HNF4G accelerates glioma progression by facilitating NRP1 transcription

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Abstract. Hepatocyte nuclear factor 4γ (HNF4G) is considered to be a transcription factor and functions as an oncogene in certain types of human cancer. However, the precise functions and the potential molecular mechanisms of HNF4G in glioma remain unclear. Therefore, the present study aimed to elucidate the role of HNF4G in glioma and the underlying mechanism. Western blotting and reverse transcription-quantitative PCR (RT-qPCR) demonstrated that HNF4G was highly expressed in glioma tissues and cell lines. The overexpression of HNF4G in LN229 and U251 glioma cells promoted cell proliferation and cell cycle progression, and inhibited apoptosis, while the knockdown of HNF4G suppressed cell proliferation, cell cycle progression and tumor growth, and induced apoptosis. A significant positive association was detected between HNF4G and neuropilin-1 (NRP1) mRNA expression in glioma tissues. Bioinformatics analysis, chromatin immunoprecipitation-RT-qPCR and promoter reporter assays confirmed that HNF4G promoted NRP1 transcription in glioma by binding to its promoter. NRP1 overexpression facilitated glioma cell proliferation and cell cycle progression, and suppressed apoptosis *in vitro*, while the knockdown of NRP1 inhibited cell proliferation and cell cycle progression, and facilitated apoptosis. NRP1 overexpression reversed the effects induced by HNF4G knockdown on glioma cell proliferation, cell cycle progression and apoptosis. In summary, the present study demonstrated that HNF4G promotes glioma cell proliferation and suppresses apoptosis by activating NRP1 transcription. These findings indicate that HNF4G acts as an oncogene in glioma and may thus be an effective therapeutic target for glioma.

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Introduction

Glioma is the most common and lethal intracranial tumor, posing a severe threat to human health and life (1); it is characterized by high morbidity and relapse rates, and a poor survival rate. The annual morbidity rate of cerebral glioma is ~6.1 per 100,000 individuals (2). Depending on its origin, glioma can be classified into astrocytoma or oligodendroglioma. Glioma is divided into grades I-IV by the World Health Organization (WHO); the median survival rates of patients with grades III and IV glioma are 2 and 1 year, respectively (3). Despite numerous advances being made in surgical excision, chemotherapy, radiotherapy, targeted therapy and gene therapy, the currently available therapeutic methods are not sufficient to completely eliminate the glioma, and the therapeutic effect remains unsatisfactory. Therefore, a more in-depth understanding of the precise mechanisms responsible for the development of gliomas is urgently required, which may be beneficial for the identification of novel therapeutic targets.

Hepatocyte nuclear factor 4 (HNF4), which includes HNF4A and HNF4G isoforms, belongs to the nuclear hormone receptor superfamily (4). HNF4G is considered to be a transcription factor. HNF4G is detected in the human kidneys, stomach, pancreas, lungs, bladder and testicles (5). Previous studies have demonstrated that HNF4G is associated with glucose metabolism and hyperuricemia (6,7). In addition, several studies have observed that HNF4G functions as an oncogene, regulating cell growth, apoptosis and invasion in certain types of human cancer, including pancreatic, prostate, bladder, liver and lung cancer (8-11). However, the function of HNF4G in several other types of tumors, including glioma, has not yet been fully elucidated. In particular, the possible molecular mechanisms underlying the role of HNF4G in gliomas remain unclear.

Therefore, the present study measured the expression of HNF4G in patients with glioma and investigated the function and molecular mechanisms of HNF4G in glioma progression. The present study compared HNF4G expression levels in glioma specimens and cell lines with those in adjacent non-tumor tissues and normal cells, respectively. The association of the mRNA expression of HNF4G and the clinicopathological features of patients with glioma was investigated. In addition, the effect of HNF4G on glioma cell proliferation was examined *in vitro* and *in vivo*. Furthermore, molecular mechanistic analyses were conducted to investigate whether HNF4G affected glioma cell proliferation and tumor growth via neuropilin-1 (NRP1).

Materials and methods

Human glioma samples. Human glioma specimens and adjacent non-tumor tissues were obtained during glioma surgery in 59 patients (38 men and 21 women; 33 cases ≥50 years old, 26 cases <50 years old; mean age, 52 years old) between April 2019 and October 2020 at the Department of Pathology, Xi'an Gaoxin Hospital (Xi'an, China). Written informed consent was obtained from each patient. The patients had not been treated with radiotherapy, chemotherapy or other treatment prior to the surgery. Three small sections from each tissue were promptly frozen and stored at -80˚C for use in the following experiments. The patient samples were divided into high and low expression groups based on the median gene expression levels (12). The present study was approved by the Ethics Committee of Xi'an Gaoxin Hospital (Xi'an, China; approval no. GXYY-XA-H-2022-068).

Cells and cell culture. The human glioma U87, LN229 and U251 cell lines were purchased from Procell Life Science &Technology Co., Ltd. and immortalized normal human astrocytes (NHAs) were purchased from Shanghai BinsuiBio Co., Ltd. The U87 cell line is not the original glioblastoma cell line established in 1968 at the University of Uppsala; it is the U87 MG ATCC version (CVCL_UE09), which is most probably a glioblastoma, but whose origin is unknown. The cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2.

Animals. A total of 4 male BALB/c nude mice (5 weeks old; 29.1±1.7 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., and fed under pathogen-free conditions. Mice were maintained at a temperature of 26˚C, with 50% relative humidity, ventilation 13 times/h and a 10-h light and 14-h dark cycle/day. The food was autoclaved and the drinking water was sterile water with a mixture of vitamins, and both were available ad libitum. The Institutional Animal Care and Use Committee of Xi’an Gaoxin Hospital approved the animal experiments (approval no. GXYY-XA-A-2022-039).

Plasmid construction and transfection. Full-length DNA sequences of HNF4G or NRP1 were incorporated into the pCMV2-GV146 plasmid (Genechem Co. Ltd.), respectively. A reporter plasmid (pGL3-NRP1-luc; Beijing AuGCT DNA-SYN Biotechnology) was also constructed, which included the 490-bp DNA fragment 33484619-33485108 relative to the NRP1 promoter, which was located upstream of the firefly luciferase reporter gene in pGL3-luc. The pGL3-luc and pGL3-NRP1-luc plasmids were transfected into LN229 and U251 glioma cells at 37˚C for 48 h using Jet Prime (Polyplus-transfection SA).

Transfection with small interfering RNA (siRNA). siRNAs were purchased from Shanghai GenePharma Co., Ltd., for the knockdown of HNF4G and NRP1 gene expression. Human HNF4G siRNA-1 (sense, 5'-CGGCACUACAUAAAUGUGATT-3' and antisense, 5'-UCACAUUUAUGUAGUGCCGTT-3'), HNF4G siRNA-2 (sense, 5'-CGAGUGAGAGAAACACAUUTT-3' and antisense, 5'-AAUGUGUUUCUCUCACUCGTT-3'), NRP1 siRNA-1 (sense, 5'-GGUUUCUCAGCAAACUACATT-3' and antisense, 5'-UGUAGUUUGCUGAGAAACCTT-3'), NRP1 siRNA-2 (sense, 5'-CUGGCAUAUCUAUGAGAUUTT-3' and antisense, 5'-AUCUCAUAGAUAUGCCAGTT-3') and negative control siRNA (NC-siRNA sense, 5'-UUCACCGACUUUGUCACGUTT-3' and antisense, 5'-ACGUGACAAAGUCGGUGAATT-3') were transiently transfected into LN229 and U251 cells at the concentration of 50 nM at 37˚C for 24, 48 or 72 h using Jet Prime (Polyplus-transfection SA).

Cell proliferation assay. The LN229 and U251 cells were independently suspended at 20,000 cells/ml in DMEM with 10% fetal calf serum and seeded in 96-well plates (200 μl/well). These cells were transiently transfected with the control vector, HNF4G overexpression vector, NRP1 overexpression vector, NC-siRNA (80 nM), HNF4G siRNA-1, HNF4G siRNA-2, NRP1 siRNA-1, NRP1 siRNA-2, HNF4G siRNA-2 + control vector, or HNF4G siRNA-2 + NRP1 overexpression vector for 24, 48 or 72 h. Cell growth was measured using an MTT assay (MilliporeSigma). MTT diluent (20 μl) was added to each well followed by culturing for 4 h. Dimethylsulfoxide (150 μl) was then added to dissolve the formazan salt. The optical density at 492 nm was examined using a microplate reader (BMG Labtech GmbH).

Cell cycle assay. The LN229 and U251 glioma cells were harvested at 24 h following transfection. The cells were washed using PBS and immobilized with 70% ethyl alcohol at 4˚C. The cells were then washed with PBS and prepared as a single cell suspension. Propidium iodide (PI; 0.05 mg/ml; MilliporeSigma) containing RNase A (0.1 mg/ml) was added to each group of cells followed by incubation at room temperature for 10 min for staining. Cell cycle analysis was performed by a flow cytometer (EPICS XL; Beckman Coulter, Inc.) using SYSTEM II Software (version 3.0; Beckman Coulter, Inc.).

Apoptosis assay. At 48 h post-transfection, the LN229 and U251 cells were collected. The cells were washed twice with PBS. The cells were then prepared as single cell suspensions with binding buffer and stained using an Annexin-V-FITC/PI Apoptosis Detection kit (Abcam). The number of apoptotic cells was detected and quantified by a flow cytometer (EPICS XL; Beckman Coulter, Inc.) using SYSTEM II Software (version 3.0; Beckman Coulter, Inc.).

Lentiviral construction. HNF4G short hairpin RNA (shRNA) was incorporated into a lentiviral vector (GV118; Shanghai GeneChem Co., Ltd.) for the silencing of HNF4G expression. The sequences were as follows: Negative control (sh-Ctrl), 5'-AAAAGAGGCTTGCACAGTGCATTCAAGACGTGC

ACTGTGCAAGCCTCTTTT-3'; and HNF4G shRNA, 5'-TCGAGTGAGAGAAACACATTTCTCGAGAATGTGTTTCTCTCACTCGTTTTTC-3'. The U251 cells were cultured in 12-well plates. The solution of lentiviral vector (0.5 ml $4x10^8$ TU/ml) was then used to infect the U251 cells with Polybrene 5 μg/ml (Shanghai GeneChem Co., Ltd.) for 10 h at 37˚C, after which the culture medium was replaced with DMEM containing 10% fetal calf serum. At 2 days post-infection, puromycin (cat. no. P9620; 25 μg/ml; MilliporeSigma) was added in infected cells and the culture was continued for 6 days for the tumor transplantation experiment.

Tumorigenicity assay. Following infection with sh-Ctrl or HNF4G shRNA, the U251 cells were prepared as a single-cell suspension in DMEM. Tumorigenicity was detected using 6-week-old BALB/c nude mice (n=4/group). The infected U251 cells ($2x10^6$) were resuspended in 100 μl DMEM and injected subcutaneously into the posterior flanks of the mice. The transplanted tumors were measured using a Vernier caliper every third day. The length (L) and width (W) of the tumors were used to calculate the tumor volume (V) using the following formula: $V=(L \times W^2)/2$. The nude mice were euthanized by anesthesia with 3% isoflurane followed by the injection of pentobarbital sodium (150 mg/kg) 31 days after the injection of the cells. The cessation of breathing and heartbeat were considered as confirmation of death. The tumors were then isolated and weighed, after which the tumor tissues were frozen for use in further assays.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the glioma samples, mouse tumor tissues and glioma cells using an RNA Extraction Kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The RNA was reversed transcribed into cDNA using the PrimeScript™ II 1st strand cDNA kit (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was then performed using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.). Reaction conditions were as follows: Initial denaturation at 95˚C for 5 min, followed by denaturation at 95˚C for 10 sec, annealing at 55˚C for 25 sec and extension at 72˚C for 10 sec, for 45 cycles. The primer sequences used were as follows: HNF4G forward, 5'-ACAGAATAAGCACCAGAAG-3' and reverse, 5'-TCACAGACATCACCAATAC-3'; NRP1 forward, 5'-CGTGGAAGTCTTCGATGGAG-3' and reverse, 5'-AAGAAATGGCCCTGAAGACA-3'; and GAPDH forward, 5'-GCCGTATCGCTCAGACAC-3' and reverse, 5'-GCCTAATACGACCAAATCC-3'. The qPCR was performed using an IQ™5 Multicolor qRT-PCR Detection System (Bio-Rad Laboratories, Inc.). The $2^{-\Delta\Delta Cq}$ method (13) was used to analyze the expression of the target genes using GAPDH as the reference gene.

Western blot analysis. Protein was extracted from the glioma samples, mouse tumor tissue and glioma cells using RIPA buffer (MilliporeSigma) with protease inhibitors. Protein quantification was performed with a BCA kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Equal amounts of protein samples (30 μg) were subjected to sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis followed by transfer onto nitrocellulose membranes. After blocking the membranes with skimmed milk (5%) at room temperature for 2 h, primary antibodies were added followed by incubation at 4˚C for 10 h. The primary antibodies comprised HNF4G antibody (cat. no. HPA005438; 1:1,000; MilliporeSigma), NRP1 antibody (cat. no. sc-5307; 1:1,000; Santa Cruz Biotechnology, Inc.) and GAPDH antibody (cat. no. sc-47724; 1:1,000; Santa Cruz Biotechnology, Inc.). The appropriate anti-rabbit (cat. no. sc-2357) or anti-mouse (cat. no. sc-2005) horseradish peroxidase-linked secondary antibody (both 1:2,000; Santa Cruz Biotechnology, Inc.) was then added followed by incubation at room temperature for 4 h. The membranes were finally incubated with enhanced chemiluminescence reagent (GE Healthcare; Cytiva) for chemiluminescence detection. The protein bands were scanned using Syngene G:BOX Chemi XX6 system (Syngene) and quantified using GeneTools software (version 3.06.02; Syngene). GAPDH was used to normalize the protein expression data.

Chromatin immunoprecipitation (ChIP)-RT-qPCR. After crosslinking the LN229 and U251 cells with formaldehyde (1%) at room temperature for 18 min, glycine was added for quenching. The cells were resuspended in SDS lysis buffer [50 mM Tris-HCl (pH 8.1), 10 mM EDTA and 1% SDS with freshly added PIC at 1 μl PIC/800 μl total volume]. The LN229 and U251 cells were then sonicated using an ultrasonic processor and nuclear lysates were extracted, which contained chromatin that had been broken into ~200-bp DNA fragments. HNF4G or IgG (cat. nos. HPA005438 and I8765; both 1:100; MilliporeSigma) antibodies were incubated with the extracted DNA fragments for 13 h at 4˚C. Dynabeads Protein A (200 μl; Thermo Fisher Scientific, Inc.) was added in 500 μl lysis buffer and agitated in a shaker for 2 h. The beads were centrifuged at 12,000 x g for 1 min at room temperature. The supernatant was removed and transfered to a new 1.5-ml microfuge tube. The DNA-protein complexes were washed in TE buffer at 65˚C. Subsequently, the crosslinking was reversed for 8 h at 65˚C. The QIAquick® PCR purification Kit (Qiagen GmbH) was used to extract the binding DNA fragments. The predicted binding NRP1 DNA fragments (UCSC Genome Browser; Human Feb. 2009, GRCh37/hg19) (14) were verified using RT-qPCR according to the aforementioned protocol. The primer sequences used were as follows: Primer 1 forward, 5'-GCATTGACTTAGCCAGAAGGTGACA-3' and reverse, 5'-GCTTTCCTGTTTCTCCATTGTCTGA-3'; primer 2 forward, 5'-ATGACTCAGACAATGGAGAAACAGG-3' and reverse, 5'-ACAAGTTCAATCCAAACCACGCGGG-3'; primer 3 forward, 5'-GTAGACCCGCGTGGTTTGGATTGAA-3' and reverse, 5'-GCCAGTCGGTCCTGTCACAGAGTCT-3'; primer 4 forward, 5'-CAGTAGACTCTGTGACAGGACCGAC-3' and reverse, 5'-ATTGTCAGAGCAGGAGCGGTTTTGT-3'; and primer 5 forward, 5'-TAACAAAACCGCTCCTGCTCTGACA-3' and reverse, 5'-GTTAAAAAAAATAAAAGTGAACAAC-3'. The target areas of the primers are shown in Fig. S1.

Luciferase reporter gene assay. The LN229 and U251 cells were plated into 96-well plates. Each group was established in six parallel wells. The LN229 and U251 cells were transfected with pGL3-luc or pGL3-NRP1-luc, and co-transfected

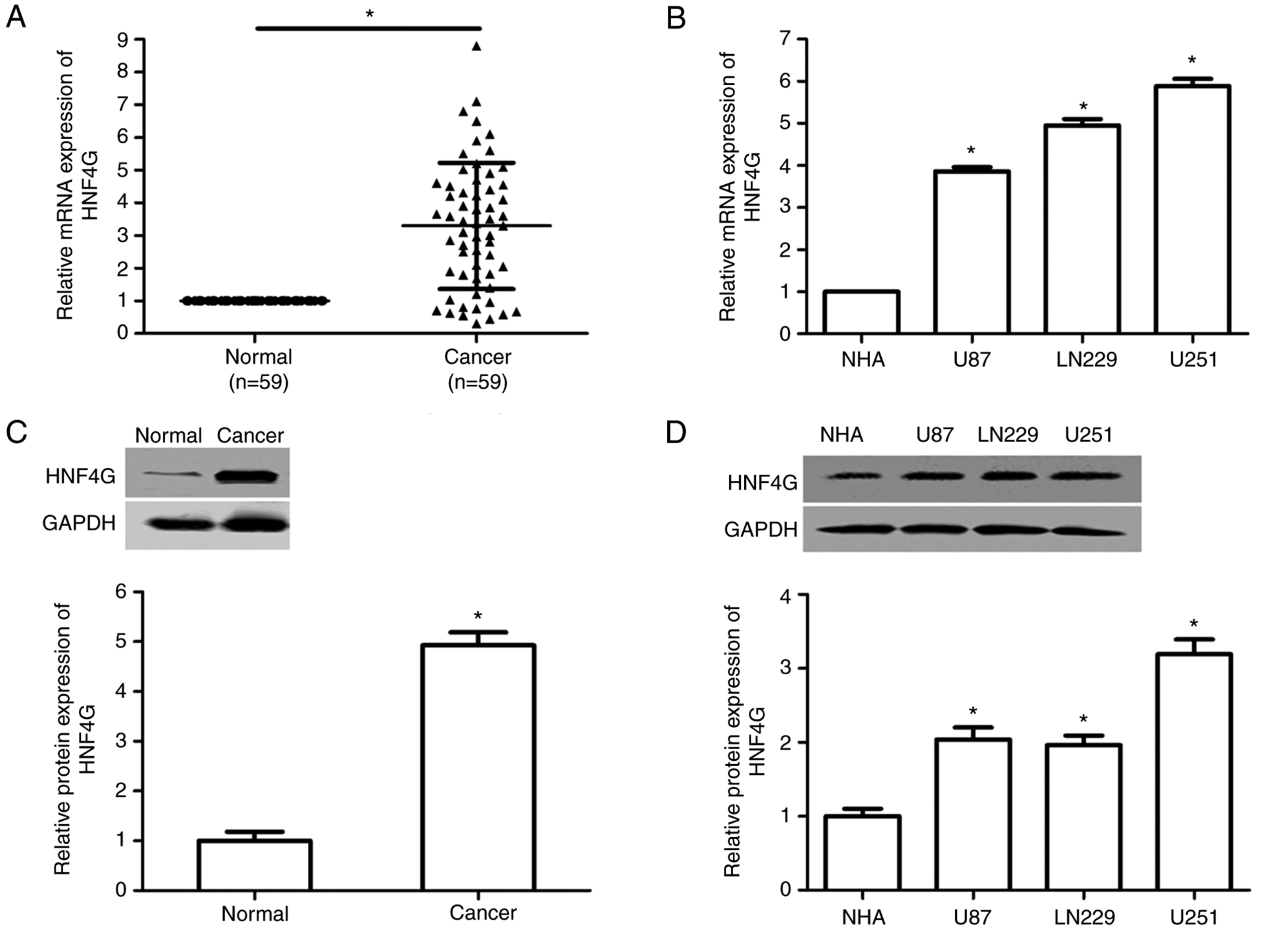

Figure 1. HNF4G expression is increased in glioma specimens and cell lines. HNF4G mRNA expression was significantly upregulated in (A) glioma samples compared with normal tissues and in (B) glioma cell lines compared with NHAs. HNF4G protein expression was upregulated in (C) glioma samples compared with normal tissues and in (D) glioma cell lines compared with NHAs. *P<0.001 vs. respective control. HNF4G, hepatocyte nuclear factor 4γ; NHA, normal human astrocyte.

with pGL3-NRP1-luc and HNF4G siRNAs or HNF4G overexpression vector at 37˚C for 2 days. The luciferase activity was detected using a Dual-Luciferase Reporter Assay System (Promega Corporation). Luciferase activity was normalized to Renilla luciferase activity.

The cancer genome atlas (TCGA) data analysis. To verify that HNF4G regulates NRP1, microarray data were collected from the public database, TCGA (http://gepia.cancer-pku.cn/detail.php?gene=HNF4G), and the correlation between HNF4G and NRP1 expression was analyzed by using simple linear regression.

Statistical analysis. All statistical data were analyzed using SPSS 25.0 software (IBM Corp.). All experiments were performed with at least three independent assays. Data are presented as the mean ± standard deviation. Unpaired Student's t-test or one-way ANOVA was performed to analyze the differences between two or multiple independent groups, respectively. The ANOVA test followed by the Bonferroni post hoc test was used for pairwise comparisons. Paired Student's t-test was used to analyze differences in HNF4G and NRP1 mRNA levels between glioma tissue and normal tissue. Fisher's exact test was used to analyze the association between HNF4G mRNA expression and clinicopathological features. Pearson's correlation analysis was conducted to analyze the correlation between HNF4G and NRP1 expression. P<0.05 was considered to indicate a statistically significant difference.

Results

HNF4G expression is upregulated in human glioma samples and cell lines. To explore the role of HNF4G in glioma, the expression of HNF4G in glioma specimens and cell lines was evaluated using RT-qPCR and western blot analysis. The results revealed that the mRNA and protein expression levels of HNF4G were significantly upregulated in human glioma samples compared with those in adjacent normal tissues (P<0.001; Fig. 1A and C). Moreover, the high mRNA expression of HNF4G was associated with the WHO pathological grade, isocitrate dehydrogenase [NADP(+)] 1 (IDH1) mutation, tumor size and the Karnofsky Performance Scale (KPS) score (all P<0.001; Table I). The HNF4G mRNA and protein expression levels were also significantly higher in the U87, LN229 and U251 human glioma cell lines than in the NHAs (P<0.001; Fig. 1B and D).

Table I. Association between HNF4G mRNA expression and clinicopathological features in patients with glioma.

Characteristics	Patients (n)	HNF4G mRNA expression High (n=49)	HNF4G mRNA expression Low (n=10)	[a]P-value
Sex				0.907
Male	38	31	7	
Female	21	18	3	
Age, years				0.839
≥50	33	27	6	
<50	26	22	4	
WHO grade				<0.001
I + II	20	12	8	
III + IV	39	37	2	
IDH1				<0.001
Mutation	27 (Nod, 13; Cod, 16)	25	2	
Wild-type	32 (Nod, 28; Cod, 6)	24	8	
1p/19q				0.065
No deletion	38	31	7	
Codeletion	21	18	3	
Tumor size, cm				<0.001
≥5	35	32	3	
<5	24	17	7	
KPS score				<0.001
<80	23	17	6	
≥80	36	32	4	

[a]P-values calculated by Fisher's exact test. HNF4G, hepatocyte nuclear factor 4γ; WHO, World Health Organization; IDH1, isocitrate dehydrogenase [NADP(+)] 1; Nod, no deletion; Cod, codeletion; KPS, Karnofsky Performance Scale.

HNF4G promotes glioma cell proliferation in vitro and in vivo. To investigate the biological function of HNF4G in human glioma, LN229 and U251 cells were stably transfected with HNF4G overexpression plasmid, control empty plasmid, HNF4G siRNAs or NC-siRNA, independently. The HNF4G overexpression plasmid significantly upregulated the mRNA and protein expression levels of HNF4G in the LN229 and U251 cells; by contrast, the HNF4G siRNAs significantly downregulated the mRNA and protein expression levels of HNF4G (P<0.001; Fig. 2A and B). The results of MTT assays revealed that HNF4G overexpression significantly promoted glioma cell proliferation (P<0.001; Fig. 2C) after 48 and 72 h, whereas the silencing of HNF4G significantly suppressed glioma cell proliferation at these time points (P<0.001; Fig. 2D). Cell cycle analysis revealed that HNF4G overexpression led to a significant reduction in the number of cells in the G_0/G_1 phase and significant increases in the number of cells in the S and G_2/M phases (P<0.001; Figs. 2E and S2A); by contrast, the silencing of HNF4G resulted in the significant accumulation of cells in the G_0/G_1 phase and a corresponding reduction in the number of cells in the S and G_2/M phases (P<0.001; Figs. 2F and S2B). Moreover, analysis of cell apoptosis using flow cytometry revealed that HNF4G overexpression reduced the rates of early and late apoptosis (Figs. 2G and S2C), whereas the silencing of HNF4G enhanced the rate of early- and late-apoptotic cells to a significant extent (P<0.001; Figs. 2H and S2D). Subsequently, an HNF4G shRNA lentiviral vector was constructed and stably transfected U251 gliomas were generated. Nude mice were subcutaneously injected in the posterior flanks with HNF4G shRNA-infected or sh-Ctrl-infected U251 cells and tumor growth was monitored for 31 days. Observation of the excised tumors revealed that HNF4G shRNA markedly inhibited tumor growth compared with that in the sh-Ctrl group, in which the largest tumor diameter was 9 mm (Fig. 2I). The volumes and weights of the tumors derived from HNF4G shRNA-transfected cells were significantly lower than those derived from sh-Ctrl-transfected cells (P<0.001; Fig. 2J and K). The results also revealed that HNF4G shRNA significantly suppressed the mRNA and protein expression levels of HNF4G in the xenograft tumors (P<0.001; Fig. 2L and M). These results suggest that HNF4G facilitated glioma cell proliferation and tumor growth, and suppressed cell apoptosis.

HNF4G activates NRP1 transcription by binding to its promoter. To determine the mechanisms underlying the effect of HNF4G in the modulation of glioma development, the UCSC Genome Browser was used to predict and select the downstream target gene of HNF4G. The results indicated that HNF4G binds to the promoter region of the NRP1 gene

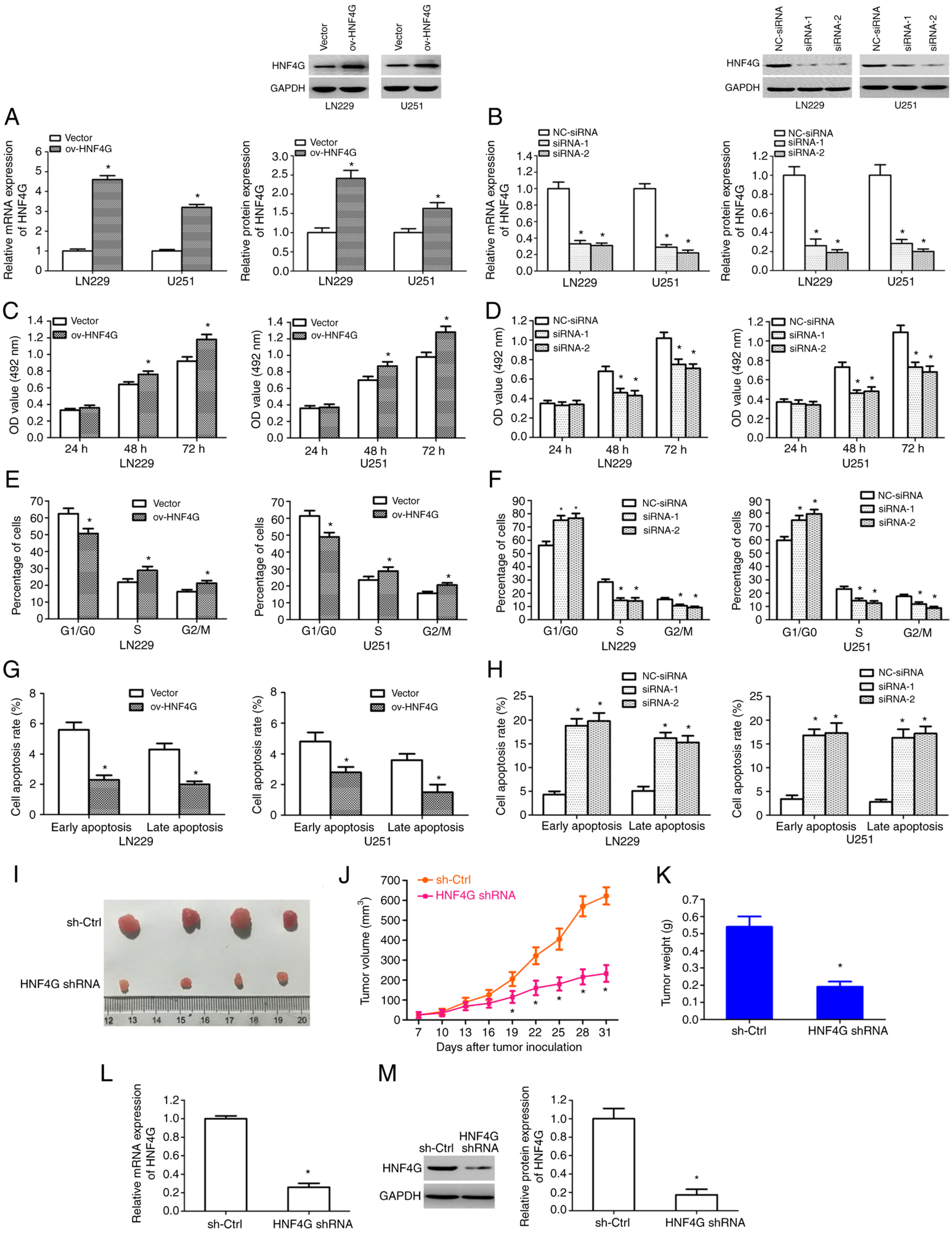

Figure 2. HNF4G promotes glioma cell growth *in vitro* and *in vivo*. HNF4G mRNA and protein expression in glioma cells transfected with (A) HNF4G overexpression vector and (B) HNF4G siRNAs. MTT assays revealed (C) increased glioma cell proliferation following transfection with HNF4G overexpression vector and (D) reduced cell growth following transfection with HNF4G siRNA. Flow cytometry was used for examination of the cell cycle following transfection with (E) HNF4G overexpression vector and (F) HNF4G siRNA. Cell apoptosis was examined using flow cytometry following (G) transfection with HNF4G overexpression vector and (H) HNF4G siRNA. (I) Morphology of tumors following resection from nude mice. (J) Growth curve of tumor volume. (K) The weight of tumors was measured following excision. (L) HNF4G mRNA and (M) HNF4G protein expression in xenograft tumors. *P<0.001 vs. respective control. HNF4G, hepatocyte nuclear factor 4γ; ov, overexpression; siRNA, small interfering RNA; NC, negative control; sh-Ctrl, short hairpin control; shRNA, short hairpin RNA; OD, optical density.

(Fig. 3A). ChIP-RT-qPCR further confirmed that HNF4G bound to the promoter regions of NRP1 in LN229 and U251 cells, and primarily bound to the segments of primers 3-5 (P<0.001; Fig. 3B). Promoter reporter assays were used to verify whether HNF4G regulates NRP1 transcription by binding to its promoter. The binding sequence of NRP1 was inserted downstream of the luciferase gene. The LN22 and U251 cells were transfected with the constructed plasmids for 2 days and the luciferase activity was detected. The results revealed that the luciferase activity in the cells transfected with pGL3-NRP1-luc was significantly higher than that in the cells transfected with pGL3-luc (P<0.001; Fig. 3C). When the glioma cells were co-transfected with pGL3-NRP1-luc and the HNF4G overexpression vector, the luciferase activity was significantly enhanced compared with that in the control vector group (P<0.001; Fig. 3D). A significant reduction in luciferase activity was observed in the cells co-transfected with pGL3-NRP1-luc and HNF4G siRNA compared with that in the cells co-transfected with pGL3-NRP1-luc and NC-siRNA (P<0.001; Fig. 3E).

The analysis of patient tissues showed that the mRNA expression of NRP1 was also significantly upregulated in glioma samples compared with that in the adjacent normal tissues (P<0.001; Fig. 3F). In addition, a significant positive association between HNF4G and NRP1 mRNA expression was detected in the glioma tissues (P<0.001; Fig. 3G). TCGA data also revealed that HNF4G expression was positively associated with NRP1 expression in glioma (P<0.001; Fig. 3H). HNF4G overexpression significantly upregulated the mRNA and protein expression levels of NRP1 in LN229 and U251 cells, while HNF4G siRNA significantly downregulated them (P<0.001; Fig. 3I, K and L). Furthermore, HNF4G shRNA significantly suppressed the mRNA and protein expression levels of NRP1 in xenograft tumors (P<0.001; Fig. 3J and M). Together, these data demonstrate that HNF4G promoted NRP1 transcription by binding to its promoter in glioma cells.

NRP1 promotes glioma cell proliferation. To explore the role of NRP1 in human glioma, the LN229 and U251 cells were stably transfected with NRP1 overexpression plasmid, control empty plasmid, NRP1 siRNAs or NC-siRNA, independently. NRP1 overexpression significantly increased the mRNA and protein expression levels of NRP1 in the cells, whereas the knockdown of NRP1 significantly suppressed the mRNA and protein expression levels of NRP1 (P<0.001; Fig. 4A and B). The results of MTT assays revealed that NRP1 overexpression markedly promoted glioma cell proliferation after 48 and 72 h (P<0.001; Fig. 4C), while the knockdown of NRP1 substantially inhibited glioma cell growth at these time points (P<0.001; Fig. 4D). Flow cytometry revealed that NRP1 overexpression induced significant changes in the cell cycle, resulting in a marked significant reduction in the proportion of cells in the G_0/G_1 phase and an increase in the proportion of cells in the S and G_2/M phases (P<0.001; Figs. 4E and S3A). However, the knockdown of NRP1 led to the significant accumulation of cells in the G_0/G_1 phase and a reduction in the proportion of cells in the S and G_2/M phases (P<0.001; Figs. 4F and S3B). In addition, flow cytometry revealed that NRP1 overexpression significantly decreased the rates of early and late apoptosis (P<0.001; Figs. 4G and S3C), whereas the knockdown of NRP1 significantly increased the rates of early and late apoptosis (P<0.001; Figs. 4H and S3D). These results demonstrate that NRP1 had oncogenic effects.

HNF4G facilitates glioma cell growth by upregulating NRP1 expression. To validate the previous findings which indicated that HNF4G enhances glioma cell proliferation by modulating NRP1 transcription, HNF4G siRNA-2 and NRP1 overexpression vector were co-transfected into LN22 and U251 cells. The knockdown of HNF4G decreased the NRP1 mRNA and protein expression levels in LN229 and U251 cells, whereas co-transfection with the NRP1 overexpression vector reversed this effect (P<0.001; Fig. 5A and B). Furthermore, MTT assay results revealed that NRP1 overexpression reversed the effects of HNF4G knockdown on cell proliferation (P<0.001; Fig. 5C). Cell cycle analysis confirmed that HNF4G knockdown led to a significant accumulation of cells in the G_0/G_1 phase and a reduction in the proportion of cells in the S and G_2/M phase, while co-transfection with NRP1 overexpression vector counteracted the effects of HNF4G knockdown on the cell cycle (P<0.001; Figs. 5D and S4A). Furthermore, apoptosis analysis showed that while the knockdown of HNF4G significantly increased the proportions of early- and late-apoptotic cells, co-transfection with NRP1 overexpression vector eliminated the effects of HNF4G siRNA-2 on apoptosis (P<0.001; Figs. 5E and S4B). These findings confirm that HNF4G promoted glioma cell proliferation and suppressed cell apoptosis by promoting NRP1 expression.

Discussion

As a major transcription factor, HNF4G is frequently upregulated in several types of cancer and functions as a pivotal oncogene (8,11). Previous studies have demonstrated that HNF4G facilitates the proliferation and invasion of bladder cancer cells (10,15). Wang *et al* (8) found that the overexpression of HNF4G promoted the progression and metastasis of pancreatic ductal adenocarcinoma. In addition, Shukla *et al* (9) observed that the HNF4G/HNF1A transcription loop accelerated prostate cancer cell proliferation and oncogenesis. Furthermore, Wang *et al* (11) demonstrated that the expression of HNF4G was significantly upregulated in lung cancer tissues, and that the overexpression of HNF4G facilitated lung cancer cell proliferation and tumorigenesis. In the present study, it was observed that the expression of HNF4G was upregulated in human glioma tissues and cell lines. The high mRNA expression of HNF4G was associated with the WHO pathological grade, IDH1 mutation, tumor size and KPS score. HNF4G promoted glioma cell proliferation *in vitro*, as well as cell cycle G_1-S phase transition and tumor growth. Moreover, experiments in which HNF4G was overexpressed or knocked down revealed that HNF4G markedly suppressed apoptosis. The previous studies found that HNF4G facilitated the proliferation, invasion and metastasis in certain cancer types, and the present study revealed similar findings for glioma; specifically, HNF4G promoted glioma cell proliferation and cell cycle transition, and suppressed apoptosis. However, metastasis was not evaluated. These findings indicate that HNF4G functions as an oncogene in human glioma, and thus has potential for use as a novel therapeutic target for glioma.

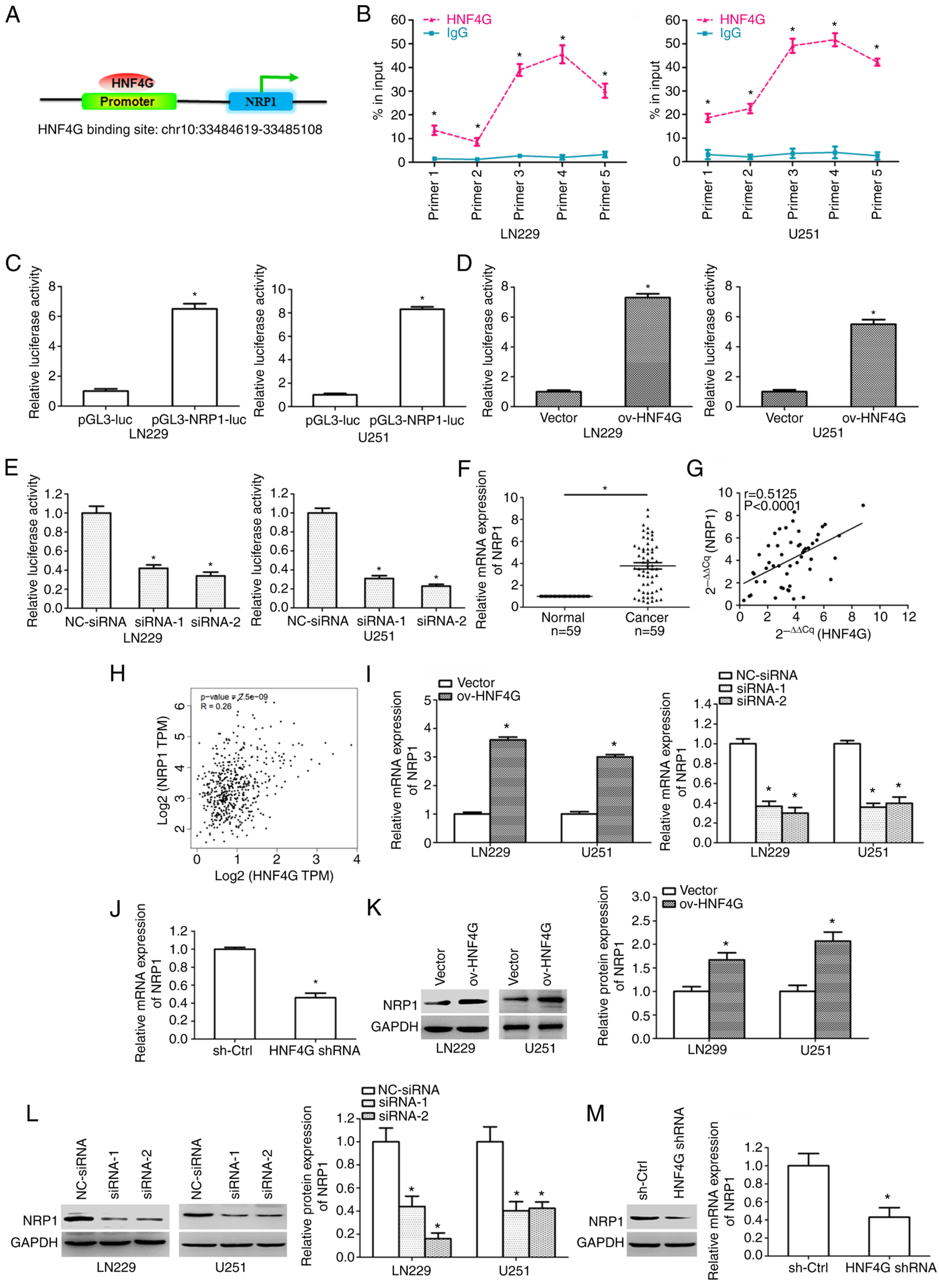

Figure 3. HNF4G promotes NRP1 transcription by binding its promoter region in glioma cells. (A) The HNF4G binding site in the NRP1 promoter was analyzed using the UCSC Genome Browser. (B) Chromatin immunoprecipitation-reverse transcription-quantitative PCR confirmed that HNF4G bound to the promoter region of NRP1 in LN229 and U251 cells. (C) Luciferase activity in LN229 and U251 cells 2 days after transfection with pGL3-NRP1-luc. Luciferase activity in LN229 and U251 cells after co-transfection with (D) pGL3-NRP1-luc and HNF4G overexpression vector, and (E) pGL3-NRP1-luc and HNF4G siRNA. (F) NRP1 mRNA expression was significantly upregulated in glioma samples compared with that in normal tissues. (G) A notable positive association was detected between HNF4G and NRP1 mRNA expression in glioma tissues. (H) The Cancer Genome Atlas data revealed that HNF4G expression was positively associated with NRP1 expression in glioma. (I) NRP1 mRNA expression in glioma cells transfected with HNF4G overexpression vector or siRNA. (J) NRP1 mRNA expression in xenograft tumors. NRP1 protein expression in glioma cells transfected with (K) HNF4G overexpression vector and (L) HNF4G siRNA. (M) NRP1 protein expression in xenograft tumors. *$P<0.001$ vs. respective control, n=3. HNF4G, hepatocyte nuclear factor 4γ; NRP1, neuropilin-1; luc, luciferase; ov, overexpression; siRNA, small interfering RNA; NC, negative control; sh-Ctrl, short hairpin control; shRNA, short hairpin RNA.

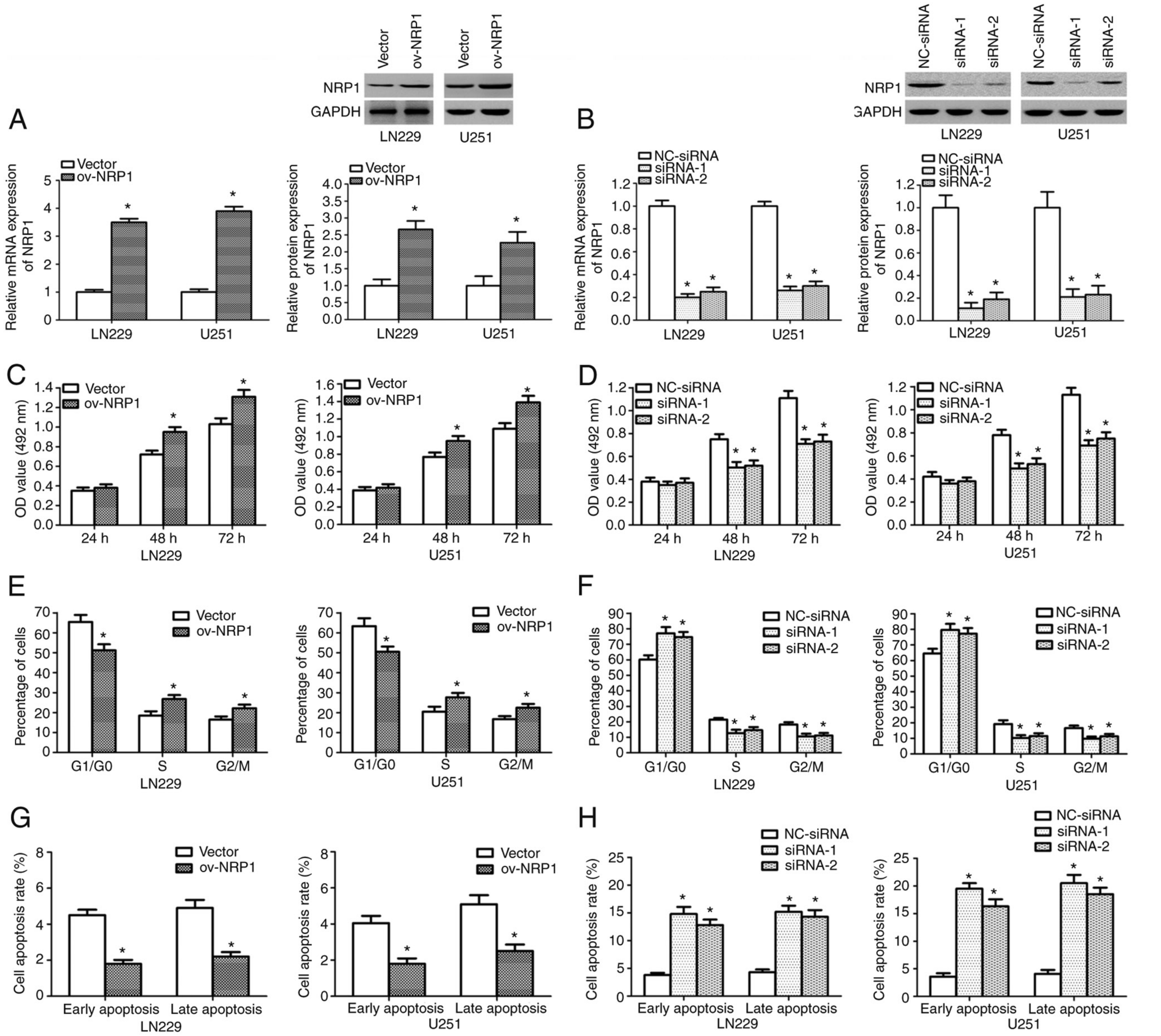

Figure 4. NRP1 promotes glioma cell proliferation *in vitro*. (A) NRP1 mRNA and protein expression in glioma cells following transfection with NRP1 overexpression vector. (B) NRP1 mRNA and protein expression in glioma cells transfected with NRP1 siRNA. MTT assays revealed (C) increased glioma cell proliferation following transfection with NRP1 overexpression vector and (D) a reduction in glioma cell proliferation following transfection with NRP1 siRNA. Flow cytometry was performed to examine the cell cycle following transfection with (E) NRP1 overexpression vector and (F) NRP1 siRNA. Cell apoptosis was detected using flow cytometry following transfection with (G) NRP1 overexpression vector and (H) NRP1 siRNA. *P<0.001 vs. respective control. NRP1, neuropilin-1; ov, overexpression; siRNA, small interfering RNA; NC, negative control; OD, optical density.

HNF4G promotes bladder cancer progression by facilitating the transcription of hyaluronan synthase 2 (10). It has been reported that HNF4G and HNF1A activate enhancer chromatin and upregulate the expression of gastrointestinal transcriptome genes (9,16). In the present study, to investigate the potential molecular mechanism of HNF4G in glioma, NRP1 was predicted as a downstream target gene of HNF4G by bioinformatics analysis. ChIP-RT-qPCR and promoter reporter assays verified that HNF4G upregulated NRP1 transcription by binding to its promoter. Furthermore, HNF4G expression positively associated with NRP1 expression in glioma. The overexpression and silencing of HNF4G revealed that HNF4G promoted NRP1 expression in glioma. These findings confirm the prediction that HNF4G upregulates NRP1 expression by binding to its promoter region in glioma cells.

NRP1 is a transmembrane glycoprotein and a multi-functional co-receptor for several signaling pathways, such as hepatocyte growth factor (HGF), semaphorins, platelet-derived growth factor and vascular endothelial growth factor (VEGF) (17-19). NRP1 has a pivotal role in embryonic angiogenesis and neurogenesis (20,21). Moreover, NRP1 can regulate cell mitogenesis, migration, motility, proliferation, survival and apoptosis by binding to VEGF and HGF (22-26). There is evidence to indicate that NRP1 is involved in tumorigenesis and progression. For example, studies have shown that the upregulation of NRP1 promotes pancreatic cancer progression by modulating the HGF/c-Met pathway (27), VEGF-A/NRP1 signaling promotes breast cancer metastasis (28), and NRP1 facilitates oral squamous cell carcinoma cell growth and invasion by interacting with

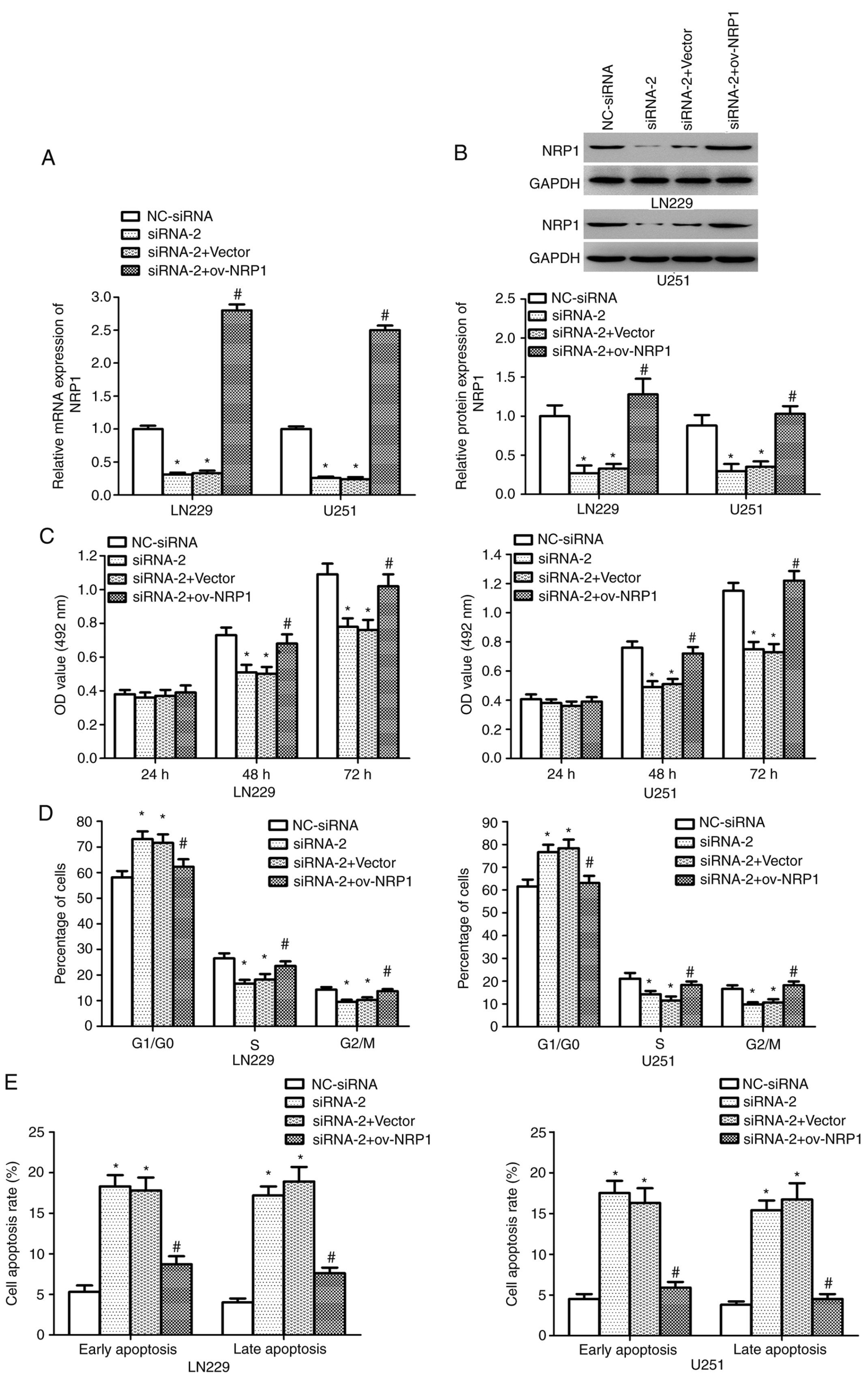

Figure 5. HNF4G promotes glioma cell proliferation by regulating NRP1 expression. (A) Reverse transcription-quantitative PCR was used to examine NRP1 mRNA expression following co-transfection with HNF4G siRNA-2 and NRP1 overexpression vector. (B) Western blot analysis was used to detect NRP1 protein expression following co-transfection with HNF4G siRNA-2 and NRP1 overexpression vector. (C) Following co-transfection with HNF4G siRNA-2 and NRP1 overexpression vector, cell viability was measured in LN229 and U251 cells using MTT assays. (D) The cell cycle was detected following co-transfection with HNF4G siRNA-2 and NRP1 overexpression vector. (E) Cell apoptosis was determined following co-transfection with HNF4G siRNA-2 and NRP1 overexpression vector. *P<0.01, compared with the NC-siRNA group; #P<0.01, compared with the HNF4G siRNA-2 group or HNF4G siRNA-2 + vector group; n=3. HNF4G, hepatocyte nuclear factor 4γ; NRP1, neuropilin-1; ov, overexpression; siRNA, small interfering RNA; NC, negative control; OD, optical density.

CMTM6 (29). In addition, the VEGFR-NRP1 axis has been demonstrated to promote the angiogenesis, growth and metastasis of gastric cancer (30). Furthermore, NRP1 has been shown to facilitate nasopharyngeal carcinoma cell migration and invasion (31). In glioma, previous studies have established that NRP1 promotes glioma cell proliferation, invasion, migration and tumor growth, and suppresses cell apoptosis (32-34). In the present study, it was confirmed that NRP1 promoted glioma cell proliferation and inhibited cell apoptosis. Additionally, NRP1 overexpression reversed the effects induced by HNF4G knockdown on cell proliferation, cell cycle progression and apoptosis. These findings demonstrate that HNF4G promoted glioma cell proliferation and suppressed cell apoptosis by promoting NRP1 transcription.

In conclusion, HNF4G is considered as an oncogene in glioma. In the present study, the results demonstrated that HNF4G expression was upregulated in glioma. HNF4G facilitated cell proliferation and cell cycle progression, and inhibited apoptosis by promoting NRP1 transcription. These results indicate that HNF4G may be an effective therapeutic target for glioma.

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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

HC and LZ designed the experiments. HC, QZ and LZ conducted the experiments. ZL provided research materials and analyzed data. LZ, HC and LZ wrote the manuscript. All authors read and approved the final version of the manuscript. HC and LZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Xi'an Gaoxin Hospital (approval no. GXYY-XA-2022-052). All patients provided written informed consent. The Institutional Animal Care and Use Committee of Xi'an Gaoxin Hospital approved the animal experiments (approval no. GXYY-XA-A-2022-039).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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