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Overexpression of SCUBE2 Inhibits Proliferation, Migration, and Invasion in Glioma Cells

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Signal peptide CUB EGF-like domain-containing protein 2 (SCUBE2), a member of the SCUBE family of proteins, was recently found to play an important role in cancer development. However, little is known regarding its biological function in glioma. In the present study, we investigated the effect of SCUBE2 on glioma and explored its relevant mechanisms. The study showed that SCUBE2 had a low expression in glioma tissue and cell lines. SCUBE2 overexpression inhibited glioma cell proliferation in vitro and in vivo as well as suppressed glioma cell migration and invasion in vitro. Furthermore, we found that the Sonic hedgehog (Shh) signaling pathway was involved in the inhibitory effect of SCUBE2 overexpression on glioma cells. In light of the results obtained from our study, SCUBE2 may be regarded as a potential therapeutic target for glioma.

Key words: Signal peptide CUB EGF-like domain-containing protein 2 (SCUBE2); Proliferation; Migration; Invasion; Glioma

INTRODUCTION

Glioma, a common type of brain tumor, is characterized by aggressiveness[1,2]. According to statistics, the incidence of glioma is 5 in 100,000, and this high rate makes glioma a great threat to public health[3]. Glioma often causes a high mortality rate because of its high incidence of malignancy[4,5]. A majority of patients with glioma are diagnosed at an advanced stage and suffer from a poor outcome[3,6]. What makes the situation worse are such obstacles as radioresistance, multidrug resistance, insufficient preclinical models, impermeable blood–brain barriers, and an incomplete understanding of the pathogenesis[7]. Therefore, we desperately need to explore new mechanisms underlying glioma progression, thus realizing more effective diagnosis and treatment of glioma.

The signal peptide CUB EGF-like domain-containing protein (SCUBE) family comprises three members, which are SCUBE1, SCUBE2, and SCUBE3[8–11]. All the family members are conserved evolutionarily from zebrafish to humans and encode secreted proteins associated with cell surface[12–14]. Recently, several reports demonstrated a significant role of the SCUBE family in cancer development. For example, SCUBE1 was found to be highly expressed in gastric cancer patients and was suggested as a new biological marker for gastric cancer[15]. Additionally, SCUBE2 was reported to be linked with progression of several kinds of cancers. Cheng et al. proved the tumor-suppression effect of SCUBE2 on breast cancer cells[16]. Lin et al. drew a similar conclusion that SCUBE2 served as a tumor suppressor in breast cancer by inhibiting cell migration and invasion[17]. In colorectal cancer, SCUBE2 was suggested to be a potential therapeutic target for its inhibitory effect on colorectal cancer cell proliferation, migration, and invasion[18]. Despite these studies on the role of SCUBE2 in cancer progression, there have been no reports on its biological function in glioma.

In the present study, we investigated the effect of SCUBE2 on glioma and explored its relevant mechanisms. The study showed that SCUBE2 had a low expression in glioma tissue and cell lines. SCUBE2 overexpression inhibited glioma cell proliferation, migration, and invasion. Furthermore, we found that SCUBE2 overexpression exerted an inhibitory effect on glioma cells via regulating the Sonic hedgehog (Shh) signaling pathway.

MATERIALS AND METHODS

Tissue Specimen Collection

Glioma tissue and normal brain tissue were collected from patients of The Second Hospital of Hebei Medical University (P.R. China). Tissue was frozen in liquid nitrogen directly after collection and then stored at −80°C for future use. All patients provided written consent before

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their participation in the study. All experiments were carried out with approval of the ethics committee of The Second Hospital of Hebei Medical University.

Cell Culture

Human glioma cell lines (U87 and A172) and human astrocytes (HA1800) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). After culturing in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), streptomycin (100 μg/ml), and penicillin (100 U/ml), all cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Quantitative RT-PCR

Isolation of total RNA from tissue or cells was performed with the TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA). Reverse transcription of RNA into cDNA was conducted with the Gene Amp PCR System 9700 (Life Technologies, Carlsbad, CA, USA). Quantitative RT-PCR was performed using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) with the following primers: SCUBE2, 5′-CCCCCAAGCGCCGCATCCTGA-3′ (forward) and 5′-TATTGAGTGGCACGTGGGCTGAGT-3′ (reverse); GAPDH, 5′-GCCAAAAGGGTCATCATCTC-3′ (forward) and 5′-ACCACCTGGTGCTCAGTGTA-3′ (reverse). The conditions for PCR amplification were as follows: 5 min of initial denaturation at 95°C, 30 s of denaturation for 35 cycles at 56°C, 60 s of annealing at 60°C, 60 s of elongation at 72°C, and 7 min of final elongation at 72°C. The relative mRNA levels were normalized to GAPDH, and fold change was calculated using the comparative CT method ($2^{-\Delta\Delta}$CT)[19].

Western Blot

Lysis buffer was used to extract total protein from tissue or cells. The BCA Protein Assay Kit (Pierce Chemical, Rockford, IL, USA) was applied to determine the protein concentration. Lysates were separated by 10% SDS-PAGE and then transferred to PVDF membranes. After blocking in 5% skim milk for 1 h, membranes were incubated overnight at 4°C with primary antibodies against SCUBE2, Gli1, Ptch1, or GAPDH. After washing three times with PBST, the membranes were incubated with HRP-conjugated secondary antibody. Protein bands were detected using ECL detection reagents (Bio-Rad, Hercules, CA, USA). The relative protein expression was analyzed using the Image-Pro plus software 6.0.

Plasmids and Transfection

The pcDNA3.1-SCUBE2 expression vector and the empty pcDNA3.1 vector were purchased from Gene Pharma Co., Ltd. (Shanghai, P.R. China). U87 and A172 cells were transfected with pcDNA3.1-SCUBE2 or the empty vector using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instruction. After 48 h, the transfection results were checked via Western blot analysis.

MTT Assay

MTT assay was performed to examine cell proliferation ability. Cells were placed into 96-well plates at a density of 5×10^3 cells/well and then cultured for 48 h. After 20 μl of MTT (5 mg/ml; Sigma-Aldrich) was added to each well, cells were cultured for an additional 4 h. Following removal of the culture medium, 150 μl of dimethyl sulfoxide (Sigma-Aldrich) was added. The optical density was determined at 450 nm using a microplate reader (Molecular Devices, Menlo Park, CA, USA).

Colony Formation Assay

Cells were seeded in a six-well plate at a density of 200 cells per well and then cultured at 37°C with 5% CO_2 in a humidified incubator for 12 days. Subsequently, cells were stained with 1% crystal violet, and the number of colonies (>50 cells/colony) was counted.

Transwell Assay

Transwell chambers with polycarbonate membranes (8-μm pore size) were used to test cell migration and invasion. For the migration assay, 5×10^3 cells were resuspended in serum-free medium and placed in the upper chamber. DMEM containing 20% FBS was added to the lower chamber. Following 24 h of incubation at 37°C, cells on the upper surface of the membrane were wiped off by cotton swabs, and cells that migrated to the lower surface of the membrane were fixed with methanol and stained with 0.05% Giemsa. The number of migrated cells was counted under a microscope (200×). For the invasion assay, the same procedure as that for the migration assay was followed, except that polycarbonate membranes were coated with Matrigel.

Tumor Xenograft Formation Assay

Four-week-old male BALB/c nude mice were obtained from the National Laboratory Animal Center (Beijing, P.R. China). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Hebei Medical University. U87 cells (5×10^6) transfected with pcDNA3.1-SCUBE2 or the empty vector were subcutaneously injected into the left flank of each mouse. Tumor volume was measured every 5 days. After 30 days, mice were euthanized, and tumors were weighed.

Statistical Analysis

Experimental data were shown as means ± standard deviation (SD). The GraphPad Prism V5.0 software was

used for graphing. Student's *t*-tests were used to analyze the differences between groups. A value of $p<0.05$ was considered statistically significant.

RESULTS

SCUBE2 Was Lowly Expressed in Glioma Tissue and Cell Lines

To define the role of SCUBE2 in glioma, we performed both RT-PCR and Western blot to measure the expression levels of SCUBE2 in glioma tissue. The results showed that SCUBE2 had a much lower expression level in glioma tissue than in normal brain tissue (Fig. 1A and B). We further assessed the expression levels of SCUBE2 in glioma cell lines (U87 and A172) and human astrocytes (HA1800). SCUBE2 was also lowly expressed in glioma cell lines, compared to the HA1800 cells (Fig. 1C and D).

Overexpression of SCUBE2 Inhibited Glioma Cell Proliferation In Vitro and In Vivo

To investigate the biological function of SCUBE2 in glioma, we established a SCUBE2 overexpression model via transfection of pcDNA3.1-SCUBE2 into U87 and A172 cells. The Western blot assay showed a successful transfection (Fig. 2A and B).

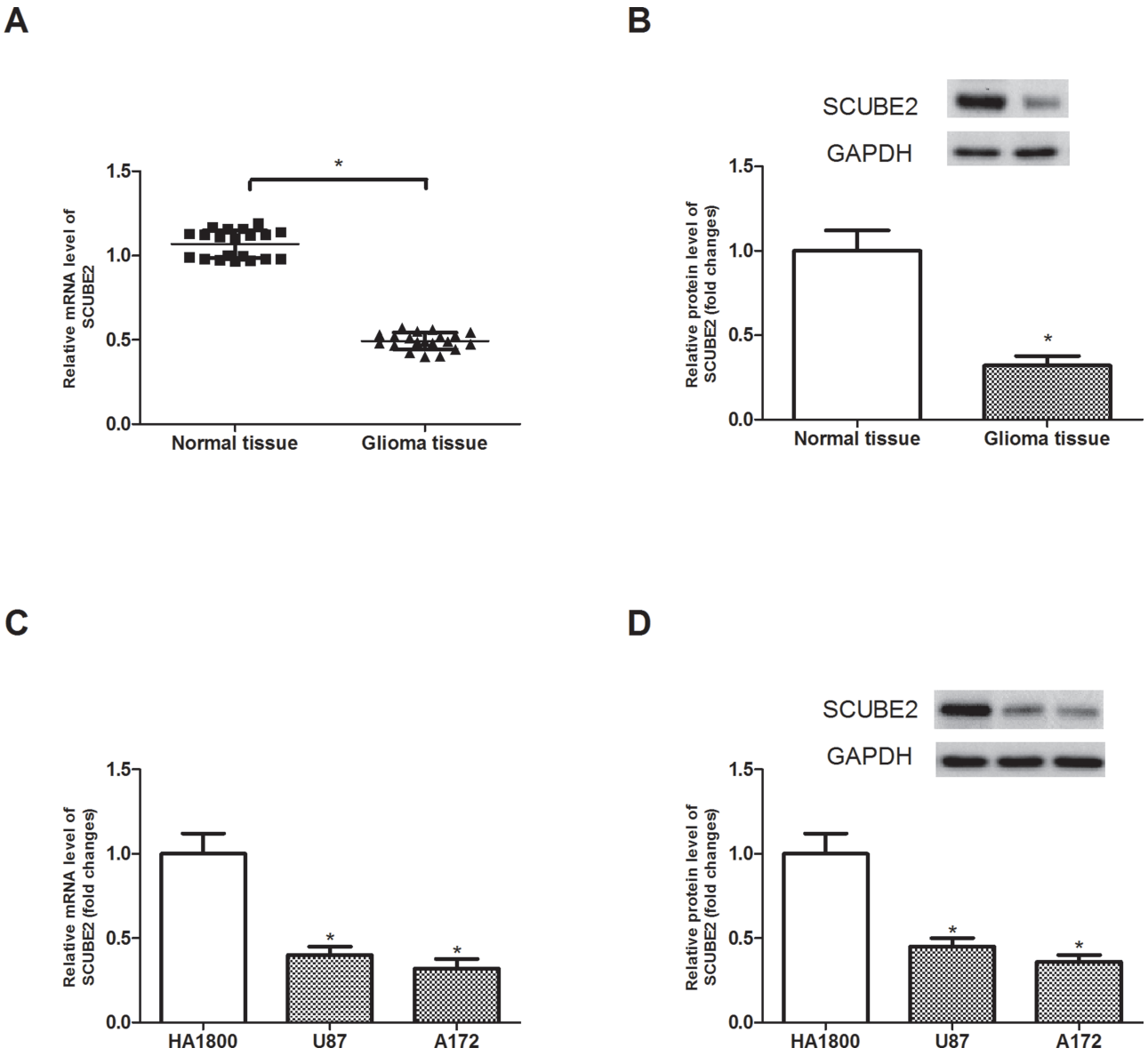

Figure 1. SCUBE2 was lowly expressed in glioma tissue and cell lines. (A, B) RT-PCR and Western blot analysis of SCUBE2 expression in glioma tissues ($n=20$) and normal brain tissues ($n=20$). (C, D) RT-PCR and Western blot analysis of SCUBE2 expression in glioma cell lines and astrocytes (HA1800). *$p<0.05$.

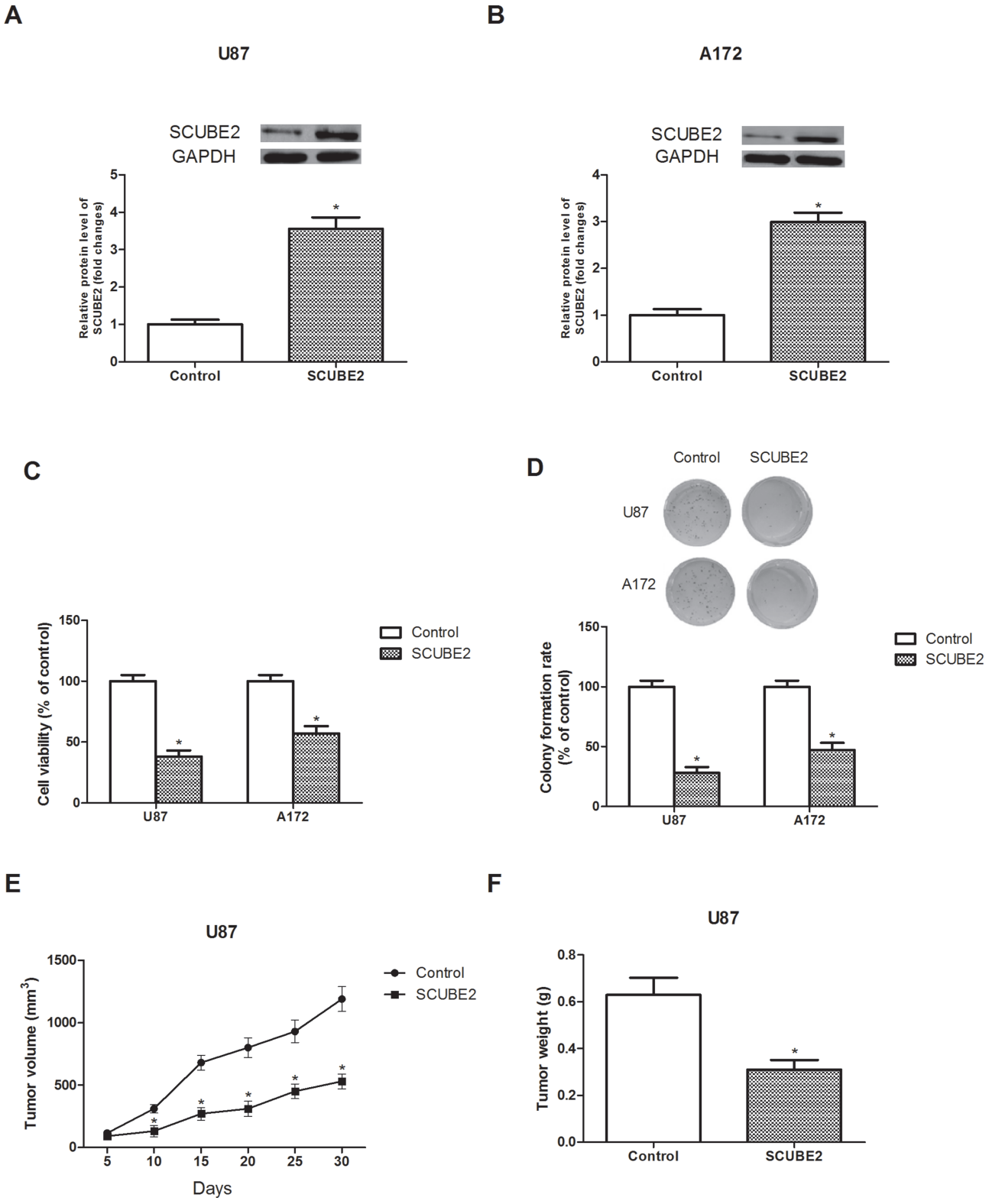

Figure 2. Overexpression of SCUBE2 inhibited glioma cell proliferation in vitro and in vivo. (A, B) Relative protein expression levels of SCUBE2 in U87 and A172 cells after transfection of pcDNA3.1-SCUBE2. (C, D) The effect of SCUBE2 overexpression on U87 and A172 cell proliferation was determined by MTT and colony formation assays. (E) The volume of tumors was measured every 5 days after injection of SCUBE2-transfected U87 cells into nude mice. (F) The weight of tumors was measured 30 days after injection of SCUBE2-transfected U87 cells into nude mice. *$p < 0.05$.

The effect of SCUBE2 overexpression on glioma cell proliferation in vitro was detected via MTT and colony formation assays. The MTT assay indicated that SCUBE2 overexpression remarkably inhibited cell viability in comparison with the control group (Fig. 2C). Similarly, the colony formation assay showed that SCUBE2 overexpression significantly decreased the number of colonies in comparison with the control group (Fig. 2D).

To verify the in vitro results, we performed in vivo experiments. After subcutaneous injection of SCUBE2-transfected U87 cells into nude mice, we measured tumor growth. The volume and weight of tumors were obviously reduced in the SCUBE2-transfected group in comparison with the control group (Fig. 2E and F).

Overexpression of SCUBE2 Inhibited Glioma Cell Migration and Invasion In Vitro

We also explored the effect of SCUBE2 overexpression on glioma cell migration and invasion. The Transwell assay showed that SCUBE2 overexpression markedly attenuated the migratory and invasive abilities of U87 cells (Fig. 3A and B). We obtained similar results for A172 cells (Fig. 3C and D).

Overexpression of SCUBE2 Inhibited the Activity of the Shh Signaling Pathway

Many studies have demonstrated the important role of SCUBE2 in the regulation of the Shh signaling

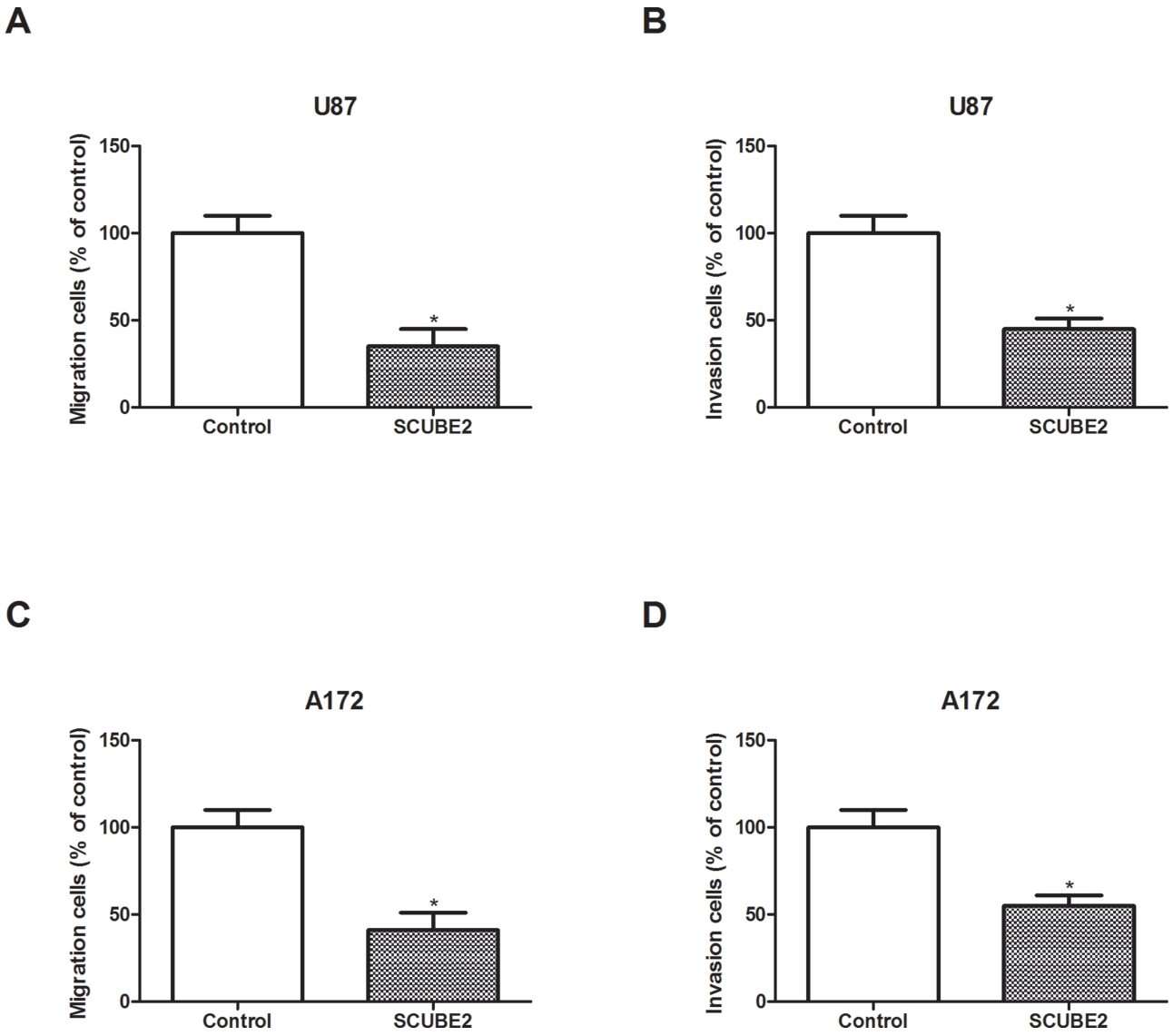

Figure 3. Overexpression of SCUBE2 inhibited glioma cell migration and invasion in vitro. (A, B) Transwell assay was performed to evaluate the effect of SCUBE2 overexpression on cell migration and invasion of U87 cells. (C, D) Transwell assay was performed to evaluate the effect of SCUBE2 overexpression on cell migration and invasion of A172 cells. *$p<0.05$.

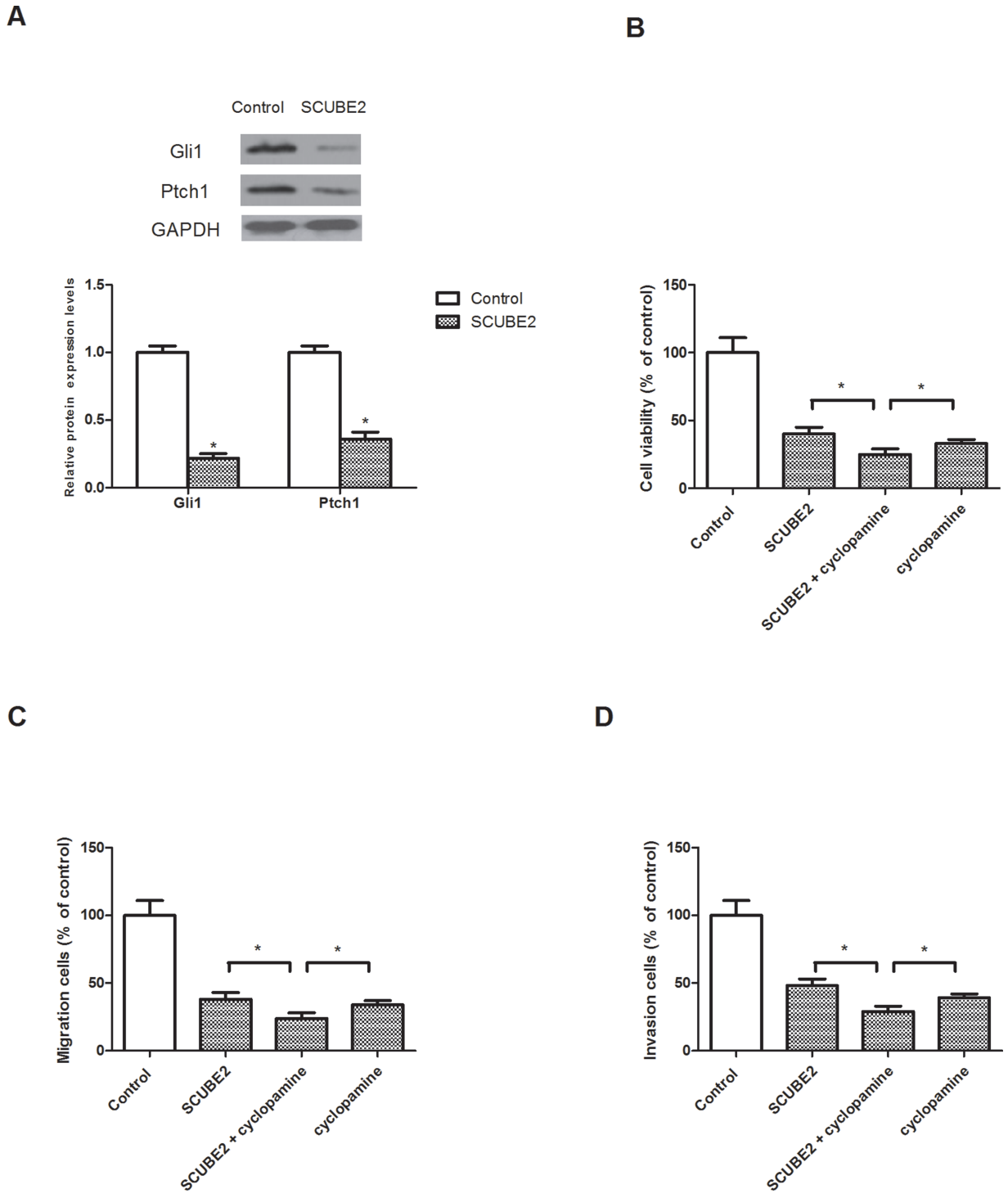

Figure 4. Overexpression of SCUBE2 inhibited activation of the Shh signaling pathway. (A) Western blot assay showed that SCUBE2 overexpression decreased the protein expression of Gli1 and Ptch1 in U87 cells. (B) U87 cells were transfected with pcDNA3.1-SCUBE2 or the empty vector in the presence or absence of cyclopamine (100 nM) for 48 h. MTT assay was performed to measure U87 cell proliferation. (C, D) Transwell assay was performed to detect U87 cell migration and invasion. *$p<0.05$.

pathway[20,21]. Therefore, we determined whether SCUBE2 exerted its inhibitory effect on glioma cells via the Shh signaling pathway. SCUBE2 overexpression remarkably decreased the protein expression levels of Gli1 and Ptch1 in U87 cells in comparison with the control group (Fig. 4A). We also investigated the effect of cyclopamine (an inhibitor of the Shh signaling pathway) on glioma cell proliferation, migration, and invasion mediated by SCUBE2 overexpression. The results showed that cyclopamine obviously potentiated SCUBE2 overexpression-inhibited U87 cell proliferation (Fig. 4B), migration (Fig. 4C), and invasion (Fig. 4D).

DISCUSSION

As a great threat to public health, glioma is a problem that urgently needs to be solved. To provide more effective therapeutic strategies, great effort has been put into the exploration of new mechanisms underlying glioma progression.

SCUBE2, a member of the SCUBE family of proteins, was recently found to have an inhibitory effect during cancer development[16–18]. Despite the findings on the functional significance of SCUBE2 in cancer, little is known regarding its role in glioma. In this study, we investigated the biological function of SCUBE2 in glioma. The study results showed that SCUBE2 was lowly expressed in glioma tissue and cell lines. In addition, SCUBE2 overexpression inhibited glioma cell proliferation, migration, and invasion in vitro. These results were consistent with a previous study where SCUBE2 had a decreased expression in colorectal cancer, and its upregulation exerted an inhibitory effect on colorectal cancer development[18]. In addition, SCUBE2 was found to serve as a tumor suppressor in breast cancer[16,17]. In our study, we also conducted in vivo experiments to confirm our in vitro results. In line with our expectation, SCUBE2 overexpression greatly reduced the volume and weight of tumors formed by SCUBE2-transfected U87 cells in nude mice in comparison with the control group. Based on these observations, we suggested the tumor-inhibiting function of SCUBE2 in glioma.

The hedgehog signaling pathway, a highly conserved system, plays a key role in tissue patterns as well as in cell proliferation and differentiation during embryonic development[22,23]. As a mammalian counterpart of the hedgehog pathway, the Shh signaling pathway shares similar functions[24]. It is activated by binding to the Ptch–Smo membrane–receptor complex[25]. There have been findings that the Shh signaling pathway is abnormally activated in various cancers, and many studies have suggested the contribution of its abnormal activation to carcinogenesis[26–31]. Recently, several studies reported the correlation between the Shh signaling pathway and glioma[32,33]. Increasing evidence has also demonstrated the importance of the Shh signaling pathway for self-renewal, proliferation, and tumorigenesis of glioma[34,35]. More importantly, SCUBE2 has been found to play a significant role in regulating the Shh signaling pathway[20,21]. From the above studies, we inferred that SCUBE2 had a fair chance of inhibiting glioma cell proliferation, migration, and invasion via the Shh signaling pathway. To verify our assumption, we examined the effect of SCUBE2 overexpression on Gli1 and Ptch1, which are important components of the Shh signaling pathway. The results showed that overexpressed SCUBE2 remarkably decreased the protein expression levels of Gli1 and Ptch1 in glioma cells. In addition, we investigated the effect of cyclopamine (an inhibitor of the Shh signaling pathway) on glioma cell proliferation, migration, and invasion mediated by SCUBE2 overexpression. The assay results indicated that cyclopamine greatly inhibited glioma cell proliferation, migration, and invasion, supporting the involvement of the Shh signaling pathway. Besides, cyclopamine markedly enhanced the suppressive effect of SCUBE2 overexpression on glioma cells, suggesting that SCUBE2 inhibited glioma cell proliferation, migration, and invasion partly through suppressing the Shh signaling pathway. Further studies are needed to reveal the definite mechanism behind this effect of SCUBE2.

Taken together, we demonstrated that SCUBE2 was lowly expressed in glioma tissue and cell lines. SCUBE2 overexpression inhibited glioma cell proliferation in vitro and in vivo as well as suppressed glioma cell migration and invasion in vitro. Furthermore, we found that the Shh signaling pathway was involved in the inhibitory effect of SCUBE2 overexpression on glioma cells. In light of the results obtained from our study, SCUBE2 may be regarded as a potential therapeutic target for glioma.

ACKNOWLEDGMENT: The authors declare no conflicts of interest.

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