Neuroglobin promotes the proliferation and suppresses the apoptosis of glioma cells by activating the PI3K/AKT pathway

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Abstract. Our previous study demonstrated that neuroglobin (Ngb) functions as an independent predictive indicator of the prognosis of patients with glioma and promotes cancer cell growth by suppressing apoptosis. However, the understanding of the mechanisms underlying the survival-enhancing function of Ngb in glioma is limited. In the present study, KEGG PathwayFinder by gene correlation analysis was performed on the R2: Genomics Analysis and Visualization Platform, which revealed a high association between Ngb and the phosphatidylinositol 3-kinase (PI3K)/AKT pathway using glioma data (GSE4290) from the Gene Expression Omnibus database. Furthermore, western blotting experiments were performed in U251 and U87 glioma cells, and Ngb knockdown using short hairpin RNA reduced the protein levels of phosphorylated (p)-AKT, p-mammalian target of rapamycin (mTOR) and antiapoptotic factor Bcl-2, and increased the expression of the proapoptotic protein Bcl-2-associated X, in U251 cells. In addition, Ngb overexpression promoted the activation of the PI3K/AKT pathway in U87 cells. MK2206, a PI3K/AKT signaling inhibitor, reduced the expression of p-AKT and increased the levels of apoptosis-associated proteins, including cleaved poly(ADP-ribose) polymerase 1 and cleaved caspase-3/7/8, in Ngb-overexpressing U87 cells. Furthermore, MK2206 treatment reduced the proliferation and induced the apoptosis of Ngb-overexpressing U87 cells, as indicated by the results of MTT, colony formation and flow cytometry assays. In addition, insulin-like growth factor-1, a PI3K/AKT signaling activator, reversed Ngb knockdown-induced growth arrest and apoptosis in U251 cells. In conclusion, the results of the present study indicate that Ngb may facilitate a malignant phenotype of glioma cells by activating the PI3K/AKT pathway.

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Introduction

Glioma, the most common form of brain malignancy, is one a major contributor to cancer-associated deaths worldwide (1). The strategies employed for the diagnosis and treatment of glioma have improved over the past several decades (2). However, the prognosis of patients with glioma remains poor, with a survival time of 12-18 months post-diagnosis (3). Therefore, the identification of the exact mechanisms underlying the malignant phenotype of glioma cells is required.

Neuroglobin (Ngb), a novel tumor-associated protein, functions as an oncogene or tumor suppressor in human cancer. Previous reports have demonstrated that overexpression of Ngb enhances reactive oxygen species scavenging and reverses oxidative stress-induced cell death in neuroblastoma cells (4-6). The expression of Ngb is upregulated under hypoxic conditions in glioblastoma cells and tumor xenografts, indicating a potential role of Ngb in cancer cell survival in hypoxic microenvironments (7,8). In addition to brain tumors, aberrant expression of Ngb has also been reported in other types of malignancies. For example, Ngb is reported to be overexpressed in certain non-small cell lung cancer cases, particularly in squamous cell carcinomas (9). Notably, 17β-estradiol induces Ngb upregulation, which renders cancer cells, including MCF-7, HepG2, SK-N-BE, HeLa and DLD-1, resistant to oxidative stress (10-13). However, Ngb expression is downregulated in hepatocellular carcinoma tissues and its silencing promotes the proliferation and cell cycle progression of cancer cells (14). Our previous study demonstrated that Ngb functions as an independent prognostic biomarker for patients with glioma and promotes the growth of cancer cells by suppressing apoptosis (15). However, the mechanisms underlying the survival-enhancing effect of Ngb in glioma remains a challenge, therefore, the present study aimed to investigate the effect and mechanisms of Ngb in glioma.

The results of the present study demonstrated that Ngb promoted the proliferation and inhibited the apoptosis of glioma cells, which may occur through effects on the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. To the best of our knowledge, the presents study is the first to indicate that Ngb may be a potential therapeutic target for glioma.

Materials and methods

Cell culture and transfection. U87MG ATCC and U251MG human glioma cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultivated in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C in a humidified incubator containing 5% CO_2.

Short hairpin RNA (shRNA) targeting Ngb (5'-GUGAGU CCCUGCUCUACAU-3') and non-targeting (NT) shRNA (5'-GCCACACGAUUGCUGUCUU-3') were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Vectors (1 μg) were transfected into cells at 50-70% confluency using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Retroviral vector pMMP-Ngb was generated, packaged and transduced as previously described (16). pMMP is a MFG-based vector with modifications from the myeloproliferative sarcoma virus (17) and primer binding sequence (18) vector systems. pMMP vector alone was used as the control for Ngb overexpression experiments. AKT inhibitor, MK2206 (1 μM; 37˚C for 48 h; Selleck Chemicals, Houston, TX, USA), and insulin-like growth factor-1 (IGF-1; 10 ng/ml; 37˚C for 48 h; Sigma-Aldrich; Merck KGaA) were used to treat glioma cells 24 h post-transfection, according to the manufacturer's protocol. Control cells were treated with dimethylsulphoxide (DMSO; EMD Millipore, Billerica, MA, USA).

Bioinformatics analysis. KEGG PathwayFinder by gene correlation analysis in the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) was performed to investigate the association between Ngb and various signaling pathways according to glioma data (GSE4290) (19).

MTT assay. Glioma cells (2x10^3 cells/well) were seeded in 96-well plates containing 100 μl DMEM per well. U87 cells were treated with either DMSO or MK2206 (1 μM; 37˚C for 48 h) 24 h following Ngb retrovirus infection. U251 cells were treated with either DMSO or IGF-1 (10 ng/ml; 37˚C for 48 h) 24 h post-transfection with Ngb shRNA. Following transfection for 24, 48, 72 and 96 h time intervals, 10 μl of MTT was added into each well and incubated at 37˚C for 4 h. Subsequently, 150 μl DMSO was added per well and the absorbance was determined using microplate reader at 490 nm.

Colony formation assay. Glioma cells (1x10^3 cells/well) that were transfected with the corresponding vectors were cultured in 6-well plates and maintained at 37˚C in humidified cell incubators containing 5% CO_2 for 14-21 days. U87 cells were treated with either DMSO or MK2206 (1 μM; 37˚C for 48 h) 24 h following infection with Ngb retroviruses. U251 cells were treated with either DMSO or IGF-1 (10 ng/ml; 37˚C for 48 h) 24 h following transfection with Ngb shRNA. The formed cell colonies were stained with crystal violet (0.05%; 20 min at room temperature) and counted to represent the cell proliferation of glioma cells.

Apoptosis analysis. Apoptosis in glioma cells following transfection was detected by using an Annexin V/propidium iodide (PI) kit (BD Pharmingen; BD Biosciences, San Jose, CA, USA). U87 cell were treated with either DMSO or MK2206 (1 μM; 37˚C for 48 h) 24 h post-infection with Ngb retroviruses. U251 cells were treated with DMSO or IGF-1 (10 ng/ml; 37˚C for 48 h) 24 h post-transfection with Ngb shRNA. Briefly, glioma cells were resuspended in 1X binding buffer at a concentration of 1x10^6 cells/ml. A total of 100 μl of solution (1x10^5 cells) was transferred to a 5 ml culture tube and supplemented with 5 μl of Annexin V-fluorescein isothiocyanate and 5 μl of PI. Following incubation for 15 min at 25˚C in the dark and with supplementation of 1X binding buffer (400 μl), the percentage ratio of apoptotic glioma cells was detected using FACSCalibur flow cytometer (BD Biosciences) and CellQuest Pro software (version 5.1.1; BD Biosciences).

Western blotting. U87 cells were treated with either DMSO or MK2206 (1 μM; 37˚C for 48 h) 24 h following infection with Ngb retroviruses. U251 cells were treated with either DMSO or IGF-1 (10 ng/ml; 37˚C for 48 h) 24 h post-transfection with Ngb shRNA. The transfected cells were lysed by radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology) for total protein extracts, followed by quantification with a Bradford Protein assay kit (Beyotime Institute of Biotechnology). Cell lysates (40 μg/lane) were separated by 10% SDS-PAGE. After being transferred to polyvinylidene fluoride membranes (Sigma-Aldrich; Merck KGaA) and blocked with 5% non-fat milk for 1 h at room temperature, the membranes were incubated with primary antibodies against GAPDH (1:5,000; cat. no. G8140-01; US Biological, Salem, MA, USA), Ngb (1:1,000; cat. no. ab37258; Abcam, Cambridge, MA, USA), AKT (1:1,000; cat. no. 9272; Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated (p)-AKT (Ser473; 1:2,000; cat. no. 4060; Cell Signaling Technology, Inc.), mammalian target of rapamycin (mTOR; 1:1,000; cat. no. 2983; Cell Signaling Technology, Inc.), p-mTOR (Ser2448; 1:1,000; cat. no. 5536; Cell Signaling Technology, Inc.), Bcl-2 (1:1,000; cat. no. 15071; Cell Signaling Technology, Inc.), Bcl-2-associated X (Bax; 1:1,000; cat. no. 5023; Cell Signaling Technology, Inc.), cleaved poly(ADP-ribose) polymerase 1 (PARP; 1:1,000; cat. no. 5625; Cell Signaling Technology, Inc.), cleaved caspase-3 (1:1,000, cat. no. 9664; Cell Signaling Technology, Inc.), cleaved caspase-7 (1:1,000; cat. no. 8438; Cell Signaling Technology, Inc.) and cleaved caspase-8 (1:1,000; cat. no. 9496; Cell Signaling Technology, Inc.) at 4˚C overnight. Subsequently, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit and horse anti-mouse secondary antibodies for 1 h at room temperature (1:1,000; cat. nos. 7074 and 7076, respectively; Cell Signaling Technology, Inc.). GAPDH was employed as a loading control. Luminata Fe Western HRP Substrate (EMD Millipore, Billerica, MA, USA) was used to visualize proteins. A Bio-Rad Gel imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to quantify western blotting data. Image J software (version 1.41; National Institutes of Health, Bethesda, MD, USA) was used to quantify protein levels.

Table I. KEGG PathwayFinder by Gene correlation of the GSE4290 dataset.

Group	In_Set	Total	Percentage, %	P-value
Retrograde_endocannabinoid_signaling	79	92	85.90	$1.30x10^{-6}$
Nicotine_addiction	33	35	94.30	$6.10x10^{-5}$
PI3K_Akt_signaling_pathway	167	225	74.20	$6.70x10^{-5}$
GABAergic_synapse	65	79	82.30	$1.30x10^{-4}$
Synaptic_vesicle_cycle	51	60	85.00	$1.60x10^{-4}$
Glutamatergic_synapse	83	106	78.30	$3.20x10^{-4}$
Dopaminergic_synapse	88	114	77.20	$4.90x10^{-4}$
Axon_guidance	94	123	76.40	$5.70x10^{-4}$
DNA_replication	32	36	88.90	$6.70x10^{-4}$
Circadian_entrainment	65	82	79.30	$8.30x10^{-4}$
Morphine_addiction	66	84	78.60	$1.10x10^{-3}$
Long_term_potentiation	49	60	81.70	$1.20x10^{-3}$
Spliceosome	89	118	75.40	$1.60x10^{-3}$
Cholinergic_synapse	74	97	76.30	$2.40x10^{-3}$
Endocytosis	167	236	70.80	$2.80x10^{-3}$
Pancreatic_cancer	52	66	78.80	$3.50x10^{-3}$
Protein_processing_in_endoplasmic_reticulum	110	151	72.80	$3.50x10^{-3}$
Serotonergic_synapse	67	88	76.10	$4.20x10^{-3}$
Amphetamine_addiction	46	58	79.30	$4.80x10^{-3}$
Oxytocin_signaling_pathway	102	140	72.90	$4.90x10^{-3}$
Long_term_depression	45	57	78.90	$6.20x10^{-3}$
Amyotrophic_lateral_sclerosis__ALS_	40	50	80.00	$6.60x10^{-3}$
Aldosterone_synthesis_and_secretion	50	65	76.90	$9.60x10^{-3}$
Fc_gamma_R_mediated_phagocytosis	63	84	75.00	$9.80x10^{-3}$
Hepatitis_B	98	136	72.10	$9.90x10^{-3}$

In_Set, number of genes in pathway that significantly correlate with Ngb expression; Total, total number of genes in pathway; Percentage, %, ratio of Ngb-associated genes in total number of genes in pathway.

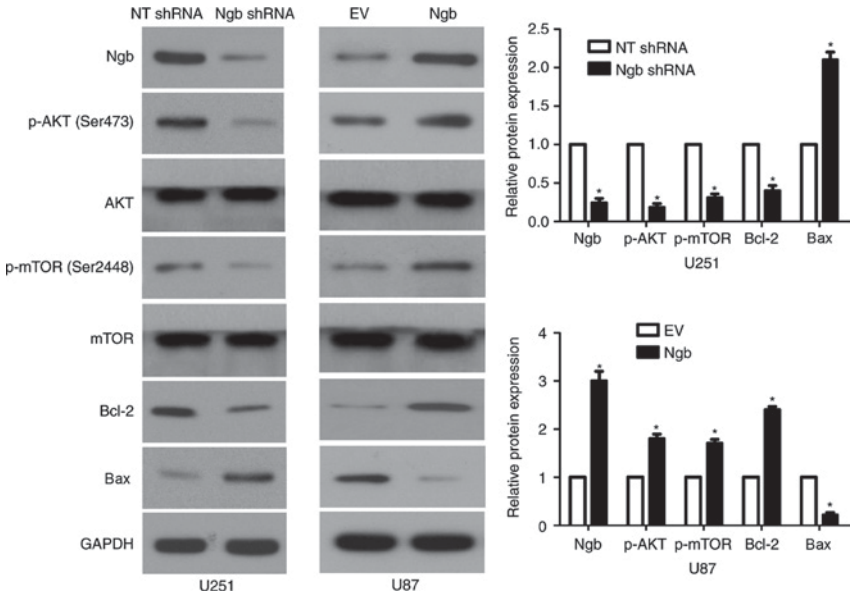

Figure 1. Ngb regulates the activation of the PI3K/AKT pathway in glioma cells. U251 cells that were transfected with NT shRNA and Ngb shRNA were subjected to western blot analysis. Ngb knockdown led to decreased levels of p-AKT, p-mTOR and Bcl-2, and increased Bax expression, in U251 cells. U87 cells that were transfected with EV and Ngb overexpression vector were subjected to western blot analysis. Ngb overexpression enhanced the activation of the PI3K/AKT pathway in U87 cells, as p-AKT levels were increased compared with the EV group. *P<0.05 vs. NT shRNA group for U251 cells; *P<0.05 vs. EV group for U87 cells. PI3K, phosphatidylinositol 3-kinase; NT, non-targeting; shRNA, short hairpin RNA; Ngb, neuroglobin; p-, phosphorylated-; mTOR, mammalian target of rapamycin; Bax, Bcl-2-associated X; EV, empty vector.

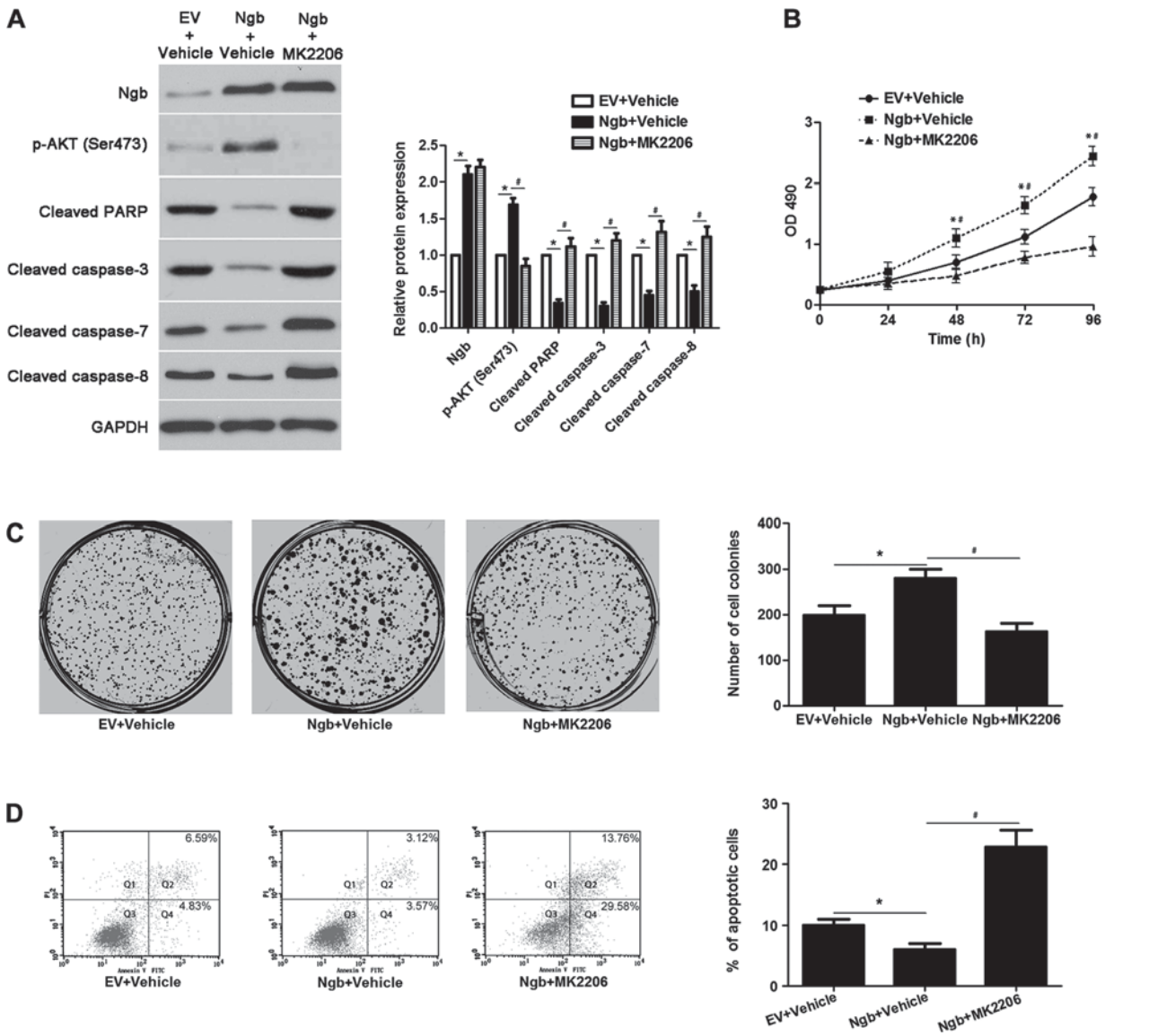

Figure 2. MK2206 treatment abolishes the effects of Ngb in U87 cells. (A) Ngb-overexpressing U87 cells that were treated with vehicle or the AKT inhibitor MK2206, respectively, were detected by western blotting. MK2206 treatment reduced p-AKT (Ser473) expression, and increased the levels of cleaved PARP and cleaved caspase-3/7/8, without affecting Ngb expression. *P<0.05 vs. EV + vehicle group; #P<0.05 vs. Ngb + vehicle group, as indicated; n=3. (B) MK2206 treatment restrained the proliferation of Ngb-overexpressing U87 cells. *P<0.05 vs. EV + vehicle group; #P<0.05 vs. Ngb + vehicle group; n=3. (C) The colony formation ability of Ngb-overexpressing U87 cells was weakened by MK2206 treatment. *P<0.05 vs. EV + vehicle group; #P<0.05 vs. Ngb + vehicle group; n=3. (D) The proportion of apoptotic cells was increased following MK2206 treatment in Ngb-overexpressing U87 cells. Q2 and Q4 quadrants were considered to indicate apoptotic cells. *P<0.05 vs. EV + vehicle group; #P<0.05 vs. Ngb + vehicle group; n=3. Ngb, neuroglobin; p-, phosphorylated-; PARP, poly(ADP-ribose) polymerase 1; EV, empty vector; OD, optical density; PI, propidium iodide; FITC, fluorescein isothiocyanate.

Statistical analysis. Data are presented as the mean ± standard deviation and were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Student's t-test or one-way ANOVA followed by the post hoc Tukey's test were employed to analyze continuous variables. P<0.05 was considered to indicate a statistically significant difference.

Results

PI3K/AKT pathway is a candidate target of Ngb in glioma. To investigate the potential mechanisms underlying the survival-enhancing effect of Ngb in glioma, KEGG PathwayFinder by gene correlation analysis using the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) was performed to investigate the association between Ngb and various signaling pathways in glioma. Base on the Gene Expression Omnibus (GEO) data (GSE4290) from the R2: Genomics Analysis and Visualization Platform, the results demonstrated that Ngb was strongly associated with the PI3K/AKT pathway in glioma (P<0.001; Table I). Therefore, the PI3K/AKT pathway is a candidate target of Ngb in glioma.

Ngb regulates the PI3K/AKT pathway in glioma cells. Subsequently, the present study investigated the activation of the PI3K/AKT pathway following modulation of Ngb levels by transfection. Ngb was silenced in U251 cells following transfection with shRNA targeting Ngb, compared with the NT shRNA group, as protein levels were reduced (P<0.05;

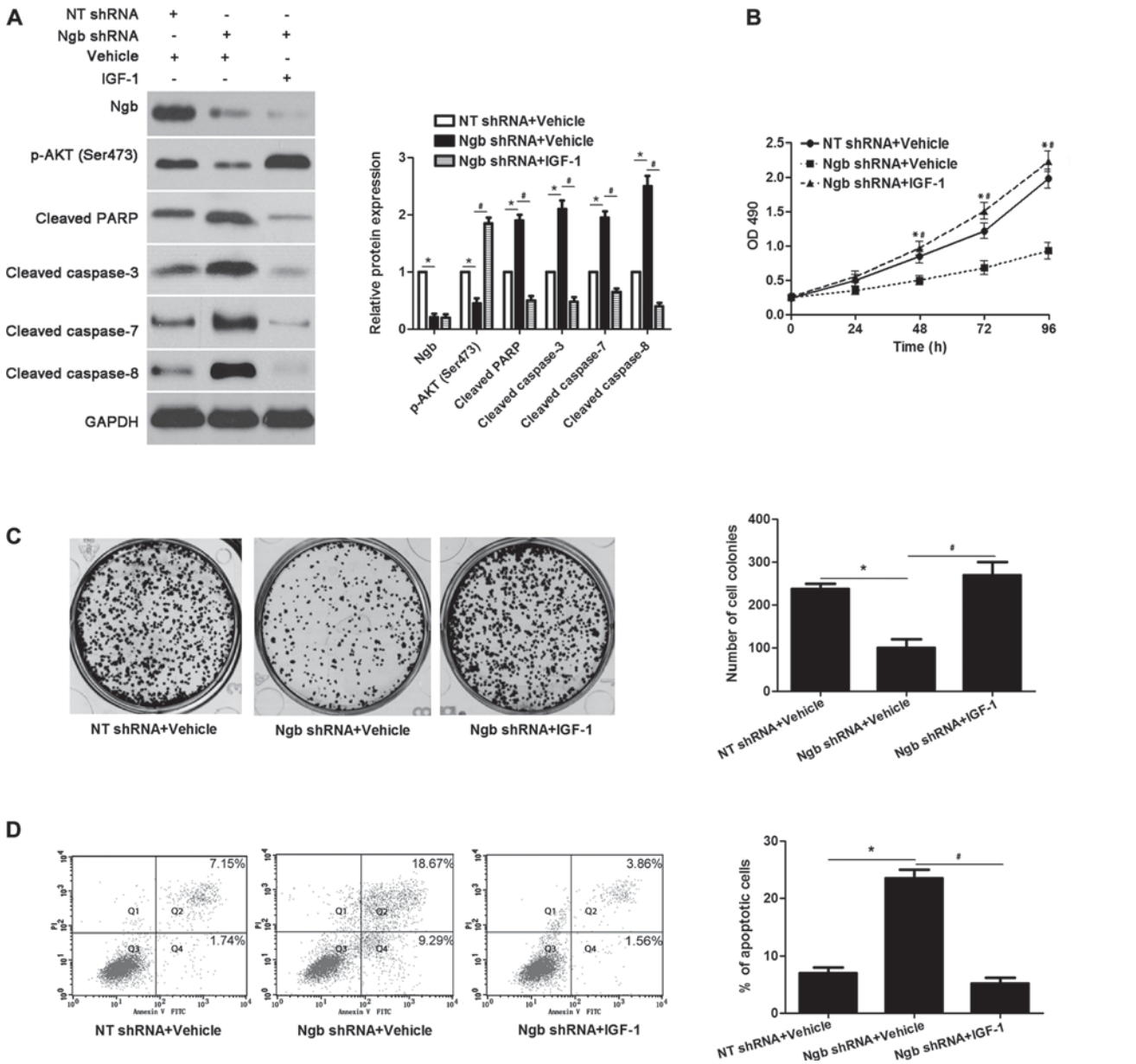

Figure 3. IGF-1 treatment reverses the effects of Ngb knockdown in U251 cells. (A) U251 cells with knockdown of Ngb using shRNA were treated with IGF-1, which is an activator of the PI3K/AKT pathway, or vehicle. Western blotting revealed that IGF-1 treatment enhanced the activation of the PI3K/AKT pathway, and reduced levels of cleaved PARP and cleaved caspase-3/7/8, in Ngb-knockdown U251 cells. *P<0.05 vs. NT shRNA + vehicle group; #P<0.05 vs. Ngb shRNA + vehicle group, as indicated; n=3. (B) IGF-1 treatment facilitated the proliferation of Ngb-knockdown U251 cells. *P<0.05 vs. NT shRNA + vehicle group; #P<0.05 vs. Ngb shRNA + vehicle group; n=3. (C) The colony formation ability in Ngb-knockdown U251 cells was increased following IGF-1 treatment. *P<0.05 vs. NT shRNA + vehicle group; #P<0.05 vs. Ngb shRNA + vehicle group, as indicated; n=3. (D) IGF-1 treatment significantly decreased the apoptosis of Ngb-knockdown U251 cells. Q2 and Q4 quadrants were considered to indicate apoptotic cells. *P<0.05 vs. NT shRNA + vehicle group; #P<0.05 vs. Ngb shRNA + vehicle group; n=3. IGF-1, insulin-like growth factor-1; Ngb, neuroglobin; shRNA, short hairpin RNA; PI3K, phosphatidylinositol 3-kinase; PARP, poly(ADP-ribose) polymerase 1; NT shRNA; non-targeting shRNA; p-, phosphorylated-; OD, optical density; PI, propidium iodide; FITC, fluorescein isothiocyanate.

Fig. 1). In addition, Ngb knockdown notably reduced the level of p-AKT (Ser473) compared with the NT shRNA group, without affecting total AKT levels, in U251 cells (P<0.05; Fig. 1). Furthermore, p-mTOR (Ser2448), Bcl-2 and Bax, which are downstream targets of the PI3K/ATK pathway, were also modulated by Ngb knockdown. Ngb knockdown led to reduced levels of p-mTOR (Ser2448) and Bcl-2, and increased BAX expression, in U251 cells, compared with the NT shRNA group (P<0.05; Fig. 1). By contrast, Ngb overexpression promoted the activation of the PI3K/AKT pathway, with increased levels of p-AKT (Ser473), p-mTOR (Ser2448) and Bcl-2, and decreased BAX expression, in U87 cells, compared with the empty vector control cells (P<0.05; Fig. 1). These data indicate that Ngb may promote the activation of the PI3K/AKT pathway in glioma cells.

Ngb promotes cellular malignant phenotypes of glioma by targeting the PI3K/AKT pathway. The present study further investigated whether Ngb regulated the proliferation and apoptosis of glioma cells through targeting the PI3K/AKT pathway. Overexpression of Ngb led to increased p-AKT expression, and reduced levels of cleaved PARP and cleaved

caspase-3/7/8, in U87 cells, compared with cells transfected with empty vector (P<0.05; Fig. 2A). Functionally, Ngb overexpression promoted the proliferation and reduced the apoptosis of U87 cells, compared with cells transfected with empty vector (P<0.05; Fig. 2B-D). An AKT inhibitor, MK2206, was employed to block the activation of PI3K/AKT in Ngb-overexpressing U87 cells. MK2206 treatment led to reduced p-AKT expression and increased levels of cleaved PARP, cleaved caspase-3, cleaved caspase-7 and cleaved caspase-8 in Ngb-overexpressing U87 cells (P<0.05; Fig. 2A). Furthermore, MK2206 treatment reduced the proliferation and induced the apoptosis of Ngb-overexpressing U87 cells (P<0.05; Fig. 2B-D). Ngb knockdown using shRNA led to reduced expression of p-AKT, and increased levels of cleaved PARP and cleaved caspase-3/7/8, in U251 cells, compared with cells transfected with NT shRNA (P<0.05; Fig. 3A). In addition, Ngb knockdown reduced the proliferation and induced the apoptosis of U251 cells, compared with cells transfected with NT shRNA (P<0.05; Fig. 3B-D). IGF-1, an activator of the PI3K/AKT pathway, increased p-AKT levels, and decreased cleaved PARP and cleaved caspase-3/7/8 expression, in Ngb-knockdown U251 cells (P<0.05; Fig. 3A). Furthermore, IGF-1 treatment resulted in enhanced proliferation and reduced apoptosis in Ngb-knockdown U251 cells (P<0.05; Fig. 3A-D). Therefore, these results further confirm that Ngb may promote a cellular malignant phenotype in glioma, which may occur via the PI3K/AKT pathway.

Discussion

The expression and role of Ngb in human cancer is a novel and controversial topic. Several studies have reported that Ngb may protect against oxidative stress-induced cell injury in brain cancer (4,5,7). In addition, our previous study demonstrated that Ngb was overexpressed in glioma tissues compared with normal brain tissues, and its overexpression was associated with poor prognostic features and shorter overall survival (15). Ngb has also been reported to promote the proliferation and inhibit the apoptosis of glioma cells *in vitro* and *in vivo* (15). However, the potential mechanisms underlying the effects of Ngb in glioma are yet to be established. The present study investigated the molecular mechanisms involved in the anti-apoptotic effect of Ngb in glioma cells. KEGG PathwayFinder by gene expression analysis using the R2: Genomics Analysis and Visualization Platform revealed that Ngb was associated with the PI3K/AKT pathway in glioma tissues from the GSE4290 dataset of the GEO database. Further experiments in the current study demonstrated that Ngb enhanced the activation of the PI3K/AKT pathway in glioma cells.

Aberrant activation of the PI3K/AKT pathway has been widely reported in various cancers types during progression, including glioma (20-22). The PI3K/AKT pathway has roles in the cell proliferation, cell cycle progression and apoptosis resistance of glioma cells via its downstream targets, which include mTOR, Bcl-2, Bax, cyclin D1 and Bcl-2-like 1 (20,23-26). mTOR was reported to have an essential role in the cell survival, proliferation and apoptosis of glioma cells (27,28). Bcl-2 is an antiapoptotic protein, while Bax is a proapoptotic factor. Altered Bcl-2/Bax expression was associated with altered apoptosis levels glioma cells (29). In the present study, treatment with the AKT inhibitor MK2206 blocked the activation of the PI3K/AKT pathway, and subsequently resulted in decreased proliferation and increased apoptosis in Ngb-overexpressing U87 cells. Furthermore, IGF-1 treatment in U251 cells with Ngb knockdown enhanced the PI3K/AKT pathway activation, cell proliferation and apoptosis resistance. Therefore, Ngb may promote malignant phenotypes of glioma cells by targeting the PI3K/AKT pathway.

In conclusion, the results of the present study demonstrated that Ngb enhanced the activation of the PI3K/AKT pathway in glioma cells. Furthermore, Ngb regulated the proliferation and apoptosis of glioma cells, and these effects may occur via the PI3K/AKT pathway. Therefore, Ngb may serve as a potential target for the treatment of glioma.

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