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Bex2 regulates cell proliferation and apoptosis in malignant glioma cells via the c-Jun NH2-terminal kinase pathway

Xiuping Zhou [a,b,c,*,1], Qingming Meng [a,d,1], Xuebin Xu [d], Tongle Zhi [d], Qiong Shi [a,b,c], Yong Wang [d], Rutong Yu [a,b,c,*]

[a] Department of Neurosurgery, Affiliated Hospital of Xuzhou Medical College, Xuzhou, Jiangsu, China
[b] Lab of Neurosurgery, Xuzhou Medical College, Xuzhou, Jiangsu, China
[c] Key Laboratory of Brain Disease Biology, Affiliated Hospital of Xuzhou Medical College, Jiangsu, China
[d] The Graduate School, Xuzhou Medical College, Xuzhou, Jiangsu, China

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ABSTRACT

The function of Bex2, a member of the Brain Expressed X-linked gene family, in glioma is controversial and its mechanism is largely unknown. We report here that Bex2 regulates cell proliferation and apoptosis in malignant glioma cells via the c-Jun NH2-terminal kinase (JNK) pathway. The expression level of Bex2 is markedly increased in glioma tissues. We observed that Bex2 over-expression promotes cell proliferation, while down-regulation of Bex2 inhibits cell growth. Furthermore, Bex2 down-regulation promotes cell apoptosis and activates the JNK pathway; these effects were abolished by administration of the JNK specific inhibitor, SP600125. Thus, Bex2 may be an important player during the development of glioma.

1. Introduction

Malignant glioma is the most lethal tumor of the central nervous system and has highly proliferative capacity [1–3]. Despite advances in surgery and adjuvant therapy, the median survival of patients has changed little over recent decades [4,5]. Therefore, it is important to elucidate the mechanisms of glioma development and to find key molecular targets for the development of effective therapies.

Bex2 belongs to the Brain Expressed X-linked gene family, which was originally found to be involved in the development of the nervous system and neurological diseases [6,7]. For example, members of the Bex family have been shown to act as olfactory marker proteins that play important roles in the differentiation and function of olfactory receptor neurons [8]. Bex3 encodes NADE, which interacts with the death domain of p75NTR and mediates apoptosis in neural cells in response to nerve growth factor; NADE may also play an important role in the pathogenesis of neurogenetic diseases [9]. Recently, Bex1 was found to bind to a p75NTR intracellular domain and acts as a link between neurotrophin signaling, the cell cycle, and neuronal differentiation [10].

However, recent studies strongly suggest that Bex genes are involved in cancer biology. For example, identified as a neuroendocrine-specific tumor gene, Bex1 may be involved in the tumorigenesis [11]. Bex2 is up-regulated in a subset of primary breast cancers and mediates apoptosis induced by nerve growth factor/nuclear factor-kappa B inhibition in breast cancer cell lines [12]. Furthermore, Bex2 not only may regulate mitochondrial apoptosis and the G1 cell cycle but also has a functional interplay with c-Jun/JNK and p65/RelA and participates in a feedback loop with ErbB2 in breast cancer [13–15]. In addition, Bex2 has been identified as a candidate tumor suppressor gene in malignant glioma by a genome-wide analysis of epigenetic silencing [16]. However, it has been reported that over-expression of Bex2 promotes the growth of glioma LN-229P cells, whereas Bex2 down-regulation sensitizes LN-229P and 229Res cells to a dominant positive variant of p53 induced cell death [17].

In summary, the role of Bex2 in glioma remains controversial and its mechanism is largely unknown. In this study, we investigated the function and mechanism of Bex2 in glioma cell proliferation and apoptosis. We found that Bex2 is up-regulated in malignant glioma samples and Bex2 down-regulation promotes cell apoptosis by regulating the JNK signaling pathway.

Abbreviations: Bex2, Brain Expressed X-linked 2; JNK, c-Jun NH2-terminal kinase.

* Corresponding authors. Address: Department of Neurosurgery, Affiliated Hospital of Xuzhou Medical College, 99 West Huai-hai Road, Xuzhou, Jiangsu 221002, China. Fax: +86 0516 85802496 (R. Yu), Lab of Neurosurgery, Xuzhou Medical College, 84 West Huai-hai Road, Xuzhou, Jiangsu 221002, China (X. Zhou).
E-mail addresses: xpzhou@xzmc.edu.cn (X. Zhou), yu.rutong@163.com (R. Yu).

[1] These authors contributed equally to this work.

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2. Materials and methods

2.1. Glioma and non-tumor human brain tissues

A cohort of 32 human glioma tissue samples (surgical resection) and 15 cases non-tumor brain tissues (internal decompression in cerebral trauma) were obtained from the Affiliated Hospital of Xuzhou Medical College (Xuzhou, China). The gliomas were histologically diagnosed based on the World Health Organization grading system [18]. As controls, the non-tumor human brain tissues were obtained from patients who underwent decompressive surgery after physical injuries to the brain. Written informed consent was obtained from the patients and the ethics committees of the hospitals approved the study.

2.2. Cell culture and reagent

U251 and U87 cell lines were purchased from the Shanghai Cell Bank, Type Culture Collection Committee, Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium and F-12 (DMEM/F-12) (Gibco), supplemented with 10% fetal bovine serum (Sijiqing Biological Engineering Materials Co.) and grown in a humidified incubator with 5% CO_2 at 37 °C. Cells were treated with SP600125 (Sigma), a selective inhibitor of JNK, at 20 μmol/l final concentration 1 h before the Bex2 siRNA transfection.

2.3. Antibodies

The following antibodies were purchased: mouse monoclonal anti-c-Jun (G-4); rabbit polyclonal anti-p-c-Jun (Ser 63/73); mouse monoclonal anti-JNK (D-2) and mouse monoclonal anti-p-JNK (G-7) (all from Santa Cruz Biotech); rabbit polyclonal anti-caspase-3; anti-cleaved caspase-3 (Asp175); anti-cleaved caspase-9 (Asp315) (all from Cell Signaling); mouse monoclonal anti-Bex2 [9E1237] (Abcam); mouse monoclonal anti-GFP(7G9) (Abmart); and mouse monoclonal anti-β-actin (Millipore).

2.4. Transient transfection of glioma cells with Bex2 and Bex2 small interfering RNAs

Transfection of Bex2 and its siRNAs were performed using the Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. The Bex2 construct was generated by cloning the human Bex2 cDNA into the expression vector pEGFP-N1 at the EcoR I and BamH I restriction sites. Three sets of siRNA duplexes (Shanghai GenePharma Co.) targeting human Bex2 are listed here: Bex2-si429 (5′-CAGUAUAGAUGGGACAUAATT-3′); Bex2-si472 (5′-CAAGGAUGAGAGAGGAGAATT-3′); and Bex2-si535 (5′-GGAGCAAGUUGCUAAUAAATT-3′). All of the transfections were performed three times independently.

2.5. Reverse transcription–polymerase chain reaction

Total RNA from glioma cells was extracted using the TRIzol Reagent (Tiangen Biotech CO.). First-strand cDNA was synthesized using the Reverse Transcription Reagents (TaKaRa RNA PCR Kit (AMV) Ver. 3.0) according to the manufacturer's instructions. Primers (Sangon Biotech Co.) for Bex2 were as follows: sense: 5′-CGGAATTCATGGAGTCCAAAGAGGAACG-3′ and antisense: 5′-CGGGATCCGTGGGCATAAGGCAAAACTCATCG-3′. The primers for β-actin were: sense: 5′-CTGGGACGACATGGAGAAAA-3′ and antisense: 5′-AAGGAAGGCTGGAAGAGTGC-3′. The relative band intensities were determined using Image J software.

2.6. Cell growth assay

A cell growth assay was used to study the effect of Bex2 on glioma cell proliferation. Cell growth curves were obtained by detecting the cell viability with the Cell Counting Kit-8 (CCK-8, Beyotime) every 24 h after Bex2-siRNA transfection according to the manufacturer's instruction. Cell viability measurements at individual time points were normalized to those taken at 0 h in each group.

2.7. Flow cytometry analysis to examine cell apoptosis

Flow cytometry assays were used to study the effect of Bex2 on cell apoptosis, which was carried out using the Vybrant Apoptosis Assay Kit #2 (Invitrogen). After being transfected with three sets of Bex2-siRNA duplexes, the cells were harvested and stained with PI and Annexin V according to the manufacturer's protocol. In each sample, 1×10^6 cells were assayed on a FACSCalibur (Becton–Dickinson) and analyzed by CellQuest Pro software (Becton–Dickinson).

2.8. Western blot analysis

At the designated time, the cells were lysed and equal amounts of protein lysates were subjected to 10% SDS–PAGE, transferred to a PVDF membrane (Millipore), and probed with primary antibodies (p-JNK, JNK, p-c-Jun, c-Jun, Bex2, GFP, cleaved caspase-3, cleaved caspase-9, caspase-3 or β-actin) and secondary antibodies. Bound antibodies were detected by the Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific Inc.) and exposed to X-ray films. Band densities were quantified by Image-Pro Plus Software (Media Cybernetics, Inc.). The relative amount of proteins was determined by normalizing the densitometry value of interest to that of the internal loading control.

2.9. Immunocytochemistry (ICC)

Forty-eight hours after Bex2 over-expression, cells were fixed with 4% paraformaldehyde and blocked with 10% goat serum (Gibco). The cells were incubated with mouse monoclonal anti-Bex2, anti-GFP or PBS (no-IgG, blank control) followed by HRP-conjugated goat anti-mouse IgG (Invitrogen). Then, the cells were incubated with peroxidase substrate 3, 3′-diaminobenzidine (DAB) chromogenic reagent (Zhongshan Goldenbridge Biotech CO.). The coverslips were counterstained with hematoxylin to stain the nucleus and dehydrated by incubation in increasing concentrations of alcohol, followed by 100% xylene, before coverslips were mounted onto the slides with neutral gum. The cells were observed under an Olympus IX-71 inverted microscope (Olympus), and images were taken.

2.10. Statistical analysis

Data are presented as the mean ± SEM. Statistical significance was determined using Student's *t*-test, with $P < 0.05$ considered significant.

3. Results

3.1. Bex2 expression in human glioma tissues and the effects on glioma cell proliferation

To study the possible role of Bex2 in the development of human glioma, we first examined its protein level in clinical glioma samples. As is shown in (Fig. 1A and B), the protein level of Bex2 in glioma samples was approximately 2.73-fold higher than in

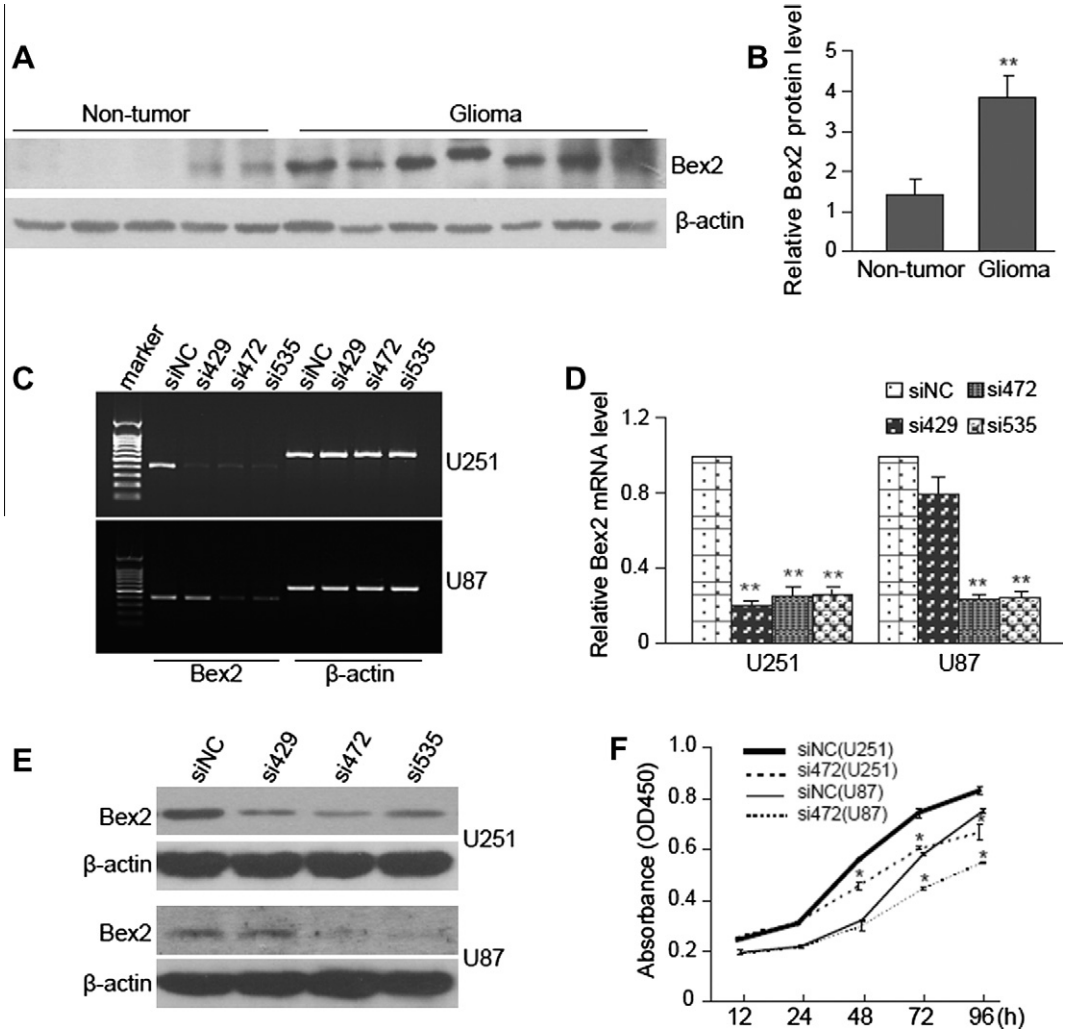

Fig. 1. Bex2 expression in human glioma tissues and the effect of Bex2 down-regulation on cell proliferation. (A) Representative immunoblots of the total extracts isolated from human glioma (seven samples shown) specimens and human non-tumor (five samples shown) brain. (B) Quantification of Bex2 protein levels in glioma tissues ($n = 33$) and non-tumor brain tissues ($n = 17$) from the immunoblots. $^{**}P < 0.01$ vs non-tumor. (C) Representative RT-PCR analysis of total RNA extracted from U251 and U87 glioma cells transfected with Bex2 si429, si472 and si535 or nontargeting siRNA (siNC). (D) Quantitative analysis of the relative mRNA levels of Bex2 normalized to those of β-actin. Data are represented as the mean ± SEM of three independent experiments. $^{**}P < 0.01$ vs siNC. (E) Representative Western blot analysis of total cell lysates isolated from U251 and U87 glioma cells transfected with indicated siRNAs. (F) Bex2 downregulation inhibited human U251 and U87 cell proliferation measured by the CCK-8 assay at the indicated time. The results are shown as the mean ± SEM of three independent experiments in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$ vs siNC.

non-tumor tissues. These results suggest that Bex2 expression is up-regulated in human glioma, which provided us the initial evidence that Bex2 may play a role in the development of human glioma.

Because the Bex2 level was markedly increased in glioma tissues, we used the RNA interference approach to down-regulate Bex2 expression and to observe its effect on cell proliferation. Three sets of siRNA duplexes (si429, si472, si535) were screened for their efficacy in suppressing Bex2 expression, and nontargeting siRNA (siNC) was used as a control. We found that Bex2 was effectively knocked down 70–80% by all three sets of siRNA duplexes, but Bex2-si429 had no obvious effect in U87 cells (Fig. 1C and D). Thus, Bex2-si429 in U87 cells was a good negative control. The suppression efficacy of the siRNAs was further confirmed using Western blot (Fig. 1E). Because si472 showed the highest down-regulation efficacy among the three siRNA duplexes, we used si472 to examine the effect of Bex2 down-regulation on cell growth. As shown in (Fig. 1F), the cell numbers were reduced by 19.1% after Bex2 siRNA transfection for 48 h in U251 and 27.6% after transfection for 72 h in U87 cells, ($P < 0.05$). This result indicates that Bex2 may play an important role in glioma cell growth.

Furthermore, we tested the effect of Bex2 on cell growth by over-expressing Bex2. First, we transfected pEGFP-N1-Bex2 and examined its expression level using immunocytochemistry and Western blot. As shown in Fig. 2A, compared with the pEGFP-N1 vector and U251 untreated groups, there was significant positive signal for both the GFP and Bex2 antibodies after pEGFP-N1-Bex2 transfection. PBS was used as a no-IgG control. Additionally, the exogenous Bex2 (GFP-Bex2 fusion protein, 48 KD) had high activity by Western blot analysis with the GFP and Bex2 antibodies (Fig. 2B). After transfection for 72 h, the reproductive activity was detected by a CCK-8 assay. We found the absorbance at the 450 nm wavelength (OD450) for the pEGFP-N1-Bex2 vector group was approximately 1.49-fold higher than that of pEGFP-N1 vector group ($P < 0.05$, Fig. 2C), suggesting that Bex2 is indeed important for cell proliferation.

3.2. The effects of Bex2 on cell apoptosis

Because proliferation and apoptosis are tightly linked, we then examined the effect of Bex2 on apoptosis using an RNA interference approach combined with flow cytometry analysis.

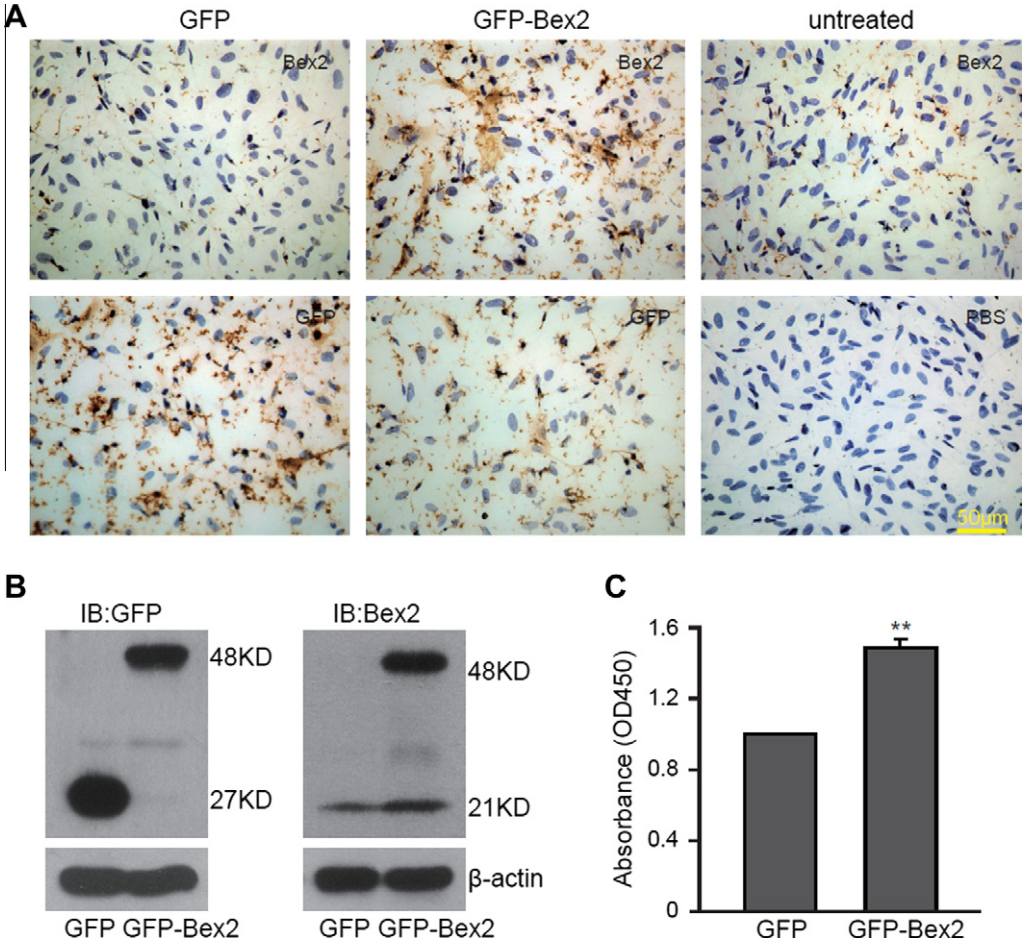

Fig. 2. The effect of Bex2 over-expression on cell proliferation. (A) Immunocytochemistry staining of Bex2 using Bex2 and GFP antibodies, respectively after Bex2 over-expression. Scale bar = 50 μm. (B) Western blot analysis of Bex2 over-expression efficiency using Bex2 and GFP antibodies, respectively. (C) Bex2 over-expression promotes cell proliferation as detected by the CCK-8 assay. Data are represented as the mean ± SEM of three independent experiments performed in triplicate. $^{*}P < 0.05$ vs GFP group.

Forty-eight hours after Bex2 specific siRNA transfection, the average apoptosis rate for the three sets of siRNAs were 10.24%, 10.19%, and 11.51% compared with siNC (6.27%) in U251 cells. The average apoptosis rates in U87 cells were 6.74%, 9.90% and 10.04%, respectively, compared with siNC (6.15%) (Fig. 3A). A quantitative analysis of the percentage of apoptotic cells in the early stage was normalized to that of the siNC group ($P < 0.01$, Fig. 3B). In line with its non-knockdown effect on the Bex2 level (Fig. 1C and D), si429 did not increase the apoptosis of U87 cells.

In addition, we investigated the effect of Bex2 on the activation of caspase3 and caspase9. We found that the levels of cleaved-caspase3 and cleaved-caspase9, the activated forms of caspase3 and caspase9, significantly increased after Bex2 down-regulation compared with the siNC group. Meanwhile, the level of total caspase3 decreased in both cell lines (Fig. 3C–E). These data sufficiently prove that Bex2 plays an important role in modulating glioma cell apoptosis in vitro.

3.3. The effect of Bex2 down-regulation on JNK signaling activity

Next, we asked how Bex2 regulates apoptosis of glioma cells. Naderi et al. reported that in breast cancer down-regulation of Bex2 inhibits JNK/c-Jun activation and promotes cell apoptosis [15]. The JNK family, which includes JNK1, JNK2, and JNK3, plays a critical role in apoptotic pathways. In response to specific stimuli, JNKs were activated through phosphorylation by JNK-activating kinase [19]. JNKs in turn activate apoptotic signaling through the transactivation of specific transcription factors, such as c-Jun. Thus, we assessed the levels of activated JNK and c-Jun (phospho-JNK, phospho-c-Jun) after Bex2 down-regulation. We observed a significant increase in the JNK1/2 and c-Jun phosphorylation following Bex2 down-regulation in both cell lines, but Bex2-si429 had no obvious effect in U87 cells (Fig. 4A).

To determine whether the JNK pathway is downstream of Bex2 in affecting cell apoptosis, we pretreated the cells with the selective JNK inhibitor SP600125 to examine whether it reverses the apoptosis we observed with Bex2 down-regulation. SP600125 is a cell-permeable compound and inhibits the phosphorylation of c-Jun in a dose-dependent manner [20]. Forty-eight hours after pretreatment, the activation effects of JNK and c-Jun, which were previously induced by Bex2 down-regulation, were eliminated (Fig. 4B). Furthermore, the pro-apoptotic effect of Bex2 down-regulation disappeared after SP600125 pretreatment (Fig. 4C and D), indicating JNK is downstream of Bex2. All these data suggest that Bex2 regulates the apoptosis of glioma cells via the c-Jun NH2-terminal kinase pathway.

4. Discussion

In this study, we show that Bex2 contributes to glioma development by regulating the c-Jun NH2-terminal kinase pathway. Several lines of evidence support this conclusion. First, we observed that Bex2 is highly expressed in human glioma. Second, down-regulation of Bex2 suppressed glioma growth but Bex2

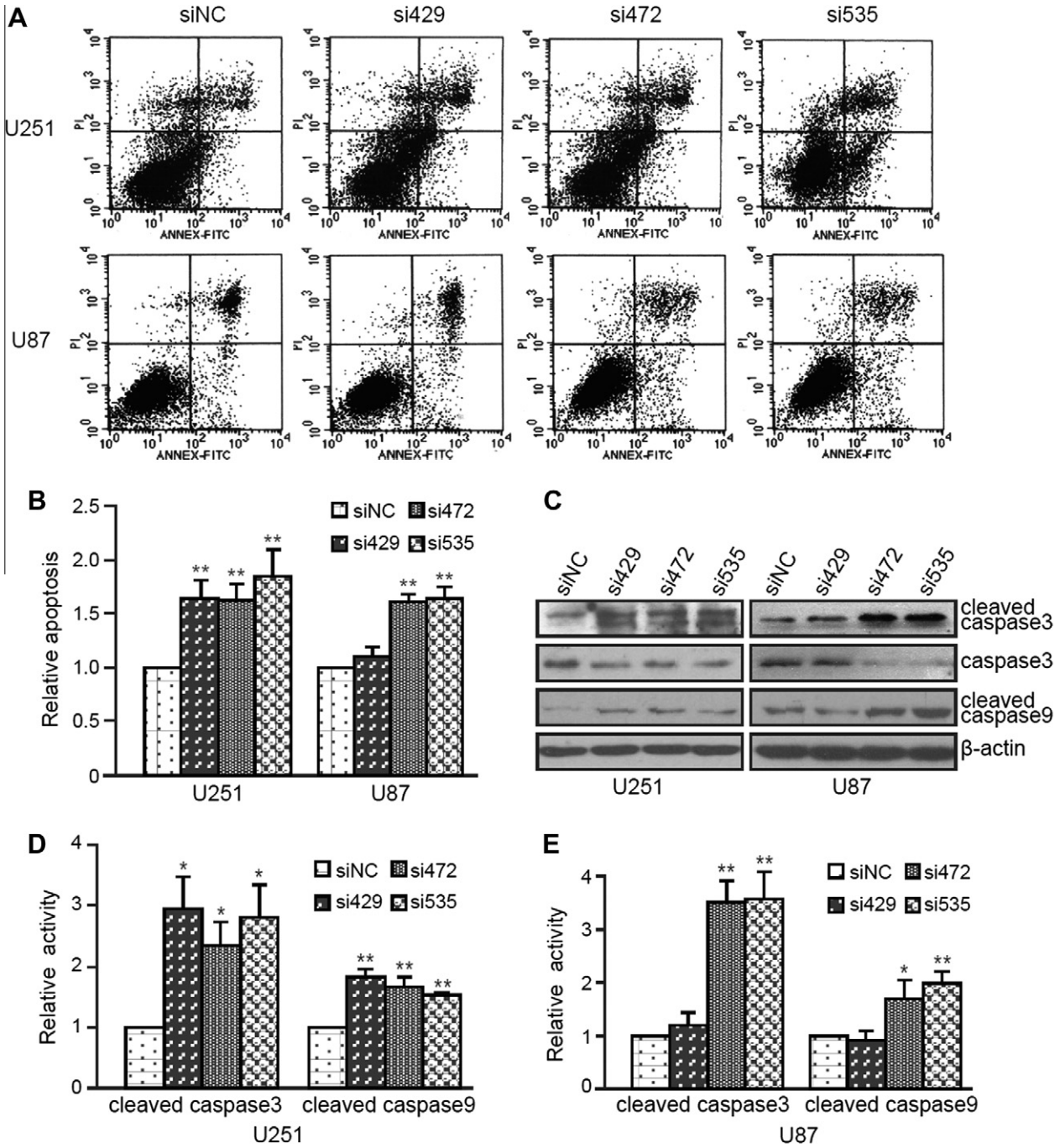

Fig. 3. The effect of Bex2 down-regulation on cell apoptosis. (A) Representative flow cytometry analysis of cell apoptosis co-stained with Annexin V/PI after Bex2 down-regulation for 48 h in U251 and U87 cells. Control cells were transfected with siNC. The lower right quadrant in the dot plot represents early stage apoptotic cells. (B) Quantitative analysis of the percentage of apoptotic cells in the early stage normalized to that of the siNC group. (C) Bex2 knockdown activated caspase3 and caspase9. Representative Western blot analysis of total cell lysates isolated from U251 cells transfected with Bex2 siRNA using the indicated antibodies. (D and E) Quantitative analysis of the relative protein levels of cleaved caspase3 and cleaved caspase9 normalized to those of β-actin. Data are given as the mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs siNC.

over-expression promoted it. Third, down-regulation of Bex2 induced apoptosis and promoted phosphorylation of JNK and its downstream target, c-Jun. Finally, the JNK inhibitor SP600125 abolished JNK/c-Jun activation and the pro-apoptosis effect on glioma cells that had previously been induced by Bex2 down-regulation.

A previous study showed that human Bex2 has high expression in the pituitary, cerebellum, and temporal lobe, but has low expression in cancer cell lines [21]. Several studies have reported that Bex2 and other Bex family members, Bex1 and TCEAL7, are silenced by promoter hypermethylation in malignant glioma [16] and ovarian cancer [22], supporting the role of Bex2 as a tumor suppressor. However, Naderi et al. reported that Bex2 is highly expressed in a subset of primary breast cancers and inhibits cell apoptosis, and the authors could not detect any correlation between Bex2 methylation and expression [12]. Le Mercier et al. also did not detect Bex2 promoter methylation in Hs683 oligodendroglioma cells [23].

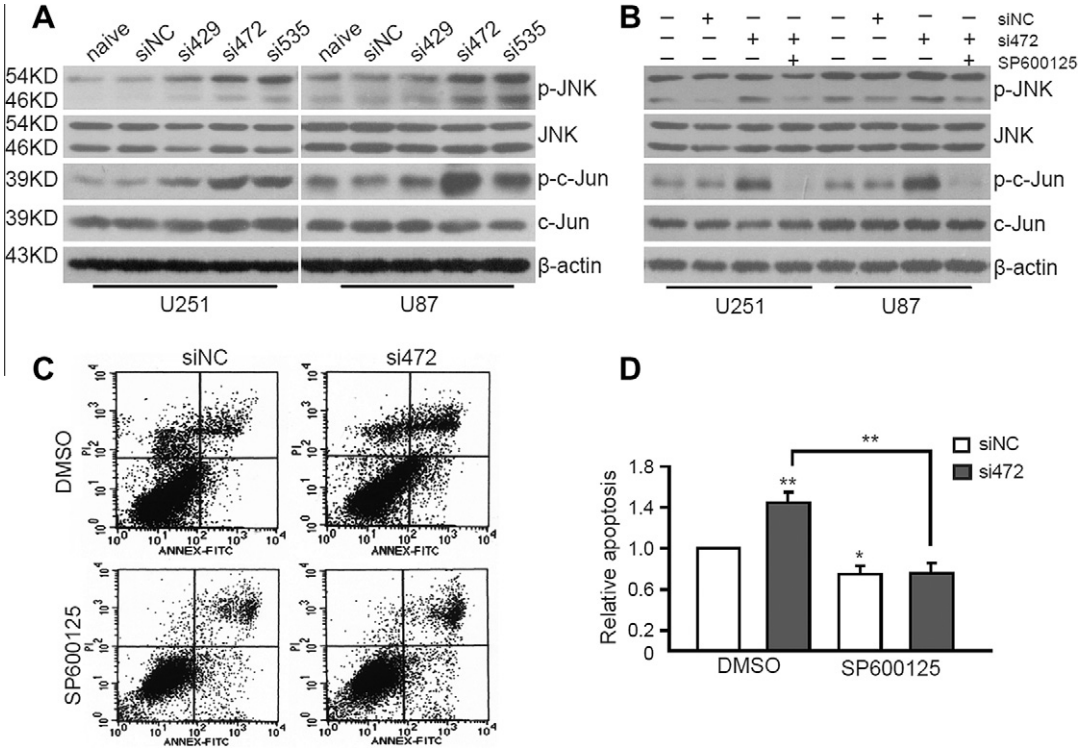

Fig. 4. The involvement of JNK and c-Jun in Bex2 down-regulation induced cell apoptosis. (A) Representative Western blot analysis showing that Bex2 knockdown activates JNK and c-Jun. (B) Representative Western blot analysis showing that SP600125 treatment eliminates Bex2 down-regulation induced JNK/c-Jun activation. (C) Representative flow cytometry analysis of cell apoptosis after Bex2 si472 transfection with or without SP600125 pretreatment in U251 cells. (D) Quantitative analysis of the percentage of apoptotic cells. Data are represented as the mean ± SEM of three independent experiments in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$.

Although Bex2 has been detected at nucleotide level by several studies in glioma [15,16], the protein level of Bex2, which is more closely related to its function in cells, has not previously been examined. In this study, we first detected the expression of Bex2 in surgical specimens of glioma. We found the level of Bex2 in glioma tissues is higher than in normal brain tissues (Fig. 1A and B). Malignant glioma often has increased cell proliferation, reduced apoptosis, infinite abnormal cell cycle regulation and significant angiogenesis [24]. It has been proposed that a tumor develops when the balance between cell growth and cell death is destroyed [25]. In our study, we found that Bex2 down-regulation inhibits cell proliferation (Fig. 1F) and over-expression of Bex2 results in the opposite result (Fig. 2C). In addition, Bex2 down-regulation significantly increased the apoptosis rate (Fig. 3A and B) and the levels of cleaved caspase 9 and cleaved caspase 3, the apoptosis activator and effector [25,26] (Fig. 3C–E). Thus, Bex2 plays a great role in cell proliferation and apoptosis, which is consistent with Naderi's research on breast cancer [13,15].

It has been widely reported that JNKs, a member of mitogen-activated protein kinases (MAPKs) family [27], participate in the process of cell proliferation, apoptosis and DNA damage repair in the cell life cycle and other processes [28,29]. JNK signals activated by acute stimuli led to cell growth, while those activated by chronic stimuli caused cell apoptosis [19,30]. Studies show that after being activated by chronic stimuli, phospho-JNK moves from the cytoplasm to the nucleus and then activates transcription factors, such as c-Jun, c-Fos, and Elk-1, through phosphorylation. The phosphorylation can adjust the expression of the downstream genes, such as FasL and TNF, which are related to apoptosis [31], and then induce the death receptor pathway of apoptosis. It can also raise the expression of Bim and Bid, which can activate Bax, promoting apoptosis through the mitochondrial pathway [32].

Naderi reported that Bex2 down-regulation decreases the c-Jun phosphorylation in breast cancer cells [15]. However, in our system, we found Bex2 down-regulation, which can act as a type of chronic stimulus, promotes JNK and c-Jun phosphorylation (Fig. 4A); this is in contrast to the report by Naderi [15], but it is consistent with the reports that JNK signaling promotes cell apoptosis after chronic stimuli [19,30]. We think this disagreement between studies may be caused by our use of different cell types or experiment systems (72 h after siRNA transfection in breast cancer cells vs 48 h after siRNA transfection in glioma cells). Additionally, pretreatment with the specific JNK inhibitor SP600125 effectively eliminated the JNK and c-Jun activation as well as reversed the increase in the cell apoptosis rate that was otherwise induced by Bex2 down-regulation (Fig. 4B–D). These results suggest that the JNK signaling pathway is required in cell apoptosis induced by Bex2 down-regulation.

In summary, we found that Bex2 regulates glioma cell proliferation and apoptosis through inhibiting the JNK signaling pathway. Our study is the first to describe the Bex2 pro-survival function and mechanism in glioma development, suggesting Bex2 may be a potential therapeutic target for human glioma. However, we did not investigate whether Bex2 is really important for glioma development in vivo due to limitations set by the animal center. Answering the above question will provide a better understanding of how Bex2 influences glioma development.

Conflict of interests

The authors declare that they have no competing interests.

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