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Quercetin Induces Apoptosis in Glioblastoma Cells by Suppressing Axl/IL-6/STAT3 Signaling Pathway

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Abstract: Gliomas are the mostly observed form of primary brain tumor, and glioblastoma multiforme (GBM) shows the highest incidence. The survival rate of GBM is fairly poor; thus, discovery of effective treatment options is required. Among several suggested targets for therapy, the Axl/IL-6/STAT3 signaling pathway has gained recent interest because of its important role within cancer microenvironment. Quercetin, a plant flavonoid, is well known for its anticancer action. However, the effect of quercetin on Axl has never been reported. Quercetin treatment significantly reduced cell viability in two GBM cell lines of U87MG and U373MG while keeping 85% of normal astrocytes alive. Further western blot assays suggested that quercetin induces apoptosis but does not affect Akt or mitogen-activated protein kinases, factors related to cell proliferation. Quercetin also decreased IL-6 release and phosphorylation of STAT3 in GBM cells. In addition, gene expression, protein expression, and half-life of synthesized Axl protein were all suppressed by quercetin. By applying shRNA for knockdown of Axl, we could confirm that the role of Axl

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was crucial in the apoptotic effect of quercetin on GBM cells. In conclusion, we suggest quercetin as a potential anticancer agent, which may improve cancer microenvironment of GBM via the Axl/IL-6/STAT3 pathway.

Keywords: Quercetin; Glioblastoma; Axl; IL-6; STAT3; Apoptosis.

Introduction

Brain cancer has increased over the past 30 years and shows very poor survival rates. Up till the World Health Organization (WHO) Classification of Tumors of the central nervous system (CNS) (Louis *et al*., 2007), brain cancers were categorized only based on their histological characteristics with no regard on the clinical relevance. However, during the recent decade, intensive research on brain cancers revealed alternations in different molecular pathways, allowing WHO to re-define the categorization of cancers in the CNS (Louis *et al*., 2016). Among brain cancers, gliomas account for more than 70%, and the most frequent and malignant histological type of gliomas is glioblastoma multiforme (GBM). The probability of survival of GBM patients is 10%, and the other 90% of patients die within 3 years of initial diagnosis (Louis *et al*., 2016). A cohort study reported that GBMs have an estimated 10-year survival rate of 0.71% (Tykocki *et al*., 2018), proving that GBM remains one of the most lethal neoplasms of all human cancers. In these GBM patients, adjuvant radiation therapy and chemotherapy are generally proposed as treatment options after surgical resection, and recent studies also suggest the possible application of immunotherapy (Thomas *et al*., 2012).

In the treatment of overexpressed cancer cells, such as lung cancer (Shieh *et al*., 2005), melanoma (Konieczkowski *et al*., 2014), leukemia (Dufies *et al*., 2011) or GBM (Onken *et al*., 2017), the importance of TAM receptors has gained focus because of their significant role in the initiation and progression of cancer. Among them, Axl is used as a diagnostic marker and appears to be a potentially new biomarker that plays an important role in tumor formation (Gay *et al*., 2017). Axl is potentially followed by phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR), RAS/RAF/Mitogen-activated protein kinase/ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK), Janus kinase (JAK)/signal transducers and activators of transcription (STAT) and nuclear factor kappa B (NF-κB) signaling pathways, which all lead to induction of cell proliferation through effector molecules (Linger *et al*., 2008). Throughout the complicated cascades, STAT3 primarily affects multiple tumorigenic signaling pathways due its role in both cancer cells and the surrounding microenvironment. STAT3 activation induces cancer cell survival, proliferation, angiogenesis, invasion, anti-apoptosis, and immunosuppressive activity in various carcinomas (Fathi *et al*., 2018). Recent interest in STAT3 has pointed out that STAT3 may be an attractive biomarker for targeted therapy (Tan *et al*., 2019) or immunotherapy (Chang *et al*., 2017) in GBM patients. Along with STAT3, Axl is considered as another promising immune checkpoint target for cancer therapy (Zhu *et al*., 2019). Thus, promising attempts to regulate Axl-STAT3 axis in the pathophysiology of cancer have been well-described in previous studies (Woo *et al*., 2014; Suh *et al*., 2015).

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) is a naturally occurring plant flavonoid well-known for its anticancer action. Quercetin exerts a pro-apoptotic feature by binding to several receptors that play important roles in carcinogenesis (Jana *et al*., 2018). It is also known to interact with PI3K/Akt and STAT3-related pathways and is therefore involved in carcinogenesis in GBM (Michaud-Levesque *et al*., 2012). However, up to date, the role of Axl in the beneficial effect of quercetin is yet to be confirmed. We therefore conducted a study to investigate the effect of quercetin on GBM cell death, focusing on the role of the Axl-STAT3 pathway. For this, we selected two widely used GBM cell lines, U87MG and U373MG, which display *PTEN* abnormality either by mutation (U87MG) or deficiency (U373MG) (Lee *et al*., 2011).

Materials and Methods

Reagents

Quercetin (≥95%, MW 302.24), Cycloheximide (CHX) and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), Rosewell Park Memorial Institute (RPMI) 1640 (LM011-01), penicillin and streptomycin were obtained from WELGNE (Gyeongsan, Republic of Korea). Fetal bovine serum (FBS) was obtained from GR scientific (Bedford, UK).

Cell Culture

Human glioblastoma cell line U87MG cells and U373MG cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Normal immortalized human astrocytes (T0280) were purchased from Applied Biological Materials Inc. (Richmond, BC, Canada). The U87MG cells and astrocyte cells were cultured in DMEM medium and U373MG cells were cultured in RPMI1640 medium at 37 °C and 5% CO_2. All media was supplemented with 10% FBS and 1% penicillin-streptomycin. Recombinant human Interukin-6 (rhIL-6) (Sigma-Aldrich, St. Louis, MO, USA) was treated at 5 ng/mL together with quercetin in cell culture media.

Crystal Violet

U87MG, U373MG and normal astrocyte cells were seeded at 10^6 cells/well in a 6-well plate and treated with vehicle or quercetin for 24 h. Then, the cells were stained using Crystal Violet Dye (Sigma-Aldrich, St. Loius, MA, USA) according to the manufacturer's instructions.

Cell Viability Assay

U87MG, U373MG and normal astrocyte cells were seeded in a 96-well plate (1×10^4 cells/well), incubated overnight, treated with quercetin for 24 h, and a 3-[4, 5-dimetylthiazol-2-yl]-2,

5-diphenyltetrazoliumbromide (MTT) assay was performed as instructed by the manufacturer. Concentrations of quercetin was decided based on previous reports (Kim *et al*., 2013; Pan *et al*., 2015).

Western Blot Assay

For western blot assays, briefly, samples were loaded (20 μg), and electrophoresis was carried out. Transfer to a nitrocellulose membrane was performed at 80 V for 2 h followed by blocking with 5% skimmed milk for 1 h. The membrane was incubated with following primary antibodies overnight in 4°C: Axl (#8661S), STAT3 (#30835S), p-STAT3 (#9131S), cleaved-caspase 3 (Asp175) (#9661S), cleaved-caspase 7 (#9491S), cleaved-caspase 9 (Asp330) (#9501S), cleaved-PARP (#9542S), Akt (#9272S), p-Akt (#9271S), ERK (#9102S), p-ERK (#9101S), p38 (#9212S), p-p38 (#9211S) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#2118S), all purchased from Cell Signaling technology (Danvers, MA, USA). Subsequently, the membranes were incubated with appropriate secondary antibodies (1:1000) for 1 h at RT, immunoblots were detected by enhanced chemiluminescence (ECL) solution, and blots were quantified using ImageJ Software (National Institutes of Health, Bethesda, MD, USA).

CHX Chase Assay

Briefly, U87MG cells were cultured in DMEM medium containing 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO_2. Subsequently, CHX (100 μg/mL) was added into the media followed by quercetin treatment.

Flow Cytometry Assay

U87MG or U373MG cells were seeded (1×10^5 cells) in a 60-mm dish, treated with 50 μM of quercetin, and incubated at 37 °C and 5% CO_2 for 24 h. Then, the cells were stained with Annexin-V-FITC and 7-aminoactinomycin D (7-AAD) to analyze apoptotic cell death by flow cytometry using BD FACScan System (BD Biosciences, San Jose, CA, USA) with Annexin-V-FITC (BD Biosciences, San Jose, CA, USA) and 7-AAD (Sigma-Aldrich, St. Louis, MO, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed using an IL-6 ELISA kit (Catalog No. 555240) from BD Biosciences (San Jose, CA, USA).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted using an R&A blue™ Total RNA Extraction Kit (iNtRON Biotech, Korea). Nanodrop 1000 (Thermo Fisher scientific, Waltham, MA, USA) was used

for RNA concentration measurement. cDNA was prepared from 1 μg of total RNA using a cDNA synthesis kit (Takara Bio Inc., Kusatsu, Japan). 1 μl of cDNA were used for RT-PCR assays. The sequence for Axl was (F: 5′-GTTTGGAGCTGTGATGGAAGGC-3′, R: 5′-CGCTTCACTCAGGAAATCCTCC-3′), IL6 was (F: 5′-AACCTTCCAAAGATGGCTGAA-3′, R: 5′-CAGGAACTGGATCAGGACTTT-3′) and GAPDH was (F: 5′-GTCTCCTCTGACTTCAACAGCG-3′, R: 5′-ACCACCCTGTTGCTGTAGCCAA-3′).

Lentivirus Production and Infection

The pLKO.1 puro lentiviral shRNA plasmid targeting Axl (shAxl$^{\#1}$ F: 5′-CCGGGATTTGGAGAACACACTGACTCGAGTCAGTGTGTTCTCCAAATCTTTTTG-3′, R: 5′-AATTCAAAAAGATTTGGAGAACACACTGACTCGAGTCAGTGTGTTCTCCAAATC-3′; shAxl$^{\#2}$ F: 5′-CCGGCGAAGCCCATAACGCCAAGGCTCGAGCCTTGGCGTTATGGGCTTCGTTTTTG-3′, R: 5′-AATTCAAAAACGAAGCCCATAACGCCAAGGCTCGAGCCTTG GCGTTATGGGCTTCG-3′) and the nonspecific scramble shRNA plasmid (5′-CAACAAGATGAAGAGCACCAA-3′) were transfected into HEK293T cells (Korean Cell Line Bank of Seoul National University, Seoul, Republic of Korea) using Lipofectamine 3000 transfection reagent (Thermo Fisher, USA) with packaging plasmid psPAX2 and envelope plasmid pMD2G. Supernatant containing lentivirus particles was harvested at 24 h and 48 h after transfection. To silence the Axl gene with shRNA, U87MG and U373MG cells were cultured on six well plates until 70%–80% confluence. Then, lentivirus-containing supernatant (1 mL) was directly overlaid on cells in the presence of polybrene (Merck, Germany) at a final concentration of 4 μg/mL. About 24 h later, the supernatants were changed with fresh medium, and GBM cells were cultured for additional two days for further experiments.

Statistical Analysis

Results are expressed as the mean ± standard error (S.E.) of three or more experiments. Statistical analyses were performed using Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA) by one-way ANOVA followed by a Tukey test. Values with $p < 0.05$ were determined as statistical significance.

Results

Quercetin Decreases Cell Viability in GBM Cell Lines U87MG and U373MG

Quercetin is consisted of 15 carbons, 10 hydrogens and 7 oxygens (Fig. 1A), and it is known to exert anticancer activity in GBM (Tavana *et al*., 2019). Astrocytes and GBM cell lines U87MG and U373MG were treated with quercetin 100 μM and live cells were stained with crystal violet. Quercetin 100 μM did not seem to fatally affect viability of normal astrocytes, as they showed over 85% portion of live cells, but they did lead to highly decreased live cell numbers in U87MG and U373MG cells (Fig. 1B). In addition,

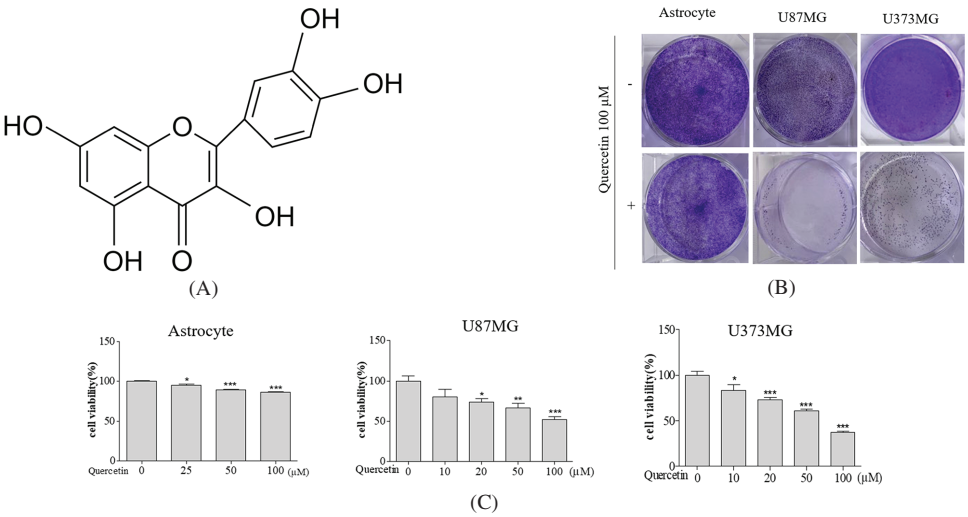

Figure 1. Effect of quercetin on cell viability of U87MG, U373MG and normal astrocytes. (A) Molecular structure of quercetin. (B) Crystal violet staining and (C) MTT assays were performed in quercetin-treated U87MG, U373MG and normal astrocytes. MTT results are displayed as mean ± S.E. of three or more separate experiments. *$p < 0.05$ vs. untreated cells, **$p < 0.01$ vs. untreated cells, ***$p < 0.005$ vs. untreated cells.

by an MTT assay, we could conclude that quercetin induces dose-dependent cytotoxicity in U87MG and U373MG cell lines but not in normal astrocytes (Fig. 1C).

Quercetin Induces Apoptotic Cell Death in GBM Cells

First, the effect of quercetin on representative markers of cell survival, proliferation and migration, such as Akt, ERK and p38, was measured. Unexpectedly, quercetin did not affect phosphorylation of the three MAPKs in normal astrocytes (Supplementary Fig. S1A), nor in GBM cells (Figs. 2A and 2B). Next, to assess the possible apoptotic response induced by quercetin in GBM, we treated quercetin (0, 25, 50, 100 μM) in U87MG and U373MG for 48 h and measured expression levels of cleaved-caspase 3, cleaved-caspase 7, cleaved-caspase 9 and cleaved-poly (ADP-ribose) polymerase (PARP) using a western blot assay. The results showed that quercetin drove the apoptotic pathway only in GBM cells (Figs. 2C and 2D), not in normal astrocytes (Supplementary Fig. S1B). An additional FITC-Annexin V and 7-AAD stain flow cytometry determined the apoptotic effect of quercetin. As shown in Fig. 2E, quercetin significantly increased the apoptotic cell death ratio up to around 40% to 50% in both U87MG cells (Control vs. quercetin-treated cells, $p = 0.008$) and U373MG cells (Control vs. quercetin-treated cells, $p = 0.015$).

Quercetin Suppresses IL-6/STAT3/Axl Signaling Pathway in GBM Cells

In the tumor microenvironment, IL-6/STAT3 signaling acts to induce proliferation, survival and metastasis of cancer cells, and studies demonstrated a correlation of IL-6/STAT3

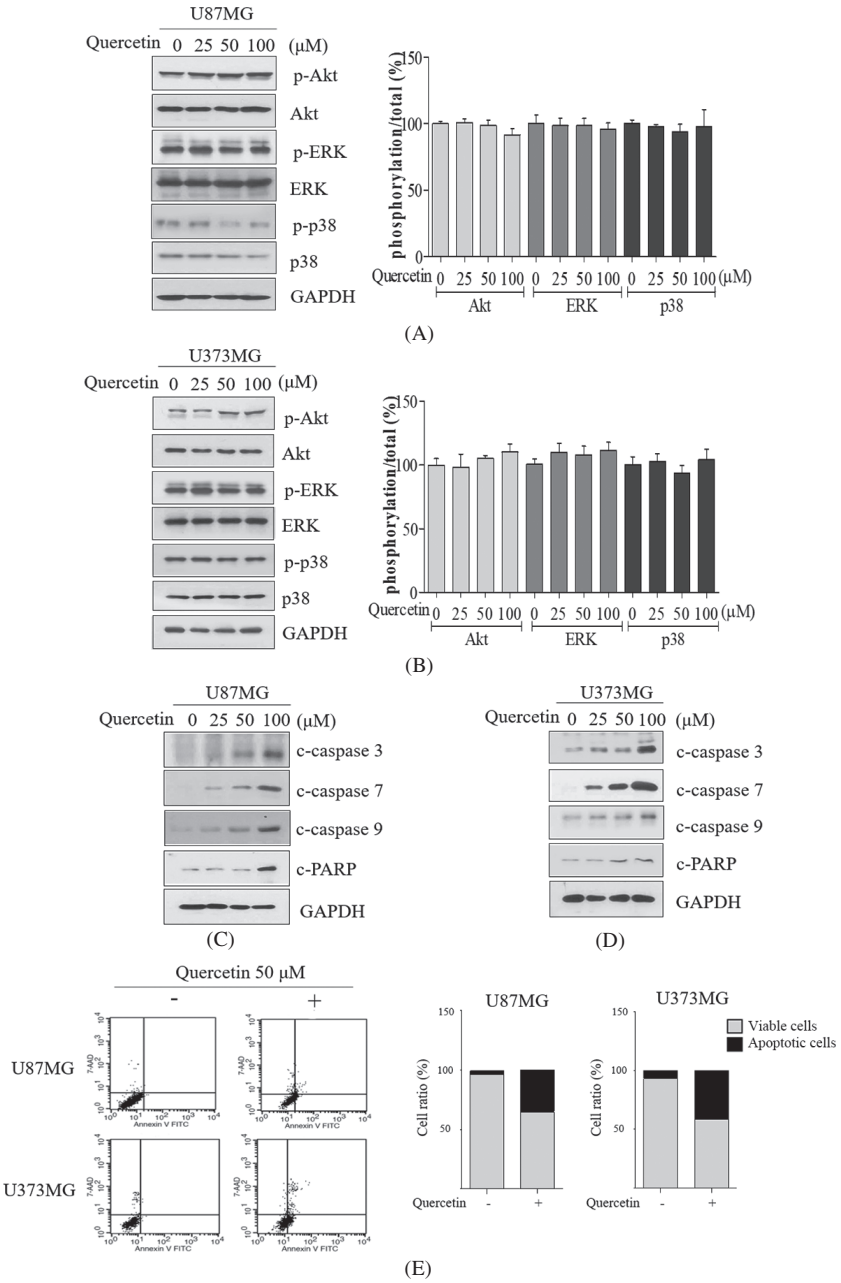

Figure 2. Effect of quercetin on apoptotic, proliferative and survival markers in U87MG and U373MG cells. Western blot assays were performed to detect expressions of p-Akt, p-ERK and p-p38 in quercetin-treated (A) U87MG and (B) U373MG cells. Western blot assays were performed to detect expressions of cleaved-caspase 3, cleaved-caspase 7, cleaved-caspase 9 and cleaved-PARP in quercetin-treated (C) U87MG and (D) U373MG cells. (E) Annexin V assay was performed in quercetin-treated U87MG and U373MG cells. Representative images of western blot assays are shown. GAPDH was used as endogenous control. Results are displayed as mean ± S.E. of three or more separate experiments. *$p < 0.05$ vs. untreated cells, **$p < 0.01$ vs. untreated cells, ***$p < 0.005$ vs. untreated cells.

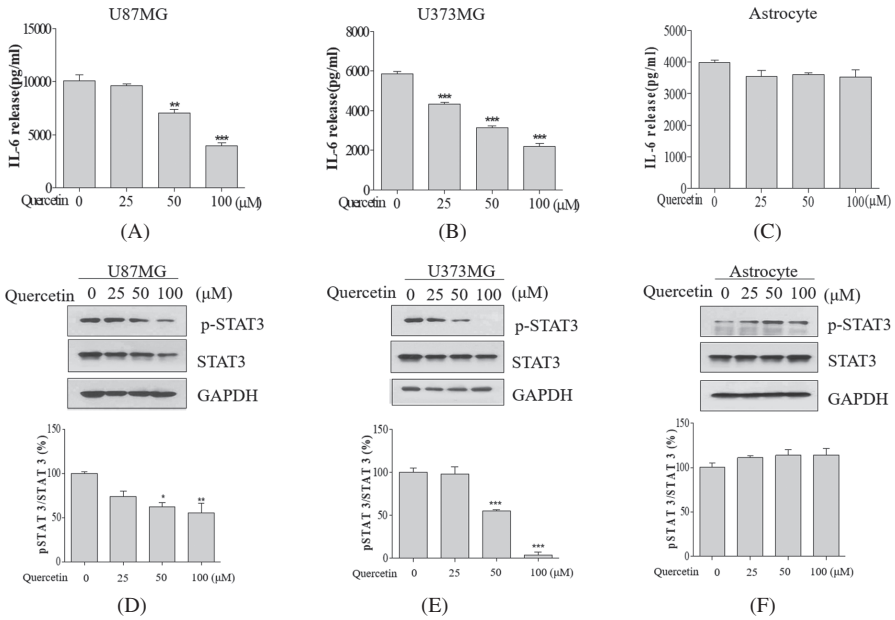

Figure 3. Effect of quercetin on IL-6/STAT3 pathway in U87MG, U373MG and normal astrocytes. ELISA on IL-6 release in quercetin-treated (A) U87MG, (B) U373MG and (C) normal astrocytes. Western blot assays on p-STAT3 were performed in quercetin-treated (D) U87MG, (E) U373MG and (F) normal astrocytes. Representative images of western blot assays are shown. GAPDH was used as endogenous control. Results are displayed as mean ± S.E. of three or more separate experiments. *$p < 0.05$ vs. untreated cells, **$p < 0.01$ vs. untreated cells, ***$p < 0.005$ vs. untreated cells.

expression with glioma tumor grade and overall survival (West *et al*., 2018). We measured IL-6 release U87MG and U373MG cells to find out quercetin decreased IL-6 levels dose-dependently in GBM cells (Figs. 3A and 3B), but not in normal astrocytes (Fig. 3C). This was due to a quercetin-induced decrease in *IL6* gene level in both U87MG and U373MG GBM cells (Supplementary Figs. S2A and S2B). Consequently, as the amount of IL-6 released was decreased, STAT3 phosphorylation was also reduced in quercetin-treated U87MG and U373MG cells (Figs. 3D and 3E). Normal astrocytes did not display such result by quercetin (Fig. 3F).

Next, we examined whether quercetin was able to affect Axl receptor tyrosine kinase (RTK) in GBM cells and normal astrocytes. We used U87MG cells to identify the time-dependent change in Axl protein level induced by quercetin 100 μM. As shown in Fig. 4A, Axl protein level was decreased significantly in GBM cells by quercetin, showing almost no expression after 24 h. Next, to verify whether quercetin affects the half-life of the Axl receptor protein, we treated the translation inhibitor CHX in GBM cells to create a non-translating condition. U87MG cells were treated with 50 μM of quercetin for 8 h, then incubated with or without CHX for 0, 6, 12, 24 h before harvest. Axl expression decreased to nearly half after 24 h, but quercetin co-treatment significantly accelerated the decrease of the Axl protein (Fig. 4B). The downregulation of Axl protein by quercetin treatment

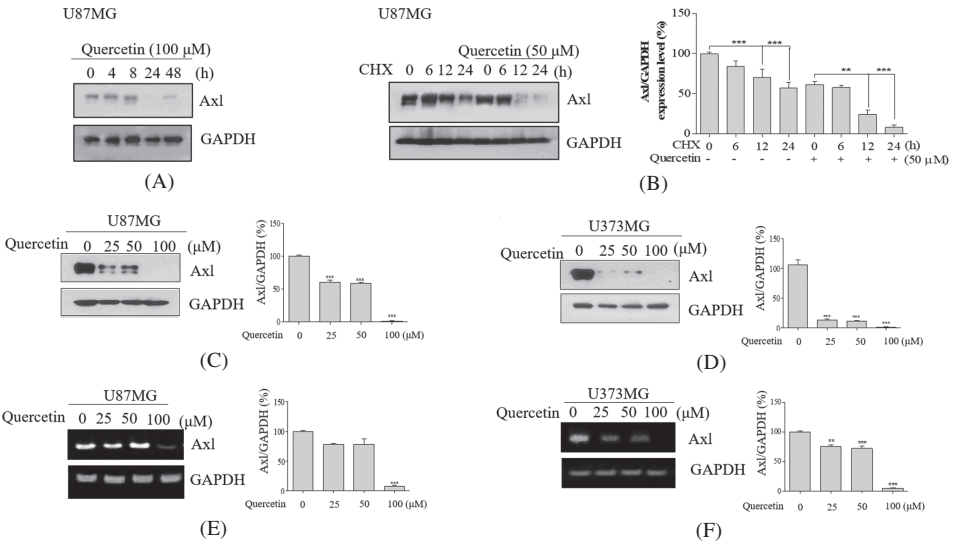

Figure 4. Effect of quercetin on Axl receptor expression in U87MG and U373MG cells. (A) Time-dependent change of Axl expression was measured by western blot in quercetin-treated U87MG cells. (B) CHX chase assay was performed in quercetin-treated U87MG cells and protein degradation of Axl were measured. Dose-dependent change of Axl protein expression was measured by western blot analysis in quercetin-treated (C) U87MG and (D) U373MG cells. Axl gene expression was measured by RT-PCR in quercetin-treated (E) U87MG and (F) U373MG cells. Representative images of western blot assays and RT-PCR assays are shown. GAPDH was used as endogenous control. Results are displayed as mean ± S.E. of three or more separate experiments. $^{**}p < 0.01$ vs. untreated cells, $^{***}p < 0.005$ vs. untreated cells.

was found out to be dose-dependent as well. Quercetin induced the decrease of Axl protein expression in both U87MG and U373MG cells (Figs. 4C and 4D), while impact on normal astrocytes was minimal and insignificant (Supplementary Fig. S3A). We then examined mRNA expression level of Axl using an RT-PCR assay. U87MG and U373MG showed decreased Axl mRNA levels in a dose-dependent manner with quercetin treatment (Figs. 4E and 4F), but astrocytes did not (Supplementary Fig. S3B). Axl mRNA levels were drastically changed especially when quercetin concentration was increased from 50 μM to 100 μM. Through these results, we concluded that quercetin suppressed gene transcription, protein synthesis and turnover of Axl.

Direct Role of Axl-STAT3 Axis in Apoptotic Potential of GBM Cell Lines U87MG and U373MG

Previous studies state that the Axl receptor acts as a receptor responsible for overall survival in GBM (Onken *et al*., 2017; Sadahiro *et al*., 2018). Furthermore, the relevance of Axl and IL-6/STAT is also well-described in terms of aggravation of cancer (Paccez *et al*., 2013). However, to date, the exact action mechanism of the IL-6/STAT3/Axl signaling pathway has not been fully elucidated. Therefore, we selected two different kinds of short hairpin RNA (shRNA) for Axl, then transfected GBM cell lines U87MG and U373MG

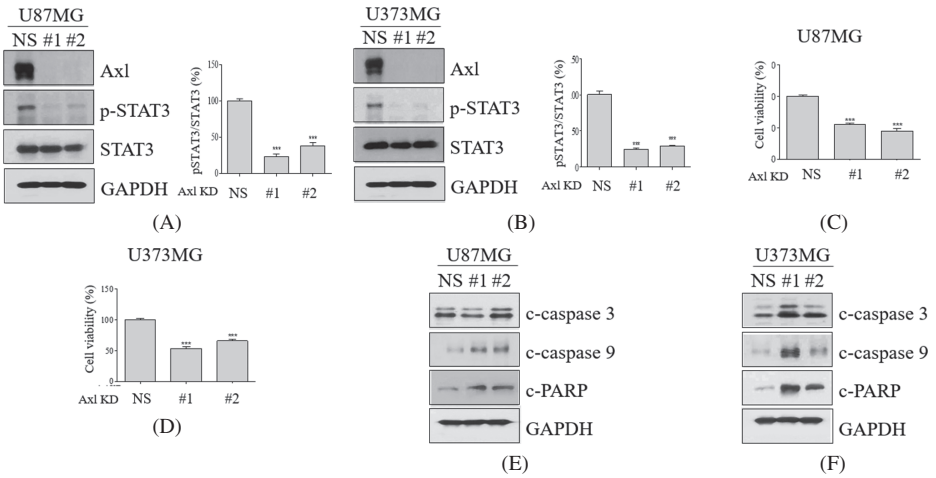

Figure 5. Changes induced by Axl knock-down in U87MG and U373MG cells. (A) Protein expressions of Axl and p-STAT3 were measured in (A) U87MG and (B) U373MG cells treated with scramble shRNA, shAxl$^{\#1}$ and shAxl$^{\#2}$. Cell viability in (C) U87MG and (d) U373MG cells were measured by an MTT assay. Protein expression of cleaved-caspase 3, cleaved-caspase 9 and cleaved-PARP in (E) U87MG and (F) U373MG cells was measured. Representative images of western blot assays are shown. GAPDH was used as endogenous control. Results are displayed as mean ± S.E. of three or more separate experiments. ***$p < 0.005$ vs. scramble shRNA-treated cells.

along with normal astrocytes to investigate the exact role of Axl in GBM regarding STAT3. shAxl$^{\#1}$ and shAxl$^{\#2}$ successfully induced knock-down of the Axl receptor in U87MG and U373MG cells. Phosphorylation of STAT3, the downstream target of the Axl receptor, was also decreased by shAxl treatment (Figs. 5A and 5B).

MTT results indicated that knock-down of Axl led to a significant decrease in cell viability (Figs. 5C and 5D). We then examined the changes which Axl knock-down may induce in GBM cells through the Western blot assay to determine its role on proliferation, survival and apoptosis of cells. Since we showed decreased cell viability by shRNA treatment, we next verified the apoptotic markers using western blot assays. U87MG and U373MG cells treated with shAxl showed increased apoptotic markers of cleaved-caspase 3, cleaved-caspase 9 and cleaved-PARP (Figs. 5E and 5F), indicating deletion of Axl gene resulted in cell death due to increased apoptosis no matter what type of cells. On the other hand, phosphorylation of MAPKs, which are used as proliferative or survival markers, were unaffected by either shAxl in GBM cells (Supplementary Figs. S2C and S2D). shAxl treatments also suppressed Axl protein expression and affected phosphorylation of STAT3 (Supplementary Fig. S3C), cell viability (Supplementary Fig. S3D) and apoptotic markers (Supplementary Fig. S3E) in normal astrocytes as well.

Apoptotic Effect of Quercetin in GBM Cells is Dependent on Axl/IL-6/STAT3 Signaling Pathway

We performed further experiments to verify whether the Axl/IL-6/STAT3 pathway involves the action mechanism of quercetin in GBM. An MTT assay showed that knock-down of

Axl abolishes the cytotoxic effect of quercetin in GBM cells (Fig. 6A). By treating U87MG cells with shAxl$^{\#1}$ and subsequently with quercetin, we observed decreased protein expressions of Axl and phosphorylated STAT3, as expected. We then measured the changes in key apoptosis factors such as cleaved-caspase 3, cleaved-caspase 9 and cleaved-PARP. Consistent with the MTT results, knock-down of Axl gene resulted in loss of apoptotic function from quercetin (Fig. 6B). Similar results were observed in U373MG cells as well (Supplementary Fig. S2D).

Both quercetin and shAxl treatment caused a significant decrease in IL-6 release (Fig. 6C). Then, we next evaluated the effect of quercetin in the presence of excess IL-6 by treating U87MG cells with recombinant human IL-6. As a result, while pSTAT3 was notably enhanced by rhIL-6 treatment, quercetin was able to repress this increase and subsequently induce regulators of apoptosis, including cleaved-caspase 9 and cleaved-PARP (Fig. 6D).

Discussion

GBM, the invasive and most common malignant brain tumor, occurs in about 1 in 50,000 brain cancer patients worldwide (Dolecek *et al*., 2012). The clinical symptoms of GBM depend on which area of the brain the cancer occurs in and the function of the involved area: i.e., persistent weakness, numbness, loss of vision, language disorder, mood disorder, fatigue, and mild memory loss (Alexander *et al*., 2017). According to the WHO classification of CNS cancer, GBMs are subcategorized into GBM IDH-mutant, GBM NOS and GBM IDH-wildtype, the last one further categorized into giant cell GBM, gliosarcoma and epithelioid GBM (Louis *et al*., 2016). Despite the intensive efforts to fully understand this disease, the cause of most cases of GBM is not known. However, risk factors include sex (male > female), age (50 years old or older) and genetic factors (Alexander *et al*., 2017).

Craniotomy surgical resection followed by combination of radiotherapy and chemotherapy is still the standard therapeutic strategy for GBM (Alexander *et al*., 2017). Temozolomide, an alkylating agent inducing cell cycle arrest (Alonso *et al*., 2007), has been utilized for the therapeutic approach in GBM for decades. However, application of temozolomide chemotherapy is not effective to at least 50% of GBM patients (Goellner *et al*., 2011). In additional chemotherapy options, apoptosis by targeting cancer cell receptors is the mainly used pathway. There are a variety of targets, such as epidermal growth factor receptor (EGFR), Her2, vascular endothelial growth factor (VEGF), tyrosine kinase inhibitors (TKIs) and platelet-derived growth factor (PDGFR), working effectively (Ben Kridis *et al*., 2020; El Guerrab *et al*., 2020). Especially, TAM receptors, composed of Tyro3, Axl, and Mer, are known to affect adult brain neuron development and show promising results. TAM receptor signaling plays a particularly important role in the swallowing and phagocytosis of apoptotic cells and membranes in adult tissues (Lemke *et al*., 2008).

Quercetin, a plant flavonoid derived from various fruits and vegetables (Tavana *et al*., 2019), is well known as a strong anticancerous agent from nature. Several studies state the beneficial effect of quercetin also in GBM. Park and Min showed that quercetin can reduce proliferation and invasion of U87 glioma cells via phospholipase D (Park *et al*., 2011), and

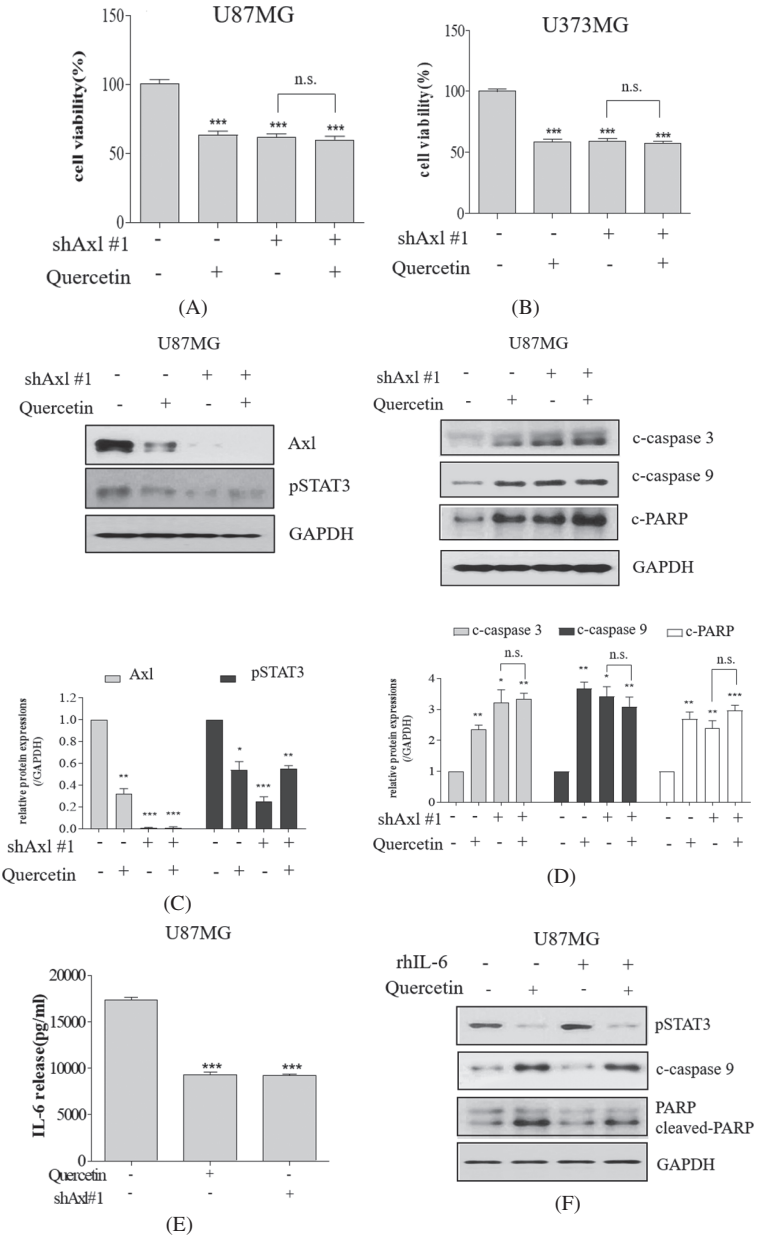

Figure 6. Effect of quercetin in Axl knocked-down environment of U87MG and U373MG cells. Cell viability in (A) U87MG and (B) U373MG cells was measured. Protein expressions of (C) Axl, p-STAT3, (D) cleaved-caspase 3, cleaved-caspase 9, cleaved-PARP were measured in quercetin-treated U87MG and U373MG cells after knock-down of Axl. (E) IL-6 release was measured. (F) Protein expressions of p-STAT3, cleaved-caspase 9 and cleaved-PARP were measured in U87MG after recombinant human IL-6 and quercetin co-treatment. Representative images of western blot assays are shown. GAPDH was used as endogenous control. Results are displayed as mean ± S.E. of three or more separate experiments. *$p < 0.05$ vs. scramble shRNA-treated cells, **$p < 0.01$ vs. scramble shRNA-treated cells, ***$p < 0.005$ vs. scramble shRNA-treated cells.

Kim *et al*. reported a pro-apoptotic and pro-autophagic effect of quercetin in U373MG cells (Kim *et al*., 2013). Quercetin also potentiates the anticancer effect of other agents such as sorafenib (Jakubowicz-Gil *et al*., 2014) and temozolomide (Sang *et al*., 2014), suggesting its possible use as a supplemental product with conventional anticancer therapy. Moreover, Michaud-Levesque *et al*. showed that quercetin inhibited IL-6-induced STAT3 in T98G and U87 GBM cell lines and thus repressed proliferation, migration and the cell cycle (Michaud-Levesque *et al*., 2012). However, the role of Axl in the anticancerous action of quercetin has not been ascertained. Thus, due to the rising interest on the Axl/STAT3 pathway in anticancer therapy, we attempted to show whether the Axl receptor along with STAT3 activation in GBM cells are involved in the action mechanism of quercetin.

The expression of Axl is responsible for important pathways inducing cell survival, proliferation, migration and invasion of cancer cells, and thus when expressed abnormally in GBM, Axl is associated with malignant tumor metastasis and poor prognosis (Onken *et al*., 2017). Accordingly, attempts targeting Axl are suggested to develop therapeutic agents in cancer treatment. Axl-targeted inhibition blocks cell growth, metastasis, invasion, epithelial-mesenchymal metastasis (EMT), angiogenesis, drug resistance and immune regulation in cancer cells (Zhu *et al*., 2019). In our results, quercetin significantly suppressed the expression of the Axl receptor in two different GBM cell lines, U87MG and U373MG time- and dose-dependently. On the other hand, Axl expression in normal astrocytes were not affected as much.

A novel approach was made in 2014 by suggesting Axl as a prognostic marker and a possible target for inhibition of the IL-6/STAT3 signaling axis (Suh *et al*., 2015). IL-6/STAT3 signaling pathway has a key role in the growth of many cancers. In the pathogenesis of cancer, elevated IL-6 stimulates activation of STAT3 signaling, which often indicates poor outcomes. Compared to the normal brain, the level of phosphorylation of STAT3 is overexpressed in almost 90% of GBM (Luwor *et al*., 2013). In addition, STAT3, composed of Tyr-705 and Ser-727, has been shown to block regeneration and cell growth of GBM cells upon inhibition of phosphorylation (Piperi *et al*., 2019). In the overall cancer microenvironment, IL-6 is produced from multiple origins, including immune cells, stromal cells, and cancer itself (Kumari *et al*., 2016). The secreted IL-6 directly stimulates cancer cells to induce STAT3 and its target genes, such as cyclin D1 or BCL families, leading to proliferation and survival of cancer. STAT3 also increases factors that promote angiogenesis, invasiveness or metastasis, such as VEGF and matrix metalloproteinases and immunosuppression (Yu *et al*., 2009). Moreover, in addition to direct effects on cancer cells, IL-6/STAT3 signaling also affects immune cells, which compose and have a significant role within the cancer microenvironment. Hyperactivated STAT3 in neutrophils, natural killer cells, T cells, and dendritic cells is frequently observed, suggesting the importance of STAT3 activation in anticancer immunity (Yu *et al*., 2007). In our study, quercetin influenced STAT3 activation and IL-6 level dose-dependently, consequently increasing apoptotic cell death. Further verification with shAxl study showed two facts: the Axl/IL-6/STAT3 signaling pathway is crucial in the proliferation and development of GBM, and this pathway is importantly involved in the anticancer effect of quercetin. When Axl was knocked down

by shRNA, STAT3 expression was also reduced, suggesting the close connection between these two factors. Furthermore, quercetin, which showed similar effect as shAxl treatment, lost its anticancerous potential when shAxl was pre-treated in GBM cell lines U87MG and U373MG. These results indicate that Axl/IL-6/STAT3 signaling cascade is responsible for the apoptotic effect of quercetin in GBM.

Overall, we hereby show the relevance and importance of the Axl/IL-6/STAT3 signaling pathway in the development of GBM. Through these results, we suggest quercetin as an effective yet safe anticancer agent to improve GBM and other gliomas via regulation of Axl-STAT3 pathway.

Acknowledgments

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Supplementary Figures

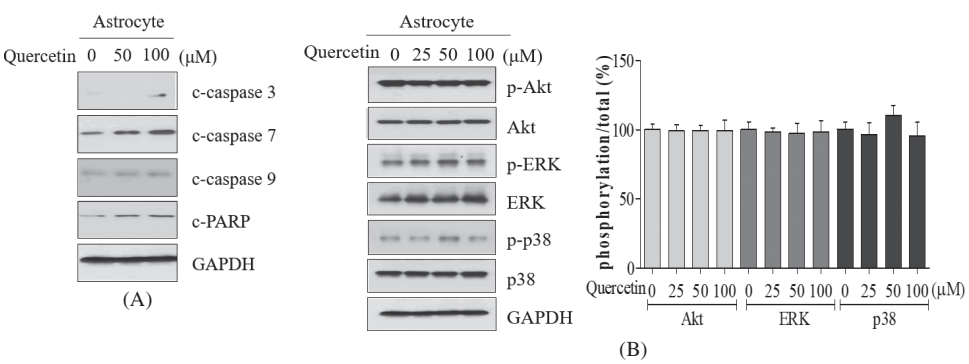


Figure S1. Effect of quercetin on apoptosis and proliferation markers in normal astrocytes. (A) Protein expression of cleaved-caspase 3, cleaved-caspase 7, cleaved-caspase 9 and cleaved-PARP in normal astrocytes was measured. (B) Protein expression of p-Akt, p-ERK and p-p38 was measured. Representative images of western blot assays are shown. GAPDH was used as endogenous control.

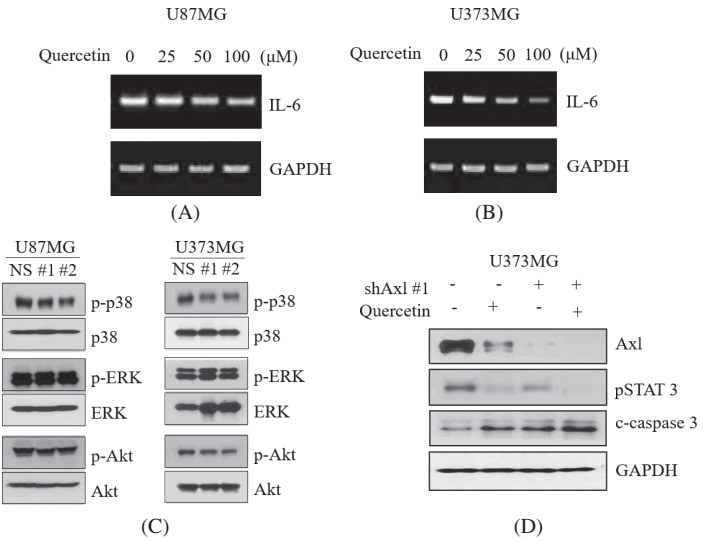


Figure S2. Effect of quercetin on IL-6/STAT3/Axl pathway in GBM cells. Changes in IL6 gene expression induced by quercetin in (A) U87MG and (B) U373MG cells were measured by ELISA. (C) Changes on proliferation markers such as p-p38, p-ERK and p-Akt induced by Axl knock-down in U87MG and U373MG cells were measured by western blot analysis. (D) Effect of quercetin on Axl, pSTAT3 and cleaved-caspase 3 in Axl knock-downed environment of U373MG cells were evaluated.

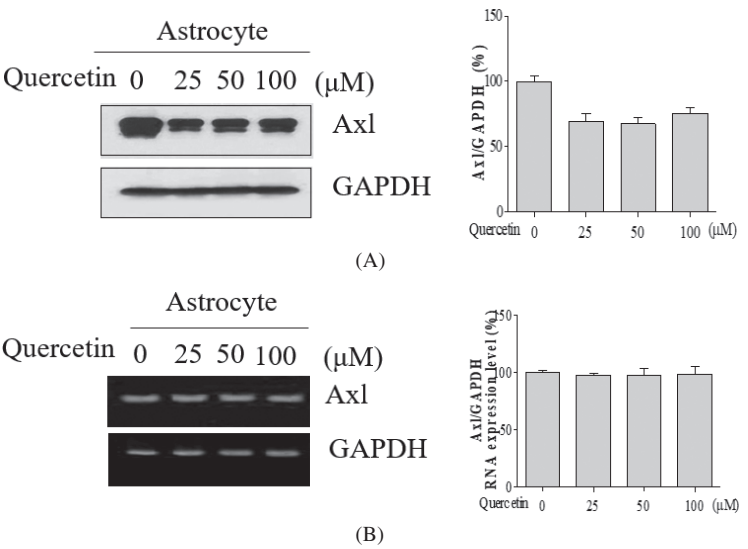
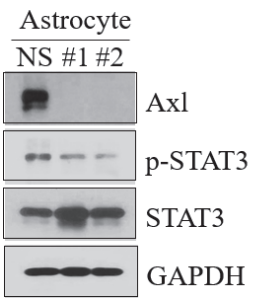


Figure S3. Effect of quercetin on Axl receptor expression in normal astrocytes. (A) Protein level and (B) mRNA level of Axl receptor was measured in quercetin-treated normal astrocytes. Changes on (C) protein expressions of Axl and p-STAT3 and (D) cell viability induced by Axl knock-down in normal astrocytes were evaluated. (E) Effect of quercetin on cleaved-caspase 3, cleaved-caspase 9 and cleaved-PARP in Axl knock-downed environment of U373MG cells. ***$p < 0.005$ vs. scramble shRNA-treated cells.



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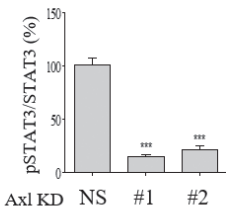

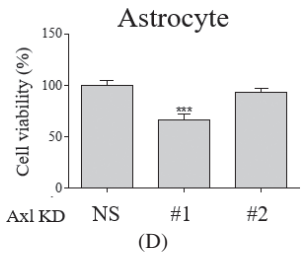

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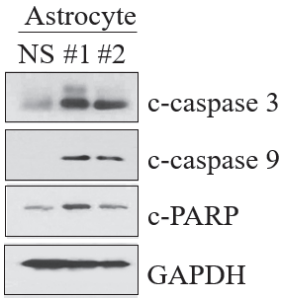

(E)

Figure S3. (*Continued*)