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Original Article

Baicalein suppresses EGF-induced proliferation and migration of glioma cells via EGFR/Akt signaling pathways

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Abstract: Background: Baicalein, a Traditional Chinese herb, has been reported to inhibit proliferation and invasion of several malignant tumors *in vitro* and *in vivo*. However, the anti-tumor effects of baicalein on glioblastoma multiforme (GBM) are not fully understood. The current study examined the inhibitory effects of baicalein on EGF-induced proliferation and migration of glioma cells, exploring whether EGFR/Akt signaling pathways act on the anti-tumor effects of baicalein. Methods: Cell viability, wound healing assays, Transwell migration assays, and cell proliferation were explored in glioblastoma multiforme U251 cells. EGFR and Akt signaling were evaluated in U251 cells. Results: Results showed that baicalein strongly reduced proliferation and migration of U251 cells, in a dose- and time-dependent manner. EGFR inhibitor AG1478 decreased proliferation and migration of U251 cells, suggesting that EGFR/Akt pathways are responsible for EGF-induced tumor proliferation and migration. Moreover, baicalein significantly decreased the phosphorylation of EGFR and Akt. Present findings were supported by the results of immunofluorescence staining. Conclusion: Baicalein may reduce cell proliferation and migration via inhibition of EGFR/Akt signaling pathways, implying that baicalein is a potential therapeutic medication for GBM.

Keywords: Baicalein, glioma cells, epidermal growth factor, EGFR/Akt signaling pathway

Introduction

Grade IV malignant gliomas or glioblastoma multiforme (GBM), the most common and lethal form of human brain cancer, have invasive and infiltrative characteristics. They account for about 10% of tumors in the central nervous system (CNS), having one of the highest mortality rates among cancers. Highly aggressive gliomas have the characteristics of rapid proliferation, migration, and invasion into surrounding normal tissues, near and far from the primary tumor [1]. Therapeutic methods for GBM include radiotherapy, surgery, and chemotherapy. However, survival times in most GBM patients treated with radiation and chemotherapy are < 15 months [2-4]. High morbidity and mortality rates of GBM, combined with common undesirable side effects observed after chemotherapy, demand the discovery of new agents from natural sources in enhancing treatment efficacy.

Bioflavonoids are a group of polyphenolic compounds usually found in plants. Herbs rich in flavonoids are generally utilized in Traditional Chinese Medicines. Increased dietary flavonoid intake has been associated with reduced risks of breast [5] and prostate cancer [6], according to epidemiologic studies. Baicalein (5,6,7-trihydroxyflavone), a natural flavonoid derived originally from the root of *Scutellaria baicalensis* georgi ("Huang-Qin" in Chinese), has an exact chemical structure (**Figure 1A**). It has been widely used in Traditional Chinese Medicines. Some biological effects of baicalein, including anti-inflammatory, anti-viral [7-10], and anti-tumor capabilities, have been realized. Baicalein has been reported to inhibit proliferation and invasion of several malignant tumors *in vitro* and *in vivo,* including human breast cancer [11, 12], lung cancer [13], hepatocellular carcinoma [14-17], prostate cancer [18, 19], osteosarcoma [20], and colorectal cancer [21, 22].

It has been reported that baicalein inhibits cell proliferation and promote cell apoptosis by reducing expression of HIF-1α, VEGF, and VEGFR2 in U87 glioma cells, acting as a therapeutic agent for malignant tumors [23]. Another study showed that baicalein acts on GPR30 signaling pathways by reducing phosphorylation of EGFR tyrosine and phosphorylation of ERK and AKT, inhibiting migration, adhesion, and invasion of breast cancer cells [24, 25]. However, the effects of baicalein on proliferation and migration in U251 glioma cells, along with related mechanism(s), are not fully understood. The current study utilized glioma cell line U251 to verify the hypothesis that baicalein can inhibit EGF-induced proliferation and migration of human GBM cells via EGFR/PI3K/Akt signaling pathways *in vitro*.

Material and methods

Reagents and antibodies

Baicalein (purity > 98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Baicalein was dissolved in dimethylsulfoxide (DMSO) as a 0.1 M stock solution and stored at -20°C. Human recombinant epidermal growth factor (hEGF) was obtained from PeproTech (USA). TUNEL Apoptosis Detection Kit (FITC) was purchased from Yeasen (Shanghai, China). The following primary antibodies were used: 1) Goat anti-rabbit IgG horseradish peroxidase-conjugated antibodies and Cell Counting Kit (CCK) were purchased from Beijing Fanbo Biotechnology (Beijing, China); 2) Phospho-Akt (Ser473; Cell Signaling Technology, Billerica, CA, USA); 3) Phospho-EGFR (Tyr1173; Abcam, Cambridge, UK); and 4) β-actin, EGFR, and AKT (1+2+3; Beijing Biosynthesis Biotechnology, Beijing, China). EGFR specific inhibitor AG1478 was purchased from Sigma-Aldrich.

Cell lines and culturing

Human glioma cell line U251, purchased from the Shanghai Cell Resource Center of the Chinese Academy of Sciences (Shanghai, China), was cultured in Dulbecco's modified Eagle's medium (DMEM). It was replenished with 10% FBS at 37°C in a 5% CO_2 incubator.

Cell viability assay

Cell viability was determined using CCK assays. Briefly, 96-well plates were seeded with cells at a density of 5×10^3 cells per well. After culturing for 24 hours, cells were pre-treated with baicalein (20, 40, and 80 μM) for 4 hours. They were then incubated with hEGF (20 ng/mL) for 12, 24, 36, and 48 hours. Investigating the roles of EGFR signaling pathways in the proliferation of glioma cells, U251 cells were pre-treated with EGFR inhibitor AG1478 (10 μM) for 1 hour. They were then incubated with hEGF (20 ng/mL) for 24 hours.

Treated cells were subsequently treated with 10 μl of the CCK indicator and cultured at 37°C for 2 hours, according to manufacturer instructions. Absorbance values were determined at 450 nm using a microplate reader. Relative cell viability levels were calculated using the following formula: Relative cell viability (%) = [(As-Ab)/(Ac-Ab)]×100, with As, Ab, and Ac denoting absorbance of experimental, blank, and control groups, respectively.

Wound healing assay

U251 cells were seeded at a density of 5×10^4 cells per well in 24-well plates. After culturing for 24 hours, every well was hand-scratched with a 200 μl plastic pipette tip, creating a uniform wound. They were washed three times with phosphate-buffered saline (PBS). Cells were pre-treated with baicalein (20, 40, and 80 μmol/L) for 4 hours, then incubated with hEGF (20 ng/mL) for 24 hours. Investigating the roles of EGFR signaling pathways in the migration of glioma cells, U251 cells were pre-treated with EGFR inhibitor AG1478 (10 μmol/L) for 1 hour. They were then incubated with hEGF (20 ng/mL) for 24 hours. Photographs of at least 3 random fields were obtained after incubation for 24 hours. The distance between 2 cell edges was detected using Image-pro plus software.

Transwell migration assays

Transwell assays were performed in 24-well Transwell chambers (Millipore, USA) including filters with 8 μm pores. Cells were seeded in the upper chambers at a density of 1×10^5 cells/well. They were cultured with baicalein at different concentrations and no FBS for 4 hours, then incubated with hEGF (20 ng/mL) for 12 hours. The lower chambers were added with medium containing 10% FBS. After incubation for 12 hours, the upper chambers were gently wiped with a cotton swab. They were then fixed with 4% paraformaldehyde for staining with 0.1% crystal violet.

TUNEL staining

Cells were washed in PBS, then fixed in 4% paraformaldehyde for 30 minutes at 4°C. Fixed cells were then washed in PBS three times. They were treated with permeabilization solution (0.2% Triton X-100 in PBS) at room temperature for 25 minutes. Next, the cells were dyed with TUNEL, according to manufacturer instructions (TUNEL Apoptosis Detection Kit (FITC), green, Yeasen, China). Afterward, Vector Laboratories containing DAPI (4', 6-diamidine-2-phenylindole) were used. VECTASHIELD of Burlingame (CA) was used to re-stain the cells, identifying the nucleus (blue). The cells were observed under a fluorescence microscope.

Western blot analysis

U251 cells were lysed in RIPA buffer containing 1% protease inhibitors. An equal amount of proteins in each sample were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. They were then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Membranes were placed in a blocking solution (5% non-fat milk in Tris-buffered saline [TBS] containing 0.1% Tween-20) at 37°C for 1 hour. Next, the membranes were incubated overnight at 4°C with antibodies against β-actin, p-Akt, p-EGFR, Akt, and EGFR. Antibodies were diluted in the blocking solution. Membranes were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies for 2 hours at 37°C. Bands were detected using an intensive chemiluminescence detection kit (Millipore) and exposed to X-ray film. Density analysis was executed using Image J software.

Immunofluorescence analysis

Cells were seeded at a density of 2×10^4 cells per dish in confocal culture dishes. After incubation for 24 hours, the cells were pre-treated with baicalein (80 μM) for 4 hours. They were then incubated with hEGF (20 ng/mL) for 24 hours. The cells were washed in PBS and fixed in 4% paraformaldehyde for 30 minutes at 4°C. Fixed cells were washed three times in PBS and treated with permeabilization solution (0.2% Triton X-100 in PBS) at room temperature for 25 minutes. Cells were blocked using 10% goat serum in PBS for 30 minutes at room temperature, then cultured with p-Akt or p-EGFR monoclonal antibodies overnight at 4°C and washed three times in TBS. They were further incubated with FITC-conjugated goat anti-rabbit secondary antibody for 1 hour in the dark. Nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI). Finally, the cells were observed under a fluorescence microscope.

Statistical analysis

Results are expressed as mean ± SD of at least three independent experiments. Differences between experimental groups were evaluated by one-way ANOVA analysis of variance. $P < 0.05$ indicates statistical significance.

Results

Baicalein inhibits proliferation and migration of U251 cells

The structure of baicalein is shown in **Figure 1A**. Investigating whether baicalein inhibits migration and proliferation of U251 cells, CCK, wound healing assays, and Transwell assays were conducted. Anti-proliferative effects of baicalein on U251 cells are shown in **Figure 1B**, as follows: 1) Following treatment with hEGF alone, proliferation of cells was promoted; and 2) Combination treatment with baicalein and hEGF resulted in obvious inhibition of cell proliferation, in a dose- and time-dependent manner. Anti-migration effects of baicalein on U251 cells showed no significant differences in gap distances existed between the groups immediately after scratching (**Figure 1C** and **1E**). After incubation for 24 hours, gap distances in the hEGF group nearly disappeared. However, gap distances in the baicalein group were blocked in a dose-dependent manner. Similarly, after incubation for 12 hours, treatment with baicalein inhibited migration of U251 cells, playing a role in a dose-dependent manner (**Figure 1D** and **1E**).

hEGF promotes proliferation and migration of U251 cells via EGFR/Akt pathways

Investigating the roles of EGFR signaling pathways in proliferation and migration of glioma cells, CCK and wound healing assays were conducted. Immunoblotting results showed that inhibition of EGFR activity with AG1478 downregulated levels of EGFR and Akt phosphorylation (**Figure 2A-C**). In addition, combination

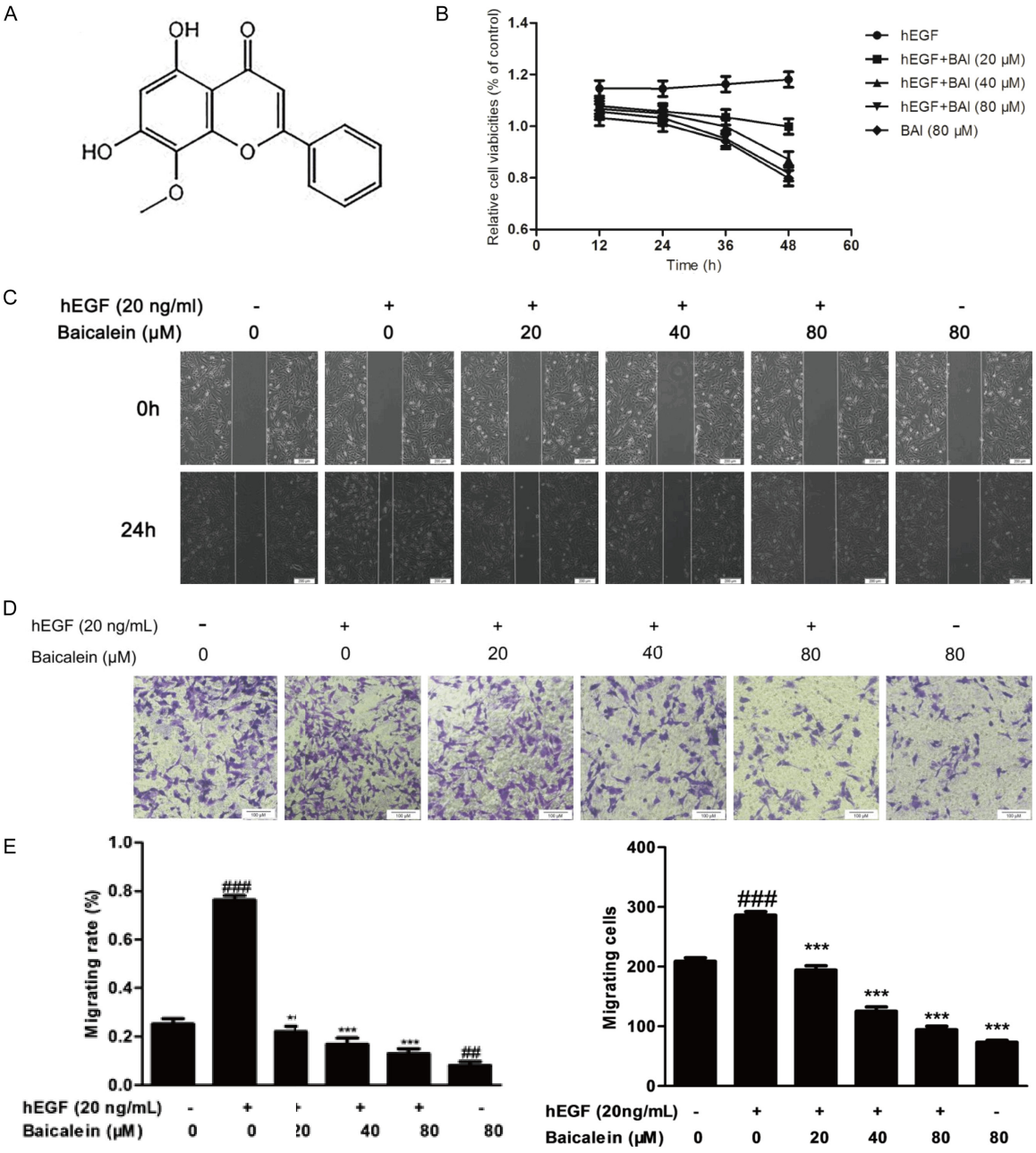

Figure 1. Effects of baicalein on U251 cell proliferation and migration. (A) Chemical structure of baicalein; (B) CCK assay; Cells were incubated with baicalein (20, 40 and 80 μM) and/or hEGF (20 ng/ml) for 12, 24, 36, and 48 hours. (C) and (E) Wound healing assay; Representative images captured under a phase contrast microscope at 0 and 24 hours of treatment with baicalein (20, 40, and 80 μM) and/or hEGF (20 ng/mL). The scale bar represents 200 μm. The inhibition of migration was transformed to the percentage of the initial distance between the two edges. The baicalein-treated cells showed a lower rate of wound closure than the control cells. (D) and (E) Transwell assays; cells were treated with baicalein (20, 40 and 80 μM, pretreated-for 4 h) and/or hEGF (20 ng/mL) for 12 hours (D). The scale bar represents 100 μm. The migration was transformed to the stained cells quantities (E). Data are represented as the mean ± SD for three independent experiments. ***P<0.001 vs. hEGF alone group; ##P<0.01, ###P<0.001 vs control group.

treatment with AG1478 and hEGF on cells resulted in obvious inhibition of cell proliferation and migration in a dose- and time-dependent manner (**Figure 2D-F**).

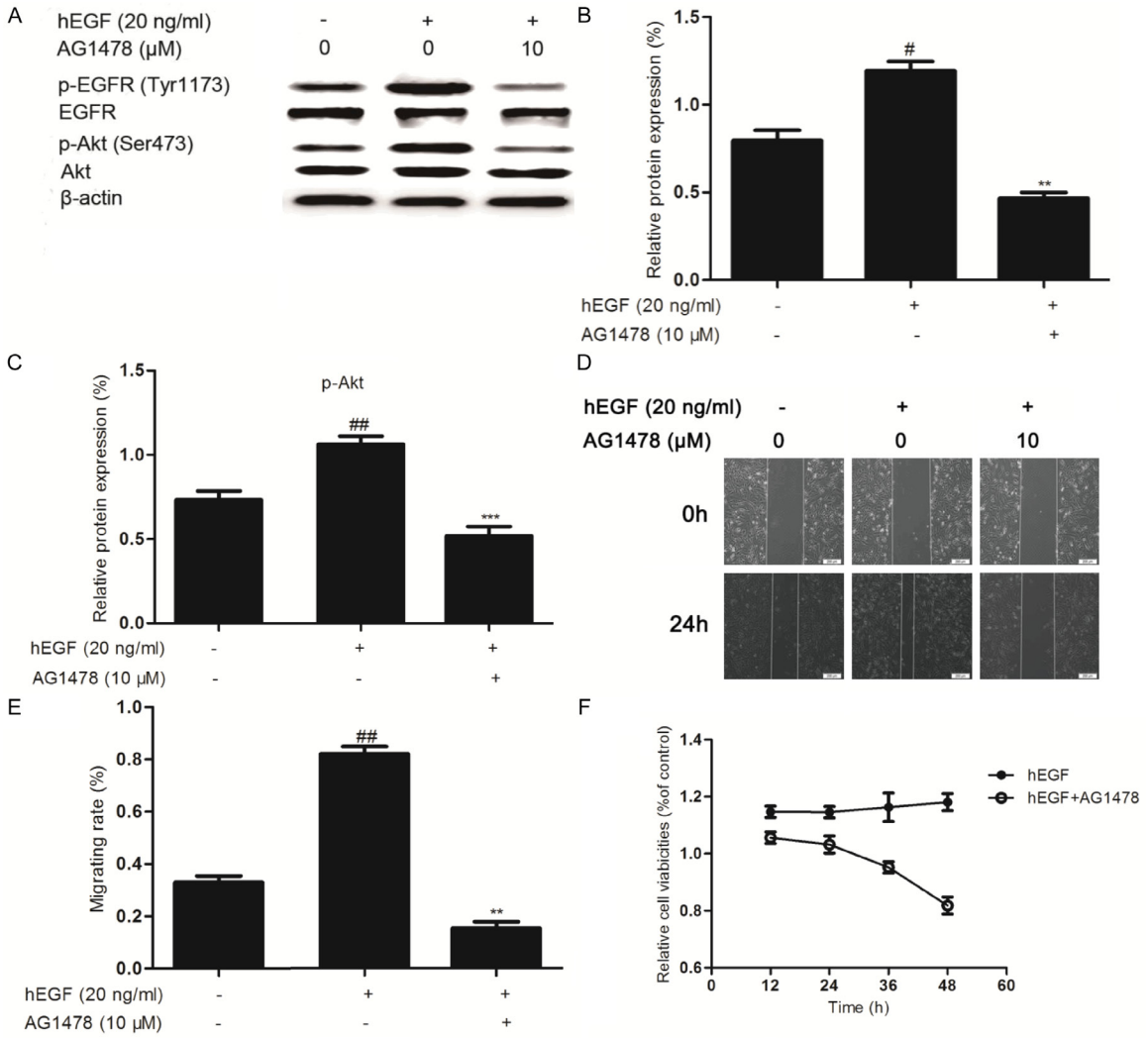

Figure 2. Blocking of EGFR/Akt pathways could suppress the proliferation and migration of U251 cells; A. Cells were pre-treated with the EGFR inhibitor, AG1478 (10 μM), for 1 hour, then incubated with hEGF (20 ng/mL) for 24 hours. EGFR and Akt phosphorylation were assessed by Western blot; B and C. Relative protein expression of p-EGFR and p-Akt were analyzed and presented by histograms; D. Wound healing assay; representative images captured under a phase contrast microscope at 0 and 24 hours of treatment with AG1478 (10 μM) and/or hEGF (20 ng/mL). The scale bar represents 200 μm. E. Inhibition of migration was transformed to the percentage of the initial distance between the two edges. AG1478-treated cells showed a lower rate of wound closure than the control cells; F. CCK assay; cells were pre-treated with the EGFR inhibitor, AG1478 (10 μM), for 1 hour, then incubated with hEGF (20 ng/mL) for 12, 24, 36, and 48 hours. All data are represented as the mean ± SD for three independent experiments. **P<0.01, ***P<0.001 vs. hEGF alone group; #P<0.05, ##P<0.01 vs. control group.

Baicalein suppresses migration and proliferation of U251 cells via EGFR/Akt pathways

Immunoblotting was used to detect the underlying mechanisms, explaining how baicalein exerts its actions. Treatment of U251 cells with baicalein (10, 20, 40, and 80 μM) and/or hEGF (20 ng/mL) for 24 hours resulted in marked downregulation of levels of EGFR and Akt phosphorylation (**Figures 3B**, **4A**). P-EGFR-to-EGFR and p-Akt-to-Akt ratios were also reduced in a dose-dependent manner (**Figures 3C**, **4B**). This study also treated U251 cells with baicalein (80 μM) and/or hEGF (20 ng/mL) for 6, 12, 24, and 36 hours. EGFR and Akt phosphorylation levels were markedly decreased at 24 and 36 hours (**Figures 3D**, **3E**, **4C**, **4D**). There was no apparent apoptosis in U251 cells treated with hEGF and baicalein for 24 hours (**Figure 3A**). In addition, U251 cells were treated with hEGF and/or

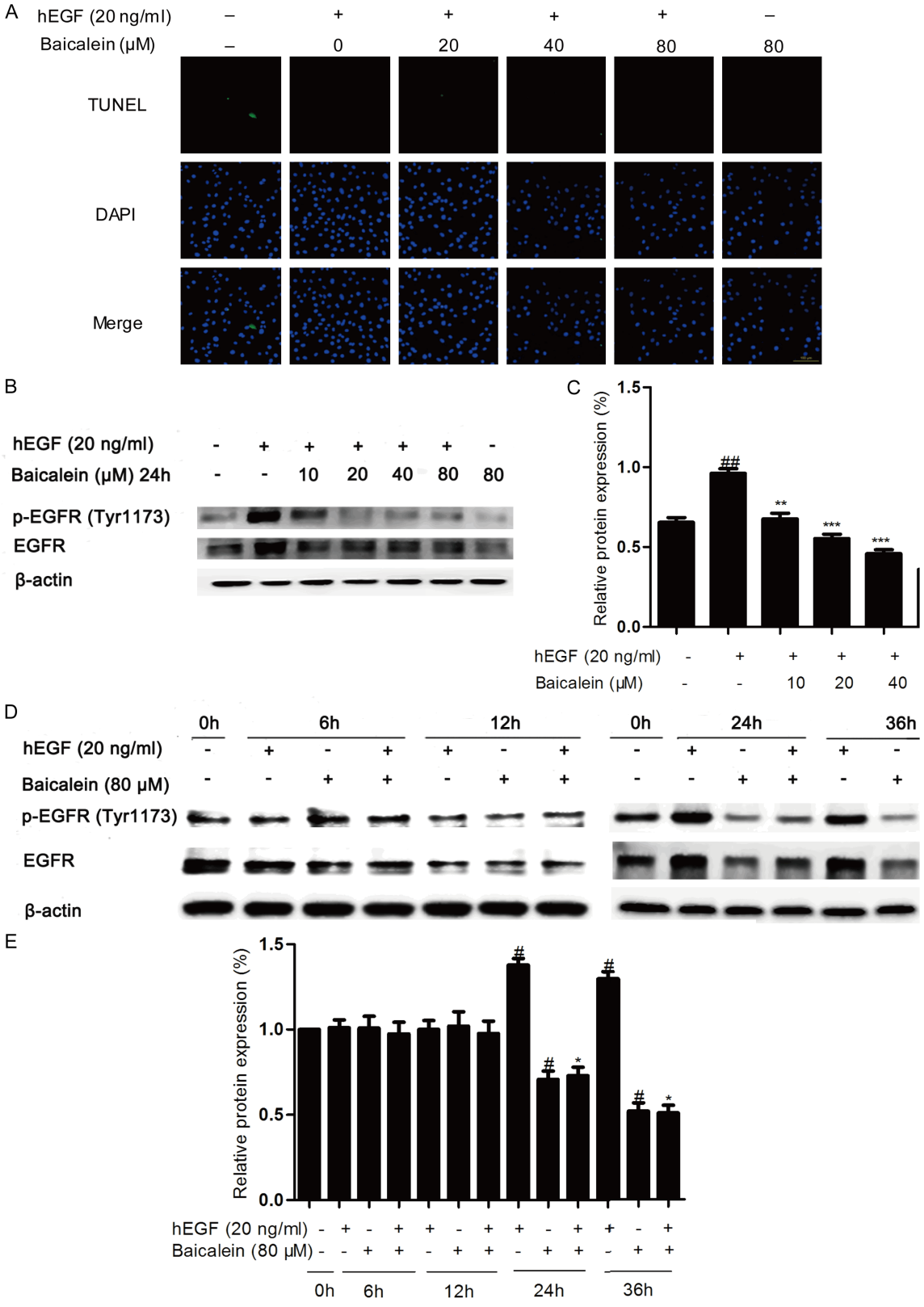

Figure 3. Effects of baicalein on hEGF-stimulated activation of p-EGFR and EGFR in U251 cells; A. TUNEL assays; Cells were treated with baicalein (20, 40 and 80 μM, pretreated-for 4 hours) and/or hEGF (20 ng/mL) for 12 hours. Apoptotic cells were detected by terminal dexynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assays. Green, TUNEL-positive nuclei; blue, 4',6-diamidino-2-phenylindole-stained nuclei. The scale bar repre-

sents 100 μm. B. Cells were treated with baicalein (10, 20, 40, and 80 μM, pre-treated for 4 hours) and/or hEGF (20 ng/mL) for 24 hour, then the expression of p-EGFR and EGFR protein was measured by Western blot; D. Western blotting to measure the protein expression of p-EGFR and EGFR. Cells treated with baicalein (80 μM, pre-treated for 4 hours) and/or hEGF (20 ng/mL) for 6, 12, 24, and 36 hours; C and E. Pixel densities of proteins were analyzed and presented by histograms. Data are represented as the mean ± SD for three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. hEGF alone group; #P<0.05, ##P<0.01 vs. control group.

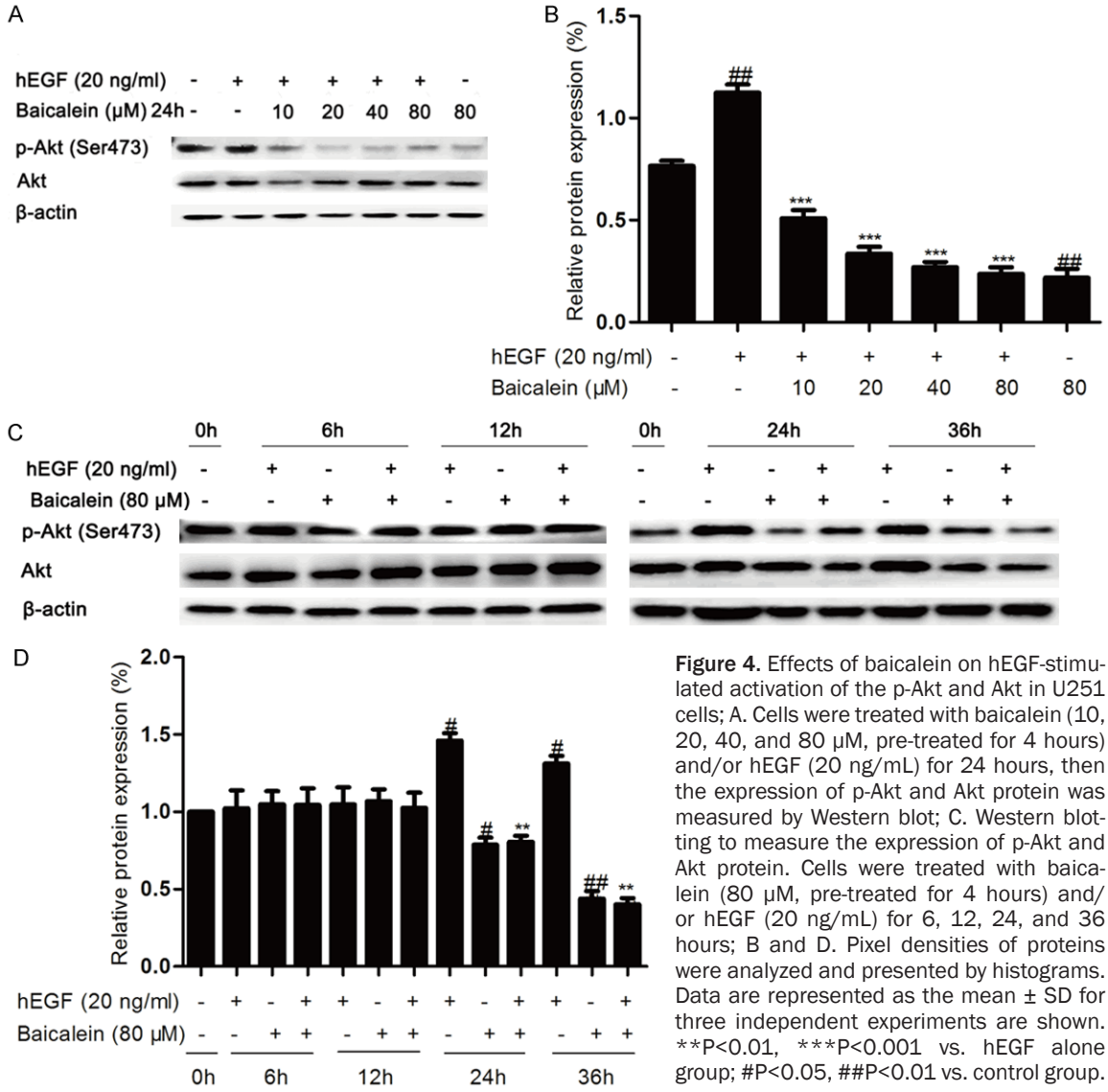

Figure 4. Effects of baicalein on hEGF-stimulated activation of the p-Akt and Akt in U251 cells; A. Cells were treated with baicalein (10, 20, 40, and 80 μM, pre-treated for 4 hours) and/or hEGF (20 ng/mL) for 24 hours, then the expression of p-Akt and Akt protein was measured by Western blot; C. Western blotting to measure the expression of p-Akt and Akt protein. Cells were treated with baicalein (80 μM, pre-treated for 4 hours) and/or hEGF (20 ng/mL) for 6, 12, 24, and 36 hours; B and D. Pixel densities of proteins were analyzed and presented by histograms. Data are represented as the mean ± SD for three independent experiments are shown. **P<0.01, ***P<0.001 vs. hEGF alone group; #P<0.05, ##P<0.01 vs. control group.

baicalein at the same concentration for 24 hours. Thereafter, expression of p-EGFR and p-Akt was measured using immunofluorescence staining. The fluorescence intensity of U251 MG after treatment with hEGF was higher than that in the control group. However, treatment with baicalein and hEGF resulted in significant downregulation of levels of EGFR and Akt phosphorylation (**Figure 5A**, **5B**).

Discussion

GBM is very difficult to cure using traditional therapeutic methods, including radiotherapy, surgery, and chemotherapy. Indeed, GBM often recurs, largely because of its highly aggressive and chemo-resistant properties. Proliferation and metastasis are the main causes of deaths in patients with GBM. Metastasis is a compli-

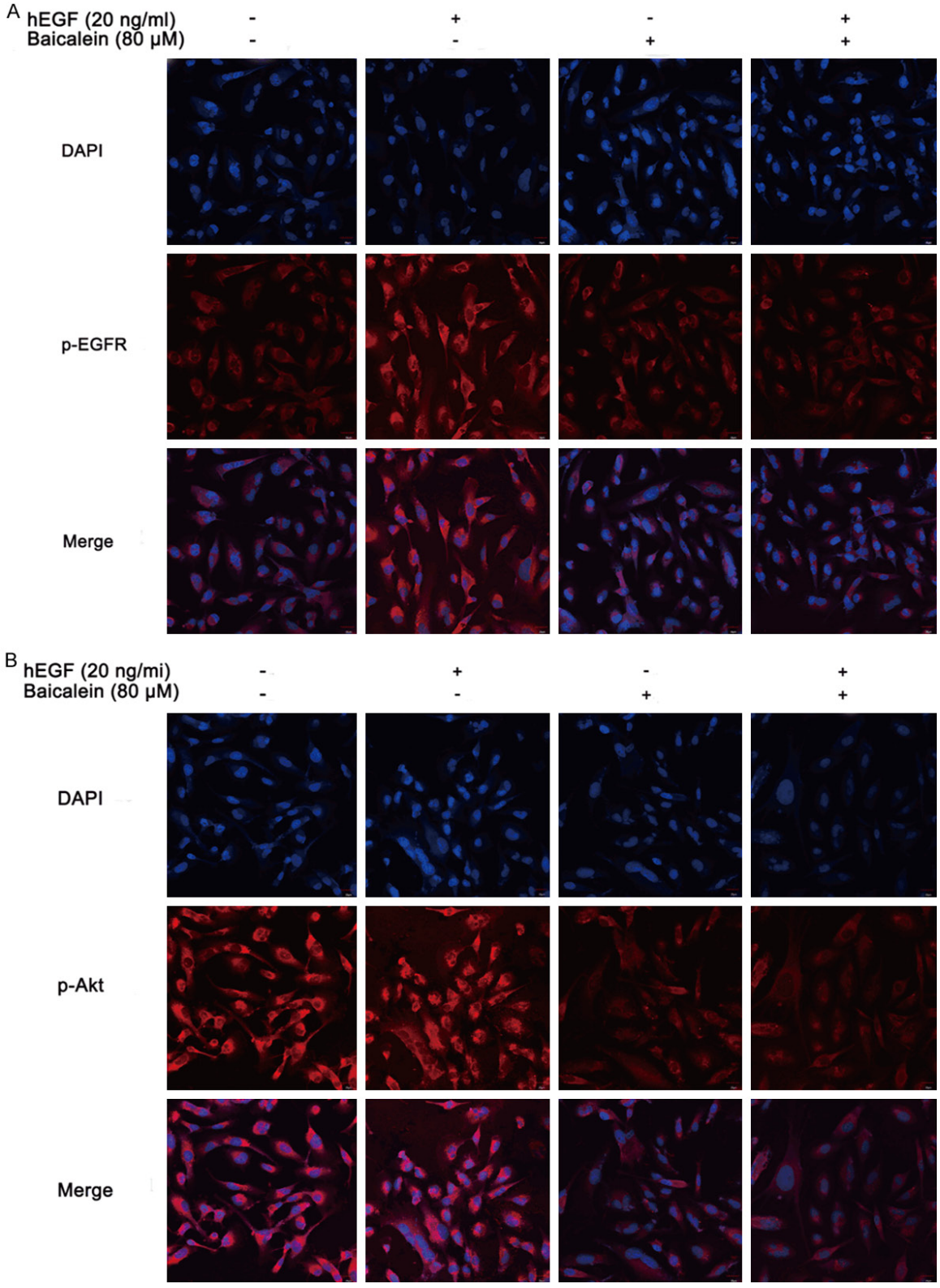

Figure 5. Effects of baicalein on expression of p-EGFR and p-Akt in U251 cells. Cells were treated with baicalein (80 μM, pre-treated for 4 hours) and/or hEGF (20 ng/mL) for 24 hours. Expression of p-EGFR (red, A) and p-Akt (red, B) was determined after immunofluorescent staining. DAPI (blue) was used for nuclei staining. The scale bar represents 20 μm.

cated multi-step process associated with cell migration and invasion. Therefore, severance of these steps constitutes an anti-metastatic treatment [26].

Growth, survival, and migration of cells are dependent on the intricate regulation of signaling cascades mediated through growth factors, cytokines, and their receptors. Many growth factors have been shown to promote the progression of diverse cancers by induction of proliferation, migration, invasion, and angiogenesis. Of these growth factors, epidermal growth factor (EGF) has been thought to play important roles in gliomagenesis, as EFG can promote proliferation and survival and inhibit differentiation of neural stem cells [3]. GBMs express endogenous low molecular weight peptide ligands, such as EGF or TGF-α [27, 28], which activate EGFR and are consistent with the existence of an autocrine growth stimulatory loop.

Many studies have confirmed that EGFR/Akt signaling pathways are hyperactive in a variety of cancers [29-31]. The most common receptor tyrosine kinase (RTK), EGFR is activated when the extracellular domain binds with its ligands, giving rise to recruitment of PI3K to cell membranes. Activated PI3K phosphorylates phosphatidynositol-4,5-bisphosphate (PIP2) to generate the second messenger phosphatidylinositol-3-phosphate (PIP3). This functions as a high-affinity binding ligand to recruit and activate downstream effector molecules, such as Akt (protein kinase B), giving rise to cell proliferation and promoting cell survival by suspending apoptosis. Abnormal activation in this signaling pathway may result in impaired apoptosis, uncontrolled proliferation, and neoplastic transformation [32-34]. EGFR genes are amplified and overexpressed at high frequencies in primary GBM. Amplification occurs in approximately 45% and overexpression in > 60% of cases [35-37]. Prominent overexpression of EGFR in GBMs suggests that EGFR and downstream signaling pathways, such as PI3K/Akt, RAF/MEK/ERK, and JAK/STAT, are critical for proliferation and migration of GBM. Inhibition of this pathway has been considered a target for cancer therapy [38-41]. The current study confirmed that there is a persistent phosphorylation phenomenon of EGFR in U251 cells at its normal proliferation. Following stimulation by EGF, levels of EGFR phosphorylation and its downstream signal (Akt) were markedly increased, compared with control cells. In contrast, AG1478, an EGFR inhibitor, treatment effectively downregulated levels of EGFR and Akt phosphorylation, inhibiting proliferation and migration of U251 cells stimulated by hEGF treatment. These results suggest that EGF induces excessive proliferation and migration through EGFR/Akt pathways, playing a crucial role in the growth of U251 cells.

Many studies have shown that baicalein has beneficial effects on human health and treatment. Baicalein has been shown to have antitumor effects in various types of cancers [11-19, 21, 22, 42, 43], with no or less toxic effects on normal epithelial cells [13, 15, 21]. The definite molecular mechanisms of baicalein inhibition of tumor cell growth remain unknown. In hepatocellular carcinoma, PI3K/Akt and MEK-ERK signaling pathways are involved in baicalein-induced cancer growth arrest. Baicalein inhibits activation of ERK1/2, MEK1, and Bad phosphorylation *in vivo* and in baicalein-treated xenograft tumors. This leads to mitochondrial signaling-related apoptosis [15]. Inhibition of this pathway may also result in attenuated cell migration and invasion by blocking multiple proteases degrading the extracellular matrix [17]. Another study confirmed that baicalein can induce G0/G1-phase arrest, inhibit phosphorylated Akt levels, and promote degradation of β-catenin and cyclin D1, without activation of GSK-3β in the hepatocellular carcinoma cell line, H22, and murine xenograft tumors [16]. ROS has been thought to participate in baicalein-induced inhibition of proliferation in colorectal cancer. Proteomic approaches have illustrated that baicalein can increase expression of levels of peroxiredoxin-6 (PRDX6), using siRNAs specific for PRDX6 results in ROS production and proliferation in colorectal cancer cells [21]. Aryal, P. et al. [44] offered a new mechanism, suggesting that baicalein suppresses the growth of human cancer cells by inducing autophagic cell death in human prostate and breast cancer cells. This progress involves AMP-activated protein kinase (AMPK)-ULK1 activation through modulating expression levels of anti-autophagic molecules, such as mTOR and Raptor.

However, the effects of baicalein on proliferation and migration of tumor cells in GBM, as well as associated mechanisms, have not been elucidated. As shown, based on CCK and wound

healing assays, 80 μM baicalein, alone, markedly inhibited migration and proliferation of U251 cells, compared with control cells. This is in agreement with previous studies concerning other cancer cells [17, 18, 22]. Baicalein, at concentrations between 20 μM and 80 μM, antagonized proliferation- and migration-promotingeffects of hEGF, in a concentration-dependent manner. Considering the important roles of EGFR/Akt signaling pathways in the regulation of glioma cell proliferation stimulated by hEGF, the present study focused on EGFR/Akt signaling to explain baicalein-induced anti-glioma properties. After treatment with hEGF, phosphorylation of EGFR and Akt was significantly activated, leading to fast proliferation and migration in U251 cells (**Figures 1B** and **4**). Baicalein was shown to reduce overexpression of P-EGFR and p-Akt, in a dose- and time-dependent manner, resulting in inhibition of proliferation and migration.

EGFR class III variant (EGFRvIII), which lacks a portion of the extracellular ligand-binding domain, exists in approximately 30% of patients with GBMs [45]. EGFRvIII is constitutively autophosphorylated. Thus, it plays an important role in tumorigenicity of gliomas [3, 46]. In the present study, effects of baicalein on expression of EGFRvIII were not observed. Therefore, further research is warranted.

Conclusion

In summary, present data indicates that baicalein can inhibit migration and proliferation of U251 cells by reducing expression of p-EGFR and p-Akt. Results also suggest that baicalein is a potential therapeutic target for treatment of GBM. Additional studies concentrating on the specificity of baicalein *in vivo* are necessary.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

GBM, glioblastoma multiforme; CNS, central nervous system; DMSO, dimethylsulfoxide; hEGF, human recombinant epidermal growth factor; CCK, cell counting Kit; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; EGFR, epidermal growth factor receptor; Akt, protein kinase B.

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