Article

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Genistin Represses the Proliferation and Angiogenesis While Accelerating the Apoptosis of Glioma Cells by Modulating the FOXC1-Mediated Wnt Signaling Pathway

Xingyu Lan[1,†], Zhihong Gui[2,†], Taotao Chen[3], Mosheng Tang[4], Huafu Wang[5,*]

[1]Department of Medical Education, Yunhe County People's Hospital, 323600 Lishui, Zhejiang, China
[2]Department of Nephrology, The Sixth Affiliated Hospital of Wenzhou Medical University, The First Affiliated Hospital of Lishui University, Lishui People's Hospital, 323000 Lishui, Zhejiang, China
[3]College of Medicine, Lishui University, 323020 Lishui, Zhejiang, China
[4]Department of Operation and Maintenance, The Sixth Affiliated Hospital of Wenzhou Medical University, The First Affiliated Hospital of Lishui University, Lishui People's Hospital, 323020 Lishui, Zhejiang, China
[5]Department of Pharmacy, The Sixth Affiliated Hospital of Wenzhou Medical University, The First Affiliated Hospital of Lishui University, Lishui People's Hospital, 323000 Lishui, Zhejiang, China
*Correspondence: wanghuafu_whf@163.com (Huafu Wang)
[†]These authors contributed equally.
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Background: Glioma is a tumor originating from glial cells and is the most common primary brain tumor. At present, the main treatment methods for glioma include surgical resection and radiotherapy and chemotherapy, but the treatment effect is not very ideal. Genistin (GS) inhibits breast cancer cell growth while promoting apoptosis, but its effect and detailed molecular mechanism on glioma are yet to be defined. In addition, forkhead box C1 (FOXC1) has been found to be involved in the growth, invasion, and angiogenesis processes of glioma cells.

Methods: Human glioma cells in the Control, GS-6.25, GS-12.5, and GS25 (GS) groups were treated with 0, 6.25, 12.5, and 25 µM of Genistin, respectively, for 72 hours, and cells in the GS + NC (negative control) and GS + FOXC1 groups were transfected with negative control or forkhead box C1 (FOXC1) overexpression plasmids, respectively, prior to Genistin (25 µM) treatment for 72 hours. Next, the viability, proliferation, apoptosis, and angiogenesis of treated glioma cells were detected using Cell Counting Kit-8 (CCK-8), 5-ethynyl-2′deoxyuridine (EdU) proliferation, flow cytometry, and tube formation assays. Meanwhile, the half-maximal inhibitory concentration (IC_{50}) of Genistin in the treated glioma cells was calculated. Afterwards, quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot quantified the levels of FOXC1, Wnt1, Wnt3a, glycogen synthase kinase-3β (GSK3β), and phosphorylated GSK3β (p-GSK3β).

Results: Genistin inhibited viability, proliferation, and angiogenesis while promoting the apoptosis of glioma cells ($p < 0.05$, $p < 0.001$). Also, Genistin decreased the levels of FOXC1, Wnt1, and Wnt3a while increasing p-GSK3β levels in glioma cells ($p < 0.05$, $p < 0.01$, $p < 0.001$). FOXC1 was up-regulated in glioma cells and tissues, and overexpressed FOXC1 overturned the effects of Genistin on the abovementioned factors in glioma cells ($p < 0.05$, $p < 0.001$).

Conclusions: Genistin inhibits viability, proliferation, and angiogenesis while accelerating glioma cell apoptosis by modulating the FOXC1-mediated Wnt signaling pathway.

Keywords: Genistin; glioma; angiogenesis; apoptosis; forkhead box C1

Introduction

Glioma, one of the most widespread primary intracranial tumors, is usually derived from central nervous system glial cells and is one of the most challenging diseases in cancer treatment [1,2]. Relevant data show that in the United States, 6 cases of glioma patients are diagnosed per 100,000 people annually [3]. Modern technologies provide options for diagnosing and treating gliomas more accurately and conveniently. However, surgical glioma resection is the only viable option for some patients [4]. Still, because of the high tumor recurrence rate after surgical resection, the prognosis of patients is generally poor [5–7]. In recent years, molecular targeted therapy for glioma has gradually attracted much attention, and studies have found that glioma progression can be inhibited by modulating some gene expressions [8].

Over the last decades, it has been found that many active components extracted from plants in nature have

anti-cancer effects. For instance, in glioma, many traditional Chinese medicines and active components extracted from plants suppress the metastasis and growth of glioma cells, which is achieved by the regulation of the corresponding gene expressions, such as Shezhi Huangling decoction, Isoliquiritigenin, and Ginsenoside Rg3 [9–11]. Similarly, Genistin, an isoflavone glycoside widely distributed in nature, is present in various dietary plants, especially in kudzu (Japanese arrowroot) and soybean, and many studies have shown that Genistin has anti-apoptotic, anti-oxidative, and anti-cancer effects [12]. Hwang *et al.* [13] have highlighted that Genistin restrains the growth and accelerates the apoptosis of breast cancer cells via modulation of the ERalpha signaling pathway. Choi *et al.* [14] have indicated that Genistin inhibits cell proliferation and induces apoptosis of human ovarian SK-OV-3 cells by inhibiting the G1 or G2/M phase of the cell cycle. However, the effect and mechanism underlying Genistin in glioma is unknown.

Mutations in epigenetic regulator genes are identified as key drivers of glioma progression [15]. Forkhead box C1 (FOXC1), from the forkhead box family of transcription factors, is implicated in the apoptosis and proliferation of different cancer cells [16]. In addition, FOXC1 is highly expressed in glioma tissues, and miR-133 can negatively regulate the invasion and growth of glioma cells by down-regulating the level of FOXC1 [17]. Likewise, silencing FOXC1 suppresses glioma cells' epithelial-to-mesenchymal transition (EMT) [18]. In addition, FOXC1 can regulate the Wnt signaling pathway, which is proven to be involved in glioma progression [19]. Interestingly, Genistein inhibits the FOXC1 level and stimulates apoptosis of colon cancer cells [20]. Therefore, we speculated that Genistin modulates the Wnt signaling pathway by inhibiting FOXC1, thereby hindering glioma progression.

The present study delved into the effect of Genistin and evaluated the level of FOXC1 in glioma. Furthermore, we explored the modulatory mechanisms of Genistin in glioma. This research demonstrated a new anti-tumor mechanism of Genistin in glioma cells, and these results provide new research direction for the treatment of glioma.

Materials and Methods

Clinical Tissue Sampling

28 cases of glioma tissue samples and 28 cases of normal tissues adjacent to cancer tissues were collected from diagnosed glioma patients. All patients with cancer were diagnosed at Lishui People's Hospital between October 2020 and February 2021, and all patients did not receive chemoradiotherapy or immunotherapy before surgery.

Cell Culture

The human glioma cell line U-87 (bio-105821) was purchased from Biobw (Beijing, China). Normal human astrocytes (NHA) cells (MZ-1246) and human glioma cell lines A172 (MZ-0012), U251 (MZ-0186), and LN-229 (MZ-1620) were purchased from Ningbo Mingzhou Biological Technology (Ningbo, China). All cells (U-87, NHA, A172, U251, and LN-229) were seeded within Dulbecco's Modified Eagle Medium (PM150235, Mingzhou Biological Technology, Ningbo, China) including 10% of fetal bovine serum (FSP500, ExCell Bio., Shanghai, China) and 1% of Penicillin-Streptomycin Solution (PB180120, Procell, Wuhan, China) at 37 ℃ with 5% CO_2. All cells were routinely tested for short tandem repeat (STR) identification and mycoplasma contamination and were confirmed to be mycoplasma-free.

Cell Transfection

FOXC1 overexpressed plasmids cloned into pcDNA3.1 vector (VT1001) and its negative control (NC) were synthesized by YouBio (Changsha, China). Lipofectamine 3000 reagent (L3000-001) purchased from Invitrogen (Carlsbad, CA, USA) was applied to transfect FOXC1 overexpressed plasmid or NC into U-87 cells at a confluence of 70–80% for 48 hours. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) were assessed for transfection efficiency.

Cell Treatment

Genistin (HY-N0595, MedChemExpress, Monmouth Junction, NJ, USA) was dissolved by DMSO (HY-Y0320, MedChemExpress, USA) to prepare the stock solution. As previously described [13], U-87 cells were administrated with 0, 6.25, 12.5, 25, 50, and 100 μM Genistin for 72 hours. In another test, the transfected U-87 cells were treated with 25 μM Genistin for 72 hours, followed by subsequent studies.

Cell Viability Analysis

In this work, a Cell Counting Kit-8 (CCK-8; C0037, Beyotime, Shanghai, China) was used to evaluate the viability of treated U-87 cells. Briefly, U-87 cells (2000 cells/well) were treated as described above and incubated (37 ℃, 5% CO_2) in the incubator. Then 10 μL of CCK-8 solution was added into every well, followed by a 1 hour incubation. A microplate reader (EnSight, PerkinElmer, Waltham, MA, USA) assessed the absorbance (at 450 nm). Afterward, the half-maximal inhibitory concentration (IC_{50}) of Genistin on the cells was analyzed by SPSS 20.0 (IBM, Armonk, NY, USA) based on the experimental results.

Cell Proliferation Analysis

5-ethynyl-2′deoxyuridine (EdU) staining assay was performed in this part, as previously described [21]. In this work, EdU Cell Proliferation Kit (C0078), Phosphate-Buffered Saline (PBS; C0221A), 4% Paraformaldehyde (P0099), Immunol Staining Blocking Buffer (P0102), and Enhanced Immunostaining Permeabilization Buffer

Table 1. All primers in qRT-PCR experiments in this study.

ID	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
FOXC1	GGCGAGCAGAGCTACTACC	TGCGAGTACACGCTCATGG
GAPDH	GAAATCCCATCACCATCTTCCAGG	GAGCCCCAGCCTTCTCCATG

qRT-PCR, quantitative real-time polymerase chain reaction; FOXC1, forkhead box C1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(P0097) were purchased from Beyotime (China). U-87 cells were maintained in 6-well plates, and 20 µL of EdU working solution was added into the sample well, followed by a 2 hour incubation. Next, the treated cells were fixed with 4% Paraformaldehyde at room temperature (RT) for 15 minutes and were washed with Immunol Staining Blocking Buffer. Later, treated U-87 cells were incubated in Enhanced Immunostaining Permeabilization Buffer for 10 minutes at RT, followed by washing of the Immunol Staining Blocking Buffer. Subsequently, cells were treated with Click Additive solution for 30 minutes in the dark, and then nuclear staining was performed using the Hoechst 33342 solution. Finally, the fluorescence intensity of treated U-87 cells was measured using an N-STORM fluorescence microscope (Nikon, Tokyo, Japan) at ×200 magnification.

Cell Apoptosis Analysis

Annexin V-fluorescein isothiocyanate (FITC)/Propidium Iodide Apoptosis Detection Kit (C1062M, Beyotime, China) was used to quantify the apoptosis of U-87 cells. 5×10^4 of Treated U-87 cells were centrifuged and resuspended with 195 µL of Annexin V-FITC conjugated solution and treated with 5 µL of Annexin V-FITC working solution, 10 µL of Propidium iodide staining solution, and incubated in dark at RT for 20 minutes. Finally, the apoptosis of cells was assessed using a flow cytometer (1026, Beamdiag, Changzhou, China) and Cell Quest software (BD Biosciences, San Diego, CA, USA).

Tube Formation Assay

As previously described, the angiogenesis of treated U-87 cells was calculated by tube formation assay [22]. Prior to the tube formation assay, Matrigel Basement Membrane Matrix (M8370) was ordered from Solarbio (Beijing, China) and used to add to 96-well plates. After the Matrigel was solidified, U-87 cells were seeded on a Matrigel-coated well (20,000 cells/well). Later, the culture media from treated U-87 cells were loaded onto cell-seeded wells and incubated for another 24 hours. Later, the formation of tube-like structures was observed and captured (under ×100 magnification) using a TS100 microscope (Nikon, Japan), and the data on tube length were quantified with ImageJ software (v. 1.52, National Institutes of Health, Bethesda, MA, USA).

qRT-PCR

qRT-PCR was performed as previously described [23]. Total RNA was extracted from treated U-87 cells and tissue samples by TRIzol Reagent (B0201, HaiGene, Harbin, China). The concentration of isolated RNA samples was detected by spectrophotometer (S-060, Onlab Instrument Co., LTD, Shanghai, China). After that, cDNA was synthesized and qRT-PCR reactions mix solution was prepared with the help of One Step SuperRT-PCR Mix Kit (T2240, Solarbio, China) and then the qRT-PCR reactions mix solution was performed using a real-time PCR instrument (CFX96 Touch, Bio-Rad, Hercules, CA, USA) under the conditions: reverse-transcription (50 ℃ for 15 minutes), pre-denaturation (95 ℃ for 2.5 minutes), and 40 cycles of denaturation (95 ℃ for 20 seconds), annealing (50–65 ℃ for 25 seconds), and extension (72 ℃ for 60 s/kb). The $2^{-\Delta\Delta ct}$ method [24] was employed to analyze relative data, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control of FOXC1. The involved sequences are listed in Table 1.

Western Blot

Total protein from treated U-87 cells was firstly isolated through radioimmunoprecipitation assay (RIPA) Lysis Buffer (CW2333, Cowin Biotech, Nanjing, China) enriched with a protease and phosphatase inhibitor cocktail (P002, NCM Biotech, Suzhou, China). A bicinchoninic acid (BCA) Protein Assay Kit (E112-01, Vazyme, Nanjing, China) was utilized to quantify protein concentration. Later, 20 µL of protein was electrophoresed with the help of an sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel preparation kit (BB-3702, BestBio, Nanjing, China). Equal amounts of protein on the gel were transferred to 0.45 µm polyvinylidene fluoride membranes (AC17619, Acmec Biochemical, Shanghai, China). Then, the membranes were blocked with 5% skim milk (P0216, Beyotime, China) in 1× Tris Buffered Saline with Tween-20 (TBST, AC17148, Acmec Biochemical, China) for 1 hour at RT. Primary antibodies and loading control GAPDH were utilized to incubate the membranes at 4 °C overnight, and a 2 hour incubation with horseradish peroxidase-conjugated secondary antibodies was performed at RT. Later, the membrane was developed in supper ECL western blotting substrate (SL1350, Coolaber, Beijing, China), and ScanLater™ western blot detection system (Molecular Devices, LLC., San Jose, CA,

Table 2. All antibody information and sources in Western blot in this study.

ID	Catalog number	Company (country)	Molecular weight	Dilution ratio
FOXC1	ab227977	Abcam (Cambridge, UK)	57 kDa	1/1000
Wnt1	ab15251	Abcam (Cambridge, UK)	41 kDa	1/1000
Wnt3a	ab219412	Abcam (Cambridge, UK)	39 kDa	1/1000
p-GSK3β	ab75814	Abcam (Cambridge, UK)	47 kDa	1/10000
GSK3β	ab32391	Abcam (Cambridge, UK)	46 kDa	1/5000
GAPDH	ab181602	Abcam (Cambridge, UK)	36 kDa	1/10000
Rabbit IgG	ab205718	Abcam (Cambridge, UK)		1/5000

FOXC1, forkhead box C1; p-GSK3β, phosphorylated glycogen synthase kinase-3β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

USA) and Image J software was used for the further analysis of the corresponding data. The antibodies used in this study are presented in Table 2.

Statistical Analysis

SPSS 20.0 (IBM, Amonk, NY, USA) was performed for statistical analysis. Measured data were expressed as mean $\pm$ standard deviation. The data in multiple groups were compared by one-way analysis of variance. Differential gene expression between the two groups was compared by paired sample *t*-test, with the post-hoc analysis of the Dunnett-*t* test and Bonferroni test. $p < 0.05$ was considered statistically significant.

Results

Genistin Inhibited Viability, Proliferation, and Angiogenesis While Promoting the Apoptosis of Glioma Cells

In this work, we aimed to unveil Genistin's effect and mechanism in glioma. At first, collected human glioma cell line U-87 were treated with 0, 6.25, 12.5, 25, 50, and 100 µM Genistin for 72 hours, and the results of the CCK-8 experiment exhibited that Genistin suppressed the viability of glioma cells (Fig. 1A, $p < 0.001$). Afterward, the half-maximal inhibitory concentration (IC_{50}) value of Genistin in the U-87 cells was calculated by SPSS based on the experimental results, which indicated that the IC_{50} value of Genistin in U-87 cells was 26.902 $\pm$ 1.430 µM (Fig. 1A). Therefore, in subsequent studies, 6.25, 12.5, and 25 µM of Genistin were chosen as the concentrations for next treatment. Later, the proliferation (Fig. 1B), apoptosis (Fig. 1C), and angiogenesis (Fig. 1D) of the treated glioma cells were measured. The results of the EdU cell proliferation assay uncovered that Genistin treatment diminished the number of EdU-positive cells, which indicated that Genistin significantly reduced cell proliferation (Fig. 1B). Afterwards, we also found that Genistin overtly accelerated apoptosis (12.5 and 25 µM) while inhibiting angiogenesis of glioma cells (Fig. 1C,D, $p < 0.05$, $p < 0.001$). Taken together, Genistin inhibited the viability, proliferation, and angiogenesis while promoting the apoptosis of glioma cells; however, further studies are needed to determine the underlying mechanisms.

Genistin Decreased the Levels of FOXC1, Wnt1, and Wnt3a, While Increasing p-GSK3β Level in Glioma Cells

To explore the mechanism of Genistin, we examined the effect of Genistin on FOXC1 expression, and the results indicated that Genistin notably reduced the level of FOXC1 (Fig. 2A,B, $p < 0.05$, $p < 0.001$). As previously described, up-regulation of FOXC1 promoted the expressions of Wnt pathway-related factors Wnt1 and Wnt3a while down-regulating the p-GSK3β level [19]. In addition, we found that Genistin decreased the expression levels of Wnt1 (Fig. 2C, $p < 0.001$) and Wnt3a (Fig. 2D, $p < 0.05$, $p < 0.001$) while increasing the ratio p-GSK3β/GSK3β (Fig. 2E, $p < 0.01$, $p < 0.001$) in glioma cells. These data hinted that Genistin may inhibit glioma progression by regulating FOXC1 expression and Wnt-related pathways.

FOXC1 was Up-Regulated in Glioma

The expression of FOXC1 was higher in glioma tissue than in normal tissues (Fig. 3A, $p < 0.001$), suggesting that FOXC1 abnormal expression was implicated in glioma progression. Similarly, the expression of FOXC1 was enhanced in glioma cell lines (U87, A172, U251, and LN-229) compared to the NHA cells (Fig. 3B,C, $p < 0.001$). Due to the highest mRNA and protein expressions of FOXC1 in U87 cells, this cell line was selected for subsequent experiments.

Overexpressed FOXC1 Abolished the Inhibitory Effect of Genistin on Viability, Proliferation, and Angiogenesis and Overturned the Promotive Effect of Genistin on Apoptosis of Glioma Cells

To further analyze the mechanism of Genistin in glioma, glioma cells were transfected with or without FOXC1 overexpression plasmid and further treated with Genistin. By examining the FOXC1 expression in the treated glioma cells, we found that Genistin decreased the FOXC1 level in comparison to the Control group

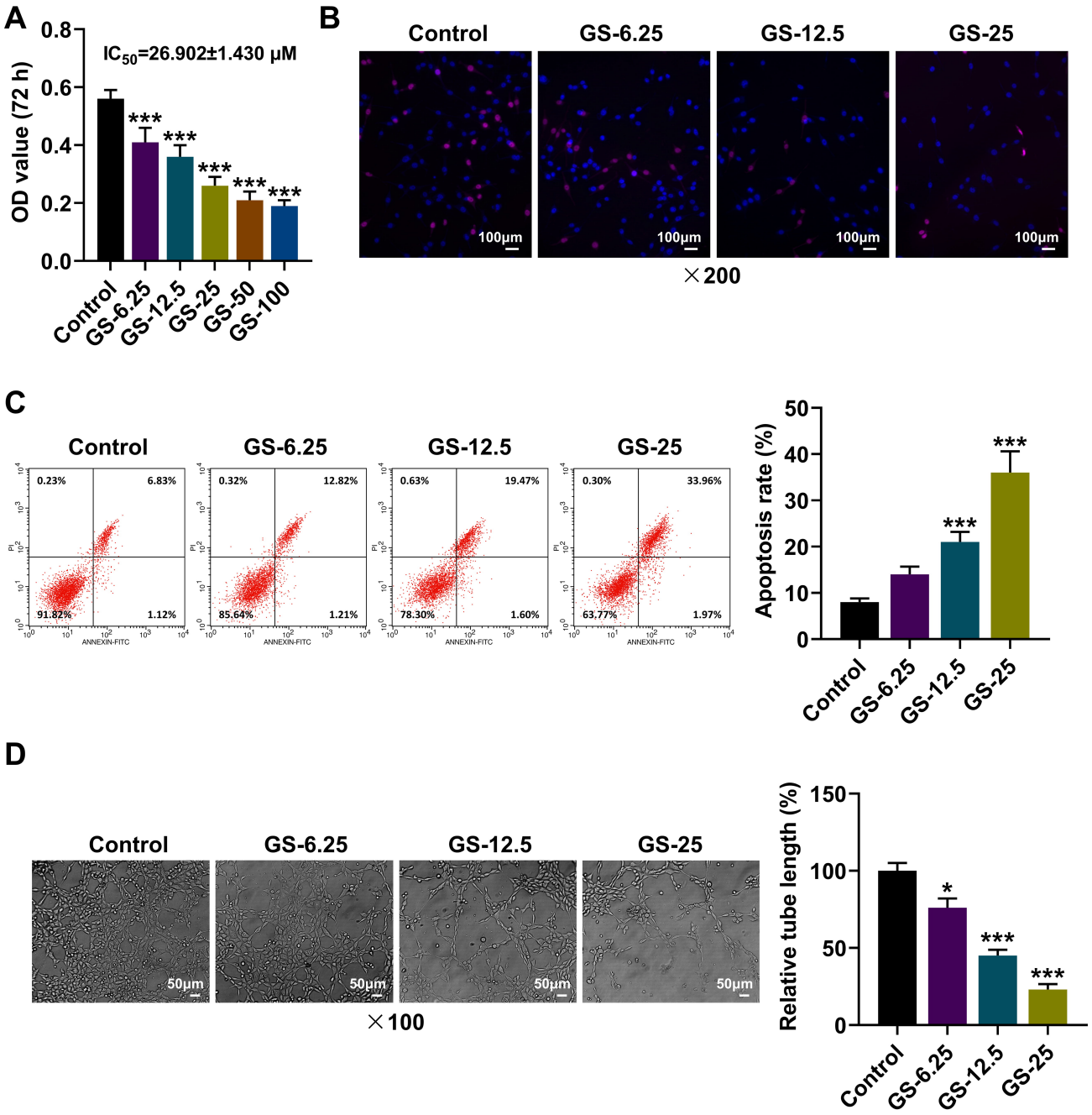

Fig. 1. Genistin suppressed viability, proliferation, and angiogenesis while promoting glioma cell apoptosis. (A–D) Glioma cells U-87 were treated with Genistin (0, 6.25, 12.5, 25, 50, 100 μM) for 72 hours, then we measured the viability using CCK-8 assay and calculated the IC_{50} value of Genistin in the U-87 cells by SPSS 20.0 (A); and then the proliferation of treated glioma cells was measured by EdU cell proliferation assay (under ×200 magnification, Scale bar = 100 μm) (B); treated glioma cell apoptosis was detected using a flow cytometer (C); the angiogenesis of treated glioma cells was tested by Tube formation assay (under ×100 magnification, Scale bar = 50 μm) (D). Data presented as mean ± standard deviation. *$p < 0.05$, ***$p < 0.001$ vs. Control. n = 3. GS, Genistin; CCK-8, Cell Counting Kit-8; IC_{50}, half-maximal inhibitory concentration; EdU, 5-ethynyl-2′deoxyuridine.

(Fig. 4A,B, $p < 0.001$); in contrast, the FOXC1 level remarkably increased by plasmids overexpressing FOXC1 in glioma cells compared to the GS (Genistin) + NC (negative control) group (Fig. 4A,B, $p < 0.001$). After that, we evaluated the viability (Fig. 4C), proliferation (Fig. 4D), apoptosis (Fig. 4E), and angiogenesis (Fig. 4F) of treated and transfected glioma cells. We found that overexpression of FOXC1 could alleviate the inhibitory effects of Genistin on viability and proliferation in glioma cells in contrast to the GS + NC group (Fig. 4C,D, $p < 0.001$). Moreover, overexpressed FOXC1 reversed the promotive effect of Genistin on apoptosis of glioma cells (Fig. 4E, $p < 0.001$) and over-

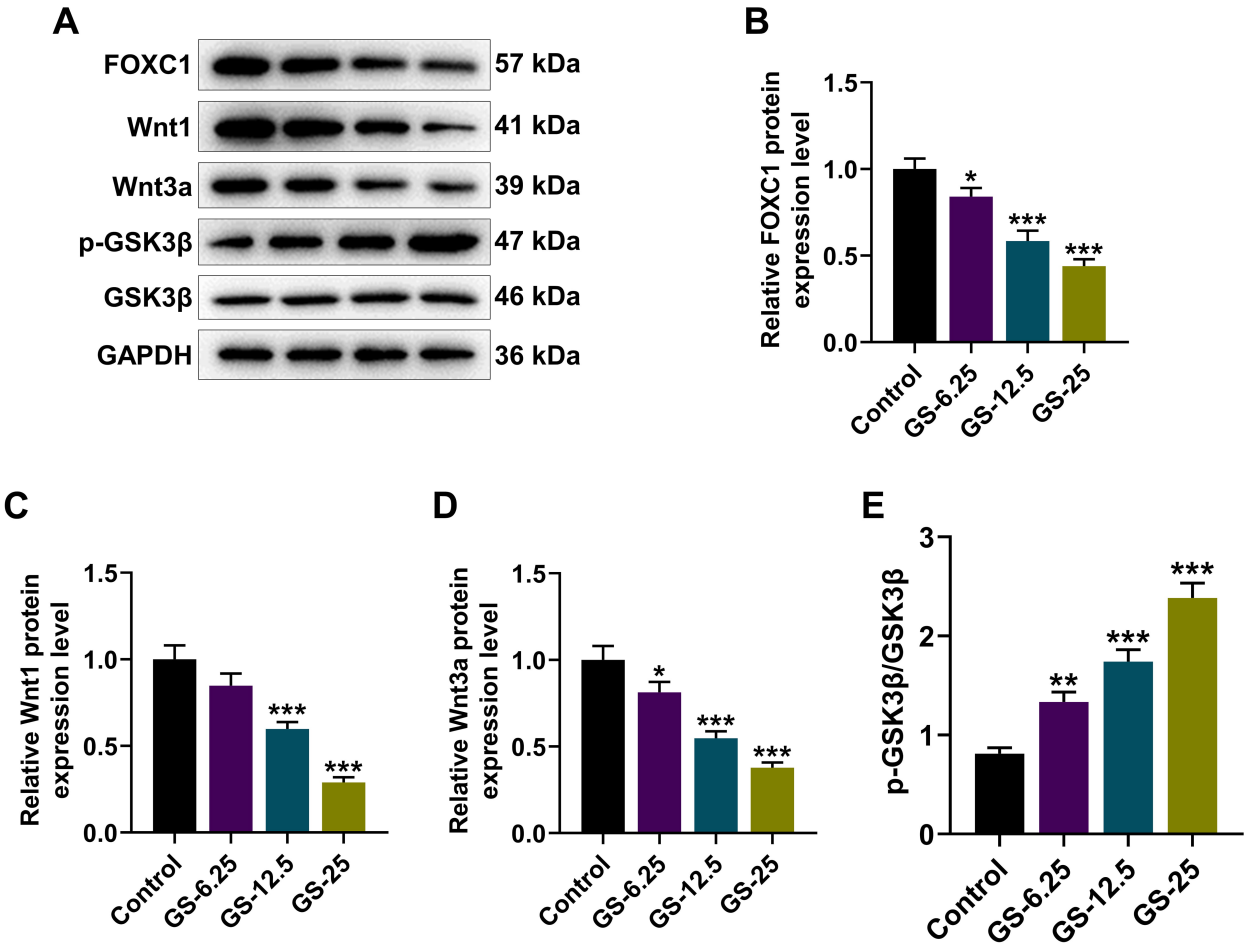

Fig. 2. Genistin decreased FOXC1, Wnt1, and Wnt3a levels while increasing p-GSK3β/GSK3β value in glioma cells. (A–E) Glioma cells U-87 were treated with Genistin (0, 6.25, 12.5, 25 μM) for 72 hours, then the levels of FOXC1 (B), Wnt1 (C), Wnt3a (D), and p-GSK3β/GSK3β (E) were detected using Western blot. Data presented as mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control. n = 3. FOXC1, forkhead box C1; p-GSK3β, the activated form of GSK3β.

turned the inhibitory effect of Genistin on angiogenesis of glioma cells, when compared with the GS + NC group (Fig. 4F, $p < 0.001$).

Overexpressed FOXC1 Increased the Genistin-Decreased the Levels of Wnt1 and Wnt3a, While Inhibiting Genistin-Increased p-GSK3β/GSK3β Value in Glioma Cells

Wnt1, Wnt3a, p-GSK3β, and GSK3β levels in treated and transfected glioma cells were also examined using Western blot assay. We found that Genistin inhibited Wnt1 and Wnt3a expressions (Fig. 5A–C, $p < 0.001$); while promoted p-GSK3β/GSK3β value (Fig. 5D, $P < 0.001$); besides, overexpressed FOXC1 increased the Genistin-decreased the levels of Wnt1 and Wnt3a, while inhibited Genistin-increased p-GSK3β/GSK3β value in glioma cells (Fig. 5A–D, $p < 0.05$, $p < 0.001$). The abovementioned finding indicated that Genistin inhibited cell viability, proliferation, and angiogenesis and promoted the apoptosis of glioma cells by inhibiting FOXC1 mediated-Wnt pathway.

Discussion

Increasing plant-extracted compounds have been widely studied and used in cancer treatment [25]. Genistin is an isoflavone, and its role has been frequently reported in different diseases [12]. Previous studies have shown that Genistin inhibits cancer progression mainly by promoting apoptosis, altering cell cycle progression, and reducing angiogenesis of cancer cells [12,13,26]. Hamdy *et al.* [27] have demonstrated that administration of Genistin inhibited the levels of oxidative stress and tumorigenicity in breast cancer model rats. Hwang *et al.* [13] have indicated that Genistin inhibited breast cancer cell viability and facilitated the apoptosis. Related studies have found that the occurrence of glioma is accompanied by massive angiogenesis, while Genistin has an anti-tumor angiogenic effect [2,13]. In this study, we, for the first time, uncovered that Genistin acted an inhibitory role in glioma cells' viability, proliferation, and angiogenesis, yet promoting cell viability. However, its modulatory mechanisms are unknown.

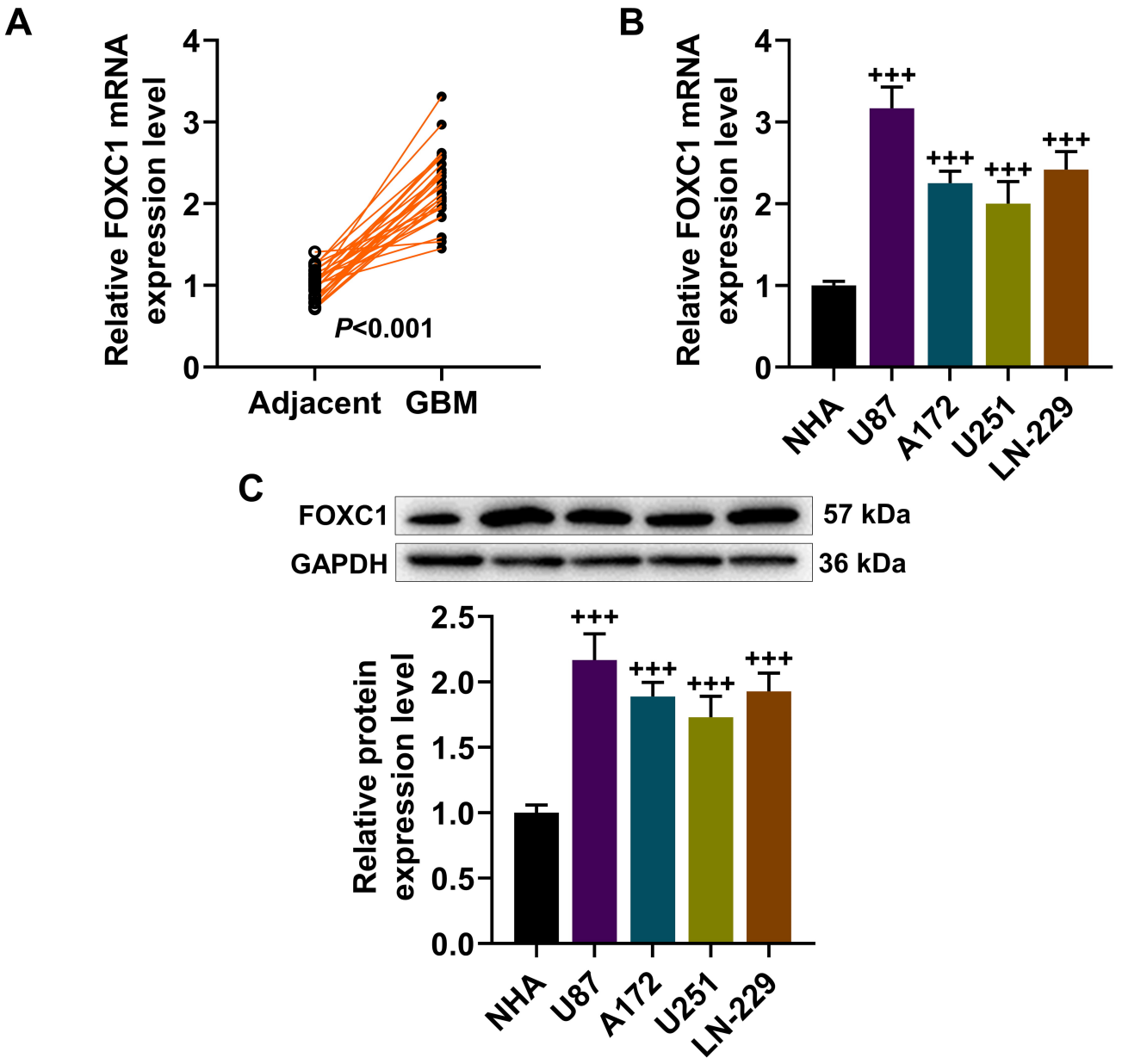

Fig. 3. FOXC1 was up-regulated in glioma. (A) FOXC1 expression in glioma tissue (n = 28) and normal tissue samples (n = 28) was examined using qRT-PCR. (B) FOXC1 expression in glioma and NHA cells was examined using qRT-PCR. (C) The level of FOXC1 in glioma cells and normal human astrocyte cells was examined using Western blot. $^{+++}p < 0.001$ vs. NHA. n = 3. Data presented as mean ± standard deviation. qRT-PCR, quantitative real-time polymerase chain reaction; NHA, normal human astrocytes; GBM, glioblastoma.

The level of FOXC1 was previously reported to be elevated in glioma tissue [17]. In agreement with what was previously reported, we also confirmed that the FOXC1 expression level was elevated in glioma tissue and cells. Furthermore, FOXC1 is up-regulated in various cancers, and aberrant FOXC1 expression is relevant to cancer cells' proliferation, angiogenesis, and apoptosis [16]. In addition, FOXC1 enhances glioma angiogenesis by promoting the promoter activity and level of chemokine receptor 7b [28]. Besides, in non-small cell lung cancer, Docosahexaenoic acid represses the cell viability and cell invasion ability by decreasing the expression of FOXC1 [29]. In basal-like breast cancer, high FOXC1 expression enhances the cancer cell migratory ability and proliferation and promotes the EMT of basal-like breast cancer cells [30]. Interestingly, Genistein stimulated colon cancer cell apoptosis by inhibiting the FOXC1 level [20]. In this study, we first uncovered that Genistin decreased the FOXC1 level in glioma cells, and overexpressed FOXC1 reversed the inhibitory role of Genistin on viability, proliferation, and angiogenesis, and overturned the promoting effect of Genistin on apoptosis of glioma cells. These results suggested that Genistin could suppress proliferation and angiogenesis while promoting glioma cell apoptosis by inhibiting the FOXC1 level.

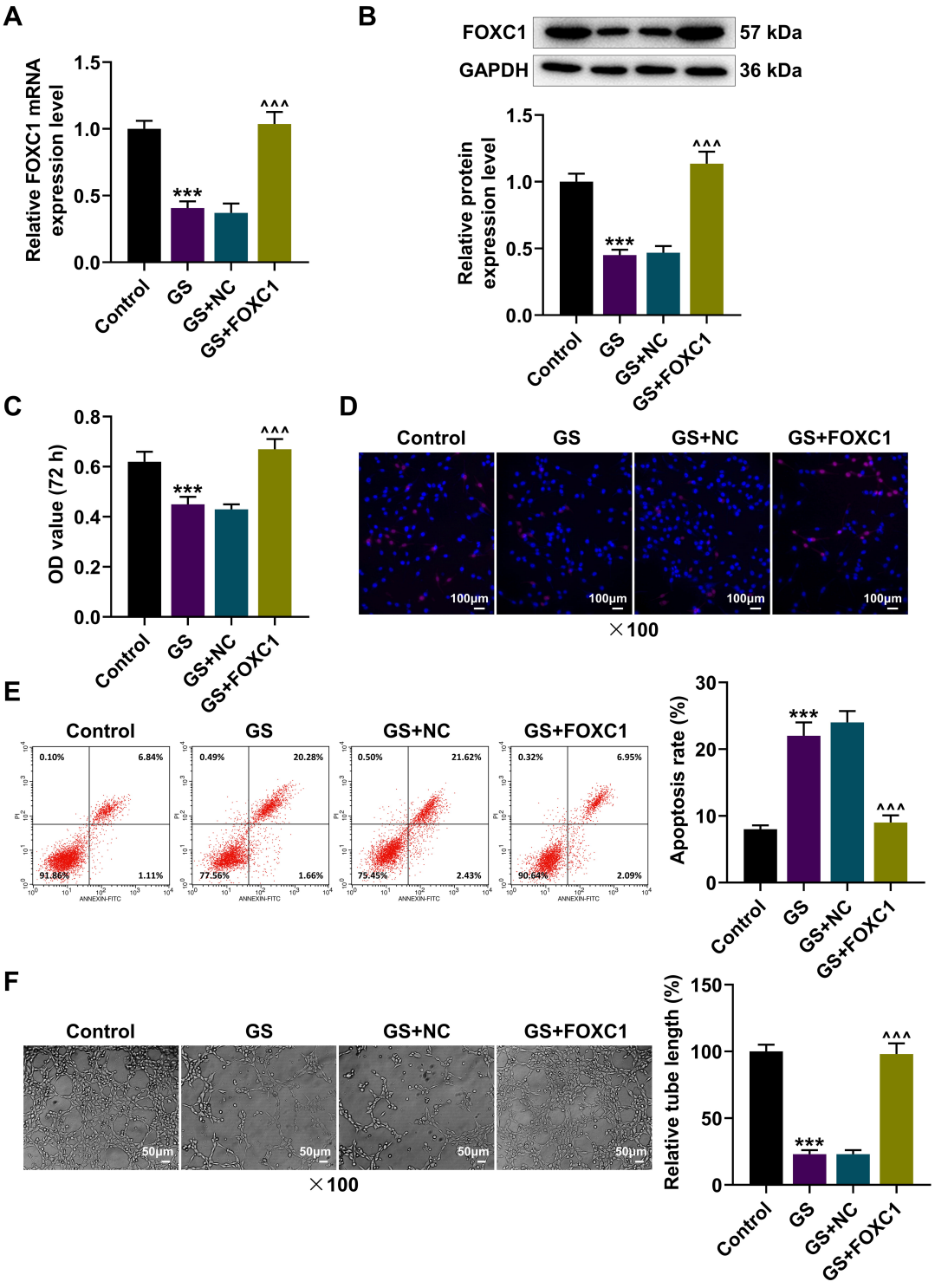

Fig. 4. Overexpressed FOXC1 reversed the inhibitory effect of Genistin on viability, proliferation, and angiogenesis and overturned the promoting effect of Genistin on apoptosis of glioma cells. (A–F) Glioma cells were transfected with FOXC1 or its negative control and further treated with 25 μM of Genistin for 72 hours, then the level of FOXC1 in treated glioma cells was examined using qRT-PCR (A); the expression of FOXC1 in treated glioma cells was examined using Western blot (B); the viability of treated glioma cells was examined using CCK-8 assay (C); the proliferation of treated glioma cells was measured by EdU cell proliferation assay (under ×100 magnification, Scale bar = 100 μm) (D); the apoptosis of treated glioma cells was detected using a flow cytometer, and the results were exhibited using the Kaluza C software (E); the angiogenesis of treated glioma cells was evaluated by Tube formation assay (under ×100 magnification, Scale bar = 50 μm) (F). Data presented as mean ± standard deviation. ***$p < 0.001$ vs. Control; ^^^$p < 0.001$ vs. GS + NC. n = 3. NC, negative control.

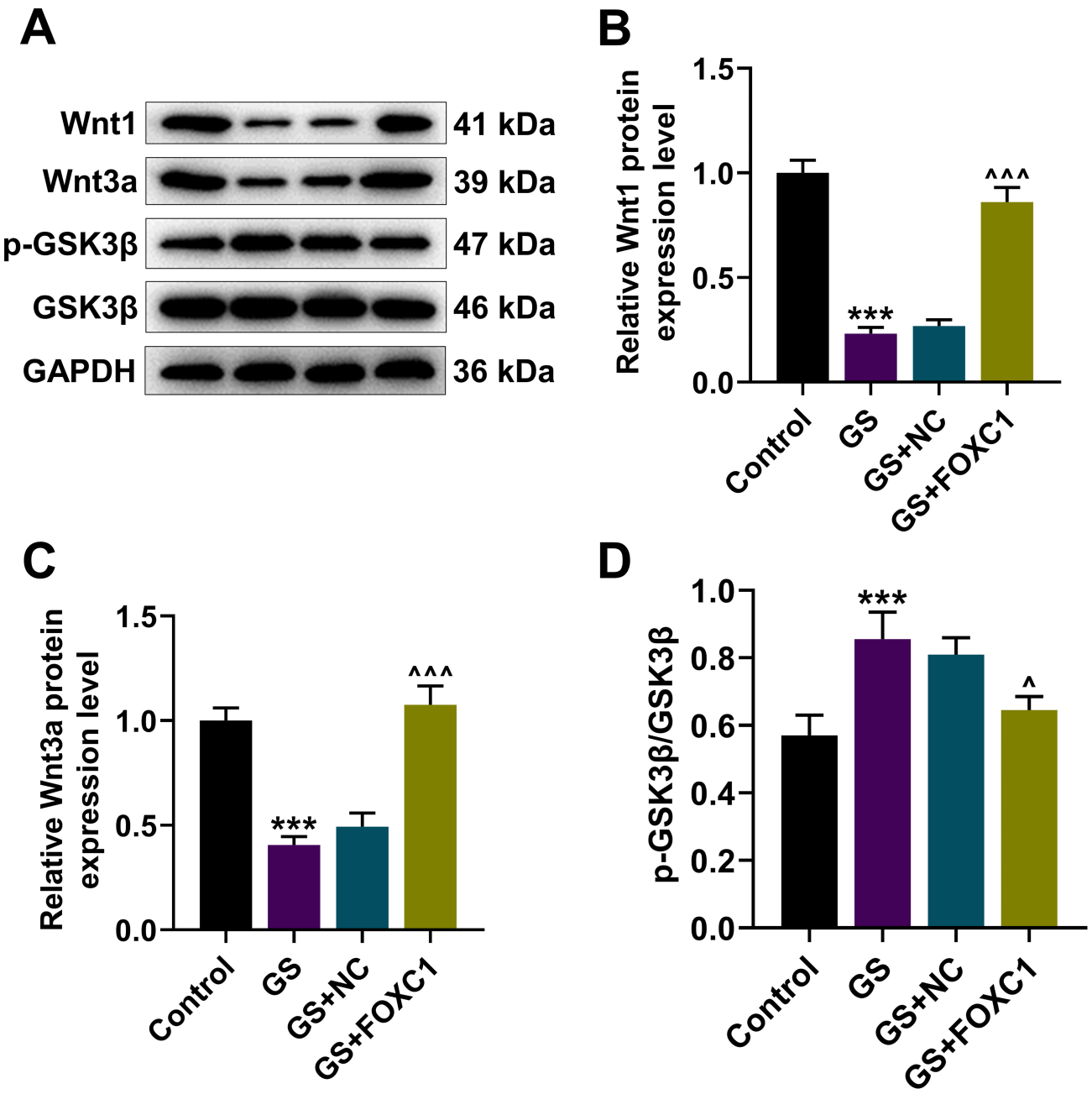

Fig. 5. Overexpressed FOXC1 increased the Genistin-decreased the levels of Wnt1 and Wnt3a, while inhibiting Genistin-increased p-GSK3β/GSK3β value in glioma cells. (A–D) Glioma cells were transfected with FOXC1 or its negative control and further treated with 25 μM of Genistin for 72 hours. Then, the levels of Wnt1 (B), Wnt3a (C), and p-GSK3β/GSK3β (D) in treated glioma cells were also examined using Western blot assay. Data presented as mean ± standard deviation. $^{***}p < 0.001$ vs. Control; $^{\wedge}p < 0.05$, $^{\wedge\wedge\wedge}p < 0.001$ vs. GS + NC. n = 3.

FOXC1 acts as a transcription factor for glutathione peroxidase 8 (GPX8), affecting cellular physiological activities and developmental processes by mediating the expression of GPX8 [19]. FOXC1 transcriptionally regulates GPX8 and facilitates the invasion and growth of gastric cancer cells via activating the Wnt signaling pathway [19]. Besides, in glioma, the aberrant expressed Wnt-related pathway is associated with glioma malignancy and suppresses cancer progression by inhibiting the activation of Wnt-related pathways [31]. Studies have shown that the Wnt signaling pathway can induce epithelial-to-mesenchymal transition by inhibiting GSK3β-mediated phosphorylation [32,33]. Wnt3a and wnt1 are involved in oncogenesis; the p-GSK3β can inhibit the activity of the Wnt pathway [19]. As previously described, high FOXC1 level up-regulated the expressions of Wnt pathway-related factors (Wnt1 and Wnt3a) while down-regulated the p-GSK3β level [19]. In this research, overexpressed FOXC1 elevated

Genistin-decreased levels of Wnt1 and Wnt3a while inhibiting Genistin-increased p-GSK3β/GSK3β value in glioma cells, indicating that Genistin was able to inhibit the progression of glioma by downregulating FOXC1 to inactivate the Wnt signaling pathway. Additionally, we should be aware that our study only demonstrated the effects of Genistin in glioma at the cellular level, so our future studies will focus on both pre-clinical and clinical models to verify its specific role in glioma.

Conclusions

In conclusion, we revealed that Genistin represses viability, proliferation, and angiogenesis while accelerating glioma cell apoptosis by modulating the FOXC1-mediated Wnt signaling pathway. Therefore, Genistin can be a potential drug for targeted therapy of glioma.

Availability of Data and Materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Author Contributions

XYL and ZHG designed the research study; XYL and ZHG performed the research; TTC, MST and HFW collected and analyzed the data. All authors have been involved in drafting the manuscript and have been involved in revising it critically for important intellectual content. All authors give final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The present study was approved by the Ethics Committee of Lishui People's Hospital (LLW-FO-403) and was conducted in accordance with the Declaration of Helsinki. Written informed consent was also collected from all glioma patients.

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Conflict of Interest

The authors declare no conflict of interest.

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