

The Function of LINC00662/miR-340-5p/STAT3 Regulation Loop in Promoting Tumorigenesis and Development of Glioma

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Abstract

Background: Long noncoding RNAs(lncRNAs) have been reported to be associated with tumorigenesis and development of glioma. LINC00662 has been implicated in pathogenesis of various human cancers. However, role of LINC00662 in glioma remains unknown.

Methods: Bioinformatics methods were used to analysis the expression of LINC00662 in glioma. RT-qPCR was performed to examine the expression levels of LINC00662 in glioma tissues and cell lines. The effect of LINC00662 in cell proliferation and invasion was evaluated by Cell Counting Kit-8(CCK-8), clone colony formation and transwell assay. Luciferase reporter assays were performed to investigate the interaction between miR-340-5p and LINC00662, 3'UTR of STAT3. CHIP-qPCR and Luciferase reporter assays were used to demonstrate the interaction between STAT3 and the promoter region of LINC00662. Rescue assays and Tumor xenografts in nude mice were applied to verify the effect of LINC00662 in modulating miR-340-5p/ STAT3 signal pathway.

Results: LINC00662 was frequently high expressed and associated with malignant phenotype of glioma. LINC00662 knockdown inhibited proliferation, invasion and glioma-genesis of glioma. Moreover, LINC00662 knockdown repressed development of glioma by inhibiting the expression and activation of STAT3 pathway. Mechanically, LINC00662 acted as a ceRNA sponging miR-340-5p to protect the expression of STAT3. More importantly, LINC00662 was one of direct target genes of STAT3.

Conclusions: There was a positive regulation loop between LINC00662 and STAT3. LINC00662 might be an oncogene in glioma. Targeting LINC00662 was a potential strategy in glioma therapy.

Background

Glioma derives from glia cells, involving a broad pathological type including astrocytoma, oligodendrogloma and glioblastoma (GBM)[1]. Despite the advances in microsurgery, radio- and chemotherapy, the mean survival time of glioma rarely exceeds 15 months, for aggressiveness, infinite proliferation and rapid relapse and therapeutic resistance[2]. Therefore, clarifying the potential mechanisms of glioma and identifying novel potential therapeutic targets are of great importance for the long-term prognosis.

Long-non coding RNA (lncRNA), a type of non-coding RNA (ncRNA), is characterized by larger than 200 nucleotides length with less or no encoding protein capability[3]. In recent years, lncRNAs have attracted much attention for their important roles in various physiological and pathological processes, including immunity, organ development, organismal viability, tumorigenesis and tumor progression[4]. LncRNAs exert their roles by regulating gene transcription, modulating mRNA processing, mediating chromatin remodeling and histone modification, generating endogenous small-molecule RNA, acting as a structural component, combining with specific proteins, changing the cellular localization of the protein, maintaining protein activity and stability[5]. Nowadays, increasing studies have shown that dysregulated expressions of lncRNAs are involved in tumorigenesis and development of glioma[6]. For example,

Notch-lncRNA axis plays important role in regulating self-renewal of glioblastoma stem-like cells (GSCs) [7]. A peptide encoded by circular form of LINC-PINT suppresses oncogenic transcriptional elongation in glioblastoma[8]. Lnc-TALC promotes O6-methylguanine-DNA methyltransferase expression in glioma via regulating the c-Met pathway by competitively binding with miR-20b-3p[9]. FOXM1-AS maintains tumorigenicity of GSCs via promoting the interaction of FOXM1 nascent transcripts with ALKBH5[10]. To date, although lots of LncRNAs have been annotated, the biological significance and the key target genes of these lncRNAs in glioma remain elusive.

Signal transducer and activator of transcription 3 (STAT3) is an important mediator in glioma-genesis and progression[11]. Persistent activation of STAT3 is found in a high percentage of glioma cells and microenvironments. Activated STAT3 induces infinite proliferation, invasion, migration, angiogenesis, stem like cell maintenance and tumorigenesis of glioma cells[12]. Importantly, STAT3 is an interaction points of multiple signal pathways, including epidermal growth factor receptor (EGFR), heregulin-2/neuregulin receptor (Her2/Neu), platelet-derived growth factor receptor (PDGFR), phosphoinositol 3-kinase (PI3K)/Akt/mTOR, c-Met, interleukin-6 receptor (IL-6R/gp130) pathways, as well as various kinases, such as the Abelson leukemia protein (ABL) family of kinases and the Src family of kinases[13–15]. This makes STAT3 an attractive therapeutic target in glioma.

In this study, we observed that LINC00662 was significantly upregulated in glioma cells and glioma samples, and was associated with poor survival of patients. Functional analysis showed that knockdown of LINC00662 inhibited proliferation, invasion and tumorigenesis of glioma cells. Further mechanical investigations demonstrated that LINC00662 promotes the expression of STAT3 by sponging miR-340-5p. Moreover, LINC00662 was the target gene of STAT3. Blocking the positive feed loop of LINC00662/miR-340-5p /STAT3 inhibited the development of glioma.

Materials And Methods

Cells culture and glioma samples

Human glioma cell lines (U87, T98G, U118 and U251), human astrocyte (AS) and 293T cell were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA) under an atmosphere of 5% CO₂ and 95% air at 37 °C. Glioma samples and adjacent tissues were collected and flash frozen. The diagnosis were pathologically confirmed. The informed consent was got from each patient, and all of the protocols were reviewed by the Ethics Committee of the hospital and performed in accordance with national guidelines.

Online cancer database analysis

GSE4290 data set was downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?>), which contains 157 patients and 23 non-tumor controls with emphasis on LINC00662 gene expression (ID = GDS1962:209886_s_at). The

correlations of LINC00662 with STAT3 or its target genes (CCND1, VEGFA, MMP2, SOX2, BCL2L1) were analyzed. A large cohort analysis for the correlations and survival was performed using GEPIA (<http://gepia.cancer-pku.cn/>).

Invasion assay

Invasion assays were performed using transwell plates (Corning, Corning, NY) according to the manufacturer protocol. Briefly, 40 μ L matrigel (Becton Dickinson, (Bedford, MA)) was added to the upper chamber to coat filters, then 160 μ L serum-free medium with 1 \times 10⁵ glioma cells were added into the upper chamber. The lower compartments were filled with 500 μ L culture medium. After incubating for 24-48h in cell incubator, the cells that had not invaded were cleaned, and the cells that had invaded were fixed with paraformaldehyde and stained with hematoxylin. The images were scanned by Invitrogen EVOS FL AUTO (Thermo Fisher).

Cell viability assay and colony formation assay

Cell viability was assessed by the CCK-8 assay according to the manufacturer protocol. Briefly, cells were seeded into each well of 96-well plates, and the absorptions of the cells were tested using a CCK-8 kit (Dojindo, CK04) at different indicated time points. For the colony formation assay, 1000 indicated cells per well were seeded into six-well plates and cultured for 14 days. The colonies were fixed with paraformaldehyde and stained with 0.1% crystal violet. The images were scanned by Scan Wizard EZ and the number of clones was counted using Image J software.

Tumor xenografts in nude mice

Four-week-old, female, BALB/c nude mice were obtained from the Shanghai Animal Center, Chinese Academy of Sciences and were housed in a specific pathogen-free environment at Wuxi people's hospital. To initiate tumors, 5 \times 10⁶ indicated cells were injected subcutaneously into the flank of each nude mouse. Tumor volume was monitored by measuring tumor diameter every 4 days. Tumor volumes were calculated using the following formula: tumor volume (mm³) = (length \times width²)/2. At 3 weeks, mice were euthanized and tumor volumes and weights were recorded. Ethical approval was obtained from the Ethics Committee of the hospital.

Terminal Deoxynucleotidyl Transferasemediated dUTP Nick-End Labeling (TUNEL)

TUNEL assays were performed according to the manufacturer protocol (KeyGEN BioTECH). Briefly, the frozen sections were fixed with 4% paraformaldehyde for 20-30 min, and were washed three times with PBS for 5 min each at room temperature. Then, sections were immersed in PBS containing 1% Triton X-100 for 3-5min, followed with PBS washing. Each sample was incubated with 50 μ L TdT enzyme reaction solution, which was consisted of 45 μ l Equilibration Buffer, 1.0 μ l biotin-11-dUTP and 4.0 μ l TdT Enzyme, for 60 min at room temperature. After PBS washing, 50 μ l Streptavidin-TRITC Labeling Buffe was

added for 30min. Then, sections were covered with DAPI for 10 min at RT. Finally, the sections were coverslipped and scanned by Invitrogen EVOS FL AUTO (Thermo Fisher).

RNA Extraction and Real-Time Quantitative PCR

Briefly, total RNA was extracted from cells or tissues using TRIzol reagent (Invitrogen, USA), following the manufacturer's protocol. The RNA was reverse-transcribed into cDNA using a PrimeScriptTM RT reagent kit (TaKaRa, China). Quantitative PCR (qPCR) analyses were conducted using a SYBR Green RT-PCR kit (TaKaRa, China). All experiments were repeated at least three times. GAPDH was used as an internal control. Primers used for qPCR are: STAT3 Forward: TCGGCTAGAAACTGGATAACG, Reverse: TGCAACTCCTCCAGTTCTTAA; LINC00662: Forward: GTGGCTTCATGACTTGTGC, Reverse: AGCGTGTAGGCACGCTTAT; CCND1 Forward: AGAGGCGGAGGAGAACAA, Reverse: GAGAGGAAGCGTGTGAGG; BCL2L1 Forward: CCTGGGTTCCCTTCCCTT, Reverse: TCCTGGTCCTTGCATCTT; The primers of has-miR340 were purchased from ThermoFisher Scientific. The primers of GAPDH were purchased from Sangon Biotech (Shanghai, China).

Vector construction and transduction

The short hairpin RNAs (shRNAs) of human LINC00662 in lentivirus gene transfer vector encoding green fluorescent protein (GFP) were purchased from GeneChem (Shanghai, China). The shRNA sequences were: for LINC00662 KD1: GCTGCTGCCACTGTAATAA, LINC00662 KD2: CCTGCAGGCGTACAACCAA; siRNA of human STAT3 was purchased from HIPPOBIO (Huzhou, China). The siRNA sequences were: sense: GCCUCCAGUUUCACUACUAATT, antisense: UUAGGUAGUGAACUGGACGCCG. For LINC00662, STAT3 or miR-340-5p overexpression experiments, the indicated cells were infected with lentivirus containing the LINC00662-GV502, STAT3-GV358 plasmids synthesized by GeneChem (Shanghai, China). All transfections were conducted following the manufacturer's instructions. MiRNA-340 mimics and inhibitors were obtained from GeneChem (Shanghai, China). The transfections were conducted using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions.

Cellular fractionation

The cytoplasmic and nuclear RNA were isolated and purified using the Cytoplasmic and Nuclear RNA Purification Kit (Norgen, Canada) following the manufacturer's instruction. The expression level of LINC00662 was tested by qRT-PCR. GAPDH and U6 were used as internal control (Sangon Biotech).

Western Blot

Cells or tissues were lysed in lysis buffer. Equal amounts of protein were electrophoresed and transferred to PVDF membranes (sigma, UK). After blocking with skimmed milk, they were incubated with primary and secondary antibodies (STAT3, CCND1\VEGF, MMP2\SOX2\BCL2L1). Protein bands were visualized by FluorChem Q (Proteinsimple). GAPDH was used as a normalization control.

Luciferase reporter assays

The fragments from LINC00662 or STAT3 mRNA 3'UTR containing the predicted miR-340-5p binding site or the corresponding mutants, as well as the fragments from LINC00662 promoter containing the predicted STAT3 protein binding site or the corresponding mutants were synthesized and cloned into plasmids. The indicated plasmids or miRNA mimics were co-transfected into 293T cells using Lipofectamine 3000 (Invitrogen, USA). The luciferase activity of transfected cells was tested by the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

Chromatin immunoprecipitation (ChIP)

ChIP assays were conducted using the Magna ChIP Kit (Millipore) according to the manufacturer's instructions. In brief, the crosslinked chromatin was sonicated into 200–300bp fragments. Next, the lysate was immunoprecipitated with antibody against STAT3 (CST), or with control IgG (abcam). The precipitated chromatin DNA was recovered and assessed by qRT-PCR with specific primers for LINC00662, CCND1 and BCL2L1.

RNA immunoprecipitation (RIP) assay

RIP was conducted using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) according to manufacturer instructions. Briefly, the indicated cells were harvested and lysed with protease inhibitors cocktail (Roche, Switzerland) and RNase inhibitors (Invitrogen, USA). Next, they were incubated with magnetic beads coated with Ago2 antibodies (Millipore, Billerica, MA, USA) overnight at 4°C. Subsequently, RNA was isolated by TRIzol (Invitrogen, USA) and reverse-transcribed into cDNA. RT-qPCR was used to determine the levels of precipitated RNAs.

Statistical analysis

Comparisons were performed using the Kaplan–Meier method with log-rank test, χ^2 -test, Spearman's rank correlation analysis, two-tailed Student's t-test. An F test was used to test variance equality. Differences were considered significant when $P < 0.05$. SPSS 16.0 package (IBM) and Graphpad prism 5.0 software (GraphPad Software) were used for all statistical analyses and data graphing, respectively.

Results

The expression of LINC00662 in glioma and its clinical significance

In the silico analysis for GEO (GSE-4290) data, we found that LINC00662 was highly expressed in glioma and was significantly associated with tumor grades (Fig. 1A). To confirm the significance of LINC00662 in glioma, in silico analysis by GEPIA, we also found LINC00662 was highly expressed both in GBM and LGG (low grade glioma) than normal brain tissue (Fig. 1B). In survival analysis, higher LINC00662 predicted poorer prognosis (Fig. 1C). Next, we compared the expression of LINC00662 in 24 fresh GBM samples and their adjacent tissues. We found that the expression of LINC00662 in GBM was significantly higher than in adjacent tissues (Fig. 1D). The expression of LINC00662 in glioma cell lines was tested. Compared to the normal astrocytes, the expression of LINC00662 in glioma cell lines increased

significantly, especially in U251(Fig. 1E). Those results implied that LINC00662 may play an important role in glioma development.

LINC00662 acts as an oncogene in glioma

To explore the function of LINC00662 in glioma, we established LINC00662 stable knockdown (KD) or overexpression (OE) cell lines by lentivirus-delivered LINC00662-shRNAs or plasmids in U251 cell. The expression level of LINC00662 was tested by RT-qPCR (Fig. 2A, B). We found shRNA2 showed the most suppressive effect, which was used for further study. Then the proliferation ability of glioma cells were tested by CCK-8 assay and clone formation. Results showed that LINC00662 KD significantly attenuated the proliferation of U251 cells (Fig. 2C, E, F). To determine the effect of LINC00662 KD on invasion, transwell assays were performed. As shown in Fig. 2E-H, the invasion of U251 cells were remarkably inhibited by LINC00662 KD. Tumor xenografts in nude mice were used to demonstrate the role of LINC00662 in vivo level, the result showed that LINC00662 KD inhibited the development of glioma (Fig. 2G). TUNEL staining of glioma tissue sections showed that LINC00662 KD increased cell apoptosis significantly (Fig. 2H).

LINC00662 mediates STAT3 pathway in glioma

Persistent activation of STAT3 has been found in a high percentage of GBM cells and tumor microenvironments. STAT3 is a critical mediator of tumorigenesis, tumor progression, stem cell maintenance, angiogenesis and immune evasion in GBM. To examine whether LINC00662 contributes to glioma-genesis and progression by STAT3 pathway, we first analyzed the correlation between LINC00662 and STAT3 downstream genes in GSE4290. The results showed that LINC00662 positively correlated to mRNA level of STAT3 (Fig. 3A) and its target genes in glioma, including SOX2, VEGFA, CCND1, MMP2, BCL2L1 (Fig. 3B-F). Since a positive correlation between LINC00662 and STAT3 pathway was found in glioma by bioinformatic analysis, it was reasonable to validate the effect of LINC00662 on the expression of STAT3 mRNA and protein in glioma cell lines. We tested the effect of LINC00662 KD or OE on the mRNA and protein expression of STAT3 pathway in cell lines. As shown in Fig. 3G-I, LINC00662 KD decreased mRNA expression of STAT3 target genes in U251 cells, and the effect of LINC00662 KD on protein levels of STAT3 target genes were in accordance to their mRNA levels in cells.

miRNA-340-5p is the target of LINC00662 and acts as a cancer suppressor in glioma

In order to investigate the molecular mechanism by which LINC00662 regulated STAT3 pathway, we first tested the subcellular distribution of LINC00662 in glioma cells and found that LINC00662 mainly located in cytoplasm and partially in nucleus of U251 (Fig. 4A). This implied that LINC00662 may modulate STAT3 pathway by interacting with miRNAs. ENCORI was used to predict miRNAs which can interact with both LINC00662 and 3' UTR of STAT3 mRNA. The venn diagram showed that LINC00662 and STAT3 mRNA have the same potential interacted miRNAs, one of which was miRNA-340-5p (Fig. 4B). To investigate the function of miRNA-340-5p in glioma cells, we first tested the expression of miRNA-340-5p in glioma cell lines. Interestingly, the expression of miRNA-340-5p was contrary to the expression of

LINC00662 in glioma cell lines (Fig. 4C). What's more, the expression of miR-340-5p was lower in GBM samples than their adjacent tissues (Fig. 4D), and was negatively correlated to the expression of LINC00662 (Fig. 4E). To determine whether the function of miRNA-340-5p was opposite to LINC00662, we tested the effect of miRNA-340-5p on the cell proliferation and invasion by over-expressing miRNA-340-5p in U251. As shown in Fig. 4F-I, the malignant phenotypes were inhibited by ectopic expression of miRNA-340-5p in U251. So, protecting the expression of miRNA-340-5p may be beneficial to inhibit the progression and development of glioma.

LINC00662 modulates the expression of STAT3 mRNA by sponging miRNA-340-5p

As miR-340-5p may be the target of LINC00662, it was reasonable to propose that there may exist a ceRNA mechanism between LINC00662 and miR-340-5p/ STAT3. To investigate whether there was a reciprocal regulation between LINC00662 and miRNA-340-5p, we first tested the expression of miRNA-340-5p in glioma cells with LINC00662 OE or KD. As shown in Fig. 5A, the expression of miRNA-340-5p upregulated in U251 with LINC00662 KD, while downregulated with LINC00662 OE. Reciprocally, miRNA-340-5p mimics decreased the expression of LINC00662 and STAT3 in U251 (Fig. 5B). Then we demonstrated if LINC00662 regulated the expression of STAT3 through miRNA-340-5p. U251 cells with LINC00662 OE was transfected with miRNA-340-5p mimics and U251 cells with LINC00662 KD was transfected with miRNA-340-5p inhibitor, RT-qPCR and Western Blotting were used to detect the expression of STAT3 mRNA and protein level. We found that miRNA-340-5p mimics decreased the upregulation of STAT3 caused by LINC00662 OE (Fig. 5C), while miRNA-340-5p inhibitor increased the downregulation of STAT3 resulted from LINC00662 KD (Fig. 5D). To determine whether miR-340-5p directly binded with LINC00662 and 3'UTR of STAT3 mRNA, we performed dual luciferase reporter assays. We firstly constructed wild-type reporter plasmids containing sequence of LINC00662 and 3'UTR of STAT3 mRNA, and mutated type plasmids containing mutated sequence (Fig. 5E). The luciferase reporter assays showed that miR-340-5p attenuated the luciferase activity of wild type plasmids, but no obvious changes in mutated type plasmids were observed (Fig. 5F). Then, the rescue experiments were performed. We found LINC00662 OE rescued the decreased luciferase activity of STAT3 WT reporter plasmid caused by miR-340-5p mimics (Fig. 5G). In addition, anti-Ago2 RIP assay was employed to reveal that the Ago2 protein could bind to LINC00662 and miR-340-5P directly in glioma cells (Fig. 5H). Cell colony formation assay and invasion assay showed that miR-340-5p mimics and STAT3 KD attenuated the increased cell proliferation and invasion caused by LINC00662 OE (Fig. 5I, J). The expression of miR-340-5p, STAT3 and target genes of STAT3 in tissues from tumor xenografts were detected by RT-qPCR and Western Blotting, results showed that LINC00662 regulated miR340-5p/ STAT3 pathway in vivo (Fig. 5K, L). Those data indicated that LINC00662 could regulate the expression of STAT3 by competitively sponging miR-340-5p both in vitro and in vivo.

LINC00662 is a direct target gene of STAT3

AS we have found the positive correlation between LINC00662 and STAT3, we wondered whether STAT3 has an effect on LINC00662 expression. As shown in Fig. 6A, the expression of LINC00662 was

upregulated in U251 after STAT3 OE or stimulating with IL-6 (activator of STAT3), but S3I-201 (inhibitor of STAT3) could attenuate this upregulation. As STAT3 is a transcription factor, it was reasonable to speculate whether it regulated the expression of LINC00662 in transcriptional level. We forecasted the binding motif of STAT3 using JASPAR (Fig. 6B ; <http://jaspar.genereg.net>). Next, we got the genomic sequence upstream regions (-2000 bp upstream) of LINC00662 using UCSC (<http://genome.ucsc.edu/>) and predicted the binding site of STAT3 on the promoter region of LINC00662 using PROMO (<http://alggen.lsi.upc.es/>). We found the sequence of predicted sites keeps high similarity to the predicted motif of STAT3 (Fig. 6C). To investigate whether STAT3 directly binded to the predicted site, we generated firefly luciferase reporter plasmids with DNA fragments containing wild-type or mutant-type binding sequence of LINC00662 promoter region (Fig. 6D). As expected, we observed that luciferase activity from the reporters with two individual wild-type sequence was increased by ectopic expression of STAT3 in 293T cells. Whereas, the activity of the reporters with mutant sequence was failed to be induced (Fig. 6E). Then, the Chip-qPCR assay was performed to determine whether STAT3 directly binded to the two predicted promoter sites of LINC00662. As shown in Fig. 6F, STAT3 could directly bind to the promoter of LINC00662, and the STAT3 DNA-binding ability was increased by IL-6 stimulation.

Discussion

As the most common and malignant intracranial tumors, the pathogenesis of glioma is still unclear, and the methods of treatment are very limited, which are the main reasons for its poor prognosis[16]. Previously, lncRNAs were considered as transcriptional noise. Recently, accumulating studies have shown that lncRNAs are involved in multiple biological processes[17, 18]. Up to date, growing evidence have revealed that lncRNAs are detected as biomarkers for the diagnosis and prognosis in glioma samples[19–21]. Therefore, verifying the regulation mechanism of lncRNAs in glioma is crucial to discover new treatment methods in future[22].

Aberrant expression of LINC00662 has been found in several tumors and associated with prognosis of tumors, including prostate cancer, oral squamous cell carcinoma, gastric cancer and lung cancer[23–27]. In function analysis, some studies report that LINC0062 acts as an oncogene in tumors, such as promoting proliferation, migration and invasion of cancer cells, and maintaining stem cell-like phenotypes[23, 24, 26]. However, the expression pattern of LINC0062 in glioma has not been investigated. In present study, we reported that LINC00662 was significantly upregulated in GBM as compared with the adjacent tissues. And in silico analysis, we found that LINC00662 expression was significantly correlated with tumor grade and prognosis. Those imply that LINC00662 may also play an important role in gliomagenesis. Furtherly, the functional analysis showed that LINC00662 knockdown inhibited the proliferation, invasion and tumorigenesis of glioma. Those evidences indicated that LINC00662 acted as an oncogene in glioma.

STAT3, as an important transcription factor, plays critical role in tumorigenesis and progression of glioma[11]. It can be activated by a variety of stimuli, including cytokines, growth factors and interferons[28]. STAT3 drives the transcription of a variety of genes which affect numerous aspects of

cell survival and growth[29]. Several lncRNAs have been documented to influence STAT3 pathway, including TSLNC8, OLA1P2, DILC, LncSox4 and LncRNA-p21[30–34]. In this study, we found LINC00662 expression was significantly correlation with expression of STAT3 mRNA and its target genes (CCND1, VEGF, MMP2, SOX2, BCL2L1). Those genes play important roles in mediating the proliferation, anti-apoptosis, migration and GSC maintaining of glioma[28, 35]. Knockdown of LINC00662 inhibited the expression of these genes which implied that LINC00662 promoted the development and progression of glioma through STAT3 pathway. Thus, inhibition of LINC00662 expression may be a new therapeutic method for glioma through indirectly inhibition of STAT3 pathway.

The competitive endogenous RNA (ceRNA) hypothesis reveals a new mechanism of RNA interaction[36]. It is known that miRNAs can cause gene silencing by binding mRNAs, while ceRNA can regulate gene expression by competitively binding microRNAs, which reveals the existence of an RNA-microRNA regulatory pathway and has great biological significance[37]. In this case, non-coding RNAs may act as competitive ceRNAs to regulate the expression level of miRNA targets by sponging miRNAs[18]. Recently, accumulating evidences have shown that the lncRNAs can act as ceRNAs to participate in the regulation of biological processes, thereby involving in the occurrence and development of tumors.[38] To further understand how LINC00662 regulated STAT3 mRNA, we analyzed the possible regulatory mechanism using bioinformatics methods. The prediction showed that LINC00662 could function as a ceRNA binding to miR-340-5p to regulate STAT3. In correlation analysis of glioma samples, we found expression of LINC00662 was negatively associated with expression of miR-340-5p. Then, our data confirmed that there was a direct interaction between miR-340-5p and LINC00662 or STAT3 3'UTR. LINC00662 regulated STAT3 by acting as a ceRNA and competitively binding to miR-340-5p to block interaction between miR-340-5p and STAT3 3'UTR.

Inflammatory microenvironment of tumors plays an important role in promoting the development and progression of tumor[39]. IL-6 is one of important pro-inflammatory factors in microenvironment, which binds to its IL-6 receptor (IL-6R) or gp130 to activate multiple signaling pathways. Among those pathways, the JAK/STAT3 pathway is one of the most studied pathways, which leads to JAK2 recruitment and STAT3 phosphorylation, Phosphorylated STAT3 alters the transcription of many genes[29, 40, 41]. Interestingly, in this study, we found the expression of LINC00662 could be induced under inflammatory environment, such as IL-6. Furtherly, ectopic STAT3 promoted the expression of LINC00662, which could be blocked by STAT3 inhibitor. In silico analysis, we predicted the binding motif of STAT3, and found there were binding sites of STAT3 in the promoter area of LINC00662. Then, luciferase assay and chip assay convinced that STAT3 could binding the promoter of LINC00662 and promote the transcription. Those data indicated that there was a reciprocal regulation between LINC00662 and STAT3.

MiRNAs are RNA molecules with length of about 20–24 nucleotides that are transcribed from DNA but cannot be further translated into protein[42]. MiRNAs specifically bind to target mRNA to inhibit post-transcriptional gene expression, which play an important role in regulating gene expression[43]. In present study, we found that the expression of miR-340-5p was downregulated in GBM tissues compared with its

expression in adjacent tissues. Furthermore, ectopic miR-340-5p significantly inhibited cell proliferation and invasion in vitro. Our evidences indicated that miR-340-5p acts as a tumor suppressor in glioma. Those data were in accordance with the previous reports that miR-340 functioned as a tumor suppressor in several cancers, including gastric cancer, breast cancer, squamous cell carcinoma, ovarian cancer, osteosarcoma, multiple myeloma and non-small cell lung cancer[44, 45]. In this study, we found that the expressions of LINC00662, STAT3 and STAT3 target genes were downregulated with miR-340-5p OE in U251 cells. This data indicated that miR-340-5p may play its role through blocking the reciprocal regulation between LINC00662 and STAT3.

In summary, our study showed that LINC00662 was significantly upregulated in glioma and associated with the poor prognosis. LINC00662 promoted the development of glioma by acting as a ceRNA and sponging miR-340-5p to protect the expression of STAT3. Furthermore, our study also showed that there was a reciprocal regulation between LINC00662 and STAT3, and blocking the positive feed loop of LINC00662/STAT3 could inhibit the tumorigenesis of glioma cells. Therefore, LINC00662 may be considered as an useful tumor biomarker in glioma and targeting LINC00662 may be a novel therapeutic strategy for patients with glioma.

Conclusions

Our study provides the first evidence that LINC00662 promotes cell proliferation, invasion and tumorigenesis of glioma by targeting miR-340-5p/STAT3. Simultaneously, STAT3 interacts with the region of LINC00662 promoter and promotes the expression of LINC00662 (Fig. 7). Our research provides a new mechanism of glioma progression and a promising prognostic biomarker of glioma.

Abbreviations

lncRNAs: Long noncoding RNAs; CCK-8:Cell Counting Kit-8; ceRNA: competing endogenous RNA; GBM: glioblastoma; ncRNA: non-coding RNA; GSCs: glioblastoma stem-like cells; STAT3: Signal transducer and activator of transcription 3; EGFR: epidermal growth factor receptor; PDGFR: platelet-derived growth factor receptor; PI3K:phosphoinositol 3-kinase; TUNEL: Terminal Deoxynucleotidyl Transferase mediated dUTP Nick-End Labeling; RT-qPCR: Real-Time Quantitative PCR; ChIP: Chromatin immunoprecipitation; RIP: RNA immunoprecipitation.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Wei Ji, Zhengwei Li and Likun Song design the study. Chao Cheng, Jun Sun, Yuankun Liu, Weiyi Huang, Jin Huang, Jin Liu and Bin Xu performed all the experiments. Jiantong Jiao, Junfei Shao and Hongyi Liu write and revise the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used during this research are available from the corresponding authors upon reasonable request.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Wuxi People's Hospital of Nanjing Medical University. All patients provided written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

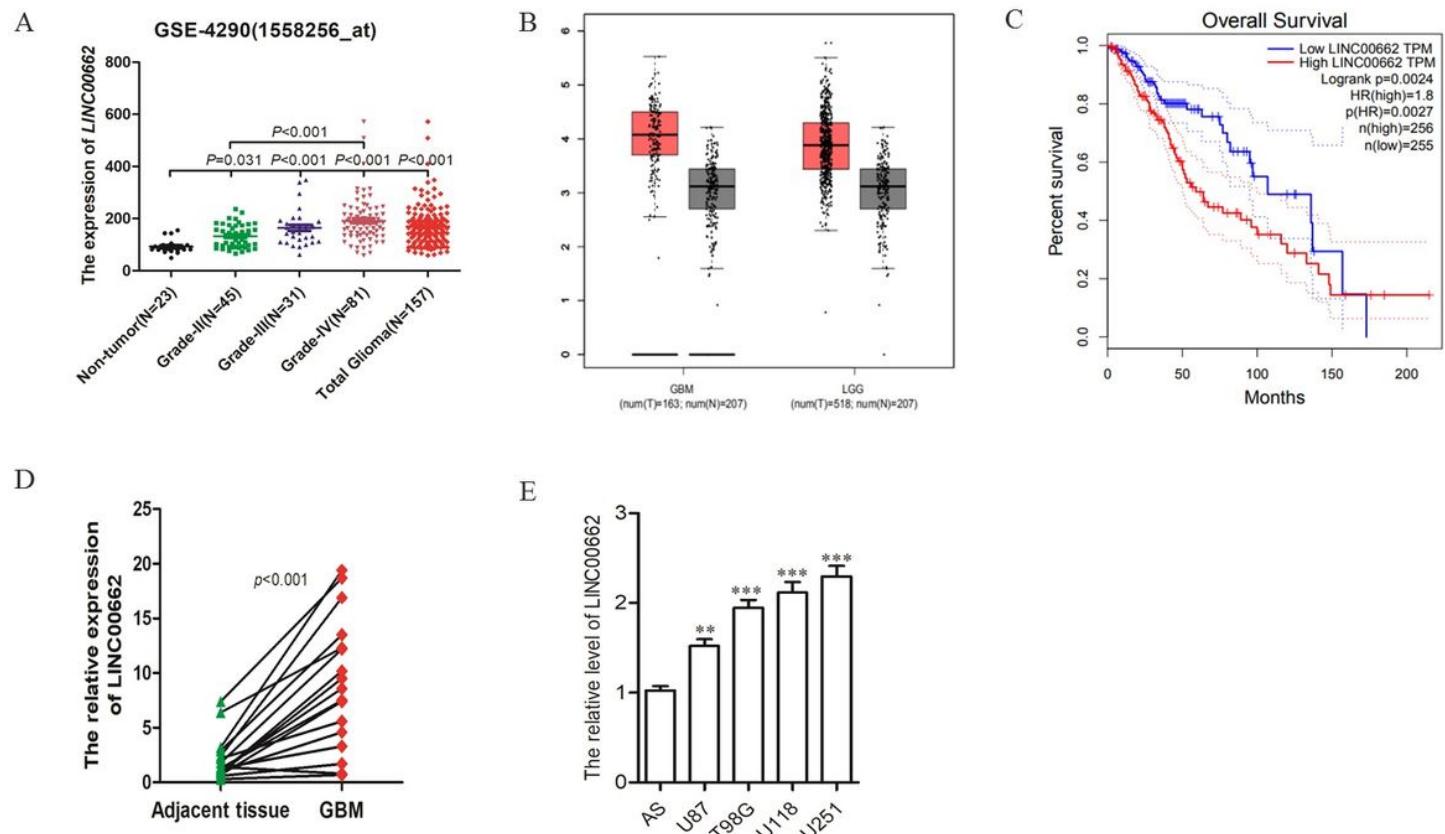


Figure 1

LINC00662 was significantly upregulated in glioma and predicted poor prognosis. A. The expression of LINC00662 in the silico analysis for GEO (GSE-4290) data. B-C. The expression and survival analysis of LINC00662 in GEPIA dataset. D. The expression of LINC00662 in 24 pairs of GBM and adjacent tissues. E. The expression of LINC00662 in glioma cell lines and human astrocyte (**p<0.01, ***p<0.001).

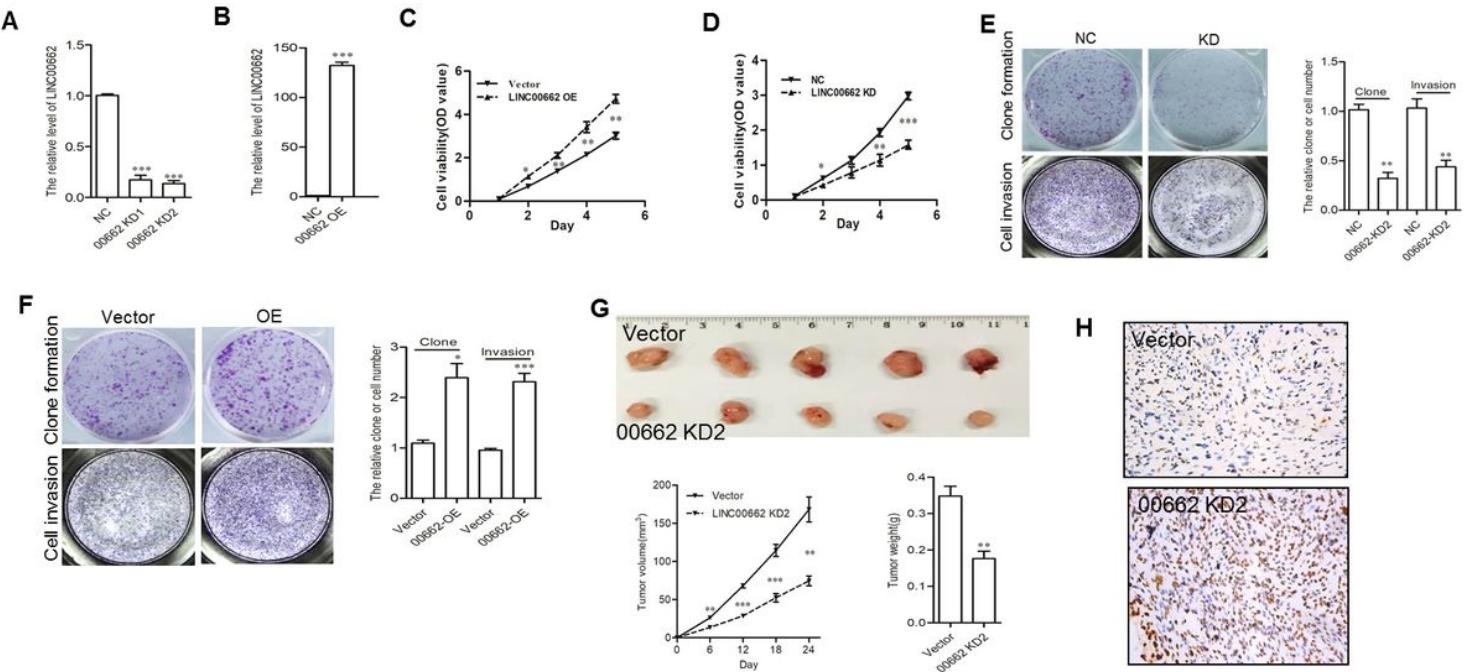


Figure 2

LINC00662 acts as an oncogene in glioma. A-B. LINC00662 was knocked down or overexpressed in U251 (**p<0.001). C-D. CCK-8 was used to assessed the proliferation of U251 with LINC00662 OE or KD (*p<0.05, **p<0.01, ***p<0.001). E-F. The proliferation and invasion of U251 with LINC00662 OE or KD was evaluated by cell clone formation and transwell assay (*p<0.05, **p<0.01, ***p<0.001). G. Tumor xenografts in nude mice showed that LINC00662 KD suppressed the development of glioma (**p<0.01, ***p<0.001). H. TUNEL staining of glima tissue sections was employed to demonstrate the effect of LINC00662 KD in cell apoptosis.

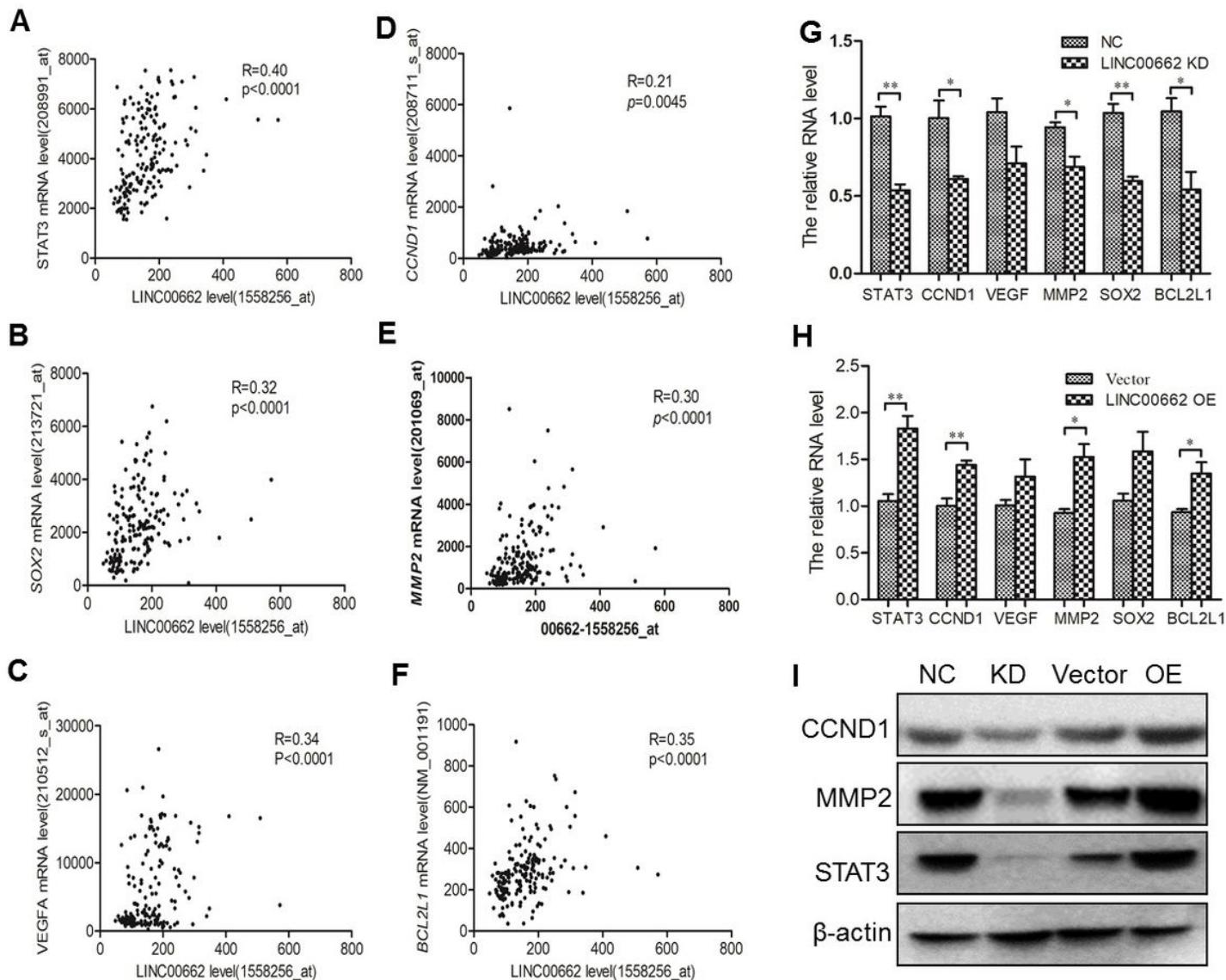


Figure 3

LINC00662 modulated STAT3 pathway in glioma. A-F. Correlation analysis was performed between LINC00662 and STAT3 downstream target genes in GSE-4290. G-H. The effect of LINC00662 KD or OE on the mRNA expression of STAT3 and its target genes (CCND1, VEGF, MMP2, SOX2, BCL2L1) (*p<0.05, **p<0.01). I. Western blot analysis of STAT3 and its target genes (CCND1, MMP2) in U251 with LINC00662 alteration.

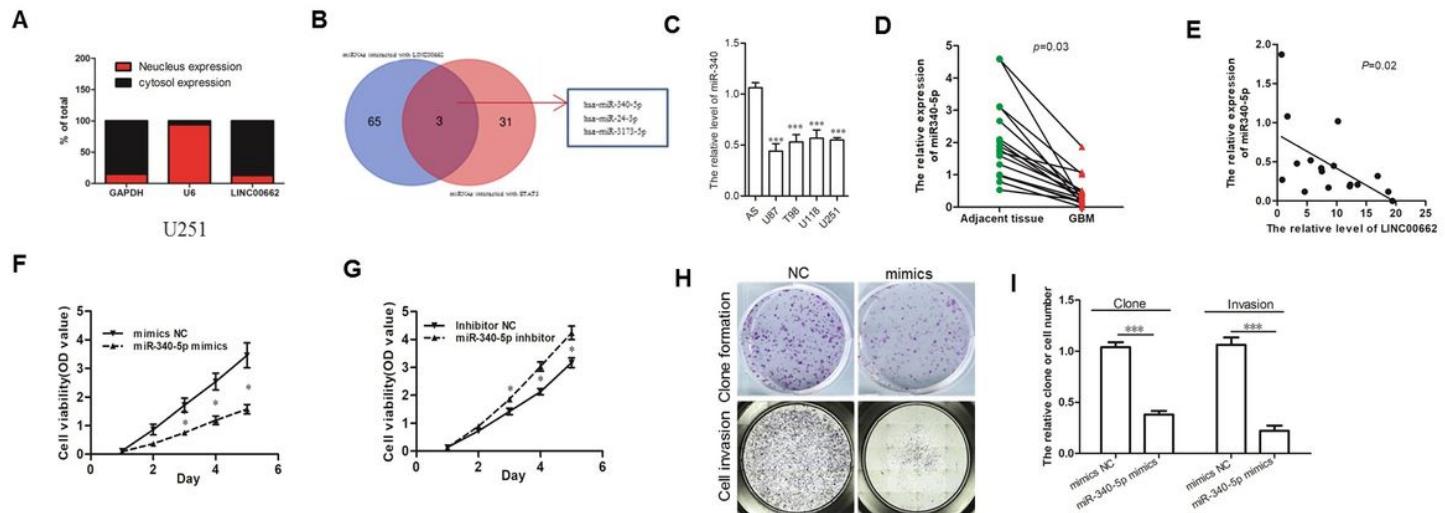


Figure 4

MiRNA-340-5p is the target of LINC00662 and acts as a cancer suppressor in glioma. A. Cellular fractionation showed that LINC00662 mainly located in cytoplasm and partially in nucleus of U251. B. ENCORI was used to predict miRNAs which can interact with both LINC00662 and 3' UTR of STAT3 mRNA. C. The expression of miR-340-5p in glioma cell lines and human astrocyte(** $p<0.001$). D. The expression of miR-340-5p in 24 pairs of GBM and adjacent tissues. E. Correlation analysis between the expression of LINC00662 and miR340-5p in GBM tissues. F-G. The influence of miR-340-5p OE or KD on cell proliferation was assessed by CCK-8 (* $p<0.05$). H-I. The proliferation and invasion of U251 with miR-340-5p OE was evaluated by cell clone formation and transwell assay (** $p<0.001$).

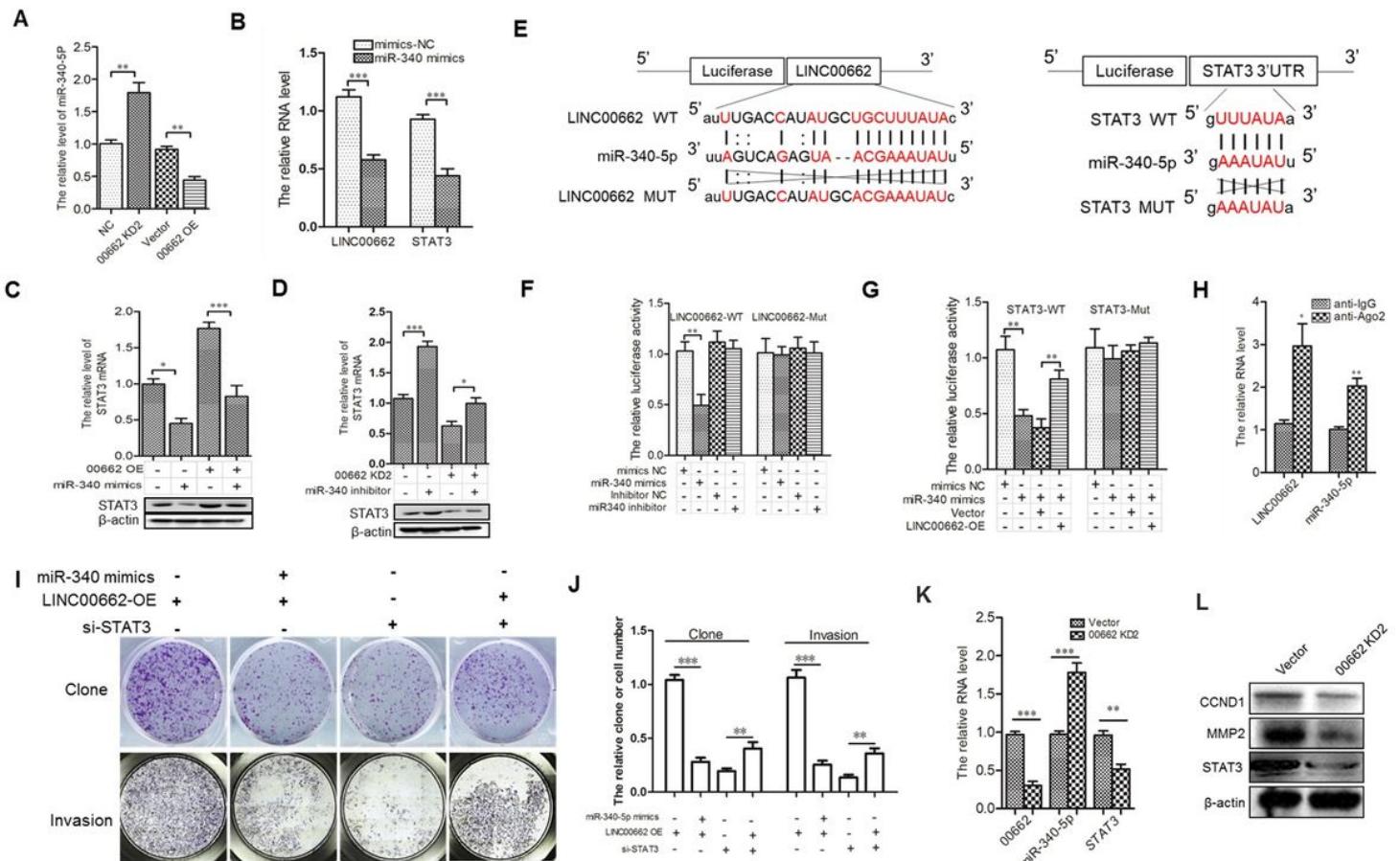


Figure 5

LINC00662 regulated the expression of STAT3 mRNA by sponging miRNA-340-5p. A. RT-qPCR analysis the effect of LINC00662 KD or OE on the expression of miR-340-5p in U251 (**p<0.01). B. The expression of LINC00662 and STAT3 mRNA with miRNA-340-5p OE in U251 (**p<0.001). C-D. miRNA-340-5p reverse the influence of LINC00662 alteration on the mRNA and protein level of STAT3. E. Construction of Luciferase reporter plasmids. F-G. Luciferase reporter assays as indicated were performed in 293T cells (**p<0.01). H. Ago antibody was used to perform RIP (*p<0.05, **p<0.01). I-J. miRNA-340-5p and STAT3 KD abolished the promotion of cell proliferation and invasion mediated by LINC00662 overexpression in U251 cells (**p<0.01, ***p<0.001). K-L. The expression of miRNA-340-5p, STAT3, CCND1 and MMP2 were downregulated in tumor xenografts with LINC00662 KD.

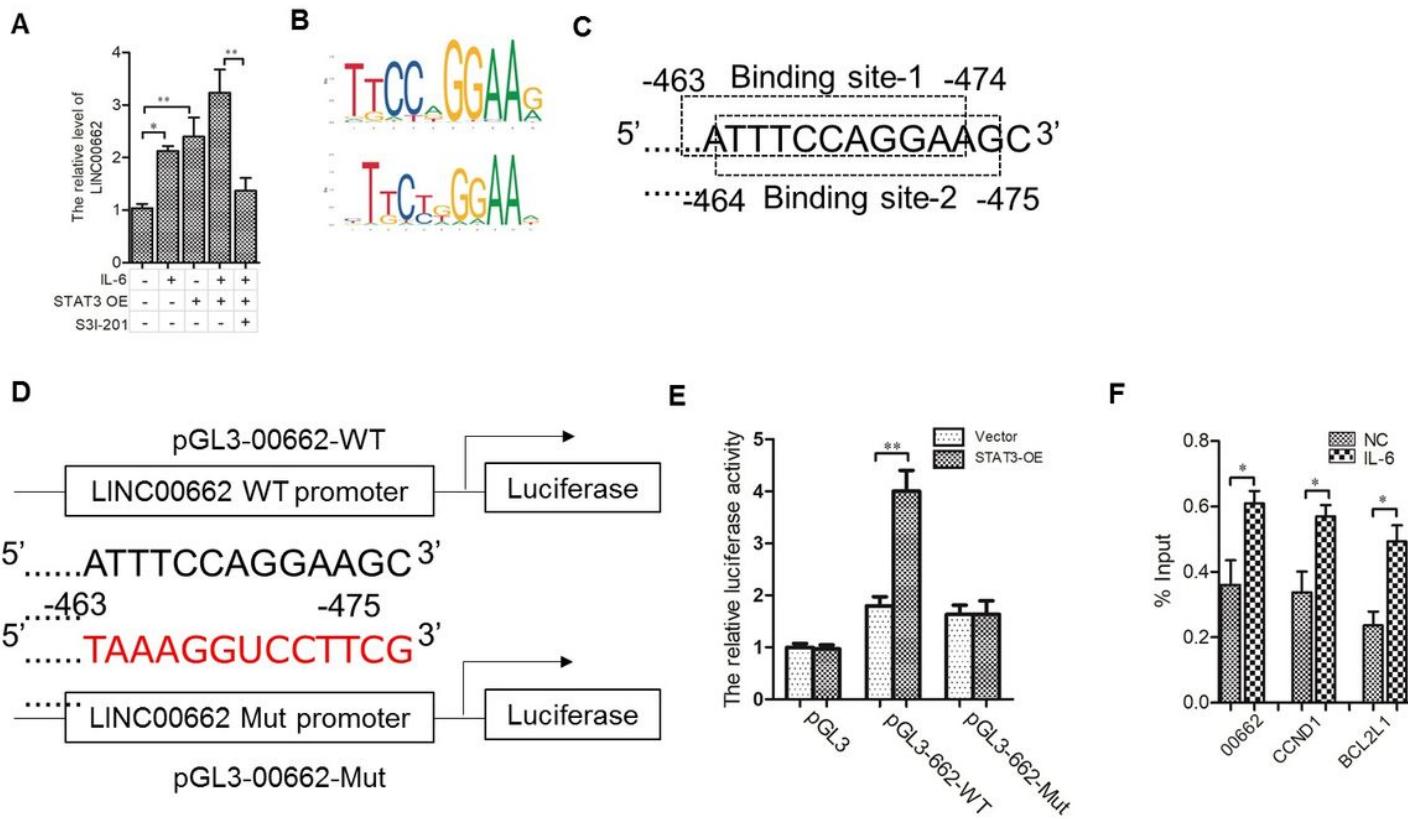


Figure 6

LINC00662 is a direct target of STAT3. A. The expression of LINC00662 in U251 cells with IL-6 or S3I201 stimulated or STAT3 OE ($*p<0.05, **p<0.01$). B-C. JASPAR and PROMO were used to forecast the binding motif between STAT3 and the promoter region of LINC00662. D. Construction of Luciferase reporter plasmids which contains wild-type or mutant-type of LINC00662 promoter region. E. Luciferase reporter assays were employed to demonstrate that STAT3 could upregulate the transcription of LINC00662 by binding to its promoter region ($**p<0.01$). F. ChIP-qPCR assay was performed to detect the binding between STAT3 and LINC00662 ($*p<0.05$).

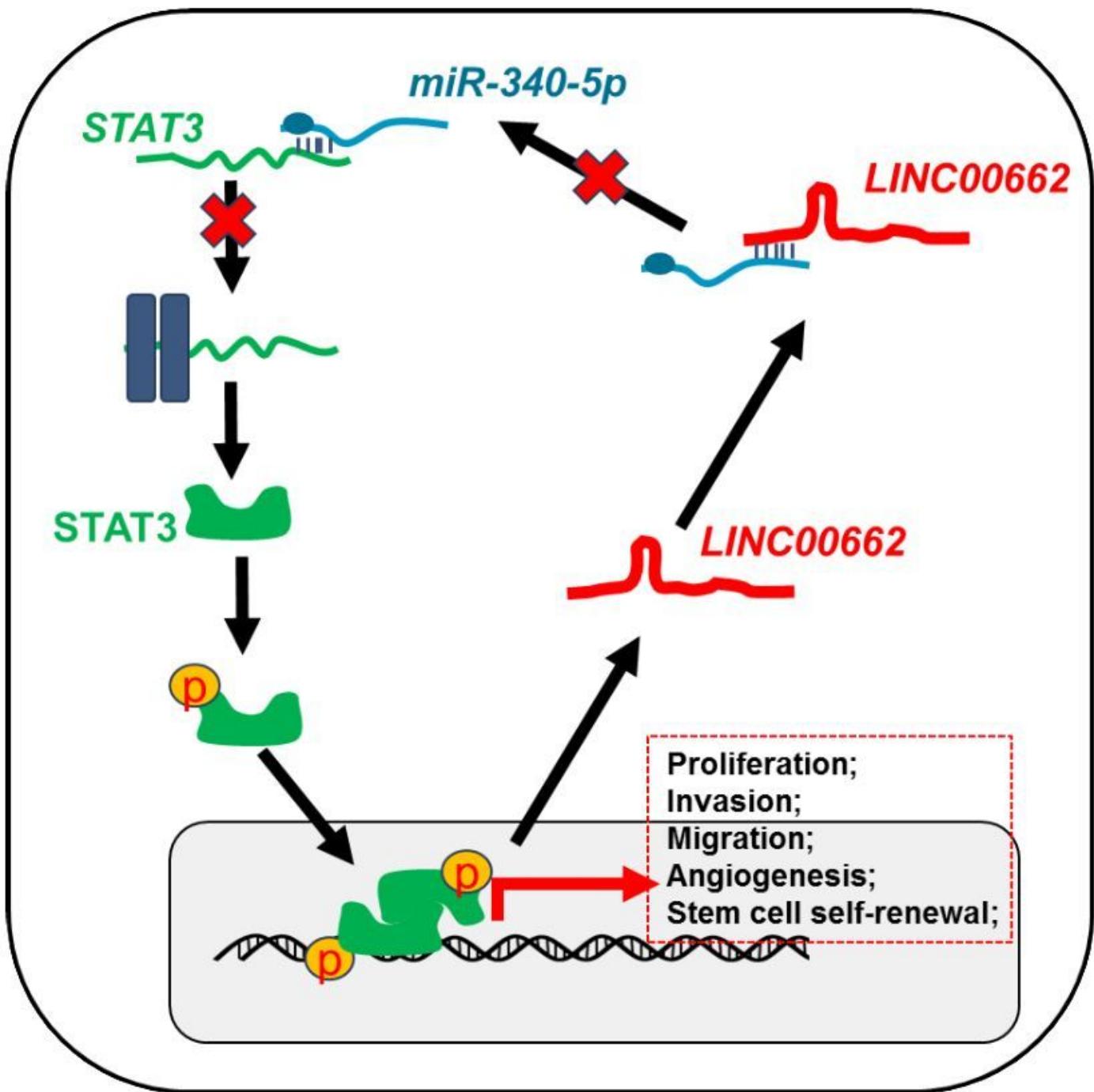


Figure 7

The chart of *LINC00662*/*miR-340-5p*/*STAT3* feedback loop in glioma. *LINC00662* promotes cell proliferation, invasion and tumorigensis of glioma by targeting *miR-340-5p*/*STAT3*. Simultaneously, *STAT3* interacts with the region of *LINC00662* promoter and promotes the expression of *LINC00662*.