Lnc-THOR silencing inhibits human glioma cell survival by activating MAGEA6-AMPK signaling

Jun Xue1, Shan Zhong1, Bo-min Sun2, Qing-Fang Sun1, Liang-Yun Hu2 and Si-Jian Pan1

Abstract

Long non-coding RNA THOR (Lnc-THOR) binds to IGF2BP1, essential for its function. We here show that Lnc-THOR is expressed in human glioma tissues and cells. Its expression is extremely low or even undetected in normal brain tissues, as well as in human neuronal cells and astrocytes. We show that Lnc-THOR directly binds to IGF2BP1 in established and primary human glioma cells. shRNA-mediated Lnc-THOR knockdown or CRISPR/Cas9-induced Lnc-THOR knockout potently inhibited cell survival and proliferation, while provoking glioma cell apoptosis. Contrarily, forced overexpression of Lnc-THOR promoted glioma cell growth and migration. Importantly, Lnc-THOR shRNA or knockout activated MAGEA6-AMPK signaling in glioma cells. AMPK inactivation, by AMPKα1 shRNA, knockout, or dominant-negative mutation (T172A), attenuated Lnc-THOR shRNA-induced A172 glioma cell apoptosis. Moreover, CRISPR/Cas9-induced IGF2BP1 knockout activated MAGEA6-AMPK signaling as well, causing A172 glioma cell apoptosis. Significantly, Lnc-THOR shRNA was ineffective in IGF2BP1 KO A172 cells. In vivo, Lnc-THOR silencing or knockout potently inhibited subcutaneous A172 xenograft tumor growth in mice. MAGEA6 downregulation and AMPK activation were detected in Lnc-THOR-silenced/ko A172 tumor tissues. Taken together, Lnc-THOR depletion inhibits human glioma cell survival possibly by activating MAGEA6-AMPK signaling.

Introduction

Glioma is among the most aggressive human malignancies, causing significant human mortalities each year1–3, and in the late/advanced stages with extremely poor prognosis. In the clinical practices, gliomas are commonly diagnosed at late/advanced stages with extremely poor prognosis. Molecularly targeted therapies are essential for better glioma prognosis. Our group has been exploring novel therapeutic targets for this devastating disease. Non-coding RNAs (ncRNAs), including microRNAs, long non-coding RNAs (LncRNA), and circular RNAs, are originally known as transcriptional noise. Recent studies have implied that LncRNAs, and other ncRNAs, play pivotal roles in initiation and progression of human glioma and many other cancers.

A recent study by Hosono et al. has reported a conserved LncRNA, THOR (“Lnc-THOR”)15. Its expression is detected in testis, and also in a number of different human cancers15–20. Lnc-THOR knockdown or knockout (KO) potently inhibited human cancer cell survival15. Lnc-THOR directly associates with insulin-like growth factor 2 (IGF2) mRNA-binding protein 1 (IGF2BP1), a conserved RNA-binding family protein15. Lnc-THOR association is essential for IGF2BP1’s function, as well as stabilization of IGF2BP1 target mRNAs, including IGF2, Gli1 (glioma-associated oncogene homolog 1), Myc, and CD4415,20.

Our previous studies have implied that forced activation of AMP-activated protein kinase (AMPK) can inhibit human glioma cells21,22. Thr-172 phosphorylation of AMPKα1 is essential for AMPK activation. AMPK activation inhibits mammalian target of rapamycin (mTOR) complex 1 (mTORC1), a key oncogenic cascade. In human cancer cells, activated AMPK could also induce...
growth inhibition and cell-cycle arrest by stabilizing and activating p53. Moreover, AMPK activation is shown to trigger autophagy and degradation of multiple growth factor receptors (i.e., epidermal growth factor receptor [EGFR] and platelet-derived growth factor receptor α [PDGFRα]), thereby causing cancer cell inhibition.

AMPKα1 expression is often sequestered in human cancer cells. Pineda et al. showed that MAGEA6-TRIM28 complex is a cancer-specific ubiquitin ligase, responsible for degradation AMPKα1 only in cancer cells. We have previously shown that MAGEA6 knockdown by targeted short hairpin RNA (shRNA) restored AMPKα1 expression, causing glioma cell death and apoptosis. In the present study, we will show that Lnc-THOR-IGF2BP1 cascade is essential for MAGEA6 expression in glioma cells. Inhibition of Lnc-THOR-IGF2BP1 cascade will induce MAGEA6 downregulation, AMPKα1 expression, and AMPK signaling activation, inhibiting glioma cell survival in vitro and in vivo.

**Materials and methods**

**Chemicals and reagents**

Puromycin and neomycin were obtained from Sigma-Aldrich (St. Louis, MO). Cell culture reagents were provided by Gibco-BRL (Grand Island, NY). The anti-MAGEA6 antibody (ab38495) was purchased from Abcam (Shanghai, China). All other antibodies were provided by Cell Signaling Tech (Shanghai, China). TRIzol reagents for RNA assays, Lipofectamine 2000, and other transfection reagents were obtained from Invitrogen (Shanghai, China).

**Cell culture**

 Cultures of HCN-1a human neuronal cells, A172 and U251MG (“U251”) human glioma cells, as well as the primary human astrocytes, were described earlier. The human glioma cells, derived from two primary glioma patients, were provided by Dr. Cao, which were named as “Pri-1/Pri-2,” and cultured as previously described. The protocols of studying human cells and tissues were approved by the Ethics Review Board of Shanghai Jiao-Tong University School of Medicine, according to Declaration of Helsinki.

**Human tissues**

As reported earlier, a total of five glioma tissues, along with paired surrounding normal brain tissues, were acquired and stored in liquid nitrogen. Tissues were separated, thoroughly washed, minced, and homogenized by the tissue lysis buffer (BiYunTian, Wuxi, China). Written informed consent was obtained from each participant.

**Quantitative real-time reverse transcriptase polymerase chain reaction (qPCR)**

As reported, 500 ng RNA of each sample was applied in the reverse transcription (RT) reaction with specific RT primers and superscript III reverse transcriptase (Invitrogen). Afterwards, 100 ng obtained complementary DNA (cDNA) template was mixed with SYBR Master Mix (Applied Biosystem) and 200 nM primers. We utilized ABI Prism 7600H Fast Real-Time PCR system for qPCR assays. The primers are listed in Table 1. qPCR quantification was through the following formula: $2^{-\Delta\Delta Ct}$ method using the following formula: $2^{-\Delta\Delta Ct}$ = $2^{-\Delta Ct}$ (Ct of target gene – Ct of reference gene).

**Lnc-THOR shRNA**

A set of two shRNAs, against non-overlapping sequence of Lnc-THOR (“Seq1/2,” designed and verified by Genechem, Shanghai, China), were individually inserted into GV248 construct. The construct, along with the lentivirus package plasmids (Genechem), were transfected to HEK-293 cells to generate Lnc-THOR shRNA lentivirus. The virus was enriched, filtered, and added to glioma cells (plated at a density of $1 \times 10^5$ cells/well into 6-well plates).

| Table 1 Primers utilized in this study |
|---------------------------------------|
| **Gene name** | **Forward primers** | **Reverse primers** |
| GAPDH | 5′-CACACACATGGAGAAGGCTGG-3′ | 5′-GAAGTCAGAAGGAGACCCACCTG-3′ |
| MAGEA6 | 5′-TGGAGGACAGAAGGCCC-3′ | 5′-CAGGATGATATTACAGGAAGCCTG-3′ |
| Lnc-THOR | 5′-CAAGGTTGTCCTCTCCGGATT-3′ | 5′-GCCAAAGTCAATTTGTTGGATAT-3′ |
| L6 | 5′-CTGCGCTCCGGAGCAGCAGC-3′ | 5′-ACGGCTCACAAATTTGGCTTGC-3′ |
| AMPKα1 | 5′-AGGAGAATCTCGTGCACAC-3′ | 5′-CGGATCTCTCTGGAGAAGCAGATG-3′ |
| Gli1 | 5′-ACGCTTCAATAGCAGGACT-3′ | 5′-GTCAAGGAGCTCACTTCTCTT-3′ |
| Myc | 5′-CTGCGCTCCCGTACGAGGACAC-3′ | 5′-CAGACCTCTGCACCATTTGGCGAC-3′ |
| IGF2 | 5′-TGGGATCTGTTGGAGAGTCTG-3′ | 5′-ACGGGCTATCTGGGGAAGTGT-3′ |

Official journal of the Cell Death Differentiation Association
Cells were then subjected to selection by using puromycin (2.5 μg/mL, for 10–12 days). In stable cells, Lnc-THOR knockdown was verified by qPCR assay.

**Lnc-THOR KO**

The CRISPR/Cas9 Lnc-THOR KO construct (with sgRNA, 5′-CACCAGGGTTAGCCGCGTGA-3′, R: 5′-AAACCTCAGCCCGCTACACCCCTc-3′[15]) was provided by Dr. Liang, which was transfected to glioma cells (plated at a density of 1 × 10⁵ cells/well into 6-well plates) by Lipofectamine 2000 reagents. Fluorescent-activated cell sorting (FACS)-mediated sorting of the green fluorescent protein (GFP)-positive cells were performed to select monoclonal cells, which were then cultured in the puromycin-containing complete medium to achieve stable cells. Lnc-THOR KO was verified by qPCR assay.

**Lnc-THOR overexpression**

The full-length Lnc-THOR was amplified by the described primers[15] and inserted to the GV248 lentiviral construct (Genechem). The lentiviral GV248-Lnc-THOR construct (“LV-Lnc-THOR”) was transfected to glioma cells (plated at a density of 1 × 10⁶ cells/well into 6-well plates), followed by selection using puromycin (2.5 μg/mL) for 10–12 days. In stable cells, Lnc-THOR overexpression was verified by qPCR assay.

**Cell viability assay**

Briefly, cells were plated at a density of 3 × 10³ cells/well into 96-well plates. Following culture of 96 h, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT; 5 mg/mL, 20 μL/well, dissolved in phosphate-buffered saline (PBS)) was added, cells were further incubated for additional 2 h, and its optical density (OD) was tested at 590 nm.

**Cell proliferation assays**

For the soft agar colony-formation assay, A172 cells (5000 cells of each treatment) were re-suspended in agar (0.5%)-containing complete medium (with fetal bovine serum (FBS)) and added on the top of 10-cm culture dishes. After incubation for 10 days, A172 cell colonies were stained and manually counted. The detailed protocol for the 5-ethyl-2′-deoxyuridine (EdU) staining assay was reported earlier[32].

**Apoptosis assays**

The detailed protocols of apoptosis assays, including Histone DNA enzyme-linked immunosorbent assay and Annexin V FACS, as well as terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) staining assay and caspase-3/caspase-9 activity assays, were described in previous studies[33,34].

**“Transwell” in vitro migration assay**

A172 glioma cells (3 × 10⁵ cells in 300 μL medium) were seeded into the upper part of the “Transwell” chambers (12-μm pore size, BD Biosciences). The lower compartments were filled with complete medium with 10% FBS. After 48 h, on the upper surfaces the non-migrated A172 cells were removed. On the lower surfaces, the migrated cells were fixed, stained, and counted.

**Western blotting analysis**

The detailed protocol of western blotting assay was described in our previous studies[9,10]. Briefly, for each treatment 40 μg of protein lysates (in each lane) were separated in denaturing 10–12% polyacrylamide gels and transferred to a polyvinylidene difluoride blots. After blocking (in 10% milk PBST solution) and three washes in TBST, blots were incubated with the indicated primary and secondary antibodies. Immuno-reactive proteins were detected by an enhanced chemiluminescence kit (Amer sham, Shanghai, China) and analyzed through autoradiography. ImageJ software (NIH) was utilized for the quantification of the protein band, which was always normalized to the loading control.

**AMPKa1 shRNA**

As described[21], the lentiviral AMPKa1 shRNA was added to A172 cells (plated at a density of 1 × 10⁵ cells/well into 6-well plates) for 48 h. Puromycin (2.5 μg/mL)-containing complete medium was added to select stable cells for 5–6 days. Control cells were infected with the lentiviral scramble control shRNA (“sh-C”). AMPKa1 silencing in the stable cells was confirmed by western blotting.

**AMPKa1 dominant-negative mutation**

The dominant-negative AMPKa1 (dnAMPKa1, T172A, as reported[21]) or the empty vector (pSuper-neo-Flag) was transfected to A172 cells (plated at a density of 1 × 10⁵ cells/well into 6-well plates) by Lipofectamine 2000. Neomycin (1.0 μg/mL) was added to select stable cells for 5–6 days. Expression of the mutant AMPKa1 was verified by western blotting.

**AMPK activity assay**

Following the treatments, 200 μg of total cellular lysates were first incubated with anti-AMPKa1 antibody. The AMPK activity was examined in the kinase assay buffer by adding AMP-[γ-32P] ATP mixture and AMPK substrate SAMS (HMRSAMSGLHLKVRR) peptide[35]. Phospho-cellulose paper was added afterwards, stopping the reactions. The AMPK radioactivity was examined by a scintillation counter, and its value was normalized to control level.
IGF2BP1 or AMPKa1 KO  
A172 cells were seeded onto 6-well tissue culture plates at a density of $1 \times 10^5$ cells/well. The lenti-CRISPR/Cas9-IGF2BP1-KO-GFP construct (provided by Dr. Zhao36) or the lenti-CRISPR/Cas9-AMPKa1-KO-GFP construct (from Dr. Li37) was transfected to A172 cells through Lipofectamine 2000 protocol. FACs-mediated sorting of the GFP-positive cells were performed to select the monoclonal cells, which were then cultured in the puromycin-containing complete medium to achieve stable cells. IGF2BP1 or AMPKa1 KO in the stable cells was confirmed by western blotting and/or qPCR assays.

Ectopic IGF2BP1 overexpression  
The recombinant adenovirus encoding IGF2BP1 expression construct (provided by Dr. Zhao36) was added to cultured A172 cells (plated at a density of $1 \times 10^5$ cells/well into 6-well plates) for 48 h. Cells were thereafter subjected to puromycin (2.5 µg/mL) selection for another 5–6 days. IGF2BP1 overexpression was confirmed by western blotting.

RNA immunoprecipitation (RIP)  
RIP experiments were carried out through a described protocol38. Briefly, glioma cells were trypsinized, washed, and incubated with 0.3% formaldehyde and glycine39. Afterwards, glioma cells were washed, and resuspended, with the pellets dissolved in the RIP buffer38. The lysates were then incubated with the anti-IGF2BP1 antibody. Pellets were washed, re-suspended, and incubated with proteinase K-containing buffer. IGF2BP1-bound Lnc-THOR and MAGEA6 mRNA was tested by qPCR, with its level normalized to internal controls.

RNA Pull-Down assay  
RNA Pull-Down was carried out using a previously described protocol39. In short, the biotin-labeled full-length Lnc-THOR (provided by Dr. Wang39) was folded in RNA structure buffer and incubated with cleared nuclei lysates of the glioma cells together with Dynabeads MyOne Streptavidin C1 magnetic beads (“Beads,” again provided by Dr. Wang39). Beads were washed, with the retrieved proteins examined by western blotting.

Xenograft assay  
As reported21, the female severe combined immuno-deficient (SCID) mice were housed under standard procedures. Lnc-THOR shRNA-bearing stable A172 cells, Lnc-THOR KO stable A172 cells, or the parental control A172 cells ($5 \times 10^5$ cells in 200 µl of Matrigel gel, no serum, each mouse) were subcutaneously (s.c.) injected to the flanks. When the volume reached approximately 100 mm$^3$ for each tumor (“Day-0”), the recordings were started. Tumor volumes were calculated as described21.

All animal procedures were approved by IACUC of Shanghai Jiao-Tong University School of Medicine.

Statistical analysis  
All statistics were calculated by using the SPSS 18.0 statistical software (SPSS, Chicago, IL). Descriptive statistics including mean and standard deviation (SD) along with one-way analyses of variance were applied to determine significant differences. Two-tailed unpaired T test (Excel 2013) was applied to test significance between the two treatment groups. $p < 0.05$ was considered significant.

Results  
Lnc-THOR expression in human glioma tissues and cells  
First, we tested the expression of Lnc-THOR in human glioma tissues. As described in our previous studies21, a total of five pairs of human glioma tissues (“T”) and paired surrounding normal brain tissues (“N”) were analyzed, and qPCR assay results in Fig. 1a show that Lnc-THOR levels are high in human glioma tissues, whereas its levels in normal brain tissues are, however, extremely low (Fig. 1a). Further studies show that Lnc-THOR is expressed in established (A172 and U251MG lines) and primary human glioma cells (derived from two different patients, “Pri-1/-2”) (Fig. 1b). Its expression is almost undetected in the primary human astrocytes22 and HCN-1a neuronal cells22 (Fig. 1b). These results confirm unique Lnc-THOR expression in human glioma tissues and cells.
Lnc-THOR silencing or KO inhibits human glioma cell progression in vitro

In order to study the function of Lnc-THOR in human glioma cells, two lentivirus-encoded Lnc-THOR shRNAs, with non-overlapping sequences (“Seq1/2”), were individually transfected to A172 glioma cells. Following puromycin selection, the stable cells were established (“sh-Lnc-THOR” cells). Moreover, the lenti-CRISPR/Cas9 Lnc-THOR-KO construct (see “Methods” section) was transfected to A172 cells. Stable cells (“KO-THOR” cells) were established by FACS sorting of GFP cells and puromycin selection. Analyzing Lnc-THOR expression in the stable cells, by qPCR, confirmed that Lnc-THOR levels were dramatically downregulated in the stable cells with Lnc-THOR shRNA or Lnc-THOR KO construct (Fig. 2a). Lnc-THOR binds to IGF2BP1 to ensure mRNA stabilization of key pro-cancerous genes, including IGF2, Gli1, and Myc.15,16,18,20. In A172 glioma cells, mRNA levels of IGF2, Gli1, and Myc proteins were downregulated as well (Fig. 2b). IGF2, Gli1, and Myc proteins were downregulated as well (Fig. 2c). Lnc-THOR shRNA or KO did not affect

---

Fig. 2 Lnc-THOR silencing or KO inhibits human glioma cell survival and proliferation. The genetically modified stable A172 cells, with Lnc-THOR shRNA (“sh-Lnc-THOR,” with non-overlapping sequences, “Seq1/2”), scramble non-sense control shRNA (“sh-C”), or the lenti-CRISPR/Cas9 Lnc-THOR-KO construct (KO-THOR), were established, the expression of Lnc-THOR and listed genes in the stable cells and in parental control A172 cells (“Ctrl”) were tested by qPCR and western blotting assays (a–c). Cells were further cultured for the indicated time, and cell viability was tested by MTT assay (d). Cell proliferation was analyzed by EdU staining (e) and soft agar colony-formation (f) assays; Cell migration was tested by “Transwell” assays (g). U251MG (U251) and primary human glioma cells (“Pri-1/Pri-2”) were transfected with lentiviral Lnc-THOR shRNA (“sh-Lnc-THOR, “Seq1”) or “sh-C” and stable cells were established via puromycin selection. Lnc-THOR levels were tested by qPCR assay (h). Cell survival and proliferation were tested by MTT assay (i) and EdU staining assay (j), respectively. Western blotting assay of the IGF2BP1 protein retrieved by in vitro-transcribed Lnc-THOR in A172 cells and primary human glioma cells (k); qPCR analyses of Lnc-THOR expression enriched by the IGF2BP1 protein in A172 cells and primary human glioma cells (l). For all the in vitro function assays, the exact same amount of viable cells was initially seeded in each well/dish (same for all figures). Blot data were quantified and normalized to the corresponding loading control (c). Data are presented as mean ± SD (n = 5). *p < 0.05 vs. “sh-C” cells. Experiments in this figure were repeated three times, and similar results were obtained.
IGF2BP1 mRNA (Fig. 2b) and protein expression (Fig. 2c). The scramble non-sense control shRNA (“sh-C”) had no significant effect on the expression of Lnc-THOR-IGF2BP1 pathway genes (Fig. 2a–c).

MTT, EdU staining, and soft agar colony-formation assays were performed to test glioma cell functions. When compared to control A172 cells, in Lnc-THOR-silenced or Lnc-THOR-KO A172 cells, MTT OD values (Fig. 2d), EdU percentages (Fig. 2e), and the number of colonies (Fig. 2f) were significantly decreased. A172 cell in vitro migration, tested by the “Transwell” assays, were significantly inhibited by Lnc-THOR shRNA or KO (Fig. 2g). In U251MG cells and primary human glioma cells (“Pri-1/-2”), transfection of the lentiviral Lnc-THOR shRNA (“Seq1”) induced >90% reduction of Lnc-THOR expression (Fig. 2h), causing reduced MTT OD (Fig. 2i) and EdU ratio (Fig. 2j). These results show that Lnc-THOR silencing or KO inhibits glioma cell growth and migration.

Lnc-THOR-IGF2BP1 binding has been reported in other cancer cells. To test the direct association between Lnc-THOR and the IGF2BP1 protein in glioma cells, we employed a Lnc-THOR pull-down assay. Results demonstrated that the IGF2BP1 protein is co-precipitated with the in vitro-transcribed biotinylated Lnc-THOR (provided by Dr. Wang) in both A172 cells and primary human glioma cells (“Pri-1/-2”) (Fig. 2k). In addition, the RIP assay results show again the direct binding between Lnc-THOR and the IGF2BP1 protein in A172 cells and the primary human glioma cells (Fig. 2l).

Lnc-THOR silencing or KO induces apoptosis activation in human glioma cells

The potential effect of Lnc-THOR on glioma cell apoptosis was studied. As shown, in the A172 cells with Lnc-THOR shRNA (“Seq1/-2”) or Lnc-THOR-KO construct (“KO-THOR” cells, see Fig. 2), the activities of caspase-3 and caspase-9 were significantly increased (compared to control A172 cells, Fig. 3a). Furthermore, Lnc-THOR silencing or KO in A172 cells induced cleavages of caspase-3, caspase-9, and PARP (poly ADP-ribose polymerase) (Fig. 3b), as well as accumulation of histone-bound DNA (Fig. 3c). In addition, Annexin V percentages (Fig. 3d, e) and nuclear TUNEL ratios (Fig. 3f) were significantly increased in Lnc-THOR-silenced or -KO cells. In U251MG and primary human glioma cells (“Pri-1/-2”), the lentiviral Lnc-THOR shRNA (“Seq1”) similarly induced increases of nuclear TUNEL ratios (Fig. 3g). Taken together, these results clearly show that Lnc-THOR silencing or KO provokes apoptosis activation in human glioma cells.

Lnc-THOR overexpression promotes human glioma cell survival and proliferation

Since Lnc-THOR silencing or KO inhibited glioma cell growth and migration, we hypothesized that forced overexpression of Lnc-THOR shall exert opposite functions. To test this hypothesis, the lentivirus encoding Lnc-THOR expression construct (see “Methods”) was transduced to A172 glioma cells. Following selection using the puromycin-containing medium, two A172 cell lines were established (“Line1/2”). Testing Lnc-THOR expression, by qPCR, confirmed that Lnc-THOR levels were significantly increased in the stable cells with Lnc-THOR construct (“OE-Lnc-THOR” cells). Consequently, mRNA and protein expression of IGF2BP1 targets, IGF2, GlI1, and Myc, were upregulated (Fig. 4b, c). IGF2BP1 expression was again not changed (Fig. 4b, c). As compared to vector control cells, increased MTT OD values (Fig. 4d), EdU staining (Fig. 4e), and colony formation (Fig. 4f) were detected in the OE-Lnc-THOR cells. These results indicate that Lnc-THOR overexpression promotes A172 cell growth and migration. Similarly in U251MG cells and primary human glioma cells (“Pri-1/-2”), adding Lnc-THOR-expressing lentivirus increased Lnc-THOR expression (Fig. 4g), enhancing cell survival (Fig. 4h) and proliferation (Fig. 4i).

Lnc-THOR depletion activates MAGEA6-AMPK signaling in glioma cells

MAGEA3/6-TRIM28 complex is a cancer-specific ubiquitin ligase of AMPKα1. Our previous study has shown that MAGEA6 sequestrs AMPKα1 in glioma cells, causing mTORC1 overactivation and cancer cell growth. Reversely, MAGEA6 silencing inhibits human glioma cell cells via re-activation of AMPK signaling. RIP assays (same experiments as Fig. 2l) confirmed the direct binding between MAGEA6 mRNA and the IGF2BP1 protein in A172 cells and the primary human glioma cells (Fig. 5a). Importantly, Lnc-THOR silencing (by “Seq1” shRNA, see Fig. 2) or KO (see Fig. 2) downregulated MAGEA6 mRNA (Fig. 5b) and protein (Fig. 5c) in A172 cells. Consequently, AMPKα1 protein expression and AMPK activation (AMPKα1-ACC phosphorylation) were significantly increased (Fig. 5c). AMPK activity was increased as well in Lnc-THOR-silenced/-KO cells (Fig. 5c).

Activated AMPK will inhibit human cancer cells via regulating its downstream effectors, causing mTORC1 inhibition, autophagy induction, and receptor tyrosine kinase (RTK) degradation. In A172 cells, Lnc-THOR silencing or KO largely inhibited p70S6K1-56 phosphorylation, indicating mTORC1 inhibition (Fig. 5d). RTKs, including EGFR and PDGFRα, were downregulated (Fig. 5e). Significantly, AMPKα1 mRNA levels were unchanged by Lnc-THOR silencing or KO (Fig. 5f). Based on these results, we propose that Lnc-THOR depletion activates AMPK activation possibly by downregulating AMPKα1’s ubiquitin ligase MAGEA6.

To test whether AMPK activation mediated Lnc-THOR-depletion-induced cytotoxicity of glioma cells, we utilized
previously described genetic strategies\textsuperscript{21} to block AMPK activation. The lentiviral AMPK\textalpha\, shRNA, the dominant-negative AMPK\textalpha\, (“dnAMPK\textalpha,” T172A) construct, or the lenti-CRISPR/Cas9 AMPK\textalpha\, KO construct was separately transduced to A172 cells. Stable cells were established via selection (see “Methods”). As shown, Lnc-THOR shRNA (“Seq1,” see Fig. 2) induced AMPK activation or AMPK\textalpha-ACC phosphorylation was almost completely blocked by AMPK\textalpha\, shRNA, dominant-negative mutation, and KO (Fig. 5g). As a result, Lnc-THOR shRNA-induced glioma cell death (Fig. 5h) and apoptosis (Fig. 5i) were largely ameliorated. Therefore, AMPK activation mediates Lnc-THOR-depletion-induced glioma cell death.
**Fig. 4 Lnc-THOR overexpression promotes human glioma cell survival and proliferation.** The genetically modified stable A172 cells, with lentiviral Lnc-THOR expression construct ("OE-Lnc-THOR," two lines, "Line1/2") or empty vector ("Vec"), were established, and the expression of Lnc-THOR and listed genes in the stable cells and in parental control A172 cells ("Ctrl") were tested by qPCR and western blotting assay (a–c). Cells were further cultured for the indicated time, and cell viability was tested by MTT assay (d). Cell proliferation was analyzed by EdU staining (e) and soft agar colony-formation assay (f). U251MG ("U251") and primary human glioma cells ("Pri-1/Pri-2") were infected with lentiviral Lnc-THOR expression construct ("OE") or empty vector ("Vec"), and Lnc-THOR expression (g), cell viability (h), and proliferation (i) were tested. Blot data were quantified and normalized to the corresponding loading control (c). Data were presented as mean ± SD (n = 5). *p < 0.05 vs. "Vec" cells. Experiments in this figure were repeated three times, and similar results were obtained.
Lnc-THOR silencing is ineffective in IGF2BP1-KO glioma cells

Using the CRISPR/Cas9 gene-editing method (see ref. 36), we established two lines of IGF2BP1 KO A172 cells (IGF2BP1 KO, "L1/L2"). qPCR results in Fig. 6a confirmed IGF2BP1 mRNA depletion, which did not affect the Lnc-THOR expression (Fig. 6b). Importantly, in IGF2BP1 KO A172 cells, MAGEA6 mRNA (Fig. 6c) and protein (Fig. 6d) levels were significantly downregulated, accompanied with increased AMPKα1 expression (Fig. 6d) and AMPKα1-ACC phosphorylation (Fig. 6d), as well as increased AMPK activity (Fig. 6e). Therefore, IGF2BP1 is
Lnc-THOR silencing is ineffective in IGF2BP1-knockout glioma cells. The stable A172 cells with CRISPR/Cas9-IGF2BP1-KO construct ("IGF2BP1 KO, L1/L2, two lines") were established. Expression of listed mRNAs (a, c), Lnc-THOR (b), and proteins (d), as well as the relative AMPK activity (e), in IGF2BP1 KO cells and the parental control A172 cells ("Ctrl") were tested. Cells were further infected with/without Lnc-THOR shRNA virus ("kd-THOR") or the scramble control non-sense shRNA virus ("sh-C"), and cell survival (MTT assay, f), cell apoptosis (TUNEL assay, g), and Lnc-THOR expression (h, qPCR assay) were tested. Stable A172 cells with the lenti-CRISPR/Cas9 Lnc-THOR-KO construct ("KO-THOR") were further infected with or without adenovirus encoding IGF2BP1 expression construct (IGF2BP1-OE), and stable cells were established with puromycin selection; the expression of listed proteins is shown (i). After culture for applied time periods, cell survival (MTT OD, j) and apoptosis (TUNEL staining, k) were tested. Blot data were quantified and normalized to the corresponding loading control (d, i). Data are presented as mean ± SD (n = 5). *p < 0.05 vs. "Ctrl" cells (a–e, j, k). *p < 0.05 vs. "sh-C" treatment of "Ctrl" cells (f–h). Experiments in this figure were repeated four times, and similar results were obtained.
viability reduction (Fig. 6j) and apoptosis activation (Fig. 6k) in A172 cells.

Of importance for MAGEA6 expression and AMPK inactivation in glioma cells.

Xue et al. IGFBP1 KO also promoted A172 cell death (Fig. 6f) and apoptosis (Fig. 6g). Importantly, adding expression failed to reverse Lnc-THOR KO-induced viability reduction (Fig. 6h). These results confirm that Lnc-THOR silencing is ineffective in IGFBP1 KO glioma cells. Furthermore, IGFBP1 overexpression failed to reverse Lnc-THOR KO-induced viability reduction (Fig. 6j) and apoptosis activation (Fig. 6k) in A172 cells.

Lnc-THOR silencing or depletion inhibits A172 xenograft tumor growth in vivo

As described in our previous study, an A172 tumor xenograft SCID mice model was established to study the potential activity of Lnc-THOR in vivo. The genetically modified stable A172 cells with Lnc-THOR shRNA (“sh-Lnc-THOR,” “Seq1”) or the lenti-CRISPR/Cas9 Lnc-THOR-KO construct (“KO-THOR”) were injected s.c. to the flanks of female SCID mice. When the tumors reached 100 mm³ (10 mice per group), recordings were initiated (“Day-0”). Tumor volumes (a, in mm³) were monitored every 7 days; Daily tumor growth was calculated as described (b). At Day-35, tumors of all three groups were isolated and weighed individually (c). At Day-7 and Day-14, one tumor per group was isolated, and the expression of Lnc-THOR (d) and listed proteins (e) in the fresh tumor lysates was tested. Blot data were quantified and normalized to the corresponding loading control (e). Data are presented as mean ± SD. *p < 0.05 vs. “Ctrl” group (a–c, n = 10).

Fig. 7 Lnc-THOR silencing or depletion inhibits A172 xenograft tumor growth in vivo. Parental control A172 cells (“Ctrl”), the genetically modified stable A172 cells with Lnc-THOR shRNA (“sh-Lnc-THOR,” “Seq1”), or the lenti-CRISPR/Cas9 Lnc-THOR-KO construct (“KO-THOR”) were injected s.c. to the flanks of female SCID mice. When the tumors reached 100 mm³ (10 mice per group), recordings were initiated (“Day-0”). Tumor volumes (a, in mm³) were monitored every 7 days; Daily tumor growth was calculated as described (b). At Day-35, tumors of all three groups were isolated and weighed individually (c). At Day-7 and Day-14, one tumor per group was isolated, and the expression of Lnc-THOR (d) and listed proteins (e) in the fresh tumor lysates was tested. Blot data were quantified and normalized to the corresponding loading control (e). Data are presented as mean ± SD. *p < 0.05 vs. “Ctrl” group (a–c, n = 10).
tumors and “KO-THOR” tumors weighed significantly lower than “Ctrl” tumors (Fig. 7c). The body weights of the SCID mice were not significantly different between the three groups (data not shown). These results confirmed that Lnc-THOR silencing or depletion inhibited A172 xenograft tumor growth in vivo.

In order to test signaling changes in vivo, at Day-7 and Day-14, one tumor per group was isolated (total six tumors). Fresh tumor lysates were achieved and tested. When compared to “Ctrl” tumors, Lnc-THOR levels were significantly decreased in the “sh-Lnc-THOR” tumors and “KO-THOR” tumors (Fig. 7d), where MAGEA6 downregulation, AMPKα1 upregulation, and AMPK activation, as well as p-S6K1 inhibition, were detected (Fig. 7e). Total S6K1 and IGF2BP1 expression was unaffected by Lnc-THOR silencing or KO in tumor lysates (Fig. 7e). These results in vivo are therefore in line with the in vitro findings.

Discussion
The results of the current study indicate that Lnc-THOR could possibly be a novel and important therapeutic target of human glioma. Lnc-THOR is uniquely expressed in human glioma tissues and cells. Its expression is extremely low or even undetected in normal brain tissues, as well as in normal neuronal cells/astrocytes. In established (A172 cell line) and primary human glioma cells, Lnc-THOR shRNA or KO potently inhibited cell survival and proliferation, while provoking cell apoptosis. Contrarily, forced overexpression of Lnc-THOR can further promote glioma cell growth and migration. In vivo, A172 xenograft tumors with Lnc-THOR silencing or KO grew significantly slower than control tumors in SCID mice. These results are in line with recent findings proposing Lnc-THOR as a novel therapeutic oncotarget for many human cancers16–20,45.

MAGE-TRIM28 complex is a cancer-specific AMPKα1 ubiquitin ligase21,27,40,41. We have previously shown that MAGEA6, one of the key AMPKα1’s ubiquitin ligase21,27,40,41, is uniquely expressed in human glioma tissues and cells, responsible for AMPKa1 degradation and AMPK inhibition. Contrarily, MAGEA6 silencing/depletion restored AMPKa1 expression and induced AMPK activation, causing downstream mTORC1 inactivation and glioma cell death21. The regulatory mechanism of MAGEA6 expression in glioma is elusive. The results of this study suggest that Lnc-THOR-IGF2BP1 association is important for MAGEA6 expression in glioma cells. The RIP results show that MAGEA6 mRNA directly binds to the IGF2BP1 protein in A172 cells and the primary glioma cells. Significantly, Lnc-THOR silencing/KO or IGF2BP1 KO induced MAGEA6 degradation (both mRNA and protein), AMPKα1 protein accumulation, and AMPK activation in A172 glioma cells. These results suggest that Lnc-THOR-IGF2BP1 complex is important for MAGEA6 expression, causing AMPKα1 degradation and AMPK inactivation in human glioma cells.

Our study21 and others have implied that forced activation of AMPK signaling can induce human cancer cell apoptosis via regulating its downstream effectors, including mTORC1 inhibition23,24,42, autophagy induction42–44, and RTK (EGFR, PDGFR, etc) degradation10,26. We provided evidences here to support that AMPK activation mediates, at least in part, Lnc-THOR-depletion-induced glioma cell death. In A172 cells, Lnc-THOR silencing/KO induced MAGEA6 degradation, AMPKα1 elevation, and AMPK signaling activation, causing mTORC1 inhibition and EGFR–PDGFR degradation and eventually cell apoptosis. Similarly, IGF2BP1 KO also activated MAGEA6-AMPK signaling in A172 cells. Importantly, AMPK inactivation, by AMPKα1 shRNA, KO, or dominant-negative mutation, attenuated Lnc-THOR shRNA-induced A172 cell apoptosis. Significantly, AMPK blockage failed to completely reverse Lnc-THOR shRNA-induced cytotoxicity in A172 glioma cells, suggesting that both AMPK-dependent and AMPK-independent mechanisms are responsible for Lnc-THOR-silencing-induced glioma cell death (see Fig. 8, the proposed signaling pathway of the study). Therefore, although further studies are needed to explore the detailed underlying mechanisms, here we propose that Lnc-THOR-IGF2BP1 association is vital for MAGEA6 expression and AMPK inactivation in human glioma cells (Fig. 8).

Conclusion
Lnc-THOR depletion activates MAGEA6-AMPK signaling and inhibits human glioma cell survival.

Acknowledgements
This project was supported by the Fund of Shanghai Municipal Health Bureau (201640210), the Fund of Shanghai Charitable Cancer Research Center, and the Medical Cross Foundation of Shanghai Jiao-Tong University (YG2016MS59) all
References

1. Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2015. CA Cancer J. Clin. 66, 7–30 (2016).
2. Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2015. CA Cancer J. Clin. 65, 8–29 (2015).
3. Siegel, R., Ma, J., Zou, Z. & Jemal, A. Cancer statistics, 2014. CA Cancer J. Clin. 64, 9–29 (2014).
4. Westphal, M. & Lammers, K. The neurobiology of gliomas: from cell biology to the development of therapeutic approaches. Nat. Rev. Neurosci. 12, 495–508 (2011).
5. Wen, P. Y. & Reardon, D. A. Neuro-oncology in 2015: progress in glioma diagnosis, classification and treatment. Nat. Rev. Neuro. 12, 69–70 (2016).
6. Reardon, D. A. & Wen, P. Y. Glioma in 2014: unravelling tumour heterogeneity – implications for therapy. Nat. Rev. Clin. Oncol. 12, 69–70 (2015).
7. Pan, S. J. et al. Tetratraspin 8-riptor-integroin alpha3 complex is required for glioma cell migration. Int. J. Mol. Sci. 15, 5363–5374 (2015).
8. Pan, S. J. et al. Ubiquitin-protein ligase E3C promotes glioma progression by mediating the ubiquitination and degrading of Annexin A7. Sci. Rep. 5, 11066 (2015).
9. Pan, S. J. et al. Over-expression of tetratraspin 8 in malignant glioma regulates tumor cell progression. Biochem. Biophys. Res. Commun. 458, 476–482 (2015).
10. He, X. Y. et al. Gambogenic acid induces EGFR degradation and Akt/mTORC1 inhibition through AMPK-dependent-LGI1 upregulation in cultured U87 glioma cells. Biochem. Biophys. Res. Commun. 433, 397–402 (2013).
11. Park, J. Y. et al. Roles of long non-coding RNAs on tumorigenesis and glioma development. Brain Tumor Res. Treat. 1, 1–6 (2014).
12. Li, Y. et al. Circular RNA as a biomarker for cancer: a systematic meta-analysis. Oncol. Lett. 16, 4078–4084 (2018).
13. Hou, L. D. & Zhang, J. Circular RNAs: an emerging type of RNA in cancer. Int. J. Immunopathol. Pharmac. 30, 1–6 (2017).
14. Zhao, Z. J. & Shen, J. Circular RNA participates in the carcinogenesis and the malignant behavior of cancer. RNA Biol. 14, 514–521 (2017).
15. Hosono, Y. et al. Oncogenic role of THOR, a conserved cancer/testis long non-coding RNA. Cell. 171, 1559.e20–1572.e20 (2017).
16. Ye, X. T., Huang, H., Huang, W. P. & Hu, W. L. LncRNA THOR promotes human renal cell carcinoma cell growth. Biochem. Biophys. Res. Commun. 501, 661–667 (2018).
17. Song, H. et al. LncRNA THOR increases the stemness of gastric cancer cells via enhancing SOX9 mRNA stability. Biomed. Pharmacother. 108, 338–346 (2018).
18. Shang, Y. LncRNA THOR acts as a retinoblastoma promoter through enhancing the combination of c-myc mRNA and IGFBP1 protein. Biomed. Pharmacother. 106, 1245–1249 (2018).
19. Cheng, Z. et al. Long non-coding RNA THOR promotes liver cancer stem cells expansion via beta-catenin pathway. Gene 584, 95–103 (2018).
20. Chen, W. et al. Long non-coding RNA THOR promotes human osteosarcoma cell growth in vitro and in vivo. Biochem. Biophys. Res. Commun. 499, 913–919 (2018).
21. Pan, S. J. et al. MAGEA6 promotes human glioma cell survival via targeting AMPKalpha1. Cancer Lett. 412, 21–29 (2018).
22. Jiang, H. et al. GSK621 targets glioma cells via activating AMP-activated protein kinase signalings. PLoS ONE 11, e0161017 (2016).
23. Inoki, K., Zhu, T. & Guan, K. L. TSC2 mediates cellular energy response to control cell growth and survival. Cell 115, 577–590 (2003).
24. Inoki, K. et al. TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. Cell 126, 955–968 (2006).
25. Jones, R. G. et al. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. Mol. Cell 18, 283–293 (2005).
26. Chen, M. B. et al. Itraconazole-induced inhibition on human esophageal cancer cell growth requires AMPK activation. Mol. Cancer Ther. 17, 1229–1239 (2018).
27. Pinetta, C. T. et al. Degradation of AMPK by a cancer-specific ubiquitin ligase. Autophagy 11, 715–728 (2015).
28. Cui, Y., Zhao, J., Yi, L. & Jiang, Y. microRNA-153 targets mTOR2 component Rictor to inhibit glioma cells. PLoS ONE 11, e0156915 (2016).
29. Liu, Y. Y. et al. microRNA-200a downregulation in human glioma leads to G alpha1 over-expression, Akt activation, and cell proliferation. Oncogene 37, 2890–2902 (2018).
30. Cai, S. et al. Gliath3 nuclear translocation causes irradiation resistance in human glioma cells. Oncotarget 8, 35061–35068 (2017).
31. Li, Z. W. et al. Over-expression of Gliath3 in human glioma is required for Akt- mTOR activation and cell growth. Oncotarget https://doi.org/10.18632/oncotarget.10995 (2016).
32. Lv, Y. et al. Overexpression of lymphocyte antigen 6 complex, locus E in gastric cancer promotes cancer cell growth and metastasis. Cell Physiol. Biochem. 45, 1219–1229 (2018).
33. Yang, L. et al. C6 ceramide dramatically enhances docetaxel-induced growth inhibition and apoptosis in cultured breast cancer cells: a mechanism study. Exp. Cell Res. 332, 47–59 (2015).
34. Li, K. R. et al. Gansenoside Rg1 protects retinal pigment epithelium (RPE) cells from cobalt chloride (CoCl2) and hypoxia assaults. PLoS ONE 8, e84171 (2013).
35. Lee, M. et al. AMP-activated protein kinase kinase activity is critical for hypoxia-inducible factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU145 cells. J. Biol. Chem. 278, 39653–39661 (2003).
36. Liu, Z. et al. IGF2BP1 over-expression in skin squamous cell carcinoma cells is essential for cell growth. Biochem. Biophys. Res. Commun. 501, 731–738 (2018).
37. Wu, F. et al. miR-1273g silences MAGEA3 to inhibit human colorectal cancer cell growth via activation of AMPK signaling. Cancer Lett. 345, 1–9 (2018).
38. Wang, Z. et al. IncRNA epigenetic landscape analysis identifies EP1C as an oncogenic IncRNA that interacts with MYC and promotes cell-cycle progression in cancer. Cancer Cell 33, 706–720 e709 (2018).
39. Wang, S. S. et al. Triptolide inhibits human nasopharyngeal carcinoma cell growth via disrupting Linc-RNA THOR-IGF2BP1 signaling. Cancer Lett. 443, 13–24 (2019).
40. Pineda, C. T. & Potts, P. R. Oncogenic MAGEA3-TRIM20 ubiquitin ligase downregulates autophagy by ubiquitinating and degrading AMPK in cancer. Autophagy 11, 841–846 (2015).
41. Ye, X., Xie, J., Huang, H. & Deng, Z. Knockdown of MAGEA6 activates AMP-activated protein kinase (AMPK) signaling to inhibit human renal cell carcinoma cells. Cell Physiol. Biochem. 45, 1205–1218 (2018).
42. Kim, J., Kundu, M., Violet, B. & Guan, K. L. AMPK and mTOR regulate autophagy through direct phosphorylation of ULK1. Nat. Cell Biol. 13, 132–141 (2011).
43. Espin, D. F. et al. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science 331, 456–461 (2011).
44. Hsu, H. Z. et al. AMP-activated protein kinase (AMPK)/ULK1-dependent autophagic pathway contributes to C6 ceramide-induced cytotoxic effects in cultured colorectal cancer HT-29 cells. Mol. Cell. Biochem. 378, 171–181 (2013).
45. Ling, G. A. O., Xiu-Lian, C. & Hua, C. A. O. LncRNA THOR attenuates cisplatin sensitivity of nasopharyngeal carcinoma cells via enhancing cells stemness. Biochimie 152, 63–72 (2018).