

Annexin A2 regulates glioma cell proliferation through the STAT3-cyclin D1 pathway

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Abstract. Annexin A2 (ANXA2) acts as a calcium-dependent phospholipid-binding protein that is widely expressed in vertebrate cells and has abnormally high expression in various tumor cells. However, the detailed molecular mechanism underlying the effects of ANXA2 on glioma cells remains unclear. The present study aimed to investigate the role and underlying molecular mechanisms of ANXA2 in glioma cell proliferation. The results revealed that knockdown of ANXA2 inhibited the proliferation of U251 and U87 glioma cell lines and decreased phosphorylated (p) signal transducer and activator of transcription 3 (STAT3)^(Y705) and cyclin D1 expression, leading to impedance of the G₁-to-S phase transition. Furthermore, it was suggested that ANXA2 may regulate pSTAT3^(Y705) levels through direct binding with STAT3, thereby affecting STAT3-cyclin D1 pathway-mediated cell proliferation. When ANXA2 was re-expressed in ANXA2-knockdown cells, the expression of pSTAT3^(Y705) and cyclin D1 was restored. Furthermore, overexpression of ANXA2 significantly promoted the proliferation of U251 cells, as determined by an MTT assay and a tumor formation assay in nude mice, but had no statistically significant effect on colony formation rate, cell cycle progression or the STAT3-cyclin D1 pathway, suggesting that endogenous ANXA2 may be redundant. Additionally, the present study provided evidence that the overexpression of ANXA2 enhanced the expression of pSTAT3^(Y705) in the presence of epidermal growth factor (EGF), indicating that the proliferation-promoting effect of ANXA2 may be due to the accumulation and synergistic effect of paracrine EGF. Taken together, the present results indicated that ANXA2 may affect the proliferation of human glioma cells through the STAT3-cyclin D1 pathway via direct interaction with STAT3

in U251 and U87 glioma cells. ANXA2 was redundant in this pathway, but positive synergy was revealed to exist between ANXA2 and EGF.

Introduction

Glioma is the most common and aggressive form of primary malignant brain tumor, which is characterized by a high proliferation rate (1,2). Despite the continuous development of novel clinical therapies, the prognosis and survival rates of patients with grade IV glioma [glioblastoma multiforme (GBM), the most malignant form of glioma] remain poor. One of the most important reasons for this may be that the proliferation of glioma cells is a complex process regulated by a network of various regulatory molecules (3,4). Elucidation of the interaction between various molecules in this network is critical for understanding the mechanisms underlying glioma development. Emerging evidence has revealed that numerous types of protein or RNA either positively (5-7) or negatively (8,9) regulate glioma cell proliferation, whereas the molecular pathways underlying this proliferative behavior remain largely unknown or disputed.

Although there are few reports of the molecular pathways involved in the proliferation of glioma cells, it has been reported that the PI3K-AKT pathway serves an important role in the process (7,10,11). Signal transducer and activator of transcription 3 (STAT3)-cyclin D1 is another well-known pathway in cancer cells, which has been implicated in the control of cellular responses to diverse cytokines and growth factors, including cell proliferation (12). Currently, whether the STAT3-cyclin D1 pathway has a role in glioma is disputed. Overexpression of cyclin D1 has been observed in glioma cells, and is closely related to the oncogenesis and progression of glioma (13). Similarly, another study demonstrated that the levels of JAK2 and STAT3 are significantly upregulated and exhibit pairwise correlation in human glioma tissues (14). The overexpression of constitutively active STAT3 has also been observed to be accompanied by the restoration of cyclin D1 expression (15); however, there is evidence that increased STAT3 and decreased cyclin D1 protein levels may contribute to the recurrence of astrocytic tumors (16). Therefore, the exact regulation of and relationship between STAT3 and cyclin D1 in glioma remains unclear. In addition, upstream regulatory molecules of STAT3 signaling in glioma cells are worthy of further study. In breast

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cancer cells, RNA interference (RNAi)-mediated silencing of Annexin A2 (ANXA2) inhibits proliferation by downregulating cyclin D1 in the STAT3-dependent pathway (17). Additionally, ANXA2 reduction has been reported to inhibit epidermal growth factor (EGF)-induced epithelial-mesenchymal transition (EMT) in a STAT3-dependent manner (18). Therefore, ANXA2 may be considered an attractive putative upstream regulator of STAT3 signaling in glioma cells. Therefore, the present study aimed to identify the role of ANXA2 and the phosphorylated (p)STAT3-cyclin D1 pathway in the proliferation of glioma cells.

ANXA2 is a multifunctional phospholipid-binding protein that is expressed in various cell types (19). High ANXA2 expression is a common feature of numerous types of tumor cells, suggesting that it is a crucial regulator of these cells (20-22). In GBM, ANXA2 is overexpressed and is positively correlated with tumor aggressiveness and low patient survival (23), whereas ANXA2-dependent gene expression profiles are strictly correlated with the regulation of fundamental cellular features, including migration, invasion, cytoskeletal remodeling and the cell cycle, which have all been examined *in vitro* and *in vivo* in primary human GBM cells (24,25). Accumulating evidence indicates that ANXA2 is involved in the proliferation of cancer cells. Transfection of HeLa or 293T cells with an antisense ANXA2 vector results in the inhibition of cell division and proliferation with a concomitant reduction in ANXA2 signaling and protein levels (26,27). The proliferation of MHCC97-H cells is strongly suppressed by short hairpin RNA (shRNA)-mediated ANXA2 silencing *in vitro* (28). Similarly, silencing of ANXA2 in breast cancer tissue leads to the accumulation of G₀/G₁ phase cells and a reduction in S/G₂+M phase cells (17,18,29). However, there are also opposing examples of the proliferative effects of ANXA2. A previous study demonstrated that pancreatic cancer cells exhibit high ANXA2 expression, but this is inversely related to cell proliferation *in vitro* (30), indicating that the molecular mechanisms of ANXA2 vary among different types of tumor cells. In human GBM cells, numerous reports regarding ANXA2 focus on pathological specimens and the mechanisms of tumor invasion and metastasis. It has been reported that ANXA2 knockdown decreases tumor size and slows tumor progression, as evidenced by decreased invasion, angiogenesis, migration and proliferation, as well as increased apoptosis in the tumor tissue of the ANXA2 knockdown group (31,32). Nevertheless, details of the molecular mechanisms underlying tumor proliferation in glioma remain unclear.

The present study aimed to investigate the effect of ANXA2 knockdown on glioma cell proliferation. The results demonstrated that ANXA2 depletion in glioma cells significantly inhibited proliferation by decelerating cell cycle progression. The arrested G₁-to-S phase transition observed in ANXA2-silenced cells was attributed to reduced activity of the STAT3-cyclin D1 pathway, which is a classic proliferative factor in breast cancer cells (17). Rescue experiments indicated that ANXA2 may specifically and directly regulate STAT3, leading to decreased cyclin D1 levels in ANXA2-silenced glioma cells. In addition, in glioma cells, ANXA2 is usually overexpressed and in a redundant state; however, in the present study, a positive synergistic effect between ANXA2 and EGF was detected on the pSTAT3 pathway, which may

be related to cell proliferation. The present results revealed a novel mechanism by which ANXA2 may regulate glioma proliferation.

Materials and methods

Cell lines and cell culture. Human U251, U87 and 293T cells were obtained from the Cell Bank of the Chinese Academy of Sciences, Shanghai Institute for Biological Science. The U87 cell line used in the present study is the American Type Culture Collection version (glioblastoma of unknown origin). Cells were cultured in DMEM (HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator containing 5% CO₂. For serum-free, low-density culture, cells were routinely cultured to a cell density of 1×10⁴ cells/ml and were then cultured in serum-free OPTI-MEM medium (Gibco; Thermo Fisher Scientific, Inc.) for another 24 h. For EGF induction, cells were starved of serum for 12 h and were then stimulated with 500 ng/ml EGF (cat. no. P5552; Beyotime Institute of Biotechnology) for 0, 5, 15 and 30 min. All cells used in this study were passaged for <3 months.

Plasmid construction, lentivirus packaging, and stable cell line generation. A lentiviral vector, pLVX-shRNA2 (Takara Bio, Inc.), was constructed to express human ANXA2-specific shRNAs (5'-CGGGATGCTTTGAACATTGAA-3'; cat. no. TRCN0000056145; Sigma-Aldrich; Merck KGaA) or human STAT3-specific shRNAs (5'-GCACAATCTACGAAG AATCAA-3'; cat. no. TRCN0000329887; Sigma-Aldrich; Merck KGaA). A scrambled sequence (ANXA2, 5'-CCGGGACATCACGGATCATAT-3'; STAT3, 5'-TGGCCAGTTTGC TTTCCACAT-3') that does not target any known human coding sequence was used as a negative control. The lentiviral vector pLVX-IRES-ZsGreen1 (Takara Bio, Inc.) was employed to overexpress ANXA2 or FLAG-HA-ANXA2-His fusion protein in glioma cells. The ANXA2 or FLAG-HA-ANXA2-His coding sequence was synthesized by Takara Bio, Inc. and then subcloned into the *Eco*RI and *Bam*HI sites of the vector. For lentivirus packing, 1×10⁶ 293T cells were seeded into a 100-mm dish and cultured to 70-80% confluence. The cells were then cotransfected at 37°C for 10 h with 10 µg lentiviral vectors and 5 µg packaging plasmids (pMD2.G and psPAX2; Addgene, Inc.) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Virus-containing media were collected 48 and 72 h post-transfection and were then centrifuged at 2,000 × g for 5 min at 4°C to remove cell debris. The supernatant was ultrafiltrated via centrifugation in ultrafiltration columns (Merck KGaA) at 1,500 × g for 1 h at 4°C to concentrate lentiviral particles. U251 and U87 cells were seeded at a density of 1×10⁵ cells/ml in 6-well plates and cultured to 80-90% confluence. The cells were infected with lentivirus (MOI=10) in the presence of 8 mg/ml polybrene (cat. no. 107689; Sigma-Aldrich; Merck KGaA), and stable expressing cells were enriched and purified according to their green fluorescent protein fluorescence.

Western blotting. The cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology) for total protein extraction and protein concentrations were determined using the

bicinchoninic acid protein assay reagent (Beyotime Institute of Biotechnology). Equal amounts of protein (40 μ g/lane) were separated by 12% SDS-PAGE and transferred onto a PVDF membrane (Merck KGaA). The membrane was blocked at room temperature with 5% nonfat milk for 1 h and incubated with anti-ANXA2 (cat. no. sc-47696; 1:2,000; Santa Cruz Biotechnology, Inc.), anti-cyclin D1 (cat. no. sc-8396; 1:500; Santa Cruz Biotechnology, Inc.), anti-STAT3 (cat. no. 9139s; 1:1,000; Cell Signaling Technology, Inc.), anti-pSTAT3^(Y705) (cat. no. 9145s; 1:1,000; Cell Signaling Technology, Inc.), anti-FLAG/HA/6X HIS (cat. no. AF0036/AF0039/AH367; 1:1,000; Beyotime Institute of Biotechnology) and anti-GAPDH (cat. no. sc-293335; 1:5,000; Santa Cruz Biotechnology, Inc.) primary antibodies overnight at 4°C. Immunodetection was subsequently performed at room temperature for 1 h with horseradish peroxidase-linked goat anti-rabbit or goat anti-mouse IgG (cat. nos. A0208 and A0216; 1:5,000; Beyotime Institute of Biotechnology) and enhanced chemiluminescence reagents (PerkinElmer, Inc.), according to the manufacturers' protocols. The bands were detected using an ImageQuant LAS 4000 mini (GE Healthcare) and band intensities were semi-quantified using ImageJ2x software (National Institutes of Health); the relative intensities to the internal GAPDH control were calculated. Western blotting was repeated at least three times to confirm the results.

MTT assay. The cells were seeded into 96-well plates at a density of 2×10^3 cells/well. At each time-point, 20 μ l MTT solution (5 mg/ml; Beyotime Institute of Biotechnology) in PBS was added to each well and the cells were stained for 4 h at 37°C. The supernatant was then aspirated carefully. The formazan in the plate was dissolved by adding 200 μ l DMSO. Absorbance was determined at 490 nm on a micro-ELISA reader. The assays were performed using five replicates at each time-point and were repeated three times. In addition, transfected cells were treated with the STAT3 inhibitor NSC 74859 (cat. no. SD4794; Beyotime Institute of Biotechnology) at a final concentration of 50 μ M and then incubated at 37°C for 48 h. Subsequently, the MTT assay was performed as aforementioned.

Colony formation assay. The cells were seeded in 6-cm dishes at a density of 1,000 cells/dish and were then cultured for 14 days. Subsequently, the medium was removed, and the cells were washed three times with PBS, fixed with 100% methanol at 4°C for 10 min and stained with 0.5% crystal violet solution (Beyotime Institute of Biotechnology) at room temperature for 2 h. The number of colonies containing >50 cells was then counted under an inverted light microscope (Primo Vert; Carl Zeiss AG). The assay was performed in triplicate and repeated three times.

Cell cycle assay. After culturing to 70-80% confluence, the cells were collected, washed three times with PBS, and fixed with ice-cold 70% ethanol at 4°C overnight. The cells were then washed three times with ice-cold PBS and stained with 500 μ l propidium iodide (PI; BD Biosciences) containing 1 μ g/ml RNase (Beyotime Institute of Biotechnology) at 37°C for 30 min. Flow cytometric analysis was performed on a Beckman Coulter EPICS analyzer (Beckman Coulter, Inc.)

and cell cycle phase distribution was analyzed with FlowJo 10 (FlowJo, LLC) and revealed in the three major phases (G_0/G_1 vs. S vs. G_2/M). The assay was performed in triplicate and repeated three times.

Subcutaneous tumorigenesis in nude mice. BALB/c nude mice (age, 6 weeks; weight, 20 ± 3 g; Laboratory Animal Center, Fujian Medical University) were housed and bred at 18-22°C and 50-60% relative humidity under a 12-h light/dark cycle with *ad libitum* access to food and water. For injection, mice were randomly divided into four groups ($n=5$ /group) and were injected subcutaneously using U251 glioma cells with different ANXA2 expression levels. Cells were trypsinized, collected and adjusted to a concentration of 5.0×10^7 cells/ml. Subsequently, ~200 μ l cells were injected subcutaneously into one side of the axillary region of nude mice. After 2 weeks of normal feeding, the mice were euthanized by intraperitoneal injection of an overdose of pentobarbital sodium. Tumor tissues were dissected and tumor weight was statistically analyzed.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts of U251 cells were prepared using the Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology). EMSA was conducted using the EMSA/Gel-Shift kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. Briefly, the STAT3 consensus oligonucleotide probe (5'-GATCCTTCTGGGAATTCCTAGATC-3') was end-labeled with biotin. For the assay, 30 μ g nuclear protein and 0.02 μ M biotin-labeled probe (final concentration) were used in the 20- μ l reaction system. The STAT3 probe binding activity was determined using a chemiluminescent EMSA kit (cat. no. GS002; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. The specificity of the DNA-protein complex was confirmed with biotin-labeled, unlabeled and mutated STAT3 probes (cat. nos. GS083B, GS083 and GS083M; Beyotime Institute of Biotechnology) added to the mixture.

Immunofluorescence and confocal microscopy. Cells (1 ml/well) were plated at a density of 1×10^5 /ml on slides in 12-well plates. After 24 h, the cells were fixed in 4% paraformaldehyde for 30 min at room temperature. After permeabilization in PBS containing 0.2% Triton X-100 at room temperature for 10 min, the slides were blocked in 3% BSA (cat. no. ST023; Beyotime Institute of Biotechnology) for 1 h at room temperature, and then incubated with anti-ANXA2 (cat. no. sc-47696; 1:200; Santa Cruz Biotechnology, Inc.) and anti-STAT3 (cat. no. 9139s; 1:200; Cell Signaling Technology, Inc.) antibodies in a humidified chamber at 4°C overnight. After washing with PBS, the slides were incubated with rhodamine- or FITC-conjugated secondary antibodies (cat. nos. AP124R and AP124F; 1:200; Merck KGaA) at 4°C for 2 h. DNA was stained with a solution of PBS containing 10 μ g/ml DAPI for 5 min. The slides were mounted with Mowiol-based anti-fading medium and visualized under a laser-scanning confocal microscope (Leica TCS SP5; Leica Microsystems, Inc.).

Co-immunoprecipitation (IP) assay. Vectors expressing 3xFLAG-ANXA2 fusion protein were constructed using

p3xFLAG-CMVTM-10 system (cat. no. E7658; Sigma-Aldrich; Merck KGaA). Briefly, 1x10⁶ U251 or U87 cells were seeded into 100-mm dishes, allowed to grow to 80-90% confluence, and transfected at 37°C for 10 h with 10 µg fusion protein-expressing vector p3xFLAG-ANXA2-CMVTM-10 using the Lipofectamine[®] 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The blank plasmid p3xFLAG-CMVTM-10 was used as the control. At 48 h after transfection, the cells were lysed for 30 min at 4°C in 1 ml IP lysis buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 250 mM NaCl, 1% NP-40 (Beyotime Institute of Biotechnology), and protease inhibitor cocktail (Sangon Biotech Co., Ltd.). Supernatants were incubated with 1 µg normal mouse IgG (cat. no. sc-2025; Santa Cruz Biotechnology, Inc.) and 40 µl protein A&G sepharose beads (Beyotime Institute of Biotechnology) with gentle agitation on a rotator at 4°C for 2 h. After centrifugation at 12,000 x g for 10 min at 4°C, the precleared supernatants were incubated at 4°C overnight on a rotator with anti-FLAG antibodies (Beyotime Institute of Biotech). Subsequently, 40 µl protein A&G sepharose beads were added for an additional 3 h with gentle agitation at 4°C. After centrifugation at 12,000 x g for 10 min at 4°C, the beads were washed with IP lysis buffer three times and then boiled in SDS loading buffer. The final samples were assessed by western blotting with anti-ANXA2, anti-STAT3 and anti-FLAG antibodies. Whole cell lysate was used as a control.

Statistical analysis. All data are presented as the means ± standard deviation of at least three independent experiments. Statistical analyses were performed using GraphPad Prism version 6.01 software (GraphPad Software, Inc.). Differences between groups were analyzed by one-way analysis of variance and Holm-Sidak's multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

Results

Knockdown of ANXA2 expression inhibits proliferation of glioma cells. It has previously been reported that ANXA2 serves an important role in the proliferation of tumor cells (19-22); however, its role in the proliferation of glioma cells remains to be clarified. To obtain evidence regarding the function of ANXA2 on glioma cells, this study investigated the effects of ANXA2 with RNAi. When ANXA2 knockdown was performed using the lentiviral vector pLVX-shRNA2-ZsGreen, the rate of ANXA2 knockdown was 72.2±3.2 and 80.2±4.3% (P<0.01) in glioma U251/U87 cell lines, respectively (data not shown). As shown in Fig. 1A and B, the protein expression levels of ANXA2 in U251/U87 cells were significantly reduced compared with in control cells post-infection with a lentivirus expressing specific shRNAs targeting ANXA2. An MTT assay was used to detect the proliferation of each cell group from day 1 to 5 post-inoculation; significant differences between test and control cells were observed on day 3 (P<0.05) and days 4 and 5 (P<0.01), indicating that ANXA2 knockdown may inhibit the proliferation of U251 and U87 cells (Fig. 1C and D). Consistent with the results of the MTT assay, cell colony formation was reduced in ANXA2-knockdown U251 and U87 cells (Fig. 1E and F). These results suggested that ANXA2 may serve an essential role in the proliferation of glioma cells *in vitro*.

Knockdown of ANXA2 expression affects cell cycle progression. It has been hypothesized that ANXA2 may affect cell proliferation through regulation of the cell cycle. To investigate this, the present study measured the proportion of cells at different stages of the cell cycle in two ANXA2-knockdown glioma cell lines by flow cytometry. The results indicated that inhibition of ANXA2 significantly increased the proportion of G₀/G₁ phase cells (Fig. 2A and B), leading to a decrease in the cell proliferation index and a potential G₁/S block. Therefore, ANXA2 may serve a critical role in the G₁-to-S phase transition in glioma cells.

This study also aimed to determine whether ANXA2 affects cell proliferation through the pSTAT3-cyclin D1 signaling pathway. Western blotting revealed that the down-regulation of ANXA2 had little effect on total STAT3 levels; however, it did reduce the expression of pSTAT3^(Y705) and cyclin D1 (Fig. 2C-F). These findings suggested that ANXA2 may be involved in the phosphorylation of STAT3 and that knockdown of ANXA2 may inhibit cell proliferation by reducing the levels of pSTAT3^(Y705) and cyclin D1.

ANXA2 affects cell proliferation through direct binding with STAT3. ANXA2 is mainly located in the cytoplasm and cell membrane, suggesting that it may function via direct or indirect interaction with other proteins. Immunofluorescence was performed to detect protein interactions between ANXA2 and STAT3 in glioma cells. Confocal images revealed that ANXA2 was mainly located in the cytoplasm and that there was an overlap in the cytoplasmic distribution of ANXA2 and STAT3, particularly in the peripheral margin of the nucleus, suggesting colocalization of, and possible interactions between, ANXA2 and STAT3 (Fig. 3A and B). IP and western blotting further confirmed direct binding between ANXA2 and STAT3. ANXA2 and STAT3 were both detected using FLAG IP (Fig. 3C and D), thus indicating that ANXA2 may interact directly with STAT3 in glioma cells.

STAT3 knockdown inhibits proliferation by downregulating cyclin D1. It has been reported that STAT3 can regulate the transcriptional expression of cyclin D1 and further regulate cell proliferation in various types of tumor cells (12). To date, however, there have been no reports on the effect of the pSTAT3-cyclin D1 pathway on glioma cell proliferation. In the present study, the shRNA sequence of the STAT3 gene was cloned into pLVX-shRNA2 and infected into U251 or U87 cells to obtain stable cell lines. MTT and colony formation assays, and western blotting, were used to assess cell proliferation. The results revealed that knockdown of STAT3 did not reduce the expression of ANXA2, but did reduce the expression of total STAT3 and pSTAT3^(Y705), which in turn further down-regulated the expression levels of cyclin D1 (Fig. 4A and B). The MTT assay revealed that the proliferation rate in the STAT3 RNAi group from day 3 to 5 was significantly reduced compared with in the nonsense or empty control groups (P<0.01; Fig. 4C and D). The colony formation assay also demonstrated that downregulation of pSTAT3^(Y705) significantly inhibited the colony-forming ability of cells (Fig. 4E and F). These data suggested that the pSTAT3-cyclin D1 pathway may affect glioma cell proliferation.

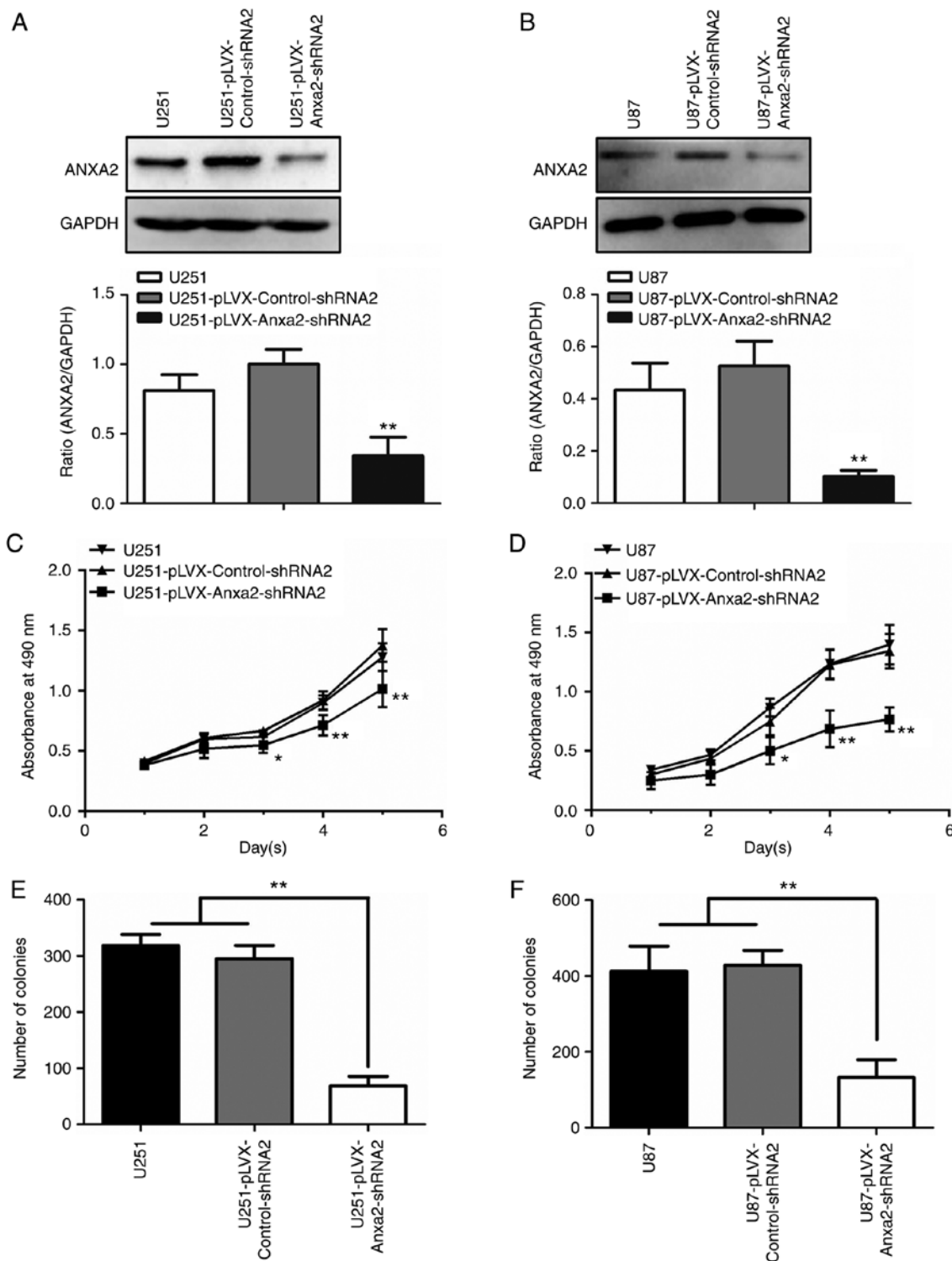


Figure 1. Knockdown of ANXA2 expression inhibits proliferation of human glioma cells. (A and B) ANXA2 expression was significantly inhibited in U251 and U87 glioma cells post-infection with a lentivirus expressing specific ANXA2-targeting shRNA (n=3). (C and D) MTT assay revealed that proliferation of ANXA2-knockdown cells was inhibited compared with control or wild-type cancer cells (n=5). (E and F) Cell colony formation was reduced in ANXA2-knockdown U251 and U87 cells (n=3) *P<0.05, **P<0.01 vs. U251/U87 or U251/U87-pLVX-Control-shRNA2 cells. ANXA2, Annexin A2; shRNA, short hairpin RNA.

Restoration of pSTAT3-cyclin D1 by ANXA2 re-expression in ANXA2-knockdown glioma cells. To further confirm that ANXA2 affects the proliferation of glioma cells through the pSTAT3-cyclin D1 pathway, ANXA2-knockdown cell lines were transiently transfected with a pLVX-ANXA2-ZsGreen expression plasmid to determine whether re-expression of ANXA2 could

restore the expression of proliferation-associated molecules that were inhibited by ANXA2 knockdown. The results revealed that ANXA2 re-expression could partially compensate for the decrease of proliferation-associated pSTAT3^(Y705)-cyclin D1 signaling caused by ANXA2 knockdown (Fig. 5A and B); however, in the presence of a STAT3 inhibitor, NSC 74859,

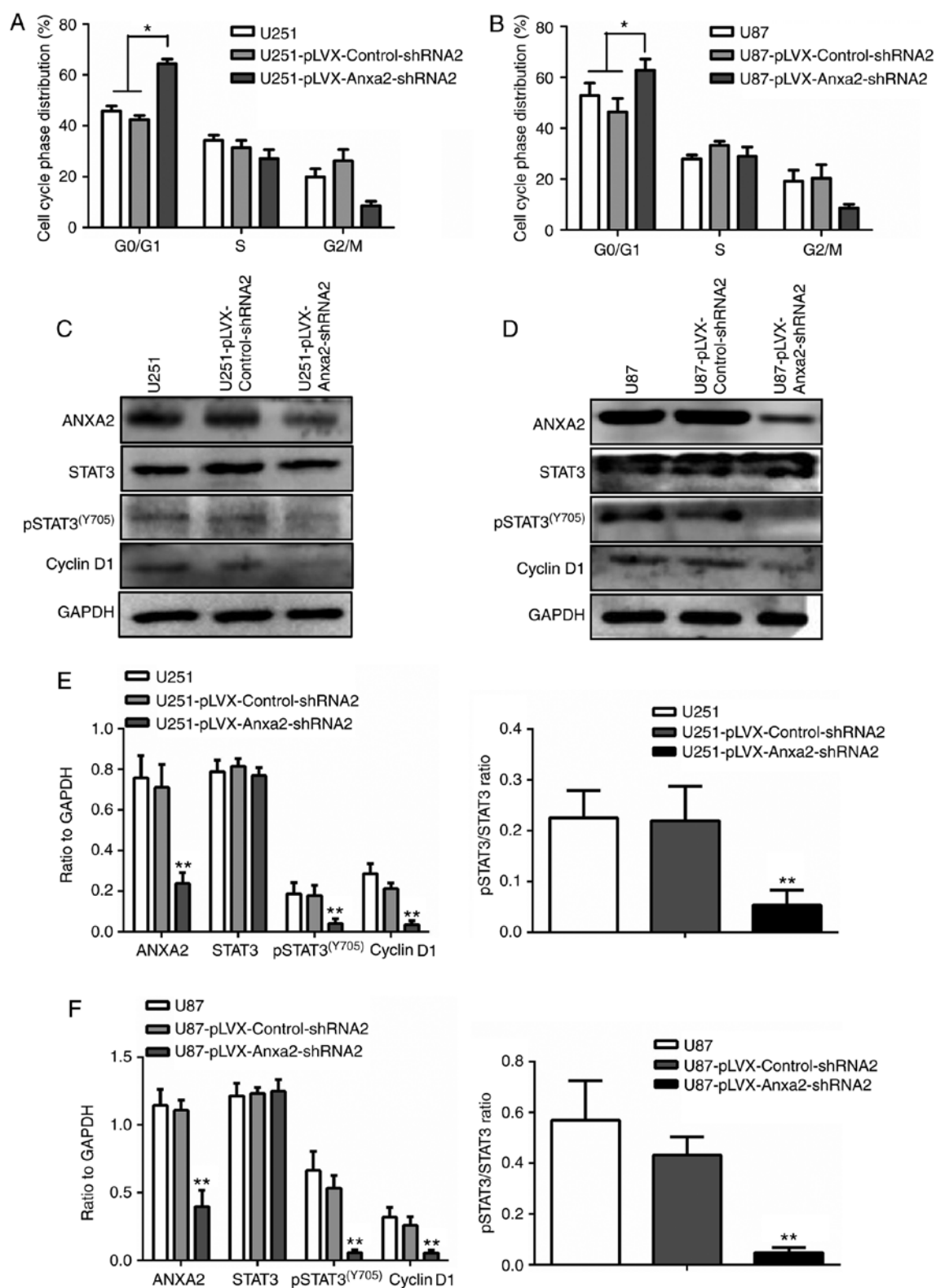


Figure 2. Knockdown of ANXA2 induces cell cycle arrest by affecting pSTAT3-cyclin D1 signaling in glioma cells. (A and B) Knockdown of ANXA2 in U251 and U87 cells induced an increase in the proportion of G₀/G₁ phase cells (n=3). (C and D) Representative western blot images of ANXA2, pSTAT3^(Y705) and cyclin D1 expression in ANXA2-knockdown U251 and U87 cells. (E and F) Relative intensities normalized to the internal control GAPDH were analyzed, and the pSTAT3^(Y705)/STAT3 ratio, normalized to GAPDH was also calculated (n=3). *P<0.05, **P<0.01 vs. U251/U87 or U251/U87-pLVX-Control-shRNA2 cells. ANXA2, Annexin A2; pSTAT3, phosphorylated STAT3; shRNA, short hairpin RNA; STAT3, signal transducer and activator of transcription 3.

the recovery of proliferation did not occur (Fig. 5C), thus suggesting that ANXA2 may have a specific role in pSTAT3-cyclin D1-mediated proliferation of glioma cells.

Effects of ANXA2 overexpression on proliferation appear to be ambiguous. This study demonstrated that ANXA2 knockdown significantly inhibited proliferation through

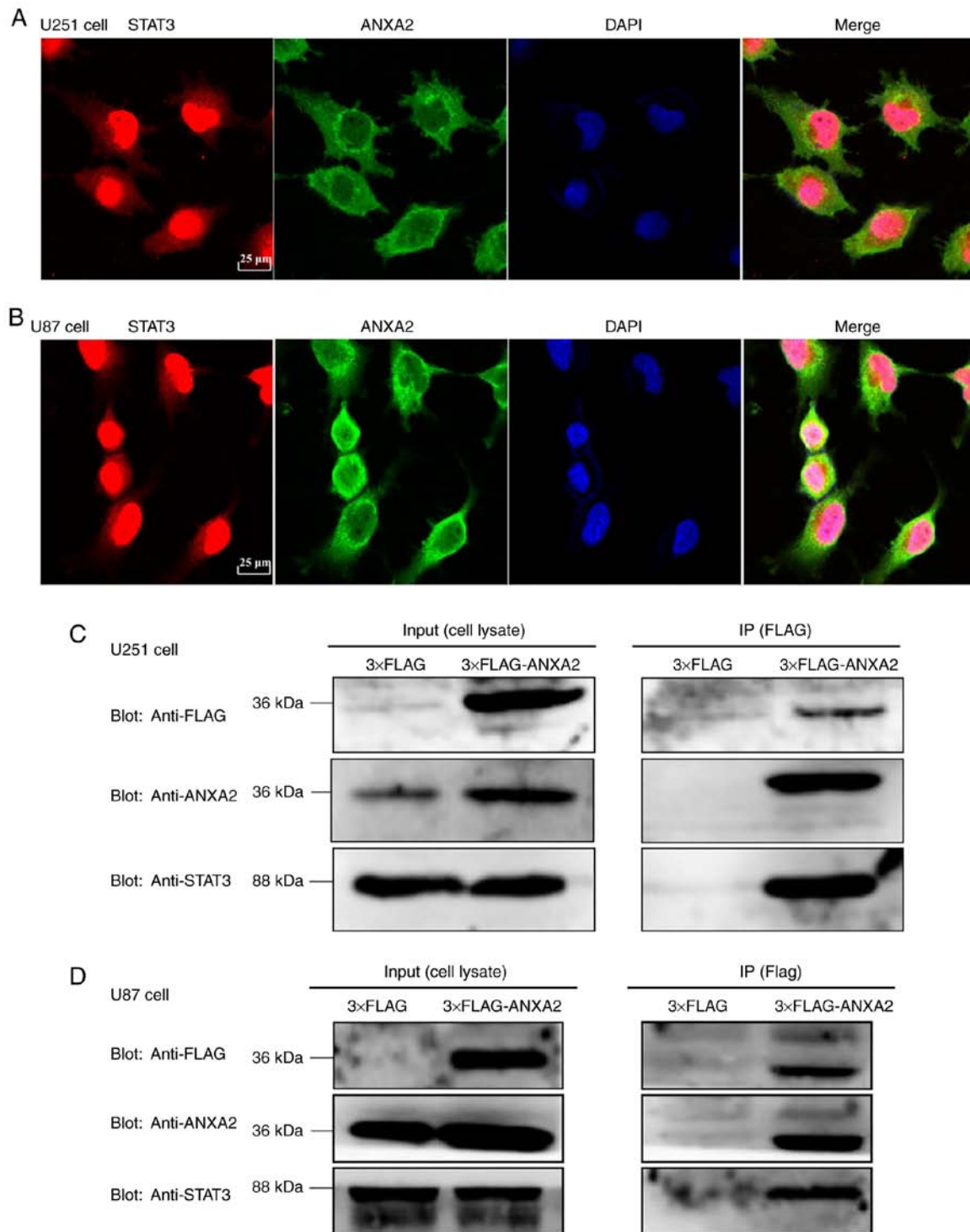


Figure 3. ANXA2 affects the cell proliferation-associated STAT3-cyclin D1 pathway by directly binding with STAT3 in glioma cells. (A and B) Immunofluorescence was performed to detect colocalization between ANXA2 and STAT3 in U251 and U87 cells. (C and D) ANXA2 containing a 3xFLAG tag was transiently expressed in U251 and U87 cells using the fusion protein expressing vector p3xFLAG-ANXA2-CMVTM-10; the blank plasmids p3xFLAG-CMVTM-10 was transfected as the control. Anti-FLAG antibody and protein A&G agarose were added to the cell lysate and the coprecipitate was detected by western blotting. ANXA2, Annexin A2; STAT3, signal transducer and activator of transcription 3.

pSTAT3-cyclin D1 signaling in glioma cells. Subsequently, this study aimed to reveal the effects of ANXA2 overexpression on proliferation. In a subcutaneous tumorigenesis model in nude mice, the volume or weight of transplanted tumors formed by ANXA2-knockdown U251 cells was smaller than those formed by control cells; however, the tumors were much larger in the ANXA2 overexpression group compared with in the control

group (Fig. 6A). The results of the MTT assay revealed that the proliferation rate of U251 cells with ANXA2 overexpression was significantly increased on days 4 and 5, whereas it was significantly decreased in U251 cells with ANXA2 knock-down, compared with in the control group ($P < 0.05$; Fig. 6B). Surprisingly, overexpression of ANXA2 had no significant effect on the colony formation rate of U251 cells and did not

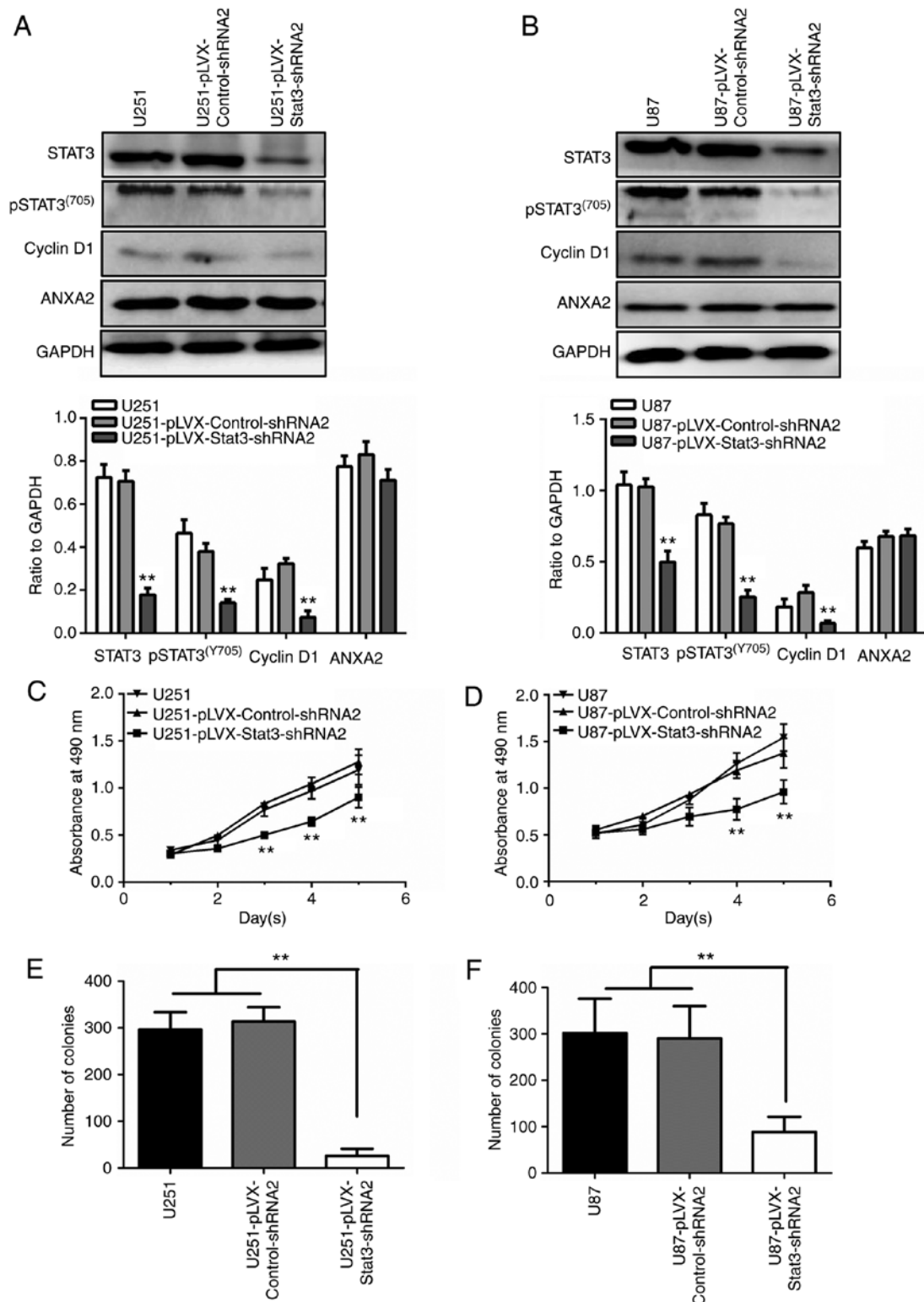


Figure 4. STAT3 knockdown inhibits proliferation by downregulating cyclin D1 in glioma cells. (A and B) STAT3 was stably knocked down in U251 and U87 cells. pSTAT3^(Y705) and cyclin D1 were downregulated in these two STAT3-RNAi glioma cell lines (n=3). **P<0.01 vs. control. (C and D) Cell proliferation rate was measured by MTT assay (n=5). **P<0.01 vs. control. (E and F) Effects of STAT3 knockdown on colony formation in U251 and U87 cell lines (n=3). **P<0.01 vs. control. ANXA2, Annexin A2; pSTAT3, phosphorylated-STAT3; STAT3, signal transducer and activator of transcription 3.

change the expression level of cycle-related proteins cyclin D1 and the proportion of G₀/G₁ phase cells (Fig. 6C-E); this is in contrast to the results obtained using ANXA2-knockdown cells. These ambiguous results may be due to complex environmental conditions *in vitro* and *in vivo*.

Overexpression of ANXA2 does not promote pSTAT3-cyclin D1 in glioma U251 cells in serum-free medium. It was hypothesized that different experimental conditions, particularly differences in extracellular paracrine factors and serum, may have led to these mixed results. Therefore, this study

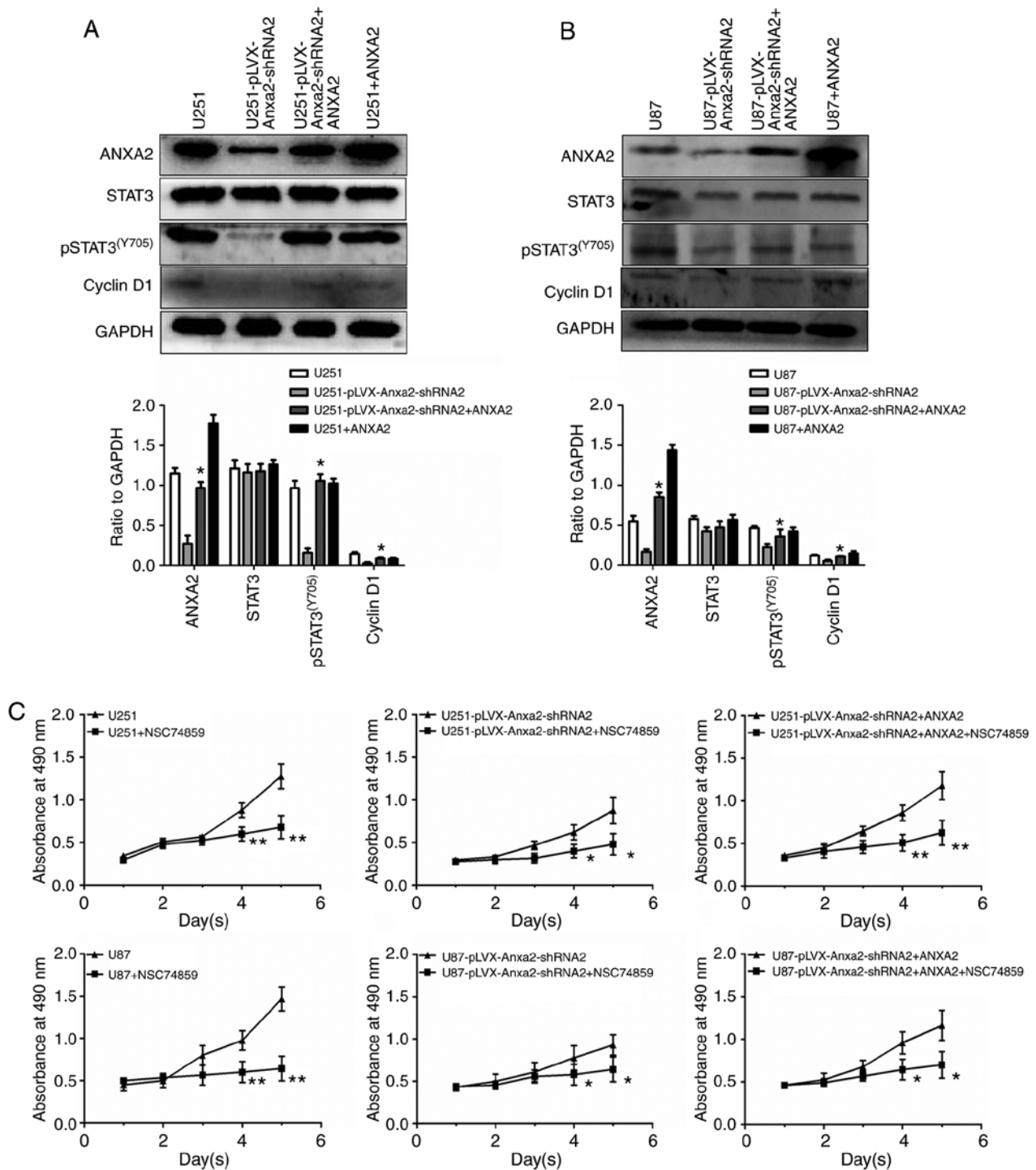


Figure 5. Re-expression of ANXA2 in ANXA2-knockdown glioma cells can partially rescue the pSTAT3-cyclin D1 pathway. (A and B) Expression levels of pSTAT3^(Y705) and cyclin D1 were partially elevated when ANXA2 was re-expressed in ANXA2-knockdown U251 and U87 cell lines. Expression levels were semi-quantified by densitometric analysis; the relative values were normalized to GAPDH (n=3). *P<0.05 vs. ANXA2-knockdown cells. (C) Cell proliferation rate was measured by MTT assay (n=5). **P<0.01, *P<0.05 vs. control. ANXA2, Annexin A2; pSTAT3, phosphorylated-STAT3; shRNA, short hairpin RNA; STAT3, signal transducer and activator of transcription 3.

aimed to further determine the effects of ANXA2 overexpression on the pSTAT3-cyclin D1 pathway in U251 cells cultured in serum-free, low-density conditions. The results of western blotting indicated that under serum-free, low-density culture, knockdown of ANXA2 significantly reduced the expression of pSTAT3^(Y705) and cyclin D1, whereas ANXA2 overexpression did not induce elevation of pSTAT3^(Y705) or cyclin D1 (Fig. 7A).

The DNA binding ability of the transcriptional factor pSTAT3^(Y705) was detected using an EMSA assay in U251 cell lines differentially expressing ANXA2. As shown in Fig. 7B, the binding of the STAT3-specific probe in the ANXA2-knockdown nucleoprotein was decreased compared with the control. However, there was no significant increase in the amount of probe binding in cells overexpressing ANXA2. To obtain more convincing evidence, FLAG-HA-ANXA2-His

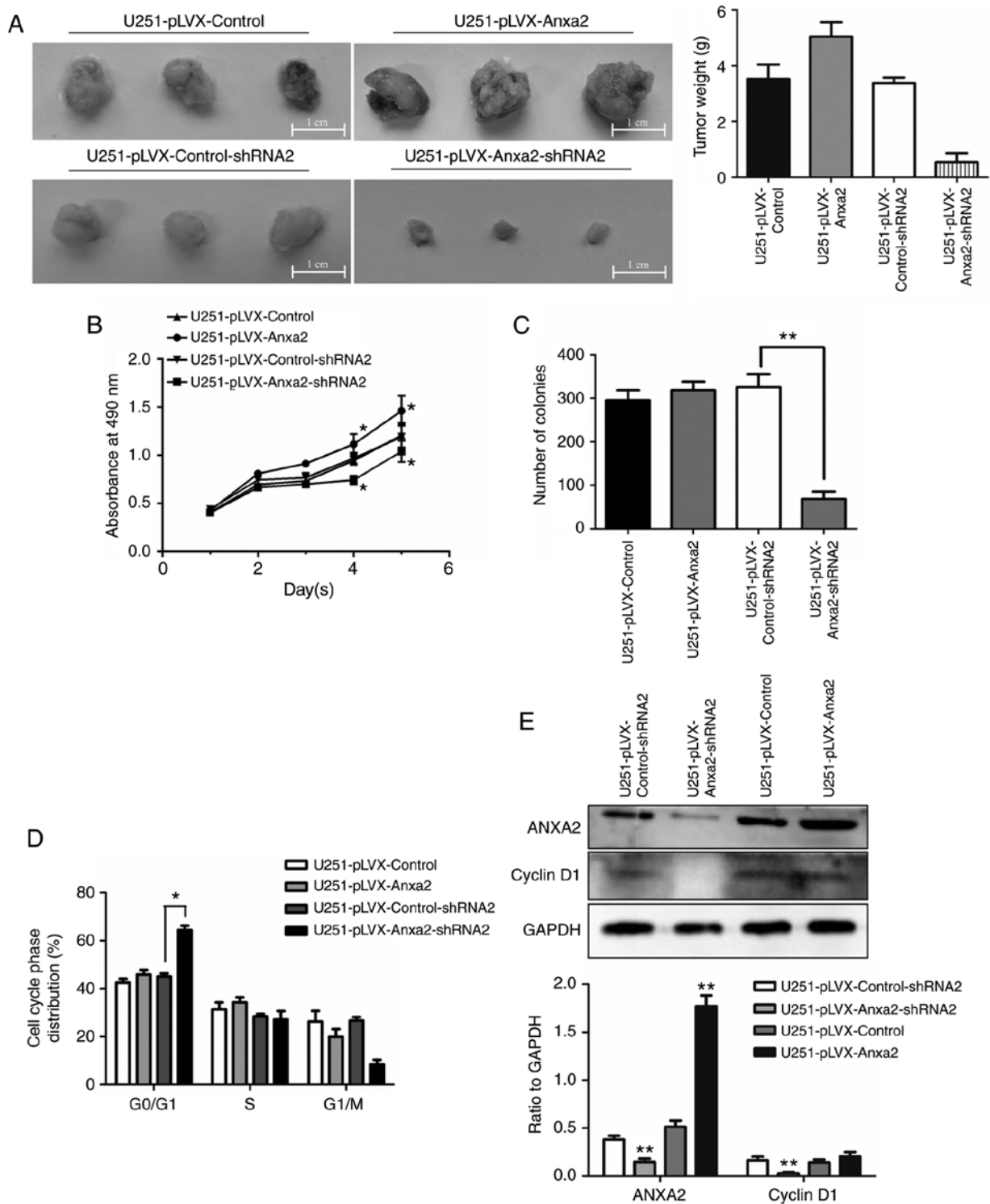


Figure 6. Effects of ANXA2 overexpression on proliferation in U251 cells. (A) Effects of ANXA2 expression on a nude mouse model of glioma. (B) Cell proliferation rate was measured by MTT assay (n=5). *P<0.05 vs. control. (C) Unlike ANXA2 knockdown, ANXA2 overexpression had no significant effect on the colony formation rate in U251 cells (n=3). **P<0.01 vs. control. (D) Knockdown of ANXA2 in U251 cells induced an increase in the proportion of G₀/G₁ phase cells; however, there was no decline in G₀/G₁ cells when ANXA2 was overexpressed. *P<0.05 vs. control. (E) Western blotting demonstrated that ANXA2 overexpression had no significant effect on cyclin D1 expression in U251 cells (n=3). **P<0.01 vs. control. ANXA2, Annexin A2; shRNA, short hairpin RNA.

fusion protein was expressed in U251 cells under serum-free, low-density conditions and the three tags were used to detect the fusion protein, in order to ensure the integrity of ANXA2. The results confirmed that FLAG, HA and His tags were all detected at 36 kDa, which was exactly the position of ANXA2, indicating that intact ANXA2 was overexpressed in

U251 cells (Fig. 7C), however, this still did not enhance the expression of pSTAT3^(Y705) or cyclin D1 (Fig. 7D). This further confirmed that overexpression of ANXA2 in U251 cells may have no significant effect on the pSTAT3-cyclin D1 pathway in low-density, serum-free conditions, thus suggesting that ANXA2 cannot activate the STAT3 pathway alone. Therefore,

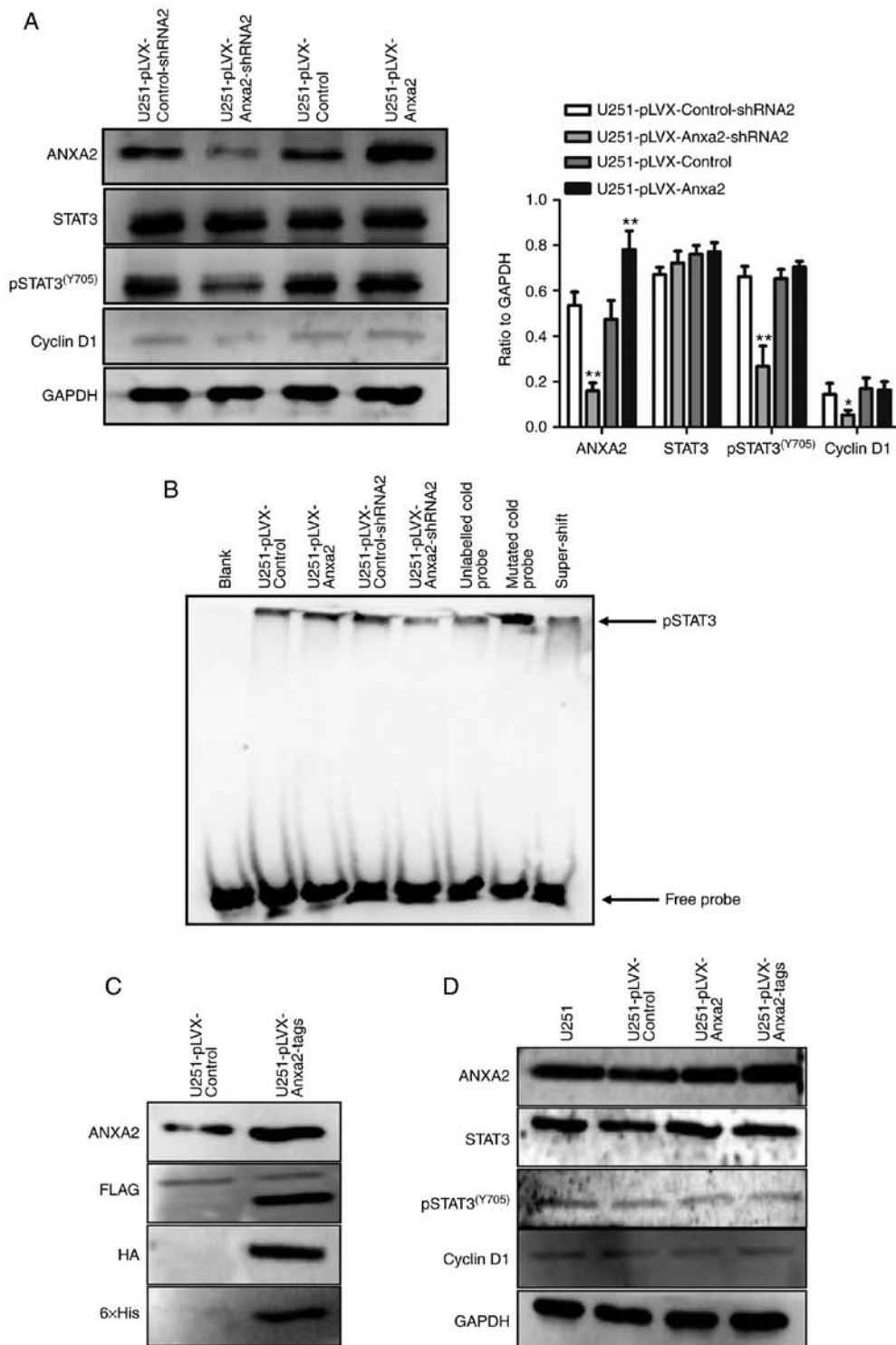


Figure 7. Effects of ANXA2 overexpression on the pSTAT3-cyclin D1 pathway in serum-free, low-density cultured U251 cells. (A) U251 cells with different ANXA2 expression levels were cultured in low-density, serum-free conditions. Cell lysates were analyzed by western blotting (n=3). *P<0.05, **P<0.01 vs. control. (B) Binding activity of nuclear extracts to the STAT3 probe was confirmed by electrophoretic mobility shift assay. (C) FLAG-HA-ANXA2-His fusion protein was expressed in U251 cells; three tags were detected to ensure the complete expression of ANXA2 by western blotting. The upper, weaker band in the FLAG blots was caused by non-specific binding of antibodies. (D) U251 cells expressing FLAG-HA-ANXA2-His fusion protein were cultured in low-density, serum-free conditions. Cell lysates were analyzed by western blotting. ANXA2, Annexin A2; pSTAT3, phosphorylated-STAT3; shRNA, short hairpin RNA; STAT3, signal transducer and activator of transcription 3.

some paracrine factors may be necessary for ANXA2 to function in cell proliferation.

ANXA2 and EGF synergistically promote pSTAT3-cyclin D1 signaling in serum-free culture. It was hypothesized that

overexpression of ANXA2 may indirectly promote cell proliferation by synergizing with a paracrine factor. In order to investigate this, ANXA2-overexpressing or -knockdown U251 cells were treated with EGF, which has been reported to effectively activate pSTAT3-cyclin D1 signaling (17). After

treatment with EGF for 0, 5, 15 and 30 min, total cellular protein was extracted and subjected to western blotting. In ANXA2-knockdown cells, the initial level of pSTAT3^(Y705) was lower than in control cells and was not significantly increased during EGF treatment (Fig. 8A). Meanwhile, in ANXA2-overexpressing cells, the expression levels of pSTAT3^(Y705) were initially not significantly different to those in control cells; however, they were significantly increased during EGF induction in a time-dependent manner (Fig. 8B). To further validate the specific synergy between ANXA2 and EGF, ANXA2 was re-expressed in ANXA2-knockdown U251 cells. Under the same conditions of EGF induction, there was a significant recovery of pSTAT3^(Y705) in ANXA2-re-expressing cells compared with the knockdown control (Fig. 8C). These findings suggested that EGF-induced activation of pSTAT3^(Y705) may depend on ANXA2 expression in glioma U251 cells. Additionally, ANXA2 overexpression alone did not affect pSTAT3-cyclin D1; however, in the presence of EGF, redundant ANXA2 can significantly activate the pathway. Therefore, ANXA2 and EGF may act synergistically in the activation of pSTAT3^(Y705), but neither can activate pSTAT3^(Y705) activity alone.

Discussion

Accumulating evidence has suggested that ANXA2 is highly expressed in numerous types of tumor cells, and serves a critical role in proliferation, migration, invasion, metastasis and angiogenesis (19-22). However, ANXA2 overexpression has an opposing role in various tumor cell types (23) and details of its function in glioma remain elusive. In the present study, it was demonstrated that ANXA2 knockdown inhibited cell proliferation by impeding the G₁-to-S phase transition via the pSTAT3-cyclin D1 pathway through a direct interaction with STAT3. The present results indicated that EGF-induced activation of pSTAT3^(Y705) may depend on the presence of ANXA2, and that ANXA2 and EGF may have a positive synergistic effect on pSTAT3-cyclin D1 signaling, which is related to proliferation of human glioma cells. Overall, the present study provided novel insights into the functions of ANXA2 as a critical molecule in glioma cells, and may improve understanding of the mechanism underlying ANXA2-mediated proliferation in cancer cells.

In glioma cells, ANXA2 can participate in invasion-associated processes (22); in a previous study, it was revealed that ANXA2 knockdown decreases glioma cell migration, tumor size and tumor progression, but does not affect proliferation (26). In addition, it has been suggested that GBM cell migration and invasion are sustained by ANXA2, and that ANXA2 impairment induces differentiation and inhibits proliferation of GBM cells (27). However, to the best of our knowledge, no further studies have been performed regarding the underlying molecular mechanisms of these processes. A previous study demonstrated that ANXA2 depletion in breast cancer cells significantly inhibits cell proliferation by decelerating progression of the cell cycle (17). In agreement with this observation, the results of the present MTT and colony formation assays demonstrated that knockdown of ANXA2 in U251 or U87 glioma cells inhibited proliferation, thus indicating that ANXA2 may serve a critical role in the proliferation of

glioma cells and encouraging us to further validate the potential underlying molecular mechanisms. Subsequently, this study demonstrated that knockdown of ANXA2 significantly decreased the expression levels of pSTAT3^(Y705) and cyclin D1, and increased the proportion of G₁ phase cells, thus reducing the cell proliferation index and suggesting that ANXA2 knockdown may lead to a G₁/S block. Cyclin D1 is a protein required for progression through the G₁ phase of the cell cycle (33), and it has been reported that G₁ phase cell cycle arrest is induced by a reduction in STAT3, which is consistent with a decrease in cyclin D1 protein expression (34). In GBM, STAT3 mutations contribute to a concomitant suppression of proliferation and survival of U251 cells (35). The present results revealed that knockdown of ANXA2 significantly reduced the expression levels of pSTAT3 and cyclin D1 in U251 and U87 cells. Furthermore, it has been reported that impairment of ANXA2 is sufficient to partially arrest GBM cells at the S-G₂/M cell cycle checkpoint (24), which differs from the results of the present study; however, there may be a potential difference between primary GBM cells and GBM cell lines.

The pSTAT3-cyclin D1 pathway is considered to serve a crucial role in the proliferation of several tumor types (12). Therefore, the arrested cell cycle progression in ANXA2-knockdown cells may be attributed to inhibition of pSTAT3^(Y705) activity. Immunofluorescence and IP suggested that a direct interaction existed between ANXA2 and STAT3, thus suggesting that ANXA2 may regulate STAT3 phosphorylation via direct binding in glioma cells, thus affecting pSTAT3-cyclin D1-mediated cell proliferation. When STAT3 was knocked down in glioma cells, the expression levels of pSTAT3^(Y705) and cyclin D1 were consistently downregulated, and cell proliferation and colony formation were also inhibited.

It has been reported that ANXA2 regulates the pSTAT3-cyclin D1 pathway in breast cancer cells, and affects breast cancer progression (17,18); however, these studies did not perform rescue experiments. To further verify the specificity of ANXA2 regulation on the pSTAT3 pathway, ANXA2 was re-expressed in ANXA2-knockdown glioma cells; as a result, the reduction in pSTAT3^(Y705) and cyclin D1 expression caused by ANXA2 deletion was partially restored. To validate the specific binding of ANXA2 and STAT3, the STAT3 inhibitor NSC 74859 was used in this rescue experiment. Similar to the previous results, re-expression of ANXA2 partially compensated for the decrease of proliferation-associated pSTAT3^(Y705)-cyclin D1 signaling caused by ANXA2 knockdown; however, in the presence of STAT3 inhibitors, there was no recovery of proliferation. This finding confirmed that ANXA2 may affect glioma proliferation by regulating the pSTAT3-cyclin D1 pathway. In summary, the present findings suggested that ANXA2 may directly bind to STAT3 and enhance its transcriptional activity, thus regulating the proliferation of glioma cells.

Few studies of gene function have investigated gene overexpression. The main reason for this is that genes are often redundant in tumor cells, and the effect of their overexpression is not obvious. ANXA2 is highly expressed in glioma cells, and its expression level is positively correlated with glioma grade (24,25). However, to the best of our knowledge, there have been no reports to date on whether or how the overexpression of ANXA2 can accelerate glioma cell proliferation.

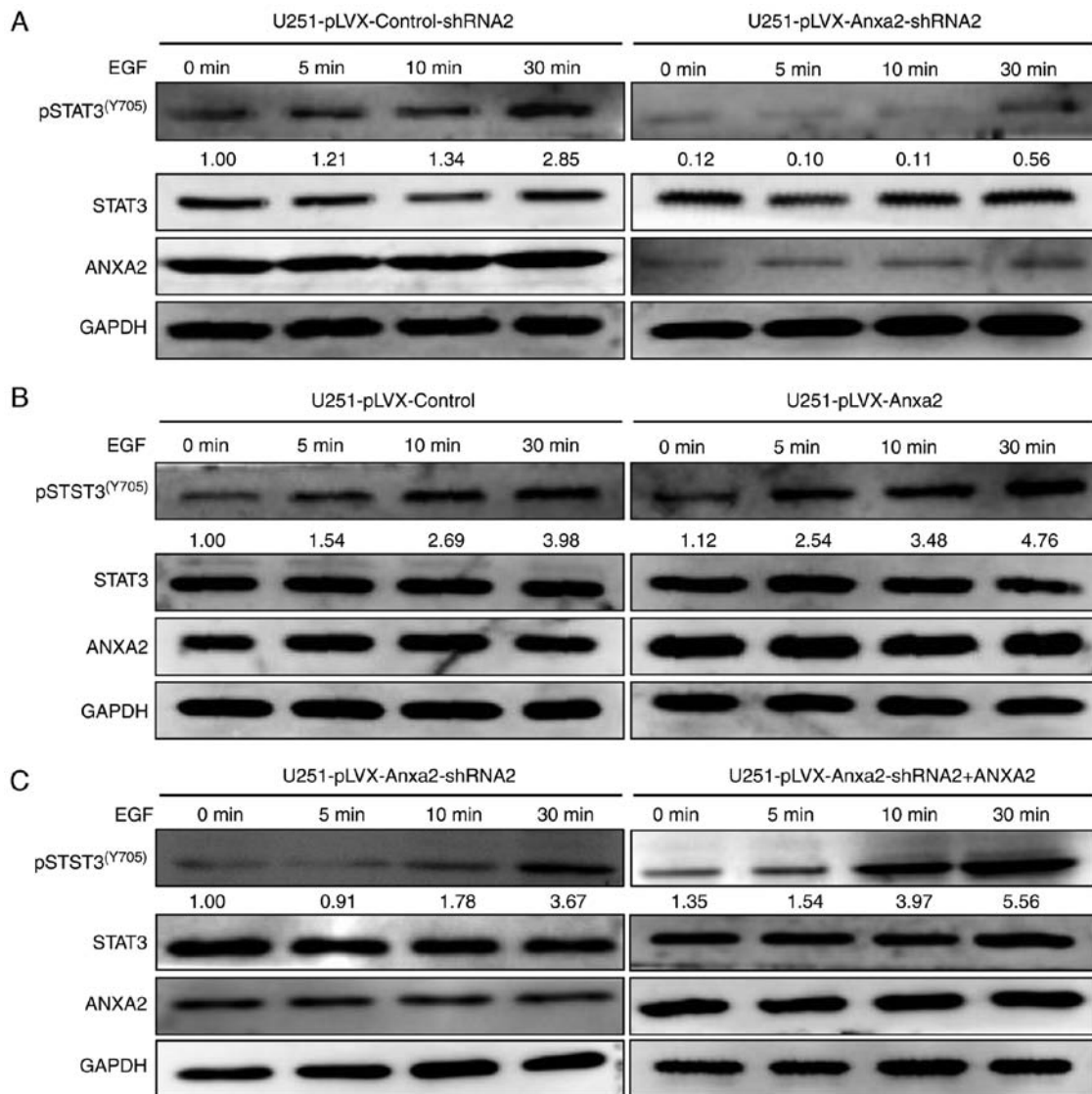


Figure 8. ANXA2 and EGF synergistically promote proliferation of U251 glioma cells. (A) Western blotting of EGF-induced phosphorylation of STAT3 at Tyr705 in control and ANXA2-knockdown U251 cells. (B) Western blotting of EGF-induced pSTAT3^(Y705) expression in control and ANXA2-overexpressing U251 cells. (C) Western blotting of EGF-induced pSTAT3^(Y705) expression in control and ANXA2-re-expressing U251 cells. The numbers below the blots indicate the relative values from densitometric analysis; the levels of pSTAT3^(Y705) were normalized to the corresponding total STAT3 and are expressed as fold changes compared to the zero-time control. ANXA2, Annexin A2; EGF, epidermal growth factor; pSTAT3, phosphorylated-STAT3; shRNA, short hairpin RNA; STAT3, signal transducer and activator of transcription 3.

Therefore, the present study aimed to elucidate the effects of ANXA2 overexpression on glioma cell proliferation. The results revealed that the overexpression of ANXA2 had no significant effect on colony formation rate, cell cycle progression or the pSTAT3-cyclin D1 pathway in the U251 glioma cell line. However, proliferation was markedly increased by ANXA2 overexpression in the MTT and subcutaneous tumorigenesis experiments. Previous studies have reported that the role of ANXA2 is associated with multiple paracrine factors, including EGF (18) and interleukin-6 (36). As for experimental methods, MTT and tumor transplant experiments may be affected by paracrine factors due to cell culture density and complex tumor formation *in vivo*. Therefore, it was hypothesized that the observed effect of overexpression on cell proliferation may be due to synergy between paracrine factors and ANXA2. To further assess whether paracrine factors are involved in ANXA2-regulated cell proliferation, serum-free medium

and low-density cell culture were used to eliminate possible interference from paracrine factors. The results revealed that knockdown of ANXA2 significantly reduced the expression levels of pSTAT3^(Y705) and cyclin D1, whereas overexpression of ANXA2 did not upregulate either pSTAT3^(Y705) or cyclin D1 in the U251 cell line in low-density, serum-free conditions. An EMSA experiment further demonstrated that knockdown of ANXA2 decreased the DNA binding activity of pSTAT3^(Y705); however, there was no obvious increase in the amount of probe binding in ANXA2-overexpressing cells. Intact expression of ANXA2 also did not enhance the expression of pSTAT3^(Y705) or cyclin D1. Furthermore, rescue experiments indicated that re-expressed ANXA2 exhibits biological activity by partially activating pSTAT3 signaling in ANXA2-knockdown cells. Together, these results demonstrated that ANXA2 may not activate the pSTAT3-cyclin D1 pathway in the absence of essential paracrine and serum components.

EGF receptor (EGFR) is a common transmembrane tyrosine kinase receptor. EGF and EGFR recognition can activate tyrosine kinase Janus kinase activity, resulting in phosphorylation of the transcription factor STAT3 at Tyr705, which may be involved in the regulation of cell proliferation (37). ANXA2 alone may not be able to activate STAT3 activity, but EGF, which is closely related to the pSTAT3-cyclin D1 signaling pathway, may be its extracellular cofactor. The latest reports in this field suggest that ANXA2 and EGF signaling have a positive synergistic effect on EMT transformation in CaSki human cervical cancer cells (38). In order to assess this possibility in glioma cells, EGF induction was performed on each cell line and on control cells. The results revealed that pSTAT3^(Y705) expression was not markedly increased when EGF was added in the absence of ANXA2. However, in cells overexpressing ANXA2, the addition of EGF markedly increased the expression levels of pSTAT3^(Y705). In addition, when ANXA2 was re-expressed in ANXA2-knockdown U251 cells, there was a significantly recovery of pSTAT3^(Y705) in response to EGF. These results suggested that EGF-induced activation of pSTAT3^(Y705) may depend on the presence of ANXA2. It was further speculated that ANXA2 and EGF may be synergistic in the activation of pSTAT3^(Y705), as neither can effectively activate pSTAT3^(Y705) activity alone. In the present MTT assay and nude mouse model, ANXA2 overexpression may have resulted in a more significant proliferation-promoting effect due to the cumulative and synergistic effect of EGF secretion.

In conclusion, the present study revealed that ANXA2 may affect the proliferation of human glioma cells via the pSTAT3-cyclin D1 pathway by direct interaction with STAT3 in U251 and U87 glioma cells. Overexpression of ANXA2 cannot activate the pSTAT3 pathway alone, while positive synergy may exist between ANXA2 and EGF. Activated EGF and elevated levels of ANXA2 are frequently observed in a large number of human malignancies, including glioma (24,25). Therefore, these findings may provide novel insight into the functions of ANXA2 as a critical molecule in glioma cells and may improve our understanding of the mechanism underlying ANXA2-mediated proliferation in cancer cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZZ was involved in study concept, design and supervision, and provided final approval of the version to be published. LC was involved in drafting of the manuscript, data analysis and interpretation, and performed experiments and obtained funding. LL assisted with the experimental design, data interpretation and acquisition of funding. NX performed experiments, and was involved in analysis and interpretation of the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was conducted in accordance with ethical standards, according to the Declaration of Helsinki and national and international guidelines, and was approved by the Ethics Committee of Fujian Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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