NFATc1 activation promotes the invasion of U251 human glioblastoma multiforme cells through COX-2

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Abstract. Recent studies have revealed that the nuclear factor of activated T-cells (NFAT) is a transcription factor that is highly expressed in aggressive cancer cells and tissues, and mediates invasion through the transcriptional induction of pro-invasion and pro-migration genes. However, the mechanisms through which nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), in particular, translocates to the nucleus and regulates the invasion of human glioblastoma multiforme (GBM) cells have not yet been fully elucidated. In the present study, to investigate the role of NFATc1 in GBM cells, we established a U251 cell line expressing a constitutively active form of NFATc1 (CA-NFATc1). On the other hand, RNA interference was used to knock down NFATc1 expression in the U251 cell line. Our results demonstrated that the expression of CA-NFATc1 promoted cancer cell invasion, while small interfering RNA (siRNA) against NFATc1 successfully inhibited the invasion ability of the U251 cell line. Moreover, we demonstrated that NFATc1 promoted U251 cell invasion through the induction of cyclooxygenase-2 (COX-2). NFAT transcriptionally regulates the induction of COX-2 induction in U251 cells and binds to the promoter. We also demonstrated that a large proportion of GBM specimens expressed NFATc1. NFATc1 expression increased according to the histopathological grade of the glioma. However, no NFATc1 staining was observed in the non-neoplastic brain tissues. These findings suggest that the inhibition of the activation of the NFATc1 pathway is an effective therapeutic strategy for the clinical management of GBM.

Introduction

Nuclear factor of activated T-cells (NFAT) is a family of transcription factors consisting of four closely related proteins, i.e., nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) (NFATc, NFAT2), NFATc2 (NFATp, NFAT1), NFATc3 (NFATx, NFAT4) and NFATc4 (NFAT3), as well as a more distant relative, NFAT5 [tonicity response element-binding protein (TonEBP)]. In the basal state, NFAT is hyperphosphorylated in the cytoplasm. Subsequent to cell stimulation and calcium release, NFAT is dephosphorylated by the phosphatase calcineurin and translocates to the nucleus, where it cooperates with other factors and co-activators to promote *de novo* gene transcription (1). A number of recent findings have pointed to the important roles of NFAT in modulating phenotypes associated with malignancy and tumor progression (1,2). NFAT isoforms are overexpressed in human solid tumors and hematological malignancies (3,4) and appear to play a role in cancer cell autonomous functions, such as invasive migration, differentiation and the survival of cancer cells. The understanding of the roles of NFAT in tumor progression may provide aid the development of effective therapeutics targeting the NFAT pathway in cancer progression and metastasis.

Cyclooxygenase-2 (COX-2) converts arachidonic acid into bioactive lipids, including prostaglandin E2 (PGE2), which has been found to be elevated in various types of tumors (5,6). Studies have documented that the localized expression of COX-2 and its catalyzed product, PGE2, are sufficient in initiating the development of and promoting the progression of tumors *in situ*. COX-2 is normally expressed at very low or undetectable levels and its expression is rapidly induced at sites of inflammation and proliferation in response to stimuli, such as growth factors and tumor promoters (7). COX-2 expression and PGE2 levels are elevated in a variety of human cancers and are associated with increased angiogenesis, tumor invasion and resistance to apoptosis (8-12). It is well known that COX-2 is frequently overexpressed in colorectal cancer, and PGE2 has been identified as the principal prostanoid promoting cell growth and survival in colorectal tumors. PGE2 is able to exert pleiotropic effects in colorectal tumors, promoting cell proliferation, survival, angiogenesis, migration and invasion (13). However, the mechanisms responsible for the malicious activity of COX-2 remain unclear in glioblastoma multiforme (GBM).

A link between NFAT activity and COX-2 is evident from previous studies. NFAT has been reported to regulate COX-2 expression in human T lymphocytes (14). Putative NFAT recognition sequences are present in the human COX-2 proximal promoter, and deletion analysis has shown that they are important for its transcriptional activation (14). A previous study also demonstrated that these sites are essential for the

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induction of COX-2 expression by NFAT in colon carcinoma cells (15). However, it is not known whether NFAT can modulate COX-2 expression in GBM.

NFATc1 is a transcription factor of the NFAT family that is regulated by calcium and the phosphatase calcineurin (16). Evidence suggests that NFAT proteins, and NFATc1 in particular, regulate important cellular processes, such as proliferation and apoptosis in different cell types (17,18). However, little information is available on the expression and activation of the NFAT transcription pathway and its function in cancer cells. In the present study, we identified NFATc1 as ectopically expressed and highly activated in U251 cells both *in vivo* and *in vitro*. The inhibition of NFATc1 signaling in cultured U251 cells by treatment with small interfering RNA (siRNA) for the knock down of NFATc1 expression led to a marked decrease in cell invasion. We also demonstrated that the invasive effect of NFATc1 is mediated by the direct binding of activated NFATc1 to the COX-2 promoter, resulting in the upregulation of COX-2 transcription and increased GBM cell invasion. These results provide important novel insight into the mechanisms of oncogenic NFATc1 activation in GBM.

Materials and methods

Cell culture. The human U251 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured as monolayers in DMEM and fetal bovine serum (FBS) (both from Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS, 100 mg/l streptomycin and 100 U/ml penicillin at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

Immunofluorescence staining. Immunofluorescence staining was carried out as previously described (19). The U251 cells were grown on Poly-D-lysine-coated, 8-chamber slides with 5,000 cells/chamber. After 24 h, U251 cells were left untreated or treated with CsA (1 μg/ml) for 60 min. Thereafter, the cells were washed with phosphate-buffered saline (PBS) and fixed for 10 min with 4% paraformaldehyde in PBS, permeabilized with 0,25% Triton X-100 in PBS and blocked for 1 h in PBS supplemented with 5% FBS. The cells were incubated overnight at 4˚C with rabbit anti-p-NFATc1 (1:100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibody, followed by washing and incubating with Cy3-labeled anti-rabbit secondary antibody (1:200 dilution; Sigma, St. Louis, MO, USA) for 2 h. In the control samples, the U251 cells were treated with an equal volume of DMEM. The nuclei were counterstained with Hoechst 33258. The results were observed and photographed under a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Production of recombinant retroviruses and infection of U251 cells. The CA-NFATc1 (constitutively active form of NFATc1) mutant (pMSCV-caNFATc1) and the control retroviral plasmids were provided by Dr Neil Clipstone (Northwestern University, Chicago, IL, USA). Recombinant retroviruses were produced as previously described (20) by co-transfecting either the pMSCV-GFP or pMSCV-caNFATc1 proviral vectors together with pVSV-G (Clontech Laboratories, Inc., Mountain View, CA, USA), encoding the glycoprotein of the vesicular stomatitis virus, into the GP293 pantropic packaging cell line (Clontech Laboratories, Inc.) using Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA). The medium was replaced after 24 h, and viral supernatants were harvested 2 days post-transfection and stored at -80˚C. For infection, $5x10^4$ cells were plated per well in a 6-well plate. The following day, the medium was replaced with 2 ml of viral supernatant containing 8 μg/ml polybrene (Sigma), and the plates were centrifuged at 2,000 rpm for 1.5 h at room temperature. After the removal of the viral supernatant, the cells were expanded in growth medium for subsequent analysis and typically used within 5-7 days of infection. To ensure reproducibility, each experiment was repeated 3 times using cells derived from independent viral infections and independently derived retroviral stocks.

Transient transfection of NFATc1 siRNA. siRNA oligos for the knockdown of endogenous NFATc1 proteins were prepared using the ON-TARGETplus SMARTpool siRNA from Dharmacon, Inc. (Lafayette, CO, USA). The cells were transfected with NFATc1 (100 nM) using the DharmaFECT siRNA transfection reagent (Dharmacon, Inc.) according to the manufacturer's instructions. ON-TARGETplus non-targeting siRNA (Dharmacon, Inc.) was used as a negative control (control siRNA) and the selective silencing of NFATc1 was confirmed by western blot analysis.

Cellular protein preparation and western blot analysis. The U251 cells were treated with CA-NFATc1 mutant or NFATc1 siRNA. Cellular protein was extracted using the Cell Protein Extraction kit; (Millipore, Billerica, MA, USA). Protein concentrations were determined using the Coomassie (Bradford) Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein were separated by SDS-PAGE using 8% separating gels followed by transfer to nitrocellulose membranes. Following transfer, the membranes were blocked using 5% non-fat dried milk in PBS; pH 7.2 and incubated overnight at 4˚C with the primary antibody (pAb), including anti-NFATc1 (sc-17834), anti-COX-2 (sc-70879) and anti-β-actin (sc-130656) antibodies (1:1,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The membranes were washed 3 times with PBS, 0,1% Tween-20 (PBST) and then incubated with secondary antibodies (horseradish peroxidase-conjugated, goat antibodies to rabbit (sc-2025) and goat antibodies to mouse (sc2027; dilution of 1:5,000; Santa Cruz Biotechnology, Inc.) for 2 h at 24˚C. Following washing 3 times with PBS, 0.1% Tween-20, the immunoreactive bands were visualized using enhanced chemiluminescence detection reagents. Autoradiograms were scanned and the labeled bands were quantified using the Sigma-Gel software (Sigma).

RNA isolation and quantitative polymerase chain reaction (qPCR). Total RNA was extracted from the cells and cDNA synthesis and amplification were performed as described in a previous study (4). Primers were designed as: COX-2 forward, 5'-CAAAAGCTGGGAAGCCTTCTCTAACC-3' and reverse, 5'-GCCCAGCCCGTTGGTGAAAG-3'; matrix metalloproteinase (MMP)-2 forward, 5'-ATGGATCCTGGC TTTCCC-3' and reverse, 5'-GCTTCCAAACTTCACGCTC-3'; MMP-9 forward, 5'-TGACAGCGACAAGAAGTG-3' and reverse, 5'-CAGTGAAGCGGTACATAGG-3'; GAPDH forward,

5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-TGGTGAAGACGCCAGTGGA-3'. Comparative qPCR was performed in triplicate, including no template controls. Relative expression was calculated using the comparative Ct method.

Reporter gene assay. Reporter gene assay was carried out as previously described (19). A 2,004-bp-long COX-2 promoter region spanning -2,069 to -66 bp upstream of the translational start site was cloned by PCR and subcloned into the pGL3 vector (pGL3-COX-2). The sequence was confirmed by DNA sequencing. The U251 cells were seeded in 12-well plates ($2x10^6$ cells/plate) overnight and transiently co-transfected with pGL3-COX-2 or the internal control, pRL-TK (internal control (Promega Corp., Madison, WI, USA), along with indicated plasmids using Lipofectamine 2000 transfection reagent (Invitrogen), following the manufacturer's instructions. Firefly and Renilla luciferase activities in the cell extracts were measured using a dual-luciferase reporter assay system (Promega Corp.). The relative luciferase activity was then calculated by normalizing COX-2 promoter luciferase activity to control Renilla luciferase activity. The results were expressed as the percentage of relative luciferase activity of the control group, which was set to 1.

In vitro Matrigel invasion assay. Polycarbonate filters (9 mm, 8.0-μm pore size) were pre-coated with 50 μl of Matrigel (Becton-Dickinson, San Jose, CA, USA), diluted to 1 mg/ml in serum-free medium (DMEM), and applied for 1 h at 37˚C until gelled, after which cell suspension was applied. The cells were grown under standard conditions (see above) and weaned from 10% fetal calf serum to serum-free medium in 2 steps to 5 and 0%, over a 24 h period. Following trypsinization, the cells were diluted so that $1x10^3$ cells were added per well. The lower chamber was filled with 500 μl 20% fetal calf serum medium. The cells subjected to the various treatments were incubated for 24 h and then washed once (5 min) with PBS (pH 7.4). Non-invading cells were removed from the upper surface of the membrane by scrubbing, and cells on the lower surface of the filter were fixed for 30 min in methanol and glacial acetic acid (3:1), air-dried briefly and stained with Giemsa. A total of 5 random fields were counted at x200 magnification. Data represent the average cells of 5 fields compared between the experimental groups and the control group. These separate wells were used per test condition, and these experiments were performed 3 times.

Tissue samples and patients. Tumor specimens were obtained from patients admitted for diagnosis and treatment at the Fourth Affiliated Hospital of Harbin Medical University (Harbin, China). The diagnosis was made according to World Health Organization criteria. The present study was approved by the Ethics Committee of the Fourth Affiliated Hospital of Harbin Medical University and was based on the criteria of the Helsinki convention. Approval for the use of tissue samples was obtained from our institutional review board. Fresh surgical samples from glioma patients and non-neoplastic brain tissues (temporal lobectomy; temporal lobe epilepsy surgery) were immediately snap-frozen in liquid nitrogen upon surgical removal. The formalin-fixed, paraffin-embedded archival tissue blocks were retrieved and the matching H&E-stained slides were screened for representative tumor regions by a neuropathologist. The tissue samples included 5 diffuse astrocytomas (grade II), 8 anaplastic astrocytomas (grade III) and 50 GBMs (grade IV astrocytoma). In addition, 10 non-neoplastic brain tissues from epilepsy surgical resections were also included.

Immunohistochemistry (IHC). The perfused brains were cryoprotected in a solution of 20% sucrose in 0.1 M of potassium phosphate buffer overnight. The brain sections were cut on a freezing microtome (Leica SM2000R) and mounted on gelatinized slides. The sections were dried at 40-50˚C for 2 h and were maintained at -20˚C until analysis. Briefly, IHC was performed as follows: the sections were incubated overnight at room temperature with the primary antibody (NFATc1, 1:100) diluted in PBS with Tween-20 (PBST). The negative controls received only PBST. The slides were washed with PBST and incubated with the secondary antibodies (1:1,000 in PBST) for 90 min. The slides were washed again with PBST and incubated with streptavidin-horseradish peroxidase (1:200 in PBST) for 60 min. The reactions were developed with 0.04% 3,3'-diaminobenzidine (DAB) + 0.03% H_2O_2. The DAB reactions were intensified with an OsO_4 solution (0.04%) for 30 min. The slides were counterstained with hematoxylin, dehydrated and mounted with Permount. The samples were visualized and images were captured using a light microscope (Olympus BX51; Olympus Optical, Tokyo, Japan). The degree of immunostaining of the sections was viewed and scored separately by 2 independent investigators, and the scores were determined by combining the proportion of positively stained tumor cells and the intensity of staining. Scores from the 2 investigators were averaged for further comparative evaluation of NFATc1 expression. The proportion of positively stained tumor cells was graded as previously described (21).

Statistical analysis. All the data are presented as the means ± standard error. All analyses were performed with one-way analysis of variance using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

NFATc1 is constitutively activated in glioma tissues. To determine whether NFATc1 is constitutively activated in glioma, the levels of NFATc1 expression were assessed by IHC using anti-NFATc1 antibody on glioma tissues. Among the glioma samples, 92.1% (58/63) demonstrated positive nuclear staining, whereas normal tissue did not stain. NFATc1 staining was predominantly nuclear *in vivo*. There was no significant difference in the constitutive activation frequency between the low- and high-grade gliomas. However, the expression levels of NFATc1 were significantly higher in the high-grade gliomas (grades III and IV) compared with the low-grade gliomas (grade II) (Fig. 1), which supports the hypothesis that NFATc1 activation is associated with the progression of glioma.

NFATc1 is activated in U251 cells and is repressible by cyclosporin A (CsA); CA-NFATc1 promotes NFATc1 activation. Immunofluorescence staining of the U251 cells indicated that NFATc1 was accumulated in the nucleus. Treatment of the U251 cells with the calcineurin inhibitor, CsA, caused the rapid

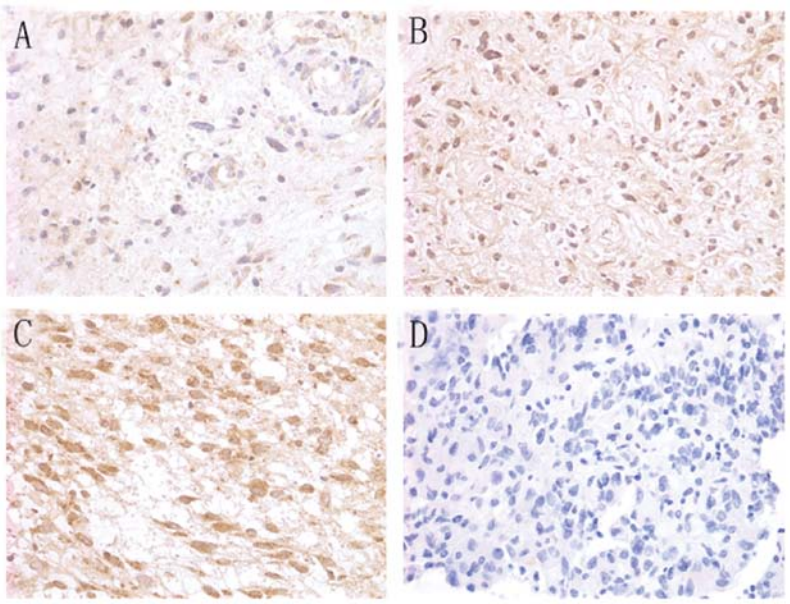

Figure 1. Immunohistochemical staining of nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) in glioma samples. (A) Grade II glioma; (B) grade III glioma; (C) grade IV glioma; (D) normal brain tissue.

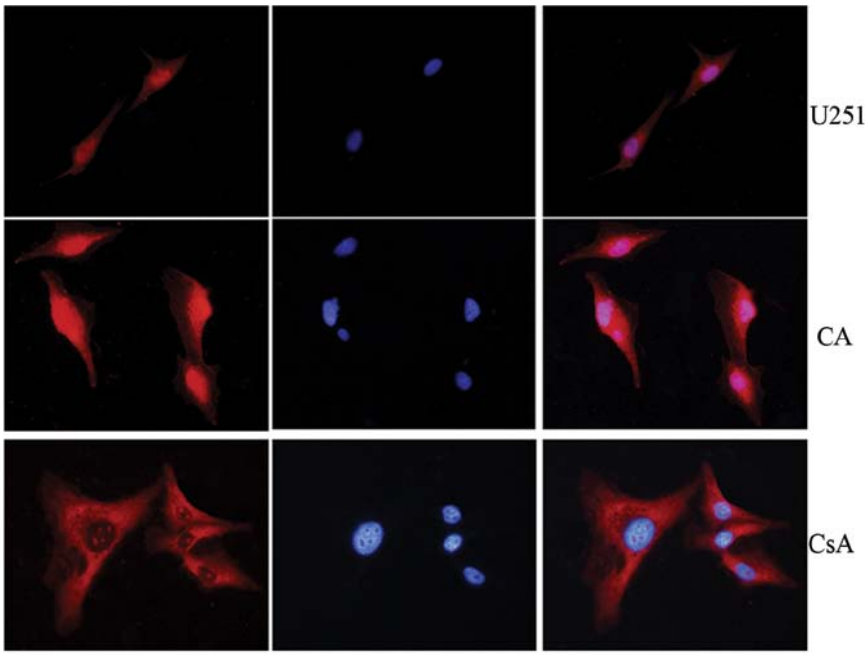

Figure 2. Immunofluorescence staining of nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) in glioblastoma multiforme (GBM) cells with constitutively active (CA)-NFATc1 and cyclosporin A (CsA) treatment. Upper panels, untransfected U251 cells; middle panels, U251 cells transfected with CA-NFATc1; lower panels, CsA treatment. Left panels, NFATc1 staining; middle panels, nuclear staining; right panels, NFATc1 and nuclear staining.

inhibition and nuclear export of NFATc1. CA-NFATc1 treatment further induced the accumulation of NFATc1 in the cell nuclei and significantly increased NFATc1 staining (Fig. 2).

NFATc1 plays an important role in the invasion of U251 cells. When the transfectants were allowed to invade the Matrigel in an *in vitro* invasion assay, the CA-NFATc1-transfected U251 cells were significantly more invasive compared with the untreated controls (Figs. 3 and 4). Conversely, transfection of the U251 cells with NFATc1 siRNA resulted in a reduction in the invasion ability compared with the controls (Figs. 3 and 4). The viability of the U251 cells was significantly affected by NFATc1 siRNA treatment (Figs. 3 and 4).

NFATc1 siRNA inhibits the induction of COX-2 expression in U251 cells, whereas CA-NFATc1 increases COX-2 expression. We examined the expression of COX-2, a gene with important implications in the progression of GBM, which has been reported to be regulated by NFAT in several cell types (22,23). qPCR analysis of COX-2 expression revealed high mRNA

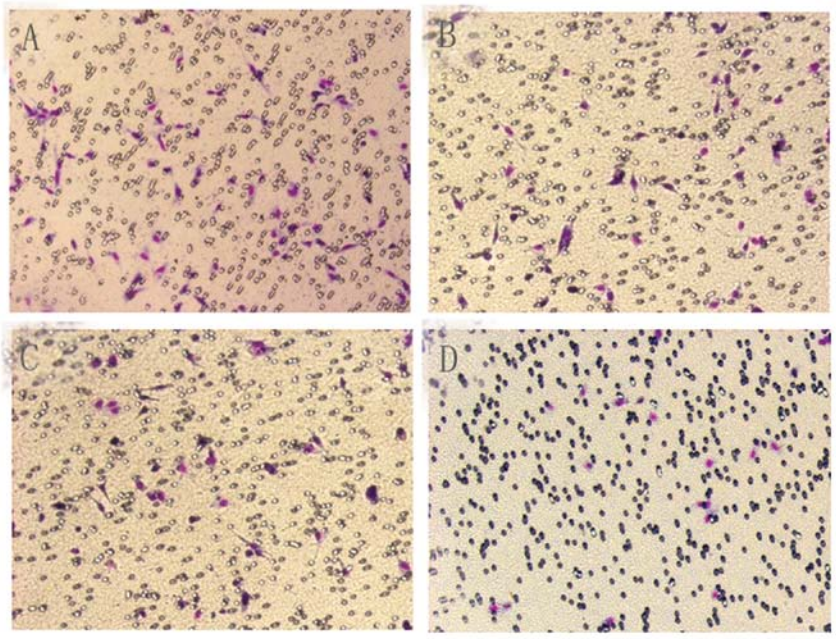

Figure 3. Effect of nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) activation and inhibition on the invasion of glioblastoma multiforme (GBM) cells. (A) U251 cells transfected with constitutively active (CA)-NFATc1; (B) untransfected U251 cells; (C) U251 cells transfected with control small interfering RNA (siRNA); (D) U251 cells transfected with NFATc1 siRNA.

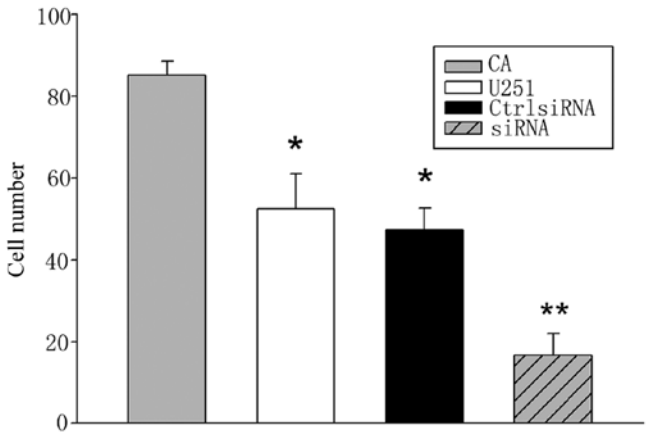

Figure 4. Number of transmembrane cells. Data are expressed as the means ± SE, n=3. *P<0.05, **P<0.05. CA, U251 cells transfected with constitutively active (CA)-NFATc1; U251, untransfected U251 cells; Ctrl siRNA, U251 cells transfected with control siRNA; siRNA, U251 cells transfected with NFATc1 siRNA.

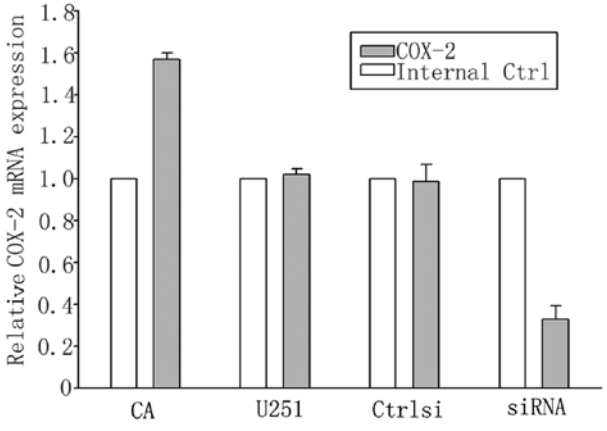

Figure 5. Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) regulates the expression of cyclooxygenase-2 (COX-2) at the transcriptional level. Normalized quantitative analysis of COX-2. Data are expressed as the means ± SE, n=3. CA, U251 cells transfected with constitutively active (CA)-NFATc1; U251, untransfected U251 cells; Ctrlsi, U251 cells transfected with control small interfering RNA (siRNA); siRNA, U251 cells transfected with NFATc1 siRNA; internal Ctrl, GAPDH.

expression levels of COX-2 in the U251 cells. Indeed, COX-2 has been identified as an inducible early gene in response to several stimuli in different cell types (24,25). We found that the induction of COX-2 mRNA expression was suppressed by NFATc1 siRNA (Fig. 5), indicating that the increase in the mRNA levels mainly occurs at the transcriptional level, requiring new RNA synthesis. On the other hand, CA-NFATc1 treatment was found to increase COX-2 mRNA expression (Fig. 5). These results support the hypothesis that COX-2 behaves as an inducible early gene, and that NFATc1 regulates the expression of COX-2 in U251 cells.

To address whether the induction of COX-2 mRNA expression is paralleled by an increase in its protein expression, western blot analysis was performed with extracts of U251 cells treated with CA-NFATc1 and NFATc1 siRNA. The COX-2 protein levels increased following treatment with CA-NFATc1, showing a pattern of induction similar to that of its mRNA expression. The inhibition by NFATc1 siRNA severely diminished the COX-2 protein levels (Fig. 6).

Involvement of NFATc1 in the transcriptional activation of the COX-2 promoter. To determine whether the induction of COX-2 mRNA expression by NFATc1 correlates with an increase in the transcriptional activity mediated by the COX-2 promoter, the U251 cells were transiently transfected with COX-2

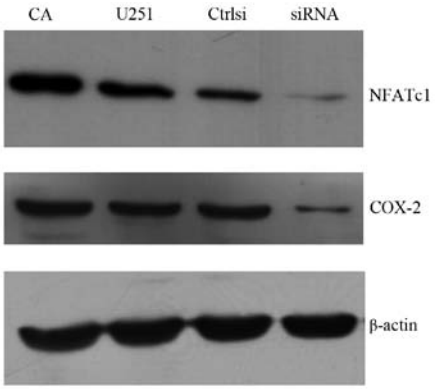

Figure 6. Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) regulates the protein expression of cyclooxygenase-2 (COX-2) . Western blot analysis of COX-2. Data are expressed as the means ± SE, n=3. CA, U251 cells transfected with constitutively active (CA)-NFATc1; U251, untransfected U251 cells; Ctrlsi, U251 cells transfected with control small interfering RNA (siRNA); siRNA, U251 cells transfected with NFATc1 siRNA.

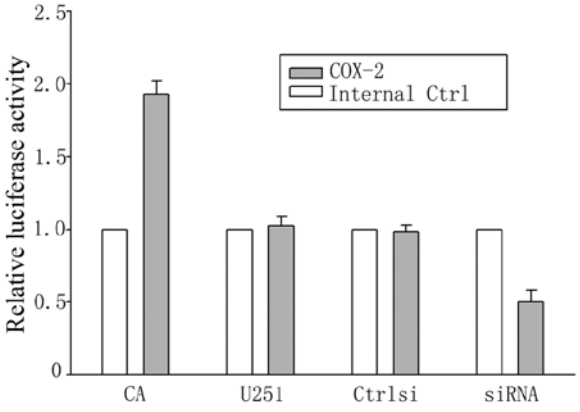

Figure 7. Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) is involved in the promoter transcriptional activity of cyclooxygenase-2 (COX-2). Luciferase activity assay of pGL3-COX-2. Data are expressed as the means ± SE, n=3. CA, U251 cells transfected with constitutively active (CA)-NFATc1; U251, untransfected U251 cells; Ctrlsi, U251 cells transfected with control small interfering RNA (siRNA); siRNA, U251 cells transfected with NFATc1 siRNA; internal Ctrl, GAPDH.

promoter. In agreement with the regulation of COX-2 mRNA levels, CA-NFATc1 strongly increased transcription driven by a construct spanning from -2,069 to -66 bp of the human COX-2 promoter (Fig. 7). To confirm the roles of NFATc1 in COX-2 expression, we also examined the effect of NFATc1 silencing by the siRNA approach in COX-2 promoter activity. The transfection of siRNA targeting NFATc1 significantly inhibited COX-2 promoter activity (Fig. 7). However, the control siRNA did not influence COX-2 promoter activity (Fig. 7). Taken together, these results strongly suggest that NFATc1 is an important mediator of COX-2 activity.

NFATc1 is involved in the regulation of MMP-2 and MMP-9 expression. To examine whether NFATc1 regulates U251 cell invasion by modulating MMP expression, we analyzed the mRNA levels of MMP-2 and MMP-9 upon NFATc1 silencing and activation. The results from qPCR revealed that the mRNA levels of MMP-2 and MMP-9 were decreased by >50% in the NFATc1-silenced cells (Figs. 8 and 9). The mRNA levels of

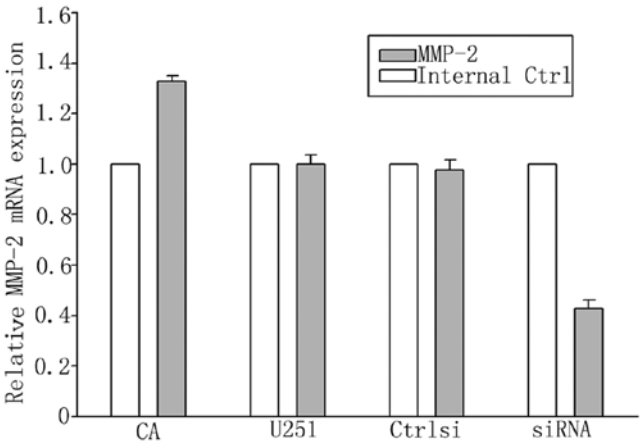

Figure 8. Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) regulates the expression of matrix metalloproteinase-2 (MMP-2) at the transcriptional level. Normalized quantitative analysis of MMP-2. Data are expressed as the means ± SE, n=3. CA, U251 cells transfected with constitutively active (CA)-NFATc1; U251, untransfected U251 cells; Ctrlsi, U251 cells transfected with control small interfering RNA (siRNA); siRNA, U251 cells transfected with NFATc1 siRNA; internal Ctrl, GAPDH.

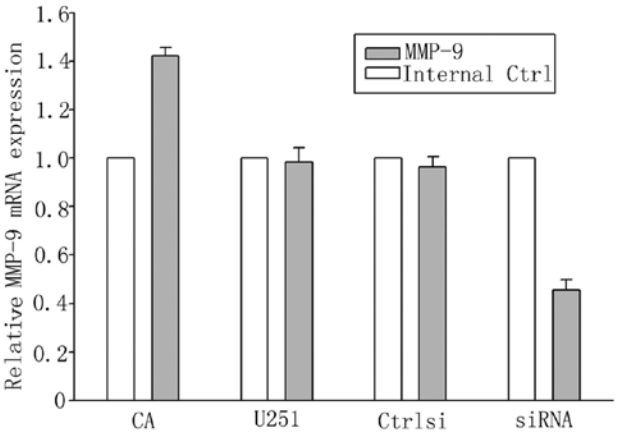

Figure 9. Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) regulates the expression of matrix metalloproteinase-9 (MMP-9) at the transcriptional level. Normalized quantitative analysis of MMP-9. Data are expressed as the mean ± SE, n=3. CA, U251 cells transfected with constitutively active (CA)-NFATc1; U251, untransfected U251 cells; Ctrlsi, U251 cells transfected with control small interfering RNA (siRNA); siRNA, U251 cells transfected with NFATc1 siRNA; internal Ctrl, GAPDH.

MMP-2 and MMP-9 in the cells transfected with the control siRNA were not altered under these conditions (Figs. 8 and 9), indicating that the downregulation of MMP-2 and MMP-9 expression by NFATc1 silencing is specific. On the other hand, CA-NFATc1 treatment was found to increase MMP-2 and MMP-9 mRNA expression (Figs. 8 and 9).

Discussion

NFAT was originally identified in immune cells as a transcription factor required for the inducible expression of cytokines critical for triggering the immune response (16,26). Previous studies have revealed that NFAT is ubiquitously expressed and regulates a plethora of transcriptional responses important

for cell survival, angiogenesis and cell growth in all cells and tissues (16,27). NFATc1 is a member of the NFAT family. The constitutive activation of NFATc1 has been found in approximately 70% of pancreatic carcinomas, and blocking NFATc1 activation with CsA has been shown to inhibit cell growth and survival in a pancreatic cancer cell line (28). In a recent study, Oikawa *et al* (29) found that the constitutively active form of NFATc1 promoted cancer cell invasion in association with changes in cell morphology in lung and breast cancer cells. However, the mechanisms responsible for the malicious activity of NFATc1 in GBM remains unclear. The present study demonstrated that NFATc1 is constitutively activated in GBM tumors. This finding indicates that NFATc1 is involved in the initiation and progression of gliomas. Moreover, we found that NFATc1 expression increased according to the histopathological grade of the glioma.

The present study revealed that NFATc1 exerted invasive effects in the majority of U251 cells. To address this issue, we developed a GBM cell line with an elevated inducible NFATc1 (CA-NFATc1) expression. As was expected, the inducible expression of NFATc1 led to increased transcriptional activity with an increase in invasion through Matrigel. We also evaluated COX-2 regulation in GBM cells by silencing NFATc1 expression using siRNA. Transfection of the U251 cells with NFATc1 siRNA resulted in a diminished effect of cell invasion, suggesting that NFATc1 is required for the invasion of GBM cells.

In recent years, a remarkable discovery for oncology is that the overexpression of COX-2 is closely associated with tumor development. COX-2 is considered a new and important target for the treatment of a variety of tumors since COX-2 inhibitors or the knockout of the COX-2 gene has been shown to inhibit tumor development and metastasis (30). Previous studies (31-33) have demonstrated that COX-2 promotes tumor cell invasion and metastasis through various means, including the regulation of downstream genes in gastrointestinal, lung and breast cancer, as well as other types of cancer. Recently, it has also been reported that COX-2 is involved in the invasion of osteosarcoma cells (34).

The induction of COX-2 by NFAT has been reported in human T lymphocytes (14). This issue has also been investigated in non-immune cells. For example, NFAT induces the transcription of COX-2 in breast epithelial cells, and this is required for the ability of NFAT to promote invasive migration (35). In addition, NFATc2 and NFAT5 proteins are expressed in invasive human ductal breast carcinomas participating in promoting carcinoma migration and invasion (3). It has also been reported that the NFAT induction of COX-2 expression contributes to tumor cell invasion and metastasis in a hepatitis virus-induced hepatocellular carcinoma model (36). Similarly, a recent study also provided evidence of COX-2 expression induced by NFAT in colon carcinoma (15). However, to the best of our knowledge, our study is the first to demonstrate the induction of COX-2 by NFATc1 in GBM cells. More importantly, we demonstrate that COX-2 is one of the genes responsible for NFAT-driven Matrigel invasion.

To the best of our knowledge, our data also demonstrate for the first time that activated NFATc1 is able to transcriptionally upregulate COX-2 through direct interaction with specific sequence elements within the COX-2 promoter. In GBM cell lines, NFAT cooperates by binding to the COX-2 promoter, leading to the expression of downstream regulators at the transcriptional level, which, as we showed, was COX-2- and NFAT-dependent. The inhibition of COX-2 *in vivo* has been found to attenuate the metastatic potential of colorectal tumors in both humans (37) and mice (38), while the overexpression of COX-2 in intestinal cells modulates their adhesive properties (39) and increases MMP activity to promote invasion (40).

One would also expect that for NFAT to function as a pro-invasion transcription factor, it would induce the transcription and secretion of MMPs that are required for efficient basement membrane proteolysis during tumor invasion and metastasis (41). While there is some evidence that NFAT is required for MMP induction in myocytes and mesangial cells, to date, no studies have addressed MMP regulation by NFAT signaling in settings of carcinoma progression. Our findings indicate that elevated expression levels of MMP-2 and MMP-9 are associated with a higher incidence of invasion in human GBM cells. MMP-2 and MMP-9 are upregulated by COX-2 and NFAT, thus promoting the invasion of GBM cells.

In conclusion, our data add to the growing body of evidence suggesting that NFATc1 transcription factors, in addition to their well-defined roles as transcriptional regulators, have the potential to control central aspects of invasion in GBM cells. NFATc1 plays a functional role in the regulation of target genes involved in the tumoral phenotype, such as COX-2. Moreover, our study provides a rationale for future research into the use of inhibitors of NFATc1 in adjuvant therapy for GBM.

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