

Original Article

FCGBP promotes malignant phenotypes of glioblastoma cells via activation of the TGF- β 1 signaling pathway

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Abstract: Objective: To investigate the expression level of IgG Fc-binding protein (FCGBP) in glioma cells, elucidate its role in malignant tumor phenotypes, and explore its relationship with the transforming growth factor- β (TGF- β) signaling pathway. Methods: The expression of FCGBP was compared between primary human astrocytes (HA) and glioma cell lines (U87, U251, LN229, and T98G). FCGBP was then silenced in U87 cells. Cell proliferation, migration, and invasion were assessed using Cell Counting Kit-8, 5-ethynyl-2'-deoxyuridine (EdU) incorporation, colony formation, wound healing, and Matrigel-coated Transwell assays. Apoptosis and cell-cycle distribution in U87 cells were analyzed by flow cytometry. Co-immunoprecipitation (Co-IP) was performed to validate the endogenous association between FCGBP and TGF- β 1-containing protein complexes. Rescue experiments were conducted using recombinant TGF- β 1 protein. Additionally, the *in vitro* findings were further validated in nude mouse xenograft models. Results: FCGBP was highly expressed in various glioma cell lines, and its knockdown reduced TGF- β levels in the culture supernatant. Knockdown of FCGBP in U87 cells significantly suppressed malignant behaviors, including proliferation, migration, and invasion, while inducing apoptosis and cell-cycle arrest. Co-IP assays suggested an endogenous association between FCGBP and TGF- β 1-containing protein complexes, and treatment with recombinant TGF- β 1 effectively reversed the inhibitory effects of FCGBP knockdown. *In vivo* experiments confirmed that silencing FCGBP markedly inhibited tumor growth in nude mice and significantly reduced systemic inflammation and TGF- β levels. Conclusion: FCGBP promotes malignant phenotypes in glioma cells (U87) and accelerates tumor growth *in vivo*, potentially through activation of the TGF- β /Smad signaling pathway.

Keywords: FCGBP, glioma, TGF- β , proliferation, invasion, apoptosis

Introduction

Among malignant intracranial tumors, gliomas are particularly aggressive [1], with glioblastoma (GBM) representing the highest grade of malignancy. Glioma cells proliferate rapidly and evade normal growth control, continuously dividing and infiltrating surrounding brain tissue [2]. Despite recent advancements in surgical resection, radiotherapy, and chemotherapy, recurrence remains frequent, and patient prognosis is poor, with a low median survival and approximately 80% of patients experiencing recurrence [3]. Therefore, further elucidation of the mechanisms underlying glioma progression is essential. Identifying molecules with potential therapeutic value has become a major focus of current research.

IgG Fc-binding protein (FCGBP) is a mucin secreted by mucosal epithelial cells. Studies have confirmed that FCGBP plays a crucial role in mucosal immune responses and helps maintain the mucus barrier [4, 5]. However, growing evidence suggests that FCGBP is abnormally expressed in various tumors and may contribute to malignant tumor behavior. For instance, integration of single-cell RNA sequencing and transcriptomic data demonstrated that tumor-associated macrophages co-expressing FCGBP and SPP1 promote the progression and subtype differentiation of primary liver cancer [6]. Importantly, elevated FCGBP levels are commonly detected in gliomas, with this expression pattern positively associated with poor patient prognosis [7]. Additionally, a recent study indicates that FCGBP enhances glioma cell prolif-

eration via activation of the JAK2/STAT3/c-Myc signaling axis, thereby accelerating tumor growth [8]. Nevertheless, it remains unclear whether the role of FCGBP in glioma involves crosstalk with other core signaling pathways.

Transforming growth factor- β (TGF- β) is a multifunctional cytokine, and its dysregulation is linked to the development of various diseases [9, 10]. TGF- β binds to the TGF- β type II receptor (T β RII) on the cell membrane, which recruits the type I receptor (T β RI), leading to phosphorylation and formation of an active receptor complex [11]. Activated T β RI directly phosphorylates receptor-regulated Smads (i.e., Smad2/3), forming a complex that translocates from the cytoplasm into the nucleus to regulate gene expression. This process influences fundamental cellular activities such as proliferation, differentiation, and apoptosis. The TGF- β pathway also plays a crucial role in the malignant progression of glioma. Prior research has shown that TGF- β activation promotes epithelial-mesenchymal transition (EMT), enhances glioma cell aggressiveness, and inhibits apoptosis [12]. Complementary studies suggest that the TGF- β /Smad pathway influences glioma progression, potentially through modulation of autophagy [13]. Given the strong association between FCGBP and tumor development, along with TGF- β 's critical role in regulating the tumorigenic features of transformed cells, we hypothesize that FCGBP may promote the oncogenic behavior of glioma cells by altering TGF- β signaling activity.

To our knowledge, only a few studies have examined the role of FCGBP in gliomas, and direct evidence regarding its role in promoting the malignant phenotype of glioma cells via the TGF- β pathway is lacking. Therefore, this study investigates how FCGBP influences proliferation, migration, invasion, and apoptosis of glioma cells through *in vitro* cell experiments and *in vivo* xenograft models in immunocompromised mice. Additionally, the relationship between FCGBP and Smad-dependent TGF- β signaling is explored.

Materials and methods

Cell culture

Primary human neural astrocytes (HA; catalog number: SNP-H239) were obtained from SUN-

NCELL (Wuhan, China) and served as the non-tumor control cells. According to the supplier, HA cells are primary human astrocytes derived from human brain tissue, prepared through enzymatic digestion combined with differential adhesion. They were identified by GFAP immunofluorescence, demonstrating a purity greater than 90%. Cells at passages 2-3 were utilized in this study. Additionally, human glioblastoma cell lines, including U251 (SNL-095), LN229 (SNL-355), and T98G (SNL-457), were obtained from SUNNCELL. Human glioblastoma cells (U87, iCell-0143a) were obtained from iCell Bioscience Inc. U87 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) using standard procedures, with medium changes every 2-3 days. We maintained all cultures at 37°C, 5% CO₂, and 95% relative humidity. The cell density used for experiments was 1×10⁶ cells/mL.

Cell transfection and grouping

In the first experiment, U87 cells in the logarithmic growth phase were stratified into control knockdown (sh-NC) and FCGBP-silenced (sh-FCGBP) arms. To each group in an EP tube, 125 μ L of Opti-MEM (Servicebio, G4568-500ML) was added, followed by transfection using 5 μ L of Lipofectamine™ 3000 reagent (Beyotime, C0526-1.5 ml) with sh-FCGBP and shRNA-Control (GENERAL BIOL). The sh-FCGBP sequence was 5'-GGGCTGTGTGGCAACTATAAT-3'. The negative control shRNA was a non-targeting scrambled sequence provided by GENERAL BIOL, and its sequence was 5'-UUCUCCGAACGUGUCACGUGGC-3'. Complete medium was added 4-6 hours after transfection, and subsequent experiments were conducted 48 hours later.

In a subsequent experiment, four groups of U87 cells were generated: sh-NC, sh-NC + TGF- β 1, sh-FCGBP, and sh-FCGBP + TGF- β 1. For the transfected cells, 10 ng/mL of recombinant TGF- β 1 protein was added. TGF- β 1 (HY-P7118) was purchased from MedChemExpress. Subsequent experiments were conducted 24 hours after treatment with the recombinant TGF- β 1 protein.

Animal experiments

A total of 12 SPF-grade BALB/c nude mice were used in this study (n=6 per group). The animals were provided by Hangzhou Ziyuan Laboratory

Table 1. Primer sequences

GAPDH-F	5'-GTGGACCTGACCTGCCGTCTAG-3'
GAPDH-R	5'-GAGTGGGTGTCGCTGTTGAAGTC-3'
FCGBP-F	5'-GCCAAGGCTGAGATGATAGGC-3'
FCGBP-R	5'-CCTGCACAGAGATGGCATAGT-3'

Animal Science and Technology Co., Ltd. The production license number and the animal quality certificate number were SCXK (Zhejiang) 2024-0004 and 20260301Abzz010500000-04, respectively. A suspension of U87 cells was prepared at a concentration of 1×10^7 cells, and each mouse received a subcutaneous injection of 100 μ L of the cell suspension into the right axillary region. The mice were monitored daily, and body weight and tumor size were measured regularly. At the experimental endpoint, mice were deeply anesthetized via intraperitoneal injection of 2% sodium pentobarbital at a dosage of 60 mg/kg. Once the absence of the pedal reflex was confirmed, terminal blood samples were collected for serum preparation and subsequent ELISA analysis. The mice were then euthanized by exsanguination under deep anesthesia. Death was confirmed by the absence of respiration, heartbeat, and reflex activity. Tumor tissues were excised and preserved for further analyses.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) experiment

Total RNA was extracted from samples collected after different treatments using TRIzol reagent (CWBIO, CW3166M). Complementary DNA (cDNA) was synthesized using NovoScript® Plus All-in-one 1st Strand cDNA Synthesis SuperMix (gDNA Purge) (novoprotein, Cat. No. E047-01B) according to the manufacturer's instructions. Quantitative real-time PCR system was performed using a real-time PCR system (FQD-96A, Hangzhou Borui Technology Co., Ltd.). Oligonucleotide primers were custom-synthesized by Sangon Biotech (Shanghai, China), and the primer sequences are listed in **Table 1**.

Western blot assay

Total protein was extracted using RIPA lysis buffer (Servicebio, G2002-30ML), and quantified using a BCA protein assay kit (Solarbio, PCO-040). Protein samples were separated by SDS-PAGE using precast gels (Epizyme Biotech, PG222) and then transferred onto PVDF mem-

branes (Servicebio, G6050-0.45). After blocking, the membranes were incubated overnight at 4°C with primary antibodies against TGF- β 1 (1:1,000, Affinity, AF1027), Smad2 (1:5,000, Proteintech, 12570-1-AP), phospho-Smad2 (1:1,000, Affinity, AF3449), Smad3 (1:3,000, Proteintech, 30130-1-AP), phospho-Smad3 (1:1,000, Affinity, AF3362), FCGBP (1:2,000, BLOSS, bs-13168R), and GAPDH (1:5,000, Proteintech, 60004-1-Ig). After incubation with the corresponding secondary antibody (1:1,000, proteintech, RGAR001) for 2 h at room temperature, immunoreactive bands were visualized using a highly sensitive ECL substrate (MA0186, MeilunBio). Images were captured using a chemiluminescence imaging system (SCG-W2000, Servicebio). Quantification of band intensity was carried out with ImageJ-based semi-quantitative densitometry (version 1.53t; Bethesda, MD, USA).

Co-immunoprecipitation (Co-IP) assay

Endogenous total protein lysates were prepared from U87 cells for Co-IP assays. Briefly, cells were lysed on ice in immunoprecipitation lysis buffer supplemented with protease and phosphatase inhibitors. Following centrifugation at $13,000 \times g$ for 10 minutes at 4°C, the supernatants were collected and quantified using a BCA assay. Equal amounts of protein were incubated overnight at 4°C with Protein A/G magnetic beads conjugated with either anti-FCGBP antibody, anti-TGF- β 1 antibody, or normal IgG. The beads were then washed, and bound protein complexes were eluted according to the manufacturer's protocol. Eluted proteins were separated by SDS-PAGE and analyzed by Western blotting, with normal IgG serving as a negative control. The antibodies used were as follows: anti-FCGBP antibody (Amerigo Scientific, C-16F11), anti-TGF- β 1 antibody (Santa Cruz Biotechnology, sc-52892), and normal IgG control antibody (Beyotime, A7001). The assay was conducted using an immunoprecipitation kit (Shanghai Lingyin Biotechnology Co., Ltd., IK-1004).

Enzyme-linked immunosorbent assay (ELISA) assay

Supernatants from U87 cells and mouse serum were collected for ELISA. Sample processing, assay procedures, and calculations were conducted in accordance with the manufacturer's

instructions. The ELISA kits used included interleukin-6 (IL-6) (MM-0163M2), interleukin-1 beta (IL-1 β) (MM-0040M2), transforming growth factor-beta (TGF- β) (MM-0689M2), tumor necrosis factor-alpha (TNF- α) (MM-0132M2), and TGF- β (MM-0090H2), all purchased from Enzyme Immunoassay Co. A BIOBASE-EL10A microplate reader was employed to record the optical density (OD) at a wavelength of 450 nm.

Cell counting kit-8 (CCK-8) assay

U87 cells were seeded into 96-well plates at a density of 4,000 cells per well and treated according to the experimental groupings. After the designated treatment period, 10 μ L of CCK-8 working solution (GLPBIO, GK10001) was added to each well and incubated for 2 h at 37°C. Absorbance at 450 nm was then measured using a microplate reader (BK-EL10A, Shandong Boguan Biotechnology Co., Ltd.).

EdU staining assay

Cell proliferation was assessed using a 5-ethynyl-2'-deoxyuridine (EdU) assay kit (AF594-labeled, Beyotime, C0071S). After fixation (Servicebio, G1011-50ML), U87 cells were stained with the EdU working solution. Nuclei were counterstained with DAPI (blue), and proliferating (EdU-positive) cells were labeled red. Red fluorescent cells were observed and counted under an inverted fluorescence microscope (DMI4000B, LEICA), and the proliferation rate was calculated.

Colony formation assay

U87 cells were seeded into six-well plates at 600 cells per well. After cell attachment, treatments were applied according to the experimental groups. Cells were cultured for 2 weeks, with medium replaced every other day. Once visible colonies formed, the cells were fixed and stained with crystal violet (Servicebio, G1014-50ML). Under microscopic observation, colonies containing more than 50 cells were counted, and images were recorded.

Wound healing assay

Cells from each group were seeded in culture plates. When cell confluence exceeded 90%, a scratch was made across the monolayer using a 10 μ L pipette tip. Images were captured im-

mediately after scratching (0 h) and again at 24 h, ensuring consistent positioning.

Transwell invasion assay

Transwell invasion assays were conducted using Matrigel-coated Transwell chambers. First, the upper chambers were pre-coated with diluted Matrigel and allowed to solidify before cell seeding. U87 cells from each group were suspended in 300 μ L of serum-free medium and seeded into the upper chamber. A total of 500 μ L of complete medium containing serum was added to the lower chamber to serve as a chemoattractant. After 48 hours of incubation, non-invading cells that remained on the upper surface of the membrane were gently removed with a cotton swab. The invading cells on the lower surface of the membrane were fixed, stained with crystal violet, photographed under a microscope (DMI4000B, LEICA), and quantified.

Cell apoptosis assay

After treatment, cells from each group were collected, and apoptosis was measured using an Annexin V-FITC apoptosis detection kit (Beyotime, C1062L). Cells were incubated in the dark with Annexin V-FITC and PI (50 μ g/mL), and the percentage of apoptotic cells was determined using a NovoCyte™ flow cytometer (RaiseCyte 2L6C, Raisecare).

Hematoxylin and eosin (H&E) staining

Tumor tissues were carefully excised and immediately fixed in paraformaldehyde (Servicebio, G1011-50ML) at room temperature for at least 24 hours. The fixed tissues were then dehydrated and embedded in paraffin. Paraffin blocks were sectioned and subjected to H&E staining, where hematoxylin stains the cell nuclei and eosin stains the cytoplasm. The sections were mounted using neutral resin and imaged using a light microscope.

Immunohistochemistry (IHC)

Paraffin sections were deparaffinized, rehydrated, and incubated in hydrogen peroxide solution to quench endogenous peroxidase activity. Sections were blocked and incubated overnight with primary antibodies against Ki-67 (1:1,000, Proteintech, 28074-1-AP) and PCNA (1:500,

Proteintech, 10205-2-AP). After washing with PBS, sections were incubated with secondary antibody solution according to the manufacturer's instructions. After mounting, positive cells were observed under a microscope.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining

Sections underwent routine deparaffinization, rehydration, and antigen retrieval, followed by staining with TUNEL reagents (Beyotime, C10-86). After washing away the staining solution, the nuclei were counterstained with DAPI. The sections were then coverslipped with antifade mounting medium and examined under a fluorescence microscope (DMI4000B, LEICA).

Statistical considerations

All statistical analyses were performed using GraphPad Prism 8.0. Data were presented as mean $\pm$ SD. Normality and homogeneity of variance were assessed prior to parametric analyses. For comparisons between two groups, an unpaired Student's t-test was used. For comparisons among multiple groups at a single time point, one-way ANOVA followed by Tukey's HSD post-hoc test was conducted. For repeated measurements or time-course data, including CCK-8 cell viability curves, tumor volume curves, and body weight curves, two-way ANOVA or two-way repeated-measures ANOVA was employed, as appropriate, followed by Bonferroni's post-hoc test for pair-wise comparisons. A two-sided *P* value of < 0.05 was considered statistically significant.

Results

FCGBP was highly expressed in glioma cells, and FCGBP knockdown reduced TGF- β secretion

To evaluate FCGBP expression in glioblastoma, we examined FCGBP expression in primary human astrocytes (HA) and four human glioma cell lines (U87, U251, LN229, and T98G). As shown in **Figure 1A, 1B**, FCGBP expression was significantly elevated in all glioma cell lines compared to normal HA cells. Based on these results, U87 cells were selected for subsequent functional experiments.

FCGBP knockdown in U87 cells was achieved using three shRNA constructs. All three sh-FCG-

BP sequences effectively reduced FCGBP expression, with sh-FCGBP-2 achieving the highest knockdown efficiency (**Figure 1C, 1D**). This sequence was therefore selected for downstream experiments and designated as the sh-FCGBP group. Additionally, ELISA results demonstrated that TGF- β level in the U87 cell culture supernatant was significantly decreased after FCGBP knockdown (**Figure 1E**), suggesting that FCGBP may regulate TGF- β secretion in glioma cells.

FCGBP knockdown significantly suppressed malignant phenotypes of glioma cells

To investigate the biological function of FCGBP in U87 cells, multiple phenotypic assays were conducted. CCK-8 demonstrated that cell viability in the sh-FCGBP group significantly decreased at 48, 72, and 96 h compared to the sh-NC group (**Figure 2A**), indicating that FCGBP knockdown inhibits U87 cell viability. EdU staining and colony formation assays further demonstrated that FCGBP knockdown decreased the percentage of EdU-positive cells and the number of colonies (**Figure 2B, 2C**), confirming that FCGBP knockdown suppresses cell viability and proliferation.

Cell motility was assessed using wound healing assay and Transwell assay. The wound closure rate at 24 hours was significantly reduced in the sh-FCGBP group (**Figure 2D**). Consistently, Transwell invasion assays confirmed a marked decrease in the number of invading U87 cells (**Figure 2E**). Flow cytometry further demonstrated that FCGBP knockdown significantly induced apoptosis, as indicated by an increased apoptotic rate in the sh-FCGBP group compared to the sh-NC group (**Figure 2F, 2G**). Cell cycle analysis showed that FCGBP knockdown led to G0/G1 phase arrest and a concomitant decrease in S phase cells (**Figure 2H**). Collectively, these results demonstrate that FCGBP knockdown effectively suppresses malignant phenotypes in glioma cells.

FCGBP regulates the TGF- β /smad signaling pathway in glioma cells

Given the reduction in extracellular TGF- β levels following FCGBP knockdown (**Figure 1E**), we further investigated whether FCGBP regulates TGF- β -induced Smad phosphorylation. Western blot analysis revealed that FCGBP knockdown

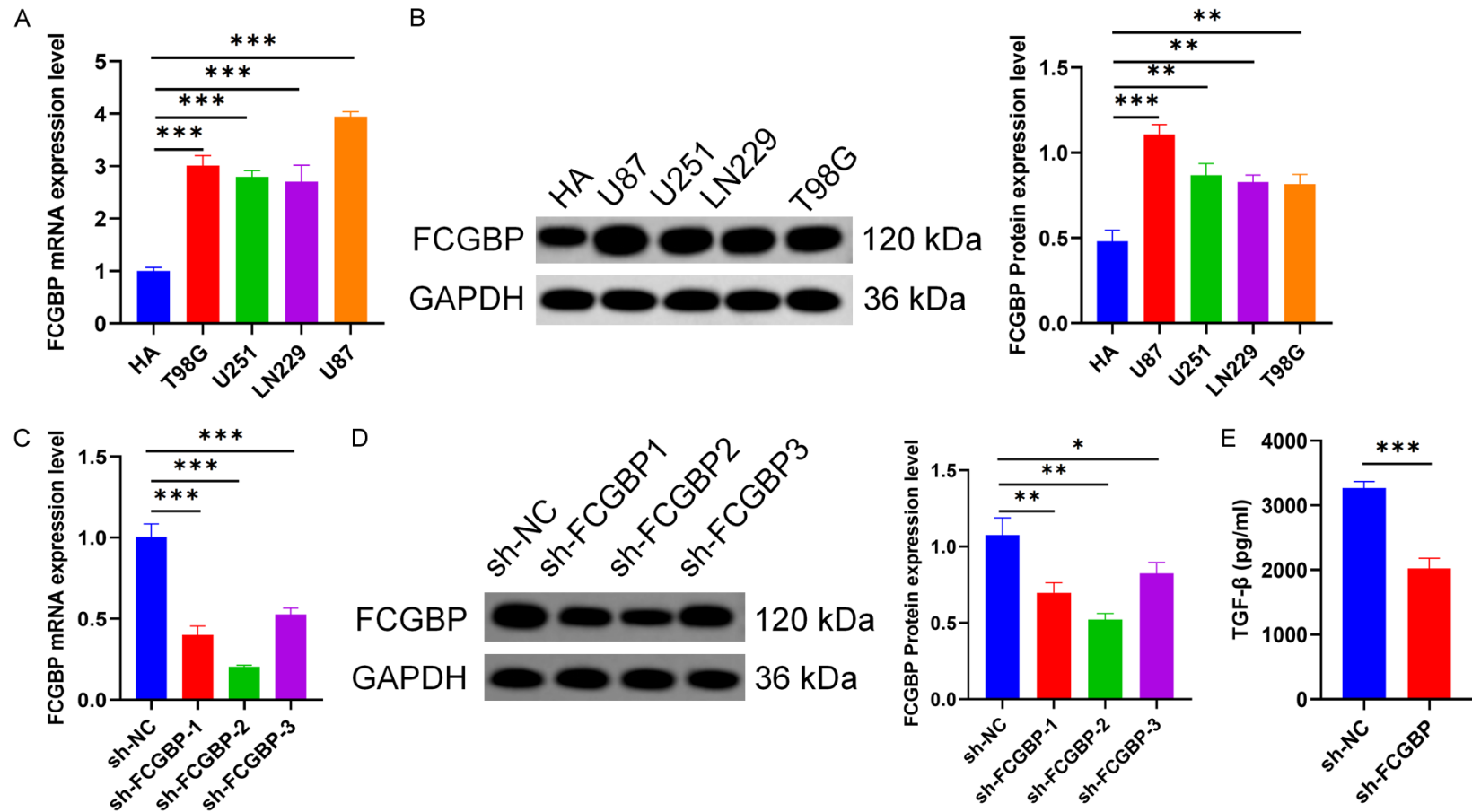
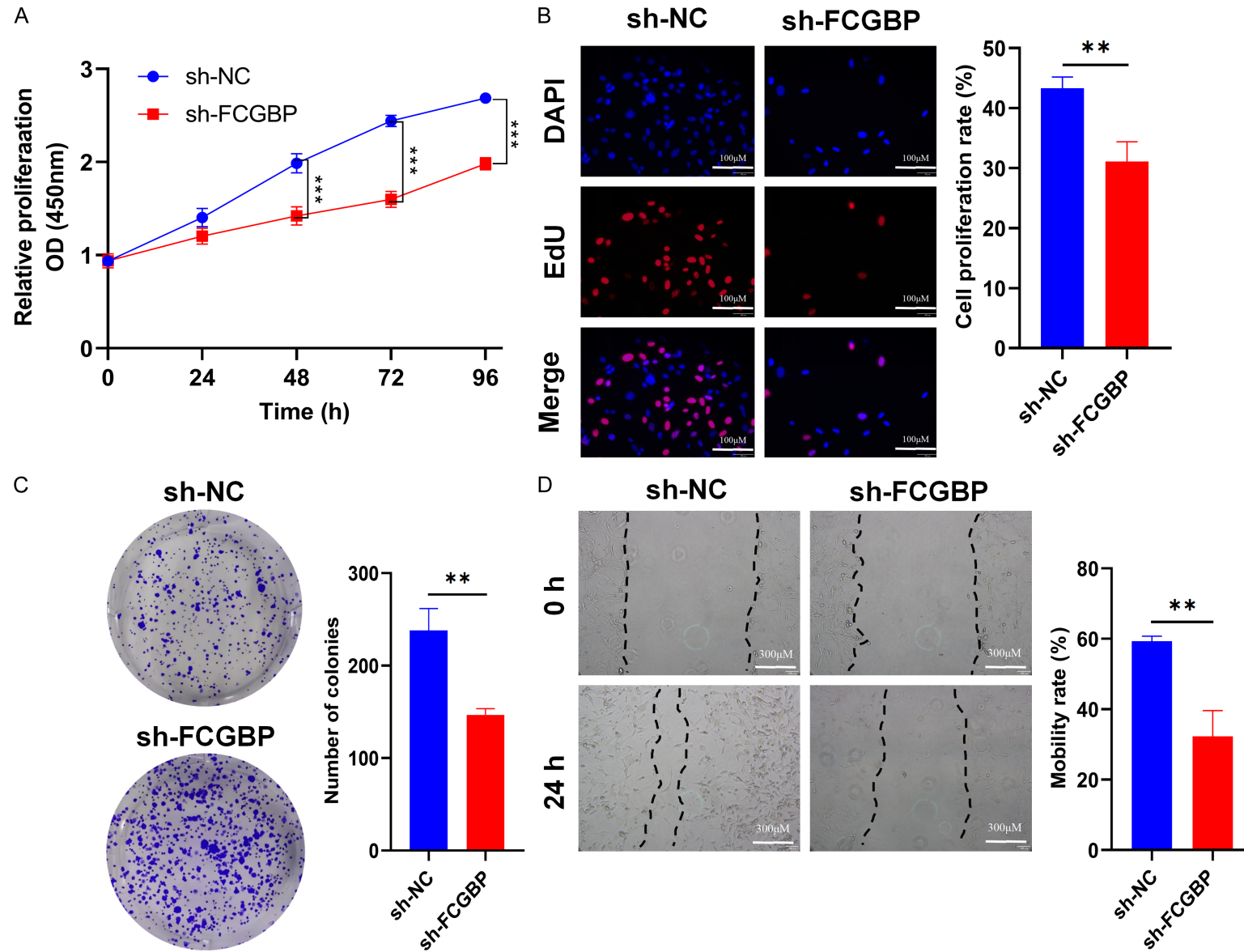


Figure 1. FCGBP is highly expressed in glioma cells, and FCGBP knockdown reduces TGF- β secretion. (A) RT-qPCR analysis of FCGBP mRNA expression in primary human astrocytes (HA) and glioma cell lines U87, U251, LN229, and T98G. (B) Western blot analysis of FCGBP protein expression in the primary human astrocytes and glioma cell lines. (C) RT-qPCR analysis of FCGBP knockdown efficiency in U87 cells. (D) Western blot analysis of FCGBP knockdown efficiency in U87 cells. (E) ELISA analysis of TGF- β levels in cell culture supernatants from the sh-NC and sh-FCGBP groups. Data are presented as mean $\pm$ SD from three independent experiments. (A-D) were analyzed by one-way ANOVA followed by Tukey's HSD. Data are presented as mean $\pm$ SD from three independent experiments. (A-D) were analyzed by one-way ANOVA followed by Tukey's HSD. (E) was analyzed by an unpaired two-tailed Student's t-test. Compared with HA, ***P < 0.001; compared with sh-NC, *P < 0.05, **P < 0.01, ***P < 0.001.



FCGBP in glioblastoma malignancy

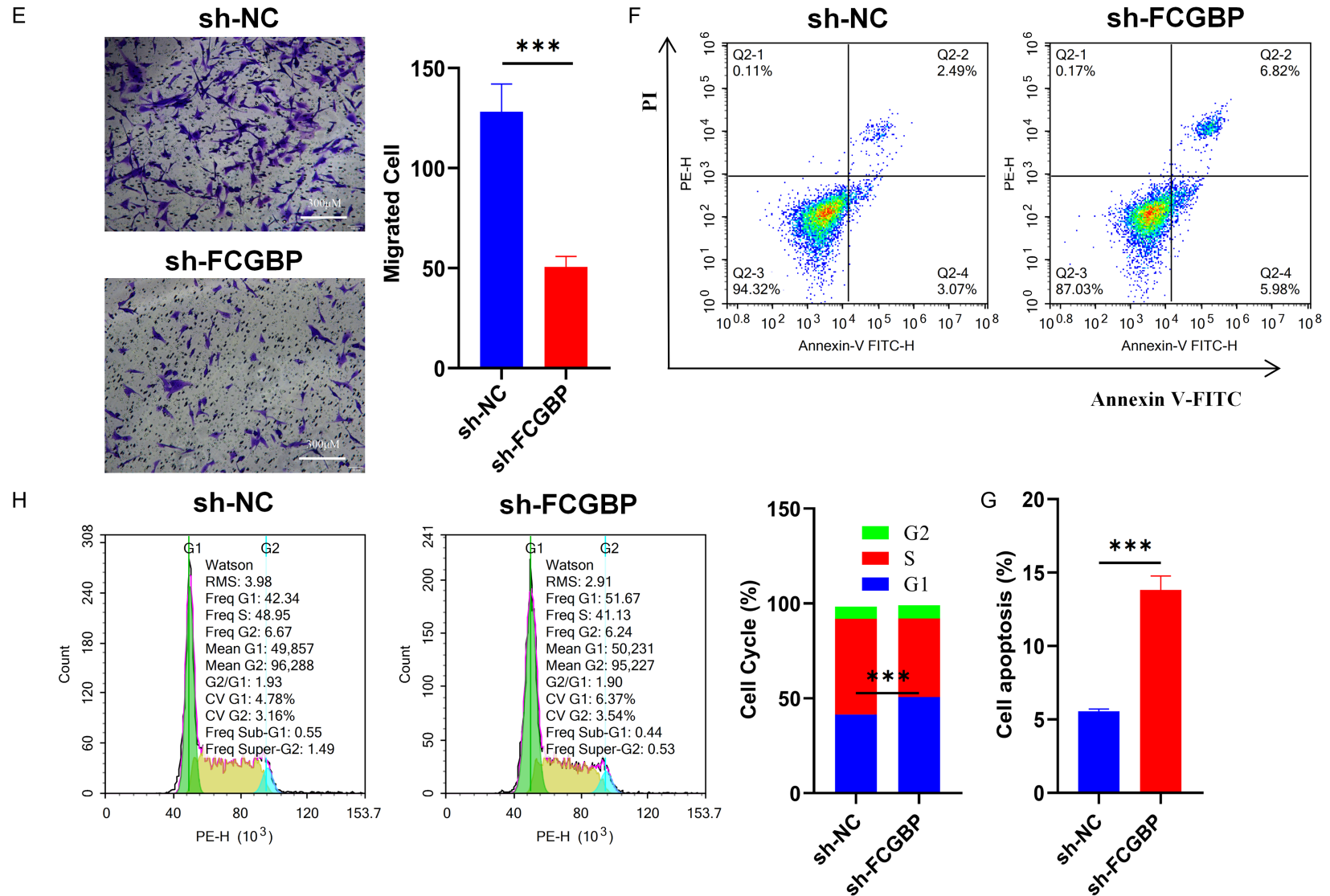


Figure 2. FCGBP knockdown significantly suppressed the malignant phenotype of glioma cells. A. CCK-8 assay assessing U87 cell proliferation at 0, 24, 48, 72, and 96 hours. B. EdU staining evaluating cell proliferation, magnification: $\times 200$. C. Colony formation assay measuring cell clonogenic capacity, magnification: $\times 100$. D. Scratch assay examining cell migration, magnification: $\times 100$. E. Transwell assay assessing cell invasion, magnification: $\times 200$. F, G. Flow cytometry analysis of cell apoptosis. H. Flow cytometry analysis of cell cycle distribution. Data are presented as mean $\pm$ SD from three independent experiments. CCK-8 time-course data were analyzed using two-way ANOVA followed by Bonferroni's post-hoc test. Other comparisons were analyzed using an unpaired Student's t-test. Comparisons with the sh-NC group show ** $P < 0.01$ and *** $P < 0.001$.

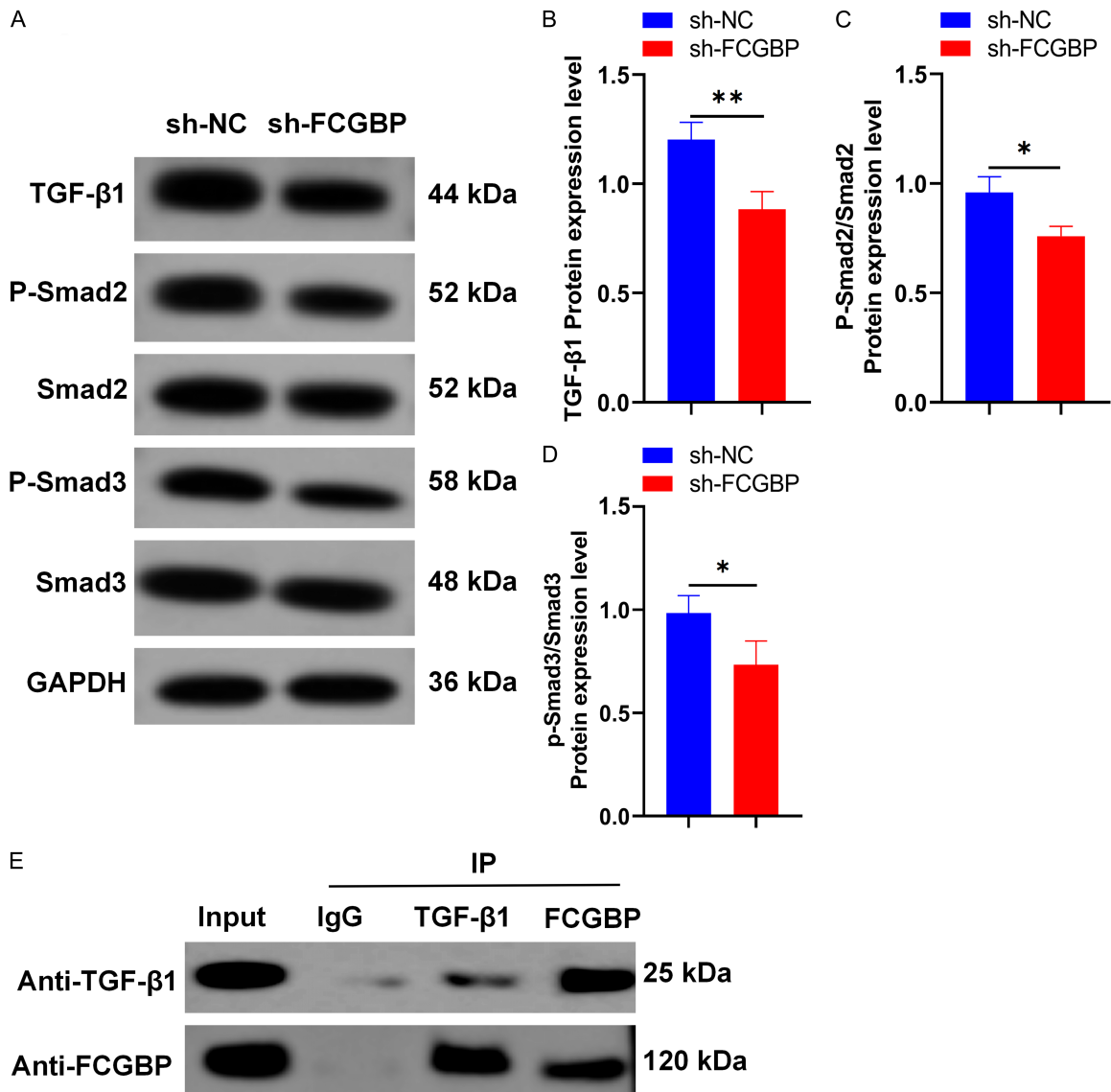


Figure 3. FCGBP regulates the TGF- β signaling pathway in glioma cells. (A) Protein levels of TGF- β 1, Smad2, p-Smad2, Smad3, and p-Smad3 analyzed by Western blot. (B-D) Densitometric quantification of TGF- β 1, p-Smad2/Smad2, and p-Smad3/Smad3 protein expression levels. (E) Endogenous Co-IP assay using total cell lysates from U87 cells, showing the association between FCGBP and TGF- β 1. IgG served as a negative control. Data are presented as mean $\pm$ SD from three independent experiments. (B-D) were analyzed using an unpaired two-tailed Student's t-test. Comparisons with the sh-NC group show * $P < 0.05$ and ** $P < 0.01$.

significantly reduced intracellular TGF- β 1 protein levels in U87 cells (**Figure 3A, 3B**). Additionally, phosphorylation of Smad2/3, the key downstream mediator of TGF- β signaling, was also markedly reduced (**Figure 3A, 3C, 3D**).

To further explore the potential interaction between these proteins, endogenous Co-IP assays were performed using total protein lysates from U87 cells. The results indicated that FCGBP and TGF- β 1 were detected in the same

immunoprecipitated protein complex, while no specific signal was observed in the IgG control group (**Figure 3E**). These findings suggest an endogenous association between FCGBP and TGF- β 1 in U87 cells. However, since total cell lysates were used, this assay does not clarify whether the association occurs during intracellular protein processing or after secretion. Together with the observed decrease in extracellular TGF- β , these findings imply that FCGBP may contribute to maintaining TGF- β 1 protein

availability and secretion, thereby contributing to Smad2/3 phosphorylation. However, whether this regulation occurs through altered TGF β 1 transcription, TGF- β 1 protein stability, secretion, or extracellular retention requires further investigation.

Activation of the TGF- β pathway reversed the suppression of malignant phenotypes caused by FCGBP knockdown

To verify the role of TGF- β signaling in FCGBP-mediated glioma progression, recombinant TGF- β 1 was added to FCGBP-knockdown U87 cells. EdU and colony formation assays revealed that the addition of recombinant TGF- β 1, following FCGBP knockdown, led to an increase in both EdU-positive cells and the number of cell colonies (**Figure 4A, 4B**), indicating enhanced proliferative capacity of U87 cells. Wound healing and Transwell invasion assays demonstrated that TGF- β 1 treatment restored cell migration and invasion in sh-FCGBP cells (**Figure 4C, 4D**). Flow cytometry results further demonstrated that TGF- β 1 reduced the elevated apoptosis rate induced by FCGBP knockdown (**Figure 4E**). Western blot analysis further confirmed that TGF- β 1 supplementation restored intracellular TGF- β 1 protein levels and Smad2/3 phosphorylation in sh-FCGBP cells (**Figure 5A-D**). Moreover, ELISA analysis showed increased TGF- β secretion into the culture supernatant following exogenous TGF- β 1 treatment (**Figure 5E**).

FCGBP knockdown inhibited the growth of subcutaneous U87 xenograft tumors in nude mice

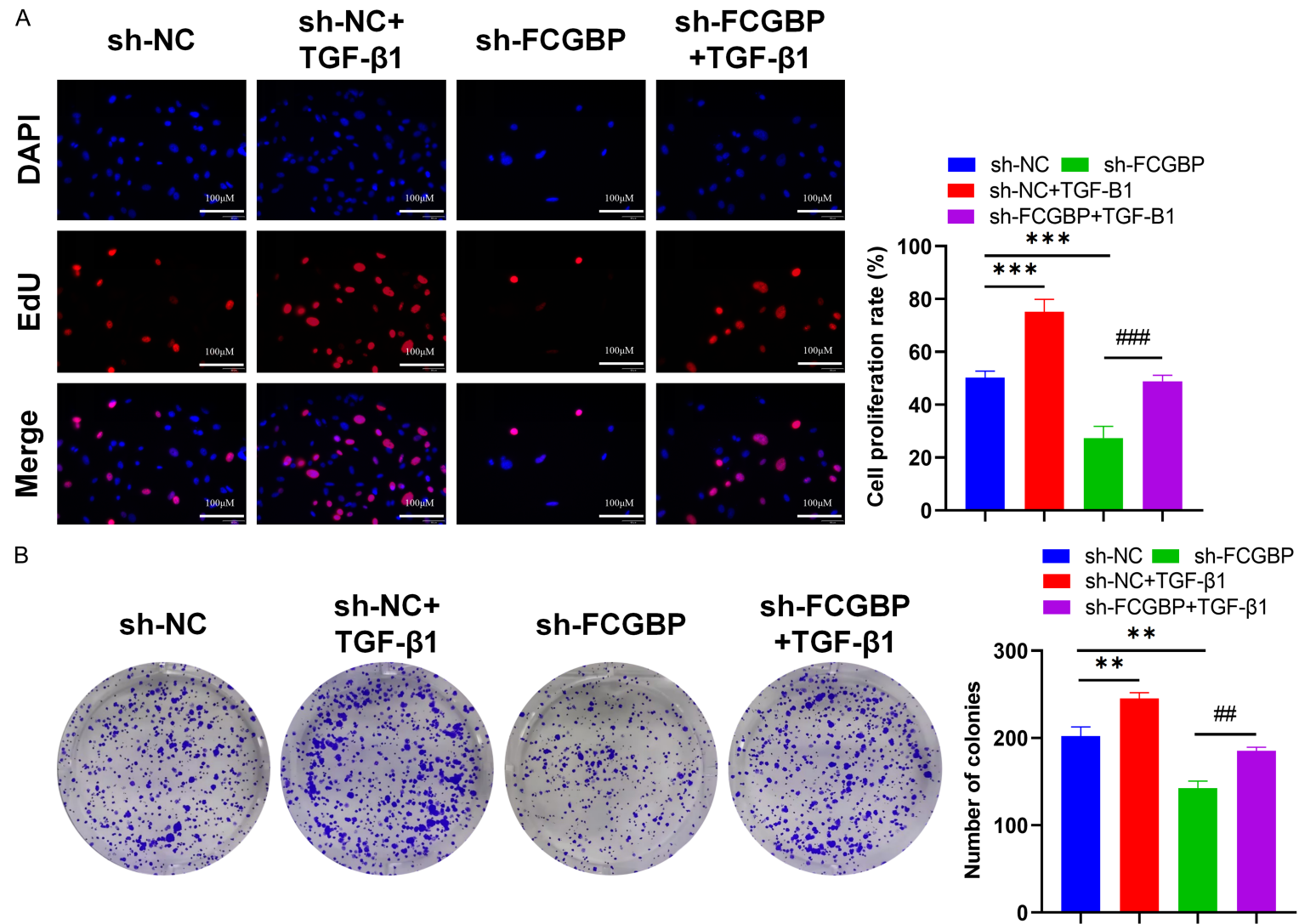
We further investigated the tumor-suppressive effects of sh-FCGBP in nude mice. Subcutaneous tumor formation assays indicated that tumors derived from sh-FCGBP-treated U87 cells exhibited significantly reduced volumes and lower weights compared with the sh-NC group (**Figure 6A-C**), demonstrating that FCGBP knockdown significantly suppresses glioma growth in vivo. Body weight measurements showed that mice in the sh-FCGBP group maintained higher body weights than those in the sh-NC group by day 15 (**Figure 6D**). H&E staining showed reduced inflammatory cell-like infiltration within the tumor stroma in the sh-FCGBP mice (**Figure 6E**). ELISA analysis of serum cytokines indicated that TGF- β , TNF- α , IL-6, and IL-1 β levels were all decreased in the sh-FCGBP

nude mice (**Figure 6F**), suggesting that FCGBP knockdown may attenuate tumor-associated inflammatory responses. Immunohistochemical analysis of proliferation-related proteins (Ki-67 and PCNA) and TUNEL staining for apoptosis confirmed consistent findings: sh-FCGBP tumors exhibited reduced Ki-67 and PCNA expression and increased apoptosis compared with sh-NC tumors (**Figure 6G, 6H**). These in vivo data demonstrate that FCGBP knockdown effectively inhibits glioma growth and promotes apoptosis in vivo.

Discussion

Identification of novel targets that play critical roles in glioma progression is of great importance [14]. In this study, we found that FCGBP is highly expressed across multiple glioma cell lines, with the most pronounced expression observed in U87 cells. FCGBP knockdown in U87 cells significantly suppressed several key malignant behaviors, including proliferation, migration, and invasion, while inducing apoptosis and G0/G1 cell-cycle arrest. Collectively, these findings suggest that FCGBP functions as a tumor-promoting factor in glioma. Although only a limited number of studies have reported the role of FCGBP in glioma [15], existing evidence indicates that FCGBP expression is markedly elevated in tumor tissues, increases with glioma grade, and is closely associated with the immune microenvironment and patient prognosis. Furthermore, FCGBP may act as a tumor-associated antigen with potential relevance for vaccine development [16]. Our findings provide direct cellular and in vivo evidence supporting the tumor-promoting role of FCGBP, extending the current understanding of its contribution to glioma progression.

The canonical TGF- β pathway is a well-established pro-tumor pathway in gliomas [17, 18]. Upon activation, TGF- β enhances tumor cell invasiveness, accelerates tumor growth, facilitates metastasis [19], suppresses apoptosis, and enables immune evasion [20]. Therefore, targeting this pathway may present a promising therapeutic strategy. Our study found that FCGBP knockdown in U87 cells significantly reduced the phosphorylation levels of TGF- β 1 and p-Smad2/3, indicating that FCGBP primarily modulates TGF- β /Smad pathway activation. Moreover, endogenous Co-IP assays demonstrated the co-immunoprecipitation of FCGBP and TGF- β 1, suggesting a potential regulatory



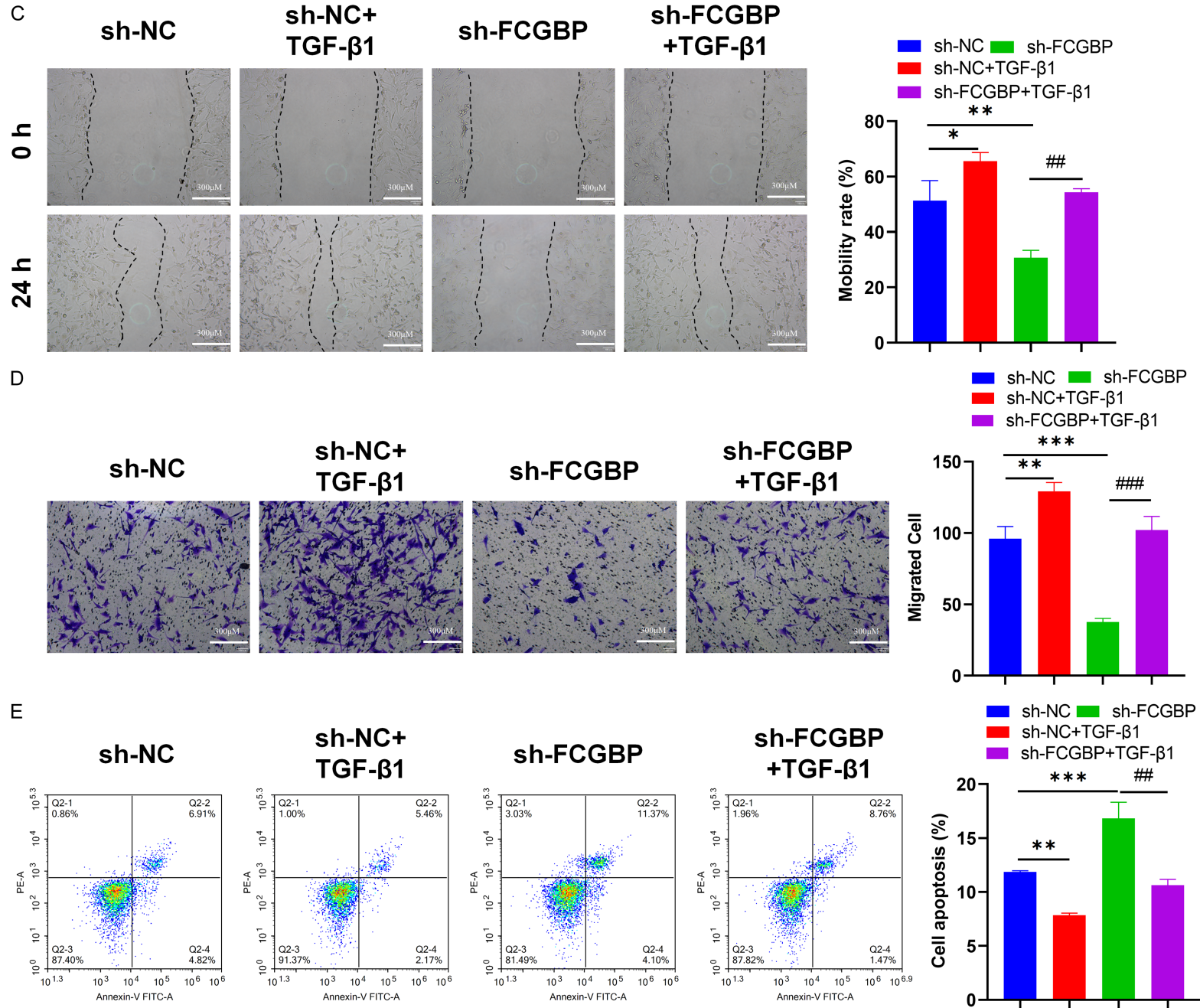


Figure 4. Recombinant TGF- β 1 partially reversed the inhibitory effects of FCGBP knockdown on glioma cell malignant phenotypes. A. EdU staining assessing cell proliferation in each group, magnification: $\times 200$. B. Colony formation assay evaluating clonogenic capacity in each group, magnification: $\times 100$. C. Wound healing assay measuring cell migration in each group, magnification: $\times 100$. D. Transwell assay evaluating cell invasiveness in each group, magnification: $\times 200$. E. Flow cytometry analyzing cell apoptosis in each group. Data are presented as mean $\pm$ SD from three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's HSD post-hoc test. Compared with the sh-NC group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; compared with the sh-FCGBP group, ## $P < 0.01$, ### $P < 0.001$.

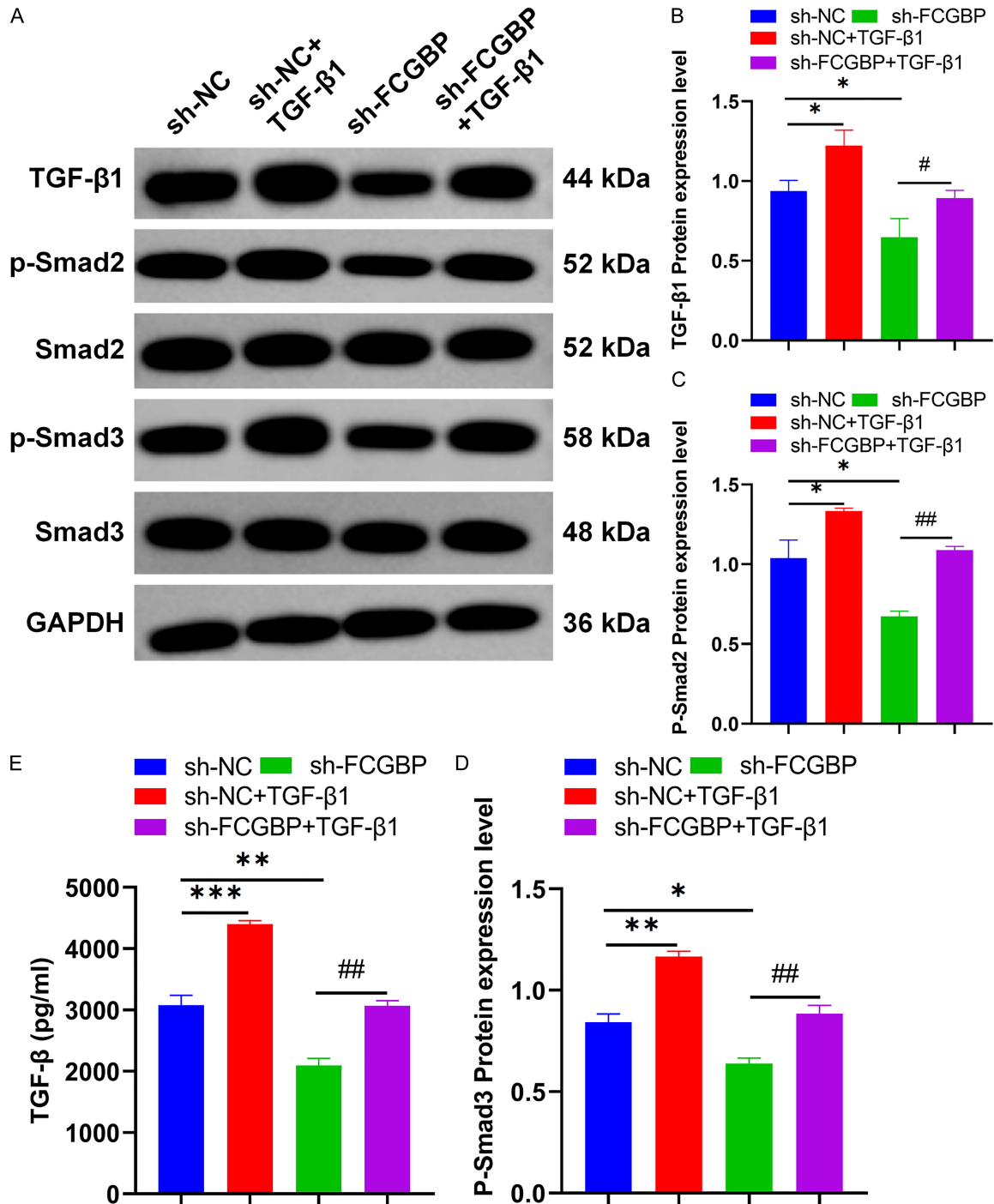


Figure 5. Activation of the TGF- β pathway partially restored TGF- β /Smad signaling in FCGBP-knockdown glioma cells. A. Western blot analysis of TGF- β 1, Smad2, p-Smad2, Smad3, and p-Smad3 protein levels in each group. B-D. Quantitative analysis of the grayscale values of the corresponding proteins. E. ELISA analysis of TGF- β levels in the culture supernatants of each group. Data are presented as mean $\pm$ SD from three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's HSD post-hoc test. Compared with the sh-NC group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; compared with the sh-FCGBP group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.

association. However, these findings do not definitively prove direct physical binding nor clarify whether this interaction occurs intracellularly or extracellularly.

It should be noted that FCGBP is a secreted mucin-like glycoprotein [4]. Therefore, the significance of the FCGBP-TGF- β 1 association requires careful interpretation. In this study, Co-IP was performed using total protein lysates from U87 cells without ectopic overexpression of either FCGBP or TGF- β 1. Consequently, the results indicate that FCGBP is associated with protein complexes containing TGF- β 1 in U87 cells. However, the use of total cell lysates limits further interpretation, as this assay cannot distinguish whether the association occurs intracellularly during FCGBP synthesis, protein processing, or vesicular trafficking, nor can it exclude the possibility that the association is established extracellularly after FCGBP secretion.

Our findings suggest a potential role for FCGBP in regulating TGF- β /Smad signaling. FCGBP knockdown reduced both intracellular TGF- β 1 protein expression and extracellular TGF- β levels in the culture supernatant. Furthermore, recombinant TGF- β 1 partially reversed the inhibitory effects of FCGBP knockdown. These results support the possibility that FCGBP contributes to maintaining TGF- β 1 availability by influencing its maturation, secretion, stability, or extracellular retention. Consequently, FCGBP depletion may decrease TGF- β 1 availability, attenuate TGF- β receptor activation, and impair downstream Smad2/3 phosphorylation. However, this model remains speculative and requires further experimental validation. Future studies should investigate TGF β 1 mRNA expression, TGF- β 1 protein turnover, proteasomal or lysosomal degradation pathways, secretion blockade models, conditioned-medium Co-IP, subcellular localization, proximity ligation assays, and extracellular protein crosslinking. These experiments may help clarify whether FCGBP regulates TGF- β 1 primarily through tran-

scriptional regulation, protein stability, secretion, or extracellular protection.

To determine whether FCGBP influences phenotypic changes in glioma cells via the TGF- β pathway, recombinant TGF- β 1 protein was introduced into U87 cells with FCGBP knockdown. Recombinant TGF- β 1 partially restored the impaired proliferation, migration, and invasion caused by FCGBP knockdown and reduced the elevated apoptosis rate in U87 cells. These findings suggest that FCGBP promotes the malignant phenotype of glioma cells, at least in part, through activation of the TGF- β /Smad pathway. Previous studies have indicated that FCGBP expression correlates with patient prognosis and tumor immune cell infiltration [7], while the TGF- β /Smad pathway has been established as a key driver of glioma progression, particularly by enhancing the malignant proliferation of tumor cells [21, 22]. However, these factors have largely been examined independently, and no research has previously established a direct regulatory relationship between the two. Our findings provide preliminary evidence that FCGBP-mediated malignant phenotypes in U87 glioma cells are, at least in part, associated with TGF- β /Smad signaling, although the precise molecular mechanism underlying FCGBP's influence on TGF- β 1 availability and Smad activation requires further clarification.

In vivo results aligned with those from *in vitro* observations. Subcutaneous implantation of FCGBP-knockdown cells in nude mice resulted in smaller tumor volumes and lower weights compared with controls. In the tumor tissue, the expression of Ki-67 and PCNA-markers of cell proliferation-decreased, while the number of TUNEL-positive cells increased, indicating cell death. These findings indicate that FCGBP promotes glioma cell proliferation and survival *in vivo*. Additionally, FCGBP knockdown decreased serum levels of several inflammatory cytokines, including TGF- β , TNF- α , IL-6, and IL-1 β , and H&E staining showed reduced infil-

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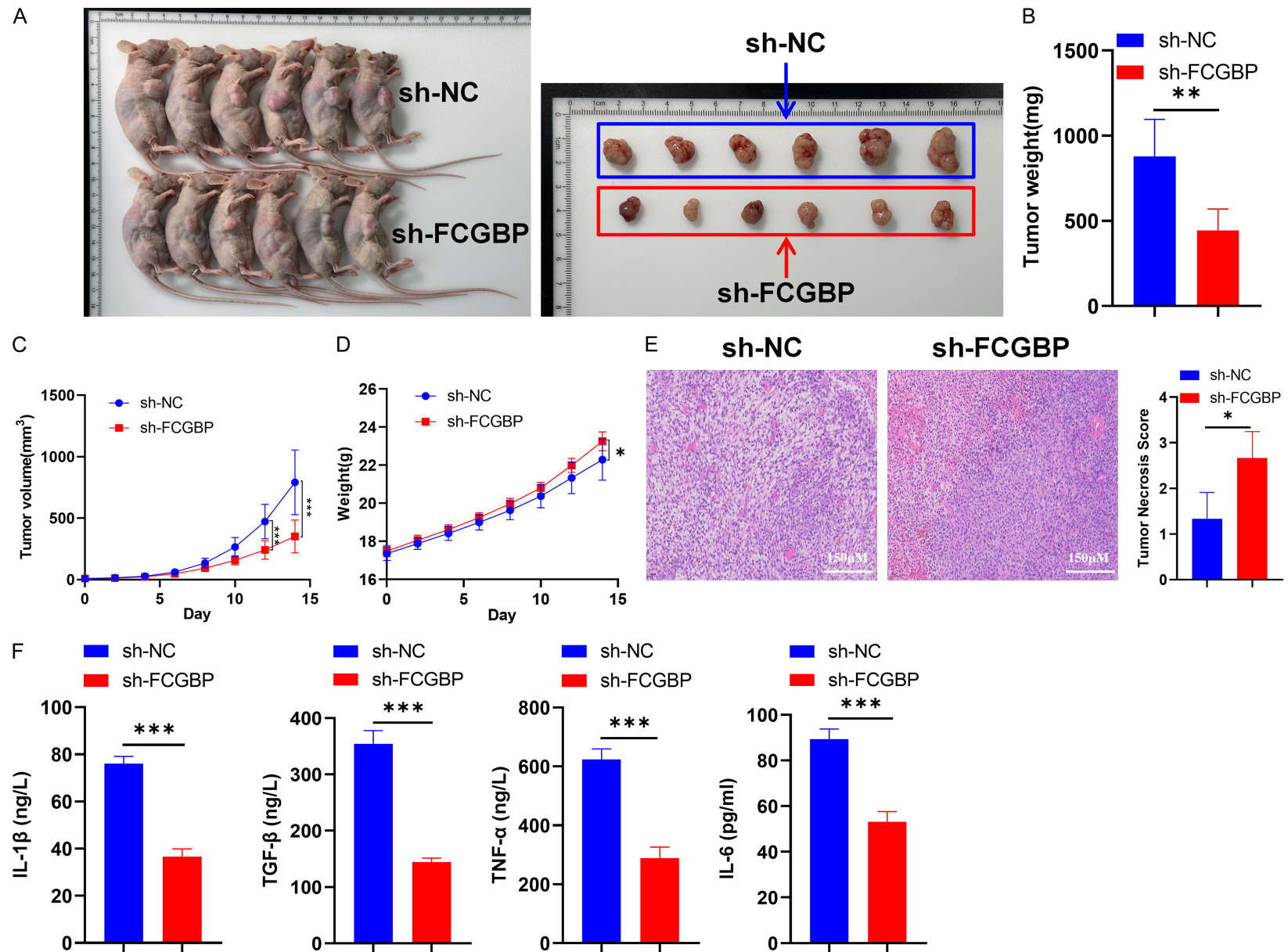




Figure 6. FCGBP knockdown suppressed the growth of subcutaneous U87 xenograft tumors in nude mice. A. Representative images of subcutaneous tumors from nude mice of the sh-NC and sh-FCGBP groups. B. Comparison of terminal tumor weight. C. Changes in tumor volume over time. D. Body weight changes in mice. E. Hematoxylin and eosin (H&E) staining of tumor tissues and quantitative analysis of tumor necrosis score, magnification: $\times 200$. F. ELISA analysis of serum levels of TGF- β , TNF- α , IL-6, and IL-1 β . G. IHC detection of Ki-67 and PCNA expression in tumor tissues, magnification: $\times 200$. H. TUNEL staining and quantitative analysis of TUNEL-positive signal intensity per unit area in tumor tissues, magnification: $\times 200$. Data are presented as mean $\pm$ SD from three independent experiments. Tumor volume and body weight curves were analyzed using two-way repeated-measures ANOVA followed by Bonferroni's post-hoc test. Other two-group comparisons were analyzed using an unpaired Student's t-test. Compared with the sh-NC group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

tration of inflammatory cells within tumor tissues. These findings suggest that FCGBP may also modulate the tumor-associated inflammatory microenvironment. In recent years, TGF- β is recognized as a key mediator of the immunosuppressive microenvironment in glioma and a central target in immunotherapy research [23-25]. Collectively, our findings suggest that FCGBP contributes to glioma progression both by promoting malignant cellular behaviors and by modulating TGF- β -related microenvironmental changes.

This study has several limitations. First, the mechanistic studies were primarily conducted in U87 cells, with insufficient validation in other glioma cell lines. Second, although the subcutaneous xenograft model is suitable for evaluating tumor growth, it does not fully recapitulate the unique intracranial glioblastoma microenvironment. Future studies utilizing orthotopic intracranial xenografts, patient-derived xenografts, or genetically engineered glioma models are warranted to confirm the effects of FCGBP on intracranial tumor progression, TGF- β /Smad signaling, inflammation, and the immune microenvironment remodeling. Finally, while this study suggests an association between FCGBP and TGF- β 1 and demonstrates that FCGBP knockdown reduces intracellular TGF- β 1 protein abundance and extracellular TGF- β levels, the precise regulatory mechanism remains unclear. Specifically, this study did not determine whether FCGBP influences TGFB1 transcription, TGF- β 1 translation, protein stability, secretion, or extracellular degradation. These questions should be addressed in future mechanistic studies.

Conclusion

FCGBP is significantly upregulated in glioma cells. FCGBP promotes malignant phenotypes, including proliferation, migration, and invasion, while suppressing apoptosis, possibly by activating the TGF- β /Smad signaling pathway. Collectively, FCGBP may represent a potential therapeutic target for glioma.

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Disclosure of conflict of interest

None.

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