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Original research

LncRNA RP1-86C11.7 exacerbates the glioma progression and oncogenicity by hsa-miR-144-3p/TFRC signaling.

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ABSTRACT

Glioblastoma (GBM) remains the most common and malignant tumor of the human central nervous system. Increasing evidence has highlighted that tumor cells with high transferrin receptor (TFRC) expression show advantages in growth. Long noncoding RNAs (lncRNAs) are related to glioma progression by mediating microRNAs (miRNAs). However, the underlying mechanism among TFRC, miRNA and lncRNA in GBM is limited. In the current study, we identified a new lncRNA-induced signaling mechanism that regulates the TFRC levels in GBM. The TFRC level was higher in glioma cell lines, and elevated TFRC expression promoted the proliferation and survival of glioma cells. Further study showed that hsa-miR-144a-3p bound to the 3′-UTR of TFRC mRNA and inhibited its expression, preventing the malignant properties of glioma cells, such as proliferation and survival. We also found that the lncRNA RP1-86C11.7 sponges hsa-miR-144-3p to suppress its protective role in glioma. RP1-86C11.7 overexpression in glioma cells elevated TFRC expression, increased the intracellular free iron level, and deteriorated oncogenicity, with a significant reduction in hsa-miR-144-3p. By contrast, silencing RP1-86C11.7 upregulated the hsa-miR-144-3p level, resulting in decreased TFRC expression and repressed glioma progression. However, the effect of silencing RP1-86C11.7 was reversed with simultaneous hsa-miR-144-3p inhibitor treatment: the TFRC level, intracellular iron level and proliferation in glioma cells increased. Mechanistically, our data indicated that RP1-86C11.7 exacerbates the malignant behavior of glioma through the hsa-miR-144-3p/TFRC axis. RP1-86C11.7 may be a potential biomarker or target to treat glioma in the future.

Introduction

Glioblastoma (GBM) is the most common and malignant tumor of the human central nervous system, accounting for 81% of brain tumors [1]. Because of its genetic heterogeneity, rapid and invasive growth, and poorly understood molecular mechanisms, glioma is challenging to treat [2]. Although numerous cancer therapies, such as surgery, chemotherapy, and radiotherapy, have been developed over the past decades, few drugs have been approved by the Food and Drug Administration (FDA) to treat glioma, resulting in limited improvements in the prognosis of glioma patients [3]. Understanding the pathogenic mechanism of glioma progression and identifying novel target biomarkers to treat glioma are necessary.

Iron is an essential element for cells involved in many biological processes, such as cell proliferation, DNA synthesis, respiration and mitosis, which are dysregulated in cancer [4]. The transferrin receptor (TFRC) regulates cellular iron uptake. TFRC controls the intracellular labile iron pool (LIP) by importing iron into cells to support various biological processes [5]. The higher level of intracellular iron promotes growth. Compared with normal cells, cancer cells require more iron to sustain their uncontrolled proliferation and rapid growth. Thus, TFRC has been applied in tumor therapy [6]. Iron is more important for the liver and brain than other organs because their biological behaviors normally depend on iron [7, 8]. Clinical studies have identified elevated iron levels and TFRC expression in glioblastoma [9, 10].

Recently, evidence has indicated that long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) regulate TFRC expression in cells. For example, miR-148a regulates TFRC expression in hepatocellular

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carcinoma [11]. Silencing lncRNA steroid receptor RNA activator (SRA) decreases TFRC expression in human erythroblasts [12]. However, many lncRNAs play an important role in the occurrence and development of glioma by sponging miRNAs to regulate gene expression at a posttranscriptional level [13]. Thus, lncRNAs or miRNAs may affect the cell growth potential and play a significant role in glioma progression by regulating TFRC expression, providing new insights into glioma therapy. However, the underlying biological mechanism among lncRNAs, miRNAs and TFRC has not been clarified in glioma.

Here, we demonstrated a new TRFC regulatory pathway in glioma. We identified that lncRNA RP1-86C11.7 accelerates glioma proliferation, survival and progression by promoting TFRC expression through sponging hsa-miR-144-3p. Additionally, lncRNA RP1-86C11.7 may be a potential biomarker or therapeutic target to treat glioma in the future.

Materials and methods

Cell culture, transfection and transduction

Glioma cell lines (U87, U251MG, TJ-905, U118, T98G, and A172) and primary normal human astrocytes (NHAs) were obtained from ATCC (Rockefeller, MD, USA). All cell lines were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco). Hsa-miR-144-3p and other mimics, miRNA control (mi-NC), hsa-miR-144-3p inhibitor (inhibitor) and its control (inhibitor NC) were purchased from Sigma. sh-TFRC, sh-lncRNA RP1-86C11.7 (sh-RP1-86C11.7) and shRNA control (sh-NC) were obtained from GenePharma (Shanghai, China).

The oligonucleotides were transfected into cells using Lipofectamine 2000 (Thermo Fisher, Grand Island, NY) following the manufacturer's instructions.

Cells stably overexpressing TFRC or shRNA were generated by lentivirus transduction. Briefly, 4×10^4 cells were seeded into 6-well plates with 2 mL of medium and cultured overnight. Next, the cells were transduced with 1 mL of medium containing 25 μL of lentiviral vector (3×10^8 TU/mL) and 4 μg/mL of polybrene. Twenty-four hours after transduction, the medium was changed, and the cells were constantly maintained for another 24 h. Puromycin (6 μg/mL) was used to screen the transduced cells. ShRNA lentiviruses were produced by cotransfection with psPAX2, pMD2G, and pLKO.1 or pCDH—CMV-Mcs-Ef1-Puro (Addgene) containing target sequences into HEK293T cells for 72 h, followed by purification by ultracentrifugation. The lentivirus containing lncRNA RP1-86C11.7 (LVV- RP1-86C11.7) was obtained from Meixuan Biological Technology Co., Ltd. (Shanghai, China).

RNA isolation and quantitative real-time PCR (qPCR)

Total RNA was extracted from tissues and cells using TRIzol Reagent (Invitrogen; Thermo Fischer Scientific, Inc., Waltham, MA). MiRNA was extracted from glioma cells using the miRNeasy Micro Kit (Qiagen, Germany) according to the manufacturer's protocol. mRNA and miRNA were reversed transcribed using the PrimeScript RT Reagent kit with gDNA Eraser (TaKaRa, Japan) and the miScript II RT kit (Qiagen, Germany). mRNA quantification was performed using TB Green® Advantage® qPCR Premix (TaKaRa, Japan), and miRNA quantification was performed using a miScript SYBR-Green PCR kit (Qiagen, Germany). The Q-PCR system was performed according to the manufacturer's protocol. GAPDH was used as the internal reference for mRNA and lncRNA, and U6 was used as the internal reference for miRNA. The relative gene expression was measured using the $2^{-\triangle\triangle CT}$ method.

The primers used for qPCR are exhibited in Table S1.

Luciferase reporter assay

The predicted sequences of lncRNA RP1-86C11.7 or TFRC were inserted into pGL4-luciferase reporter plasmids (Promega). U87 cells (1×10^4) were seeded into one well of a 96-well plate and incubated for 24 h before transfection. Next, the cells were cotransfected with 100 ng of reporter plasmids and hsa-miR-144-3p using Lipofectamine 2000 (Life Technologies). The dual-luciferase reporter assay system (Promega) was used to measure the luciferase activity in glioma cells after transfection for 48 h.

Western blot assay

Western blot analysis was performed as described previously [14]. Briefly, protein was extracted using an extraction reagent (Thermo Scientific; 78,503) supplemented with proteinase inhibitors (Sangon Biotech; C600387). Twenty micrograms of protein from each sample was separated on 10% SDS–PAGE gels and transferred onto PVDF membranes. After blocking, the membranes were incubated with primary antibodies overnight at 4 °C, followed by incubation for 1 h at room temperature with the secondary antibody. Bands were detected using the Bio-Rad ChemiDoc XRS system. Anti-GAPDH (1:1000) and anti-TFRC (1:1000) antibodies were obtained from Proteintech (Rosemont, USA). Mouse anti-rabbit HRP (1:5000) and rabbit anti-mouse HRP (1:5000) were obtained from Sangon Biotech (Shanghai, China).

Cell proliferation assay

Cell proliferation was tested using Cell Counting Kit 8 (CCK8; Beyotime, Shanghai, China). Cells overexpressing TFRC or sh-TFRC were seeded into 96-well plates at 3×10^3/well and cultured for 0, 24, 48, 72, 96 and 120 h. CCK-8 solution was added and incubated for 2 h, and then the absorbance was measured at 450 nm.

Clone formation assay

Approximately 1500 cells were resuspended in 1 mL of medium and seeded into a 6-well plate at 1 ml mixture/well. After 10 days of incubation, the colonies were fixed with 4% paraformaldehyde (Sangon Biotech, Shanghai, China), stained with GIEMSA solution (Beyotime, Shanghai, China), and dried, and then the colony numbers were determined.

RNA immunoprecipitation (RIP) assay

A Magna RIP™ RNA-Binding Protein Immunoprecipitation kit (Millipore, Billerica, MA, USA) was used to conduct the RIP assay following the manufacturer's instructions. Antibodies used for the RIP assay included anti-AGO2 (1:20) and control IgG (1:20) (Millipore). The enriched RNAs were measured by qRT-PCR, as described above.

RNA pull-down assay

RNA pull-down assays were performed as described previously [15]. Briefly, cells at 50% confluence in 6-well plates were transfected with 50 nM biotinylated miRNA mimics or nonsense control (Sigma). Next, the cells were harvested and lysed in lysis buffer at 24 h after transfection. The cell lysates were incubated with washed streptavidin magnetic beads (Life Technologies) for each binding reaction and further incubated at room temperature for 1 h. The beads were washed briefly three times, and TRIzol reagent (Invitrogen) was used to extract RNA interacting with miRNA. The coprecipitated RNAs were detected by q-PCR.

Cell cycle analysis by flow cytometry

U87 cells (1×10^6) were harvested and washed with cold phosphate-buffered saline (PBS) 3 times and then were fixed in 70% cold ethanol solution (diluted with PBS) at 4 °C overnight. After washing with PBS 3 times again, the cells were resuspended in 1 ml of PBS containing 100

μg/mL of DNase-free RNase and incubated at 37 °C for 20 min. The cells were then washed, resuspended in 100 μl of PBS containing 3 μM PI, and incubated at RT for 15 min. The cell cycle was evaluated using a flow cytometer (BD Biosciences, San Jose, CA, USA).

Intracellular free iron measurement

Calcein-AM enters viable cells and becomes fluorescent following hydrolysis by esterase; its fluorescence is quenched by LIP [16-19]. Cells at 60–70% confluence in 6-well plates were incubated with 1 ml of 100 nM calcein-AM (BioLegend) for 30 min at 37 °C in PBS. After calcein-AM loading, the cells were trypsinized, washed, resuspended in PBS without calcein-AM, and placed in 96-cell plates at 1×10^4 cells/well; the fluorescence was monitored using multifunctional enzyme markers at λex 488 nm and λem 518 nm (Thermo, USA). Low calcein-AM fluorescence intensity indicates high intracellular free iron levels.

Xenograft tumor assay

BALB/c nude mice (7 weeks old) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and randomly divided into 2 groups ($n = 6$). U87 cells (1×10^7) overexpressing RP1-86C11.7, sh-RP1-86C11.7 or their corresponding controls were subcutaneously injected into the back region of BALB/c nude mice. The tumor volume was measured every 5 days after injection using a Vernier caliper using the following formula: volume $= 0.5 \times$ length $\times$ width^2. Tumor weight was determined after 30 days.

Statistical analysis

All the data were analyzed using GraphPad Prism 7.0 (GraphPad Software, Inc., LA Jolla, CA) and presented as means±SD. The results were analyzed by unpaired 2-sided Student's *t*-test. Multiple groups were compared by one-way ANOVA with Tukey's post hoc test. $p<0.05$ was considered statistically significant. All experiments were repeated three times.

Results

TFRC is upregulated in glioma

Glioma is a cancer type with a high malignancy level and that is related to disordered iron metabolism [20]. TFRC is involved in iron metabolism [21]. To verify the role of TFRC in glioma progression, we investigated the clinical data of TFRC in glioma patients from the Gene Expression Profiling Interactive Analysis database (GEPIA: http://gepia.cancer-pku.cn/). The TFRC level was significantly higher in tumor patients than in the normal group (Fig. 1a). Thus, we analyzed TFRC expression in glioma cell lines (U87, U251MG, TJ-905, U118, T98G, and A172) and NHAs by qRT-PCR and Western blotting. An increased TFRC level was easily observed in Fig. 1b-c compared with NHAs. Consistent with the expression of TFRC in glioma cell lines, the intracellular free iron level was also elevated (Fig. 1d). These results suggested that TFRC is upregulated in glioma.

Higher expression of TFRC is relevant to glioma growth

Considering the importance of intracellular free iron in cell proliferation, we hypothesized that TFRC might promote glioma growth. Thus, we chose the U87 cell line for subsequent experiments because of its highest expression of TFRC among other cell lines. We artificially regulated TFRC expression in U87 cells by transducing them with LVV-TFRC or sh-TFRC. The mRNA and protein levels of TFRC were overexpressed or inhibited successfully (Fig. 1e-f). Because TFRC was elevated in U87 cells, intracellular free iron showed a higher level than that in controls, but it decreased with TFRC knockdown (Fig. 1g). Interestingly, the increased proliferation of U87 cells was associated with TFRC overexpression; correspondingly, TFRC reduction induced by sh-TFRC resulted in suppressed cell proliferation (Fig. 1h). These results were confirmed by the colony formation assay: TFRC overexpression enhanced the colony numbers, while TFRC inhibition decreased them (Fig. 1i-j). Additionally, cell cycle analysis showed that TFRC silencing elevated the percentage of U87 cells in the G0-G1 stage, suggesting inhibition of the cell cycle (Fig. 1(k), (l)). Taken together, the results showed that TFRC acts as a tumor oncogene and plays an essential role in glioma development.

TFRC is a target of hsa-miR-144-3p

We predicted potential miRNAs targeting TFRC within 5 algorithm programs (PITA, microT, miRanda, PicTar, and TargetScan) in starBase (http://starbase.sysu.edu.cn/index.php). We identified four miRNAs copredicted by all 5 algorithm programs—hsa-miR-7–5p, hsa-miR-141–3p, hsa-miR-144-3p and hsa-miR-200a-5p—which showed the most potential for binding to TFRC (Fig. 2a).

Therefore, we eliminated miRNA expression in glioma cell lines compared with NHAs. qPCR analysis revealed that hsa-miR-7–5p and hsa-miR-144-3p were downregulated in all 6 glioma cell lines, consistent with previous study findings [22-24]. Hsa-miR-200a-3p was upregulated only in U87, U118 and T98G cells, with no significant difference in hsa-miR-141–3p in all cell lines (Fig. 2b). Overexpressed hsa-miR-144-3p reduced the luciferase activity of TFRC (Fig. 2c), indicating that miR-144-3p is a candidate miRNA targeting TFRC. Next, we created luciferase reporters containing wild-type (WT) and mutant (MUT) sequences to confirm this result. The luciferase activity was decreased in the group cotransfected with TFRC-WT and hsa-miR-144-3p compared with that in the group cotransfected with TFRC-WT and mi-NC. No significant change in luciferase activity was found in the TFRC-MUT group with mi-NC or hsa-miR-144-3p (Fig. 2d-e). Additionally, the RIP results showed that TFRC and hsa-miR-144-3p were markedly enriched by anti-Ago2 antibody in U87 cells (Fig. 2f). The expression profile of miR-144-3p from Fig. 2b is shown in Fig. 2h. Additionally, overexpressed hsa-miR-144-3p inhibited TFRC expression at the mRNA and protein levels (Fig. 2i-k). The level of intracellular free iron was also decreased with the overexpression of hsa-miR-144-3p (Fig. 2l). Silencing hsa-miR-144-3p with an inhibitor reversed its effects on TFRC expression and intracellular free iron (Fig. 2i-l). Furthermore, the CCK-8 assay results were consistent with the intracellular free iron level. hsa-miR-144-3p overexpression markedly suppressed the proliferation of U87 cells, and the hsa-miR-144-3p inhibitor dramatically inhibited this influence (Fig. 2m). In conclusion, these results showed that hsa-miR-144-3p plays a suppressor role in glioma development by targeting TFRC and downregulating its expression.

RP1-86C11.7 regulates hsa-miR-144-3p

Considering the crucial role of lncRNAs in miRNA regulation and cancer progression, we hypothesized that hsa-miR-144-3p is regulated by an lncRNA. We used DIANA (http://diana.imis.athena-innovation.gr/DianaTools/) to predict the potential lncRNAs binding to hsa-miR-144-3p. Five hundred sixty-five lncRNAs were predicted from LncBase of DIANA. According to the p(HR) of the clinical survival curve for each lncRNA in GBM patients (data from GEPIA), we selected the top 10 lncRNAs with minimal p(HR) values (Table 1). After excluding lncRNAs reported previously in glioma because we attempted to explore a new lncRNA in glioma, 8 lncRNAs were chosen for further study (Table 1). We used a luciferase activity assay to screen these 9 lncRNAs by cotransfection with mi-NC or hsa-miR-144-3p. Three lncRNAs displayed reduced luciferase activity, and RP1-86C11.7 was the best performing lncRNA, indicating that its role in hsa-miR-144-3p-induced TFRC regulation is worth exploring (Fig. 3a). We performed a luciferase

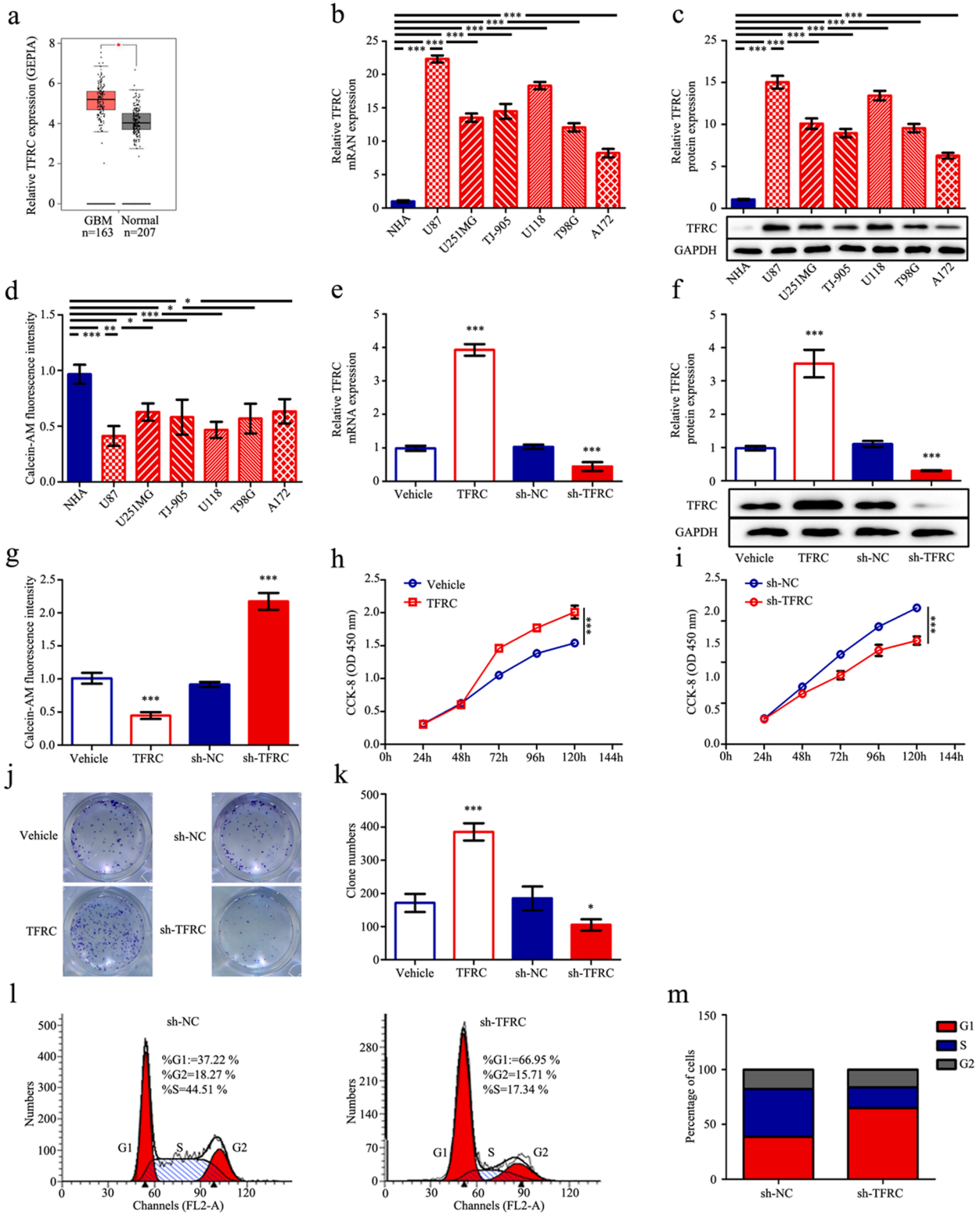

Fig. 1. TFRC is upregulated in glioma, and higher expression of TFRC is relevant with glioma growth.
(a) The relative expression of TFRC in GBM patients ($n = 163$) and normal people ($n = 207$) from clinical database (GEPIA). (b, c) The mRNA and protein levels of TFRC in NHA and glioma cell lines were detected by qRT-PCR and western blot. (d) The intercellular free iron level in NHA and glioma cell lines was examined by Calcein-AM. (e, f) Detection of TFRC expression with overexpressing or silencing of TFRC by qRT-PCR and western blot. (g) Measurement of the intracellular free iron level with overexpressing and silencing of TFRC by Calcein-AM in U87 cells. (h-k) The effect of TFRC on the proliferation of U87 cells was examined by CCK8 assay and colony formation assay. (l, m) The effect of TFRC on the cell cycle of U87 cells was examined by flow cytometry. The data are presented as the means ± SD, $n = 3$ experiments in a-l. *$p < 0.05$, **$p<0.01$, ***$p<0.005$. b-g, k, used ANOVA analyses with Tukey's post-hoc test; h, i, m used Student's *t*-test.

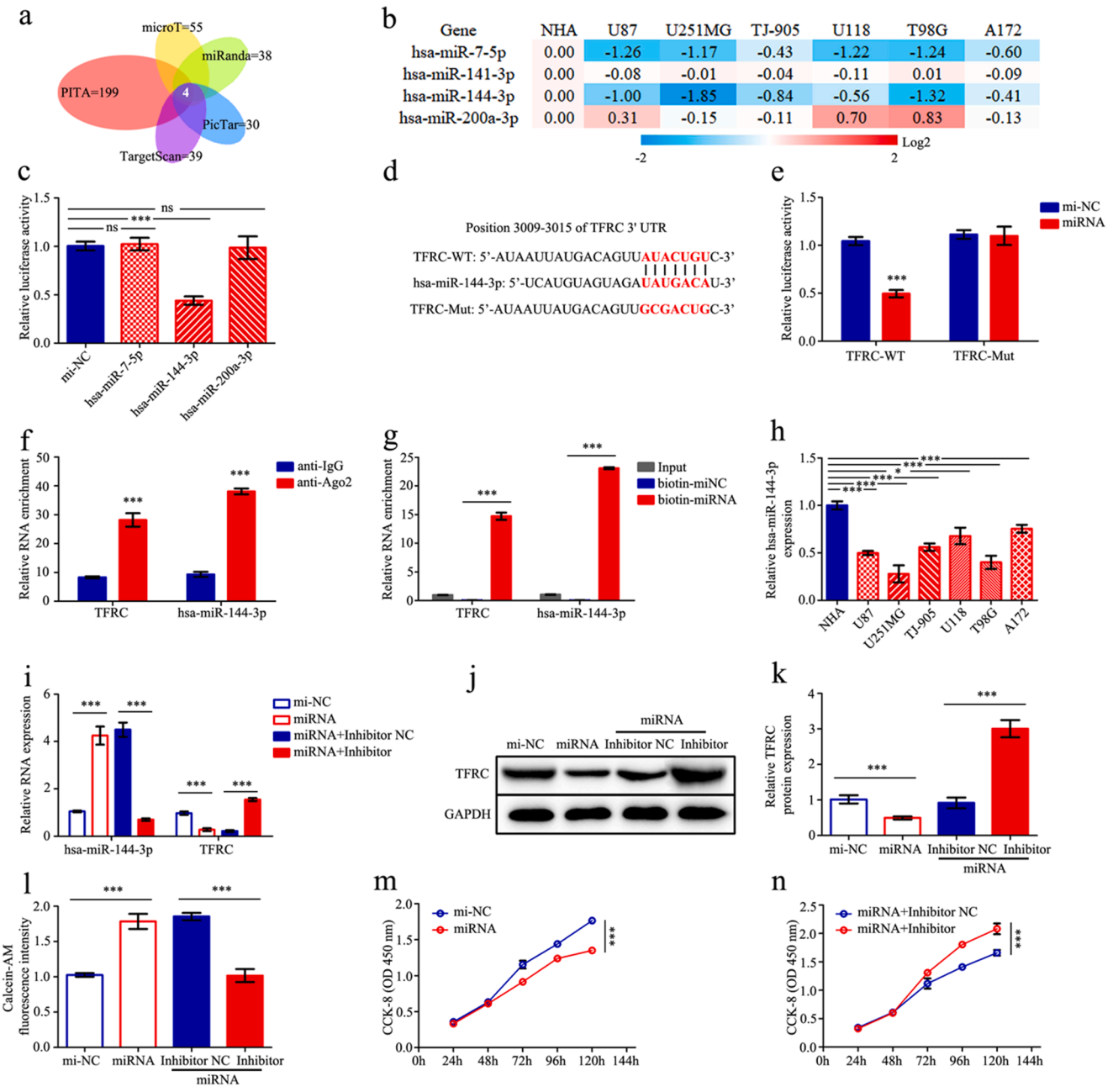

Fig. 2. TFRC is a target of hsa-miR-144-3p.

(a, b) Starbase was used to predict the miRNAs which could bind to TFRC and their expression in glioma cell lines compared with NHA. (c) Determination of luciferase activity of potential miRNAs binding with TFRC mRNA in U87 cells. (d) The binding site of TFRC and hsa-miR-144-3p. (e) Measurement of luciferase activity when U87 cells were transfected with a hsa-miR-144-3p mimic in the presence of wild-type and mutant TFRC. (f, g) The relationship between TFRC and hsa-miR-144-3p was detected by Ago2 RIP-qPCR assay and RNA-pulldown-qPCR assay in U87 cells. (h)The expression of hsa-miR-144-3p in U87 cells was detected by qRT-PCR. (I, j, k) The mRNA level and protein level of TFRC when overexpressing and silencing hsa-miR-144-3p were detected by qRT-PCR and western blot in U87 cells. (l) The effect of hsa-miR-144-3p on the intracellular free iron level was detected by Calcein-AM in U87 cells. (m, n) The effect of hsa-miR-144-3p on the proliferation of U87 cells was examined by CCK8 assay. The data are presented as the means ± SD, $n = 3$ experiments in b-m $^{*}p < 0.05$, $^{**}p<0.01$, $^{***}p<0.005$. b, c h, i, k and l used ANOVA analyses with Tukey's post-hoc test; b, e, f, g, m and n used Student's *t*-test.

activity assay by adding RP1-86C11.7-MUT to confirm the previous result (Fig. 3b-c). The clinical data from GEPIA showed a higher level of RP1-86C11.7 in GBM patients (Fig. 3d). The survival curve of RP1-86C11.7 from GEPIA is shown in Fig. 3e. RIP results showed that hsa-miR-144-3p and RP1-86C11.7 were markedly enriched by Ago2 antibody in U87 cells (Fig. 3f). Next, we used a biotinylated hsa-miR144-3p probe to pull down RP1-86C11.7. The RNA pull-down results showed that endogenous RP1-86C11.7 was enriched specifically by the hsa-miR-144-3p probe compared with the control group, suggesting that hsa-miR-144-3p is a direct target of RP1-86C11.7 (Fig. 3g). These results demonstrated that RP1-86C11.7 directly binds to hsa-miR-144-3p and plays a critical role in glioma.

RP1-86C11.7 regulates TFRC expression through hsa-miR-144-3p

In addition to the RP1-86C11.7 data in the clinic, we found upregulated RP11–86C11.7 expression in different glioma cell lines, with the highest expression in U87 cell lines (Fig. 4a). To clarify the relationship

Table 1.
Top 10 lncRNAs with minimal p(HR) value in GBM.

Gene	p(HR)	Logrank p
TMEM191C	0.00052	0.00039
RP11–214O1.3	0.0035	0.0035
RP1-86C11.7	0.0048	0.0044
AP000695.4	0.0055	0.0055
AC092198.1	0.0071	0.0076
RP11–597D13.9	0.0076	0.0065
RP11–584P21.2	0.0093	0.0084
RP11–932O9.9	0.013	0.012
DPH6-AS1	0.017	0.018
LINC00662	0.021	0.02

LncRNAs marked with **Bold** means this lncRNA has been reported before.

among RP11–86C11.7, hsa-miR-144-3p and TFRC, we also monitored TFRC expression in RP1-86C11.7-overexpressing or RP1-86C11.7-silenced cells. Overexpressing RP1-86C11.7 increased TFRC expression, while silencing RP1-86C11.7 decreased TFRC expression without any change in hsa-miR-144-3p, indicating the possibility of sponge activity (Fig. 4b-c). The intracellular free iron levels exhibited a trend similar to that of TFRC expression (Fig. 4d). Furthermore, the CCK-8 and colony formation assays showed that RP1-86C11.7 deteriorated the oncogenicity of glioma by promoting glioma cell growth (Fig. 4e-g).

To further explore the relationship between RP1-86C11.7 and TFRC, we investigated whether an hsa-miR-144-3p inhibitor could reverse the effects induced by sh-RP1-86C11.7 on U87 cells. Thus, U87 cells with RP1-86C11.7 knockdown successfully by sh-RP1-86C11.7 were transfected with hsa-miR-144-3p inhibitor NC or inhibitor. The mRNA and protein levels of TFRC were downregulated with RP1-86C11.7 knockdown, and transfection with an inhibitor increased TFRC expression (Fig. 4h-i). The level of intracellular free iron showed similar changes as TFRC expression (Fig. 4j). Thus, the inhibited proliferation and clone formation induced by sh-RP1-86C11.7 were reversed after transfecting the hsa-miR-144-3p inhibitor into U87 cells (Fig. 4k-m). Furthermore, RP1-86C11.7 knockdown in U87 cells increased the percentage of cells in G1 phase. At the same time, transfection with the inhibitor changed the percentage of cells in G1 phase close to that in the sh-NC group (Fig. 4n-o). These results indicated that RP1-86C11.7 upregulates TFRC and promotes glioma oncogenicity by sponging hsa-miR-144-3p.

Effects of RP1-86C11.7 on glioma malignancy through the hsa-miR-144-3p/TFRC axis in other cell lines

To generalize the role of RP1-86C11.7 and its corresponding pathway in glioma malignancy, we analyzed its effects in two other glioma cell lines, U251MG and A172, which express relatively high and low levels of RP1-86C11.7, respectively (Fig. 4a). We also used a second shRNA to knock down TFRC and RP1-86C11.7 (named sh-TFRC-2 and sh- RP1-86C11.7–2). First, we measured the intracellular free iron of U251MG and A172 cells with TFRC overexpression or knockdown.

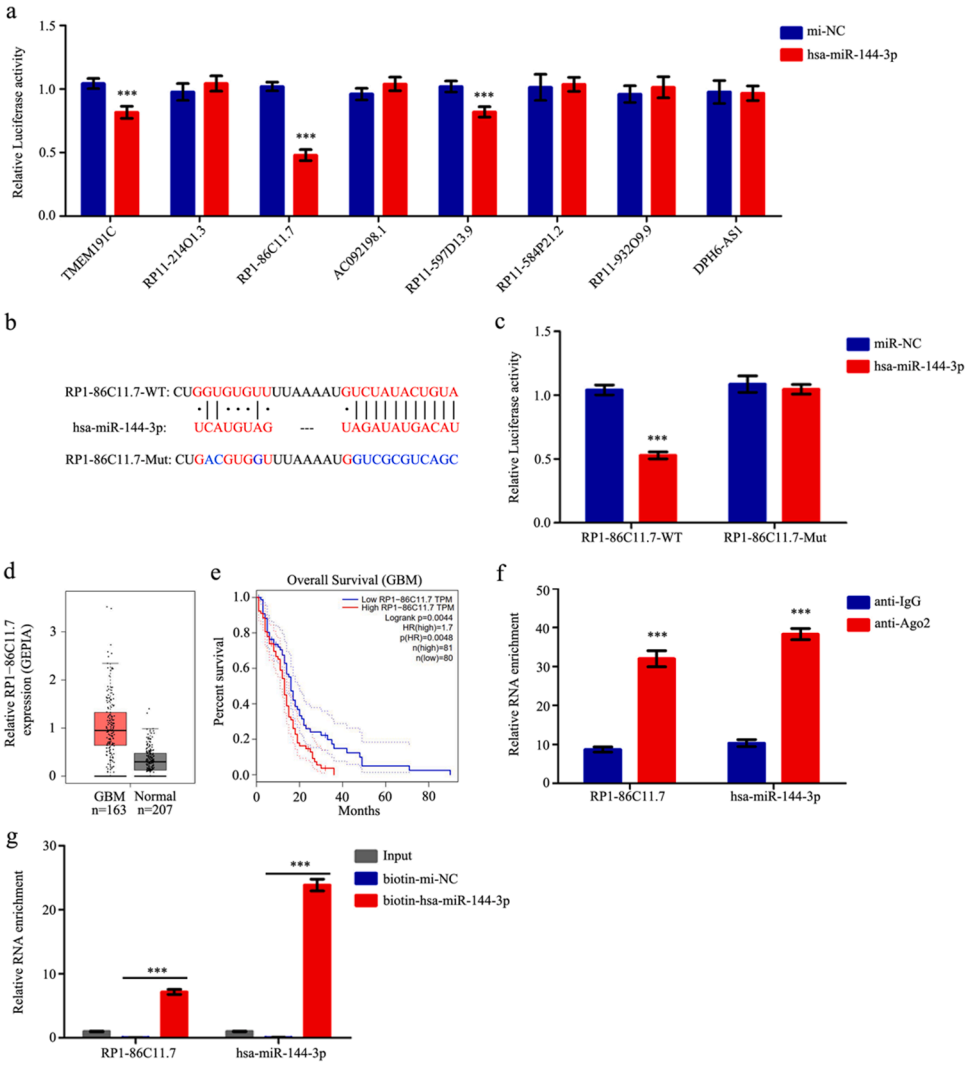

Fig. 3. RP1-86C11.7 regulates hsa-miR-144-3p.
(a) The luciferase activity of potential lncRNAs predicted by DIANA binding to hsa-miR-144-3p in U87 cells. (b) The binding site of RP1-86C11.7 and hsa-miR-144-3p. (c) Measurement of luciferase activity when U87 cells were transfected with hsa-miR-144-3p in the presence of wild-type and mutant RP1-86C11.7. (d) The relative expression of RP1-86C11.7 in GBM patients ($n = 163$) and normal people ($n = 207$) from clinical database (GEPIA). (e) Overall survival rates of GBM patients in high and low expression of RP1-86C11.7 from the clinical database (GEPIA). (f, g) The relationship between hsa-miR-144-3p and RP1-86C11.7 was detected by the Ago2 RIP-qPCR assay and biotin-labeled RNA-pulldown-qPCR assay in U87 cells. The data are presented as the means ± SD, $n = 3$ experiments in a, c, f, g. $^{*}p < 0.05$, $^{**}p<0.01$, $^{***}p<0.005$. a, c, f, g, m and n used Student's *t*-test.

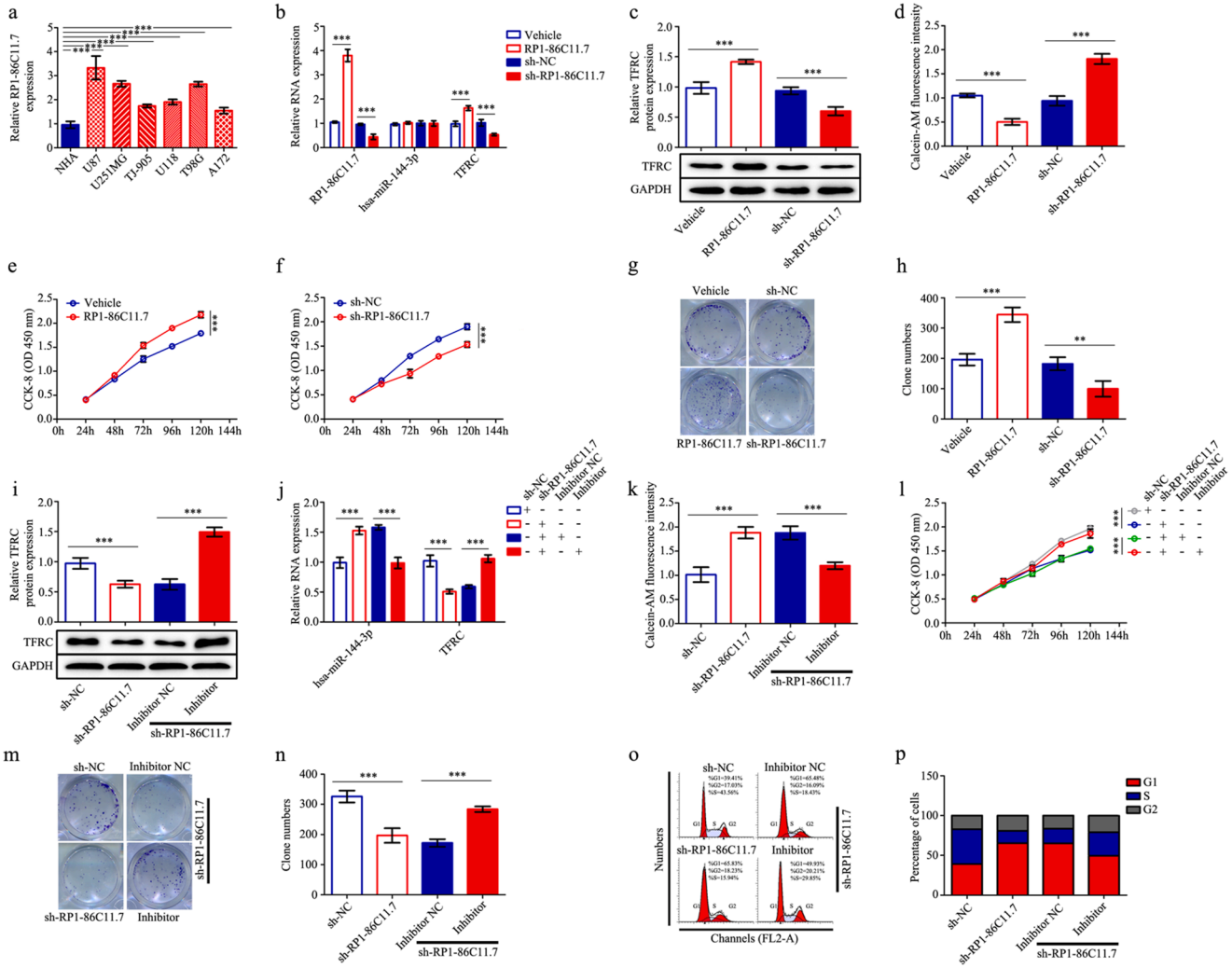

Fig. 4. RP1-86C11.7 regulates the expression of TFRC through hsa-miR-144-3p.
(a) The expression of RP1-86C11.7 in U87 cell lines was detected by qRT-PCR. (b) The mRNA level of RP1-86C11.7, hsa-miR-144-3p and TFRC with overexpressing or silencing RP1-86C11.7 were detected by qRT-PCR. (c) The protein expression of TFRC with overexpressing or silencing RP1-86C11.7 was detected by western blot in U87 cells. (d) The effect of RP1-86C11.7 on the intracellular free iron level was detected by Calcein-AM in U87 cells. (e-h) The effect of RP1-86C11.7 on the proliferation of U87 cells was examined by CCK8 assay and colony formation assay. (i) The protein expression of TFRC in sh-RP1-86C11.7 transduced U87 cells with transfecting hsa-miR-144-3p inhibitor was detected by western blot. (j) The mRNA level of hsa-miR-144-3p and TFRC in sh-RP1-86C11.7 transduced U87 cells with transfecting hsa-miR-144-3p inhibitor was detected by qRT-PCR. (k) The intracellular free iron level of sh-RP1-86C11.7 transduced U87 cells with transfecting hsa-miR-144-3p inhibitor was detected by Calcein-AM. (l-n) The cell proliferation of sh-RP1-86C11.7 transduced U87 cells with transfecting hsa-miR-144-3p inhibitor was detected by CCK8 assay and colony formation assay. (o, p) The cell cycle of sh-RP1-86C11.7 transduced U87 cells with transfecting hsa-miR-144-3p inhibitor was detected by flow cytometry. The data are presented as the means ± SD, $n = 3$ experiments in a-o. *$p < 0.05$, **$p<0.01$, ***$p<0.005$. a-d, h-k and n used ANOVA analyses with Tukey's post-hoc test; e-f, i and p used Student's *t*-test.

Overexpressing TFRC resulted in a higher level of intracellular free iron than that of the vehicle groups. However, the intracellular free iron was upregulated when TFRC was knocked down by shRNA compared with that in the sh-NC groups (Fig. 5a). Thus, the proliferation of U251MG and A172 cells was increased by TFRC overexpression and inhibited by TFRC knockdown (Fig. 5b-e). Next, the effect of hsa-miR-144-3p in the intracellular free iron steady state and cell proliferation was analyzed in U251MG and A172 cell lines. Similar to that in U87 cell lines, hsa-miR-144-3p overexpression elevated the intracellular free iron levels, and its inhibition displayed an inverse tendency (Fig. 5f). The proliferation of U251MG and A172 cells was controlled by hsa-miR-144-3p in the same manner (Fig. 5g-j). To confirm the change, we detected TFRC expression by qPCR and western blotting, which showed a lower level of TFRC in cells treated with hsa-miR-144-3p and an opposite result in cells treated with inhibitor (Fig. 5k-m).

Additionally, similar to RP1-86C11.7 in U87 cell lines, RP1-86C11.7 upregulation increased the intracellular free iron level in U251MG and A172 cells, while its downregulation induced by sh-RP1-86C11.7–2 repressed iron levels (Fig. 5n). Interestingly, the repression effect of sh-RP1-86C11.7–2 could be rescued by the hsa-miR-144-3p inhibitor (Fig. 5n), indicating that the influence of RP1-86C11.7 on intracellular free iron in U251MG and A172 cells also occurred via the hsa-miR-144-3p/TFRC axis. Proliferation also showed a similar tendency, with a promotive role in RP1-86C11.7 upregulation, a suppressive role with sh-RP1-86C11.7–2 treatment, and a rescue effect with the hsa-miR-144-3p inhibitor group compared with the sh-RP1-86C11.7–2 group (Fig. 5o-r). We measured the effect of RP1-86C11.7 on the clone formation ability of U251MG and A172 cells. The results showed that their clone forming ability was enhanced by RP1-86C11.7 overexpression, inhibited by sh-RP1-86C11.7–2 treatment and rescued by the inhibitor (Fig. 5s-v). TFRC expression was also monitored by qPCR and western blotting. Consistent with these results, RP1-86C11.7 promoted the TFRC levels, and the hsa-

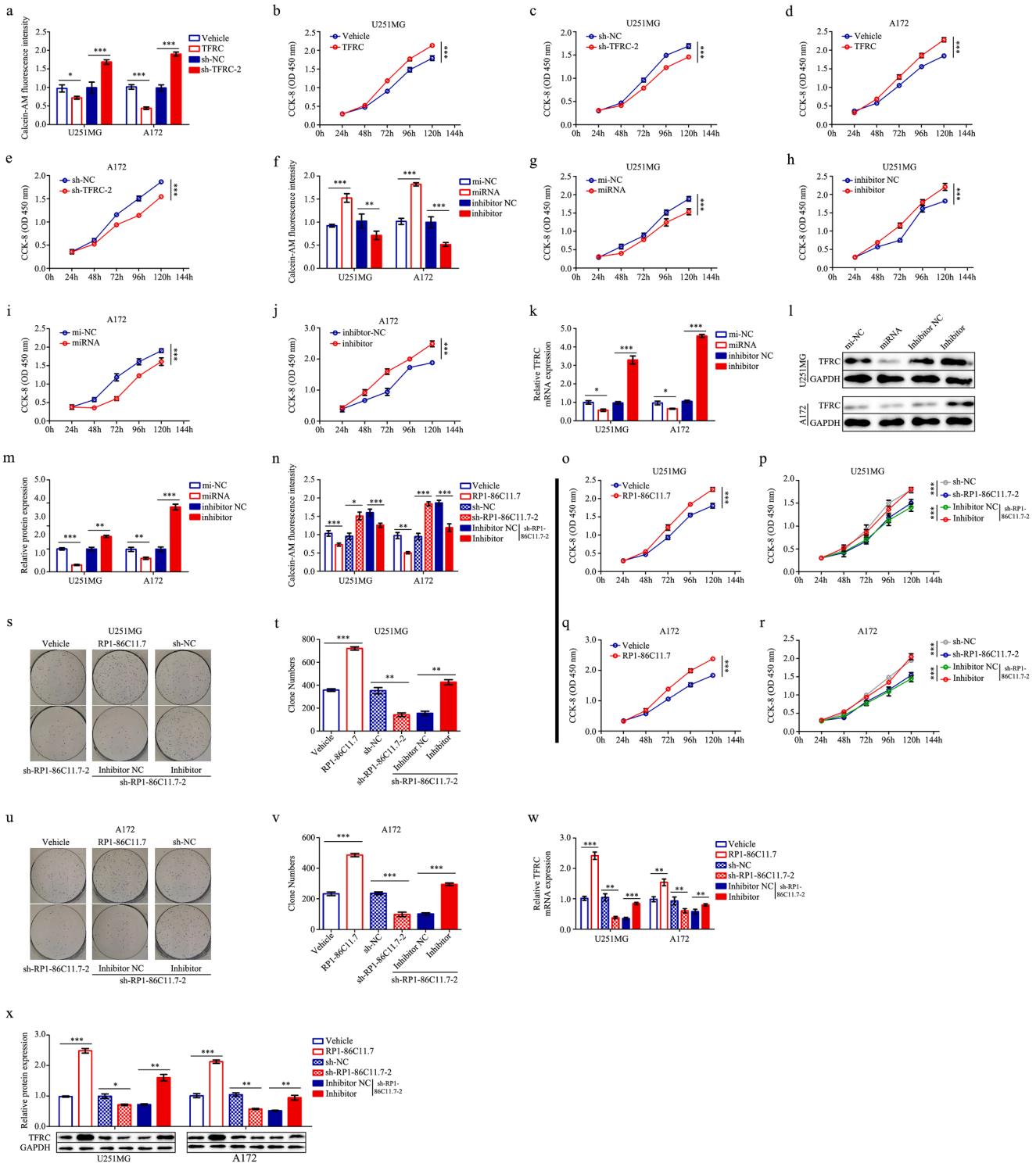

Fig. 5. Effects of RP1-86C11.7 on glioma malignance through hsa-miR-144-3p/TFRC axis in other cell lines

(a) The effect of TFRC on the intracellular free iron level was detected by Calcein-AM in U251MG and A172 cells. (b-e) The effect of TFRC on the proliferation of U251MG and A172 cells was examined by CCK8 assay. (f) The effect of hsa-miR-144-3p on the intracellular free iron level was detected by Calcein-AM in U251MG and A172 cells. (g-j) The effect of hsa-miR-144-3p on the proliferation of U251MG and A172 cells was examined by CCK8 assay. (k-m) The effect of hsa-miR-144-3p on TFRC expression analyzed by qPCR and western blot in U251MG and A172 cells. (n) The effect of RP1-86C11.7 on the intracellular free iron level was detected by Calcein-AM in U251MG and A172 cells. (o-v) The effect of RP1-86C11.7 on the proliferation of U251MG and A172 cells was examined by CCK8 assay and clone formation assay. (w-v) The effect of RP1-86C11.7 on TFRC expression analyzed by qPCR and western blot in U251MG and A172 cells. The data are presented as the means ± SD, $n = 3$ experiments in a-x. *$p < 0.05$, **$p<0.01$, ***$p<0.005$. a, f, k, m, n, t, v, w and x used ANOVA analyses with Tukey's post-hoc test; b-e, g-j and o-r used Student's *t*-test.

miR-144-3p inhibitor rescued the TFRC downregulation induced by sh-RP1-86C11.7–2 (Fig. 5w-x).

Together, these results in U251MG and A172 cells showed a coincident tendency with those in U87 cells, indicating that the general oncogenic role of RP1-86C11.7 in glioma cells.

Effect of RP1-86C11.7 on glioma tumor growth in vivo

Given the function of RP1-86C11.7 in vitro, we established a mouse xenograft model to investigate its effect on glioma growth in vivo. U87 cells stably expressing RP1-86C11.7 or vehicle, sh-RP1-86C11.7 or sh-NC were subcutaneously injected into nude mice. The xenograft tumors from sh-RP1-86C11.7-transduced U87 cells grew slower than the tumors from sh-NC transduced U87 cells, while the RP1-86C11.7-transduced groups remarkably promoted the growth of tumors compared with the vehicle-transduced groups (Fig. 6a-b) at 25 days after injection. The volume and weight measured at 25 days after injection showed similar results (Fig. 6c-d). Additionally, we detected the expression of hsa-miR-144-3p and TFRC in tumor tissues.

The expression of hsa-miR-144-3p in the sh-RP1-86C11.7-transduced group was upregulated, but it was suppressed in the RP1-86C11.7-transduced group. TFRC expression showed the opposite tendency at the mRNA and protein levels (Fig. 6e-f). The results suggested that RP1-86C11.7 displayed oncogenic activity to promote glioma growth in vivo.

Together, our results demonstrated that lncRNA RP1-86C11.7 sponges hsa-miR-144-3p, an miRNA targeting the 3′-UTR of TFRC mRNA, inhibiting its expression. Thus, RP1-86C11.7 exacerbates tumor progression and development in vitro and in vivo by upregulating TFRC expression in glioma.

Discussion

Increasing evidence suggests that iron metabolism disorder is related to brain disease [25, 26]. TFR1 encodes TFRC and plays a critical role in iron uptake and the balance of iron metabolism. TFRC is highly expressed in glioma [27] and brain endothelial cells [28]. Increased transferrin expression indicates that tumor cells can obtain more iron, which is essential for cell proliferation. Therefore, tumor cells have advantages in growth compared with normal cells [29]. The important role of TFRC in tumor progression has been reported in several cancers, such as breast cancer [30], ovarian cancer [31] and liver cancer [32]. However, confirmation of its role in glioma is limited. Here, we found that a high level of TFRC is associated with promoted proliferation and survival of glioma cells, indicating the potential effects of high levels of TFRC in GBM.

Recent studies indicate that miRNAs play a critical role in GBM and mediate cancer cell growth, apoptosis, migration, and invasion [33]. Additionally, miRNA plays a crucial role in regulating TFRC. Kamesh R Babu et al. demonstrated that miR-148a regulates transferrin receptor expression in hepatocellular carcinoma [11]. In our study, hsa-miR-144-3p regulates TFRC expression in glioma. Hsa-miR-144-3p participates in the antitumor process in glioma through different signaling pathways. Previously, low miR-144-3p expression was found in glioblastoma cell lines, and hsa-miR-144-3p serves as a tumor suppressor by targeting FZD7 or c-MET and predicts the prognosis of human glioblastoma [24, 34]. Additionally, our study is the first to clarify the relationship between hsa-miR-144-3p and TFRC in glioma.

A study in 2012 showed that the lncRNA profile in glioblastoma is significantly altered, a finding that is relevant to glioblastoma pathogenesis [35], by binding proteins or mediating miRNAs [36, 37]. With advances in research, an increasing number of lncRNAs have been found to play critical roles in glioma, particularly over the past ten years. NET1 [38], CRNDE [39], HOTAIRM1 [40], FOXD3-AS1 [41] and MIR22HG [42] were highly expressed in GBM and promoted glioma progression. Gai et al. demonstrated that lncRNA SOX21-AS1 promotes cell proliferation and invasion by upregulating PAK7 expression and sponging miR-144-3p in glioma cells [43]. These findings were consistent with those of the current study, whereby RP1-86C11.7 promoted the proliferation and survival of glioma cells by inactivating the hsa-miR-144-3p-TFRC axis. Accordingly, we hypothesized that RP1-86C11.7 is an oncogenic lncRNA in GBM. Additionally, to broaden our knowledge concerning the functional mechanisms of RP1-86C11.7 in glioma development, further study may be required to explore the regulation of RP1-86C11.7 and binding partners of RP1-86C11.7 in glioma. Identifying novel molecular mechanisms will strengthen the promising application of RP1-86C11.7 in cancers as a potential biomarker or target for treatment. For example, the self-assembled small

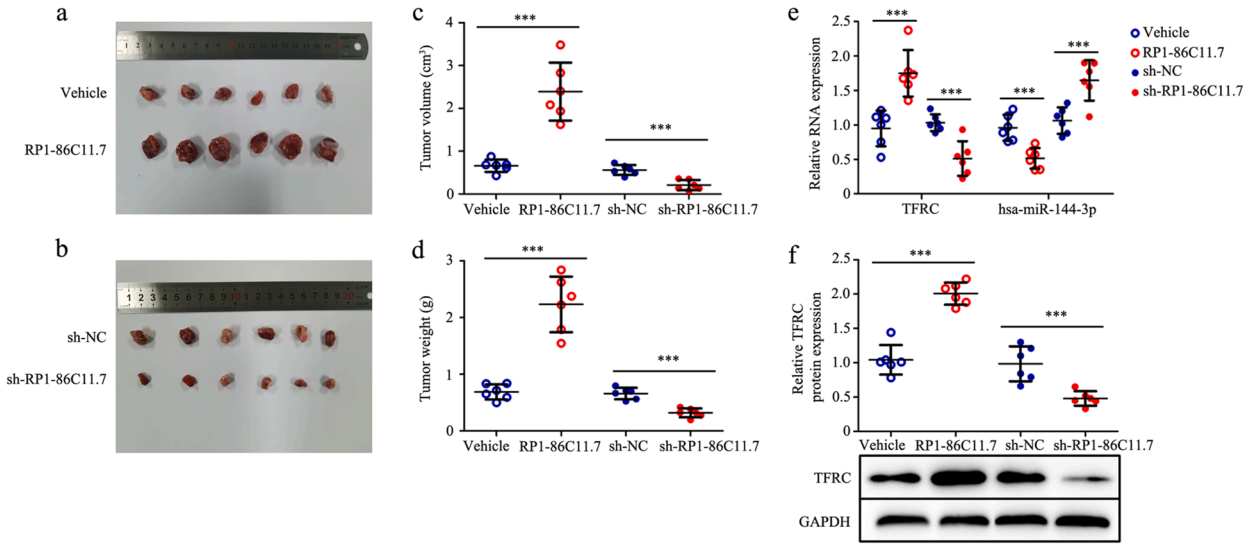

Fig. 6. Effect of RP1-86C11.7 on tumor growth of glioma in vivo.
Nude mice ($n = 6$) were subcutaneously injected with U87 cells transfected with vector and RP1-86C11.7 or shNC and sh RP1-86C11.7. (a, b) Representative tumor diagrams in different groups were shown. (c) Tumor volume was analyzed after dissecting from the nude mice at 30 d after injection using the formula: volume = 0.5 × length × width2. (d) The tumors were weighed after dissecting from the nude mice at 30 d after injection. (e) The mRNA level of TFRC and hsa-miR-144-3p were detected by qRT-PCR in different groups. (f) The protein level of TFRC in different groups was detected by western blot. The data are presented as the means ± SD, $n = 6$ experiments in c-f. *$p < 0.05$, **$p<0.01$, ***$p<0.005$. c-f used ANOVA analyses with Tukey's post-hoc test.

RNA system used by Zheng Fu et al. delivered siRNAs targeting EGFR and TNC in glioblastoma for treatment [44]. This new generation of RNAi therapeutics reprograms the host liver with genetic circuits to direct the synthesis and self-assembly of siRNAs into secretory exosomes and facilitate the in vivo delivery of siRNAs through circulating exosomes. After displaying an RVG tag on the exosome surface, exosomes can efficiently cross the blood-brain barrier and deliver siRNAs into the brain.

In summary, our study facilitates understanding the role of TFRC in GBM and identifies a new molecular mechanism to regulate TFRC, contributing to the investigation of GBM oncogenicity and potential therapeutic target development.

Conclusion

Here, we clarified the relationship between TFRC and glioma progression. Additionally, TFRC regulation is involved in hsa-miR-144-3p and lncRNA RP1-86C11.7 in glioma. Hsa-miR-144-3p binds to the 3′-UTR of TFRC mRNA and inhibits its expression, while RP1-86C11.7 promotes TFRC levels in glioma by binding to hsa-miR-144-3p directly. Therefore, a high RP1-86C11.7 level exacerbates tumor progression and accelerates tumor development in glioma. These results contribute to understanding the function and mechanism of TFRC and RP1-86C11.7 in GBM. The oncogenic potential of TFRC and RP1-86C11.7 suggests that targeting them may be good strategies in GBM treatment.

Abbreviation

GBM: Glioblastoma; TFRC: Transferrin receptor; LncRNA: Long non-coding RNA; miRNA: microRNA; FDA: Food and Drug Administration; LIP: labile iron pool; SRA: steroid receptor RNA activator; NHA: normal human astrocytes; mi-NC: miRNA control; inhibitor: hsa-miR-144-3p inhibitor; inhibitor NC: inhibitor control; sh-TFRC: short hairpin RNA target TFRC mRNA; sh-RP1-86C11.7: short hairpin RNA target RP1-86C11.7; sh-NC: short hairpin RNA control; CCK8: Cell Counting Kit 8; qPCR: quantitative real-time PCR; RIP: RNA immunoprecipitation; PBS: phosphate buffer saline; WT: wild type; MUT: mutant type.

Declaration of Competing Interest

None of the authors has any competing interests in the manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Animal Care and Use Committee of Huai'an Hospital. All animal experiments were performed in the animal laboratory center at Huai'an Hospital, and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Consent for publication

Not Applicable.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

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Authors' contributions

JL designed this study. JL supervised the study. QM and WX contributed to experiments and data analysis. QM and WX prepared the manuscript. JL revised the manuscript. All authors read and approved the final manuscript.

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Supplementary materials

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