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RESEARCH PAPER

m^{6}A RNA demethylase FTO promotes the growth, migration and invasion of pancreatic cancer cells through inhibiting TFPI-2

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ABSTRACT

Pancreatic cancer (PC) is one of the most fatal cancers with a very poor prognosis. Here, we found that N^6-methyladenosine (m^{6}A) RNA demethylase fat mass and obesity-related protein (FTO) promote the growth, migration and invasion of PC. FTO expression level is increased in human PC and is associated with poor prognosis of PC patients. Knockdown of FTO increases m^{6}A methylation of TFPI-2 mRNA in PC cells, thereby increasing mRNA stability via the m^{6}A reader YTHDF1, resulting in up-regulation of TFPI-2 expression, and inhibits PC proliferation, colony formation, sphere formation, migration and invasion *in vitro*, as well as tumour growth *in vivo*. Rescue assay further confirms that FTO facilitates cancer progression by reducing the expression of TFPI-2. Mechanistically, FTO promotes the progression of PC at least partially through reducing m^{6}A/YTHDF1 mediated TFPI-2 mRNA stability. Our findings reveal that FTO, as an m^{6}A demethylase, plays a critical role in promoting PC growth, migration and invasion, suggesting that FTO may be a potential therapeutic target for treating PC.

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Introduction

Pancreatic cancer (PC) is one of the most aggressive solid malignant tumours. The latest data showed that the 5-year survival rate of PC in the United States in 2020 was merely 9%, which was the lowest among all tumour types [1]. Surgical resection of tumour is the only possible cure for PC [2]. Unfortunately, 80% of PC patients are already in locally advanced stages of unresectable tumour or have distant metastases [3]. Furthermore, PC is insensitive to most chemotherapeutic agents [4]. Hence, it is urgent to reveal the molecular biological mechanism of PC progression and develop new effective therapeutic methods. At present, epigenomics research, including DNA methylation, long non-coding RNA and microRNA, has begun to provide new insights for PC [5]. However, N^6-methyladenosine (m^{6}A) RNA methylation in PC development is largely unknown.

m^{6}A RNA methylation, the most significant chemical modification found in eukaryotic messenger RNA (mRNA), is an emerging epigenetic molecular mechanism that regulates gene expression at the post-transcriptional level [6]. Recently, Jia et al [7]. found that FTO remove the methylation of m^{6}A on mRNA, indicating that mRNA methylation is a dynamic and reversible process. This latest discovery has open a new field of post-transcriptional gene regulation in eukaryotes [8]. Generally, m^{6}A methylation is catalysed by the 'writer' complex mainly composed of METTL3, METTL14 and WTAP, and is removed by the 'eraser' demethylase FTO or ALKBH5 [9]. The reading proteins 'reader' include the YTH domain family of proteins [10], eukaryotic initiation factor (EIF) 3 [11], and IGF2 mRNA binding protein (IGF2BP) family [12], which recognize m^{6}A, bind mRNA and determine the fate of the target mRNA. m^{6}A modification affects basic cell biological processes, including DNA damage repair, meiosis [13,14], innate immune response and tumorigenesis [15,16]. As the first m^{6}A demethylase identified, FTO

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Supplemental data for this article can be accessed here.

has been demonstrated to play a role as an oncogenic factor in leukaemia [17] and glioblastoma [18], and also plays a critical tumour-promoting role in digestive system tumours such as oesophageal cancer [19] and liver cancer [20]. However, the role and potential mechanism of FTO as m^6A demethylase in the carcinogenesis and development of PC remain unclear.

Here, we reported that m^6A demethylase FTO promotes PC cell proliferation, colony formation, sphere formation, migration, and invasion *in vitro*, and facilitates PC growth *in vivo*, and this regulatory effect is at least partially dependent on m^6A /YTHDF-1 mediated TFPI-2 mRNA stability. This novel mechanism may provide a new strategy for treating PC.

Materials and methods

Cell lines and cell culture

Normal pancreatic ductal epithelial cell lines HPDE and PC cell lines MIA PaCa-2, SW1990 and BxPC-3 were all kindly donated by Dr. Jie Wang from Renmin Hospital of Wuhan University. PC cell line PANC-1 was obtained from Shanghai Cell Bank, Chinese Academy of Sciences. PANC-1, MIA PaCa-2 and SW1990 cells were incubated in high glucose DMEM medium containing 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin. HPDE and BxPC-3 cells were incubated in RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin. All cells were cultured in a humidified incubator at 37°C, 5% CO_2.

Human PC specimens

Twenty matched cases of PC and paracancerous pancreatic tissues, and 43 paraffin-embedded PC specimens and 24 specimens of normal pancreatic tissue adjacent to carcinoma were obtained from Renmin Hospital of Wuhan University. All human specimens were studied after approval by the Ethics Committee of Renmin Hospital of Wuhan University. In addition, informed consent has been obtained from the participants involved.

RNA isolation and qRT-PCR

Total RNA was isolated from tissues or cells utilizing Trizol (Thermo Fisher Scientific, USA). qRT-PCR was performed as described in a previous study [21]. FTO, TFPI-2, YTHDF1 were normalized against GAPDH. The RNA expression level was analysed using $2^{-\Delta\Delta CT}$. The primer sequences used in this study were presented in supplemental Table S1.

Western blot

Total proteins from cells or tissues were extracted using RIPA buffer (Beyotime, China). An equivalent amount of protein was isolated using SDS-PAGE and then transferred to a PVDF membrane. The membrane was blocked with 5% skimmed milk for 1 h at 20°C, followed by primary antibodies: FTO (1:1000, Abclonal, A3861), TFPI-2 (1:1000, Abcam, ab186747), and GAPDH (1:1000, Biosharp, BL006A).

Small interfering RNA (siRNA) Transfection and lentiviral infection

siRNA (siFTO-1, 5'-GUGGCAGUGUACAGUUAUATT-3'; siFTO-2, 5'-ACACUUGGCUCCCUUAUCUTT-3'; siTFPI-2, 5'-UUCUUUAAUCUAAGUUCCAUGTT-3'; siYTHDF1, 5'-ACCUACGGACAGCUCAGUAACTT -3') and corresponding negative control siRNA (siNC, 5'-UUCUCCGAACGUGUCACGUTT-3') were obtained from Shanghai GenePharma Co., Ltd., China. Cells were transfected with siRNAs (100 nM) by PepmuteTM Transfection Buffer and Reagent (Signagen, Maryland, USA). The transfection efficiency was detected by qRT-PCR/western blot after 48 h/72 h of cell culture. FTO stable knockdown and overexpression cell lines were constructed using recombinant lentivirus infected cells. shFTO, shCtrl, FTO, vector and relative virus-containing supernatants were constructed and obtained from Shanghai GenePharma Co., Ltd. When the cells grew to about 50 ~ 60%, the appropriate volume of virus-containing supernatants was gently dropped into the medium, then polybrene was added. Puromycin (1.5

mg/mL) was used to further screen the stable expression cells.

RNA m^{6}A dot blot assay

RNA m^{6}A Dot blot was conducted as described formerly [22], to detect the level of total mRNA m^{6}A modification after knockdown of FTO.

Immunohistochemical (IHC) analysis

IHC was performed as described previously [23]. The percentage of positive cells (PP) and staining intensity (SI) were scored for the stained specimens, and the PP and SI scores were multiplied to calculate the IHC score [24].

Wound healing assay

Wound healing assay was performed as described previously with appropriate modification [21]. Cell migration rate = (0 h wound area – 48 h wound area)/0 h wound area. Experiments were performed thrice.

Migration and invasion assay

Migration and invasion assay were performed as described previously [21]. Experiments were performed thrice.

Proliferation assay

Cell Counting Kit-8 (CCK-8, Vazyme, Nanjing, China) was employed for cell proliferation experiments as described in a previous study [25]. Experiments were performed thrice.

Colony formation

Cells (500 per well) were seed into 6-well plates. After 10 days of cell culture, the cells were fixed with 4% paraformaldehyde for 30 min, at 20°C. Stained with 0.1% crystal violet solution for 60 min, washed with PBS and air-dried. The 6-well plate was placed upside down on a piece of white paper with a mesh and then counted colonies with >50 cells.

Sphere formation

Cells (2×10^3 per well) were inoculated on 24-well plate with ultra-low adhesion (Corning, USA). The cells were cultivated in DMEM/F12 medium supplemented with EGF (20 ng/mL), bFGF (10 ng/mL), B27 (10 μl/mL), insulin (5 μg/mL), and 1% penicillin/streptomycin. After 10 days of cell culture, three fields were randomly photographed under an inverted microscope and then counted microspheres with a diameter of >50 μm.

Animal experiments

The animal experiments were studied after approval by the Animal Ethics Committee of Wuhan University. Male BALB/c nude mice (4 weeks) were obtained from Shulaibao Biotech (Wuhan, China) and reared under specific pathogen-free conditions. A total of 1×10^7 shCtrl or shFTO PANC-1 cells were randomly seeded subcutaneously into the left axillary fold of nude mice, 4 mice in each group. The minimum width (W) and maximum length (L) of the tumour were measured with calipers per four days. The tumour volume was calculated following the formula $V = LW^2/2$. After 39 days, all the nude mice were sacrificed, the tumour tissues were collected, fixed in 4% paraformaldehyde and then embedded in paraffin.

Methylated RNA immunoprecipitation (MeRIP)-qPCR

MeRIP was conducted as formerly described [26]. Briefly, cells were collected and treated with RIP lysate, and then the cell lysate was added into an EP tube containing 5 μg m^{6}A antibody (Abcam, UK)-magnetic beads (MCE, China). After being rotating overnight at 4°C, the cell lysate was washed with high-salt buffer solution, and then RNA was extracted with RIP detergent. The mRNA level of m^{6}A+ TFPI-2 was measured by qPCR.

mRNA stability

Actinomycin D (2 μM) was used to inhibit mRNA transcription [6]. Each sample was collected at 0, 3 and 6 h after treatment with

actinomycin D, and RNA was extracted by Trizol for qRT-PCR analysis.

Bioinformatics analysis

GEPIA [27] and Oncomine [28] were used to analyse the target gene expression level. A Meta-analysis of the data of FTO expression in PC tissues and corresponding normal tissues from Oncomine was conducted using Stata v14.0 software. The clinical survival of the candidate gene was analysed by the R2 Genomics Analysis and Visualization Platform (https://hgserver1.amc.nl). RNA-protein interaction prediction (RPISeq) [29] was employed to predict the target protein with candidate genes mRNA probability. SRAMP [30] was employed to detect candidate genes mRNA m^6A modify site distribution. PRIdictor [31] was used to analyse the sites where the target protein could bind to the mRNAs of candidate genes. In addition, RMBase v2.0 [32] was used to analyse the m^6A motifs of target protein binding to candidate gene mRNAs.

Statistical analysis

Data were expressed as mean ± SD. All experiments were performed at least thrice. The experimental data were analysed by GraphPad Prism v8.4.0 and SPSS v25.0 software (IBM). Statistical differences were analysed utilizing either an unpaired student t-test or one-way ANOVA. The correlation between FTO expression and TFPI-2 expression was analysed by Pearson's correlation analysis. $P < 0.05$ was considered statistically significant, and the results presented as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, no statistical significance (NS).

Results

Expression of m^6A demethylase FTO is significantly upregulated in PC

To investigate the expression level of FTO in PC, we firstly analysed the FTO expression in tumour tissues and normal tissues using the GEPIA, and data analysis showed that FTO expression was abnormally upregulated in PC tissues compared to normal tissues (Figure 1a). Notably, we performed a meta-analysis on several data sets including TCGA data in the Oncomine database, and the data indicated that FTO expression was remarkably upregulated in PC (SMD 0.42, 95%CI 0.11–0.73, P = 0.008) (Figure 1b). Then, qRT-PCR analysis revealed that FTO mRNA levels in PC tissues were elevated compared with adjacent normal pancreatic tissues (Figure 1c), and western blot analysis presented the similar results (Figure 1d), which were consistent with the above database analysis. To further demonstrate the elevated expression of FTO protein in PC, IHC staining was employed to detect FTO protein level in 43 paraffin-embedded PC specimens and 24 adjacent normal tissue specimens, and our data revealed that FTO expression was dramatically elevated in PC tissues (Figure 1e). In addition, clinical outcome analysis showed that upregulated FTO was remarkably correlated with tumour stage, degree of differentiation, lymph node positivity, and perineural invasion (PNI) (Table 1). The Kaplan-Meier analysis indicated that high FTO expression was significantly correlated with shorter overall survival of PC patients (figure 1f). These results indicate that the upregulated FTO may be involved in the tumorigenesis and development of pancreatic cancer.

FTO significantly promoted the proliferation, migration and invasion of PC cells *in vitro*

The qRT-PCR data showed that FTO mRNA levels were dramatically elevated in human PC cell lines compared to human normal pancreatic duct epithelial cells HPDE (Figure S1A), and western blot also showed similar results (Figure S1B).To investigate the role of FTO in PC, PANC-1 and SW1990 were treated with small interfering RNA (siFTO) to estimate the effect of FTO in PC progression. The knockdown efficiency of FTO was determined by qRT-PCR and western blot, and the results showed that siFTO-1 and siFTO-2 had significant interference efficiency (Figure 2a and b). Furthermore, dot blot assay indicated that FTO knockdown significantly increased the m^6A modification level of the total mRNA in PC cells (Figure 2c). CCK-8 data revealed that FTO knockdown remarkably repressed the cell proliferation of PC cells (Figure 2d). Wound

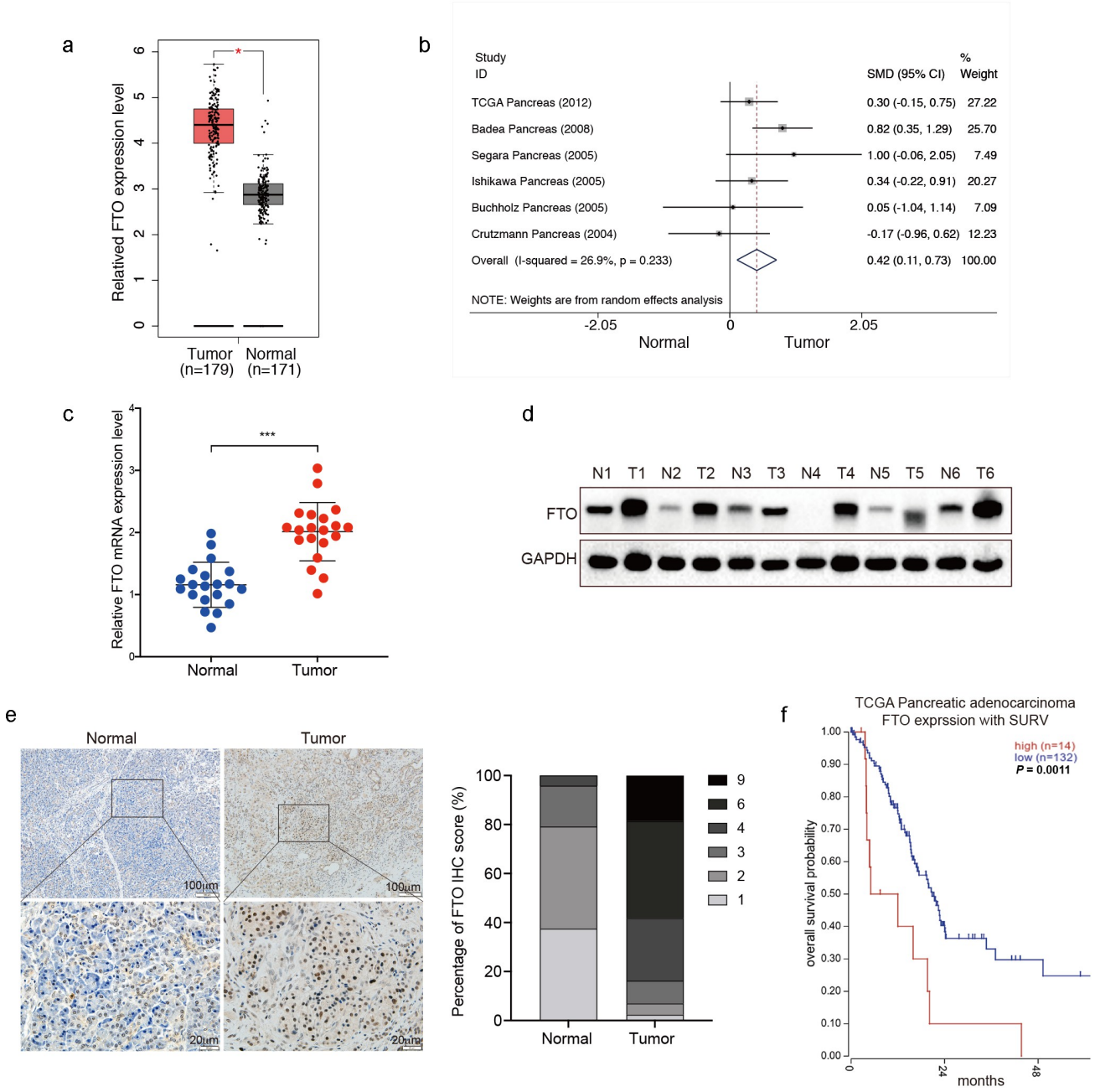

Figure 1. Expression of m^{6}A demethylase FTO is significantly upregulated in pancreatic cancer (PC). (a) FTO expression in PC tissues and normal tissues based on GEPIA database. (b) Meta-analysis of FTO expression based on Oncomine database in PC. (c) qRT-PCR and (d) western blot analysis of FTO mRNA and protein levels in PC tissues and adjacent normal pancreatic tissues. (e) IHC analysis of FTO protein expression in PC specimens and adjacent normal pancreatic tissue specimens. (f) Kaplan-Meier analysis indicated that higher FTO expression was remarkably correlated with shorter overall survival of PC patients. *P < 0.05, ***P < 0.001.

healing experiment indicated that silenced FTO notably suppressed migration of PC cells (Figure 2e), and the result was further demonstrated by subsequent migration assay (figure 2f). Invasion assay revealed that silenced FTO significantly reduced the invasion ability of PC cells (Figure 2g). These results indicate that FTO plays an important role in the proliferation, migration and invasion of PC cells.

Table 1. Relationship between FTO expression and clinicopathologic features in pancreatic cancer.

Clinicopathological features	Patients (n = 43)	FTO		P value
		Low expression	High expression	
Age (years)				
≤50	15	6	9	0.856
>50	28	12	16	
Gender				
male	24	9	15	0.515
female	19	9	10	
Histological grading				
Low differentiation	27	7	20	0.006
High differentiation	16	11	5	
Tumour diameter				
≤2 cm	13	8	5	0.085
>2 cm	30	10	20	
TNM staging				
I	11	8	3	0.043
II	17	6	10	
III	15	4	12	
Lymph node				
Positive	26	7	21	0.008
Negative	17	11	6	
PNI				
Positive	36	12	24	0.01
Negative	7	6	1	

Knockdown of FTO significantly inhibited PC growth *in vitro* and *in vivo*

To better study the role of FTO on the development of PC, we used FTO stable knockdown cell lines constructed using recombinant lentivirus to further evaluate the effect of knockdown FTO on the PC growth. Stable knockdown efficiency of FTO was determined by qRT-PCR and western blot (Figures 3A and b). Colony and sphere formation assays indicated that silenced FTO repressed the ability of PC cell colony and sphere formation (Figure 3c and d). In addition, to confirm that the growth inhibition by FTO knockdown is due to the on-target effect, we used FTO expression vector to perform a rescue experiment. More than 90% of cells with overexpression lentivirus of FTO were successfully transfected and their green fluorescent proteins were observed to be positive (Figures S2A). Overexpression efficiency of FTO was detected by qRT-PCR and western blot (Figures S2B and C). The results showed that FTO overexpression promotes the ability of PC cell proliferation, colony and sphere formation (Figure S2D-F). Furthermore, we subcutaneously injected the stable shFTO PANC-1 cells into nude mice to construct the xenograft model of PC. Animal experiments showed that knockdown of FTO significantly inhibited tumour growth (Figure 3e), tumour volume (figure 3f) and weight (Figure 3g) compared to the control group (shCtrl). Together, these results indicate that FTO plays a critical role in facilitating PC growth *in vivo* and *in vitro*.

Tissue factor pathway inhibitor 2 (TFPI-2) was identified as the downstream target of m^{6}A demethylase FTO

As shown above, we have demonstrated that m^{6}A RNA demethylase FTO could facilitate the growth and metastasis of PC. Next, we went on to get further insight into the mechanisms from our findings. TFPI-2 plays a crucial role in maintaining the structural integrity of extracellular matrix (ECM) and regulating the growth, invasion and metastasis of tumour cells [33]. Moreover, we analysed the clinical survival of TFPI-2 using R2 based on Hussain's microarray gene expression profiles (GSE62452), and the results showed that low expression of TFPI-2 was significantly associated with shorter overall survival in PC patients (Figure S3). In recent years, TFPI-2 gene related epigenomic studies have been reported, such as DNA methylation [34] and microRNA [21], while mRNA m^{6}A modification study is rarely investigated. Here, we used bioinformatics analysis to predict that TFPI-2 might be a potential target gene of m^{6}A demethylase FTO. First, five m^{6}A modify sites in TFPI-2 mRNA were identified using SRAMP, and modification of m^{6}A motif was mainly GGACU (Figure 4a). Then, RPISeq was used to predict the binding probability of FTO protein to TFPI-2 mRNA. The results suggested that there may be a significant interaction relationship between FTO protein and TFPI-2 mRNA (Figure 4b). Online software PRIdictor further elucidated the interaction between FTO protein and TFPI-2 mRNA (Figure 4c). In addition, the high-throughput sequencing results of RMBase v2.0 database showed that FTO regulates TFPI-2 mRNA m^{6}A motif, which was mainly GGACU, located at the junction of mRNA coding sequence (CDS) and 3'-untranslated region (3'-UTR) (Figure 4d). The bioinformatics analyses indicate that

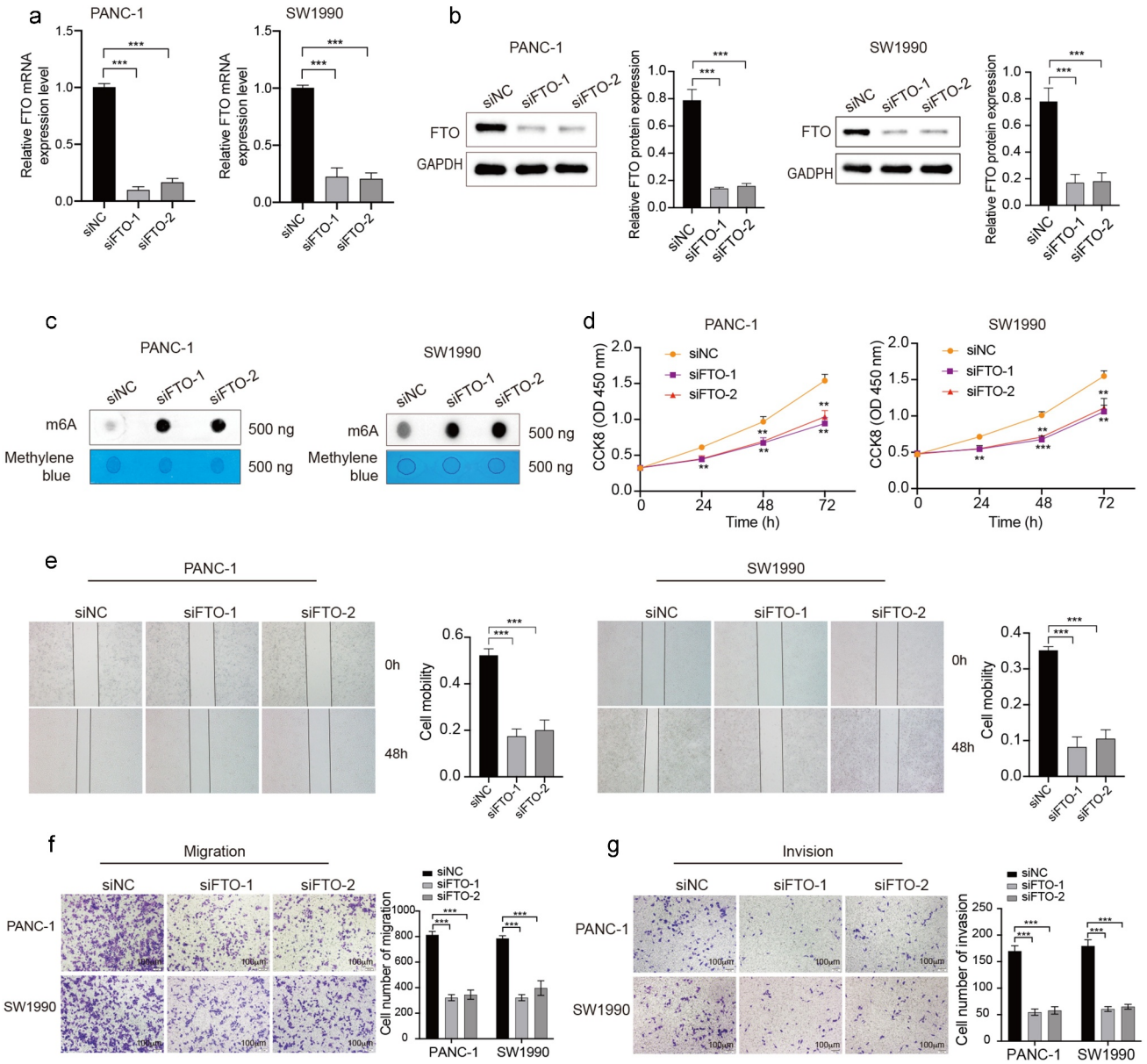

Figure 2. FTO significantly facilitated the proliferation, migration and invasion of pancreatic cancer (PC) cells *in vitro*. (a) qRT-PCR and (b) western blot were used to determine the knockdown efficiency of FTO in PANC-1 and SW1990 cells. (c) Dot blot was used to determine the effect of FTO knockdown on the m^{6}A modification level of total mRNA in PANC-1 and SW1990 cells. (d) CCK-8 assay revealed that FTO knockdown significantly inhibited the proliferation of pancreatic cancer cells. (e) Wound healing experiment indicated that silenced FTO significantly inhibited migration of PC cells. (f) Migration and (g) invasion assays revealed that silenced FTO significantly reduced the migration and invasion ability of PC cells. **P < 0.01, ***P < 0.001.

TFPI-2 is a potential target gene of m^{6}A demethylase FTO.

To verify that TFPI-2 is the downstream target gene of FTO, qRT-PCR and western blot were conducted to test mRNA and protein expression levels of TFPI-2 after silencing FTO in PANC-1 and SW1990 cells. The results showed that knockdown of FTO upregulated the expression levels of TFPI-2 mRNA and protein in PANC-1 and SW1990 cells (Figure 4e and f). To demonstrate that TFPI-2 is a true target of FTO m^{6}A modification, we performed MeRIP and analysed it by gene-specific m^{6}A-qPCR. Consistent with the expected result, FTO knockdown significantly increased m^{6}A levels of TFPI-2 mRNA (Figure 4g). Taken together, these data indicate that TFPI-2 is a downstream target gene of m^{6}A demethylase FTO.

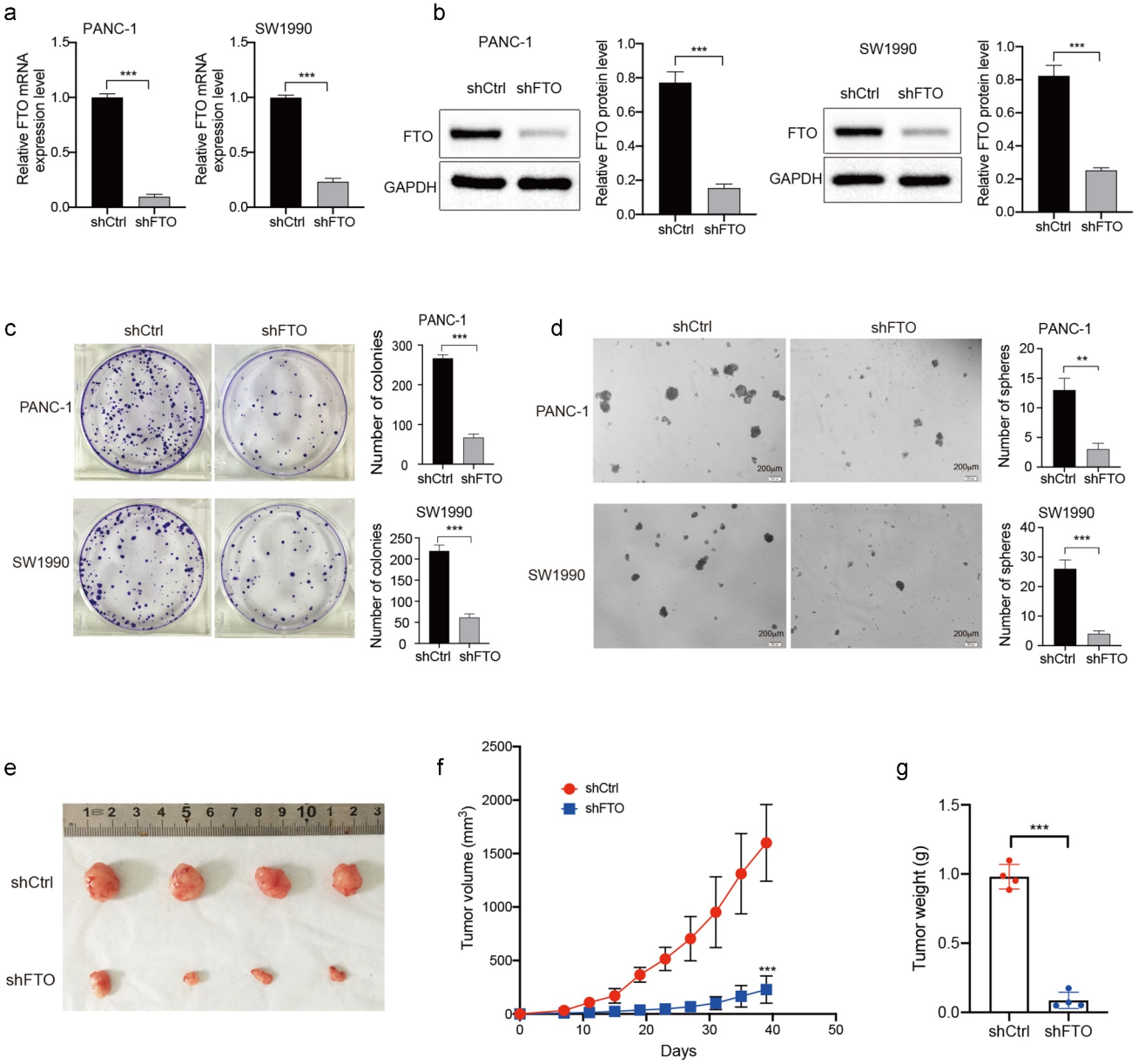

Figure 3. Silencing FTO significantly inhibited pancreatic cancer (PC) growth *in vitro* and *in vivo*. (a, b) Knockdown efficiency of FTO in PANC-1 and SW1990 cells were detected by (a) qRT-PCR and (b) western blot. (c, d) Knockdown of FTO repressed the ability of PC cell (c) colony and (d) sphere formation. (e-g) Knockdown of FTO significantly inhibited subcutaneous graft (e) tumour growth in nude mice, and reduced (f) tumour volume and (g) weight. **P < 0.01, ***P < 0.001.

TFPI-2 and FTO expression were negatively correlated in PC

To further prove the relationship between TFPI-2 and FTO, IHC was employed to assess the protein expression levels of TFPI-2 and FTO in 43 human PC tissue specimens. The results indicated that the specimens with higher FTO expression had lower TFPI-2 expression (Figure 5a). Pearson's correlation analysis of IHC scores of clinical PC specimens showed that there was a remarkable negative correlation between TFPI-2 and FTO protein expression levels in pancreatic cancer tissues ($r = -0.4932$, $P < 0.001$) (Figure 5b). Furthermore, we found that TFPI-2 was upregulated in FTO knockdown tumour samples in PC model injected subcutaneously in nude mice with PANC-1 (Figure 5c). These data indicate that TFPI-2 is negatively correlated with FTO expression in PC clinical samples.

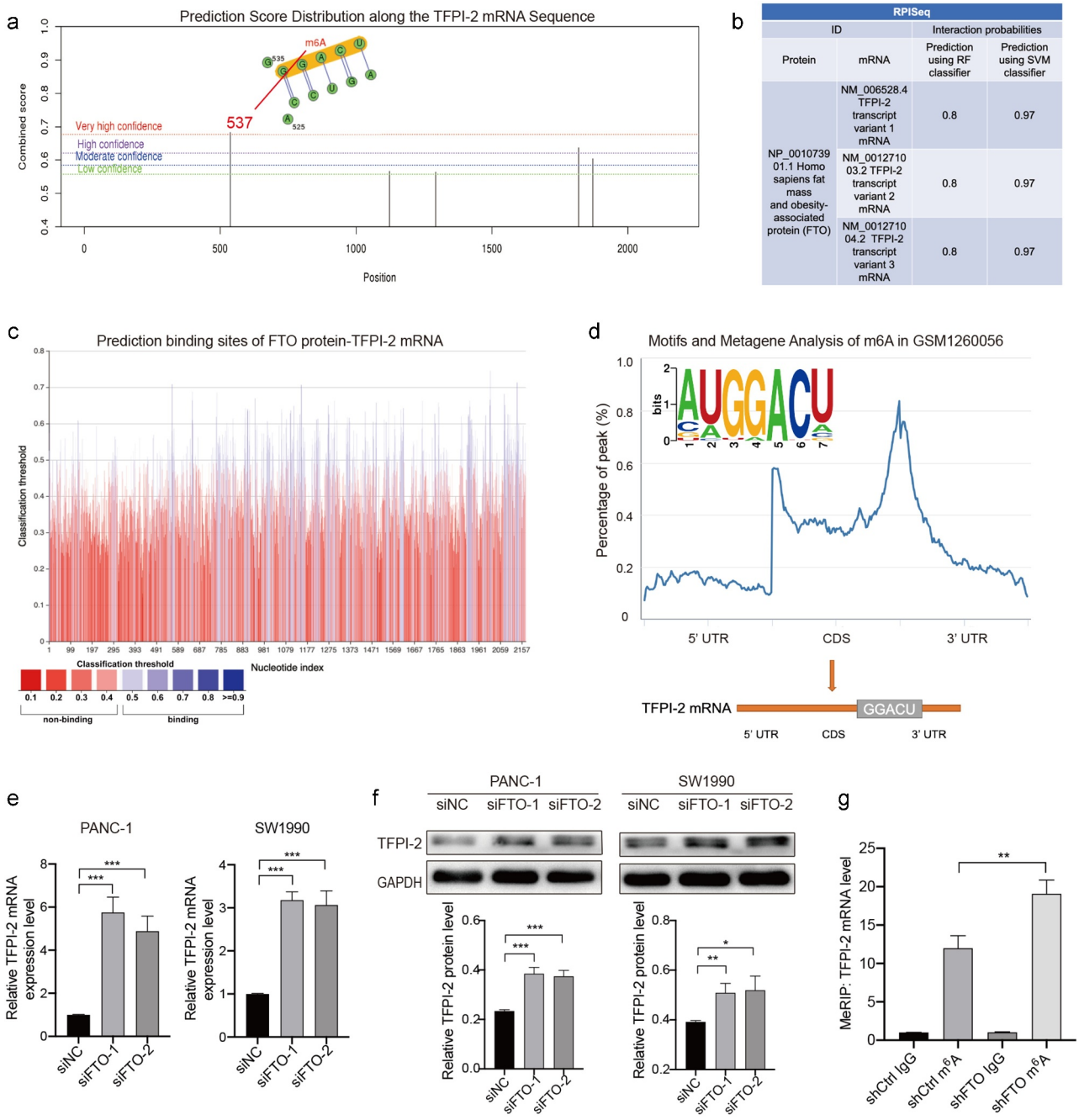

Figure 4. TFPI-2 was identified as the downstream target gene of m^{6}A demethylase FTO. (a) m^{6}A modify sites of TFPI-2 mRNA were detected by SRAMP database. (b) The binding probability of FTO protein to TFPI-2 mRNA predicted by RPISeq. (c) The interaction between FTO protein and TFPI-2 mRNA predicted by PRIdictor. (d) RMBase v2.0 database showed that FTO regulates TFPI-2 mRNA m^{6}A motif that was mainly GGACU located at the junction of mRNA CDS and 3′-UTR. (e, f) TFPI-2 mRNA and protein expression levels in silenced FTO PANC-1 and SW1990 cells detected by (e) qRT-PCR and (f) western blot. (g) MeRIP analysis showed that FTO knockdown significantly increased m^{6}A levels of TFPI-2 mRNA. *P < 0.05, **P < 0.01, ***P < 0.001.

FTO regulated the expression of target gene TFPI-2 by inhibiting the stability of TFPI-2 mRNA mediated through m^{6}A/YTHDF1

The function of m^{6}A in regulating gene expression is mainly performed through the readers, including the family proteins containing the YTH domain in mammalian cells [35]. Compared with YTHDF2 and YTHDF3, analysis of PRIdictor showed that YTHDF1 was more likely to bind to TFPI-2 mRNA, and the binding region was consistent with the

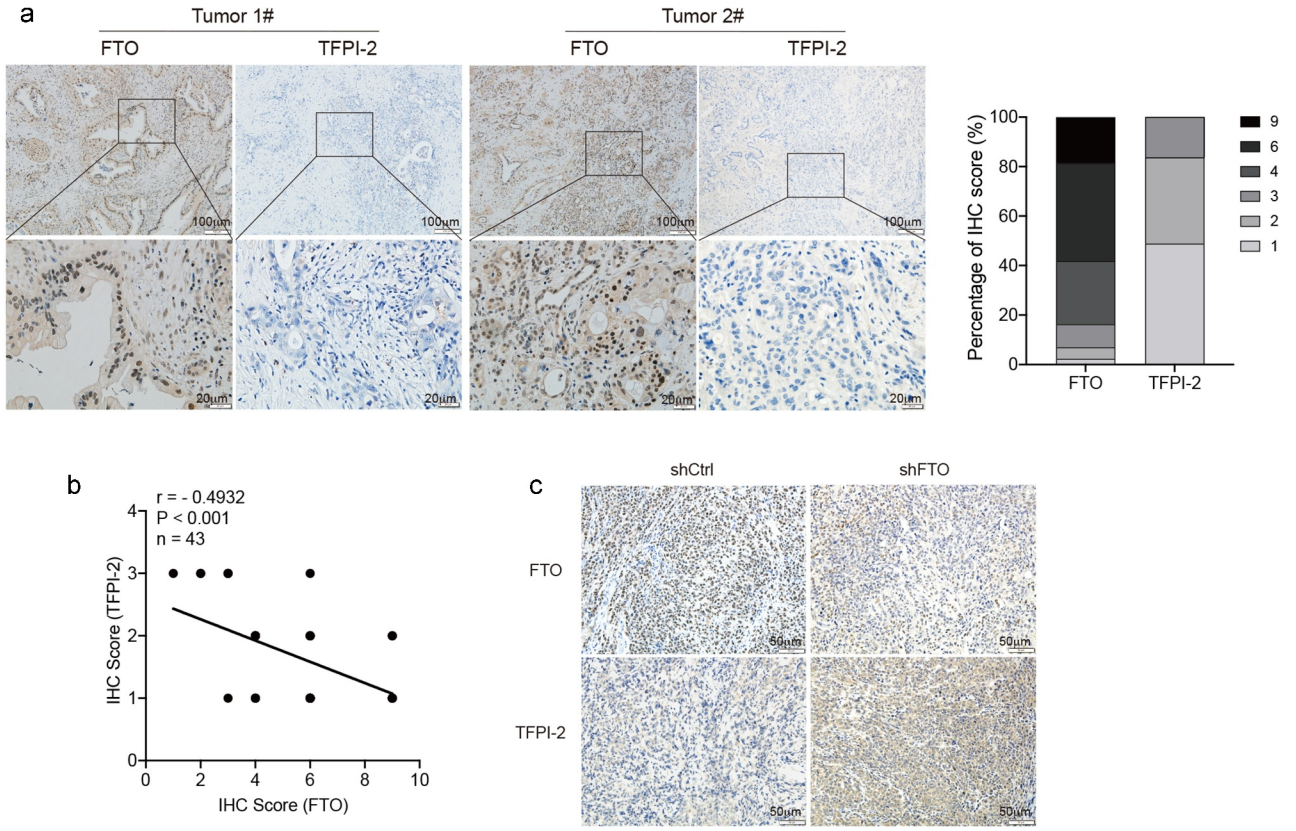

Figure 5. TFPI-2 and FTO expression were negatively correlated in pancreatic cancer (PC). (a) Protein expression levels and IHC scores of TFPI-2 and FTO by IHC analysis in human PC specimens. (b) Pearson's correlation analysis of IHC scores of clinical PC specimens. (c) IHC analysis of TFPI-2 and FTO protein expression levels in subcutaneous graft tumour with PANC-1.

distribution of the m^{6}A modified motif of TFPI-2 mRNA (Figure 6a). First, qRT-PCR analysis was employed to evaluate the knockdown efficiency of siYTHDF1 in PANC-1 cells (Figure 6b). In order to clarify the role of YTHDF1 in TFPI-2 mRNA metabolism, we tested the effect of YTHDF1 on TFPI-2 mRNA stability by mRNA stability assay. As our expectation, downregulated FTO significantly increased the stability of TFPI-2 mRNA, while knockdown of YTHDF1 inhibited the stability of TFPI-2 mRNA in shFTO PANC-1 cells (Figure 6c). These results suggest that FTO regulates the expression of target gene TFPI-2 partly by inhibiting the stability of TFPI-2 mRNA mediated via m^{6}A/YTHDF1.

TFPI-2 knockdown significantly attenuated the inhibitory effect of silenced FTO on the malignant phenotype of PC

To further elucidate the role of TFPI-2 in FTO-dependent tumour growth and metastasis, we used small interfering RNA (siTFPI-2) or siNC to treat shFTO PANC-1 cells. qRT-PCR was employed to analyse and evaluate the knockdown efficiency of siTFPI-2 (Figure 7a). CCK-8 assay indicated that knockdown of TFPI-2 effectively alleviated the repressive effect of silenced FTO on PC cell proliferation (Figure 7b). Similar effects were confirmed by plate cell colony formation and sphere formation experiments (Figure 7c and d). Furthermore, we explored the effect of TFPI-2 on FTO-dependent tumour migration and invasion using wound healing assay and Transwell assay, and the results presented that the migration and invasion ability of PC cells with TFPI-2 and FTO dual knockdown was significantly restored when compared to those with stable knockdown of FTO (Figure 7d-g). Taken together, the results suggest that TFPI-2 knockdown can significantly restore the inhibition of silenced FTO on PC growth and metastasis.

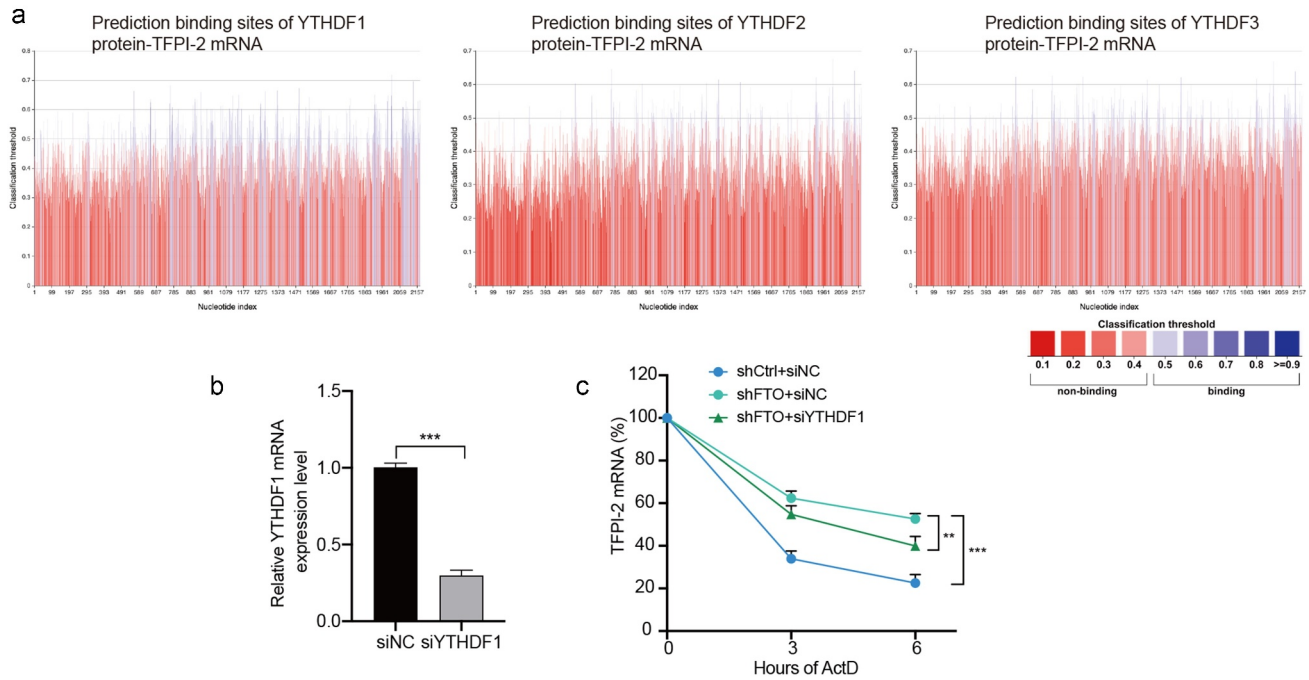

Figure 6. FTO regulated the expression of target gene TFPI-2 by inhibiting the stability of TFPI-2 mRNA mediated through m^{6}A/YTHDF1. (a) PRIdictor database predicted the reader proteins of TFPI-2 mRNA m^{6}A modification. (b) Knockdown efficiency of siYTHDF1 was evaluated by qRT-PCR analysis. (c) The effect of YTHDF1 on TFPI-2 mRNA stability was detected by RNA stability assay. **P < 0.01, ***P < 0.001.

Discussion

In recent years, the development of RNA epigenetics, especially the discovery of dynamic reversible m^{6}A RNA methylation, has provided new insights for better understanding the molecular mechanisms of cancer occurrence and progression. As the first m^{6}A RNA demethylase discovered, the role of FTO in tumour has attracted more and more attention. Here we show that m^{6}A demethylase FTO plays a critical role in PC. Abnormally upregulated FTO is associated with poor prognosis in PC patients, suggesting that FTO acts as an oncogenic gene in PC. FTO facilitates PC cell proliferation, colony formation and sphere formation, migration and invasion *in vitro*, as well as tumour growth *in vivo*. Mechanically, we found that FTO regulates the malignant phenotype of PC cells at least partially by downregulating the expression of TFPI-2, which is mainly mediated via m^{6}A/YTHDF1. Our findings reveal the role of FTO in regulating the growth, migration and invasion of PC, suggesting that FTO may be a potential therapeutic target for treating PC.

FTO is the first m^{6}A demethylase found to exhibit m^{6}A demethylation activity on mRNA [7]. Earlier studies showed that FTO was highly correlated with weight gain and obesity in children or adults [36,37]. Recent studies have found that FTO mediates the mRNA metabolism of lipogenic regulatory factors through m^{6}A demethylation [38]. In addition, Li et al. [17] showed that FTO, as an m^{6}A demethylase, plays a key role in carcinogenesis in acute myeloid leukaemia. Another study reported that FTO and its m^{6}A modification function were involved in the tumorigenesis of hepatocellular carcinoma [39]. Yan et al. [40] found that the generation of drug-resistant phenotypes during the treatment of tyrosine kinase inhibitor was determined by the reduction of m^{6}A induced by FTO overexpression in leukaemia cells, suggesting that the FTO-m^{6}A axis may become a potential therapeutic target for human cancer [41]. In the present study, we found that FTO acts as an oncogene in PC, which is consistent with the previous reports [17–19]. Here, we further confirmed that FTO promotes PC proliferation and facilitated the growth of PC through colony formation, sphere formation and *in vivo*

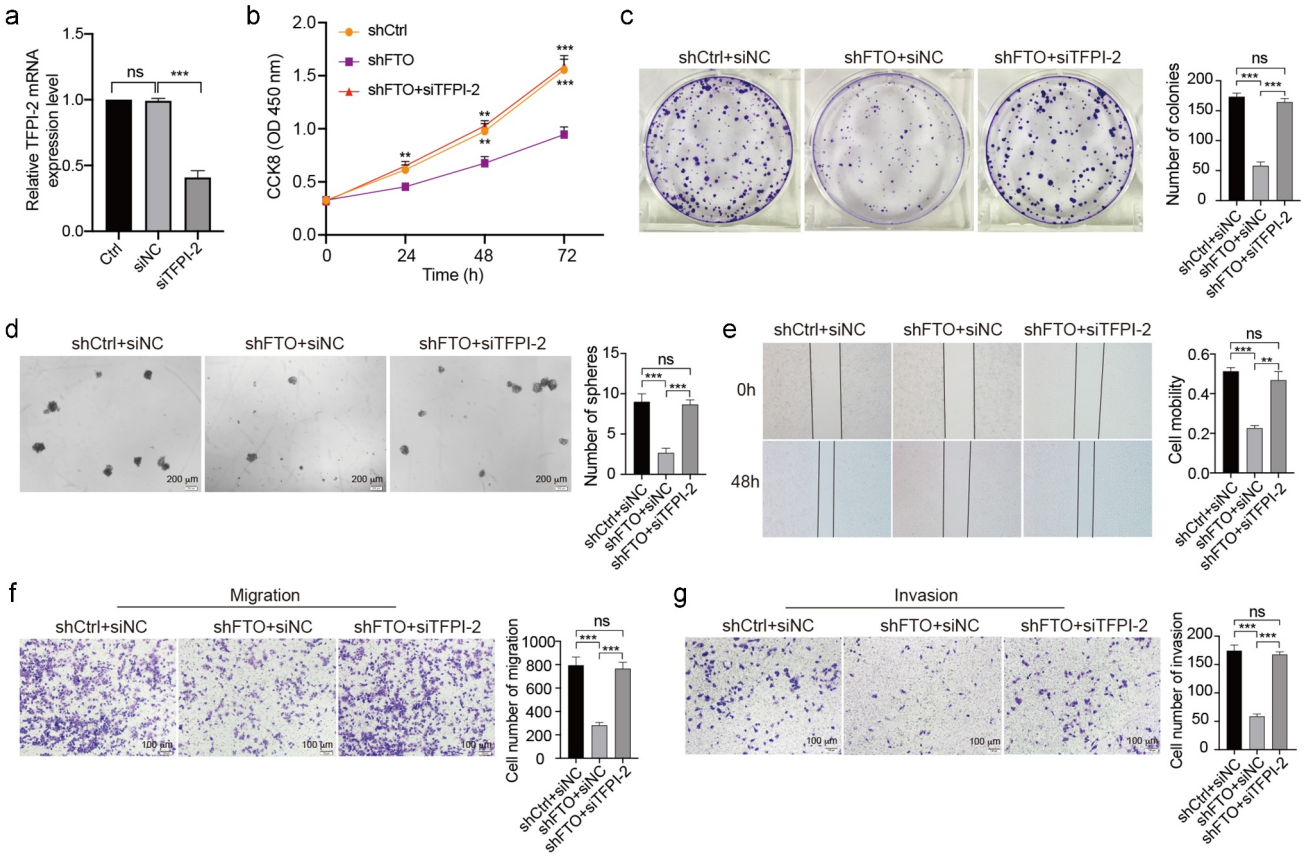

Figure 7. TFPI-2 knockdown significantly attenuated the inhibitory effect of silenced FTO on the malignant phenotype of pancreatic cancer (PC). (a) Knockdown efficiency of siTFPI-2 was evaluated qRT-PCR. (b) CCK-8 assay indicated that knockdown of TFPI-2 effectively alleviated the repressive effect of silenced FTO on cell proliferation. (c and d) knockdown of TFPI-2 effectively alleviated the repressive effect of silenced FTO on cell (c) colony and (d) sphere formation. (e-g) Knockdown of TFPI-2 effectively alleviated the repressive effect of silenced FTO on cell migration and invasion determined by (e)wound healing assay, (f) migration assay, and (g) invasion assay. **P < 0.01, ***P < 0.001, ns, no significance.

experiments. Moreover, we revealed that FTO could facilitate the migration and invasion of PC cells. Importantly, we found a novel regulatory mechanism of FTO, which contributes to a better understanding of the potential epigenetic role of FTO in the development and progression of PC.

TFPI-2 encodes a broad-spectrum serine protease inhibitor that negatively modulates ECM degradation, a necessary step for tumour invasion and metastasis [42]. Our survival analysis based on Hussain's microarray gene expression profiles (GSE62452) showed that low expression of TFPI-2 was significantly associated with shorter overall survival of PC patients. Previous studies reported that overexpression of TFPI-2 in PC cell lines can inhibit the malignant phenotype of PC *in vivo* and *in vitro* [34,43]. In the past decade, TFPI-2 has attracted continuous attention, especially in the epigenetics study. Sato et al. [34] found that the deletion of TFPI-2 expression in PC was correlated with abnormal hypermethylation of the promoter CpG Island. Nigro et al. [44] found that TFPI-2 methylated DNA was detected in serum as a sensitive and specific biomarker for metastatic melanoma. Moreover, TFPI-2 mRNA was also found to be post-transcriptional regulated by microRNAs, such as miR-616 [45,46], and miR-23a [21]. With the rapid development of bioinformatics, new high-throughput experimental frameworks provide an opportunity to integrate and generalize different mechanisms on a single gene [47]. Here, through bioinformatics analysis and validation experiments, we found that an emerging epigenetic mechanism, m^6A modification, was involved in regulating the expression of TFPI-2, and TFPI-2 was identified as the downstream target gene of FTO. Recently, Li et al. reported that up to 84.5% of potential FTO

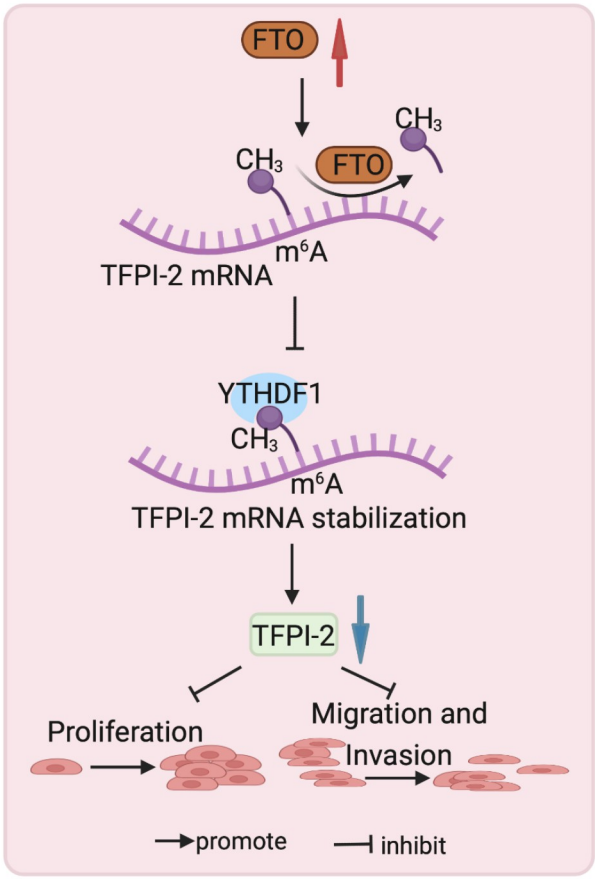

Figure 8. FTO promotes the progression of pancreatic cancer by inhibiting the expression of TFPI-2, partially through decreasing the stability of TFPI-2 mRNA mediated via m^{6}A /YTHDF1.

targets in AML cells are negatively modulated by FTO as FTO reduces the stability of these mRNA transcripts [17]. Consistent with this report, we found that silenced FTO in PC cells could significantly upregulate the m^{6}A modification level of its target TFPI-2 mRNA. And mRNA stability assay showed that knockdown of FTO could significantly increase the stabilization of TFPI-2 mRNA. Moreover, YTHDF1 knockdown significantly reduced the elevated TFPI-2 mRNA stabilization induced by FTO silencing, suggesting that FTO regulates the expression of target gene TFPI-2 by inhibiting the mRNA stabilization of TFPI-2 partially through m^{6}A /YTHDF1 mechanism.

To further confirm the role of FTO in promoting cancer mainly through regulating the downstream target gene TFPI-2, we performed rescue assay by double silencing of FTO and TFPI-2 in PC cells. The rescue assay indicated that the double silence significantly reduced the inhibition of the malignant phenotype of pancreatic cancer cells induced by FTO knockdown alone, while failed to completely restore to the level of the control group. These results indicate that FTO promotes PC progression partially by regulating the target gene TFPI-2, and there are still some other mechanisms involved in the PC progression regulated by FTO, which require further studies.

We acknowledge there were some potential limitations to consider in the present study. First of all, for the selection of downstream target genes of FTO, the present study adopted the strategy of phenotypic experiment combined with literature guidance and comprehensive bioinformatics analysis, which had certain limitations for systematically studying the downstream target genes of FTO with high throughput. As shown in the results of rescue experiment, there might be still other mechanisms involved in the regulation of FTO in PC progression. Secondly, the results of RNA stability assay showed that the double knockdown of YTHDF1 and FTO did not completely restore the elevated TFPI-2 mRNA stability caused by silencing FTO, suggesting that there might be other manner for the metabolism of FTO target gene mRNA, which needs further investigation. In addition, as previously reported, the inhibition mechanism of TFPI-2 may be DNA methylation on its promoter and regulation of miRNAs. Here, we identified a novel epigenetic modification, the m^{6}A modification, that is involved in regulating TFPI-2 expression. However, the relationship among these three epigenetic regulation modes and which one is dominant need to be further explored.

In conclusion, our findings confirmed that FTO expression is abnormally upregulated in PC and is closely associated with adverse clinicopathological features and prognosis. FTO promotes PC cell proliferation, colony formation, sphere formation, migration and invasion *in vitro*, as well as tumour growth *in vivo*. Mechanically, FTO facilitates the progression of PC at least partially by inhibiting the expression of TFPI-2 through decreasing the stabilization of TFPI-2 mRNA mainly mediated via m^{6}A /YTHDF1 (Figure 8). Taken together, we revealed the role and molecular mechanism of m^{6}A demethylase FTO in promoting PC progression, providing potential target and new strategy for PC therapy.

Abbreviations

m^6A, N^6-methyladenosine; FTO, fat mass and obesity-related protein; TFPI-2, tissue factor pathway inhibitor 2; qRT-PCR: quantitative reverse transcription polymerase chain reaction; MeRIP, methylated RNA immunoprecipitation.

Author contributions

WW and YH conceived and designed the experiments. WW and YH performed the experiments, contributed to the data analysis, and wrote the manuscript. LZ, LC, LY, and LW edited the manuscript. ZT and JN designed the study, performed critical revision and supervised all phases of the study. All authors read and approved the final manuscript.

Data Availability Statement

All data included in this study are available upon request by contact with the corresponding author.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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