EGR1 Modulated LncRNA HNF1A-AS1 Drives Glioblastoma Progression Via miR-22-3p/ENO1 Axis

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Abstract

Background: Accumulating evidences revealed that long noncoding RNAs (lncRNAs) have been participated in cancer malignant progression, including glioblastoma multiforme (GBM). Despite much studies have found the precise biological role in the regulatory mechanisms of GBM however the molecular mechanisms particularly upstream mechanisms still need further elucidated.

Methods: RT-QPCR, cell transfection, western blotting and bioinformatic analysis were executed to detect the expression of EGR1, HNF1A-AS1, miR-22-3p and ENO1 in GBM. Cell proliferation assay, colony formation assay, wound healing, migration and invasion assays were performed to detect the malignant characters of GBM cell. The molecular regulation mechanism was confirmed by luciferase reporter assay, ChIP and RIP. Finally, orthotopic mouse models were established to examine the effect of HNF1A-AS1 in vivo.

Results: In the current study, we analyzed clinical samples to show that the long non-coding antisense transcript of HNF1A, HNF1A-AS1, is upregulated and associated with poor prognosis in GBM. Functional studies revealed that knockdown of HNF1A-AS1 markedly inhibits cell proliferation, migration and invasion both in vitro and in vivo, whereas overexpression of HNF1A-AS1 exerts opposite effect. Mechanistically, the transcription factor EGR1 forced the transcription of HNF1A-AS1 by directly binding the promoter region of HNF1A-AS1. Furthermore, combined bioinformatics analysis with our mechanistic work, using luciferase reporter assays and RIP, we first demonstrated that HNF1A-AS1 functions as a competing endogenous RNA (ceRNA) with miR-22-3p to regulate ENO1 expression in GBM cells. HNF1A-AS1 directly binds to miR-22-3p and significantly inhibits miR-22-3p expression, while ENO1 expression was increased. miR-22-3p inhibitor offsets the HNF1A-AS1 silencing induced suppression in proliferation, migration and invasion of GBM cells, as well as promotion effect on ENO1 expression. ENO1 was verified as a direct target of miR-22-3p and its expression levels was negatively with the prognosis in GBM patients.

Conclusion: Taken together, our study illuminated the definite mechanism of HNF1A-AS1 in promoting GBM malignancy, and provided a novel therapeutic target for further clinical application.

Background

Glioblastoma multiforme (GBM), the most aggressive subtype of glioma in adults, highly malignant and high risk of recurrence, accounting for 47.1% of all malignant brain tumors[1]. According to the World Health Organization (WHO) classification of tumor in the central nervous system (CNS), GBM is classified as a grade IV glioma, with a poor prognosis and a 5-year overall survival rate of<10% [2, 3].Even after multimodal therapies, including maximal surgical resection, adjuvant radiotherapy, temozolomide (TMZ)-based chemotherapy or targeted therapy with rituximab, which have been widely used in patients with GBM, the patients' overall survival is still poor with a median overall survival of 12-15 months from first diagnosis[4]. Therefore, it is extremely vital for us to establish new targeted therapies and to gain a

greater understanding of the molecular mechanisms of GBM carcinogenesis for the identification of new diagnostic and prognosis markers.

LncRNAs comprise a class of transcripts that are >200 nt in length, with none protein-coding potential[5, 6]. The functions and mechanisms of lncRNAs exert their biological role through diverse ways, including chromatin modification, alternative splicing, mRNA stability, encode functional micropeptides, ceRNA molecular sponge to miRNAs, and so on[7-14]. An increasing number of reports have demonstrated that lncRNAs participate in various biological processes, such as cell proliferation, migration, invasion and metastasis, and are dysregulated in various human cancers, including GBM[15-17]. For example, LncRNA miR155HG is overexpressed in GBM, and promotes GBM growth and progression by acting as a ceRNA for the tumor suppressor miR-185 to upregulate ANXA2[18]. LncRNA AC016405.3 suppressed proliferation and metastasis through modulation of TET2 by sponging of miR-19a-5p in GBM cells[19]. LncRNA HOTAIRM1 is upregulated in GBM and this up-regulation is correlated with grade malignancy in glioma patients, and promotes GBM cell proliferation, migration, invasion and inhibit cell apoptosis by epigenetic regulation of HOXA1 gene[20]. Despite several lncRNAs have been well studied, the functional mechanism of most lncRNAs in GBM remain largely unknow.

Hepatocyte nuclear factor 1 homeobox A antisense RNA 1 (HNF1A-AS1), was first identified as a lncRNA that upregulated in oesophageal adenocarcinoma[21], and its elevated expression was significantly related with an advanced tumor stage and poor outcomes in various cancers, including oral squamous carcinoma, urothelial carcinoma of the bladder and lung carcinoma, indicating an oncogenic function of HNF1A-AS1 in tumor progression [22-24]. However, the role of HNF1A-AS1 in GBM has not been fully investigated yet. Herein, we demonstrate that HNF1A-AS1 is overexpressed in GBM tissues and GBM cells, associates with poor prognosis of GBM patients, by acting as ceRNA for miR-22 and facilitating ENO1 expression, to promote GBM malignant phenotypes. Therefore, our findings imply that HNF1A-AS1 may serve as a potential prognostic biomarker and therapeutic target for GBM.

Materials And Methods

Patient samples

72 GBM tissues, 41 low grade glioma (LGG) tissues and 15 normal brain tissues (NBT) applied in this study were obtained from the Department of Neurosurgery, the Second Affiliated Hospital of AnHui Medical University from January 2013 to October 2018. All patients signed the written informed consent, and this study was reviewed and approved by the Clinical Research Ethics Committee at the Second Affiliated Hospital of AnHui Medical University.

Cell culture

Human GBM cell lines (U251, LN18, U87 and A172) were obtained from the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) with high glucose supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher

Scientific, Inc., Waltham, MA, USA) and streptomycin (100μg/ml), penicillin (100 U/ml). Normal human astrocyte (HA) was purchased from Sun Yat-Sen University and cultured in astrocyte medium with low glucose supplemented with 10% fetal bovine serum. All cell lines were cultured at 37 °C in a humidified incubator with 5 % CO2.

Subcellular fractionation

Cytoplasmic and nuclear fractions of the LN18 cells were prepared and collected according to the manufacturer's instructions of PARIS™ Kit (Ambion, USA). GAPDH was used as the cytoplasmic endogenous control. U6 small nuclear RNA was used as the nuclear endogenous control.

Cell transfection

SiRNA targeting HNF1A-AS1 (si-HNF1A-AS1), EGR1 (si-EGR1) and scrambled negative control (si-NC) were designed and synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The sequence of si-HNF1A-AS1-1 was as follows: CCCTCCATCTAACATTCAA, si-EGR1, CAACGAGAAGGTGCTGGTG, and these fragments were transfected into U251 and LN18 cells respectively by using Lipofectamine2000 (Invitrogen, USA) according to the manufacturer's protocols. full-length of HNF1A-AS1 and EGR1 was amplified by PCR and sub-cloned into pcDNA3.1-Vector (pcDNA3.1-HNF1A-AS1 and Vector), pcDNA3.1-Vector (pcDNA3.1-EGR1 and Vector) (Sangon Biotech, Shanghai). MiR-22-3p negative control (NC), miR-22-3p mimics and miR-22-3p inhibitors were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China), and transfected into cell lines as the above described.

Total RNA isolation and quantitative Real-time PCR (RT–QPCR)

Total RNA was isolated from cultured cells or tissues with ready to use TRIzol Reagent (Invitrogen, USA), according to the manufacturer's instructions. Using a Nanodrop Spectrophotometer (IMPLEN GmbH, Munich, Germany), RNA concentration and quality were determined by the 260/280 nm absorbance. Then RNA was reversely transcribed into cDNA by using PrimeScript™ RT Master Mix (Perfect Real Time) (TaKaRa Biotechnology, Dalian, China). Maxima SYBR-Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Inc.) was used for quantitative PCR. The primers for genes were determined as follows: HNF1A-AS1 forward 5'- CAAGAAATGGTGGCTATGA-3', reverse 5'- TGGACTGAAGGACAAGGGT-3';

EGR1, forward: 5'-CAGCACCTTCAACCCTCAG-3', reverse: 5'-CACAAGGTGTT

GCCACTGTT-3'; ENO1 forward 5'- GCCTCCTGCTCAAAGTCAAC-3', reverse 5'-AACGATGAGACACCATGACG-3'; GAPDH forward 5'-AGCAAGAGCACAAGAGGAAG-3', reverse 5'-GGTTGAGCACAGGGTACTTT-3'. All-in-One™ miRNA First-Strand cDNA Synthesis kit (Genecopoeia, Guangzhou, China) was used for miRNA reverse transcription and RT–QPCR was conducted using the All-in-One™ miRNA qPCR kit (Genecopoeia) for miR-204-3p and U6 (miRQ0022693-1-1/MQP-0202, respectively; RiboBio Co., Ltd.), respectively, using ABI 7300 (Applied Biosystems, Darmstadt, Germany). GAPDH and U6 were used as loading control for HNF1A-AS1, ENO1 and miR-22. PCR Cycling conditions for mRNA was 2 min at 50 °C, 10 min at 95 °C and followed by 45 cycles of 95 °C for 15 s, 60 °C for 60s;

for miRNA was 10 min at 95 °C and followed by 45 cycles of 95 °C for 10s, 60 °C for 20s and 72 °C for 12 s. All RT–QPCR reactions were performed in triplicate. The data were determined using the 2−△△Ct method.

Cell proliferation assay

GBM cells were placed into 96-well plates at the density of 2000 cells/well. Approximately 20 μl of CCK8 regent was added to each well after transfection. Finally, the absorbance at 450 nm was measured using a ST-360 micro-plate reader (KHB, Shanghai, China) after incubated at 37 °C for 2 h.

Colony formation assay

For the clone formation assay, 48 hours after transfection, GBM cells (200 viable cells per well) were seeded in a 6-well plate and cultured with complete medium for 12 days. cells were fixed with 4 % polyoxymethylene and stained with 1.5 % methylene blue for 30 min at room temperature.

Wound healing, migration and invasion assays

For the wound healing assay, 48 hours after transfection, GBM cells (200 viable cells per well) were seeded in a 6-well plate and cultured with complete medium. a 10-μl pipette tip was used to create wound gaps, gently washed, and cultured with serum-free medium for 24 h. The wound gaps were observed at 0 and 24 h after wounding and photographed with a light microscope (IX71; Olympus, Tokyo, Japan) at × 200 magnification. Lines were drawn along the leading edges of the cells, and the gap distances of migrating cells from five different areas for each wound were measured and analyzed.

GBM Cells migration and invasion ability was tested by using 24-well chambers with 8 μm pore size (Corning, USA). A total of $2x10^4$ cells were resuspended in 150 μl serum-free medium and seeded into the upper chamber with or without pre-coated with 500 ng/ml Matrigel solution (BD Biosciences, San Jose, CA, USA), while 500 μl of 10 % FBS medium was placed in the lower chamber of Transwell plates, following incubation at 37˚C for 48 h for the migration and invasion assays. Non-migrated and non-invaded cells from the upper chamber were scraped off using a cotton swab. The migrated and invaded cells on the lower chamber membrane were fixed with 4% polyoxymethylene and stained with crystal violet (Sigma) at room temperature for 5 min, and dried. Five predetermined fields were counted under a microscope (Olympus IX71, (Olympus; x200 magnification). All assays were performed in triplicate.

Luciferase reporter assays

The fragments of HNF1A-AS1 and 3' UTR of ENO1, both containing the predicted miR-22 binding site, then the predicted wild-type (WT) binding sites of miR-22 and mutant binding sites (Mut) were cloned into a pmiRGLO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI, US), termed as pmiRGLO-HNF1A-AS1-wild-type (HNF1A-AS1-WT), pmiRGLO-HNF1A-AS1-mutated-type (HNF1A-AS1-Mut), pmiRGLO-ENO1-wild-type (ENO1-WT) and pmiRGLO-ENO1-mutated-type (ENO1-Mut). Then HNF1A-AS1-

WT or HNF1A-AS1-Mut was co-transfected with the miR-22 mimics or miR-22 NC into GBM cells by using Lipofectamie2000 (Invitrogen, USA). After 48 h transfection for luciferase assay using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol. ENO1-WT and ENO1-Mut were handled similarly as described above.

To confirm the bind relation between EGR1 and HNF1A-AS1 promoter, pGL3-HNF1A-AS1 promoter was co-transfected into cells along with si-EGR1 or si-NC using Lipofectamine 2000. The luciferase activity was measured by a Dual-Luciferase reporter assay system (Promega, USA). All assays were independently performed in triplicate.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using the EZ-Magna ChIP™ Chromatin Immunoprecipitation Kit (Millipore, Darmstadt, Germany). U251 and LN18 cells were fixed with 1% formaldehyde for 10 min at room temperature and lysed in ChIP lysis buffer, and then the DNA was sonicated for shearing DNA into 500-bp fragments. DNA samples were precipitated with anti-IgG or anti-EGR1 antibody and Protein A/G magnetic beads for overnight. Subsequently, the co-precipitated chromatin DNA was collected, and was tested by RT-QPCR.

RNA immunoprecipitation

RNA immunoprecipitation (RIP) experiments were performed by the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA), and following the manufacturer's protocol. GBM cells were lysed by RIP lysis buffer, and magnetic beads conjugated with human anti-Argonaute2 (Ago2) antibody (Millipore) or normal mouse IgG (Millipore), which was indicated as negative control. Samples were incubated with Proteinase K buffer and then immunoprecipitated RNA was isolated. Furthermore, purified RNAs were extracted and assayed by RT-QPCR.

Western blotting

Western blotting was performed as described previously (Ma et al., 2016). Briefly, cells were collected and lysed using RIPA protein extraction reagent (Beyotime, Shanghai) with a protease inhibitor cocktail (Beyotime), on ice, subjected to SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes, which were then blocked in buffer (5% nonfat milk in TBST) about 1.5h before being incubated with primary antibodies (anti-rabbit-ENO1, 1:1000, Abcam, EUGENE, USA), (anti-rabbit-EGR1, 1:1000, CST, USA) and anti-rabbit-β-actin (1:1000, Abcam, EUGENE, USA) at 4 °C overnight. Horseradish peroxidase-conjugated goat anti-rabbit (1:5000, Beyotime) was applied as a secondary antibody and incubated at 4°C for 1h, and then immune complexes were visualized by SuperSignal ® West Femto Trial Kit (Thermo Fisher, USA) and blot bands were scanned using Find-do × 6 Tanon (Tanon, Shanghai, China).

Tumor xenograft model

Female nude mice at 4–6 weeks of age were used in this study, and experiments with nude mice were conducted strictly in accordance with a protocol approved by the Administrative Panel on Laboratory Animal Care of the Second Affiliated Hospital of AnHui Medical University. The animals were free to autoclaved food and water during the study. U251 cells stably transfected with si-NC or si-HNF1A-AS1 were collected, and injected into the subcutaneous tissues of the axillary skin. The tumor volumes were measured every five days after inoculation. 40 days after injection, the mice were sacrificed, and the tumor nodules were harvested for further study.

Statistical analysis

Unless stated otherwise, all experiments were performed in triplicate and all data were presented as the mean ± standard deviation (SD). GraphPad Prism V6.01 (GraphPad Software, Inc., La Jolla, CA, USA) software was used for statistical analysis and generate figures. Differences were analyzed by SPSS statistical software (version 19.0, Armonk, NY, USA) with the Student's t-test or one-way ANOVA. Pearson's correlation was performed to analyze the relationship between the expression of HNF1A-AS1, miR-22 and ENO1 in tissues. Survival analysis was performed using the Kaplan-Meier method and log-rank tests in GraphPad Prism 6.01. Differences were considered significant if $P < 0.05$.

Results

HNF1A-AS1 is upregulated in GBM and is negatively related with patient prognosis

To identify the expression of HNF1A-AS1 in GBM, using real-time quantitative PCR (RT–QPCR) analysis, we firstly measured HNF1A-AS1 expression levels in 15 normal brain tissues, 41 low-grade glioma tissues, 72 GBM tissues. We found that HNF1A-AS1 was obviously upregulated in GBM, as compared to low-grade glioma tissues and normal brain tissues, but there was no significant difference between low-grade glioma tissues and normal brain tissues (Fig. 1A). Furthermore, we also tested HNF1A-AS1 expression levels in normal human astrocyte (HA) and four GBM cell lines (U251, LN18, U87 and A172). The results showed that HNF1A-AS1 was upregulated in four GBM cell lines in comparison with human astrocyte (Fig. 1B).

To further clarify the clinical value of HNF1A-AS1 in GBM patients. The Kaplan–Meier method and log-rank test were performed to evaluate the expression of HNF1A-AS1 in GBM TCGA date cohort and our study. As shown in GBM TCGA date cohort, GBM patients with high expression of HNF1A-AS1 was negatively associated with overall survival time (Fig. 1C). In our study, we also observed that high HNF1A-AS1 expression was significantly correlated with poor overall survival (Fig. 1D).

In addition, subcellular fractionation and RT–QPCR analyses showed that HNF1A-AS1 is localized both in the cytoplasm and nucleus of GBM cells (Fig. 1E), which indicated its complicated functions.

HNF1A-AS1 underpins GBM cells proliferation, migration, invasion

To evaluate the biofunctional role of HNF1A-AS1 in GBM cells, the GBM cells (U251 and LN18) were transfected with small interfering RNA targeting HNF1A-AS1 (si-HNF1A-AS1) or small interfering negative control (si-NC). RT–QPCR was performed to examine the knockdown efficiency of HNF1A-AS1 after transfection 48 hours (Fig. 2A). CCK-8 assays revealed that HNF1A-AS1 knockdown significantly inhibited cell proliferation compared to that of cells transfected with si-NC groups (Fig. 2B). furthermore, colony formation assays showed that the clone numbers and colony size decreased in the HNF1A-AS1 knockdown groups, suggesting that HNF1A-AS1 suppressed the anchorage-independent growth of GBM cells (Fig. 2C).

To further confirm the effect of HNF1A-AS1 on GBM cells migration and invasion. Wound healing assays indicated that the wound-healing capacity was worse and slower in si-HNF1A-AS1 groups than in si-NC groups (Fig. 2D). In addition, transwell assay showed that knockdown of HNF1A-AS1 significantly reduced migratory and invasive capacity compared with si-NC groups (Fig. 2E). On the contrary, overexpression of HNF1A-AS1 obviously promote the malignancy of GBM cells (Fig. 3A-C). Taken together, these results indicated that HNF1A-AS1 plays an important role in promoting GBM cells malignant behaviors.

EGR1 strengthen HNF1A-AS1 at transcriptional level

To further discover the regulatory mechanisms which cause the upregulation of HNF1A-AS1 in GBM. By using UCSC Genome Browser (http://genome.ucsc.edu/), we found that Early Growth Response gene 1 (EGR1) is a latent transcription factor of HNF1A-AS1. Furthermore, previous study has demonstrated that EGR1 transcriptionally activated HNF1A-AS1 in human gastric cancer[25]. According to JASPAR (http://jaspar.genereg.net/) database, the predicted transcription factor-binding site of EGR1 in the HNF1A-AS1 promoter is indicated in (Figure. 4A). Importantly, TCGA database confirmed that EGR1 is highly expressed in GBM tissues, and patients with higher HNF1A-AS1 expression indicated a poor poorer prognosis (Figure. 4B-C). To detect the effect of EGR1 on HNF1A-AS1 expression, we successfully establish EGR1 overexpression or knockdown model and was tested by RT-QPCR and western blot (Figure. 4D-F). As a result, depletion of EGR1 significantly decrease the levels of HNF1A-AS1, while EGR1 overexpressed dramatically increase the levels of endogenous HNF1A-AS1 (Figure. 5A-B). Luciferase reporter assay further demonstrated that the region of HNF1A-AS1 promoter is responsible for HNF1A-AS1 transcription (Figure. 5C-E). Moreover, ChIP assay showed that the EGR1 specifically associated with the promoter of HNF1A-AS1 (Figure. 5F). These results demonstrated that EGR1 enhance HNF1A-AS1 expression at transcriptional level by directly binding to its promoter.

HNF1A-AS1 physically bound to miR-22 and induced its degradation

To further clarify the potential molecular mechanism by which HNF1A-AS1 regulates GBM cells proliferation, migration and invasion. As we all know, lncRNAs could function as a ceRNA by competitively binding miRNAs and restrain miRNAs expression and activity[26]. Bioinformatics data such as RegRNA 2.0 (http://regrna2.mbc.nctu.edu.tw/), miRANDA (http://www.microrna.org), Diana-lncBase (http://carolina.imis.athena-innovation.gr) were performed to predict the potential miRNA targets of

HNF1A-AS1. According to the mirSVR and PhastCons scores, we found that miR-22 may have putative binding sites with HNF1A-AS1. Our studies indicated that the expression of miR-22 was significantly inverse in correlation with HNF1A-AS1 in GBM tissues (Fig. 5G). Moreover, HNF1A-AS1 knockdown dramatically increased miR-22 expression, while GBM cells transfected with PCDNA3.1-HNF1A-AS1 significantly inhibited the expression of miR-22 (Fig. 6A-B). However, no expression changed on HNF1A-AS1 when GBM cells transfected with miR-22 mimics or inhibitors (Fig. 6C-D). These results indicated that miR-22 was negatively regulated by HNF1A-AS1 in GBM.

Bioinformatics analysis predicted the potential binding sites of miR-22 on HNF1A-AS1 and the conservation of HNF1A-AS1 in the binding site of miR-22 was snapshotted from human genome in UCSC Genome Browser (Fig. 6E-F). Then, luciferase reporter assay was performed to validate the direct binding between miR-22 and HNF1A-AS1 at endogenous levels. We found that there was no significant difference in the relative luciferase activity between HNF1A-AS1-Mut+ miR-22 mimics and HNF1A-AS1-Mut+ miR-22 NC groups, but co-transfection of pmirGLO-HNF1A-AS1-WT and miR-22 mimics dramatically reduced the luciferase activity compared with HNF1A-AS1-WT+miR-22 NC groups (Fig. 6G-H). To confirm whether HNF1A-AS1 and miR-22 are in the same RNA induced silencing complex (RISC), we conducted anti-Ago2 RNA-binding protein immunoprecipitation (RIP) assay, and the results showed that Ago2 antibody enriched HNF1A-AS1 (Fig. 6I-J). These findings suggested that HNF1A-AS1 directly targeted miR-22 in GBM cells.

Downregulation of miR-22 promotes GBM cells malignant behaviors

According to the TCGA data, the expression of miR-22 was obviously decreased in GBM samples, and was negatively correlated the pathological grades of glioma (Fig. 7A), which was coincided with our study (Fig. 7B). RT–QPCR analysis were performed to test the expression level of miR-22 in HA cell and GBM cell lines, and the results showed that miR-22 expression was significantly downregulated in four GBM cell lines compared with the HA (Fig. 7C). Compared with the miR-22 NC group, the proliferation, migration and invasion ability of GBM cells were distinctly reduced when cells transfected with miR-22 mimics, and cells' malignant behavior ability were enhanced when cells transfected with miR-22 inhibitors (Fig. 7D-E). These results indicated that miR-22 acted as an anti-oncogene in GBM cells.

ENO1, a direct target of miR-22 in GBM cells

Bioinformatic tools (TargetScan and MirDB) were adopted to predict potential targets of miR-22 in GBM cells. We found that 3'UTR regions of the overlapped potential candidates both have the predicted binding sites of miR-22. Ultimately, a key glycolytic enzyme, ENO1, was identified as target gene, in view of its upregulation is associated with glioma progression and prognosis[27, 28]. A previous study showed that miR-22 suppresses the proliferation of retinoblastoma cells by inhibiting ENO1, and ENO1 was a target of miR-22[29]. As showed in Fig. 8A, the putative binding sites of miR-22 within the 3'UTR of ENO1 were predicted by TargetScan and MirDB. To confirm whether miR-22 regulated ENO1, GBM cells were transfected with miR-22 mimic or miR-22 inhibitors. The results indicated that overexpression of miR-22 significantly reduced both mRNA and protein expression levels of ENO1 compared to miR-22 NC and

conversely ENO1 expression significantly increased after inhibited miR-22 (Fig. 8B-C). Then, luciferase reporter assay showed that co-transfection of ENO1 WT and miR-22 mimics drastically reduced luciferase activity compared with the ENO1 WT + miR-22 NC group, whereas miR-22 Mut binding site within ENO1 abrogated the inhibitory effect of miR-22 mimics on the reporter gene expression (Fig. 8D-E). In addition, we found that ENO1 was significantly overexpressed in GBM tissues and its high expression was inverse correlation with overall survival time in GBM TCGA data, which was consistent with CGGA data (Fig. S1A-D). These findings indicated that miR-22 inhibited ENO1 expression in GBM cells by targeting the 3' UTR of oncogene ENO1.

Knockdown of HNF1A-AS1 induced suppression of malignant phenotype was smothered by knockdown of miR-22 in GBM cells

Previous study has confirmed that ENO1 downregulation led to suppressed cell growth, migration and invasion progression in glioma cells[27]. However, whether HNF1A-AS1 promote the malignant behaviors of GBM cells by inhibiting miR-22 remained largely unknown. miR-22 mimics and miR-22 inhibitors were transfected into si-HNF1A-AS1 GBM cells. The results showed that cell proliferation, migration and invasion ability were reduced in si-HNF1A-AS1 and miR-22 mimics groups. si-HNF1A-AS1 combined with miR-22 mimics group was strongly reduced the malignant phenotype of GBM cells, while miR-22 inhibitors reversed the suppression of HNF1A-AS1 knockdown in GBM cells (Fig. 9A-D). Therefore, these results suggested that HNF1A-AS1 exerts its biofunctional roles though miR-22 in GBM cells.

HNF1A-AS1 functions as a ceRNA for ENO1 via modulating miR-22 in GBM

To explore whether HNF1A-AS1 regulates the expression of ENO1 by inhibiting miR-22 in GBM. the mRNA and protein levels of ENO1 were detected by RT-QPCR and Western blot assays after cells transfected with si-HNF1A-AS1 combined with miR-22 mimics or miR-22 inhibitors. The results showed that HNF1A-AS1 knockdown decreased the mRNA and protein levels of ENO1 compare with si-NC, and si-HNF1A-AS1 combined with miR-22 mimics drastically decreased the expression levels of ENO1 but were reversed by co-transfection with si-HNF1A-AS1 and miR-22 inhibitors (Fig. 10A-C). Furthermore, our study first found that ENO1 expression was negatively correlated with miR-22 and positively associated with HNF1A-AS1 in 72 GBM patients (Fig. 10D-E). Hence, these results indicated that HNF1A-AS1 regulates ENO1 expression by sponging miR-22 in GBM.

HNF1A-AS1 knockdown inhibits GBM tumorigenesis in vivo

Tumor xenograft models were performed to evaluate the functional roles of HNF1A-AS1 in vivo. We inoculated subcutaneously the treated U251 cell, as showed in (Figure. 11A-C), Tumor volumes and tumor weights in the si-HNF1A-AS1 group were obviously smaller and lower compared with the si-NC group. Also, western blot indicated that ENO1 protein expression levels in nude mice tumor tissues was significantly decreased in the si-HNF1A-AS1 group than in the si-NC group (Figure. 11D).

Discussion

GBM is the most common and lethal primary malignant brain cancer in adults[30]. Although Maximal safe resection and chemoradiotherapy are known as comprehensive regimens which are the most popular mean for the treatment of patients with GBM, the overall survival time of GBM patients is still poor [31, 32]. Therefore, the diagnosis, treatment and prognosis estimation of such brain tumors remain a challenge in our clinical work. What is encouraging is that great progress has been made in research of molecular diagnosis participated in the progression of glioma such as IDH, TP53, EGFR, H3K27M and WNT[33-37].

Recently, accumulating studies have confirmed that dysregulation of lncRNAs play a considerable functional role in carcinogenesis and progression of multiple cancers. Moreover, a number of studies have demonstrated that lncRNAs could serve as a diagnostic biomarker and therapeutic target in GBM[38]. Previous studies have confirmed that lncRNAs are dysregulated in GBM. For instance, NEAT1, a glioblastoma-associated lncRNA, was an oncogenic factor that was regulated by EGFR pathway, by activating WNT/β-Catenin pathway to promote GBM cells growth and invasion[39]. High ATB expression was associated with a poor clinical outcome in glioma patients, and its knockdown suppressed glioma biological characteristics by directly repression miR-200a, and positively regulating TGF-β2 expression in glioma cells[40].

In this study, we found HNF1A-AS1 was significantly upregulated in GBM tissues and cell lines compared with normal brain tissues and HA cell. High expression of HNF1A-AS1 was negatively associated with the clinical outcomes of GBM patients. In addition, knockdown of HNF1A-AS1 inhibited cell proliferation, colony formation, migration and invasion in vitro and in vivo, while overexpression HNF1A-AS1 strengthen the malignant behaviors of GBM cells. Taken together, our results indicated that HNF1A-AS1 functions as an oncogene and may serve as a potential prognostic biomarker in GBM.

Mounting studies have demonstrated that lncRNAs expression could be activated by their upstream transcription factors. For instance, MKL1 induce the transactivation of SNHG18, which promotes NSCLC growth, invasion, and metastasis[41]. The transcription factor TEAD4 mediates MNX1-AS1 expression to drive gastric cancer progression[42]. EGR1 is a zinc finger transcription factor, belonging to the EGR family, and as a transcription activator several lncRNAs[43, 44]. In the present study, we found that EGR1 could bind with HNF1A-AS1 promoter region and transcriptionally induced HNF1A-AS1 overexpression in GBM cells, and TCGA datasets confirmed upregulated EGR1 is correlated with poor prognosis of GBM patients.

Recently, increasing evidences demonstrated that lncRNAs can function as ceRNAs by competitively binding to miRNAs and then regulate the expression of miRNA downstream target genes[8]. Liu et al. find that high HNF1A-AS1 expression serve as a ceRNA that sequesters miR-661, thereby protecting the target gene CDC34 from repression and in turn upregulating HNF1A-AS1 in in gastric cancer[25]. Cai et ai. show that HNF1A-AS1 is overexpressed in colon tissues and cell lines, and served as a ceRNA to modulate miRNA-34a expression, subsequently with repression of miR-34a/SIRT1/p53 feedback loop and activation of canonical Wnt signaling pathway in metastasis of colon cancer[45]. Here, we found that

HNF1A-AS1 located in both the nucleus and cytoplasm, indicating its complicated functions. To clarify the underlying mechanism by which HNF1A-AS1 functions as an oncogene in GBM. According to our bioinformatics analysis, we found that miR-22 might has putative binding sites with HNF1A-AS1 in GBM. Moreover, a negative association between HNF1A-AS1 and miR-22 expression in GBM tissues was confirmed from our study. HNF1A-AS1 knockdown or overexpression significantly increased or decreased the expression of miR-22 in GBM cells. In addition, dual-luciferase reporter and RIP assay demonstrated that HNF1A-AS1 acted as miRNA sponge and negatively regulates miR-22 expression in GBM cells.

MiR-22, which was an exon of the C17orf91 gene, was located at chromosome 17p13.3. Previous studies have confirmed that miR-22 is markedly downregulated and functions as a tumor suppressor miRNA in various cancers. Sun et al. find that miR-22 is downregulated in colon cancer, overexpression of miR-22 significantly inhibits cell proliferation, migration, metastasis, and epithelial-mesenchymal (EMT) transition by directly targeting BCL9L[46]. Jiang et al. show that miR-22 is significantly downregulated in AML and forced expression of miR-22 significantly suppresses leukaemic cell viability and growth, and restoration of miR-22 expression holds great therapeutic potential to treat AML[47]. Chen et al. find that miR-22 mimics suppresses cell proliferation, migration, and invasion via targeting the 3'-UTR of SIRT1 in the progression of GBM[48]. However, many other important downstream target genes of miR-22 in GBM are not clear. In our study, we found miR-22 expression was downregulated in GBM tissues and cells in comparison with normal tumor tissues and HA cell, and low miR-22 expression associated with poor pathological grade of glioma. Furthermore, overexpression of miR-22 remarkably suppressed cell proliferation, migration, and invasion, and miR-22 inhibition exhibited the opposite effects. In addition, miR-22 suppression reversed the inhibitory effects caused by HNF1A-AS1 knockdown. In a word, these findings suggested that HNF1A-AS1 promoted the biological characteristic of GBM cells by directly targets miR-22.

Alpha-enolase (ENO1), a famous glycolytic enzyme functioning during aerobic glycolysis, was found in nearly all parts of adult human, and contributed to the Warburg effect in cancer cells[49]. Previous studies showed that ENO1 was up-regulated and functioned as an oncogene in various cancer types[50, 51]. Song et al. find that elevated ENO1 expression was an independent prognostic factor, and boosted cell proliferation, migration, and invasion ability by activating the PI3K/AKT pathway in glioma cells[27]. Principe et al. show that ENO1 silencing which increased integrins and uPAR (an ECM receptor), could impeded cell adhesion, invasion, and metastasis, by acting as a plasminogen receptor on the tumor cell surface in pancreatic cancer[52]. Fu et al also demonstrate that upregulated ENO1 drastically enhanced NSCLC cell glycolysis and malignant biological behaviors by activating FAK-mediated PI3K/AKT pathway and its downstream signals to regulate the glycolysis, cell cycle, and EMT-associated genes[53]. In our study, we found both TCGA data and CGGA data validated that ENO1 was upregulated in GBM tissues and high ENO1 expression indicated a poor outcome of GBM patients. We confirmed that miR-22 directly target the 3'UTR of ENO1 and negatively regulate its expression. More importantly, we further validated that HNF1A-AS1 knockdown induced the reduction of ENO1 expression was regain via miR-22 inhibition, indicating HNF1A-AS1 could positively regulate ENO1 expression by inhibiting miR-22 expression in GBM. In addition, we found that ENO1 expression was negatively correlated with miR-22, but positively

associated with HNF1A-AS1 in GBM tissues. These results suggested that HNF1A-AS1 functioned as a ceRNA by competitively binding miR-22 and releasing ENO1 in GBM.

In conclusion, our data demonstrate that a crucial oncogenic transcription factor EGR1 mediates HNF1A-AS1 expression via binding to the promoter region of HNF1A-AS1, which is highly expressed in GBM tissues and cell lines, and was negatively correlated with GBM progression and prognosis. HNF1A-AS1 suppressed GBM malignancy by functioning as a ceRNA to sponge miR-22 and facilitates the expression of oncogene ENO1, which is a direct target of miR-22 in GBM. Therefore, our findings support that HNF1A-AS1 may become a new therapeutic target for GBM treatment.

Abbreviations

LncRNAs, Long non-coding RNAs; GBM, Glioblastoma Multiforme; TCGA, The Cancer Genome Atlas; HNF1A-AS1, Hepatocyte nuclear factor 1 homeobox A antisense RNA1; EGR1, Early Growth Response gene 1, ENO1, Enolase 1; CNS, Central nervous system; TMZ, Temozolomide; MiRNAs, MicroRNAs; HOTAIRM1, HOX antisense intergenic RNA myeloid 1; RISC, RNA induced silencing complex; CeRNA, Competing endogenous RNA; HOTAIR, HOX transcript antisense intergenic RNA; LncRNA-ATB, Long noncoding RNA activated by TGF-β; TGF-β2, Transforming growth factor-β 2; NBT, Normal brain tissue; DMEM, Dulbecco's modified Eagle's medium; FBS, Fetal bovine serum; RIP, RNA immunoprecipitation.

Declarations

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Authors' contributions

CCM and HLW conceived and designed the experiments. FY, ZHY and GZ performed the experiments. EBB, and JH analyzed the data. YYW and CCM drafted the manuscript. CCM and BZ supervised the whole work and revised the manuscript. All authors read and approved the manuscript.

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Availability of data and materials

Because of our internal policy, raw data cannot be shared.

Informed consents were obtained from all patients, and this study was approved by the Clinical Research Ethics Committee at the

Second Affiliated Hospital of AnHui Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Supplementary Material

Supplementary figure. FIGURE S1 ENO1 was obviously function as a proto-oncogene in GBM. (A-B) TCGA date showing that ENO1 was significantly upregulated in GBM only, not in LGG, and high ENO1 expression indicated a poor prognosis in GBM patients. (C-D) CGGA date further confirmed that ENO1 was significantly overexpression in GBM compared with grade glioma and grade glioma. Higher ENO1 expression was also associated with a poor prognosis for patients with GBM.

Competing Interests

The authors have declared that no competing

interest exists.

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Figures

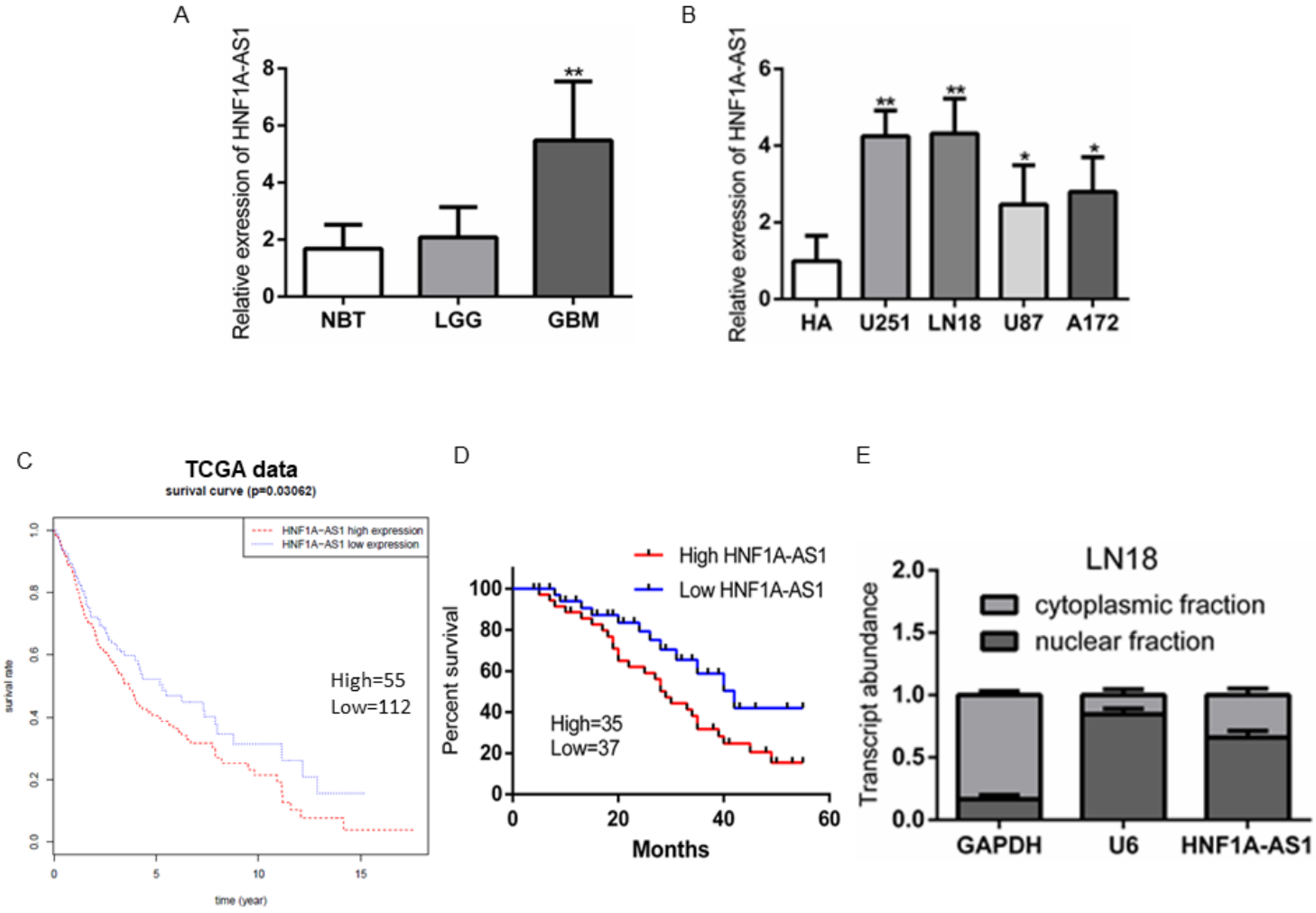

Figure 1

HNF1A-AS1 was highly expressed in GBM. (A) RT-QPCR analysis of HNF1A-AS1 expression in normal brain tissues (NBT) (n=15), low-grade glioma tissues (n = 41) and GBM tissues (n = 72). **P < 0.01 vs. NBT group. (B) RT-QPCR analysis the expression levels of HNF1A-AS1 in normal human astrocyte (HA) and four GBM cell lines (U251, LN18, U87 and A172). *P < 0.05, **P < 0.01 vs. HA group. (C) In TCGA date, the overall survival rate of GBM patients with high (HNF1A-AS1-high, n = 55) and low (HNF1A-AS1-low, n = 112) expression of HNF1A-AS1 in tumor. (D) Kaplan-Meier analyses of the associations between HNF1A-AS1 expression level and overall survival of patients with human glioma in our department (The log-rank test was used to calculate P-values). **P < 0.01 vs. low HNF1A-AS1 expression group. (E) Nuclear and cytoplasmic fractions of HNF1A-AS1 in GBM cell lysates were analyzed by RT-QPCR.

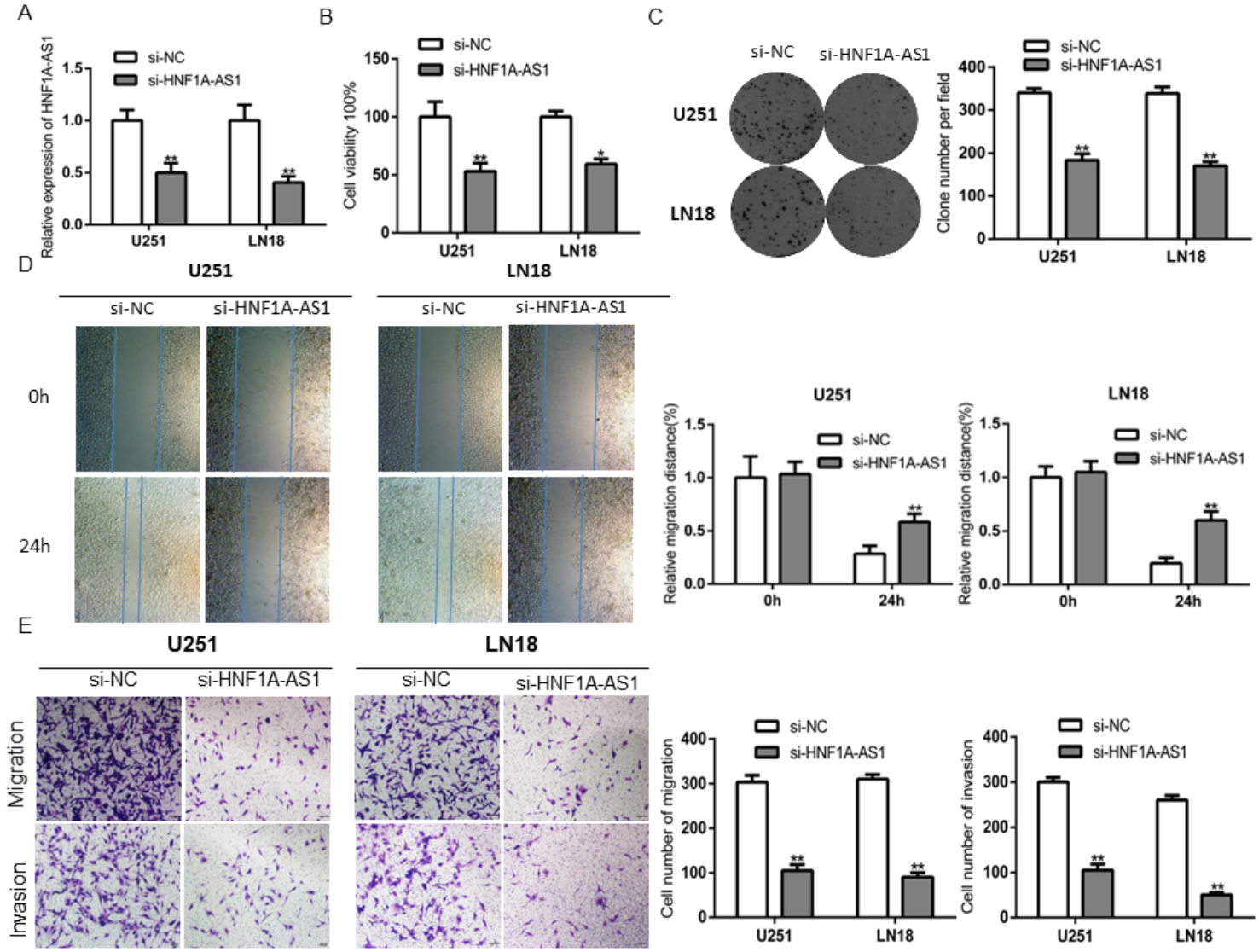

Figure 2

Knockdown of HNF1A-AS1 inhibited the proliferation, migration, and invasion of glioma cells in vitro. (A) Relative expression levels of HNF1A-AS1 after GBM cells transfected with si-HNF1A-AS1 and si-NC. **P < 0.01 vs. si-NC. (B) CCK-8 assay was performed to determine the proliferation effect of si-HNF1A-AS1 and si-NC transfected U251 and LN18 cells. *P < 0.05, **P < 0.01 vs. si-NC group. (C) Colony formation assay was performed to detect the proliferation of U251 and LN18 cells after transfected with si-HNF1A-AS1 and si-NC. **P < 0.01 vs. si-NC group. (D) Wound healing assay to evaluate the effect of HNF1A-AS1 on cell migration in U251 and LN18 cells. **P < 0.01 < 0.05 vs. si-NC group. (E) Transwell assay for testing cell migration and invasion capacity. **P < 0.01 vs. si-NC group. Data are presented as mean±SD from three independent experiments.

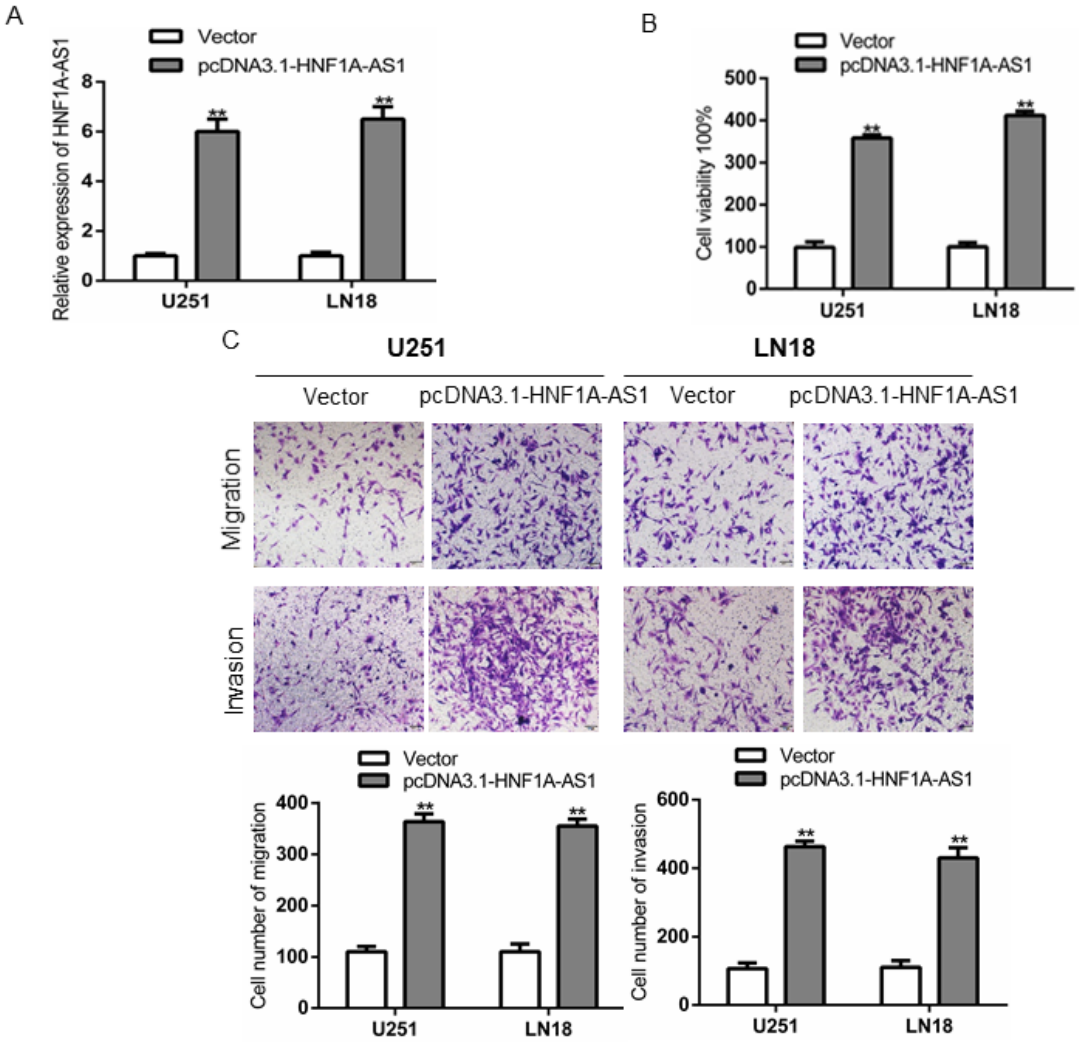

Figure 3

Overexpression of HNF1A-AS1 enhance the proliferation, migration, and invasion of glioma cells in vitro. (A) Relative expression levels of HNF1A-AS1 after GBM cells transfected with pcDNA3.1-HNF1A-AS1 and Vector. **P < 0.01 vs. Vector. (B-C) CCK-8 assay and Transwell assay was performed to determine the proliferation, migration and invasion effect of pcDNA3.1-HNF1A-AS1 and Vector after transfected with U251 and LN18 cells. **P < 0.01 vs. Vector group.

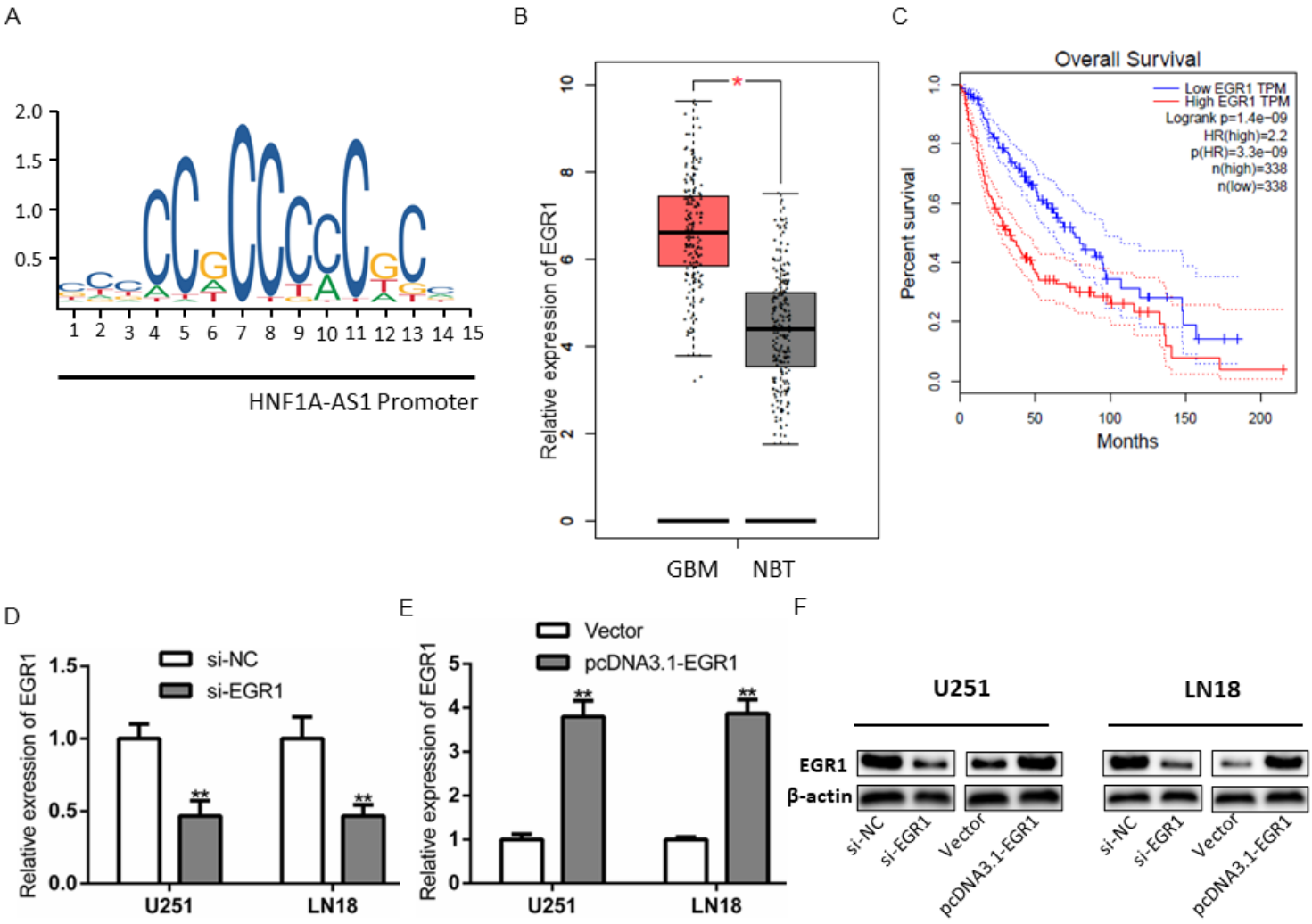

Figure 4

The transcription factor EGR1, specially activate HNF1A-AS1 expression at transcriptional level. (A) JASPAR database was adopted to predict the putative bind site of EGR1 on the promoter region of HNF1A-AS1. (B-C) TCGA date indicated that ENO1 was significantly upregulated in GBM, and high ENO1 expression indicated a poor prognosis in glioma patients. (D-F) Relative expression of EGR1 on mRNA and protein levels after GBM cells transfected with si-EGR1 or pcDNA3.1-EGR1 and si-NC or Vector. **P < 0.01 vs. si-NC or **P < 0.01 vs. Vector.

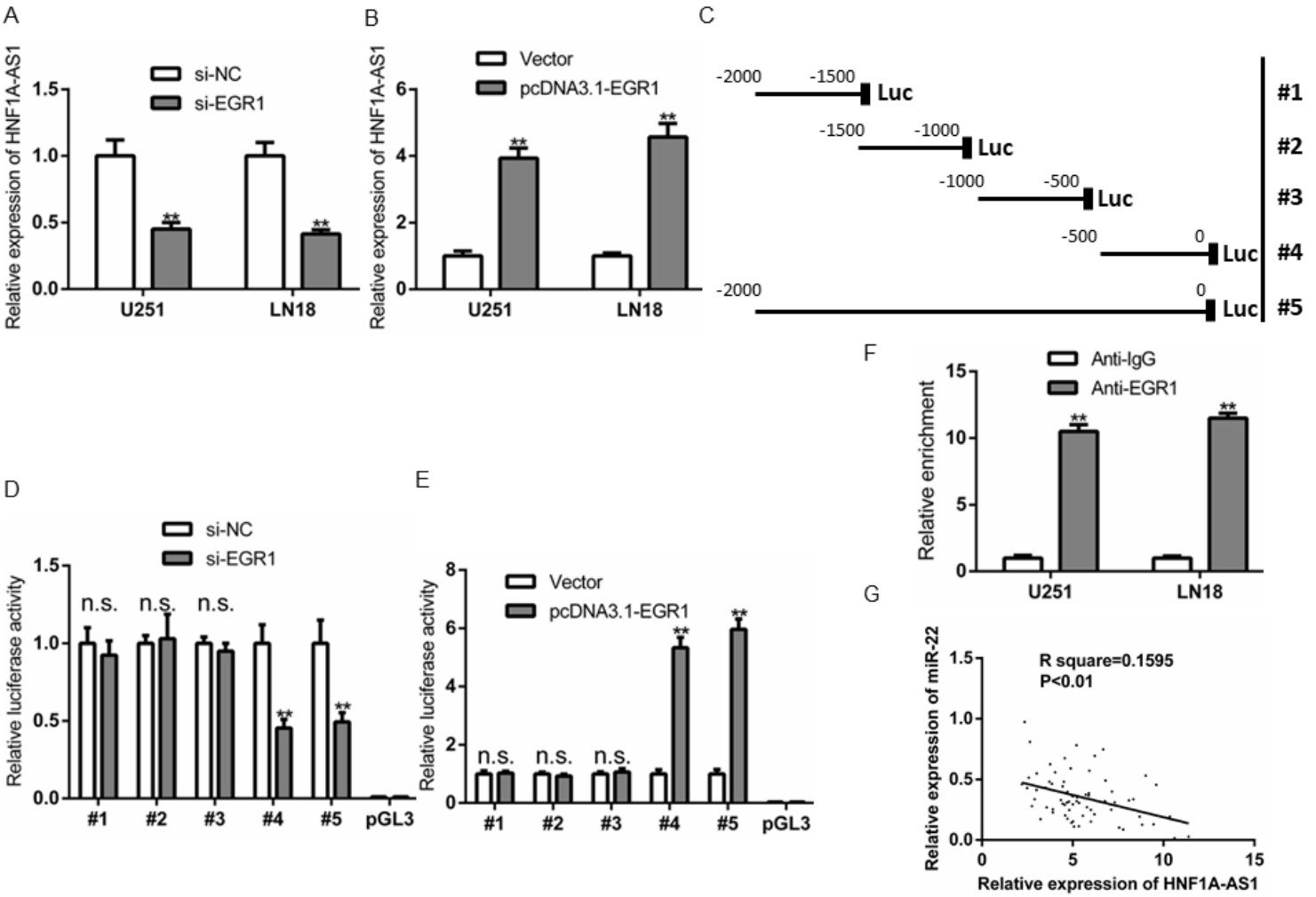

Figure 5

(A-B) The expression levels of HNF1A-AS1 was tested in GBM cells transfected with si-EGR1 or pcDNA3.1-EGR1 using RT-QPCR assay. **P < 0.01 vs. si-NC or **P < 0.01 vs. Vector. (C) Serial truncations of HNF1A-AS1 promoter fragments spanning from -2,000/ -1500/ -1000/ -500/ to 0. (D-E) This promoter fragments were cloned into pGL3-basic vectors, and dual luciferase reporter assays were performed to assess the exact EGR1 binding site on the HNF1A-AS1 promoter region. **P < 0.01 vs. si-NC or **P < 0.01 vs. Vector, n.s. indicates no significance relative to si-NC or Vector. (F) ChIP assay showed that the binding affinity between EGR1 and HNF1A-AS1 promoter region. **P < 0.01 vs. IgG. (G) Pearson's correlation was performed to analyze the relationship between HNF1A-AS1 expression and miR-22 expression in 72 GBM patients.

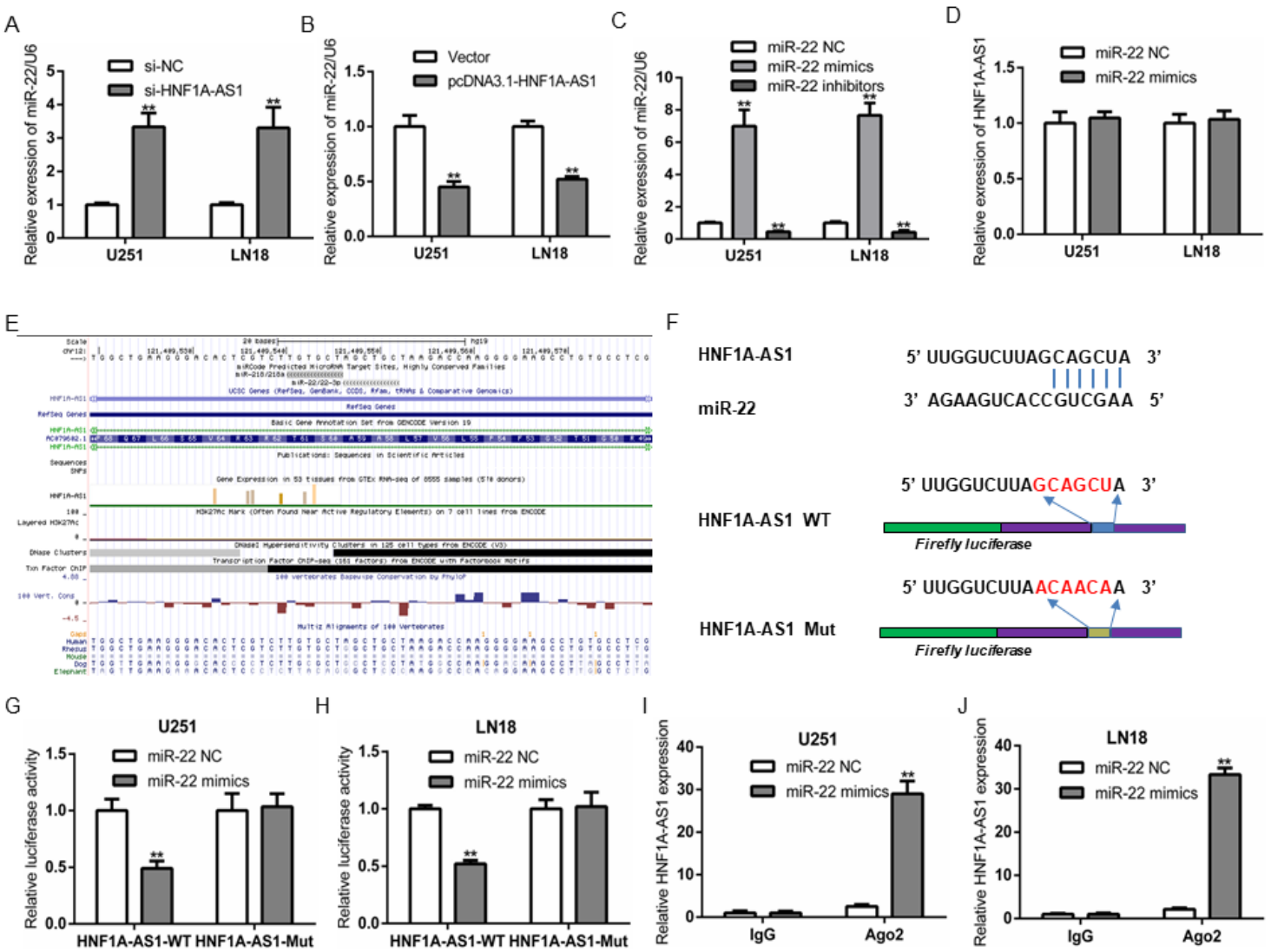

Figure 6

HNF1A-AS1 expression was negatively associated with miR-22 expression in GBM. (A-B) Relative expression of miR-22 in U251 and LN18 cells transfected with si-HNF1A-AS1, si-NC, pcDNA3.1-HNF1A-AS1 and Vector. **P < 0.01 vs. si-NC group. (C) The efficiency of miR-22 expression levels after glioma cells transfected with miR-22 NC, miR-22 mimics and miR-22 inhibitors. **P < 0.01 vs. miR-22 NC group. (D) Relative expression of HNF1A-AS1 in U251 and LN18 cells transfected with miR-22 mimics and miR-22 NC. (E-F) The conservation of HNF1A-AS1 in the binding site of miR-22 was snapshotted from human genome in UCSC Genome Browser and bioinformatics date predicted the putative binding sites of miR-22 on HNF1A-AS1. (G-H) Luciferase activity in U251 and LN18 glioma cells co-transfected with miR-22 mimics and luciferase reporters containing HNF1A-AS1-WT or HNF1A-AS1-MUT transcript. **P< 0.01 vs. miR-22 NC group. (I-J) RNA-IP with anti-antibody was performed in U251 and LN18 cells transfected with miR-22 NC and miR-22 mimics. HNF1A-AS1 expression level was detected using RT-QPCR. **P< 0.01 vs. miR-22 NC group. Data were presented as mean ± SD from three independent experiments.

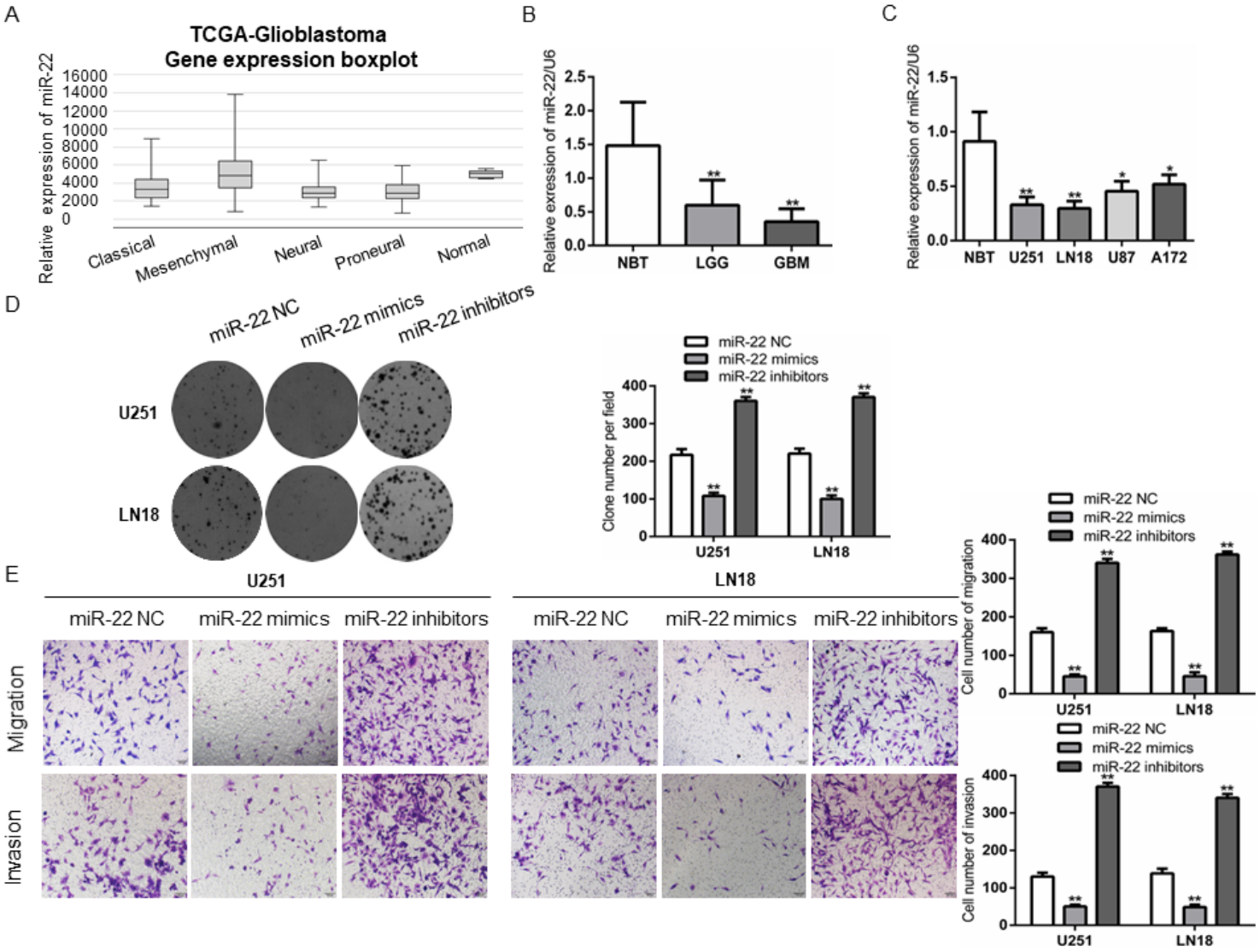

Figure 7

MiR-22 was downregulated in GBM. (A-B) TCGA date indicated that miR-22 was lowly expressed in GBM and was inversely correlated with pathological grades of glioma. **P < 0.01 vs. NBTs group. (C) RT-QPCR analysis the expression levels of miR-22 in normal human astrocyte (HA) and four GBM cell lines (U251, LN18, U87 and A172). *P < 0.05, **P < 0.01 vs. HA group. (D-E) CCK-8 assay and Transwell assay was performed to determine the proliferation, migration and invasion effect of miR-22 mimics and miR-22 inhibitors after transfected with U251 and LN18 cells. **P < 0.01 vs. miR-22 NC.

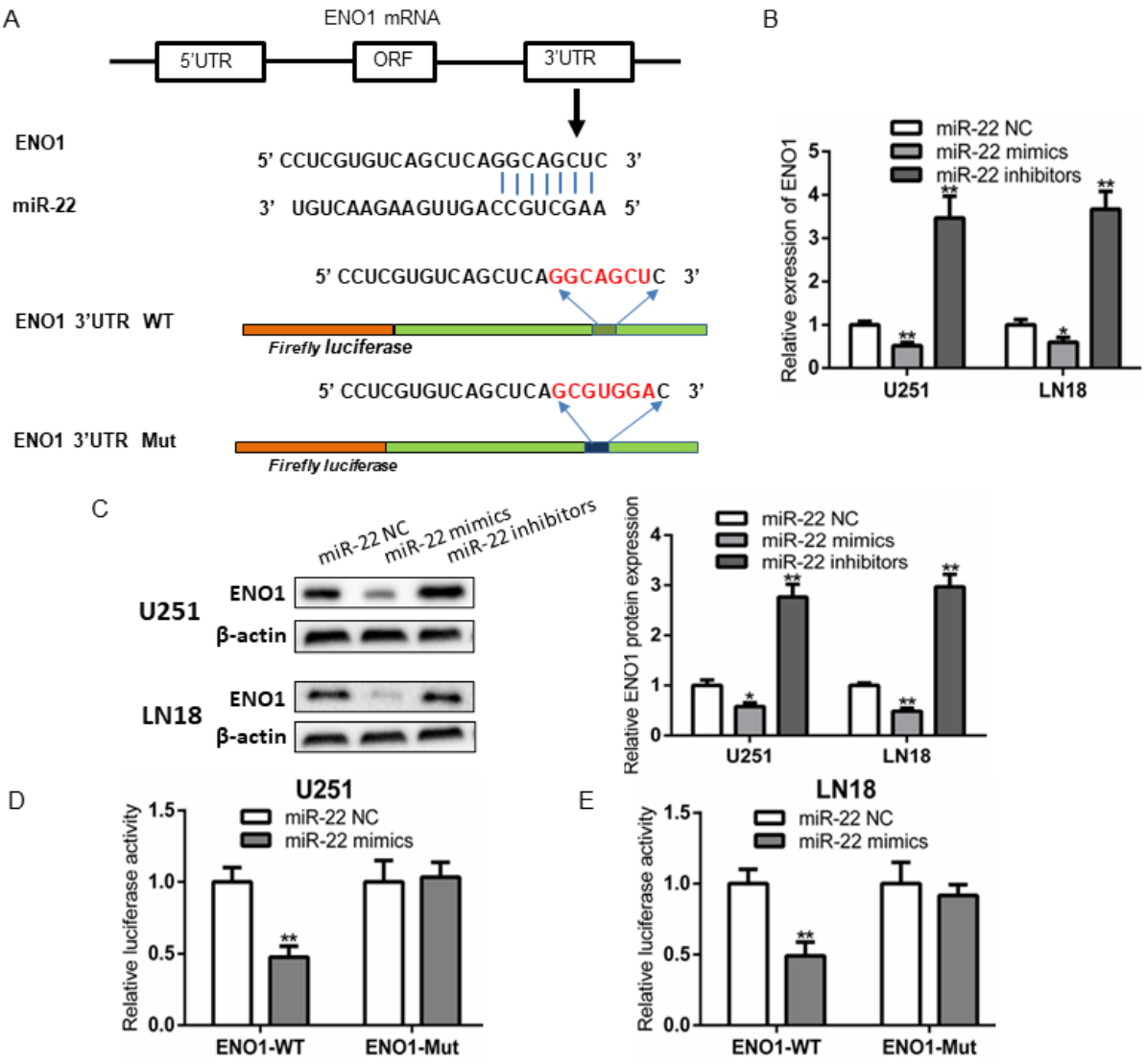

Figure 8

MiR-22 targets the 3'UTR of ENO1 and inhibits its expression in GBM. (A) Schematic diagram showing the predicted miR-22 binding sites within the 3'UTR of oncogene ENO1. (B-C) Relative expression of ENO1 mRNA and protein levels in U251 and LN18 cells after transfected with miR-22 mimics, miR-22 inhibitors, and miR-22 NC. *P< 0.05, **P < 0.01 vs. miR-22 NC group. (E-F) Luciferase activity in U251 and LN18 cells co-transfected with miR-22 mimics and luciferase reporters containing ENO1 wild type (WT) or mutant type (MUT) 3'-UTR. **P < 0.01 vs. miR-22 NC group. Data are presented as mean±SD from three independent experiments.

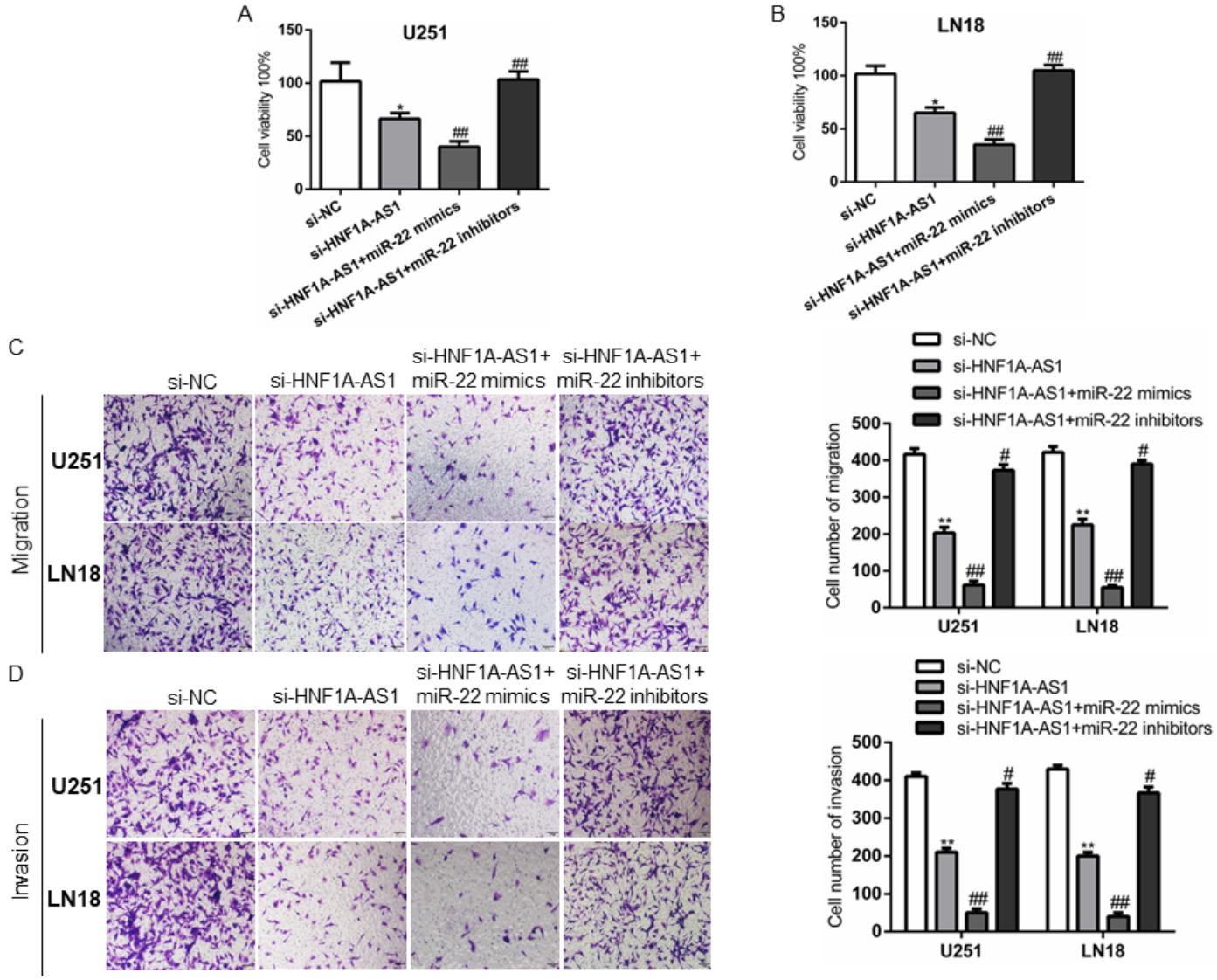

Figure 9

MiR-22 inhibition reversed the si-HNF1A-AS1 induced inhibitory effects on GBM cells. (A-B) CCK-8 assay was performed to determine the proliferation after co-transfected with si-HNF1A-AS1 and miR-22 mimics or miR-22 inhibitors. *P < 0.05 vs. si-NC group; ##P < 0.01 vs. si-HNF1A-AS1 group. (C-D) Transwell assay in U251 and LN18 cells was performed to determined cell migration and invasion ability after co-transfected with si-HNF1A-AS1 and miR-22 mimics or miR-22 inhibitors. **P < 0.01 vs. si-NC group; #P < 0.05, ##P < 0.01 vs. si-HNF1A-AS1 group. Data are presented as mean±SD from three independent experiments.

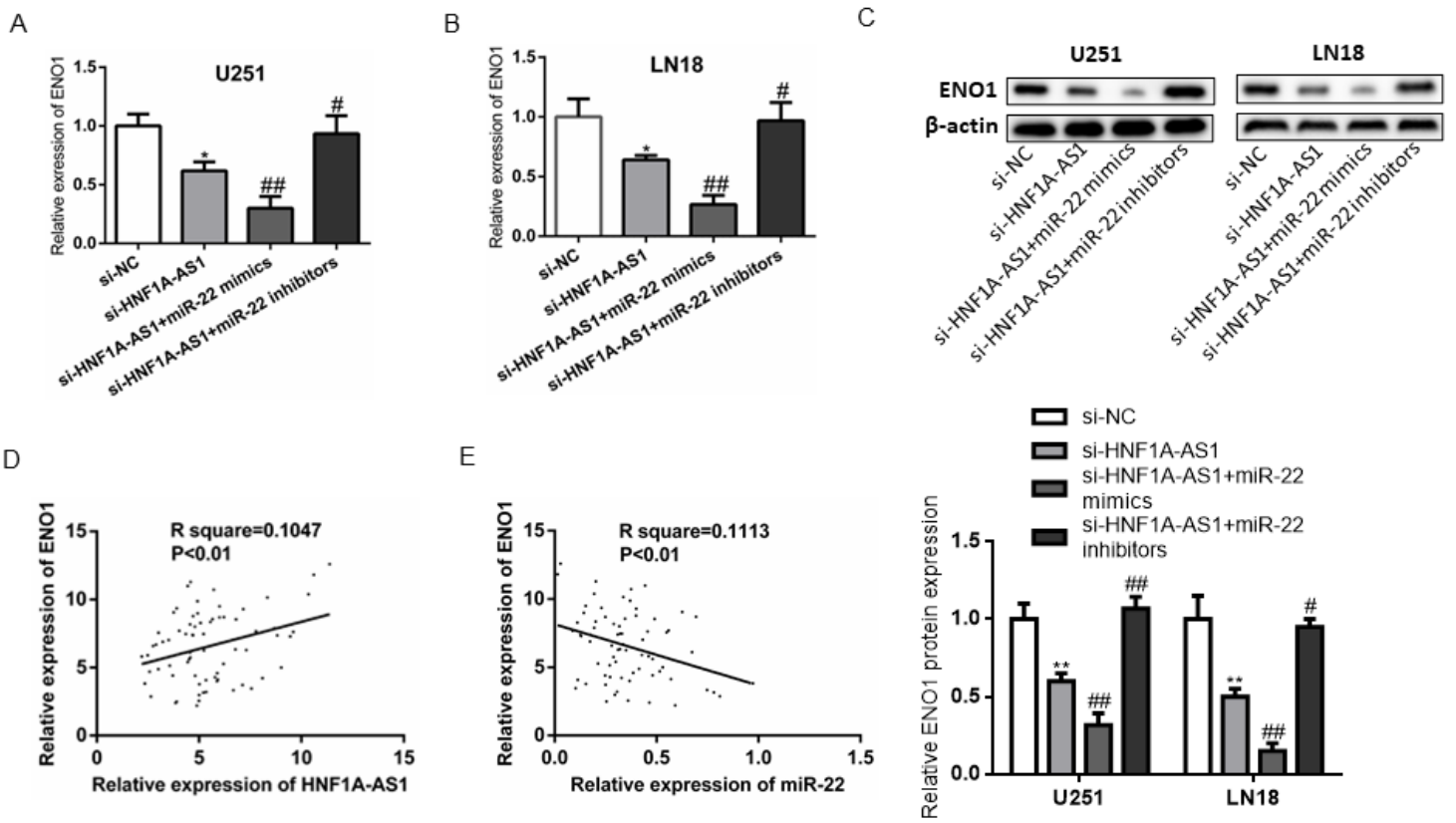

Figure 10

HNF1A-AS1 function as a ceRNA by inhibiting miR-22, and then strengthen ENO1 expression in GBM. (A-C) RT-QPCR and Western blot assays were performed to detect the mRNA and protein levels of ENO1 after cells transfected with si-HNF1A-AS1 and miR-22 mimics or miR-22 inhibitors. *P < 0.05, **P < 0.01 vs. si-NC group; #P < 0.05, ##P < 0.01 vs. si-HNF1A-AS1 group. Data are presented as mean±SD from three independent experiments. (D) Pearson's correlation analysis of the relationship between ENO1 expression and HNF1A-AS1 expression. (E) Pearson's correlation analysis of the relationship between ENO1 expression and miR-22 expression.

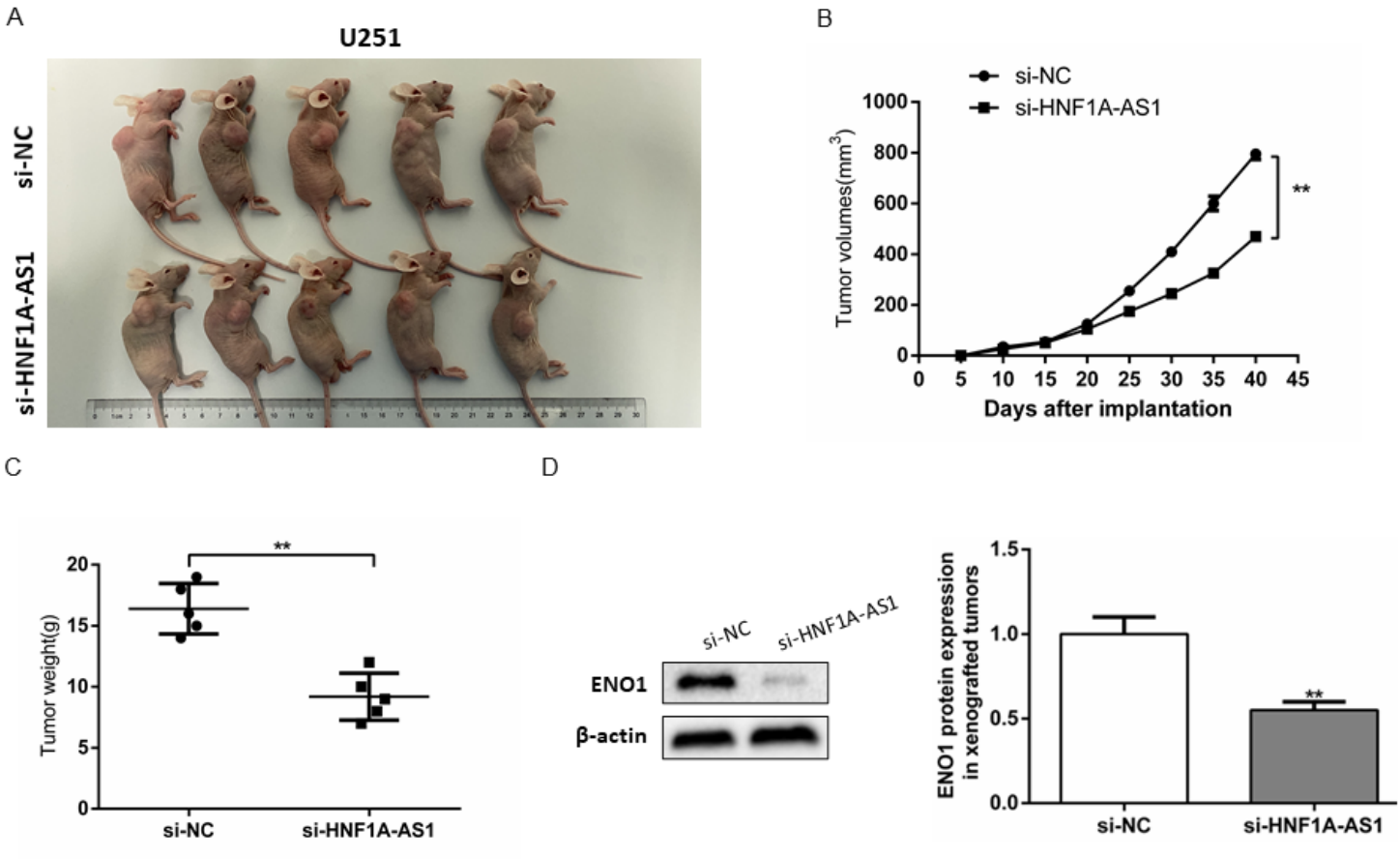

Figure 11

HNF1A-AS1 promotes tumor growth in vivo. (A) U251 cells were stably transfected with si-NC or si-HNF1A-AS1, which were injected subcutaneously into nude mice, respectively. (B) Tumor volumes were calculated every 5 days after injection. Bars indicate SD. (C) Tumor weight were significantly decreased in the si-HNF1A-AS1 group. (D) The protein levels of ENO1 in nude mice tumor tissues were tested by western blot. **P < 0.01 vs. si-NC group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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