indicate that IKKε plays a role in glioma cell migration and invasion.

Downregulation of IKKε impairs nuclear translocation of NF-κB. NF-κB is constitutively activated in several human cancers, including glioma (15,16). NF-κB activity is controlled by inhibitors (IκBs). IκBs are in turn regulated by the IKK kinase in response to various cellular stimuli, resulting in ubiquitin-mediated protein degradation of IκBs and subsequent NF-κB translocation into the nucleus (9,17). However, the mechanism underlying the increase in NF-κB activity in human glioma is not well understood. To determine whether the knockdown of IKKε affects constitutive NF-κB activity in glioma cells, we analyzed the expression of p65 in cytoplasmic and nuclear extracts of transfected cells. As shown Fig. 5A, the nuclear translocation of NF-κB p65 protein was markedly decreased in both U251 and LN229 cells treated with IKKε siRNA compared with control cells. This was further confirmed by immunocytochemical and fluorescence analysis (Fig. 5B and C). These results indicate that silencing of IKKε impairs NF-κB p65 nuclear translocation.

Silencing of IKKε affects NF-κB downstream targets. NF-κB-mediated cell proliferation and invasion is well documented in glioma (18). Targeted knockdown of IKKε in glioma cells decreased expression of NF-κB target genes including c-myc, cyclin D1, Bcl-2, MMP-9 and VEGF at the level of mRNA (Fig. 5D) and protein (Fig. 5E) (both $P<0.05$). Intriguingly, suppression of IKKε did not affect the expression of Bcl-2 in either U251 or LN229 glioma cell lines. In most cases, inactive NF-κB proteins are sequestered in the cytoplasm in a complex with an inhibitor protein, termed IκB, in particular, the IκBα isoform. Activation of NF-κB proceeds following the activation of IKKε, which phosphorylates IκBα, causing its degradation. We found that U251 and LN229 cells treated with IKKε siRNA exhibited higher levels of IκBα compared with control siRNA cells ($P<0.05$). These data support the notion that IKKε may contribute to the regulation of NF-κB signaling via the regulation of IκBα (8,10). Taken together, our results demonstrate that IKKε expression modulates the expression of NF-κB target genes involved in glioma cell invasion and proliferation.

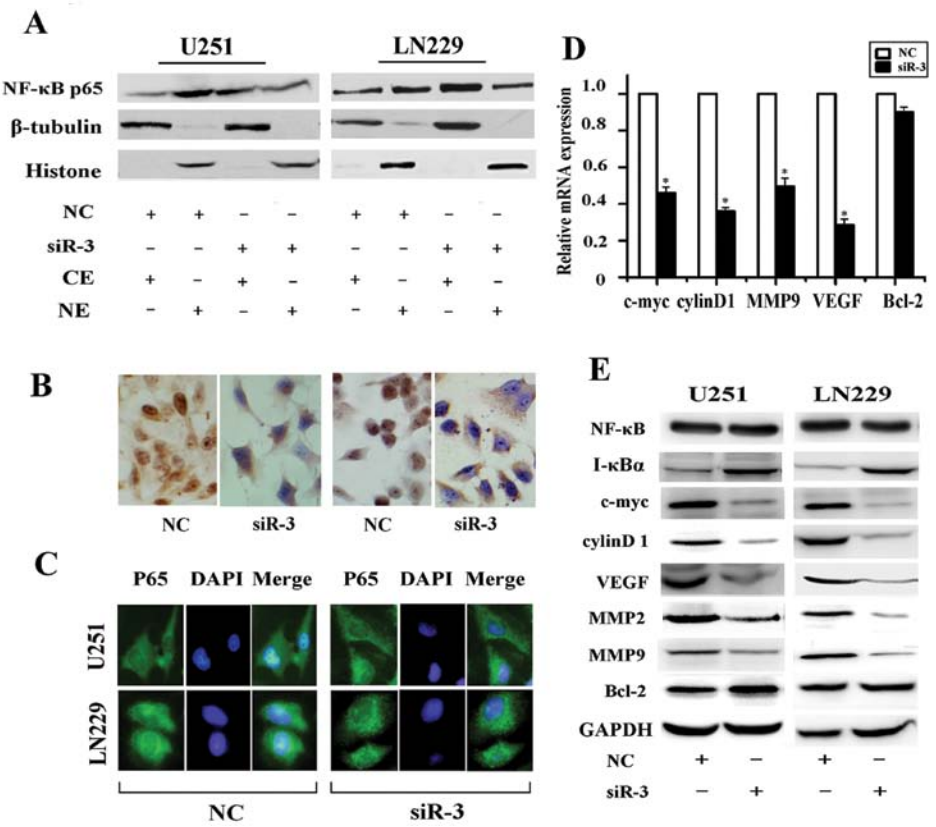

Figure 5. Knockdown of IKKε expression inactivates the NF-κB signaling in glioma cells. (A) NF-κB p65 expression was analyzed in cytoplasmic and nuclear extracts of glioma cells by western blotting. (CE, cytoplasmic extraction; NE, nuclear extraction). Histone was used as a marker for the nuclear loading control. β-tubutin was used as a cytoplasmic loading control. (B) Immunocytochemistry and (C) cell immunofluorescence assay using anti-NF-κB p65 antibody in U251 and LN229 cells. DAPI staining was employed as a nuclear counter stain (x200). (D) The level of mRNA associated with cell invasive ability, apoptosis and cell proliferation was detected by quantitative real-time PCR in U251 and LN229 cells 48 h after transfection. (E) Western blot analysis of c-myc, MMP2/9, cyclinD1, VEGF, Bcl-2, IκBα and NF-κB expression in U251 and LN229 cells transfected with IKKε siR-3 or scrambled control.

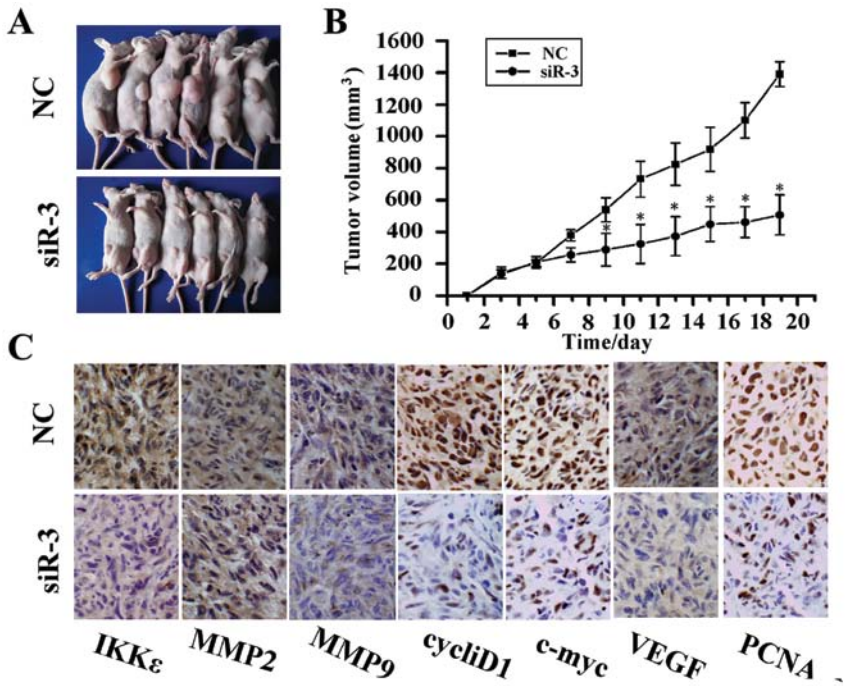

Figure 6. Treatment of nude mice bearing subcutaneous xenograft gliomas with IKKε siRNA. (A) Tumors treated with IKKε siRNA were significantly smaller than NC-siRNA treated tumors. (B) Tumor growth in nude mice treated with IKKε siRNA compared with that in NC-siRNA treated mice. (C) Immunohistochemistry analysis of expression of IKKε, PCNA, MMP2/9, VEGF, c-myc, cyclin D1 and PCNA in siR-3-treated tumors compared with NC treated tumors (x200). The results were consistent with that observed *in vitro*. IKKε protein was effectively inhibited by siRNA-3 treatment, resulting in downregulation of IKKε, PCNA, MMP2/9, VEGF, c-myc, cyclin D1 and PCNA in siR-3-treated tumors.

IKKε downregulation inhibits tumor transformation in a murine xenograft model. Since our *in vitro* data showed an anti-tumorigenic role for IKKε siRNA, we examined the therapeutic potential of IKKε knockdown *in vivo* in a mouse xenograft model. Nude mice were subcutaneously inoculated with U251 cells and monitored until the tumor cells had formed palpable tumors with an average volume of 120 mm^3 (approximately 7 days). IKKε siRNA or a control siRNA was then administered by intratumoral injections every 2 days for a total of 20 days. All mice were sacrificed on day 21. As shown in Fig. 6A-C, intratumoral delivery of synthetic IKKε siRNA induced a specific inhibitory response and robustly interfered with tumor growth compared with control mice ($P<0.05$). To correlate the therapeutic response observed with inhibition of IKKε, expression of IKKε, MMP2/9, cyclin D1, PCNA, VEGF and c-myc was assessed by immunohistochemical staining. As observed in our *in vitro* studies, IKKε protein was effectively inhibited by siRNA treatment, and downstream targets MMP-9, cyclin D1, PCNA, VEGF and c-myc were also downregulated in IKKε siRNA infected tumors.

Discussion

IKKε was shown to be upregulated in breast cancer (8,10), ovarian cancer (11) and prostate cancer (12). Recently, Guan *et al* demonstrated that IKKε is also overexpressed in gliomas, however, expression level was not related to tumor pathological grade (14). Our study revealed that elevated levels of IKKε are more frequently observed in high grade tumors, suggesting that alterations in IKKε expression are an early event in the development of some gliomas. A similar result was shown for IKKε expression in ovarian cancer progression (11). Further studies with more clinical samples are required to substantiate this observation.

Glioma is a highly invasive tumor, associated with high morbidity and mortality. Despite advances in the diagnosis and treatment of gliomas during the past several decades, the prognosis of patients remains poor. Indeed the 5-year survival rate for stage III and IV disease is only 1.9-9.8% when patients are treated with temozolomide or radiotherapy alone (19). Understanding the molecular mechanisms underlying gliomagenesis is necessary for the development of novel and promising therapeutic solutions.

Bohem *et al* confirmed that IKKε was required for the proliferation and survival of ZR-75-1 cells and also showed that knockdown of IKKi was required to diminish the viability of MCF-7 cells (10). A similar inhibitory effect on cell proliferation was also observed following IKKε knockdown in HeLa cells and ovarian cancer cells (9,11). In agreement with these findings, we found that targeted knockdown of IKKε significantly suppresses glioma cell proliferation. Previous studies by Bin *et al* also showed that inhibition of IKKε expression by siRNA reduced the ability of breast cancer cells to form anchorage-independent foci in six-well plates (20). Consistent with these observations, we show knockdown of IKKε in two IKKε-expressing cell lines, U251 and LN229, significantly impaired foci formation. Importantly, this reduction of foci forming potential correlated with inhibition of tumors in nude mice treated with IKKε siRNA (21).

Lastly, we examined the effect of IKKε on cell apoptosis. The role of IKKε in cell apoptosis remains controversial. One study reported that IKKε inhibition induced apoptosis in HeLa cells (22). A recent study revealed that shRNA-mediated knockdown of IKKε in glioma promotes cleavage and activation of caspase 3, decreasing Bcl-2 expression and inducing apoptosis via activation of NF-κB signaling (14). In contrast, knockdown of IKKε in the human A2780 ovarian cancer cell line did not affect apoptosis (11). In the current study, we observed negligible apoptosis in glioma cells after IKKε inhibition. These controversial reports suggest that apoptosis in glioma cells may be independent of IKKε-mediated signaling pathway. Since induction of apoptosis is determined by a balance of multiple pro-apoptotic and anti-apoptotic agents (23), this observation might be explained by the recruitment of other anti-apoptotic factors that may compensate for IKKε silencing.

Cell invasion, angiogenesis and tumor growth are complex mechanisms that involve a variety of biochemical and cellular processes (24). The degree of primary brain tumor growth is directly correlated with its invasive potency and angiogenesis (25). Accumulating evidence implicates NF-κB and NF-κB-related IKKs in cell invasion and tumor metastasis (26,27). For example, study of $IKK\alpha^{AA}$ knock-in mice, which express a catalytically inactive kinase mutant, revealed an important role for IKKα in the development of metastasis, in addition, Luo *et al* also demonstrated that blocking IKKα activation resulted in inhibition of prostate cancer metastasis in TRAMP mice (28). Based on this, it is of great interest to elucidate whether silencing of IKKε is associated with inhibition of glioma cell invasion. Our *in vitro* migration and invasion assays indicate that glioma cell invasion and migration properties are significantly inhibited following IKKε silencing, highlighting an important role for IKKε in these tumorigenic processes.

NF-κB is a transcription factor that plays a key role in carcinogenesis by controlling expression of several oncogenes, tumor suppressor genes, growth factors and cell adhesion molecules (29,30). NF-κB is comprised of two subunits, most commonly p65 and p50, that are typically restricted in the cytoplasm by the IκB inhibitors. IκB activity is controlled by the IKK kinase, which responds to cellular stimuli by phosphorylation of IκB, resulting in ubiquitin-mediated protein degradation. Subsequently, NF-κB is released and translocated into nucleus, stimulating an array of target genes promoting cell proliferation and invasion, and preventing apoptosis (31). Previous studies have shown that overexpression of IKKε leads to IκBα degradation (8,10). Ectopic expression of IKKε induced p65 phosphorylation, NF-κB activation and NF-κB-dependent target gene expression. In stimulated T-cells, IKKε has also been shown to enhance the activity of p65 via serine468 phosphorylation (32). Our studies show that NF-κB p65 nuclear translocation is significantly arrested in U251 and LN229 cells treated with IKKε siRNA. Moreover, IκBα expression was increased in cells treated with IKKε siRNA. Taken together, these data suggest that IKKε may promote malignant transformation, proliferation and invasiveness of glioma cells via activation of NF-κB.

PCNA expression may be used as a marker of cell proliferation because cells at the G1/S phase may be extended when proliferating (33,34). Cyclin D1 is overexpressed in various tumors and promotes the G1/S transition, leading to cell prolif-

eration (35). Previous studies observed that the expression of an inactive IKKε kinase (K38A), led to decreased activation of NF-κB element-driven cyclin D1 and relB promoters (8). IKKε is also sufficient to activate cyclin D1 in ER-negative and ER-positive ovarian cancer cells (11). Expression of Myc family members is also associated with neurogenic tumors and there is evidence of Myc gene amplification in human brain cancer (36). Superactivation of MMP-9 is associated with the pathogenesis and progression of gliomas (37,38). NF-κB can induce the expression of MMP-9 by direct promoter regulation (39). NF-κB can also regulate MMP-2 activity indirectly via control of an enzyme mediating MMP-2 post-translational processing (40).

Based on this, to further investigate the potential molecular mechanisms involved, we examined expression of VEGF, Bcl-2, Cyclin D1, c-myc and MMP-9 *in vitro*. Inhibition of IKKε resulted in a significant decrease in NF-κB target gene mRNA and protein levels, excluding Bcl-2. Our results suggest that the downregulation of IKKε results in inhibition of NF-κB downstream targets. Although IKKε overexpression has been associated with the enhanced proliferation and invasion of many tumor cell types *in vitro*, few studies have investigated the role of IKKε in promoting tumorigenesis *in vivo*. Using a U251 GBM xenograft model, we show, for the first time, that inhibition of IKKε using an IKKε-specific siRNA, suppresses tumor growth. Consistent with this phenotype, we observed decreased expression of the NF-κB targets, PCNA, cyclin D1, c-myc and MMP-2/9, suggesting that attenuated tumor progression following IKKε knockdown may be attributed to decreased activation of the NF-κB signaling pathway.

In conclusion, the key finding of our study is that IKKε expression is drastically upregulated in clinical glioma specimens compared with normal brain tissue and deregulation of IKKε is positively correlated to glioma pathological grade. Cell proliferation, viability, speed of migration, ability to form anchorage-independent colonies and invasive capability of glioma cells is reduced upon IKKε silencing, highlighting the role for IKKε in these key tumorigenic phenotypes. Mechanistically, we show that IKKε is involved in modulation of the NF-κB signaling pathway, by directly attenuating NF-κB translocation from cytoplasm to nucleus. Furthermore, our *in vivo* data demonstrate significant growth inhibition of established neurospongioma xenografts by intratumoral delivery of synthetic IKKε siRNA oligonucleotides, supporting the therapeutic potential of this novel RNAi technique.

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