



## Bradykinin B1 receptor contributes to interleukin-8 production and glioblastoma migration through interaction of STAT3 and SP-1



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### HIGHLIGHTS

- Bradykinin mediates IL-8 expression and GBM migration through the B1 receptor.
- Bradykinin transcriptionally enhances IL-8 expression via the FAK/STAT3 and SP-1 pathways.
- Phosphorylated STAT3 and acetylated SP-1 modulate bradykinin-induced IL-8 expression and GBM migration.

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### ABSTRACT

Glioblastoma (GBM), the most aggressive brain tumor, has a poor prognosis due to the ease of migration to surrounding healthy brain tissue. Recent studies have shown that bradykinin receptors are involved in the progression of various cancers. However, the molecular mechanism and pathological role of bradykinin receptors remains unclear. We observed the expressions of two major bradykinin receptors, B1R and B2R, in two different human GBM cell lines, U87 and GBM8901. Cytokine array analysis showed that bradykinin increases the production of interleukin (IL)-8 in GBM via B1R. Higher B1R levels correlate with IL-8 expression in U87 and GBM8901. We observed increased levels of phosphorylated STAT3 and SP-1 in the nucleus as well. Using chromatin immunoprecipitation assay, we found that STAT3 and SP-1 mediate IL-8 expression, which gets abrogated by the inhibition of FAK and STAT3. We further demonstrated that IL-8 expression and cell migration are also regulated by the SP-1. In addition, expression levels of STAT3 and SP-1 positively correlate with clinicopathological grades of gliomas. Interestingly, our results found that inhibition of HDAC increases IL-8 expression. Moreover, stimulation with bradykinin caused increases in acetylated SP-1 and p300 complex formation, which are abrogated by inhibition of FAK and STAT3. Meanwhile, knockdown of SP-1 and p300 decreased the augmentation of bradykinin-induced IL-8 expression. These results indicate that bradykinin-induced IL-8 expression is dependent on B1R which causes phosphorylated STAT3 and acetylated SP-1 to translocate to the nucleus, hence resulting in GBM migration.

### 1. Introduction

Glioblastoma multiforme (GBM) is one of the most deadly brain

tumors and their biological features make successful treatment difficult (Huang et al., 2010; Molina et al., 2010). The most significant obstacles to its treatment are the aggressive local invasion and migration of the

**Abbreviations:** GBM, glioblastoma; BK, bradykinin; STAT3, signal transducer and activator of transcription 3; SP-1, specific protein-1; IL-8, interleukin-8; B1R, bradykinin B1 receptor; B2R, bradykinin B2 receptor; HDAC, histone deacetylase; FAK, focal adhesion kinase; BBB, blood-brain barrier

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malignant cells from the original tumor. Chemotherapy and radiotherapy, stand-alone or in combination, have only produced a moderate increase in median survival owing to the difficulty in effectively targeting invading cells, which in addition show inherent resistance to these therapies. Despite research advances, over 90% of GBM patients succumb to their disease. While their median survival time ranges from 12 to 15 months, most patients die within 2 years following diagnosis (Clarke et al., 2010; Stupp et al., 2005). Effective treatment is urgently needed with a better understanding of the signaling pathways that stimulate GBM migration as well as the identification and specific targeting of the crucial effectors.

Bradykinin (BK), considered as a neurogenic inflammatory mediator in the brain may increase the blood-brain barrier (BBB) permeability (Abbott, 2000; Lehmberg et al., 2003). Accumulating evidence has shown that bradykinin increases astrocyte and microglia cell migration (Hsieh et al., 2008; Ifuku et al., 2007). Other reports have also shown that bradykinin is implicated in colon and gastric cancer tumorigenesis, wherein it enhances cell proliferation, migration, invasion, and tumor growth (Wang et al., 2014, 2017). Levels of bradykinins are elevated under pathophysiological conditions that correlate with tumor progression and inflammation (Ratajczak et al., 2006). There are two types of bradykinin receptors, namely bradykinin B1 (B1R) and B2 receptors (B2R). B2R is constitutively expressed and responsible for physiological responses while B1R is induced under pathological conditions (Leeb-Lundberg et al., 2005). It has also been reported that B1R is highly expressed during tissue injury or stroke, or upon the stimulation of inflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$  (Marceau et al., 1998). Moreover, gliomas are surrounded by an inflammatory zone, which is required for the sustenance of tumor progression (Badie and Schartner, 2000; da Fonseca and Badie, 2013), and also triggers the expression and activation of B1R in the microcirculatory environment of the brain tumor. On the other hand, it has been observed that the activity of selective B2R agonists through B2R increases the permeability of the BBB in a brain tumor animal model (Cote et al., 2010). In addition, several studies have reported that B1R is highly expressed in various cancers, including colon, bladder, chondrosarcoma, breast, gastric, renal and lung cancers (da Costa et al., 2014). Our previous reports have also shown that bradykinin-induced cyclooxygenase (COX)-2 expression and cell migration is mediated by B1R in GBM (Lu et al., 2010). In the present study, we further investigated in detail the role of B1R in the bradykinin signaling mediated cell migration of GBM.

Interleukin (IL)-8 is one of the CXC chemokines (CXCL8), which play multiple roles in the recruitment and activation of immune and inflammatory cells under inflammation (Moser et al., 2004). In addition, it has been shown that IL-8 is an autocrine growth factor of lung cancer, and the mitogenic activity of IL-8 in lung cancer cells (Zhu et al., 2004). Moreover, several studies have reported that IL-8 overexpression is correlated with tumorigenesis and metastasis in a variety of cancer types, such as human melanoma, pancreatic, prostate, and gastric cancer (Chen et al., 2012; Kim et al., 2001; Kitadai et al., 2000; Singh et al., 1994). Furthermore, IL-8 has been shown to increase the secretion of type IV collagenase by tumor cells, thereby leading to the invasion of the host stroma (Luca et al., 1997). Aberrant IL-8 expression occurs in response to inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  as well as cellular stresses including hypoxia and ischemia in the brain (Meeuwesen et al., 2003; Waugh and Wilson, 2008). It has also been reported that IL-8 expression is significantly correlated with the clinicopathological grades of gliomas (Zhang et al., 2015). Importantly, glioma patients with tumors presenting high IL-8 expression had a significantly poor outcome (Zhang et al., 2015).

Signal transducer and activator of transcription (STAT) 3 regulates several genes that are critical to cancer cell survival,

angiogenesis, proliferation, invasion, migration, and immune evasion (Bowman et al., 2000; Haura et al., 2005). The oncogenic role of STAT3 in gliomas is consistent with the observation while activation of STAT3 is seldom detected in normal brain (Abou-Ghazal et al., 2008; Lo et al., 2008). In gliomas, activation of STAT3 is positively associated with the pathological grade and poor prognosis (Abou-Ghazal et al., 2008; Alvarez et al., 2007). Moreover, IL-8 production in response to bradykinin stimuli is observed in various cancer cells, including neuroblastoma, liver and breast cancer. Furthermore, the inhibition of STAT3 markedly reduces bradykinin-induced IL-8 expression in liver cancer (Chan et al., 2016). Importantly, recent studies have shown that STAT3 is critical in glioma tumorigenesis and self-renewal in glioma stem-like cells (Gong et al., 2015; Xue et al., 2016). Additionally, it has been reported that phosphorylated (p)-STAT3 expression in GBM patients is correlated with the clinical outcome (Lin et al., 2014a).

In this study, we investigated how bradykinin increases GBM migration and its involvement in the underlying signaling cascade. The present study shows that bradykinin-induced phosphorylation of STAT3 and acetylation of specificity protein (SP)-1, which contributes to IL-8 expression leading to cell motility in GBM.

## 2. Materials and methods

### 2.1. Materials (reagents and antibodies)

Bradykinin, des-Arg<sup>9</sup>-[Leu<sup>8</sup>]-bradykinin (desBK), [Lys-des-Arg<sup>9</sup>]-bradykinin (LDBK), HOE140, PF-573228, Trichostatin A (TSA), and SAHA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mithramycin A (MTA) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). S3I-201 was purchased from Selleckchem (Houston, TX, USA). WP631 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies specific for FAK, STAT3, p-STAT3, SP-1, p300, and  $\beta$ -actin were purchased from Santa Cruz biotechnology. Primary antibodies specific for p-FAK, Ac-STAT3, and Ac-p65 were purchased from Cell Signaling Technology (Danvers, MA, USA). The primary antibody specific for p-SP-1 was purchased from Abcam (Cambridge, UK). The primary antibody specific for Acetyl-Lys was purchased from GeneTex (Irvine, CA, USA). The primary antibody specific for  $\alpha$ -tubulin was purchased from Sigma-Aldrich. On-Target smart pool IL-8 small interfering (si) RNA and control non-targeting siRNA were purchased from Dharmacon (Lafayette, CO, USA). The overexpressing constructs containing the coding sequence (CDS) of SP-1 (2358 bp, 98–2455) were obtained by PCR amplification of the cDNA from U251 cells as previously described (Liu et al., 2017). In brief, the PCR products were cloned into pcDNA3.1 empty vectors (Invitrogen, Carlsbad, CA, USA) between the XHOI and HindIII restriction sites.

### 2.2. Cell culture

U87 and GBM8901 human GBM cells were obtained from the Bioresource Collection and Research Center (BCRC, Taiwan). U87 was maintained in Minimum Essential Medium (Gibco, Grand Island, NY, USA) and GBM8901 was maintained in RPMI-1640 (Gibco). All cell culture medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, and incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> and 95% air. All cells used in this study were tested for mycoplasma.

### 2.3. RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from cells using TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA) and was quantified using the BioDrop

spectrophotometer (Cambridge, UK). The target mRNA levels were detected using quantitative real-time PCR. The reverse transcription (RT) reaction was performed using 2 µg of total RNA converted into cDNA using the Invitrogen RT Kit (Carlsbad, CA, USA) and amplified using the following oligonucleotide primers: IL-8: 5'-AGGTG CAGTT TTGCC AAGGA-3' and 5'-TTCT GTGTT GGCAG AGTGT-3'; B1R: 5'-TTCT ATTCC AGGTG CAAGC AG-3' and 5'-CTTTC CTATG GGATG AAGAT AT-3'; B2R: 5'-CAGCA CCTTC CTGGA TACGC TGCAT C-3' and 5'-CACCT CCCAA GACTT CTTTC GGAAG C-3'; and 36B4: 5'-AGATG CAGCA GATCC GCAT-3' and 5'-GTTCT TGCCC ATCAG CACC-3'. Quantitative real-time PCR was performed with StepOne Plus System (Applied Biosystems) using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The PCR was performed as follows: 45 cycles of 95 °C for 1 s and 60 °C for 20 s. The expression level of 36B4 was used as an internal control to normalize the expression levels of the target mRNAs. The threshold was set within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted as CT).

#### 2.4. Cell transfection

U87 was transiently transfected with smart pool siRNA against IL-8, SP-1, p300, or Control siRNA (Dharmacon, Lafayette, CO, USA) using DharmaFECT transfection reagents (Dharmacon). Either the siRNAs or the negative control were premixed with the DharmaFECT transfection reagents in a serum-free medium for 25 min and were then used to transfect the cells. After 24 h, the reagent-containing medium was replaced with fresh serum-free medium. U87 was transiently transfected with wild-type SP-1 or empty vector (pcDNA3.1) using Lipofectamine (LF)3000 (Invitrogen). Plasmid DNA and LF3000 were premixed in a serum-free medium for 5 min and were then used to transfect the cells. After 24 h of transfection, the LF3000-containing medium was replaced with fresh serum-free medium.

#### 2.5. Proteome array for measurement of cytokines and chemokines

To determine the changes in the cytokine profile of U87 and GBM8901 after bradykinin treatment, the human cytokine array (R&D Systems, Minneapolis, MN, USA) experiment was performed according to the manufacturer's instructions.

#### 2.6. Measurement of IL-8 production

Cells were seeded in 24-well culture plates ( $8 \times 10^4$  cells/well) for 24 h. Following treatment with various specific inhibitors or transfection with siRNA, they were stimulated with bradykinin for another 24 h. The medium was collected and stored at  $-80^{\circ}\text{C}$  until the assay was performed. The IL-8 level in the medium was assayed using the Human IL-8 DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

#### 2.7. Cell proliferation assays

Cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sulforhodamine B (SRB) assays. U87 and GBM8901 GBM cells were seeded at a density of  $1 \times 10^4$  cells/well in 96-well plates and incubated in a culture medium for 24 h. Then, they were treated with various concentrations of bradykinin for another 24 h.

For MTT assay, MTT (0.5 mg/ml) was added to the treated cells. After 1 h, the medium containing MTT was removed, and the cells were dissolved in dimethyl sulfoxide (DMSO) and shaken for 15 min. The absorbance was measured at a wavelength of 550 nm using a

microplate reader (Bio-Tek, Winooski, VT, USA). The absorbance indicates the enzymatic activity of mitochondria and provides information on cell viability. For SRB assay, cells were fixed with 10% trichloroacetic acid (TCA) and stained with 0.4% SRB (w/v) in 1% acetic acid at  $4^{\circ}\text{C}$  for 1 h. Unbound SRB was washed out by 1% acetic acid and SRB-bound cells were dissolved in 10 mM Trizma base. The absorbance was measured at a wavelength of 515 nm using a microplate reader (Bio-Tek). The absorbance indicates the cellular protein content and provides information on cell viability.

#### 2.8. Cell migration assay

*In vitro* migration assays were performed using Costar Transwell inserts (8-µm pore size; Costar, NY, USA) in 24-well plates as previously described (Huang et al., 2014; Lin et al., 2015) with minor modifications. Prior to the migration assay, the cells were transfected with IL-8 or control siRNA for 24 h. Approximately  $1.5 \times 10^4$  U87 and GBM8901 cells in 200 µl of serum-free medium were placed in the upper chamber and 400 µl of the same medium containing bradykinin was placed in the lower chamber. The plates were incubated for 24 h at  $37^{\circ}\text{C}$  in a 5% CO<sub>2</sub> atmosphere. The cells that migrated through the filters were fixed with 3.7% formaldehyde for 5 min, and then stained with 0.05% crystal violet for 30 min. Cells on the upper side of the filters were wiped with cotton-tipped swabs, and the cells on the underside of the filter were photographed using a digital camera mounted onto a microscope. For *in vitro* migration wound healing assay, the cells were treated with bradykinin until the indicated time points. A cell-free gap of 500 µm was generated after removing the Culture-Insert (Ibidi, München, DE, Germany). Cells with higher migration activity exhibited narrower cell-free gaps in the culture which were detected under a light microscope, and photographs were captured at 0 and 16 h using a digital camera.

#### 2.9. Western blotting

Whole-cell lysis extracts were prepared as previously described (Liu et al., 2017). In brief, the cells were lysed with radioimmunoprecipitation (RIPA) assay buffer for 30 min on ice. The supernatants were collected by centrifugation at 13,000 g for 20 min and stored at  $-20^{\circ}\text{C}$ . Protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat milk in TBST, and probed overnight with a primary antibody at  $4^{\circ}\text{C}$ . After undergoing TBST washes, the membranes were incubated with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The blots were visualized using enhanced chemiluminescence and Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY, USA). The blots were subsequently stripped by incubation in stripping buffer, and reprobed with a loading control antibody. Quantitative data were obtained using an ImageJ software.

#### 2.10. Preparation of cytosolic and nuclear extracts

Nuclear extracts were prepared as previously described (Lin et al., 2014b) with minor modifications. Briefly, cells were rinsed with cold phosphate buffered saline (PBS) and resuspended in a hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, and protease inhibitor cocktail) for 10 min on ice. The lysates were separated into cytosolic and nuclear fractions using centrifugation at 10,000 g for 2 min. The supernatants containing the cytosolic proteins were collected, and the pellets containing the nuclear fraction were resuspended in buffer C (20 mM HEPES pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 0.4 M NaCl, 25% glycerol, and protease inhibitor

cocktail) for 30 min on ice. The suspensions were centrifuged again at 13,000 g for 20 min, and the supernatants containing the nuclear proteins were collected and stored at –80 °C.

### 2.11. Chromatin immunoprecipitation (ChIP) assay

The protocol of ChIP was performed using EZ-Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (Millipore, Billerica, MA, USA), as previously described (Liu et al., 2017) using isolated nuclei from the formaldehyde-cross-linked U87 cells. Immunoprecipitation was performed using the primary antibodies, anti-SP-1 or anti-STAT3, and magnetic beads. Normal mouse IgG was used as negative control, and 1 µg of antibody was used for each reaction. The diluted chromatin was then incubated on a rotator at 4 °C for overnight, and then extracted and purified. Purified DNA was subjected to PCR amplification using the oligonucleotide primers, 5'-CCAAA TTGTG GAGCT TCAGT-3' and 5'-GCTCC GGTGG CTTTT TATAT C-3', which were used to amplify across the IL-8 promoter region. The PCR products were resolved by 2% agarose gel electrophoresis and visualized under UV light.

### 2.12. GEO glioma patients gene expression data

For this study, already available DNA microarray data were obtained from NCBI Gene Expression Omnibus (GEO, accession number [GSE4290](#)), and gene expression was re-analyzed by using the GraphPad Prism 6 software. The study cohort consisted of 180 patients from the Henry Ford Hospital with histologically confirmed gliomas of different grades: 23 non-tumors, 45 grade II (seven astrocytomas, 38 oligodendroglomas), 31 grade III (19 astrocytomas, 12 oligodendroglomas), and 81 grade IV astrocytomas (GBM) (Sun et al., 2006). The STAT3 and SP-1 gene expression values were obtained from the GSE4290 dataset to evaluate correlations with the grading of human gliomas.

### 2.13. Co-immunoprecipitation (Co-IP) assay

Co-IP was performed with whole cell lysates (500 µg) incubated with SP-1, or non-specific IgG (2 µg) antibodies using protein G Mag Sepharose Xtra (GE Healthcare, Uppsala, Sweden) and MagJET Separation Rack (Thermo Scientific, Hudson, NH, USA), according to the manufacturer's instructions.

### 2.14. Statistical analysis

Statistical analysis was done using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). Data are presented as mean ± standard error of the mean (SEM), and all experiments were performed with three biologically independent replicates. Statistical analyses between two samples was assessed by a Student's *t*-test. One-way ANOVA was used for statistical analyses among three or more independent groups. Pearson's correlation test was used to examine association between STAT3 and SP-1 expression in glioma patients. In all cases, a *p*-value < 0.05 was considered to be of statistical significance. The *n* and *p*-values are indicated in the figure legends.

## 3. Results

### 3.1. Bradykinin induces IL-8 expression through activation of B1 receptor

We first assessed the B1R and B2R expression in U87 and GBM8901 by real-time PCR. The basal expression of B1R in U87 is higher than that in GBM8901, but B2R expression in GBM8901 is moderately higher than that in U87 (Fig. 1A). Then, we applied

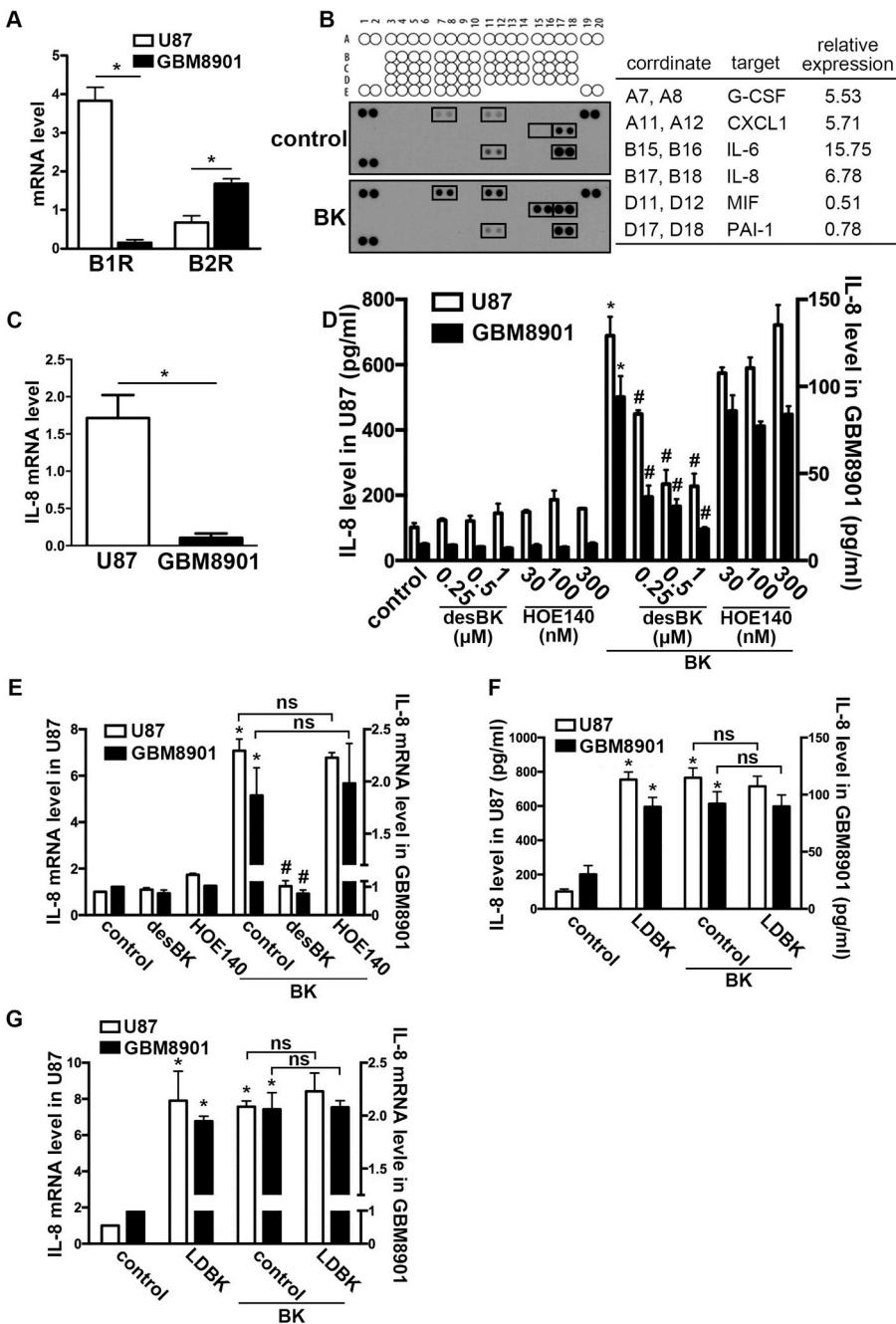
western blot-based human cytokine array analysis to identify cytokines that could be important in mediating enhanced GBM migration. We found that the most abundant cytokines and chemokines in the conditioned medium (CM) of bradykinin-treated U87 were G-CSF, CXCL1, IL-8, and IL-6 (Fig. 1B). Results show that the basal expression of IL-8 in U87 is higher than that in GBM8901 (Fig. 1C). In addition, U87 with higher B1R levels had higher IL-8 levels compared to that of GBM8901 with lower B1R levels (Fig. 1A, C). In parallel, cytokine array analysis showed that the bradykinin-induced IL-8 levels of U87 were much more than that of GBM8901 (Fig. 1B and [Supplementary Fig. 1](#)). We then examined which bradykinin receptor is predominantly involved in the bradykinin-induced IL-8 expression. As shown in Fig. 1D and E, bradykinin-induced IL-8 protein and mRNA expression were antagonized by treatment with desBK (B1R antagonist) but not by HOE140 (B2R antagonist). A previous study has reported that the presence of high doses of the B2R antagonist, HOE 140 (5 µM) reduces the number of BK-augmented glioma cells attached to blood vessels (Cuddapah et al., 2013). However, when we used a higher concentration of HOE140 (5 µM), it did not affect the bradykinin-induced IL-8 expression in U87 ([Supplementary Fig. 2](#)). In contrast, treatment with LDBK (B1R agonist) effectively increased IL-8 protein and mRNA expression in U87 and GBM8901 (Fig. 1F and G). Moreover, a combination treatment of bradykinin and LDBK did not trigger a higher IL-8 expression compared to that triggered by the stand-alone bradykinin treatment (Fig. 1F and G). These results suggest that B1R is the predominant bradykinin receptor that contributes to the bradykinin-induced IL-8 expression in GBM.

### 3.2. Bradykinin-induced IL-8 production mediates GBM migration

As shown in Fig. 2A and B, bradykinin increased the mRNA and protein expressions of IL-8 in U87 and GBM8901 in a dose-dependent manner. In addition, bradykinin treatment for varying durations also induced IL-8 expression in a time-dependent manner (Fig. 2C and D). Our results also confirm a previous report (Nicoletti et al., 2014) which revealed that bradykinin enhances cell proliferation ([Supplementary Fig. 3](#)). The transwell and wound healing assays were performed to examine whether bradykinin facilitates GBM migration. The microscopic images (Fig. 3A) indicate that following incubation with bradykinin, the GBM migratory activity is significantly increased in both U87 and GBM8901 (Fig. 3B). Representative micrographs of migrating cells are shown in Fig. 3C, wherein bradykinin increased the migration of U87 and GBM8901 in a dose-dependent manner (Fig. 3D). Moreover, transfection with siRNA against IL-8 significantly repressed the bradykinin-induced IL-8 expression (Fig. 3E) and GBM migration ability (Fig. 3F and G) in U87 and GBM8901, respectively. These results indicate that bradykinin markedly enhances GBM migration through IL-8 production.

### 3.3. FAK/STAT3 and SP-1 signaling pathways are involved in IL-8 expression and GBM migration

A diagram of the STAT3, AP-1, SP-1, and NF-κB transcription factor binding sites in the human IL-8 promoter region is shown in Fig. 4A. First, we investigated whether these transcription factors are involved in inducing IL-8 expression in GBM. Treatment with bradykinin increased phosphorylation of STAT3 in a time-dependent manner, but did not activate c-Fos and c-Jun (Fig. 4B) as well as SP-1 and p65 (Fig. 4C). Hyper activation of STAT3 contributes to tumor invasiveness by matrix metalloproteinase secretion and upregulation of focal adhesion kinase (FAK) (Wei et al., 2013). FAK is a critical molecule that modulates cytoskeleton assembly and membrane extension to regulate cell movement (Mitra et al., 2005). As shown in Fig. 5A, stimulation of

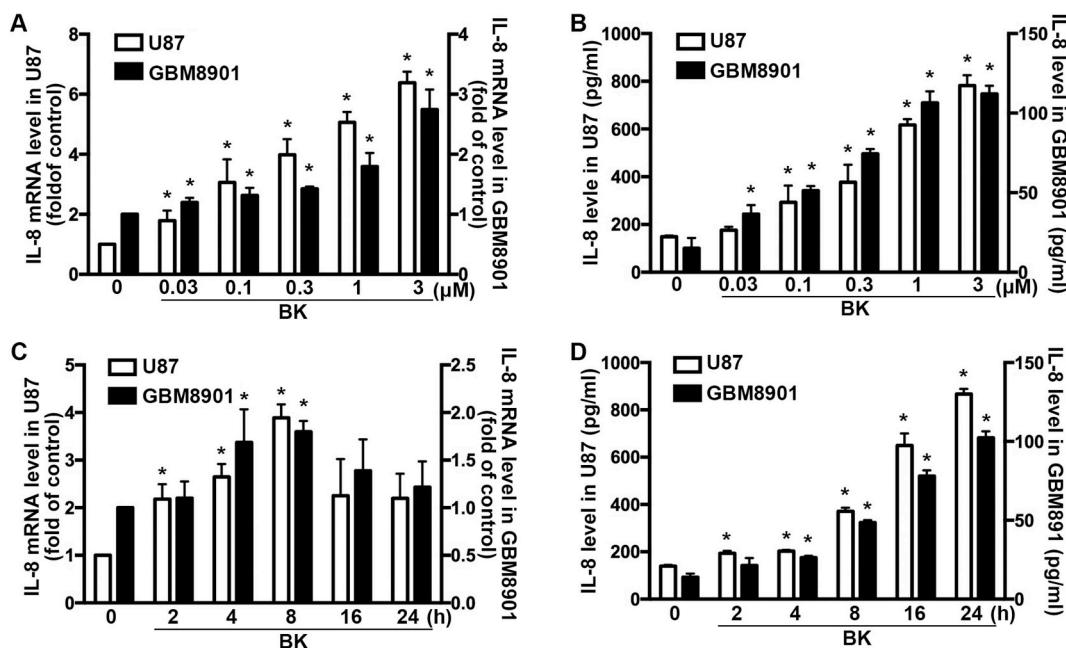


**Fig. 1.** The bradykinin B1 receptor is involved in the bradykinin-induced IL-8 expression in GBM. (A) B1R and B2R basal expression in U87 and GBM8901 were determined using real-time PCR. Expression of 36B4 was used as an internal control to normalize the mRNA expression levels. (B) U87 was treated with bradykinin (0.3 μM) for 24 h and the conditioned medium (CM) was analyzed using Human Cytokine Proteome Profiler Array. (C) IL-8 basal expression in U87 and GBM8901 were determined using real-time PCR. Expression of 36B4 was used as an internal control to normalize the mRNA expression levels. \*p < 0.05 U87 compared with the GBM8901 (Student's t-test). (D) U87 and GBM8901 were treated with desBK (0.25, 0.5, or 1 μM) or HOE140 (30, 100, or 300 nM) for 30 min and then treated with bradykinin (1 μM) for another 24 h. IL-8 production was determined using ELISA. (E) U87 and GBM8901 were treated with desBK (1 μM) or HOE140 (300 nM) for 30 min and then treated with bradykinin for another 6 h. IL-8 expression was determined using real-time PCR. U87 and GBM8901 were treated with LDBK (100 nM) for 30 min and then treated with bradykinin for another 24 h (F) or 6 h (G), and IL-8 expression was determined using ELISA (F) and real-time PCR (G), respectively. \*p < 0.05 represents comparison with the control group (Student's t-test). #p < 0.05 represents comparison with the bradykinin treatment group (Student's t-test). ns: not significant. Quantitative data are presented as mean ± SEM (representative of n = 3).

bradykinin increased phosphorylation of FAK in a time-dependent manner. Additionally, inhibition of FAK effectively antagonized bradykinin-induced phosphorylation of STAT3 (Fig. 5B, left panel). Alternatively, inhibition of STAT3 was not affected by bradykinin-induced phosphorylation of FAK (Fig. 5B, right panel). Additionally, treatment with desBK (B1R antagonist) dramatically decreased phosphorylation of FAK and STAT3 (Fig. 5C). Furthermore, inhibiting SP-1 by either WP631 or MTA did not affect bradykinin-induced phosphorylation of FAK and STAT3 (Fig. 5D). However, treatment with the FAK, STAT3, and SP-1 inhibitors reduced bradykinin-induced IL-8 protein (Fig. 5E) and mRNA expression (Fig. 5F) as well as the GBM migration activity (Fig. 5G and H). These results indicate that bradykinin-induced IL-8 expression and GBM migration are mediated by the FAK/STAT3 and SP-1 signaling pathways.

### 3.4. Activation of STAT3 and SP-1 mediate IL-8 expression and GBM migration

It has been observed that co-activation of STAT3 and SP-1 regulates gene expression with EGF treatment in hypothalamic cells (Breit et al., 2015). According to previous study and our earlier finding, we aimed to further investigate the interaction between STAT3 and SP-1 in modulation of the bradykinin stimulated in GBM. As shown in Fig. 6A and B, treatment with bradykinin resulted in translocation of p-STAT3 and SP-1 from the cytoplasm to the nucleus. Moreover, the bradykinin-mediated binding of STAT3 to the STAT3 binding site on the IL-8 promoter was repressed by S3I-201 treatment (Fig. 6C, left panel). Furthermore, bradykinin also mediated the binding of SP-1 to the SP-1 binding site on the IL-8 promoter (Fig. 6C, right panel). The bradykinin-mediated SP-1



**Fig. 2.** Bradykinin induces IL-8 expression in GBM. U87 and GBM8901 were treated with various concentrations (0.03, 0.1, 0.3, 1, or 3 μM) of bradykinin for 6 h (A) or 24 h (B), and IL-8 expression was determined using real-time PCR (A) and ELISA (B), respectively;  $p < 0.05$  by one-way ANOVA for IL-8 expression. U87 and GBM8901 were treated with bradykinin (1 μM) for indicated time periods (2, 4, 8, 16, or 24 h), and IL-8 expression was determined using real-time PCR (C) and ELISA (D), respectively;  $p < 0.05$  by one-way ANOVA for IL-8 expression. \* $p < 0.05$  represents comparison with the control group (Student's *t*-test). Quantitative data are presented as mean ± SEM (representative of  $n = 3$ ).

binding on the IL-8 promoter was repressed by PF-573228 and S3I-201 treatment (Fig. 6C, right panel). In addition, transfection with wild-type SP-1 increased IL-8 mRNA levels (Fig. 6D). Importantly, IL-8 expression (Fig. 6D) and GBM migration ability (Fig. 6E and F) were much higher with a combination treatment of SP-1 overexpression and bradykinin than those with either SP-1 overexpression or bradykinin stand-alone treatments. In addition, analysis of the GSE4290 dataset showed that levels of STAT3 (Fig. 6G) and SP-1 (Fig. 6H) are higher in the astrocytoma group (grade II to grade IV) than those in the non-tumor group. Pearson's correlation analysis also showed a positive correlation between STAT3 and SP-1 expression levels in the gene expression dataset of patients with glioma (Fig. 6I). These results suggest that bradykinin modulates IL-8 expression and GBM migration through the activation of STAT3 and SP-1, and that the higher expression levels of STAT3 and SP-1 are positively correlated with the clinicopathological grades of gliomas.

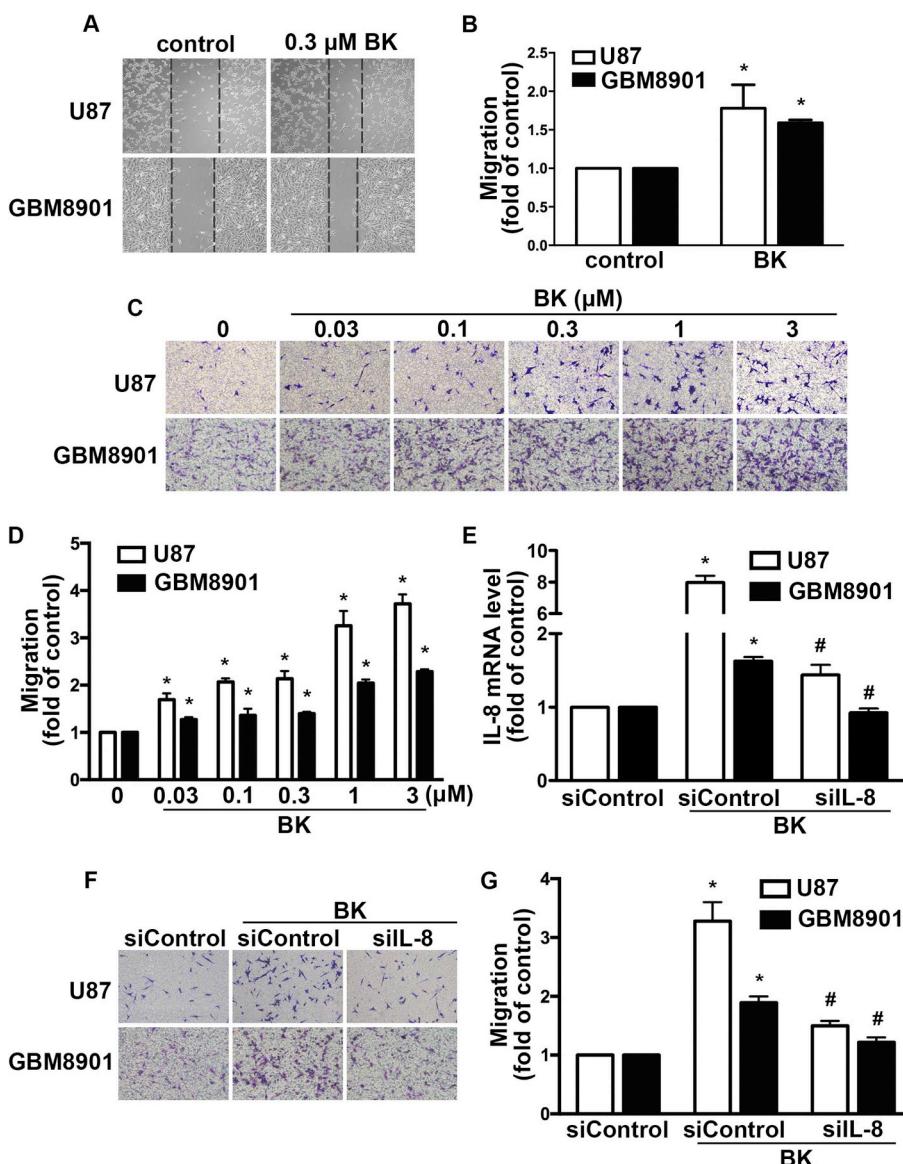
### 3.5. Acetylated SP-1 modulates bradykinin-induced IL-8 expression in GBM

The above results indicate that post-translational modification of SP-1 is involved in the bradykinin-induced IL-8 expression and GBM migration. Previous report has reported that the inhibition of histone deacetylase (HDAC) activity by the HDAC inhibitor, TSA increases IL-8 expression in breast cancer (Chavey et al., 2008). As shown in Fig. 7A and B, treatment with HDAC inhibitors alone increased IL-8 protein (Fig. 7A) and mRNA expression (Fig. 7B). Interestingly, a combination of bradykinin and HDAC inhibitors did not further promote IL-8 expression in U87 (Fig. 7A and B). In addition, treatment with bradykinin increased the acetylation of SP-1 as well as the expression of p300 acetyltransferase in a time-dependent manner (Fig. 7C), but not affect the acetylation of STAT3 and p65 (Fig. 7 D). On the other hand, we found that treatment with TSA and SAHA also induced the acetylation of SP-1 (Fig. 7E). Importantly, both FAK and STAT3 inhibitors decreased the bradykinin-induced acetylation of SP-1 (Fig. 7F).

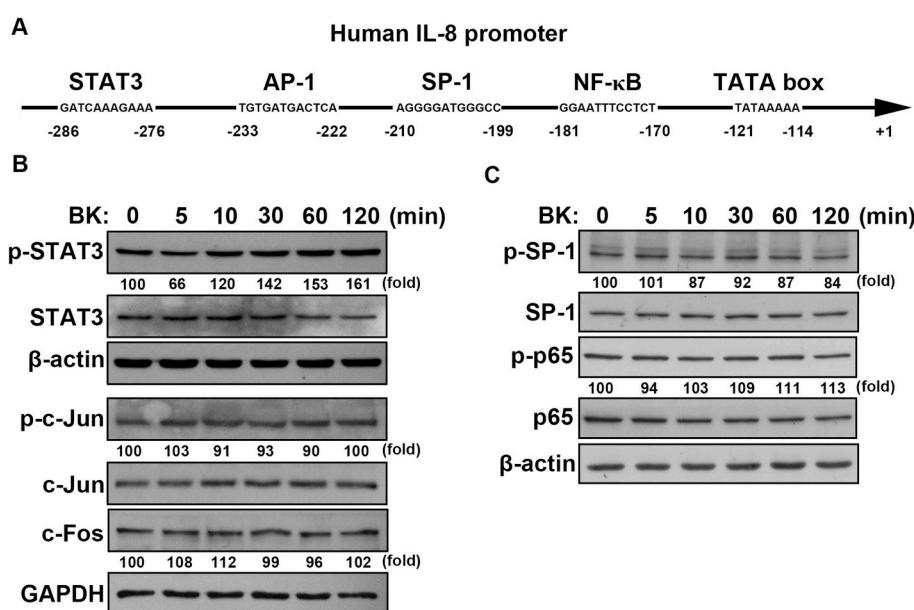
Transfection with siRNA against p300 in U87 moderately reduced the bradykinin-induced IL-8 expression (Fig. 7G). Furthermore, transfection with SP-1 siRNA decreased bradykinin-, TSA-, or SAHA-induced IL-8 expression (Fig. 7H). These data suggest that the bradykinin-induced IL-8 expression and GBM migration are regulated by the interaction of p-STAT3/Ac-SP-1.

## 4. Discussion

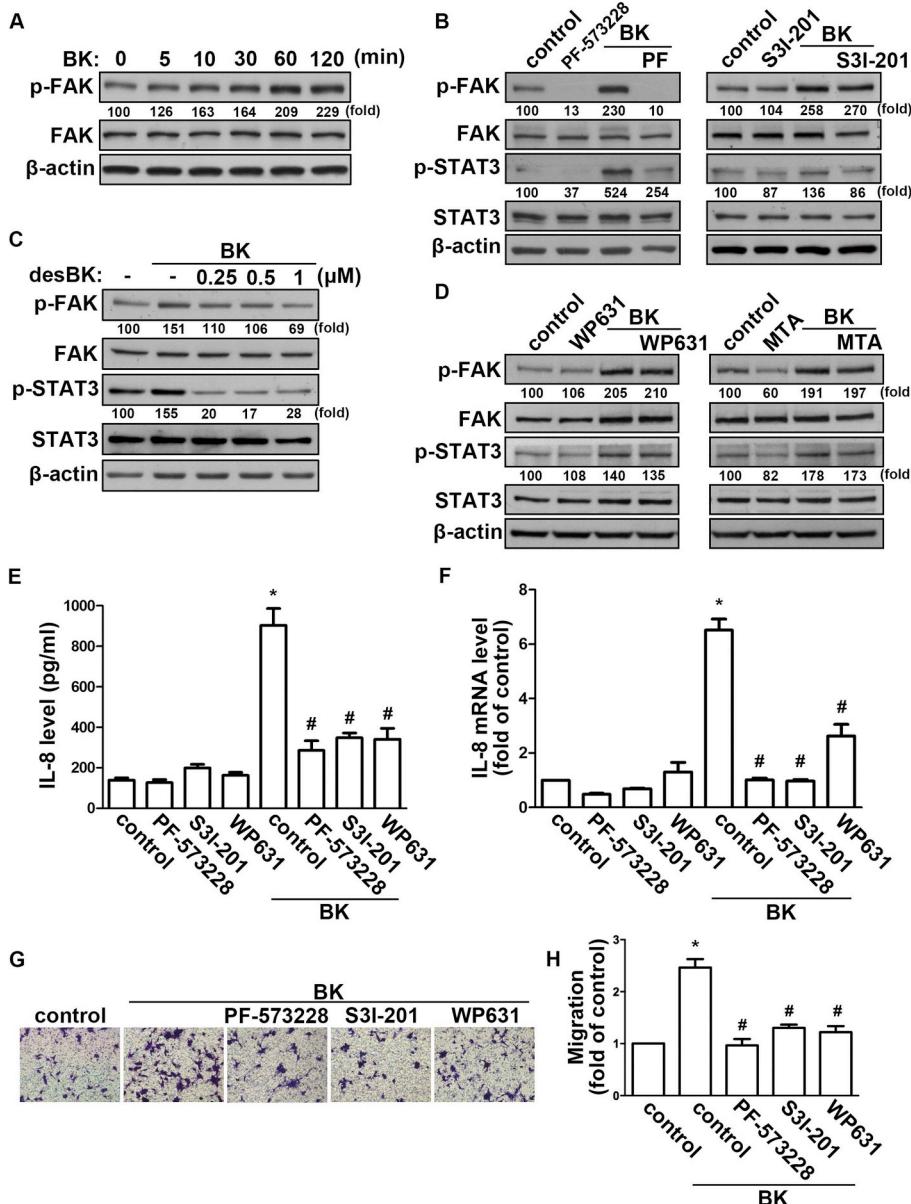
Bradykinin, a pro-inflammatory molecule, plays an important role in various inflammatory pathological conditions such as ischemia reperfusion injury and cancers (Taub et al., 2003; Wang et al., 2008b). Accumulating evidence shows that bradykinin enhances cell migration of the microglia and astrocytes in the brain (Hsieh et al., 2008; Ifuku et al., 2007). In addition, bradykinin receptors are present in all the cell types in the brain, such as microglia, neurons, oligodendrocytes, and astrocytes (Delmas et al., 2002; Ifuku et al., 2007; Stephens et al., 1993a, 1993b). It has been reported that B1R is overexpressed in the tumors of the rat glioma model and in the human glioma cell lines, including LN229, T98G, U118, U138, and U87 cells (Cote et al., 2012). Similarly, elevated levels of B1R expression has been observed in all grades of glioma patients but not in the normal brain tissues (Cote et al., 2012). In several cancer studies, it has been suggested that tumors produce bradykinin as an autocrine mediator to enhance cell proliferation, migration, and angiogenesis (da Costa et al., 2014). Moreover, bradykinin receptors have been implicated in cancer migration, invasion, and metastasis (Montana and Sontheimer, 2011; Yu et al., 2013). Finally, antagonists for bradykinin receptors have shown anti-inflammatory, anti-angiogenic, anti-proliferative, and anti-migratory properties (da Costa et al., 2014). In this study, we tested the effects of a wide range of concentrations (0.03–3 μM) of bradykinin based on the results of previous studies (Cuddapah et al., 2013; Seifert and Sontheimer, 2014; Wang et al., 2014). Our results support the findings



**Fig. 3.** Bradykinin-directed GBM migration is regulated by IL-8 expression. (A) U87 and GBM8901 were treated with bradykinin (0.3  $\mu$ M) for 16 h. The migrated cells were examined using wound healing assay and visualized using a digital camera. (B) Quantification of GBM migration by fold-change of cells that migrated into the wound area. (C) U87 and GBM8901 were treated with various concentrations (0.03, 0.1, 0.3, 1, or 3  $\mu$ M) of bradykinin and *in vitro* migration activities were measured after 24 h by the transwell assay and visualized using a digital camera. (D) Quantification of GBM migration by fold-change of cells that migrated to the underside of the filter;  $p < 0.05$  by one-way ANOVA for U87 or GBM8901. (E) U87 and GBM8901 were transfected with Control or IL-8 siRNA for 24 h and treated with bradykinin (1  $\mu$ M) for another 6 h. IL-8 expression was determined using real-time PCR. (F) U87 and GBM8901 were transfected with Control or IL-8 siRNA for 24 h and treated with bradykinin (1  $\mu$ M) and *in vitro* migration activities were measured after 24 h with transwell assay and visualized using a digital camera. (G) Quantification of GBM migration by fold-change of cells that migrated to the underside of the filter. \* $p < 0.05$  represents comparison with the control group (Student's *t*-test). # $p < 0.05$  represents comparison with the bradykinin treatment group (Student's *t*-test). Quantitative data are presented as mean  $\pm$  SEM (representative of  $n = 3$ ).



**Fig. 4.** Representation of transcription factor binding sites in the human IL-8 promoter region. (A) The binding sites of the transcription factors, STAT3, AP-1, SP-1, and NF- $\kappa$ B on the human IL-8 promoter region are indicated. U87 was treated with bradykinin (1  $\mu$ M) for indicated time periods (5, 10, 30, 60, or 120 min), and p-STAT3, p-c-Jun, c-Fos (B) p-SP-1, and p-p65 (C) expression were determined using western blot analysis.

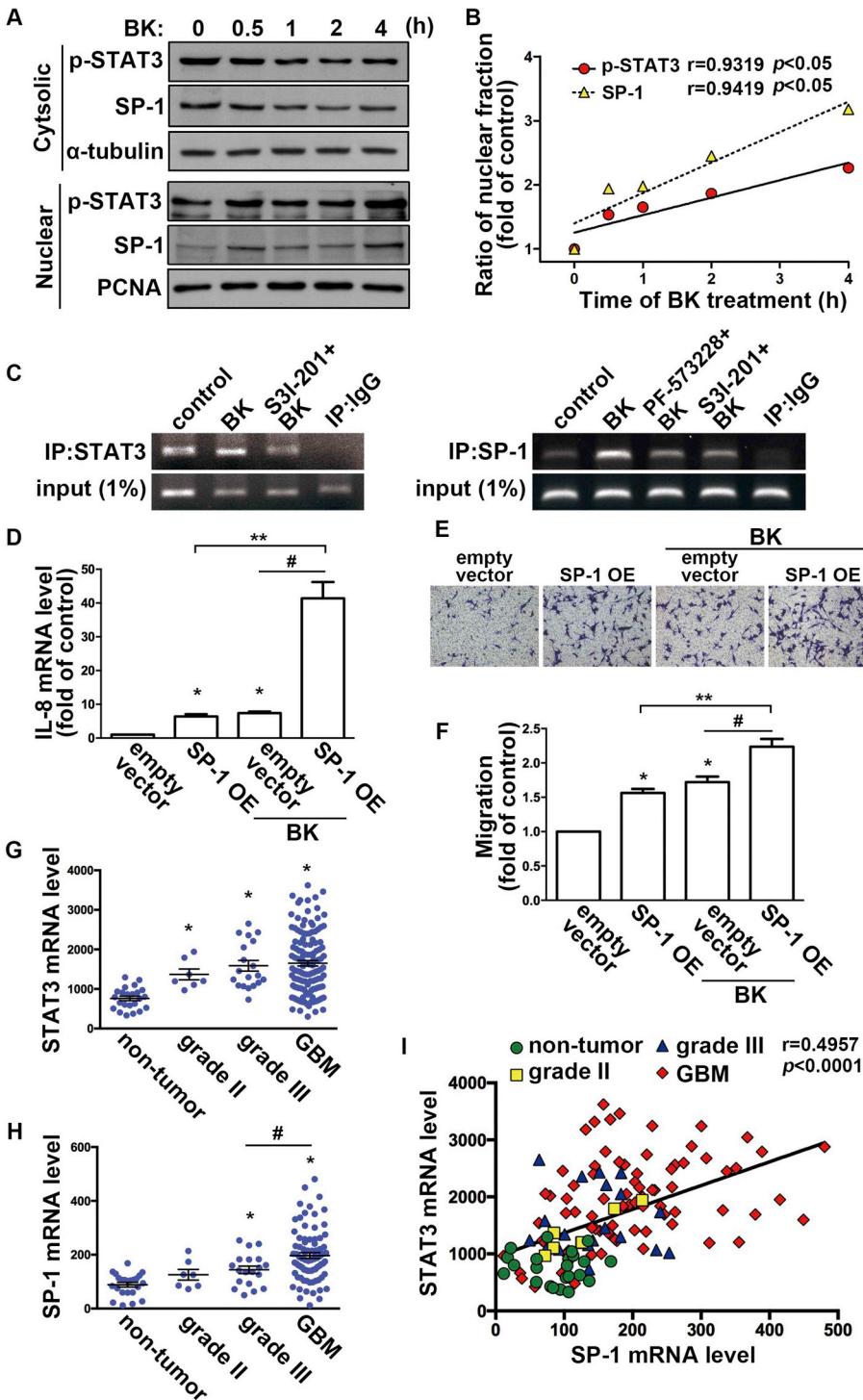


**Fig. 5.** FAK/STAT3 and SP-1 signaling pathways are involved in IL-8 expression and GBM migration. (A) U87 was treated with bradykinin (1 μM) for indicated time periods (5, 10, 30, 60, or 120 min) and p-FAK expression was determined using western blot analysis. (B) U87 was treated with PF-573228 (1 μM) or S3I-201 (30 μM) for 30 min and then treated with bradykinin (1 μM) for another 30 min p-FAK and p-STAT3 expressions were determined using western blot analysis. (C) U87 was treated with various concentrations (0.25, 0.5, or 1 μM) of desBK for 30 min and then treated with bradykinin for another 30 min p-FAK and p-STAT3 expressions were determined using western blot analysis. (D) U87 was treated with a SP-1 inhibitor (100 nM WP631, or 300 nM MTA) for 30 min and then treated with bradykinin (1 μM) for another 30 min p-FAK and p-STAT3 expressions were determined using western blot analysis. U87 was treated with PF-573228 (1 μM), S3I-201 (30 μM), or WP631 (100 nM) for 30 min and then treated with bradykinin for another 24 h (E) or 6 h (F), and IL-8 expression was determined using ELISA (E) and real-time PCR (F), respectively. (G) U87 was treated with PF-573228 (1 μM), S3I-201 (30 μM), or WP631 (100 nM) for 30 min and then treated with bradykinin. The *in vitro* migration activities were measured after 24 h by the transwell assay and visualized using a digital camera. (H) Quantification of GBM migration by fold-change of cells that migrated to the underside of the filter. \**p* < 0.05 represents comparison with the control group (Student's *t*-test). #*p* < 0.05 represents comparison with the bradykinin treatment group (Student's *t*-test). Quantitative data are presented as mean ± SEM (representative of *n* = 3).

of these studies in that, bradykinin induces IL-8 expression via bradykinin B1 receptor (B1R) and promotes GBM migration (Figs. 1 and 2). The present study also reveals that the lower IL-8 production in response to bradykinin or B1R agonists (GBM8901) may due to the low B1R expression (Fig. 1A, 1D-G).

Overexpression of IL-6 and IL-8 has been found in GBM cell lines and human tissues (Shan et al., 2015; Van Meir et al., 1992). IL-6 plays an important role in tumor migration and proliferation, and expression of IL-6 positively correlates with the progression of glioma patients (Shan et al., 2015). Our previous study showed that in GBM, bradykinin-induced COX-2 expression and cell migration is mediated by B1R (Lu et al., 2010) which might be considered to be responsible for the production of PGE2 (Park et al., 2006). In addition, PGE2 has been shown to regulate IL-6 production in macrophages and astrocytes (Chen et al., 2006; Fiebich et al., 2001). In the present study, we also found that bradykinin induced IL-6 expression from the cytokine array analysis (Fig. 1B). This reinforces the findings of our previous study as well as that of other groups in that, IL-6 production is related to the poor

prognosis of glioma and that there is an association between COX-2/PGE2 production and IL-6 synthesis. IL-8, which enhances invasiveness, is considered to be an inflammatory chemoattractant for GBM (Raychaudhuri and Vogelbaum, 2011; Wakabayashi et al., 2004). Furthermore, IL-8 is secreted by GBM cells to increase cell growth, migration, and invasion in an autocrine manner (Sun et al., 2011; Wakabayashi et al., 2004). It has been demonstrated that activated STAT3 is required to upregulate IL-8 in pancreatic adenocarcinoma cells (Trevino et al., 2006). Moreover, a study on atherosclerosis has shown STAT3 is an important molecule for regulating IL-8 expression both *in vitro* and *in vivo* (Gharavi et al., 2007). Also, administration of a STAT3 inhibitor decreases tumor vasculature and the infiltration of macrophages (de Groot et al., 2012). A recent study of ours has also revealed that STAT3 regulates adhesion molecule expression and monocyte adhesion (Liu et al., 2017). The present study supports the findings of these previous studies in that, STAT3 phosphorylation (Figs. 4 and 5) and translocation to the nucleus (Figs. 5G and H, and 6A-C) regulates IL-8-associated GBM migration.

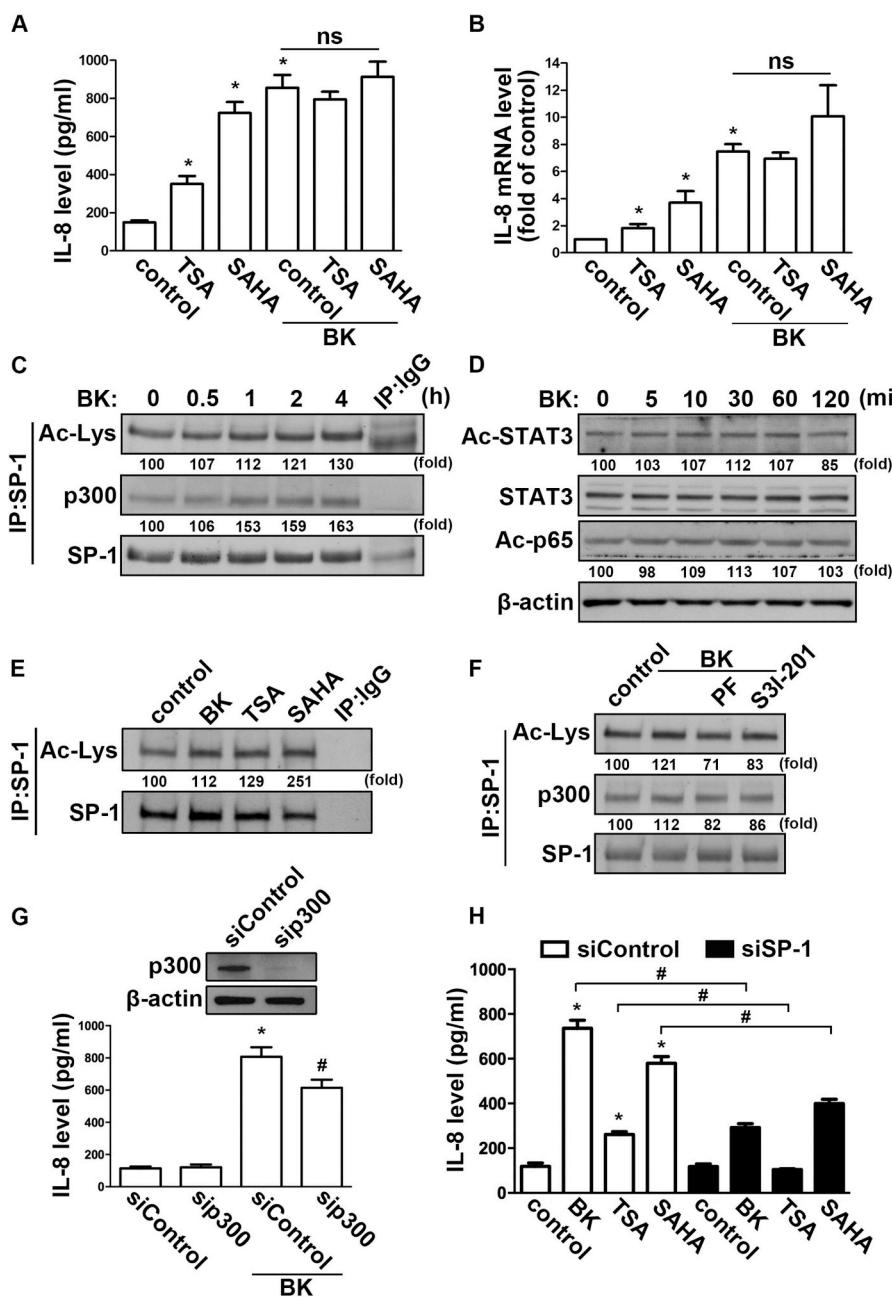


**Fig. 6.** Activation of STAT3 and SP-1 induce IL-8 expression and GBM migration. (A) The cytosolic and nuclear extracts from bradykinin-treated ( $1\ \mu\text{M}$ ; 0.5, 1, 2, or 4 h) U87 were subjected to western blotting. Expression levels of p-STAT3 and SP-1 were determined using western blot analysis. (B) Quantitative ratio of nuclear fraction. Pearson's correlation analysis between the quantitative ratio of p-STAT3 or SP-1 to time of bradykinin treatment in western blot analysis (p-STAT3:  $r = 0.9319$ ; SP-1:  $r = 0.9419$ ,  $p < 0.05$ ). (C) U87 was treated with S3I-201 ( $30\ \mu\text{M}$ ) or PF-573228 ( $1\ \mu\text{M}$ ) for 30 min and then treated with bradykinin ( $1\ \mu\text{M}$ ) for 2 h. The cells were then fixed and subjected to chromatin immunoprecipitation assay using primary antibodies to non-specific IgG, STAT3, or SP-1. Levels of immunoprecipitated chromatin fragments of the IL-8 promoter or input were examined by PCR. (D) U87 was transfected with empty vectors or wild-type SP-1 for 24 h and then treated with bradykinin ( $1\ \mu\text{M}$ ) for 6 h. The mRNA expression level of IL-8 was determined using real-time PCR. (E) U87 was transfected with empty vectors or wild-type SP-1 for 24 h and then treated with bradykinin ( $1\ \mu\text{M}$ ). The *in vitro* migration activities were measured after 24 h using the transwell assay and visualized using a digital camera. (F) Quantification of GBM migration by fold-change of cells that migrated to the underside of the filter. \* $p < 0.05$  represents comparison with the empty vector group (Student's *t*-test). # $p < 0.05$  represents comparison with the bradykinin treatment group (Student's *t*-test). \*\* $p < 0.05$  represents comparison of the SP-1 overexpression group with the combinatorial SP-1 overexpression and bradykinin treatment group (Student's *t*-test). Quantitative data are presented as mean  $\pm$  SEM (representative of  $n = 3$ ). Messenger RNA levels of STAT3 (G) and SP-1 (H) in patient specimens from human glioma microarray datasets GSE4290. \* $p < 0.05$  represents comparison with the non-tumor group (Student's *t*-test). # $p < 0.05$  represents comparison of the grade III astrocytoma group with the GBM group (Student's *t*-test). Quantitative data are presented as mean  $\pm$  SEM (representative of  $n = 3$ ). (I) Pearson's correlation analysis between STAT3 and SP-1 expression in human glioma microarray datasets GSE4290 ( $r = 0.4957$ ,  $p < 0.0001$ ).

Specific protein-1 (SP-1) is a crucial transcription factor in the regulation of multiple genes critical to tumorigenesis and cancer progression (Wang et al., 2008a; Yuan et al., 2007). Increasing evidence shows that SP-1 levels are elevated in breast and pancreatic cancers (Shi et al., 2001; Zannetti et al., 2000), and are also correlated with poor clinical outcomes. In addition, SP-1 has been reported to upregulate the downstream gene expression associated with the self-renewal capability of glioma stem cells (Gopisetty et al., 2013), and promote glioma tumorigenesis (Luo et al., 2015). It has also been found that treatment with SP-1 inhibitors effectively induces cell death and reduces cell migration in GBM (Seznec et al., 2011). Furthermore, upregulation of SP-1 in glioma cells promotes cell invasion and predicts a poor clinical

outcome (Guan et al., 2012). It has also been reported that an elevated activity of SP-1 increases IL-8 expression in endothelial cells (Florczyk et al., 2011). Our recent study has demonstrated that SP-1 translocates to the nucleus, wherein it binds with promoter region of EGFR which contributes to GBM progression (Liu et al., 2017). The present findings reinforce those of the previous studies in that, SP-1 expression is higher in patients with gliomas than in healthy brains, and levels of SP-1 significantly correlates with glioma tumor grades and is associated with the positive expression of STAT3 (Fig. 6H and I).

Collectively, our results indicate that interaction of p-STAT3 and Ac-SP-1 modulates IL-8 expression-mediated GBM migration. The present study also indicates that B1R and the p-STAT3/Ac-SP-1 signaling



**Fig. 7.** SP-1 acetylation is involved in IL-8 protein secretion and mRNA expression in GBM. U87 was treated with TSA (100 nM) or SAHA (10 μM) and then treated with bradykinin (1 μM) for another 24 h (A) or 6 h (B), and IL-8 expression was determined using ELISA (A) and real-time PCR (B), respectively. (C) U87 was treated with bradykinin (1 μM) for indicated time periods (0.5, 1, 2, or 4 h) and co-IP experiments were carried out by precipitating the whole-cell lysate (500 μg) with the SP-1 and non-specific IgG antibodies using protein G magnetic beads. Acetyl-Lys and p300 expression levels were determined using western blot analysis. Blots were striped and re-probed with the antibody used for IP to confirm the antibody specificity. (D) U87 was treated with bradykinin (1 μM) for indicated time periods (5, 10, 30, 60, or 120 min), and Ac-STAT3 and Ac-p65 expression were determined using western blot analysis. (E) U87 was treated with bradykinin (1 μM), TSA (100 nM), or SAHA (10 μM) for 4 h and co-IP experiments were carried out by precipitating the whole-cell lysate (500 μg) with the SP-1 and non-specific IgG antibodies using protein G magnetic beads. Acetyl-Lys expression were determined using western blot analysis. (F) U87 was treated with PF-573228 (1 μM) or S3I-201 (30 μM) for 30 min and then treated with bradykinin (1 μM) for another 4 h. Co-IP experiments were carried out by precipitating the whole-cell lysate (500 μg) with SP-1 antibody using protein G magnetic beads. Acetyl-Lys and p300 expression were determined using western blot analysis. (G) U87 was transfected with Control or p300 siRNA for 24 h and then treated with bradykinin (1 μM) for another 24 h. The IL-8 expression was determined using ELISA. \* $p < 0.05$  represents comparison with the control group (Student's t-test). # $p < 0.05$  represents comparison with the bradykinin treatment group (Student's t-test). ns: not significant. Quantitative data are presented as mean ± SEM (representative of  $n = 3$ ). (H) U87 was transfected with Control or SP-1 siRNA for 24 h and treated with bradykinin (1 μM), TSA (100 nM), or SAHA (10 μM) for another 24 h, and the IL-8 expression was determined using ELISA. \* $p < 0.05$  represents comparison with the control group (Student's t-test). # $p < 0.05$  represents comparison with Control siRNA group (Student's t-test). Quantitative data are presented as mean ± SEM (representative of  $n = 3$ ).

pathways could serve as therapeutic targets for developing a novel GBM therapy.

#### Author contributions

YSL and DYL conceived and designed the studies; YSL and JWH performed the plasmid constructs; YSL, HYL, and SWL performed the experiments; YSL, BRH, CFT, and DYL discussed the studies; YSL and HYL performed the data statistical analysis; YSL prepared the final figures; YSL and DYL wrote the paper; All authors read and approved the final manuscript.

#### Conflicts of interest

The authors declare no biomedical financial interests or potential conflicts of interest.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.neuropharm.2018.10.033>.

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