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LncRNA FOXD3-AS1/miR-128-3p axis-mediated IGF2BP3 in glioma stimulates cancer angiogenesis and progression

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Abstract

Introduction: *The aim of the study was to research the mechanism by which IGF2BP3 regulates glioma progression as well as its upstream regulatory axis.*

Material and methods: *The researched mRNA was determined using differential expression analysis based on bioinformatics data, and its upstream miRNAs and lncRNAs were predicted. Interaction between genes we researched was identified by dual-luciferase method. The viability, migration, invasion and angiogenesis of glioma were measured with MTT, colony formation, Transwell and Matrigel tube formation experiments, respectively. The mRNA expression of each gene was tested with qRT-PCR. IGF2BP3 level was determined via western blot and immunohistochemistry. Subcellular fractionation of FOXD3-AS1 was tested with fluorescence in situ hybridization. In vivo tumorigenesis assay was conducted on nude mice.*

Results: *IGF2BP3 high level in glioma cells correlated with patient's prognosis. Downregulation of IGF2BP3 restrained proliferation, migration, invasion and angiogenesis in glioma cells both in vitro and in vivo. There was a binding relationship between IGF2BP3 and miR-128-3p. Besides, FOXD3-AS1 as a sponge of miR-128-3p was located mainly in cytoplasm. Additionally, FOXD3-AS1 facilitated IGF2BP3 level via sponging miR-128-3p to stimulate glioma angiogenesis.*

Conclusions: *FOXD3-AS1 was a sponge of miR-128-3p through upregulating IGF2BP3 in glioma. Our findings shed light on diagnosis and treatment of glioma.*

Key words: *FOXD3-AS1, MiR-128-3p, IGF2BP3, glioma, biofunction.*

Introduction

Human gliomas are a class of brain tumours arising from ependymal cells, oligodendrocytes or astrocytes [38]. In addition, glioma is one of the most lethal human tumours [28]. Liu *et al.* [22] analysed the survival of high-grade glioma after comprehensive treatment and denoted that the 1-, 2-, 3-, and 5-year survival rates post-treatment were 87.50%, 56.25%, 40.63%, and 17.19%, respectively. Though surgery, chemotherapy and radiotherapy have remained the milestone of treatment for glioma in the last few years, overall survival of high-grade glioma patients is unsatisfactory [30,37]. Angiogenesis is a fundamental pathological feature for lethal tumours and exerts important functions in occurrence and development of tumours [1]. Malignant glioma possesses strong vascular aggressiveness, and vessels with abnormal structures and functions are related to tumorigenesis [14]. Thus, anti-angiogenesis is a crucial option for glioma therapy, which needs further investigation.

IGF2BP3 can affect RNA stability, cell growth and migration during embryonic development [10,15,19,34]. IGF2BP3 is highly associated with many cancer types, like pancreatic cancer [29], colorectal cancer [35], lung cancer [44], and ovarian cancer [12]. According to one study with microarray analysis, IGF2BP3 was correlated with aggressive tumour features and dismal prognosis [4]. Bhargava *et al.* [3] stated that IGF2BP3 mediated the translatome of glioma cells. Besides, other reports

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demonstrated that IGF2BP3 accelerated glioma cell migration *via* RElA/p65 translation. IGF2BP3 works as a specific marker of glioma which is involved in modulating IGF-2 to activate PI3K/MAPK pathway [36]. In glioblastoma multiforme (GBM), miR-129-1 acts as a tumour inhibitor that targets IGF2BP3 and MAPK1 to induce cell cycle arrest [20]. Silence of IGF2BP3 restrains angiogenesis in colorectal cancer *via* adjusting VEGF [40]. Nonetheless, the impact of IGF2BP3 on glioma angiogenesis is not yet fully understood.

The highly-expressed lncRNA FOXD3-AS1 may exert a key effect on cancer progression through ceRNA network, containing lung cancer, malignant melanoma, and colon adenocarcinoma [41]. FOXD3-AS1 is consistently up-regulated in gliomas, which has been demonstrated in clinical specimens [5]. By bioinformatics analysis, aberrantly expressed miRNAs have been screened in gliomas and miR-128-3p is noticeably decreased [32]. As a target of FOXD3-AS1, miR-128-3p has been identified in cervical cancer [39].

To date, plenty of works have been concerned with lncRNA-miRNA-mRNA ceRNA network in assorted cell functions and angiogenesis [6,11,16,33]. In view of the above, we intended to study the potential mechanisms underlying IGF2BP3 in glioma development. Using cellular biological experiments and through bioinformatics assays, we identified the impact of FOXD3-AS1/miR-128-3p/IGF2BP3 regulatory axis during glioma progression. Therefore, we assume that this axis may act as an indicator for glioma.

Material and methods

Bioinformatics analysis

Glioma mRNA and lncRNA expression data (normal: 5, tumour: 698) and miRNA expression data (normal: 5, tumour: 530) were downloaded from TCGA database. The 'edgeR' package was utilized to conduct differential analysis between normal and tumour groups ($|logFC| > 1.5$, $FDR < 0.05$) on mRNAs, miRNAs, and lncRNAs to obtain differential mRNAs, miRNAs, and lncRNAs. Combined with literature citations, the target mRNA was identified. Target gene prediction was done through starBase database, miRDB, and mirDIP, and the upstream miRNAs were identified by correlation analysis. Further, starBase was introduced to identify putative miRNAs and do correlation analysis to determine upstream regulatory lncRNAs.

Cell incubation

The human brain glial cell line HEB (BNCC358606) and human glioma cell lines T98G (BNCC338721), U251 (BNCC341988) and HUVEC (BNCC337616) were bought from BeNa Culture Collection (BNCC, Beijing, China). U87 (IM-H424) were bought from Immocell (Xiamen, China). Experiments were conducted after all cells were passaged 3 times. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma, USA) + 10% foetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) and 100 U/ml penicillin/streptomycin (Corning, New York, NY, USA) at 37°C with 5% CO_2.

Cell transfection

Plasmid vectors siRNA-FOXD3-AS1 (si-FOXD3-AS1), siRNA-IGF2BP3 (si-IGF2BP3), oe-IGF2BP3, miR-128-3p mimic (miR-mimic), miR-128-3p inhibitor (miR-inhibitor) and corresponding negative control (NC) were all constructed by GenePharma (Shanghai, China). U251 and U87 cells (6×10^5 cells/well) were plated in 6-well plates at a density that was adjusted to 70%. After being maintained for 12-16 h, cell transfection was done using Lipofectamine 3000.

qRT-PCR

Trizol (Invitrogen, Carlsbad, CA, USA) was applied for total RNA extraction. miR-128-3p was reversely transcribed to cDNA by mi Script II RT kit (Qiagen, Germany) and tested by miScript SYBR Green PCR Kit (Qiagen, Germany) with U6 as an internal reference. PrimeScript RT Master Mix (Takara, Japan) was applied to generate cDNA, and SYBR® Premix Ex Taq TM II (Takara Bio Inc., Japan) kit was used for expression detection with GAPDH as an internal reference. All qRT-PCRs were performed on Applied Biosystems® 7500 Real-Time PCR Systems (Thermo Fisher, MA). Primers were as follows: FOXD3-AS1 forward primer: 5'-GGTGGAGGAGGCGAGGATG-3', reverse primer: 5'-AGCGGACAGACAGGGATTGG-3'; miR-128-3p forward primer: 5'-GGTCACAGTGAACCGGTC-3', reverse primer: 5'-GTGCAGGGTCCGAGGT-3'; IGF2BP3 forward primer: 5'-CTTCCTGGTGAAGACTGGC-3', reverse primer: 5'-CCGAGTGCTCAACTTCTATG-3'; GAPDH forward primer: 5'-GAAGGTCGGAGTCAACGGATT-3', reverse primer: 5'-CGCTCCTGGAAGATGGTGAT-3'; U6 forward primer: 5'-ATTGGAACGATACAGAGAAGATT-3', reverse primer: 5'-GGAACGCTTCACGAATTTG-3'. Relative gene expression was compared by $2^{-\Delta\Delta Ct}$ value.

Western blot

Cells were gathered and lysed by RIPA lysis buffer (Beyotime, Haimen, China) adding 100× protease inhibitors and phosphatase inhibitors. Protein concentration was determined by BCA (Beyotime, Beijing, China). Proteins were then separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, USA). The membrane was sealed with 5% BSA for 2 h. Thereafter, membrane was incubated with the suitable primary

antibodies at 4°C overnight. Primary antibodies included: rabbit anti-IGF2BP3 (ab273131, 1 : 1000, Abcam, UK), Anti-GAPDH (ab9485, 1 : 2500, Abcam, UK). Afterwards, 1 × TBST detergent solution was applied to wash membrane at room temperature (5 min × 3 times). Secondary antibody goat anti-rabbit IgG (ab6721, 1 : 2000, Abcam, UK) labelled by horseradish peroxidase (HRP) was supplemented. Hybrid incubation was conducted at room temperature for 120 min. The ECL kit (Solarbio, Beijing, China) was adopted to visualize protein bands.

Cell proliferation assay

After 24-h transfection, U87 and U251 cells were digested by trypsin. Next, cells were plated in 96-well plates (5×10^3 cells/well). MTT solution (Procell, Wuhan, China) was supplemented to each well at 0, 24, 48 and 72 h. Cells were grown under standard culture conditions for 4 h and then treated with 150 µl dimethyl sulfoxide. After 10 min of blending, absorbance at 490 nm was measured with ultraviolet spectrophotometer.

Colony formation assay

Cells (1×10^3 cells/well) were plated in 6-well plates, with 3 parallel wells used for each group. Cells were maintained in the complete medium for 14 days until visible colonies formed. Cell colonies were fixed with 4% paraformaldehyde at room temperature for 15 min and then stained by 0.1% crystal violet (Thermo Fisher, USA) for 20 min. Each well was rinsed with sterile water to remove the excessive crystal violet stain. More than 50 cells were defined as a colony. Images were captured through a light microscope (BX53, Quantitative Imaging Corporation, Canada) equipped with MicroPublisher 3.3RTV camera (Olympus Corporation, Japan). The number of colonies in each well was counted.

Transwell assays

Following transfection, glioma cells were starved for 24 h. Then cells (2.5×10^4 cells/well) plated in 200 µl serum-free DMEM were added in the upper chamber of Transwell assay inserts (8-µm pore size, Millipore, Biollerica, MA, USA) that was coated with/without Matrigel. Thereafter, 750 µl DMEM + 10% FBS was added to the bottom chamber. After 24 h, migrating/invading cells in the bottom chamber were fixed with methanol and stained with crystal violet.

Dual-luciferase reporter gene analysis

Regulation between miR-128-3p and lncRNA FOXD3-AS1/IGF2BP3 was detected by dual-luciferase reporter analysis. pmirGLO luciferase reporter vectors (Promega, USA) were inserted with wild type (wt) or mutant (mut) FOXD3-AS1 or IGF2BP3. Human embryonic kidney cells HEK-293T (BNCC339658, BNCC, China) were cultured in 24-well plates. Cells were transfected using 100 nM miR-128-3p mimic/miR-NC, FOXD3-AS1-wt/FOXD3-AS1-mut and IGF2BP3-wt/IGF2BP3-mut plasmids to construct a set of co-transecting cell lines (miR-NC+IGFBP3-wt, miR-mimic + IGFBP3-wt, miR-NC + IGFBP3-mut, miR-mimic + IGFBP3-mut, miR-NC + FOXD3-AS1-wt, miR-mimic + FOXD3-AS1-wt, miR-NC + FOXD3-AS1-mut and miR-mimic + FOXD3-AS1-mut). 48 h later, luciferase intensity was tested on the dual-luciferase reporter system (Promega, USA).

Subcellular fractionation assay

Nucleus or cytoplasm of U251 and U87 cells was fractionated by PARIS Kit (Life Technologies, Carlsbad, USA). Next, FOXD3-AS1 expression and U6 and GAPDH in the cytoplasm and nucleus were assayed through qRT-PCR.

Immunohistochemical analysis

All tissue samples were fixed with formalin and embedded in paraffin. Then, they were cut into 3 mm thick slices. Next, the slices were dewaxed and incubated with 1% H_2O_2 to eliminate the activity of endogenous peroxidase. The slices were rinsed with phosphate-buffered saline (PBS) in triplicate and incubated for 30 min with 5% skim milk/PBS to lessen non-specific binding. Next, slices were incubated with anti-IGF2BP3 (ab273131, 1 : 1000, Abcam, UK), diluted with 5% skim milk/PBS, at 4°C for 16 h. Tissue samples were rinsed with PBS and stained with 3, 3-diaminophenylhydrazine hydrochloride (Sigma-Aldrich, USA). All slices were further stained by haematoxylin. The primary antibody was replaced with the same concentration of non-immune IgG (ab6721, 1 : 2000, Abcam, UK) as the NC group.

Tube formation assay

The transfected cells were cultivated in the medium without serum for 24 h, after which the supernatant was gathered as conditioned medium (CM), which was centrifuged at 3,000 rpm and stored at –80°C. 100 µl Matrigel (BD Biosciences, USA) was placed on pre-cooled 96-well plates for 30-min incubation at 37°C. HUVEC cells pre-cultivated in CM (added with 10% FBS) for 18 h were gathered and suspended in 100 µl CM. HUVEC cells were then seeded in wells, incubated for 6 h at general temperature, and pictured using an inverted microscope (Nikon, Tokyo, Japan).

Tumour xenograft transplantation assay

Twelve male nude mice (4 weeks; Shanghai Slack Laboratory Animal Co., Ltd, Shanghai, China) were housed in specific pathogen-free environment (12/12 h light/dark cycle, 25°C, 60-70% moisture). 2 × 10^6/ml U87 cell line with si-IGF2BP3 and si-NC was subcutaneously implanted into mice. Tumour weight and volume were assessed 1 week later. Tumour volume (cm^3) = (length × $width^2$)/2. Four weeks later, nude mice were euthanatized. Tumour weight was detected and tumour pictures were taken.

Statistical analysis

The above experiments underwent 3 repetitions. All data were treated on SPSS 22.0 statistical software (SPSS, Inc., Chicago, IL, USA). All results were denoted as mean ±SD. Inter-group comparison was assessed using *t*-test. Differences were remarkable when $p < 0.05$.

Results

IGF2BP3 presents a high expression level and is related to poor prognosis in glioma

The mRNA expression data of glioma were analysed by differential analysis, and 2,581 differentially expressed mRNAs (DEmRNAs) were screened out (Fig. 1A). Among them, IGF2BP3 expression was notably high in glioma tissues (Fig. 1B). Survival analysis revealed that upregulation of IGF2BP3 is indicative of poor prognosis in glioma patients (Fig. 1C). Thereafter, as tested by qRT-PCR and western blot, IGF2BP3 level was also remarkably upregulated in glioma cell lines (Fig. 1D, E). Therefore, it was verified that IGF2BP3 participated in modulating cell growth in glioma. Afterwards, U87 and U251 cells with high IGF2BP3 expression were chosen for the subsequent assays. U87 cells were used for *in vivo* experiments as well.

Silence of IGF2BP3 represses glioma cell phenotypes and angiogenesis

In this part, IGF2BP3 was silenced in U87 and U251 cells for further studies. qRT-PCR and western blot tested expression efficiency. IGF2BP3 expression was remarkably downregulated in U87 and U251 glioma cells following IGF2BP3 silencing (Fig. 2A, B).

As exhibited in Figure 2C, cancer cell viability of si-IGF2BP3 was markedly lower than si-NC. Colony formation assay displayed the diminished ability of colony formation after si-IGF2BP3 (Fig. 2D). As further revealed by Transwell assays, si-IGF2BP3 evidently constrained cell migration (Fig. 2E) and invasion (Fig. 2F). si-NC and si-IGF2BP3-derived CMs were utilized to treat HUVEC cells. si-IGF2BP3-derived CM treatment triggered a notable decrease in tube formation ability compared with the si-NC group (Fig. 2G). Altogether, silencing IGF2BP3 constrains the growth and angiogenesis of glioma cells, suggestive of its underlying function as a target against glioma.

MiR-128-3p inhibits IGF2BP3 expression in glioma cells

First, differential expression analysis was performed on miRNAs in TCGA database. Altogether, 178 differentially expressed miRNAs (DEmiRNAs) were acquired (Fig. 3A). Then, starBase, miRDB and mirDIP were used to predict the interaction between miRNAs and IGF2BP3. The miRNAs were overlapped with 80 downregulated DEGs, and 1 DEmiRNA (miR-128-3p) was obtained (Fig. 3B). The negative association between miR-128-3p and IGF2BP3 was discovered in Pearson correlation analysis (Fig. 3C). MiR-128-3p of glioma tissue was markedly lower than normal tissue (Fig. 3D). Survival analysis displayed a lower survival rate for patients with a decreased miR-128-3p level (Fig. 3E). qRT-PCR demonstrated the remarkable decrease of miR-128-3p in glioma cells (Fig. 3F). Moreover, IGF2BP3 mRNA and protein levels were noticeably reduced in cells with miR-128-3p overexpression (Fig. 3G, H). Bioinformatics analysis further determined relationship and binding sites between IGF2BP3 and miR-128-3p (Fig. 3I). Dual-luciferase method exhibited that miR-128-3p upregulation remarkably constrained luciferase intensity of IGF2BP3-wt, while no impact was observed on IGF2BP3-mut (Fig. 3J). Altogether, miR-128-3p restrained IGF2BP3 expression in glioma.

MiR-128-3p restrains IGF2BP3 and represses glioma cell proliferation, migration, invasion and angiogenesis

We constructed oe-NC + miR-NC, oe-IGF2BP3 + miR-NC, and oe-IGF2BP3 + miR-mimic transfection groups in U251 and U87 cell lines. Firstly, qRT-PCR uncovered that IGF2BP3 was evidently increased in glioma cell lines after the overexpression of IGF2BP3, while it was markedly downregulated upon the simultaneous upregulation of miR-128-3p and IGF2BP3 (Fig. 4A). Based on MTT assay, cell proliferative potential was conspicuously enhanced upon IGF2BP3 upregulation, while it was significantly inhibited following simultaneous overexpression of miR-128-3p and IGF2BP3 (Fig. 4B). Cell colony formation ability was significantly upregulated after overexpressing IGF2BP3, while concomitant upregulation of miR-128-3p and IGF2BP3

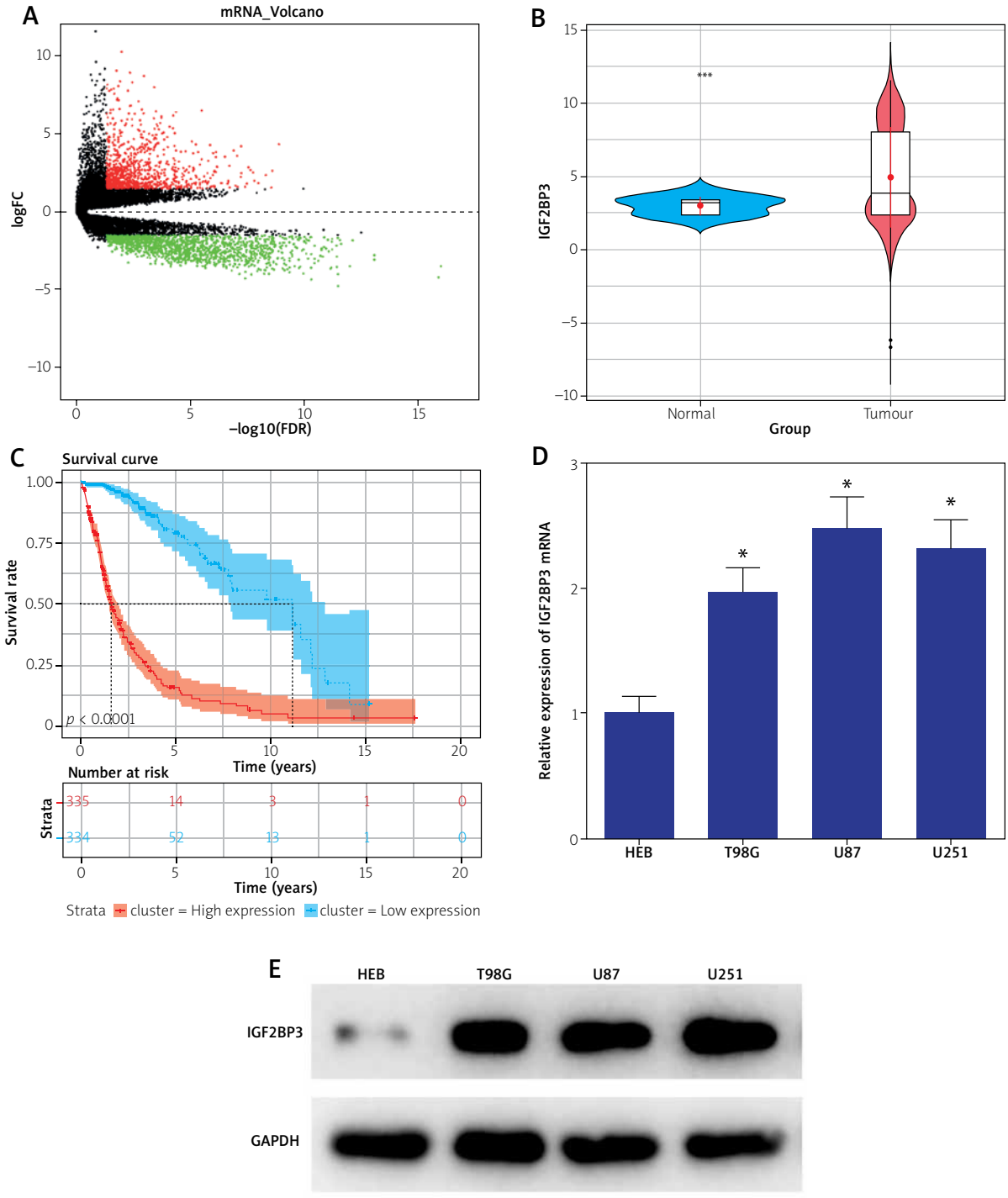

Fig. 1. IGF2BP3 is highly expressed in glioma. **A**) Volcano plot of DEmRNAs in normal and tumour groups. **B**) IGF2BP3 expression in glioma tissue and normal tissue. Y-axis: mRNA expression, X-axis: sample type. **C**) Survival analysis tested the relationship between IGF2BP3 level and patient's prognosis. Blue: low expression, red: high expression. **D, E**) IGF2BP3 mRNA and protein expression in HEB, T98G, U87 and U251 cell lines, respectively. *$p < 0.05$.

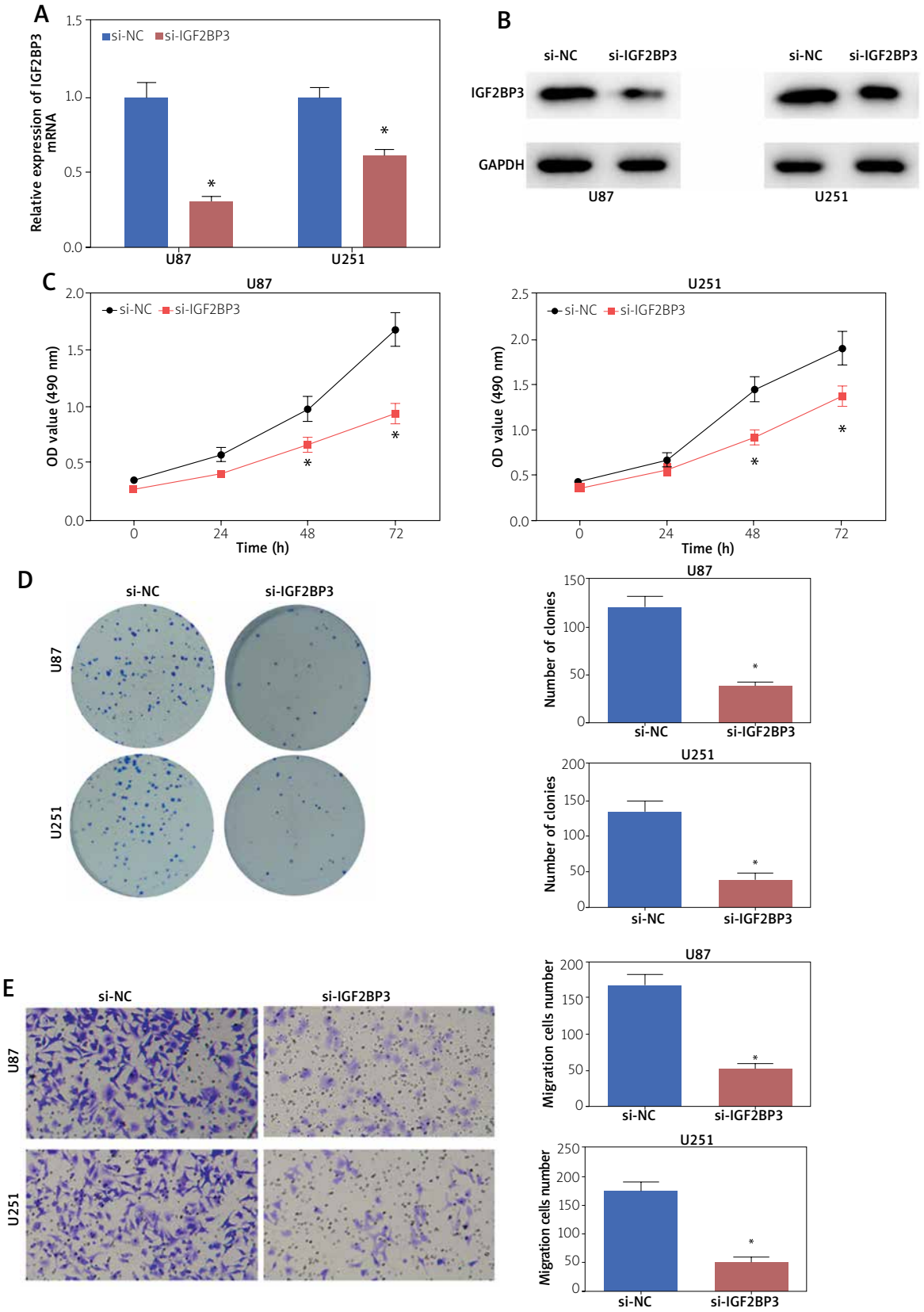

Fig. 2. Silencing IGF2BP3 suppresses growth of glioma cells. **A, B**) IGF2BP3 mRNA and protein levels in U87 and U251 cells upon silencing IGF2BP3. **C**) The viability of tumour cells regulated by IGF2BP3. **D**) The impact of silencing IGF2BP3 on colony formative ability of tumour cells. **E**) The inhibition of silencing IGF2BP3 on migration of tumour cells (100×).

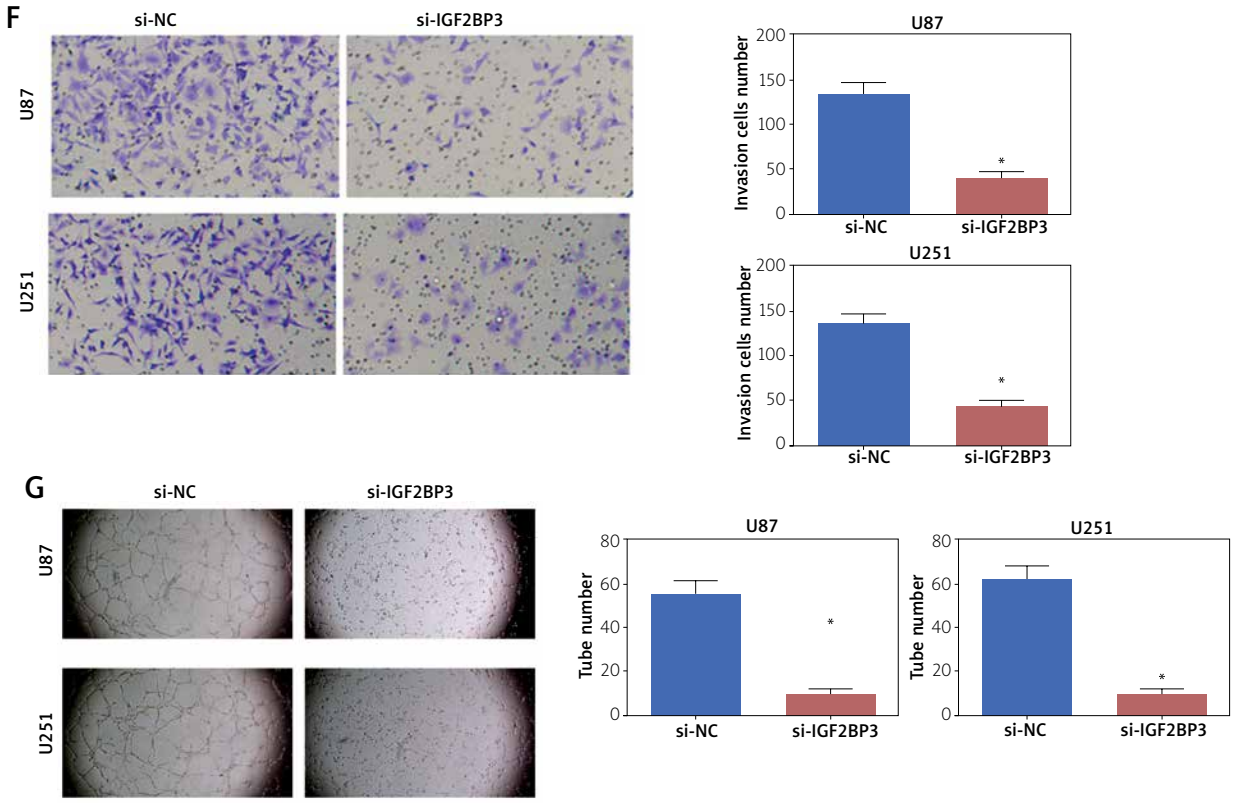

Fig. 2. Cont. F) The invasive ability of tumour cells by silencing IGF2BP3 expression (100×). **G**) IGF2BP3 knockdown influences tube formation in glioma. *$p < 0.05$.

significantly declined colony formation (Fig. 4C). Cell migration and invasion were prominently enhanced after overexpressing IGF2BP3, which were prominently downregulated following simultaneous increased miR-128-3p and IGF2BP3 (Fig. 4D, E). Likewise, with the supplementation of miR-128-3p mimic, promoting impact of IGF2BP3 upregulation on tube formation was weakened (Fig. 4F). These results suggested that miR-128-3p rescued promotion impact of IGF2BP3 on glioma cell phenotypes and angiogenesis.

FOXD3-AS1 participates in mediating miR-128-3p/IGF2BP axis in glioma cells

We first performed differential expression analysis on the lncRNAs of glioma in TCGA database. Then, we obtained 1089 differential lncRNAs (Fig. 5A). Using starBase to predict lncRNAs with binding sites with miR-128-3p and overlap with 421 differential upregulated lncRNAs, 10 differential upregulated lncRNAs with binding sites were obtained (Fig. 5B). Pearson analysis demonstrated a positive correlation between FOXD3-AS1 and IGF2BP3 ($r = 0.5$) (Fig. 5C). Expression analysis indicated that FOXD3-AS1 was evidently overexpressed in glioma tissue (Fig. 5D). As survival analysis presented, FOXD3-AS1 high level triggered patients' lower survival (Fig. 5E). Therefore, we concluded that lncRNA FOXD3-AS1 was able to facilitate glioma cell phenotypes and angiogenesis by mediating miR-128-3p/IGF2BP3 axis.

Afterwards, FOXD3-AS1 level in glioma cells was detected *via* qRT-PCR. In comparison with normal cell line (HEB), FOXD3-AS1 was dramatically stimulated in glioma cells (Fig. 5F). The nuclear/cytoplasm fractionation results uncovered that FOXD3-AS1 was expressed mainly in cytoplasm. We verified FOXD3-AS1 as a ceRNA (Fig. 5G). Then, binding sites between miR-128-3p and the 3'UTR region of FOXD3-AS1 were predicted (Fig. 5H). Dual luciferase analysis displayed that miR-128-3p high level prominently repressed luciferase intensity of FOXD3-AS1-wt but did not affect FOXD3-AS1-mut (Fig. 5I). In addition, silencing of FOXD3-AS1 significantly elevated the miR-128-3p level in glioma cells (Fig. 5J). Generally, FOXD3-AS1 could be a sponge of miR-128-3p.

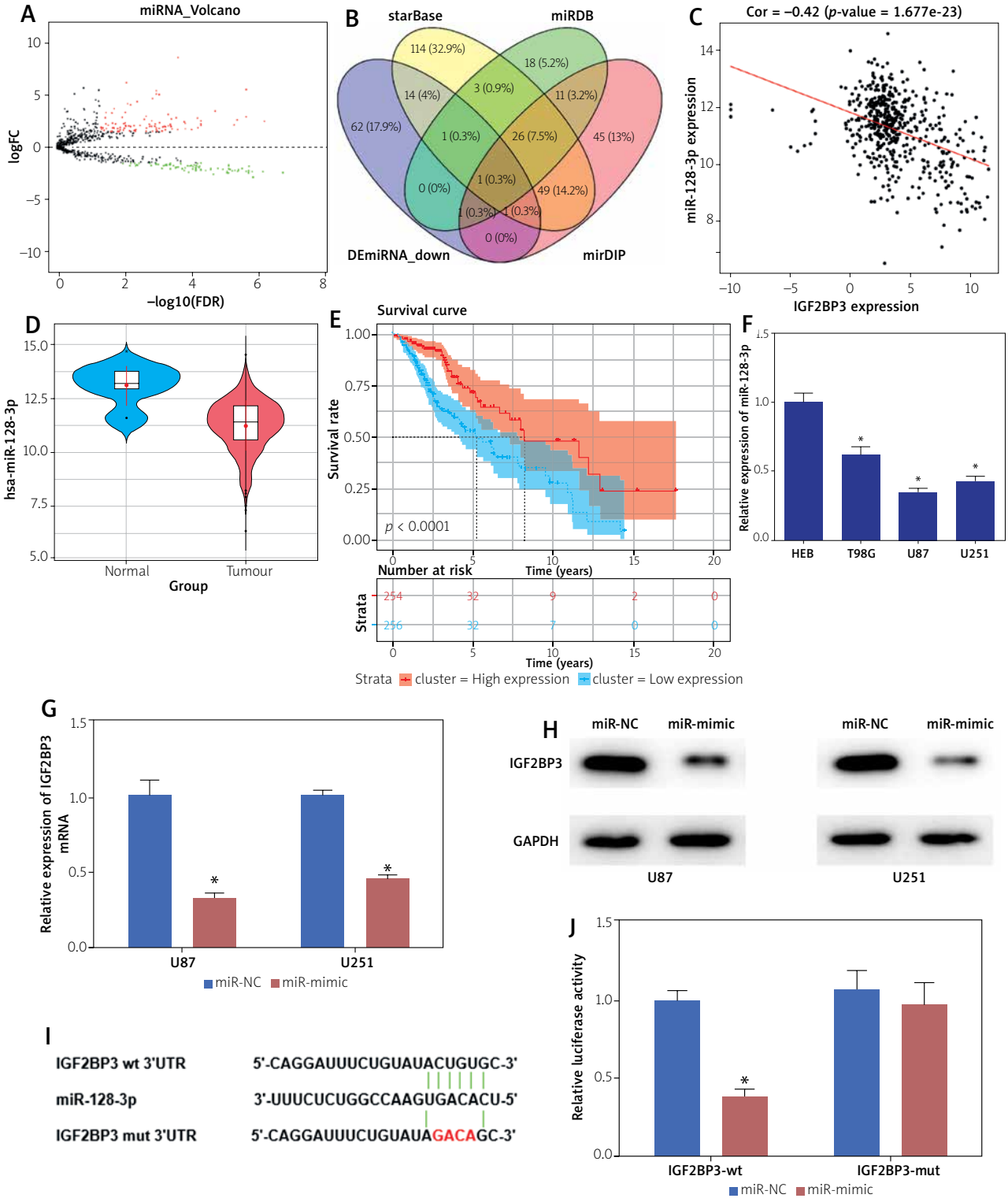

Fig. 3. miR-128-3p restrains IGF2BP3 expression in glioma. **A**) Volcano plot of DEmiRNAs in normal and tumour groups. Green: downregulated genes, red: upregulated genes. **B**) Venn diagram of target mRNAs and downregulated DEmiRNAs. **C**) Scatter plot of correlation between miR-128-3p and IGF2BP3. **D**) Violin plot of miR-128-3p level in normal (blue) and tumour group (red). **E**) Survival curve of miR-128-3p on prognosis. Blue: low expression, red: high expression. **F**) MiR-128-3p expression in glioma cells. **G, H**) The impact of overexpressing miR-128-3p on IGF2BP3 mRNA and protein levels in U87 cells and U251 cells. **I**) Binding sites between miR-128-3p and IGF2BP3. **J**) Validating the binding between miR-128-3p and IGF2BP3. *$p < 0.05$.

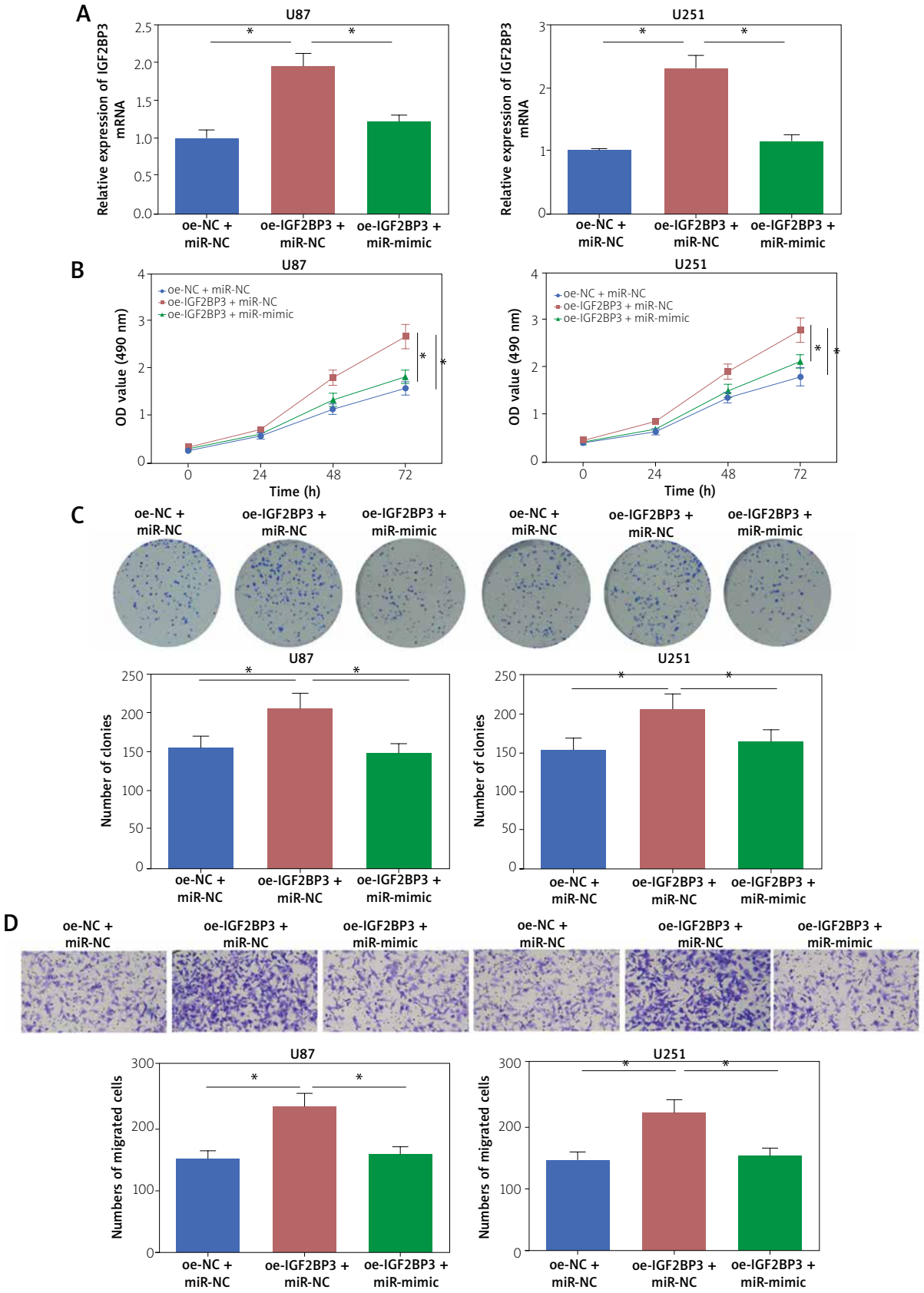

Fig. 4. MiR-128-3p targets IGF2BP3 to repress glioma progression. **A**) IGF2BP expression in different transfected cells. **B**) MiR-128-3 p/IGF2BP3 axis impact on U87 and U251 cell viability. **C**) Colony formation of U87 and U251 cells in each treatment group. **D**) The regulatory role of miR-128-3p and IGF2BP3 in glioma cell migration (100×).

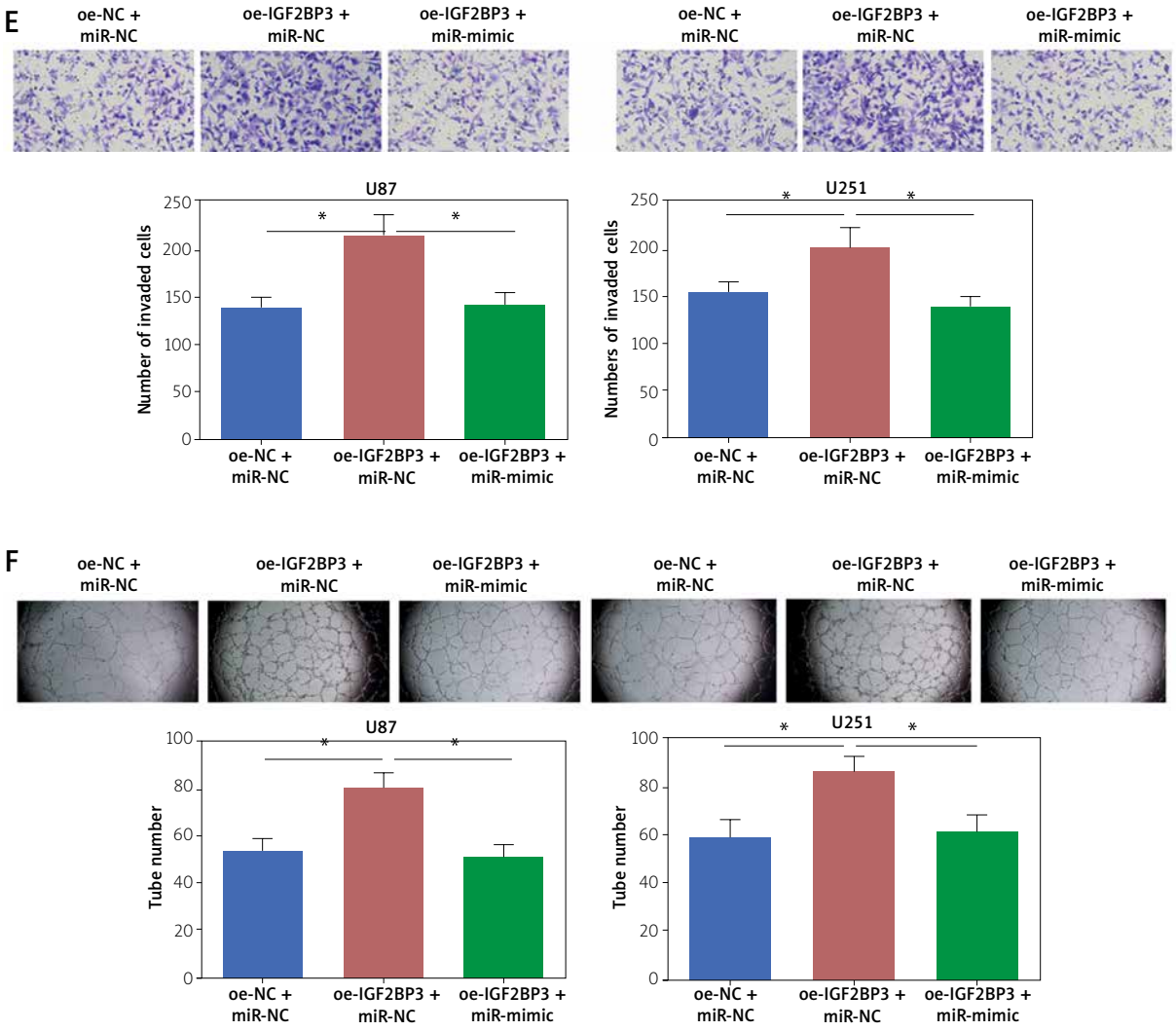

Fig. 4. Cont. **E**) The regulatory role of miR-128-3p and IGF2BP3 in glioma cell invasion (100×). **F**) MiR-128-3p and IGF2BP influence glioma angiogenesis. *$p < 0.05$.

Subsequently, we constructed different transfection groups to validate interaction between FOXD3-AS1, miR-128-3p, and IGF2BP3. As measured through qRT-PCR, the miR-128-3p level was notably upregulated whereas IGF2BP3 mRNA expression was significantly downregulated under FOXD3-AS1 silenced condition. Besides, miR-128-3p inhibitor or IGF2BP3 overexpression rescued IGF2BP3 expression under FOXD3-AS1 silenced condition (Fig. 5K). These results suggested that FOXD3-AS1 might participate in mediating miR-128-3p/IGF2BP3 axis in glioma.

FOXD3-AS1/miR-128-3p/IGF2BP3 axis affects biological functions of glioma cells

Functional experiments were conducted on U251 and U87 cells. FOXD3-AS1 knockdown impaired U251 and U87 cell proliferation, and such effect was weakened *via* miR-128-3p inhibitor or IGF2BP3 overexpression dramatically (Fig. 6A). Silencing of FOXD3-AS1 suppressed colony formation of tumour cells, which was restored with miR-128-3p inhibitor or IGF2BP3

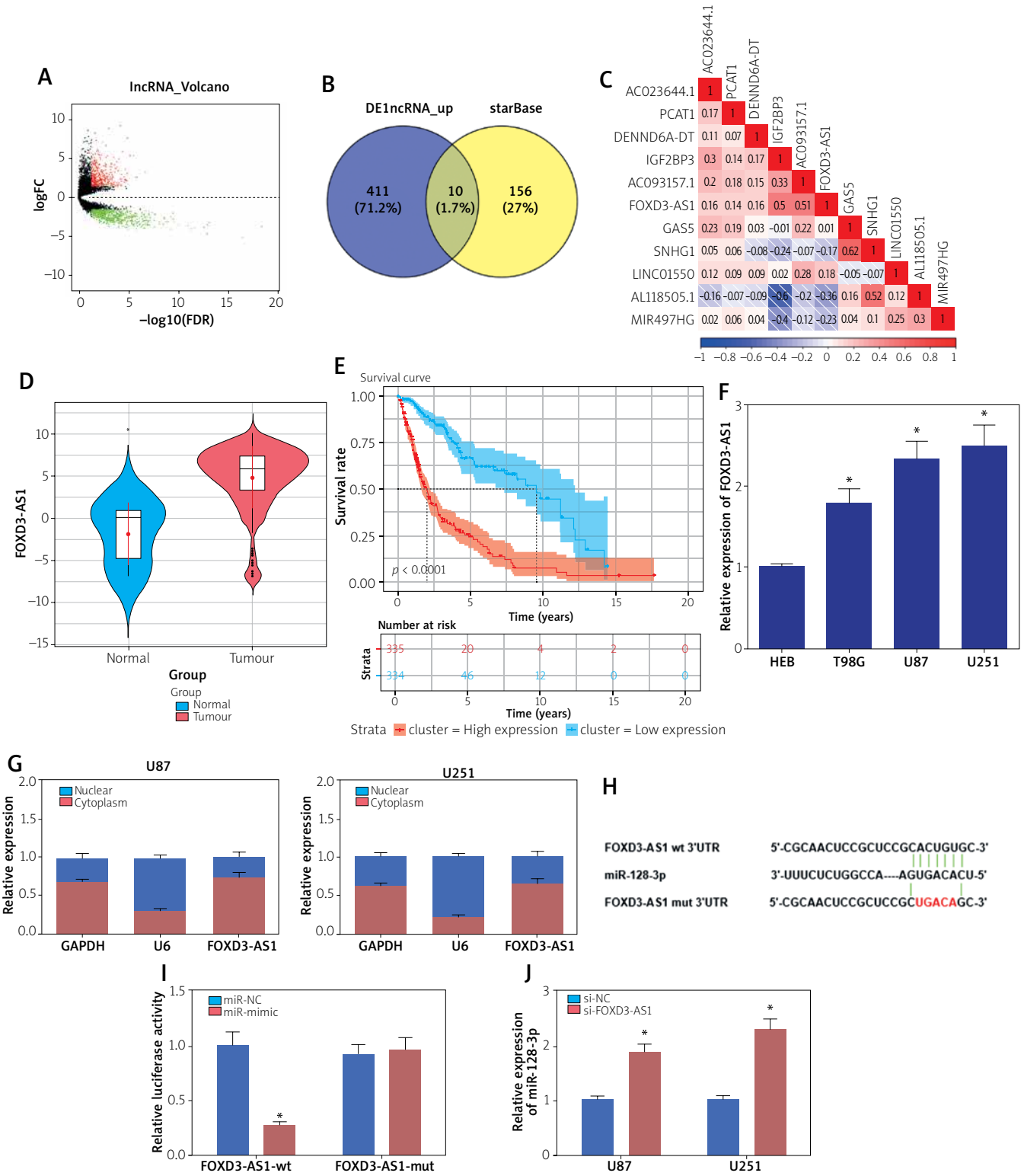

Fig. 5. FOXD3-AS1 stimulates IGF2BP3 level *via* miR-128-3p inhibition. **A**) Volcano plot of DElncRNAs in normal and tumour groups. Green: downregulated DElncRNAs, red: upregulated DElncRNAs. **B**) Venn diagram of predicted lncRNAs and upregulated DElncRNAs. **C**) Heat map of correlation between candidate lncRNAs and IGF2BP3. **D**) Violin plot of FOXD3-AS1 level in the normal group (blue) and the tumour group (red). **E**) Survival curve of prognosis of patients with different FOXD3-AS1 expressions. Red: high expression, blue: low expression. **F**) FOXD3-AS1 level in normal cells and glioma cells. **G**) Expression sites of FOXD3-AS1 in glioma cells. **H**) Binding sequences of FOXD3-AS1 and miR-128-3p. **I**) The targeting between FOXD3-AS1 and miR-128-3p. **J**) The impact of silencing FOXD3-AS1 on miR-128-3p expression. **p* < 0.05.

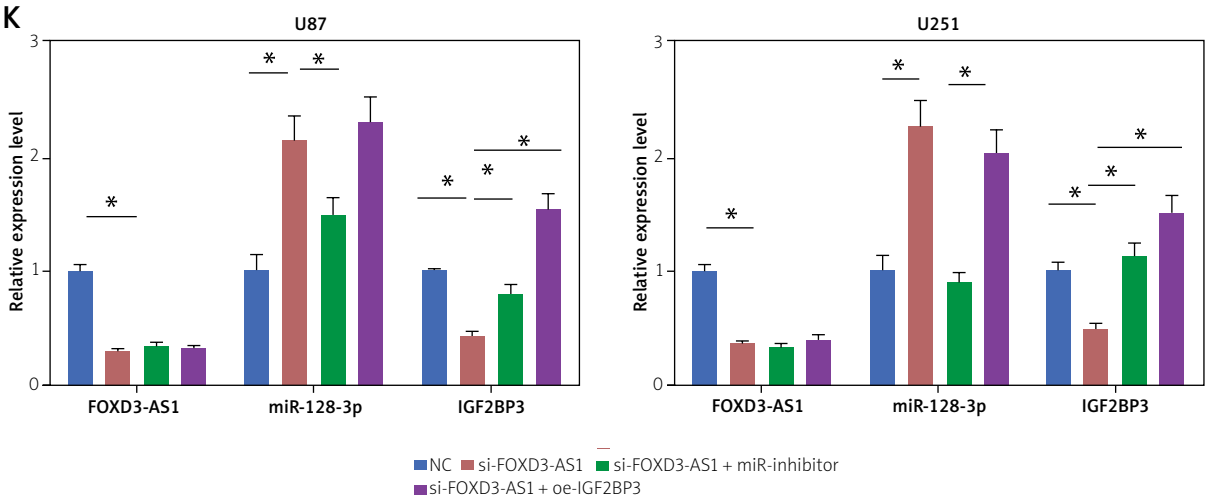

Fig. 5. Cont. **K**) FOXD3-AS1, miR-128-3p and IGF2BP3 mRNA levels in cells. *$p < 0.05$.

overexpression (Fig. 6B). FOXD3-AS1 silence significantly reduced glioma cells migration and invasion, and migration, as well as invasion, were recovered when miR-128-3p was inhibited or IGF2BP3 was overexpressed (Fig. 6C, D). FOXD3-AS1 silence evidently constrained angiogenesis capability whereas miR-128-3p inhibitor or IGF2BP3 enforced expression conspicuously under FOXD3-AS1 silenced condition (Fig. 6E). Taken together, FOXD3-AS1 could sponge miR-128-3p and improve IGF2BP3 expression, thereby regulating glioma progression and angiogenesis.

In vivo assay testified the impact of IGF2BP3 on glioma tumour growth

Here, a nude mouse-human tumour xenograft model was constructed. As depicted in Figure 7A, B, silencing of IGF2BP3 remarkably inhibited tumour weight and tumour volume. Silencing of IGF2BP3 remarkably declined the positive expression rate of IGF2BP3 and ki67, as detected by immunohistochemical (Fig. 7C). IGF2BP3 expression was remarkably decreased in tumour tissue in si-IGF2BP3 (Fig. 7D). Altogether, silencing of IGF2BP3 hampered tumour growth of glioma *in vivo*.

Discussion

The IGF2BP3 gene on chromosome 7p15.3 encodes a 69 kDa protein [26] and can adjust angiogenesis [17]. Silencing IGF2BP3 adjusts angiogenesis of colorectal cancer cells and reduces vascular endothelial growth factor (VEGF) mRNA expression and stability [40]. IGF2BP3 has been implicated in multiple aggressive cancer types. For instance, a high IGF2BP3 level is related to gastric cancer patients' poor prognosis [42]. Silencing of IGF2BP3 in ovarian cancer significantly restrains cancer cell migration, proliferation, and invasion, and promotes apoptosis [23]. We found that silencing of IGF2BP3 restrained human glioma cell migration, proliferation, and invasion, and facilitated apoptosis, showing the same trend in ovarian cancer. CDR1as epigenetic silencing drives IGF2BP3 expression to mediate metastasis and invasion of melanoma [9]. IGF2BP3 high expression stimulates aggressive phenotypes of colorectal cancer. In addition, IGF2BP3 can affect growth of cancer cells [3,18,20]. The exact mechanism underlying IGF2BP3 in glioma progression is not completely understood which awaits further investigations. In this paper, we attempted to study the molecular mechanism of IGF2BP3 in glioma development. The results showed evident IGF2BP3 upregulation in glioma tissues and cells. IGF2BP3 high level foresaw bad prognoses of patients and silence of its expression evidently restrained *in vivo* and *in vitro* growth of glioma cells. Of note, angiogenesis is of profound importance in most gliomas [8,31]. This work revealed that IGF2BP3 downregulation mediated by FOXD3-AS1/miR-128-3p repressed angiogenesis in glioma.

Bioinformatics methods were used to identify the upstream modulatory mechanism of IGF2BP3 in glioma. miR-128-3p, downregulated in glioma cells, was identified as a regulatory gene of IGF2BP3 in glioma cells. Thereafter, dual-luciferase assay validated affinity between miR-128-3p and IGF2BP3, and that IGF2BP3 level was negatively related to miR-128-3p in glioma cells. Functional assays also manifested that simultaneous overexpressing miR-128-3p and IGF2BP3 markedly reduced the promotion effect of IGF2BP3 on glioma progression. Earlier studies also indicated that miR-128-3p may work as a cancerous inhibitor in plenty of cancer cells. LncRNA MIR4435-2HG stimulates the cell biological behaviours in ovarian cancer *via* medi-

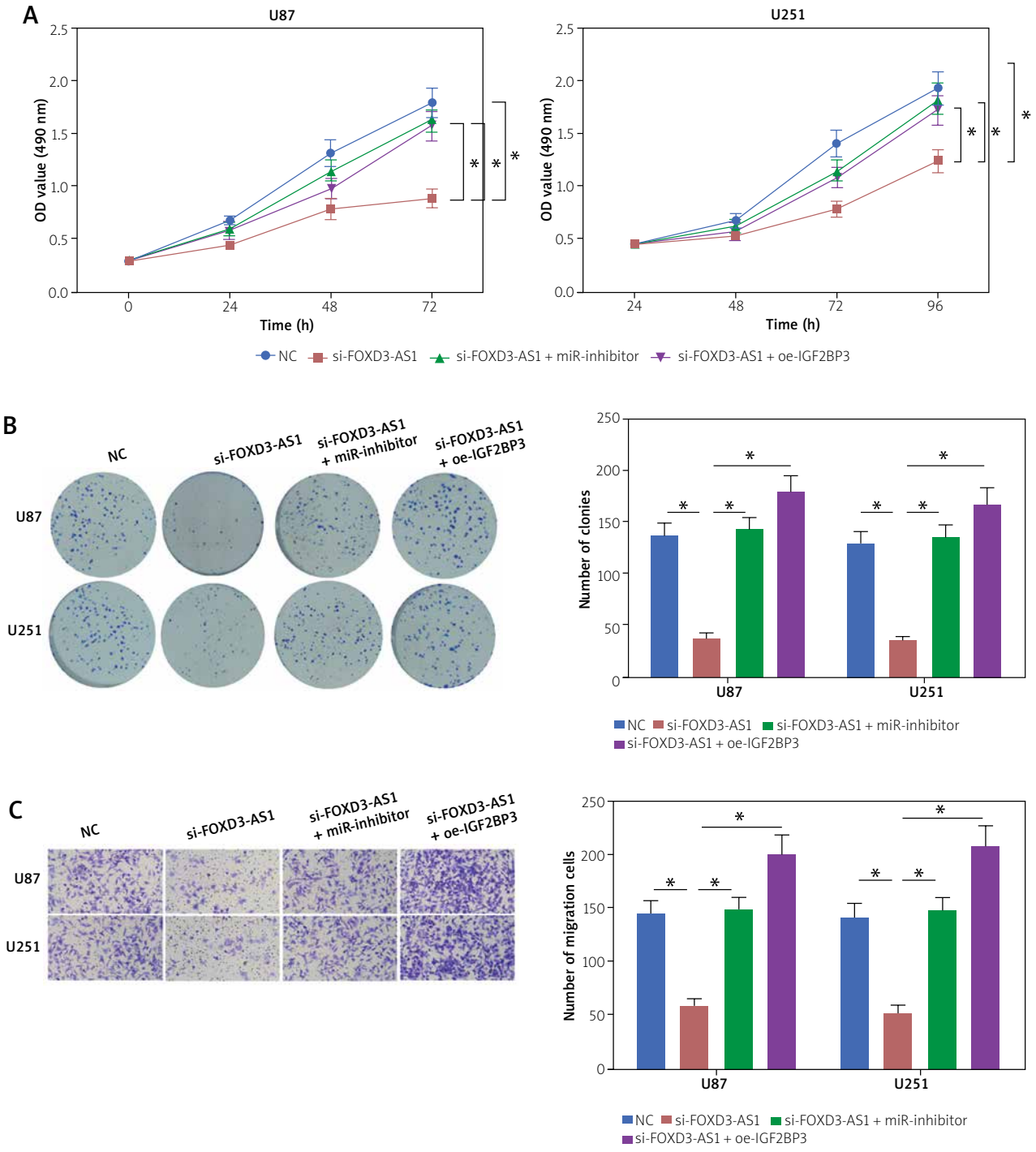

Fig. 6. FOXD3-AS1 stimulates malignant growth of glioma cells via the miR-128-3p/IGF2BP3 axis. **A**) Proliferation ability of glioma cells in different groups. **B**) The colony formation of glioma cells. **C**) The impact of different treatments on migration ability of U87 and U251 cells (100×) *$p < 0.05$.

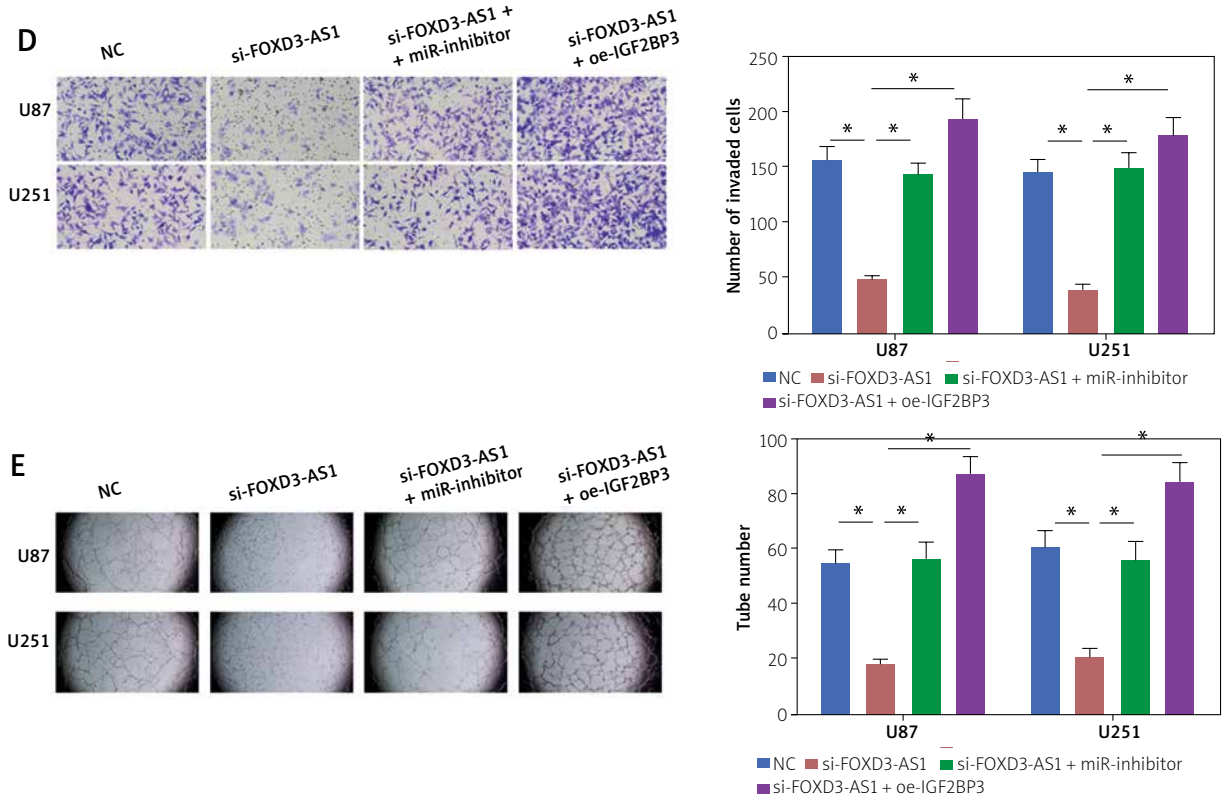

Fig. 6. Cont. **D**) The impact of FOXD3-AS1, miR-128-3p and IGF2BP3 on invasion ability of glioma U87 and U251 (100×). **E**) Angiogenesis of glioma cells. *$p < 0.05$.

ating the miR-128-3p/CKD14 axis [46]. MiR-128-3p suppresses colorectal cancer progression by inhibiting PI3K/Akt and MEK/ERK pathways [25]. MiR-128-3p can repress invasion and proliferation of melanoma cells through modulating JAG1 [27]. In glioma, miR-128-3p mediates mitochondrial dysfunction and stimulates tumour cell apoptosis by targeting PDK1 [32]. MiR-128-3p regulates the IRS-1/PI3K/Akt pathway *via* targeting NPTX1, thus inhibiting proliferation and differentiation of glioma cells [13]. LncRNA PVT1 accelerates glioma development through miR-128-3p/Grem1 axis and BMP signalling pathway [7]. This work discovered miR-128-3p constraining glioma angiogenesis through directly mediating IGF2BP3. MiR-128-3p low level pertained to poor prognoses of glioma patients. With integrative analyses of earlier works and our findings, we determined miR-128-3p as an inhibitor in glioma.

A previous report stated that lncRNAs modulated the expression of downstream genes through competitively adsorbing miRNAs [2]. Bioinformatics analysis identified FOXD3-AS1 as an upstream regulatory gene of miR-128-3p. Dual luciferase assay illustrated that FOXD3-AS1 targeted miR-128-3p in glioma. Cell functional experiments showed that FOXD3-AS1 knockdown hampered cell phenotypes and angiogenesis in glioma, while miR-128-3p inhibitor or IGF2BP3 overexpression reversed FOXD3-AS1 silencing-mediated inhibitory effect. As stated in published papers, FOXD3-AS1 plays an imperative role in cancer growth. Zeng *et al.* [41] demonstrated that the overexpressed lncRNA FOXD3-AS1 exacerbated NSCLC progression through miR-127-3p/MED28 axis. It is worth noting that FOXD3-AS1 has been long deemed as an oncogene owing to its capability of promoting tumour proliferation and metastasis rather than angiogenesis. Thus, FOXD3-AS1 downregulation constraining angiogenesis *in vitro* might be found for the first time in this study.

In summary, FOXD3-AS1 could stimulate the cell functions of glioma through the miR-128-3p/IGF2BP3 axis. The pivotal functions of FOXD3-AS1/miR-128-3p/IGF2BP3 axis performed in angiogenesis of glioma were certified by this work. This paper offers a theoretical basis for regulatory mechanism of FOXD3-AS1, miR-128-3p and IGF2BP3, and demonstrates that this axis may function as a novel biomarker for glioma patients.

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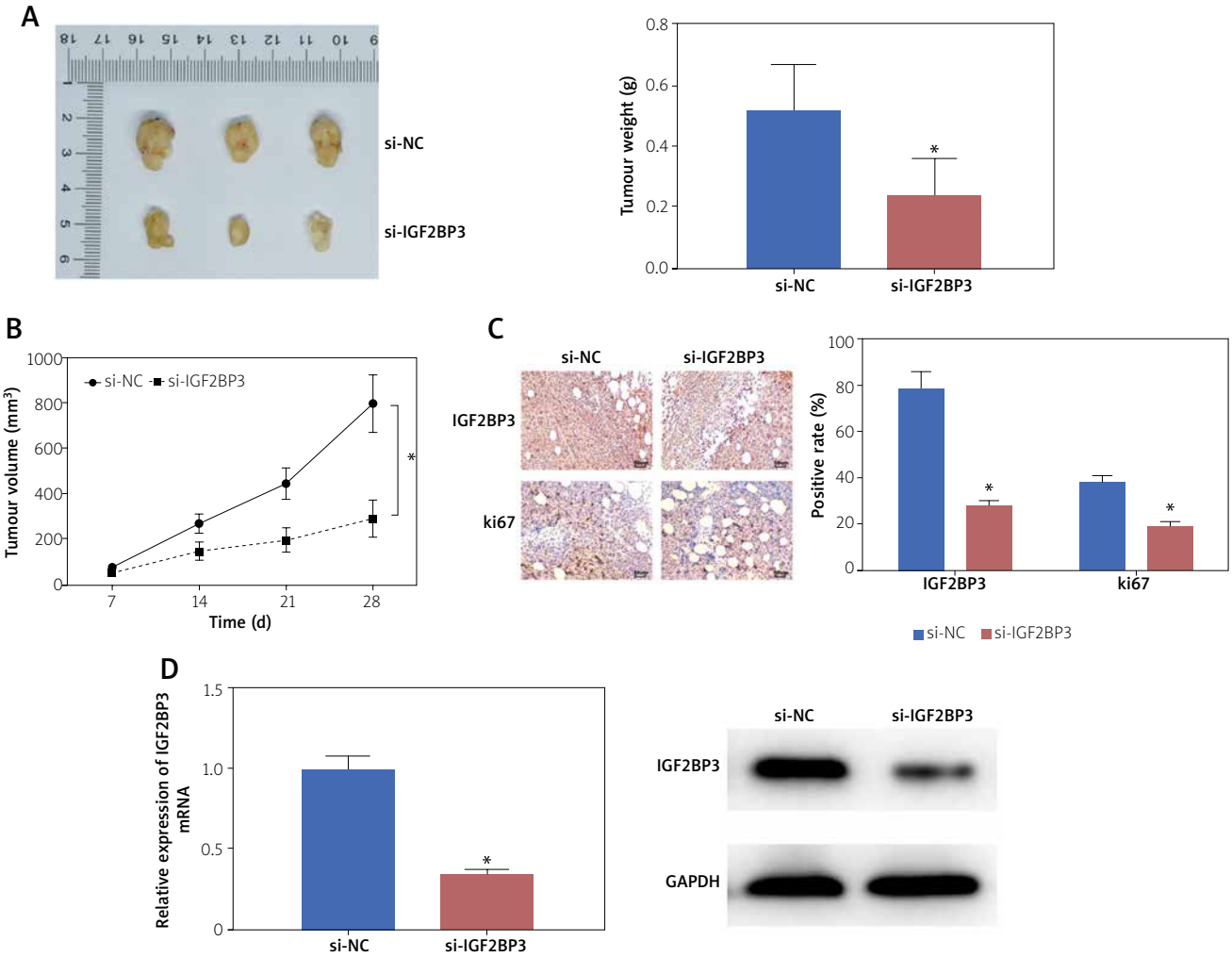

Fig. 7. Silencing IGF2BP3 inhibits tumour growth of nude mice glioma *in vivo*. **A**) Xenograft nude mice tumour growth weight in si-NC or si-IGF2BP3 in U87 cells. Mean ± standard deviation (n = 6/group). **B**) Tumour volume of glioma in si-NC and si-IGF2BP3 groups. **C**) The positive expression rates of IGF2BP3 and Ki67 in tumour tissue of nude mice after IGF2BP3 silencing (400×). **D**) IGF2BP3 mRNA and protein levels in tumour tissues of nude mice. *$p < 0.05$.

Ethics approval and consent to participate

The study was approved by the ethics committee of the Affiliated Hospital of Zunyi Medical University. The methods were carried out in accordance with the approved guidelines. Written informed consent was obtained prior to the study.

Disclosure

The authors report no conflict of interest.

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