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Original Article

Molecular mechanisms of basic fibroblastic growth factor upregulates connexin43 expression in the human glioblastoma cells

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Abstract: Decreasing Connexin43 (Cx43) expression and dysfunction of gap junction intercellular communication (GJIC) is associated with increasing proliferation and the grade of human glioma cells. Our group has previously reported that bFGF knockdown interferes connexin43 expression, however, the mechanisms underlying this interference remain unknown. We hypothesized that the bFGF signaling pathway may activate gene Cx43, and identified the signal pathway involved in bFGF-induced Cx43 expression. In the present study, we revealed that bFGF stimulates expression of Cx43 gene and that this stimulation is time-dependent. Conversely, knocking down bFGF levels using LV-bFGF-siRNA can significantly decrease both Cx43 gene and p-Cx43 expression in glioma cell line LN229. Furthermore, presence of both AG490 (STAT3 antagonist), as well as PD0325901 (inhibitor of ERK1/2 pathway) inhibited expression of gene Cx43 via blocking the bFGF stimulation effect over Cx43. This finding suggests that bFGF-induced Cx43 expression is regulated mainly via activation of STAT3 and ERK1/2 but not AKT pathway. Further, LV-bFGF-siRNA can decrease the Cx43 expression and aggravate GJIC in LN229 glioma cell. Also, Cx43 is found within the cytoplasm and nuclear membrane of LN229 cells, unlike U251 cell lines. This subcellular distribution is decreased by LV-bFGF-siRNA. The results of our study suggest that bFGF upregulates Cx43 and p-Cx43 (Ser368) expression in LN229 cell. We have indirect evidence that this upregulation occurs via the STAT3 and ERK1/2 pathway. Our study also shows that Cx43 expression and subcellular distribution in glioma cells is highly heterogeneous.

Keywords: bFGF, connexin43, GJIC, STAT3, ERK, glioma

Introduction

Malignant glioma cells frequently lack functional gap junctions. Cx43 is the most abundant protein in glial gap junctions [1, 2]. The intracellular concentration of Cx43 plays a major role in determining the nature and behavior of glioma tumors. Evidence shows that Cx43 expression is inversely proportional to the histological tumor grade and proliferation in gliomas [3]. Several authors have reported that transfecting exogenous Cx43 to glioma cells can reverse oncogenicity and increase gap-junction communication [4], suggesting that Cx43 acts as a tumor growth suppressor in gliomas. However, Cx43 expression and its function are highly heterogeneous in tumor cells. Other functions of Cx43 include the prevention of apoptosis. Exogenous expression of Cx43 has also been related to decreased activity of caspase 3 [5]. It can enhance the migration of glioma cells out of the tumor core as well [6]. Therefore, the ambivalent effect of Cx43 in glioma tumors (i.e. tumor suppressor effect and promotion of cell migration) complicates the understanding of its role in glioma tumors.

The level of basic fibroblast growth factor (bFGF), an over-expressed growth factor in malignant glioma, correlates with glioma angiogenesis, proliferation and anti-apoptosis [7]. bFGF is secreted as a pleiotropic protein and exerts functions via its specific binding to FGFR, which thereby triggers classical cell signaling

pathways (i.e. Ras/Raf/MEK/MAPK, PI3K/AKT, JAK/STAT3), and regulates numerous transcription factors and effector molecules [8, 9]. Several studies have demonstrated the correlation between bFGF and Cx43. bFGF can inhibit GJIC function and reduce the expression of bFGF in C6 glioma cells [10]. In vitro bFGF transference increased Cx43 expression in cultured rat bladder smooth muscle cells via the ERK1/2 pathway [11]. We previously reported that adenovirus-mediated delivery of bFGF small-interfering RNA increases levels of Cx43 in glioma cell line U251 [12], suggesting that bFGF interferes Cx43 expression. At present, there is strong evidence supporting the theory that bFGF plays a major role in Cx43 in glioma tumors. However, the mechanism underlying Cx43 regulation by bFGF in glioma cell lines is not clear. Therefore, we sought to identify the signaling pathways involved in bFGF regulation of Cx43 in glioma tumors.

Materials and methods

Cell culture

The human glioblastoma cell line LN229 was cultured in Dulbecco's modified Eagle medium (DMEM, Gibcol-BRL, USA) enriched with 10% heat-inactivated fetal bovine serum (FBS, Gibcol-BRL, USA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere containing 5% CO_2 at 37 degrees.

Construction and transfection of bFGF siRNA lentivirus vectors

Small interfering RNA for bFGF, and a negative control were used (Genbank, Shanghai, China). The following siRNA sequence was used for bFGF (5'-CGAACTGGGCAGTATAAAC-3'). LN229 cells immersed in serum-free DMEM were infected with either LV-bFGF-siRNA at 5 and 10 MOI (Multiplicity of Infection), or a lentivirus vector expressing green fluorescent protein (LV-GFP) as mock controls at 10 MOI for 72 h. Cells treated with dimethylsulfoxide were used as controls.

Chemical regents and antibodies

The following agents were used in this study: Human recombinant bFGF (Peprotech, NJ, USA), AG490, PD0325901 and AZD5363 (Selleckchem, Munich, Germany). Each agent was prepared at 10 mg/mL stock solution in dimethyl sulfoxide (DMSO) and stored at -20°C. The dye Lucifer Yellow (LY) (Sigma-Aldrich, MO, USA) and the primary antibodies anti-Cx43, p-Cx43 (Ser368), p-STAT3 (Tyr705), p-AKT1, p-ERK1/2, and β-actin were also used in this study (Santa Cruz CA, USA).

Western blot analysis

Western blot analysis was performed as previously described [13]. All LN229 cells (experimental and control groups) were lysed in M-PER Reagent containing the halt protease and phosphatase inhibitor cocktail. Protein (30 μg/lane), quantified with the BCA protein assay kit (Pierce, Fisher Scientific), was separated using 8-12% SDS-PAGE and transferred to Polyvinylidene Fluoride membranes. The membranes were blocked with 5% non-fat dry milk in TBST (for non-phosphorylated proteins) or 5% BSA in TBST (for phosphorylated proteins) for 1 h and then incubated with primary antibodies overnight at 4°C. After cleansing, the membranes were incubated with secondary antibodies soaked in horseradish peroxidase (1:5000) for 1 h at room temperature and developed using a ECL kit (Thermo Co., Ltd.).

Immunofluorescence staining

LN229 cells grown on coverslips were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100/PBS for 20 min. Cells were then washed twice with PBS and soaked in 1% bovine serum albumin (BSA) for 30 min prior to incubating the cells with primary antibodies recognizing Cx43 or p-Cx43 (S368) for 1 h in a humidified chamber. After repeated cleansing with PBS, cells were incubated with Cy3-conjugated secondary antibodies for 1 h. After washing with PBS, cells were stained with DAPI for 10 min and immunofluorescence was detected using a confocal laser scanning microscope.

Determination of GJIC by scrape loading

LN229 cells were seed in 6-well plates, then infected with LV-bFGF-siRNA (not including GFP) for 72 h. The GJIC level was determined using the scrape loading and dye transfer (SL/DT) technique with the fluorescent dye; Lucifer Yellow. After washing with PBS, cells were incubated with 0.1% Lucifer Yellow in PBS. Scrape loading was performed using a surgical scalpel to draw several clear straight lines on the cell

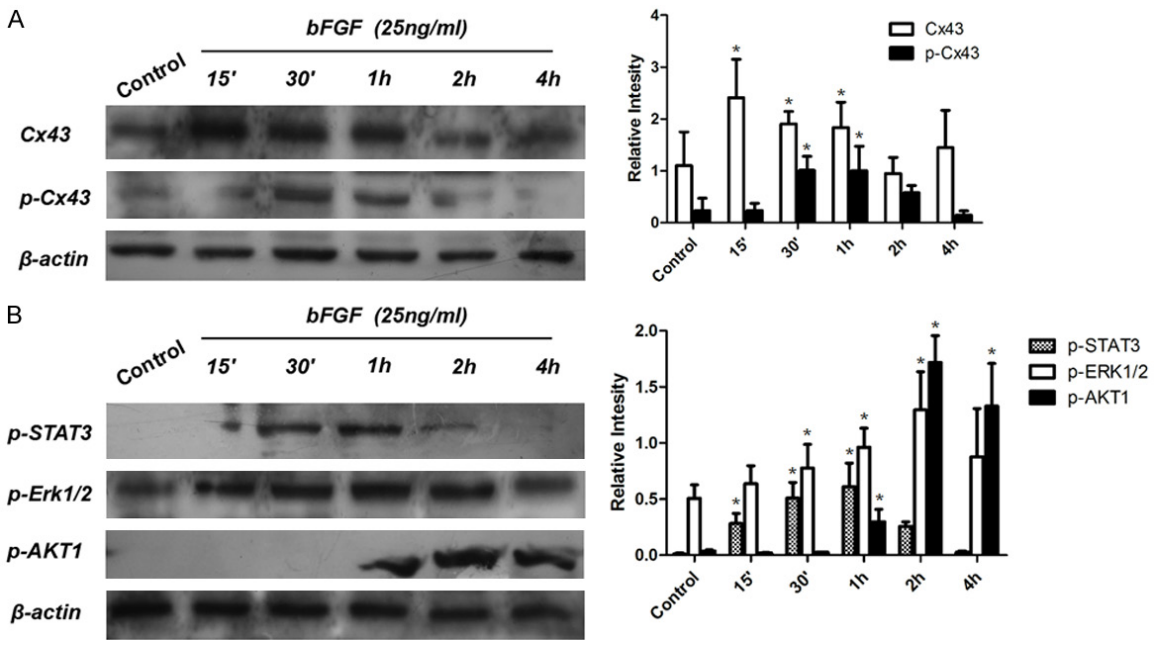

Figure 1. A. bFGF induces Cx43 expression in LN229 cell. Western blot analysis revealed that the levels of Cx43 and p-Cx43 (Ser368) are increased in a time-dependent manner. B. bFGF activation of three best-characterized receptor tyrosines kinase family members (i.e. STAT3, ERK1/2 and AKT1) are detected by Western blot. Phosphorylated STAT3, ERK1/2 and AKT1 are activated by extracellular bFGF (25 ng/mL) at different time points. Results represent the mean ± SEM of 3 separate. *indicates significant difference as compared to the untreated control group (P<0.05).

monolayer. After 5 min, the Lucifer Yellow solution was removed, cells were washed 3 times with PBS, followed by transfer of Lucifer Yellow, which was detected using an inverted fluorescence microscope. The fluorescence transmission distance was measured by image J software.

Statistical analysis

Results were analyzed using SPSS software and control-experimental groups were compared using one-way ANOVA. Data were presented as mean ± standard deviation (SD) of three independent experiments. P<0.05 was considered statistically significant.

Results

bFGF-induced Cx43 expression in LN229 cell is time-dependent

The amount of Cx43 protein and its phosphorylation pattern was examined at time intervals using western blot of cultured LN229 cells incubated with 25 ng/mL bFGF (serum-free). Cx43 increased at time =15 min, at time =2 h the expression of Cx43 came to normal (**Figure 1A**), the p-Cx43 (Ser368) level increased after 15 m and was then gradually restored to the control level after 1 h.

bFGF-induced Cx43 expression is inhibited by AG490

We examined which signaling pathways regulate bFGF-induced Cx43 expression in LN229. First, We examined bFGF activation of the three best-characterized receptor tyrosine-kinase family members by immunoblotting for the activated forms of STAT3, ERK1/2 and AKT1. As shown in (**Figure 1B**), extracellular bFGF (25 ng/mL) stimulated the phosphorylation of STAT3, ERK1/2 and AKT1 in LN229 cell at different time points. Treatment of cells with 25 ng/mL bFGF increased the phosphorylated STAT3 levels after 30 m and the level restored after 2 h. To explore whether the STAT3 pathway was involved in bFGF-induced Cx43 expression, we then used an inhibitor of STAT3 pathway: the AG490. Cells were pre-treated for 6 h with AG490 at increasing concentrations (1, 10, 50, 100 μM), prior to the addition of 25 ng/mL bFGF for 1 h. The expression level of Cx43, as analyzed using western blot, was markedly

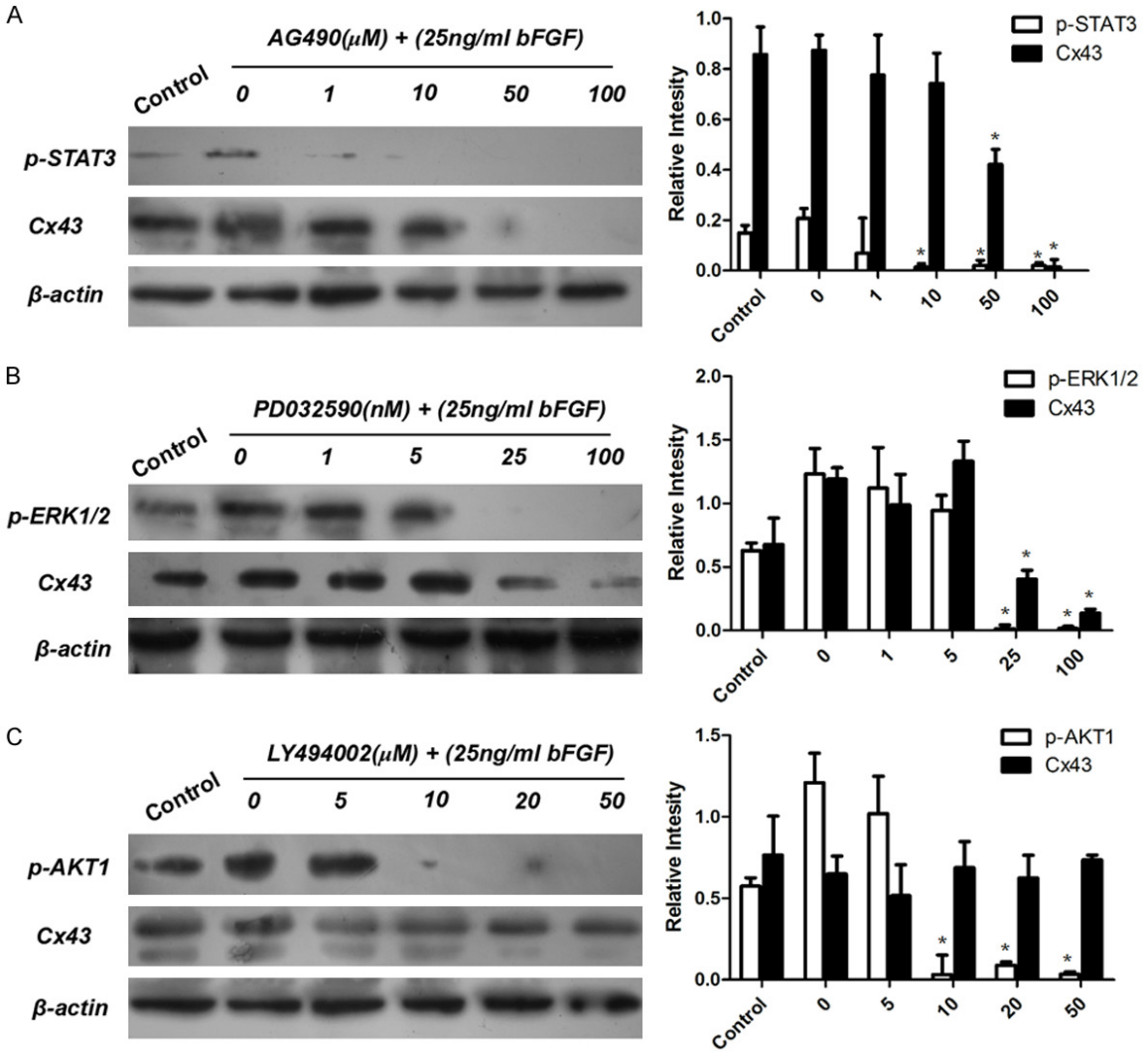

Figure 2. A. LN229 Cells were incubated by 25 ng/mL bFGF for 1 h. Then, STAT3 antagonist AG490 decreased the expression level of Cx43 in a dose-dependent manner. B. As the above method, the inhibitor of ERK1/2 pathway PD0325901 also decreased the expression level of Cx43 in a dose-dependent manner. C. The inhibitor of AKT pathway AZD5363 did not reduce the Cx43 expression with increasing concentrations. Results represent the mean ± SEM of 3 separate. *indicates significant difference as compared to the untreated control group ($P<0.05$).

decreased at 50 μM dose of AG490, and reached an undetectable level at 100 μM dose (**Figure 2A**).

bFGF-induced Cx43 expression is also inhibited by PD0325901

With 25 ng/mL bFGF stimulation, phosphorylation of ERK1/2 began to increase at 15 m, peaked at 1 h, and gradually decreased to the initial level after 2 h (**Figure 1B**). Total ERK1/2 level remained unchanged throughout the whole process. Then we explored whether the ERK1/2 pathway was involved in bFGF-induced Cx43 expression using an inhibitor of ERK1/2 pathway (i.e. PD0325901). Cells were pre-treated with PD0325901 at 1, 5, 25, 100 μM for 6 h, prior to the addition stimulation of 25 ng/mL bFGF for 1 h. Cx43 expression was markedly decreased at 25 μM, and decreased to an undetectable level at 100 μM dose (**Figure 2B**).

bFGF-induced Cx43 expression is not inhibited by AZD5363

With 25 ng/mL bFGF stimulation, phosphorylation of AKT1 began to increase at 1 h, and maintained the activated state until 4 h (**Figure 1B**). We used an inhibitor of AKT pathway (i.e. AZD5363) to detect changes in AKT pathway.

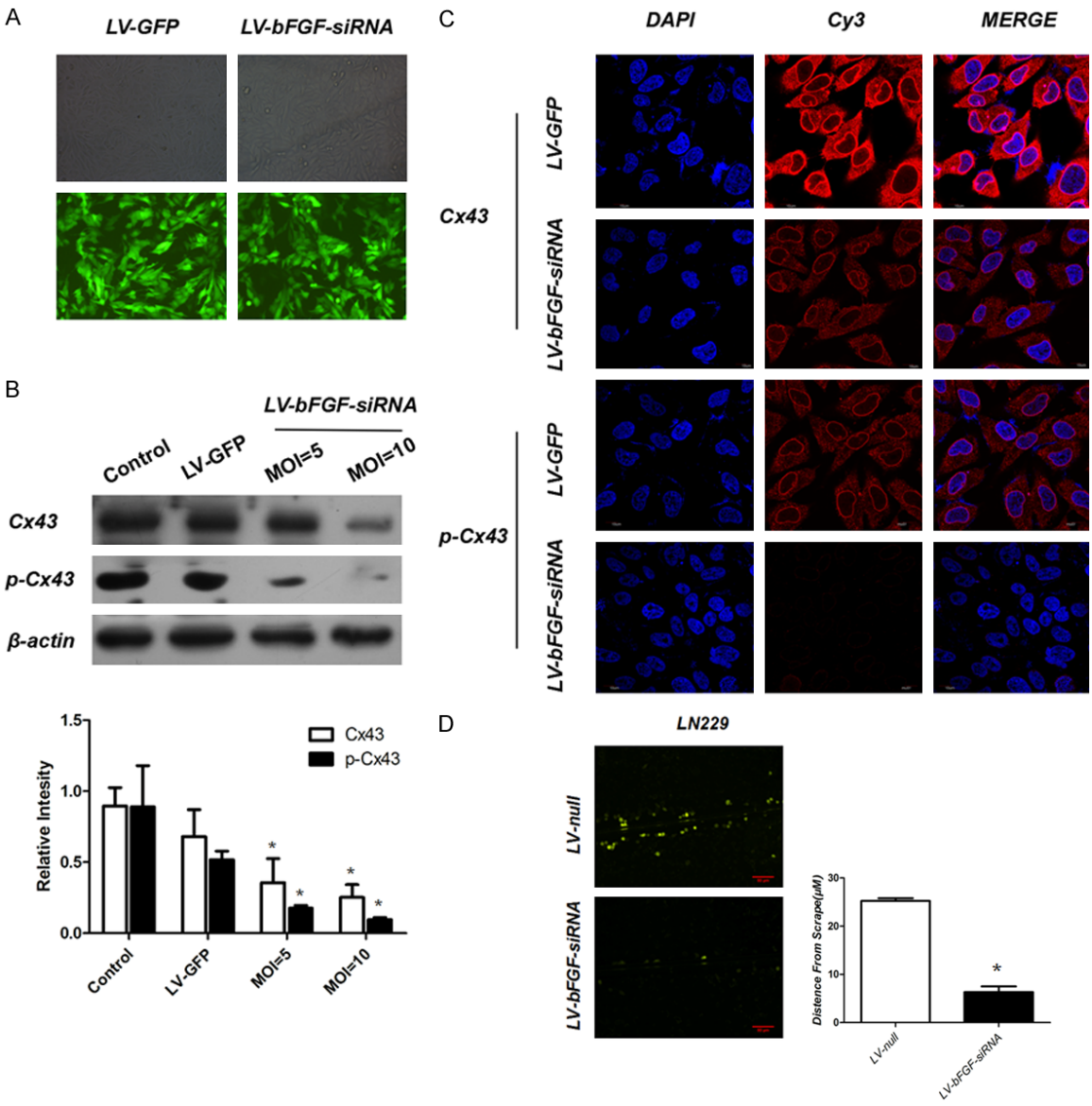

Figure 3. A. LN229 cells in serum-free DMEM were respectively infected with LV-GFP and LV-bFGF-siRNA at 10 MOI, which is an MOI at which minimized virus toxicity was observed, 80-90% infection efficiency was detected based on the expression of GFP. B. Expression of Cx43 and p-Cx43 at S368 site in control, LV-GFP, and LV-bFGF-siRNA-infected LN229 cells as measured by western blotwestern blot. C. Subcellular localization of Cx43 and p-Cx43 in LN229 cells stained with primary antibody and Cy3-conjugated secondary antibodies, DAPI staining to identify nuclei. Immunofluorescence was detected using a confocal laser scanning microscope. D. Effects of LV-bFGF-siRNA (not including GFP) and LV-null on the GJIC using by SL/DT assay. LV-bFGF-siRNA treated cells showed more aggravated GJIC and shorter Lucifer Yellow transmission distance compared with control group. Results represent the mean ± SEM of 3 separate. *indicates significant difference as compared to the untreated control group ($P<0.05$).

The dose of AZD5363 was increased progressively 5, 10, 20, 50 μM. Cells were pre-treated for 6h with AZD5363 at various concentrations, prior to adding 25 ng/mL bFGF. Cellular protein was isolated from cells and studied with western blot to detect the expression of Cx43 (see results in **Figure 2C**). While AKT1 protein level inhibited at 10 μM of AZD5363, Cx43 expression was not markedly changed after AZD5363 treatment comparing to the control group.

LV-bFGF-siRNA downregulates the Cx43 expression and GJIC in LN229 cell

We knocked down bFGF using LV-bFGF-siRNA, and the decrease in bFGF protein levels was

confirmed using western blot [13]. At a multiplicity of infection (MOI) of 10, at which virus toxicity was observed, 80-90% infection efficiency was detected, based on the expression of GFP (**Figure 3A**). Unlike in U251 cells, both expression of Cx43 and phosphorylation of Cx43 (S368) were significantly decreased LN229 cells treated in LV-bFGF-siRNA (**Figure 3B**). To further analyze this effect, we infected U87 cell with LV-bFGF-siRNA and observed an increased level of p-Cx43 (data not shown). In addition, the subcellular distribution of Cx43 and p-Cx43 in LN229 cells was examined using indirect immunofluorescence. We found that Cx43 was predominantly localized in the nuclear membrane and cytoplasm of LN229 cells (**Figure 3C**). SL/DT assays showed that the functional GJIC in LV-bFGF-siRNA treated cells were greatly collapsed. Also, LV-bFGF-siRNA treated cells showed shorter Lucifer Yellow transmission distance compared with control group (**Figure 3D**).

Discussion

In this study, we found that bFGF stimulates an increased expression in Cx43 and p-Cx43 (Ser368) protein is time-dependent. Conversely, knocking down bFGF levels using LV-bFGF-siRNA can significantly decrease the Cx43 and p-Cx43 expression in glioma cell line LN229. In addition, the our results show that AG490 (STAT3-antagonist), as well as PD0325901 (inhibitor of ERK1/2 pathway), blocked the stimulation effect of bFGF over Cx43 This finding suggests that bFGF-induced Cx43 expression is regulated mainly via activation of STAT3 and ERK1/2 but not through the AKT pathway.

Decreasing Cx43 expression and dysfunctional GJIC have been reported in human glioma cells. The loss of Cx43 expression correlates with increasing oncogenic proliferation as well as the degree of the malignancy of astrocytomas [1, 2]. Although several studies show that down-regulation of Cx43 facilitates tumor formation (add reference here), and this has been considered the hallmark of glioma tumors, evidence also shows that the regulation of Cx43 expression is highly heterogeneous. Low levels of Cx43 protein but high levels of Cx43 mRNA were observed in 32 human high-grade gliomas [14]. Additionally, data from The Cancer Genome Atlas repository revealed that although 57% of primary tumors show a more than twice times reduction of Cx43 level, the rest shows an increased Cx43 expression [15]. Further, the expression and functions of Cx43 may be dependent on the cellular status of the tumor [2].

We previously reported that knocking down bFGF with small interfering RNA increases Cx43 expression and improve GJIC in U251 cells [12]. However, the present study shows that LV-bFGF-siRNA decreases significantly Cx43 expression and aggravates GJIC in LN229 glioma cell. We also found that LV-bFGF-siRNA increases the p-Cx43 expression in U87. We observed that the subcellular distribution of Cx43 in LN229 is not predominantly localized to the cytoplasm (unlike U251), but mainly at the nuclear membrane. Aronica et al. [16] reported that high-grade glioma exhibits non-phosphorylated Cx43 protein localized within the cytoplasm, in contrast to low-grade glioma, which often exhibits increased expression within the nuclear membrane. We presume that the difference between Cx43 expression and subcellular distribution in glioma cells is related to the high heterogeneity in Cx43 and local tumor microenvironment, however there is lack of evidence to support this statement.

As a putative tumor suppressor gene, the involvement of Cx43 in apoptosis regulation is controversial, as evidence partially supports involvement of Cx43 in both the induction as well as prevention of apoptosis. The reduced activity of the anti-apoptotic protein bcl-2 has been associated with transferring exogenous Cx43 in human glioblastoma cells [17]. On the contrary, increased resistance to apoptosis by Cx43 has been reported in C6 glioma cells [18]. In addition, Nakase et al. found increased apoptosis and inflammation levels after focal brain ischemia in mice with astrocites lacking Cx43, suggesting that Cx43 may prevent cell apoptosis [19]. The ambivalent capacity to pro-apoptosis and anti-apoptosis may partly be explained by the heterogeneous expression of Cx43 in glioma cells. Our experience and results in Cx43 research suggests that the difference in bFGF-siRNA-induced Cx43 expression in glioma cells may be associated with the ambivalent characteristic of Cx43 in apoptosis regulation, however future studies are required to examine the underlying mechanisms.

Evidence suggests that Cx43 may be affected by the bFGF signaling pathway. However, the mechanisms involved remain unknown. We

investigated the underlying signaling pathway of bFGF-regulated Cx43 in glioma cells. Cx43 is regulated by several upstream kinases, including ERK, AKT, STAT3, AP1 and Wnt pathway and maintain normal GJIC between cells [20-22]. It also receives exogenous stimulation of growth factors and cytokines (e.g. ciliary neurotrophic factor, EGF, TGF-β, bFGF) [23-25] and endogenous signaling like hypoxia and apoptosis [26]. Levels of bFGF have been shown to correlate with glioma grade and clinical outcome. bFGF exerts mitogenic and angiogenic effects on human astrocytic tumors via triggering classical cell signaling including ERK, PI3K/AKT, JAK/STAT3 pathways [8, 9]. Many reports have demonstrated a tight correlation between bFGF and Cx43 in the non-tumor or/and tumor cells [27, 28]. bFGF also has been reported to inhibit the GJIC function of brain astrocytes and rat C6 glioma cells [10, 29]. In addition, bFGF expression was reduced with exogenous Cx43 gene transduction into glioma cell [30]. Within this context, we investigated the role of the bFGF-activated pathways in Cx43 regulation and effect on GJC. The results of our study demonstrated that bFGF caused a time-dependent transient increase in Cx43 protein. We also found that bFGF-induced Cx43 expression is not inhibited by LY294002, but inhibited by AG490 and PD0325901. Our findings suggest that bFGF upregulates Cx43 expression in LN229 glioma cells via activation of STAT3 and/or ERK1/2, not through the AKT pathway. STAT3 serves as the convergent transcription factor of various signaling pathways activated by multiple growth factors and/or cytokines [31, 32]. Geletu et al. reported that STAT3 is required for GJIC both in normal epithelial cells and human lung carcinoma cells [33], which is consistent with our findings. bFGF-induced Cx43 expression of glioma cells was attenuated by JAK/STAT3 inhibition, suggesting that this pathway may have important implications for bFGF-induced Cx43 expression in glioma progression. As a central component of the MAPK pathway, ERK1/2 can be activated by bFGF, and over-activated ERK1/2 which may contribute to malignant transformation [34]. Several studies have demonstrated that ERK1/2 regulates Cx43 expression. Hypoxia induces Cx43 dysregulation by modulating matrix metalloproteinases via MAPK signaling [26], and angiotensin-II induced Cx43 expression via MAPK-AP-1 signaling pathway [35]. Our data also demonstrates the role of ERK1/2 in the bFGF-induced Cx43 expression in glioma.

Collectively, we revealed that bFGF induces Cx43 expression in LN229 glioma cells mainly via activation of STAT3 and ERK1/2 pathway. This study first unveils the molecular mechanism of bFGF upregulates connexin43 expression in the human glioblastoma cells. Moreover, LV-bFGF-siRNA can decrease the Cx43 expression and aggravate GJIC in LN229 glioma cell, which is different in U251 cell. It also shows that Cx43 expression and subcellular distribution in glioma cells is highly heterogeneous Further research is required to explore the underlying mechanisms of the high heterogeneity and carcinogenesis in glioma.

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Disclosure of conflict of interest

None.

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